Molecular Detection and Phylogenetic Study of *Toxoplasma gondii* in Aborted Ovine Fetuses from Qazvin Province, Iran: A Cross-Sectional Study

Vahid Nasiri [1] , Habibollah Paykari [1*], Farnoosh Jameie[1],

Pejvak Khaki[2]

[1] Parasitology Department, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran.

[2] Microbiology Department, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran.

Vahid Nasiri

ORCID: 0000-0002-4959-890X

Phon: 09924629846

v.nasiri@rvsri.ac.ir

Habibollah Paykari

*Correspondence: h.paykari@rvsri.ac.ir

ORCID: 0000-0002-0989-4018

Phon: 09122657298

Farnoosh Jamei

ORCID: 0000-0003-3033-993X

Pejvak Khaki

ORCID: 0000-0001-8839-1023

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Abstract

Introduction: *Toxoplasma gondii*, an obligate intracellular apicomplexan parasite, is a globally significant cause of reproductive failure in small ruminants, inflicting considerable economic burdens on the livestock industry. This investigation was undertaken to determine the molecular prevalence of *T. gondii* DNA in aborted ovine fetuses originating from Qazvin province, a strategically important region in central Iran.

Methods: From 2019 to 2023, a total of 100 tissue specimens—comprising brain (n=39), placenta (n=8), embryonic membranes (n=7), cotyledons (n=7), umbilical cord (n=2), and pooled homogenates of visceral organs (n=37)—were collected from 47 aborted sheep fetuses. Following genomic DNA extraction via the phenol-chloroform technique, a nested PCR (n-PCR) assay targeting the GRA6 locus was employed for the specific identification of *T. gondii*. Positive amplicons were subsequently subjected to bidirectional sequencing for phylogenetic characterization.

Results: *Toxoplasma gondii* DNA was identified in 24 (51.1%; 95% CI: 36.8–65.4%) of the 47 aborted fetuses examined. Brain tissue exhibited the highest rate of positivity (43.6%; 17/39). A statistically significant (P = 0.009) upward temporal trajectory in infection rates was observed, escalating from 44.4% (8/18) in 2019 to 85.7% (6/7) in 2022. Phylogenetic inference based on GRA6 sequences (GenBank accession nos. OL825741–OL825744) placed the Iranian isolates within a clade containing reference strains from diverse geographic and host origins.

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Conclusion: The findings affirm a substantial prevalence (51.1%) of *T. gondii* in aborted ovine fetuses from Qazvin province, reinforcing its etiological importance in ovine abortions. Brain tissue emerges as the preferred specimen for molecular diagnostic applications.

Keywords: GRA6 gene, Iran, Nested PCR, Ovine abortion, Qazvin, *Toxoplasma gondii*.

1- Introduction

Toxoplasmosis, a globally distributed zoonosis instigated by the intracellular protozoan *Toxoplasma gondii*, affects a broad spectrum of homoiothermic vertebrates, encompassing both domestic animals and humans[1]. The life cycle of this parasite is characterized by a definitive host—members of the Felidae family, especially domestic cats—which excrete environmentally resilient oocysts into the surroundings [3,2]. Intermediate hosts, including ovine and caprine species, typically acquire the infection via oral uptake of these oocysts from tainted fodder, water supplies, or pastureland [4].

In gravid ewes, *T. gondii* constitutes a predominant trigger for reproductive wastage, manifesting as abortion, stillbirth, or the delivery of frail, non-viable offspring [6,5]. The clinical outcome is intrinsically linked to the gestational stage at the time of infection: early-gestation infections frequently culminate in embryonic resorption or mummification, mid-gestation infections commonly precipitate abortion, and late-gestation infections may yield congenitally infected lambs that appear phenotypically normal [7]. The financial ramifications of ovine toxoplasmosis are profound, encompassing both direct losses from diminished lamb yields and ancillary expenditures related to diagnostic procedures and intervention strategies [8].

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In Iran, ovine husbandry occupies a pivotal socio-economic position, particularly within rural and nomadic communities. A wealth of seroepidemiological and molecular surveys have documented variable infection rates across the country, signifying pervasive environmental contamination with *T. gondii* oocysts [11,10,9]. Meta-analytical estimates place the overall seroprevalence at approximately 31% [9], although provincial figures have reached as high as 91% in Mazandaran [12]. Nevertheless, comprehensive data focusing exclusively on aborted fetuses from central regions, notably Qazvin province, remain conspicuously scarce. Such information is indispensable for elucidating the etiological underpinnings of abortion outbreaks in these areas.

Relying solely on clinical presentation or maternal serology for diagnosing ovine toxoplasmosis is fraught with challenges, given the potential for chronic infections and the persistence of antibodies from prior gestations [13]. Molecular detection of *T. gondii* DNA within fetal tissues—especially the brain—offers a definitive and highly sensitive approach for confirming congenital transmission [14,13]. The *GRA6* locus, encoding a dense granule protein, has emerged as a robust genetic marker for both detection and genotyping of *T. gondii*, attributable to its exceptional sensitivity, specificity, and absence of cross-reactivity with phylogenetically related apicomplexans, including *Neospora caninum* [15,10].

In light of the unresolved abortion outbreaks afflicting ovine flocks in Qazvin province between 2019 and 2022, the present investigation was conceived with three primary objectives: (i) to assess the molecular prevalence of *T. gondii* in aborted ovine fetuses via a nested PCR assay targeting the *GRA6* gene; (ii) to ascertain the most suitable tissue type for diagnostic sampling; and (iii) to conduct phylogenetic analyses of the detected isolates, thereby elucidating their genetic affinities with strains circulating elsewhere.

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2. Materials and Methods

2.1. Ethical Considerations

All experimental procedures involving animals received formal approval from the Institutional Animal Care and Use Committee of the Razi Vaccine and Serum Research Institute, Alborz, Iran (Ethical Code: IR.RVSRI.REC.1399.XXX), and were executed in strict compliance with the guidelines stipulated by the National Research Council for the care and utilization of laboratory animals.

2.2. Study Site and Specimen Collection

This cross-sectional investigation was carried out between June 2019 and February 2023 within Qazvin province, situated in the north-central part of Iran. A cohort of 47 aborted ovine fetuses was obtained from privately owned farms (predominantly operating under traditional management regimes) subsequent to reports of abortion storms or isolated incidents. Necropsy was performed immediately, and tissue samples were harvested. The complete specimen set comprised 100 individual tissues, specifically: brain (n=39), placenta (n=8), embryonic membranes (n=7), cotyledons (n=7), umbilical cord (n=2), and a pooled homogenate comprising heart, liver, spleen, and gastrointestinal tract (n=37). All samples were promptly transferred to the Protozoology Laboratory at the Razi Institute and preserved at -20°C pending further processing.

The inclusion criteria for fetal selection were as follows: (i) fetuses were obtained from flocks with a documented history of abortion during the study period; (ii) the aborted fetus was submitted to the laboratory within 24 hours of expulsion; and (iii) sufficient tissue material was available for molecular analysis. Fetuses exhibiting obvious signs of autolysis or decomposition were excluded.

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Additionally, fetuses from flocks with a confirmed history of bacterial (e.g., *Brucella* spp., *Chlamydia abortus*) or viral (e.g., Border disease virus) abortion outbreaks were excluded to minimize confounding etiologies. For tissue sampling, a standardized protocol was followed: whenever possible, brain, placenta, and pooled visceral organs (heart, liver, spleen, and gastrointestinal tract) were collected from each fetus. When some tissues were unavailable due to autolysis or logistical constraints, the available tissues were sampled and recorded accordingly.

2.3. DNA extraction

Genomic DNA was isolated from approximately 50 mg of each tissue specimen using a standard phenol-chloroform protocol, following established methodologies [17,16]. The concentration and purity of the extracted DNA were evaluated via spectrophotometric measurement (NanoDrop, Thermo Scientific). Purified DNA was stored at -20°C until utilized for PCR amplification.

2.4. Nested PCR Amplification of the *GRA6* Gene

A nested PCR (n-PCR) strategy was adopted for the sensitive and specific detection of *T. gondii* DNA, employing primers targeting the *GRA6* locus as delineated in prior investigations [15,14]. The oligonucleotide sequences and thermocycling parameters are summarized in Table 1. The primary reaction mixture (25 µL) consisted of 12.5 µL of Master Mix (Amplicon, Denmark), 10 pmol of each external primer (GRA6-F1 and GRA6-R1), and 2 µL of template DNA. For the secondary amplification step, 1 µL of a 1:10 dilution of the primary PCR product was employed as the template. Each amplification run incorporated appropriate positive (RH strain *T. gondii* DNA) and negative (nuclease-free water) controls.

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Strict contamination prevention measures were implemented throughout the PCR workflow. Pre-PCR procedures (DNA extraction and master mix preparation) were performed in a dedicated clean area, while post-PCR analyses (gel electrophoresis and sequencing) were conducted in a separate laboratory space. Dedicated pipettes, aerosol-resistant tips, and separate sets of reagents were used for each area. Negative controls (nuclease-free water) were included in every amplification run to monitor for potential contamination.

Table 1. Primer sequences and thermal profiles for nested PCR amplification of the *GRA6* locus.

Step / Primer	Primer Name	Sequence (5' → 3')	Amplicon Size	Cycling Conditions
Primary PCR	*GRA6*-F1	ATTTGTGTTTCCGAGCAGGT	553 bp	95°C for 5 min; 40 cycles of (94°C for 40s, 59°C for 40s, 72°C for 70s); 72°C for 10 min
	GRA6-R1	GCACCTTCGCTTGTGGTT		
Secondary PCR	*GRA6*-F2	TTTCCGAGCAGGTGACCT	344 bp	95°C for 5 min; 35 cycles of (94°C for 30s, 57°C for 30s, 72°C for 60s); 72°C for 10 min
	GRA6-R2	TCGCCGAAGAGTTGACATAG		

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2.5. Sequencing and Phylogenetic Reconstruction

Four positive PCR products (344 bp) were selected randomly using a computer-generated random number list from the pool of positive samples, ensuring representation across different years and tissue types, and underwent purification and bidirectional Sanger sequencing (Pishgam Biotech, Iran).The resulting electropherograms were manually curated using Chromas software, and the finalized consensus sequences were deposited in the GenBank repository under accession numbers OL825741, OL825742, OL825743, and OL825744. Homology searches were performed using BLASTn against the NCBI non-redundant database. Multiple sequence alignments were generated with CLUSTALW2 and refined using BioEdit. A Maximum Likelihood (ML) phylogenetic tree, incorporating 1000 bootstrap pseudoreplicates, was constructed using MEGA X software [18].

2.6. Statistical Evaluation

Statistical analyses were conducted utilizing SPSS version 26.0 (IBM Corp., Armonk, NY, USA). Inter-year variations in infection rates were compared employing Fisher's exact test. A p-value below 0.05 was deemed statistically significant. Prevalence estimates were reported with 95% confidence intervals (Wilson method).

3. Results

3.1. Molecular detection of *Toxoplasma gondii*

Of the 47 aborted fetuses screened, *T. gondii* DNA was identified in 24 specimens, corresponding to an overall prevalence of 51.1%. The tissue-specific distribution of positive findings is delineated in Table 2. Brain tissue yielded the highest detection rate (43.6%; 17/39), followed by umbilical

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cord (50.0%; 1/2) and embryonic membrane (28.6%; 2/7), albeit the latter two were based on limited sample sizes. Table 3 provides a year-stratified breakdown of positive cases.

Table 2. Frequency of *T. gondii* DNA detection in different tissues from aborted ovine fetuses.

Tissue Type	No. Examined	No. Positive	Infection (%)	95% CI
Brain	39	17	**43.6%**	**28.8–59.4%**
Placenta	8	1	12.5%	2.2–47.1%
Embryonic Membrane	7	2	28.6%	8.2–64.1%
Cotyledon	7	1	14.3%	2.6–51.3%
Umbilical Cord	2	1	50.0%	9.5–90.5%
Tissue Mixture*	37	5	13.5%	5.9–28.0%
Total	**100**	**27**	**27.0%**	**19.3–36.3%**

*Note: The table presents tissue-level positivity. Fetus-level positivity was 51.1% (24/47). Tissue mixture includes heart, liver, spleen, and digestive tract.

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Table 3. Yearly prevalence of *T. gondii* in aborted sheep fetuses from Qazvin province.

Year	No. of Fetuses Examined	No. Positive (%)	95% CI
2019	18	8 (44.4%)	**24.6–66.3%**
2020	14	5 (35.7%)	**16.3–61.2%**
2021	6	5 (83.3%)	**43.6–97.0%**
2022	7	6 (85.7%)	**48.7–97.4%**
2023	2	0 (0.0%)	**0.0–65.8%**
Total	**47**	**24 (51.1%)**	**36.8–65.4%**

Statistical note: Trend analysis using Fisher's exact test revealed a statistically significant upward temporal trajectory (P = 0.009).

3.2. Temporal Dynamics of Infection

A marked and statistically significant escalation in the detection rate of *T. gondii* within aborted fetuses was documented over the study interval. The prevalence surged from 44.4% (8/18) in 2019 to 85.7% (6/7) in 2022 (P = 0.009) (Table 3).

3.3. Phylogenetic Characterization

The four sequenced *GRA6* amplicons (OL825741-OL825744) exhibited complete sequence identity (100%) among themselves and demonstrated high homology (97-100%) with numerous *T. gondii* isolates archived in GenBank. The ML phylogenetic tree (Figure 1) robustly grouped the Iranian ovine isolates alongside reference strains derived from diverse hosts and geographical

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177 locales, while clearly distinguishing them from other apicomplexan taxa, including *Neospora*
178 *caninum*.



179

180 **Figure 1.** Maximum Likelihood phylogenetic tree inferred from *GRA6* gene sequences. The
181 analysis comprised 47 sequences, including the four Iranian isolates (highlighted with red
182 diamonds). Bootstrap support values (1000 replicates) are indicated at branch nodes.

4. Discussion

The present investigation furnishes robust molecular evidence establishing *Toxoplasma gondii* as a principal etiological agent of ovine abortion in Qazvin province, central Iran. The aggregate detection rate of 51.1% (24/47) in aborted fetuses is substantial and congruent with earlier Iranian reports. Notably, Habibi et al. [19] documented a 66% prevalence in a parallel study from the same province, while Moazeni-Jula et al. [20] reported 69% in Tabriz, and Amouei et al. [21]found 19.7% in Mazandaran. The elevated prevalence observed in our study, consistent with prior findings from the same region [19], underscores the significant and enduring contribution of this parasite to ovine reproductive pathology in this area of Iran.

The heterogeneity in prevalence rates across Iranian studies is likely attributable to a constellation of factors, including geographic and climatic variability (with humidity and temperature profoundly influencing oocyst survival and sporulation), divergent management systems (traditional versus intensive), and the density of definitive hosts (feral cats) on farms [9,3]. The persistently high prevalence in Qazvin province across two separate studies (spanning 2012 and 2023) suggests a sustained and substantial level of environmental contamination with *T. gondii* oocysts. This phenomenon may be ascribed to the region's conducive climate and the predominance of traditional pastoral systems, wherein sheep frequently graze on pastures accessible to stray cats.

Brain tissue emerged as the most diagnostically informative specimen, with 43.6% of examined brains testing positive (Table 2). This observation aligns with the established biology of *T. gondii*, which exhibits a marked tropism for the central nervous system and musculature of intermediate hosts, where it forms latent tissue cysts [22]. Bradyzoites can persist in the brain for the host's

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lifetime without inducing overt clinical signs; however, pregnancy-associated immunosuppression can precipitate reactivation and subsequent transplacental transmission[6]. Accordingly, for routine PCR-based diagnosis of ovine toxoplasmosis in aborted fetuses, sampling of brain tissue is strongly advocated, a recommendation corroborated by other researchers [14,13].

The progressive increase in detection rates observed over the study years (from 44.4% in 2019 to 85.7% in 2022, Table 3) constitutes an intriguing and noteworthy finding. This statistically significant upward trajectory ($P = 0.009$) may reflect a period of heightened environmental contamination, potentially driven by favorable climatic conditions for oocyst sporulation, an expansion of the stray cat population, or recrudescence of latent infections in chronically infected ewes during pregnancy[6]. Alternatively, it could be attributed to a specific abortion storm predominantly caused by *T. gondii* during those years. Long-term surveillance initiatives are warranted to validate this trend and to pinpoint the underlying risk determinants.

A speculative hypothesis to account for this increasing trend—from 44.4% in 2019 to 85.7% in 2022—pertains to the underlying epidemiology of abortion outbreaks in the region. It is plausible that the initial abortion storms documented in 2019–2020 may have been precipitated by other abortifacient pathogens, particularly viruses (e.g., Border disease virus, Bovine herpesvirus-1), which typically induce explosive, self-limiting epidemics. As these viral waves subsided, the chronic, endemic causes of abortion, most notably *T. gondii*, would have become relatively more conspicuous. In this scenario, chronically infected ewes harboring latent tissue cysts in their brains may undergo reactivation of the parasite due to pregnancy-associated immunosuppression, culminating in transplacental transmission and abortion. Consequently, the lower proportion of T. gondii detection in the initial year (44.4%) might not reflect a true lower prevalence, but rather a

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"dilution" effect imposed by a concurrent viral abortion storm. However, this hypothesis remains speculative and requires further investigation through simultaneous screening of multiple pathogens in future studies.

Phylogenetic analysis (Figure 1), predicated on the *GRA6* gene, confirmed that the isolates from Qazvin province are phylogenetically related to other *T. gondii* strains circulating both globally and within Iran. The *GRA6* locus serves as a dependable marker for identification and has been effectively employed to differentiate *T. gondii* from the morphologically analogous *N. caninum* (17). The observation that four randomly selected positive samples yielded virtually identical sequences suggests the possible existence of a common source or a dominant clonal lineage circulating in the region. However, to discern the specific pathogenic and epidemiological attributes of the circulating strains, further genotyping employing markers such as SAG2, SAG3, or *GRA6*-RFLP is necessary to classify them into the classical clonal types (I, II, III) or atypical strains[24,23] .

This study is not without limitations. First, while the sample size provides meaningful insights, a larger cohort would have facilitated more detailed risk factor analyses (e.g., evaluating the impact of feline presence, feeding practices, or water sources). Second, we did not undertake genotyping to identify specific strains, which would have aided in understanding the pathogenicity of the circulating isolates—a valuable avenue for future investigations. Third, the use of pooled tissue homogenates (heart, liver, spleen, and gastrointestinal tract) may have diluted DNA from individual organs, potentially leading to an underestimation of the true prevalence in those specific tissues. This limitation should be considered when interpreting the tissue-specific prevalence data. Finally, we did not concurrently evaluate other potential abortifacient agents (e.g., *Chlamydia*

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abortus, *Neospora caninum*, *Brucella* spp.), which would have furnished a more holistic picture of the etiological spectrum of abortion in these flocks.

Notwithstanding these limitations, the elevated prevalence documented in this study carries significant practical implications. Control strategies for ovine toxoplasmosis should be implemented, focusing on: (i) preventing feline access to sheep feed, water storage areas, and barns to curtail oocyst contamination; (ii) proper removal and disposal of aborted fetuses and placentas to interrupt the transmission cycle; (iii) considering the deployment of the commercial live vaccine (Toxovax®) in susceptible flocks, particularly in high-prevalence areas[25]; and (iv) educating farmers regarding the zoonotic risks inherent in handling aborted materials from infected ewes.

5. Conclusion

The present investigation demonstrates a high prevalence (51.1%) of *Toxoplasma gondii* infection in aborted ovine fetuses from Qazvin province, Iran, thereby confirming its status as a primary infectious cause of abortion in this region. Brain tissue was identified as the most reliable specimen for PCR-based diagnosis. These findings underscore the urgent need for implementing integrated control strategies to mitigate the economic impact of toxoplasmosis on the Iranian sheep industry.

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Authors' Contribution

Habibollah Paykari, Vahid Nasiri, Farnoosh Jameie and pejvak khaki: were involved in the conception of the research idea and methodology design, supervision, performed data analysis and interpretation and in the methodology and data analysis; **Vahid Nasiri and Farnoosh Jameie** prepared and critically revised the manuscript for publication and revision. Each and every writer perused and authorized the conclusive version of the manuscript.

Conflict of interests

The authors declare no conflict of interest.

Ethics approval

All experimental procedures involving animals received formal approval from the Institutional Animal Care and Use Committee of the Razi Vaccine and Serum Research Institute, Alborz, Iran (Ethical Code: IR.RVSRI.REC.1399.XXX), and were executed in strict compliance with the guidelines stipulated by the National Research Council for the care and utilization of laboratory animals**.**

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Data Availability

All data generated or analyzed during this study are included in this article. The sequences have been deposited in GenBank under accession numbers OL825741-OL825744.

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