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

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Abstract

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

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

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 all ways in which infection can be acquired (2). Moreover, the tachyzoite form of the parasite can be

transmitted to the fetus through the placenta. The transmission of *Toxoplasma gondii* infection varies globally, and it is recognized as a zoonotic pathogen. Toxoplasmosis affects approximately 30% of the global population (3).

The pathogenicity of *Toxoplasma gondii* parasite depends on the host's resistance, different strains of the parasite, and antigenic changes of the parasite. *Toxoplasma gondii* antigens include membrane antigens, cytoplasmic antigens, and excretion-secretion antigens as their primary features (4). Excretion-secretion (ES) antigens play a significant role in stimulating the immune system among these antigens. The microneme, rhapterium, and dense granules of the parasite secrete substances that produce ES antigens. The organelles' contents are accountable for cell identification and attachment, formation of the parasitophorous vacuole (PV), parasite proliferation, and intracellular survival (5).

Most of *Toxoplasma gondii* strains comprises of 2 or 3 clonal lineages (types I, II and III), which exist in both animals and humans (6). Furthermore, unique dimorphic allelic compounds as “atypical” are abundantly identified in the *Toxoplasma gondii* genotype which not fitting among the three dominant lineages (7). A commonly employed technique to detect the *Toxoplasma gondii* parasite involves cultivating the parasite in cell culture such as Vero cells. The detection of diseases is achieved through serological methods by measuring the quantity and type of antibodies produced against the parasite in the body. These methods are capable of determining whether the infection is recent or ancient. Polymerase chain reaction (PCR) is the most sensitive and definitive diagnostic method, which involves amplifying millions of copies of a small piece of DNA or RNA. The PCR reaction involves heating the double-stranded DNA and then annealing the primers at a lower temperature. The Taq polymerase enzyme is used to synthesize a new DNA strand. Various types of PCR reactions include nested PCR, RAPD, RFLP, microsatellite 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.

2. Materials and Methods

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

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. 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

continued with 35 cycles including 10 seconds of denaturation at 94°C, 20 seconds of annealing at 61°C for Gra20 gene and 59°C for Cytochrome b gene and 30 seconds of extension at 72°C. The final extension cycle at 72 °C for 5 minutes ended the reaction.

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 PCR product. The PCR product obtained from each sample with cytochrome b genes and Gra20 gene was sent to Kowsar Technology Company for sequencing. MEGA X software was used to evaluate the obtained sequences and create a phylogenetic tree.

Table 1. Sequences of the primers used for the amplification of the genes

Primers	Sequences	Product length (bp)	Ref.
Gra20-outer primer	F: 5′-ATGCATAGCCGGAACTGCGTC-3′	1242	**(11)**
	R: 5′-TCACGCGGGCTTTCTACGG-3′		
Gra20-inner primer	F: 5′-ACAGGAAGAAACGCTACGGG-3′	302	**Designed**
	R: 5′-CCAATTGCTCGATTTGCCGT-3′		
Cytb–outer primer	F: 5′-CGGGCACACCTTGTCTTTTAT-3′	741	**Designed**
	R: 5′-TGGTGTTACGAACCGGTTGAC-3		
Cytb-inner primer	F: 5′-ACTACCGCTTGGATGTCTGG-3′	317	**Designed**
	R: 5′-AAAGGCAACTTTAAGCGCGG-3′		

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 85 bp and 540 bp (10).

3. Results

3.1. PCR results: The PCR analysis was conducted on various DNA samples, including tachyzoite type I, II, and III, rooster and snake brain tissue, cat heart tissue, cat oocyst, and sheep aborted embryo brain tissue. The PCR was performed using the outer primer of cytochrome b, and the resulting DNA fragments were visualized on an agarose gel. Only the samples of type I, type III, and the positive control showed a band of the expected size (740 bp). There were no bands detected in the other samples (Figure 1-A). Another PCR was performed on the PCR product of the previous step with the internal primer of cytochrome b and the expected band of 317 bp was observed on agarose gel in all samples and positive control (Figure 1-B). In the results of PCR performed with the Gra20 outer primer, no bands were observed on the agarose gel except for the positive control. Gra20 internal primer was used in the second PCR reaction, and the desired 302 bp band was observed in all samples (Figure 1-C and 1-D).

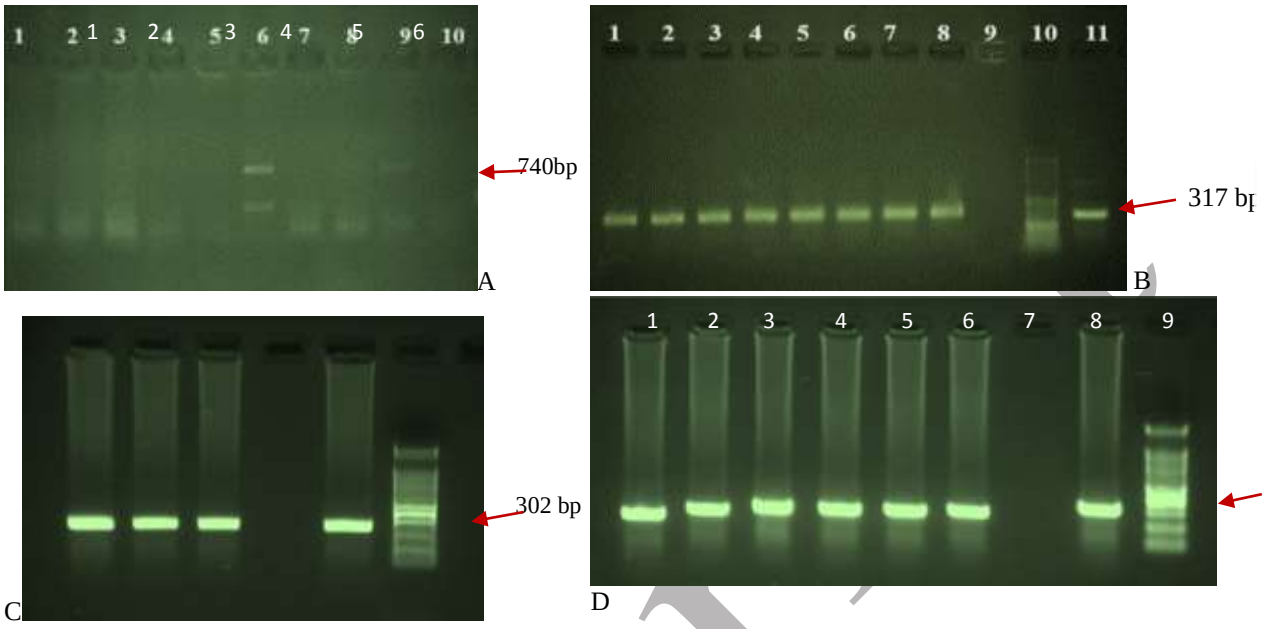

Figure 1. A: PCR product electrophoresis of samples with outer cytochrome b primer (1: Cat heart sample, 2: Rooster brain sample, 3: Snake brain sample, 4: Cat oocyst sample, 5: Sheep fetal brain, 6: Tachyzoite type I, 7: Tachyzoite type II, 8: Tachyzoite type III, 9: Positive control, 10: Negative control).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

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

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

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	*Toxoplasma gondii* 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	*Toxoplasma gondii* isolated from cat heart tissue	Gra20	**MW660541**
11	*Toxoplasma gondii* isolated from rooster brain	Gra20	**MW660542**

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

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

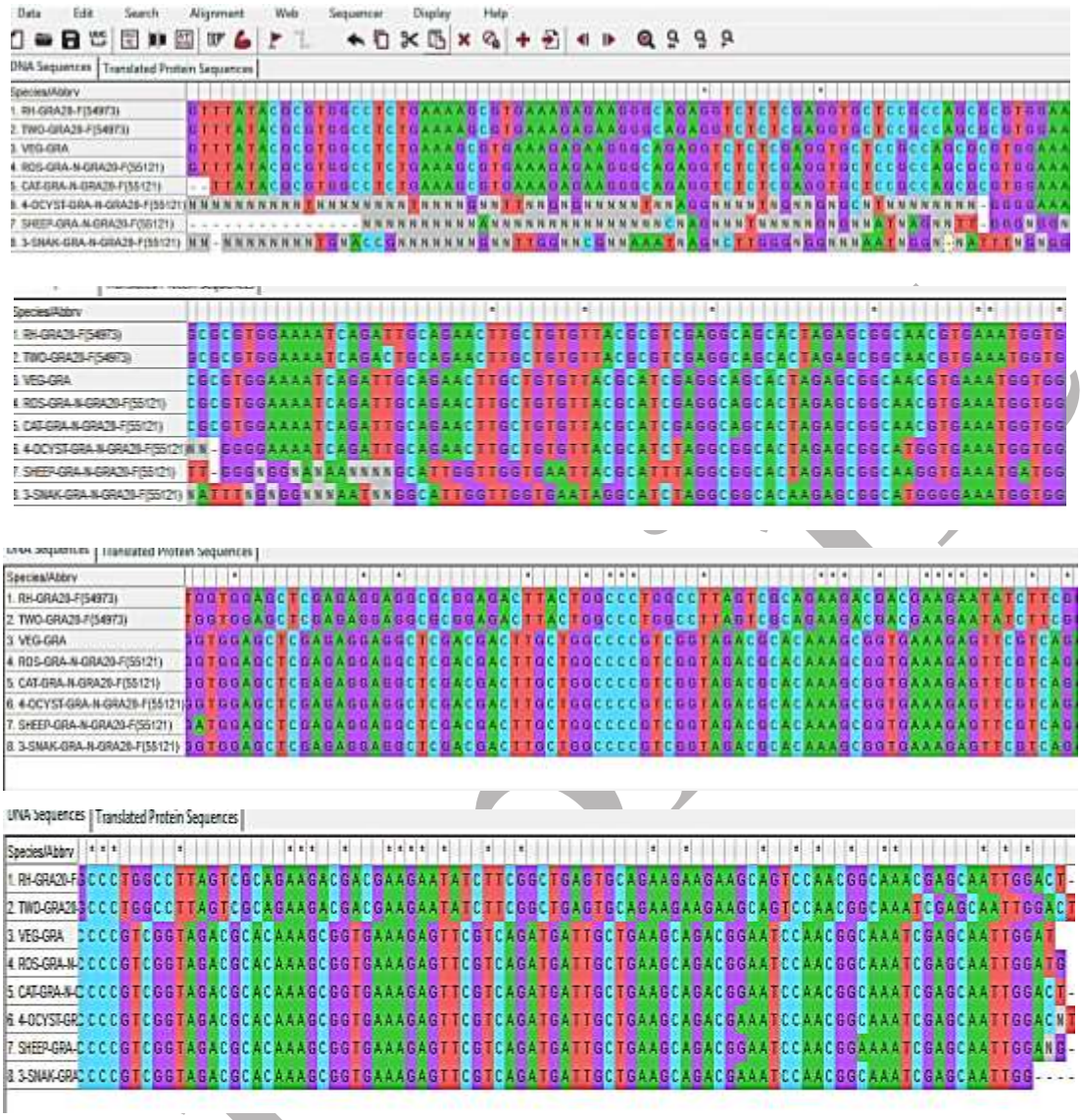

Figure 3. The result of multiple alignment of amplified samples with Gra20 gene by MEGA X software

3.4. Phylogenetic assessment: The phylogenetic tree was constructed using the nucleotide sequences of the cytochrome b and Gra20 genes from both the samples in the current study and those recorded in the databases. The phylogeny tree of the samples from the current study, which were recorded in the NCBI, was constructed using the neighbor-joining method and bootstrap 100, utilizing the Mega X program. In Figure 4, a phylogenetic tree was constructed using the nucleotide sequence of the cytochrome b gene of the *Toxoplasma gondii.* Our study of samples from Iran revealed that they have similarities with samples from France, Norway, and the United States, as well as with the protozoan *Sarcocystis*. They were clustered together in a group. *Neospora* and *Hammondia* were the outgroup. The length of branches is a measure of the genetic distance between different sequences. The genetic distance between the samples in the present study has been calculated as zero, and the largest distance (0.49) has been calculated between the *Hammondia* sample and the other samples. In the phylogenetic tree constructed using the nucleotide

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

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

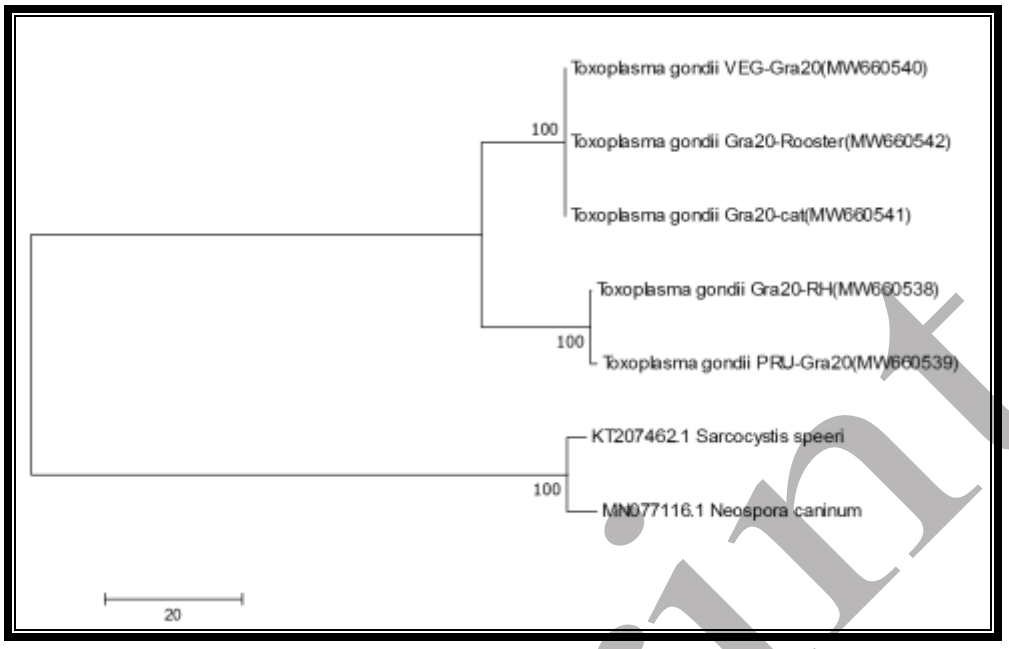

Figure 5. Phylogenetic tree drawn with Mega X software based on the nucleotide sequence of Gra20 gene of protozoan *Toxoplasma gondii*

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 are the same pattern.

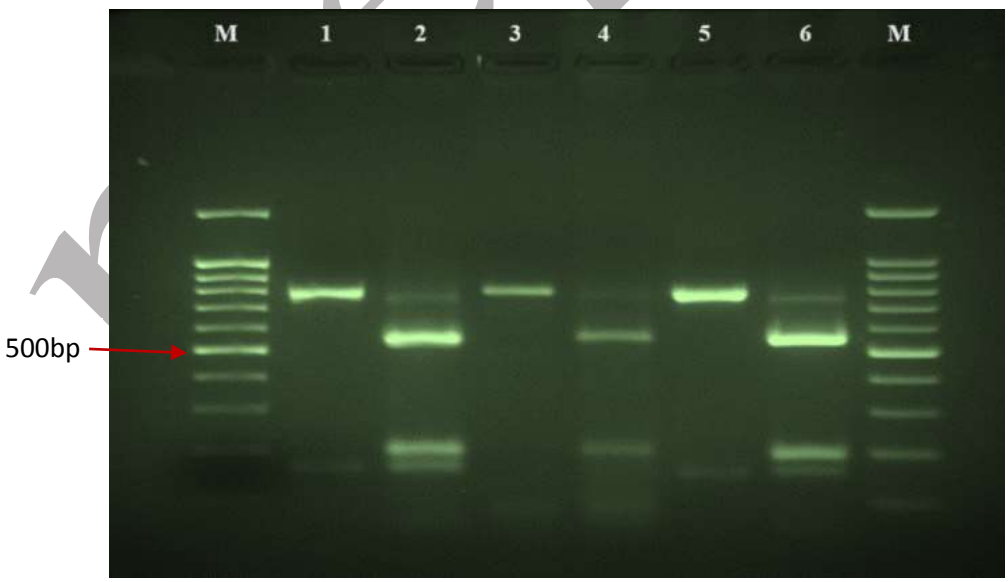

Figure 6. Electrophoresis of enzymatic digestion (Mse1 enzyme) of samples amplified with Gra6 gene (M: marker 100bp, 1: type I sample before enzymatic digestion, 2: type I sample after enzymatic digestion, 3: cat

heart samples before enzymatic digestion, 4: Cat heart sample after enzymatic digestion, 5: rooster brain samples before enzymatic digestion, 6: Rooster brain samples after enzymatic digestion)

4. Discussion

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

This study compared the nucleotide sequences of cytochrome b genes and Gra20 gene based on their positions in the phylogenetic tree. Distinct clusters were observed in the phylogeny tree diagram constructed based on the nucleotide sequences of the Gra20 gene. One cluster consisted of isolates from rooster brain and cat heart, along with samples of type III. Another cluster contained samples of protozoan types I and II. In the corresponding phylogenetic tree, the closeness of the Iranian isolates to the French (European) sample was demonstrated.

In a study conducted in China in 2015 by Ning et al. (11) the Gra20 gene was used to examine 16 *Toxoplasma gondii* isolates from different parts of the world. *Toxoplasma gondii* types I and II were found to be grouped together in a single branch and two close related clusters while building the phylogenetic tree. Nevertheless, *Toxoplasma gondii* type III was categorized into a distinct branch and cluster. In this study, the Gra20 gene was introduced as a marker to show genetic mutations in *Toxoplasma gondii* during phylogenetic analysis.

Sequencing the cytochrome b region of the mitochondrial genome is a highly valuable technique for establishing the evolutionary connection between closely related species (12). The nucleotide sequence of the cytochrome b gene was obtained from the NCBI in the present study. In the analysis of the obtained phylogenetic tree, *Toxoplasma gondii* isolates and samples of protozoan types I, II, and III were placed in a branch and also in a large cluster. The protozoan *Sarcocystis* was clustered with *Toxoplasma gondii* isolates, while *Neospora* and *Hammondia* were mapped in a separate clade.

In Sercundes et al.'s research (2016), apicoplast (pseudo-organelle) and mitochondrial molecular markers were used. These researchers used the apicoplast genes clpC and rpoB along with the widely used cytochrome b gene for phylogenetic study. The phylogenetic trees obtained showed that *Toxoplasma gondii* and *Hammondia heydorni* were well differentiated among different strains (RH, CTG, PTG). To evaluate the genetic relationships of the Sarcocystidae family, it is recommended to use the three genes mentioned above. Also, in their study, they showed the value of organelle genes in distinguishing the *Sarcocystis* genus from the Toxoplasmatinae family (13).

The phylogenetic tree constructed using the cytochrome b gene revealed the proximity of the isolates from the present study (Iranian isolates) to both the European isolate (French isolate) and the American continent. The tree constructed using the Gra20 gene revealed that *Toxoplasma gondii* type I tachyzoite showed a genetic similarity to French type II, whereas in other isolates from Iran, it had a close genetic relationship to French type III tachyzoite.

In Sercundes et al.'s research, the cytochrome b marker was used to identify the Sarcocystidae family. Their research involved amplifying the cytochrome b gene in all samples. Furthermore, following the amplification of the cytochrome b gene from samples of *Sarcocystis*, *Neospora*, and *Toxoplasma gondii*, a phylogenetic tree was constructed, which resulted in the placement of all members of the Sarcocystidae family inside a single branch (13). Gjerde et al. (2013) used cytochrome b and cytochrome c oxidase genes to identify *Toxoplasma gondii*, *Neospora* and *Hammondia* protozoa. Moreover, the phylogenetic tree constructed using these genes revealed that all three protozoa were grouped together in a single cluster. This finding demonstrates the effectiveness of cytochrome b and cytochrome c oxidase genes in identifying *Toxoplasma gondii*, *Neospora*, and *Hammondia* protozoa (14).

Here the Gra20 gene specificity was determined by placing *Toxoplasma gondii* samples next to *Sarcocystis* and *Neospora* samples. The electrophoresis of the PCR product revealed the amplification of the Gra20 gene exclusively in *Toxoplasma gondii* protozoa, while no presence of this gene was detected in the samples of *Sarcocystis* and *Neospora bandi*. Based on these results, it can be concluded that the Gra20 gene is specific in separating Toxoplasma protozoa from *Sarcocystis* and *Neospoa* (Sarcocystidae family). It is notably that no other studies have been found that examine the use of the Gra20 gene to assess the *Toxoplasma gondii*, as well as other protozoa from the Sarcocystidae family such as *Sarcocystis* and *Neospore*. Only Ning et al. (2015) utilized this gene to determine the phylogenetics of these protozoans (11).

The RFLP was used to identify *Toxoplasma gondii* protozoans in samples. Fazaeli's study (2000) compared the fragment sizes to determine the type of *Toxoplasma gondii* parasite. They performed the RFLP test based on the coding region of the Gra6 gene and using the Mse1 endonuclease to identify three different protozoan types (10). Norouzi et al. (2016) used the Gra6 marker for the genotyping of *Toxoplasma gondii* protozoa in the blood samples of patients with ocular toxoplasmosis (52 patient blood samples) in Tehran. *Toxoplasma gondii* type III was present in the results obtained from the MseI's enzymatic digestion (15).

The findings of this study indicate that the nucleotide sequence of the Gra20 gene can serve as a reliable method to differentiate *Toxoplasma gondii* protozoa from related species like *Sarcocystis* and *Neospora*. The nucleotide sequence of the cytochrome b gene can be utilized as a universal gene for identifying members of the Sarcocystidae family. Several issues were encountered in this investigation, such as limited availability of samples (brain tissue and isospora oocysts in cat feces), challenges in establishing Vero culture for tachyzoite propagation, and financial constraints for genetic studies and sequencing. Hence, it is recommended to gather additional specimens (isolates) of the *Toxoplasma gondii* protozoa from other regions within the country, and ideally from the Asian countries as well, by establishing communication with pertinent centers and organizations and by lessening the imposed limitations. A comprehensive analysis and comparison should be conducted by utilizing the aforementioned genes as well as incorporating additional genes.

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Authors' Contribution

Study concept and design: Gh. H. and S. N.

Acquisition of data: S. B. ,A. A. ,A. SH. and V. N.

Analysis and interpretation of data: Gh. H. ,S. N. and S. B.

Drafting of the manuscript: S. B.
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.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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