https://doi.org/10.52973/rcfcv-e35547 Revista Científica, FCV-LUZ / Vol. XXXV

Isolation and Molecular Characterization of Bovine Viral Diarrhea Virus in Calves with Congenital Malformation in Türkiye

Aislamiento y caracterización molecular del virus de la diarrea viral bovina en terneros con malformaciones congénitas en Turquía

Sebahattin Akça[1,2], Mehmet Özkan Timurkan[3]*, Ömer Faruk Küçükkalem[1], Meryem Güloğlu[1,4], Hakan Aydın[3], İbrahim Sözdutmaz[5]

[1]*Veterinary Control Institute, Republic of Türkiye Ministry of Agriculture and Forestry. Erzurum, Türkiye.*
[2]*Atatürk University, Health Science Institute, Department of Virology, Erzurum, Türkiye*
[3]*Atatürk University, Faculty of Veterinary Medicine, Department of Virology. Erzurum, Türkiye.*
[4]*Atatürk University, Health Science Institute, Department of Pathology, Erzurum, Türkiye*
[5]*Erciyes University, Faculty of Veterinary Medicine, Department of Virology. Kayseri, Türkiye.*
Corresponding author: motimurkan@atauni.edu.tr

ABSTRACT

This study aims to identify the causative agent in cases of abortion on a cattle farm in Erzurum province, Türkiye. Samples from the farm were sent to the Erzurum Veterinary Control Institute, Ministry of Agriculture and Forestry. The analysis revealed the presence of Bovine Viral Diarrhea Virus (BVDV) in a newborn calf exhibiting congenital malformations. Subsequently, blood and serum samples were collected for four weeks post-abortion to assess the acute/persistent infection status on the farm. Using the ELISA method, antigen, and antibody positivity were detected in both the cattle and the aborted calf. Six blind passages were conducted in the MDBK cell line to isolate the virus from cerebrospinal fluid. Confirmation of isolation was carried out through regular CT increase in Real-Time RT-PCR due to the non-cytopathogenic nature of the detected virus. The isolate (EVE-BVDV-2023) was identified as belonging to the BVDV-1d genotype through partial genome analysis of the 5'UTR gene. This study conclusively confirms the presence of BVDV infection in cattle herds in the eastern region of Türkiye, particularly in Erzurum province. Future studies should continue efforts to control and eradicate infectious agents in cattle herds, with a particular emphasis on addressing BVDV infection.

Key words: Abort; BVDV; isolation; molecular characterization; Türkiye

RESUMEN

Este estudio tuvo como objetivo identificar el agente causal en casos de aborto en una granja de ganado en la provincia de Erzurum, Turquía. Las muestras de la granja se enviaron al Instituto de Control Veterinario de Erzurum, Ministerio de Agricultura y Silvicultura. El análisis reveló la presencia del virus de la diarrea viral bovina (BVDV) en un ternero recién nacido que presentaba malformaciones congénitas. Posteriormente, se recogieron muestras de sangre y suero cuatro semanas después del aborto para evaluar el estado de infección aguda/persistente en la granja. Utilizando el método ELISA, se detectó positividad de antígeno y anticuerpo tanto en el ganado como en el ternero abortado. Se realizaron seis pases ciegos en la línea celular MDBK para aislar el virus del líquido cefalorraquídeo. La confirmación del aislamiento se llevó a cabo mediante el aumento regular de CT en RT-PCR en tiempo real debido a la naturaleza no citopatógena del virus detectado. El aislado (EVE-BVDV-2023) se identificó como perteneciente al genotipo BVDV-1d a través del análisis parcial del genoma del gen 5'UTR. Este estudio confirma de manera concluyente la presencia de infección por BVDV en rebaños de ganado vacuno de la región oriental de Turquía, en particular en la provincia de Erzurum. Los estudios futuros deben continuar los esfuerzos para controlar y erradicar los agentes infecciosos en los rebaños de ganado vacuno, con especial énfasis en abordar la infección por BVDV.

Palabras clave: Abortar; BVDV; aislamiento; caracterización molecular; Turquía

Recibido: 27/10/2024 Aceptado: 25/12/2024 Publicado: 11/03/2025

INTRODUCTION

Bovine Viral Diarrhea Virus (BVDV) infection is typically asymptomatic in immunocompetent cattle (*Bos taurus*). However, infections caused by the cytopathogenic type of the virus, leading to mucosal disease (MD), can escalate to fatality, causing respiratory, digestive, and genital issues. This causative agent predominantly affects cattle, sheep, and pigs, significantly disrupting animal production and resulting in substantial economic losses [1 , 2]. BVDV, classical swine fever, and border disease belong to the Pestivirus genus within the Flaviviridae family [2 , 3 , 4 , 5]. The virus possesses a positive-strand RNA, approximately 12.3 kb in length [5]. A conserved non-coding 5' untranslated region (UTR) region at the genome's beginning and end serves as a highly conserved region used in DNA typing. The genome comprises a single open reading frame (ORF) encoding structural and non-structural proteins [6 , 7].

Transmission of BVDV infection occurs both directly and indirectly. Indirect transmission involves secretions (nasal discharge, tears, saliva, genital discharge, etc.), semen, milk, and feces. The virus can localize in the genital tract, causing reproductive issues like abortion and infertility [6]. Placental transmission to the fetus can result in abortion, malformations, or persistent infection in immune-tolerant young animals [6]. Infected animals may develop fatal mucosal disease (MD), either due to mutations of the non-cytopathogenic biotype (NCP) or ingestion of cytopathogenic BVDV from external sources. In addition, the virus's suppression of the immune system renders infected animals susceptible to secondary infections.

According to the International Committee on Taxonomy of Viruses, pestiviruses are categorized as follows: Bovine Viral Diarrhea Virus (BVDV), BVDV-1 (Pestivirus A), BVDV-2 (Pestivirus B), Classical Swine Fever (Pestivirus C), Border Disease (Pestivirus D) [8 , 9 , 10 , 11], Pronghorn Antelope Pestivirus (Pestivirus E), Porcine Pestivirus (Pestivirus F), Giraffe Pestivirus (Pestivirus G), HoBi-like Pestivirus (Pestivirus H), Aydin-like Pestivirus (Pestivirus I), Rat Pestivirus (Pestivirus J), and Atypical Porcine Pestivirus (Pestivirus K) [8 , 9 , 10]. BVDV is also divided into subtypes. The subtypes within BVDV-1 are classified into 21 (1a-1u), BVDV-2 into 4 (2a-2d), and BVDV-3 into 4 (3a-3d) subtypes [5 , 6 , 7]. BVDV-1 and BVDV-2 genotypes exhibit two biotypes, non-cytopathogenic (NCP) and cytopathogenic (CP), based on their effects in cell culture [3 , 12 , 13 , 14 , 15]. The identification of these subtypes relies on analyzing partial sequences of the 5'UTR, Npro, and E2 regions [3 , 6]. Three types of BVD have been documented in Türkiye (BVDV-1, BVDV-2, BVDV-3). However, the presence of BVDV-1 has been extensively reported [16].

In addition to acute and chronic clinical conditions, diverse clinical manifestations of BVDV have been observed, including continuous virus shedding by persistently infected (PI) calves, congenital anomalies, and abortions [17 , 18]. The severity of clinical signs and the localization of infection vary. In pregnant cattle, due to the insufficient connection between the maternal epithelium and fetal trophoblasts during the first 30-35 days (d) of the BVD virus, vertical transmission occurs, and intrauterine infection ensues with the development of cotyledons, resulting in early embryonic deaths [13 , 19]. Fetal deaths occur because the virus damages the maternal placenta directly, leading to a failure to transport substances vital to the fetus. Furthermore, due to the insufficient development of the fetus's immune system during this period, it displays immune intolerance to the virus [13]. Consequently, beyond fetal death, mummification, premature birth, and abortion resulting from transplacental infection in the first trimester of pregnancy, persistent infection emerges in the fetus due to non-cytopathogenic (NCP) biotype infection after 30 days of gestation [3 , 13]. Persistently infected calves continuously shed the virus throughout their lives and remain constantly viremic [13].

Various diagnostic methods, including Peroxidase-Linked Antibody (PLA), Direct and Indirect Enzyme-Linked Immunosorbent Assay (ELISA), Immunofluorescence (IF), Virus Neutralization Test (VNT), Neutralizing Peroxidase-Linked Antibody (NPLA), Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) [20], and Real-Time RT-PCR, are commonly employed to diagnose BVDV in clinical specimens [21].

This study aimed to identify the etiology of the causative agent on a farm with a history of abortion cases with congenital anomalies. It also sought to isolate the identified pathogen and confirm the isolation through real-time RT-PCR. The isolated virus was sequenced at the 5'UTR gene region to determine its relationship with strains detected in Türkiye and globally for establishing control and management strategies.

MATERIALS AND METHODS

Ethical statement

This research was conducted in accordance with ethical principles with due respect for animal rights. Approval was obtained from an ethics commission (Animal Experimental Local Ethics Committee on 21 September 2023 (Number 13067196/124).

Sample collection

Upon reviewing the anamnesis, it was determined that there were no previous instances of abortion. The herd's feeding strategy involved grass, herbage, and cultivated feed in pastures during summer and in barns during winter. According to reports, the herd received vaccination exclusively against foot-and-mouth disease. Additionally, it was noted that there are 66 farms with a total of 617 cattle in the area, all of which are in good overall condition.

This study included cattle that had experienced abortion, along with calves displaying anomalies. The main subject of the investigation was a calf sample (protocol number 33, animal identification number: TR254623145) sent from Aşkale district, Erzurum province, in January 2023; the sample was received by the Erzurum Veterinary Control Institute Directorate under protocol number EVE/2023/36 (FIG. 1). Whole blood and serum were also collected from the cattle.

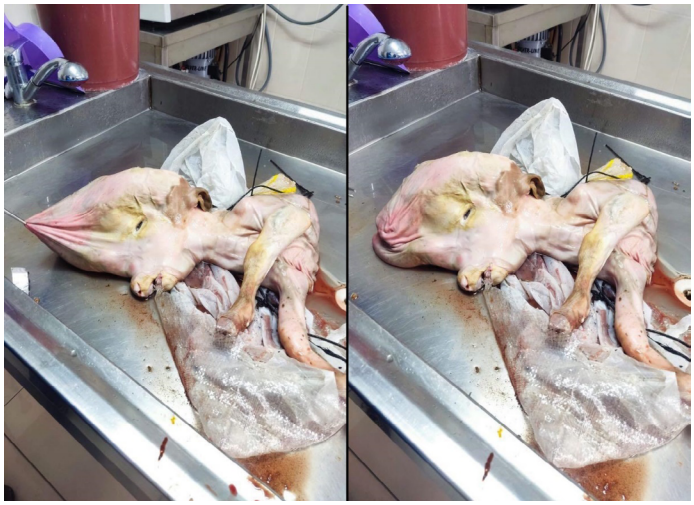

FIGURE 1. Abortion bovine with congenital malformations caused by BVDV-1d

The virology laboratory of the institute conducted tests on the aborted material for various viral agents, including Akabane virus, Schmallenberg virus, Bovine Ephemeral Fever virus, Bovine Herpes virus-1 (BoHV-1), and BVDV. The etiologic agent responsible for the abortion was identified as BVDV using Real-Time RT-PCR. To determine whether the abortion case caused by BVDV was an isolated incident or a herd-wide problem, a visit to the cattle farm in question was conducted; and, an investigation was performed, based on anamnesis information, for checking for other animals experiencing abortion, verifying vaccination practices beyond the foot-and-mouth disease vaccine, confirming the supplementation of vitamins and minerals, and assessing the nutritional status of the animals. Farms in the surrounding region were also evaluated.

Macroscopic analysis

Necropsy and macroscopic examination were performed on the abnormally aborted calf. Following the necropsy of the aborted sample, cerebrospinal fluid was collected for further analysis.

A necropsy was conducted on the calf with anomalies that arrived at our institute for a thorough postmortem assessment. Macroscopically, an external hydrocephalus, resembling a sac filled with cerebrospinal fluid, was observed in the cranial region. The calvarium was cut easily, and the brain was rudimentary. Examination of the facial region revealed a short lower jaw (micrognathia inferior). In addition, incomplete development of teeth in the oral cavity was noted. While both anterior limbs were present, both posterior limbs were absent (amelia posterior). Furthermore, the anterior right leg was observed to be shorter than the left one. No internal organ formation was detected in the peritoneal cavity of the calf (FIG. 1). The cattle tested positive in both indirect and direct ELISA.

Virological detection (Antigen ELISA and Real-Time RT-PCR)

Antigen ELISA

The blood sample underwent centrifugation at 1500 G for 10 minutes (min) using a Thermo centrifuge (SL16R, Thermo Scientific, Germany) [22]. Subsequently, the buffy coat was subjected to direct antigen (Ag) ELISA using the BVDV Antigen Test Kit/Serum Plus (IDEEX, Germany).

In addition, blood collected in an Ethylenediaminetetraacetic acid (EDTA)-free tube was centrifuged at 1500 G for 10 min, and the serum sample was then analyzed through indirect antibody (Ab) ELISA using the BVDV/MD/BDV p80 Protein Antibody Test Kit (IDEXX, Germany).

RNA Extraction

Cerebrospinal fluid samples, stored in antibiotic PBS (PBS, pH 7.4, Gibco, USA) (at +4°C, were centrifuged at 2000 G for 15 min. For nucleic acid isolation, 200 µl of the supernatant prepared from cerebrospinal fluid and blood samples were collected, and RNA extraction was performed using a robotic isolation device (Indimag 48S, Indical, Germany) with the Indical nucleic acid isolation kit (Cat No: F202300039), following the kit procedure. The obtained RNAs were stored at -80°C (Ultra freezer, Lexicon, Esco, Singapore). The quality and quantity of the isolated RNAs were determined using a spectrophotometer (Nanodrop, Thermo Scientific, Germany).

Real-time RT-PCR and RT-PCR

For real-time RT-PCR analysis (Rotor-Gene Q 5 plex, Qiagen, USA), we employed primers and probes reported by Baxi *et al.* [21]. For sequencing, BVDV-positive samples were analyzed by conventional RT-PCR with the primers and methods reported by Vilcek *et al*. [23] in (TABLE I).

TABLE I.
Nucleotide Information of Primers and Probes Used for Real-time and Conventional RT-PCR

Target PCR	Primer-Prob	Sequence (5'–3')	Referens No
Real Time RT-PCR	Forward	CTA GCC ATG CCC TTA GTA G	[21]
	Rewerse	CGT CGA ACC AGT GAC GAC	
	Probe (FAM/BHQ3)	TAG CAA CAG TGG TGA GTT CGT TGG	
	Probe (TXR/BHQ3)	TAG CGG TAG CAG TGA GTT CGT TGG ATG GCC	
RT-PCR	324	ATG CCC W (AT) TA GTA GGA CTA GCA	[23]
	326	TCA ACT CCA TGT GCC ATG TAC	

Phylogenetic analyses

Samples from calves and cattle that tested positive for BVDV via real-time RT-PCR underwent reanalysis through conventional RT-PCR using 5'UTR gene-specific primers. Sequencing was performed on positive amplicons (TABLE I) [23]. Based on the obtained sequence data, we conducted phylogenetic analysis, incorporating strains from GenBank and those documented from Türkiye in GenBank by Tamura *et al*. [24].

Virus isolation

Virus isolation was carried out using the Madin-Darby Bovine Kidney (MDBK) cell line obtained from Erciyes University, Faculty of Veterinary Medicine, Department of Virology, Kayseri Türkiye. In brief, supernatants of tissue homogenates (Tissue Lyser LT, Qiagen, USA) were inoculated into a 25 cm^2 flask using the adsorption method (1 h). Control wells were inoculated with PBS as a negative control. Following the adsorption period, the flask was replenished with sufficient medium and incubated (Celculture CO_2 Incubatore, Esco, Singapore) at 37°C with 5% CO_2 for 6 days. Virus titers for each blind passage's supernatant were tested by real-time RT-PCR (FIG. 2).

The samples inoculated into the cell culture from the brain suspension were monitored for 6 d. This process was repeated through 6 serial blind passages. No cytopathic effect (CPE) formation was observed at the end of the passages, indicating a non-cytopathogenic biotype. However, due to the non-cytopathogenic nature of the virus, Real-Time RT-PCR was performed to assess the reproductive status and isolation of the virus. The Cycle Threshold (CT) values for each passage of the virus in cell culture were determined as follows: 25.94 (1st passage), 26.54 (2nd passage), 27.58 (3rd passage), 25.40 (4th passage), 26.34 (5th passage), and 26.76 (6th passage) (FIG. 2). Therefore, while the virus amount showed a relative decrease in the first 3 passages (indicating a new adaptation of the virus to the cell culture), the increase in the CT value from the 4th passage suggested that the virus tended to multiply.

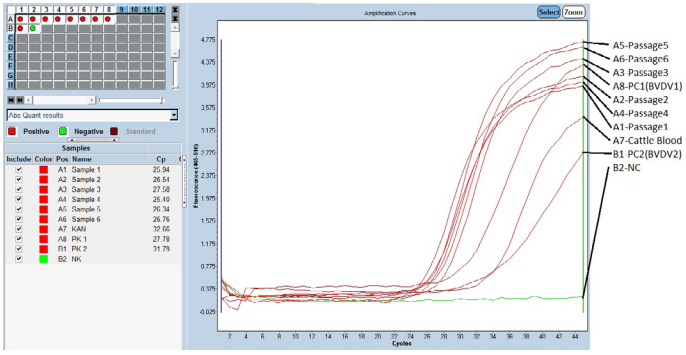

FIGURE 2. Real-Time RT-PCR result (A1: 1st passage, A2: 2nd passage, A3: 3th passage, A4: 4th passage, A5: 5th passage, A6: 6th passage, A7: Maternal blood sample, A8: BVDV-1 PC, B1: BVDV-2 PC, B2: NC)

RESULTS AND DISCUSSIONS

After PCR and electrophoresis, the obtained amplicons were sequenced. The raw data from the sequence analysis were compared with the nucleic acid sequence of reference strains obtained from the gene bank using the bioinformatics programs BioEdit version 7.0.5 and Clustral W software. The isolate in the study was identified as BVDV-1d (FIG. 3).

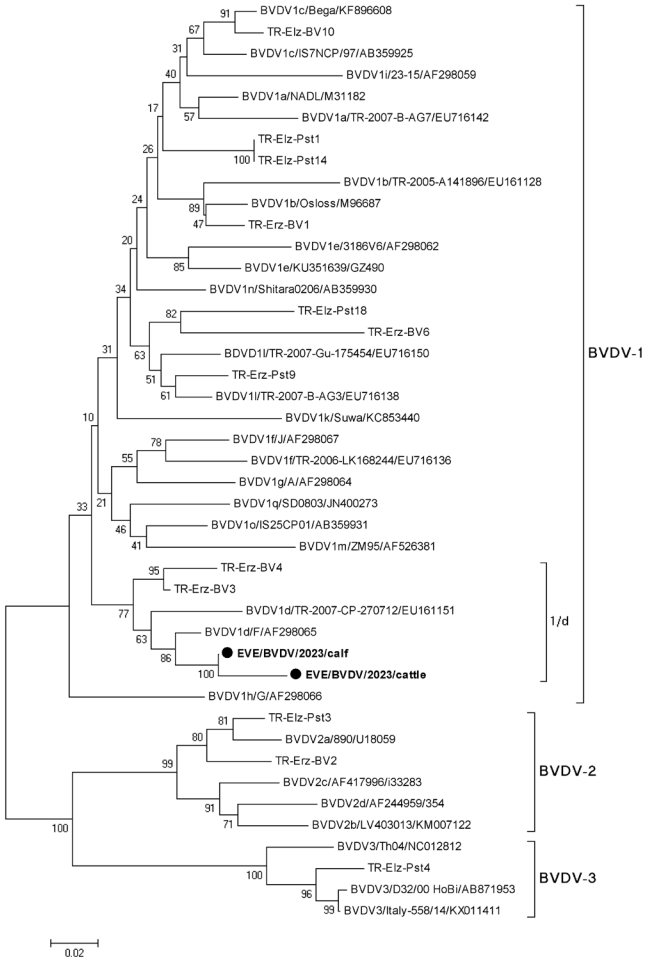

FIGURE 3. The MEGA v.11 was used to analyze BVDV 5'UTR gene sequences via the neighbor-joining method with 1,000 bootstrap datasets based on the ClustalW algorithm. A circular shape (●) indicates the phylogenetic location of our study strain.

BVD infection is a global concern in cattle, causing various reproductive and health issues, including abortion, stillbirth, fetal resorption, congenital malformations, fetal infections, growth retardation, decreased pregnancy rates, and the birth of underdeveloped calves. Moreover, it leads to respiratory and digestive tract infections, making cattle more susceptible to secondary agents due to immunosuppression worldwide [25 , 26]. A high incidence of the disease in cattle populations has been observed in various countries around the globe through serologic and virologic studies [27]. In a study by Deng *et al.* [28] in China, the surge in demand for beef and dairy products triggered significant investments in the cattle industry, fostering rapid sector growth, resulting in increased cattle transport and the swift spread of BVDV across the country due to a lack of effective protection (mandatory vaccination) and eradication efforts. Meanwhile, various European countries have initiated voluntary or mandatory BVD control programs, with some achieving disease-free status [29]. Hence, it is crucial for Türkiye to establish BVD infection-free herds and implement preventive control measures.

Understanding Bovine Viral Diarrhea (BVD) is greatly aided by molecular typing studies. BVDV is classified into three genotypes

(BVDV-1, BVDV-2, BVDV-3), and extensive global research has focused on these genotypes. BVDV-1 is the most frequently studied genotype, with prevalent subgenotypes 1b, 1a, and 1c. European countries exhibit remarkable diversity in BVDV sub-genotypes. This suggests that there exists genetic diversity throughout the continent [30], emphasizing the importance of molecular studies for effective prevention and control.

In Europe, the BVDV-1d sub-genotype was identified in 281 out of 3,101 samples [30]. A country-specific study [31] revealed the dominance of subtypes 1b and 1d near the Danish border in Germany, 1d and 1f in Slovenia, 1b in Spain, 1a in Northern Ireland, and 1f, 1h, 1b, and 1d in Austria. The 1d genotype identified in this study aligns with strains recognized as subtype 1d in Europe. This underscores the need to consider imports of animals, animal products, and biological products from these countries.

In a study by Kuta *et al.* [32] in Poland to determine BVDV types, all 65 BVDV samples were confirmed positive by RT-PCR. Of these samples, 31 were subtype 1b, 24 were 1d, 8 were 1f, and 2 were 1g. The herds with 1b and 1f types were vaccinated with BVDV-1a for 2 years, but by the end of vaccination, 1f type was found in calves. In their study in Poland, Mirosław and Polak [33] identified subtypes 1b, 1g, 1f, 1d, 1r, and 1e. Another study by Kucer *et al.* [34] in Croatia marked the first detection of BVDV 1d in that country. Our identification of type 1d in this study suggests that vaccines containing type 1a in Türkiye may be insufficient for protection and control, and vaccines used in Türkiye should also include type 1d.

Scharnböck *et al.* [35] analyzed 325 studies in 73 countries from 1961 to 2016 using the meta-analysis method and found that some animals infected with BVDV were Persistently Infected (PI), some were viremic, and some were antibody-positive. The prevalence of PI animals was higher in countries with fewer control/eradication studies. This study underscores the significance of control and eradication practices in Türkiye, emphasizing the importance of symptomatic and prophylactic treatment for animals at risk, since minimizing the prevalence of BVDV is crucial for herd health and welfare.

In a review study of BVDV prevalence by Yeşilbağ *et al.* [30], 1d subtype was detected in 20 out of 91 samples on the African continent, with 1d positivity ranking second only to 1a and tying with 1c subtype. Fulton *et al*. [36] reported that the 1d subtype is predominant in southern Africa and causes respiratory tract infections. Vuuren [37] suggested that BVDV-1 subtypes in Africa are similar to the classical BVDV-1 of American origin. This implies that BVDV-1 may have spread through animals and animal products (semen, biological products, etc.) transported from the USA to the African continent.

In the Americas, subtype 1d was detected in 24 out of 1055 samples, ranking as the third most common subtype after 1b and 1a [30]. A study on a Angus herd in Brazil by Bianchi *et al*. [25] revealed that the herd grazed on natural pastures, established breeding conditions through natural selection and that the animals were housed in barns during winter; although the animals were vaccinated against foot-and-mouth disease (FMDV), rabies, and clostridia, they were not vaccinated against BVDV. The study reported an outbreak of mucosal disease caused by BVDV-1d in the herd, resulting in high morbidity and lesions limited to the upper digestive tract and skin (there were no lesions in the intestines); the study also reported that the outbreak led to early embryonic loss, abortions, and stillbirths. In the current study, although no clinical findings were observed in the cattle, in the aborted fetus experienced an anomaly.

Nogueira *et al.* [38] conducted a study to uncover new aspects of pestivirus epidemiology in human in Brazil, and performed protein analysis on samples from the brains of infants who died of Zika virus disease and detected peptides from the polyprotein of BVDV. The same study found BVDV RNA nucleic acid in amniotic fluid collected from mothers of infants with Zika and microcephaly during the Paraiba outbreak. This suggests that the Zika virus may not be the alone etiologic agent, emphasizing the importance of considering the risks of BVDV, especially for veterinarians, veterinary technicians, and individuals involved in animal husbandry. Therefore, it should be kept in mind, as with any disease, that mixed infections can always be detected when one agent is found in etiologic investigations.

In Australia, subtype 1d was not identified in any of the 439 samples [30]. Nevertheless, the potential for intercontinental transmission should not be ruled out.

In Asia, subtype 1d was detected in 13 out of 1411 samples [30]. Numerous studies in Türkiye have explored the presence of BVDV, revealing a widespread distribution of antibodies against pestiviruses throughout the country. Alpay *et al.* [39] asserted that BVDV entered Türkiye through the trade of animals and animal products from other countries, this claim was also supported by another study [16] with the identification of BVDV-1c (Australia-specific strain) in a herd imported from Australia.

Genetic and molecular characterization studies in Türkiye have identified various BVDV subtypes, including BVDV-1a, 1b, 1d, 1f, 1h, 1i, 1l, as well as BVDV-2a, 2b, and BVDV-3. A study conducted between 2005 and 2007 by Oğuzoğlu *et al* . [40] found that the prevalent BVDV types were BVDV-1l (18/40), 1f (10/40), 1b (7/40), 1d (3/40), and 1a (2/40). Subtype 1l predominated in Ankara, BVDV-1f (8/10) in cattle samples from the Marmara region, BVDV-1b (6/7) in the Central Anatolia region, BVDV-1d (3/3) in Şanlıurfa, and BVDV-1a (2/2) in the Marmara region. Timurkan and Aydın [10] identified 7 different subtypes (BVDV-1l, -1a, -1d, -1b, -1c, -2a, -3) in abortion cases from Erzurum and Elazığ provinces. The commonality of the 1d subgenotype in our province, as observed by Timurkan and Aydın [10], suggests that this occurrence may be attributed to animal movements.

Toker *et al.* [41] identified the presence of respiratory pathogens, including BPIV-3, BRSV, BVDV (1l and 1r), and BoHV-1, in northwestern Turkish provinces (Bursa, Balıkesir, Kütahya, Bilecik, and Kocaeli) through phylogenetic analyses conducted between 2012 and 2017. Their findings indicated similarities to other subtypes in Türkiye, excluding the detection of the BVDV-1d subtype. In a separate study, Cağırgan *et al.* [16] identified 9 BVDV-positive cases from 117 aborted fetal materials in western Türkiye between 2017 and 2020. Among them, 3 different BVDV-1 types were detected, with 4 classified as the dominant type 1d, 3 as 1l, and 2 as 1f. Considering the prevalent circulation of BVDV-1d and 1f types in European countries (Germany, Italy, Austria, and Poland), the study suggested allowing the import of animals and animal products from these countries after necessary analyses.

Yeşilbağ *et al.* [2] conducted a study across 15 provinces of Türkiye between 1997 and 2005, focusing on cattle and sheep, and found that out of the 30 virus isolates, 29 were from cattle

[BVDV-1l (n=15), BVDV-1a (n=4), BVDV-1b (n=4), BVDV-1d (n=3), BVDV-1f (n=2), and BVDV-1h (n=1)], and 1 was BVDV-2 in sheep. While acknowledging that this study may not fully represent Türkiye, it is noteworthy that BVDV-1d was found in samples from other provinces.

Sarıkaya *et al.* [7] identified 53 BVDV-positive cases (BVDV-1a, BVDV-1b, and BVDV-2) out of 267 samples in their study conducted in the Central Anatolia region between 2009 and 2010. Although the BVDV-1d type was not detected, its absence in these regions doesn't conclusively rule out its presence, more intensive surveillance studies could reveal the potential existence of type 1d. Although type 1d has been sporadically identified in various surveillance studies in Türkiye, it has not been linked to reports of abnormalities; only abortion events have been mentioned. This study sheds light on an important aspect of BVDV pathogenesis. Previous research has emphasized the significance of imported animal movements, emphasizing the need for more comprehensive investigations in this region.

In this study, the non-cytopathogenic nature of BVDV prompted the use of the Real-Time RT-PCR method to assess virus reproduction accuracy through six passages in cell culture. While immunoperoxidase or IF methods generally yield more precise results, it was suggested that Real-Time RT-PCR could serve as an alternative method for reproductive control, a possibility that future studies may further support.

Using the ELISA method, it was determined that the sample taken from the cattle, who entered the facility after 4 weeks, tested positive for both antigen and antibodies. Pestivirus biotypes, known to be both cytopathogenic (CP) and non-cytopathogenic (NCP), undergo biotype determination in cell culture. CP and NCP biotypes of pestiviruses cause high morbidity, a short clinical course, lesions limited to the digestive system and skin, and abortions in both cattle and sheep. The *Flaviviridae* family's structural and non-structural proteins are crucial for viral replication [42]. Notably, the formation of the non-structural protein NS3, antigenically linked to NS2-3, is a significant genetic difference observed only in cells infected with CP viruses [16 , 42]. In this study, samples from the cattle and aborted calf were identified as BVDV-1d subtype and NCP biotype. Our results are compatible with previous studies indicating that most abortions are caused by the NCP pestivirus biotype.

CONCLUSION

Consequently, it is crucial to conduct essential analyses on imported animals, animal and biological products such as FBS, semen, vaccines etc. Continuous monitoring of BVDV outbreaks, systematic research, and classification of newly identified isolates can significantly improve discussions within the pestivirus family. These measures are necessary for future epidemiologic studies to be effective. In addition, incorporating subtype 1d into vaccines used in Türkiye (BVDV-1a, BVDV-1b subtypes, and BVDV-2 subtypes) could contribute to defining new strategies for control and eradication programs.

Author contributions

SA: Drafting manuscript, conceptualization, formal analysis, investigation, MÖT: Drafting manuscript, critical revisions, Conceptualization, Formal analysis, ÖFK: Conceptualization, Formal analysis, data collection, MG: Formal analysis, data curation HA: Critical revisions, IS: Critical revisions.

Conflict of interest

The authors declared no conflict of interest.

Funding

This study did not receive a grant from any institutions.

BIBLIOGRAPHIC REFERENCES

[1] Vilcek S, Nettleton PF. Pestiviruses in wild animals. Vet. Microbiol. [Internet]. 2006; 116(1–3):1–12. doi: https://doi.org/fhh5b8

[2] Yesilbag K, Forster C, Bank-Wolf B, Yılmaz Z, Alkan F, Ozkul A, Burgu I, Rosales, SC, Thiel HJ, Konig M. Genetic heterogeneity of bovine viral diarrhoea virus (BVDV) Isolates from Turkey: Identification of a New Subgroup in BVDV-1. Vet. Microbiol. [Internet]. 2008; 130(3–4):258–267. doi: https://doi.org/bnzgs9

[3] Wu Y, Zhang G, Jiang H, Xin T, Jia L, Zhang Y, Yang Y, Qin T, Xu C, Cao J, Ameni G, Ahmad A, Ding J, Li L, Ma Y, Fan X. Molecular Characteristics of Bovine Viral Diarrhea Virus Strains Isolated from Persistently Infected Cattle. Vet .Sci. [Internet]. 2023; 10(7):413. https://doi.org/n44x.

[4] Sevik M. The Role of Pestiviruses (BDV and BVDV) in Ruminant Abortion Cases in the Afyonkarahisar Province. Kocatepe Vet. J. [Internet]. 2018; 11(3):238-244. doi: https://doi.org/n4qd

[5] Spetter MJ, Louge-Uriarte EL, Armendano JI, Morrell EL, Canton GJ, Verna AE, Dorsch MA, Pereyra SB, Odeón AC, Saliki JT, González-Altamiranda EA. Detection methods and characterization of bovine viral diarrhea virus in aborted fetuses and neonatal calves over a 22-year period. Brazilian J. Microbiol. [Internet]. 2020; 51(4):2077–2086. doi: https://doi.org/n4qg

[6] Britto-de Oliveira PS, Silva-Júnior JVJ, Weiblen R, Flores EF. A new (old) bovine viral diarrhea virus 2 subtype: BVDV-2e. Arch. Virol. [Internet]. 2022; 167(12):2545–2553. doi: https://doi.org/n4qk

[7] Sarikaya B, Azkur AK, Gazyagci S, Aslan ME. Genetic variability of bovine viral diarrhea virus in the 5'-UTR in the Central Anatolia of Turkey. Acta. Sci. Vet. [Internet]. 2012[cited 22 Sept 2024]; 40(1):1013. Available in: https://n9.cl/nbedfm

[8] Gürçay M, Kececi H, Öztürk M. Determination of presence and prevalence of bovine viral diarrhea virus ınfection in cattle herds in Bingöl province. Etlik. Vet. Mikrobiyol. Derg. [Internet]. 2020; 31(1):34–38. doi: https://doi.org/n4q2

[9] Postel A, Smith DB, Becher P. Proposed update to the taxonomy of pestiviruses: eight additional species within the genus pestivirus, family flaviviridae. Viruses. [Internet]. 2021; 13(8):1-12. doi: https://doi.org/n4q3

[10] Timurkan MO, Aydın H. Increased genetic diversity of BVDV strains circulating in Eastern Anatolia, Türkiye: first

detection of BVDV-3 in Turkey. Trop. Anim. Health. Prod. [Internet]. 2019; 7(51):1953–1961. doi: https://doi.org/n4q4

[11] Weber MN, Silveira S, Machado G, Groff FHS, Mosena ACS, Budaszewski RF, Dupont PM, Corbellini LG, Canal CW. High frequency of bovine viral diarrhea virus type 2 in Southern Brazil. Virus. Res. [Internet]. 2014; 191:117–124. doi: https://doi.org/f6k9ng

[12] Brodersen BW. Bovine Viral Diarrhea Virus Infections: Manifestations of Infection and Recent Advances in Understanding Pathogenesis and Control. Vet. Pathol. [Internet]. 2014; 51(2):453–464. doi: https://doi.org/ghdcvv

[13] Dogan F, Dagalp SB. Sığırlarda viral nedenli abort olgularının etiyopatogenezi. Sağlık Bilim Enst. Derg. [Internet]. 2017; 5(1):66–77. doi: https://doi.org/n4q5

[14] Garoussi MT, Mehrzad J, Nejati A. Investigation of persistent infection of bovine viral diarrhea virus (BVDV) in Holstein dairy cows. Trop. Anim. Health. Prod. [Internet]. 2019; 51(4):853–858. doi: https://doi.org/n4q6

[15] Tuncer-Göktuna P, Yesılbag K. Bovine viral diarrhea virus propagates in cell culture after ınoculation of ınactivated sera from persistently ınfected calves. Uludag. Univ. Vet. Fak. Derg. [Internet]. 2016; 35(1-2):7–10. doi: https://doi.org/g7gmps

[16] Cagırgan AA, Kaplan M, Pekmez K, Van-Schalkwyk A, Arslan F, Timurkan MO. The Status of Bovine Viral Diarrhea Virus (BVDV) in Western Türkiye: Detection of Three Subtypes. Kafkas. Univ. Vet. Fak. Derg. [Internet]. 2022; 28(6):709-715. doi: https://doi.org/n4q9

[17] Atasever A, Mendil AS, Timurkan MO. Detection of bovine viral diarrhea virus and bovine herpes virus type 1 in cattle with and without endometritis. Vet. Res. Forum. [Internet]. 2023; 14(10):541-548. doi: https://doi.org/n4rb

[18] İssi M, Gülaçtı İ, Kızıl Ö, Karapınar T, Bulut H, Gül Y. The mucosal disease cases observed in our clinic and confirmed with reverse transcription -polymerase chain reaction (RT-PCR). Fırat. Univ. Sağlık. Bilim. Vet. Derg. [Internet]. 2006[Cited 22 Sept 2024]; 20(3):253-258. Available in: https://goo.su/noqHr

[19] Usta Z, Dıstl O. Hydrocephalus internus and other neural malformations, brachyury in German Holstein twin calves. MAEU. Vet. Fak. Derg. [Internet]. 2019; 4(1):37–40. doi: https://doi.org/n4rf

[20] Ozkaraca M, Timurkan MO. Erzurum Yöresinde Pnömonili Sığırlarda BVDV' nin Teşhisinde İmmunohistokimya ve PCR Yöntemleri. Van Vet. J. [Internet]. 2016[Cited 02 July 2024]; 27(2):85-89. Available in: https://goo.su/Nd4vl

[21] Baxi M, McRae D, Baxi S, Greiser-Wilke I, Vilcek S, Amoako K, Deregt D. A one-step multiplex real-time RT-PCR for detection and typing of bovine viral diarrhea viruses. Veterinary Microbiology [Internet]. 2006; 116(1-3), 37-44. https://doi.org/10.1016/j.vetmic.2006.03.026

[22] Ozkul A. The role on infertility eases of IBR/IPV and BVD virus infections eneountered in dairy eattle herds. Ankara Univ. Vet. Fak. Derg. [Internet]. 1995; 42(03):381-387. doi: https://doi.org/n4rj

[23] Vilček S, Herring AJ, Herring JA, Nettleton PF, Lowings JP, Paton DJ. Pestiviruses isolated from pigs, cattle and sheep can be allocated into at least three genogroups using polymerase chain reaction and restriction endonuclease analysis. Arch. Virol. [Internet]. 1994; 136:309-323. doi: https://doi.org/cmvdqh

[24] Tamura K, Stecher G, Kumar S. MEGA11: Molecular evolutionary genetics analysis, Version 11. Mol. Biol. Evol. [Internet]. 2021; 38(7):3022–3027. doi: https://doi.org/gkknc5

[25] Bianchi MV, Konradt G, de Souza SO, Bassuino DM, Silveira S, Mosena ACS, Canal CW, Pavarini SP, Driemeier D. Natural outbreak of BVDV-1d–ınduced mucosal disease lacking ıntestinal lesions. Vet. Pathol. [Internet]. 2017; 54(2):242–248. doi: https://doi.org/n4rk

[26] Walz PH, Grooms DL, Passler T, Ridpath JF, Tremblay R, Step DL, Callan RJ, Givens MD. Control of bovine viral diarrhea virus in ruminants. J. Vet. Intern. Med. [Internet]. 2010; 24(3):476–486. doi: https://doi.org/ctv3p5

[27] Erol N, Gur S, Acar A. A Serological ınvestigation for bovine viral diarrhea virus ınfection in and around Afyonkarahisar province, West Anatolia. Kocatepe. Vet. J. [Internet]. 2014; 7(2):17–21. doi: https://doi.org/n4rm

[28] Deng M, Chen N, Guidarini C, Xu Z, Zhang J, Cai L, Yuan S, Sun Y, Metcalfe L. Prevalence and genetic diversity of bovine viral diarrhea virus in dairy herds of China. Vet. Microbiol. [Internet]. 2020; 242:108565. doi: https://doi.org/gtjqvt

[29] Yesilbag K, Alpay G, Tuncer P. Control and Elimination of Bovine Viral Diarrhoea Virus in a Dairy Herd. Uludag. Univ. Vet. Fak. Derg. [Internet]. 2012[cited 24 Sept 2024]; 31(1):11–17. Available in: https://goo.su/vVycKd

[30] Yesilbag K, Alpay G, Becher P. Variability and global distribution of subgenotypes of bovine viral diarrhea virus. Viruses. [Internet]. 2017; 9(128):1-19. doi: https://doi.org/gsk824

[31] Uttenthal A, Stadejek T, Nylın B. Genetic diversity of bovine viral diarrhoea viruses (BVDV) in denmark during a 10-year eradication period. Acta. Path. Microbiol. Immunol. Scand. [Internet]. 2005; 113(7–8):536–541. doi: https://doi.org/fbr56n

[32] Kuta A, Polak MP, Larska M, Żmudziński JF. Predominance of bovine viral diarrhea virus 1b and 1d subtypes during eight years of survey in Poland. Vet. Microbiol. [Internet]. 2013; 166(3–4):639–644. doi: https://doi.org/f5cfkd

[33] Mirosław P, Polak M. Increased Genetic Variation of Bovine Viral Diarrhea Virus in Dairy Cattle in Poland. BMC. Vet. Res. [Internet]. 2019; 15(1):1–12. doi: https://doi.org/g6cdgc

[34] Kucer N, Markovıc E, Prpıc J, Rudan D, Jemersıc L, Hajek Z, Skaro K, Pavlak M, Rudan N. The Epidemiology of bovine viral diarrhea virus ınfection on a dairy farm-clinical signs, seroprevalence, virus detection and genotyping. Vet. Arch. [Internet]. 2022; 92(2):119–126. doi: https://doi.org/n4rn

[35] Scharnböck B, Roch FF, Richter V, Funke C, Firth CL, Obritzhauser W, Baumgartner W, Käsbohrer A, Pinior B. A meta-analysis of bovine viral diarrhoea virus (BVDV) prevalences in the global cattle population. Sci. Rep. [Internet]. 2018; 8(14420):1-15. doi: https://doi.org/gfchz9

[36] Fulton RW, Ridpath JF, Saliki JT, Briggs RE, Confer AW, Burge LJ, Purdy CW, Loan RW, Duff GC, Payton ME. Bovine viral diarrhea virus (BVDV) 1b: predominant bvdv subtype in calves with respiratory disease. Can. J. Vet. Res. [Internet]. 2002; 66(3):181-190. PMID: 12146890. Available in: https://pubmed.ncbi.nlm.nih.gov/12146890/

[37] Vuuren MV. Bovine viral diarrhoea virus ınfection in livestock in southern Africa. Perspect. Agric. Vet. Sci. Nutr. Nat. Resour. [Internet]. 2006; 1(9):1-5. doi: https://doi.org/fnhkbp

[38] Nogueira FCS, Velasquez E, Melo ASO, Domont GB. Zika virus may not be alone: proteomics associates a bovine-like viral diarrhea virus to microcephaly. Bio. Rxiv. [Internet]. 2015; 21:1885-1886. doi: https://doi.org/bm4c

[39] Alpay G, Toker EB, Yeşilbağ K. Persistent BVD virus ınfections in offspring from imported heifers. Trop. Anim. Health. Prod. [Internet]. 2019; 51(2):297–302. doi: https://doi.org/n4rp

[40] Oğuzoğlu TC, Muz D, Yılmaz V, Timurkan MO, Alkan F, Akça Y, Burgu I. Molecular characteristics of bovine virus diarrhoea virus 1 ısolates from Turkey: approaches for an eradication programme. Transbound. Emerg. Dis. [Internet]. 2012; 59(4):303–310. doi: https://doi.org/d56k57

[41] Toker EB, Yeşilbağ K. Molecular characterization and comparison of diagnostic methods for bovine respiratory viruses (BPIV-3, BRSV, BVDV, and BoHV-1) in field samples in Northwestern Turkey. Trop. Anim. Health. Prod. [Internet]. 2021; 53(79):1-11. doi: https://doi.org/n4rq

[42] Klemens O, Dubrau D, Tautz N. Characterization of the determinants of NS2-3-ındependent virion morphogenesis of pestiviruses. J. Virol. [Internet]. 2015; 89(22):11668–11680. doi: https://doi.org/g8dsjh