

# Detection of bovine viral diarrhea virus in bovine semen using nested-PCR

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## Abstract

A rapid and sensitive reverse transcription polymerase chain reaction (RT-PCR) and nested-PCR were used to detect bovine viral diarrhea virus 1 (BVDV-1) in bull semen. Selected primers could amplify a part of the 5'UTR of the BVDV genome. A 294 bp DNA fragment was amplified and specificity of the results was confirmed by direct sequencing of the PCR product. Prior to RNA extraction, the seminal inhibitors were eliminated using a simple dilution method. Therefore, a sensitivity of  $3 \times 10^2$  50% tissue culture infective dose (TCID<sub>50</sub>) was achieved when experimentally infected semen was used for RNA extraction. In nested-PCR a 160 bp fragment was amplified and sensitivity of the test was increased to 3 TCID<sub>50</sub>. This technique can be used as a rapid and sensitive method of BVDV-1 detection in bovine semen.

**Keywords:** Bovine Viral Diarrhea Virus; Semen; Nested-PCR

## INTRODUCTION

Bovine viral diarrhea virus (BVDV), an economically important viral pathogen of cattle, belongs to the genus Pestivirus of the family Flaviviridae (van Regenmortel *et al.*, 2000). The genome of BVDV is a single stranded (+) RNA of about 12.5 kb in length without a poly (A) tail (Collett *et al.*, 1988). BVDV isolates consist of two different genotypes; BVDV-1 and BVDV-2. BVDV is associated with several clinical symptoms including mild diarrhea, respiratory disease, congenital malformations, reproductive failures, and mucosal disease (Baker, 1987). Infection of bovine fetuses with BVDV-2 during the first 120

days of pregnancy can result in the birth of persistently infected offspring that are immunotolerant to the virus (Charleston *et al.*, 2001; Givens *et al.*, 2003). Persistently infected animals are difficult to be identified because of their normal appearance. Serum samples from these animals fails to react in most serological tests and the animals shed large amounts of virus in all body secretions and excretions over prolonged periods (Barlow *et al.*, 1986). The prevalence of BVD 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 BVD incidence has been reported (Mirshamsy *et al.*, 1970). In a study on slaughtered cattle in Tehran province, 58.51% of animals were found to be seropositive (Kargar *et al.*, 1995). In a later survey of the cattle population in Iran, 3000 serum samples were tested. Results showed that about 39.6% of young animals were seropositive. The prevalence of antibody and the proportion of seropositive animals rose markedly with age up to 62% (Sedighi-nejad, 1996). To our knowledge there is no published data on prevalence of infected bovine semen with BVDV or infections caused by infected semen through artificial insemination in Iran. However, an important outcome of viral infection is that the semen produced by persistently infected bulls may contain virions that can be transmitted either by natural or artificial insemination to the offspring, causing important economic losses (McGowan and Kirkland, 1995). The existence of apparently healthy bulls persistently infected with BVDV emphasizes the need for rigorous screening of all animals in artificial insemination centers to identify the animals carrying the infectious agent. Serological procedures, antigen detection and virus isolation are the preferred methods for diagnosis of BVDV infected animals. However, negative serological results must be interpreted with caution since

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persistently infected bulls have no neutralizing antibodies or antibodies only at low titers and may appear healthy with acceptable semen quality. The detection of BVDV-1 by PCR amplification is an alternative to conventional tests. RT-PCR method is being used for detection of viral nucleic acid as a specified method which is fast enough to detect the virus at a maximum of 8 hours (Givens *et al.*, 2003; Da silva *et al.*, 1995; Belak and Ballagi-pordany, 1991; Brock, 1991). Detection of BVDV-1 and BVDV-2 in extended semen sample (diluted and treated semen for cryo preservation) is 10 times more sensitive compared to raw semen (fresh ejaculates and untreated semen) (Givens *et al.*, 2003). Accurate and inexpensive methods for the detection of virus in semen of bulls infected with BVDV would be ideal, since it would allow prevention of BVDV dissemination on farms. Due to the economic impact of BVDV transmission via semen this study was conducted by using nested-PCR to detect extended bull semen infected with BVDV-1 in order to contribute to the elimination of this important route of transmission of the disease.

## MATERIALS AND METHODS

**Viral strains and cell culture:** The BVDV-1 NADL 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 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 was used as the negative control.

**Preparation of semen samples before RNA extraction:** Twenty semen samples from uninfected bull were obtained. Five samples were kept as negative controls and 15 were inoculated with BVDV-1 and in order to eliminate seminal inhibitors prior to RNA extraction, an equal volume of DEPC-dH<sub>2</sub>O was added to inoculated semen samples. The mixture was subjected to three cycles of freezing and thawing and 300 µl was then used in RNA extraction. Uninoculated samples were also used for RNA extraction.

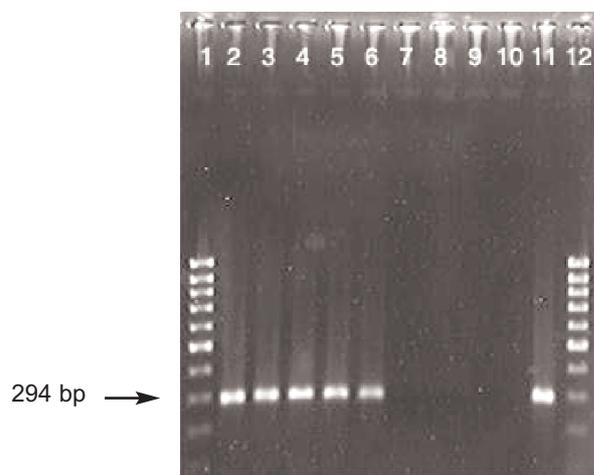
**RNA extraction:** Viral RNA was extracted using a phenol-chloroform-thiocyanate based method described by Chomczynski and Sacchi, 1987. Briefly, 1 ml of RNA extraction solution was added to 300 µl of inoculated semen sample in a 1.5 ml microfuge tube. This was followed by adding 200 µl of chloroform and subsequent centrifugation at 10000 ×g for 5 minutes. Supernatant was transferred to a new tube and an equal volume of cold isopropanol was added. After centrifugation (10000 ×g for 15 minutes) the resulting pellet was washed with 70% ethanol, dried and resuspended in 20 µl of DEPC-dH<sub>2</sub>O. Extracted RNA was immediately used or stored at -70°C until needed.

**Reverse transcription- polymerase chain reaction (RT-PCR):** Oligonucleotide primers from 5'UTR of virus genome (PF1 5'-ATGCCCTTAGTAGGACTAGC-3' and PR1 5'-ACTCCATGTGCCATGTACAG-3') were used for RT reaction and subsequent PCR amplification as previously described (Sasaki *et al.*, 1996). The mixture was subjected to the following program using a Techne thermocycler (UK): 94°C for 3 min (1 cycle), 94°C for 30 sec, 52°C for 30 sec, 72°C for 30 sec (30 cycles) and 72°C for 10 min (1 cycle). The samples were analyzed in 1% (W/V) agarose gel in Tris acetate-EDTA buffer.

**Sequencing of the PCR products:** The PCR product was cloned into a T-vector (pTZ57R) based on the manufacturer's instructions (Fermentas, Lithuania). Recombinant plasmids were sequenced and data were compared to that of the NADL strain using the DNASTAR software package.

**Sensitivity of PCR:** In order to determine the sensitivity of PCR, an uninfected extended semen sample was inoculated with 3×10<sup>6</sup> TCID<sub>50</sub> /ml of BVDV-1 and ten fold serial dilutions were prepared. Uninoculated semen sample was used as a negative control. Total RNA extraction and RT-PCR were performed as above.

**Nested-PCR assay:** In order to specifically amplify a part of the 5'UTR of the virus genome, oligonucleotide primers to be used in nested-PCR were chosen from sequence data of the PCR product. The primers used in this study were Pesti-N-F 5'-GATGGCTTAAGCCCTGAGTA-3' and Pesti-N-R 5'-GCTGTATTTCGTAACAGTCGG-3'. Nested-PCR was performed in 50 µl reaction volume containing, 0.5 µl of first round PCR, 0.5 U *Taq* DNA polymerase, 30



**Figure 1.** Sensitivity of PCR for detection of BVDV-1 RNA extracted from 10 fold serial dilutions of virus in extended semen. Lane 1 and 12: DNA size marker, ladder 100. Lane 2-9: Correspond to  $3 \times 10^6$ ,  $3 \times 10^5$ ,  $3 \times 10^4$ ,  $3 \times 10^3$ ,  $3 \times 10^2$ ,  $3 \times 10^1$ , 3 and 0.3 TCID<sub>50</sub>, respectively. Lane 10: Negative control. Lane 11: Positive control.

pmoles of each primer, 1.5 mM MgCl<sub>2</sub>, 200 mM dNTPs, 10 mM Tris-HCl and 50 mM KCl. Amplification was performed in a Techne thermocycler (UK). The PCR thermal program was used for nested-PCR. Negative controls from the first round of PCR and a nested-PCR negative control lacking template DNA were also included.

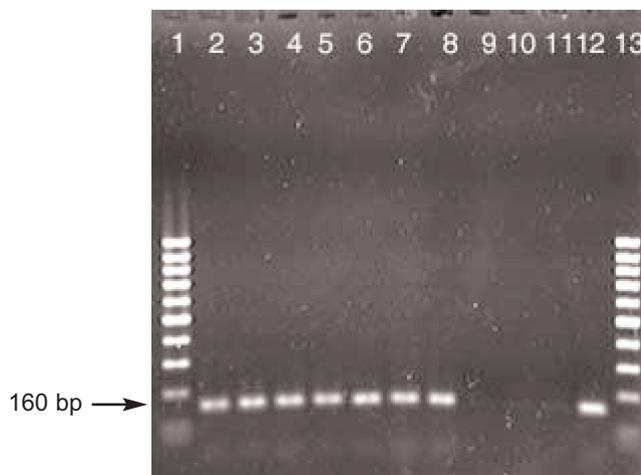
**Sensitivity of nested-PCR:** One microlitre of PCR products from tested semen samples were used as template in nested-PCR. In addition to PCR negative control, a nested-PCR negative control was also included.

## RESULTS

A highly conserved region within the virus genome was amplified by the RT-PCR and a single DNA fragment of 294 bp was observed while no DNA bands were produced in the negative control sample.

**Specificity of the RT-PCR:** The nucleotide sequence for this amplicon was determined by direct sequencing (Acc. No. AY954693). Results were compared with the published sequence (Acc. No. AJ133739) of the NADL strain. The two sequences were identical.

**Sensitivity of PCR:** DNA bands were detected covering a range from  $3 \times 10^6$  to  $3 \times 10^2$  TCID<sub>50</sub>. No signal was observed when RNA was extracted from lower dilutions or uninoculated samples (Figure 1).



**Figure 2.** Sensitivity of nested-PCR for detection of BVDV-1 RNA in 10 folds serial dilutions of virus in extended semen. Lane 1 and 13: DNA size marker, ladder 100. Lane 2-9: Correspond to  $3 \times 10^6$ ,  $3 \times 10^5$ ,  $3 \times 10^4$ ,  $3 \times 10^3$ ,  $3 \times 10^2$ ,  $3 \times 10^1$ , 3 and 0.3 TCID<sub>50</sub>, respectively. Lane 10: PCR negative control, Lane 11: Nested-PCR negative control, Lane 12: Nested-PCR positive control (160 bp).

**Sensitivity of nested-PCR:** An expected 160 bp amplicon was observed in the positive control. Positive results were observed in a range from  $3 \times 10^6$  to 3 TCID<sub>50</sub>/ml of inoculated samples. No DNA bands were observed in semen samples inoculated with 0.3 TCID<sub>50</sub>/ml of virus or in PCR and nested-PCR negative controls (Figure 2).

## DISCUSSION

Detection of BVDV strains in sick animals is based on virus isolation or by measuring specific antibodies using complement fixation test, ELISA, immunoperoxidase and immunofluorescence assays (Afshar *et al.*, 1991). Molecular techniques have also been used to detect the presence of virus in blood and serum samples (Barlow *et al.*, 1986; Baker, 1987). Comparison of RT-PCR with ELISA and cell culture immunoperoxidase tests for the detection of ruminant pestivirus infections revealed that RT-PCR is more sensitive than the other tests (Horner *et al.*, 1995). Monitoring of virus in semen samples of seronegative and nonviremic bulls is suggested (Fray *et al.*, 2000, Voges *et al.*, 1998). Due to the virucidal effects of semen, viral isolation is not efficient and can fail to detect virus in samples from infected bulls (Kahrs *et al.*, 1980; Voges *et al.*, 1998). The sensitivity of RT-PCR for detection of virus in semen sample is higher than viral isolation (Givens *et al.*, 2003). Extraction of intact RNA is a crucial factor in the

RT-PCR assay. The sensitivity of RT-PCR relies on the ability to extract, purify and recover RNA from clinical materials including semen. Many samples submitted to diagnostic laboratories are often contaminated with a number of micro-organisms. Cell lyses are common in biological samples and a number of cellular fractions such as different proteins, RNases and enzyme inhibitors are also present which often interfere with RNA extraction, cDNA synthesis or amplification (Hale *et al.*, 1996). Therefore it is important to adopt a nucleic acid extraction method which removes inhibitory factors and recovers most of the desired RNA. Extraction of viral RNA from semen was achieved by diluting the sample with DEPC-dH<sub>2</sub>O prior to total RNA extraction. This technique reduces the inhibitory factors in semen and extracted RNA is suitable for further molecular experiments such as RT-PCR. However, sensitivity of the test could be reduced by diluting the samples. The sensitivity of PCR was increased 100 fold by nested-PCR and this may compensate the dilution factor. This procedure was rapid, reproducible and could be practiced without using extra purification steps which are usually tedious or time consuming. To achieve high sensitivity in the test, other workers have extracted RNA from semen using chromatography steps to solve this problem (Da silva *et al.*, 1995). However, this may not be applicable when large numbers of semen samples are being tested. Detection of BVDV-1 in semen by our nested-PCR was found to be sensitive and sequencing results also confirmed its specificity. This technique can be an effective tool for detection of virus in the semen of infected bulls in the field as well as in artificial insemination centers.

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