ORIGINAL ARTICLE

Veterinary Research Forum. 2023; 14 (10) 541 - 548
doi: 10.30466/vrf.2023.1999091.3830
Journal Homepage: vrf.iranjournals.ir

Veterinary Research Forum

Detection of bovine viral diarrhea virus and bovine herpes virus type 1 in cattle with and without endometritis

Ayhan Atasever[1], Ali Sefa Mendil[1]*, Mehmet Ozkan Timurkan[2]

[1] Department of Pathology, Faculty of Veterinary Medicine, Erciyes University, Kayseri, Türkiye; [2] Department of Virology, Faculty of Veterinary Medicine, Ataturk University Erzurum, Türkiye.

Article Info

Article history:

Received: 31 March 2023
Accepted: 17 June 2023
Available online: 15 October 2023

Keywords:

Bovine herpes virus type 1
Bovine viral diarrhea virus
Cattle
Uterus

Abstract

This study aimed to investigate the potential presence of bovine herpes virus type 1 (BHV-1) and bovine viral diarrhea virus (BVDV) in cattle uteri that did not display any clinical and macroscopic signs of infection. Virus detection involved polymerase chain reaction (PCR) test, double immunohistochemistry (IHC), and double immunofluorescence (IF). One hundred cornu uterus samples were collected from cattle aged 1 year and older. The BVDV was detected by PCR or by double IHC/IF in the collected samples from slaughterhouses in Kayseri city (Central Anatolia, Türkiye) from 2021 - 2022. By contrast, BHV-1 was detected by PCR and double IHC/IF at a rate of 16.00% and 21.00%, respectively. In the IHC and IF detection, BHV-1 was detected in endometrial epithelial cells and in some mononuclear cells in the lamina propria, periglandular areas and myometrium. Although no macroscopic lesion was found in the BHV-1-positive samples (n = 21), histopathological detection showed that two had acute endometritis, eight had subacute endometritis, eight had chronic endometritis and the three others showed no signs of endometritis. This prevalence study demonstrated for the first time that even while BVDV could not be detected in the samples, BHV-1 posed a critical potential reproductive risk in pregnant animals, as it can specifically cause abortions when it resides in cattle uteri that do not show clinical or macroscopic and even microscopic signs of infection. Additionally, this study was the first to combine PCR and double IHC/IF for BHV-1 and BVDV detection in cattle uteri.

Introduction

Diseases of the reproductive system of cattle cause economic losses in a multifaceted way and the most important factors for these economic losses are uterine infections that make huge economic losses by decline in fertility. The occurrence of uterine infections is influenced by the degree of contamination, the defense mechanism of the uterus and the ability of infectious agents to survive and multiply in uterine tissues.[1] The infectious agents affecting the uterus leading to reproductive problems are bacterial, fungal, protozoal, and viral pathogens,[2] bovine herpes virus type 1 (BHV-1) and bovine viral diarrhea virus (BVDV) are among the viral pathogens that cause the most significant reproductive problems in cattle including metritis and endometritis depending on the infection severity.[3] Additionally, repeat breeding is one of the most common causes of reproductive disorders in cattle.[1]

The BVDV is an RNA virus classified under the family *Flaviviridae* and genus *Pestivirus*.[4] In BVDV-infected animals, this virus was found in macrophage-like cells in the endometrium 7 - 16 days after infection.[5] Given the serious problems associated with this virus, in particular persistent immunodeficiency, cattle must be kept free from BVDV infection to ensure herd health.[6] In addition, BVDV infection is hazardous at all stages of pregnancy as it also causes reproductive problems associated with early embryonic deaths. It prevents implantation of the embryo into the uterus resulting in infertility.[7] Methods such as virus isolation, the polymerase chain reaction (PCR) test, and antigen enzyme-linked immunosorbent assay are used in BVDV detection.[8] Other techniques that reveal infections in tissues include immunohistochemistry (IHC) and immunofluorescence (IF).[9,10]

Another virus that resides in the uterus is BHV-1 is a double-stranded DNA virus classified under the family

***Correspondence:**

Ali Sefa Mendil. DVM
Department of Pathology, Faculty of Veterinary Medicine, Erciyes University, Kayseri, Türkiye
E-mail: 4022140002@erciyesuni.edu.tr

Herpesviridae, subfamily *Alphaherpesvirinae*, and genus *Varicellovirus* and causes genital diseases.[11] The incubation period for the genital form of diseases is 1 - 3 days.[12] When the virus remains latent in sensory ganglion neurons, it can maintain persistent infection.[13] Moreover, studies have shown that BHV-1-infected male animals can transmit the virus to female animals through mating resulting in endometritis and abortions.[14] According to genetic diversity and clinical symptom variety, BHV-1 isolates are classified into three subtypes: BHV-1.1, BHV-1.2a and BHV-1.2b.[15] Methods such as cell culture, serology and electron microscopy are used to detect BHV-1.[16] Virus neutralization tests have also been routinely employed for the detection of BHV-1 and BVDV specific antibodies.[17]Another detection method is PCR which is an isolation method.[18] Diagnosis can also be made through IF, radio immunoprecipitation, immunoperoxidase staining and immunoblotting techniques.[19,20]

This study aimed to investigate the potential presence of BHV-1 and BVDV in cattle uteri that did not display any macroscopic signs of infection by using PCR, double IHC and double IF.

Materials and Methods

Animals. Samples were obtained from cattle in slaughterhouses in Kayseri and nearby areas from 2021 - 2022. The animals were 1 year or older and diverse in terms of their species, sex, weight and other characteristics. A total number of 100 uterine cornu samples were collected from either the left or right uterine cornu that did not display any macroscopic signs of infection. This study was approved by the Erciyes University Animal Experiments Local Ethics Committee (EUHHADYEK-2022/17).

Polymerase chain reaction test. The uterine cornu samples were first subjected to nucleic acid isolation using High Pure Viral Nucleic Acid Kit (Roche, Rotkreuz, Switzerland) according to the manufacturer's recommendations. The obtained nucleic acid suspension was kept at – 20.00 ˚C until the PCR test. The BVDV detection involved the screening of RNA-based infectious agents isolated from the uterine cornu samples. Viral infections in the RNA genome character must be translated into complementary DNA (cDNA) before PCR analysis. Thus, reverse transcriptase enzyme and random hexamer primers were used. The cDNA was stored at – 20.00 ˚C until the PCR test which involved the use of a specific primer pair to investigate the presence of BHV-1 and BVDV in the samples. For the reverse transcription-polymerase chain reaction (RT-PCR), the primers and optimization conditions for BVDV (324– ATGCCCWTAGTA GGACTAGCA, 326 - TCAACTCCATGTGCCATGTAC) were adopted from Timurkan and Aydın[21] and for BHV-1 (gC-F-CGGCCACGACGCTGACGA, gC-R-CGCCGCCGAGTACTACCC, gB-F-TACGACTCGTTCGCGCTCTC, gB-R-GGTACGTCTCCAA GCTGCCC) from Dagalp *et al.*[22] The heat cycle using a thermal cycler (Veriti; Applied Biosystems; Waltham, USA) used in the reaction was selected according to the studies in which the primers were utilized. The amplicons obtained were evaluated by gel electrophoresis (GT; Bio-Rad, Hercules, USA). The resulting amplification products were transferred onto ethidium bromide-treated 2.00% agarose gel as support medium. The PCR products were separated based on the distance they have moved in the appropriate buffer pool as a function of their molecular weight. The appropriately sized bands were considered positive at 478 bp (for gB gene of BHV-1), 575 bp (for gC gene of BHV-1) and 288 bp (5'UTR gene of BVDV) under Ultraviolet light.

Histopathological evaluations. Tissue samples taken for double IHC, double IF and Hematoxylin and Eosin (H&E) staining were fixated in a 10.00% neutral formalin solution for 48 hr. They were then washed for 12 hr. After washing, the tissues were passed through a routine alcohol xylol series, placed in paraffin blocks, and the sections taken with a microtome were transferred to polylysine slides for staining. For the microscopic classification of endometritis, the positive samples as evaluated by PCR, IHC, or IF were stained with Hematoxylin and Eosin. Each sample was examined and classified as acute endometritis (mononuclear infiltration in the lamina propria, stromal edema, desquamation of endometrial epithelial/glandular epithelial cells), subacute endometritis (mononuclear infiltration in the lamina propria, hyperplasia in endometrial epithelial/glandular epithelial) or chronic endometritis (mononuclear infiltration and fibrosis in the lamina propria, atrophy of glands, medial hypertrophy of blood vessels) according to the criteria in the study by Bhadaniya *et al.*[23]

For double IHC, the 5.00 µm-thick sections of the polylysine slides were stained using the double IHC method. The sections were placed in alcohol–xylene and subsequently deparaffinized. The sections were kept in 3.00% H_2O_2 (Merck, Darmstadt, Germany) solution for 10 min, washed with PBS and then subjected to heat treatment (500 W) for 10 min using a citrate-containing antigen retrieval solution (pH 7.00) to release the antigen (Santa Cruz Biotechnology, Dallas, USA). Subsequently, they were incubated in protein block (Thermo Scientific, Waltham, USA) solution at room temperature for 5 min to prevent nonspecific binding. The sections were incubated with BHV-1 primary antibody (dilution ratio 1/1000; VMRD, Pullman, USA) which was the first antibody to be used at room temperature for 45 min, washed with PBS, incubated with Biotinylated Rabbit Anti-Goat IgG (dilution ratio 1/200, Abcam, Cambridge, UK) antibody at room temperature and washed again with PBS. Then, the sections were incubated with streptavidin-HRP (Thermo Scientific, Waltham, USA) solution and stored at room temperature for 30 min and subsequently washed with PBS to facilitate the binding of the BHV-1 antibody. For the

chromogenic test, the sections were incubated with 3-Amino-9-Ethylcarbazole (AEC; Thermo Scientific, Waltham, USA) solution and then washed with PBS. Prior to incubation with the second primary antibody, the sections were incubated at room temperature and washed for 5 min with protein block solution to prevent nonspecific binding. The sections were subsequently incubated with BVDV primary antibody (the second primary antibody; dilution ratio 1/100) at room temperature for 45 min, washed with PBS, incubated with Biotinylated Goat Anti-Polyvalent (Thermo Scientific, Waltham, USA) and then washed again with PBS. Subsequently, they were incubated with Streptavidin-AP (Abcam, Cambridge, UK) solution for 30 min and were washed to promote binding of the BVDV primary antibody. Then, they were incubated with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium chromogen (Amsbio, Abingdon, UK), (6.60 µL NBT and 3.30 µL BCIP for 1.00 mL) and washed again for microscopic observation. The sections were counter-stained with Methyl Green for 30 sec and then sealed with a water-based adhesive. The BHV-1 and BVDV positivity were indicated by red and purple fluorescence, respectively, as seen under the microscope.

The double IF procedure performed until the antigen retrieval stage was the same as in the IHC analysis. The subsequent steps were as follow: The sections were incubated in 2.00% bovine serum albumin (BSA)- phosphate buffered saline (PBS) solution at room temperature for 10 min to prevent nonspecific binding. The antibodies used were the same as those in the IHC staining. The BHV-1 (dilution ratio 1/1000) and BVDV (dilution ratio 1/100) primary antibodies were diluted in equal volumes and dripped onto the sections which were stored at 4.00 ˚C overnight and then washed with PBS three times. Rabbit Anti-Goat IgG-Texas Red (Biorbyte, Cambridge, UK) and Goat Anti-Rabbit IgG- Fluorescein Isothiocyanate (FITC; Jackson Immunoresearch, Cambridge, UK) antibodies which were used as secondary antibodies were mixed with 0.10% BSA - PBS in equal volumes at a dilution rate of 1/100 and then dripped onto the sections. The sections were left in a dark environment for 2 hr at room temperature and subsequently washed with distilled water three times. 4′,6-diamidino-2-phenylindole (DAPI; Abcam) was dripped onto the sections for examination under a fluorescence microscope with FITC, Texas Red and DAPI filters (Axio-Zeiss, Wetzlar, Germany). The nucleus, BHV-1 and BVDV appeared as blue, red and green, respectively.

Results

Polymerase chain reaction findings. This study examined the viral factors of BHV-1 and BVDV infections in 100 uterine tissues. BHV-1 nucleic acid was detected in 16.00% (16/100) of the samples. By contrast, BVDV was not detected in the molecular analysis (Fig. 1).

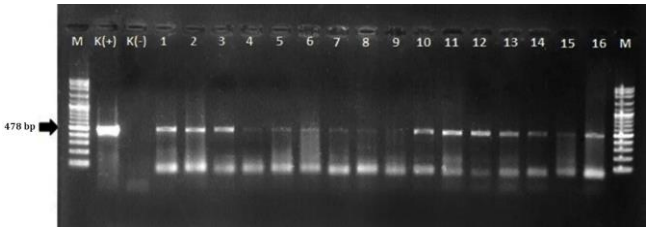

Fig. 1. Agarose gel image of the polymerase chain reaction products (M: DNA ladder (100 bp), K(+): Positive control, K(−): Negative control and 1-16: Positive samples.

Immunohistochemical and immunofluorescence findings. The BHV-1 was detected in 21 out of the 100 samples through IHC and IF staining, whereas, BVDV was not found. Specifically, BHV-1 was detected intracyto-plasmically in endometrial epithelial cells, lamina propria, periglandular areas and in some mononuclear cells in the myometrium (Fig. 2).

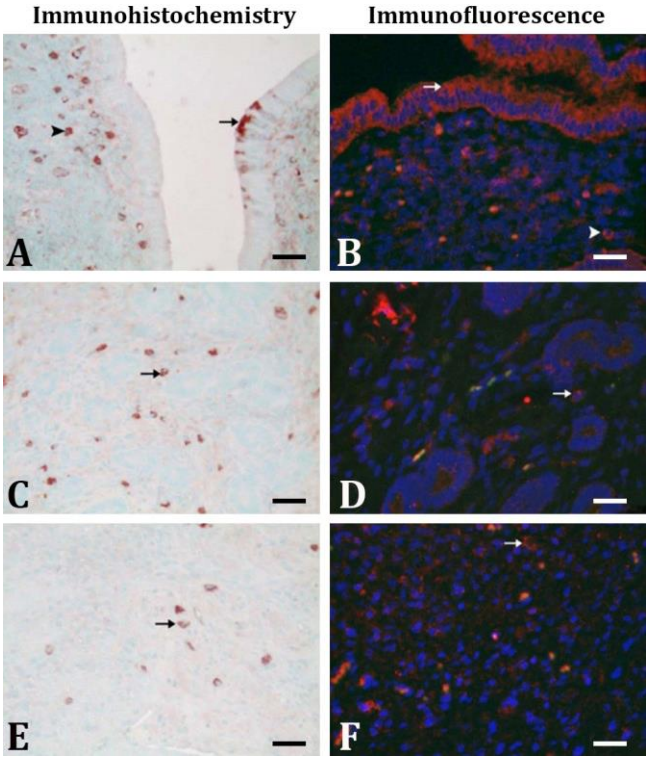

Fig. 2. A and **B)** Bovine herpes virus type 1 positivity was detected in endometrial epithelial cells (arrow) and mononuclear cells in the lamina propria (arrow head), **C** and **D)** Mononuclear cells in glandular areas (arrow), **E** and **F)** Mononuclear cells in the myometrium (arrow), (bars = 20.00 µm)

Histopathological findings. A total number of 16 and 21 BHV-1-positive samples were identified using PCR and IHC/IF, respectively. Histopathological assessment revealed that among the BHV-1-positive samples, two had acute endometritis, eight had subacute endometritis, and eight had chronic endometritis. Three samples were free of the disease (Table 1).

Table 1. Diagnosis of BHV-1 by polymerase chain reaction (PCR), immunohistochemistry (IHC), immunofluorescence (IF) methods and classification of endometritis.

Samples	PCR	IHC	IF	Classification
1	+	+	+	Healthy
2	+	+	+	Subacute
3	+	+	+	Acute
4	+	+	+	Subacute
5	+	+	+	Chronic
6	+	+	+	Subacute
7	+	+	+	Healthy
8	+	+	+	Subacute
9	+	+	+	Chronic
10	+	+	+	Subacute
11	+	+	+	Subacute
12	+	+	+	Chronic
13	+	+	+	Chronic
14	+	+	+	Chronic
15	+	+	+	Subacute
16	+	+	+	Healthy
17	-	+	+	Chronic
18	-	+	+	Subacute
19	-	+	+	Chronic
20	-	+	+	Chronic
21	-	+	+	Acute
Total	**16**	**21**	**21**	*

* Totally, Acute = 2, Subacute = 8, Chronic = 8, and Healthy = 3.

In samples with acute endometritis, mononuclear cells were observed in the lamina propria. Moreover, the mononuclear cell infiltrations had a diffuse distribution in the lamina propria and they consisted mostly of lymphocytes and macrophages. The other findings in these samples were the presence of stromal edema and the occasional shedding of epithelial and glandular epithelial cells. In samples with subacute endometritis, the microscopic findings were hyperplasia in the endometrial and glandular epithelia and mononuclear cell infiltrations in the lamina propria. In samples with chronic endometritis, the microscopic formations detected were the atrophy of the endometrial glands, hypertrophy of the medial layers of blood vessels and mononuclear cell infiltrations in the lamina propria along with the presence of fibrotic areas associated with fibroblast activation in the lamina propria (Fig. 3).

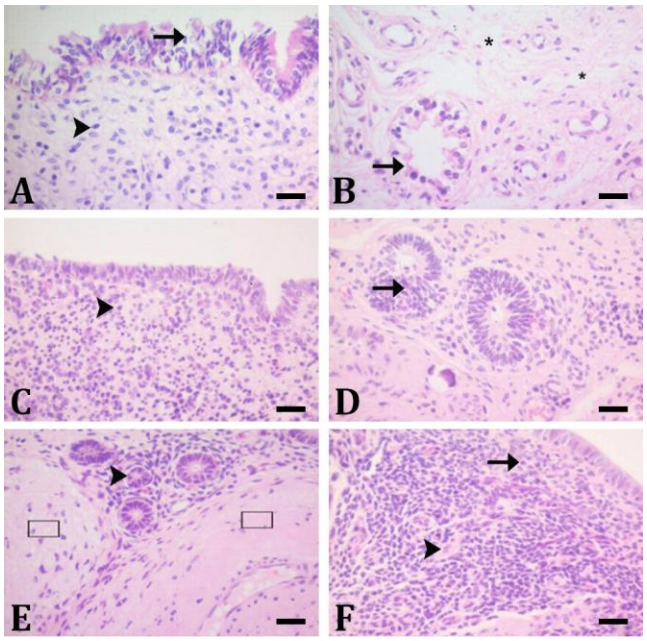

Fig. 3. A) Acute endometritis. Shedding of endometrial epithelial cells (arrow) and mononuclear cell infiltrations in the lamina propria (arrow head), **B)** Acute endometritis. Stromal edema (*) and shedding of glandular gland epithelium (arrow head), **C)** Subacute endometritis. Mononuclear cell infiltrations in the lamina propria (arrow head), **E)** Subacute endometritis. Hyperplasia of glandular epithelial cells (arrow), **E)** Chronic endometritis. Atrophy of the endometrial gland (arrow head) and medial hypertrophy of vessels (rectangle), and **F)** Chronic endometritis. Fibrocyte/ fibroblast growth in the lamina propria (arrow head) and mononuclear cell infiltrations (arrow). (H&E; bars = 50.00 μm).

Discussion

Endometritis, the most important disease of the uterus, negatively affects cattle breeding and related industries worldwide, leading to economic losses. For instance, it decreases milk yield, a negative impact on ovarian activity and significant treatment costs. Bacterial and viral pathogens are the primary causes of endometritis.[24]

The BHV-1, which have a worldwide distribution, can be detected serologically (antibody search/screening).[18] Such serological methods have shown that BHV-1 seroprevalence is widespread in Türkiye and the countries in the region. For example, in a study covering all geographical regions of Türkiye, an average of 53.20% seropositivity was determined,[25] while seropositivity rates of 31.30% in Syria[26] and 39.70% in Iran[27] were detected in cattle. It is thought that the differences in rates vary based on the efficacy of control and eradication programs.

Another method used in the diagnosis of the disease is PCR which is the foremost analysis method that can characterize BHV-1 genomically and thus has been used in many studies.[18] The PCR was first employed for BHV-1 detection in the 1990s. For instance, Vilcek *et al.*[28] infected the respiratory system with BHV-1 and successfully employed PCR to detect the viral antigen from secretion and excretion fluids collected using a nasal swab and from semen samples. Later, PCR was used mostly for BHV-1 detection in semen samples.[28-31] In contrast, few studies have performed PCR-based detection of BHV-1 in the uterus and these studies aimed to investigate disease pathogenesis instead of obtaining epidemiological data.[29,30] Queiroz-Castro *et al.*[32] have shown that BHV-1 can reside in the uterus. Moreover, studies have revealed male animals infected with BHV-1 can transmit the virus to females through mating leading to the development of of chronic endometritis and eventually abortions.[14]

In Türkiye, few studies have been conducted related to diagnosing BHV-1 by the PCR method. In one of these studies, BHV-1 was investigated in blood samples collected from animals with metritis, but it was not found.[33] In terms of the genital system, two studies were conducted on blood samples collected from aborted cattle, one of which found BHV-1 positivity at a rate of 0.39%,[34] while no positivity was found in the other.[35] In our study, 16.00% positivity was detected by PCR in tissue samples. The higher rate of positivity compared to other studies suggested that it might be related to the duration of the virus in the tissues and blood and the virus was localized in the tissues for a more extended period, so it was found at a higher rate compared to other studies.

Our literature review showed that research on the relationship between BHV-1/BVDV and the uterus remains limited. Additionally, a prevalence study involving PCR and double IHC/IF for BHV-1/BVDV detection in bovine uteri that do not show any macroscopic sign of infection has never been conducted. Thus, this study is the first of its kind.

In this study, 21.00% of the uterine samples infected with BHV-1 were identified by dual IHC/IF. Studies on IHC detection of BHV-1 have focused mainly on abortions.[36,37] The only study involving bovine uteri was conducted by Queiroz-Castro *et al.*[32] who examined 450 uteri, oviducts, and ovaries. Using IF, they examined the presence of BHV-1 in 75 samples. They detected BHV-1 seropositivity, with the highest positivity rate observed in the uterus at 54.70%. The positivity rates for oviducts and ovaries were 40.00% and 32.00%, respectively. In the present study, the virus was found in the genital system, particularly in the uterus. The uterus is important as it is one of the places where the virus enters the body and replication occurs.[18] Additionally, the virus has been reported to cause abortions and has been found in aborted fetuses. In a study on abortions related to BHV-1, PCR and IHC analyses have revealed the presence of the virus in the placenta as well as in the brain, liver, lungs, and heart of the investigated fetus. While PCR is considered the most reliable detection method, IHC demonstrates limited sensitivity.[38] detection rates of 16.00% and 21.00% for PCR and IHC/IF in the present study, respectively, suggested that the sensitivity of the IHC/IF method was indeed limited and that non-specific staining rendered diagnosis difficult. The positive samples identified through IHC/IF staining were found mainly in mononuclear cells as well as in endometrial epithelial cells. Other studies have also demonstrated that the virus infects mononuclear cells such as monocytes, macrophages and lymphocytes,[39-41] consistent with the present finding that the virus is localized in immune cells. Meanwhile, it has been speculated that BHV-1 alters the nature of endometritis depending on the duration of infection as most of the 21 positive samples detected by IHC/IF had symptoms of subacute (n = 8) and chronic (n = 8) endometritis. Three of the positive samples had no signs of endometritis suggesting that the virus had just infected the uterus and endometritis would progress into acute, subacute and chronic endometritis over time.

The BVDV is a common infection in cattle herds worldwide. While the rate of persistent infection (PI) animals was 0.50 - 2.50% in the present study, a variable prevalence for BVDV (ranging from 28.00 - 66.00%) has been determined in herd-based studies.[42] These rate variations suggest the use of PI animals which are known to transmit the disease primarily through semen[43] in artificial insemination, but this may not always be the case because in research conducted in Türkiye and neighboring countries, BVDV seroprevalence was found at similar rates. In a study conducted in the border region covering the southeastern part of Türkiye, BVDV seropositivity was found to be 48.05%,[44] while in a study conducted in the north of Syria, it was 58.00%,[26] and in research undertaken in Iran, 55.40%[27] seropositivity was found. A few studies conducted in Türkiye for detecting BVDV by the PCR method found quite different values. In one study, BVDV was not detected in blood samples collected from animals with metritis.[33] However, in two studies conducted on blood samples collected from aborted cattle, 3.55%[34] and 41.73%[35] BVDV positivity was detected. Yıldız and Babaoğlu attributed the high rate they found to traditional animal breeding and the lack of vaccination programs.[35] However, we believe that future studies in different regions will provide more information about positivity levels.

The BVDV was not detected in this study. However, it has been reported that BVDV infection commonly occurs in the uterus. Studies have shown that this organ, especially the endometrium, is a key area in the spread and persistent presence of BVDV.[45] Animals with persistent infection can transmit the virus transplacentally to their offspring which can cause death and malformations in the offspring, depending on the gestation stage during the time of infection or they can transmit the virus to the environment by giving birth to an offspring that is a carrier.[43] Studies have reported that the presence of BVDV in the uterus is related to the duration of infection. Bielanski *et al.* and McGowan *et al.* have detected the virus in uterine tissues 7 days after inoculation and that it persisted for up to 24 days.[5,46] How long the virus may remain in the uterus is unknown, but evidence has shown that it may persist for several weeks.[47] BVDV was not detected in the uterus possibly because months had already elapsed since the infection or because the samples were not infected.

In conclusion, this study was the first to investigate the presence of BHV-1 and BVDV in bovine uteri using PCR and double IHC/IF. Although BVDV was not detected, BHV-1, another infectious pathogen that causes abortions, had a high occurrence rate (21.00%) in the

samples that did not show any clinical or macroscopic signs of infection. From the perspective of reproductive health, BHV-1 which resides in bovine uteri is considered a significant risk factor especially in pregnant cattle as it eventually causes abortions making it a phenomenon that warrants further investigation.

Acknowledgments

The authors would like to acknowledge the support extended by the Scientific Research Unit of Erciyes University (Project No: TDK-2022-11686).

Conflict of interest

There is no declared conflict of interest.

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