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RESEARCH ARTICLE

The Status of Bovine Viral Diarrhea Virus (BVDV) in Western Türkiye: Detection of Three Subtypes

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Abstract: Bovine viral diarrhea virus (BVDV) is a viral pathogen that causes significant economic losses in cattle, especially by causing abortions. Globally, BVDVs are classified into three genetically distinct types: BVDV-1, BVDV-2 and BVDV-3. Despite the presence of all three groups in Türkiye, BVDV-1 is by far the most prevalent. The aim of the study was to determine the genetic diversity of BVDV detected in materials from aborted fetus between 2017 and 2020 in western Türkiye. Sequence and phylogenetic analyzes were performed based on the 5'-UTR and N^{pro} gene regions of BVDVs from samples, which tested positive using real time RT-PCR. According to pairwise similarity and cluster analysis the samples clustered into three different sub-types, with one dominant subtype 1d (n=4). The remaining samples clustered within subtype 1l (n=3) and 1f (n=2). In this study, different subtypes were found in abortion materials submitted from the same region. Since different subtypes of BVDV were identified even in a small geographical area of Türkiye, it is essential to prepare control and eradication programs through specific vaccination, diagnostic and mitigation programs coordinated by national government, to prevent the spread of these viruses.

Keywords: *Abortion, Bovine viral diarrhea virus, Cattle, Genetic variation, Türkiye*

Türkiye'nin Batısında Sığır Viral Diyare Virusunun (BVDV) Durumu: Üç Alt Grubun Tespiti

Öz: Bovine viral diyare virusu (BVDV), sığırlarda özellikle abortların görülmesiyle birlikte önemli ekonomik kayıplara neden olan viral bir patojendir. Küresel olarak, BVDV genetik olarak BVDV-1, BVDV-2 ve BVDV-3 olmak üzere farklı üç tipte sınıflandırılır. Türkiye'de her üç grubun varlığına rağmen, BVDV-1 açık ara en yaygın olanıdır. Bu çalışmanın amacı, Türkiye'nin batısında 2017-2020 yılları arasında sığırlardan elde edilen abort materyallerinde tespit edilen BVDV'nin genetik çeşitliliğini belirlemektir. Real Time RT-PCR kullanılarak pozitif test edilen abort örneklerinin 5'-UTR ve N^{pro} gen bölgelerine dayalı olarak dizi ve filogenetik analizleri yapıldı. İkili benzerlik ve küme analizine göre örnekler, bir baskın alt tip 1d (n=4) ile üç farklı alt tipte kümelendi. Kalan örnekler 1l (n=3) ve 1f (n=2) alt tipinde kümelenmiştir. Bu çalışmada aynı bölgeden gönderilen abort materyallerinde farklı alt tipler bulunmuştur. Türkiye'nin küçük bir coğrafi bölgesinde bile BVDV'nin farklı alt tipleri tespit edildiğinden, bu virusların yayılmasını önlemek için ulusal hükümet tarafından koordine edilen spesifik aşılama, teşhis ve hafifletme programları ile kontrol ve eradikasyon programlarının hazırlanması esas olmalıdır.

Anahtar sözcükler: *Abort, Bovine viral diyare virus, Sığır, Genetik varyasyon, Türkiye*

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Introduction

Bovine viral diarrhea virus (BVDV), which is in the *Pestivirus* genus, is of great importance due to the damage they cause to the world livestock economy. Until now, long-term programs for the control and eradication of pestivirus infections have not been developed and applied in many countries. Although BVDV frequently presents as subclinical cases, it causes respiratory, digestive and infertility disorders [1,2].

BVDV is transmitted horizontally by secretions and excreta (saliva and faces). In addition, the virus replicates in the placenta in susceptible pregnant cattle and vertically infects the fetus [3]. The consequences of intrauterine infection vary depending on the period of pregnancy, causing malformations, abortions, and immune-tolerant persistent infections. Chronically infected animals are either born weak or seemingly healthy but clinically undiagnosed, yet these animals are likely to develop Mucosal disease (MD) with anorexia, gastrointestinal erosion, and persistent diarrhea at some point in their lives [4,5].

This enveloped virus is 40-60 nm in diameter and contains a single-stranded RNA genome 12.3 kb in length. It belongs to the genus *Pestivirus* in the family *Flaviviridae* and has been divided in two biotypes: cytopathic (CP) and non-cytopathic (NCP) according to the morphological changes it produces in cell culture [6]. There is an untranslated region (UTR) at both the beginning (5') and end (3') of the genome [7]. The 5'-UTR is not capped and there is no poly(A)-tail at the 3' end of the viral RNA. The viral genome consists of four structural proteins (Core (C) and Envelope: Erns, E1 and E2) and seven non-structural proteins (N^{pro} NS2, NS3, NS4A, NS4B, NS5A, NS5B) [1]. Molecular characterization and phylogenetic studies of genetically diverse BVDVs are based on the 5'-UTR, N^{pro}, NS3 and E2 gene regions [8-12]. Recently, the International Committee on Taxonomy Viruses (ICTV) has renamed Pestiviruses and sub-divided them into 11 distinct species. These groups are called BVDV-1 (Pestivirus A), BVDV-2 (Pestivirus B), border disease (Pestivirus D), classical swine fever (Pestivirus C) and HoBi-like (Pestivirus H). In BVDV-1, 21 subtypes were identified and designated between 1a to 1u, whilst four subtypes of BVDV-2 were described and designated from 2a to 2d. In recent years BVDV-3, also called pestivirus H or HoBi-like pestivirus, has been identified as genetically and antigenically distinct disease in cattle and buffalo [13].

Previous studies identified BVDV-1 to be more prevalent than BVDV-2 in Türkiye [10-12]. In addition, it was recently reported that BVDV-3 was also present in Türkiye [12]. Whilst subtypes 1a, 1b, 1c, 1d, 1f, 1h have all been identified in Türkiye, it is reported that subtype 1l is the predominant subtype with the widest distribution [11,12].

Abortion cases due to BVDV infection are an important cause of economic loss to the livestock industry. No official vaccination, control and eradication program has been proposed or implemented for pestivirus cases in Türkiye, despite various studies indicating the presence and importance of BVDV in Türkiye. Therefore, early detection and subsequent elimination of diseases, such as BVDV, affecting herd health and well-being are critical in supporting a viable livestock industry. In order to achieve this, the fast and accurate identification of virus species, as well as the molecular characterization and phylogenetic classification of subtypes are necessary for BVDV in Türkiye.

In this study, the BVDV subtypes were determined based on sequence analysis using the 5'-UTR and N^{pro} gene regions of isolates obtained from abortion cases observed in western Türkiye.

Material and Methods

Samples and Preparation

In this study, nine pestivirus positive samples detected from 117 aborted fetus submitted to Izmir/Bornova Veterinary Control Institute for routine diagnosis between 2017-2020 were used. Internal organ samples of bovine abortions (spleen, liver, lung and lymph nodes) were transported and stored at 4°C. The samples were pooled and 1 g was crushed in a sterile porcelain mortar with sterile sand. It was homogenized by adding 5 mL of Eagle's Minimum Essential Medium (EMEM) and the homogenate was centrifuged at 3500 rpm at 4°C for 15 min. The supernatant was removed and used during subsequent RNA extraction. The provinces from where samples originated are indicated in *Fig. 1* and additional metadata of the samples are provided in *Table 1*.

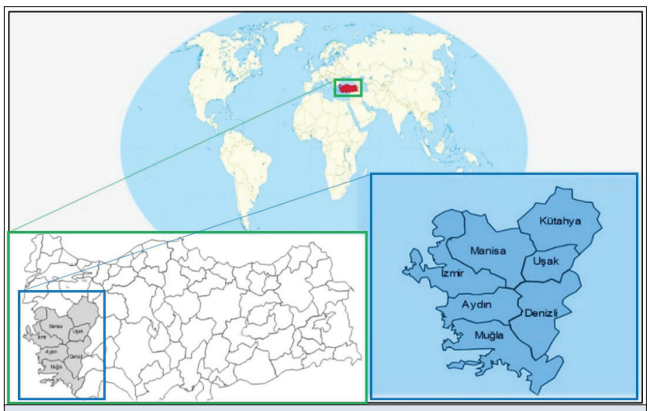

Fig 1. Geopolitical position of Türkiye and provinces where samples were submitted

Table 1. Information about BVDVs obtained from this study

Location	Sample Type	Year	Isolate Name
Uşak	Fetus	2017	BVDV-1_Bor1_Türkiye_2017
İzmir	Fetus	2019	BVDV-1_Bor2_Türkiye_2019
Aydın	Fetus	2018	BVDV-1_Bor3_Türkiye_2018
Muğla	Fetus	2019	BVDV-1_Bor4_Türkiye_2019
İzmir	Fetus	2018	BVDV-1_Bor5_Türkiye_2018
Denizli	Fetus	2020	BVDV-1_Bor6_Türkiye_2020
Kütahya	Fetus	2019	BVDV-1_Bor7_Türkiye_2019
Manisa	Fetus	2020	BVDV-1_Bor8_Türkiye_2020
Aydın	Fetus	2020	BVDV-1_Bor9_Türkiye_2020

RNA Extraction and Real Time PCR

Viral RNA extraction was performed using the Roche MagNA Pure LC 2.0 instrument and the MagNA Pure LC Total Nucleic Acid isolation kit according to the manufacturer's instructions (Roche, Basel, Switzerland). Real time RT-PCR were performed using primers and probe previously described by Hoffmann et al.[14] and the extracted RNA as template. For both the reverse transcription and DNA amplification reaction, the Real Time ready RNA Virus Master kit (Roche, Germany) was used. The RT-qPCR was performed in a 20 µL reaction volume containing 5 µL template RNA, 0.5 pmol of each primer and 0.25 pmol probe in a Roche LightCycler® 480. Following cDNA synthesis for 6 min at 50°C, the reaction progress for 40 cycles by 57°C annealing temperature.

Detection of Pestivirus Biotypes

The RT-PCR method described by Greiser-Wilke et al.[15] was used to determine biotypes (CP or NCP) of BVDV. For amplification, the Xpert One-Step RT-PCR Kit (Grisp Research Solutions, Porto, Portugal) was used. The reaction was performed in a reaction volume of 25 µL and primer final concentration as 0.4 mM.

Phylogenetic Tree and Molecular Characterization

Sequencing based on the 5'UTR were performed using the primers 324 and 326 as previously described by Vilcek et al.[8]. Additional analysis based on the N^{pro} gene region were performed using the primers BD1, BD2, and BD3 as described by Vilcek et al.[16]. All PCR products were visualized on an 1.5% agarose gel stained with ethidium bromide in TAE buffer and photographed using equipment from Vilber Lourmat (France). Amplified products were sequenced using both Forward and Reverse primers incorporated during the generation of the amplicons, at the commercial laboratory (Microsynth AG, Balgach, Switzerland). The nucleotide sequence results were evaluated and edited using DNADynamo Software. The consensus nucleotide sequences were verified using the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI). Individual sequence alignments of the 5'-UTR (220 bp) and N^{pro} protein start (403bp) regions, containing the nine samples from Türkiye as well as sequences from GenBank (total n = 185 and n = 195), were generated using CLC Genomics Workbench v.9.5 (Qiagen, www.clcbio.com). Each of the alignments were used to determine the phylogenetic relatedness of the viruses, by generating individual maximum likelihood trees in Mega 6 [17]. Each individual phylogenetic tree was constructed under General Time Reversal (GTR) (G+I, G = 4) with 1000 bootstrap iterations.

Results

Nine pestivirus positive samples were identified as noncytopathic (NCP) biotype using RT-PCR. The nine pestivirus isolates originated from the aborted samples and the sequences corresponding to each of the samples were submitted to GenBank under the accession numbers provided in *Table 2*. These BVDV samples were submitted between 2017 and 2020 and their phylogenetic relatedness towards previously published BVDV sequences were analyzed by comparing the 220bp 5'UTR and 404bp N-terminal region of the polyprotein. The two maximum likelihood phylogenetic trees generated by utilizing each of the previously described data sets, displayed congruence concerning the clustering of the new BVDV samples *(Fig. 2, Fig. 3)*. While all nine samples were identified as BVDV-1, they were grouped to clusters BVDV-1d, BVDV-1f and BVDV-1l. Samples Bor6_Türkiye_2020, Bor7_Türkiye_2019, Bor8_Türkiye_2020 and Bor9_Türkiye_2020 grouped in cluster BVDV-1d, sharing a more recent common ancestor with BVDV samples from China in 2012 - 2013, than BVDV sample described in Türkiye in 2016 *(Fig. 1, Fig. 2)*. In contrast, samples Bor3_Türkiye_2018 and Bor4_Türkiye_2019 clustered phylogenetically closer to previously identified samples from Türkiye, than BVDV samples obtained in

Table 2. *Genetic classification and accession number of the field strains in the present study*

Samples	Subtype	Biotype	Gene Region	Accession no.
BVDV-1_Bor1_Türkiye_2017	1l	NCP	5'-UTR	OM223845
			N^{pro}	OM223836
BVDV-1_Bor2_Türkiye_2019	1l	NCP	5'-UTR	OM223846
			N^{pro}	OM223837
BVDV-1_Bor3_Türkiye_2018	1f	NCP	5'-UTR	OM223847
			N^{pro}	OM223838
BVDV-1_Bor4_Türkiye_2019	1f	NCP	5'-UTR	OM223848
			N^{pro}	OM223839
BVDV-1_Bor5_Türkiye_2018	1l	NCP	5'-UTR	OM223849
			N^{pro}	OM223840
BVDV-1_Bor6_Türkiye_2020	1d	NCP	5'-UTR	OM223850
			N^{pro}	OM223841
BVDV-1_Bor7_Türkiye_2019	1d	NCP	5'-UTR	OM223851
			N^{pro}	OM223842
BVDV-1_Bor8_Türkiye_2020	1d	NCP	5'-UTR	OM223852
			N^{pro}	OM223843
BVDV-1_Bor9_Türkiye_2020	1d	NCP	5'-UTR	OM223853
			N^{pro}	OM223844

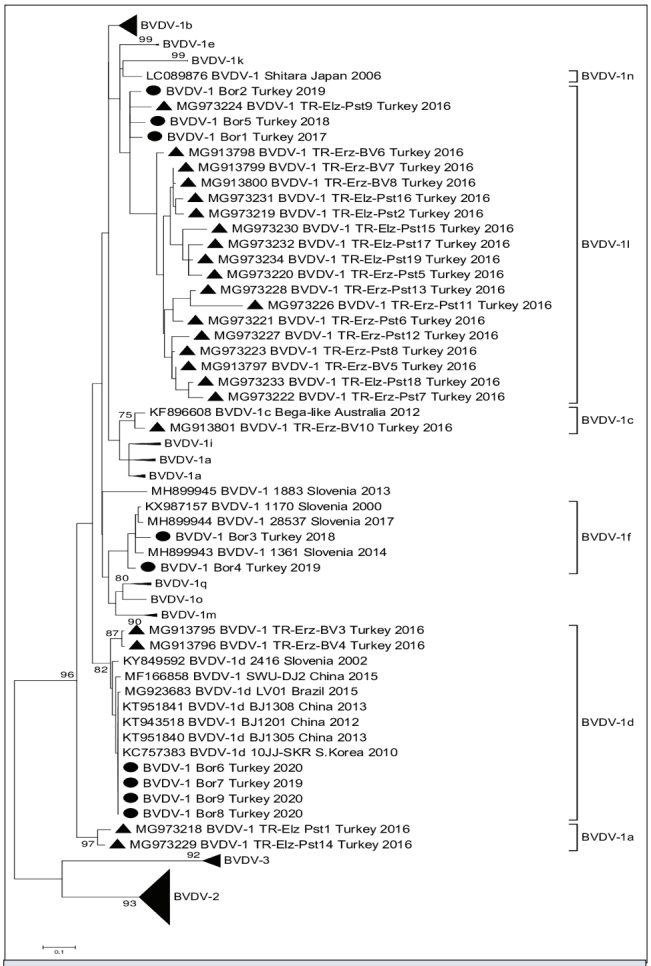

Fig 2. Maximum likelihood phylogenetic tree constructed with the 220bp 5'UTR region of BVDV-1, BDVD-2 and BVDV-3 sequences. The isolates were aligned by the ClustalW, and the phylogenetic tree was constructed using the MEGA6 package with a bootstrap value of 1.000 replicates. The sequences obtained in this study are marked with a round black spot (●), and the sequences obtained from different studies in Türkiye are marked with a black triangle (▲)

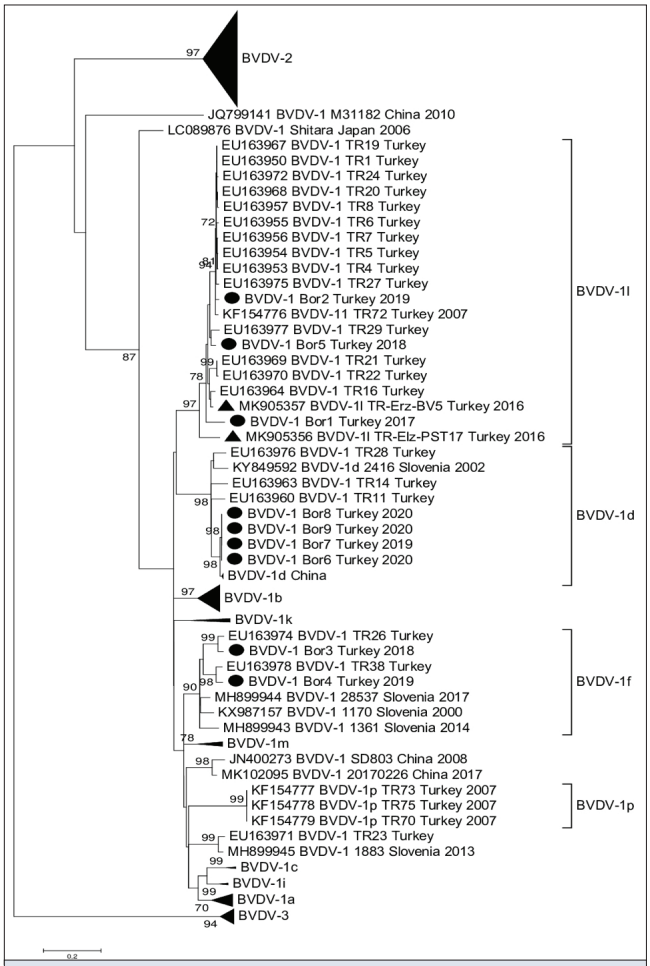

Fig 3. Maximum likelihood phylogenetic tree constructed with the 403bp N-terminal region of the polyprotein from BVDV-1, BDVD-2 and BVDV-3 sequences. The isolates were aligned by the ClustalW, and the phylogenetic tree was constructed using the MEGA6 package with a bootstrap value of 1.000 replicates. The sequences obtained in this study are marked with a round black spot (●), and the sequences obtained from different studies in Türkiye are marked with a black triangle (▲)

Slovenia belonging to cluster BVDV-1f *(Fig. 2, Fig. 3)*. The remaining three samples Bor1_Türkiye_2017, Bor2_Türkiye_2019 and Bor5_Türkiye_2018 grouped with the BVDV samples described in Türkiye between 2007 and 2017 in sub cluster BVDV-1l *(Fig. 2, Fig. 3)*. Subtyping based on phylogenetic study of the 5'-UTR and N-terminal region produced similar results.

Discussion

According to current statistical data, the cattle population in Türkiye is approximately 18 million [18]. Pestiviruses are endemic among livestock in Türkiye and causes significant economic losses by causing abortions in sheep, goats and cattle. In recent years, numerous studies on the typing of pestiviruses have been conducted in other parts of Türkiye, yet information concerning the subtyping of pestiviruses responsible for causing abortion in the west of the country is still deficient. This study was conducted to investigate the genetic diversity of pestiviruses circulating in western Türkiye, which cause abortions and subsequent economic losses in cattle. For this purpose, sequence analyzes were performed based on the 5'-UTR and N^{pro} gene regions of abortion samples, which were identified as BVDV positive using real time RT-PCR. Based on the resulting phylogenetic trees and pairwise percentage sequence identity, the nine samples were divided into three different subtypes. The most prevalent subtype identified from cattle abortion in the west of Türkiye was 1d (n=4), followed by 1l (n=3) and 1f (n=2).

Previous molecular characterization studies conducted in Türkiye, identified BVDV-1l as the dominant subtype amongst BVDV-1 types [19-22]. Yesilbag et al.[19] was the first to identify BVDV-1l as a unique subtype and that it is the most dominant subtype in Türkiye (in 5 out of 15 locations). This result was confirmed when 18 (45%) of the 40 positive samples, from 15 different farms representing 5 regions of Türkiye, identified BVDV-1l as the predominant genotype in Türkiye [22]. Similarly, 19 of the 28 samples (67.8%) described by Timurkan and Aydin [12], belonged to BVDV-1l subtype. Since all the previously mentioned studies were conducted in the Eastern Anatolia region of Türkiye, it was imperative to determine if the prevalence of BVDV-1l is restricted to the eastern Anatolia region or if it could be extrapolated to the whole of Türkiye.

In this study BVDV-1l (n=3), BVDV-1d (n=4) and 1f (n=2) subtypes were identified, originating from western Türkiye. Based on all the genetic and molecular characterization studies conducted in Türkiye, BVDV 1a, 1b, 1d, 1f, 1h, 1i, 1l as well as BVDV 2a, 2b and BVDV-3 have been identified [12,19-22]. Therefore, the 1d, 1l, 1f detected in this study correlates and clusters with sequences from previous identified samples in the country. It is also important to emphasize that the subtype, BVDV-1l, seems to be unique to Türkiye and circulates countrywide [10]. Considering the geographic position of Türkiye as a bridge between Europe and Asia, the importance of subtypes 1d and 1f detected in this study should be noted, since these two types were the dominant subtype identified in various Europe countries (Germany, Italy, Austria and Poland) [16,23-28]. Therefore, trade in animals and animal products between countries may increase the probability of observing different subtypes.

It has been reported that subtype BVDV-1b has a frequent and global distribution, yet upon considering the global spread of BVDV-1 the following observations are made. BVDV-1a and 1b occur in the Americas, 1c in Australia, 1a in Africa, whilst 1b, 1a, 1c, and 1m occur in Asia and 1a, 1b, 1e, 1f, 1h and 1d in Europe [11]. Although this explains the reason for 1a and 1b subtypes inclusion in the current vaccines, it is important to additionally evaluate the level of protection afforded by these vaccines, especially in the content of the isolates circulating in the western part of Türkiye, as described in this study.

BVDV-2 and BVDV-3 types were not detected in this study. Currently, BVDV-3 has only been described in a single study in Türkiye, which is not surprising considering the relatively recent global description of this virus as new species. In contrast, various studies have reported the identification of BVDV-2 in Türkiye [10,19,29]. These studies indicated that this type, which has a global distribution, not only occurs in Türkiye but has novel genetic differences based on spatial and temporal distributions.

Knowing the genetic heterogeneity and phenotypic differences of BVDV infection is important for a rapid diagnosis, control and eradication of the diseases caused by pestiviruses. In this study conducted in the west of Türkiye, 1d, 1l and 1f types were re-identified in cattle herds. The paucity of information on the true prevalence of this infection across Türkiye (cattle, sheep-goat and swine data) hinders efforts to estimate the true impact of pestiviruses on cattle populations. For this infection, systematic molecular epidemiological studies should be carried out throughout the country and prevention-control programs should be developed, including national trade regulations for live animals, animal products and biological products.

Both CP and NCP pestiviruses are involved in the pathogenesis of mucosal disease, a deadly disease induced by the development of CP virus in cattle that have been infected with NCP virus for a long time. Pestivirus biotypes are typically detected using cell cultures in which CP biotypes cause cytopathic effects. The creation of the non-structural protein NS3, which is antigenically linked to NS2-3 in cells infected with CP viruses alone, has been

found to constitute a major genetic difference between the CP and NCP biotypes [15,30]. Pestivirus cultivation in cell cultures is time-consuming and labor-intensive. As a result, the RT-PCR method described by Greiser-Wilke et al.[16] was used to differentiate biotypes in this study. Pestiviruses of the CP and NCP biotypes can cause abortion in cattle and small ruminants. However, based on the RT-PCR assay, all positive pestiviruses in this study were found to be the NCP biotype. This is consistent with previous research, which found that the majority of abortions in the field are caused by the NCP biotype of pestiviruses [31,32].

In conclusion, pestiviruses are an infection that causes serious economic losses (persistent infections) worldwide as well as in Türkiye. Since all pestivirus infections cause similar clinical symptoms, they will continue to cause significant economic losses as they enter new parts of the world. For this reason, it is necessary to prepare important control and eradication programs to prevent the spread of viruses by using specific diagnostic and control approaches that can be enforced by the state.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author (A.A. ÇAĞIRGAN) on reasonable request.

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Ethical Approval

Ethics committee approval is not required as it is used with abortion material.

Competing Interests

There is no conflict of interest.

Author Contributions

AAÇ: design of the study; AAÇ, AS, MOT: drafting of the manuscript; AAÇ, MK, KP, FA: design of the study, performing the experiment, and drafting the manuscript; AAÇ, MK, KP, FA: sampling. All authors read and approved the final version of the manuscript.

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