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Özgür Kanat

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Molecular and histopathologic investigation of Pestivirus, *Chlamydophila abortus* and *Listeria monocytogenes* infections in aborted sheep fetuses

Ö. Kanat 

Department of Pathology, Faculty of Veterinary Medicine, University of Hatay Mustafa Kemal, Hatay, Turkey

ABSTRACT: The aims of this study was to investigate the presence of pestiviruses, *Chlamydophila abortus* (*C. abortus*) and *Listeria monocytogenes* (*L. monocytogenes*) and histopathological findings caused by these agents in aborted sheep fetuses. A total of 52 aborted sheep fetuses, aged between 1 to 5 months of gestation, were collected from Konya province in Turkey. Molecular techniques were used for the detection of pestivirus RNA, *C. abortus* and *L. monocytogenes* DNA in investigated samples. Pestivirus RNA was detected in 6 (11.5%) of the 52 aborted sheep fetuses whereas *C. abortus* DNA was determined in 8 (15.4%) fetuses. However, *L. monocytogenes* DNA was not detected in investigated samples. The significant histopathological findings were hypomyelination, degeneration and necrosis of neurons in the brain, interstitial pneumonia, mononuclear infiltrations in the liver, hyperemia and proximal tubule degeneration in the cortex of kidney in pestivirus positive samples and multifocal purulent-necrotic foci, diffuse neutrophils and mononuclear infiltrations in the liver and spleen, hyperaemia, bleeding, intramyelinic and perivascular oedema, gliosis, neuronophagia in the brain, hyperemia, proximal tubule degeneration and neutrophil granulocyte infiltration in the cortex of kidney were observed in *C. abortus* positive samples. The results of the study show that *C. abortus* and pestivirus infections play an important role in abortion in sheep.

Keywords: Border disease virus; *Chlamydophila abortus*; Ewe; *Listeria monocytogenes*; Pathology

Corresponding Author:
Özgür Kanat, Department of Pathology, Faculty of Veterinary Medicine, University of Hatay Mustafa Kemal, 31090 Hatay, Turkey
E-mail address: vetstorm@gmail.com

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INTRODUCTION

Small ruminants significantly contribute to the national socio-economic development through their high meat or milk yield, their rapid generational turnover, and their high number, which exceeds 38 million according to 2018 data in Turkey (TÜİK, 2019). However, in livestock enterprises, the most important risk factor that threatens sustainability is abortion. Previous studies have shown that pestiviruses, *Chlamydophila abortus* (*C. abortus*) and *Listeria monocytogenes* (*L. monocytogenes*) are important infectious agents that cause abortion in small ruminants in Turkey (Bulut et al., 2018; Kalender et al., 2013; Karaca et al., 2007).

Border disease virus (BDV) and bovine viral diarrhoea virus (BVDV), which infect sheep and goats are members of the *Pestivirus* genus in the *Flaviviridae* family. These two pestivirus infections are globally distributed, and the presence of immunotolerant, persistently infected animals lead to significant economic losses and reproductive losses as well as to respiratory disease and diarrhoea in ruminants. Pestivirus infections have been more widely reported in sheep than in goats. Congenital infection of small ruminants can lead to abortion, malformations and birth of persistently infected offsprings (Bulut et al., 2018; Constable et al., 2017; Feknous et al., 2018; Sozzi et al., 2019). Histopathologically, pestivirus infections are characterized by central myelination deficiency, along with demyelination and acute necrotising and inflammatory lymphoproliferative lesions (Constable et al., 2017).

Chlamydophila abortus (previously named *Chlamydia psittaci*), an intracellular microorganism and zoonotic agent, causes abortion in sheep by infecting epithelial cells and macrophages. *C. abortus* can persist for several months in non-pregnant sheep without any clinical signs. The principal sources of this microorganism include vaginal discharges, foetuses and placentas (Livingstone et al., 2017). Histopathologically, necrotic and inflammatory foci can be found in the liver of infected foetuses, and small focal necrosis can be found in the lungs, spleen and rarely in the brain (Kalender et al., 2013).

Listeria monocytogenes, a facultatively anaerobic, gram-positive microorganism found in silage and soils, causes listeriosis, a serious and life-threatening disease affecting a wide range of animals (Wang et al., 2018). Listeriosis in ruminants is characterized by encephalitis, abortion and neonatal septicaemia with

miliary visceral abscesses. In cases of utero foetal death with autolysis, no recognisable lesions can be seen. However, in some foetuses, multifocal hepatocellular necrosis or microabscesses are commonly numerous in the liver but occur less in the spleen (Constable et al., 2017). Similar lesions are present in the lung, kidney, spleen and brain and are usually only visible microscopically (Schlafer and Foster, 2016).

In Turkey, the seroprevalence of pestiviruses, *C. abortus* and *L. monocytogenes* in small ruminants varied between 0 - 90.27%, 1.6 - 29.3% and 34.69 - 58.39%, respectively (Gökçe et al., 2007; Kalender et al., 2013; Karaca et al., 2007; Ural and Erol, 2017). These infections have not received enough attention in abortion cases in Turkey. The epidemiological screen is one of the main appropriate control strategies to limit the spread of these infections. The objective of this study were to investigate presence of pestiviruses, *C. abortus* and *L. monocytogenes* in abortion cases of sheep and describe the histopathological lesions caused by these agents in sheep foetuses.

MATERIAL AND METHODS

Samples

The current study was performed during the lambing season of 2018. The Konya province, according to the data of Turkish Statistical Institute for the year 2018 ranked second with sheep populations of about 2 million. A total of 52 aborted sheep foetuses were collected from the Konya province in Turkey. Sheep foetuses aborted were aged between 1 to 5 months of gestation. At necropsy, tissue samples (lung, liver, spleen, kidney and brain) were collected from aborted sheep foetuses, and placed in PBS for molecular analyses and in 10% buffered formalin for histopathological examination. The study was approved by the Animal Ethical Committee of Hatay Mustafa Kemal University (Approval No. 2017/1-1).

Nucleic Acid Extraction for PCR

Foetal tissue samples were homogenized in PBS using mortar and pestle. DNA extraction was performed from the tissue homogenates using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the kit's instructions. RNA extraction was carried out from the homogenized foetal tissue samples using the RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Both DNA and RNA extracts were stored at -85 °C until required for PCR analyses. DNase/RNase-free distilled water

was used as negative control in detection of agents.

Detection of *C. abortus*

A PCR method described by Thiele et al., (1992) was used to detect 16S rRNA sequence of *C. abortus*. PCR was performed in a final volume of 25 µl of a mixture containing 2A: 5'-GCTTTTCTAATTACACC-3' and 2B: 5'-ATAGGGTTGAGACTATCCACAT-3' primers. Amplification was performed using Techne Thermal Cycler (Bibby Scientific Limited, Staffordshire, UK) with the following conditions: 40 cycles of 94 °C for 2 minutes, 50 °C for 1 minute and 72 °C for 75 seconds, and final extension at 72 °C for 7 minutes. Amplified DNA products were electrophoresed at 90V for 1 hour in 1.5% agarose gel stained with ethidium bromide and examined under UV illumination. A 116 bp fragment was deemed positive for *C. abortus* (Thiele et al., 1992).

Detection of *L. monocytogenes*

Real time PCR was performed using *L. monocytogenes* prfA gene specific primers and probe described by Rossmanith et al. (2006) with a probe master mix kit (Roche, Germany). Real time PCR reactions were performed in Lightcycler 2.0 PCR machine (Roche, Indianapolis, USA) with the following conditions: an initial denaturing step at 95 °C for 10 minutes, followed by 45 cycles of 94 °C for 15 seconds, 64 °C for 1 minute. The samples that had a Ct value <35 were considered positive (Rossmanith et al., 2006).

Detection of Pestiviruses by RT-PCR

One-step RT-PCR was used for the detection of pestivirus RNA. The assay was carried out in a 25 µl of a mixture containing primers 324: 5'-ATGCCCTAGTAGGACTAGCA-3' and 326: 5'-TCAACTC-CATGTGCCATGTAC-3', which amplify 288 bp region of the 5' UTR region (Vilcek et al., 1994), with using one step RT-PCR kit (Qiagen, Hilden, Germany). Amplification was performed using Techne Thermal Cycler (Bibby Scientific Limited, Staffordshire, UK) with the following conditions: reverse transcription step at 50 °C for of 30 minutes and initial PCR activation step at 95 °C for 15 minutes, followed by 35 cycles of 94 °C for 1 minute, 57 °C for 1 minute and 72°C for 1 minute, and final extension at 72 °C for 10 minutes. Amplified PCR products were analysed on 1.5% agarose gel stained with ethidium bromide after electrophoresis at 90 V for 60 min. A 288 bp fragment was deemed positive for pestiviruses

(Vilcek et al., 1994).

Histopathological evaluation

The tissues (lung, liver, spleen, kidney and brain) were fixed 10% buffered formalin for histopathology. Tissues were routinely processed and paraffin-embedded. The sections in 5 µm thickness from paraffin blocks were prepared and stained with Haematoxylin and Eosin stain (HE). These samples were observed under the light microscope (Olympus BX50-F4, Tokyo, Japan). For significant cases, microscopic images were photographed and transferred into digital camera system (Olympus DP12-BSW, microscopic, Tokyo, Japan).

RESULTS

Detection of *C. abortus* and *L. monocytogenes* by PCR

Out of 52 aborted sheep fetuses, 8 were found positive for *C. abortus* by PCR, which was directly performed on tissue samples obtained from the aborted fetuses. The age of the *C. abortus* positive fetuses were 2.5 months old (n=2) and 4-5 months old (n=6). *L. monocytogenes* DNA was not detected in the investigated samples.

Detection of Pestivirus RNA by RT-PCR

Pestivirus RNA was detected in 6 (11.5%) out of the 52 aborted sheep fetuses. The age of the pestivirus positive fetuses were 1 month old (n=1), 2 months old (n=2), 3 months old (n=2) and 4 months old (n=1). In this study, dual infection was not detected in the investigated samples.

Histopathological Findings in Foetal Tissues

Histopathological examination of six pestivirus-infected fetuses aborted at 3-5 months of gestation revealed the presence of severe hyperaemia, areas of multifocal haemorrhage, oedema and hypomyelination (Figs. 1A-C), degeneration and necrosis of neurons, neuronophagia (Fig. 1B), gliosis in brain, hyperaemia in the meninges and infiltrations of mononuclear cells (especially of lymphocytes in one abortion) (Fig. 1D). In the lungs, we observed severe hyperaemia, mononuclear cell infiltration in the interalveolar septum, and in one abortion, enlargement of the interlobular septum and oedema. In the liver, considerably intense mononuclear cell infiltration in the portal area as well as severe hyperaemia and hydropic degeneration in hepatocytes were detected only in one abortion (Fig. 1E). Renal hyperemia was detected in

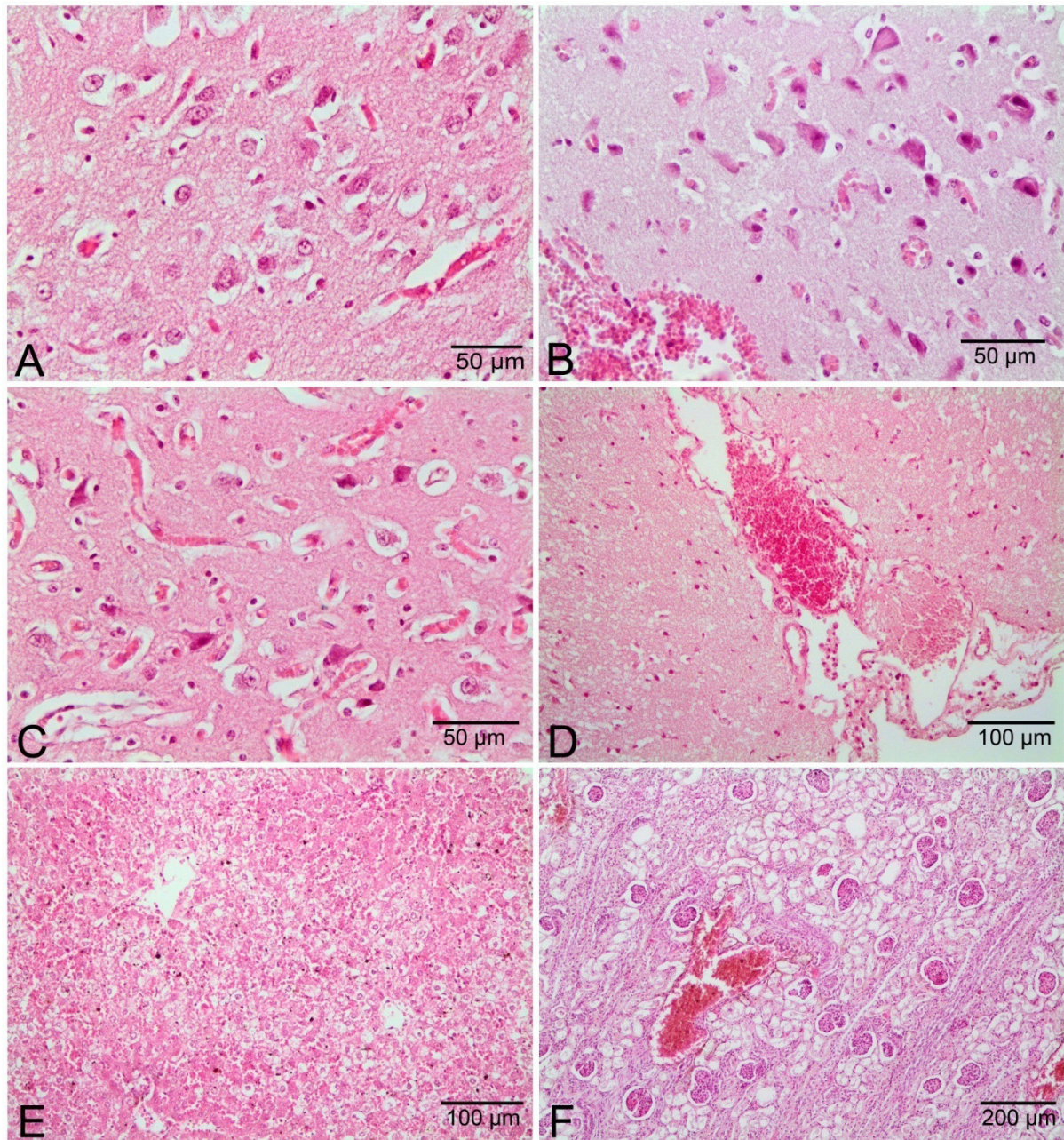


Figure 1. Hypomyelination (A), haemorrhage, degeneration and necrosis in neurons (B), hyperaemia, intramyelinic oedema (C), hyperaemia and mononuclear cells in the meninges (D), hydropic degeneration in hepatocytes (E), tubule degenerations and metanephros or atrophy of some glomeruli (F). HE staining

all cases; in one abortion, haemorrhage and tubule degenerations were observed, whereas metanephros or atrophy of some glomeruli was observed in another abortion (Fig. 1F). Hyperaemia was also observed in the spleen of the aborted foetuses.

Histopathological examination of tissues obtained from eight *C. abortus*-infected foetuses aborted at 2.5-5 months of gestation revealed hyperaemia, degeneration and desquamation in the epithelium of

bronchi and bronchioles as well as severe neutrophil granulocyte, lymphocyte, macrophage infiltration and mild neutrophil granulocyte infiltration in the alveol and interalveolar septum (Fig. 2A). In the liver, we observed hyperaemia, multifocal purulent-necrotic foci, diffuse neutrophil granulocyte and mononuclear cell infiltrations of limited number of lymphocytes and plasma cells and of macrophages and hepatocyte degenerations; in two abortions, degeneration and necrosis of hepatocytes were observed, whereas in

four abortions, intense neutrophil granulocyte infiltration was observed especially in the portal area and around the vena centralis (Figs. 2B-C). In the spleen, neutrophil granulocyte infiltration and multifocal purulent-necrotic foci were detected (Fig. 2D). In the central nervous system, hyperaemia in the brain and meninges, bleeding, intramyelinic and perivascular

oedema, gliosis, neuronophagia and ischemic neuronal changes were observed; in one abortion, mononuclear cell infiltration was noticed in the brain (Fig. 2E). In the kidney, hyperaemia, proximal tubule degeneration and neutrophil granulocyte infiltration in the cortex and pelvis were observed in one abortion (Fig. 2F).

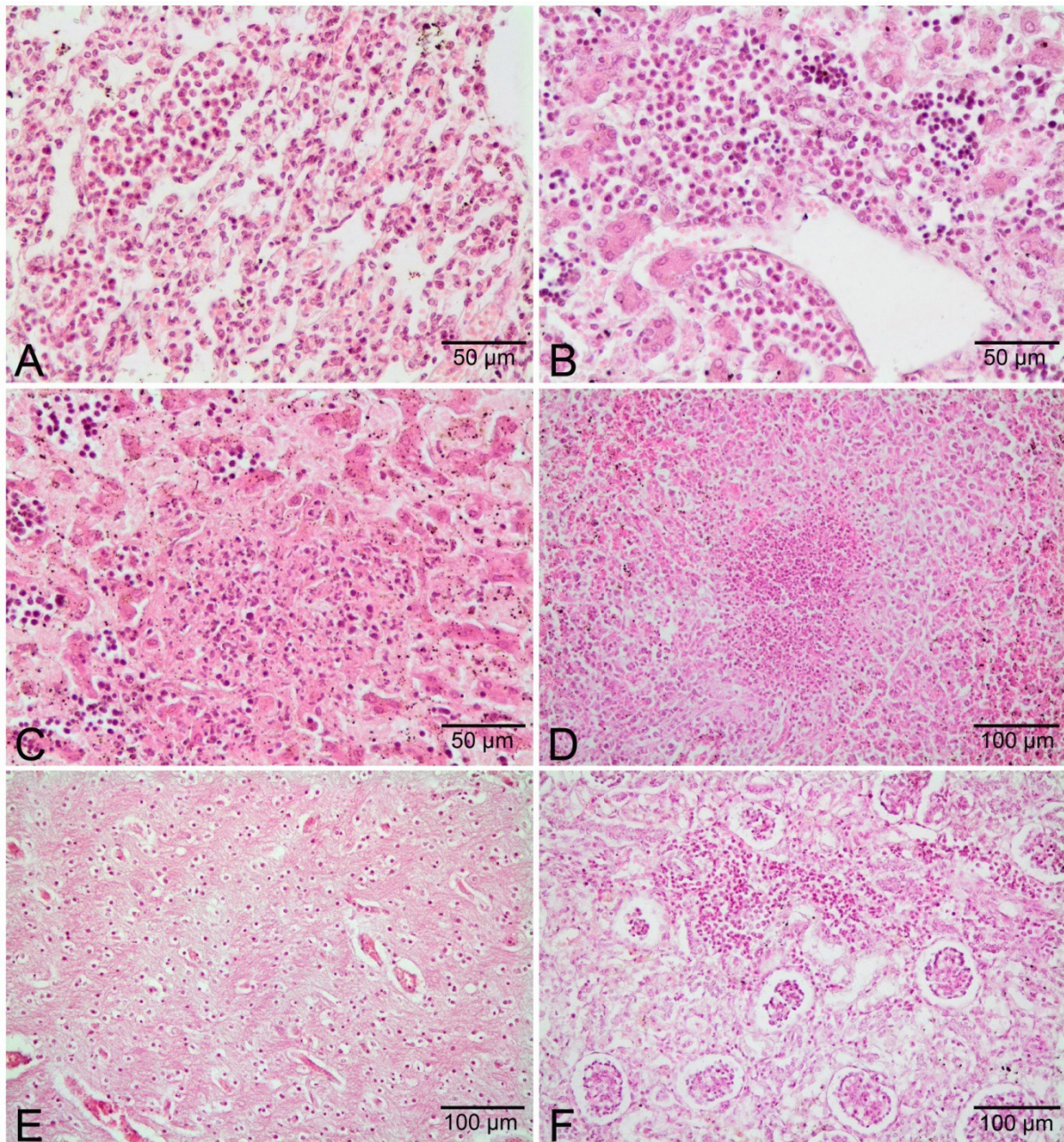


Figure 2. Neutrophil granulocyte infiltration in the alveol and interalveolar tissue (A), intense neutrophil infiltration around the vena centralis (B), multifocal purulent-necrotic foci in the liver (C) and in the spleen (D), hyperaemia and intramyelinic oedema in the brain (E), proximal tubule degeneration and neutrophil infiltration in the kidney (F). HE staining

DISCUSSION AND CONCLUSION

When *C. abortus*, which is the etiologic agent of enzootic abortion in sheep, is introduced into naive flocks, abortion rates can reach up to 25%-60% (Pugh et al., 2020). Serological studies conducted in different regions of Turkey have shown that the presence of *C. abortus*-specific antibodies among flocks that have history of abortion ranged from 1.6% to 46.6% (Gökçe et al., 2007). In this study, *C. abortus* DNA was detected in 8 (15.3%) of the 52 aborted sheep foetuses. This finding is consistent with a previous report (Arif et al., 2020; Heidari et al., 2018), although the current prevalence rate of *C. abortus* in aborted sheep foetuses is higher than the reported rates (3.49% and 6%) (Kalender et al., 2013). Possible explanations for this discrepancy include the number of sampled animals, time of sampling, individual differences and farm management.

Ewes infected with *C. abortus* are generally aborted within the last 2-3 weeks of gestation (Pugh et al., 2020). In the current study, six of the *C. abortus*-positive foetuses were aborted at 4-4.5 months of gestation. However, two *C. abortus*-positive foetuses were aborted at less than 3 months of gestation. Similar results were obtained by a previous study (Longbottom et al., 2013). Longbottom et al., reported that *C. abortus*-infected ewes are aborted at 74-138 days of gestation. This condition can be explained by latent infection with *C. abortus*, presence of secondary infections, individual differences or non-infectious factors. Microscopically, necrotic and inflammatory foci can be found in the liver, spleen and rarely in the brain of *C. abortus* positive sheep foetuses (Buxton et al., 2002; Kalender et al., 2013). Similarly, multifocal purulent-necrotic foci in the liver and spleen are observed in this study. There may be mononuclear cell infiltration in hepatic portal areas and multifocal areas of hepatitis (Buxton et al., 2002; Constable et al., 2017; Kalender et al., 2013; Longbottom et al., 2013; Navarro et al., 2004; Schlafer & Foster, 2016). However, in our study, intense neutrophil granulocyte infiltration was seen in the portal area and around the vena centralis in four of the foetuses infected with *C. abortus*. Thickening by mononuclear cell in interalveolar septum (Kalender et al., 2013; Longbottom et al., 2013; Navarro et al., 2004; Schlafer and Foster, 2016), infiltration of alveolar spaces by histiocytes and rare neutrophils in some cases in the lung (Miller et al., 1990) and in the brain, mild meningoencephalitis with vasculitis and hemorrhage (Kalender et al., 2013; Schlafer and Foster, 2016), small foci of leuco-

malacia, confined to the cerebral white matter cores, minimal focal microgliosis in the thalamus and midbrain (Buxton et al., 2002) have also been reported. In our study, mild neutrophil granulocyte infiltration in the interalveolar septum and changes in the epithelium of bronchi and bronchioles were observed. While mononuclear cell infiltration was noticed similarly in only one abortion in the brain, in other infected foetuses, bleeding, intramyelinic and perivascular oedema, gliosis and ischemic neuronal changes were observed in the brain.

Listeria monocytogenes can also cause abortion in sheep (Shoukat et al., 2014). The detection rates of *L. monocytogenes* in sheep abortions are 2.83% in Kashmir Region in India (Shoukat et al., 2014), 8.3% in Denmark (Agerholm et al., 2006) and 25% in Austria (Wagner et al., 2005). In Turkey, the presence of *L. monocytogenes* has been frequently identified serologically in cattle and small ruminants. The reported seroprevalence of *L. monocytogenes* in sheep is 25.8% in Bursa (Kennerman et al., 2000), whereas that in goats is 34.69%-60.81% in different provinces of Turkey (Karaca et al., 2007; K. Ural et al., 2009). We investigated the presence of *L. monocytogenes* in tissue samples of aborted sheep foetuses by using real-time PCR; however, *L. monocytogenes* DNA was not detected in investigated samples.

Both BDV and BVDV can infect small ruminants and cause abortion (Sozzi et al., 2019). Therefore, in this study only detection of pestiviruses was performed and genetic characterization of pestiviruses was not performed. In this study, the rate of pestivirus infections in sheep abortions was 11.5% (6/52). This finding is consistent with previous report (Şevik, 2018), although the current rate was higher than the reported rates (0.93%-3%) (Çokçalışkan, 2002; Oguzoglu et al., 2009). Possible explanations for this discrepancy include the detection method, the number of sampled animals and farm management. Ewes infected with pestiviruses generally abort at 60-85 days of gestation. Additionally, abortion may result if infection occurs beyond day 85 of gestation (Menzies, 2007). In the current study, three of the pestivirus-positive foetuses were aborted at 3-4 months of gestation. However, three pestivirus-positive foetuses were aborted at less than 3 months of gestation. This situation can be explained by strain of pestivirus, infection period and immune status of infected animals.

Chlamyphila abortus and *L. monocytogenes* DNA and pestivirus RNA were not detected in 38

aborted sheep fetuses. Abortion in these cases may be related with other infectious agents such as *Brucella sp.*, *Salmonella sp.*, akabane virus and bluetongue virus (Pugh et al., 2020) or may be related with non-infectious factors (nutritional and management factors).

In histopathological examination of pestivirus infected animals, hypomyelination, which usually occurs without signs of inflammation, is seen in all parts of the brain and spinal cord (Schlafer and Foster, 2016). It has been reported necrotizing and/or non-suppurative meningo-encephalomyelitis, often accompanied by hypomyelinogenesis in the brain. The most consistent finding are periventricular leukomalacia with perivascular mononuclear cell infiltration and gliosis (Krametter-Froetscher et al., 2010; Oguzoglu et al., 2009). In this study, severe hyperemia, areas of multifocal haemorrhage, intramiyelinic oedema and similarly, hypomyelination, degeneration and necrosis in some neurons and gliosis were observed in the brain of the aborted fetuses. A moderate leucocytostasis of the sinusoids has been seen in the liver and kidneys showed the moderate pigment nephrosis (Krametter-Froetscher et al., 2010). In our study, considerably intense mononuclear infiltration in the portal area, severe hyperaemia in several cases and hydropic degeneration in hepatocytes were detected in the liver of one aborted fetus. While renal hyperemia was detected in all cases, haemorrhage, tubule degenerations and metanephros or atrophy of some glomeruli were observed in another abortion. Metanephrosis and atrophy could not be distinguished in this study. Interstitial pneumonia characterised by mono-

nuclear cell infiltration was seen in the lung (Krametter-Froetscher et al., 2010; Oguzoglu et al., 2009). We observed severe hyperaemia, mononuclear infiltration in the interalveolar tissue and also, enlargement of the interlobular septum and oedema in one abortion in the lungs. Although our findings violently demonstrated the common association of pestiviruses and *C. abortus* in sheep abortions, this does not rule out the possible involvement of other pathogens as the cause of the abortions in sheep. However, further studies are needed to determine detailed epidemiologic screening for other pathogens associated with sheep abortions.

In conclusion, our study investigated the prevalence of *C. abortus*, *L. monocytogenes* and pestivirus infections in sheep abortions. The current findings are only specific for the surveyed province of Turkey and may not be representative for other regions of the country. Our results show that *C. abortus* and pestivirus infections play important role in abortion in sheep. Further studies on different infectious agents that can induce abortion in large number of small ruminants will provide a deeper understanding of the role of infectious agents in abortion cases.

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CONFLICT OF INTEREST

None declared by the authors.

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