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### Original Article

## Genetic Diversity of *Toxoplasma gondii* by Serological and Molecular Analyzes in Different Sheep and Goat Tissues in Northeastern Iran

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#### **Abstract**

**Background:** Toxoplasmosis is a parasitic disease caused by compilation protozoan agent *Toxoplasma gondii*, leading to significant financial and quality-adjusted life-year losses. Overcooked or raw meat consumption has been a considerable transmission route. The present study was conducted to determine the seropositivity rate of *T. gondii* in sheep and goats by serological and molecular tests and genotyping of obtained isolates in northeast Iran.

**Methods:** Blood and tissue samples (diaphragm, heart) of 296 animals (including 168 sheep and 128 goats) were collected from the slaughterhouse in Quchan Country from august 2016 to April 2017. Modified agglutination test (MAT) and the PCR method performed to detect parasite DNA on tissues. PCR-RFLP method of *GRA6* gene was used to determine the genotype of *T. gondii*. In addition, sequencing analysis was performed to evaluate the *Toxoplasma* type strains.

**Results:** Serum positive for MAT results were found in 27.4% of sheep and 23.4% of goats. Positive PCR of *B1* gene results in diaphragm and heart tissues of sheep and goats was 47.8% and 26.1%, 40% and 23.3%, respectively. PCR of *GRA6* gene results were positive in 10 samples that RFLP technique results using *MseI* enzyme revealed genotype I. Sequencing and phylogenetic analysis revealed DNA of all samples was closely related to *Toxoplasma* type I.

**Conclusion:** Concerning the high seropositivity rate of toxoplasmosis, undertaking an appropriate preventive program for reducing the prevalence of *T. gondii* infection by raw or undercooked meat consumption of livestock is recommended. Our study supports the notion that these animals' consumption of raw and undercooked meat can be a probable source of human toxoplasmosis.



## Introduction

*Toxoplasma gondii* is an obligate intracellular protozoan widely prevalent in humans and other animals (1,2). Felines, mainly cats, are definitive hosts in the life cycle of *T. gondii* and excrete millions of resistant oocysts after primary infection into the environment. Almost all warm-blooded plays a role in transmission cycle as intermediate hosts such as sheep, goat, cattle, pigs and camels or aberrant hosts as humans (3).

Even though most cases of human infection are asymptomatic or mild clinical symptoms, the parasite can cause severe complications such as encephalitis in congenitally infected children and immunocompromised individuals (4,5). Reactivations of latent infection in immunocompromised individuals can cause fatal toxoplasmic encephalitis, pneumonitis and myocarditis. Acquired infections during pregnancy are associated with severe damage to the fetus including stillbirths or abortions. Humans are usually infected by consuming undercooked meat containing tissue cysts or cyst contaminated water (6,7).

*T. gondii* is broadly spread among farm animals and humans. Overall assessed frequency is reported with variable seroprevalence rates of 75% in dogs, 11–36% in pigs, 11–61% in goats, less than 10% in cows, 35–73% in cats, and 35–73% in humans (8,9). In comparison, this value in Iran and humans has been reported 29% to 55% (10). In Khorasan Razavi Province, the seroprevalence rate of toxoplasmosis in sheep was found 15.5% (11). Also, another recent study in Sabzavar City in Khorasan Razavi Province revealed that 60% of sheep, 52.5% of goats and 65% of camels were infected by *T. gondii* (12).

According to the published data related to the census of animal husbandry in Iran, (<https://www.amar.org.ir/>), Khorasan Razavi Province is the most important provinces in livestock breeding, and Quchan City is the

animal husbandry hub of this province. Therefore, it is essential to evaluate the prevalence of *T. gondii* in livestock as humans' food. Lack of accurate and comprehensive data concerning to livestock prevalence of toxoplasmosis in this area is the main obstacle to control and design preventive plan. Therefore, the present study was conducted to survey the frequency of *T. gondii* in livestock meat (sheep and goats) by using Modified Agglutination Test (MAT) test and compare with PCR methods as well as to determine genetically diversity infecting strains of *T. gondii*.

## Materials and Methods

### *Ethics approval*

The study was approved by the Ethics Committee of the Kerman University of Medical Sciences in Iran (Ethical number: 94/389).

### *Study area*

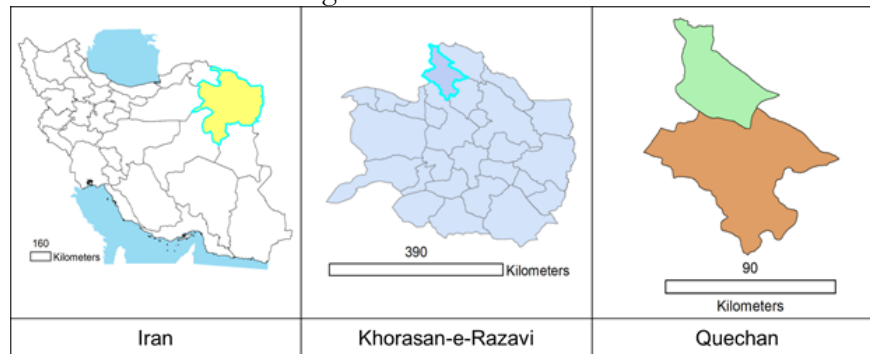
This Cross-Sectional study that was conducted in Quchan City in Khorasan Razavi Province in northeastern Iran. It has an area of about 523400 hectares and a population of over 180,000 peoples. The city is located in a mountainous area, elevation 1149 meters above sea level and has cold winters and mild summers. Its rainfall is 200-150mm/year and lies between 37.11° latitude and 58.51 ° E longitude. (Available at <https://www.worldatlas.com/as/ir/30/where-is-quchan.html>, <https://en.wikipedia.org/wiki/Quchan#Geography>). Fig.1 shows ArcGIS geographic location of Iran and Quechan.

### *Sample collection*

Based on statistical advice and previous studies, 296 animals including 168 sheep and 128 goats were sampled from the slaughterhouse in Quchan City for this study.

This descriptive cross-sectional study was implemented from August 2016 to April 2017 and samples were collected in four stages in

the spring, summer, autumn and winter seasons.



**Fig. 1:** The situation of Khorasan Razavi Province in Iran and location of study area in Quchan

According to the seasonal pattern, 65, 68, 104 and samples were collected in spring; summer, autumn in winter respectively. Livestock was numbered and randomly selected, whereas their blood samples (jugular vein) were obtained from numbered livestock. At the same time, age and gender were also recorded. We had from each livestock a blood sample, a heart sample and a diaphragm sample. Blood samples were centrifuged without anticoagulant tubes at 8000 rpm for 5-10 minutes and sera were transferred to 1.5 ml micro-tubes. Until to performing serological and molecular tests, all sera and tissue samples were stored at -20 degree.

### **Serological examination**

Sera of sheep and goats were examined for anti- *Toxoplasma gondii* antibodies by the modified agglutination test (MAT) (Toxo screen DA, bi-omerieux®, France) as described by Dubey and Desmonts(13).

### **DNA extraction**

All positive samples of MAT test were investigated by PCR assay on the heart and diaphragm the same animals. The DNA extraction was performed using (Gene All, Exgene, Cell SV mini, Korea) kit and according to the manufacturer's instruction.

### **Nested-PCR for B1 gene**

The Nested-PCR assays were accomplished using two repeated genomic targets, *B1* to detect *T. gondii* DNA in contaminated tissues (13). Two PCR primer pairs of the *B1* gene, *S1* (5'-CGACAGAAAGGGAGCAAGAG-3') and *AS1* (5'-ACGCTGTGTCTCCTCTAGGC-3'), *S2* (5'-TCTTCCCAGACGTGGATTTC-3') and *AS2* (5'-CTCGACAATACGCTGCTTGA-3'), eventually amplifying a 531 bp fragment were used. The first amplification was carried out in 20 µl of reaction mixture containing 1 µl of each primer (*S1* and *AS1*), 10 µl Master mix (Ampliqon Company, Denmark), 2 µl extracted DNA from heart or diaphragm samples and 6µl Distilled water sterilized. The first PCR was performed in a thermocycler (Flex Cycler) for initial denaturation at 94 C for 3 min, this step was followed by 35 cycles of denaturation at 94 degree for 30 s, annealing at 60 degree for 30 s, extension at 72 degree for 2 min and a final extension step at 30 degree for 1 min. The second amplification was performed in 20 µl reaction mixture. The first PCR product was diluted with a ratio of 1:40 to distilled water and then used as a template. Twenty-µl reaction mixture was containing 1 µl of each primer (*S2* and *AS2*), 8µl Master mix (Ampliqon Company, Denmark), 1 µl of our new template and 9µl distilled water sterilized. The second PCR was performed in 30 cycles.

### **Nested PCR for *GRA6* gene**

The positive samples of Nested-PCR of *B1* gene included in analyzing by Nested-PCR of *GRA6* gene. *GRA6*, a highly polymorphic gene is repeated in the genome of the *T. gondii*. This gene is suited to distinguish between three types I, II and III from each other, primarily type III that is close to type I. Two PCR primer pairs of the *GRA6* gene, *GRA6FO* (5'-GGCAAACAAAACGAAGTG-3') and *GRA6RO* (5'-CGACTACAAGACATAGAGTG-3') used in first amplification, and *GRA6R* (5'-GTAGCGTGCTTGTGGCGAC-3') and *GRA6* (5'-TACAAGACATAGAGTGCCCC-3') used in second amplification. The first amplification was carried out in 25 µl of reaction mixture containing 1 µl of each primer (*GRA6FO* and *GRA6RO*), 8 µl Master mix (Ampliqon Company, Denmark), 5 µl extracted DNA of heart or diaphragm samples and 10 µl Distilled water sterilized. The first PCR was performed in a thermocycler (Flex Cycler) for initial denaturation at 94 degree for 5 min, this step was followed by 35 cycles of denaturation at 94 degree for 30 s, annealing at 54 degree for 60 s, extension at 72 degree for 90 s and a final extension step at 72 degree for 7 min [14]. The second amplification was performed in 25 µl reaction mixture. The first PCR product used as a template while diluted with a ratio of 1:10 to distilled water. Twenty-five microlitres reaction mixture contained 1 µl of each primer (*GRA6R* and *GRA6*), 8 µl Master mix, 1 µl of our new template and 14 µl Distilled water sterilized.

The second PCR was performed at the annealing temperature of 60 degree for the 60s [15]. The PCR products were electrophoresed in a 1.5% agarose gel in tris-borate-EDTA 0.5X (TBE 0.5X) buffer and stained with ethidium bromide. To differentiate the three types (I, II, III) of *T. gondii*, all positive samples of Nested PCR for *GRA6* gene were used to performing PCR-RFLP technique.

### **PCR-RFLP Assay**

The *GRA6* gene amplified product was digested with *MseI* with *MseI* restriction

endonuclease (10 U/µl, 300 units), (Fermentas, Thermo Scientific, USA).

### **Sequencing**

The *GRA6* gene amplified product (with suitable quality in PCR-RFLP) was sent to MacroGen Company (South Korea) for sequence analysis and to obtain more accurate results from the genotype of the *T. gondii* (I, II, III). Outcomes were aligned with BioEdit and sequence Scanner program and compared to the following sequence data available from GenBank: [AJ635332](#), [AF239283](#), [AF239292](#) and [AF239284](#). The maximum-likelihood analysis was employed to estimate phylogenetic relationships among genotypes. Additionally, Mega6 and BioEdit software were used to construct the phylogeny tree to compare our collected isolates against types submitted in GenBank as well as to demonstrate homology of obtained sequences respectively.

### **Statistical Analysis**

Differences in *T. gondii* prevalence with variables such as season, sex and age were analyzed using Pearson Chi-square test and crosstab. Statistical analysis was performed using SPSS version 23 (IBM Corp., Armonk, NY, USA). The *P*-values less than 0.05 were considered statistically significant.

## **Results**

### **Serological, molecular and risk factor**

*T. gondii* antibodies (MAT titers  $\geq 1:20$ ) were found in 46 (27.4%) of 168 sheep and 30 (23.4%) of 128 goats (Table 1). The samples were assayed at dilution from 1:20 to 1:640 (Table 2). In addition, positive results were categorized at dilution of  $\geq 1:20$  based on the seasons, sex and age (Tables 3 and 4).

The comparison of collected seropositivity data and different seasonal patterns in sheep indicate significant differences ( $P = 0.002$ ), while there was no significant difference in goats. Our analyzed data showed a statistically significant difference between age associations and

seropositivity in sheep and goats ( $P$ -value = 0.001). In addition, the results of statistical analysis showed a significant difference between

sex associations with seropositive in sheep ( $P$ -value = 0.0001); whereas there was no significant difference in goat ( $P$ -value = 0.01).

**Table 1:** *Toxoplasma* infection in sheep and goats by MAT method at  $\geq 1:20$  dilution

<i>Animal</i>	<i>MAT</i>		<i>Total</i>	<i>Percent (%)</i>
	Positive	Negative		
Sheep	46	122	168	27.4
Goats	30	98	128	23.4

**Table 2:** *Toxoplasma* infection in sheep and goats by MAT at 1:20 to 1:640 dilution

<i>Variable</i>	<i>Sheep</i>	<i>Goats</i>
1:20	18	8
1:40	13	10
1:80	9	8
1:160	4	3
1:320	1	-
1:640	1	1
Total	46	30

**Table 3:** *Toxoplasma* infection in sheep and goats by MAT method at  $\geq 1:20$  dilution based on seasons and sex

<i>Variable</i>	<i>Season</i>				<i>Sex</i>		
	Spring	Summer	Autumn	Winter	Male	Female	
Sheep	Positive	5	8	17	16	20	26
	Negative	40	32	38	12	88	34
	Total	45	40	55	28	108	60
	Seroprevalence (%)	11.1	20	30.9	57.1	18.5	43.3
Goats	Positive	2	5	13	10	15	15
	Negative	18	23	36	21	65	33
	Total	20	28	49	31	80	48
	Seroprevalence (%)	10	17.9	26.5	32.3	18.8	31.3

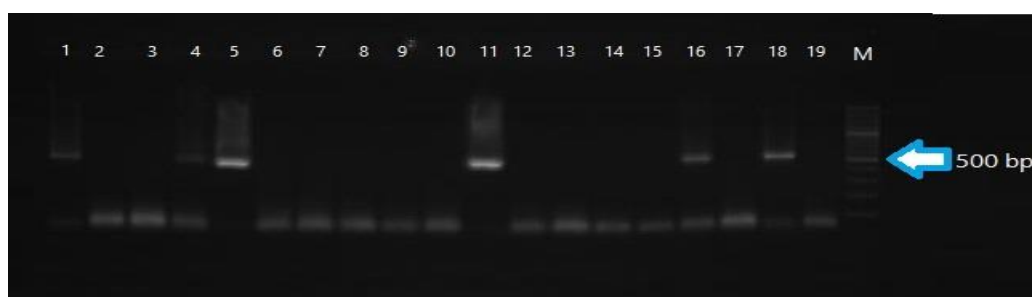
**Table 4:** *Toxoplasma* infection in sheep and goats by MAT method at  $\geq 1:20$  dilution based on ages

<i>Age(yr)</i>	<i>Sheep</i>			<i>Goat</i>		
	<i>MAT Positive</i>	<i>Negative</i>	<i>Percent (%)</i>	<i>MAT Positive</i>	<i>Negative</i>	<i>Percent (%)</i>
<1	1	24	4	1	32	3
1-3	15	59	20.3	13	44	22.8
3-5	21	32	39.6	8	13	38.1
>5	9	7	56.3	8	9	47.1

Total	46	122	27.4	30	98	23.4
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**Table 5:** Results of Nested-PCR for B1 gene on the heart and diaphragm tissues that their MAT results at  $\geq 1:20$  dilution were positive

Animal	No.	Tissue	No. tissue	No. positive samples at Nested-PCR with using B1 gene	Percent (%) infection in each tissue	No. infection in each animal at Nested-PCR with using B1 gene	Percent(%) in each animal
Sheep	46	Diaphragm	46	22	47.8	27	58.7
		Heart	46	12	26.1		
Goats	30	Diaphragm	30	12	40	16	53.3
		Heart	30	7	23.3		



**Fig. 2:** Electrophoretic pattern of the PCR products of B1 gene (531bp) from tissue samples. Lines 1-6: diaphragm tissue of sheep, lanes 7-11: heart tissues of sheep, lanes 12-16: diaphragm tissues of goats, lanes 17&18: heart tissues of goats, lane 18: positive control, lane 19: negative control, lane M: DNA marker

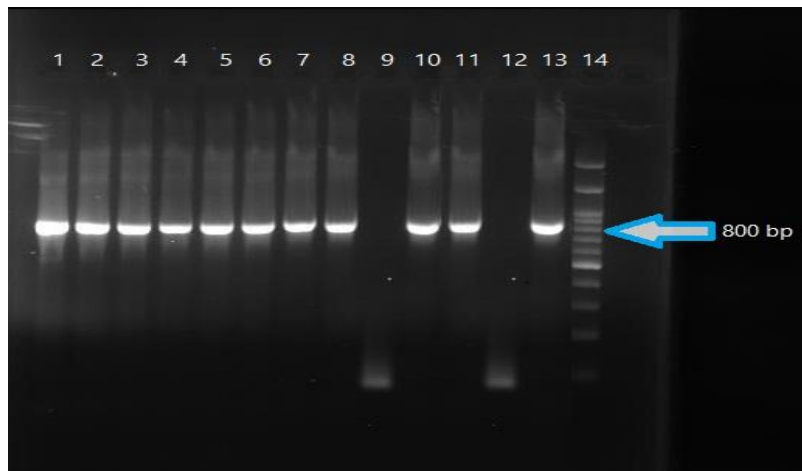
Table 5 and Fig. 2 depict the results of Nested-PCR for *B1* gene on tissue samples of the same animals with previously positive MAT reports. The analyzed data indicated a significant difference between serum dilutions and positive results of Nested-PCR for *B1* gene in sheep and goats ( $P$ -value  $< 0.05$ ). Table 6 and Fig.3 demonstrate the related results about Nested-PCR for *GRA6* gene that was performed on positive samples of Nested-PCR of *B1* gene. Fig.

4 exhibits the results PCR-RFLP technique (to determine the genotypes of *T. gondii*) that carry out on positive samples of Nested-PCR for *GRA6* gene.

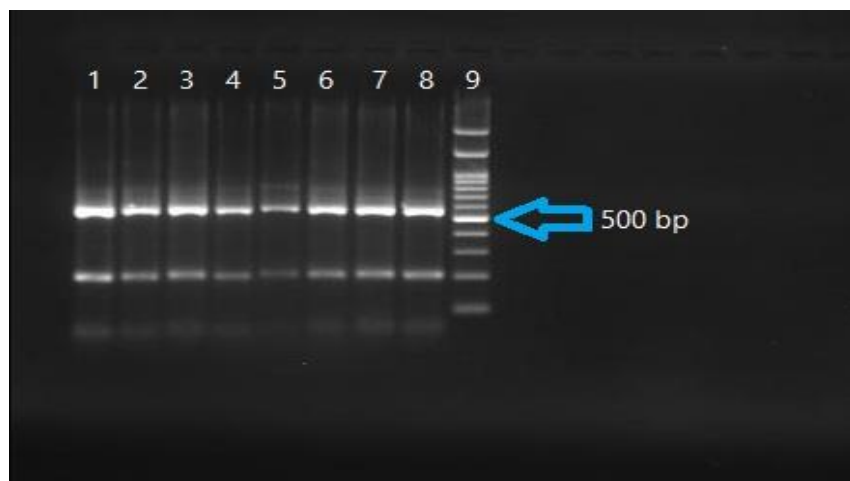
Additionally, our analyzed results represent a significant difference between dilutions of serums and positive results of Nested-PCR for *GRA6* gene in sheep and goats ( $P$ -value  $=0.02$ ).

**Table 6:** Nested-PCR results for *GRA6* gene on the positive samples of Nested-PCR of *B1* gene

Animal	No.	Tissue	No. tissue	No. positive samples at Nested-PCR with using <i>GRA6</i> gene
Sheep	27	Diaphragm	22	6
		Heart	12	1
Goats	16	Diaphragm	12	3
		Heart	7	-



**Fig. 3:** Electrophoretic pattern of the PCR products of GRA6 gene from tissue samples. Lanes 1-6: diaphragm tissue of sheep, lane 7: heart tissues of sheep, lanes 8-11: diaphragm tissues of goats, lane 12: negative control, lane 13: positive control, lane M: DNA marker



**Fig. 4:** PCR-RFLP analysis of GRA6 gene coding region with MseI endonuclease. Lane 9 is DNA marker, Lanes 1-8 are *Toxoplasma gondii*, type I (RH)

#### Sequencing and phylogenetic analysis

As shown in Fig.5 phylogenetic analysis of 10 sequenced products confirmed that all isolates belonged to type1 with high similarity in sister clade and their sequences are available (Fig. 6).

in GenBank with accession numbers: MG976038 to MG976047. Homology of identified sequence compared with gene-bank sequences

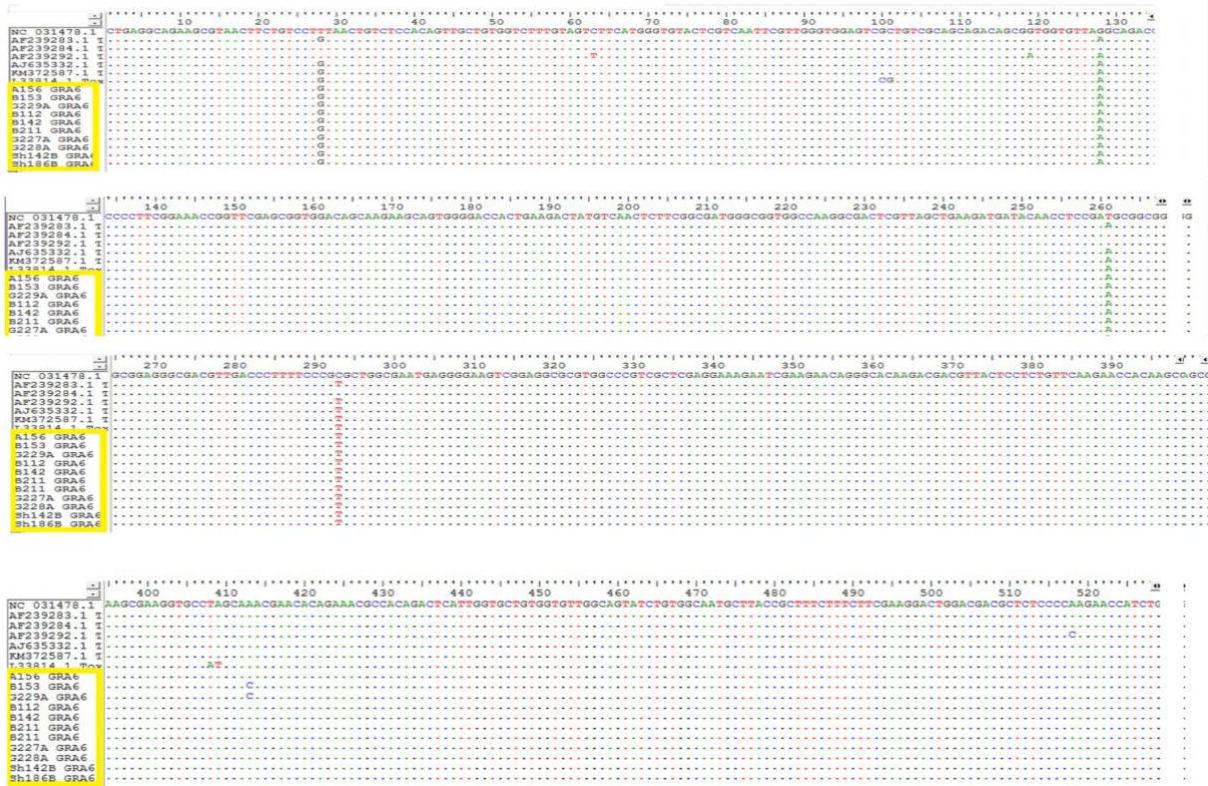


Fig. 5: The comparison of sequencing of GRA6 gene isolated from sheep and goats with gene bank sequences

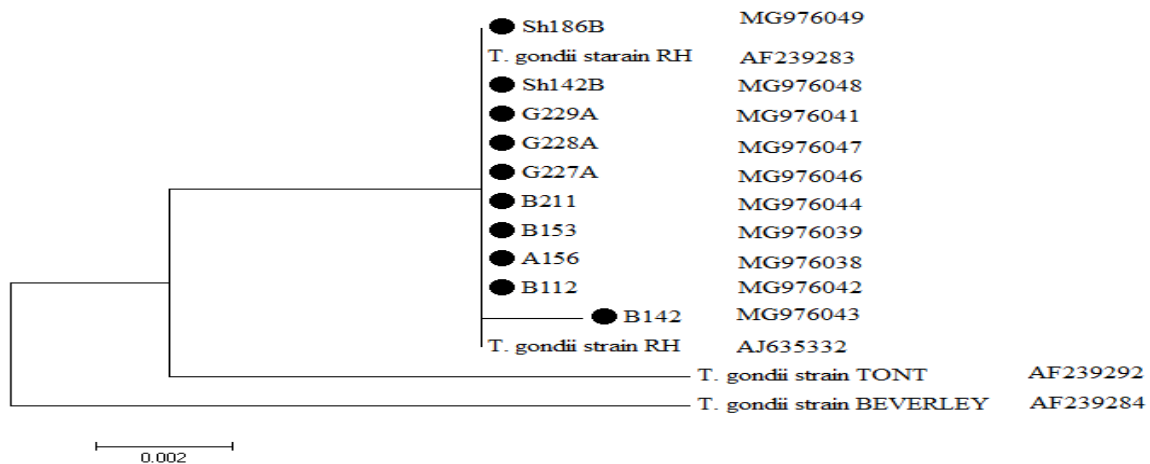


Fig. 6: The phylogenetic tree was constructed by maximum likelihood method using the nucleotide sequence of reference strains and our isolates (indicated with colorful shapes behind them). The scale bar indicates a 2% nucleotide difference



## Discussion

This study was conducted to determine the seropositivity and molecular detection of *T. gondii* in sheep and goats in northeastern Iran. Goats and sheep are the most important livestock in societies such as Iran and their products consider as main food sources for humans. Despite some progress in diagnosing and treating toxoplasmosis, the disease remains a significant public health and economic loss.

Based on a previous comprehensive study, maximum and minimum worldwide seropositivity of sheep was reported 4.4% (in China), 99.2% (in France) and the same result for goats was founded in ranged from 3.7 to 81% (16). In contrast, these values in Iran for sheep and goats were recorded between 13-35% and 13-30%, respectively [17].

Our study revealed that the seropositivity of *Toxoplasma* in sheep and goats was 27.4% and 23.4%, respectively. These values are in agreement with finding in various area of Iran including 18.8% and 29.5% in Fars province, 24.7% and 15.8% in Kerman province for sheep and goats respectively; as well as 21.1 % of sheep in Urmia (18–20). Comparing our findings with previous studies from other parts revealed more or fewer similarities/differences. For instance, in two studies of China serologic evidence of infection was found in 12.71% of sheep and 20.3% of goats (24,25). Additionally, in an effort by Dubey et al. to estimate seropositivity of 234 goats in USA, it was shown 53.4% of goats were positive (13).

It is well known that discrepancies in overall seropositivity results for toxoplasmosis in animals may be attributed to kind of used serological test, sample size, ecological status, management and hygienic standards and other factors. Generally, MAT was selected as a first choice method to evaluate seropositivity of *Toxoplasma* in animals, whereas in several studies molecular methods were used to determine the rate of toxoplasmosis in animals (18).

In this regard, our molecular results *via B1* gene shows 58.7% and 53.3% of studied sheep and goats were infected with *T. gondii* and this is in agreement with the finding of 56.66% and 44.16 % for sheep and goats in Kerman province respectively (23). In line with our finding in Iran, 38% of studied sheep in Chaharmahal va Bakhtiary province were infected with *Toxoplasma* (18). As well as similar results were taken in the Fars Province in the investigation of 56 sheep and 22 goats tissue samples with total molecular prevalence of *Toxoplasma* was 33.3% (24).

Our molecular prevalence data are comparable with other worldwide reports; for example in Tunisia, 33.3% and 32.5% of *B1* gene Nested PCR tests were positive for ewes and goats, respectively (25). Routinely, genotyping as a crucial determinant for pathogenesis and virulence of *Toxoplasma* was performed in recent studies. There was a correlation between of *T. gondii* genotype and clinical symptoms and pathogenesis profile of the parasite (26,27). Besides, genotype I known as acutely virulent, whereas genotype II and III are significantly less virulent that can establish latent toxoplasmosis (28).

In our study, the Nested-PCR of *GRA6* gene for 10 tissue samples (7 sheep and 3 goats) was positive and RFLP technique approved all of them cluster into genotype I. These results are in agreement with two previous surveys of different parts of Iran (29,30). Predominant genotype in animal especially ruminants in Iran is genotype II; Whereas prior European investigation demonstrated the most predominant genotype belongs to type III (31). In our study, variables analysis in sheep and goats showed that infection rate of toxoplasmosis increased with age. This age-related variation can be because older animals have been exposed to risk factors for a longer time. In addition, infection rate in sheep is higher in dry than wet seasons. Likewise, female sheep has more chance of acquiring toxoplasmosis than male. Infection occurs more often in the wet

season, and since IgG antibodies can persist a long time, so the high infection in dry season might be due to the carry-over effect from preceding wet season infections. Analysis of age and sex variables showed that seropositivity was higher in adults than younger animals and also higher in females than males (8).

The finding related to influence of age and seasons on seropositivity was verified by parallel researches (32,33). Collectively, discovering series factors such as seropositivity of toxoplasmosis, the biological properties of parasite and other risk factors in sheep and goats as main human foods are tied to establish efficient prevention and control health programs against toxoplasmosis.

## Conclusion

This study can serve as a road map and valuable information source to clarify the quantitative risk assessment of toxoplasmosis in humans as a foodborne disease. The presence of *T. gondii* DNA in the tissues of sheep and goats from northeastern Iran implicates that meat consumption might pose the risk of human infection. This investigation depicts new perspectives about the genotyping map of *T. gondii*, which is an indispensable factor for evaluation of meaningful control and prevention strategies.

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## Conflict of Interest

The authors declare that there is no conflict of interests.

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