Pathological and molecular investigation of *Mycoplasma agalactiae* infection in aborted fetuses of small ruminants northwest Iran

Running title: *Mycoplasma agalactiae* infection in aborted fetuses

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Abstract

Introduction: *Mycoplasma* infection mainly affects small ruminants, leading to mastitis, arthritis, keratoconjunctivitis, and abortion. This disease not only reduces reproductive efficiency but can also negatively impact milk production and the overall health of the flock. Therefore, the present study was carried out to recognize *Mycoplasma* infection caused by *Mycoplasma agalactiae (M.*

agalactiae) in aborted fetuses of small ruminants (sheep and goats) from East Azerbaijan province, northwest Iran, using pathological and molecular studies.

Materials & Methods: A total of 62 aborted fetuses were collected from local sheep and goat flocks in autumn and winter 2023. Tissue samples were collected during necropsy for molecular (PCR) and pathological studies. A conventional PCR assay was carried out using specific primers (MYC1 and MYC2) to detect the genome of *M. agalactiae* genome (373 bp). Besides, the collected tissue samples were routinely prepared for histopathological studies and stained with hematoxylin and eosin (H&E).

Results: PCR results revealed the presence of the *M. agalactiae* genome in 38 out of 62 samples (61.29%, and 95%CI: 0.49 - 0.73). Histopathological examinations showed multifocal hepatitis, interstitial pneumonia, and multifocal nephritis in addition to vascular hyperemia and focal hemorrhage in the heart and brain.

Conclusion: The high prevalence of *Mycoplasma* infection in aborted fetuses indicates that this infection can play a remarkable role in causing abortions in small ruminants in East Azerbaijan province. Therefore, rapid diagnosis of these infections is necessary for effective prevention, vaccination strategies, control and treatment of disease outbreaks in livestock herds.

Keywords: Abortion, Economic losses, Goat, Mycoplasmosis, Sheep.

1. Introduction

Mycoplasmas, which typically cause chronic endemic diseases, are the smallest microorganisms, about 300 nm in diameter that can live independently and are classified under the class *Mollicutes*. This class includes eight genera, five of which are typically associated with animals: *Mycoplasma*, *Acholeplasma*, *Ureaplasma*, *Anaeroplasma*, and *Asteroplasma*. Among these, *Mycoplasma* and

Ureaplasma are known to cause more diseases in animals compared to the others [1-3]. These bacteria lack cell walls, rendering them resistant to antibiotics that target the cell wall [4, 5]. Numerous pathogenic species have been detected, some of which also affect humans [6]. *Mycoplasma* species are associated with economically important diseases in ruminants worldwide. Since animal husbandry is one of the main pillars of developing countries and plays important economic, social, and cultural roles, abortion caused by these bacteria leads to a significant economic loss in this profession [7-9] *Mycoplasma*-induced abortion in sheep is mainly associated with *Mycoplasma ovis* (*M. ovis*) and *Mycoplasma agalactiae* (*M. agalactiae*) species, which are non-zoonotic [4]. *Mycoplasma agalactiae* is one of the main causes of abortion in sheep, reported in countries such as India, Egypt, Iran, and some European countries [10, 11]. Small ruminants play an important role in Iran's agricultural economy [10], and abortion has a significant economic impact on their herds [12], impacting reproductive efficiency, milk production, and the overall health of the herd [13]. *Mycoplasma agalactiae* generally leads to contagious agalactia disease (CA), characterized by mastitis in 60 to 80% of lactating females, along with pneumonia, arthritis, keratoconjunctivitis, and abortion in fewer than 10% of affected animals. These reproductive health issues manifest in various ways, often linked to uterine inflammation and granular vulvovaginitis in females, while swelling of the testes in males contributes to 90% and 100% of outbreaks in goats and sheep, respectively [13, 14]. The major risk factors linked to infection and abortion include flock size and vaccination against *M. agalactiae*. Though it may seem illogical for abortion to occur more frequently in vaccinated flocks, only flocks at risk of *M. agalactiae* infections may be vaccinated [11, 14]. Poor livestock management, such as herd size, mixed farming, grazing systems, and contact with other animals, also contribute to abortion rates. Additionally, inadequate compliance with hygiene principles is known as one of the effective

factors that lead to an increase in *Mycoplasma* infections and, as a result, abortion in ewes [15]. Herein, mycoplasmosis infection caused by *M. agalactiae* was evaluated in aborted fetuses of sheep and goats in Iran.

2. Materials and Methods

2.1. Study location and sample collection

The current study was conducted across 7 cities in the East-Azerbaijan province where located in northwest of Iran, which include Tabriz, Khoda Afrin, Charuymaq, Jolfa, Bostan Abad, Heris, and Mianeh. This study presents findings on *Mycoplasma* infection as part of a broader investigation into the infectious and non-infectious factors contributing to abortion in small ruminants in East Azerbaijan province, northwest Iran (under review or published data in Supplementary file). To achieve this, between November 2023 and February 2024, a total of 62 aborted fetuses were collected in the mentioned regions, as reported by the owners. In the area we studied, semi-intensive production systems are the most common. These systems combine agricultural production with small ruminant farming. From spring to mid-autumn, sheep and goats graze on natural pastures, but during the winter months, they are kept indoors and fed mainly with forage and crop residues. We studied a total of 43 sheep flocks, which were classified into three categories according to size: small flocks (1–100 sheep) (n = 7), medium flocks (101–300 sheep) (n = 19), and large flocks (over 300 sheep) (n = 17).

Initially, the age of the aborted fetuses was calculated [16]. After a systematic necropsy, 50 mg of abomasal contents was transferred in a 2 mL microtube and preserved at -70 °C for subsequent molecular analysis. In addition, tissue samples from different organs were collected and fixed in 10% formalin solution for further histopathological examination.

2.2. Pathological study

The tissue samples were fixed in a 10% neutral buffered formalin solution for a minimum of two days. They were then processed routinely, embedded in paraffin, and sectioned at 4-5 µm thickness. Finally, these sections were stained with hematoxylin and eosin (H&E) and examined under a light microscope (Olympus, CH-30, Japan).

2.3. Molecular analysis (DNA extraction and PCR assay)

Genomic DNA (gDNA) was extracted from the abomasal contents utilizing a commercial DNA extraction kit® (Pishgam Sanjesh, Iran) in accordance with the manufacturer's guidelines. The DNA's quality and quantity were assessed using a NanoPhotometer® NP80 (IMPLEN, Germany). The PCR reactions were done in a final volume of 25 µL containing Taq DNA Polymerase Master Mix RED® (Ampliqon, Denmark) and 3 µL template DNA. In addition, specific primers were used for detection of *M. agalactiae* genome using the PCR test: MYC1 (Forward: 5'-AAAGGTGCTTGAGAAATGGC-3') and MYC2 (Reverse: 5'- GTTGCAGAAGAAAGTCCAATCA -3'), targeting a 373 base pair (bp) sequence. Subsequently, the reaction conditions (with 40 cycles and an annealing temperature of 54°C) run (18, 19) using a T100 Thermal Cycler (Bio-Rad, USA). Finally, the PCR products were visualized by electrophoresis on 1.5% agarose gels stained with a safe DNA stain (SinaClon, Iran).

2.4. Statistical analyses

The Chi-Square test was employed to assess the correlation between infection rate and fetal age groups (four categories). A p-value less than 0.05 was considered statistically significant. All statistical analyses were conducted using IBM SPSS Statistics software version 22, and results were reported with a 95% confidence interval (CI).

3. Results

3.1. Molecular findings

The PCR findings based on four age groups are shown in Figure 1 and Table 1. The genome of *M. agalactiae* was detected in a 373 bp PCR product from 38 out of 62 samples (61.29%, 95%CI: 0.49 - 0.73) from the examined fetuses, with higher infection rates found in fetuses aged 4 to 5 months. However, there were no significant differences in infection rates among the four age groups.

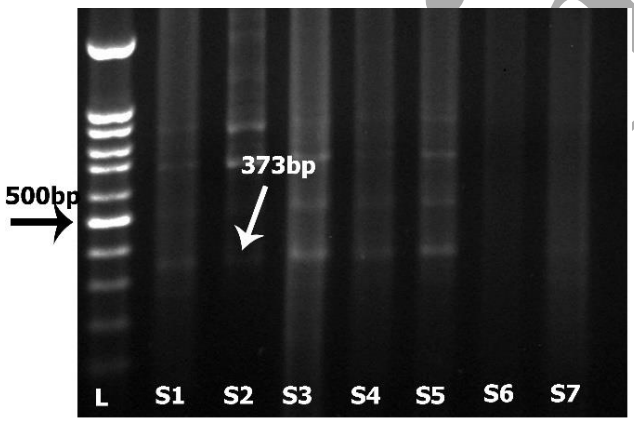

Figure 1. Molecular findings for *M. agalactiae* genome detection in aborted fetuses. The PCR products with a 373 bp band. L: ladder 100 bp; S1, S2, S3, S4, S5: the positive samples with a 373 bp band; S6 and S7: represent negative results.

Table 1. Molecular detection of *Mycoplasma agalactiae* in aborted fetuses (N = 62).

Age groups	No. of the positive samples (%)	95% CI
2-3 m: 5/62 (8.06%)	3/5 (60%)	0.17-1.03
3-4 m: 14/62 (22.58%)	4/14 (28.57%)	0.05-0.51
4-5 m: 36/62 (58.06%)	27/36 (75%)	0.16-0.89
Over 5 m: 7/62 (11.29%)	4/7 (57.14%)	0.21-0.93
Total: 62	38/62 (61.29%)	0.49-0.73

3.2. Pathological findings

At necropsy, there was notable interstitial pneumonia with various patterns, associated with pale foci and hyperemia in the liver and kidneys (Figures 2A and B). Also, there was vascular hyperemia in the brain and meninges. Microscopic examinations primarily showed interstitial pneumonia characterized by infiltration of various inflammatory cells with a predominance of neutrophils in the lungs. Additionally, there were focal areas of cellular necrosis in the liver and kidneys, surrounded by inflammatory cells, vascular hyperemia, and focal hemorrhage. There were diffuse gliosis and vascular hyperemia in the brain (Figures 2C-F).

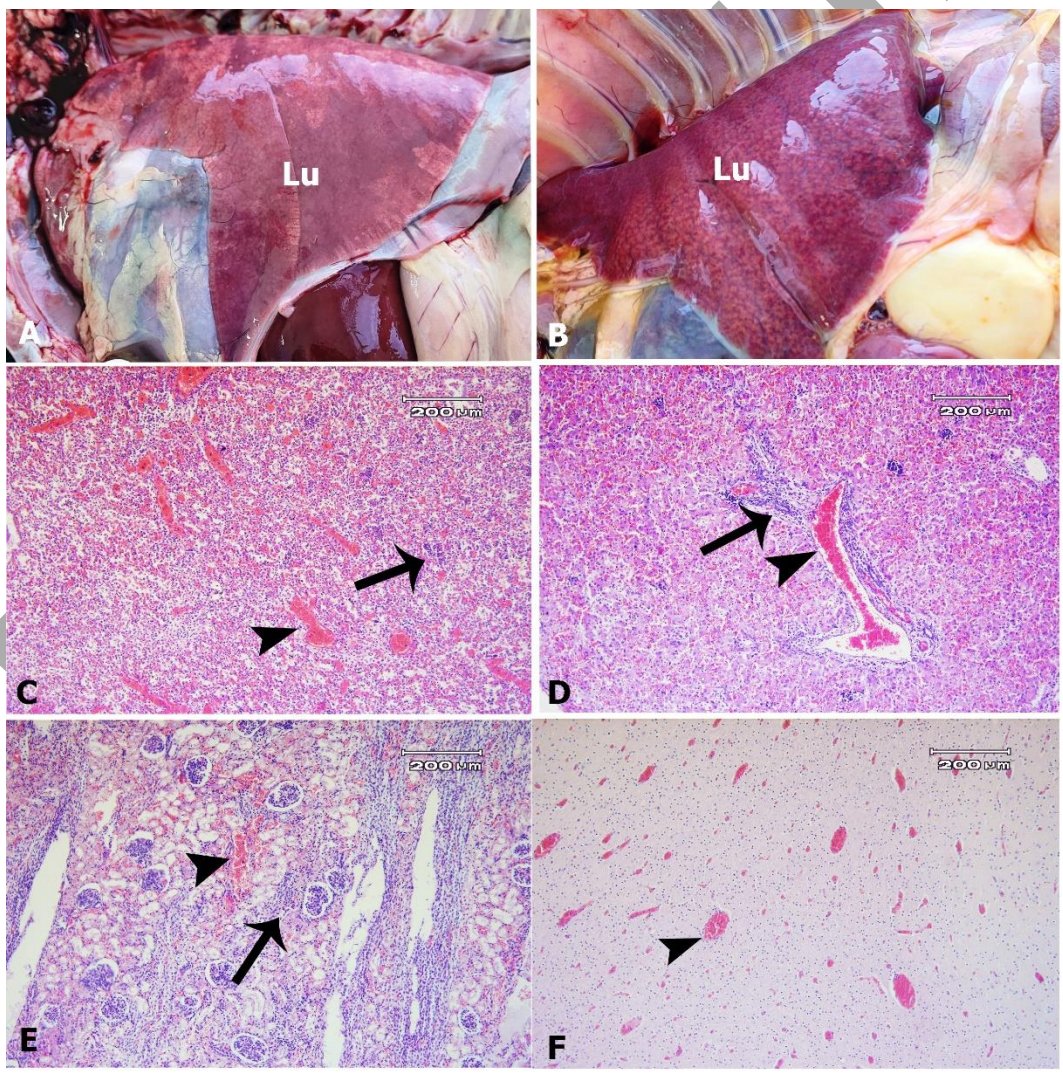

Figure 2. A and B: Lung with interstitial pneumonia associated with diffuse hyperemia. C: Lung: the presence of mixed inflammatory cells in the parenchyma and airways (arrow), associated with hyperemia in the pulmonary vessels (arrowhead). D: Liver: there are focal areas of hepatocyte necrosis, surrounded by mononuclear cells (arrow) and vascular hyperemia (arrowhead). E: Kidney: infiltration of inflammatory cells (arrow) associated with focal hemorrhage (arrow). F: Brain: there are diffuse gliosis and notable hyperemia (arrowhead). H&E.

4. Discussion

In the present study, *Mycoplasma* infection was detected in 61.3% of dead fetuses across various ages by PCR and pathology, indicating a very high infection rate. Given the rapid and highly contagious nature of this infection, this rate is expected. In this regard, a cross-sectional study conducted in southern Iran found *M. agalactiaefrom* in 3% of samples from 183 sheep and 117 goats using PCR, highlighting its status as a neglected abortifacient in small ruminants [6]. Similarly, another study detected *Mycoplasma* in 14 out of 33 (42.4%) sheep samples in Tehran province in both PCR and bacterial culture [17]. Kheirabadi et al examined milk and conjunctiva samples from ewes using PCR technique and reported 19.8% (20 out of 101) of the samples positive for *Mycoplasma agalactiae* [18]. Additionally, recent research in Turkey identified a 4.47% positivity rate for *Mycoplasma* in aborted fetuses of cows and sheep. A recent research in Turkey reported a 4.47% positive sample for the *Mycoplasma* genome in aborted fetuses of cows and sheep by PCR [19]. Another similar study conducted in the United States found an average prevalence of *M. ovis* in the blood samples of domestic sheep herds at 73.3% in 2001, decreasing to 23.2% in 2011, with higher prevalence rates observed in sheep flocks experiencing abortions and grazing with other breeds [11]. Moreover, a previous study in the Ili region of northwest China

reported an overall seroprevalence of *Mycoplasma* infections in sheep at 6.42%, with abortion rates identified as 30.40% among seropositive cases [15]. Growing evidence indicates that the economic impact of *Mycoplasma* infections is significant, resulting in losses due to complications such as pneumonia, arthritis, keratoconjunctivitis, and abortion, accompanied by reduced reproductive efficiency (in males and females), and milk production [10-14].

In the present study, the lungs were firm on palpation with marked interstitial pneumonia at microscopic evaluation. Also, there were multifocal necrotic hepatitis and nephritis associated with lymphocytic mononuclear cell infiltrates, consistent with previous reports [20]. Herein, we did not take samples from conjunctiva or placenta.

The present results with a significantly higher infection rate are in agreement with previous findings. Variations in the results of the studies can be due to differences in sample type, sample size, testing method, herd size, vaccination practices, management, geographic location, and the presence of other infections in the area. The high infection rate observed in the present study may be attributed to the fact that all samples were collected from aborted fetuses, and not from blood or vaginal samples of adults. Taken to gather, *Mycoplasma* is one of the main causes of abortion in small ruminants, leading to significant economic losses for the affected farms. Therefore, diagnosis of this infection is necessary for preventing and controlling outbreaks and developing vaccination strategies.

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Author's contribution

Study concept and design: M.Kh, H.S, J.Sh

Acquisition of data: M.Kh, H.S, J.Sh, AR.J, F.JA,Y.JKh, F.M, A.M

Analysis and interpretation of data: M.Kh, H.S, J.Sh, AR.J, F.JA,Y.JKh, F.M, A.M

Drafting of the manuscript: M.Kh, H.S, J.Sh, AR.J, F.JA, Y.JKh, F.M, A.M

Critical revision of the manuscript for important intellectual content: M.Kh, H.S, J.Sh, AR.J, F.JA, Y.JKh

Statistical analysis: M.Kh, H.S

Administrative, technical, and material support: M.Kh

Study supervision: M.Kh

Ethics

All applicable international, national, and institutional regulations regarding the care and use of animals, were adhered to, including the protocol sanctioned by the Animal Research Ethics Committee of the University of Tabriz (ID: IR.TABRIZU.REC.1403.049).

Conflicts of Interest

There is no conflict of interest.

Funding Statement

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Data availability

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

AI (artificial intelligence) statement

This paper does not use artificial intelligence.

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