Applied Biological Research 14 (2): 215-219, 2012 **Short Communication**

MOLECULAR AND ANTIBODY BASED DIAGNOSIS OF BOVINE VIRAL DIARRHEA VIRUS (BVDV) ASSOCIATED WITH CATTLE FROM MATHURA, UTTAR PRADESH (INDIA)

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ABSTRACT

Bovine viral diarrhea (BVD) infection causing great reproductive and productive losses is an immunosuppression seen in all age groups of ruminants world-wide. In present study out of 78 peripheral blood leucocyte (PBLs) samples 4 samples were positive for BVDV antigen by pestivirus antigen-capture ELISA, while none of the faecal samples was positive for BVDV antigen by AGID and RT-PCR. The use of RT-PCR on all PBLs samples was restricted to the above 4 positive samples which yielded a 288 bp amplicon, while rest were found negative. The BVDV positive samples were confirmed by restriction endonuclease analysis. The study revealed the presence of BVDV in Mathura region of Uttar Pradesh.

Keywords: BVDV, RT-PCR, PBL's, ELISA

INTRODUCTION

Bovine viral diarrhea (BVD) infection is characterized by fever, persistent diarrhea, coughing, immuno-suppression, reproductive disorder, congenital defects, reduction in milk yield and lameness (Houe, 2003; Ghazi *et al*., 2008). The Bovine viral diarrhea virus (BVDV) infection may be noticed in ruminants of all age groups throughout the world causing great reproductive and productive losses (Chase *et al*., 2003; Mishra *et al*., 2009). BVDV of ruminants and ovine, and hog cholera virus (HCV) of swine reportedly have close antigenic and biologic relationship (Buckwold and Beer, 2003; Mishra *et al*., 2007). BVDV belongs to the genus *Pestivirus* of family *Flaviviridae* and contains a single stranded positive sense RNA genome with icosahedral symmetry. On the basis of antigenic variation and sequence difference in 5′ UTR the virus has two genotypes BVDV1 and BVDV2 (Vilcek and Nettleton, 2006; Mishra *et al*., 2007). BVDV has the ability to cross placenta, invade the fetus and set up persistent infection (PI). Calves become immunotolerant to the virus and may be born as PI and also pose constant threat of BVDV infection in cattle and buffaloes (Ridpath, 2003; Mishra *et al*., 2005).

In recent years several diagnostic assays based on BVDV isolation and their antibody/antigen detection have been in use (Lindberg, 2003; Robert *et al*., 2005; Jaruwan *et al*., 2007). But these methods require either specific monoclonal antibody or cell line so are expensive and time-consuming. Some workers evaluated the antigen-capture ELISA and reverse transcription-PCR (RT-PCR) test for diagnosis of persistently infected animals and observed RT-PCR test relatively improvised diagnostic tool for demonstrating the presence of BVDV genome in peripheral blood, organs and milk (Sentsui *et*

al., 2001; Derget *et al*., 2002). One of our studies showed seroprevalence of BVDV among all age groups of ruminants (Varshney *et al*., 2009). In present study an attempt was made to detect BVDV antigen from peripheral blood leukocytes (PBLs) and faecal samples of cattle population.

MATERIALS AND METHODS

Seventy eight blood samples in heparin and 78 faecal samples were collected from the same animals reared at Dairy Demonstration Farm and Madhuri Kund Farm of DUVASU, Mathura (UP). The samples were transported to laboratory on ice. PBLs were separated from the blood samples and used as test sample for detection of BVDV antigen by antigen capture ELISA (Ingezim BVD Das K.2, Ingenasa, Spain). The faecal samples were screened for detection of BVDV antigen by agar gel immuno-diffusion (AGID) test. The samples were confirmed by RT-PCR for molecular detection of BVDV genome. Positive samples for BVDV antigen were subject to RT-PCR for molecular detection of BVDV genome. The serum samples, found positive for BVDV antibodies by ELISA in our earlier study (Varshney *et al*., 2009), were employed as known positive BVDV antiserum.

Detection of BVDV antigen (p80/125) in PBLs samples

The BVDV p80/125 antigen was detected in PBLs by using ELISA kit (Ingezim BVD Das K.2, Ingenasa) as per the manufacturer's instruction. Briefly, 100 µL of positive and negative control samples and test samples of PBL was added in respective wells of ELISA plate in duplicate. The plate was incubated at room temperature (RT) for 18 h, then washed 6 times with washing solution and 100 µL of conjugate 1 (1:5000) added in each well. The plate was then incubated at 37°C for 1 h and again washed 6 times. Similarly, 100 µL conjugate 2 (1:5000) was added in each well, incubated at RT for 30 min and washed 6 times with washing solution. Finally, 100 µL of substrate was added to each well and after incubation in dark for 5 min the reaction was stopped by adding 100 µL stop solution to each well. Optical density (OD) was measured at 450 nm by ELISA reader and cut off value calculated as per the protocol of ELISA kit. The samples showing OD more than cut off value plus 15% was considered positive for BVDV antigen.

Detection of BVDV antigen in faecal samples

Agar gel immuno-diffusion (AGID) test was used for detection of BVDV antigen. Briefly, 1.5% agarose gel was prepared in 0.9% NaCl and poured into petriplates. After solidification, a set of 7 wells (1 central and 6 peripheral) was made with the help of gel punch. Serum samples positive for BVD antibodies by ELISA were used as source of antibodies. Positive serum (>1:100) was placed in central well and supernatant of faecal samples (viral antigens) centrifuged at 5000 rpm for 15 min were placed in peripheral wells. These petriplates were kept in a humidified chamber at RT for 5 days and observed for presence or absence of precipitation line.

Molecular detection of BVDV by RT-PCR

Viral RNA from PBLs was isolated by SV total RNA isolation system [Promega] (Misra *et al*., 2007). The purified RNA was stored at -70°C till further use. The cDNA was constructed by Access RT-PCR System (Promega) in one step RT-PCR of total RNA. RT-PCR was carried out as per standard protocol of HSADL Bhopal (Misra *et al*., 2004). The 2 primers used were HSBVD 40 (F) and HSBVD 41 (R) having 5′ to 3′ sequence as ATGCCATAGTAGGACTAGCA and CAACTCCATGTGCCATGTC, respectively, with 5′ UTR region. A PCR reaction mixture (25 µL) [containing template RNA (3 µL), 5x AMV-tfl reaction buffer (5 µL), 10 mM dNTP mix (1 µL), 25 mM $MgSO_4$ (2.5 µL), AMV RT (2.5U), Tfl DNA polymerase (2.5U), 25 ρmol each primer and nuclease-free water (10.5 µL)] was added and immediately placed in thermal cycler (Hybaid Touchdown). Reverse transcription and amplification was done as per cyclic conditions viz., initial reverse transcription at 48°C for 45 min. and reverse transcriptase inactivation at 94°C for 2 min., followed by 35 cycles of denaturation at 94°C for 30 sec., annealing at

60°C for 45 sec., extension at 72°C for 1 min. and at 68°C for 10 min. and soak cycle at 4°C. The amplified products were separated by agarose gel electrophoresis (1.5% agarose in 0.5 x TBE) at 80V for 1 h and stained with ethidium bromide (0.5 µg mL^{-1}). PCR products were observed by UV transilluminator.

For restriction endonuclease analysis of PCR product, the amplicons generated by employing forward HSBVD40 and reverse HSBVD41 primers were purified by using Wizard PCR prep-DNA purification system (Promega). Purified PCR products were subjected to restriction endonuclease enzyme digestion using *EcoR1* restriction endonuclease. The digestion with *EcoR1* was performed as per the manufacturer's protocol using specific conditions and buffer supplied. Briefly, 2 µL purified PCR product was taken in a 0.5 ml microcentifuge tube, containing 1 µL (10 U) *EcoR1* and 1 µL compatible buffer. The volume was made upto 10 µL with nuclease-free water. Digestion was carried out at 37°C for 2 h in water bath and reaction stopped by orange (O^+) loading dye. The digested products alongwith DNA ladder were electrophoresed in 1.2% agarose gel in 1xTAE buffer at 80 V for 3 h using electrophoresis apparatus. After completing electrophoresis, the gel was examined under UV transilluminator and photographed.

RESULTS AND DISCUSSION

Out of 78 PBLs samples 4samples showed presence of p80/125 BVDV antigen. Among them 1 sample was seropositive. Out of the 4 positive PBLs samples 3 belonged to cattle aged between 1 to 3 years and the remaining one was obtained from 15 year old cattle. Two samples of these 4 positive samples were recovered from male and 2 from female. None of the 78 faecal samples was positive for BVDV antigen by AGID. All the 4 BVDV antigen-positive animals were apparently healthy and showed no clinical symptoms. Many investigators have done their work on antigen capture ELISA and found it is more specific and sensitive diagnostic test for detection of viral antigen (Rossmanith *et al*., 2001; Orhan *et al*., 2006).

RT-PCR was used for detection of BVD viral RNA in sera and leukocytes. RT-PCR proved a rapid and sensitive method to detect BVDV nucleic acid. The technique has previously been used for the detection of pestivirus including BVDV using oligo-nucleotide/ primers located in 5′ UTR region of viral genome (Rossmanith *et al*., 2001; Sentsui *et al*., 2001). An amplicon of 288 bp was amplified from 5′ untranslated region (UTR) using HSBVD 40 and HSBVD 41 primers (Fig. 1). RE analysis of 288 bp purified PCR product revealed similar and identical band pattern by *EcoR1* digestion which cut the amplicons into two fragments of 243 and 45 bp size (Fig. 2).

BVD virus contains a single stranded RNA genome of 12.3 kb and is flanked by 5′ and 3′ untranslated region (5′ and 3′ UTR). Genetic typing of pestivirus has mostly been based on sequence comparison of the 5′ UTR, Npro and E2 regions. BVDV strains have been classified as genotype 1 and 2 on the basis of antigenic and genetic variation. In the present study an amplicon of 288 bp encompassing 5' UTR of BVDV was amplified from four animals by RT-PCR. Similar band pattern 288 bp in RT-PCR (Fig. 1) has also been observed by other workers through primers specific for 5′ UTR region (Vilcek *et al*., 2003; Mishra *et al*., 2005).

The authenticity of RT-PCR as 10 times more sensitive as

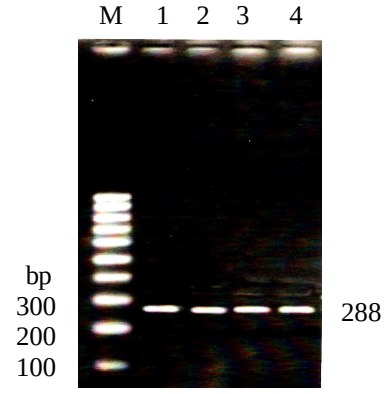

Fig. 1: Agarose gel electrophoresis of 5' or 3' untranslated region of BVD virus RT-PCR product (288 bp)

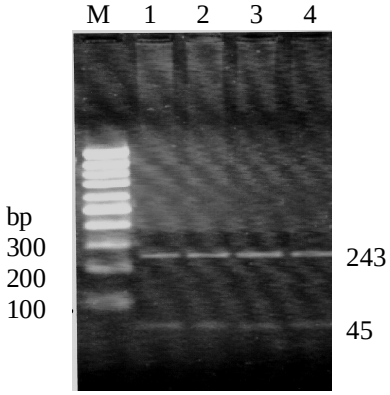

Fig. 2: Agarose gel electrophoresis of *EcoR1* digested RT-PCR product of BVD virus

the virus isolated has been advocated by many workers and they developed a RT-PCR to differentiate the BVDV from other pestiviruses and to determine the genotype of BVDV isolates. The primers specific to the 5′ UTR region was also used by them in the study. RT-PCR has been reported to be a sensitive and specific technique for rapid detection of BVD virus (Renshaw *et al*., 2000; Mishra *et al*., 2005). Based on the present findings, the presence of BVDV antigens in cattle in PBLs and identification of viral genome in PBLs by RT-PCR appears to be important in carry out the future control programme.

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