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. None 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 the 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 crosses 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
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 bulk milk tanks and evaluate a screening and complementary test to

Table 1. Oligonucleotide primers used for PCR.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Direction</th>
<th>Sequence</th>
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</thead>
<tbody>
<tr>
<td>PCR primer F1</td>
<td>Forward</td>
<td>5′- CAT- GCC-CCT-AGT-AGG-ACT-AGC-3′</td>
</tr>
<tr>
<td>PCR primer R1</td>
<td>Reverse</td>
<td>5′- TCA-ACT-CCA-TGT-GCC-ATG-TAC-3′</td>
</tr>
<tr>
<td>Nested primer F2</td>
<td>Forward</td>
<td>5′- AGG-GTA-GTC-GTC-AGT-GGT-TGG-3′</td>
</tr>
<tr>
<td>Nested primer R2</td>
<td>Reverse</td>
<td>5′- CTC-TGC-AGC-ACC-CTA-TCA-3′</td>
</tr>
</tbody>
</table>
10 \mu l distilled water) and 16.125 \mu l of d-H_2O. 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_2O was used as the negative control.

The nested PCR products were separated on 3% agarose gel. The agarose gels contained 10 \mu g/ml of ethidum bromide to allow visualization of the products under an ultraviolet transiluminator.

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 (11.11%) of 18 case herds were found to be virus positive. The investigation 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

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

<table>
<thead>
<tr>
<th>BVD virus</th>
<th>Small 30-100 (%)</th>
<th>Medium 101-250 (%)</th>
<th>Large &gt;250</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>1 (12.5%)</td>
<td>4 (100%)</td>
<td>1 (16.6%)</td>
<td>2 (11.11%)</td>
</tr>
<tr>
<td>-</td>
<td>7 (87.5%)</td>
<td>-</td>
<td>5 (83.33%)</td>
<td>16 (88.88%)</td>
</tr>
<tr>
<td>Total</td>
<td>8</td>
<td>4</td>
<td>6</td>
<td>18</td>
</tr>
</tbody>
</table>

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**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.
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 sheddors 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.

References


