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### Original Article

# Phylogenetic Analysis and Genetics Polymorphisms Evaluation of *ROP8* and *B1* Genes of *Toxoplasma gondii* in Livestock and Poultry Hosts of Yazd, Qom and Golestan Provinces of Iran

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#### **Abstract**

**Background:** A high correlation is observed between specific clonal lineages and host types in toxoplasmosis. The main objectives of this study were comparing polymorphism and evolutionary analysis of the *B1* and *ROP8* genes, as well as the evaluation of phylogenetic and *Toxoplasma gondii* isolates obtained from different hosts and regions.

**Methods:** Overall 96 brain/ diaphragm tissue samples of livestock and poultry from three provinces of Iran (cows: 9 from Yazd, 9 from Qom; sheep: 19 from Yazd, 7 from Qom; goats: 7 from Yazd, 4 from Qom; one camel from Yazd and 37 chickens, 2 roosters and one duck from Golestan) were tested during 2018-19. A nested PCR and PCR-PCR methods were developed with the *B1* and *ROP8* genes. Evaluation of genetic proximity, genetic diversity and evolutionary analysis were done using MEGA-X and DnaSP5 software. Thirty samples of both genes were sequenced (18 *B1* and 12 *ROP8* genes), and submitted to the GenBank (MN275903-MN275932).

**Results:** Tajima's D index analyses showed that both genes were in the negative direction of evolution. The *B1* gene was more sensitive than the *ROP8* gene. The *ROP8* gene showed better and more acceptable results in terms of the relationship between the host and the genotyping of the samples.

**Conclusion:** The *B1* gene was only an attractive target for rapid detection of *T. gondii* parasites, whereas the *ROP8* gene due to a high level of polymorphism was able to isolate the three clonal lineages (type I, II and III), inter-types and even atypical strains from different isolates of *T. gondii*.



## Introduction

**T** *Toxoplasma gondii* is an obligate intracellular parasite and the causative agent of toxoplasmosis that considered as an important public health concern not only for humans but also in veterinary field and animal husbandry worldwide (1,2). The definitive host of *T. gondii* is feline species and other animals as intermediate hosts could be infected by ingesting oocysts from food or water sources (3). Livestock and poultry as a source of human nutrition have a great economic importance in most countries. Therefore, toxoplasmosis infection in them is considered as a source of disease for humans and other carnivores. *T. gondii* parasite causes abortion in livestock and humans, and use of undercooked and raw meat in pregnant women is one of the most important risk factors of abortion during pregnancy (4, 5).

The majority of *T. gondii* strains consists of 2 or 3 clonal lineages (types I, II and III), which found in both animals and humans (6,7). Furthermore, unusual dimorphic allelic compounds as “atypical” are abundantly observed in the *T. gondii* genotype which not fitting within the three dominant lineages (8,9). The seroprevalence of *T. gondii* infection is 39.3% among Iranian general population, 31% in sheep, 27% in goat, 18.1% in cattle, 0.36% in cats (2,10,11). Clinical manifestations of *T. gondii* infection are non-specific and unreliable for diagnosis (3).

Various methods are used to identify and diagnose *T. gondii* including serological assay, bioassay, microscope examination, conventional PCR, nested PCR, real-time PCR etc. (12,13).

Repetitive gene regions in the parasite genome including the Glycerol-3-phosphate dehydrogenase (*B1*) gene, the 529 bp repeat element and the internal transcribed spacer (ITS-1) or 18S rDNA sequences are commonly used for rapid identification of *T. gondii* (12). However, some genetic markers, which are

more polymorphic, can be used for genetic diversity and polymorphism analysis such as the rhostry protein (ROPs) genes (14-17).

The objective of this study was to compare genetic diversity in two *B1* and *ROP8* genes in different types of *T. gondii*, and phylogenetic and evolutionary analyses among *T. gondii* isolates obtained from different hosts and regions.

## Materials and Methods

### Sampling

In this cross sectional study, 96 animal isolates were collected from different hosts, including 56 diaphragm muscle tissue samples of livestock and 40 poultry brain tissue samples from slaughterhouses located in three provinces (Yazd, Qom and Golestan) of Iran (18 cows: 9 from Yazd, 9 from Qom; 26 sheep: 19 from Yazd, 7 from Qom; 11 goats: 7 from Yazd, 4 from Qom; one camel from Yazd and 37 chickens, 2 roosters and one duck from Golestan). The samples were randomly collected in different days during 10 Jun 2018 to 21 Jan 2019. Three reference strains of *T. gondii* (Strain RH (type I), PRU (type II) and VEG (type III) strains) were obtained from the Biological Resource Center for *Toxoplasma*, Limoges University, Limoges, France.

This study was approved by the Ethics Committee of Qom University of Medical Sciences, IR.MUQ.REC.1398.054.

### Digesting and DNA extraction

Briefly, all samples were digested using the acid pepsin digestion solution (18). Then, 200 µl of final pellet was used for the DNA extraction by a FavorPrep Tissue Genomic DNA Extraction Mini Kit (Favorgen Biotech Corp, Ping-Tung, Taiwan), following the manufacturer's instructions. Total DNA was eluted from the column in 50 µl of elution buffer, and stored at -20 °C for further durability.

### Gene analysis and primer design

The *B1* and *ROP8* genes were targeted for this study. Several reference sequences of different types of *ROP8* gene were derived from GenBank and loaded into the CLC Genomics Workbench 12 software. Genetic makeup and amount of genetic diversity of two genes were evaluated for selecting and designing suitable primers. A pair of conventional PCR primers were designed for *ROP8* gene. The conventional PCR and nested PCR primers of *B1* gene were as previously described (19).

### PCR-PCR product and Nested PCR amplification

In order to increase sensitivity of gene regions for detection, *T. gondii* was used from nested PCR and PCR-PCR procedures for *B1* and *ROP8* gene regions, respectively.

The conventional PCR primers of *B1* gene were Tg1 (5' TGT TCT GTC CTA TCG CAA CG) and Tg2 (5' ACG GAT GCA GTT CCT TTC TG) to amplify a 580 bp fragment. The PCR primers of *ROP8* gene were ROP8.F (5' CCG ACC AGG AGA CAG GAG AA) and ROP8.R (5' TCG CAC CAG ATG TTC AAA GC) to amplify a 488 bp fragment. PCR reactions were carried out in a total volume of 20 µL, including 10 µL Master mix 2x (YTA, Co, IRAN), 0.8 µL each of forward and reverse primers (5 pmol concentration), 3 µL DNA template and 5.4 µL of PCR-grade H<sub>2</sub>O. Both PCR was performed using the Applied Biosystems Veriti™ Thermal Cycler (Applied Biosystem, CA, USA), under the following conditions: initial denaturation at 95 °C for 5 min, followed by 45 cycles of denaturation at 94 °C for 45 s, annealing at 58 °C for 15 s, extension at 72 °C for 30 s and a final extension at 72 °C for 5 min.

To perform nested PCR and PCR-PCR, assay was used from diluted PCR products (1:1000) in nuclease free water as DNA template. In second round of nested PCR, a 531 bp DNA fragment of *B1* gene with an internal primer pair were amplified; Tg3 (5' TCT TCC

CAG ACG TGG ATT TC) and Tg4 (5' CTC GAC AAT ACG CTG CTT GA). In addition, the second round of PCR amplification of *ROP8* gene was done by same conventional PCR primers and PCR product as template. Reaction protocol and cycling conditions (except for the nested PCR primer pairs and DNA templates) were similar to the first round of amplification. PCR products were visualized using a 1% agarose gel and photographed using a gel documentation system (Cambridge, Warwickshire, UK).

### Sequencing, genetic polymorphisms and phylogenetic analysis

After purification, thirty positive samples were sent for sequencing by an ABI PRISM™ 310 automated sequencer (Applied Biosystems, USA), using internal reverse and forward primers. The sequencing results were analyzed using the BLAST output to evaluate nucleotide similarity (20). Genetic diversity and percentage of genetic proximity was evaluated between different types of *T. gondii*. Genetic proximity analysis was performed using DnaSP 5.10.01 software (21). All the sequences with several reference sequences were imported into Molecular Evolutionary Genetic Analysis version 10.0.5 (MEGA-X) software for phylogenetic analysis. Multiple Sequence Alignment was used to align the sequences using Clustal W. Phylogenetic tree using maximum Likelihood (ML) method was constructed to depict genetic relationship and phylogeny of isolates. Bootstrap test of 1000 replicates was performed on each tree to determine the reliability and robustness.

### Statistical analysis

Chi-square test was applied to compute the qualitative data. Kappa coefficient was used to evaluate the agreement between two *B1* and *ROP8* genes results for the detection of *T. gondii* infection using MedCalc statistical software. Natural selection, genetic diversity between sequences and several tests of neutrality were done for the assessment of genetic dis-

tances using DnaSP 5.10.01 software. The ratio of non-synonymous substitution (dN) to synonymous substitution (dS) was evaluated by DnaSP 5.10.01 software (21).

## Results

### *Toxoplasma gondii* identification

Overall, the *B1* and *ROP8* genes identified 65 and 49 positive samples out of 96 isolates using nested PCR and PCR-PCR methods, respectively. To evaluate the degree of agreement between the two genes with the two methods for detecting *T. gondii* infection, the

kappa coefficient was estimated to be about 0.13 (with a 95% confidence interval of -0.05 to 0.32 and the standard error of 0.093), which indicates poor agreement or association between the two genes or two techniques.

### Genetic polymorphisms evaluation

Thirty positive samples of two genes were sequenced (18 *B1* and 12 *ROP8* genes), analyzed and submitted in GenBank (Access number: MN275903-MN275932) (Table 1). Both genes were amplified in the expression region but only 219 bp of *B1* gene amplicon is the expression (Fig. 1).

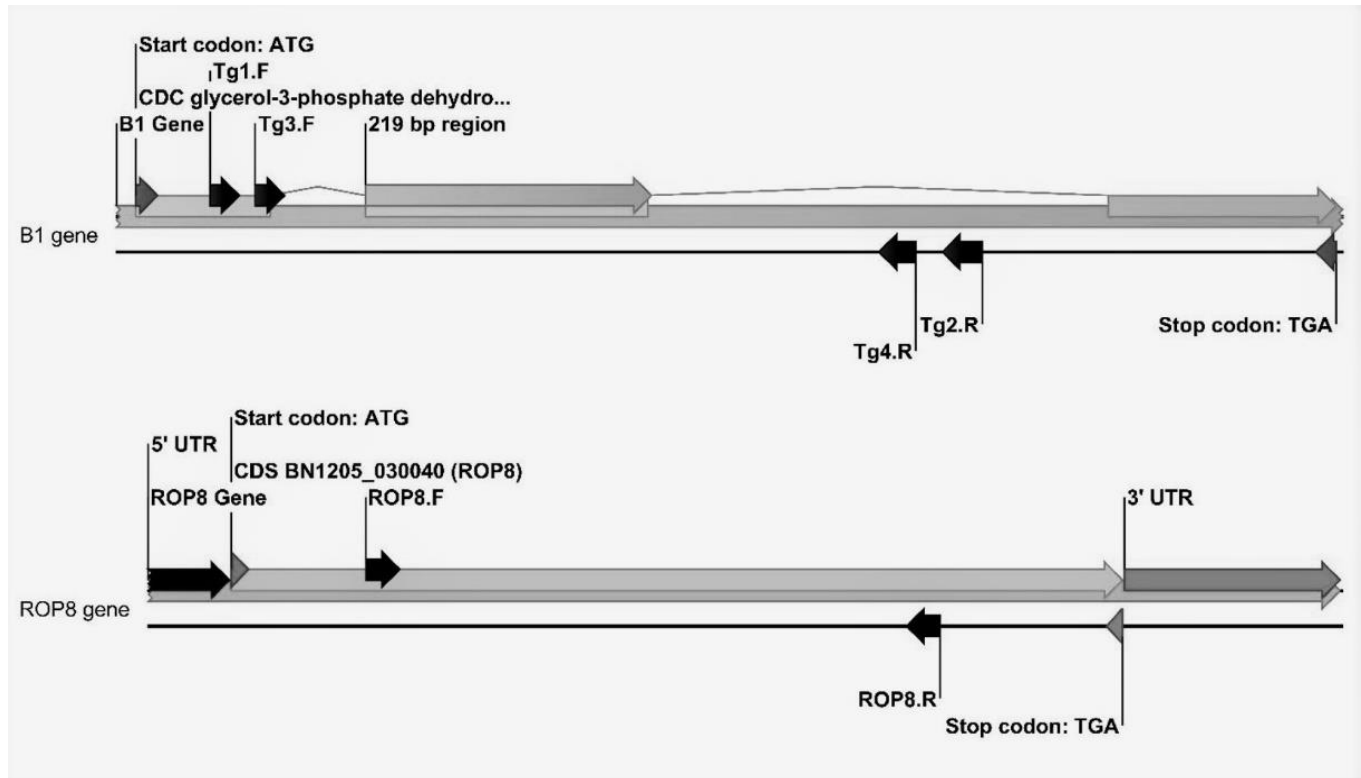
**Table 1:** Sequenced and submitted samples of *Toxoplasma gondii* by the *B1* and *ROP8* genes to the GenBank

<i>Host</i>	<i>Sample ID</i>	<i>Origin</i>	<i>B1 gene</i>		<i>ROP8 gene</i>	
			Parasite type	accession no	Parasite type	accession no
Reference strain	RH	France	I	MN275918	III	MN275930
	PRU	France	II	MN275919	II	MN275931
	VEG	France	III	MN275920	III	MN275932
Sheep	SY2	Meybod/Yazd	1	MN275903	II	MN275921
	SY4	Meybod/Yazd	II	MN275904	III	MN275922
	SY5	Meybod/Yazd	II	MN275905	II	MN275923
	SY12	Meybod/Yazd	II	MN275906	III	MN275924
Cow	CY4	Ashkezar/Yazd	I	MN275907	ND	ND
	CQ7	Qom	II	MN275910	ND	ND
Goat	GY2	Ashkezar/Yazd	I	MN275908	ND	ND
	GY3	Ashkezar/Yazd	I	MN275909	ND	ND
	GY4	Ardakan/Yazd	I	MN275911	ND	ND
	GQ2	Qom	I	MN275912	ND	ND
Poultry	GQ3	Qom	I	MN275913	ND	ND
	CG7	Golestan	ND	ND	II	MN275926
	CG19	Golestan	I	MN275915	ND	ND
	CG21	Golestan	III	MN275916	III	MN275927
	CG36	Golestan	ND	ND	III	MN275928
	CG37	Golestan	I	MN275917	III	MN275929
	D1	Golestan	I	MN275914	III	MN275925

ND: not done, B1: glycerol-3-phosphate dehydrogenase, ROP8: rhostry protein 8

To evaluate polymorphisms, variations of *B1* gene only were considered in this region. Comparison of polymorphism analysis of two gene regions with DnaSP software shown in Table 2. In the *ROP8* gene, the number of

polymorphic sites was significantly higher than monomorphic sites, and the *B1* gene was reversely. The highest and lowest variations were observed in the *ROP8* and *B1* genes (227 vs. 9), respectively.



**Fig. 1:** Schematic illustration of the two genes of *B1* and *ROP8* amplified with specific primers (small black arrows) for *Toxoplasma gondii* using CLC Genomic workbench 12 bioinformatics software

The most informative sites were related to the *ROP8* gene (95.15%); however, the *B1* gene had the most non-informative sites (77.8%). Overall, non-synonymous variants of *ROP8* gene (69.8%) were more than *B1* gene (66.7%). The comparison of synonymous mutations in silent sites and non-synonymous mutations in replacement sites showed no significant difference between different types of *T. gondii* in two expression regions. Tajima's D index was calculated to be -1.78 and -0.64 for two *B1* and *ROP8* genes. This indicated that evolution was negative in two expression regions. Total number of mutations (Eta) of *ROP8* gene were greater than the *B1* gene (245 vs. 9) (Table 2).

#### Phylogenetic proximity

The phylogenetic analysis was done to select gene regions in different types of *T. gondii*.

Maximum Likelihood (ML) statistical method was applied to draw phylogenetic trees based on the nucleotide and amino acid sequences. Kimura's two-parameter and Jones-Taylor-Thornton (JTT) matrix-based models were used as the nucleotide and amino acid substitution models, respectively. Molecular phylogenetic analysis of *B1* gene was done with 24 partial genome sequences of different types. In addition, under similar conditions, phylogenetic analysis of *ROP8* gene was conducted by 32 partial genome sequences of common clonal lineages. Totally, 530 and 486 nucleotide positions in their final dataset were presented in *B1* and *ROP8* genes, respectively (Table 2). The phylogenetic trees of *B1* gene were not able to isolate the common types of *T. gondii*, especially trees based on amino acid sequences. However, the topology of the *ROP8* trees revealed that this gene caused the

separation of three clonal lineages, inter-types and even atypical strains (Fig. 2 and 3). During the studies of phylogenetic and genetic diversity, the majority of the investigated samples

using *B1* gene were type I and II, and all samples that analyzed using *ROP8* gene were type II and III (Table 1).

**Table 2:** The role of selected DNA mutations in the different types of *Toxoplasma gondii* by employing expression regions of the *B1* and *ROP8* genes

Genes	Types	No. of Seq.	S	Eta	k	$\pi$	$\Theta$ per nucleotide position	$\Theta$ pre seq.	Tajima's D index	dS	dN	V (%)	Total Singleton variables (%)	Total Parsimonious variable (%)
<i>B1</i> (219 bp region)	1 & 2	16 <sup>a</sup> (14) <sup>b</sup>	1	1	0.125	0.00057	0.00138	0.30	-1.16	1	0	9 (4.1)	7 (77.8)	2 (22.2)
	3	3 (2)	1	1	0.67	0.00304	0.00304	0.67	-	0	1			
	Other	4 (2)	0	0	0	0	0	0	0	0	0			
	All	24 (18)	9	9	1.101	0.00503	0.01101	2.41	-1.78	3 (33.3)	6 (66.7)			
<i>ROP8</i>	1	12 (1)	30	30	6.61	0.01359	0.02044	9.94	-1.51	3	27	227 (52.9)	11 (4.85)	216 (95.15)
	2	9 (3)	7	7	1.72	0.00354	0.0053	2.58	-1.48	1	6			
	3	9 (8)	28	28	11.83	0.02435	0.0212	10.30	0.74	3	25			
	Other	2	0	0	0	0	0	0	0	0	0			
	All	32 (12)	171	245	47.03	0.09677	0.12518	60.84	-0.64	55 (30.2)	127 (69.8)			

S: Segregation site, Eta: Total number of mutations, K: Average number of pairwise nucleotide differences between pairs of sequences,  $\pi$ : Nucleotide diversity,  $\Theta$ : The amount of genetic variation, Tajima's D index: The statistical test proposed by (22), dS: Synonymous site, dN: Non-synonymous site, V: Variable (polymorphic) nucleotide site.

<sup>a</sup> Total number of sequences used in this study

<sup>b</sup> Number of sequencing without reference sequences in each gene

## Discussion

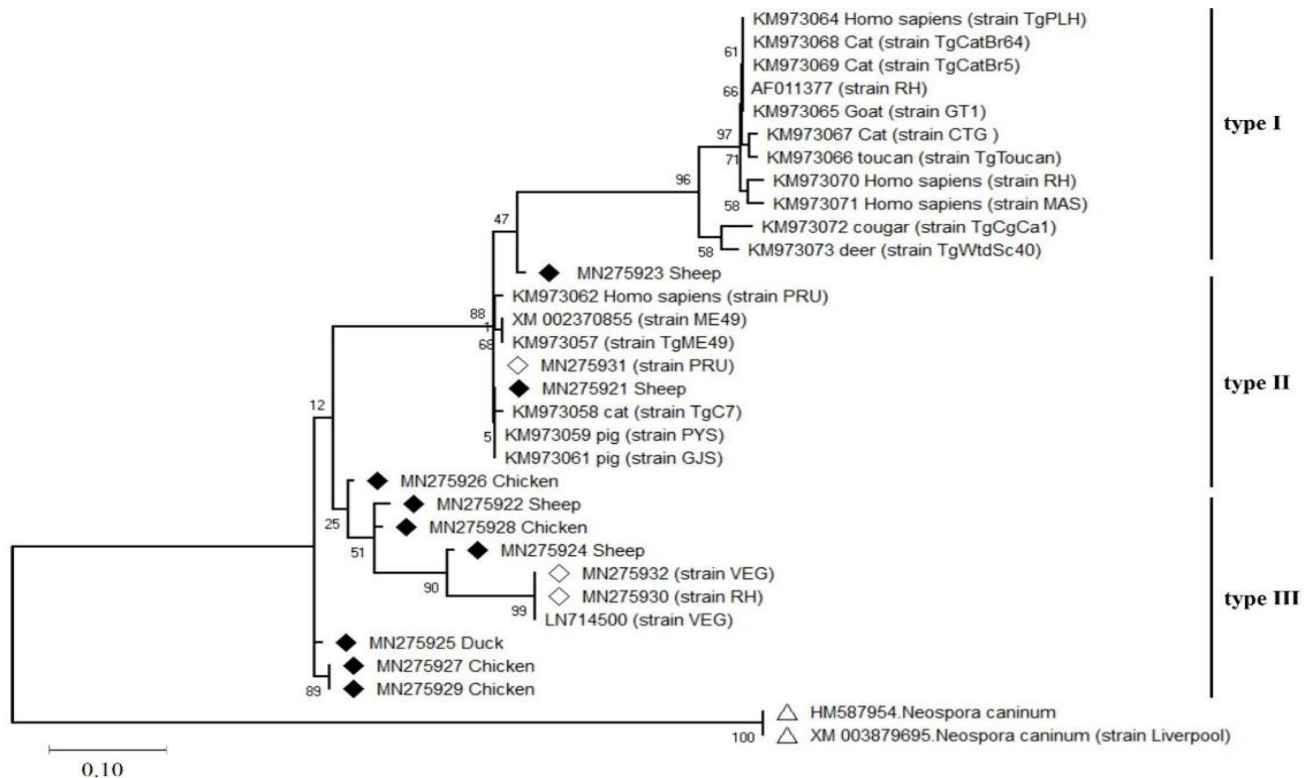
*T. gondii* strains were identified by targeting, two expression genes, and haplotype diversity with polymorphisms and variations of two genes in three main clonal lineages of *T. gondii* were recognized. Various genetic comparisons were evaluated for phylogenetic and evolutionary analyses of both genes among isolates and different types of *T. gondii*. Each gene of three predominant lineages had specific, clear and readable sequences. There was poor agreement and association (kappa coefficient = 0.13) between the two genes and both techniques (22). The targeted *B1* gene is a multi-

copy gene region (35-fold) and high conserved in the parasite genome. Thus, the *B1* gene was showed higher sensitivity than *ROP8* gene for identification and molecular diagnosis of the parasite (23). In this study, 67.71% and 51% of the samples were identified, *T. gondii* positive, using the *B1* and *ROP8* genes, respectively. The parsimony-informative site is a position related to a set of sequences, which there are at least two different types of nucleotides (or amino acids) at that point in the sequences. On the contrary, any nucleotide site at which only unique nucleotides (singletons) exist is

not informative (21, 24). In this study, most informative mutations were related to *ROP8* gene, and great non-informative mutations was showed in the *B1* gene.

The ratio of dN (amino acid altering substitutions) and dS (substitutions that do not alter amino acids) (dN/dS) has been widely used as

an indicator of selection pressure (25,26). In our study, the ratio of dN/dS was high in the *ROP8* compared to *B1* gene (2.3 vs. 2).



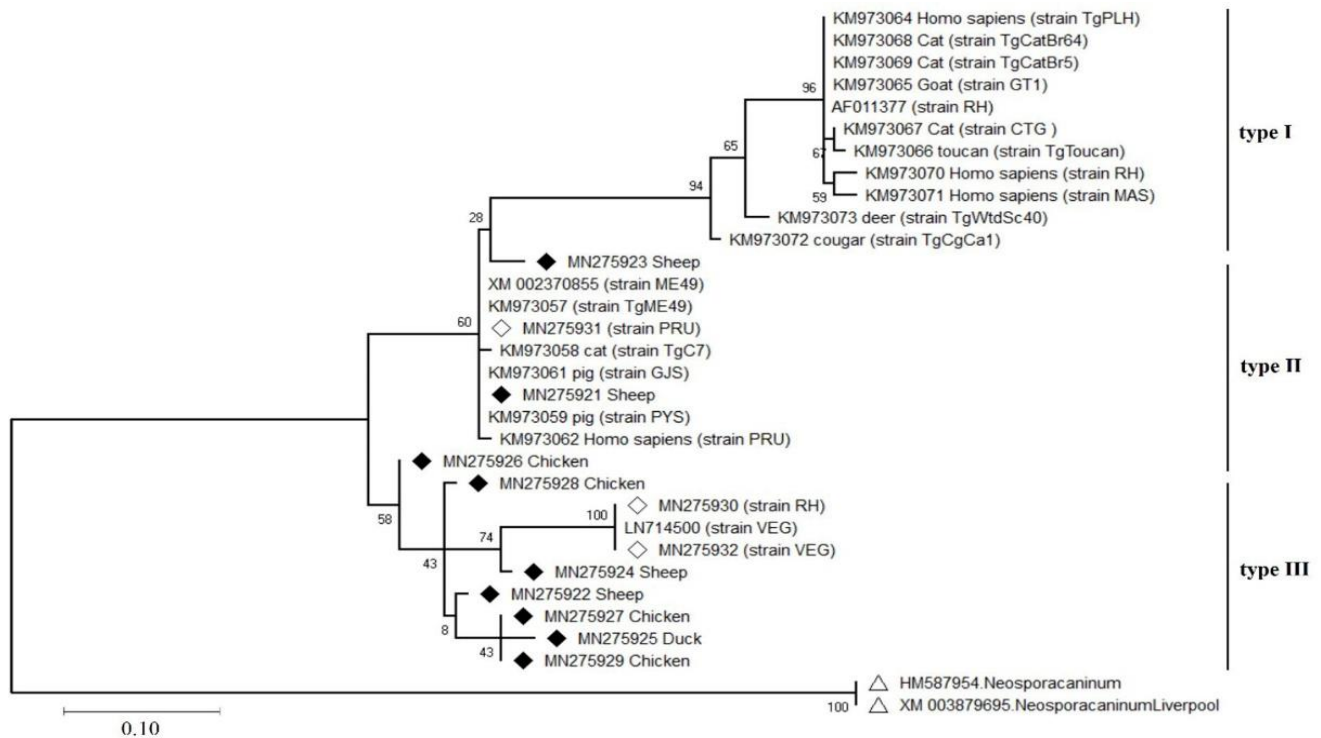
**Fig. 2:** Molecular phylogenetic analysis of different types of *Toxoplasma gondii*, using the *ROP8* gene region by the Maximum Likelihood statistical method and the Kimura 2-parameter model. The rhombuses black represents the sequences in the present study, while the white rhombuses denote reference sequences. Unmarked sequences represent similar sequences from the GenBank, and the white triangles denote indicative outgroup sequences for comparison. Evolutionary analyses were conducted in MEGA-X

Tajima's D is a population genetic statistical test. Tajima's D is computed as the difference between two measures of genetic diversity, the mean number of pairwise differences and the number of segregating sites (22, 27, 28). Tajima's D index was negative in two expression regions, except for type III population in *ROP8* gene. The *B1* gene had less genetic diversity than polymorphic gene of *ROP8*. The *B1* gene due to a low level of polymorphism

was not able to determine the clonal lineages of different isolates of *T. gondii* (29). So far, based on performed studies, most of the high repetitive markers that provide a high sensitivity (i.e., B1, 529 bp repeat, rRNA genes) could not be used for genotyping and distinguishing different types of *T. gondii* (29,30). Topologic evaluation of phylogenetic trees confirmed the high accuracy using ML method in both genes. Evaluation of both genes by phylogenetic

analysis and genetic diversity to distinguish different types of *T. gondii* had different results. The correlation of specific clonal lineages with the type of toxoplasmosis in the different host is very important in the process of diagnosis and treatment. The *ROP8* gene showed better and more acceptable results in terms of the relationship between the host and the geno-

typing of the samples. Accordingly, in accordance with the results of other studies, all the studied samples based on their host (livestock and poultry) were types II and III by employing the *ROP8* gene (31, 32).



**Fig. 3:** Molecular phylogenetic analysis of different types of *Toxoplasma gondii*, using the *ROP8* gene region by the Maximum Likelihood statistical method and the JTT matrix-based model. The rhombuses black represents the sequences in the present study, while the white rhombuses denote reference sequences. Unmarked sequences represent similar sequences from the GenBank, and the white triangles denote indicative outgroup sequences for comparison. Evolutionary analyses were conducted in MEGA-X

In addition, the phylogenetic analysis indicated that *ROP8* unlike *B1* gene are able to isolate the three clonal lineages, inter-types and even atypical strains of *T. gondii*. Although, Li et al did not achieve the desired results for the differentiation of the three classical genotype strains using the *ROP8* gene by polymer-

ase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (17).

Different regions of gene can be show different genotyping results in detecting strain type (33,34). Sequencing and phylogenetic studies of the reference RH strain using the *ROP8* gene showed that this strain was very similar to the VEG strain, while the strain



phenotype and the *B1* gene analysis confirm it. It is possible that this gene region has mutated during serial passages (35-37).

The time-consuming and costly meat digestion process, low parasitic load, lack of identification and definitive diagnosis of the parasite by conventional PCR method and the use of nested PCR and PCR-PCR techniques, as well as focus on preventing infection due to the use of these methods were the limitations and challenges of this study.

## Conclusion

The majority of livestock and poultry populations studied in Yazd, Qom and Golestan provinces of Iran were infected to the *T. gondii* parasite. The most *T. gondii* isolates were identified using the *B1* and *ROP8* genes. The *B1* gene was only an attractive target for rapid detection of *T. gondii* parasites, whereas the *ROP8* gene due to having higher sequence variation could be a more suitable marker to isolate the three clonal lineages (type I, II and III), inter-types and even atypical strains from different isolates of *T. gondii*.

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## Conflict of interest

The authors declare that there is no conflict of interest.

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