# Molecular characterization and phylogenetic analysis of bovine viral diarrhea virus in dairy herds of Fars province, Iran

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## Summary

Bovine viral diarrhea virus (BVDV) is one of the most important viral pathogens of cattle worldwide. The aim of present study was to determine the molecular characterization and phylogenetic analysis of BVDV infection in dairy herds of Fars province, Iran. For initial screening, a total of 400 blood samples were collected from 12 industrial dairy herds with previous history of diarrhea, abortion or birth of weak calves and analyzed using reverse transcription-polymerase chain reaction (RT-PCR) on buffy coat. In the next step, blood samples and also ear notch biopsies were collected from 100 cattle of infected farms three weeks later which were subsequently tested by antigen capture ELISA (ACE), RT-PCR and immunohistochemistry (IHC). The results of nested RT-PCR were successful in 16 out of 400 buffy coat samples (4%) in the initial screening. Also, 8 out of 100 samples (8%) were positive by all practiced tests including RT-PCR, ACE and IHC on buffy coat, serum and skin samples, respectively. Immunoreactivity for bovine BVDV antigen as brown, coarsely to finely granular was observed within the cytoplasm of epidermic epithelial cells, hair follicles and subcutaneous stromal cells. Genetic sequence analyses showed both genotypes, BVDV-1 and BVDV-2. The new isolates were identified as BVDV1-FarsA, BVDV1-FarsB and BVDV2-FarsA in the phylogenetic tree. Since both genotypes of the virus are present in the region, our findings emphasize the importance of monitoring BVDV infection in cattle and suggest detection and elimination of PI animals for controlling and eradication of BVDV in Fars province.

**Key words:** Bovine viral diarrhea virus, Cattle, Molecular characterization, Phylogenetic analysis

## Introduction

Bovine viral diarrhea virus (BVDV) is one of the most important viral pathogens of cattle worldwide and is classified as a member of the genus *Pestivirus* within *Flaviviridae* family (Paton, 1995; Houe, 1999; Heinz *et al*., 2000). Two genotypes of BVDV-1 and BVDV-2 are recognized, causing acute and persistent infections (Carman *et al*., 1998). While BVDV-1 has been recognized for many years and is widely spread all over the world, BVDV-2 was first identified in the 1990's in North America (Pellerin *et al*., 1994; Ridpath *et al*., 1994) and has been only sporadically detected in other countries such as Japan (Nagai *et al*., 1998), Germany (Wolfmeyer *et al*., 1997; Doll and Holsteg, 2014), Belgium (Letellier *et al*., 1999) and the United Kingdom (Courtenay *et al*., 2007).

Furthermore, isolates of BVDV can be separated into non-cytopathogenic and cytopathogenic biotypes (Ridpath *et al*., 1994; Baker, 1995). Prenatal infection in the first four months of pregnancy with non-cytopathogenic biotype of BVDV can result in the birth of immunotolerant and persistently infected (PI) animals. Persistently infected cattle generally remain life-long virus carriers, shedding large quantities of virus in most bodily excretions and secretions and are the main factor of the virus continuation within herds (Brock *et al*., 1998; Lindberg and Alenius, 1999; Wittum *et al*., 2001; Brock *et al*., 2005; Lindberg and Houe, 2005).

Accurate detection and elimination of PI cattle is essential for controlling the transmission of the virus (Alenius *et al*., 1996; Bhudevi and Weinstock, 2003). Various methods such as immunohistochemistry or IHC (on skin biopsies), antigen capture ELISA or ACE (on serum and skin samples), virus isolation (VI), and reverse transcription-polymerase chain reaction (on a variety of samples, including blood, serum and ear-notch supernatant) are used to detect PI cattle (Dubovi, 1996; Thür *et al*., 1997; Brodersen, 2004; Saliki and Dubovi, 2004; Cornish *et al*., 2005; Sandvik, 2005; Hilbe *et al*., 2007). The ACE, available as a commercial test kit, uses monoclonal antibodies to capture viral antigen Erns (gp48) and detects antigen-antibody complexes with enzyme-conjugated antibody by spectrophotometry (Goyal, 2005). RT-PCR has been proved to be a rapid and sensitive method to detect viral nucleic acids and this technique has been used for the detection of pestiviruses using oligonucleotide primers located in conserved regions of the viral genome (Tajima *et al*., 1995; Canal *et al*., 1996). RT-PCR assays are more rapid and are appropriate for detection of persistent infections in young calves because of the problem of colostral antibody

interference seen in virus isolation (VI) and ELISA (Deregt *et al*., 2002; Kozasa *et al*., 2005). Pooling samples for RT-PCR is a popular method to screen many animals for BVDV at a reduced cost. If the pooled sample is positive by RT-PCR, the originally submitted samples are tested individually. IHC staining of formalin-fixed, paraffin-embedded skin biopsies has been used as a method for the early detection of persistent BVDV infection in cattle (Brodersen, 2004; Hilbe *et al*., 2007).

In Iran, there are a few reports that show the prevalence of antibodies against BVDV from 16% to 69% in cattle populations (Mirchamsy *et al*., 1970; Sedighinejad, 1996). More recently, a preliminary study by ACE on primary sera samples of the present study showed BVD antigen in 4% of studied cattle and also histopathological and immunohistochemical characteristics of naturally-occurring BVDV infection in two cattle were described (Farjani Kish *et al*., 2013; Khodakaram-Tafti *et al*., 2015). There is no report about the genotyping and prevalence of acute and persistent BVDV infection in dairy populations of Fars province so far. Therefore, the aims of this study were to determine the molecular characterization and genetic analysis of BVDV infected dairy cattle of Fars province, Iran.

## Materials and Methods

### Sampling

In the initial screening, a total of 400 blood samples were collected from calves and cattle between 3 months and 5 years of age in 12 industrial dairy herds between 2013 and 2014. The herds had previous history of diarrhea, abortion or birth of weak calves and the population between 200 and 600 cattle in each farm (Table 1). Pooled buffy coats (usually each pool contained 20 samples) were tested by RT-PCR. Then, the buffy coats of each positive pooled sample were retested to determine the individual infected animals. In the next step, blood samples and also ear notch biopsies were collected from 100 cattle of infected farms after three weeks. In this step the whole blood (with and without EDTA) was centrifuged at 250 g for 15 min so the buffy coat and sera were isolated and stored at -20°C and -70°C, respectively. The sera and buffy coats were tested by ACE and RT-PCR, respectively. The ear notches were tested by ACE and IHC (Table 1).

### Antigen capture ELISA (ACE) on skin biopsies

The skin samples, 1 × 1 cm in size, were obtained from the hairless area under the ear of each calf with an ear notcher. In the laboratory, prior to the test, the biopsies of skin were delicately dissected for better soaking. Ear notch tissues were placed in individual sterile tubes with 2 ml of "Ear Notch Soaking Buffer" at room temperature for 24 h and mixed. Then 50 µL of the buffer was directly added into the ELISA microplates wells. All soaked ear notches were assayed for BVDV by ACE per manufacturer's instructions (IDEXX Laboratories, Switzerland).

### PCR

*RNA extraction*

RNA isolation (from pooled samples and each buffy coat individually) was performed using the Cinnapure RNA extraction Kit according to the manufacturer's instructions (Cinnagen, Iran). Briefly, 100 µL buffy coats was transferred to a 2 µL tube (included spin column with collection tube) then 400 µL of lysis buffer and 300 µL of precipitant were added and centrifuged at 16000 g for 1 min. In the next step, the spin column was placed in a new collection tube followed by two step washing by 400 µL of buffers I and II which was subsequently centrifuged for 2 min. Thereafter, 100 µL of RNase free water was poured in the center of the column and incubated for 3-5 min at 55°C. Finally, the tube was centrifuged at 16000 g for 1 min to elute the RNA.

*cDNA synthesis and RT-PCR*

For cDNA synthesis, 5 µL of extracted RNA, 1 µL of specific reverse primer and 14 µL of DEPC treated water were added to the lyophilized master mix contained in Bioneer AccuPower$^{TM}$ RT PreMix kit (Korea). The mixture was incubated at 42°C for 60 min. cDNA synthesis was terminated by incubation at 95°C for 5 min.

Amplification of 5' UTR (288 bp) was carried out on pooled buffy coat (each pool contained 20 samples) RNA using the primers 324 (5´-ATG CCC WTA GTA GGA CTA GCA-3′) and 326 (5′-TCA ACT CCA TGT GCC ATG TAC-3′) to detect pestivirus infection as described by Vilcek *et al*. (1994).

In individual samples, amplification of cDNAs by PCR was performed using the primer pairs 0I 100 (5′-CAT GCC CWY AGT AGG ACT AGC-3′)/1400R (5′-ACC AGT TGC ACC AAC CAT G-3′) as described by Becher *et al*. (1999) and BD1 (5′-TCT CTG CTG TAC ATG GCA CAT G-3′)/BD2 (5′-TTG TTR TGG TAC ARR CCG TC-3′) (nested PCR) as described by Vilcek *et al*. (1997) and BD1/BD3 (5′-CCA TCT ATR CAC ACA TAA ATG TGG T-3′) and BD1/BD4 (5′-CCA TCC ACG CAT ACG TAG ATG TG-3′) to detect the strains of BVDV as described by Vilcek *et al*. (2001). In the nested PCR, in the first PCR, the outer primers 0I 100 and 1400R were amplified and in the second PCR, 3 µL of the first-round PCR product was amplified with primers BD1/BD2 using the same number of cycles and the same thermal profile to obtain a 738 bp DNA fragment. In addition, to amplify a 428 bp amplicon to detect the BVDV strains NADL and UK the primers BD1/BD3 and BD1/BD4 were employed, respectively. All oligonucleotide primers were obtained from a commercial source (Bioneer, INC., Korea).

PCR was carried out in a total volume of 30 µL containing 3 µL of 10 x PCR buffer, 0.5 µL of dNTPs (0.16 mM), 1 µL of cDNA, 1 µL of each primer (10 pmol), 1.2 µL of $MgCl_2$ (2 mM), 0.3 µL of Taq DNA polymerase (1.5 U) and 22 µL of DNase/RNase free distilled water. Reactions were performed in an automated thermal cycler (Bio-Rad gradient Thermal Cycle).

Cycle parameters for PCR were as follows: initially 95°C for 5 min followed by thirty-five cycles in 3 continuous phases including 94°C for 30 s, 55°C for 100 s, and 72°C for 2 min, and finally terminated by a single cycle of a final extension at 72°C for 10 min. The RT-PCR-amplified products were examined by electrophoresis in a 1.5% agarose gel, stained with a 1% solution of ethidium bromide and visualized using a UV transilluminator.

*Sequencing of PCR products*

To determine the genotypes of BVDV and sequencing, the PCR products obtained with the primers BD1/BD2 (372-1109 based on the strain BVDV-1 SD-19 with the accession number: KR866116) and BD1/BD3 (372-799 as well) were delivered to Bioneer/Korea. Alignment of the sequences and phylogenetic trees were realized using the Internet Software Multalin with hierarchical clustering method (Corpet, 1988).

## Histopathology and IHC procedures

Tissue samples of skin biopsies were fixed in 10% netural buffered formalin, routinely processed, and stained with hematoxylin and eosin (H&E) for light microscope examination. Consecutive sections to those used for the histopathological examination were subjected to IHC analysis.

The slides were deparaffinized in xylol, rehydrated, and treated with 3% hydrogen peroxide solution for 10 min at room temperature to quench the endogenous peroxides. The antigen retrieval was conducted by pretreatment by microwaving (power 100 for 10 min; then, power 20 for 20 min) using a 10-mmol/L concentration of citrate buffer (pH = 6.0).

The primary antibody (anti-BVDV monoclonal antibody, VMRD.INC) was applied for 1 h (diluted 1:100). The detection system used was Envision+ (DakoCytomation, Glostrup, Denmark) and developed with diaminobenzidine (DakoCytomation). Diaminobenzidine-hydrogen peroxide was applied as the chromogen. The slides were then counterstained with Mayer's haematoxylin, dehydrated, and coverslipped.

## Results

In the initial screening, a single RT-PCR method employing the panpestivirus reactive 324/326 primers amplified a 288 bp DNA fragment from 5 of the analyzed pooled samples (each pool contained 20 samples). To identify the BVDV infection in each cow, all the samples in the positive pools were tested individually. Therefore, the nested RT-PCR (outer primers 0I100/1400R and inner primers BD1/BD2) was successful in 16 out of 400 (4%) buffy coats samples in the initial screening (Fig. 1, Table 1). Eight animals that were positive in the initial screening were also found positive by RT-PCR on buffy coats, ACE on serum and also ACE and IHC on ear notch biopsies in the next step sampling. No histopathological lesion was found in the skin samples but the positive immunoreactivity for BVDV as brown, coarsely to finely granular within the cytoplasm of basal and spinosum cells, keratinocytes, hair follicles epithelial cells and a small number of subcutaneous stromal cells, particularly endothelial cells of vasculature were found in these animals (Figs. 2 and 3). The PCR, employing the primers (BD1/BD3&BD1/BD4) showed that two samples were BVDV NADL.

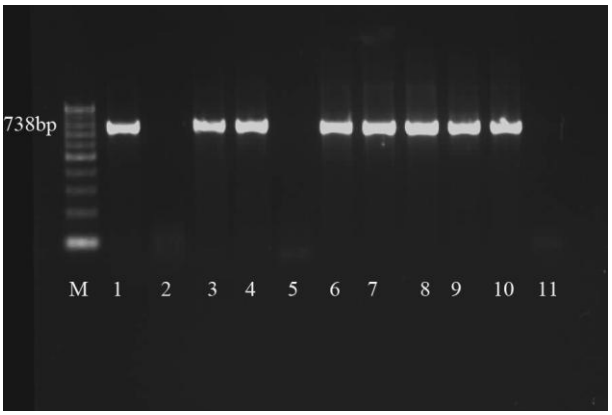



**Fig. 1:** The final products of the nested RT-PCR on buffy coat samples. M is 100 bp ladders, 1, 3, 4, 6, 7, 8, 9 are positive samples, 2 and 5 are negative samples, 10 is positive control, and 11 is blank. The amplicon size was 738 bp

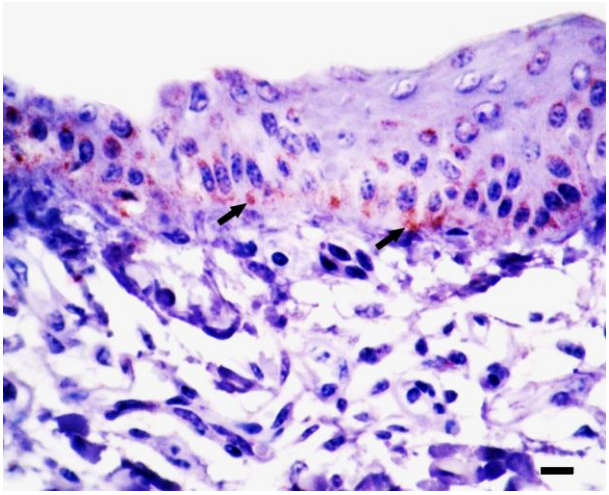


**Fig. 2:** Ear-notch skin from a persistently infected calf. Immunopositivity for BVDV as finely brown cytoplasmic granules are observed in the cytoplasm of basal cells (arrows) and other cells of epidermis, (IHC, bar = 100 µm)

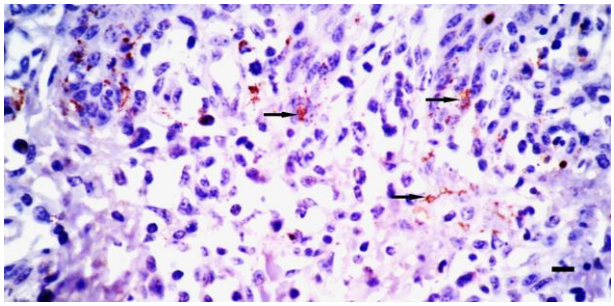
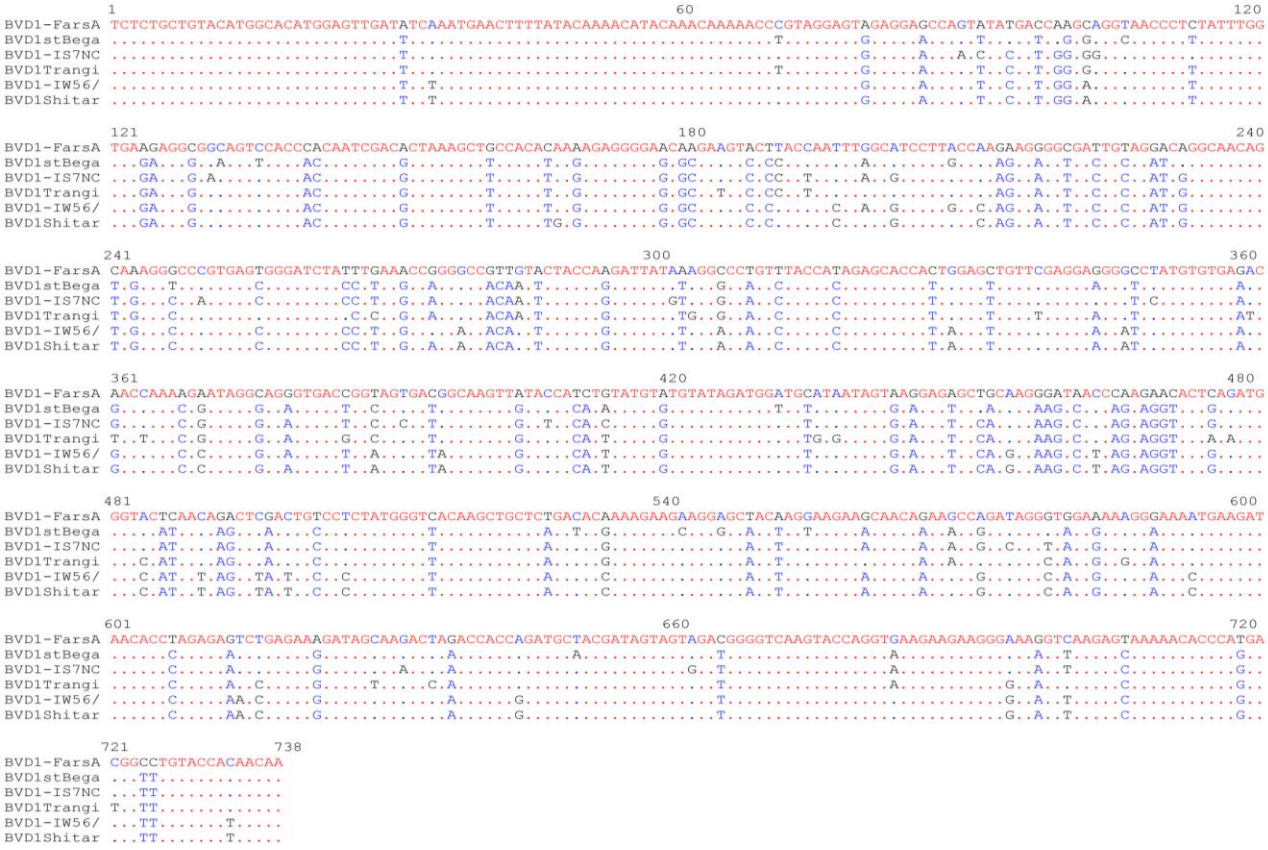


**Fig. 3:** Ear-notch skin from a persistently infected calf. Positive immunoreaction for BVDV antigen are seen in the cytoplasm (arrows) of subcutaneous stromal cells, (IHC, Bar = 100 µm)

**A**


1 60 120
BVD1-FarsA TCTCTGCTGTACATGGCACATGGAGTTGATATCAAATGAACTTTTATACAAAACATACAAACAAAAACCCGTAGGAGTAGAGGAGCCAGTATATGACCAAGCAGGTAACCCTCTATTTGG
BVD1stBega
BVD1-IS7NC
BVD1Trangi
BVD1-IW56/
BVD1Shitar
121 180 240
BVD1-FarsA TGAAGAGGCGGCAGTCCACCCACAATCGACACTAAAGCTGCCACACAAAAGAGGGGGAACAAGAAGTACTTACCAATTTGGCATCCTTACCAAGAAGGGGCGATTGTAGGACAGGCAACAG
BVD1stBega
BVD1-IS7NC
BVD1Trangi
BVD1-IW56/
BVD1Shitar
241 300 360
BVD1-FarsA CAAAGGGCCCGTGAGTGGGATCTATTTGAAACCGGGGCCGTTGTACTACCAAGATTATAAAGGCCCTGTTTACCATAGAGCACCACTGGAGCTGTTCGAGGAGGGGCCTATGTGTGAGAC
BVD1stBega
BVD1-IS7NC
BVD1Trangi
BVD1-IW56/
BVD1Shitar
361 420 480
BVD1-FarsA AACCAAAAGAATAGGCAGGGTGACCGGTAGTGACGGCAAGTTATACCATCTGTATGTATGTATAGATGGATGCATAATAGTAAGGAGAGCTGCAAGGGATAACCCAAGAACACTCAGATG
BVD1stBega
BVD1-IS7NC
BVD1Trangi
BVD1-IW56/
BVD1Shitar
481 540 600
BVD1-FarsA GGTACTCAACAGACTCGACTGTCCTCTATGGGTCACAAGCTGCTCTGACACAAAAGAAGAAGGAGCTACAAGGAAGAAGCAACAGAAGCCAGATAGGGTGGAAAAAGGGAAAATGAAGAT
BVD1stBega
BVD1-IS7NC
BVD1Trangi
BVD1-IW56/
BVD1Shitar
601 660 720
BVD1-FarsA AACACCTAGAGAGTCTGAGAAAGATAGCAAGACTAGACCACCAGATGCTACGATAGTAGTAGACGGGGTCAAGTACCAGGTGAAGAAGAAGGGAAAGGTCAAGAGTAAAAACACCCATGA
BVD1stBega
BVD1-IS7NC
BVD1Trangi
BVD1-IW56/
BVD1Shitar
721 738
BVD1-FarsA CGGCCTGTACCACAACAA
BVD1stBega
BVD1-IS7NC
BVD1Trangi
BVD1-IW56/
BVD1Shitar

**B**


1 60 120
BVD1-FarsB TCTCTGCTGTACATGGCACATGGAGTTGATCACAAATGAACTTTTATATAAAACATACAAACAAAAACCCACTGGAGTGGAGGAACCAGTCTACGACCAAGCTGGTAACCCTTTGTTTGG
BVD1-799nc
BVD1-iso12
BVD1-839nc
BVD1-CC13B
BVD1-isol9
BVD1-isoAU
121 180 240
BVD1-FarsB AGAAAGAGGAGAGATTCATCCGCAGTCAACGCTAAAACTGCCACATAAAAGAGGGGAGCGTGAAGTCCCCACCAATTTGGCTTCTTTACCAAAAAGAGGTGACTGCAGGTCAGGTAATAG
BVD1-799nc
BVD1-iso12
BVD1-839nc
BVD1-CC13B
BVD1-isol9
BVD1-isoAU
241 300 360
BVD1-FarsB CAAGGGACCTGTGAGTGGAATTTATTTAAAACCAGGGCCACTATTCTACCAGGATTATAAAGGACCCGTCTATCACAGAGCCCCATTGGAGTTTTTTGAAGAGGCGTCTATGTGTGAGAC
BVD1-799nc
BVD1-iso12
BVD1-839nc
BVD1-CC13B
BVD1-isol9
BVD1-isoAU
361 420 480
BVD1-FarsB AACTAAAAGAATTGGGAGAGTAACTGGTAGTGACAGCAAATTATACCACATTTATGTATGTATTGACGGGTGCATAATAGTTAAGAGTGCTACAAGAGATCACCAAAAAGTACTCAAATG
BVD1-799nc
BVD1-iso12
BVD1-839nc
BVD1-CC13B
BVD1-isol9
BVD1-isoAU
481 540 600
BVD1-FarsB GGTTCACAACAAGCTAAACTGCCCCCTATGGATTTCAAGCTGCTCCGACACAAAGGAGGAGGGGGCGGTGAGAAAGAAGCAACAAAAGCCAGATAGGGTGGAAAAGGGGAGAATGAAGAT
BVD1-799nc
BVD1-iso12
BVD1-839nc
BVD1-CC13B
BVD1-isol9
BVD1-isoAU
601 660 720
BVD1-FarsB AACACCTAAGGAGTCAGAGAAAGATAGTAAGACCAAGCCACCAGATGCCACAATAGTGGTAGATGGAGTCAAGTATCAGGTTAAGAAAAAGGAAAAGTCAAGAGCAAGAATACCCAGGA
BVD1-799nc
BVD1-iso12
BVD1-839nc
BVD1-CC13B
BVD1-isol9
BVD1-isoAU
721 738
BVD1-FarsB CGGCCTGTACCACAACAA
BVD1-799nc
BVD1-iso12
BVD1-839nc
BVD1-CC13B
BVD1-isol9
BVD1-isoAU

**C**


**Fig. 4:** Alignment of nucleotide sequences of the isolates with those published for BVDV. A: Alignment of nucleotide sequences from the BVD1-FarsA, B: Alignment of nucleotide sequences from the BVD1-FarsB, and C: Alignment of nucleotide sequences from the BVD2-FarsA

**Table 1:** Details of regional distribution, comparative results of used tests in initial and next samplings and isolated genotypes of BVDV in this study. NO: Number, and BC: Buffy coat

| Farm number | Region | Lnitial | Sampling | After 3 weeks RT-PCR (Bc) | Next sampling | | | | Isolated genotype |
|---|---|---|---|---|---|---|---|---|---|
| | | No. of samples | RT-PCR (Bc) | | No. of samples | ACE (serum) | ACE (ear skin) | IHC (ear skin) | |
| 1 | Naghsh rostam | 20 | 2 | 1 | 20 | 1 | 1 | 1 | BVDV1/FarsA |
| 2 | Marvdasht | 20 | 0 | 0 | 0 | 0 | 0 | 0 | - |
| 3 | Marvdasht | 20 | 1 | 0 | 0 | 0 | 0 | 0 | - |
| 4 | Zarghan | 29 | 4 | 2 | 20 | 2 | 2 | 2 | BVDV1/FarsA |
| 5 | Zarghan | 27 | 3 | 2 | 20 | 2 | 2 | 2 | BVDV1/FarsA, BVDV2/FarsA |
| 6 | Zarghan | 35 | 2 | 2 | 20 | 2 | 2 | 2 | BVDV1/FarsB, BVDV2/FarsA |
| 7 | Sepidan | 25 | 0 | 0 | 0 | 0 | 0 | 0 | - |
| 8 | Shiraz | 23 | 0 | 0 | 0 | 0 | 0 | 0 | - |
| 9 | Shiraz | 29 | 0 | 0 | 0 | 0 | 0 | 0 | - |
| 10 | Kheramah | 66 | 1 | 0 | 0 | 0 | 0 | 0 | - |
| 11 | Beiza | 69 | 2 | 1 | 20 | 1 | 1 | 1 | BVDV1/FarsA |
| 12 | Shiraz | 37 | 1 | 0 | 0 | 0 | 0 | 0 | - |
| Total | | 400 | 16 | 8 | 100 | 8 | 8 | 8 | |

Animals that were positive only in the RT-PCR in the first step but negative in all tests of the second step were regarded as acutely infected. Animals that were positive in both steps were considered as persistently infected.

Eight PCR products (PI animals) for N (pro) region were successfully sequenced. Genetic sequence analysis showed that 6 of 8 PI cattle were infected with the genotype BVDV-1 and 2 of them were infected with the genotype BVDV-2.

All strains were amplified with BD1/BD2 and BD1/BD3 primers. Alignment of nucleotide sequences of the isolates was identified as BVDV1-FarsA (5), BVDV1-FarsB (1) and BVDV2-FarsA (2) in comparison to those published for BVDV in GenBank. The sequences alignments of these BVDV-1 and BVD-2 isolates are presented in Figs. 4A-C, respectively. The results of this study are summarized in Table 1.

A phylogenetic tree was constructed as shown in Fig.

5. Although BVDV1-FarsA and BVDV1-FarsB were divided to two clades in the tree, they have close genetic proximity to each other.

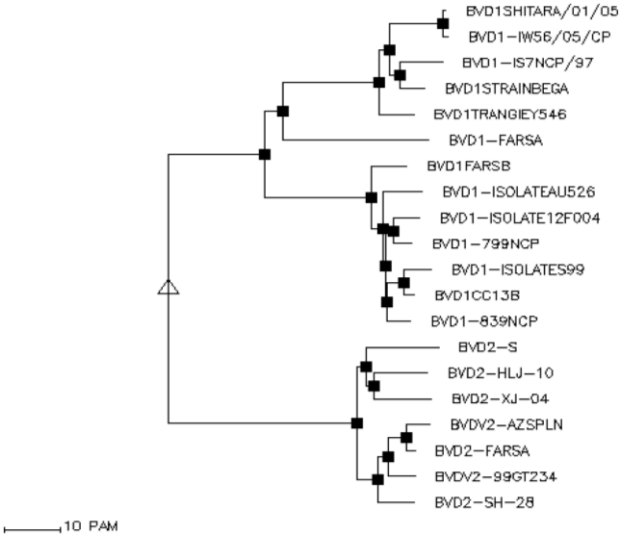



**Fig. 5:** Phylogenic tree of BVDV isolates detected in the PI cattle of Fars province/Iran and some related isolates obtained from GenBank

## Discussion

Studies investigating the molecular epidemiology of BVDV can provide invaluable information about the diversity of viral strains present in a population and, in turn, inform control programs, drive vaccine development and determine likely infection sources (Booth *et al*., 2013). In this study, the predominant BVDV sub-genotype identified on the five of eight infected farms was BVDV-1 FarsA. BVDV-1 FarsB was isolated from one farm. Also, genotype BVDV2 was identified during this study on the two infected farms. Isolated occurrences of types 1 and 2 BVDV have been reported in many countries (Courtenay *et al*., 2007; Booth *et al*., 2013; Strong *et al*., 2013).

Several tests are currently used by diagnostic laboratories to detect BVD PI cattle. Each method to detect BVDV has advantages, disadvantages, and applicability for different diagnostic situations. The results of the present study indicate that IHC, ACE, and RT-PCR can all be reliable tests for the detection of BVDV in PI cattle. In diagnosis of BVDV, IHC is a popular technique because of the convenience of sample collection, and reliability (Cornish *et al*., 2005). Even ACE is a quick and reliable method, but false negative results by test serum samples in young calves before 30 days of age and also in some cases false positive results can be obtained (Hilbe *et al*., 2007).

In the present study, at least 4% BVDV infection in examined dairy populations was almost consistent with the other reports in the dairy herds of European and North American countries (Drew *et al*., 1999; Graham *et al*., 2001; Kim and Dubovi, 2003).

Eradication programs against BVD are based on the strict control of cattle movements and on the elimination of persistently infected animals from herds. The experience with eradication program against the disease in a number of countries has shown that the RT-PCR method is a useful tool to reveal persistently infected animals in cattle herds (Falcone *et al*., 2003; Hurtado *et al*., 2003).

Because PI animals often are less than 1% of animals within the herd, pooling strategies to detect BVDV PI cattle are popular alternatives because of reduced testing fees (Radwan *et al*., 1995; Kennedy *et al*., 2006). In the present study, the RT-PCR assay was used for the detection of BVDV in bovine buffy coat pooled. Each buffy coat sample within a positive pool was retested individually to identify the BVDV-infected animals. This method reduces the testing cost per cow and provides the opportunity for widespread participation in BVD surveillance and control programs.

The ACE has several advantages over the other diagnostic tests. Antigen capture ELISA can be performed within hours of skin specimen collection, permitting the feedlot veterinarian and manager to make decisions on biosecurity and control. Calves with positive ACE results can be isolated while additional samples are being collected and submitted for further testing. Antigen capture ELISA gives results for individual cattle, whereas serum samples used for RT-PCR assays are pooled. Although RT-PCR assay is useful for detection of PI cattle if results are negative, further testing is required for all individual samples in the pool if results are positive. Therefore, additional time would be required to identify PI cattle.

An ear-notch skin sample is a convenient tissue for detection of bovine viral diarrhea virus (BVDV) persistently infected (PI) cattle because it is easy to collect and requires minimal supplies and equipment. Ear-notch skin also offers the flexibility of testing by IHC, RT-PCR and ACE. All three tests have high sensitivity and specificity for detecting PI cattle (Njaa *et al*., 2000; Grooms and Keilen, 2002; Cornish *et al*., 2005; Fulton *et al*., 2006), and none appear to be negatively affected by passively acquired antibodies when performed on skin samples.

Immunohistochemistry on skin biopsies for PI animal's identification has been used as a parallel test to antigen ELISA and RT-PCR. In present study, results of 400 buffy coat samples examined by RT-PCR were in agreement and correlated among the two antigen detection methods (IHC and ACE). Based on the IHC results, the virus had a tropism for epithelial cells, vascular endothelial and dermal cells, similar to previous studies (Haines *et al*., 1992; Njaa *et al*., 2000; Cornish *et al*., 2005; Khodakaram-Tafti and Miller, 2006; Luzzago *et al*., 2006; Hilbe *et al*., 2007; Bedekovic *et al*., 2011).

With IHC method persistently infected animals in a herd can be easily detected and eradicated (Hilbe *et al*., 2007). The results of this study have shown that IHC staining for BVDV in formalin-fixed, paraffin-embedded skin is an effective method for the diagnosis of PI cattle. Immunohistochemistry has been shown to be an accurate

method for detection of neonatal PI calves and occasionally acute infections (Njaa *et al*., 2000; Brodersen, 2004; Cornish *et al*., 2005). The use of IHC in BVD diagnosis offers several advantages including reduced time for making a diagnosis and the capability to make the diagnosis (Allan *et al*., 1989; Haines and Chelack, 1991; Haines *et al*., 1992). Results of this study indicated that serological and molecular tests can be successfully replaced by IHC in diagnosis of PI cattle, although the number of samples was limited. IHC and ACE both showed high sensitivity for detecting BVDV infected calves (Cornish *et al*., 2005). Current IHC and ACE tests are useful for early detection and removal of PI calves, but should not be used as the only means to eradicate BVDV within a large population of cattle (Larson *et al*., 2005). In this study, the RT-PCR and ACE identified infected cows and calves in initial screening. Also, the results were subsequently reconfirmed by IHC and ACE performed on ear notch biopsies. On the basis of comparative results of ACE, IHC and RT-PCR assays, all tests detected nearly 100% of PI animals.

In one study involving 59 PI Angus calves, skin biopsy samples (ear notches) were collected and the results of IHC and ACE were compared. Both IHC and ACE detected almost all of the PI calves (Cornish *et al*., 2005). Therefore, the accuracy of IHC in detecting BVDV PI animals was confirmed in the present study, and ACE using ear notch samples was also an equivalent method for diagnosis of persistent infections. The results of this study showed IHC or ACE on skin biopsies are good, fast and sensitive methods for detection of persistently infected animals of BVDV infection in dairy herds. Accurate detection and elimination of PI cattle is essential for controlling the transmission of virus. Considering that persistently infected cattle are the main source of the virus, it is crucial to identify and remove these animals from the herd (Houe, 1999; Lindberg and Alenius, 1999; Mainar-Jaime *et al*., 2001).

In present study, the sequence analysis of the samples showed higher identity with BVDV. Phylogenetic tree analysis of the BVDV detected from the PI cattle and BVDV sequences obtained from Genbank revealed that they belonged to both genotypes, BVDV-1 and BVDV-2. Genotypes BVDV1 (6) and BVDV2 (2) isolates were further analyzed by sequencing the amplification DNA fragment. These results are in agreement with other reports that BVDV infections in most cases belonged to genotype 1 (Cornish *et al*., 2005; Booth *et al*., 2013; Strong *et al*., 2013). Recently, phylogenetic analysis of 5´ UTR region of viral RNA extracted from 316 blood samples of infected cattle in England and Wales demonstrated existence of five subtypes of BVDV-1 (a, b, e, f, i) and in comparison, only three subtypes (a, b, i) were detected in 1999, resulting in increase of genetic diversity of BVDV-1 subtypes during the past 10 years (Strong *et al*., 2013). Also, in another investigation, phylogenetic and nucleotide sequence analysis of 5´ UTR and Npro regions of 104 isolates from six different regions of the United Kingdom identified five subtypes of BVDV-1 (a, b, e, i, d), of which BVDV-1a was the predominant subgenotype (Booth *et al*., 2013).

In conclusion, for the first time, genetic sequence analyses showed genotypes BVDV-1 and BVDV-2 exist in dairy herds of this area. The new isolates were identified as BVDV1-FarsA, BVDV1-FarsB and BVDV2-FarsA in the phylogenetic tree. Since both genotypes of the virus are present in the region, our findings emphasize the importance of monitoring BVDV infection in cattle and suggest early detection and elimination of PI animals for control and eradication of BVDV in the dairy herds of Fars province.

## Conflict of interest

The authors declare no conflict of interest.

## References

**Alenius, S; Lindberg, A and Larsson, B** (1996). A national approach to the control of bovine viral diarrhoea virus. *Proceedings of the Third ESVV Symposium on the Control of Pestivirus Infections*. Lelystad, The Netherlands, September, 1996. PP: 162-169.

**Allan, GM; McNulty, MS; Bryson, D; Mackie, D and Platten, M** (1989). Demonstration of bovine virus diarrhea virus antigen in formalin fixed, paraffin embedded tissue using a streptavidin biotin technique. Res. Vet. Sci., 46: 416-418.

**Baker, JC** (1995). The clinical manifestations of bovine viral diarrhea infection. Vet. Clin. N. Am. Food Anim. Pract., 11: 425-445.

**Becher, P; Orlich, M; Kosmidou, A; Konig, M; Baroth, M and Thiel, HJ** (1999). Genetic diversity of pestiviruses: identification of novel groups and implication for classification. Virology. 262: 64-71.

**Bedekovic, T; Lemo, N; Lojkic, I; Beck, A; Lojki, M and Madic, J** (2011). Implementation of immunohisto-chemistry on frozen ear notch tissue samples in diagnosis of bovine viral diarrhea virus in persistently infected cattle. Acta Vet. Scand., 5: 53-65.

**Bhudevi, B and Weinstock, D** (2003). Detection of bovine viral diarrhea virus in formalin fixed paraffin embedded tissue sections by real time RT-PCR (Taqman). J. Virol. Methods. 109: 25-30.

**Booth, RE; Thomas, CJ; El-Attar, LM; Gunn, G and Brownlie, J** (2013). A phylogenetic analysis of Bovine Viral Diarrhea Virus (BVDV) isolates from six different regions of the UK and links to animal movement data. Vet. Res., 44: 43-57.

**Brock, KV; Grooms, DL and Givens, MD** (2005). Reproductive disease and persistent infections. In: Goyal, SM and Ridpath, JF (Eds.), *Bovine viral diarrhea virus: diagnosis, management and control*. (1st Edn.), Blackwell Publishing Professional. PP: 145-156.

**Brock, KV; Grooms, DL; Ridpath, J and Bolin, SR** (1998). Changes in levels of viremia in cattle persistently infected with bovine viral diarrhea virus. J. Vet. Diagn. Invest., 10: 22-26.

**Brodersen, BW** (2004). Immunohistochemistry used as a screening method for persistent bovine viral diarrhea virus infection. Vet. Clin. North Am. Food Anim. Pract., 20: 85-93.

**Canal, CW; Hotzel, I; de Almeida, LL; Roehe, PM and**

**Masuda, A** (1996). Differentiation of classical swine fever virus from ruminant pestiviruses by reverse transcription and polymerase chain reaction. Vet. Microbiol., 48: 373-379.

**Carman, S; van Dreumel, T; Ridpath, J; Hazlett, M; Alves, D; Dubovi, E; Trembla, R; Bolin, S; Godkin, A and Anderson, N** (1998). Severe acute bovine viral diarrhea virus infection in Ontario, 1993-1995. J. Vet. Diagn. Invest., 10: 27-35.

**Cornish, TE; van Olphen, AL; Cavender, JL; Edwards, JM; Jaeger, PT; Vieyra, LL; Woodard, LF; Miller, DR and O'Toole, D** (2005). Comparison of ear notch immunohistochemistry, ear notch antigen-capture ELISA, and buffy coat virus isolation for detection of calves persistently infected with bovine viral diarrhea virus. J. Vet. Diagn. Invest., 17: 110-117.

**Corpet, F** (1988). Multiple sequence alignment with hierarchical clustering. Nucl. Acids Res., 16: 10881-10890.

**Courtenay, AE; Henderson, RG; Crawell, MP and Sandvik, T** (2007). BVD virus type 2 infection and severe clinical disease in a dairy herd. Vet. Rec., 160: 706-707.

**Deregt, D; Carman, PS; Clark, R; Burton, KM; Olson, WO and Gilbert, SA** (2002). A comparison of polymerase chain reaction with and without RNA extraction and virus isolation for detection of bovine viral diarrhea virus in young calves. J. Vet. Diagn. Invest., 14: 433-437.

**Doll, K and Holsteg, M** (2013). BVD virus type 2 outbreak in Germany. http://bvd-day2013.eu/wp-content/uploads/2013/09/26-En-Doll_Holsteg_BVDV-2-Outbreak-Germanyx.pdf.

**Drew, TW; Yapp, F and Paton, DJ** (1999). The detection of bovine viral diarrhoea virus in bulk milk samples by the use of a singletube RT-PCR. Vet. Microbiol., 64: 145-154.

**Dubovi, EJ** (1996). Laboratory diagnosis of bovine viral diarrhea virus infections. Vet. Med., 91: 867-872.

**Falcone, E; Cordioli, P; Tarantino, M; Muscillo, M; La Rosa, G and Tollis, M** (2003). Genetic heterogeneity of bovine viral diarrhoea virus in Italy. Vet. Res. Communic., 27: 485-494.

**Farjani Kish, GH; Khodakaram-Tafti, A and Mohammadi, A** (2013). Serological survey of bovine viral diarrhea virus by antigen capture ELISA in dairy herds in Fars province, Iran. Bulgarian J. Vet. Med., 16: 217-222.

**Fulton, RW; Hessman, B; Johnson, BJ; Ridpath, JF; Saliki, JT; Burge, LJ; Sjeklocha, D; Confer, AW; Funk, RA and Payton, ME** (2006). Evaluation of diagnostic tests used for detection of bovine viral diarrhea virus and prevalence of subtypes 1a, 1b, and 2a in persistently infected cattle entering a feedlot. J. Am. Vet. Med. Assoc., 228: 578-584.

**Goyal, SM** (2005). Diagnosis. In: Goyal, SM and Ridpath, JF (Eds.), *Bovine viral diarrhea virus: diagnosis, management, and control*. (1st Edn.), Ames, IA, Blackwell Publishing. PP: 197-208.

**Graham, DA; German, A; McLaren, IE and Fitzpatrick, DK** (2001). Testing of bulk tank milk from Northern Ireland dairy herds for viral RNA and antibody to bovine viral diarrhoean virus. Vet. Rec., 149: 261-265.

**Grooms, DL and Keilen, ED** (2002). Screening of neonatal calves for persistent infection with bovine viral diarrhea virus by immunohistochemistry on skin biopsy samples. Clin. Diagn. Lab. Immunol., 9: 898-900.

**Haines, DM and Chelack, BJ** (1991). Technical considerations for developing enzyme immunohistochemical staining procedures on formalin-fixed paraffin-embedded tissues for diagnostic pathology. J. Vet. Diagn. Invest., 3: 101-112.

**Haines, DM; Clark, EG and Dubovi, EJ** (1992). Monoclonal antibody based immunohistochemical detection of bovine viral diarrhea virus in formalin-fixed, paraffin-embedded tissues. Vet. Pathol., 29: 27-32.

**Heinz, F; Collett, M; Purrchell, R; Gould, E; Howard, C; Houghton, M; Moorman, R; Rice, C and Theil, HJ** (2000). Family Flaviviridae. In: van Regnmortel, MHV; Fanqute, CM; Bishop, DHL; Carstens, EV; Estes, MK; Lemon, SM; Manilott, J; Mayo, MA; McGeoch, DJ; Pringle, CR and Wickner, RB (Eds.), *Virus taxonomy, Proceedings of the Seventh Report of International Committee on Taxonomy of Viruses*. Academic Press. PP: 859-878.

**Hilbe, M; Stalder, H; Peterhans, E; Haessig, M; Nussbaumer, M; Egli, Ch; Schelp, Ch; Zlinszky, K and Ehrensperger, F** (2007). Comparison of five diagnostic methods for detecting bovine viral diarrhea virus infection in calves. J. Vet. Diagn. Invest., 19: 28-34.

**Houe, H** (1999). Epidemiological features and economical importance of bovine viral diarrhoea virus (BVDV). Infect. Vet. Microbiol., 64: 89-107.

**Hurtado, A; Garcia-Perez, AL; Aduriz, G and Juste, RA** (2003). Genetic diversity of ruminant pestiviruses from Spain. Virus Res., 92: 67-73.

**Kennedy, JA; Mortimer, RG and Powers, B** (2006). Reverse transcription-polymerase chain reaction on pooled samples to detect bovine viral diarrhea virus by using fresh ear-notch-sample supernatants. J. Vet. Diagn. Invest., 18: 89-93.

**Khodakaram-Tafti, A and Miller, L** (2006). The comparative evaluation of cellular localization of viral antigens with microscopic changes in the ileum of cattle infected with bovine viral diarrhea. Comp. Clin. Pathol., 15: 90-93.

**Khodakaram-Tafti, A; Mohammadi, A and Farjani Kish, GH** (2015). Histopathological and immunohistochemical findings from bovine viral diarrhea virus infection in cattle. Online J. Vet. Res., 19: 317-324.

**Kim, SG and Dubovi, EJ** (2003). A novel simple one-step single-tube RT-duplex PCR method with an internal control for detection of bovine viral diarroea virus in bulk milk, blood, and follicular fluid samples. Biologicals. 31: 103-106.

**Kozasa, T; Tajima, M; Yasutomi, I; Sano, K; Ohashi, K and Onuma, M** (2005). Relationship of bovine viral diarrhea virus persistent infection to incidence of diseases on dairy farms based on bulk tank milk test by RT-PCR. Vet. Microbiol., 106: 41-47.

**Larson, RL; Miller, RB; Kleiboeker, SB; Miller, MA and White, BJ** (2005). Economic costs associated with two testing strategies for screening feeder calves for persistent infection with bovine viral diarrhea virus. J. Am. Vet. Med. Assoc., 226: 249-254.

**Letellier, C; Kerkhofs, P; Wellemans, G and Vanopdenbosch, E** (1999). Detection and genotyping of bovine viral diarrhea virus by reverse transcription-polymerase chain amplification of the 50 untranslated region. Vet. Microbiol., 64: 155-167.

**Lindberg, A and Alenius, S** (1999). Principles for eradication of bovine viral diarrhoea virus (BVDV) infections in cattle populations. Vet. Microbiol., 64: 197-222.

**Lindberg, A and Houe, H** (2005). Characteristics in the epidemiology of bovine virus diarrhoea virus (BVDV) of relevance to control. Prev. Vet. Med., 72: 55-73.

**Luzzago, C; Frigerio, M; Tolari, F; Mazzei, M; Salvadori, C; Del Piero, F and Arispici, M** (2006). Indirect immunohistochemistry on skin biopsy for the detection of persistently infected cattle with bovine viral diarrhoea virus in Italian dairy herds. New Microbiol., 29: 127-131.

**Mainar-Jaime, RC; Berzal-Herranz, B; Arias, P and Rojo-Vazquez, FA** (2001). Epidemiological pattern and risk factors associated with bovine viral-diarrhoea virus (BVDV) infection in a nonvaccinated dairy-cattle population from the Asturias region of Spain. Prev. Vet. Med., 52: 63-73.

**Mirchamsy, H; Shafyi, A and Bahrami, M** (1970). The occurrence of bovine virus diarrhea-mucosal disease in Iran. Arch. Inst. Razi. 22: 197-201.

**Nagai, M; Sato, M; Nagano, H; Pang, H; Kong, X; Murakami, T; Ozawa, T and Akashi, H** (1998). Nucleotide sequence homology to bovine viral diarrhea virus 2 (BVDV 2) in the 50-intranslated region of BVDVs from cattle with mucosal disease or persistent infection in Japan. Vet. Microbiol., 60: 271-276.

**Nettleton, PF and Entrican, G** (1995). Ruminant pestiviruses. Br. Vet. J., 151: 615-642.

**Njaa, BL; Clark, EG; Janzen, E; Ellis, JA and Haines, DM** (2000). Diagnosis of persistent bovine viral diarrhea virus infection by immunohistochemical staining of formalin-fixed skin biopsy specimens. J. Vet. Diagn. Invest., 12: 393-399.

**Paton, DJ** (1995). Pestivirus diversity. J. Comp. Pathol., 112: 215-236.

**Pellerin, C; van den Hurk, J; Lecomte, J and Tijssen, P** (1994). Identification of a new group of bovine viral diarrhea virus strains associated with severe outbreaks and high mortalities. Virology. 203: 260-268.

**Radwan, GS; Brock, KV; Hogan, JS and Smith, KL** (1995). Development of a PCR amplification assay as a screening test using bulk milk samples for identifying dairy herds infected with bovine viral diarrhea virus. Vet. Microbiol., 44: 77-91.

**Ridpath, J; Bolin, SR and Dubovi, EJ** (1994). Segregation of bovine viral diarrhea virus into genotypes. Virology. 205: 66-74.

**Saliki, JT and Dubovi, EJ** (2004). Laboratory diagnosis of bovine viral diarrhea virus infections. Vet. Clin. N. Am. Food Anim. Pract., 20: 73-75.

**Sandvik, T** (2005). Selection and use of laboratory diagnostic assays in BVD control programmes. Prev. Vet. Med., 72: 3-16.

**Sedighinejad, S** (1996). A survey on bovine viral diarrhea-mucosal disease in Iran. Res. Cons. 30: 128-131.

**Strong, R; Errington, J; Cook, R; Ross-Smith, N; Wakeley, P and Steinbach, F** (2013). Increased phylogenetic diversity of bovine viral diarrhea virus type 1 isolates in England and Wales since 2001. 23: 315-320. doi: 10.1016/j.vetmic.2012.09.006.

**Tajima, M; Kirisawa, R; Taguchi, M; Iwai, H; Kawakami, Y; Hagiwara, K; Ohtsuka, H; Sentsui, H and Takahashi, K** (1995). Attempt to discriminate between bovine viral-diarrhea virus strains using polymerase chain reaction. J. Vet. Med., 42: 257-265.

**Thür, B; Hilbe, M; Strasser, M and Ehrensperger, F** (1997). Immunohistochemical diagnosis of pestivirus infection associated with bovine and ovine abortion and perinatal death. Am. J. Vet. Res., 58: 1371-1375.

**Vilcek, S; Herring, AJ; Herring, JA; Nettleton, PF; Lowings, JP and Paton, DJ** (1994). 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., 136: 309-323.

**Vilcek, S; Nettleton, PF; Paton, DJ and Belak, S** (1997). Molecular characterization of ovine pestiviruses. J. Gen. Virol., 78: 725-735.

**Vilcek, S; Paton, DJ; Durkovic, B; Strojny, L; Ibata, G; Moussa, A; Loitsch, A; Rossmanith, W; Vega, S; Scicluna, MT and Palfi, V** (2001). Bovine viral diarrhoea virus genotype 1 can be separated into at least eleven genetic groups. Arch. Virol., 146: 99-115.

**Wittum, TE; Grotelueschen, DM; Brock, KV; Kvasnicka, WG; Floyd, JG; Kelling, CL and Odde, KG** (2001). Persistent bovine viral diarrhoea virus infection in US beef herds. Prev. Vet. Med., 13: 83-94.

**Wolfmeyer, A; Wolf, G; Beer, M; Strube, W; Hehnen, HR; Schmeer, N and Kaaden, OR** (1997). Genomic (50-UTR) and serological differences among German BVDV field isolates. Arch. Virol., 142: 2049-2057.