High prevalence of Toxoplasma gondii infection in ovine aborted fetuses in Gilan Province, Iran: Molecular detection and genotype characterization

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Abstract

Introduction: Sheep farming is one of the most important economic aspects of the livestock industry in Gilan province, Iran. Toxoplasmosis is a prevalent zoonotic disease and a major cause of abortion, congenital infection, and stillbirth in animals and humans. Previously, it was assumed that sheep are mostly infected with *Toxoplasma gondii* (*T. gondii*) after birth. Nevertheless, recent studies have shown that *T. gondii* congenital transmission is more prevalent than previously speculated. Therefore, determining the genotypes of this parasite in the intermediate host plays an important role in human infections and prevention programs. This cross-sectional study aimed to determine the *T. gondii* genetic diversity in aborted ovine fetuses during the lambing season (2018-2019) in Gilan province, Iran.
Materials and Methods: Molecular detection of *T. gondii* was performed in 44 brain tissue samples, collected from aborted ovine fetuses, using a nested polymerase chain reaction (nested-PCR) assay, to target the *GRA6* gene. Also, the nested-PCR products belonging to the *GRA6*-positive samples underwent genotyping with the help of *Tru1I* (*MseI*) restriction enzyme using the PCR-restriction fragment length polymorphism (PCR-RFLP) method to classify strains into one of the three major lineages of *T. gondii.*
Results: The results revealed infection in 30 (68.18%) out of 44 brain samples of aborted ovine fetuses, based on the nested-PCR assay with *GRA6* gene. Also, the PCR-RFLP results demonstrated the predominance of type II *T. gondii* in all of the isolates.
Conclusion: Overall, the present results revealed the high incidence of *T. gondii* infection through congenital transmission.This is the first molecular detection and genotyping of *T. gondii* in ovine aborted fetuses in Gilan Province, Iran.

Keywords: Toxoplasmosis, Sheep, Abortion, Genotyping, Iran

Introduction

Toxoplasmosis is a highly prevalent zoonotic disease, caused by an obligate intracellular protozoan, called *Toxoplasma gondii*, with a worldwide distribution (1). It is usually asymptomatic in individuals with a healthy immune system, but can be a major life-threatening risk among immunocompromised patients, including

HIV and AIDS patients, organ recipients, and cancer patients (2). This disease may result in abortion, stillbirth, and congenital infection among humans and animals and cause various health-related and economic problems in animal husbandry (3).

The *T. gondii* sexual cycle is limited to feline hosts, while the asexual cycle occurs in humans and other intermediate hosts. The produced oocysts are excreted through the cat feces and remain resistant in the environment for 12 to 18 months, depending on the weather conditions (4). Sheep become infected by ingesting sporulated oocysts, as well as congenital transmission through the placenta. Previously, it was assumed that sheep mostly acquired *T. gondii* infection after birth. However, recently, researchers have concluded that *T. gondii* transmission in sheep through the placenta may be more prevalent than previously speculated (5).

Tachyzoites may cross the placenta and enter the growing fetus with various consequences, depending on the stage of pregnancy (6, 7). Acquisition of infection during early pregnancy (before 50 days) can cause death and reabsorption of the fetus, while infections acquired around the middle months of pregnancy (70-90 days) are associated with a high risk of abortion or stillbirth. Also, If infection with *T. gondii* occurs in the late months of pregnancy (>110 days), ewes may give birth to healthy, congenitally infected (8).

The *T. gondii* strain is one of the important factors in the severity of toxoplasmosis. *Toxoplasma gondii* has three main strains with different patterns of pathogenesis. The highest severity of toxoplasmosis is related to type I *T. gondii* strains, causing severe infections in humans. The RH strain is also one of the most well-known type I strains. Type II strains are common among animals and humans, and often cause chronic diseases, especially if the host's immune system is compromised. In previous studies, these strains were isolated from patients with congenital and ocular toxoplasmosis. Generally, infections caused by type III strains of *T. gondii* are less severe than those caused by type I and type II strains and are more common in birds (9). Moreover, some atypical strains have been identified, To better represent the patterns do not belong to any strain types (I, II, and III) of *T. gondii*. Infections caused by atypical strains are more severe and mostly reported from South America (10).

Recently, researchers have applied several diagnostic molecular techniques, including polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay, multi-locus enzyme electrophoresis, multi-locus DNA sequencing, and microsatellite markers for *T. gondii* genotyping. Previous studies mostly analyzed genetic markers with the use of multi-locus PCR-RFLP and microsatellite markers. However, DNA extracted from parasites may be insufficient and fail to produce the desired result. Among various markers, the dense granule protein gene (*GRA6* gene) functions as a single copy gene with a higher polymorphic rate than other markers and can distinguish three different types of *T. gondii* genotypes, using PCR-RFLP assay with *Tru1I* (*MseI*) enzyme (11).

In Iran, the overall estimate of toxoplasmosis prevalence among sheep is 31% (12), and few studies have been performed on *T. gondii* genotyping among these animals. Since identification of parasite genotypes in intermediate hosts plays an important role in human infections, it is important to determine the biological characteristics of these parasites and develop preventive programs, such as DNA vaccination. Therefore, the present study aimed at genotyping and molecularly detecting *T. gondii* strains in aborted ovine fetuses in Gilan Province, Northern Iran.

Materials and Methods

Study Area

Gilan is a northern province in Iran, with a population of approximately 2,500,000 (13). It is located at 37° 27' north latitude and 49° 58' east longitude from the meridian. Its altitude above sea level is variable in different regions, and its area is 14,711 km^2, with an average annual rainfall of 1275 mm. In terms of geographical location, Gilan is divided into three regions: plain with a mild climate, hillside with a semi-humid climate, and highlands with a cold alpine climate(14).

Sample Collection

This cross-sectional study was performed on 44 brain tissue samples of aborted ovine fetuses Which were collected from sheep herds of different areas marked in of Gilan province during 2018-2019. To sample the brains of aborted fetuses, after disinfecting the skull area with 70% alcohol and separating the skin, an incision was made in the frontal bone, using sterile scalpel. Next, 1cm^3 of the brain frontal lobe was separated and placed in a microtube. After labeling the samples, they were kept in a freezer at -20°C and stored until transfer to the central laboratory university of Guilan for DNA extraction and PCR assays.

DNA Extraction

To extract DNA from the brain tissues of aborted ovine fetuses, the Molecular Biological System Transfer (MBST) Genomic DNA Kit (Institute of Molecular and Biological Transmission Systems, Tehran, Iran) was used, according to the manufacturer's instructions.

DNA Purity Quantitation and Determination

The accuracy of DNA extraction was quantified at 260 and 280 nm by a spectrophotometer (Epoch 2, BioTek) and verified, using B-actin sense primer: 5'-ACCCACACGGTGCCCATCTA-3' and B-actin antisense primer: 5'-CGGAACCGCTCATTGCC-3' sequences via agarose gel electrophoresis. The cycling conditions are presented in Table 1.

Table 1. PCR cycling conditions for determination of purity of DNA extraction.

Reaction stage	Time and temperature	Number of cycles
Initial denaturation	5 min, 94°C	1
Denaturation	30 s, 94°C	35
Annealing	30 s, 57°C	35
Extension	30 s, 72°C	35
Final extension	5 min, 72°C	1

T. gondii Infection Detection with Nested-PCR Assay

The PCR assay was performed with the use of a *T. gondii* primer pair: *GAR6*-ex-F1: 5'-ATTTGTGTTTCCGAGCAGGT-3' and *GAR6*-ex-R1: 5'-GCACCTTCGCTTGTGGTT-3'. Next, nested-PCR was conducted with specific primers (*GAR6*-int-F2: 5'-TTTCCGAGCAGGTGACCT-3' and *GAR6*-int-R2: 5'-TCGCCGAAGAGTTGACATAG-3')(15, 16). Amplification was carried out using a reaction mixture with a final 20 µL volume, containing 1.5 mM $MgCl_2$ (Cat. No.: A180301, Ampliqon, Denmark), 10 µL Taq DNA Polymerase 2X Master Mix Red, 6 µL distilled water, 2 µL template DNA, and 10 pmol of each primer.

The nested-PCR assay made use of one µL of the first-round PCR product as the template. One negative control (i.e., double-distilled water) as well as one positive control (i.e.,

DNA extracted from the *T. gondii* RH strain) were added to each reaction. Amplification was carried out as follows: a five-minute initial denaturation at 94°C, a 30-second denaturation with 35 cycles at 94°C, a 30-second annealing at 56°C in the first round and at 58°C in the nested-PCR assay, a 30-second extension at 72°C, and a final five-minute extension at 72°C. After each PCR assay ended, nested-PCR products (100 bp DNA ladder) with an amount of 5 µL underwent electrophoresis using agarose gel (1.5%), staining using safe stain (Sinaclon, Iran) with Tris-Borate-EDTA (TBE) buffer, and visualization using a UV transilluminator (17).

Genotyping of *T. gondii* Positive Samples with PCR-RFLP Assay

Genotypes of *T. gondii* positive samples were established, using *GRA6* marker by the PCR-RFLP method(1). Briefly, nested-PCR products belonging to the *GRA6*-positive samples were digested with the use of the *Tru1I* (*MseI*) restriction enzyme (Cat. No.: ER0981, Thermo Fisher Scientific, USA)(15, 18), according to the manufacturer's protocol. The products underwent digestion using the reaction mixture with an ultimate volume of 15.5 µL. The mixture contained nested-PCR products with the amount of 5 µL, Tru1I enzyme with the amount of 0.5 µL, 10X Buffer R with the amount of 1 µL, and nuclease-free water with the amount of 9 µL. The reaction mixture was spun down for a short period of time, and subsequently, was incubated for three hours at 65°C. Finally, 10 µL of the digested products underwent electrophoresis on 3% agarose gel using TBE buffer and was visualized under a UV transilluminator.

This study was ethically approved by the Ethics Committee of the Science and Research Branch of Islamic Azad University of Tehran, Iran. We declare that all stages of this study were in accordance with the ethical standards.

Results

Detection of *T. gondii* DNA with Nested-PCR Assay

Based on the results, *T. gondii* infection with *GRA6* gene was detected in 68.18% (30/44) of brain tissue samples, collected from aborted ovine fetuses. The positive samples in the nested-PCR method showed 344-bp fragments (Figure 1).

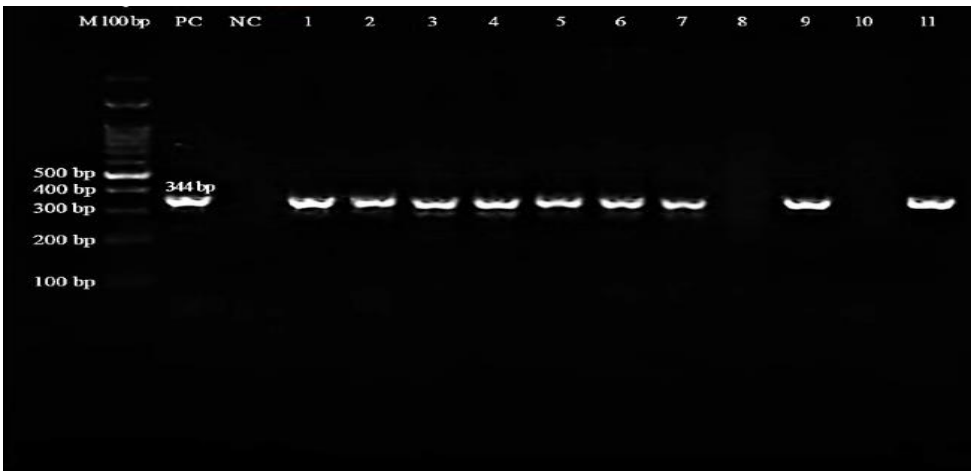

Figure 1. The nested-PCR products of *T. gondii* samples with *GRA6* gene. M: 100-bp DNA marker; PC: positive control; NC: negative control; lanes 1-6, 9 & 11: positive samples; and lanes 8 & 10: negative samples.

Genotyping of Sheep Isolates by *GRA6* Gene

All 30 positive isolates were genotyped, using the PCR-RFLP method. The *GRA6*-positive nested-PCR products underwent digestion with the *Tru1I* (*MseI*) restriction enzyme, and then, the genotypes were detected, based on the digestion patterns. To better represent the genotype patterns, *GRA6* sequences were obtained from the GenBank and the online NEBcutter website (http://nc2.neb.com/NEBcutter2/), which belonged to RH type I, ME49 type II, and NED type III as three well-known *T. gondii* types (Figure 2)(16) .

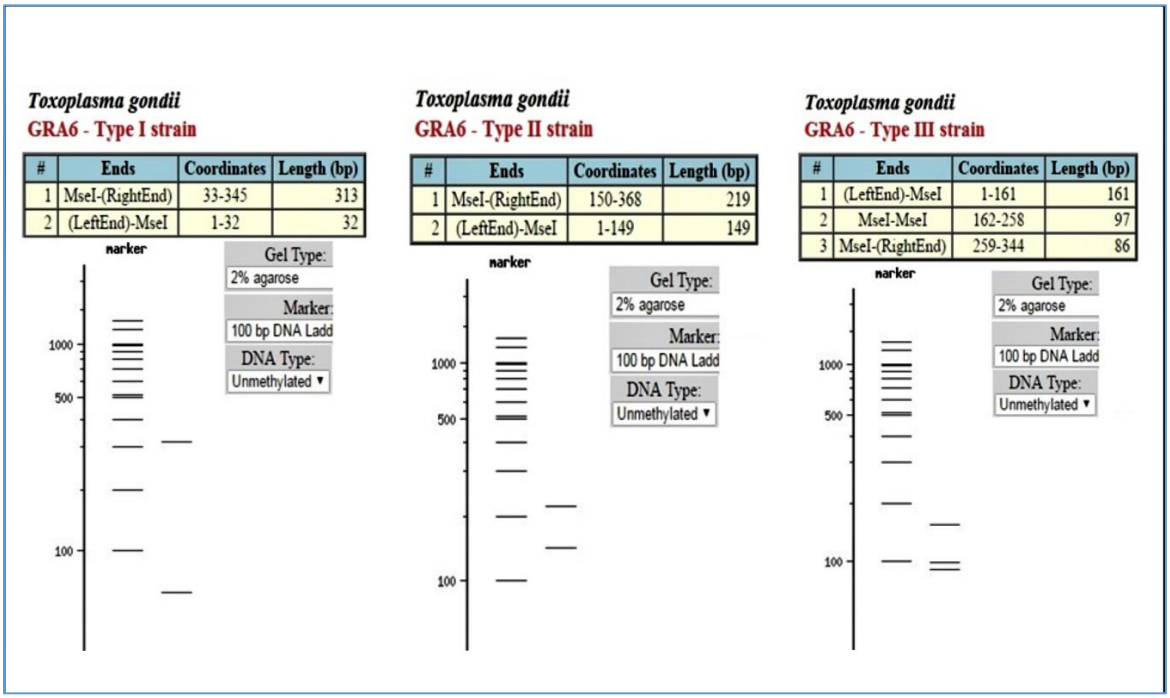

Figure 2. The patterns of *T. gondii* genotypes, *GRA6* sequences were obtained from the GenBank and the online NEBcutter website (http://nc2.neb.com/NEBcutter2/), which belonged to RH type I, ME49 type II, and NED type III as three well-known *T. gondii* types.

The DNA fragments of all 30 positive samples was cleaved from two regions by *Tru1I* (*MseI*) restriction enzyme, which was similar to the digestion pattern of type II genotypes (Figure 3).

Discussion

Toxoplasmosis is a significant reason for abortion among Iranian sheep, causing significant damage to the country's animal husbandry industry (19, 20). According to previous studies, brain tissue is one of the most susceptible tissues to the formation of *T. gondii* cysts (21). Therefore, in this study, we aimed to diagnose toxoplasmosis in the brain tissues of sheep.

In the present study, which was performed on brain samples belonging to some aborted ovine fetuses, the prevalence of *T. gondii* infection was 68.18% (30/44), based on the molecular nested-PCR method.

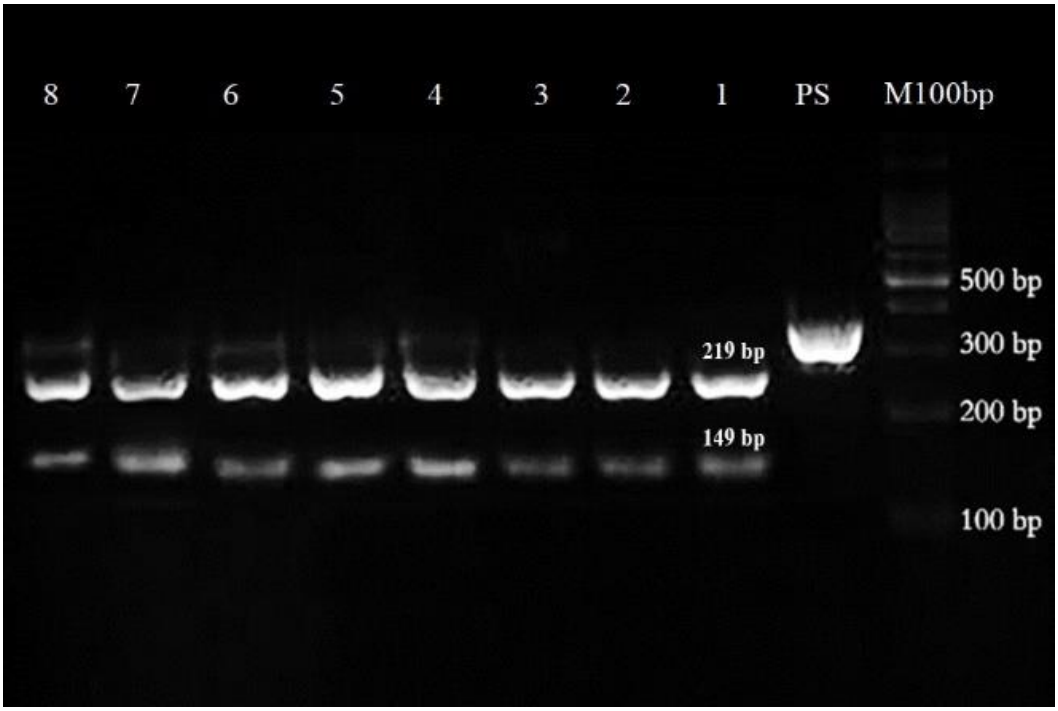

Figure 3. The PCR products undergoing digestion with the *Tru1I* (*MseI*) restriction enzyme using agarose gel electrophoresis. M: DNA marker of 100-bp; PS: positive samples undigested; lanes 1-8: type II *T. gondii* genotype.

The results of the present study are consistent with the study of Habibi et al. In Qazvin and Moazeni Jula et al. In Tabriz, which were performed on the brain of ovine aborted fetuses by nested PCR method and showed 66% and 69% of *T. gondii* infection, respectively (22, 23). The findings of the studies of Chaechi et al., Havakhah et al., Which showed a high seroprevalence in Gilan province, confirm the findings of the present study (4, 24).

The results of the present study indicate that the prevalence of *T. gondii* in Gilan province compared to other provinces of Iran (17, 19, 25-28) shows a higher rate, which is probably due to the temperate and humid climate that makes oocysts to survive longer in the environment, as well as the traditional breeding of sheep and the presence of stray and wild cats.

The pathogenesis of toxoplasmosis depends on a variety of factors, including the host's immune system, genetic background, and parasite strains. One of the most important factors in the severity of toxoplasmosis is the virulence of parasite strains (29). *Toxoplasma gondii* has three main strains with different patterns of pathogenesis. The majority of known *Toxoplasma* strains belong to these three clonal lineages. Type I strains show the greatest severity and usually cause major infections. Type II strains are common among animals and humans and often cause chronic diseases. On the other hand, type III strains are less prevalent than type I and II strains and are more commonly found in birds (9, 29). The strains, which do not belong to these three types, are atypical. These strains are associated with a high severity of infection and are mostly reported from South America (10).

The PCR-RFLP method is one of the most common genotyping methods of *T. gondii* strains. So far, several genes have been used for this purpose (30). In the present study, the *GRA6* gene was used for genotyping *T. gondii* isolated from sheep. The results of PCR-RFLP assay with the *GRA6* gene revealed that all isolates, obtained from the brains of aborted ovine fetuses, showed type

II *T. gondii* patterns. Also, genotyping of 57 isolates from sheep in the United States showed that 45.6% (26/57) of isolates were type II, 15.7% (8/57) were type III, and 22 samples were atypical (31). Moreover, in a study from Brazil, 22 isolates, collected from the sheep brain, heart, diaphragms, and lungs, were genotyped. The parasite type was not identified in nine isolates, while type II and type III *T. gondii* were detected in 13 samples (32).

Furthermore, *T. gondii* isolates from the heart samples of sheep, goats, and cattle with positive sera were genotyped and mostly showed type II *T. gondii* patterns; in some isolates, both type I and II patterns were identified (33). Also, *T. gondii* isolates obtained from sheep, cattle, and pigs underwent genotyping in Switzerland, showing that type II pattern was more common in sheep, while type I, type III, and atypical genotypes were more common in the cattle and pigs (34). Additionally, 17 isolates were genotyped from different hosts (humans, sheep, pigs, and cats) in China and showed the presence of type I, type II, and type III genotypes (35).

In Iran, various studies have been performed to diagnose toxoplasmosis, using molecular methods (19, 20, 36) . Nevertheless, information is limited about *T. gondii* genotypes in Iran. In 2007, Zia Ali et al. examined the genotypes of 16 samples, isolated from sheep, goats, domestic chickens, geese, and cats, using the microsatellite method. All isolates obtained from birds (six isolates from domestic chickens and one isolate from a duck) belonged to type III *T. gondii*, while type II genotypes were found in cat isolates (two isolates). Also, type II and type III genotypes were found in humans (two isolates), and also, were observed in sheep (four isolates); however, type I genotype was not detected (37).

In a study by Shahbazi et al. from Ardabil, Iran, the brains of 75 aborted ovine fetuses using the nested-PCR method with *GRA6* gene, the prevalence of positive samples was reported to be 64%(38). In another study, 39 brain samples of aborted ovine fetuses were evaluated, using the nested-PCR assay with *B1* gene. The results showed that 54% of samples were positive. Also, genotyping of positive samples using the PCR-RFLP method with the *GRA6* gene showed that the samples were type I *T. gondii* (11). Moreover, in a study by Tavassoli et al. from Urmia, Iran, positive samples were evaluated, using *AluI* restriction enzyme to detect *T. gondii* strains via enzymatic digestion. The results revealed that the three positive samples had similar enzymatic digestion patterns; therefore, the sheep were infected with *T. gondii* genotype I (36).

In another study from south of Iran performed with the use of the PCR-RFLP method with *SAG2* and *GRA6* markers, type I genotypes were the most common *T. gondii* genotype (72%) in 125 positive samples, collected from the sheep diaphragm and heart (39) . In addition, in a study by Habibi et al. from Qazvin province, Iran, nested-PCR-RFLP assay was performed on 18 brain samples of aborted ovine fetuses. The results revealed that 66% of aborted sheep embryo samples were infected with type I *T. gondii* (22).

Conclusions

According to the present results, brain samples belonging to the aborted ovine fetuses were observed to possess *T. gondii* DNA, which suggests the high prevalence of congenital transmission of toxoplasmosis in Gilan province, Iran. One of the most important reasons for the high prevalence of this infection is the temperate climate and high humidity of this region. Our results showed that all of the isolates of *T. gondii* in our study had a Type II allele at the GRA6

marker. In conclusion, it is helpful to determine predominant *T. gondii* types in high-prevalence regions, including Gilan Province, to control, prevent, and design effective vaccine programs for reducing the incidence of infection in human and animal populations.

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