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Detections of *Brucella* infection in serum samples and aborted fetuses of small ruminants in East Azerbaijan province (northwest Iran)

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Abstract

Brucellosis, a zoonotic disease caused by *Brucella species*, results in significant economic losses in both animal production and human health. The aim of the present study was to evaluate the presence of *Brucella* infection in aborted fetuses and serum samples from adults with history of recent abortion using serological, molecular, and pathological studies in East Azerbaijan Province. A total of 62 aborted fetuses and 373 blood samples were collected from sheep and goat flocks. The conventional PCR method was employed for the detection of *Brucella* infection following DNA extraction from the abomasal contents of the aborted fetuses. The serum samples from the adults were evaluated using the Rose Bengal Plate Test (RBPT), Wright, and 2-mercaptoethanol (2-ME). Moreover, the formalin-fixed tissue samples from the aborted fetuses were conducted for histopathological examinations. Molecular and serological findings revealed that *Brucella* infection was present in 88.7% (56 out of 62) of aborted fetuses and 79.09% (272 out of 373) of serum samples. Histopathological studies revealed necrotic and inflammatory responses associated with severe hyperemia and hemorrhagic lesions in the tissue sections, particularly in the brain, lung, liver, and kidney. In conclusion, the detection of *Brucella* infection in both aborted fetuses and blood samples indicates of its significant role in sheep and goat abortions in East Azerbaijan. More importantly, it remains one of the most common zoonotic diseases worldwide. Therefore, effective management and vaccination strategies are crucial for preventing and controlling the disease from a public health perspective.

Introduction

Brucella is a small and gram-negative bacteria that causes Malta fever in humans, which is one major cause of abortion during late pregnancy in sheep and goats (1, 2). Brucellosis in small ruminants is caused by *Brucella*

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melitensis (B. melitensis) and *Brucella abortus (B. abortus)* with some clinical signs including retained placenta, stillbirth, infertility, and abortion (3). Consumption of contaminated milk, close contact with the placenta, uterus, contaminated fetal tissues and fluids are the usual sources of transmission of this bacteria to humans (4). Due to the traditional and nomadic systems of keeping sheep and goat herds, it is difficult to control and eradicate *Brucella* infection (2). The geographical distribution of brucellosis is constantly changing with new foci emerging or re-emerging (5). The disease occurs worldwide, except in those countries where bovine brucellosis has been eradicated. The worldwide economic losses due to brucellosis are extensive not only in animal production but also in human health (5). Although a number of successful vaccines are being used for the immunization of animals, no satisfactory vaccine against human brucellosis is available. When the incidence of brucellosis is controlled in the animal reservoirs, there is a corresponding and significant decline in the incidence in humans (5). In Iran, brucellosis of small ruminants is responsible for heavy economic losses (2). In this regard, several studies have evaluated the presence of *Brucella* in different samples such as aborted fetuses (6), serum and milk samples (7-13) from various regions of Iran including, Kerman (6, 12), Mashhad (7), Kurdistan (8), Tabriz (9), Hamedan (10, 11) and Lorestan (13), using PCR and serological assays. Also, there are similar reports from other countries like Tunisia (14), Mexico (15), Eritrea (16), and Jordan (17).

Although the gold standard for the diagnosis of brucellosis is bacterial isolation, screening tests for Brucella are generally based on serological tests (18). Rose Bengal Plate Test (RBPT), 2-Mercaptoethanol (2-ME), Standard Agglutination Test (SAT), Complement Fixation Test (CFT), and indirect ELISA test (i-ELISA) are generally used to detect *Brucella* antibodies. The RBT test is widely used for the initial screening of brucellosis, but it is recommended that the results of the RBT test be confirmed by other serological tests (18). The aim of the present study was to evaluate the presence of *Brucella* infection in the aborted fetuses and serum samples from adults with a recent history of abortion using serological (RBPT, Wright, and 2-ME), molecular (conventional PCR), and pathological studies in East Azerbaijan Province, northwest Iran, where is one the most important area for small ruminant breeding in the country.

Materials and methods

Study Area and Sampling

This study was conducted in Tabriz, Marand, Charuymaq, Khoda Afarin, Jolfa, Heris, Bostan Abad, Mianeh, and Hashtrud cities in East-Azerbaijan province, northwest Iran (Figure 1). The findings focus on *Brucella* infection, as part of a larger investigation into the infectious and non-infectious causes of abortion in small ruminants in this region with the published and unpublished data. For this purpose, a total of 62 aborted fetuses and 373 blood samples were collected from sheep and goats in these regions between November 2023 and February 2024, following reports of abortions by their owners. All herds were maintained under traditional conditions. Five milliliters of anticoagulated (EDTA) blood samples were obtained from aborted and pregnant animals for serological tests. Whole blood was centrifuged at 6000 rpm for 10 minutes to separate the sera, which was then transferred to sterile 1.5-ml microcentrifuge tubes and stored at -70 °C for later analyses. About aborted fetuses, 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.

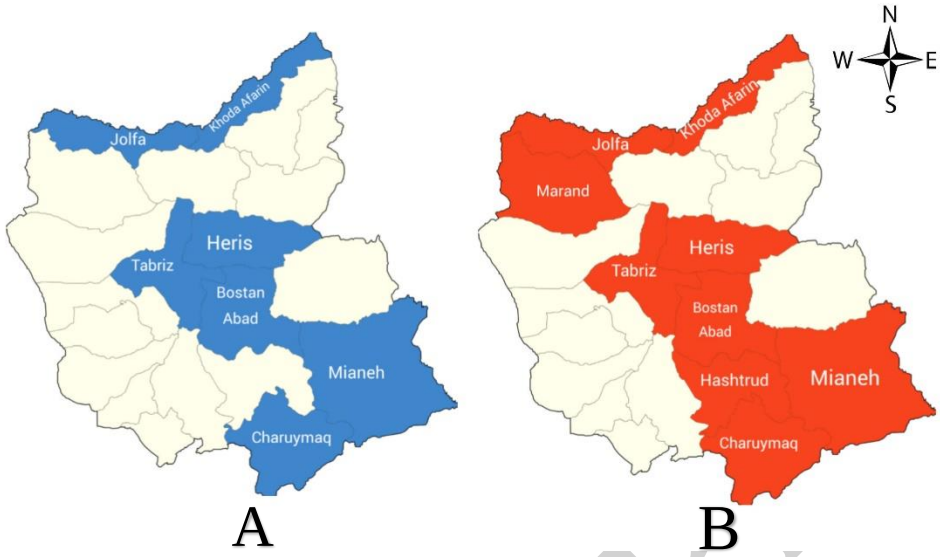

Figure 1. Map of East-Azerbaijan province (northwest Iran) showing the location of the study area for detections of Brucella infection in serum samples and aborted fetuses of small ruminants.
A: Seven cities which fetus samples were received.
B: Nine cities which blood samples were received.

Serological Assays

Rose Bengal Plate Test (RBPT)

All sera samples were screened for the anti- *Brucella* antibodies presence using the RBPT. Briefly, equal volumes (70 µL) of RBPT antigen (Pasteur Institute, Tehran, Iran) and serum were poured into a slide and thoroughly mixed with the applicator. The plate was placed on the rotator for about 4 min to do the interactions of antigen and antibody and then assessed for agglutination.

Standard Tube Agglutination Test

The STAT was conducted using the Wright tube antigen of *B. melitensis* obtained from the Razi Vaccine and Serum Research Institute in Iran. A total of 19 tubes were used, with ten tubes in the first row and nine tubes in the second row. The tubes in the first row were used to prepare serial dilutions of serum samples from 1:20 to 1:2560, the last tube was chosen as a negative control. The second row contained serum tubes for positive control samples. To prepare the tubes, 0.9 mL of normal saline was added to the first tube and 0.5 mL of normal saline to each subsequent tube in the first row. In the negative control tube, 0.9 mL of normal saline was added along with 0.1 mL of negative control serum. Next, 0.1 mL of the serum sample was added to the first tube and mixed, and 0.5 mL from the first tube was poured into the second tube and mixed. This process was continued until the last tube, from which 0.5 mL was discarded. The same procedure was followed for positive control tubes with positive control serum. Subsequently, 0.5 mL of Wright tube antigen was added to each tube, and they were incubated at 37 °C for 24 hours. The tubes were then examined for agglutination. The last tube in which agglutination was observed at the bottom was considered the test titer.

2-Mercaptoethanol Test

For the 2-ME test, the procedure was similar to STAT, except that 2-ME buffer (0.1 M) was used instead of normal saline. The Wright tube antigen of *B. melitensis* used for this test was obtained from the Razi Vaccine and Serum Research Institute in Iran**.**

Molecular Studies (DNA extraction and PCR assay)

The genomic DNA (gDNA) from the abomasal contents was extracted using a DNA extraction kit (Pishgam Sanjesh, Tehran, Iran) following the manufacturer's instructions. 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 of DNA/cDNA, 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). To perform PCR test, the specific primers for BRU1 (Forward: 5'-TGGAGGTCAGAAATGAAC-3') and BRU2 (Reverse: 5'-GAGTGCGAAACGAGCGC-3') targeting 282 base pair (bp) were used (17), applying 35 cycles with an annealing temperature 51°C (17).

Pathological study

The tissue samples were kept in a 10% neutral buffered formalin solution for at least 48 hours. They were then processed using a DS2080/H tissue processor (Didsabz, Iran), embedded in paraffin, cut into 5 µm thick sections, and stained with common hematoxylin and eosin (H&E). Finally, the sections were studied under a light microscope (Olympus, CH-30, Japan), and the observed lesions were recorded.

Statistical analyses

The Chi-Square test was used to determine the correlations between infections and two age groups of the fetuses (less than and more than 4 month-olds). Differences were considered significant at $P < 0.05$. The analyses were performed with IBM SPSS Statistics v.22 software, and a 95% confidence interval (CI) was applied.

Results

Serological findings

The serological examinations results of the adult serum samples are presented in Table 1. In summary, the results show an infection rate ranging from 0.0% to 96.60% in the examined serum samples.

Table 1. The serological and molecular results obtained from the serum and fetus samples, respectively of small ruminants in East Azerbaijan province, northwest Iran.

	Serum samples (n = 373**)**		Fetus samples (**n = 62**)	
City	**% positive samples**	**CI*95%**	**% positive samples**	**CI95%**
Marand	0%	0.0	0%	0.0
Heris	23.8%	0.05-0.41	50%	0.19-1.19
Charuymaq	69.40%	0.61-0.77	90.62%	0.59-1.21
Bostan Abad	75.67%	0.63-0.87	92.85%	0.79-1.05
Hashtrud	80%	0.65-0.95	0%	0.0
Mianeh	80.82%	0.72-0.88	100%	1.0
Tabriz	90.47%	0.79-1.01	80%	0.45-1.15
Khoda Afarin	94.73%	0.84-1.04	100%	1.0
Jolfa	96.60%	0.90-1.02	100%	1.0
Total	79.09%	0.75-0.83	88.70%	0.80-0.98

*CI: Confidence Interval

Molecular findings

The results of the molecular study related to the cities are presented in Table 1. Briefly, the genomes of *Brucella* were detected in 88.70% of the examined fetuses with the presence of sharped target bands at 282 bp (Figure 2). Of the positive samples from aborted fetuses, 16 out of 56 (28.57%, CI95%: 0.17-0.39) were from less than 4- month-old, whereas 40 out of 56 (71.42%, CI95%:0.60-0.82) were from more than 4- month-old group. There was significant difference between the two age groups ($P < 0.05$). However, the infection rate was higher in the older fetuses.

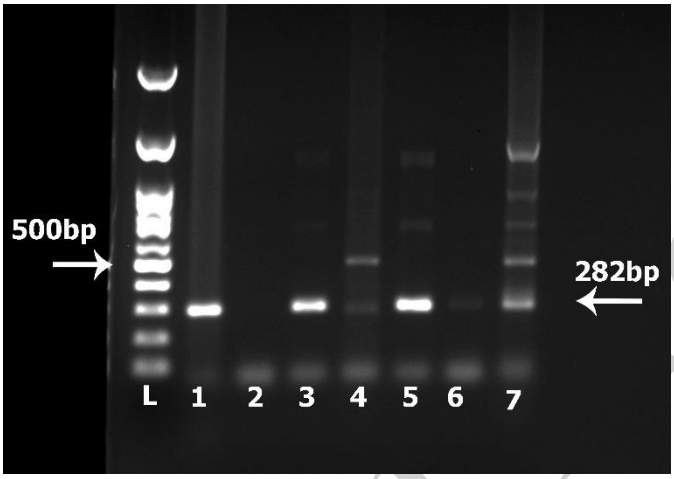

Fig. 2. Molecular findings for detecting the *B. melitensis* genome in aborted fetuses of small ruminants in East Azerbaijan province, northwest Iran. The PCR products with a 282 bp band were detected through electrophoresis on agarose gels stained with a safe DNA stain. L: ladder 100 bp; S1: positive control; S2: negative sample; S3, S4, S5, S6, and S7: the samples with positive results with a 282 bp band.

Pathological findings

At necropsy (Figure 3A and B), there was hemorrhagic fluid in the abdominal and thoracic cavity associated with pale to yellowish round foci and hyperemia in the liver (necrosis and inflammation), interstitial pneumonia in the lung, and notable hyperemia in the kidney, meninges, and brain. In microscopic examinations, pathological lesions were found in different examined tissues (Figure 3C-F). In the lung, there was suppurative bronchopneumonia with the presence of neutrophils in the airways. Diffuse gliosis and severe vascular hyperemia were observed in the examined brains. In the kidney, hemorrhagic and multifocal tubular necrosis accompanied by acute interstitial nephritis were found in the kidneys. Moreover, in the liver, the main histopathological lesions included focal to multifocal necrotic hepatitis associated with remarkable sinusoidal hyperemia. Of note, all of the examined aborted fetuses (100%) showed the pathological lesion of brucellosis in the studied tissues.

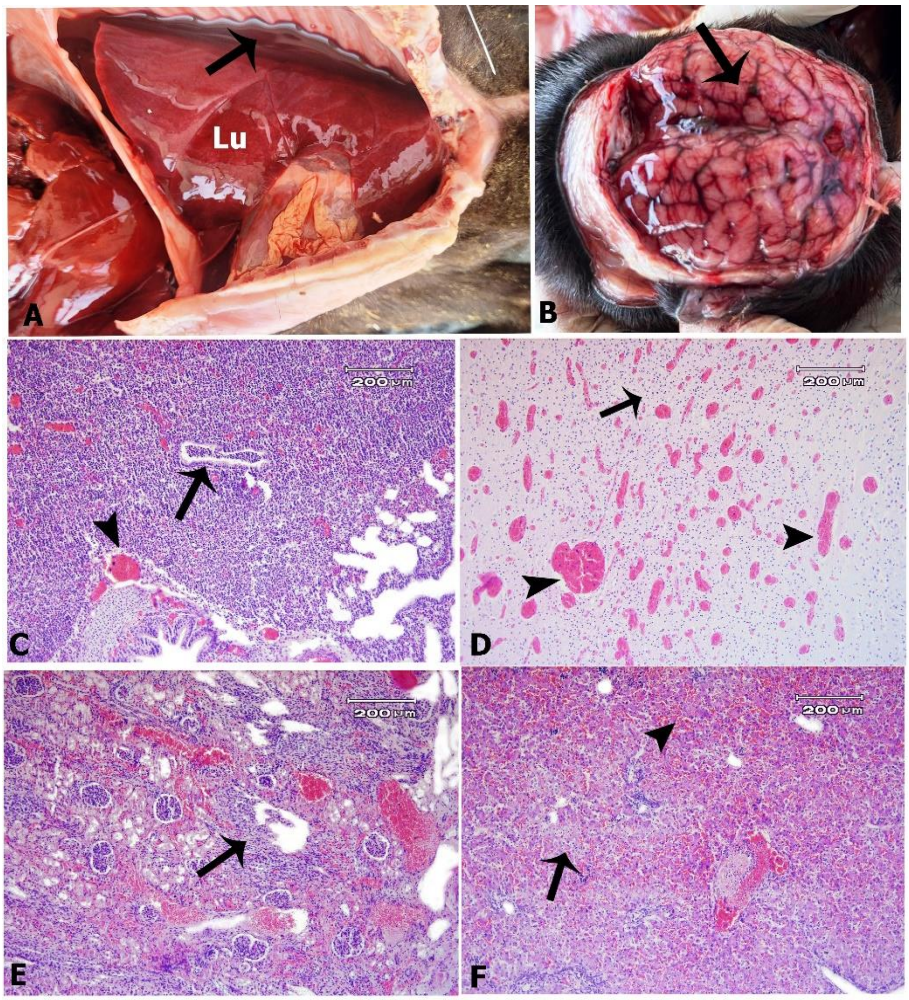

Fig. 3. Brucellosis aborted fetuses (lamb) of small ruminants in East Azerbaijan province, northwest Iran. A: Hemorrhagic fluid in the thoracic cavity (arrow) associated with interstitial pneumonia in the lung (Lu); B: Extensive hyperemia (arrow) in the meninges and brain; C: Interstitial purulent bronchopneumonia with the presence of neutrophils (arrow) and pulmonary vascular hyperemia (arrow head) in the lung; D: Severe vascular hyperemia accompanied by diffuse gliosis in the brain; E: Acute interstitial nephritis associated with inflammatory cell infiltration (arrow) in the kidneys (arrow); F: Cellular necrosis associated with notable hyperemia (arrow) in the liver. H&E.

Discussion

The present results indicate a significant infection rate of Brucellosis in aborted fetuses and adult small ruminants in East Azerbaijan province, identified by molecular (Conventional PCR, 90.32%), serological (RBT, Wright, 2-ME79.09%), and pathological examinations (100%). These findings demonstrated a high infection rate, which may be due to the abortion history of the samples examined. A similar study conducted in Kerman province (southeast Iran), used PCR to analyze 50 aborted fetuses, detecting *Brucella* species in 15 samples (30%), including 13 sheep (28%) and 2 goats (50%) (6). In another study in Mashhad (northeast Iran), serum and milk samples from 100 sheep aged three to five years with a history of abortion were examined. They reported the prevalence of Brucellosis 32, 42, and 44% using RBT, bacterial culture, and qPCR respectively (7). In contrast, a study in Hamedan province indicated 1.38% and 1.2% positive results in

the serum samples of small ruminants using serology and PCR assays (10). Another previous study in Tabriz (northwest Iran), found an overall brucellosis prevalence of 18.5% in sheep, with rates of 2% in rams and 16.5% in ewes (9). Moreover, the seroprevalence of Brucellosis in the serum samples of sheep and goats in Kurdistan, western Iran, was reported to be 5.8%, 5%, and 1.2% based on the ELISA method (8). In this regard, previous studies in Kerman, Lorestan (western Iran), and Hamedan provinces determined the prevalence of Brucellosis in milk as 1.28%, 26.5%, and 73.4%, respectively, using the PCR method (12, 13). Also, several studies from other countries provide insights into this issue. In Mexico, 345 sera from sheep were screened, revealing a *Brucella* serum prevalence of 70.7% using RBPT (15). In Jordan, 250 biological samples collected from 188 animals (81 sheep and 107 goats) during the lambing season showed a PCR positivity rate of 41.9% (17). The differences in the results in different studies can be due to different laboratory methods, types of samples, sampling methods, different geographical conditions, animal management practices, hygiene level, and general health conditions. In the present study that showed a significantly higher infection rate, all samples were taken from the animals with recent abortion histories in herds with traditional housing and lower hygiene level.

Herein, the pathological studies presented both macroscopic and microscopic features of acute Brucellosis in the examined aborted fetuses, which included fluid accumulation in body cavities, severe hyperemia in brain, diffused bronchopneumonia, and enlarged hyperemic kidney and liver. Microscopic examination confirmed the necropsy findings, showing severe vascular hyperemia in the brain associated with purulent bronchopneumonia, acute interstitial nephritis, and focal necrotic hepatitis. These findings have been previously described by others in cases of fetal *Brucella* infection (19, 20). Generally, we observed the most severe gross and microscopic lesions in the brain. Moreover, previous studies indicated that aborted fetuses may exhibit signs of bronchopneumonia, splenomegaly, and fibrinous peritonitis or pleuritic, often associated with fibrinous necrotizing placentitis in adults (21-23). The worldwide economic losses due to brucellosis are extensive not only in animal production but also in human health. Although a number of successful vaccines are being used for the immunization of animals, no satisfactory vaccine against human brucellosis is available. When the incidence of brucellosis is controlled in animal reservoirs, there is a corresponding and significant decline in the incidence in humans (5). Despite the implementation of brucellosis eradication programs in Iran, this disease is still endemic and highly prevalent among ruminants, particularly small ruminants, which contribute to the persistence of disease in herds.

Conclusion

Herein, the detection of *Brucella* infection in both aborted fetuses and blood samples indicates of its significant role in sheep and goat abortions in East Azerbaijan, Iran. Of note, this disease causes abortion in livestock and in this way leads to a decrease in the capacity of livestock producers to supply sufficient meat and healthy dairy products. Malta fever has great economic and health consequences as well. The appearance of Malta fever in new areas and its transmission from wild and domestic animals is very important in terms of epidemiological aspects. Regular and careful surveillance is essential to determine the true picture of brucellosis, especially in high-prevalence areas.

Ethical approval

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.

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Conflicts of Interest

There is no conflict of interest.

Artificial Intelligence Statement

The authors declare that no artificial intelligence tools were used in the preparation of this manuscript.

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