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Case Report
Olgu Sunumu

Multietiological abortion due to *Brucella melitensis* and *Chlamydia abortus* in a sheep fetus

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Abstract: In this case report, multietiological abortion due to *Brucella melitensis* (*B. melitensis*) and *Chlamydia abortus* (*C. abortus*) agents defined in sheep abortion. Agents, was defined in sheep abortion, fetus of which was submitted to Konya Veterinary Control Institute (KVCI) from a sheep farm in Nigde province *Brucella spp.* presence was determined by bacterial isolation method and confirmed by slide agglutination test with monospecific A and M sera. *C. abortus* was detected by real-time PCR and immunohistochemistry (IHC) methods. In histopathological examinations, hyperemia, haemorrhage and bronchopneumonia were observed in the lung; degeneration and necrosis of heart muscle cells were observed in the heart; hyperemia, haemorrhage, oedema, necrosis and mononuclear cell infiltrations were observed in the placenta. In this case report, it was emphasized that multietiological abortions involving more than one factor should be taken into consideration, as well as an etiological agent in the fight against sheep abortions, In this context, It would be appropriate to use multidisciplinary diagnostic methods to determine the abortion factors, thus contributing to the prevention of abortions, which is still an essential problem in sheep breeding.

Keywords: *Brucella melitensis, Chlamydia abortus*, histopathology, immunohistochemistry, multietiological abortion, PCR.

Bir koyun fetusunda *Brucella melitensis* ve *Chlamydia abortus* nedeniyle oluşan multietiyolojik abort

Özet: Bu olgu raporunda, Konya Veteriner Kontrol Enstitüsü Müdürlüğüne (KVKEM) 2021 yılında, Niğde ilindeki bir koyun işletmesinden gönderilen koyun abort olgusunda *Brucella melitensis* (*B. melitensis*) ve *Chlamydia abortus* (*C. abortus*) etkenlerine bağlı multietiyolojik abort tanımlandı. *Brucella spp.* varlığı bakteriyel izolasyon yöntemi ile belirlendi ve monospesifik A ve M serumları ile yapılan lam aglutinasyon testi ile doğrulandı. *C. abortus* ise real time PZR ve immunohistokimya (İHK) yöntemleri ile tespit edildi. Histopatolojik incelemelerde ise akciğerde; hiperemi, kanama ve bronkopnömoni; Kalpte kalp kası hücrelerinde dejenerasyon ve nekroz ile plasentada hiperemi, kanama, ödem, nekroz ve mononükleer hücre infiltrasyonları dikkati çekti. Bu olgu raporunun, koyun abortları ile mücadelede tek bir etiyolojik ajanın yanında birden fazla etkenin karıştığı multietiyolojik abortların da dikkate alınması gerektiği, abort etkenlerini belirlemek için de multidisipliner teşhis yöntemlerinin kullanılmasının uygun olacağını belirtmek ve böylelikle koyun yetiştiriciliğinde halen önemli bir sorun olan abortların önlenmesine katkı sağlaması açısından yayımlanması uygun bulunmuştur.

Anahtar kelimeler: *Brucella melitensis, Chlamydia abortus*, histopatoloji, immunohistokimya, multietiyolojik abort, PZR.

Introduction

To maintain profitability in small cattle breeding, it is aimed to have at least one offspring per year. Abortion is one of the most important factors that negatively affect this aim and cause significant economic losses in ovine breeding (Wu et al. 2014). The fact that the abortion rate is more than 5% in a flock reveals the necessity of significant measures (Ay 2017). Abortion factors that can occur at any stage of pregnancy are classified as infectious and non-infectious causes (Brom et al. 2012). Non-infectious causes include care and nutritional disorders, environmental conditions, and misuse of hormones and drugs (Ay 2017). Among the infectious causes that play a much more significant role in the aetiology of abortion there are bacterial, viral, parasitic, and fungal factors. Despite the different prevalences between countries, the most important infectious abortion factors in sheep in Türkiye are *Brucella melitensis*, *Campylobacter fetus subsp. fetus*, *Chla-*

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mydia abortus, *Salmonella abortusovis*, *Akabane virus*, *Border Disease virus (BDV)*, *Blue Tongue Virus*, *Toxoplasma gondii*, *Coxiella burnetii,* and *Neospora caninum*. Some of these pathogens are zoonotic agents that cause abortion and stillbirth, not only in domestic animals but also in humans (Gulaydın et al. 2023).

In addition to abortions caused by an etiological agent, sometimes abortions caused by more than one factor can be seen (Gulaydın et al. 2023). De Angelis et al. (2022) detected a co-infection caused by *Listeria monocytogenes* and *Toxoplasma gondii* in abortion and lamb deaths in a flock of sheep. Ramo et al. (2022) reported abortions with other abortion factors accompanying *Coxiella burnetii* in small ruminant flocks. In the reports prepared in Türkiye, Sevik et al. (2017a) revealed co-infection of *Peste des Petits Ruminants Virus (PPR)* and *Brucella melitensis* in an aborted sheep fetus, and in another report (Sevik et al. 2017b) co-infection of *Border Disease Virus (BDV)* and *Brucella melitensis* in a sheep abortion.

The aim of this case report was to describe a multietiological abortion case due to *B. melitensis* and *C. abortus* detected in a sheep and to draw attention to multietiological abortions in the abortion struggle.

Materials and Methods

The sample selection

Aborted fetus and fetal placenta were brought to KVCI in 2021 from a sheep herd in Nigde province. According to the information given by the herd owner, it was stated that abortions occurred in the enterprise, especially in the last period of pregnancy. A systemic necropsy was performed on the aborted fetus, and gastric contents, lung, heart, and placenta samples were taken for laboratory studies.

Bacteriological examination

Stomach contents were inoculated into blood agar (CM0271) in which *Brucella* selective supplement (Oxoid, SR0083A) was added. It was then incubated at 37°C and 10% CO_2 in an oven for 8-10 days (Woah 2022a). Gram staining was performed on the colonies observed because of incubation, and they were classified according to their morphological characteristics at the genus level. Results were confirmed by a slide agglutination test with monospecific A and M antisera (A+M Positive Control Antiserum, Pendik Veterinary Control Institute Directorate, Istanbul). A Vitek 2 device was used to determine the isolate at the species level.

DNA extraction and real-time PCR analysis

Lung, heart, and placental tissues were frozen at -20˚C after necropsy. Then, the frozen tissues were cut into small pieces and placed in an Eppendorf tube containing 450 µl of ultrapure water (Lonza, Belgium). Then, the tissues were fragmented with the help of a tissue shredding device (Qiagene, Germany). Eppendorf tubes with fragmented tissue were centrifuged at 5.000 rpm for 4-5 min. DNA extraction was performed from the supernatant part in an automatic extraction device (QIAcube, Qiagene, Germany) in accordance with the kit protocol (IndiSpin Pathogen Kit, Indical Bioscience, Germany).

Real-time PCR was performed on a Qiagen Rotor-Gene Q (Qiagen, Germany) device. Analysis was performed according to modified company protocols using the primer-probe set (Pantchev et al. 2009) and LightCycler 480 Probe Master Kit (Roche, USA) targeting the *ompA* gene indicated in Table 1.

Procedures performed for differential diagnosis

Brucella spp. in order to reveal the presence of other aerobic and microaerophilic bacterial abortion factors (*Campylobacter spp.*, *Salmonella spp.*, *Listeria spp.*) other than stomach contents, lung, heart, and placenta samples were cultured (Aydin et al. 2007; Dhama et al. 2015; Woah 2022b). Molecular tests were applied to investigate the presence of *Akabane*, *Border disease,* and *Blue tongue virus* as well as *PPRV*, *Neospora caninum,* and *Toxoplasma gondii* (Muller et al. 1996; Akashi et al. 1999; Costa et al. 2000; Vilček and Paton 2000; Hofmann et al. 2008; Li et al. 2016).

Histopathological examination

Necropsy of the sheep fetus was performed and tissue samples were collected, fixed in 10% buffered formalin solution, embedded in paraffin, sectioned at 5 µm and stained routinely with Hematoxylin-Eosin (H-E). All sections of the tissues were examined under a light microscope, and pathomorphological changes were determined according to the organs.

Immunohistochemical examination

Sections of 4-5 µm thickness were taken from the paraffin tissue blocks on polylysine slides (Isotherm, Türkiye). After this stage, staining was performed automatically on the Ventana Benchmark XT device with the biotin-free indirect IHC staining method in accordance with the kit procedure (ultraVIEW Universal DAB Detection Kit, USA). Mouse monoclonal antibody specific to the *Chlamydiaceae* family (Progen Biotechnic GmbH, Germany) was used

as an antibody in IHC staining. Mayer's hematoxylin included in the kit was used for background staining. All tissue sections were examined under a light microscope. Positive control preparations obtained from the pathology laboratory of the Faculty of Veterinary Medicine, University of Zurich, were used as IHC positive controls. Accordingly, cellular-associated granular or homogeneous brown staining on a blue background, sometimes in the nucleus, in the cytoplasm and sometimes in both areas, was considered positive. PBS-treated preparations were used as negative controls instead of antibodies.

Results

Gross pathology

Necropsy of the aborted fetus revealed macroscopic autolysis of the brain, liver and intestines. Apart from this, during necropsy, subcutaneous oedema and watery or bloody contents in the abdominal and thoracic cavities were observed. Necrosis was found mainly in the cotyledons and the intercotyledonous region in the placental tissue of the fetus.

Bacteriological isolation

In this case, *Brucella spp.* was isolated. The result was confirmed by a slide agglutination test with monospecific A and M antisera (A+M Positive Control Antiserum, Pendik Veterinary Control Institute Directorate, Istanbul). The isolate was determined to be *Brucella melitensis* on the Vitek 2 device.

Real-time PCR analysis

C. abortus DNA was detected in PCR performed using the extract prepared from fetal tissues (Table 1).

Table 1. Primer-probe sets used in real-time PCR (Pantchev et al. 2009).

Primary / Probe Name	Primer and Probe (FAM/TAMRA) Index 5'-3'	Amplicon Size (bp)
C. abortion	F: (5' - GCAACTGACACTAAGTCGGCTACA - 3') R: (5' - ACAAGCATGTTCAATCGATAAGAGA - 3') P:(FAM - AAATACCACGAATGGCAAGTTGGTTTAGCG - TAMRA)	82

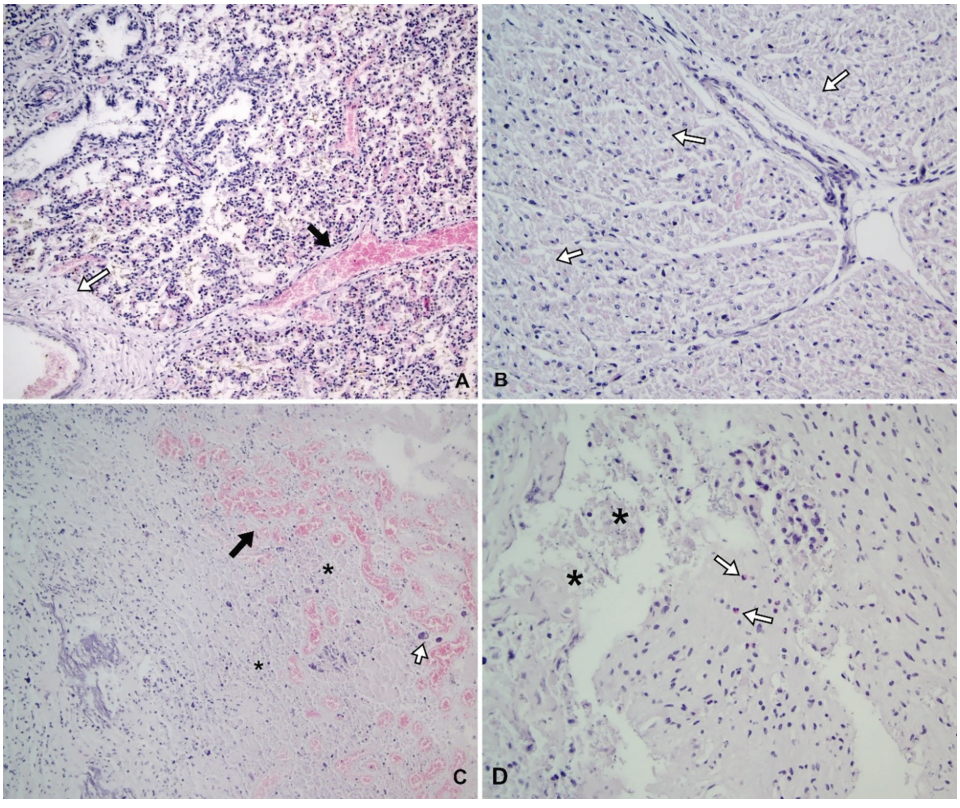

Figure 1. A) Lung. Dilated and hyperemic vessels (black arrow) and perivascular oedema (white arrow), H-E, 100X. B) Heart. Degenerative and necrotic areas (arrows), H-E, 200X., C) Placenta. Severe hyperemia (black arrow), necrosis (*), and a trophoblast with inclusion body (white arrow) H-E, 100X. D) Placenta. Necrotic areas (*) and granulocytes (white arrows), H-E, 200X.

Differential diagnosis for other abortion agents:

As a result of bacteriological culture and molecular analysis, *Campylobacter spp.*, *Salmonella spp.*, *Listeria spp.*, *Akabane virus*, *Border disease virus*, *Bluetongue virus*, *PPRV*, *Neospora caninum,* and *Toxoplasma gondii* were found negative.

Histopathology

The fetal lung showed catarrhal or mucopurulent bronchopneumonia with varying degrees of hyperemia, oedema and desquamation of bronchial/bronchiolar epithelium. The heart showed myocarditis with degeneration and necrosis of varying severity in the heart muscle cells, hyperemia, haemorrhage and mononuclear cell infiltration in interstitial areas. The placenta showed acute placentitis dominated by granulocytes and chronic placentitis dominated by mononuclear cells, including necrosis, hyperemia, oedema and haemorrhage of varying severity. Histopathological findings of the lung, heart and placenta are presented in Figure 1.

Immunohistochemistry

In IHC staining of aborted fetus lung, heart, and placenta, *Chlamydia spp.* immunopositivity was found. Localization of *Chlamydial* antigen was determined in alveolar and peribronchiolar macrophages in the lung, inflammatory infiltration in the interstitial area of the heart, and trophoblasts in the placenta. Figures of positive staining for *Chlamydial* antigen are presented in Figure 2.

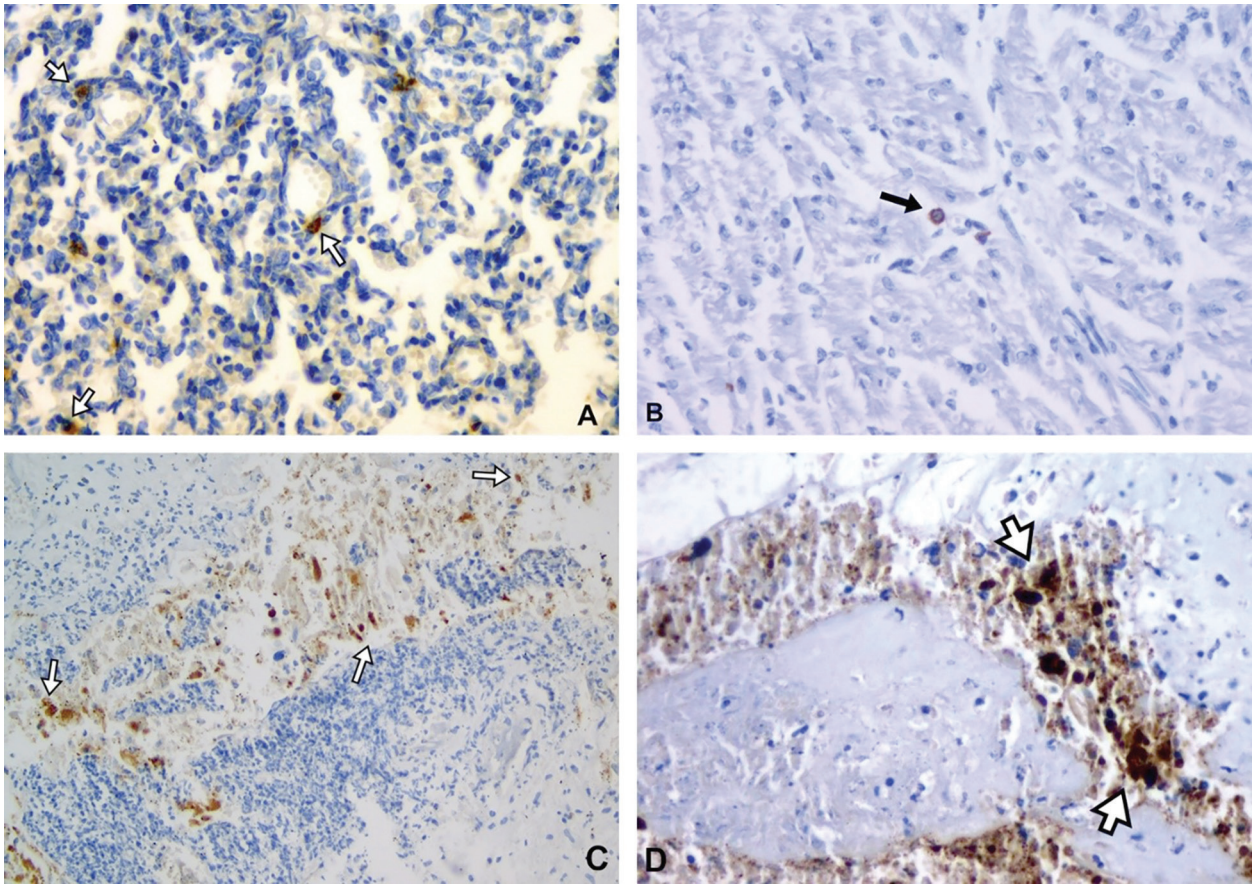

Figure 2. Immunopositive staining of *Chlamydia spp.* in alveolar macrophages of the lung (A), in inflammatory cells of the heart (B) and trophoblasts in the placenta (C and D). Original magnifications: A, B, D=400X, C=200X, Indirect IHC staining.

Discussion and conclusion

B. melitensis is one of the most common causes of sheep abortions in Türkiye (Sakmanoglu et al. 2021; Gulaydın et al. 2023). Although studies on *C. abortus* are fewer compared to *B. melitensis*, *C. abortus* is also one of the essential abortion factors in Türkiye (Malal and Turkyılmaz 2021; Yeni 2022). Due to

the inability to obtain healthy offspring, abortions continue despite strict vaccination programs in Türkiye as well as all over the world and cause severe economic losses by disrupting sustainable livestock breeding. This suggests that multietiological abortions are more than predicted, and abortions belong to a single etiologic agent. As a matter of fact, as mentioned in the introduction, abortion cases in which different etiologic agents are seen together have been reported recently (Sevik et al. 2017a; Sevik et al. 2017b; De Angelis et al. 2022; Ramo et al. 2022).

In this case report, a multietiological abortion caused by *B. melitensis* and *C. abortus* was described in a sheep abortion.

When the studies on sheep abortions in Türkiye were examined, no multietiological abortion case was reported together with *B. melitensis* and *C. abortus*. In the studies carried out worldwide Hailat et al. (2018), both *B. melitensis* and *C. abortus* positivity were reported in 5 placenta samples in the study performed by IHC and PCR on placenta samples of small ruminants. The histopathological findings obtained in this case were found to be compatible with previous data (Yesilmen et al. 2018; Westermann et al. 2021; Filikci 2022; Kanat 2022). In addition, experimental and field studies were conducted in terms of *Chlamydial* antigen localization in IHC staining (Maley et al. 2009; Longbottom et al. 2013; Livingstone et al. 2017).

In line with the studies and our report presented, it should not be overlooked that pets are not constantly exposed to a single abortion factor and that different etiological agents may play a role together and lead to multietiological abortions. Therefore, in the diagnosis of abortion cases, it is thought that a multidisciplinary approach in terms of multiple factors rather than an etiologic agent is essential in diagnosis. Knowing this situation and making a complete diagnosis will ensure that more perfect protection measures are taken in terms of human and animal health.

In this case, *B. melitensis* was diagnosed by bacteriological isolation, and *C. abortus* was diagnosed by real-time PCR and IHC methods. For this reason, it would be helpful to test many factors with multidisciplinary diagnostic methods in an abortion case.

In conclusion, a multiethological abortion case due to *B. melitensis* and *C. abortus* in sheep abortions was described for the first time in Türkiye, and it was evaluated that similar abortion patterns should be considered in the fight for abortion. Considering that these infectious agents are also essential zoonoses, it was concluded that it would be appropriate to focus on them seriously and to investigate the agents one by one with more comprehensive diagnostic methods without depending on limited methods in laboratory diagnosis.

Ethics committee for the use of experimental animals and other ethical committee decisions and permissions: It has been understood that there is no need for the permission of the Ethics Committee in accordance with the article "Procedures performed with dead animals or their tissues, slaughterhouse materials, waste fetuses are not subject to the permission of HADMEK" in the legislation on the Working Procedures and Principles of Animal Experiments Ethics Committees.

With the official letter of the Ministry of Agriculture and Forestry, General Directorate of Food and Control, dated 31.03.2023 and numbered E-71037622-399-9408343, this case report was allowed to be published.

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