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Reverse Transcriptase PCR Assay for Detection of *Bovine viral diarrhea virus* (BVDV) Infection in Iranian Bull's Semen Samples

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Abstract: *Bovine viral diarrhea virus* (BVDV) is a single-stranded RNA virus, belongs to the genus *Pestivirus* of the family *Flaviviridae*. This virus is an economically important infectious disease of cattle and significant agent to reduced milk production, decreased reproductive performance, growth retardation and increased occurrence of other diseases in cows. The purpose of present study was to detect BVDV RNA in Iranian bull's semen specimens that used for artificial insemination using RT-PCR assay. 172 bull's semen specimens were obtained from Iranian animal husbandries and artificial insemination centers and viral RNA was extracted from semen samples. Total RNA was reverse transcribed to cDNA using kit. The oligonucleotide primers were selected to amplified 5' untranslated region (UTR) of the BVDV genome and 290 base pair target sequence from BVDV cDNA was amplified by RT-PCR method. Then, amplified products were visualized in a 1% agarose gel electrophoresis. BVDV RNA was detected in 32 of the 172 (18.60%) of bull's semen specimens that used for artificial insemination in Iran. The results showed high presence of BVDV infection in Iranian bull's semen samples and RT-PCR analysis is a rapid and cost-effective method for the screening of viral infection in cattle. The present study indicated that semen samples are important transmission sources of BVD virus infection in Iranian cattle. These findings indicated that control and eradication programs such as vaccinations programs for prevent and reduce of economic loses of BVDV infection in Iranian cattle it seems to be necessary and fast detection and sensitivity of RT-PCR technique might be of relevance for studies on epidemiological and pathogenesis of infection with BVD virus.

Abbreviations: BVDV, *Bovine viral diarrhea virus*; UTR, untranslated region; RT-PCR, Reverse transcription PCR

Key words: *Bovine viral diarrhea virus* (BVDV) · RT-PCR · UTR region · Bulls · Semen · Artificial insemination

INTRODUCTION

Bovine viral diarrhea virus (BVDV) is a *pestivirus* in the family *Flaviviridae* and is closely antigenically related to Border Disease Virus (BDV) and Classical Swine Fever Virus (CSFV) [1]. BVDV mainly infects cattle but infections among other animals such as swine, sheep, goat and wild animals are also reported [2, 3]. There are four recognized species within the *pestivirus* genus. These species are BVDV-1, BVDV-2, BDV of sheep and CSFV, previously known as hog cholera virus and causes a contagious haemorrhagic disease of pigs of worldwide importance [4].

BVDV genome is a single stranded positive sense RNA of approximately 12.5 kb [5]. It has a single open reading frame (ORF) that is flanked by a non-coding region (NCR) at the 5' and 3' ends. The ORF is translated into a polyprotein of about 4000 amino acids, which is co- and post-translationally processed by cellular and viral proteases [6]. The mature viral proteins are arranged as follows: NH2-Npro, C, Erns, E1, E2, p7, NS2-3, NS4A, NS4B, NS5A and NS5B-COOH [7, 8].

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BVDV-1 and BVDV-2, which may be differentiated from each other and from other *pestiviruses* by monoclonal antibodies (MAb) directed against the E2 protein, or by genetic analysis of different regions of the genome [9-11]. Recently, an atypical isolate 'HoBi', which was found in a batch of pooled foetal calf serum, has been suggested to be of a third genotype of BVDV [12]. Whereas BVDV-1 is the dominant genotype found worldwide, BVDV-2 appears to prevail mainly in the US and Canada. In Europe, Asia and South America, BVDV-2 has been reported sporadically [13-15]. BVDV-2 infections have been associated with outbreaks of a severe acute infection that cause a haemorrhagic syndrome [16, 17]. However, subclinical infections are common with both genotypes. By molecular studies, BVDV-1 and BVDV-2 are further subdivided into at least 11 subtypes for BVDV-1 and 2 subtypes for BVDV-2 [4, 6].

BVDV infections appear to have a worldwide distribution. The prevalence of infection can be expressed in terms of antibody carriers or persistently infected (PI) animals. The prevalence of BVDV antibody positive animals is usually between 60 and 80% with the proportion of PI animals being around 1 to 2%. The prevalence of herds with current or recent infection often ranges from 70 to 100% [18, 19]. However, there are some differences between regions and countries, which may be related to differences in cattle densities, housing, vaccination and management systems, as well as animal trading activities. The herd level prevalence was estimated at less than 1% in 1993 in Finland and 30% in Norway [20]. A few studies have looked at the prevalence of herds with PI animals. A survey of all cattle in 19 Danish dairy herds with unknown status of BVDV infection showed that 10 herds (53%) had PI animals in varying numbers [21]. Another survey of 20 herds in the USA showed that 15% of the herds had PI animals [22, 23]. Vaccination had been carried out in 15 of the 20 American herds but in none of the Danish herds. In a study carried out in Germany, 45% of 329 herds had PI animals [24].

PI animals are considered the epidemiologically most important sources of infection excreting high amounts of virus continuously via nasal discharge, saliva, semen, urine, faeces, tears and milk. Serum samples diluted up to 106 may still yield the virus when examined by virus isolation techniques [25, 26]. In PI bulls, virus titers up to 10^7 have been found in semen [27]. PI animals have been shown to experimentally infect 60 to 63% of susceptible animals within 24 hours where a heifer and her PI calf were put in the same yard with these animals [28]. However, the rate of spread at pasture may be quiet low, in the order of 1%. Acutely infected animals may excrete the virus for 3 to 10 days but the amount of virus they excrete is low compared to PI animals with very little infection to susceptible animals resulting [15]. With the acute infection there is pyrexia, a leukopenia from about days 3 to 7 post infection and a mild nasal discharge. This is followed by a rise in antibodies, which peaks at 10 to 12 weeks post infection and immunity is assumed to last a lengthy period, possibly the animals' life time [29, 30].

The losses due to BVDV infections include reduced milk production, reduced conception rates and abortions, congenital defects and growth retardation after foetal infection, respiratory disorders, other diseases and even death among animals acquiring acute infection [23]. In addition, PI animals that result from foetal infection are often small and unthrifty, have increased susceptibility to other diseases and frequently succumb to mucosal disease (MD) [30]. The exact quantification of the clinical and pathological damages after infection in a population is difficult because most descriptions in the literature are based on selected clinical outbreaks and are thus not representative of the broad spectrum of the disease [18]. Only a few studies have examined the effect of BVDV in herds not selected on the basis of clinical outbreaks. Reviews of the clinical and pathological effects of BVDV infections and quantification of some of the damage have recently been published [22, 31]. An epidemiological study examining the effects of BVDV infection on the general health of cattle herds showed BVDV associated with increased risk of clinical mastitis, retained placenta, oestrus-stimulating treatments and longer calving intervals [32]. Calculations of the economic losses are complex and the losses in a herd from an outbreak vary according to the initial herd immunity, pregnancy status of the cow at the time of infection and the virulence of the infecting virus strain. Accordingly, calculations of economic losses in individual herd outbreaks may vary from a few hundred to several thousand dollars [18]. Estimates of economic losses from infection with highly virulent strains in a 500 cow dairy herd have ranged from 40000 to 100000 US$ per herd [17].

The 5' NCR, Npro, UTR, Erns, E2 and NS2/3 genome regions have been used to identify *pestiviruses*, by means of reverse transcription-polymerase chain reaction (RT-PCR) complemented by sequencing for phylogenetic analysis. In particular, the 5 ? NCR, UTR and Npro regions show a high degree of conservation among *pestiviruses*, making them suitable for detection of a broad range of viruses and for strain discrimination studies [33, 34]. The E2 protein is a highly genetically and antigenically variable glycoprotein and possesses neutralizing epitopes

and thus is used to study the molecular and serological diversity of BVDV [34].

Laboratory diagnosis is an important component in both the diagnosis and of BVDV infections [25]. Diagnosis based on clinical findings is difficult because the signs are usually non-specific and mild in acute infections. It is also very difficult to diagnose PI animals based on clinical observations [22, 35]. Only MD can be diagnosed on the basis of clinical and postmortem findings but this needs to be confirmed by isolation of CP BVDV from sites such as gastrointestinal tract [36].

Recently developed molecular techniques have provided many assays for BVDV diagnosis. Diagnosis of BVDV infection is based primarily on virus or antibody detection [3]. With the availability of BVDV-specific monoclonal antibodies, several different antigen capture enzyme-linked immunosorbent assays (ELISA) have been developed [25]. The test is rapid, takes about 2 days and is less costly as it does not involve cell cultures. ELISA methods have been widely used especially in the Nordic countries [3]. Whole blood collected in EDTA tubes from live animals is the most convenient sample for antigen detection by ELISA and spleen and lymph nodes tissues can be used post mortem. Compared to virus isolation, ELISA has relatively low sensitivity [13].

Polymerase chain reaction (PCR) involves the direct detection of nucleic acids of the viral genomic RNA. This requires that the RNA be extracted from samples before detection, which is very difficult to extract RNA from samples. The ability to detect BVDV RNA by reverse transcription polymerase chain reaction (RT-PCR) amplification of cDNA has been reported by many laboratories [30, 37]. This has advantages over virus isolation because of the lack of potential interference with neutralizing antibodies. Using RT-PCR amplification, BVDV can be detected up to 12 to 14 days post infection and in most cases RT-PCR can detect from 101 to 103-fold lower virus level than virus isolation techniques [37].

The objective of present study was to detection of *Bovine viral diarrhea virus* (BVDV) infection in Iranian bull's semen samples that used for artificial insemination using reverse transcription PCR (RT-PCR) assay.

MATERIALS AND METHODS

Semen Sampling: In this study, one hundred and seventy-two bull's semen specimens were obtained from Iranian animal husbandries and artificial insemination centers, between August 2010 and March 2011, being especially careful to avoid cross-contamination with bacteria present in the prepuce. Prior to taking the samples, the prepuce was washed with detergent, warm water and 1% benzalkonium chloride solution drying with sterile cotton. Semen samples were diluted according to standard procedures and sent to the Biotechnology Research Center of Islamic Azad University of Shahrekord Branch in refrigerated boxes. All semen specimens were stored at -70°C for further use.

Cell Culture for Preparation of BVDV Positive and Negative Controls: The BVDV-1 NADL reference strain and bovine turbinate (BT) cell culture were obtained from the Razi Institute (Karaj, Iran). The virus was propagated in BVDV free BT cells at 37°C in minimum essential medium (MEM) supplemented with 10% (V/V) fetal bovine serum (FBS). The BT cells and FBS were free of adventitious virus. The FBS was also free of antibodies against BVDV. Cells infected with the BVDV-1 isolate were incubated at 37°C and usually 48 hours after incubation the extensive cytopathic effect (CPE) was observed. The virus was harvested from infected cells after two cycles of freezing and thawing. Uninoculated cell culture media was used as the negative control.

RNA Extraction: Viral RNA was extracted from semen specimens using QIAamp viral RNA Mini Kit (QIAGEN, Crawley, UK) according to the manufacturer's protocol. Extracted RNA was immediately used or stored at -70°C until needed. The total viral RNA was measured at 260 nm optical density according to the method described by Sambrook and Russell [38].

Reverse Transcription -Polymerase Chain Reaction (RT-PCR): RNA was reverse transcribed to cDNA with a first strand cDNA synthesis kit (Fermentas, Germany) according to the manufacturer's instruction. The Oligonucleotide primers (5'-TAGCCATGCCCTTAGTAGGAC-3' and 5'-ACTCCATGTGCCATGTACAGC-3' described by Weinstock et al. were used for amplification of *5′ UTR* gene of BVDV in cDNA samples [39]. For gene amplification PCR reaction were performed in a final volume of 25 µL volume in 0.5 ml tubes containing 1 µg of template cDNA, 20 pmole of each primer, 10X PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl), 1 mM $MgCl_2$, 0.25 mM of dNTPs and 1 U of *Taq* DNA polymerase (Roche applied science). The tubes contain this solution inserted in a Gradient Palm Cycler (Corbett Research, Australia) and then initially incubated for 5 min at 94°C, followed by 32 cycles of 1 min denaturation at 94°C, 1 min annealing at 62°C and 1 min extension 72°C, with a final extension at 72°C for 5 min.

Analysis of RT-PCR Products: The amplified cDNA products were detected in 1% agarose gel electrophoresis. The electrode buffer was TBE (Tris-base 10.8 g 89 mM, Boric acid 5.5 g 2 mM, EDTA (pH 8.0) 4 ml of 0.5 M EDTA (pH 8.0), combine all components in sufficient H_2O and stir to dissolve). Aliquots of 10 µl of PCR products were applied to the gel. Constant voltage of 80 V for 30 min was used for products separation. The 100 bp DNA ladder (Fermentas, Germany) was used as a molecular weight marker to determine the length of the amplified fragment. After electrophoresis, the gel was stained with ethidium bromide and examined under UV light and photographed were obtained in UVIdoc gel documentation systems (UK).

Statistical Analysis: All data for presence of BVDV infection in semen samples of Iranian bulls were analyzed by the chi-square test using the SPSS 17 (SPSS Inc. Chicago, IL, USA) software.

RESULTS

In this study 172 semen samples of bulls were examined for the presence of *Bovine viral diarrhea virus* (BVDV) infection. Analysis of PCR products for presence of *5′ UTR* gene of BVDV on 1% agarose gel revealed a 290 bp fragment (Figure 1).

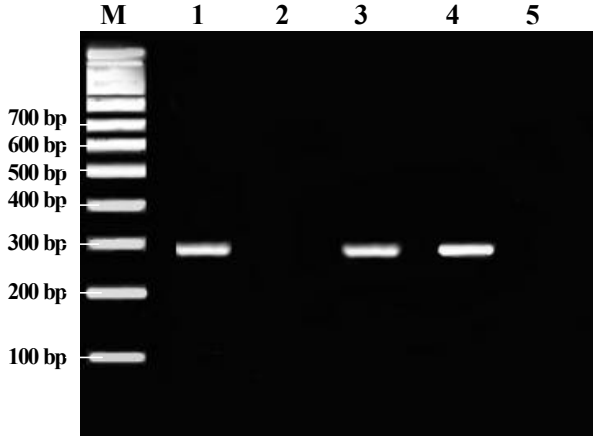

Fig. 1: Agarose gel electrophoresis of RT-PCR amplification products for detection of BVDV cDNA in bull's semen samples (Line M is 100 bp DNA marker (Fermentas, Germany), line 1 is positive control (BVDV-1 NADL), line 2 is PCR negative control sample with no cDNA template, lines 3-4 are positive specimens from infected bull's semen and line 5 is negative specimen)

Specimens producing a band of the expected size were considered positive and matched to the positive control but no product observed for negative control. Viral cDNA was detected in 32 of the 172 (18.60%) of bull's semen specimens. The results showed a high presence of BVDV infection in bull's semen samples that were used for artificial insemination in Iran.

DISCUSSION

The clinical syndromes associated with BVDV infection result in severe economic losses to the cattle industry of Iran. BVDV has also been isolated from other ruminants, sheep and goats, camelids, rabbits, deer, pigs and other animals [3, 26]. BVDV transmission from PI cattle to a pregnant sheep resulting with a PI lamb that then transmitted BVDV to pregnant cattle had been demonstrated. The role of other species in BVDV infection transmission is not clear at the moment [27].

Vaccination programs are essential to decreasing losses to BVDV. The goal of any vaccination program is to prevent fetal infection and increase colostral immunity [26]. The two types of vaccine available are modified live and inactivated (killed) and controversy exists over which is better. Modified live vaccines (MLV) offer more cross-protection against different strains and the immunity conferred by them is longer lasting and stronger [26, 40]. Modified live vaccines should be used with caution, however, as they may cause immunosuppression, fetal infection, or revert to virulence [4]. Inactivated vaccines are not immunosuppressive, do not infect feti and have minimal risk. However, the immune response they generate is weaker, of shorter duration and may not crossprotect as well as MLV. Cattle receiving inactivated vaccine must also have a booster 3 to 4 weeks after the first vaccination. Neither MLV nor inactivated vaccines give lifelong protection and yearly boosters are required with both [41]. There is no one vaccination program for all situations. Producers should consult their veterinarian for a program tailored to their herd [40, 42].

Bulls that are persistently infected usually have poor quality, highly infective semen and, as a result, reduced fertility [43, 44]. All bulls used for natural or artificial insemination should be screened for persistent BVD infection. A rare event, possibly brought about by acute infection during pubescence, can result in persistent infection of the testes and thus strongly seropositive bulls [32, 45]. This phenomenon has also been observed following vaccination with an attenuated virus [46].

Female cattle used as embryo recipients should always test negative for BVD viraemia before first use.

Detection of BVDV infection in cattle and fresh and frozen semen samples of bulls are important and based on variety of laboratory assays such as agar gel immunodiffusion test (AGID), virus isolation or by measuring specific antibodies using complement fixation test, ELISA, immunoperoxidase and immunofluorescence assays. These methods are time consuming and difficult [47].

Bovine viral diarrhea virus is difficult to detect for several reasons. The virus, particularly the NCP grows slowly in cell culture and may require several passages before sufficient viral antigen is expressed to allow detection by antibodies. On the other hand, detection by antibody-based techniques is also difficult because of antigenic variation. Molecular techniques such as RT-PCR have also been used to detect the presence of virus in blood and serum samples and sensitivity of PCR is so high, that other methods could not compete with this method anymore [42, 48].

The aim of current study was to detection of BVDV RNA in semen specimens of infected bulls using RT-PCR assay. In present study high presence (18.60%) of BVDV infection in bull's semen samples that were used for artificial insemination were observed and indicated that this viral infection is a significant agent to reduced milk production, decreased reproductive performance, growth retardation, increased occurrence of other diseases, unthriftiness, early culling and increased mortality among young stock.

Many studies were performed about BVDV infection in bull's semen and described its correlation with decreased of milk production, reduced conception rates, respiratory disorders, abortions and congenital defects in cattle.

The prevalence of BVDV in Iran has been mainly reported on the basis of the detection of antibody against BVDV. In early investigations a range of 20-90% of BVDV incidence has been reported [49]. In a study on slaughtered cattle in Tehran province (Iran), 58.51% of animals were found to be seropositive [50]. Ahmad *et al*., in 2011 showed the prevalence of BVDV in 12 Holstein cattle dairy herds of Charlottetown, Canada was 0.85% and 2.03%, respectively. Their results demonstrates that the prevalence of PI animals in dairy herds of Prince Edward Island is similar to that, reported from other surveys carried out in other countries and reveal the necessity of further studying BVDV infection in the area [51]. Their findings showed low prevalence of infection and different to the result of present research.

Schmitt *et al*. [52] in 1994 showed BVDV can be detected by PCR directly out of tissue homogenates generated in a diagnostic setting. Polak and Zmudzinski [53] in year 1999 performed a study on prevalence of BVDV infection in bulls in artificial insemination centers in Poland. In their study neutralizing antibodies were found in 86% of the bulls and PI was detected in 0.9% of the analyzed blood samples. Their method is different from the technique of present study, but both research showed high presence of BVDV infection in bulls. Weinstock and coworkers were evaluated 60 dairy cattle herds located in Pennsylvania by the m icroplate virus isolation method and pooled sera were analyzed by RT-PCR. Similar results regarding the sensitivity of PCR for BVDV detection in bovine pooled serum were reported by Weinstock *et al*. [39]. They showed that RT-PCR was sensitive and specific for analysis of pooled sera and provides a rapid and cost-effective method for the screening of cattle herds for the presence of animals persistently infected with BVDV [39].

Radwan [42] in year 2002, showed PCR amplification-probe hybridization assay may be a useful addition in developing new rapid and sensitive tests for detection of BVDV. The speed and the sensitivity of this method might be of relevance for studies on epidemiological and pathogenesis of infection with BVD virus. The study of Daliri *et al*. [48] in Iran on detection of BVDV in bovine semen using nested-PCR showed this technique can be used as a rapid and sensitive method of BVDV-1 detection in bovine semen. The molecular methods that used in their study same to the present research and these findings confirmed the high presence of BVDV in semen samples of Iranian bulls.

Virus detection is the only certain way of identifying PI animals and may be very important for all BVDV control programs without use of vaccinations. RT-PCR assays detect small quantities of virus, potentially leading to fewer false negative tests in recently exposed animals and it can used for detection of BVDV in Iranian bull's semen samples. According to results of this study we observed high presence of BVDV infection in Iranian bull's semen samples used for artificial insemination. These results showed that RT-PCR assays could provide a powerful tool in studies on the epidemiology and pathogenesis of animal infections with BVD virus and this study indicated

that semen specimens are important transmission sources of BVD virus in Iranian cattle. Furthermore, the findings of present study suggested that control and eradication programs such as vaccinations programs for prevent and reduce of economic loses of BVDV infection in Iranian cattle it seems to be necessary.

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