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Research Paper

Investigation of *Listeria* Infection in the Aborted Fetuses of Small Ruminants in East Azerbaijan Province, Northwest Iran

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ABSTRACT

Introduction: Listeriosis, an important food-borne zoonotic disease, is caused by the member of Listeriaceae family. The aim of the present study was to detect the presence of *Listeria* infection in aborted fetuses and serum samples from adults with a recent history of abortion using serological, molecular, and pathological methods in East Azerbaijan Province.

Materials & Methods: A total of 62 aborted fetuses and 288 vaginal swabs were collected from sheep and goat flocks across nine cities. For molecular analyses, conventional polymerase chain reaction (PCR) was employed to detect the Listeria genome after extracting DNA from the abomasal contents of examined aborted fetuses. Histopathological examinations were also conducted on formalin-fixed tissue samples from the aborted fetuses.

Results: In microbiological study, the organism was isolated in 1.24% of abomasal contents, 2.43% of vaginal swaps, and 3.72% of the fetus's brain. Molecular analyses showed that *Listeria* infection was present in 41.93% (95% CI, 0.41%, 0.12%) of the aborted fetuses. Pathological examinations revealed white foci in the liver, lung, and myocardium, accompanied by severe hyperemia in the brain. In addition, microscopic studies indicated remarkable necrotic and inflammatory responses in the tissue sections, particularly in the brain (encephalitis), lung (pneumonia), liver (hepatitis), and heart (myocarditis).

Conclusion: In conclusion, the detection of *Listeria* infection in aborted fetuses at a much higher infection rate indicates that this infection plays a notable role in abortion among sheep and goats in East Azerbaijan. More importantly, it remains a major zoonotic diseases worldwide. Therefore, effective public health management strategies are crucial for its prevention and control.

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1. Introduction

Listeria is a group of rod-shaped bacteria [1] in the Listeriaceae family [2], consisting of 21 species [3, 4]. Among these species, *Listeria monocytogenes* is the primary cause of listeriosis in several animal species [5, 6]. During the 1980s, several epidemic outbreaks occurred in North America and Europe, and listeriosis was recognized as an important food-borne illness linked to contaminated food [7, 8]. Sheep are usually infected by consuming feed contaminated with common environmental factors such as silage, soil, water, and decaying plants, which also pose serious health risks to humans and animals [9]. Sheep and goats of any age or sex may be affected; symptoms can appear rapidly, and death may occur [10]. Fetal infection is considered to occur via hematogenous spread from the placenta, with an incubation period of about 5-12 days [10]. Importantly, this disease exhibits a high mortality rate of about 20% in humans. It can carry and spread the pathogen without showing symptoms, releasing bacteria into their environment through feces [11-13].

In addition, *L. monocytogenes* causes reproductive problems such as late abortion, stillbirth [14], and poor offspring [15]. Usually, abortions are sporadic, but sometimes 50% of pregnant animals may abort [10, 15]. In small ruminants, listeriosis can manifest as a neurological disease due to encephalitis and septicemia, increasing the risk of third-trimester miscarriages [15]. Abortion caused by listeriosis in sheep results in a significant economic losses, including higher weaning rates and production costs, as well as increased expenses for slaughtering, food, labor, and veterinary care [16, 17]. Currently, there is no specific vaccine for listeriosis in sheep and goats [5, 10].

Therefore, effective management and prevention strategies are very important to avoid infection in flocks. The aim of this study was to investigate the infection rate of listeriosis in aborted sheep and goat fetuses using bacterial culture, molecular, and pathological examinations, thereby enhancing our understanding of this animal disease and its health implications.

2. Materials and Methods

2.1. Study area and sampling

The present study was performed in seven cities of East-Azerbaijan Province, northwest Iran, including Tabriz, Charuymaq, Khoda Afrin, Jolfa, Heris, Bostan Abad, and Mianeh. This study presents findings on *Listeria* infection as part of a larger investigation into the infectious and non-infectious causes of abortion in small ruminants in East Azerbaijan Province. From November 2023 to February 2024, a total of 62 aborted fetuses and 288 vaginal swaps were collected from small ruminants including sheep and goats in the mentioned regions with a recent history of abortion. All samples belonged to the herds under traditional conditions. Indeed, in the studied area, semi-intensive production systems predominate, where agricultural production coexists with sheep and goat farming. Sheep and goats graze in pastures from spring to mid-autumn but are housed and fed indoors during winter, relying on forage and crop residues as primary feed sources.

Vaginal samples were stored in sterile normal saline and transferred to the microbiology laboratory. The age of the aborted fetuses was estimated using the Equation 1:

1. $(X+17)\times 1/2$, where X represents the fetal size in centimeters measured from forehead to tail. Then, a systematic necropsy was performed, and pathological lesions were recorded. Finally, 50 mg of the abomasal contents were placed in a 2-mL microtube and stored in a freezer at -70 ˚C for further molecular studies. Additionally, tissue samples from various organs, including the brain, liver, heart, and lungs, were collected and transferred to a 10% formalin solution for histopathological analysis.

2.2. Bacterial culture (*L. Monocytogenes* detection)

Fetal tissues (brain, lung, and liver) and abomasal contents were cultured for testing. The fetal tissue samples were flame- sterilized and directly plated on tryptose soy agar (TSA) with 5% sheep's blood agar (Merck, Germany). Cervico-vaginal swabs and fetal abomasal contents were also directly plated on the same agar medium as the tissues. All media were incubated at 37 ˚C with 5% CO_2 for 18-24 hours and then examined according to standard operating procedures. Bacterial colonies that resembled morphology members of the genus *Listeria* were sub-cultured onto 5% sheep's blood agar for purity. These suspected *Listeria* colonies then underwent gram staining, catalase testing, and phenotypic analysis, using CAMP, gas production, H2S production, hemolysis, indole, motility, and methyl red (MR) tests [18].

2.3. Pathological study

Tissue samples were kept in a 10% neutral buffered formalin solution for at least 48 hours. The tissues were then processed routinely using a DS2080/H tissue processor

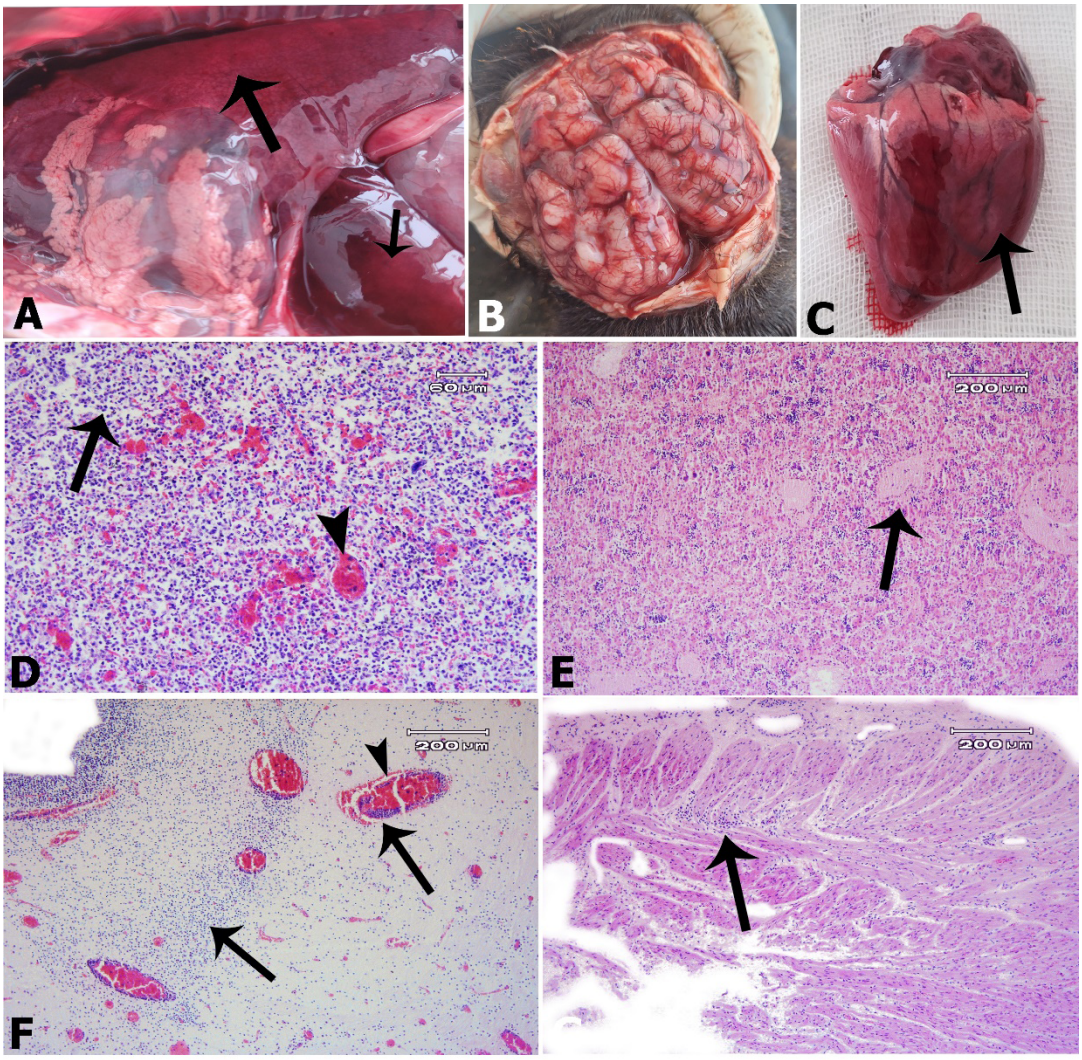

Figure 1. An aborted fetus with infection of listeriosis (H&E)

A) White to yellow foci in the liver (short arrow) and lung (long arrow) associated with diffuse hyperemia; B) Remarkable hyperemia and edema in the brain; C) white foci in the heart (arrow) associated with diffuse hyperemia; D) infiltration of inflammatory cells (interstitial pneumonia) (arrow) with severe pulmonary hyperemia (arrowhead); E) focal hepatic necrosis (arrow) surrounded by inflammatory cells; F) Diffuse infiltration of inflammatory cells in the brain parenchyma and around the vessels (perivascular cuffing) accompanied by hyperemia (arrowhead), G) Focal inflammatory cell infiltration in the heart (arrow)

(Didsabz, Iran). Subsequently, the tissues were embedded in paraffin, cut into 5 µm thick sections, and stained with hematoxylin and eosin (H&E). Finally, the sections were studied under a light microscope (Olympus, CH-30, Japan), and the observed lesions were recorded.

2.4. Molecular studies (DNA extraction and Polymerase chain reaction (PCR) assay)

Genomic DNA (gDNA) from the abomasal contents was extracted using a DNA extraction kit® (Pishgam Sanjesh, Tehran, Iran) according to the manufacturer's instructions. The genome's quality and quantity were analyzed using a 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 3 µL of DNA/cDNA, using the T100 Thermal Cycler (Bio-Rad, USA). To perform PCR test, specific primers for HLYA1 (forward: 5'- ATCAGTGAAGGGAAAATG-CAAGAAG-3') and HLYA2 (reverse: 5'- TTGTATA-ACCAATGGGAACTCCTGG -3'), targeting 451 base pair (bp) fragment, were used. The reaction included 40 cycles with an annealing temperature of 59 ˚C. Besides, ATCC-19115 was used as the positive control. Amplified products were detected through electrophoresis on 2% agarose gels stained with a safe DNA stain (Sina-Clon, Iran).

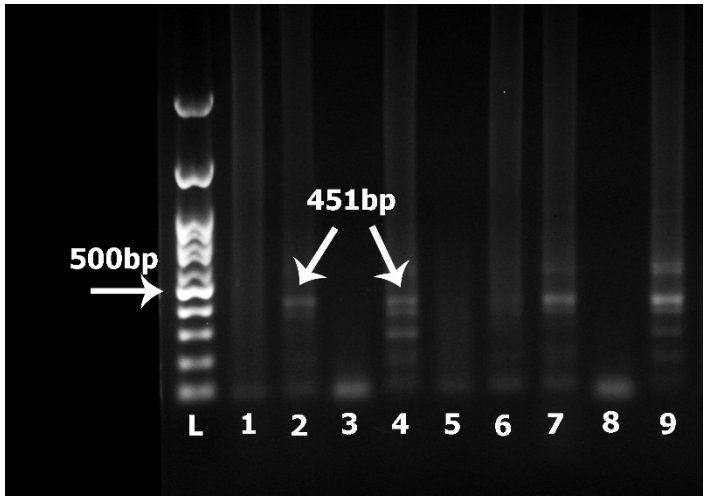

Figure 2. Molecular findings of the present study for detecting the Listeria genome in aborted fetuses

Note: The PCR products with a 451 bp band were detected through electrophoresis on agarose gels stained with a safe DNA stain. L: Ladder 100 bp; S1: Negative control; S3, S5, S6, and S8: The samples with negative results; S2, S4, S7, and S9: Positive samples with a 451 bp band.

2.5. Statistical analyses

The chi-square test was used to determine correlations between infections and age groups (four groups, including 2-3, 3-4, 4-5, and over 5 month-olds) of the fetuses. Differences were considered significant at $P<0.05$. Analyses were performed with IBM SPSS statistics software (version 22), with a 95% confidence interval (CI).

3. Results

3.1. Bacterial culture

Out of 288 vaginal samples, seven (2.43%; 95% CI, 0.24%, 0.048%) were contaminated with *Listeria* species. Also, it was isolated from two (1.24%; 95% CI, 0.12, 0.08%) stomach content (which was also positive by PCR) and six (3.72%; 95% CI, 0.37%, 0.12%) brain samples (out of 62, individually). No isolates were obtained from lung or liver samples.

3.2. Pathological findings

At necropsy, gross lesions consisted of white to yellow foci in the liver, lung, and heart, associated with diffuse hyperemia. Additionally, there were remarkable hyperemia and edema in the brain. Microscopic examinations revealed multifocal hepatitis and hepatic necrosis were observed in the liver. Similar foci with cellular necrosis, small numbers of degenerating neutrophils, and mononuclear cells associated with hyperemia and focal hemorrhage were observed in the lung and myocardium. Importantly, there were diffuse gliosis, meningoencephalitis with perivascular cuffing, and notable hyperemia in the brain (Figure 1).

3.3. Molecular findings

The genome of *Listeria* was detected in 41.93% (26 out of 62) (95% CI, 0.41%, 0.12%) of the examined fetuses, as indicated by distinct 451 bp target bands (Figure 2). The results of the molecular study related to age groups are presented in Table 1. Briefly, the most positive samples were detected in the 4-5 month-old group. However, there was no statistically significant difference between the four age groups ($P<0.05$).

4. Discussion

The present findings demonstrate a concerning 41.93% listeriosis infection rate among aborted fetuses of sheep and goats in northwest Iran, as detected using molecular and pathological studies. These findings highlight the serious effect of *L. monocytogenes* on sheep and goat abortion, especially in pregnant ewes. One of the high prevalence herein reported could be due to the selection of only aborted animals. In ruminants, listeriosis is often linked to cerebral localization and encephalitis. However, localization also occurs in the pregnant uterus, where abortion or stillbirth are then the common signs [19]. Alongside abortion, encephalitis is the most common symptom of *Listeria* infection in sheep [19, 20]. In this regard, a study in southern Iran reported the clinical case of ovine listeriosis encephalitis using histopathological, bacteriological, and PCR methods to diagnose *L. monocytogenes* infection. They reported meningoencephalitis

Table 1. PCR results for detection of *Listeria* in the aborted fetuses (n=62)

Age Groups (m)	No. of Fetus in Age Group (%)	No. of the Positive Samples (%)	95% CI
2-3	5/62 (8.06)	2/5 (40)	0.4, 0.43
3-4	14/62 (22.58)	3/14 (21.42)	0.214, 0.215
4-5	36/62 (58.06)	18/36 (50)	0.5, 0.163
>5	7/62 (11.29)	3/7 (42.85)	0.428, 0.366
Total	62	26/62 (41.93)	0.419, 0.123

and tissue degeneration in microscopic examination. Notably, spoiled corn silage had been identified as the source of contamination [20]. In the present study, none of the examined animals were fed spoiled corn silage, and other sources of contamination and infection transmission likely played a role in the affected animals in this study.

The pathophysiology of this disease involves bacterial invasion of the placenta, causing inflammation and miscarriage. Studies have shown that this bacterium can be isolated from placental and fetal tissue of aborted lambs, confirming its role in reproductive failure [21]. By crossing the placental barrier, *L. monocytogenes* can cause severe infections such as septicemia and placental necrosis. In experimental studies, pregnant ewes exposed to highly virulent strain of *Listeria* exhibited a high rate of abortion [22]. In addition, a cross-sectional study conducted on 50 dairy farms in New York State examined various sources for the presence of *L. monocytogenes*, including cow feces, milk, environmental samples, internal milk filters, and bulk tank milk. The findings showed that *L. monocytogenes* was present in composite milk (13%) and stool samples (43%), with higher rates in winter and summer. All of the evaluated samples herein were collected in autumn and winter. This bacterium was generally found in feed bins, water tanks, and litter.

This study emphasized the importance of improving sanitation and hygiene practices during milk collection to control the spread of this pathogen [23]. Another study showed that the incidence of listeriosis as a cause of abortion in sheep varied significantly by region. In Australia, for example, about a quarter of sheep abortions investigated between 2000 and 2018 were attributed to listeriosis [24]. Another cross-sectional study identified *Listeria* species in 7 out of 544 flocks, accounting for 1.3% of cases with approximately 2% abortion rate [25].

The dead fetus is typically expelled within 5 days; by this time autolytic changes mask minor gross lesions produced by the organism [19]. Herein, both macroscopic and microscopic examinations revealed severe lesions in the brain, liver, and lungs. Emerging evidence indicates that common pathological lesions of listeriosis in the dead fetus of small ruminants [19] are in agreement with the present findings. Grossly and microscopically, there are commonly yellow pinpoint foci in the liver. These foci frequently have a central area of cellular necrosis surrounded by small numbers of degenerating neutrophils and mononuclear inflammatory cells. Similar foci, sometimes only visible microscopically, may also occur in the lungs, myocardium, kidney, adrenal glands, spleen, and brain.

In near-term fetuses, there may be severe diffuse cerebrospinal meningitis [19, 20]. In the present study, there were various degrees of necrotic hepatitis, interstitial pneumonia, myocarditis, and meningoencephalitis in the aborted fetuses. In conclusion, this disease causes abortion or stillbirth in livestock, leading to a decrease in the capacity of livestock producers to supply sufficient meat and healthy dairy products. Notably, it is considered as a food-borne pathogen with zoonotic potential. On the other hand, as previously described, spoiled corn silage could be as the source of contamination in farm animals.

5. Conclusion

In conclusion, the detection of *Listeria* infection in aborted fetuses with a higher infection rate indicates that this infection plays a notable role in abortion among sheep and goats in East Azerbaijan. The higher prevalence herein reported could be due to the selection of only aborted animals, production systems, and cold and humid climate of the study area during autumn and winter. Given that the risk of disease in human may increase with higher infectious doses of the bacterium, the occurrence of the listeriosis should be considered a priority in public health programs.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Animal Research Ethics Committee of the University of Tabriz, Tabriz, Iran (Code: IR.TABRIZU.REC.1403.049). All relevant international, national, and institutional guidelines for the care and use of animals were followed.

Data availability

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

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Authors' contributions

Conceptualization: Monireh Khordadmehr, Hassan Sadri, and Jafar Shirazi; Software: Farinaz Jigari-Asl, Katayoon Nofouzi, Saba Skandari; Methodology, review, editing, and final approval: All authors; Writing the original draft: Monireh Khordadmehr, Hassan Sadri, Farinaz Jigari-Asl, Saba Skandari; Supervision, project administration and funding acquisition: Monireh Khordadmehr.

Conflict of interest

The authors declared no conflict of interest.

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