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Molecular identification and genotyping of *Toxoplasma gondii* isolated from sheep and cattle in northern Iran

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Abstract

Toxoplasmosis, a foodborne disease, in human occurs commonly after the ingestion of tissue cysts via the raw and/or undercooked meat of different infected intermediate hosts such as sheep and cattle. The current study aimed to detect the genetic structure of *Toxoplasma gondii* isolated from various organs of sheep and cattle in the north of Iran. Conventional PCR was carried out by B1 and REP-529 genes of *T. gondii*. Nested and RFLP-PCR were performed for all positive samples using SAG2 and GRA6 genetic markers. Amplicons from second round of nested-PCR were sequenced and analyzed with NCBI database. Among of 179 examined samples, 38(21.20%) were positive. The highest of positive cases were found in kidney (28.60%). PCR-RFLP of SAG2 and GRA6 genes demonstrated the alleles of clonal type III in the all of isolates. Sequence analysis of the amplicons revealed the alleles of clonal type III and atypical isolates (Tg-67, Tg-100 and Tg-106). Phylogenetic analyses showed separate clade for the atypical isolates from others in the present study and the reference strains clades. In conclusion, the genetic characterization of *T. gondii* isolates from sheep and cattle showed high genetic diversity compared with standard type I, II and III genotypes. These results support the hypothesis of the existence of polymorphic and overlapping strains within livestock in Iran. It also suggested the necessity of increased genotyping and sampling efforts to accurately estimate *T. gondii* intra specific genetic diversity

Introduction

Toxoplasma gondii is an obligate intracellular coccidian parasite able to infect humans and all warm blooded animals. *Toxoplasma* infection may lead to severe medical problems such as cerebral and ocular damage in newborns through congenital transmission and in immune-compromised individuals via acquired infection. Several risk factors influence the prevalence of toxoplasmosis in humans including age, geographical location, nutritional habits, and keeping of hygienic standards.[1] This infection has been considered as a foodborne disease since humans become infected by the ingestion tissue cysts via the raw meat of intermediate hosts and/or through the ingestion of water or food contaminated by oocysts released from the definitive hosts.[2,3] However, alive *T. gondii* parasites have been isolated from fresh meat, meat products and different organs of herbivorous animals and therefore they are regarded as one of the important risk factors of *T. gondii* infection for human.[4,5] The global seroprevalence rate of *T. gondii* in human is between 30.00% and 50.00% depending on geographic and climatic factors, socio-economic status, cultural habits and the nutritional customs.[6] In Iran, the prevalence of latent toxoplasmosis in human has been estimated 40.00%,[7] with high frequency in northern regions.[8] The global seroprevalence of *T. gondii* infection in sheep and cattle flocks have been reported up to 100% and 28.00%, respectively.[9] Moreover, *T. gondii* infection was 31.00% in sheep and 18.10% in cattle from different regions of Iran.[10, 11]

There is one species in *Toxoplasma* genus which has a clonal population structure. The most isolated *T. gondii* throughout the world, particularly in Europe and north America, are classified into three clonal lineages (type I - III).[12-14] Other clonal lineages were also found and characterized based on geographic origins which differ from the common three lineages and known as unique genotype or atypical strains.[14,15]

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The significances of *T. gondii* infection is related to the host species and parasite genotypes. Generally, type I lineages are uniformly lethal in mice, but the type II and III genotypes are significantly less virulent. The disease signs vary widely in humans ranging from asymptomatic to severe acute toxoplasmosis. Type II genotype is the predominant lineage found in humans.[16] The proportion of predominant genotypes of this parasite is different in sheep and cattle in global scale. The genotypes II and III is found in different regions of Iran.[17-19] In addition, one study performed in East Azerbaijan Province reported that all isolates belonged to type I.[20]

The genetic diversity of *Toxoplasma* isolates are not well understood in Iran. Therefore, this study was conducted to find the genetic structure of *T. gondii* isolated from various organs of sheep and cattle in the north of Iran.

Materials and Methods

Sample collection. A total of 179 independent samples including skeletal muscle tissue (n = 70), heart (n = 22), liver (n = 33), kidney (n = 21) and brain (n = 33) were randomly collected from butchery and slaughterhouse in different parts of Mazandaran province, northern Iran, during July to December 2019. Samples were kept at – 20.00 ˚C until laboratory experiments were performed.

DNA extraction and amplification. Total DNA was extracted from the specimens, tachyzoites and tissue cysts by PCRBIO Rapid extract PCR kit (PCR Biosystems, London, UK) according to manufacturer's instructions. Conventional PCR was carried out for all extracted DNA using specific primers to amplify 321 bp fragment of B1 (forward, 5' ATAGGTTGCAGTCACTGACG 3' and reverse, 5' CTCCTCTTC GCGAAACCTCA 3') and 182 bp fragment of Rep-529 genes (forward, 5' TGTGCTTGGAGCCACAGAAG 3' and reverse, 5' GCAGCCAAGCCGGAAACAT 3') of the parasite in order to screen the collected samples. The primer pairs and PCR temperature cycling conditions were in according to a previous study.[21] PCR was performed with denaturation at 94.00 ˚C for 5 min, followed by 35 cycles of denaturation at 94.00 ˚C for 30 sec annealing at 59.00 ˚C for 30 sec, and extension at 72.00 ˚C for 30 sec. The amplification was carried out with final volume of 20.00 µL, consisting of 2.00 µL of each primer (10.00 pmol of each primer), 5.00 µL of distilled water, 10.00 µL of Taq DNA polymerase 2X Master Mix (2.00 mM $MgCl_2$; PCR Biosystems), and 3.00 µL of diluted DNA (100 ng genomic DNA).

PCR-RFLP of SAG2 and GRA6. The positive samples were analyzed at the 5' and 3'ends of 5'-SAG2; and GRA6 genes by nested-PCR approach. The 5' end of the loci were amplified by standard PCR for 40 cycles with the external primers pairs of SAG2 [forward, 5' GGAACGCGAACAATGA GTTT 3'; reverse, 5' GCACTGTTGTCCAGGGTTTT 3' (729bp fragment)] and GRA6 [forward, ATTTGTGTTTCCGAGCA GGT 5' 3'reverse, 5' GCACCTTCGCTTGTGGTT 3'; (546 bp fragment)] at an annealing temperature of 58.00 ˚C and 56.00 ˚C, respectively.[16] The resulting amplification products were diluted 1:2 in water, and a second amplification was performed with the internal primers [5'-SAG2: forward, 5' GAAATGTTTCAGGTTGCTGC 3'; reverse, 5' GCAAGAGCGAACTTGAACAC3' (242 bp)] and [GRA6: forward, 5' TTTCCGAGCAGGTGACCT 3'; reverse, 5' TCGC CGAAGAGTTGACATAG 3' (344bp)] of the abovementioned genes of *T. gondii*. The target DNA was amplified using the following conditions: one cycle of 4 min initial denaturation at 95.00 ˚C followed by 40 cycles for 30 sec, 56.00 ˚C for 1 min, 72.00 ˚C for 2 min, and was ended by 1 cycle of extension at 72.00 ˚C for 7 min. In each amplification, the final volume was 20.00 µL, consisting of 2.00 µL of each primer (10.00 pmol of each primer), 5.00 µL of distilled water, 10.00 µL of Taq DNA polymerase 2X Master Mix white (2.00 mM $MgCl_2$, Amplicon, Odense, Denmark), and 3.00 µL of diluted DNA (100 ng genomic DNA). The *T. gondii* RH strain DNA was used as positive control, while the negative control was the nuclease-free water.[16] The positive nested-PCR products were digested using MboI restriction enzymes (Jena Bioscienc, Jena, Germany) for 5´-SAG2 genes and the Tru1I (MseI) restriction enzyme (Thermo Fisher Scientific, Waltham, USA) for GRA6 gene. 10.00 µL of the nested-PCR products were digested using 1.00 U of restriction enzymes, 2.00 µL of Universal in a total volume of 30.00 µL. The restriction digestions were performed at 60.00 ˚C for 1 hr for MboI and at 37.00 ˚C for 30 min for MseI. All digestion procedures were performed according to the manufacturer's instructions. The digestion products were analyzed on a 2.00% agarose gel and assessed by UV light using a gel documentation system (Vilbert, Lourmat, France). The isolates were assembled based on their RFLP patterns, and a typical of each group was selected for sequencing. In all PCR analysis, *T. gondii* RH and Tehran strains were used as positive controls and distilled Nuclease-free water was applied as negative control.

DNA sequencing and phylogeny analysis. Direct sequencing of the partial genomes was carried out by an ABI 3500 automated sequencer (Pishgam Biotech. Co., Tehran, Iran) using the same forward and reverse primer as used for the PCR reaction. Approximately, all sequences were condensed faintly at both ends and thus the majority of sequences started and ended at the same homologous nucleotide positions. The sequences were analyzed using Mega Software (version 6.0; Biodesign Institute, Tempe, USA) and subjected to BLAST (http://www.blast.ncbi.nlm.nih.gov). A multiple sequence align-ment was produced with a gap opening penalty of 10 and a gap extension penalty of 1 for the pair wise and multiple alignments, in that order. The Mega Software was also used to perform phylogenetic analysis using maximum likelihood method and bootstrap of 1,000 replicates. *Sarcocystis cruzi* was used as outgroup to anchor the tree.

Results

Of 179 samples, 59 were belonged to cattle and 120 were from sheep. The *T. gondii* DNA was detected in 38 (21.20%) samples either by PCR analysis of B1 gene or 529-gene (Table 1).

The successful amplification of 5´-SAG2 gene was observed in 20 out of 38 positive DNA samples. GRA6 was amplified in 21 out of 38 isolates. PCR-RFLP of 5´-SAG2 and GRA6 genes identified all the positive samples as *T. gondii* type III (Fig. 1). Direct sequencing of the PCR product of 10 samples was also performed for each gene. Blast analysis of the partial sequence of SAG2 gene of the isolates showed 99.00 - 100% homology with *T. gondii* type III reference sequences in GenBank® (MG588010 and MH704613). Multiple sequence alignment showed 5 nucleotide substitutions in our isolates. Analysis of the amino acid sequence showed that two nucleotide substitutions were synonymous.

Analysis of the partial sequence of GRA6 gene of the isolates showed 99.30% homology with a genotype III isolate from Spain (TgShSp24, MT370490), in all cases except for the Tg-67, Tg-100 and Tg-106. An obvious polymorphism was found for the isolates Tg-67, Tg-100 and Tg-106 which had 84.90% homology with TgShSp24 isolate (MT370490) while had 84.20 - 86.50% homology with *T. gondii* strains NED, VEG, and C5, (AF239286, JX044209-10) and 83.90 - 86.10% with Me49, Beverley and PRU strains (AF239284-5, JX044216) as well as 83.90% homology with RH strain (AF239283). Multiple sequence alignment revealed 45 nucleotide substitutions in our isolates. Deletion did not occur in 7 substitutions for the isolates Tg-67, Tg-100 and Tg-106 compared to previously described (Fig. 2A). Analysis of the amino acid sequence showed that 29 nucleotide substitutions change were synonymous. Of 29 amino acid changes, 24 were found similar for the all the 3 isolates (Tg-67, Tg-100 and Tg-106) and four of them were observed only for isolate Tg-100.

Table 1. Summarized data on *Toxoplasma*-positive tissue samples.

No.	Origin	Host	PCR+	GRA6+	SAG2+	Genotype MSe	Genotype MboI	Sequencing GRA6	Sequencing SAG2	Accession No.
16	Heart	Sheep	+	+	+	III	III	III	III	MT926078, MT926073
21	Kidney	Sheep	+	+	+	III	III	-	-	-
23	Heart	Sheep	+	+	+	III	III	III	III	-
26	Heart	Sheep	+	+	+	III	III	-	-	
27	Kidney	Sheep	+	+	+	III	III	III	III	-
36	Kidney	Sheep	+	-	-	-	-	-	-	
40	Kidney	Sheep	+	+	+	III	III	-	-	-
44	Heart	Cattle	+	-	-	-	-	-	-	-
45	Kidney	Cattle	+	-	-			-	-	-
49	Kidney	Cattle	+	+	+	III	III	-	-	-
55	Liver	Cattle	+	+	+	III	III	III	III	-
67	Muscle	Cattle	+	+	+	III	III	III	III	MT926079, MT926074
72	Muscle	Cattle	+	+	+	III	III	-	-	-
83	Muscle	Sheep	+	-	-	-	-	-	-	-
92	Brain	Sheep	+	-	+		III	-	-	-
96	Brain	Sheep	+	+	+	III	III	-	-	-
97	Brain	Sheep	+	+	+	III	III	III	III	-
100	Brain	Sheep	+	+	+	III	III	III	III	MT926080, MT926075
102	Brain	Cattle	+	-	-					
106	Muscle	Sheep	+	+	+	III	III	III	III	MT926076
107	Muscle	Sheep	+	-	-	-	-	-	-	-
108	Muscle	Sheep	+	-	-	-	-	-	-	-
110	Muscle	Sheep	+	+	+	III	III	-	-	-
111	Muscle	Sheep	+	-	-	-	-	-	-	-
128	Muscle	Cattle	+	-	-	-	-	-	-	-
131	Brain	Sheep	+	-	-	-	-	-	-	-
136	Muscle	Sheep	+	-	-	-	-	-	-	-
137	Muscle	Cattle	+	+	+	III	III	-	-	-
139	Muscle	Sheep	+	-	-	-	-	-	-	-
140	Muscle	Cattle	+	+	+	III	III	III	III	
143	Brain	Sheep	+	+	+	III	III	-	-	-
144	Muscle	Sheep	+	-	-	-	-	-	-	-
151	Muscle	Sheep	+	+	+	III	III	-	-	-
154	Muscle	Sheep	+	-	-	-	-	-	-	-
155	Muscle	Sheep	+	+	+	III	III	III	III	MT926081, MT926077
170	Heart	Cattle	+	-	-	-	-	-	-	-
171	Heart	Cattle	+	-	-	-	-	-	-	-
173	Muscle	Sheep	+	-	-	-	-	-	-	-

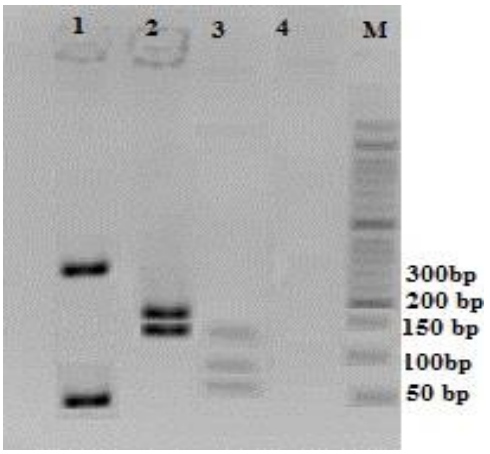

Fig. 1. PCR-RFLP analysis of GRA6 gene coding region with MseI endonuclease. Lane M: DNA size marker VI (between 2176 and 174 bp). Lanes 1-3: *Toxoplasma gondii*, type I (RH), type III (C56), and type II (BEVERLEY), respectively, lane 4: negative control.

Only one amino acid alteration was observed for isolates Tg-16, Tg-27, Tg-140 and Tg-155. Furthermore, the most frequent amino acid changes were observed for proline (alanine, isoleucine, leucine, phenylalanine and valine at position 2, 6, 24, 30 and 38 replaced by proline, respectively), (Fig. 2B).

The nucleotide sequences of 5´-SAG2 gene of the isolates in the current study were deposited in the GenBank® database under accession numbers MT92607881. The accession numbers for GRA6nucleotide sequences were MT926073-77.

Furthermore, phylogenetic tree was created based on the partial GRA6 and SAG2 sequences of our isolates, in addition to those from the clonal reference strains included. Predictably, Tg-67, Tg-100 and Tg-106 isolates formed a separate clade from other isolates in the present study and the reference strains (the type III alleles) as obtained by GRA6 sequences (Fig. 3).

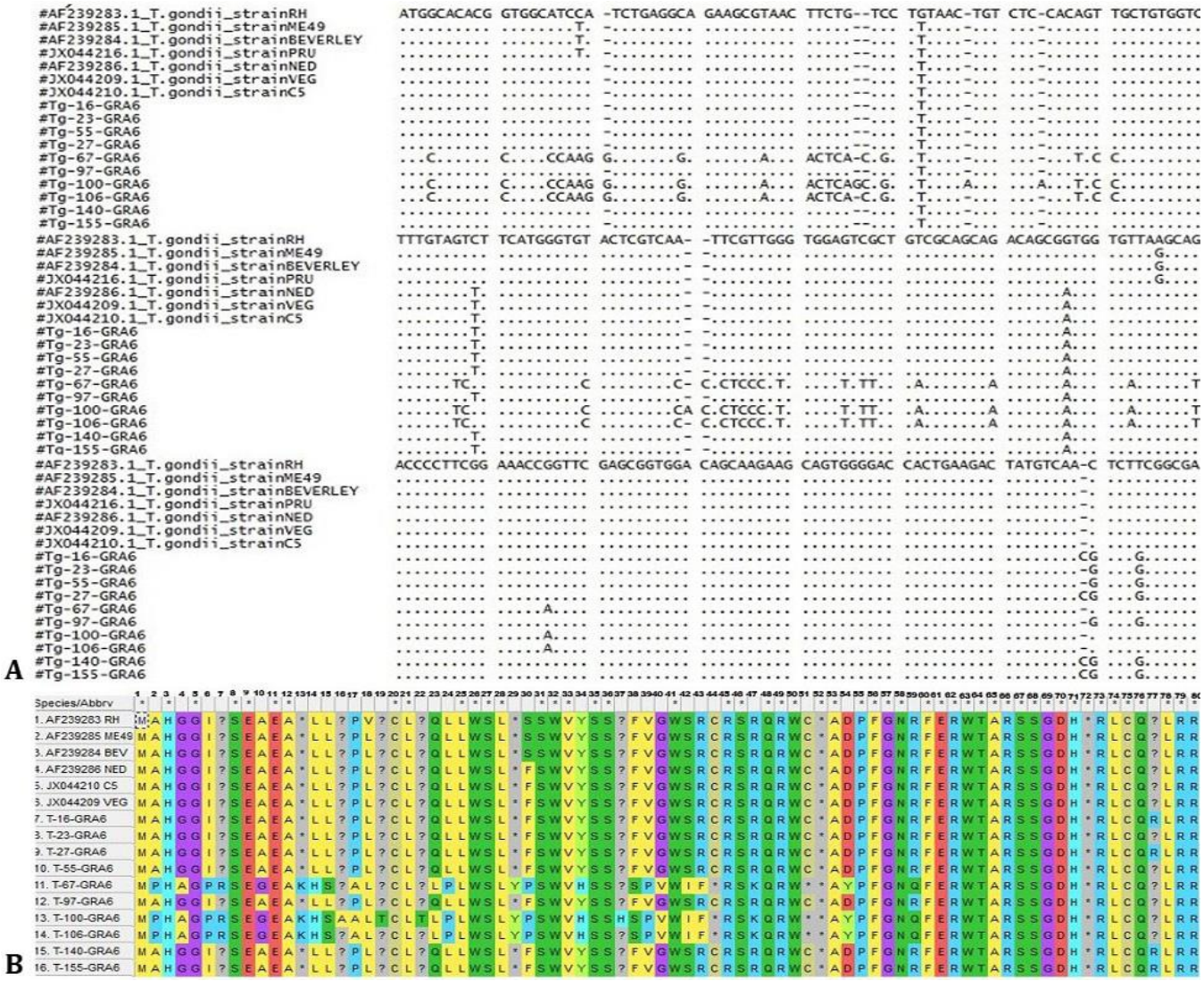

Fig. 2. A) Multiple sequence alignments and **B)** amino acid changes of *T. gondii* GRA6 coding region from different organs of sheep and cattle isolated in the present study with other reference sequences in GenBank®.

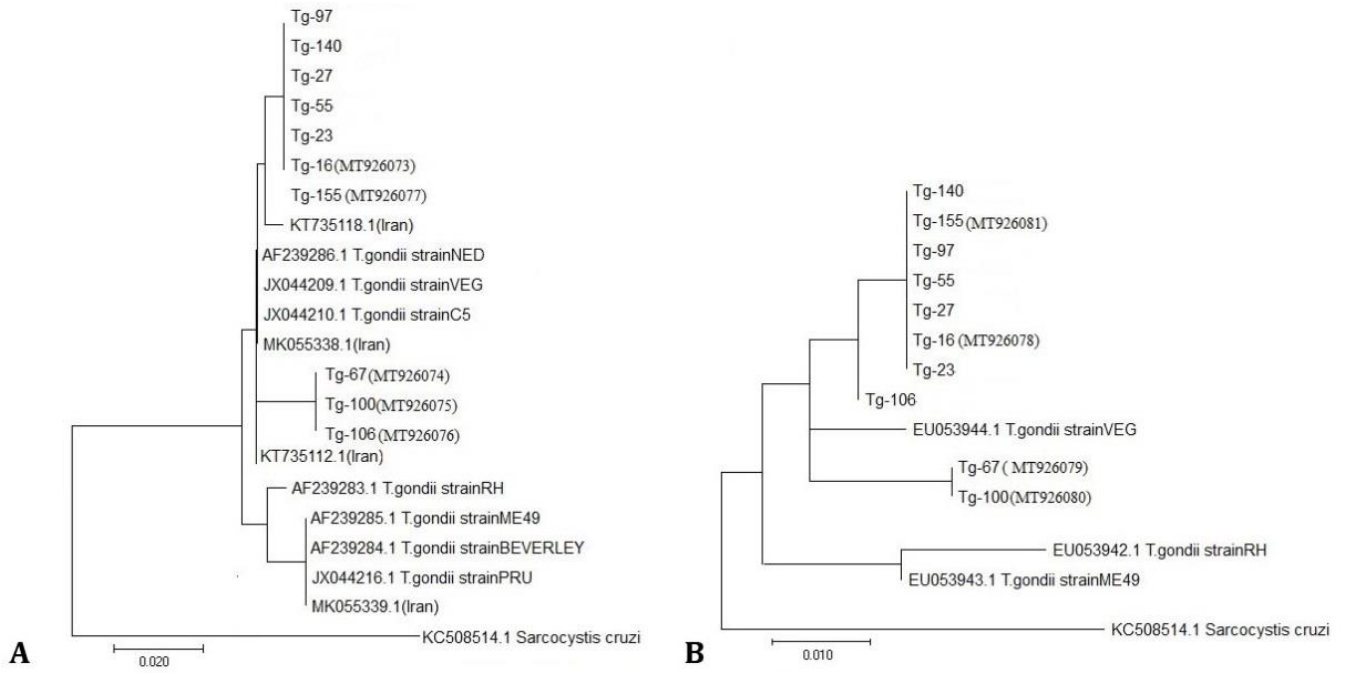

Fig. 3. The The phylogenetic tree of *T. gondii* based on **A)** GRA6 and **B)** SAG2 genes sequences from different organs of sheep and cattle isolated in the north of Iran and other reference sequences in GenBank® using maximum likelihood algorithm and 1,000 bootstrap resampling. *Sarcocistis cruzi* was considered as outgroup branch.

Discussion

In Iran, the prevalence rate of *T. gondii* infection was estimated to be 31.00% (5.00 - 61.00%) in sheep and 18.10% (9.20 - 28.50%) in cattle.[10,11] The present study, similar to other studies, showed that this infection is widespread in above mentioned animals in the north of Iran. The frequency of toxoplasmosis in the sheep and cattle (21.80%) was similar to a previous report in Iran.[18] Fallah *et al*. found a higher prevalence (32.00%) among meat product in the north-west of Iran.[20] The higher prevalence rate of this infection using PCR has also been reported by several studies.[22,23] In contrast, the prevalence of *T. gondii* infection was much lower in other reports of Iran[24] and other parts of the world compared with our results.[25-27] Several factors such as weather conditions, high prevalence of infected felids and contact with *Toxoplasma* oocysts could affect the distribution of toxoplasmosis in different population.[9]

In the present study, the highest infection rate was observed in kidney (28.20%) followed by heart muscle (27.70%). *Toxoplasma* tissue cyst formation preferentially often occurs in brain tissue, the skeletal and heart muscle although it can happen in any other organs particularly heart muscle.[9,28] However, kidney should be considered as an important infection source for humans.

In the present work, some of B1and rep-529- PCR-positive samples failed to be genotyped and only partial data were obtained. This problem was observed in several studies of genotyping of various organismes.[29,30] This could be resulted from low DNA concentration, DNA degradation and low copy number of the subjected gene.[31] PCR-RFLP assay was performed on the products of second steps of nested-PCR of SAG2 and GRA6 genes for identifying the *T. gondii* genotypes. According to previous data, *T. gondii* isolates have been categorized in three main lineages (types I, II and III) and atypical strains around the world. The distribution of *T. gondii* genotypes varies in different area and also they are different in virulence. The results obtained by PCR-RFLP of SAG2 and GRA6 showed that all *T. gondii* isolates belonged to type III genotype. These results are supported by other studies performed on soil, cat feces, meat products which reported that type III was the predominant genotype.[18,32,33] But, our findings are in contrast with other documents that found type I or type II were the predominant genotypes.[20,34-36]

The SAG2 nucleotide sequence of 10 isolates which were characterized as genotype type III were completely identical with *T. gondii* isolate NED (AF357579) and *T. gondii* isolate C56 (AF249698.1) strains. Alignment of these sequences with the reference strains showed that 9 nucleotide substitution occurred which 5 of them led to amino acid alterations. Moreover, phylogenetic analyses demonstrated that our isolates particularly Tg-67 and Tg-100 formed separates clades originated from *T. gondii* isolate NED and might be considered as atypical strains (Fig. 3).

The GRA6 nucleotide sequence of 10 isolates which were characterized as genotype type III were not completely indistinguishable with previous sequences in

GenBank®. The isolates Tg-67, Tg-100 andTg-106 had homology about 84% with *T. gondii* isolate NED and *T. gondii* isolate C56 (AF249698) strains and 85.2% with *T. gondii* isolate H121 from Iran (KT735118). Alignment of these sequences with the reference strains showed that 45 nucleotide substitution occurred in these isolates which 29 of them led to amino acid alterations (Fig. 2). Additionally, phylogenetic analyses revealed that these isolates formed separates clade from the references strains (*T. gondii* type III genotype) as well as from other isolates found in the current study (Fig. 3). These findings indicated that the ratio of non-synonymous to synonymous nucleotide changes was very high and positive selection pressure on the GRA6 gene in *T. gondii* seemed to be strong. Our findings are in agreement with previous published data indicating that such variations, particularly within the parasitophorous vacuole in the host cell, occur in the protein.[37] Some genetic characteristics such as surface and secretory antigens are potentially variable loci which encode the virulence or survival factors of the parasites. Hence, they have been commonly used to infer possible genetic population structure models, transmission patterns and evolutionary relationships between *T. gondii* populations and reservoirs. [38-40]

The most frequent amino acid changes were replacement of proline with alanine, isoleucine, leucine, phenylalanine and valine. It seems that replacement of more proline in amino acid sequence of GRA6 may provide the parasite with more survival fitness within the host by stabling tubular network with the aids of other GRAs.[41] This explanation is supported by other studies demonstrating that proline is multifunctional amino acid and has several protective roles in living agents.[42] *Toxoplasma* GRA4 and GRA8 are predicted to contain proline-rich domains which are released into parasitophorous vacuole (PV) during the host cell invation.[41]

In the current study, we conducted PCR-RFLP and sequencing of two polymorphic genes, SAG2 and GRA6, which is considered as a limitation of our study.

In conclusion, genetic characterization of 10 *T. gondii* isolates from sheep and cattle showed high genetic variability compared with standard type I, II and III genotypes. These results support the hypothesis of the existence of polymorphic and overlapping strains within livestock in Iran. It also suggested the necessity of increased genotyping and sampling efforts to accurately estimate *T. gondii* intraspecific genetic diversity. Moreover, our findings revealed a relatively high prevalence of *T. gondii* infection in sheep and cattle in the north of Iran.

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Conflict of interest

The authors declare that they have no competing interests.

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