Investigation of *Listeria* infection in the aborted fetuses of small ruminants in East Azerbaijan Province, northwest Iran

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Abstract

Listeriosis, an important food-borne zoonotic disease, is caused by the *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 studies in East Azerbaijan Province. For this purpose, a total of 62 aborted fetuses and 288 vaginal swabs were collected from sheep and goat flocks from nine cities. For molecular analyses, conventional PCR was employed for the detection of *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. At microbiological study, the organism was isolated in 1.24% of abomasal contents, 2.43% from vaginal swaps, and 3.72% from the fetus's brain. Molecular analyses showed that *Listeria* infection was present in 41.93% (CI95%: 0.41 ± 0.12) of the aborted fetuses. At pathology, gross examination revealed white foci in the liver, lung, and myocardium, associated with 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). In conclusion, the detection of *Listeria* infection in aborted fetuses with a much higher infection rate indicates that this infection plays a notable role in the abortion of sheep and goats in East Azerbaijan. More importantly, it remains one of the zoonotic diseases worldwide. Therefore, effective public health management strategies are crucial for its prevention and control.

Keywords: small ruminant, infection, abortion, zoonotic disease, economic losses

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* stands out as the cause of listeriosis in several animal species (5, 6). During 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 be by hematogenous spread from the placenta with an incubation period of about 5-12 days (10). Importantly, this disease exhibits a high mortality rate of 20% in humans. It can carry and spread without showing symptoms, releasing bacteria into their environment through feces (11-13). Also, *L. monocytogenes* causes reproductive problems, such as late abortion, stillbirth (14), and poor offspring (15). Usually, the abortions produced 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, enhancing our understanding of this animal disease and its animal 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, northwest Iran. For this purpose, 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. The vaginal samples were stored in sterile normal saline and transferred to the microbiology lab. The age of the aborted fetuses was estimated using the formula $(X + 17) \times 1/2$, where X is the fetal size in centimeters measured from forehead to tail. Then, a systematic necropsy was performed and the 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 histopathology analysis.

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

Fetal tissues (brain, lung, and liver) andabomasal 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 h, 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 suspect *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

The tissue samples were kept in a 10% neutral buffered formalin solution for 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 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 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 (19), the specific primers for HLYA1 (Forward: 5'-ATCAGTGAAGGGAAAATGCAAGAAG-3') and HLYA2 (Reverse: 5'-TTGTATAACCAATGGGAACTCCTGG -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 for the positive control. The amplified products were detected through electrophoresis on 2% agarose gels stained with a safe DNA stain (SinaClon, Iran).

2.5. *Statistical analyses*

The Chi-Square test was used to determine the 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 $P < 0.05$. The analyses were performed with IBM SPSS Statistics v.22 software, 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 positive by PCR too) and six (3.72%; 95%CI: 0.37 ± 0.12) brain samples (out of 62, individually), but not 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. Besides, there were remarkable hyperemia and edema in the brain. At microscopic examinations, multifocal hepatitis and hepatic necrosis were observed in the liver. Similar foci with cellular necrosis and 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).

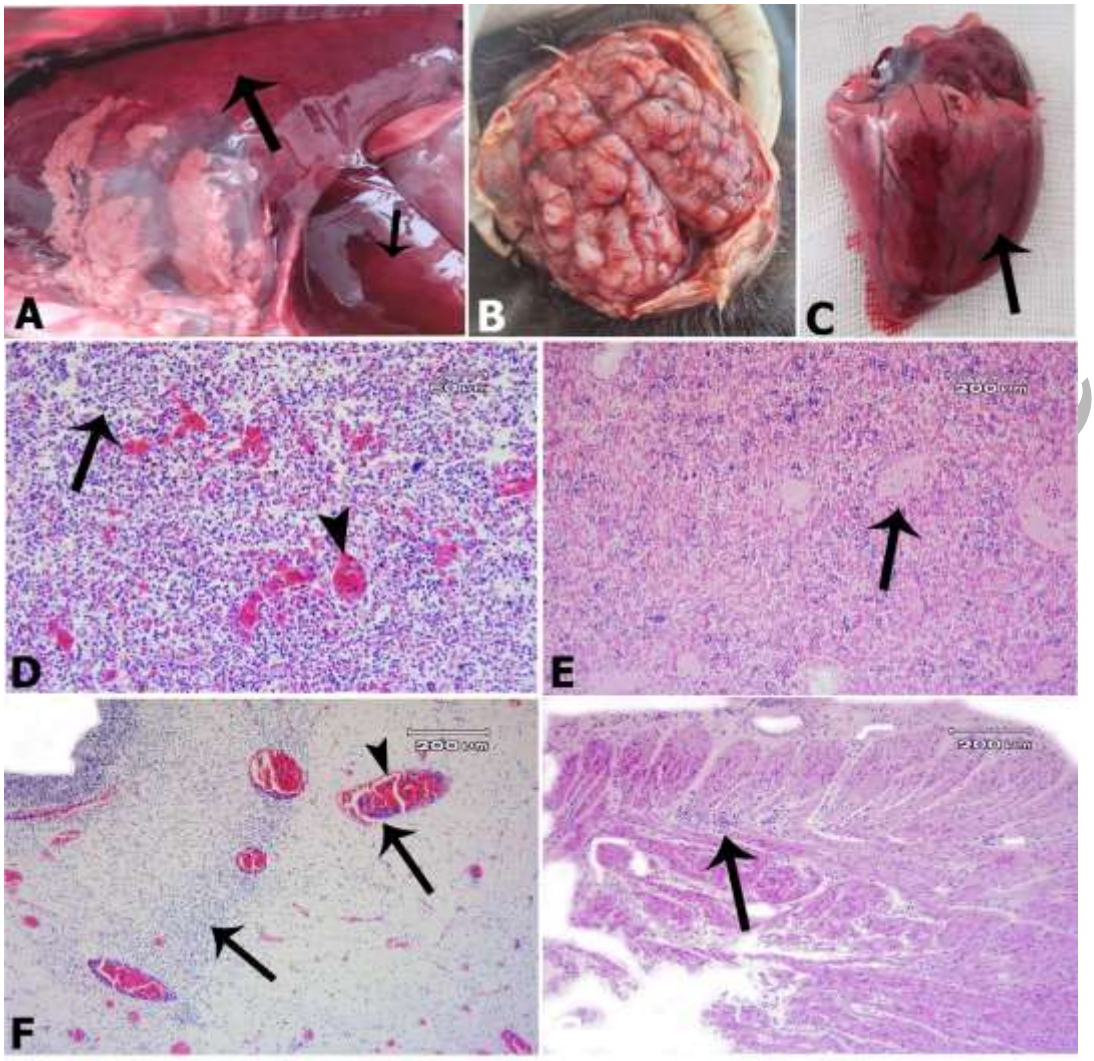

Figure 1. An aborted fetus with infection of listeriosis. A: white to yellow foci in the liver (short arrow) and lung (long arrow) associated with diffuse hyperemia. B: remarkable hyperemia and edema in the brain. C: white foci in the heart (arrow) associated with diffuse hyperemia. D: infiltration of 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). H&E.

3.3.Molecular findings

The genome of *Listeria* was detected in 41.93% (26 out of 62) (CI95%: 0.41 ± 0.12) of the examined fetuses, 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 significant difference between the four age groups ($P < 0.05$).

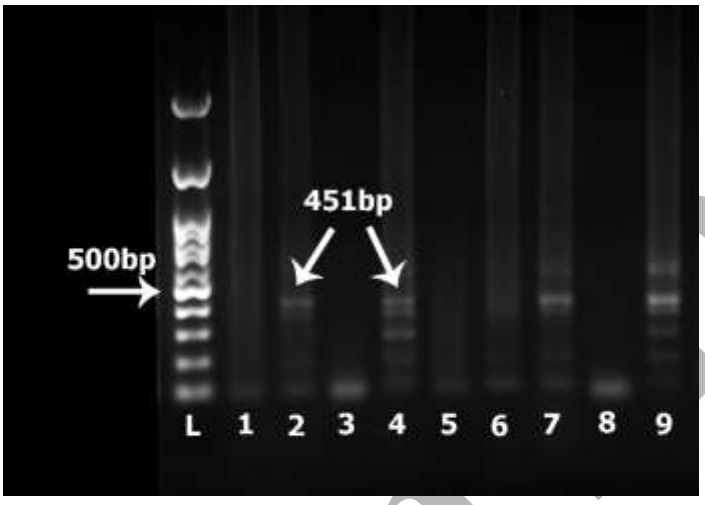

Figure 2. Molecular findings of the present study for detecting the *Listeria* genome in aborted fetuses. The PCR products with a 451 bp band were detected through electrophoresis on agarose gels stained with a safe DNA stain. L: ladder 100 bp; S1: negative control; S3, S5, S6, and S8: the samples with negative results; S2, S4, S7, and S9; positive samples with a 451 bp band.

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

Age groups	No. of fetus in age group	No. of the positive samples (%)	95% CI
2-3 m	5/62 (8.06)	2/5 (40%)	0.4±0.43
3-4 m	14/62 (22.58%)	3/14 (21.42%)	0.214±0.215
4-5 m	36/62 (58.06%)	18/36 (50%)	0.5±0.163
Over 5 m	7/62 (11.29%)	3/7 (42.85%)	0.428±0.366
Total	62	26/62 (41.93%)	0.419±0.123

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, and abortion or stillbirth are then the common signs (20). Along with the symptoms that can cause abortion, encephalitis is the most common symptom of *Listeria* infection in sheep (20, 21). 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 and tissue degeneration in microscopic examination. Notably, spoiled corn silage had been identified as the source of contamination (21). 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 that leads to miscarriage. Studies have shown that this bacterium can be isolated from the placenta and fetal tissue of aborted lambs and confirm its role in reproductive failure (22). Knowing that *L. monocytogenes*, by crossing the placental barrier, can cause severe infections such as septicemia and placental necrosis, in an experiment involving pregnant ewes exposed to a highly virulent strain of *Listeria*, and a significant percentage resulted in abortions (23). 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 (24). 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 (25). Another cross-sectional study identified *Listeria* species in 7 out of 544 flocks, accounting for 1.3% of cases with approximately 2% abortion rate (26).

The dead fetus is expelled within approximately 5 days; by this time autolytic changes mask minor gross lesions produced by the organism (20). 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 (20), 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, but usually only visible microscopically, may also present in the lungs, myocardium, kidney, adrenal glands, spleen, and brain. In near-term fetuses, there may be severe diffuse cerebrospinal meningitis (20, 21). 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 (27, 28) with zoonotic potential. On the other hand, as previously described, spoiled corn silage could be as the source of contamination in farm animals.

In conclusion, the detection of *Listeria* infection in aborted fetuses with a higher infection rate indicates that this infection plays a notable role in the abortion of 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 in autumn and winter. As the risk of disease in human may increase with higher infectious doses of the bacterium, the occurrence of the listeriosis should be considered in public health programs.

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Author contributions

Conceptualization: MKh, HS, JSh; Methodology: MKh, HS, JSh, SB, HA, FJA, KN, SS, and HH; Software: FJA, KN, SS; Writing/preparation of original draft: MKh, HS, FJA, SS; Writing, review and editing: MKh, HS, HA, JSH, FJA, KN, and HH; Supervision, project administration and funding acquisition: MKh; All authors have read and approved the final version of the manuscript.

Ethics

All relevant 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.

Conflicts of Interest

The authors declare that they have no conflict of interest.

Data availability

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

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