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 (Patton, 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., 1995), Germany (Wolfmeier 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; Thir et al., 1997; Brodersen, 2004; Saliki and Dubovi, 2004; Cornish et al., 2005; Sandvik, 2005; Hibbe 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., 1976; Sedighinejad, 1996). More recently, a preliminary study by ACE on primary sera samples of the present study showed BVDV 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). Pooleduffy 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™ 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’SUTR (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 GTA GCA-3′) and 326 (5′-TCA ACT CAG TTT GCC ATG TAC-3′) to detect pestivirus infection as described by Vilecek et al. (1994). In individual samples, amplification of cDNAs by PCR was performed using the primer pairs 01 100 (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 AGT TAC ATG GCA CAT G-3′)/BD2 (5′-TTG TCA ATG TAC ACC CCG TC-3′) (nested PCR) as described by Vilecek et al. (1997) and BD1/BD3 (5′-CCA TCT ATR CAC ACA TAA ATC TGG TAC-3′) and BD1/BD4 (5′-CCA TCC AGC CAT AGC TAG ATG TG-3′) to detect the strains of BVDV as described by Vilecek et al. (2001). In the nested PCR, in the first PCR, the outer primers 01 100 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 MgCl2 (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% neutral 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 01l00/1400R 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.
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[www.SID.ir](http://www.SID.ir)
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

<table>
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<tr>
<th>Farm number</th>
<th>Region</th>
<th>Initial No. of samples</th>
<th>RT-PCR (Bc)</th>
<th>After 3 weeks RT-PCR (Bc)</th>
<th>Next sampling No. of samples</th>
<th>ACE (serum)</th>
<th>ACE (ear skin)</th>
<th>IHC (ear skin)</th>
<th>Isolated genotype</th>
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<td>1</td>
<td>Naghsh rostam</td>
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<td>2</td>
<td>1</td>
<td>20</td>
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<tr>
<td>2</td>
<td>Marvdasht</td>
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<td>BVDV1-FarsA</td>
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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.

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 (Courtenant 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 buffalo coy pocketed. Each buffalo 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 buffalo 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-Taufi 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, phylgenetic 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.

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