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Original Article

Frequency of *Toxoplasma gondii* in Livestock Meats Using Nested PCR and RFLP in Sabzevar City, Iran

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Abstract

Background: We investigated the prevalence of *Toxoplasma gondii* in slaughtered livestock (sheep, goats, camels) in Sabzevar, Eastern Iran using nested PCR methods.

Methods: Samples of diaphragm and heart tissue were collected from 40 sheep, 40 goats, and 40 camels at local slaughterhouses. DNA was extracted from the samples and analyzed using nested PCR targeting the *B1* gene of *T. gondii*.

Results: The results indicate an overall prevalence of 60% (24/40) in sheep, with 37.5% (15/40) of diaphragm samples and 22.5% (9/40) of heart samples testing positive. In goats, the overall prevalence was 52.5% (21/40), with 35% (14/40) of diaphragm and 17.5% (7/40) of heart samples positive. Camels showed the highest prevalence at 65% (26/40), with 45% (18/40) of diaphragm and 20% (8/40) of heart samples infected. Diaphragm tissue showed higher infection rates compared to the heart in all species. No significant difference was found in infection rates between male and female animals. RFLP analysis using XhoI on selected positive PCR products differentiated genotypes based on digestion patterns. Among analyzed samples (19 per species), genotypes II/III were identified in 57.9% (11/19) of sheep, 36.8% (7/19) of goats, and 47.4% (9/19) of camels, with the remainder likely genotype I or undigested II/III.

***Conclusion*:** The high prevalence rates observed, especially in diaphragm tissue and in camels, have important implications for public health and food safety. Enhanced meat inspection, public education, and further research on effective control measures are recommended.

Introduction

Toxoplasmosis, a disease spread from animals to humans, is caused by protozoan parasite *Toxoplasma gondii* and is a major health problem worldwide. The prevalence of *T. gondii* in approximately one-third of humans has significant effects on public health and economic security (1). The intricate life cycle of the parasite includes final hosts like cats and numerous middle hosts like humans and other mammals (2). Different ways in which transmission can happen include consuming contaminated water or inadequately cooked meat, passing from mother to fetus during pregnancy, and meeting oocysts found in cat feces (3,4).

Toxoplasmosis in humans can cause serious problems, especially for those with weakened immune systems and unborn babies, causing eye issues, brain disorders, and birth defects (5). The significant consequences of toxoplasmosis on the economy and health are considerable. The illness results in major economic losses in livestock farming because of reproductive issues like abortion, stillbirths, and neonatal death (6). Additionally, *T. gondii* found in livestock meat can directly endanger human health, since consuming raw or undercooked infected meat is a significant way for the parasite to spread to humans (7). Livestock, such as sheep, goats, and camels, are essential in the spread of *T. gondii*, acting as intermediary hosts and transmitting the infection to humans with the consumption of raw or undercooked meat as part of traditional culinary practices (8). Previous research has explored how prevalence of *T. gondii* in livestock using different serological tests, like ELISA and MAT, and molecular techniques such as PCR in various techniques (Conventional PCR, real-time PCR, nested PCR) and different markers for identifying *T. gondii* infection in livestock (9,10).

The *B1* gene and the 529 bp repeat element are frequently used markers in nested PCR to detect *T. gondii*. These markers are both repetitive and highly conserved in the *T. gondii* genome (11–16). Bezerra et al. found a 14.3% prevalence in 126 sheep meat samples in Brazil by using nested PCR to target the *B1* gene (14). Similarly, El-Nawawi et al. examined 300 meat samples (100 from each sheep, goat, and camel) in Egypt with nested PCR focused on the *B1* gene, finding prevalence rates of 8%, 6%, and 4% in sheep, goats, and camels, respectively. The sensitivity of the nested PCR assay was shown to be 96.7%, with a specificity of 95.2% when compared to the modified agglutination test (MAT) (13). In Mashhad, Iran, Tavakoli Kareshk et al. tested 150 meat samples (50 from each sheep, goat, and camel) from slaughterhouses, finding a total prevalence of 6.7% with positivity rates of 8%, 6%, and 6% in sheep, goats, and camels, respectively (12). A different study found infection rates of 14.5% in sheep, 12% in goats, and 3% in camels (15).

Sabzevar, a city in the Razavi Khorasan Province of Iran, has livestock farming activities including sheep, goats, and camels, though the prevalence of toxoplasmosis in these animals remains to be investigated. Therefore, we aimed to fill this gap by employing the highly sensitive and specific nested PCR technique to detect *T. gondii* DNA in sheep, goat, and camel meat samples from Sabzevar.

Materials and Methods

Ethical Considerations

All procedures were conducted in accordance with the ethical guidelines for research involving animal samples. The study protocol was reviewed and approved by the Ethics Committee of the Kerman University of Medical Sciences (Ethical Approval Code: 930490). Since the samples were collected from livestock already slaughtered for public consumption at a municipal slaughterhouse, no animals were harmed or sacrificed specifically for the purposes of this research.

Geographic Location and Characteristics

The study was conducted in Sabzevar, located in Razavi Khorasan Province, northeastern Iran (coordinates: 36.2126° N, 57.6741° E) (Fig. 1). Sabzevar is known for its significant livestock population, making it an ideal location for this research.

Meat Samples Collection

A cross-sectional study design was employed. We used a traditional slaughterhouse that operates under the supervision of the Sabzevar Veterinary Department. The sample size was determined to be 280 meat samples collected randomly, including heart and diaphragm from 120 slaughtered animals (40 sheep, 40 goats, 40 camels) and some ground sheep meat samples (Table 1).

Fig. 1: Geographical divisions of Sabzevar and surrounding regions

Table 1: Characteristics of animal species and tissue samples for toxoplasmosis diagnosis in Sabzavar city, Iran

Animal Type	Tissue Tested	Number
Sheep	Diaphragm muscle Heart muscle	40 40
Goat	Diaphragm muscle Heart muscle	40 40
Camel	Diaphragm muscle Heart muscle	40 40
Sheep & Goat	Ground meat	25
Camel	Ground meat	15
Total		280

Sample collection took place over a three-month interval, from February 2018 to late April 2018, covering the transition from late winter to early spring. All samples were collected with the cooperation of the Sabzevar Veterinary Office and the on-site veterinarian at the slaughterhouses. Some ground meat samples were also purchased from authorized retailers in the city to determine contamination levels and compare them with slaughterhouse meat. We recorded all relevant animal characteristics (species, sex) and sample details

(tissue type, date of collection) to ensure traceability and statistical reliability. Approximately 50 grams of diaphragm and heart tissue were collected from each animal using disposable scalpels. Samples were placed in disposable plastic bags, labeled with unique codes, and immediately transferred to a -20 °C freezer. Subsequently, the frozen samples were transported to Kerman and stored at -20 °C in the Parasitology Department of Afzalipour Medical School until further processing.

Sample Preparation and DNA Extraction

The frozen samples were thawed and approximately 5 grams of tissue were placed in a disposable petri dish. The tissues were finely chopped with a disposable scalpel until a homogenous mixture was obtained. DNA was extracted using the GeneAll Exgene Cell SV kit (GeneAll Biotechnology, Seoul, Korea) according to the manufacturer's instructions. 200 µL of the homogenized tissue sample was mixed with 200 µL of CL buffer and 20 µL of Proteinase K (PK) solution. The mixture was vortexed briefly and incubated at 56 °C for 24 hours to ensure complete lysis. For modifications to the Standard Protocol, fresh kiwi juice was added to the homogenized samples to enhance tissue breakdown (17). This step involved adding 20 mL of kiwi juice, incubating at room temperature for 1 hour, followed by vortexing for 10 minutes. The concentration and purity of the extracted DNA were assessed using a NanoDrop spectrophotometer (Thermo Scientific, USA).

Nested PCR and RFLP–PCR Technique

Detection of *T. gondii* was performed using nested PCR targeting the B1 gene, as previously described (18). Two rounds of amplification were carried out using specific outer (AS1, F;5'-CGACAGAAAGGGAGCAAGAG-3', R; 5'-ACGCTGTGTCTCCTCTAGGC-3') and inner (AS2 F; 5'-CTCGACAATACGCTGCTTGA-3', R; 5'-TCTTCCCAGACGTGGATTTC-3') primer sets. Briefly, PCR amplification was conducted under standard conditions, including initial denaturation at 94 °C, followed by denaturation, annealing at 60 °C, and extension at 72 °C for 30–35 cycles in each round. Positive (*T. gondii* RH strain DNA) and negative (sterile distilled water) controls were included in all reactions. PCR products were analyzed by agarose gel electrophoresis, and samples showing the expected band size. Genotyping of *T. gondii*–positive samples was performed using PCR-RFLP analysis of the *B1* gene with the XhoI restriction enzyme, as previously described (18). Briefly, PCR products were digested with XhoI, and the resulting fragments were analyzed by agarose gel electrophoresis. Genotypes were determined based on the observed digestion patterns, where type II and III strains produced characteristic fragments, while type I remained undigested.

Statistical Analysis

Data analysis was performed using SPSS version 20 (IBM Corp., Armonk, NY, USA). Chi-square tests were used to compare the prevalence of *T. gondii* among different livestock species, with a significance level set at $P< 0.05$.

Results

A total of 280 samples were randomly collected from the slaughterhouse of domestic animals in Sabzevar city over a period of five months. The samples were obtained from sheep, goats, and camels, specifically from the heart and diaphragm muscles of the slaughtered animals. Additionally, to assess the contamination of meat available in the city and compare it with slaughterhouse meat, ground meat samples were purchased from authorized sellers within the city. *T. gondii* infection was detected in sheep, goats, and camels, with variation observed between animal species and tissue types. Detailed prevalence data are presented in Table 2.

Table 2: Comparison of toxoplasmosis infection in the heart and diaphragm of sheep, goats, and camels in Sabzevar city.

Animal	Tissue	No. Infected	Total Tested	Infection %	*P* value
Sheep	Diaphragm Heart	15 9	40 40	37.5 22.5	*P* =0.012*
Goat	Diaphragm Heart	14 7	40 40	35 17.5	*P* =0.052
Camel	Diaphragm Heart	18 8	40 40	45% 20%	*P* =0.011*
Sheep & Goat Camel	Ground meat	7 4	25 15	28 26.6	*P* =0.07

As illustrated in Table 2, the degree of contamination within the diaphragm muscles exhibited a more pronounced increase across all animal subjects, with this disparity achieving statistical significance in both sheep and camels. No statistically significant difference was observed in the infection rates between ground meat from sheep/goats and camels (*P*=0.07). Furthermore, in all three animal species, a significant difference (*P* =0.048) was observed in the contamination levels of meat samples procured from the station and wheel, with a notable escalation detected in minced meat. The prevalence of *T. gondii* infection was compared between male and female animals across different species. No significant difference in infection rates was observed between sexes (*P*= 0.795) (Table 3).

Table 3: *Toxoplasma gondii* infection rates by PCR in livestock (sheep, goats, camels) by sex in Sabzevar City, Iran in 2018

Animal	Sex	No. Infected	Infection %	Tissue Tested	Total Infection %
Sheep	Male Female	9 15	22.5 37.5	Diaphragm & Heart	60
Goat	Male Female	11 10	27.5 25	Diaphragm & Heart	52.5
Camel	Male Female	15 11	37.5 27.5	Diaphragm & Heart	65

Our results showed that the Nested PCR products detecting *T. gondii* in the diaphragm and heart muscles of sheep, goats, camels, and in-ground meat samples. Positive bands of 531 bp can be seen in the infected samples (Fig. 2).

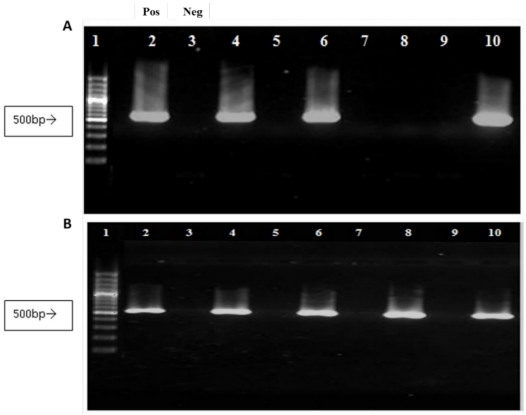

Fig. 2: Electrophoresis image of Nested PCR product for *T. gondii* in all livestock species. A: Sheep diaphragm muscle, B: Sheep heart muscle. Line 1: Ladder 500bp. Line 2: positive control. Line 3: Negative control. Line 4 to 10: PCR product obtained from *B1* gene amplification in samples

A total of fifty-five PCR products positive for the *B1* gene were selected for RFLP analysis using XhoI restriction enzyme. Differentiation was based on digestion patterns: XhoI cleaves the *B1* amplicon of genotypes II and III into 2-3 fragments, while genotype I remains undigested; however, some II/III may also remain undigested due to incomplete enzymatic activity. Variation in digestion patterns was observed among sheep, goat, and camel isolates, indicating the presence of different genotypes across animal species (Fig. 3 and Table 4).

Table 4: Characteristics of *Toxoplasma gondii* genotypes from animal types using RFLP on Nested PCR

Animal Type	Num of Samples	Genotype II & III	Possibly Genotype I or Undigested Genotype II & III
Sheep	19	11	8
Goats	19	7	12
Camels	19	9	10

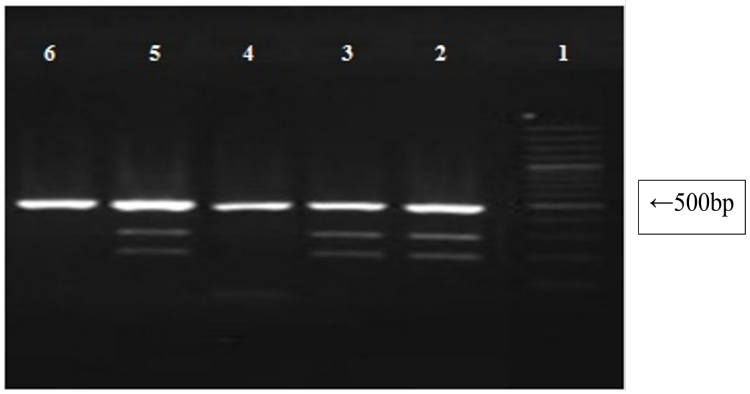

Fig. 3: Electrophoresis image of the RFLP assay on the *B1* gene PCR product using the Xho1 enzyme Line 1: Ladder 500bp.Line 2 to 6: PCR product using the Xho1 enzyme

Discussion

T. gondii is a globally important zoonotic parasite transmitted primarily through ingestion of oocyst-contaminated water or consumption of undercooked meat containing tissue cysts (2, 4). In this context, assessing its occurrence in livestock is essential for understanding regional transmission dynamics and associated public health risks.

The findings of the present study confirm the widespread presence of *T. gondii* in livestock from the study area, indicating a considerable potential for foodborne transmission. Variation in detection among animal species was observed, with camels showing a relatively higher level of contamination compared to other livestock. This may have important public health implications, particularly in regions where camel meat is commonly consumed as part of traditional diets. The elevated detection in camel meat is consistent with previous reports from similar settings (19) and may reflect differences in environmental exposure, feeding behavior, or management practices. Given the cultural importance of camel meat and the possibility of its consumption in undercooked forms, these findings highlight the need for increased awareness regarding safe meat handling and adequate cooking practices.

The present study demonstrated a consistently higher detection of *T. gondii* in diaphragm samples compared to heart tissues across all examined livestock species. This finding agrees with previous studies suggesting a preferential distribution of the parasite in highly active and well-perfused muscular tissues, such as the diaphragm (2, 20). Such tissue-specific differences may be attributed to biological and physiological characteristics that favor cyst formation and persistence. The overall prevalence observed in this study is broadly consistent with reports from regions with comparable environmental and climatic conditions, where *T. gondii* infection remains widespread in livestock populations (21, 22). However, variations in reported prevalence across different countries are notable and may reflect differences in environmental factors, animal management systems, and diagnostic methodologies.

Compared to some European settings, where relatively lower prevalence has been reported in domestic animals, the higher levels observed in the present study may be explained by increased environmental exposure, particularly in regions with extensive grazing systems and closer contact with definitive hosts. Similarly, studies conducted in Iran and neighboring regions have reported variable prevalence rates, highlighting the influence of geographic and ecological factors on parasite distribution (24–27). However, these findings support the notion that *T. gondii* infection in livestock is shaped by a complex interplay of tissue characteristics, environmental conditions, and regional husbandry practices.

The present findings suggest variation in *T. gondii* detection across different tissue types, with lower detection in minced meat compared to specific organs such as the diaphragm and heart. This observation may be related to differences in tissue tropism and parasite distribution, as previous studies have reported lower parasite burdens in skeletal muscles relative to other tissues (28).

No significant association was observed between animal sex and *T. gondii* contamination, indicating that sex is not a major determinant of infection risk. Although some variation in infection patterns between males and females was noted across species, these differences are inconsistent and likely influenced by external factors rather than intrinsic biological susceptibility. Previous studies have similarly reported variable and sometimes higher infection rates in female sheep, potentially due to longer lifespan and increased environmental exposure associated with breeding practices (29, 30). These findings suggest that management conditions, environmental exposure, and husbandry practices are more influential

than host sex in shaping infection dynamics in livestock populations.

Environmental conditions, animal husbandry practices, and cultural habits play important roles in shaping the transmission dynamics of *T. gondii*. Contamination of pastures and water sources with oocysts shed by felids may be facilitated by grazing systems and local ecological conditions (31, 32). In addition, inadequate hygiene in slaughterhouses and meat processing environments may further contribute to contamination of meat products (33). The use of nested PCR in the present study enabled highly sensitive detection of *T. gondii* DNA, allowing identification of low parasite burdens. However, despite its high sensitivity and specificity, this method does not provide information on parasite viability, which limits direct assessment of the actual risk of transmission (34, 35). Genotyping analysis using PCR-RFLP indicated the presence of multiple *T. gondii* lineages among the examined samples, including patterns consistent with type II/III as well as undigested profiles suggestive of type I or incomplete enzymatic digestion. These findings are in line with the well-established classification of *T. gondii* into three major clonal lineages (types I, II, and III), which differ in virulence and host distribution (36). Type I strains are typically associated with high virulence and severe clinical manifestations, whereas types II and III are more frequently reported in livestock and are generally linked to chronic or subclinical infections (37–39). The predominance of type II/III patterns observed in the present study is consistent with previous reports indicating that these genotypes are widely distributed among food animals.

Studies conducted in different regions of Iran have demonstrated variable genotype distributions, reflecting geographic heterogeneity. For instance, previous investigations have identified type II and III genotypes in livestock using molecular approaches (24), while other studies have reported the predominance of type I strains in certain regions based on alternative genetic markers (16). In contrast, findings from northern Iran have often highlighted the dominance of type III genotypes in animal hosts (40).

Such discrepancies may be attributed to differences in study design, sample type, and genotyping methods, particularly the use of single-locus versus multilocus approaches. Additionally, environmental conditions, host species, and transmission dynamics may contribute to the observed variability in genotype distribution. Overall, the results of the present study suggest the circulation of multiple *T. gondii* genotypes in the study area, with a predominance of type II/III lineages. However, given the limitations of single-marker PCR-RFLP analysis, further studies employing high-resolution genotyping methods and larger sample sizes are required to better characterize the population structure of *T. gondii* in this region.

A challenge of this study is the relatively limited sample size, especially when categorizing by various livestock species and tissue types. Increasing the size of the sample would lead to more precise estimates of prevalence and enable a thorough examination of the risk factors linked to *T. gondii* infection in livestock. Studying *T. gondii* samples from livestock may offer valuable insights. Furthermore, studying the information, beliefs, and behaviors of farmers, slaughterhouse employees, and consumers about *T. gondii* and meat safety could help develop specific educational programs.

Conclusion

The present study confirms the presence of *T. gondii* in meat from different animal species, highlighting the importance of continuous surveillance and improved food safety practices. These findings contribute to the growing body of evidence emphasizing the role of meat as a potential source of human toxoplasmosis and support the need for coordinated control strategies at both veterinary and public health levels.

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Conflict of Interests

The authors declare no conflict of interest.

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