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Molecular and pathological studies on *Leptospira* and *Campylobacter* infections in aborted fetuses from northwest Iran

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Abstract

This study was performed to identify *Leptospira* and *Campylobacter* infections in aborted fetuses of small domestic ruminants, including sheep and goats in East Azerbaijan province, northwest Iran. For this purpose, a total of 62 aborted fetuses were collected from sheep and goat flocks. At necropsy, tissue samples were collected for histopathological (brain, liver, kidney, and lung) and molecular studies (abomasal content). The conventional PCR method using specific primers was conducted for the molecular evaluations. Additionally, the formalin-fixed tissue samples were routinely processed for histopathological examinations. The genomes of *Leptospira* and *Campylobacter* were detected in 19.35% and 22.58% of the examined fetuses, respectively. And, three out of 62 (4.83%) aborted fetuses demonstrated positive results for both infections. Histopathological studies demonstrated multifocal gliosis and nonsuppurative meningoencephalitis in the brain, multifocal necrotic hepatitis in the liver, multifocal tubular necrosis in the kidney, and suppurative bronchopneumonia in the lung. In conclusion, the identification of *Leptospira* and *Campylobacter* genomes in aborted fetuses with high prevalence rates indicates that these infections play a notable role in the abortion of sheep and goats in East Azerbaijan. Therefore, preventing and controlling these infections in this region is necessary, particularly regarding their zoonotic potential and public health.

Introduction

Leptospirosis is a common and zoonotic disease that affects animals and has significant consequences according to its economic impact. This infection leads to reduced milk production, increased spontaneous abortion rates, and decreased fertility levels. Additionally, it poses a significant public health risk, often associated with socioeconomic conditions, flood events, and specific occupational exposures (1, 2). It should be

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noted that abortion is the most common symptom of leptospirosis in sheep (3). The genus *Leptospira* is part of the Leptospiraceae family and the Spirochaetales order. They possess a protein-based outer membrane and a periplasmic flagellum that enables their movement (4, 5). This zoonotic disease has more than 250 pathogen strains in 32 serological groups. The genus Leptospira includes 69 species, 41 of which are pathogenic and infect different hosts. The acute and subacute forms result from *L. pomona* and *L. hardjo*, leading to reproductive issues such as abortion. Serovars such as *L. interrogans*, Bratislava, Balum, and Pomona can also lead to reproductive problems. Among these, *L. hardjo* is the main cause (6, 7). Understanding the pathogenesis of leptospirosis began with experimental studies more than 100 years ago (8). *Leptospira* infection is usually chronic in ruminants and pigs, resulting in transient bacteremia following long-term colonization in the kidney or uterus. The severity of the disease varies depending on the infecting serovar and the species affected, but there are many common aspects. The acute phase of infection is mostly asymptomatic, and the main economic loss is caused by chronic infection, which leads to reproductive problems. Clinical signs of leptospirosis vary among species, ranging from severe disease in dogs to reproductive failure in pigs and ruminants to asymptomatic or no clinical disease in Norway rats and humans (6).

Campylobacter is part of the *Campylobacteraceae* family and *Campylobacter fetus* (*C. fetus*) was the first species recognized. *Campylobacter* species have been associated with various animal diseases like diarrhea and septic abortion found in cows and sheep. Smith associated them closely with diseases among livestock back in 1918 when he isolated similar bacteria from aborted cow fetuses. Since they took on a somewhat different shape, they were originally placed in a category of Vibrio because of their shape and Smith referred to them as Vibrio embryos (9-11). The species *C. fetus* includes two subspecies involved in the reproductive failures of ruminants. *C. fetus* subsp. Fetus infection in sheep and cattle is caused by gastrointestinal colonization, Besides, *C. jejuni* was implicated in abortions among sheep (12). *Campylobacter* species are important in the health of sheep because they invade via the gut, infecting the intestinal lining; these infections could move into the bloodstream, leading to serious infections and placentitis, which seriously disturbs the functioning of the placenta, leading to fetal distress and miscarriages. Even though the sheep's immune system tries to fight the infection, *Campylobacter* has developed ways to avoid these defenses. This leads to continued harm, such as tissue death in the placenta and possible loss of the fetus (13-16). In New Zealand, *Campylobacter* spp. and *Salmonella brandenburg* are predominant (17), while *C. abortus* is significant in North America (18) and Iran (19). Abortions caused by *Campylobacter* are frequently introduced into a flock by acquiring an infected animal (20).

The aim of the current study was to molecularly detect and investigate the pathology of *Leptospira* and *Campylobacter* infections in aborted fetuses of domestic small ruminants, including sheep and goats in East Azerbaijan province, northwest Iran.

Materials and methods

Study area and sampling

The present study was performed in seven cities of the East-Azerbaijan province in northwest Iran, including Tabriz, Charuymaq, Khoda Afrin, Jolfa, Heris, Bostan Abad, and Mianeh (Figure 1). From November 2023 to February 2024, a total of 62 aborted fetuses were collected from sheep and goat flocks in the mentioned regions, which their owners had contacted for abortion in their farms. All samples belonged to the herds with the traditional conditions. At first, the age of the aborted fetuses was estimated using the formula $(X + 17) \times 1/2$, where X is the size of the fetus in centimeters, which were measured from forehead to tail. Then, a systematic necropsy was performed and the pathological lesions were recorded. Next, 50 mg of the abomasal content was placed in a 2 mL

microtube and stored in a freezer at -70 °C for further molecular studies. Additionally, tissue samples of the various organs including the brain, liver, kidney, and lung were collected and transferred to 10% formalin solution for histopathology purposes.

Fig. 1. Map of the East-Azerbaijan province showing the location of the study area.

Pathological study

The tissue samples were kept in a 10% neutral buffered formalin solution for at least 48 hours, then processed using a DS2080/H tissue processor (Didsabz, Iran). The tissues were subsequently embedded in paraffin, cut into 5 µm thick sections, and stained with 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)

Genomic DNA (gDNA) was extracted from abomasal content using a DNA extraction kit® (Pishgam Sanjesh, Tehran, Iran) following the manufacturer's instructions. The quality and quantity of genome were assessed using NanoPhotometer® NP80 (IMPLEN, Germany). All PCR assays were performed in a final volume of 25 µL using Taq DNA Polymerase Master Mix RED® (Ampliqon, Denmark), which included 12.5 µL master mix, 1 µL forward primer, 1 µL reverse primer, 7.5 µL water, and 3 µL DNA using a T100 Thermal Cycler (Bio-Rad, USA). The amplified products were detected through electrophoresis on 2% agarose gels stained with a safe DNA stain (SinaClon, Iran). Details of the primers and PCR conditions are presented in Table 1 (3, 19).

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 5–6-month-olds) of the fetuses. Differences were considered significant at $P < 0.05$. The analyses were performed with IBM SPSS Statistics v.22 software. Also, the data was assessed using a 95% confidence interval (CI).

Table 1. Characteristics of the primers and PCR conditions used in this study.

Bacteria	Sequence (5'-3')	Product size (bp)	Annealing temperature (°C)	Cycles
Leptospira sp.	F: TAAAGCCAGGACAAGCGCC R: TACGAACTCCCATTTCAGCG	102	56	40
Campylobacter fetus	F: GCACCTGTCTCAACTTTC R: CCTTACCTGGGCTTGAT	78	50	40

Results

Pathological findings

At necropsy (Figures 2A and B), macroscopic pathological lesions were observed in the examined organs. There was hemorrhagic fluid in the abdominal and thoracic cavities associated with pale to yellowish round foci in the liver, interstitial pneumonia in the lung, focal hemorrhage in the kidney, and notable hyperemia in the brain.

In microscopic studies (Figures 2C-F), pathological lesions were observed in different examined tissues. In the PCR-positive fetuses for *Leptospira*, diffuse gliosis and nonsuppurative meningoencephalitis were observed in the brains accompanied by hemorrhagic and multifocal tubular necrosis in the kidneys. In the PCR-positive fetuses for *Campylobacter*, the main histopathological lesions included focal to multifocal necrotic hepatitis in the liver, hemorrhagic necrosis in the cortex of the kidneys, and suppurative bronchopneumonia with the presence of neutrophils in the lung.

Molecular findings

The molecular study results associated with the age groups are presented in Table 2 and Figure 3. Briefly, the genomes of *Leptospira* and *Campylobacter* were detected in 19.35% and 22.58% of the examined fetuses, respectively. Of note, three out of 62 (4.83%) aborted fetuses demonstrated positive results for both infections. Higher infection rates were found in the 4-5 and 2–3-month-old groups for *Leptospira* and *Campylobacter*, respectively. However, there was no significant difference between the four age groups.

Discussion

In the present study, *Leptospira* and *Campylobacter* infections were detected in 19.35% and 22.58% of the examined aborted fetuses of sheep and goats in East Azerbaijan province, Iran. Also, the macroscopic and microscopic pathological findings confirmed the presence of *Leptospira* and *Campylobacter* infections in the aborted fetus. Both infections are important for public health due to their zoonotic potential. In this regard, a serological survey conducted in Tabriz and its surrounding areas reported a titer of *Leptospira* antigen in 10% of aborted ewes, while the *Leptospira* genome was detected in 57.8% of aborted fetuses from the liver, kidney, lung, spleen, heart, and placental (7). In another study that was conducted to identify *Leptospira* infection in sheep with an abortion history, two out of 150 (1.3%) vaginal swab samples reported positive using the PCR method (3). In a systematic review from 1998 to 2017, the prevalence of leptospirosis in sheep and goats was reported as 17.38% and 12.18%, respectively (21).

In Turkey, a study was conducted to diagnose leptospirosis in ruminants (1252 aborted fetuses including 750 lambs, 218 goats, and 284 calves) in the Marmara region between 2013 and 2018 using immunohistochemistry (IHC), Levaditi staining, and histopathological examinations. They demonstrated *Leptospira* infection in 12.77% of the examined samples. Moreover, they stated that the IHC method is more sensitive than the Levadity staining method (22). In another study carried out in Mexico's highland regions, researchers employed artificial neural networks to investigate how infectious agents, such as *Leptospira* spp., influence abortion rates in ewes. The findings revealed that

55.2% of the ewes had been exposed to *Leptospira*, and there was a notable relation between specific environmental factors and infection rates (23).

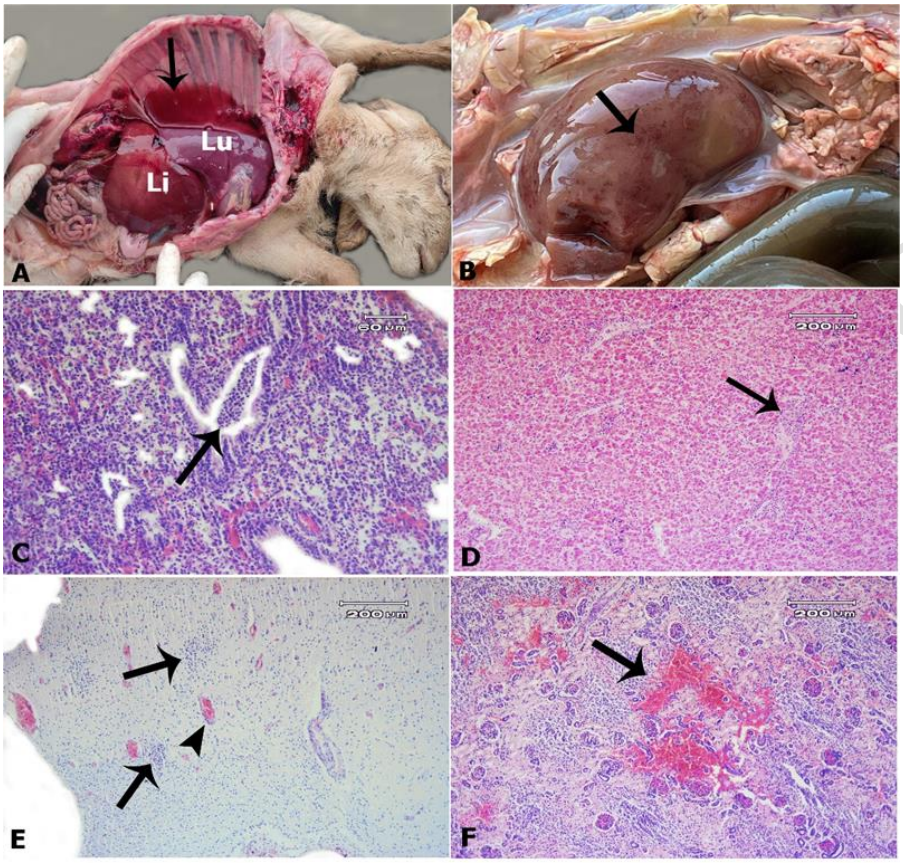

Fig. 2. A: Hemorrhagic fluid in the thoracic cavity associated with hyperemia in the lung (Lu) and liver (Li). B: Focal hemorrhage (arrow) in the cortex of the kidney. C: Interstitial purulent bronchopneumonia with the presence of neutrophils (arrow). D: C: Liver: focal necrosis associated with inflammatory cell infiltration (arrow). E: Brain: multifocal gliosis (arrows) with notable hyperemia (arrowhead). F: Tubular necrosis associated with focal hemorrhage in the kidneys (arrow). H&E.

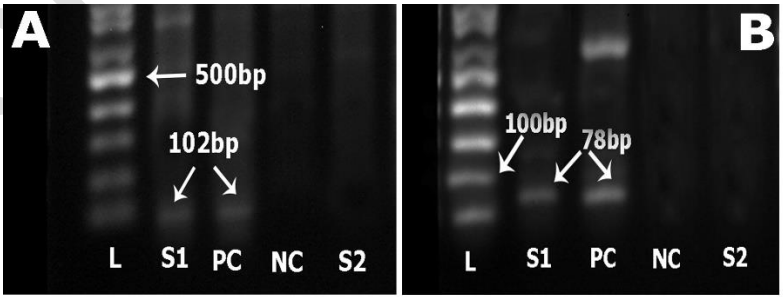

Fig. 3. The PCR products were detected through electrophoresis on agarose gels stained with a safe DNA stain for *Leptospira* (A) and *Campylobacter* (B). **A**: L: ladder 100 bp; S1: a sample with the positive result; PC: positive control with a 102 bp band; NC: negative control; S2: a sample with a negative result. **B:** L: ladder 50 bp; S1: a sample with a positive result; PC; positive control with a 78 bp band; NC: negative control.

Table 2- Infection rates based on PCR results for detection of *Leptospira* and *Campylobacter* in the aborted fetuses (N = 62).

Age	Infection No. (%)	*Leptospira*	*Campylobacter*
2-3 m	5/62 (8.06%)	1/5 (20%) (95% CI:0.20±0.35)	2/5 (40%) (95% CI:0.4±0.43)
3-4 m	14/62 (22.58%)	2/14 (14.28%) (95% CI:0.14±0.18)	1/14(7.14%) (95% CI:0.07±0.10)
4-5 m	36/62 (58.06%)	8/36 (22.22%) (95% CI:0.22±0.135	9/36 (25%) (95% CI:0.25±0.141)
5-6 m	7/62 (11.29%)	1/7 (14.28%) (95% CI:0.14±0.29)	2/7 (28.57%) (95% CI:0.28±0.33)
Total	62	12/62 (19.35%) (95% CI:0.19±0.097)	14/62 (22.58%) (95% CI:0.22±0.103)

In the current study, 19.35% of the examined aborted fetus samples were positive for leptospirosis, which shows a high percentage compared to previous studies in Iran. Additionally, meningoencephalitis and nephritis were found in the current study as the most significant pathological lesions due to *Leptospira* infection in aborted fetuses, which are in agreement with the previous findings (22). A study of 100 abortion-related samples, including fetuses and placenta, from sheep flocks in Uruguay between 2015 and 2021 found antibodies against one or more *Leptospira* serovar in 23.8% of the fetal samples using a microscopic agglutination test. However, *Leptospira* was not detected by qPCR, culture, fluorescent antibody test, or immunohistochemistry (13). In addition, The investigation revealed that among *Campylobacter* infections, 5 cases (5%) were caused by *C. fetus* subsp. *fetus*, and 1 case (1%) wasattributed to an unidentified species of *Campylobacter* (13).

Emerging evidence demonstrated a link between *Campylobacter* infection rates and sheep abortion in various regions of Iran. The highest percentage of *Campylobacter* contamination was reported in Zanjan province, which examined 129 vaginal swabs of Afshari ewes using the PCR method, finding the *Campylobacter* genome in 51.9% of the examined samples (24). In other studies, the contamination rates observed in flocks from Isfahan province ranged from 0.6% to 13.3% (25), 4% around Shiraz (26), 0.44% in Hamedan (27), and 4.9% in samples collected from Chahar Mahal and Bakhtiari, Khorasan Razavi, and Isfahan (28), with 1.4% in the Markazi province (29). Also, Campylobacteriosis was reported in Tabriz at 10.59% (30) and 7.7% in the Sistan region (31) among aborted sheep embryos. Also, *C. fetus* was isolated from all 8 cases of aborted fetuses of a sheep flock in Tehran (32). Notably, few studies have been able to isolate *Campylobacter* from aborted fetus samples (33). In the present study, *Campylobacter* disease was detected in 22.58% of fetal samples, which shows a higher prevalence than the previous report from Tabriz (10.59%). In this regard, one study investigated *C. fetus* from aborted fetuses and vaginal swab samples collected from sheep flocks in Sulaymaniyah province of Iraq by PCR. The pathogen was identified in clinical samples using conventional PCR, and *C. fetus* was isolated in 42.1% and 18.6% of aborted fetuses and vaginal swabs from aborted ewes, respectively (34). In another similar study conducted in the Van region of Turkey, the multiplex-PCR (m-PCR) method was evaluated for diagnosing sheep campylobacteriosis from samples containing the milk of aborted sheep

fetuses. A total of 116 aborted sheep fetuses from 98 different sheep flocks were tested, and *C. fetus* was isolated from 8 samples (6.9%) by cell culturing, while 13 samples (11.2%) tested positive using the PCR method (35). In South America, abortions caused by *Campylobacter* species, are rarely reported. In 2017, Fiorentino et al. reported the prevalence of abortion in 7 out of 205 Pampinta ewes from La Pampa, Argentina (36). Both pathogens, *Leptospira* and *Campylobacter,* are resilient pathogens that can persist in aquatic environments for a long time (37), posing significant risks to public health and the environment.

Conclusions

In the present study, the identification of *Leptospira* and *Campylobacter* genomes in 19.35% and 22.58% of aborted sheep and goats' fetuses, respectively, associated with severe pathological lesions indicates their significant role in the abortion of sheep and goats in East Azerbaijan. Both pathogens can persist in aquatic environments for a long time. Therefore, effective management, prevention, and control of these infections are necessary in this province, particularly regarding their zoonotic implications and public health.

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Ethical approval

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

Conflict of interest statement

The authors declare that there is no conflict of interest to disclose.

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