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^γ Phylogenetic study of protozoan *Toxoplasma gondii* using Cytochrome b and Gra20 genes

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۱٤ Abstract

10 Toxoplasma gondii is an obligate intracellular protozoan in the phylum Apicomplexa. In addition to ١٦ humans, toxoplasmosis can also cause serious diseases in livestock, leading to significant economic losses. ۱۷ The use of molecular methods with high sensitivity has made it possible to detect and study ۱۸ microorganisms. In this study the cytochrome b and dense granule 20 (Gra20) genes were utilized for ۱٩ studying Toxoplasma gondii parasites. After collecting 29 animal samples of Toxoplasma gondii, the PCR ۲. method was applied to evaluate the presence of Gra20 and cytochrome b genes. The sequence of 11 samples ۲١ was acceptable and submitted in the NCBI database. Mega X software was utilized to create the ۲۲ phylogenetic tree. In addition, the genetic diversity was studied using the RFLP-PCR assay for Gra6 gene. ۲۳ Assessment of the bands obtained from PCR showed that the bands related to cytochrome b are found in ۲٤ all species of the Sarcocystidae family, but the bands related to the Gra20 gene are specific to Toxoplasma ۲0 gondii. In the phylogenic tree, there was a closer relationship between Iranian isolates of Toxoplasma gondii ۲٦ and French strain of Toxoplasma gondii. Type I was determined as the genetic type of protozoan ۲۷ Toxoplasma gondii isolated from Iran. Based on the results, the cytochrome b gene was considered as a ۲۸ general gene for recognizing different species of the Sarcocystidae family, however divergence was seen ۲۹ in the Gra20 gene, which is considered a particular gene for *Toxoplasma gondii*.

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Keywords: *Toxoplasma gondii*, dense granule 20 (GRA20), cytochrome b, phylogenetic analysis.

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۳۳ **1. Introduction**

A broad range of warm-blooded vertebrates are infected by *Toxoplasma gondii*, a protozoan that is obligately intracellular and belongs to the Apicomplexa phylum (1). The *Toxoplasma gondii* parasite is transmitted through the feces of its primary host, the Felidae family. Sporulated oocysts present in water and food, as well as infected tissue containing tissue cysts harboring *Toxoplasma gondii* bradyzoites, are

^{γ_{Λ}} all ways in which infection can be acquired (2). Moreover, the tachyzoite form of the parasite can be

ransmitted to the fetus through the placenta. The transmission of *Toxoplasma gondii* infection varies

 ε globally, and it is recognized as a zoonotic pathogen. Toxoplasmosis affects approximately 30% of the

 ε global population (3).

٤٢ The pathogenicity of *Toxoplasma gondii* parasite depends on the host's resistance, different strains of the ٤٣ parasite, and antigenic changes of the parasite. Toxoplasma gondii antigens include membrane antigens, ٤٤ cytoplasmic antigens, and excretion-secretion antigens as their primary features (4). Excretion-secretion 20 (ES) antigens play a significant role in stimulating the immune system among these antigens. The ٤٦ microneme, rhapterium, and dense granules of the parasite secrete substances that produce ES antigens. ٤٧ The organelles' contents are accountable for cell identification and attachment, formation of the ٤٨ parasitophorous vacuole (PV), parasite proliferation, and intracellular survival (5). ٤٩ Most of Toxoplasma gondii strains comprises of 2 or 3 clonal lineages (types I, II and III), which exist in

٥. both animals and humans (6). Furthermore, unique dimorphic allelic compounds as "atypical" are 01 abundantly identified in the Toxoplasma gondii genotype which not fitting among the three dominant ٥٢ lineages (7). A commonly employed technique to detect the Toxoplasma gondii parasite involves ٥٣ cultivating the parasite in cell culture such as Vero cells. The detection of diseases is achieved through 0 2 serological methods by measuring the quantity and type of antibodies produced against the parasite in the 00 body. These methods are capable of determining whether the infection is recent or ancient. Polymerase ٥٦ chain reaction (PCR) is the most sensitive and definitive diagnostic method, which involves amplifying ٥٧ millions of copies of a small piece of DNA or RNA. The PCR reaction involves heating the double-stranded ٥٨ DNA and then annealing the primers at a lower temperature. The Tag polymerase enzyme is used to ٥٩ synthesize a new DNA strand. Various types of PCR reactions include nested PCR, RAPD, RFLP,

- wicrosatellite marker method, and the Real-Time PCR (8).
- The aim of this study is to acquire a genetic profile for *Toxoplasma gondii*. The phylogenetic structure of this pathogen was also determined using cytochrome b and Gra20 genes in the present study.
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2. Materials and Methods

٦٥ 2.1. Sample collection and preparation: In this research, 29 animal samples were collected. Among these, ٦٦ 14 samples of cat oocysts (feces), 6 samples of heart, liver and kidney of cats, 2 samples of brain of aborted ٦٧ sheep and 2 samples of rooster brain were obtained from animal husbandry, slaughterhouse and veterinary ٦٨ clinic. Two samples of snake brain and one sample of the standard RH strain (Type I) were prepared from ٦٩ Razi Vaccine and Serum Institute in Karaj. Also, one sample of the French PRU strain (Type II) and one ٧. sample of the French VEG strain (Type III) were obtained from the Department of Parasitology at ۷١ Mazandaran University. The oocyst sample was prepared using the Sheather's sugar solution flotation ۲۷ method (9). Brain samples from aborted sheep fetus, rooster brain samples, cat hearts, and snake brains ۷۳ were prepared using an autopsy method. The Type I, II, and III strains were cultured on Vero monolayer ٧٤ cells using the tachyzoite growth method.

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2.2. Genome amplification and phylogenetic study: The sample genomes were extracted using a proteinase K method. The quality of extracted DNA was analyzed by a 0.8% agarose gel electrophoresis.
VV Using a thermocycler (Corbett-CGI-96), the Gra20 (XM 002372037: GenBank) and Cytochrome b (GenBank: JX 473253.1) genes were amplified during the PCR reaction. For this purpose, 16 µl of Master Mix, 1 µl of Taq DNA Polymerase 1 U/µl, 1 µl of each of the forward and reverse primers, as well as 1.5 µl of DNA sample were mixed together and brought to a volume of 20 µl with double distilled water. The temperature profile of the reaction began with a denaturation cycle lasting 90 seconds at 95°C. Then

 $\Lambda\gamma$ continued with 35 cycles including 10 seconds of denaturation at 94°C, 20 seconds of annealing at 61°C

- ^Λ^π for Gra20 gene and 59°C for Cytochrome b gene and 30 seconds of extension at 72°C. The final extension
- $\Lambda \xi$ cycle at 72 °C for 5 minutes ended the reaction.

A° 2.3. The primers used: The primers (Table 1) required by TakapouZist were made by BioNeer (South

- Korea) on order. A gel electrophoresis was performed on agarose with a percentage of 1.5% to assess the
- AV PCR product. The PCR product obtained from each sample with cytochrome b genes and Gra20 gene was
- ^{AA} sent to Kowsar Technology Company for sequencing. MEGA X software was used to evaluate the obtained
- ^{A9} sequences and create a phylogenetic tree.
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Primers	Sequences	Product length (bp)	Ref.
Gra20-outer	F: 5'-ATGCATAGCCGGAACTGCGTC-3'	1242	(11)
primer	R: 5'-TCACGCGGGCTTTCTACGG-3'		
Gra20-inner	F: 5'-ACAGGAAGAAACGCTACGGG-3'	202	Destand
primer	R: 5'-CCAATTGCTCGATTTGCCGT-3'	— 302	Designed
Cytb-outer	F: 5'-CGGGCACACCTTGTCTTTTAT-3'	741	Designed
primer	R: 5'-TGGTGTTACGAACCGGTTGAC-3	/41	Designed
Cyth-inner	F: 5'-ACTACCGCTTGGATGTCTGG-3'		
primer	R: 5'-AAAGGCAACTTTAAGCGCGG-3'	— 317	Designed

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 2.4. Restriction Fragment Length Polymorphism (RFLP-PCR): Different samples, including type I strain, rooster brain tissue, and cat heart tissue, were analyzed through PCR for Gra6. The PCR product was subjected to enzymatic cleavage using MseI enzyme and R buffer. This enzyme cuts the 795 bp fragment of the Gra6 gene into two regions. The Type I strain divides into fragments of around 540 bp, 170 bp, and 85 bp. Type II strain is divided into fragments of 640 bp and 85 bp, while Type III strain is divided into fragments of 640 bp and 85 bp, while Type III strain is divided into fragments of 85 bp and 540 bp (10).

99 3. Results

۱.. 3.1. PCR results: The PCR analysis was conducted on various DNA samples, including tachyzoite type I, 1.1 II, and III, rooster and snake brain tissue, cat heart tissue, cat oocyst, and sheep aborted embryo brain tissue. 1.1 The PCR was performed using the outer primer of cytochrome b, and the resulting DNA fragments were 1.5 visualized on an agarose gel. Only the samples of type I, type III, and the positive control showed a band 1.5 of the expected size (740 bp). There were no bands detected in the other samples (Figure 1-A). Another 1.0 PCR was performed on the PCR product of the previous step with the internal primer of cytochrome b and 1.7 the expected band of 317 bp was observed on agarose gel in all samples and positive control (Figure 1-B). ۱.۷ In the results of PCR performed with the Gra20 outer primer, no bands were observed on the agarose gel ۱.۸ except for the positive control. Gra20 internal primer was used in the second PCR reaction, and the desired

1.9 302 bp band was observed in all samples (Figure 1-C and 1-D).



117 Figure 1. A: PCR product electrophoresis of samples with outer cytochrome b primer (1: Cat heart sample, 2: ۱۱۳ Rooster brain sample, 3: Snake brain sample, 4: Cat oocyst sample, 5: Sheep fetal brain, 6: Tachyzoite type I, 115 7: Tachyzoite type II, 8: Tachyzoite type III, 9: Positive control, 10: Negative control). B: PCR product 110 electrophoresis of samples with internal cytochrome b primer (1: Cat heart sample, 2: Rooster brain sample, 117 3: Snake brain sample, 4: Cat oocyst sample, 5: Fetal brain Sheep, 6: Tachyzoite type I, 7: Tachyzoite type II, 117 8: Tachyzoite type III, 9: Negative control, 10: 500bp marker, 11: Positive control). C: PCR product 114 electrophoresis of samples with internal Gra20 primer (1: Tachyzoite type I, 2: Tachyzoite Type II, 3: 119 Tachyzoite type III, 4: Negative control, 5: Positive control 6: 500bp marker). D: PCR product electrophoresis 17. of samples with internal Gra20 primer (1: Cat heart sample, 2: Rooster brain sample, 3: Snake brain sample, 171 4: Cat oocyst sample, 5: Sheep fetal brain, 6: Positive control, 7: Negative control, 8: Positive control 9: 500bp ۱۲۲ marker).

۱۲۳ 3.2. Sample sequencing: The PCR product of the cytochrome b and Gra20 genes was sent to Kowsar ١٢٤ Technology Company for sequencing. Upon receiving the nucleotide sequences, an evaluation revealed 170 that the sequence readings of five samples were conducted improperly. Therefore, the sequences of 11 177 samples were registered in the national center for biotechnology information (NCBI) data bank after editing. ۱۲۷ Table 2 presents the accession numbers and specifications of the samples registered in the NCBI. The ۱۲۸ cytochrome b gene in the samples analyzed in this study exhibited complete similarity (100%) with the 129 cytochrome b sequences of Toxoplasma gondii strains ME49, RH, and VEG. Therefore, the cytochrome ۱۳. gene can be considered a universal gene for many forms of Toxoplasma gondii protozoa. Also, 100% ۱۳۱ similarity with Sarcocystis and 97% similarity with Neospora was observed. Gra20 primer amplified ۱۳۲ nucleotide sequences were compared by blasting with the sequences in the NCBI. The type I sample showed ۱۳۳ a similarity of 99.67% with the ME49 gene. The type II sample showed a 100% similarity with the ME49 ١٣٤ gene, while the type III sample showed a 100% similarity with the VEG strain gene. Also, 100% similarity 100 with the gene of VEG strain was observed in five nucleotide sequences of rooster brain, snake brain, sheep 137 fetal brain, cat oocyst, and cat heart.

Table 2. Accession numbers of the sequences registered in the NCBI

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No.	Sample	Amplified gene	Accession numbers in GenBank
1	Toxoplasma gondii type I (RH strain)	Cytochrome b	MW620206
2	Toxoplasma gondii isolated from snake brain	Cytochrome b	MW620207
3	<i>Toxoplasma gondii</i> isolated from the brain of aborted sheep	Cytochrome b	MW620208
4	Toxoplasma gondii type III (VEG strain)	Cytochrome b	MW620209
5	Toxoplasma gondii type II (PRU strain)	Cytochrome b	MW620210
6	Toxoplasma gondii isolated from cat oocyst	Cytochrome b	MW620211
7	Toxoplasma gondii type I (RH strain)	Gra20	MW660538
8	Toxoplasma gondii type II (PRU strain)	Gra20	MW660539
9	Toxoplasma gondii type III (VEG strain)	Gra20	MW660540
10	<i>Toxoplasma gondii</i> isolated from cat heart tissue	Gra20	MW660541
11	Toxoplasma gondii isolated from rooster brain	Gra20	MW660542

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3.3. Multiple alignment of nucleotide sequences: The ClustalW system of MEGA X software was utilized 12. 151 to align multiple nucleotide sequences. The nucleotide sequences of the amplified samples were aligned 157 using multiple alignment, with the exception of the rooster brain and cat heart samples. The sequences of 157 these samples were not included due to errors in reading the sequences. The alignment results of cytochrome 122 b sequences can be shown in Figure 2 and all the sequences completely overlap with each other. The 120 multiple alignment of nucleotide sequences for the Gra20 gene is shown in Figure 3. The nucleotide 127 sequence of three samples of snake brain, fetal sheep brain, and cat oocyst has not been completely 157 deciphered. The sequence of type III sample was found to have a greater resemblance to diverse samples ١٤٨ than the sequences of type I and type II after comparing them.

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Figure 2. The result of multiple alignment of amplified samples with cytochrome b gene by MEGA X software

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7 SHEEP-ORA-M-ORA20-F(56121)		NNNNN		RHRHHHH	NNNNCHA	INNNTWN	N N H ON ON H	ATNACIST
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VEG-GRA	COCOTOGAAAA	TCAGATTEC	AGAACTTOC	TETETTAC	CATCOA	GOCASCA	TAGAGEG	CAACGIGA
RDS-GRA-N-GRA20-F(\$5121)	COCOTOGAAAA	TCAGATIGCA	AGAACTTGC	TOTOTTAC	GCATCGA	GCAGCA	TAGAGCO	CAACGTGA
5 CAT-GRA-N-GRA2N-FISS(21)	COCOTOGAAAA	TCAGATTECA	AGAACTIC	TETETTAC	SCATCGA	GELAGEA	TAGAGEG	CAACGTEA
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- 17. Figure 3. The result of multiple alignment of amplified samples with Gra20 gene by MEGA X software
- 171 ١٦٢
- ١٦٣ **3.4.** Phylogenetic assessment: The phylogenetic tree was constructed using the nucleotide sequences of 175 the cytochrome b and Gra20 genes from both the samples in the current study and those recorded in the 170 databases. The phylogeny tree of the samples from the current study, which were recorded in the NCBI, 177 was constructed using the neighbor-joining method and bootstrap 100, utilizing the Mega X program. In ١٦٧ Figure 4, a phylogenetic tree was constructed using the nucleotide sequence of the cytochrome b gene of ۱٦٨ the Toxoplasma gondii. Our study of samples from Iran revealed that they have similarities with samples 179 from France, Norway, and the United States, as well as with the protozoan Sarcocystis. They were clustered ۱۷. together in a group. Neospora and Hammondia were the outgroup. The length of branches is a measure of 171 the genetic distance between different sequences. The genetic distance between the samples in the present ۱۷۲ study has been calculated as zero, and the largest distance (0.49) has been calculated between the ۱۷۳ Hammondia sample and the other samples. In the phylogenetic tree constructed using the nucleotide

١٧٤ sequence of the Gra20 gene (Figure 5), the type III tachyzoite sample, the rooster brain sample, and the cat 140 heart sample were grouped together in a Clustal (cluster), indicating their genetic similarity. Type I and II ۱۷٦ tachyzoite samples were located together in a separate cluster with high homology. In the tree topology, the 177 protozoa Neospora and Sarcocystis were considered as outgroups. For Gra20 gene, the genetic distance ۱۷۸ between Neospora and Sarcocystis was very low (0.01), but the distance between Neospora and Sarcocystis 119 protozoa and Toxoplasma gondii protozoan samples was high (2.44). The distance between type I and II ۱۸۰ tachyzoite samples was found to be zero. In general, there was a difference and gap between Toxoplasma ۱۸۱ gondii type I and II tachyzoite samples extracted from rooster brain and cat heart, as well as tachyzoite type ۱۸۲ III samples.



- ۱۸۳
- **Figure 4. Phylogenetic tree drawn with Mega X software based on the nucleotide sequence of the cytochrome**
- b gene of the protozoan *Toxoplasma gondii*
- ۱۸٦



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Figure 5. Phylogenetic tree drawn with Mega X software based on the nucleotide sequence of Gra20 gene of protozoan *Toxoplasma gondii*

19.

191 **3.5. PCR-RFLP results:** At first, a PCR reaction was performed on *Toxoplasma gondii* type 1 samples,

rooster brain tissue, and cat heart tissue using the Gra6 gene. The Mse1 enzyme was utilized for digesting.

Before enzymatic digestion, all the samples showed a 795bp band upon electrophoresis on an agarose gel

(Figure 6). The enzymatic digestion pattern for each sample showed that the fragments of all three samples

۱۹۰ are the same pattern.



۱۹٦

Figure 6. Electrophoresis of enzymatic digestion (Mse1 enzyme) of samples amplified with Gra6 gene (M: marker 100bp, 1: type I sample before enzymatic digestion, 2: type I sample after enzymatic digestion, 3: cat 199 heart samples before enzymatic digestion, 4: Cat heart sample after enzymatic digestion, 5: rooster brain ۲.. samples before enzymatic digestion, 6: Rooster brain samples after enzymatic digestion)

۲.۱ 4. Discussion

۲.۲ Molecular phylogeny involves the analysis of nucleotide sequences to accomplish objectives such as ۲.۳ identifying the nearest relatives of a microorganism, estimating the time when species diverged, and ۲. ٤ tracking the origin of a gene. In this study, the amplified fragments of different samples were sequenced ۲.0 and the obtained sequencing results were evaluated. The accuracy of nucleotide sequences submitted for ۲.٦ sequencing is crucial when selecting sequences for phylogenetic studies. This study used a two-way ۲.۷ approach to assess the identification of nucleotide sequences. To avoid errors or mistakes, the overlapping ۲۰۸ regions of the sequences were used to select the definitive nucleotide sequence. The nucleotide sequences ۲.٩ of cytochrome b and Gra20 genes were compared with the NCBI and found to be completely similar ۲١. (100%). Consequently, 11 of the 16 nucleotide sequences for cytochrome b and Gra20 genes from the 117 present study were submitted in GenBank.

- ۲۱۲ This study compared the nucleotide sequences of cytochrome b genes and Gra20 gene based on their
- ۲۱۳ positions in the phylogenetic tree. Distinct clusters were observed in the phylogeny tree diagram constructed
- 212 based on the nucleotide sequences of the Gra20 gene. One cluster consisted of isolates from rooster brain
- 110 and cat heart, along with samples of type III. Another cluster contained samples of protozoan types I and
- ۲۱٦ II. In the corresponding phylogenetic tree, the closeness of the Iranian isolates to the French (European) ۲۱۷
- sample was demonstrated.
- ۲۱۸ In a study conducted in China in 2015 by Ning et al. (11) the Gra20 gene was used to examine 16
- ۲۱۹ Toxoplasma gondii isolates from different parts of the world, Toxoplasma gondii types I and II were found
- ۲۲. to be grouped together in a single branch and two close related clusters while building the phylogenetic
- 221 tree. Nevertheless, Toxoplasma gondii type III was categorized into a distinct branch and cluster. In this
- 222 study, the Gra20 gene was introduced as a marker to show genetic mutations in Toxoplasma gondii during
- ۲۲۳ phylogenetic analysis.
- ۲۲٤ Sequencing the cytochrome b region of the mitochondrial genome is a highly valuable technique for
- 220 establishing the evolutionary connection between closely related species (12). The nucleotide sequence of
- 222 the cytochrome b gene was obtained from the NCBI in the present study. In the analysis of the obtained 222 phylogenetic tree, Toxoplasma gondii isolates and samples of protozoan types I, II, and III were placed in
- ۲۲۸ a branch and also in a large cluster. The protozoan Sarcocystis was clustered with Toxoplasma gondii
- 229 isolates, while *Neospora* and *Hammondia* were mapped in a separate clade.
- ۲۳. In Sercundes et al.'s research (2016), apicoplast (pseudo-organelle) and mitochondrial molecular markers ۲۳۱ were used. These researchers used the apicoplast genes clpC and rpoB along with the widely used ۲۳۲ cytochrome b gene for phylogenetic study. The phylogenetic trees obtained showed that Toxoplasma gondii ۲۳۳ and Hammondia heydorni were well differentiated among different strains (RH, CTG, PTG). To evaluate ۲۳٤ the genetic relationships of the Sarcocystidae family, it is recommended to use the three genes mentioned 170 above. Also, in their study, they showed the value of organelle genes in distinguishing the Sarcocystis genus
- ۲۳٦ from the Toxoplasmatinae family (13).
- ۲۳۷ The phylogenetic tree constructed using the cytochrome b gene revealed the proximity of the isolates from
- ۲۳۸ the present study (Iranian isolates) to both the European isolate (French isolate) and the American continent.
- ۲۳۹ The tree constructed using the Gra20 gene revealed that *Toxoplasma gondii* type I tachyzoite showed a
- ۲٤. genetic similarity to French type II, whereas in other isolates from Iran, it had a close genetic relationship
- 251 to French type III tachyzoite.

٢٤٢ In Sercundes et al.'s research, the cytochrome b marker was used to identify the Sarcocystidae family. Their ٢٤٣ research involved amplifying the cytochrome b gene in all samples. Furthermore, following the

- ۲٤٤ amplification of the cytochrome b gene from samples of Sarcocystis, Neospora, and Toxoplasma gondii, a
- 720 phylogenetic tree was constructed, which resulted in the placement of all members of the Sarcocystidae
- 252 family inside a single branch (13). Gjerde et al. (2013) used cytochrome b and cytochrome c oxidase genes
- ۲٤٧ to identify Toxoplasma gondii, Neospora and Hammondia protozoa. Moreover, the phylogenetic tree ۲٤٨
- constructed using these genes revealed that all three protozoa were grouped together in a single cluster. 7 2 9 This finding demonstrates the effectiveness of cytochrome b and cytochrome c oxidase genes in identifying
- 10.
- Toxoplasma gondii, Neospora, and Hammondia protozoa (14).
- 101 Here the Gra20 gene specificity was determined by placing Toxoplasma gondii samples next to Sarcocystis 101 and Neospora samples. The electrophoresis of the PCR product revealed the amplification of the Gra20 100 gene exclusively in Toxoplasma gondii protozoa, while no presence of this gene was detected in the samples 702 of Sarcocystis and Neospora bandi. Based on these results, it can be concluded that the Gra20 gene is 100 specific in separating Toxoplasma protozoa from Sarcocystis and Neospoa (Sarcocystidae family). It is 207 notably that no other studies have been found that examine the use of the Gra20 gene to assess the 101 Toxoplasma gondii, as well as other protozoa from the Sarcocystidae family such as Sarcocystis and ۲٥٨ Neospore. Only Ning et al. (2015) utilized this gene to determine the phylogenetics of these protozoans
- 209 (11).
- ۲٦. The RFLP was used to identify Toxoplasma gondii protozoans in samples. Fazaeli's study (2000) compared 221 the fragment sizes to determine the type of *Toxoplasma gondii* parasite. They performed the RFLP test 222 based on the coding region of the Gra6 gene and using the Mse1 endonuclease to identify three different
- ۲٦٣ protozoan types (10). Norouzi et al. (2016) used the Gra6 marker for the genotyping of Toxoplasma gondii
- 225 protozoa in the blood samples of patients with ocular toxoplasmosis (52 patient blood samples) in Tehran.
- 220 Toxoplasma gondii type III was present in the results obtained from the MseI's enzymatic digestion (15).
- ۲٦٦ The findings of this study indicate that the nucleotide sequence of the Gra20 gene can serve as a reliable 221 method to differentiate Toxoplasma gondii protozoa from related species like Sarcocystis and Neospora. ۲٦٨ The nucleotide sequence of the cytochrome b gene can be utilized as a universal gene for identifying 229 members of the Sarcocystidae family. Several issues were encountered in this investigation, such as limited ۲٧. availability of samples (brain tissue and isospora oocysts in cat feces), challenges in establishing Vero ۲۷۱ culture for tachyzoite propagation, and financial constraints for genetic studies and sequencing. Hence, it ۲۷۲ is recommended to gather additional specimens (isolates) of the Toxoplasma gondii protozoa from other ۲۷۳ regions within the country, and ideally from the Asian countries as well, by establishing communication ۲۷٤ with pertinent centers and organizations and by lessening the imposed limitations. A comprehensive 200 analysis and comparison should be conducted by utilizing the aforementioned genes as well as 272 incorporating additional genes.
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- ۲۷۹ The authors would like to thank for Dr. Mirjalili for the help in preparation of the samples and for helpful ۲۸۰ advice and feedback.
- 111

۲۸۲ **Authors' Contribution**

- ۲۸۳ Study concept and design: Gh. H. and S. N.
- ۲۸٤ Acquisition of data: S. B., A. A., A. SH. and V. N.
- ۲۸٥ Analysis and interpretation of data: Gh. H., S. N. and S. B.

- Drafting of the manuscript: S. B.
- ^{YAV} Critical revision of the manuscript for important intellectual content: Gh. H. and S. N.
- Statistical analysis: Gh. H. and S. B.
- Administrative, technical, and material support: Gh. H. and S. N.
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- ۲۹۱
- **T**97 **Ethics**
- We hereby declare all ethical standards have been respected in preparation of the submitted article.

Y95Conflict of interest statement

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