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REGULAR ARTICLES

Investigation of persistent infection of bovine viral diarrhea virus (BVDV) in Holstein dairy cows

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Abstract

The aim of this study was to investigate the persistent infection (PI) of bovine viral diarrhea virus (BVDV) along with its coexistence between BVDV antibody titer and BVD virus in blood of Holstein dairy cows. Only large commercial farms (each contained < 1000–3000 unvaccinated cows) were included. There were 11 dairy cattle herds. They included nearly 20,000 dairy cows. Totally, 140 cows, > 3 months to almost 10 years old, were randomly sampled. Indirect enzyme-linked immunosorbent assay (ELISA) and reverse transcription-polymerase chain reaction (RT-PCR) were used to detect BVDV antibody and virus, respectively. The percent positivity (PP) < 14 and ≥ 14 values are interpreted negative and positive, respectively. Simultaneously, whole blood samples pooled in groups of 10 animals were used for molecular detection of BVDV. The results revealed that 138 (98.56%) out of 140 cows were positive for BVDV antibody, while the BVDV antigen was detected only in 2 (1.42%) cows, which were negative for BVDV antibody and so were considered as persistent infection (PI) cows. They were also retested 3 weeks apart. Since the results showed the strong coexistence between seropositivity and BVD virus, in the infected dairy cattle herds, the combination of simple ELISA and pooled whole blood RT-PCR strategy could be an achievable approach to detect PI animals.

Keywords ELISA · PCR · BVDV · Persistent infection

Introduction

Bovine viral diarrhea virus (BVDV) is a single-standard RNA virus which belongs to the genus *Pestivirus* and family of *Flaviviridae*. BVDV is one of the most common pathogens in dairy herds (Vilcek and Nettleton 2006). It causes huge economic losses such as drop in milk yield, decreased reproductive performance, immune-suppression, and congenital defective calf (Talebkhan Garoussi 2007; Moennig et al. 2005). Also, birth of persistent infection (PI) calves is very important consequences of this disease which are crucial for the spread of the virus. This virus is classified into two biotypes designed as non-cytopathic (NCP) and cytopathic (CP) depending on their effect on tissue culture cells (Talebkhan Garoussi and Mehrzad 2011; Denise Goens 2002).

The NCP biotype is most commonly isolated in the field. It replicates in cultured cells without inducing cell death and crosses the placenta to establish a persistent and lifelong infection if the fetus is infected in the first 125 days of gestation and survives after birth (Constable et al. 2017; Grooms 2004; Munoz-Zanzi et al. 2003). BVD virus can cause bovine reproductive disorders that can severely affect the developing embryo and fetus (Constable et al. 2017; Talebkhan Garoussi and Mehrzad 2011; VanLeeuwen et al. 2010). Postnatal infection by NCP BVDV is transient and is followed by the development of long-lasting antibodies. Prenatal infection can result in the birth of immunotolerant and PI individuals. The PI animals generally remain lifelong virus carriers, shedding large quantities of virus in most bodily excretions and secretions. PI animals 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

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element of many BVD control strategies (Harkness 1987; Alenius et al. 1997).

In contrast, the CP biotype induces apoptotic cell death in culture cells causing cytopathic effect (CPE) (Zhang et al. 1996); this biotype is usually encountered in association with clinical cases of mucosal disease (MD). Although CP BVDV is able to cross the placenta and infect the fetus, it does not establish PI and, therefore, does not have a mechanism to maintain its presence within the herd (Brownlie et al. 1989; McClurkin et al. 1984). The implementation of a program to control the infection must be based on, firstly, the identification of animals and herds either free of infection or the presence of active infection, secondly, the clearance of virus shedder(s) from the infected herds and thirdly, control measures to prevent the transmission of the virus within herds (Graham et al. 2014; Stahl and Alenius 2012; Presi and Heim 2010; Talebkhan Garoussi et al. 2008, 2009; Lindberg and Houe 2005).

The main objective of this study was to investigate the coexistence between BVDV antibody (Ab) titer and BVD virus using ELISA and RT-PCR in blood of Holstein dairy cows to specifically detect the PI cows.

Materials and methods

Sampling and dairy herd management

In the present study, 11 large industrial dairy farms of Holstein breed were included, the herds' sizes were about < 1000–3000 dairy cows, animals were from 3 months to 10 years old (4 years old on average), all animals were housed in intensive systems, and almost 20,000 cows were covered. Approximately 60% of the herds used the free-stall system, and cows were typically fed alfalfa, corn silage, and concentrate in various proportions using the Totally Mixed Ration (TMR) system. Cows were milked three times daily using milking machines. Newborn calves were kept in individual boxes for 2–3 months. Male and female calves were reared separately in an intensive system. All dairy cows and heifers were artificially inseminated. All cows within the herds were not vaccinated against BVDV.

Five farms with 900 milking cows each referred reproductive problems. One herd referred mastitis problem and monster case in recent calving.

Nutrition and reproduction management of the herds were controlled using computerized herd health management.

Randomized blood samples were taken from dairy cattle herds according to a proportional geographical distribution in various parts of the suburb of Mashhad, Iran, using a lottery method to select individual animals (Rogan and Gladen 1978). The sample size required estimating the seroprevalence of BVDV in the population of herds with level of confidence of 95%, desired absolute precision 5%, and expected prevalence of 90% was at least 138 dairy cows using the relevant formula as follows (Thrusfield 2005):

$$n = \frac{(1.96)^2 \times P_{\text{exp}} \times \left(1 - P_{\text{exp}}\right)}{d^2}$$

where n, P_{exp}, and d were the required sample size, expected prevalence, and desired absolute precision, respectively.

Therefore,

$$n = \frac{(1.96)^2 \times (0.9) \times (1 - 0.9)}{(0.05)^2} = 138$$

Totally, 140 Holstein dairy cows were included randomly in this study. At the beginning of sampling, the owners of the herds did not state the aims for not having bias. However, after sampling, they were informed for assurance and continuing the related affairs of the study.

Assays

ELISA and RT-PCR were used to detect BVDV antibody and virus, respectively. Serum samples were used individually to detect antibodies against BVDV and determine percent positivity (PP) value by means of indirect ELISA. Samples were assayed using commercial indirect ELISA-kit (SVANOVA Biotech AB, Uppsala, Sweden) in which microtiter plates were coated with BVDV Ag. They were used for the detection of antibodies against BVDV in serum samples according to the procedure of the manufacturer and validated protocol. Before the interpretation of the results, all optical density (OD) values were corrected by subtracting the ODs for the control Ag from the samples' ODs (OD sample − OD control = OD corrected). The result could be read visually where the OD was measured at 450 nm. The PP values were evaluated. All corrected OD values for the test samples as well as the negative control are related to the corrected OD values of the positive control as follows:

$$\text{PP} = \frac{\text{Test sample or negative control (OD corrected)}}{\text{Positive control (OD corrected)}} \times 100$$

As per the manufacturer, the PP < 14 and ≥ 14 values were interpreted negative and positive, respectively.

Simultaneously, whole blood samples were pooled in groups of 10 animals for Ag detection using RT-PCR.

After positive results, another RT-PCR was performed as pool in seropositive samples, the remaining two groups of 10 animals each were then divided into four groups with a pool of five blood samples in each for another RT-PCR to finally

reach the individual cow results. The molecular detection of BVDV (Talebkhan Garoussi et al. 2007) was performed by RNA extraction from blood samples, and then cDNA was made using random hexamer primer. The primers were designed using CLC main workbench software (CLCbio Co., Aarhus, Denmark) for BVDV with specific R and F sequences, annealing temperature, and amplicon size (Fig. 1). After optimization of PCR reaction for the designed BVDV primers, the annealing temperature of 61 °C was used. Each PCR reaction was done in a 25-μl final volume containing specific R and F primers (each 1 μl, final concentration of 10 pM), 5 μl PCR master mix, 1 μl of cDNA template, and 17 μl ddH2O. The PCR reaction for BVDV detection was carried out in Thermocycler (Bioer GenePro Thermal Cycler) with cycling program, including holding 5 min at 94 °C followed by cycling 45 times at 94 °C (20 s), 61 °C (30 s), and 72 °C (20 s) for each temperature with one cycle at 72 °C (10 min). Finally, the PCR product was detected and confirmed by agarose gel electrophoresis (Fig. 1).

The primers were designed using CLC main workbench software (CLCbio Co., Aarhus, Denmark) for BVDV with specific R and F sequences, annealing temperature, and amplicon size (Fig. 1). The locations of the primers and sequences are as follows: forward primer 99 5′-AGGC TAGCCATGCCCTTAGT-3′ and 5′-TCTGCAGCACCCTA TCAGG-3′ reverse primer 342.

Results

The results revealed that 97.14% (136/138) of tested cows had PP > 14 values in ELISA test (i.e., positive serological results) and only 1.44% of cows (2/138) presented PP < 14 values

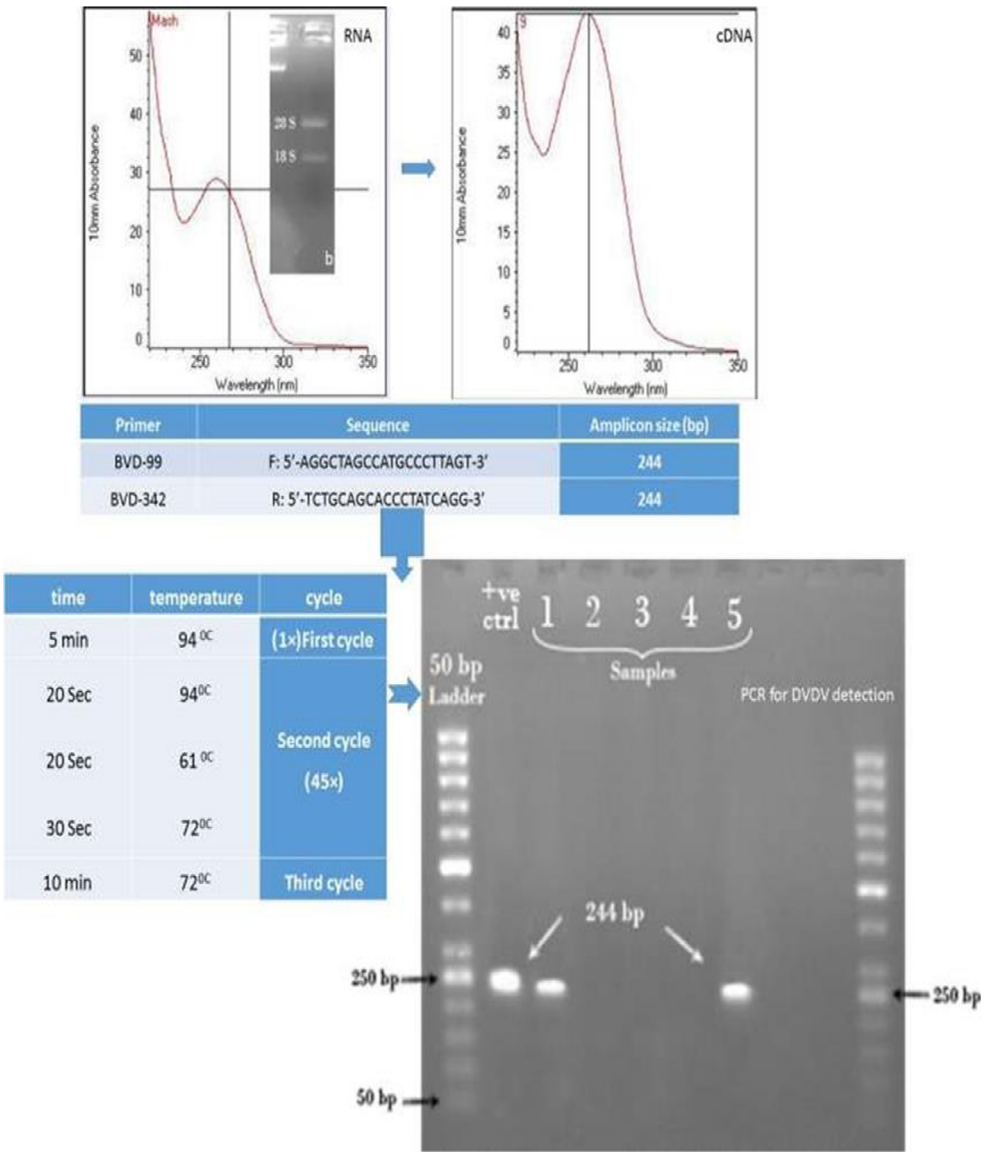

Fig. 1 Molecular analyses for detection of BVDV in blood samples of dairy cows. Representative figure of spectrophotometric nanodrop for confirmation of quantity and quality of extracted RNA from leukocytes (upper left, with insert agarose gel electrophoresis of RNA), cDNA (upper right), specific primers designed for BVDV (centered table), PCR condition program (lower left) with annealing temperature of 61 °C and PCR products in 1.5% agarose gel electrophoresis (lower right) from 140 screened samples of this study. The specific 244-bp BVDV PCR products/bands are seen in blood samples from two cows in lanes 1 and 5

(i.e., negative serological results). Between seropositive cows, 60.02% (83/138) presented 15–125 PP values (Table 2).

Regarding the herd size, 71.01% (98/138) of seropositive cows were from the herd with 1001–2000 dairy cows and a herd size of less than 1000, and over 2000–3000 dairy cows presented 13.76% (19/138) and 15.21% (21/138) seropositive cows, respectively (Table 2).

Only 2 of the 138 studied cows (18.18%) out of 11 herds presented negative on serological test and positive on PCR test for BVDV, simultaneously, thus indicating PI condition of these animals (Fig. 1). PI cows were aged almost 6 years old and during their lifetime, they calved four times and were detected in herd sizes of 1001–2000 and 2001–3000 dairy cows. For confirmation of PI condition, animals were retested 3 weeks later, maintaining the same previous results in both tests, thus removing confounding inaccurate results with the possibility of these cows presenting a transient infection condition.

Discussion

In the present study, it was confirmed that 2 out of 11 (18.18%) randomly selected dairy cattle herds in the suburb of Mashhad, Iran, contained PI cows. Several other studies have also estimated the proportion of herds with PI cows. For example, a BVDV prevalence was estimated at 10% in Portugal, based on a finding that the probability of PI cattle being present was 20% for herds with antibody-positive bulk tank milk (BTM) samples and 4% for those with doubtful or negative BTM antibody test results (Niza-Ribeiro and Pereira 2004). However, the prevalence of PI cows ranged between 0 and 16% (Constable et al. 2017; Presi et al. 2011). It is shown that PI cows can have a normal life and may breed successfully with an apparently normal pregnancy and can persistently establish BVDV in several generations. Production of PI cows provides one of the major mechanisms for maintenance of the BVDV within the herds.

In the present study, indirect ELISA test demonstrated that 98.57% (138/40) of tested cows were seropositive (Tables 1 and 2) and RT-PCR-based assay detected that 1.42% (2/140)

Table 1 The frequency of BVDV infection according to percent positivity (PP) in indirect ELISA Holstein cows of 11 herds of industrial dairy in the suburb of Mashhad, Iran

Herd size	Percent positivity (PP)				Total
	< 14 (%)	15–75(%)	76–125(%)	> 125(%)	
< 1000	1(0.72)	6 (4.34)	11 (7.97)	1 (0/72)	19 (13.76)
1001–2000	–	18 (13.04)	61 (44.2)	19 (13.76)	98 (71.01)
2001–3000	1(0.72)	4 (2.89)	11 (7.97)	5 (3.62)	21 (15.21)
Total	2(1.44)	28 (20.28)	83 (60.14)	25 (18.11)	138

Table 2 Distribution of persistent infection (PI) cows according to ELISA and RT-PCR assays Holstein cows of 11 herds of industrial dairy in the suburb of Mashhad, Iran

Assay	Results		Number of tested animals
	Positive (%)	Negative (%)	
ELISA	138 (98.57%)	2 (1.42%)	140
RT-PCR	2 (1.42%)	138 (98.57%)	140

of PI cows aged approximately 6 years old were seropositive; to avoid errors about transient infections, these animals were retested 3 weeks apart for PI reconfirmation.

Since, the studied herds were not immunized against BVDV, therefore, the high prevalence of BVDV Ab within the herds can occur naturally. Natural infection in cattle is considered to be lifelong (Talebkhan Garoussi et al. 2011). High presence (98.57%) of seropositive dairy cows indicates that PI animals are present within the herds (Constable et al. 2017) with an average age of 4 years, suggesting the lifelong BVDV condition. In natural infection of seronegative cows, BVD virus does not cause sever disease; they would be TI animals, which remained with detectable antigens for many months (Niskanen and Lindberg 2003). Indeed, TI animals may be a source of horizontal infection, and a few reports suggest that BVDV may persist in a herd in absence of PI animals (Moennig et al. 2005; Moerman et al. 1993). In our study, TI animal(s) may be the source of seroprevalence within the herds. Therefore, serological response reflected natural infection. Research studies based on the BVDV Ab detection, either in individual animals or in bulk milk, have shown that the prevalence of infected herds ranged 70 to 100% (Taylor et al. 1995; Obando et al. 1999). It was shown that the prevalence of seropositive animals in herds with one or more PI animals was 87%; however, it was 43% in herds without PI animals (Houe 1999). Indeed, there is a possibility of the existence of PI and TI animals in the present study, and PI cows are more likely to shed BVD virus within the herds compared to TI cows. That is why in this study, the PI cows are the major cause of infection. Also, PI animals are young (Talebkhan Garoussi et al. 2011) and to avoid MD condition, they should be culled.

PI cattle are the main source for transmission of the BVDV (McClurkin et al. 1984). However, acutely infected cattle as well as other ruminants, either acutely or PI, may transmit the virus (Vilcek and Nettleton 2006). The prevalence of BVDV infections has been investigated in several cross-sectional studies as reviewed previously (Alenius et al. 1986; Alenius et al. 1997).

When infections of the fetus occur before approximately 125 days of gestation and before immunocompetence, the calf may be born PI (Constable et al. 2017). PI cows will shed large quantities of virus in all bodily fluids throughout their life (Coria and McClurkin 1978; McClurkin et al. 1984). Due to an impaired immunity caused by BVDV, the infected

animals will be susceptible to other infections, which partly explains the high mortality during young age, compared to non-infected calves (Houe 1992, 1999). Some PI animals, however, remain clinically unaffected and may breed successfully (McClurkin et al. 1979) and will then transmit the infection to the fetus, which will always be PI. In most bovine populations, the prevalence of PIs is estimated to be around 1%, although variation occurs (Houe 1995). In the present study, it was shown that the prevalence is more than 1% (1.42%) (Table 2).

Furthermore, PI animals are already young enough to give birth to further PI animal(s) (Constable et al. 2017; Talebkhan Garoussi et al. 2011). Two studies have shown that the BVD virus can circulate in a herd for 2–3 years despite no PI animals being present and no direct contact with PI cows occurring (Barber and Nettleton 1993; Moerman et al. 1993). Therefore, the actual prevalence of PI animals in this study may be higher than the obtained results.

In this study, the unvaccinated herd sizes are relatively large and testing every individual animal is neither logistically nor economically feasible for detection of PI cow(s). Although, testing strategies for pooled samples have been developed for efficiently replacing the unnecessary testing for all individuals (Kennedy et al. 2006).

Based on the obtained data, the BVDV seropositive cows present widely in industrial dairy cattle herds with different sizes in the suburb of Mashhad, Iran. Antibody screening would only provide information that BVD virus is circulating in the herds (Sayers et al. 2015). These herds have had a recent or an ongoing infection most likely due to the presence of PI animal(s) (Talebkhan Garoussi et al. 2007; Houe and Meyling 1991).

It is concluded that the prevalence of PI animals was not high. On the basis of the current and previous works, the high levels of seropositivity among dairy herds in this region could be attributed to the presence of PI animals that constantly shed the BVD virus into the herds. It was revealed that there is a coexistence between the prevalence of seroinfection and the presence of BVD virus within the herds. Overall, preventative measures using pooled whole blood samples for RT-PCR are recommended in order to minimize the laboratories detection charges and the economic losses caused by this disease, and, if possible, introduce another study that used the pool technique to identify PI animals in cattle herds.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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