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ORIGINAL ARTICLE

Frequency of different genotypes of *Giardia duodenalis* in slaughtered sheep and goat in east of iran

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Abstract *Giardia duodenalis* is one of the most common and important protozoan parasites of the gastrointestinal tract in humans and animals, especially in developing countries. The purpose of this study was determining prevalence of *Giardia* genotypes specially zoonosis genotypes in sheep and goat in eastern of iran slaughterers.This cross-sectional study was conducted during April to November 2019. 300 fecal samples were collected from the rectum of sheep and goats. The samples were subjected to DNA extraction after sucrose gradient purification. A fragment of the glutamate dehydrogenase gene (*gdh*) was amplified by semi-nested PCR and genotype diagnosis was performed by digestion of the secondary PCR product with restriction enzymes *Rsa*I and *Nla IV*. The prevalence of *Giardia* was found as (274/300) by the molecular method. Restriction endonuclease digestion of the nested-PCR product showed; among 274 positive isolates, 95 were typed as assemblage E, 15 as assemblage B, 87 assemblage AI, 45 assemblage AII, and 32 assemblege C. In this study, frequency of different assemblages of *G. duodenalis* was determined in sheep and goats by *gdh* gene and PCR-RFLP method. Same of other studies, assemblage E was dominant genotype in sheep and goats. Isolation of zoonotic assemblages as AI, AII, and BIII showed that sheep and goats should be considered as a source for human infection.

Keywords *Giardia* · Genotype · Sheep · Goat · PCR-RFLP

Introduction

Giardia is a flagellate protozoa of Phylum Sarcomastigophora, Class Zoomastigophora, Family Hexamitidae and Genus *Giardia* which in its life cycle have two stages of trophozoite and cysts. *Giardia duodenalis* is one of the most common gastrointestinal parasitic and waterborne infections especially in developing countries. Contamination occurs by eating at least 10 parasite cysts and this protozoa is one of the major causes of diarrhea in humans, especially in children, and one of the health problems in the world, including Iran. Its prevalence in Iran is estimated at 9–30%. Symptoms of the disease range from mild to severe, and include mild diarrhea, abdominal bloating, anorexia, abdominal cramping pains, epigastric sensitivity, steatorrhoea, and malabsorption syndrome (Lalle et al. 2005).Types of *Giardia* genetic groups are A to G, Groups A (including A1 to A8) and B (including B1 to B6) are zoonoses the other types include groups C and D in Dogs, group E in Ruminants, F in Cat and G in Rodents (Thompson and Monis 2004; Monis 2003, 2009). Different animal species can be infected with Giardiasis isolates from other animals as well as human cysts.Therefore, it is possible that humans and animals could infect each other (Erlandsen et al. 1988). Many studies have shown that Giardiasis isolated from different hosts is similar, and this strengthens the zoonoticity of this protozoan parasite (Thompson and Hunter 2005). Although all isolates

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derived from humans belong to the two assemblies A and B, they have been identified in domestic animals such as ruminants, dogs, cats and other wildlife animals (Itagaki et al. 2005). Therefore, some researchers believe that *G. duodenalis* can be an important zoonotic parasite from animals such as cows (Wilson Jolaine and Hankenson 2010; Mendonça et al. 2007), dogs (Leonhard et al. 2007; Paoletti et al. 2008), cats (Overgaauw et al. 2009), wild mice (Robertson et al. 2007), sheep, farm animals, and Wildlife (Overgaauw et al. 2009) for humans. So, understanding of parasite reservoirs, the prevalence and determination of genotypes of *G. duodenalis* could be necessary to control, prevention and inhibit many health and economic problem to society.

In order to know the epidemiology of *Giardia* infection to determine the control methods and determine whether *G. duodenalis* can infect human zoonotic pathway this study tries to evaluate the frequency of different genotypes of *G. duodenalis* in sheep and goats slaughterhouses using PCR method.

Materials and methods

Study design and sample size

In this descriptive study based on similar study in the area, 300 samples of sheep and goat stool were collected from the rectum of slaughter sheep and goat from different slaughter houses during April to November 2019.then transferred to parasitology laboratory at 4 °C.

Direct microscopy test

A drop of saline was applied to a clean smear with the help of an applicator from a suspension stool and a lamella was placed on it. Expansion was prepared using optical microscopy with a magnification of 10% and 40% in terms of the presence of trophozoites or Giardiasis duodenal cysts.

Floatation test

Samples were separately suspended in 100 ml PBS solutions and passed through a four-layered gauze and centerfuged for 10 min at 1000*g* at 4 °C. The sediment was centrifuged again with PBS and suspended in 30 ml of PBS. In a test tube, 20 ml of sucrose 0.85M was added and 30 ml of suspended stool poured in to the tube gradually until two distinct phases were formed, then centerfuged for 5 min at 800*g* at 4 °C and the cloudy layer containing *Giardia* cysts was removed between the sucrose and stool layer by pasteur pipette and transferred to another tube, to remove sucrose from *Giardia* cysts the test tube was centrifuged after adding PBS at about 1000*g* for 10 min with three replicates. The sediment was transferred to a 2 ml tubes and centrifuged with PBS for 5 min, at 1000*g*. The resulting precipitate containing condensed cysts without excess feces was suspended in 1 ml of distilled water and stored at − 20 °C.

DNA extraction

The tubes containing sediment were placed in 100 °C water bath for 3 min then immediately placed in liquid nitrogen for 5 min and repeated these two steps 5 time (Freeze-thaw stage) then with QIAgen DNA extraction kit and according to its instruction DNA was extracted.

PCR detection

PCR reaction was performed on all specimens by specific primers of glutamate dehydrogenase (gdh) gene using Nested-PCR (Read et al. 2004). Internal and reverse primers (Table 1) in 25 µl were performed according to Table 2 for the first and second round of the PCR reaction with the program according to Table 3 of DNA replication. In the second step, 1 µl of the PCR product obtained from the first PCR was used as a template (Read et al. 2004).

Then the PCR product was electrophoresed on 2% agarose gel and DNA extracted from a *G. duodenalis* trophozoite sample as standard DNA was used to optimize PCR. 5 µl of PCR product electrophoresis on 2% agarose gel in TAE buffer in voltage between 45 and 50 for 1 h and the gel was photographed using UV light in a trans-illuminator device.

PCR-RFLP

To determine the genotypes of *Giardia* to 10 µl of the PCR product in positive samples, 2 µl of enzyme buffer was added and, after mixing, 1–2 µl of the enzyme (Table 4) was added. The tube was placed in a 37 °C hot block for 2 h. To evaluate the components obtained from the enzyme cleavage according to Table 5, the results were evaluated.

Finally 5 µl of PCR product was electrophoresis on 5% agarose gel in TAE buffer for about 1 h then the gel was photographed using UV light in a trans-illuminator device. PCR products were sent to South Korea's Bioneer Company to determine the sequence after extraction of DNA from the gel by using the Qiagen kit.

Table 1 External and internal primer sequence in this study

Primer	Sequence	PCR product (bp)
External forward primer	TCA ACG TYA AYC GYG GYT TCC GT	432
Internal forward primer	CAG TAC AAC TCY GCT CTC GG	432
Reverse primer	GTT RTC CTT GCA CAT CTC C	432

Table 2 Volume and materials required in the first and second PCRs

Material	First stage PCR (µl)	Second stage PCR (µl)
Premix	5/12	5/12
Work primer-1	2	4
Templete	2 µl extracted DNA	1 µl PCR product first stege
DW. water	5/8	5/7
Total volume	25	25

Table 3 Thermocyclere program for DNA replication

Stage	First stage PCR			Second stage PCR		
	Tempreture (°C)	Time (s)	Cycle	Tempretur (°C)	Time (s)	Cycle
Initial denaturation	94	300	1	94	300	1
Denaturation	94	30	30	94	30	15
Annealing	61	20		60	20	
Extention	68	20		65	20	
Final extention	68	420	1	65	420	1

Table 4 Restriction site of restriction enzyme

Restriction enzyme	Restriction site
Nla IV	5′…… GGN / NCC …...…3′
	3′……..CCN/NGG.....…0.5′
*Rsa*I	5′....….....GT/AC.....…0.3′
	3′….....……CA/TG.....5′

Results

Out of 300 samples, 150 samples collected from sheep and 150 samples collected from goats In east province of iran. And. in direct microscopy test, 53 specimens (17.66%) were positive for *G. duodenalis*.Out of these 53 positive samples, 38 samples related to sheep and 15 of them related to goat.(Fig. 1).

Molecular results

The nested-PCR of *Giardia* glutamate dehydrogenase (*gdh*) gene confirmed the presence of 432 bp fragment in 274 out of the 300 samples (Fig. 2).

RFLP

The results of enzymatic digestion showed that out of 274 positive samples, 95 isolates related to E genotype; were 25 isolates of them is unmixed E genotype and 70 isolates

Table 5 Cleavage pattern of different genotypes of *Giardia* with Nla IV and RsaI enzymes

Genotype	Enzyme	Fragments after digestion with the enzyme (bp)	Distinguished fragments (bp)
A I	*Nla IV*	16, 18, 39, 87, 123, 149	90, 120, 150
A II	*Nla IV*	16, 18, 39, 72, 77, 87, 123	70, 80, 90, 120
B III	*Nla IV*	18, 123, 291	120, 290
B IV	*Nla IV*	18, 123, 291	120, 290
C	*Nla IV*	18, 31, 72, 123, 187	70, 120, 190
D	*Nla IV*	18, 39, 126, 249	120, 250
E	*Nla IV*	16, 18, 72, 106, 216	80, 100, 250
B III	*Rsa I*	2, 133, 297	130, 300
B IV	*Rsa I*	2, 430	430

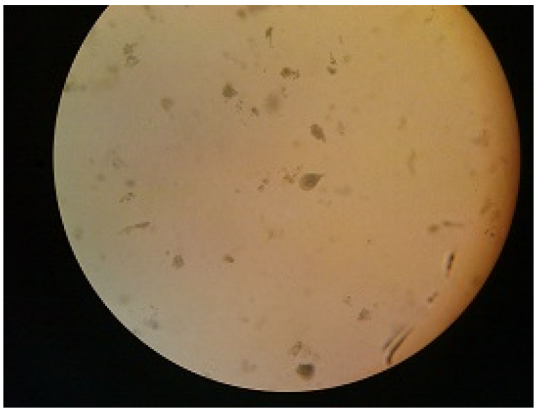

Fig. 1 *Giardia duodenalis* in direct microscopic test

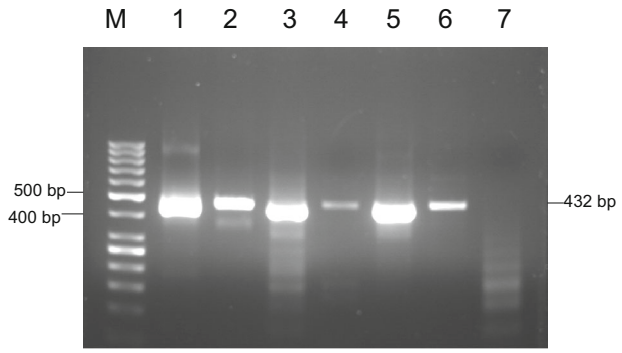

Fig. 2 Electrophoresis of the product of PCR on 1.5% agarose gel column M: molecular marker 50 bp, column 1 positive control, columns 2–6 PCR product positive samples, column 7 negative control

were mixed with other genotypes. 15 samples related to B genotype and all of them were BIII and mixed with other genotypes. 87 isolates related to AI genotype were 43 isolates of them unmixed AI genotype and 44 isolates were mixed with other genotypes.45 samples related to AII genotype were 25 isolates of them unmixed AII genotype and 20 isolates were mixed with other genotypes. 32 samples related to genotype C were 2 isolates of them unmixed C genotype and 30 isolates were mixed with other genotypes (Fig. 3; Table 6).

Column M: 50 bp molecular marker, column 1: genotypes E (80,100, 220 bp) mixe with B (120, 290 bp), column 2: genotype E (80,100, 220 bp), column 3: genotypes E (80,100, 220 bp) and B (120, 290 bp), column 4: genotypes E (80,100, 220 bp) and AII (70, 80, 90, 120 bp), column 5: genotypes E (80,100, 220 bp) and AI (90, 120, 150 bp), column 6: genotype E (80,120, 220 bp) mixe with B ((120, 290 bp), column 7: genotype E (80,100, bp 220), column 8: genotype C (70, 120, 190 bp), column 9: genotype E (80,100, 220 bp) mixe with B (F (120, 290 bp), column 10: genotype E (80,100, 220 bp).

Discussion

Briefly in the result of study, out of 300 samples, 274 samples were positive for *G. duodenalis*; that 95 isolates were E genotype, 15 samples were B genotype, 87 isolates were AI genotype, 45 samples were AII genotype and 32 samples related to C genotype; in these case some were unmixed and in some they mixed with other genotypes.

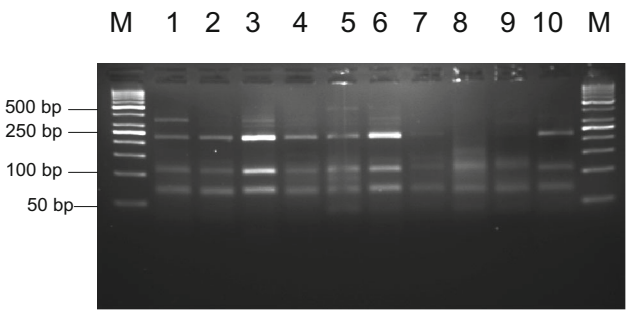

Fig. 3 Electrophoresis of enzyme digestion on 5% agarose gel (different genotypes)

Table 6 Genotypes isolates along and mixe with their source of animal

C/MIX	C	A11/MIX	A11	A1/MIX	A1	B/MIX	B	E/MIX	E	Genotype Animal
20	2	15	17	35	31	11	0	63	20	Sheep
10	0	5	8	9	12	4	0	7	5	Goat

And, in direct microscopy test, 53 specimens were positive for *G. duodenalis* in this samples, Accordingly Nucleic acid-based methods are powerful and reliable tools for identifications of parasites, including *Giardia*, in fecal and environmental samples.

Most of the genes used in various studies can group *Giardia* into an assemblage and some may be able to identify the types of AI and AII, while only a few of the locus can differentiate subtypes within the B assemblage. For example, some molecular markers, such as *SSURNA* and *elfa1-α* genes, can only be used to differentiate the main assemblies (A and B) While they are unable to identify subgroups of these assemblies (Abe et al. 2003; Sulaiman et al. 2003) otherwise, various sequences of Glutamate dehydrogenase gene were selected by some researchers and successfully identify the genotypes of *G. deodenalis*, The glutamate dehydrogenase enzyme plays an important role in the metabolism of carbohydrates, ammonia absorption, synthesis or catabolism of amino acids. In *Giardia*, this vital enzyme is coded by a copy of the gdh gene dependent on the NADP coenzyme this gen is continuous and has no intron in its sequence and able to identify *Giardia* subgroups such as AI, AII, BIII and BIV with RFLP testing without sequencing (Sulaiman et al. 2003; Thompson 2004). Different methods are used to detect parasites but the common method detects parasite with the direct microscopic examination of the stools, Compared with direct microscopic testing, other tests such as ELISA and immunofluorescence have a sensitivity of between 90 and 99% and specificity of between 95 and 100%. Although these tests are fast, but they are qualitative and unable to differentiate genotypes and lacking enough sensitivity to detection of low levels of infection. Molecular methods such as PCR are a good substitute for pathogen identification in the stool and in combination with RFLP they are also used to classify *Giardia* genotypes (Thompson and Hunter 2005) these methods have been developed for direct genetic typing of *Giardia* cysts in stool samples and eliminate the need for *Giardia* cultivation in vitro and in in vivo And are able to identify genotypes that are found in nature with high precision (Thompson and Hunter 2005; Leonhard et al. 2007; Abe et al. 2003).

In another study, by using gdh gene *G. deodenalis* genotypes were determined using RFLP test (Abe et al. 2005) and later Itagaki et al. used a sequence of 177 bp of gdh to detect *G.duodenalis* genotypes in animals using Nested-PCR (Itagaki et al. 2005) Also Read et al. introduced the gdh gene as a very suitable marker for genetic identification of *Giardia* genotypes (Read et al. 2004).

Among them RFLP is a test with high reliability, simple and fast which has the power to differentiate between genotypes using different genetic locus such as gdh and tpi genes (Abe et al. 2005) and also detects the presence of mixed genotypes (Saebi 1998). Regarding the above mentioned, in this study the gdh gene was used as a molecular marker and RFLP method for grouping *G. duodenalis* successfully.

According to the results of the studies, it seems that AI and BIII genotypes have more potential for zoonotic transmission than AII and BIV genotypes. Also, the first two genotypes have a wider range of hosts. These genotypes have different abundances in different areas, and this difference can be attributed to different populations studied, specific geographical locations and the relationship between human and animal (Thompson and Hunter 2005; Thompson 2004).

Various studies have shown different genotypes in animals, as in studies reported in Italy the AI genotype dominant in sheep, In the United States in sheep the dominant genotype was E and in one case the genotype was A, in Belgium, the dominant genotype in sheep and goats were E. Zare et al. For the first time identified the genotype of human *Giardia* isolates by PCR-RFLP method in Iran (Zare et al. 2002). In 2005, Castro-Hermida et al. examined the prevalence of *Giardia* by sampling 200 goats from 20 farms in France, the results showed that 38% of goats were infected with *Giardia* (Castro-Hermida et al. 2005). In 2007 Sant'In et al. examined 32 sheep in the United States for *Giardia* genotypes, the results showed that 4% were infected, The E genotype was dominant and 1 case was A genotype (Santin et al. 2007). Yang et al. In Australia in 2009 examined 447 sheep in 5 farms for *Giardia* genotypes, the results showed that 11.1% of sheep were infected. 36 cases of genotype E, 5 cases of genotype A and 11 cases of combination of genotype A and E (Yang et al. 2009). In 2016 Fantinatti et al. studying the genotypes of 44 children concluded that most of the samples were

related to the A genotype, strangely, genotype E was observed in 15 samples. And observing E introduces a new zeonototic pathway in humans. In 2017 Skhal et al. concluded that 40% of the specimens were genotype of *Giardia* in Damascus. 65% of the samples were A, 27.5% B and 7.5% E By studying the genotype of *Giardia* in Damascus on 40 samples, concluded that 65% of the samples were A genotype, 27.5% B and 7.5% mixture genotype of A and B.In this study, as in most similar studies, it was found that the dominant assemblage in the examined animals were the E assemblage, Also, the prevalence of AI genotype is higher than AII genotype, And, infections with mixed genotypes constitute a high percentage of infections.

In conclusion, it can be stated that the study of the frequency of *G.* duodenalis in livestock and other animals is important, because it enhances our understanding the means of transmission and prevention as well as the molecular epidemiology of the parasite, and the zoonotic aspect of the parasite is determined, And finally, it's important to answer questions about the health of people, especially children who are at risk of infection.

This study was able to determine the prevalence of different genotypes of *G.*duodenalis in Iran in sheep and goats. Separation of zoonosis genotypes of AI, AII and BIII shows that these animals can be considered as an important source of infection for humans. The study also recommended for use of the PCR-RFLP method by the gdh & tpi gene for the detection and differentiation of *Giardia*'s genetic groups and subtypes in a similarly designed study.

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Authors' contributions All authors contributed to the study design. ATK was leader of the research. AF, SS and NF carried out experimental tests and prepared the Manuscript. All authors read and approved the final version of the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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