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Molecular detection and genetic diversity of *Toxoplasma gondii* in different tissues of sheep and goat in Eastern Iran

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Abstract. This study was designed to detect parasitic DNA in tissues from sheep and goats raised and slaughtered in the southeastern Iran as well as to genetically characterize infecting strains of *T. gondii*. A total of 240 tissue samples consisting of heart, brain, and diaphragm were obtained from sheep (n=40) and goats (n=40) slaughtered in abattoirs from three provinces located in southeastern Iran including Kerman, Razavi Khorasan, and South Khorasan Provinces between February to October 2015. Nested PCR amplified the B1 and GRA6 genes. To determine the genetic characterization of positive samples, all genotyped positive samples were examined by PCR-RFLP. Sequencing analysis was performed to evaluate the prevalence of type strains (I, II and III). A total of 68(56.66%) tissue samples of sheep and 53(44.16%) from goats were found to be positive for *T. gondii* B1 gene, that included 11(27.5%) diaphragm, 21(52.5%) heart, and 36(90%) brain of sheep; and 20(50%) diaphragm, 11(22%) heart and 22(55%) brain of goats. Moreover, 22(18.3%) tissue samples of sheep and 20(16.6%) tissue samples of goats were found positive with GRA6 gene for *T. gondii*. There are three genotypes and mix genotype using *mseI* enzyme among all positive samples. The results demonstrated the presence of *T. gondii* DNA in tissues of sheep and goats from southeast of Iran. Control of *Toxoplasma* infection animal products are important in consumer protection.

INTRODUCTION

Toxoplasma gondii is an obligate intracellular protozoan parasite that infects humans and a broad spectrum of warm-blooded vertebrates (Hill & Dubey, 2002). Cats, both wild and domestic, are the only definitive hosts for *T. gondii*. A great variety of homeo thermic animals as well as humans act as intermediate hosts (Gangneux & Darde, 2012). Carnivores including human can become infected when they eat raw or undercooked tissues containing tissue cysts or occasionally tachyzoites (Hill & Dubey, 2002). Both herbivores and carnivores may ingest infective oocysts in food or water (Gangneux & Darde, 2012). *T. gondii* can cross the placenta in some species, particularly sheep, goats, humans and small rodents. Toxoplasmosis cause significant reproductive and economic problems in animals and have become a public health concern as consumption of contaminated meat and milk can damage human health (Sukthana, 2006; Dubey, 2009a).

According to the previous studies, prevalence of *T. gondii* in sheep and goats are considerable because of the continuous contamination of pastures by *T. gondii* oocysts and that makes this parasite a

common infectious agent among these animals (Dubey 2009b, Jittapalapong *et al.*, 2005). Epidemiological studies have demonstrated that the seroprevalence of toxoplasmosis is between 13-35% for sheep, 13- 30% for goats, 0-16% for cattle, 4-10% for buffaloes and 11.5% for horses in different parts of Iran (2006).

Several investigations with multilocus PCR-RFLP or microsatellite markers have demonstrated that *T. gondii* has a clonal population structure consisting of three genetic lineages i.e., Type I, Type II and Type III (Quan *et al.*, 2008). Reviews have shown a predominance of genotype II in domestic animals from various parts of the world including France, Spain, Switzerland and Germany (Dumètre *et al.*, 2006; Montoya *et al.*, 2008; Herrmann *et al.*, 2009; Berger-Schoch *et al.*, 2011). However, to the best of our knowledge and according to a survey of the literature there are few studies on genetic characterization of *T. gondii* isolates in domestic animals such as sheep and goats in Iran. Therefore, the present study was designed for detecting parasitic DNA in tissues from sheep and goats raised and slaughtered in the Eastern Iran as well as to genetically characterize infecting strains of *T. gondii*.

MATERIALS AND METHODS

Sample collection

A total of 240 tissue samples consisting of heart (n=80), brain (n=80), and diaphragm (n=80) were obtained from sheep (n=40) and goats (n=40) slaughtered in abattoirs from three provinces located in Eastern Iran including Kerman, Razavi Khorasan and South Khorasan Provinces between February to October 2015. All the animals examined were born and raised in southeastern Iran and were intended for human consumption. All tissue samples were stored at -20ºC until DNA extraction. This study was approved by the Kerman University of Medical Sciences Ethical Committee as complying with the Iran legislation for the protection of animals (No. 94/389).

DNA extraction

Tissue samples were digested in a trypsin solution for 2 hours at 37ºC. The pellets were stored at -20ºC until use. Genomic DNA was extracted from tissue specimens using Qiagen DNA extraction kit (Qiagen, Germany) according to the manufacturer's instructions. Extracted DNA samples were stored in -20ºC before using for molecular analysis.

B1 Nested-PCR

Nested-PCR was performed in a final volume of 25 µl using the Taq DNA Pol 2.0 × master mix with an $MgCl_2$ concentration adjusted to 1.5 mM; 100 ng/µL of DNA was used for the PCR assay. Each sample was tested in duplicate for each method. The nucleotide sequences of the primers for the nested-PCR assays targeting the B1 and GRA6 gene are listed in Table 1 [R. Chiabchalard *et al.*, 2005; Q.M. Kong *et al.*, 2012). The PCR master mixture consisted of 2.5 µl 10x PCR buffer, 1.5 mMol/lMgCl2, 200 µMol/l dNTP (Thermo Scientific, USA), 10 pMol of each primer, 0.5 U/µl Taq DNA polymerase (Thermo Scientific, USA), 2 µl DNA template and distilled water to adjust the final volumeto 25 µl. PCR amplification was performed in a FlexCycler (Analytik Jena, Germany). The amplification started with an initial denaturation at 94ºC for 5 min, followed by 30 cycles of 20 s at 94ºC, 20 s at 63ºC and 20 s at 72ºC and a final extension of 5 min at 72ºC. A PCR negative-control sample omitted template DNA, which was replaced by sterile water and a positive-control sample that used extracted DNA from *T. gondii* tachyzoites RH-strain. (Fallahi *et al.*, 2014a)

GRA6 Nested PCR

Nested PCR was done to amplify the coding region of the GRA6 gene. PCR amplification was performed with 1 µl of DNA template in 50 µl of a reaction mixture containing 10 mMTris-HCl, pH 8.3; 50 mMKCl; 2 mM MgCl2; 200 µM dNTPs; 0.5 µM each of oligonucleotide primers; 1.25 units of Taq DNA polymerase (Takara, Japan); and 50 pmol of each primer. All PCRs were performed in an automatic DNA thermal

Table 1. Primers for the nested-PCR assays targeting the B1 and Gra6 used for *T. gondii* molecular diagnosis

Assay	Oligonucleotide sequence	Nucleotide positions
B1-nested PCR	5'TCAAGCAGCGTATTGTCGAG	663–682
	5'CCGCAGCGACTTCTATCTCT	949–930
	5'GGAACTGCATCCGTTCATGAG	694–714
	5'-TCTTTAAAGCGTTCGTGGTC	887–868
GRA6- nested PCR	5'-GGCAAACAAAACGAAGTG-3'	223–240
	5'-CGACTACAAGACATAGAGTG-3'	1166–1147
	5'- GTAGCGTGCTTGTTGGCGAC-3'	372–391
	5'- TACAAGACATAGAGTGCCCC-3'	1162–1143

cycler (Model TP600 Takara). The first step of amplification was 5 min of denaturation at 94°C. This step was followed by 35 cycles, with 1 cycle consisting of 30 s at 94°C, 60 s at the annealing temperature for each pair of primers, and 90 s at 72°C. The final cycle was followed by an extension step of 7 min at 72°C. The PCR primer pair was designed from the GRA6 gene sequence (Lecordier *et al*., 1995), GRA6FO: 5'-GGCAAACAAAACGAAGTG-3' and 1106 S. GRA6RO: 5'-CGACTACAAGACA TAGAGTG-3' (positions 223–240 and 1166–1147, respectively), used in the initial PCR reaction at an annealing temperature of 54°C. The resulting amplification products were diluted to 1/10 in water, and a second amplification was performed with the internal primers described by Fazaeli *et al.* (Fazaeli A *et al*., 2000), GRA6R: 5'-GTAGCGTGCTT GTTGGCGAC-3' and GRA6; 5'-TACAAGA CATAGAGTGCCCC-3' (positions 372–391 and 1162–1143, respectively), using 1 µl of the diluted product as the template at an annealing temperature of 60°C. The PCR amplification is expected to yield 791 bp GRA6 positive reaction.

PCR-RFLP

The GRA6 amplification product was exposed to *MseI* enzyme for digestion. Briefly, 10ml of PCR product was digested using 1.5 U of *MseI* endonuclease and 2 U buffer R and incubated at 65°C for 4 hr in accordance with the manufacturer's protocol. The restriction fragments were separated by electrophoresis in 1.6% agarose gel followed by staining with ethidium bromide and visualization under UV. The cut position of *MseI* in GRA6 genes of types I, II and III was 168 bp and 712 bp, 71 bp and 694 bp and 71 bp, 168 bp and 712 bp, respectively (Danehchin *et al.*, 2016).

Sequencing

In the present study, sequence analysis for comparison of B1 and GRA6 genetic profiles was performed to determine type of *T. gondii* (I, II, III) isolates in comparison to RFLP method. Nested PCR products and internal primers sent to Macrogene Company of South Korea. Results were aligned with BioEdit and sequene Scanner program and compared to the following sequence data available from GeneBank: AB235428, AB235433, AF239283 RH type I, AF239284, AB235430 Beverley type II and AF239286, AB235429NED type III, (GenBank). The phylogenetic relationships among genotypes were estimated using Maximum-likelihood analysis. Mega 6 software was also used to construct the phylogeny tree to compare our isolates with types submitted in Genebank. *Hammondia hammondi* (GenBank accession numbers: XM 008888391) was employed as the outgroup to root the resulting trees.

Analysis of data

The results were analyzed by SPSS software (version 20), using Chi-square test and a P value <0.05 was considered as statistically significant.

RESULTS

Figures 1 and 2 indicate gel electrophoresis after PCR amplification with B1 and GRA6

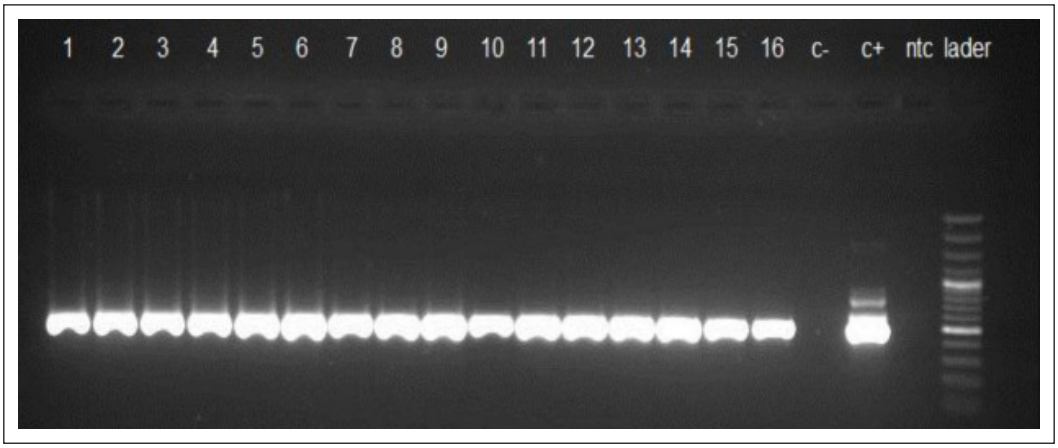

Figure 1. Gel electrophoresis after PCR amplification with B1 primers. Ladder with size marker of 100 bp; Lane 1-16, positive samples (529 bp); ntc, negative sample; C-, negative control; C+, positive control (529 bp).

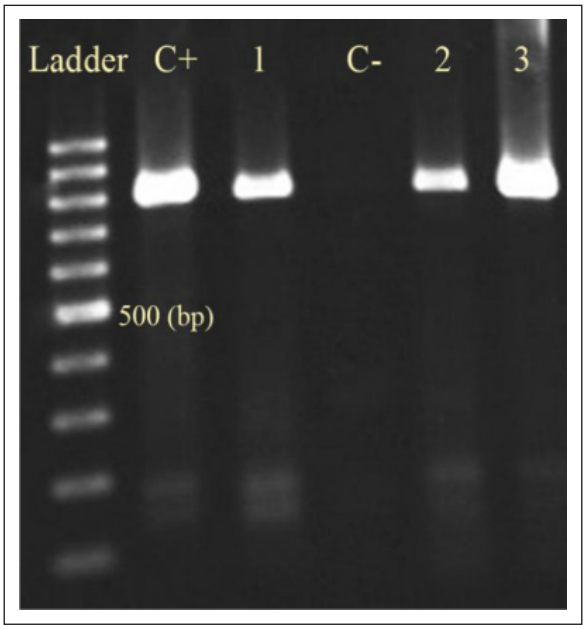

Figure 2. Gel electrophoresis after PCR amplification with GRA6 primers. Ladder with size marker of 100 bp; Lane 1-3, positive samples (791 bp); C-, negative control; C+, positive control (791 bp).

respectively. Table 2 shows PCR results of 240 tissues from sheep and goat samples. Totally, 68(56.66%) tissue samples of sheep and 53(44.16%) tissue samples of goats were found positive for the *T. gondii* B1 gene, including 11(27.5%) diaphragm, 21(52.5%) heart, and 36(90%) brain of sheep; and 20(50%) diaphragm, 11(22%) heart and 22(55%) brain of goats. As shown in Table 2, 22(18.3%) tissue samples of sheep and 20(16.6%) tissue samples of goats were found positive with GRA6 gene for *T. gondii*. The highest infected tissue was brain (72.5%, 58 out of 80 animals). A significant correlation

Table 2. PCR results in the 240 tissue samples including heart (n=80), brain (n=40), and diaphragm (n=80) obtained from sheep (n=40) and goats (n=40) slaughtered in abattoirs from Eastern Iran

		Sheep		Goat		Total	
		No.	%	No.	%	No.	%
B1 gene	Brain						
	Positive	36	90	22	55	58	72.5
	Negative	4	10	18	45	14	17.5
	Heart						
	Positive	21	52.5	11	27.5	32	40
	Negative	19	47.5	29	72.5	48	60
	Diaphragm						
	Positive	11	27.5	20	50	31	38.75
	Negative	29	72.5	20	50	49	61.25
GRA6 gene	Brain						
	Positive	17	42.5	15	37.5	32	40
	Negative	23	57.5	25	62.5	48	60
	Heart						
	Positive	3	7.5	1	2.5	4	5
	Negative	37	92.5	39	97.5	76	95
	Diaphragm						
	Positive	2	5	4	10	6	7.5
	Negative	38	95	36	90	74	92.5
Age	1-2 year	24	60	12	30	36	45
	2-4 year	8	20	15	37.5	23	28.75
	4-6 year	8	20	11	27.5	19	23.75
	6-8 year	0	0	2	5	2	2.5
Gender	Male	13	32.5	14	35	27	33.75
	Female	27	67.5	26	65	53	66.25

was found between the infection rate in brain and diaphragm tissues by B1 gene ($P<0.05$) and animal type, but it wasn't significant by GRA6 gene. Results also revealed that there was not asignificant association ($p>0.05$) between the infection rate in various tissues by B1 gene and age as well as sex among sheep and goats.

Genetic characterization

To determine the genetic characterization of isolates, genotyping of positive samples were examined by PCR-RFLP (Fig. 3). As shown in Table 3, there are three genotypes and mix genotype using *MseI* enzyme among all positive samples.

Sequencing and phylogenetic analysis

The phylogenetic analysis demonstrated that two samples 8a and 11 were identified similar to type RH; whereas, two sample 4a and 5a were found similar to type I but on another cluster. Three samples 5B, 7B and 16A were found similar to type II genotype with high similarity in sister clade (Fig. 4).

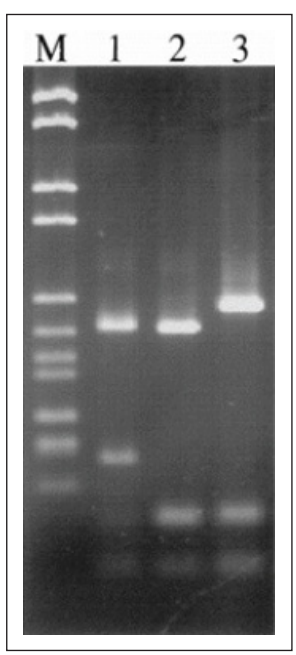

Figure 3. PCR-RFLP analysis of *GRA6* gene with *Mse*I endonuclease. Lane L, DNA size marker (between 2176 and 174 bp). Lanes 1–3 are *Toxoplasma gondii*, type I (RH), type III (C56), and type II, respectively.

Table 3. Genetic characterization of positive samples examined by PCR-RFLP

Animal	Genotype I / II, III No. (%)	Genotype II	Genotype III	Genotype II / III
Sheep	40 (33.3%)	6 (5%)	6 (5%)	16 (13.3%)
Goat	15 (18.78%)	10 (12.5%)	6 (7.5%)	–

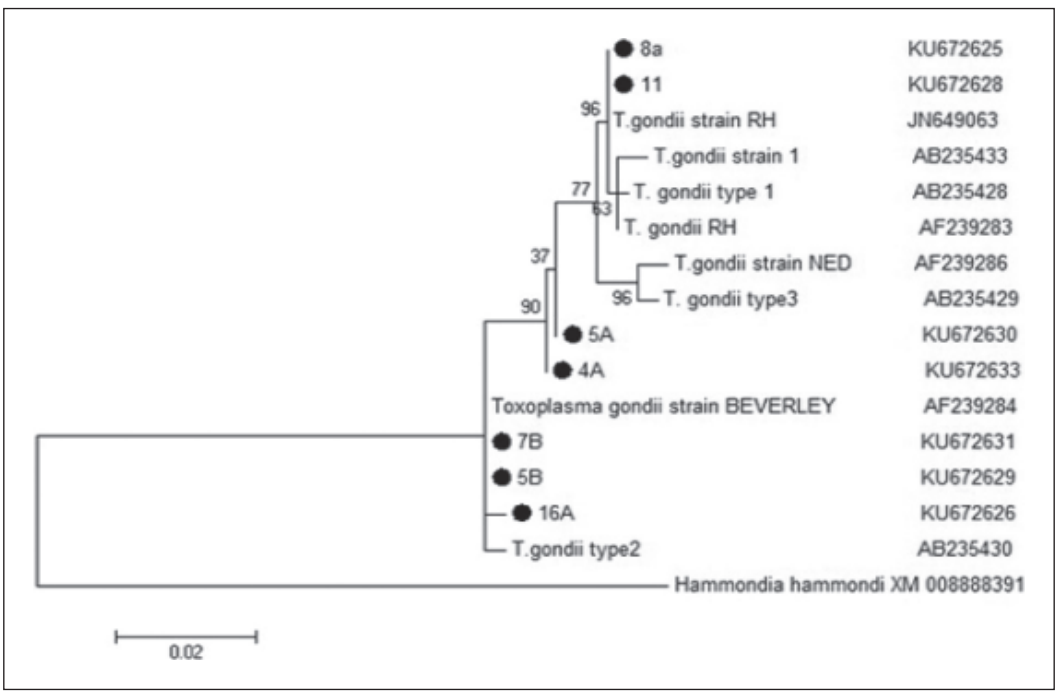

Figure 4. Molecular phylogenetic tree of *T. gondii* isolates in sheep and goats from Eastern Iran.

Representative nucleotide sequences obtained in this study were deposited in the GenBank database under the accession numbers: KU672625-31, KU672633-35, KU672637-41, KU672642-44, KU672646-52.

DISCUSSION

Sheep and goats are the main livestock in developing countries such as Iran, and their products including meat and milk are widely used as important sources of food for humans around the world (Dubey, 2009b). Normally, sheep and goats become infected with *T. gondii* by ingesting oocyts from food or water sources. Toxoplasmosis may cause serious complications including early embryonic death, abortion, stillbirth, and neonatal death. Moreover, toxoplasmosis in sheep and goat is a source of infection for humans and carnivorous animals (Sukthana, 2006). The prevalence of toxoplasmosis among animals was found to be 28.3% and 22.08% in sheep and goats respectively using PCR assay with B1 gene.

Tissue cysts have a high affinity for neural and muscular tissues. They are located predominantly in the central nervous system, as well as skeletal and cardiac muscle (Tenter, 2009). In our study, the highest rate of infection was found in brain followed by heart or diaphragm of animals. Also in consistent with the other studies, *Toxoplasma*

infection in various tissues was more frequent in sheep compared with goat (Esteban-Redondo, 1999).

According to previous studies the overall prevalence rate of toxoplasmosis in Iran was estimated to be 31.0% and 27.0% in sheep and goats, respectively (Sharif *et al*., 2015). Recently, Tavassoli M *et al.* (2013) reported that among 124 and 113 goats and sheep slaughtered in the Urmia, Northwest of Iran, PCR products of three samples (1.26%) taken from sheep were positive and for goats negative (Tavassoli M *et al*., 2013a). In the other study conducted by Tavassoli *et al.* (2013) demonstrated that among 625 examined milk samples (345 sheep and 280 goats), 16(4.63%) sheep and 3(1.07%) goat milk samples indicated a specific *T. gondii* B1 fragment (529 bp), of which specific *T. gondii* B1 fragment detected [Tavassoli *et al*., 2013b]. Khamesipour *et al.* demonstrated that the overall prevalence of *T. gondii* DNA in 95 sheep from Isfahan and Chaharmahal va Bakhtiary provinces was 17.9% (Khamesipour F *et al*., 2014).

In line with our results, Azizi *et al.* demonstrated that 38% studied sheep from Chaharmahal va Bakhtiary province, Central Iran were infected with *T. gondii*; they also indicated that the highest infection rate was found in brain (32.0%) (Azizi *et al*., 2014). Asgari *et al.* (2011) showed that in tissues of 56 sheep and 22 goats in Fars province, Iran, The total prevalence of *Toxoplasma* infection was found to be 33.3%. The highest infected tissue was tongue (21.8%) followed by brain (19.2%), femoral and intercostal muscles (17.9%) (Asgari *et al*., 2011). Reviews have reported that the nested PCR method provides the highest sensitivity and specificity for the detection of toxoplasmosis. In adition, B1 gene has been shown to have higher copies in genome of *T. gondii* and also high specificity and sensitivity and is the appropriate tool for detection of *T. gondii* (Jones *et al*., 2000, *et al*., 2014a, Fallahi *et al*., 2015). PCR-RFLP is an appropriate technique to identify the genotypes of *T. gondii* isolates using analysis of the pattern derived from the cleavage of its DNA (Su *et al*., 2006, Fallahi *et al*., 2014b). In a study by Zahra Arab *et al*. it was concluded that rGRA7 can be used as a potential immunogenic antigen for developing immunodiagnostic tools for immunodiagnosis of toxoplasmosis in patients including patients with cancer. (Arab-Mazar *et al*., 2016). Another study showed 12.6% seroprevalence of *Toxoplasma gondii* infection among childbearing aged women in Kerman city using ELIZA method. (Tavakoli *et al*., 2015). The results of our study showed three genotypes in all positive samples by using *MseI* enzymes. Similarly, Lopes *et al*. demonstrated three genotypes I, II and III of *T. gondii* in cattle, sheep, goats and swine from the North of Portugal (Lopes *et al*., 2015). Zia-Ali *et al*., 2007 had found type II and III in 4 isolates from sheep according to microsatellite and GRA6 gene sequence analysis in Iran (Zia-Ali *et al*., 2007). Moreover, some studies demonstrated that type I in sheep resulted in abortion of fetuses determined by using PCR-RFLP based on GRA6 gene in Iran (Habibi *et al*., 2012). In our study, the GRA6 sequencing and phylogenetic analysis confirmed two samples were identified similar to type RH and two samples for type I. Three samples were found similar to type II genotype. Since sheep and goats are the most important food sources in Iran, there may be a high risk of contamination through consumption of products from these hosts due to their susceptibility to the infection. Therefore control of the infection in food animals is important for consumer protection.

CONCLUSION

Findings of this study showed the presence of *Toxoplasma gondii* DNA in the tissues of sheep and goats from Eastern of Iran. The results confirmed that the combination of B1 and GRA6 genes nested PCR is more sensitive than B1 or Gra6 alone. Study also showed that using different tissues of hosts increasing the chance of detection in infected animals. Finally, for prevention of human infections, health standards in sheep breeding, education of environmental health workers, and standardization of preparation and handing of meats are required.

Conflict of interest

We declare that we have no conflict of interest.

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