

## Short Communication

# Detection of bovine viral diarrhea virus using a nested RT-PCR assay in bulk milk samples of dairy cattle herds in suburb of Mashhad-Iran

Massoud Talebkhan Garoussi\*, Mohammad Reza Bassami, Seyed Ehsan Afshari

Department of Clinical Sciences, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad,  
P.O. Box 91775-1793, Mashhad, I.R. Iran

## Abstract

Bovine viral diarrhoea virus (BVDV) is an important pathogen of dairy cattle. In this study, bulk milk samples representing a total of 4105 milking cows, from 18 dairy cattle herds in the suburb of Mashhad- Iran, were tested for presence of BVDV by the use of a nested reverse transcription polymerase chain reaction (Nested RT- PCR) assay. Non of the cows in the herds had been vaccinated against BVDV. RNA was extracted from somatic cell pellets of bulk milk tank samples. Oligonucleotide primers were selected based on the 5' untranslated region of the BVD virus genome. BVD virus was detected in 2 (11.1%) out of 18 samples, representing 742 lactating cows. These results indicate that nested RT-PCR analysis of bulk milk samples may provide a rapid and sensitive screening method for the detection of BVDV infections in non-vaccinated dairy cattle herds.

**Keywords:** BVD virus; Nested RT- PCR; Bulk milk; cattle.

Bovine viral diarrhoea virus (BVDV), a member of *Pestivirus* genus of the family *Flaviviridae*, is an important pathogen of dairy cattle. It causes multiple clinical syndromes including bovine viral diarrhoea, mucosal disease and fetal infection, leading to major widespread economic losses. This *Pestivirus* is classified into two biotypes designated as non-cytopathic (NCP) and cytopathic (CP), depending on their effect on tissue cell culture. The NCP biotype is most commonly isolated in the field.

It replicates in cultured cells without inducing cytopathic effects. The virus cross the placenta to establish a persistent and a lifelong infection. In contrast, the CP biotype induces apoptotic cell death in culture cells causing CPE (Zhang *et al.*, 1996). Postnatal infection is transient and is followed by the development of long lasting antibodies. Prenatal infection can result in the birth of immunotolerant and persistently infected (PI) individuals. The PI animals generally remain lifelong virus carriers, shedding large quantities of virus in most bodily excretions and secretions. They are therefore a very significant reservoir and the main source of infection for other non-infected cattle. That is why identification and culling of such cows is an important element of many BVD control strategies (Harkness, 1987; Alenius *et al.*, 1997). BVDV infection was detected by Sohrab in Iran in 1966 (Mirshamsy *et al.*, 1970). The BVD virus infection was determined in Khorassan province using a serum neutralisation (SN) test by Mirshamsy and colleagues in 1970.

In the past, detection of PI animals was based on virus isolation in individual animals, sometimes accompanied by serological assays. Due to necessity of having a fast and a cheap serological assay, the enzyme-linked immunosorbent assay (ELISA) was developed (Radwan *et al.*, 1995). This assay for the detection of viral antigens (Ag) has made testing fast and somehow cheaper. More recently, selective strategies have been employed, involving antibody tests on bulk milk or sera in infected cattle. If a group of cattle are antibody negative, these can indicate whether a PI animal is present or not. However, an antibody positive bulk milk test does not necessarily mean that a PI animal is still present, since anti-BVDV antibodies can

\*Correspondence to: Massoud Talebkhan Garoussi, DVM, Ph.D.  
Telefax: +98 511 8763852  
E-mail: garoussi@ferdowsi.um.ac.ir

persist for many years after removal of all PI cattle. Nor does a low value indicate absence of a PI animal, since it can have been recently introduced. A reverse transcriptase-polymerase chain reaction (RT-PCR) technique has previously been described for the detection of BVDV in tissues (Belak and Ballagi Pordany, 1991) and in cell cultures (Hertig *et al.*, 1991). More recently, a combined RT-PCR has been described for its detection in whole blood and tissues (Hamel *et al.*, 1995) and has also been applied to the detection of PI animals in milking herds, through examination of somatic cells from bulk milk (Radwan *et al.*, 1995).

Mashhad Suburb in the Khorassan Razavi province is a major producer of livestock in east of Iran. Bulk milk sampling is straightforward, and a large number of adult cattle can be screened simultaneously. This reduces the need for individual blood testing of the milking cows in a herd, which should only be necessary in the case of a virus positive bulk milk test result. However, the purpose of this study was: to develop and evaluate a screening and complementary test to detect virus in bulk milk present in the bulk milk tanks of dairy cattle herds in the suburb of Mashhad-Iran.

Eighteen Holstein dairy cattle herds were sampled in suburb of Mashhad-Iran. The population of herds varied from 32 up to 680 milking cows. Vaccination against BVDV had not been practiced in these dairy herds. Within six months, a bulk milk sample was obtained from each herd. Bulk milk samples from herds were collected in 500 ml volumes in sterile bottles after finishing the whole milking process. The samples were transported directly to the laboratory, on ice.

Each bulk milk sample was divided into 3 equal parts in polypropylene tubes. The samples were centrifuged at 3500  $\times g$  for 15 min at 4°C and the cream and milk supernatants were removed. The cell pellets of all 3 equal parts of each sample were pooled in a 50 ml of sterile Falcon. They were washed in 25 ml of ice cold 0.01 M phosphate buffered saline (PBS) and centrifuged at 600  $\times g$  for 10 min at room temperature. The cell pellets were then resuspended in 1.5 ml of PBS transferred to a 1.5 ml tube and centrifuged at room temperature as before. After centrifugation, the supernatants were removed and somatic cell pellets were

resuspended in 140  $\mu l$  of PBS. Somatic cell samples were then stored at -70°C till RNA extraction.

Total RNA was extracted from somatic cell pellet samples using Trizol reagent following directions instructed by the manufacturer. Briefly, 1 ml of reagent was used to homogenize cell pellet samples. Following chloroform extraction and isopropanol precipitation, RNA was resuspended in 15  $\mu l$  of Diethylpyrocarbonate (DEPC) treated water. To evaluate the quality of the extracted RNA, 5  $\mu l$  of RNA was electrophoresed through agarose gel. The gel was stained with ethidium bromide and visualized on a gel documentation apparatus.

The PCR and nested-PCR oligoprimers sequences are presented in Table 1. The nested primers amplified a 290 base pair (bp) fragment from the 5' non-coding region of all pestiviruses (Trevor *et al.*, 1999) (Table 1).

Total RNA (3  $\mu l$ ) was mixed with 1.5  $\mu l$  ml of reverse primer (10  $\mu M$  /  $\mu l$ ) and incubated at 70°C for 5 min followed by chilling on ice. The rest of the reaction mixture contained 4  $\mu l$  of 5X first strand buffer, 2  $\mu l$  of dNTPs (10 mM), 20 U of RNasin (20 U/ $\mu l$ ), 200U (200 U/ $\mu l$ ) of Moloney Murine Leukemia Virus (M-MuLV) (Fermentas) and 7.5  $\mu l$  d-H<sub>2</sub>O was added, followed by an incubation at 42°C for 60 min cDNA synthesis was terminated by incubation at 70°C for 10 min. PCR was performed in a 25  $\mu l$  reaction mix. The final concentration of the reagent was as follows: PCR buffer (1 $\times$ time) (Cinagen, Iran), dNTP 0.2 mM), MgCl<sub>2</sub> (1.5 mM), each primer (0.5  $\mu M$ ), *Taq* DNA polymerase (0.625). Reactions were performed in an automated thermal cycler (Bio-Rad gradient Thermal Cycle). Cycle parameters for PCR were as follows: one cycle at 95°C for 5 min followed by thirty five cycles in 3 continuous phases including 94°C for 30 sec, 55°C for 100 sec, and 72°C for 2 min, and finally terminated by a single cycle of a final extension at 72°C for 10 min and stored at 4°C till the nested-PCR tests.

Nested-PCR was also performed in a 25  $\mu l$  reaction mix containing 2.5  $\mu l$  of 10X PCR buffer (Cinagen, Iran), 1.5 mM MgCl<sub>2</sub>, 200  $\mu M$  of dNTPs, 0.5  $\mu M$  of each nested primer, 0.625 U of *Taq* DNA polymerase (5 U/ $\mu l$ ), and 2  $\mu l$  of diluted PCR product (diluted 1 in

**Table 1.** Oligonucleotide primers used for PCR.

Primer	Direction	Sequence
PCR primer F1	Forward	5'- CAT- GCC-CCT-AGT-AGG-ACT-AGC-3'
PCR primer R1	Reverse	5'- TCA-ACT-CCA-TGT-GCC-ATG-TAC-3'
Nested primer F2	Forward	5'- AGG-GTA-GTC-GTC-AGT-GGT-TCG-3'
Nested primer R2	Reverse	5' CTC-TGC-AGC-ACC-CTA-TCA-3'

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10 µl distilled water) and 16.125 µl of d-H<sub>2</sub>O. Reactions were also performed in the same automated thermal cycler. Conditions for thermal cycler in nested RT-PCR procedure were as follows: one cycle at 95°C for 5 min, thirty five cycles in 3 continuous phases which included: 94°C for 30 sec, 58°C for 100 sec, and 72°C for 2 min, a final extension at 72°C for 10 min. The PCR products were then stored at 4°C until gel electrophoresis were performed.

The positive control was the BVD virus which was isolated from cell culture. The sterilized d-H<sub>2</sub>O was used as the negative control.

The nested PCR products were separated on 3% agarose gel. The agarose gels contained 10 µg/ml of ethidium bromide to allow visualization of the products under an ultraviolet transilluminator.

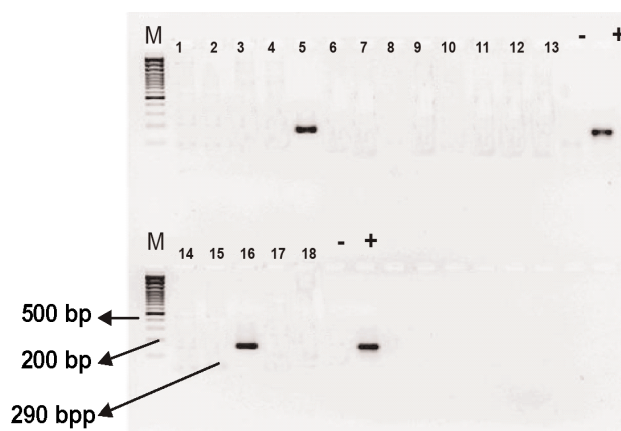
The intensity of the ribosomal RNA bands together with the smear of mRNA indicated the quality and the integrity of the isolated RNA. As no internal control target was used in this study, the integrity of RNA extracted was evaluated as a quality control measure.

The nested RT-PCR products were considered positive if a specific band of the expected size (290 bp) was observed after electrophoresis (Figure 1). Since, no results were obtained by RT-PCR, therefore, only the visualized results of nested RT-PCR are shown.

The BVD virus genome was detected by nested RT-PCR in 2 (11.1%) out of 18 bulk milk samples. In nested RT-PCR positive herds, the number of animals contributing to the milking process on the day ranged from 30 to >250 dairy cows (Table 2).

Confirmation of the sensitivity was not performed. A more direct quantitative determination of the sensitivity of the method is therefore required.

This is the first herd-level survey of BVDV using bulk tank milk samples by nested RT-PCR in Iran. The selection of herds in this study was not based on randomization and we can not expect to generalize this prevalence from our samples. Using nested RT-PCR on somatic cells from bulk milk, 2 (11.1%) of 18 case herds were found to be virus positive. The investiga-



**Figure 1.** Nested RT-PCR product visualized after agarose gel electrophoresis. Arrows show 290 bp fragments. Negative (-) and positive (+) control samples are indicated. M= 100 bp ladder.

tion of the influence of herd size on BVDV bulk tank distribution revealed that only one small and one large herd size were infected by BVDV, respectively (Table 2). These results show that it can be found, at its minimum, a recent or an ongoing viremic most likely due to presence of PI milking cows which can distribute the BVD virus in the herds (Trevor *et al.*, 1999).

The infection status of a herd may be established by testing the bulk milk sample for BVDV by RT-PCR amplification (Radwan *et al.*, 1995). These types of assays are best used as an initial screening test to identify herds in which further diagnostic test should be performed to identify the source of virus spread (i.e PI cows). In some situations, it is difficult to convince herd owners that the expense of herd testing is warranted; therefore, positive identification of BVDV by preliminary PCR screening from a bulk milk sample would give good justification for further herd test by virus detection. Although the PCR bulk milk test could not be used to rule out BVDV infection, a positive result would provide useful information. Positive PCR results would be weighted more heavily than negative results. The use of this assay may be most beneficial as a method of focusing on or justifying BVDV-positive

**Table 2.** Distribution of Bulk tank milk BVD virus by herd size in the dairy cattle herds in suburb of Mashhad-Iran.

BVD virus	Herd size (cow numbers)			Total
	Small 30-100 (%)	Medium 101-250 (%)	Large >250	
+	1 (12.5%)	4(100%)	1(16.6%)	2(11.11%)
-	7 (87.5%)	-	5(83.33%)	16(88.88%)
Total (herds)	8	4	6	18

herds for development of control strategies and not as a definitive test to ensure BVDV negative herd.

Naturally, comprehensive control and eradication of the infection will have to be based on: (1) identification of the herds with active infection; (2) clearance of shedders from the herds; and (3) infection control measures for all positive herds including both transmission to herds previously without infection and reinfection of herds cleared of the infection. Effective tests for detection of both active and earlier infection in animals and herds are essential.

Since only lactating dairy cows were screened, other methods such as ELISA should be done to determine the true presence of PI animals among dry cows and other categories of stock such as calves, heifers and bulls. It is envisaged that the RT-PCR bulk milk method could be used in conjunction with antibody tests to help establish herd status with respect to BVDV, because there is a greater probability of finding PI animals in herds with high bulk milk antibody levels (Houe *et al.*, 1995). Therefore, bulk milk samples could first be tested by Ab ELISA, and only those with a high level of antibody can be tested by the RT-PCR procedure. However, a nested RT-PCR test on pooled bloods might be one way of screening these animals.

In contrast, the nested RT-PCR test on bulk milk has been demonstrated to have a potential value as a sensitive method for detection of virus positive lactating cows.

There is a need to quantify its sensitivity, both in terms of quantity of target RNA and in its ability to detect individual PI animals in herds of different sizes and composition. The latter will prove costly, since it requires a large amount of individual testing to accumulate the necessary data, so the best approach would be to run the test in parallel with conventional methods in areas where control or eradication programmes are running. Once validated, its employment in strategies for BVDV control will lead to a reduction in cost of tests without compromising the efficacy of the strategy. However, if eradication is the aim, antibody status of individual animals may still be necessary, in order to provide a comprehensive picture of the situation with regard to BVDV within the herd.

It can be concluded that BVDV infection is an important disease in dairy cattle herds of Mashhad suburb, Iran. These herds have had a recent or an ongoing infection the likely due to presence of PI animals (Houe and Meyling, 1991). These results indicate that nested RT-PCR analysis of bulk milk samples may

provide a rapid and sensitive screening method for the detection of BVDV infections in non-vaccinated dairy cattle herds. They also indicate that BVDV may be highly prevalent in dairy cattle herds in suburb of Mashhad-Iran.

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