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Molecular diagnosis of neglected infectious agents of heep and attle abortions: the prevalences of *Coxiella burnetii, Francisella tularensis* and *Chlamydophila abortus* at a glance

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Abstract: Bacterial abortive agents such as *Brucella* spp., *Salmonella* spp., *Campylobacter* spp., *Listeria* spp. cause serious infections that lead to significant economic losses in cattle and sheep breeding. These bacteria can be easily cultured under *in vitro* laboratory conditions. Abortions caused by intracellular bacteria such as *Coxiella burnetii (C. burnetii), Chlamydophila abortus (C. abortus)* and *Francisella tularensis (F. tularensis)* are less prevalent in abortive cases and the diagnosis of these bacteria, many of which need to cell culture for cultivation and biosafety level-3 laboratory facilities for safe working, can be made by PCR. In this study, it was aimed to determine the molecular prevalence of *C. burnetii, C. abortus* and *F. tularensis* agents, which were neglected during the diagnosis of abortions, in cattle and sheep collected from different regions of Türkiye. A total of 395 clinical materials were analyzed via agent-specific commercial Real-Time PCR. As a result, the molecular prevalence of *F. tularensis, C. burnetii* and *C. abortus* was determined as 14%, 2.9% and 2.28%, respectively. *F. tularensis* in cattle clinical samples and *C. burnetii* in sheep clinical samples were not found. This is the first report that *F. tularensis* was found as an agent in an abortive material in Türkiye. Although it differs in terms of prevalence, it has been determined that these microorganisms, which are neglected in routine diagnosis, can be spread with aborted materials, especially vaginal discharge, which may pose a risk of transmission.
Keywords: Abortion, *Chlamydophila abortus*, *Coxiella burnetii*, *Francisella tularensis*, real-time PCR.

Koyun ve sığır abortlarında ihmal edilen enfeksiyöz ajanların moleküler tanısı: bir bakışta *Coxiella burnetii, Francisella tularensis* ve *Chlamydophila abortus* prevalansları

Özet: *Brucella* spp*., Salmonella* spp*., Campylobacter* spp*., Listeria* spp*.* gibi bakteriyel atık etkenleri sığır ve koyun yetiştiriciliğinde önemli ekonomik kayıplara yol açan infeksiyonlar oluşturmaktadır. Bu etkenler *in vitro* laboratuvar koşullarında kolaylıkla kültüre edilebilmektedirler. *Coxiella burnetii (C. burnetii), Chlamydophila abortus (C. abortus), Francisella tularensis* (*F. tularensis*) gibi intrasellüler etkenlerden kaynaklanan atık olguları ise daha az sıklıktadır. Kültürel analizleri için çoğunun hücre kültürüne ihtiyaç duymaları ve güvenli çalışma için biyogüvenlik seviyesi-3 laboratuar gereksinimleri nedeniyle bu etkenlerin teşhisi genellikle PCR ile yapılmaktadır. Bu çalışmada, Türkiye'nin farklı bölgelerinden toplanan, atık yapmış sığır ve koyun örneklerinden, teşhiste göz ardı edilen, *C. burnetii, C. abortus* ve *F. tularensis* etkenlerinin moleküler prevalanslarının belirlenmesi amaçlandı. Toplam 395 klinik materyal, etken spesifik ticari Real-Time PCR ile analiz edildi. Çalışma sonucunda, atık olgularında *F. tularensis, C. burnetii* ve *C. abortus* moleküler prevalansları sırasıyla %14, %2,9 ve %2,28 olarak belirlenirken, sığır klinik örneklerinden *F. tularensis* ve koyun klinik örneklerinden *C. burnetii* etken varlığına rastlanılmadı. Bu, *F. tularensis*'in Türkiye'de abort materyalinde etken olarak bulunduğuna dair ilk rapordur. Yaygınlık açısından farklılık gösterse de rutin teşhiste göz ardı edilen bu etkenlerin bulaş riski oluşturabilecek şekilde atık materyalleri ile özellikle de vajinal ekstretleri ile saçılabilecekleri belirlenmiştir.
Anahtar sözcükler: Abort*, Chlamydophila abortus, Coxiella burnetii, Francisella tularensis*, real-time PCR.

Introduction

Infectious abortions are the main problems that are encountered in cattle and sheep husbandry and cause significant economic losses. Infections caused by many bacteria, viruses and parasites are not only limited to abortion cases but also cause problems such as embryonic deaths, weak offspring or stillbirths and infertility in pregnant animals. Although their prevalence varies in cattle and sheep, abortion cases in the case of bacterial agents such as *Brucella* spp*., Salmonella* spp*.,*

Campylobacter spp., *Listeria monocytogenes* rank first in terms of prevalence (13, 28). The fact that these microorganisms can be easily cultured under *in vitro* laboratory conditions has contributed positively to the increasing reports on their prevalence. Cattle and sheep abortions caused by obligate intracellular bacteria such as *Coxiella burnetii (C. burnetii), Chlamydophila abortus (C. abortus)* and facultative intracellular pathogens such as *Francisella tularensis (F. tularensis)* are mostly ignored in diagnosis since they cannot be cultured in routine laboratories and therefore their reports are less prevalent (19, 22, 24).

The importance of these microorganisms, which lead to early embryonic deaths, stillbirths or abortions, is based not only on economic losses in animal production but also on their zoonotic potential (16).

Chlamydia are obligate intracellular bacteria and cause several clinically and economically important diseases such as abortion, urinary system infections, pneumonia, conjunctivitis and, enteritis in livestock (12). The species responsible for chlamydial infections are *C. abortus*, *C. pecorum* and, *C. psittaci. C. abortus* is commonly responsible for sheep and cattle abortions. Abortions generally coincide with the last few months of gestation and are formed as a result of diffuse placentitis. The agent, in the form of elementary bodies that are infectious but non-replicating, is scattered by abortive materials such as placenta, uterine and vaginal fluids and fetal materials. The scattered agent is usually taken through the digestive system and can cause new infections (25).

Q fever is a common zoonotic disease in humans and animals caused by *C. burnetii* which is an obligate intracellular bacterium (14). The agent can be scattered abundantly with the milk, vaginal secretions, feces and, placenta of the aborted animals (8, 15). The disease can be transmitted by direct contact with these aborted materials, especially in sheep, cattle and goats. Several arthropods, primarily ticks, also play a role in the epidemiology of *C. burnetii* (23). Although they are widely asymptomatic carriers, *C. burnetii* causes placentitis, abortion, stillbirth and infertility in cattle and sheep (15).

Tularemia is a zoonotic disease caused by *F. tularensis*, a facultative intracellular bacterium, that causes different clinical findings in many animal species such as rabbits, sheep, cats, dogs, horses and pigs, especially rodents, and in humans. The presence of the disease in sheep, which ranks first among farm animals in terms of susceptibility to *F. tularensis*, has mostly been reported by serologically (20).

Infection in humans has a widespread transmission characteristic through the handling of infected animals, contact with or consumption of contaminated food or water, aerosol pathway and vector (ticks, flies, and mosquitoes) bites (9, 18). *F. tularensis* infections in humans are mostly case reports involving clinical complaints and cultural, serological or moleculardiagnosis of risky contacts. There are scarcely any studies on the adverse effects of *F. tularensis* on fetal development during the pregnancy. Although there are few reports of abortion and premature birth in untreated cases of *F. tularensis* in humans (5, 10), most of the cases consist of concerns of the characterization of complications and limiting the use of antibiotics due to their teratogenic effects (35). There is a similar situation in sheep as well, and late term abortions or neonatal lamb deaths caused by *F. tularensis* have been reported (27). The abortion and lamb deaths caused by this infectioncan reach 50% if left untreated. Although seroprevalence studies in cattle, which are more resistant to the infection than sheep, suggest *F. tularensis* infection, common clinical findings were not encountered in these animals and most of the cases were asymptomatic (20, 32). Considering the difficulties encountered in isolation and the drawbacks such as the presence of cross- reactions in serological analysis, Real-Time PCR can be used as a reliable, highly sensitive and practical diagnostic method in diagnosis (2, 6, 11).

In this study, it was aimed to investigate the molecular prevalence of *Chlamydophila abortus*, *Coxiella burnetii*, *Francisella tularensis* by Real-Time PCR in clinical materials of aborted cattle and sheep collected from different regions of Türkiye.

Materials and Methods

Study material: Clinical samples of cattle and sheep with abortion encountering in the first three months of 2021 which were sent to the laboratories of the Veterinary Control Central Research Institute of the Ministry of Agriculture and Forestry (Türkiye) were examined. The samples represented the three geographic regions of Türkiye, Central Anatolia Region, Black Sea Regionand and Eastern Anatolia Region. In this context, 345 cattle samples including 92 blood sera, 99 vaginal swabs and 154 aborted fetus stomach contents and 50 sheep samples including 28 blood sera, 10 vaginal swabs and 12 aborted fetus stomach contents were analyzed (Table 1).

Cultural analysis: The abortive materials (vaginal swabs and fetal stomach contents) were examined for the presence of abortive bacterial agents such as *Brucella* spp., *Campylobacter* spp., *Salmonella* spp. and *Listeria* spp. that could be cultured *in vitro* on the agent specific media reported before (1, 7). The samples were cultured for *F. tularensis* on Francis media which was prepared with Brain Heart Infusion Agar (Oxoid, UK), 8-9% defibrinated sheep blood, 1% Dextrose (Difco, USA), 0.1% L-Cysteine (Sigma-Aldrich, USA), *Helicobacter pylori* Selective Supplement (Dent) (Oxoid, UK) and antibiotics (Penicillin G 1ml/100 IU, Cycloheximide

L/100mg, Polymixin B l/8×10[4]). *F. tularensis* subsp. *holarctica* (NCTC 10857) was used as positive control (19).

Real-Time PCR analysis: DNA extraction from clinical samples was carried out using the cador Pathogen 96 QIAcube HT Kit (QIAcube HT Plasticware, Qiagen) in accordance with the manufacturer's instructions. The Real-Time PCR process was performed with CFX96 Touch Real-Time PCR Detection System (Bio-Rad). In Real-Time PCR analyzes, Bio-Speedy® Tularemi Real-Time PCR detection kit (Bioeksen R&D Technologies Ltd.® Istanbul) for *F. tularensis* and Bio-Speedy® Chlamydia-Coxiella Real-Time PCR detection kit for *C. burnetii* and *C. abortus* (Bioeksen R&D Technologies Ltd.® Istanbul) were used. Real-Time PCR analyzes were performed in the presence of positive controls (*C. abortus* S26/3, *C. burnetii* NM/2017-11 P6, *F. tularensis* NCTC 10855) and negative control (nuclease-free water). Real-Time PCR reaction was created with 11 µL volume for each sample and consisted of 5 µL qPCR mix (2X), 3 µL agent specific oligo mix (*F. tularensis* or *Chlamydia-Coxiella* spp.), 1 µL internal control and 2 µL template DNA. Real-Time PCR was performed as pre-denaturation at 95 °C for 5 min, 45 cycles consisting of denaturation at 95 °C for 15 sec and binding and elongation at 60 °C for 40 sec. When reading the results, *F. tularensis* targeted reactions in the FAM channel of the Real-Time PCR instrument were evaluated for *F. tularensis*. For *Chlamydia-Coxiella* spp., FAM was evaluated for *C. abortus* and ROX for *C. burnetii*. All samples were evaluated for the internal control target in the HEX channel. At the end of the Real-Time PCR analysis, samples was evaluated according to the amplification curves and Ct (threshold value cycle) data. Amplification with a sigmoidal curve and a significant logarithmic phase at Ct ≤ 37 was directly evaluated as positive. The result was considered negative as indicated in the kit procedure when amplification did not occur or when the cycle threshold (Ct) value exceeded 37 cycles (Figure 1).

Statistical analysis: Data were analysed via IBM SPSS Statistics 20.0. program. Chi-square test was used and a P-value of <0.05 was considered statistically significant.

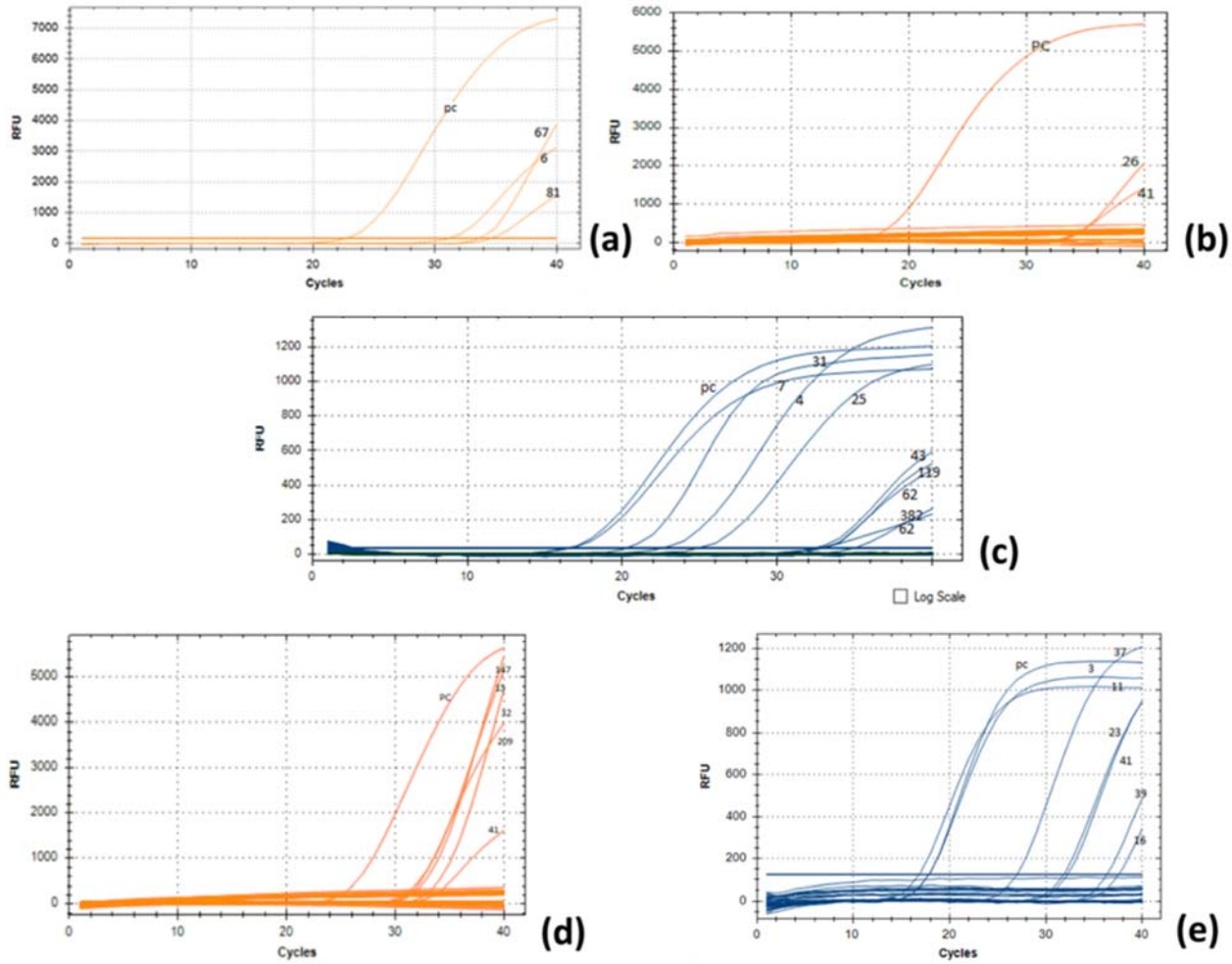

Figure 1. Amplification curves of Real-Time PCR reactions of cattle and sheep clinical materials.
(a): *C. burnetii* positivity in cattle vaginal swabs, (b): *C. burnetii* positivity in cattle blood serum, (c): *C. abortus* positivity in cattle and sheep vaginal swab and cattle fetus stomach content, (d): *C. burnetii* positivity in cattle fetus stomach content, (e): *F. tularensis* positivity in sheep vaginal swab and blood sera.

Table 1. Sample types and agent-specific RT-PCR positive results.

The microorganism	Cattle			Sheep			Total
	V. swab (n: 99)	Fetus (n: 154)	Blood sera (n: 92)	V. swab (n: 10)	Fetus (n: 12)	Blood sera (n: 28)	
F. tularensis	-	-	-	2 (20%)	-	5 (17.86%)	7 (14%) *
C. burnetii	3 (3.03%)	5 (3.25%)	2 (2.17%)	-	-	-	10 (2.9%) **
C. abortus	5 (5.05%)	2 (1.3%)	-	2 (20%)	-	-	7 (2.03%) ** 2 (4%) * 9 (2.28%) ***
Total	8 (8.08%)	7 (4.55%)	2 (2.17%)	4 (40%)	-	5 (17.86%)	26 (6.58%)

* Only for sheep samples, ** Only for cattle samples, *** Values determined for cattle and sheep samples.

Results

All aborted materials cultured *in vitro* on the relevant media in terms of specific bacteria were found to be culture negative. As a result of the Real-Time PCR analysis, the individual molecular prevalence of obligate or facultative intracellular agents in abortion was calculated as 4.93% in cattle and 14% in sheep, and it was determined as 6.58% in total. The findings in terms of the presence of all the neglected abortive agents were statistically significant based on the host preference ($P<0.05$). In total, the prevalence of *F. tularensis*, *C. burnetii* and *C. abortus* was 14%, 2.9% and 2.28%, respectively (Table 1). However, no significant relationship was found on the basis of a host in terms of the individually carrying these microorganisms ($P>0.05$).

C. burnetii was detected in 10 (2.9%) clinical samples of cattle, including 3 (3.03%) vaginal swab, 5 (3.25%) fetal stomach content and 2 (2.17%) blood serum (Table 1, Figure 1a,b,d). *C. abortus* was detected in 7 (2.03%) clinical samples of cattle, including 5 (5.05%) vaginal swab and 2 (1.3%) fetal stomach contents (Table 1, Figure 1c). *F. tularensis* DNA was not detected in any of the material of cattle (Table 1).

F. tularensis was detected in 7 (14%) of the clinical specimens belonging to sheep, including 2 (20%) vaginal swabs and 5 (17.86%) blood serum (Table 1, Figure 1e). *C. abortus* was detected in 2 (20%) vaginal swab samples of sheep (Table 1, Figure 1c). No *C. burnetii* DNA was detected in any of the abortive materials of sheep (Table 1).

Discussion and Conclusion

C. burnetii, F. tularensis and *C. abortus* infections are common worldwide and cause important zoonotic diseases. However, research of these microorganisms on the epidemiology of abortions in domestic animals is extremely limited. This situation is valid in both research laboratories and veterinary services of Türkiye and other countries. With obligate or facultative intracellular localization, these microorganisms can lead to infectious abortions, early embryonic deaths and infertility in cattle and sheep. Since most laboratories that make routine diagnosis do not have living environments such as cell or tissue culture and experimental animals, the diagnosis of these agents is inadequate or often overlooked. However, it is certain that agent isolation to be achieved after cultural analysis will be beneficial for further identification, characterization of molecular and antigenic, virulence trials and vaccine studies. PCR techniques based on enzymatic amplification and imaging of agent-specific protected gene regions provide more practical and reliable results in the diagnosis of obligate intracellular bacteria such as *F. tularensis* whose *in vitro* culture is quite difficult or requires high biosecurity measures. In this context, Real-Time PCR method is seen as an advantageous technique that provides results simultaneously with the amplification of gene regions (2, 6, 11, 33).

In a study in Switzerland, the analysis of *C. burnetii, C. abortus* and *Leptospira* spp., which were ignored in the diagnosis of cattle abortions, was performed by the serological, molecular, bacteriological, histopathological and immunohistochemical methods and the Real-Time PCR supported by the histopathological analysis was found the recommended method in diagnosis (33). As a result of this study, the molecular prevalence of obligate and facultative intracellular bacteria was found to be 6.58% from various clinical samples of aborted cattle and sheep. The presence of these neglected agents was significantly higher in cattle ($P<0.05$). Interpretations regarding the agent-specific prevalence and probabilities were discussed in the following sections.

Among the chlamydial agents, *C. abortus* is the species that it is widely responsible for sheep and cattle abortions. Abortions caused by *C. abortus* can sometimes occur as abort storms and the rate can reach up to 30% in naive herds. The advantages of PCR techniques have been reported both in terms of diagnosis and in the management of such outbreaks (7). In a study conducted by Kılıç et al. (21) on *C. abortus* in aborted cattle fetuses by PCR, a 6.3% positivity was obtained. Aras et al. (4) detected *C. abortus* in 2 (3%) of 65 stomach contents of aborted cattle fetuses by PCR in Konya region (Türkiye). Livingstone et al. (24) evaluated the epidemiology and potential effect of an enzootic abortion caused by *C. abortus* in sheep, and a small number of *C. abortus* specific genomes were detected by the Real-Time PCR in vaginal swabs of sheep

after abortion. In the studies, it has been emphasized that *C. abortus* should also be taken into account in combating infectious abortion in cattle and sheep. In this study, *C. abortus* DNA was detected in 9 (2.28%) of the samples belonging to cattle and sheep and these findings are relatively similar to other studies (4, 21, 24). The samples with *C. abortus* showed diversity known as important sources of infection in the transmission of the disease, including the vaginal swab of aborted animals and the stomach contents of the aborted fetus.

Q Fever cases caused by *C. burnetii* have been reported from all over the world except Antarctica and New Zealand (3, 26). An important part of these reports are studies in which the agent analysis is carried out by PCR from various aborted materials (tissue, blood, blood serum and milk samples) belonging to cattle and sheep. Erdenliğ et al. (11) obtained a 1.5% *C. burnetii* positivity in aborted cattle samples and 2.7% in aborted sheep samples by Real-Time PCR. In addition to the zoonotic importance of Q Fever, the researchers emphasized the diagnostic advantage of the Real-Time PCR in rapid and safe detection of this agent. In a study conducted by Selim et al. (30) in sheep, Q Fever was investigated by both ELISA and RT-PCR. Interestingly, 42 samples detected seronegative by ELISA were found to be positive by RT-PCR and thus, although ELISA is more advantageous as a screening test in the preliminary diagnosis of Q Fever, RT-PCR has been reported to give more reliable and accurate results. Although it is a generally low amount (4.3-6%), the molecular prevalence of Q Fever has been reported at different rates in abortion in ruminants (8, 22). In the detection of *C. burnetii*, which is an absolute intracellular agent, from abortions, blood serum samples have been reported to be indispensable clinical materials in addition to placenta, vaginal secrets and fetal tissue (29). Hence, in this study, *C. burnetii* positivity was obtained from 2 blood serum samples in cattle. Considering its general prevalence, the prevalence of *C. burnetii* in abortive cases was found to be low, similar to other studies (8, 22). The absence of sheep was interpreted as the abortion cases evaluated in this study may not be caused by *C. burnetii.*

Although *F. tularensis* is listed among the agents that can cause abortion and premature birth (35), it is not included in the diagnostic manual of many countries. Moreover, *F. tularensis* infections are contented with being defined as episodes that can occur during pregnancy in both humans and animals and have not been associated with the disease (27, 35). Nevertheless, it was emphasized that *F. tularensis* should be considered in the differential diagnosis in cases of abortion and neonatal death corresponding to the late period of pregnancy in places where the disease is endemic and tick activity is intense (27).

In the differential diagnosis, the histopathological changes observed in sheep abortions, alleged to be caused by *F. tularensis*, were described cursory. Macroscopic lesions consist of focal necrosis found in many organs such as the lung, liver, kidney, however, the bacteria could not be identified in smear prepared from these areas and stained with hematoxyline-eosin or Gram stain. Diagnosis can only be made on a limited number of samples by immunohistochemical (IHC), serological, PCR, and cultural methods (27). This confusion regarding gross macroscopy and other analysis methods could not be interpreted in this study because of the low culturability even if positive by PCR (17, 31). *F. tularensis* positivity has already been detected only by the Real-Time PCR.

Detecting of *F. tularensis* in intravascular monocytic cells with IHC in some abortive materials and the presence of its extracellular niches in the circulatory system indicates that the agent can be found in the circulatory system in abortion cases (27). Therefore, detection of *F. tularensis* from sheep blood serum not only serologically, but also by PCR in blood serum samples of 5 sheep, representing the circulatory system, showed the clinical importance of these samples in diagnosis. Although the histopathological analysis of aborted materials is not performed, the determination of the agent in blood will contribute to solving the mystery of the role of *F. tularensis* as a constructive agent in sheep abortions, which is theoretically known but scarcely encountered as clinical cases.

In the study, the agent was detected by the Real-Time PCR in the vaginal discharges of 2 aborted sheep. This is the first report that *F. tularensis* was found as an agent in an abortive material in Türkiye. In line with these findings, we think that *F. tularensis*, which is not a vaginal floral agent, may be scattered with vaginal secretions following the abortion, similar to that the probability of bacteria scattering with nasal or ocular discharges in the form of oculoglandular tularemia infection in human (34), and thus may be a possible abortion agent unless otherwise specified.

In conclusion, the molecular prevalence of *C. abortus, C. burnetti* and *F. tularensis* in cattle and sheep abortion was determined as 6.58%. Although their prevalences are similar to that obtained from many countries, it was determined that these agents, which are mostly ignored during the diagnosis, can be scattered with aborted materials especially vaginal discharges in a way that may pose a risk for animal and human. In areas where infections are endemic, these agents should be considered as a part of the diagnosis in cases with a history of disease and gross pathology. It can be also said that the Real-Time PCR is a usable method that provides fast, sensitive and reliable results in this direction.

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Ethical Statement

The ethical permission was ensured from The Animal Experiments Local Ethics Committee of Veterinary Control Central Research Institute (Türkiye) with a code of "2021-01". A publication permit was granted by the Ministry of Agriculture and Forestry (Türkiye) with the document dated 26.06.2020 and numbered 71037622-824.01.03-E.1750551.

Conflict of interest

The author(s) declare no potential conflicts of interest.

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