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Original Article

Molecular and Pathological Detection of *Toxoplasma gondii* in Aborted Fetuses of Sheep and Goats in East Azerbaijan Province, Northwest Iran

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Abstract

Background: *Toxoplasma gondii* is recognized as one of the most important causes of abortion in small ruminants globally. This study was conducted to evaluate *T. gondii* in aborted fetuses of sheep and goats in East Azerbaijan Province, northwest Iran.

Methods: A total of 62 aborted fetuses were collected from sheep and goat flocks from 2023-2024. The tissue samples following a systematic necropsy were obtained from the brains, livers, and lungs for histopathology and molecular studies. The conventional PCR method using specific primers was performed for molecular evaluations. Additionally, the formalin-fixed tissue samples were routinely conducted for histopathological examinations.

Results: *T. gondii* was present in 40.32% (25/62) of the aborted fetus. In more detail, one, 7, and 15 positive samples were found in the lungs, livers, and brains, respectively. Additionally, one fetus was positive in both the liver and brain, and one fetus was positive in both lung and liver tissues. Histopathological studies demonstrated moderate to severe hyperemia and focal hemorrhage associated with focal to multifocal gliosis, nonsuppurative meningoencephalitis, focal to multifocal mononuclear hepatitis, and nonsuppurative pneumonia. Notably, the *Toxoplasma* tissue cysts were observed in the livers, but not in the lungs and brains.

Conclusion: The detection of *T. gondii* genome in the aborted fetuses with high prevalence rate indicates that this infection plays a notable role in the abortion of sheep and goats in East Azerbaijan. Therefore, fundamental management is necessary for the prevention and control of the disease in this region, particularly regarding zoonotic potential.

Introduction

Toxoplasma gondii, a protozoan parasite found in warm-blooded animals (1), is zoonotic (2), and can be dangerous for humans and animals. In many parts of the world, especially among animals, such as sheep and goats, this is recognized as one of the important causes of abortion (3, 4).

Toxoplasmosis can lead to serious problems in the reproduction of livestock, and this issue causes abortions. This can negatively affect the production. It also affects animals' health and welfare and may challenge the small ruminant farming industry globally (5). On the other hand, toxoplasmosis can also be dangerous for public health, because it is possible to be transmitted to humans through contact or consumption of contaminated meat and milk (5). Immunocompromised people and pregnant women are also at risk of toxoplasmosis (6). It has an asexual reproduction life-cycle in intermediate hosts such as mammals and a sexual reproduction in the intestine of cats, the definitive host for the parasite. Infected cats can expel a large number of oocysts, which can remain in the environment for a long time (7).

In non-pregnant ewes, this disease does not cause clinical symptoms, therefore farmers are less concerned about it (8). But, if sheep and goats get infected with this parasite during pregnancy, they may face an increase in stillbirths and the birth of weak lambs (9). More importantly, toxoplasmosis in sheep causes abortion and white spots on the placenta. Early infection may result in death or abortion, but cellular protective immunity is established. In late pregnancy, due to the relative development of the fetal immune system, clinical effects may not be evident, or lambs may be born healthy but still infected. Abortion caused by toxoplasmosis in sheep is often linked to contamination of their feed with cat feces (10).

Using the PCR technique to identify parasite DNA in various tissues is one of these methods to control and prevent the spreading of this infection in pastures. Proper management of food and water resources, along with vaccination using a live vaccine, is implemented in countries where the vaccine is available (8, 10).

The aim of the present study was the molecular detection and pathological investigation of *T. gondii* infection in the aborted fetus of sheep and goats in East Azerbaijan province of Iran.

Materials and Methods

Ethical approval

All applicable international, national, and institutional guidelines for the care and use of animals, including the protocol approved by the Animal Research Ethics Committee of the University of

Tabriz (ID: IR.TABRIZU.REC.1403.049) were followed.

Study area and sample collection

A total of 62 aborted fetuses were collected from sheep and goats in the East Azerbaijan Province (Fig. 1) (Seven counties, including Tabriz, Charuymaq, Khoda Afrin, Jolfa, Heris, Bostan Abad, and Mianeh, which have mostly a tropical and subtropical steppe climate), northwest Iran, from November 2023 to February 2024. Indeed, this study presents findings on *Toxoplasma* infection as part of a larger investigation into the infectious and non-infectious causes of abortion in small ruminants in East Azerbaijan province, northwest Iran. In the area we studied, semi-intensive production systems are the most common. We studied a total of 43 sheep flocks, which were categorized by size: 7 small flocks (1–100 sheep), 19 medium flocks (101–300 sheep), and 17 large flocks (over 300 sheep).

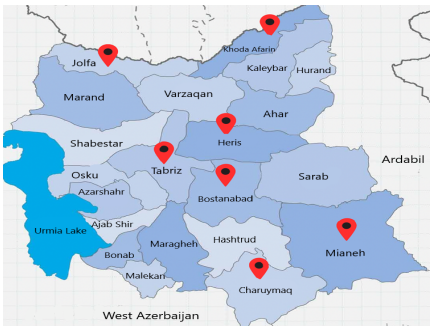

Fig. 1: Study area in the present study

The samples were transferred to the Pathology Lab. At first, a systematic necropsy was performed for each aborted fetus. The samples were collected from the liver, brain, and lung, which were divided into two parts; one part approximately 50 mg was placed in a microtube and stored at -70 °C for further molecular analyses. Another part was collected for histopathology, which was placed in the 10% neutral buffered formalin solution.

Pathological study

The tissue samples were kept in10% neutral buffered formalin solution at least 48 hours. After that, the processing of the tissues was conducted routinely using a DS2080/H tissue processor (Didsabz, Iran). Subsequently, the tissues were embedded in paraffin, cut into 5 µm thick sections, and stained with common hematoxylin and eosin (H&E). Finally, the sections were studied by a light microscope (Olympus, CH-30, Japan), and the observed lesions were recorded.

Molecular studies: DNA extraction and PCR assay

The genomic DNA (gDNA) of the tissue samples including the brain, liver, and lung was extracted, using a DNA extraction kit® (Pishgam Sanjesh, Tehran, Iran) based upon the manufacturer's instructions. Positive DNA was also prepared of tachyzoites extracted from the peritoneal fluid of infected laboratory mice using the same kit after some freeze-thaw cycles. The genome's quality and quantity were analyzed using NanoPhotometer® NP80 (IMPLEN, Germany). All PCR assays were performed in a final volume of 25 µl with Taq DNA Polymerase Master Mix RED® (Ampliqon, Denmark) and 3µl DNA/cDNA, using the T100 Thermal Cycler (Bio-Rad, USA). To perform PCR test, the specific primers for TgTox4F (Forward: 5'-CGCTGCAGGGAGGAAGAC-GAAAGTTG-3') and TgTox4R (Reverse: 5'-CGCTGCAGACACAGTGCATCTGGATT-3') (11, 12), targeting 529 base pair (bp) fragment were used. Herein, TgTox gene was chosen because of its presence in high number of copies and proven sensitivity, and specificity to detect *Toxoplasma* infection. The reaction included 35 cycles with an annealing temperature of 55°C. The amplified products were detected through electrophoresis on 2% agarose gels stained with a safe DNA stain (SinaClon, Iran).

Statistical analyses

The Chi-Square test was used to determine the correlations between infections and age groups (under three months, between 3-4 months, and greater than four months old) of the fetuses. Differences were considered significant at $P < 0.05$. The analyses were performed with IBM SPSS Statistics v.26 software (IBM Corp., Armonk, NY, USA).

Results

Pathological findings

At macroscopic examination (Fig. 2), there were small white foci due to the presence of *Toxoplasma* tissue cysts in the cotyledons. Indeed, we could examine the placenta and cotyledons only in the five affected animals, which two out of them presented this marked lesion. At necropsy, there were extensive hyperemia and interstitial pneumonia in the lungs associated with severe hyperemia in the liver. Moreover, severe hyperemia was observed in the brains and meninges. Subsequently, tissue sections of the brains, livers, and lungs were examined and studied for histopathological lesions.

Fig. 2: Necropsy of aborted fetus naturally infected with *Toxoplasma gondii*. A: At first, the external inspection and measurement of the fetus's body were performed to estimate the age. B: Small white foci (arrow) due to the presence of *Toxoplasma* tissue cysts in cotyledons. C: There were extensive hyperemia and interstitial pneumonia (large arrow) in the lung associated with severe hyperemia in the liver (small arrow). D: Severe hyperemia was observed in the brain and meninges (arrow)

At microscopic studies (Fig. 3), the lesions observed in the brain tissue included moderate to severe hyperemia, mild perivascular and perineuronal edema, focal to diffused gliosis, focal necrosis, and nonsuppurative meningoencephalitis. Parasite cysts were not observed in histological sections of the brains (0/15; none out of 15 PCR positive samples). In the livers, focal mononuclear hepatitis accompanied by focal necrosis and mild to moderate hyperemia were found. Notably, *Toxoplasma* parasite cysts were observed in the livers (3/7; three out of seven PCR positive samples). In the lung, diffuse infiltration of mononuclear inflammatory cells (nonsuppurative pneumonia) along with vascular hyperemia and focal hemorrhages were observed. No parasite cysts were observed in the lung sections (0/1; none out of one PCR positive sample).

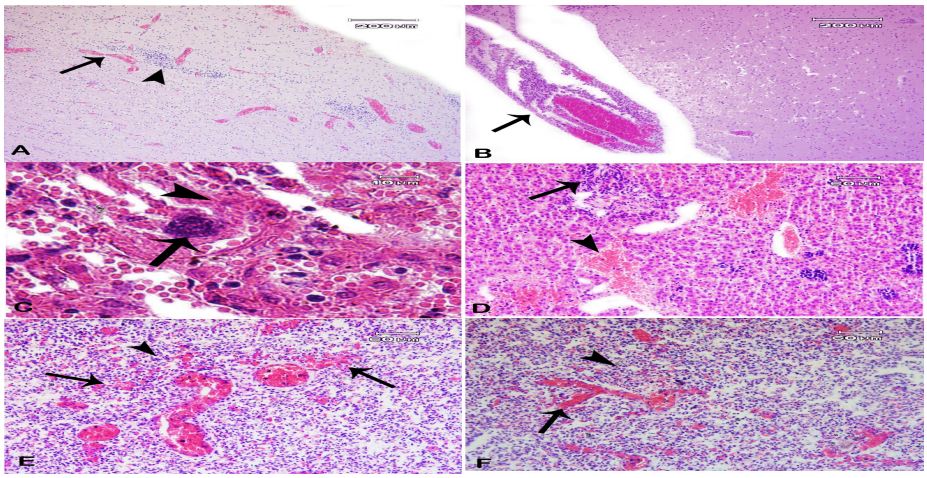

Fig. 3: Aborted fetus with natural infection of toxoplasmosis. A: Brain: focal gliosis (arrowhead) is observed with severe hyperemia (arrow). B: Brain: Hyperemia of meningeal vessels with infiltration of inflammatory cells (non-purulent meningitis) (arrow). C: Liver: *Toxoplasma* cyst (arrow) with focal hepatocyte necrosis (arrowhead). D: Liver: A focal hepatitis (arrow) associated with focal hemorrhage (arrowhead). E: Lung: Focal hemorrhage (arrows) with infiltration of mononuclear cells (non-purulent pneumonia) (arrowhead). F: Lung: severe hyperemia of pulmonary vessels (arrow) with infiltration of mononuclear cells (non-purulent pneumonia) (arrowhead). H&E

Molecular findings

PCR results indicated the presence of the *T. gondii* genome in the 25 (40.32%) of the examined fetus with the presence of the 529 base pair bands (Fig. 4). As more detail, among the 25 positive fetus, in 15, 7, and one positive fetus, *T. gondii* genome were found in the brains, livers, and lungs, respectively. Additionally, one fetus was positive in both the liver and brain, and one fetus was positive in both lung and liver tissues.

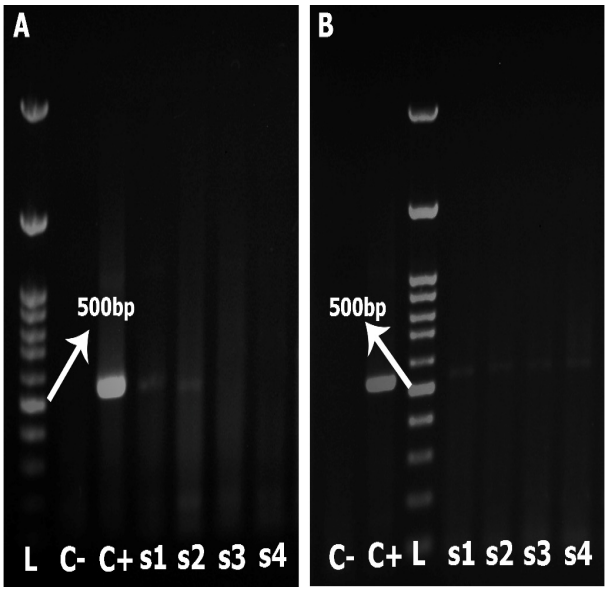

Fig. 4: PCR findings of the present study. The amplified products were detected through electrophoresis on 2% agarose gels stained with a safe DNA stain. L: ladder 100 bp; C-: negative control; C+; positive control with a 529 bp band. **A:** s1 and s2 show positive samples in the lung, and s3 and s4 show negative samples in the lung. B: s1 and s2 show positive samples in the brain, and s3 and s4 show positive samples in the liver

Statistical analyses

These results present in Table 1 and Fig. 5. Although the infection rate was higher at older ages, no statistically significant difference was observed between different age groups (P = 0.403). Also, there was no significant difference between different seven counties (P =0.742). Since the age of the aborted fetuses was greater than or equal to (≥) 2 months, three age groups were determined as the beginning of pregnancy (<3 months), the middle of pregnancy (3-4 months), and the end of pregnancy (>4 months).

Table 1: PCR results and the age groups of the aborted fetuses (n=62) with no significant difference.

Age (month)	0-3 m	3-4 m	4-6 m	*P value*
Number of aborted fetuses	8	25	29	-
Number of Positive samples (%)	3 (4.84%)	8 (12.90%)	14 (22.58%)	0.403

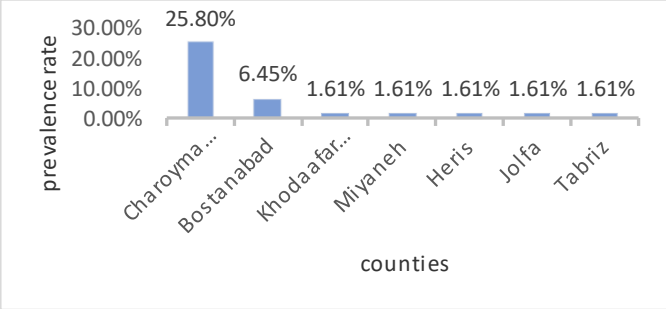

Fig. 5: PCR results in different counties with no significant difference (*P value*= 0.742).

Discussion

The present findings in East Azarbaijan province demonstrated the presence of *T. gondii* infection in small ruminants with a notable infection rate, which is consistent with the previous studies regarding toxoplasmosis infection conducted in different provinces of Iran. In this regard, growing evidence shows some differences with each other, which can be related to the geographical region, climate and weather, management methods, health level, and laboratory diagnostic methods.

As previously described, toxoplasmosis is important as a common disease between humans and animals, which is pathogenic in immunocompromised people and pregnant women (12). Despite the low prevalence of clinical symptoms, the conducted studies all indicate different and relatively high *Toxoplasma* infections based on different methods. In Iran, studies have been conducted on abortions related to toxoplasmosis. The important point in the research is the variety of methods used to investigate this disease. In a previous study conducted in East Azerbaijan province, using the ELISA method, the serum prevalence of toxoplasmosis in sheep in the villages around Tabriz was reported to be about 13.45% (13). Also, in other investigations conducted on ovine with a history of abortion in rural areas of Tabriz, using the indirect fluorescent antibody test, it was found that 34.6% of these sheep have toxoplasmosis-specific immunoglobulin G (14).

Other research in Tabriz has been done using different laboratory methods, such as dye tests and ELISA, which have shown different results about the spread of this disease. For example, in one study the prevalence was reported as 56.4% (15) and in another study 6.98% (16). Also, another report using the dot blot method in the villages of Tabriz showed a prevalence of 9.14% (17). It is interesting to note that the level of infection in the ovine was higher than in the ram, but this difference was not statistically significant (18).

Serological and molecular studies have consistently reported significant infection rates in sheep and goat populations in Iran, with prevalence ranging from 12.2 to 21.74% in sheep and from 4.4 to 10.6% in goats (18, 19). Furthermore, molecular investigations, including PCR analysis of fetal tissues and blood samples, have shown even higher infection rates, highlighting the significant impact of this parasite on the abortion of sheep and goats (20). There are geographical differences in the prevalence of toxoplasmosis in Iran, with central and southern regions having higher infection rates than western regions (18). In a study conducted in Qazvin, on 18 brain samples obtained from sheep aborted fetuses, *T. gondii* DNA (*B1* gene) was detected in 12 out of 18 samples (66.66%) by nested-PCR (21). In Khorasan Razavi Province, North-East of Iran, a study was conducted on

37 brain samples, and *T. gondii* DNA (*B1* gene) was detected in 20 out of 37 samples (54%) by semi-nested PCR method (22). In another study in this region on 200 brain samples of aborted fetuses by IFA and nested-PCR, *T. gondii* DNA (*B1* gene) was detected in 27 of 200 samples (13.5%), and a positive test for anti- *T. gondii* antibodies was detected in 31 of 200 samples (15.5%). In Ardebil Area (northwest of Iran), another study conducted on 75 brain samples of sheep aborted fetuses, *T. gondii* DNA (*GRA6* gene) was detected in 48 of 75 samples (64%) by nested-PCR (23). In a study in the Iran-Iraq border areas (2020) conducted on 111 brain samples obtained from aborted sheep and goat fetuses, *T. gondii* DNA (*B1* gene) was detected in 9 of 111 (8.1%) by nested-PCR (24). In addition, in North Khorasan province, emerging evidence has shown that more than half of abortion cases (53.10%) in sheep were related to *Toxoplasma* (25).

According to the meta-analysis conducted to estimate the global prevalence of *T. gondii* infection in aborted and stillborn fetuses of sheep and goats in 2021, the overall prevalence of infection in sheep and goats was 42% and 31%, respectively, by molecular methods and was 16% and 27%, respectively, using serological tests. In addition, the overall prevalence of *T. gondii* infection in aborted sheep and goats was 56% (95% CI: 35–76%) and 50% (95% CI: 6%–94%), respectively (5). Extensive research in Switzerland farms has shown that there was a high percentage of *T. gondii* infections in livestock, such that the percentage of serum contamination in sheep reached 66.3% and in goats 50.5%. Also, *T. gondii* DNA was found in 6.1% of aborted sheep fetuses, which shows a direct relationship between infection and abortion (26). Another systematic review indicates that about 10% of women who had spontaneous abortions were infected with this parasite (21). Recent infection with *Toxoplasma* can increase the risk of miscarriage. Therefore, this study emphasizes the importance of further research on the risk factors associated with toxoplasmosis and suggests that more tests be conducted to investigate the role of this infection in abortion cases (27).

Herein, histopathological studies indicated remarkable lesions like focal to multifocal gliosis accompanied with nonsuppurative meningoencephalitis, focal to multifocal mononuclear hepatitis, and nonsuppurative pneumonia in all PCR positive samples. However, *Toxoplasma* tissue cysts were observed only in the three examined livers. The present findings are in continence with others that previously reported similar histopathological lesions from Brazil (23), Spain (24), and Switzerland (25) with 14.3%, 5.4%, and 17.64% infection rates, respectively. Another similar study in Italy determined *Toxoplasma* infection in the placenta of sheep and goats with 31.5% and 8.7% using PCR and histopathology methods (26), respectively. Taken to gather, increasing evidence demonstrated acute inflammatory reactions associated with vascular disorders and cellular necrosis in the affected tissues by *T. gondii.*

In the statistical analyses, there were no significant differences between infection rate and various age groups or different seven counties. While some studies suggest a possible relationship between infection rates and the age or gender of the animals (27), more research is needed to clarify these relationships. According to the present results, the infection rate increased in the older fetus, but, there was no significant between the age groups.

Conclusion

The northwest region of Iran, especially East Azerbaijan, is one of the rich climates for the breeding of the livestock population. Therefore, it is very important to investigate the prevalence of infectious diseases linked to abortion, like toxoplasmosis, in the animals of this region. A series of studies have shown

that *T. gondii* infection, especially during early pregnancy, is associated with an increased risk of abortion. This issue has a significant economic impact on animal husbandry and requires efficient management plans to reduce contact with this parasite. These programs can include environmental controls and monitoring of wildlife that may act as parasite reservoirs. To better understand and reduce the effects of toxoplasmosis in livestock as well as its consequences for human health, further research and improved surveillance systems are necessary.

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Conflicts of Interest

There is no conflict of interest.

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