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Original Article

Phylogenetic study of the 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 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 sequences of 11 samples were acceptable and submitted to the NCBI database. MEGA X software was utilized to create the phylogenetic tree. In addition, genetic diversity was studied using the RFLP-PCR assay for the 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 phylogenetic tree, there was a closer relationship between Iranian isolates of *Toxoplasma gondii* and the French strain of *Toxoplasma gondii*. Type I was determined as the genetic type of the protozoan *Toxoplasma gondii* isolated from Iran. Based on the results, the cytochrome b gene was considered 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*.

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1. Introduction

A broad range of warm-blooded vertebrates are infected by *Toxoplasma gondii*, a protozoan that is obligately intracellular and belongs to the phylum Apicomplexa (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 in the parasite. *Toxoplasma gondii* antigens include membrane antigens, cytoplasmic antigens, and excretion-secretion antigens as their primary features (4). Among these antigens, excretion-secretion (ES) antigens play a significant role in stimulating the immune system. The microneme, rhapterium, and dense granules of the parasite secrete substances that produce ES antigens. The organelles' contents are responsible for cell identification and attachment, formation of the parasitophorous vacuole (PV), parasite proliferation, and intracellular survival (5).

Most *Toxoplasma gondii* strains comprise 2 or 3 clonal lineages (types I, II and III), which exist in both animals and humans (6). Furthermore, unique dimorphic allelic compounds referred to as "atypical" are abundantly identified in the *Toxoplasma gondii* genotype that do not fit 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 the disease 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 long-lasting. 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 real-Time PCR (8). The aim of this study was 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), six samples of cat heart, liver, and kidney, two samples of brain tissue from aborted sheep, and two samples of rooster brain were obtained from animal husbandry, slaughterhouses, and veterinary clinics. Two samples of snake brain and one sample of the standard RH strain (Type I) were prepared from the 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 Sheather's sugar solution flotation method (9). Brain samples from aborted sheep fetuses, 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 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. It then continued with 35 cycles, including 10 seconds of denaturation at 94°C, 20 seconds of annealing at 61°C for the Gra20 gene and 59°C for the 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. Gel electrophoresis was performed on 1.5% agarose to assess the PCR product. The PCR product obtained from each sample with cytochrome b and GRA20 genes was sent to Kowsar Technology Company for sequencing. MEGA X software was used to evaluate the obtained sequences and create a phylogenetic tree.

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 the 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. The Type II strain is divided into fragments of 640 bp and 85 bp, while the 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 Type I, II, and III tachyzoite, rooster and snake brain tissue, cat heart tissue, cat oocysts, and brain tissue from aborted sheep embryos. 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 from the previous step using the internal primer of cytochrome b, and the expected band of 317 bp was observed on agarose gel in all samples and the 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. The 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).

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) databank 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 b 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 using BLAST against sequences in the NCBI. The Type I sample showed a similarity of 99.67% with the ME49 strain gene. The Type II sample showed a 100% similarity with the ME49 strain gene, while the Type III sample showed a 100% similarity with the VEG strain gene. Also, 100% similarity with the VEG strain gene was observed in five nucleotide sequences from rooster brain, snake brain, sheep fetal brain, cat oocyst, and cat heart.

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 sequence reading. The alignment results of cytochrome b sequences are 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 sequences of three samples — snake brain, fetal sheep brain, and cat oocyst — have 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 comparison.

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 phylogenetic tree of the samples from the current study, which were recorded in the NCBI, was constructed using the neighbor-joining method with 100 bootstrap replicates,

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´ R: 5´-TCACGCGGGCTTTCTACGG-3´	1242	(11)
Gra20-inner primer	F: 5´-ACAGGAAGAAACGCTACGGG-3´ R: 5´-CCAATTGCTCGATTTGCCGT-3´	302	Designed
Cytb–outer primer	F: 5´-CGGGCACACCTTGTCTTTTAT-3´ R: 5´-TGGTGTTACGAACCGGTTGAC-3	741	Designed
Cytb-inner primer	F: 5´-ACTACCGCTTGGATGTCTGG-3´ R: 5´-AAAGGCAACTTTAAGCGCGG-3´	317	Designed

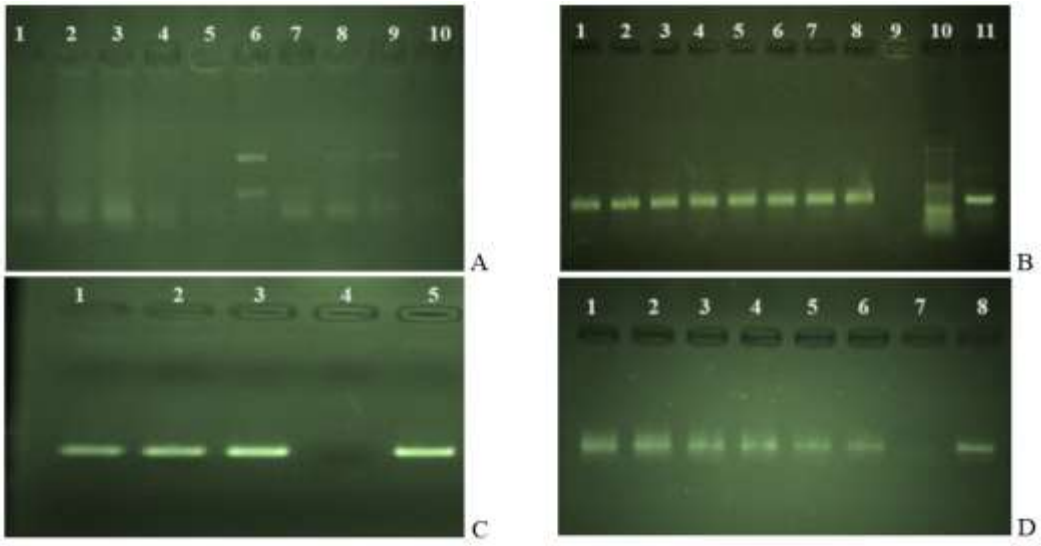


Figure 1. A: PCR product electrophoresis of samples with outer cytochrome b primer (1: Cat heart sample, 2: Rooster brain sample, 3: Snake brain sample, 4: Cat oocyst sample, 5: Sheep fetal brain, 6: Tachyzoite type I, 7: Tachyzoite type II, 8: Tachyzoite type III, 9: Positive control, 10: Negative control).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

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

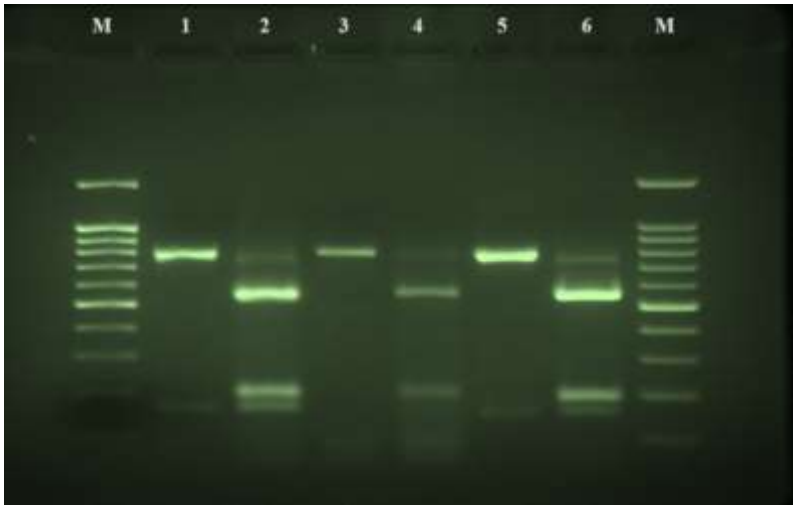
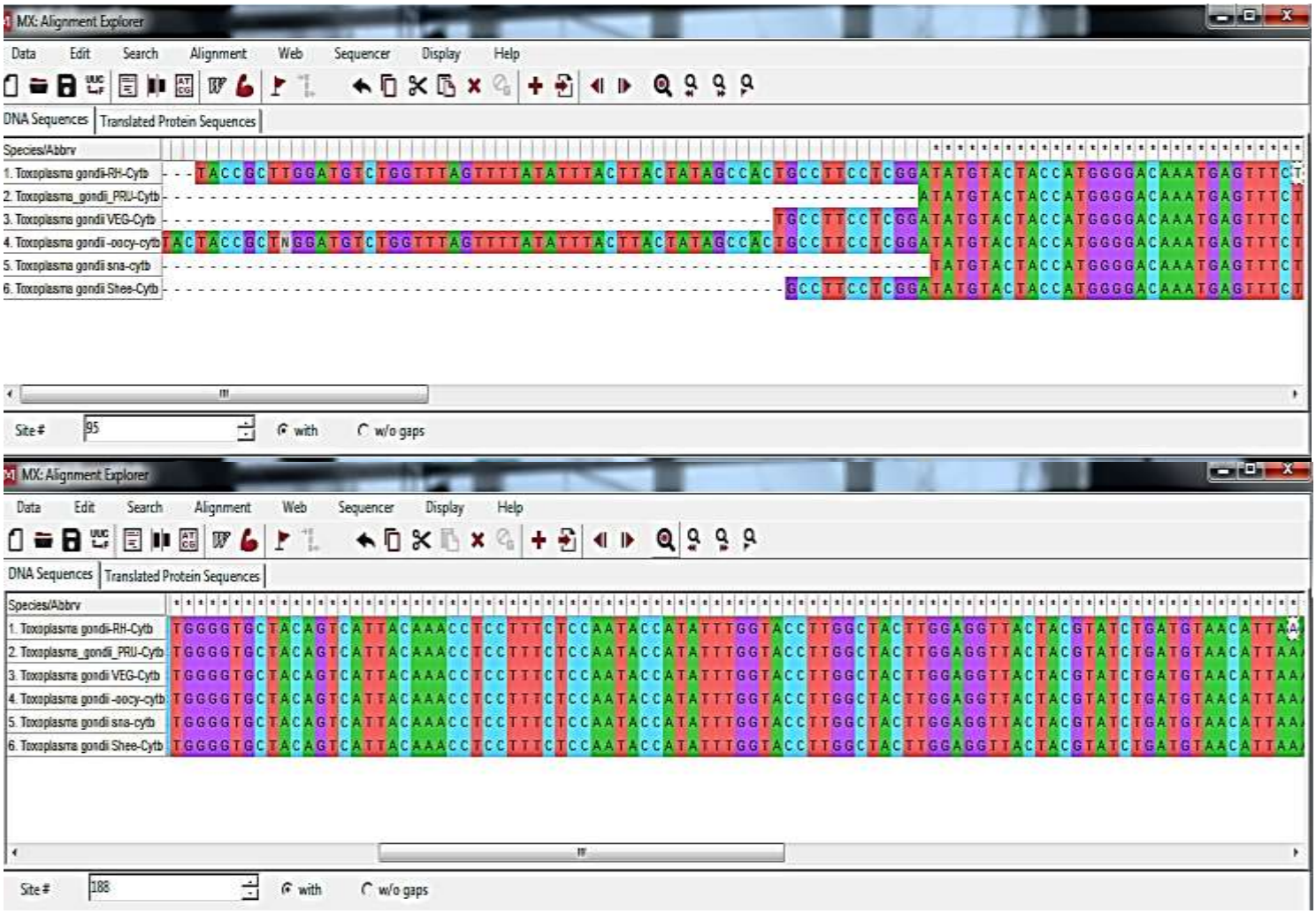


Figure 2. Electrophoresis of enzymatic digestion (Mse1 enzyme) of samples amplified with Gra6 gene (M: marker 100bp, 1: type I sample before enzymatic digestion, 2: type I sample after enzymatic digestion, 3: cat heart samples before enzymatic digestion, 4: Cat heart sample after enzymatic digestion,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



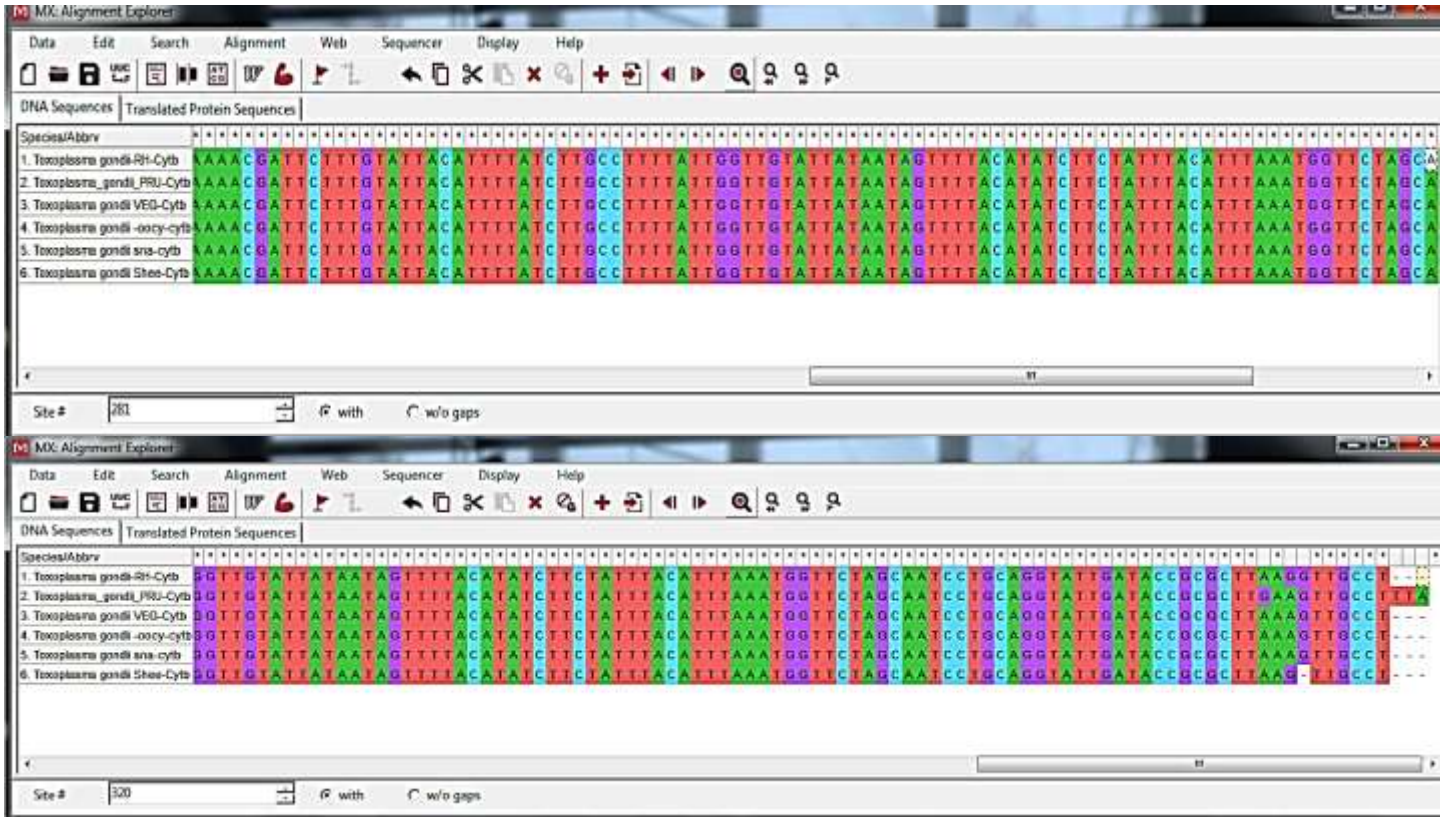


Figure 3. The result of multiple alignment of amplified samples with cytochrome b gene by MEGA X software.

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 outgroups. The length of branches is a measure of the genetic distance between different sequences.

The genetic distance between the samples in the present study was calculated as zero, and the largest distance (0.49) was 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 Type II tachyzoite samples were located together in a separate cluster with high homology. In the tree topology, the protozoa *Neospora* and *Sarcocystis* were considered 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 Type II tachyzoite samples was found to be zero. In general, there was a difference and gap between *Toxoplasma gondii* Type I and Type II tachyzoite samples extracted from rooster brain and cat heart, as well as tachyzoite Type III samples.

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 digestion. Before enzymatic digestion, all the samples showed a 795 bp band upon electrophoresis on an agarose gel (Figure 6). The enzymatic digestion pattern for each sample showed that the fragments of all three samples followed the same pattern.

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

Figure 4. The result of multiple alignment of amplified samples with Gra20 gene by MEGA X software.

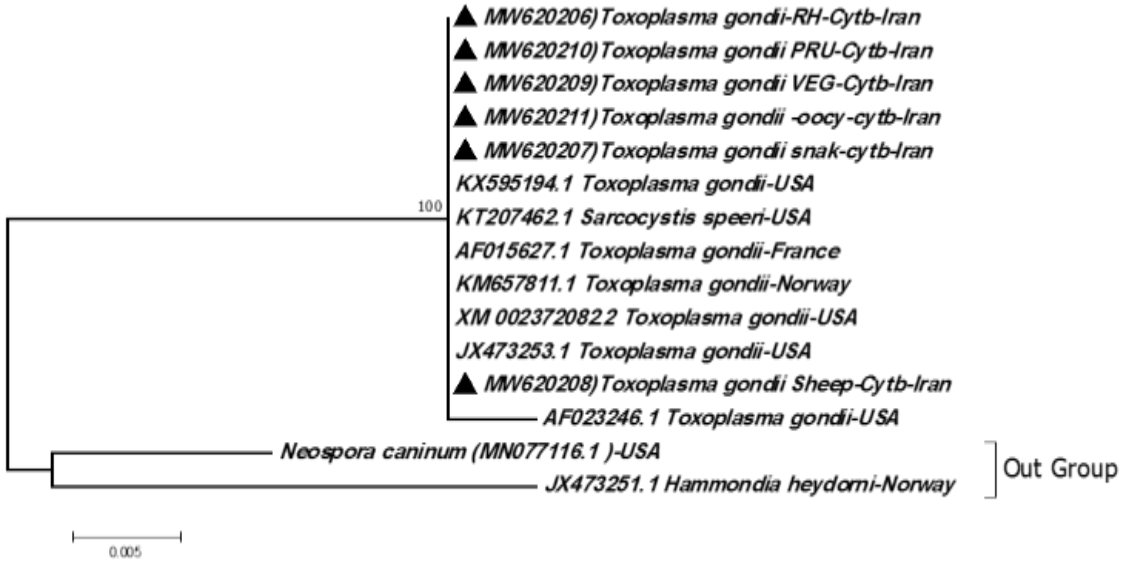


Figure 5. Phylogenetic tree drawn with Mega X software based on the nucleotide sequence of the cytochrome b gene of the protozoan *Toxoplasma gondii*

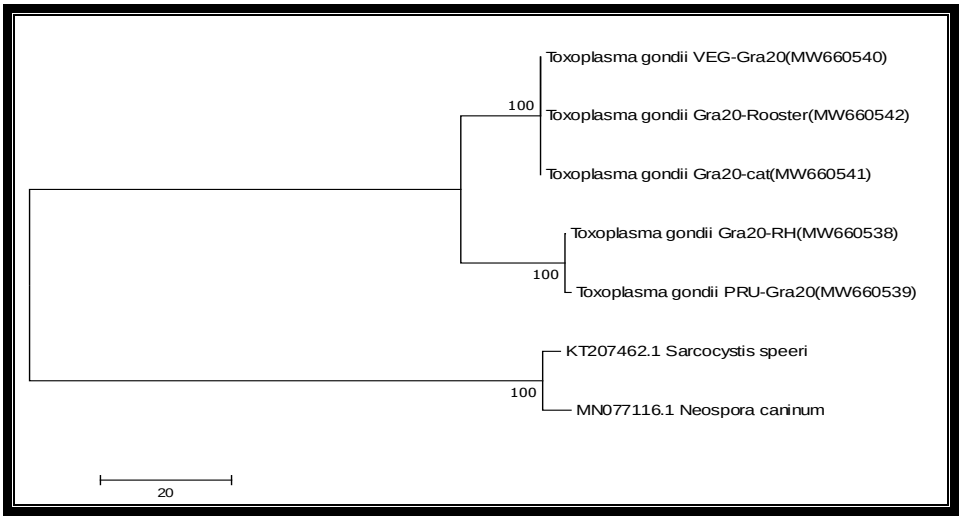


Figure 6. Phylogenetic tree drawn with Mega X software based on the nucleotide sequence of Gra20 gene of protozoan *Toxoplasma gondii*.

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, the overlapping regions of the sequences were used to select the definitive nucleotide sequence. The nucleotide sequences of the cytochrome b and GRA20 genes were compared with those in the NCBI database and found to be completely similar (100%). Consequently, 11 of the 16 nucleotide sequences for the cytochrome b and GRA20 genes from the present study were submitted to Gen Bank.

This study compared the nucleotide sequences of the cytochrome b and GRA20 genes based on their positions in the phylogenetic tree. Distinct clusters were observed in the phylogenetic tree diagram constructed from the nucleotide sequences of the GRA20 gene. One cluster consisted of isolates from rooster brain and cat heart, along with Type III samples. 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 closely related clusters during the construction of 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 connections between closely related species (12). In the present study, the nucleotide sequence of the cytochrome b gene was obtained from the NCBI database. In the analysis of the obtained phylogenetic tree, *Toxoplasma gondii* isolates and protozoan Types I, II, and III samples of 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 to 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 resulting phylogenetic trees showed that *Toxoplasma gondii* and *Hammondia heydorni* were well differentiated among different strains (RH, CTG, PTG). To evaluate the genetic relationships within the Sarcocystidae family, it is recommended to use the three genes mentioned above.

Also, their study 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 those from the American continent. The tree constructed using the GRA20 gene revealed that *Toxoplasma gondii* Type I tachyzoite showed genetic similarity to French Type II, whereas other Iranian isolates demonstrated a close genetic relationship to the French Type III tachyzoite. In Sercundes et al (2016), research the cytochrome b marker was used to identify members of the Sarcocystidae family. Their research involved amplifying the cytochrome b gene in all samples. Furthermore, following amplification of the cytochrome b gene from samples of *Sarcocystis*, *Neospora*, and *Toxoplasma gondii*, a phylogenetic tree was constructed, which placed all members of the Sarcocystidae family within a single branch (13). Gjerde et al. (2013) used the 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. Electrophoresis of the PCR product revealed the amplification of the GRA20 gene exclusively in *Toxoplasma gondii* protozoa, with no detectable presence of this gene in *Sarcocystis* and *Neospora bandi* samples. Based on these results, it can be concluded that the GRA20 gene is specific in separating Toxoplasma protozoa from *Sarcocystis* and *Neospoa* (Sarcocystidae family). Notably, no other studies have been found that examine the use of the GRA20 gene to assess *Toxoplasma gondii* alongside other members of the Sarcocystidae family, such as *Sarcocystis* and *Neospore*. Only Ning et al. (2015) utilized this gene to determine the phylogenetics of these protozoans (11).

RFLP analysis was used to identify *Toxoplasma gondii* protozoans in the samples. Fazaeli's study (2000) compared fragment sizes to determine the type of *Toxoplasma gondii* parasite. They performed The RFLP test was performed based on the coding region of the GRA6 gene and the Mse1 endonuclease, to identify three different protozoan types (10). Norouzi et al. (2016) used the Gra6 marker for genotyping *Toxoplasma gondii* protozoa in blood samples from patients with ocular toxoplasmosis (52 patient blood samples) in Tehran. The results obtained from enzymatic digestion with MseI revealed the presence of *Toxoplasma gondii* Type III (15). The findings of this study indicate that the nucleotide sequence of the GRA20 gene serve as a reliable method for differentiating *Toxoplasma gondii* protozoa from related species such as *Sarcocystis* and *Neospora*. The nucleotide sequence of the cytochrome b gene can be utilized as a universal genetic marker for identifying members of the Sarcocystidae family.

Several challenges were encountered during this investigation, including limited availability of samples (brain tissue and isospora oocysts from cat feces), difficulties in establishing Vero cell cultures 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 across the country, and ideally from the Asian countries, through collaboration with relevant research centers and organizations, and by lessening the imposed limitations. A comprehensive analysis and comparison should be conducted using the aforementioned genes along with additional molecular markers.

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Authors' Contribution

Study concept and design: Gh. H, S. N.
Acquisition of data: S. B, A. A, A. SH, V. N.
Analysis and interpretation of data: Gh. H, S. N, S. B.
Drafting of the manuscript: S. B.
Critical revision of the manuscript for important intellectual content: Gh. H, S. N.
Statistical analysis: Gh. H, S. B.
Administrative, technical, and material support: Gh. H, S. N.

Ethics

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

Conflict of Interest

The authors declare that they have no conflict of interest.

Data Availability

The data that support the findings of this study are available on request from the corresponding author.

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