



Detection of bovine viral diarrhoea virus (BVDV) in peripheral blood mononuclear cells (PBMCs) of BVDV antibody positive Indian cattle following mitogen stimulation

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Bovine viral diarrhoea (BVD), an economically important viral disease of cattle and sheep prevalent worldwide, is caused due to bovine viral diarrhoea 1 (BVDV-1) and bovine viral diarrhoea 2 (BVDV-2) belonging to the genus *Pestivirus* in the *Flaviviridae* family (Thiel *et al.* 2005). BVDV-1 strains predominate in cattle populations worldwide, while BVDV-2 strains occur more sporadically. BVD control and eradication efforts are mostly focused on the identification and removal of persistently infected (PI) animals arising after in utero infection with ncp BVDV during first trimester of gestation (McClurkin *et al.* 1984). However, acute infections with BVDV can persist for months or years after the removal of the PI source despite repeated screening for PIs and tight biosecurity measures (Houe 1999).

Prolonged BVDV infection can be maintained within testicular tissue, ovarian tissue, central nervous system's tissue and circulating white blood cells (Voges *et al.* 1998, Grooms *et al.* 1998, Blas-Machado *et al.* 2004, Gogorza *et al.* 2005, Collins *et al.* 2009) suggesting the possibility of a form of chronic persistence. Persistence of BVDV infection following acute infection (98 dpi) was recently reported in UK cattle, having the potential to remain infectious for BVDV in naive animals (Collins *et al.* 2009). Mitogen stimulation of leukocytes *in vitro* could detect BVDV in seropositive cattle (Gogorza *et al.* 2005, Collins *et al.* 2009) and hepatitis C virus in humans (Pham *et al.* 2005), which remained undetectable earlier.

In India, BVD is prevalent and both BVDV-1 and BVDV-2 were identified in cattle (Mishra *et al.* 2004, Behera *et al.* 2011). Serological studies indicated prevalence of widespread BVDV infection in Indian cattle. Although reported in

Argentina and UK, no effort has yet been made in possible detection of BVDV in leukocytes of BVDV neutralizing antibody positive cattle. Hence, the aim of this study was to detect BVDV in PBMCs of antibody positive naturally infected Indian cattle following phytohaemagglutinin A (PHA) stimulation.

Indian cattle BVDV-1b isolate, Ind S-1449 (Mishra *et al.* 2004) and Indian cattle BVDV-2a isolate, Ind 141353 (Behera *et al.* 2011) were used in this study. Madin-Darby bovine kidney (MDBK) cells, obtained from Collection of Cell Lines in Veterinary Medicine, Riems, Germany (kind gift by Roland Riebe) and maintained in our laboratory were used for virus propagation and virus neutralization tests. The cells were grown in Eagle's minimum essential medium containing 10% horse serum and maintained at 37°C with 5% atmospheric CO₂. The cells were checked for other adventitious pestivirus contamination by RT-PCR using panpestivirus specific primers.

Blood samples (250) collected from cattle of different geographic areas of India (Ludhiana, Punjab; Yamunanagar and Fatehabad, Haryana; Lucknow, Uttar Pradesh; Rajnandgaon, Chhatisgarh) from the samples obtained for an ongoing contract research project on BVD at HSADL, Bhopal during October 2012 and April 2013 were used. The sera separated from blood were tested for the presence of BVDV antibodies by virus neutralization test following previously reported method (Mishra *et al.* 2008) using Indian BVDV-1 and BVDV-2 isolates, MDBK cells and immune-peroxidase monolayer assay (IPMA). Positive and negative serum controls, virus controls and uninfected cell controls were always included in each plate. The VN titre for each serum is the dilution at which BVDV is completely neutralized in 50% of the wells. Animals showing neutralization at 1:5 initial serum dilution (1:10 final) were considered to possess BVD virus neutralizing antibodies.

The PBMCs of BVDV immune cattle were isolated from

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2 ml of blood by density gradient centrifugation method using Histopaque following the protocol of Collins *et al.* (2009) with the modification that 0.8% ammonium chloride was used for lysis of RBCs instead of ultrapure water. The washed PBMCs were suspended initially in 2 ml of pre-warmed RPMI-1640 medium, the cell viability was checked by dye exclusion method using 0.4% trypan blue.

PHA stimulation of PBMCs was carried out following Collins *et al.* (2009). Isolated PBMCs obtained from 2 ml blood were finally re-suspended in 4 ml of RPMI-1640 medium containing BVDV and BVDV antibody free 10% FCS, glutamine 200 mmoles, penicillin 100 IU/ml, streptomycin 100 µg/ml with 5 µg/ml phytohaemagglutinin and were incubated for 72 h at 37°C. After PHA stimulation, PBMCs were pelleted by centrifugation at 400 × g for 10 min and were re-suspended in 0.5 ml of EMEM before being subjected for RNA extraction or virus isolation.

For detection of BVDV RNA in PHA stimulated PBMCs of BVDV immune cattle, total RNA from 0.25 ml of PHA stimulated PBMCs was extracted using Trizol following the manufacturer's protocol. The pelleted RNA was resuspended in 30 µl of nuclease free water and was stored at -80°C until use. The RNA extracted from the PHA stimulated PBMCs was subjected to one-step RT-PCR using the panpestivirus specific primers 190F and V326 (Vilcek *et al.* 1994, Hoffmann *et al.* 2006) targeting 5'-UTR of BVDV genome and one-step RT-PCR system with platinum taq (Invitrogen high fidelity, following the manufacturer's recommendations). The RT-PCR amplified product was subjected to submarine agarose gel electrophoresis in TAE buffer.

For detection of infectious BVDV in PHA stimulated PBMCs, MDBK cells in 96-well TC plates were infected with the frozen-thawed PBMC lysates followed by immunostaining in IPMA as described previously (Mishra *et al.* 2008) using anti-BVDV polyclonal antibodies.

Out of the 250 cattle tested, 93 cattle (37.2%) were found positive for BVDV neutralizing antibodies. The neutralizing antibody titre in cattle varied from 1:10 to 1:320 (low, intermediate and high) indicating that in the field animals have been exposed with BVDV for variable periods of time. These 93 BVDV immune cattle were selected for further studies on PHA stimulation of PBMCs. When PBMCs of all 93 immune cattle were tested for viability by dye exclusion method, an average viability of 95–98% was found which was in conformity with previous studies (Gogorza *et al.* 2005, Collins *et al.* 2009).

Of the 93 BVDV immune field cattle tested, BVDV RNA was detected only in PHA stimulated PBMCs of 3 cattle (Fig. 1), all from the same dairy farm of Chhattisgarh, while no infectious BVDV could be recovered from any of the PHA stimulated and un-stimulated PBMCs of immune cattle by virus isolation. A moderate neutralizing antibody titre (1: 160) was evident in one of the 3 cattle in which BVDV

RNA was detected.

In a previous study, BVDV was detected in the peripheral blood leukocytes from 15 of 229 seropositive cattle from 1 beef and 3 dairy farms in Argentina using antigen capture ELISA following stimulation of samples with phytohemagglutinin-M and culture on MDBK cells which were treated with polycation (Gogorza *et al.* 2005). Besides, neutralizing antibodies against BVDV were detected in sera from each animal that yielded virus from peripheral blood leukocytes. In a subsequent study, BVDV was detected from the peripheral blood leukocytes from 10 of 104 seropositive cattle from 2 dairy farms following culture on polycation-treated MDBK cells by antigen capture ELISA (Gogorza *et al.* 2006). But BVDV could not be detected in whole blood or plasma. A study in cattle showed that the BVDV RNA can be detected intermittently in PBMCs for prolonged periods when infectious virus cannot be recovered in BVDV immune cattle and BVDV could be detected in the *ex-vivo* stimulated (with PHA) white blood cells of some of the acutely infected calves following the development of neutralizing antibodies providing evidence that BVDV can persist in PBMCs of BVDV immune cattle (Collins *et al.* 2009).

The detection of replicating virus despite the presence of specific neutralizing antibody also was observed in pigs chronically infected with another pestivirus, classical swine fever virus (Choi *et al.* 2003).

Presence of BVDV RNA and absence of infectious virus in PBMCs of 3 BVDV immune cattle found in our study is not surprising, since the use of more sensitive detection techniques such as RT-PCR can extend the period when

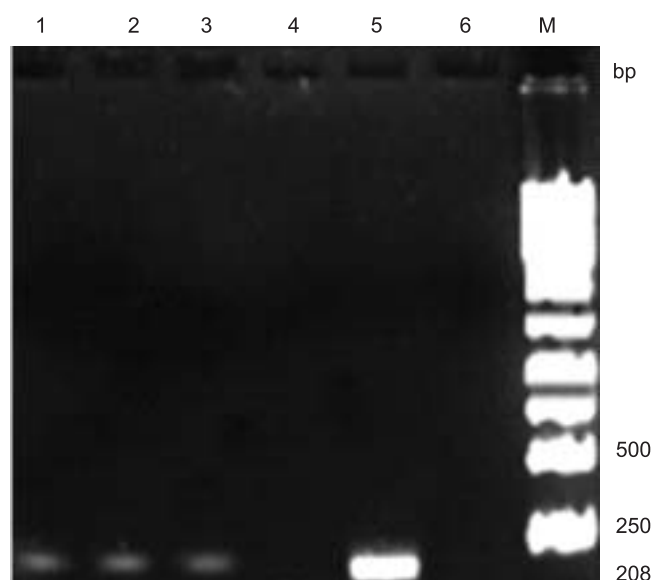


Fig. 1. Detection of BVDV RNA in PHA stimulated PBMCs by one step RT-PCR. Lanes 1–3: test (positive) samples; lane 4: test (negative) sample; lane 5: positive control; lane 6: negative template control; lane M: 1kb DNA ladder.

BVDV RNA can be detected but isolation of infectious virus is generally not possible beyond 14–21 days post infection as the animal develops significant levels of BVDV neutralising antibodies. However, the amplified products in this study displayed very weak BVDV RNA signals in PHA stimulated PBMCs of 3 immune cattle and further amplification of the product was unsuccessful to carry out nucleotide sequencing of the product. Collins *et al.* (2009) made a similar observation. Thus, animals with prolonged infections in white blood cells might serve as a source of continued infection even despite the presence of neutralizing antibodies.

Although more work needs to be done in the future, this is for the first time that BVDV RNA was detected in PBMCs from BVDV antibody positive Indian cattle following mitogen stimulation. The result provides evidence in that long term persistence of BVDV in the PBMCs can occur in some of the acutely infected immune animals and remain undetectable unless stimulus is provided by mitogens. In field situation, the stimulus can be provided by probable stress, over-crowding, recurrent infection or transportation. Persistent infection of BVDV in PBMCs of some BVDV immune cattle may have epidemiological significance.

SUMMARY

The aim of the present study was possible detection of BVDV in peripheral blood mononuclear cells (PBMCs) of antibody positive naturally infected Indian cattle following phytohaemagglutinin A (PHA) stimulation. Of 250 serum samples from cattle in organized dairy farms in India tested by virus neutralization test (VNT), 93 cattle (37.2%) were found positive for BVDV neutralizing antibodies. The PBMCs from BVDV-immune cattle were stimulated with 5µg/ml of PHA for 72 h prior to BVDV RNA detection by RT-PCR or infectious virus detection by virus isolation. Of the 93 BVDV immune field cattle tested positive, BVDV RNA was detected only in PHA stimulated PBMCs of 3 cattle, while no infectious BVDV could be detected by virus isolation. This is for the first time, that BVDV RNA was detected following mitogen (PHA) stimulation of PBMCs in BVDV antibody positive Indian cattle.

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