ATATÜRK ÜNİVERSİTESİ YAYINLARI
ATATURK UNIVERSITY PUBLICATIONS

Research Article / Araştırma Makalesi DOI: 10.5152/VetSciPract.2023.22056

Molecular and Pathological Survey of Bovine Viral Diarrhea Virus in the Testis of Bulls

Boğaların Testislerinde Bovine Viral Diyare Virüsünün Moleküler ve Patolojik Olarak Araştırılması

Mustafa ÖZKARACA[1]
Mehmet Özkan TİMURKAN[2]
Yavuz Selim SAĞLAM[3]
Selçuk ÖZDEMIR[4]
Hakan AYDIN[2]
Serdar ALTUN[3]

[1]Department of Veterinary Pathology, Cumhuriyet University, Sivas, Türkiye
[2]Department of Veterinary Virology, Atatürk University, Erzurum, Türkiye
[3]Department of Veterinary Pathology, Atatürk University, Erzurum, Türkiye
[4]Department of Veterinary Genetics, Atatürk University, Erzurum, Türkiye

ABSTRACT

Bovine viral diarrhea virus (BVDV) is an important pathogen that causes diseases in the gastrointestinal, respiratory, and reproductive systems. It also leads to a decrease in reproductive performance and consequently continuous economic losses in cattle management. The presence of BVD virus in bull testes was investigated in this study using indirect immunofluorescence (IF), immunohistochemistry (IHC), and Reverse Transcription polymerase chain reaction (RT-PCR) methods. The tissue distribution of BVDV was examined pathologically and virologically in 100 bull testis tissue samples. For this purpose, IF, IHC, and RT-PCR methods were employed. Positive IF staining was detected in 21 (21%) testis samples using the indirect IF method. In IHC staining, 16 (16%) samples were found to be positive. In RT-PCR, 13 (13%) samples tested positive. The results of the presented study demonstrate that BVDV can infect different cell types in bull testes and that the virus can primarily be transmitted through natural or artificial insemination. Therefore, bulls are an important epidemiological factor in the transmission of the disease.

Keywords: Bovine viral diarrhea virus, immunoflourescence, immunohistochemistry, RT-PCR, testis

ÖZ

Bovine viral diarrhea virus (BVDV), gastrointestinal, solunum ve üreme sistemlerinde hastalıklara neden olan önemli bir patojendir. Ayrıca, üreme performansında azalmaya yol açar ve bu nedenle sığırların yönetiminde sürekli ekonomik kayıplara neden olur. Bu çalışmada, BVDV'nin boğa testisindeki varlığı indirekt immunofloresan (IF), immunohistokimya (IHC) ve ters transkriptaz polimeraz zincir reaksiyonu (RT-PCR) yöntemleriyle araştırıldı. Çalışmada, 100 boğa testis dokusu örneğinde BVD virusunun doku dağılımı patolojik ve virolojik olarak incelendi. Bu amaçla, IF, IHC ve RT-PCR yöntemleri kullanıldı. İndirekt IF yöntemiyle 21 (%21) testis örnek, IHC yöntemiyle 16 (%16) örnek pozitif bulundu. RT-PCR'da ise 13 (%13) örnek pozitif bulundu. Sunulan çalışmanın sonuçları, BVDV'nin boğa testisinde farklı hücre tiplerine enfekte olabileceğini ve virüsün öncelikle doğal veya suni tohumlama yoluyla bulaşabileceğini göstermektedir. Bu nedenle, boğalar hastalığın yayılmasında önemli bir epidemiyolojik faktördür.

Anahtar Kelimeler: Bovine viral diyare virüsü, immunfloresans, immunohistokimya, RT-PCR, testis

Geliş Tarihi/Received: 02.10.2022
Kabul Tarihi/Accepted: 14.12.2022
Yayın Tarihi/Publication Date: 16.08.2023

Sorumlu Yazar/Corresponding author: Mustafa Özkaraca
E-mail: mustafaozkaraca@cumhuriyet.edu.tr

Atıf: Özkaraca M, Timurkan MÖ, Sağlam YS, Özdemir S, Aydın H, Altun S. Boğaların testislerinde bovine viral diyare virüsünün moleküler ve patolojik olarak araştırılması. *Vet Sci Pract*. 2023;18(2):47-51.

Cite this article as: Özkaraca M, Timurkan MÖ, Sağlam YS, Özdemir S, Aydın H, Altun S. Molecular and pathological survey of bovine viral diarrhea virus in the testis of bulls. *Vet Sci Pract* 2023;18(2):47-51.

INTRODUCTION

Bovine viral diarrhea (BVD) is a viral cattle disease that occurs worldwide, resulting in significant economic losses.[1] The responsible virus is part of the *Flaviviridae* family of viruses under the genus *Pestivirus*. It is an RNA virus with a positive-strand structure and has 3 classes: type I (BVD1), type II (BVD2), and type III (BVD3).[2] Two distinct biotypes, that is, the non-cytopathic (NCP) biotype and the cytopathic (CP) biotype, are known to be manifested by all genotypes of BVD1 and BVD2 viruses.[3,4] Significant effects of the NCP-BVD become noteworthy in the periods of breeding and pregnancy. Persistently infected (PI) calves are the most important outcomes of this condition.[5,6]

Bovine viral diarrhea can display various clinical manifestations affecting respiratory, gastrointestinal, reproductive, and fetal tissues and manifest in acute, transient, mucosal, or thrombocyt openic/hemorrhagic forms. Persistently infected cattle are also a result of BVD.[7] It is considered that transiently infected (IT) cattle are epidemiologically insignificant compared to PI cattle as they are the prime means of transmission.[8-10] The virus can be found in the testicular tissue and semen of bulls with a BVD infection history.[11] Different approaches can be utilized for the detection of infected specimens. However, the most effective approaches are the precise diagnosis of the acute form and the elimination of PI cattle in a given herd.[12] Cattle worldwide are under the threat of BVD infection.[13] It has been reported that the serological prevalence of bovine viral diarrhea virus (BVDV) ranges between 2.3% and 64.7%.[14,15] It is stated that intensive breeding of dairy cattle generates less than 0.25% PI cattle in establishments.[16] Persistently infected cattle have higher quantities of the virus in their tissues, and established methods can effectively diagnose it, for example, isolation, enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR), immunofluorescence (IF), and immunohistochemistry (IHC).[17-20] Two objectives of the present study were as follows: (1) comparative detection of BVD virus in testicular specimens using PCR, IHC, and IF methods and (2) determination of viral distribution and localization of antigens in testicular tissue.

MATERIALS AND METHODS

Sample Collection

This study was carried out in accordance with the Declaration of Helsinki. Macroscopically examined 100 bulls' (showing no clinical signs) testes were collected. Collected testis tissues were separated into 2 pieces. Half of the sections were fixed in 10% neutral formaldehyde solution for IHC and IF process. The other half of testis samples that were suspected of having persistent infection were homogenized for reverse transcription (RT)-PCR in a tissue disruptor in phosphate buffer saline (PBS) containing 1% IU of 10 000 IU penicillin/mL, 10 mg streptomycin/mL, and 0.025 mg/mL amphotericin B, and then, tissues were centrifuged at 3000 rpm for 15 minutes at +4°C. This supernatant was passed through a 0.45 µm diameter injector filter in a sterile cabinet and each specimen was transferred to sterile tubes of 2 mL volume. Samples were stored at −80°C until RT-PCR testing was performed.

Nucleic Acid Extractions

A commercial kit (GF-1 Viral Nucleic Acid Extraction Kit (proteinase K included), 100 preps, GF-RD-100, Vivantis, Malaysia) was used for RNA isolation. After frozen testis tissue samples were thawed at room temperature, RNA extractions were performed per the manufacturer's instructions and the samples were passed to RT process.

Reverse Transcription

Because BVD contained a genome in RNA, RT was performed before PCR. Reverse transcriptase enzyme was used for this purpose. First-strand complementary DNA (cDNA) synthesis kit (Thermo Scientific, USA) was used. The kit was applied according to recommendations from the manufacturing company.

Polymerase Chain Reaction

The sample was subjected to PCR process using cDNA template obtained after RT. Bovine viral diarrhea virus-specific primer pair was used to investigate the presence of the BVDV genome in the samples. The heat cycles, primers, and optimization conditions to be used in the reaction were maintained as described in a previous study.[21] Bovine viral diarrhea virus-positive tissue samples, confirmed by sequence reaction, were used as positive control. The amplicons obtained from the PCR were evaluated by gel electrophoresis and 288 bp product reactions were evaluated as positive.

Immunohistochemistry and Indirect Immunofluorescence Examination

Bulls' testis samples were washed with tap water before routine serial treatment of samples with graded alcohol and xylene was performed, and these tissues were embedded in paraffin. After the routine histopathology process, sections of 5 µm in thickness were cut with a rotary microtome and mounted on glass slides that were precoated with poly-L-lysine. After deparaffinization, 3% H_2O_2 solution (hydrogen peroxide; 18304-1L, Sigma, Mo, USA) was applied dropwise on each slide for 10 minutes to inactivate endogenous peroxidase activity. Then, the slides were immersed in antigen retrieval solution (ab96674, Abcam, USA) (pH 6.0) and heated in a microwave for 10 minutes to unmask the antigens. After cooling, sections were incubated for 10 minutes with a protein block solution (Cat. no. ab80436, Abcam) to prevent non-specific binding. Sections were incubated for IF staining with primer antibody (BVDV anti-viral antiserum (Cat. No. 210-70-BVD, VMRD, USA)) at room temperature for 30 minutes. Sections were incubated for IF staining with a secondary antibody—anti-caprine immunoglobulin G-FITC (fluorescein isothiocyanate) (Cat. No. CJ-F-CAPG-10 mL VMRD, USA) in the dark for 45 minutes at room temperature. Sections were then examined with fluorescence microscope at magnification of 40× and closed by fluoroshield mounting medium with 4,6-diamino-2-phenyl indole (DAPI).

In IHC staining, sections were incubated with primary antibody (BVDV anti-viral antiserum, (Cat. No. 210-70-BVD, VMRD) at room temperature for 20 minutes. Labeled Streptavidin-Biotin (LSAB)+System-HRP (Carpinteria, USA) IHC kit was used and sections were incubated with 3,3′-diaminobenzidine and chromogen. The slides were counterstained with hematoxylin, and entellan was dropped on the tissue sections, which were then marked as positive (+) or negative (−) under a light microscope.

Statistical Analysis

The specificity and sensitivity of the tests were calculated using International Business Machine' Statistical Package for Social Science (SPSS) 20.0 software (IBM Corp.; Armonk, NY, USA). The sensitivity and specificity of the test were evaluated using RT-PCR as a relative gold standard. The level of agreement between the results of the PCR, IF, and IHC staining was measured at a 5% level of significance.

RESULTS

Positivities concerning PCR, IHC, and IF are given in Table 1. Pestivirus nucleic acid was detected in 13 (13%) of 100 tissue samples that were suspected of being infected with BVDV (Figure 1). Bovine viral diarrhea virus viral antigens were found in 16 of the

Table 1. Numbers of Positivity of BVDV Antigens Using PCR, Immunohistochemical, and Immunofluorescence

	PCR	IHC	IF
Positive sample number	13/100	16/100	21/100

BVDV, bovine viral diarrhea virus; IF, immunofluorescence; IHC, immunohistochemistry; PCR, polymerase chain reaction.

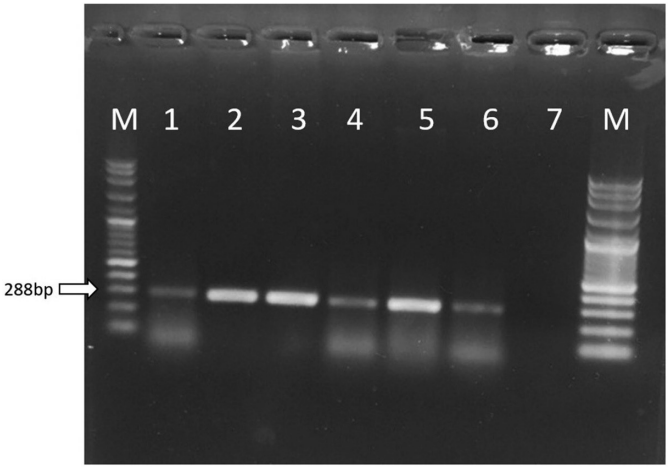

Figure 1. RT-PCR product visualized in agarose gel electrophoresis. Arrows show 288 bp fragments. 1: positive control, 2-6: BVDV-positive samples, and 7: negative control. M = 100 bp ladder. BVDV, bovine viral diarrhea virus; RT-PCR, reverse transcription-polymerase chain reaction.

100 samples examined in IHC staining and in 21 of the 100 samples examined in IF staining. Bovine viral diarrhea virus positivity was detected by IF and IHC methods in all 13 samples that tested positive for PCR. In addition, BVDV positivity was detected in 3 samples with IHC and 8 samples with IF. Bovine viral diarrhea virus positivity determined according to cell types is given in Table 2. Immunohistochemically, BVDV viral antigens were detected in spermatocytes, spermatids, and Sertoli cells in the seminiferous tubules (Figure 2A and C). Immunopositivity was observed as intracytoplasmic at severe level in spermatocytes and spermatids, moderate level in Leydig cells in intertubular areas, and mild level in Sertoli cells. In IF staining, BVDV viral antigen positivity was observed as intracytoplasmic at the severe level in spermatids and Leydig cells in intertubular areas (Figure 2B), moderate in spermatocytes, and mild in Sertoli cells (Figure 2D). Using PCR as the relative gold standard, the IF had a sensitivity of 85.7% and specificity of 87.3% and IHC had a sensitivity of 81.2% and a specificity of 88.1%.

DISCUSSION

With over 70% incidence rate, BVD infection is a significant risk for cattle reproductive health worldwide in all herds. Studies investigating the seroprevalence of BVD disease found seropositivity between 12% and 86% in herds from different countries.[22-26] Seroprevalence studies for BVDV in Türkiye have reported seropositivity between 50% and 94%.[27,28] Different results were seen in the determination studies of BVDV antigen positivity with IHC, IF, or PCR in Türkiye. In the study performed on the testis, BVD virus was not obtained from testicular tissues.[29] In our study, BVDV antigen positivity by PCR, IHC, and IF at 13%, 16%, and 21%, respectively, was seen.

Table 2. BVDV Antigen Positivity Observed in Different Cell Types by IHC and IF Methods

	IHC	IF
Spermatid	16	21
Leydig cells	7	12
Spermatocytes	12	21
Sertoli cells	16	21

BVDV, bovine viral diarrhea virus; IF, immunofluorescence; IHC, immunohistochemistry.

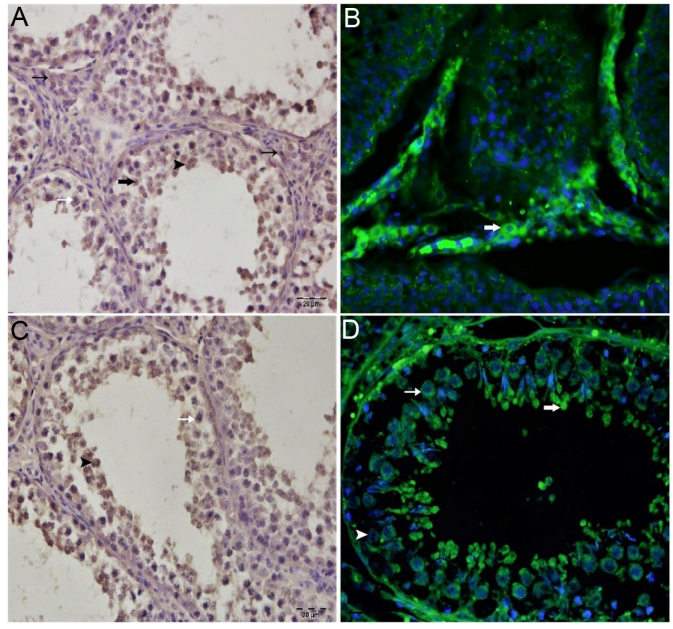

Figure 2. Photomicrograph showing antigenic localization in BVDV-infected testis tissues using immunohistochemistry and immunofluorescence staining (A-D). (A) BVDV antigen positivity in spermatocytes (white arrow), spermatids (arrowhead), Sertoli cells (thick arrow), and Leydig cells (thin arrow) IHC ×20. (B) Intracytoplasmic BVDV antigen positivity in Leydig cells (arrow) IF. (C) BVDV antigen positivity in spermatocytes (white arrow) and spermatids (arrowhead) IHC ×20. (D) BVDV antigen positivity in spermatocytes (thin arrow), spermatids (thick arrow), and Sertoli cells (arrowhead) IF×20. BVDV, bovine viral diarrhea virus

Reproductive results of BVD infection include death in embryonic and fetal stages, defective calves at birth, and PI calves.[30] Again, the BVD virus can be persistent in semen in cases where bulls are significantly seropositive yet non-viremic.[31] It has been observed that PI bulls' entire genital tract, including the testes, has significant amounts of the BVD virus. Acute infection in bulls provided the virus to accessory glands and epididymis but not the testes.[11] However; the presented study found BVDV antigen positivity in bulls' testes. This state shows that the bulls are infected during the formation of blood–testis barriers; so the virus can replicate in the testis and escape from the barrier.[31]

In terms of pathogenesis, persistent infection is formed by transfer of virus from mother to fetus during pregnancy in female animals. Our aim in this study was to investigate the pestiviruses incidentally found in the testis which is one of the places where the virus would persist in male individuals. In this context, pestivirus nucleic acid was searched in testicular tissue of 100 animals cut in a slaughterhouse, and 13 cases (13%) were detected positively. Persistent infection rates were determined between 0.07% and 3.07% in studies conducted.[27,32] The rate, which is quite high compared to these studies, may be due to the fact that our study was carried out in the testis, and RT-PCR was selected as the diagnostic method instead of the Ag-ELISA method used in other studies.[33] The persistence situation could not be fully explained in this study because the animal has to be sampled at least 21 days later again and antigen positivity must be provided for this situation to be revealed.[34] However, since the testicular tissue of the animals that died as the material is selected and single sampling is performed, the status of persistent infection cannot be determined. Antigen positivity in BVDV

infections identifies 2 conditions. Positivity may indicate both acute infection and persistent infection.[12] However, since the findings obtained in the study are the determination of antigen positivity in randomly sampled animals, it is not known whether the animals are in the period of acute infection or persistent infection.

Even though organ-level distribution of virus was present in different research,[35,36] few studies have been performed on prevalence and the localization of virus in the bulls' testis.[37,38] Viral antigens were demonstrated in different tissues in lung, thymus, heart, pancreas, placenta, ovarium, uterine, skin, sinusoids of spleen, and mucosa of the digestive system in many BVD studies.[39-41] In the present study, BVD virus was observed in bulls' testes using IHC and IF methods, and the accuracy of these tests was proven by PCR. Viral antigens were observed to be intracytoplasmic as reported in the literature.[38,42,43] In studies regarding the BVD virus distribution in the testes, Givens et al[44] demonstrated the presence of the antigen in the Sertoli cells but not in Leydig cells and seminiferous tubules adjoining the basal membranes by using 2 bulls that were artificially infected. In studies performed, viral antigen immunopositivity was detected in Sertoli cells and spermatogonia, but not in Leydig cells. In the present study, BVDV immunopositivity was found in Sertoli cells, spermatocytes, spermatogonia, and Leydig cells. Positivity in Leydig cells has been associated with BVDV agent that can settle into Leydig cells, depending on the increased amount of viral antigen. While the results were incompatible with the observations from cell culture, 65 bulls did not provide BVD virus in the following tissues: testes, epididymis, seminal vesicle, and the prostate gland.[29] Positive staining was detected as 21%, 16%, and 13% by indirect IF, IHC, and PCR, respectively. In IHC and IF testing, several factors that may play a role in different outcomes have been reported. One notable drawback of the IF method is its subjectivity in interpretation, even by the experts. It should be noted that the IHC staining enables the detection of virus particles concealed in the regular FA method due to utilizing proteolytic enzymes.[45]

In conclusion, in this study on the investigation of BVDV antigens in bovine abortions in Türkiye where the work was carried out, while 8 (14.28%) samples were detected positive by direct IF method, 6 (10.71%) samples were found to be positive by IHC staining in fetal tissues.[46] Results of the presented study are in accordance with this study and it has determined that bulls are an important epidemiological factor in the transmission of the disease. Bovine viral diarrhea virus-positive bulls are widely used both in natural mating and in artificial insemination; they can cause a significant increase in the percentage of abortions that occur. Bovine viral diarrhea virus antigen positivity was found at 21%, 16%, and 13% by indirect immunofluorescence, immunohistochemistry, and RT-PCR, respectively. Cellular localization of the virus was observed to be testicular cytoplasms. Obtained results indicate that bulls are a transmission source of infection, whether they are persistently infected or in the acute phase, and have an important place in the spread and prevention of the disease.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – M.O.; Design – M.O., M.O.T.; Supervision – Y.S.S.; Resources – H.A.; Data Collection and/or Processing – S.O.; Analysis and/or Interpretation – H.A., S.A.; Literature Search – M.O.; Writing Manuscript – M.O., M.O.T.; Critical Review – S.O.

Declaration of Interests: The authors have no conflicts of interest to declare.

Funding: The authors declared that this study has received financial support from Atatürk University Scientific Research Projects (PRJ2016/81).

Hakem Değerlendirmesi: Dış bağımsız.

Yazar Katkıları: Fikir– M.O.; Tasarım – M.O., M.O.T.; Denetleme – Y.S.S.; Kaynaklar – H.A.; Veri Toplanması ve/veya İşlemesi – S.O.; Analiz ve/veya Yorum – H.A., S.A.; Literatür Taraması – M.O.; Yazıyı Yazan – M.O., M.O.T.; Eleştirel İnceleme – S.O.

Çıkar Çatışması: Yazarlar çıkar çatışması bildirmemişlerdir.

Finansal Destek: Yazarlar bu çalışmanın Atatürk Üniversitesi Bilimsel Araştırma Projeleri (PRJ2016/81) tarafından finansal olarak desteklendiğini beyan etmişlerdir.

REFERENCES

1. Yeşilbağ K, Alpay G, Becher P. Variability and global distribution of subgenotypes of bovine viral diarrhea virus. *Viruses*. 2017;9(6):128. [CrossRef]
2. Timurkan MÖ, Aydın H. Increased genetic diversity of BVDV strains circulating in eastern Anatolia, Turkey: first detection of BVDV-3 in Turkey. *Trop Anim Health Prod*. 2019;51(7):1953-1961. [CrossRef]
3. Brock KV. The persistence of bovine viral diarrhea virus. *Biologicals*. 2003;31(2):133-135. [CrossRef]
4. Kalaycıoğlu AT. Bovine viral diarrhoea virus (BVDV) diversity and vaccination. A review. *Vet Q*. 2007;29(2):60-67. [CrossRef]
5. McDougall S. Effect of calf age on bovine viral diarrhea virus tests. *J Vet Diagn Invest*. 2021;33(3):528-537. [CrossRef]
6. Akagami M, Seki S, Kashima Y, et al. Risk factors associated with the within-farm transmission of bovine viral diarrhea virus and the incidence of persistently infected cattle on dairy farms from Ibaraki Prefecture of Japan. *Res Vet Sci*. 2020;129:187-192. [CrossRef]
7. Walz PH, Chamorro MF, Falkenberg SM, et al. Impact of species and subgenotypes of bovine viral diarrhea virus on control by vaccination. Bovine viral diarrhea virus: an updated American College of Veterinary Internal Medicine consensus statement with focus on virus biology, hosts, immunosuppression, and vaccination. *J Vet Intern Med*. 2020;34(5):1690-1706.
8. Nickell JS, White BJ, Larson RL, Renter DG, Sanderson MW. A simulation model to quantify the value of implementing whole-herd Bovine viral diarrhea virus testing strategies in beef cow–calf herds. *J Vet Diagn Invest*. 2011;23(2):194-205. [CrossRef]
9. Sarrazin S, Dewulf J, Mathijs E, Laureyns J, Mostin L, Cay AB. Virulence comparison and quantification of horizontal bovine viral diarrhoea virus transmission following experimental infection in calves. *Vet J*. 2014;202(2):244-249. [CrossRef]
10. Reardon F, Graham DA, Clegg TA, Tratalos JA, O'Sullivan P, More SJ. Quantifying the role of trojan dams in the between-herd spread of bovine viral diarrhoea virus (BVDV) in Ireland. *Prev Vet Med*. 2018;152(1):65-73. [CrossRef]
11. Kirkland PD, Richards SG, Rothwell JT, Stanley DF. Replication of bovine viral diarrhoea virus in the bovine reproductive tract and excretion of virus in semen during acute and chronic infections. *Vet Rec*. 1991;128(25):587-590. [CrossRef]
12. Goto Y, Yaegashi G, Fukunari K, Suzuki T. An importance of long-term clinical analysis to accurately diagnose calves persistently and acutely infected by bovine viral diarrhea virus 2. *Viruses*. 2021;13(12):2431. [CrossRef]
13. Prosser NS, Green MJ, Ferguson E, et al. Cattle farmer psychosocial profiles and their association with control strategies for bovine viral diarrhea. *J Dairy Sci*. 2022;105(4):3559-3573. [CrossRef]
14. Alkan F, Özkul A, Bilge-Dağalp S, et al. Virological and serological studies on the role of PI-3 virus, BRSV, BVDV and BHV-1 on

respiratory infections of cattle. I. The detection of etiological agents by direct immunofluorescence technique. *Dtsch Tierarztl Wochenschr*. 2000;107(5):193-195.
15. Tan MT, Karaoğlu MT, Erol N, et al. Serological and virological investigations of bovine viral diarrhoea virus (BVDV) infection in dairy cattle herds in Aydın province. *Turk J Vet Anim Sci*. 2006;30(3):299-304.
16. Burgu I, Alkan F, Yeşilbağ K. Prevalence of persistent BVD virus infection in cattle in Turkey. *Ank Univ Vet Fak Derg*. 1991;46:169-177.
17. Çomakli S, Sağlam YS, Timurkan MÖ. Comparative detection of bovine herpesvirus-1 using antigen ELISA, immunohistochemistry and immunofluorescence methods in cattle with pneumonia.*Turk J Vet Anim Sci*. 2019;43(3):306-313. [CrossRef]
18. Njaa BL, Clark EG, Janzen E, Ellis JA, Haines DM. Diagnosis of persistent bovine viral diarrhea virus infection by immunohistochemical staining of formalin-fixed skin biopsy specimens. *J Vet Diagn Invest*. 2000;12(5):393-399. [CrossRef]
19. Dubovi EJ. Laboratory diagnosis of bovine viral diarrhea virus. *Biologicals*. 2013;41(1):8-13. [CrossRef]
20. Özkaraca M, Timurkan MÖ. Immunohistochemistry and PCR methods for the diagnosis of BVDV in cattle with pneumonia in Erzurum Region. *Van Vet J*. 2016;27(2):85-89.
21. Vilček S, Paton DJ, Durkovic B, et al. Bovine viral diarrhoea virus genotype 1 can be separated into at least eleven genetic groups. *Arch Virol*. 2001;146(1):99-115. [CrossRef]
22. Solis-Calderon JJ, Segura-Correa VM, Segura-Correa JC. Bovine viral diarrhoea virus in beef cattle herds of Yucatan, Mexico: seroprevalence and risk factors. *Prev Vet Med*. 2005;72(3-4):253-262. [CrossRef]
23. Msolla P, Sinclair JA, Nettleton P. Prevalence of antibodies to bovine virus diarrhoea-mucosal disease virus in Tanzanian cattle. *Trop Anim Health Prod*. 1988;20(2):114-116. [CrossRef]
24. Polak MP, Zmudzinski JF. Prevalence of bovine viral diarrhea virus infection in bulls in artificial insemination centers in Poland. *Vet Microbiol*. 1999;64(2-3):253-257. [CrossRef]
25. Selvaraj J, Manohar BM, Balachandran C, et al. Seroprevalence of bovine viral diarrhoea in buffaloes at Chennai. *Indian Vet Pathol*. 2007;31(2):180-180.
26. Sausker EA, Dyer NW. Seroprevalence of OHV-2, BVDV, BHV-1, and BRSV in ranch-raised bison (Bison bison). *J Vet Diagn Invest*. 2002;14(1):68-70. [CrossRef]
27. Ak S, Firat I, Bozkurt HH. The prevalence of bovine viral diarrhoea virus (BVDV) infections in cattle and existence of persistently infected cattle in the Trakya region. *Turk J Vet Anim Sci*. 2002;26(2):245-248.
28. Okur-Gumuşova S, Yazıcı Z, Albayrak H, et al. Seroprevalence of bovine viral respiratory diseases. *Acta Vet Beograd*. 2007;57(1):11-16.
29. Fırat I, S, Bozkurt HH, et al. Distribution of bovine viral diarrhoea virus (BVDV) in the genital system tissues of cattle. *Vet Arh*. 2002;72(5):235-248.
30. Evans CA, Pinior B, Larska M, et al. Global knowledge gaps in the prevention and control of bovine viral diarrhoea (BVD) virus. *Transbound Emerg Dis*. 2019;66(2):640-652. [CrossRef]
31. Read AJ, Gestier S, Parrish K, et al. Prolonged detection of bovine viral diarrhoea virus infection in the semen of bulls. *Viruses*. 2020;12(6):674. [CrossRef]
32. Burgu İ, Alkan F, Özkul A, et al. Türkiye'de Süt Sığırcılığı Işletmelerinde Bovine Viral Diarrhea Virus (BVDV) Enfeksiyonunun Epidemiyolojisi Ve Kontrolü. *Ankara Üniv Vet Fak Derg*. 2003;50(2):127-133.
33. Hilbe M, Stalder H, Peterhans E, et al. Comparison of five diagnostic methods for detecting bovine viral diarrhea virus infection in calves. *J Vet Diagn Invest*. 2007;19(1):28-34. [CrossRef]
34. Yeşilbağ K, Alpay G, Tuncer P. Bir süt sığırcılığı işletmesinde bovine viral diarrhoea (BVD) virus enfeksiyonunun kontrol ve eliminasyonu control and elimination of bovine viral diarrhoea virus in a dairy herd". *Uludag Univ Vet Fak*. 2012;31(1):11-17.
35. Oguejiofor CF, Thomas C, Cheng Z, Wathes DC. Mechanisms linking bovine viral diarrhea virus (BVDV) infection with infertility in cattle. *Anim Health Res Rev*. 2019;20(1):72-85. [CrossRef]
36. Yitagesu E, Jackson W, Kebede N, Smith W, Fentie T. Prevalence of bovine abortion, calf mortality, and bovine viral diarrhea virus (BVDV) persistently infected calves among pastoral, peri-urban, and mixed-crop livestock farms in central and Northwest Ethiopia. *BMC Vet Res*. 2021;17(1):87. [CrossRef]
37. Givens MD, Riddell KP, Walz PH, et al. Noncytopathic bovine viral diarrhea virus can persist in testicular tissue after vaccination of peri-pubertal bulls but prevents subsequent infection. *Vaccine*. 2007;25(5):867-876. [CrossRef]
38. Borel N, Janett F, Teankum K, Zlinszky K, Iten C, Hilbe M. Testicular hypoplasia in a bull persistently infected with bovine diarrhoea virus. *J Comp Pathol*. 2007;137(2-3):169-173. [CrossRef]
39. Taniyama H, Hirayama K, Kagawa Y, et al. Immunohistochemical demonstration of bovine viral diarrhoea virus antigen in the pancreatic islet cells of cattle with insulin-dependent diabetes mellitus. *J Comp Pathol*. 1999;121(2):149-157. [CrossRef]
40. Fredriksen B, Press CM, Løken T, Odegaard SA. Distribution of viral antigen in uterus, placenta and foetus of cattle persistently infected with bovine virus diarrhoea virus. *Vet Microbiol*. 1999;64(2-3):109-122. [CrossRef]
41. Lamm CG, Broaddus CC, Holyoak GR. Distribution of bovine viral diarrhea virus antigen in aborted fetal and neonatal goats by immunohistochemistry. *Vet Pathol*. 2009;46(1):54-58. [CrossRef]
42. Falkenberg SM, Dassanayake RP, Terhaar B, Ridpath JF, Neill JD, Roth JA. Evaluation of antigenic comparisons among BVDV isolates as it relates to humoral and cell mediated responses. *Front Vet Sci*. 2021;8(8):685114. [CrossRef]
43. Baszler TV, Evermann JF, Kaylor PS, Byington TC, Dilbeck PM. Diagnosis of naturally occurring bovine viral diarrhea virus infections in ruminants using monoclonal antibody-based immunohistochemistry. *Vet Pathol*. 1995;32(6):609-618. [CrossRef]
44. Givens MD, Heath AM, Brock KV, Brodersen BW, Carson RL, Stringfellow DA. Detection of bovine viral diarrhea virus in semen obtained after inoculation of seronegative postpubertal bulls. *Am J Vet Res*. 2003;64(4):428-434. [CrossRef]
45. Ellis JA, Martin K, Robert NG, Haines DM. Comparison of detection methods for bovine viral diarrhea virus in bovine abortions and neonatal death. *J Vet Diagn Invest*. 1995;7(4):433-436. [CrossRef]
46. Oruç E, Saglam YS, Sözdutmaz I, et al. The investigation of bovine viral diarrhoea virus antigens with immunofluorescence and immunohistochemical methods in bovine abortions. *Pak Vet J*. 2015;35(4):426-429.