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Peripheral blood mononuclear cells from field cattle immune to bovine viral diarrhea virus (BVDV) are permissive *in vitro* to BVDV

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Summary. – The aim of this study was to determine the *in vitro* permissivity of peripheral blood mononuclear cells (PBMCs) from bovine viral diarrhea virus (BVDV)-immune field cattle to homologous and heterologous BVDVs. PBMCs from seventeen BVDV-naïve and sixteen BVDV-immune animals were infected with noncytopathic BVDV-1 or BVDV-2. The immune status of cattle was indicated by the presence of virus neutralizing antibodies, while viral load of PBMCs was determined by real-time RT-PCR. The results revealed that the PBMCs from naïve or immune animals were permissive to either BVDV-1 or BVDV-2, but the viral load was significantly higher for the naïve than for the immune animals. Furthermore, the load of homologous virus in PBMCs from immune animals was lower than that of heterologous virus. Our results provide evidence that the PBMCs from BVDV-immune cattle in field are susceptible to reinfection with homologous or heterologous BVDV, albeit to a lower extent in the former case.

Keywords: bovine viral diarrhea virus; peripheral blood mononuclear cells; cattle; immunity; virus permissivity in vitro

Introduction

Bovine viral diarrhea (BVD), an economically important viral disease of cattle is prevalent worldwide. The causative agents of BVD, bovine viral diarrhea virus 1 (BVDV-1) and bovine viral diarrhea virus 2 (BVDV-2) together with border disease virus (BDV) and classical swine fever virus belong to the genus *Pestivirus* in the family *Flaviviridae* (King *et al*., 2011). Additionally, atypical bovine pestivirus, a candidate for BVDV-3 species (Liu *et al*., 2009) has been identified recently. BVDV-1 strains are prevalent in cattle populations around the world, while BVDV-2 strains occur sporadically except for few exceptions. The BVDV genome is approximately 12.3 kb long single-stranded positive-sense RNA containing one large ORF that encodes a single poly-protein of about 3,900 aa flanked by UTR at both ends which is cleaved into four structural and 7–8 nonstructural proteins by viral and host cell proteases (Meyers and Thiel, 1996). Based on genetic characterization, BVDV-1 species has been divided into at least 16 subtypes and BVDV-2 species into 2 subtypes (Vilcek *et al*., 2001; Jackova *et al*., 2008).

On the basis of their effect on cultured cells, BVDV strains have been classified into two biotypes, cytopathic and noncytopathic (ncp). Ncp BVDVs are the prominent biotypes in the field and cause persistent infection, vital for transmission and survival of BVDV. BVDV infection in susceptible cattle can lead to either acute infection in immuno-competent animals, persistent infection in developing, immunologically naïve bovine fetus or a chronic infection within immune-privileged sites (testicular tissue, ovarian tissue and central nervous system's tissue) following transient infection (Voges *et al*., 1998; Grooms *et al*., 1998; Givens *et al*., 2003; Blas-Machado *et al*., 2004). Cattle with

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Abbreviations: BVD = bovine viral diarrhea; BVDV-1 = BVD virus 1; BVDV-2 = BVD virus 2; ncp = noncytopathic; PBMCs = peripheral blood mononuclear cells; p.i. = post infection

prolonged BVDV infection will develop a significant immune response accompanied by high neutralizing antibody titers (Voges *et al*., 1998; Givens *et al*., 2003).

Both ncp/cytopathic BVDVs infect peripheral blood mononuclear cells (PBMCs), which are pivotal in control of the innate and acquired immune response in cattle. These cells include granulocytes, monocytes, null cells, macrophages, antigen-presenting cells, myeloid cells, CD4+ and CD8+ T-lymphocytes and B cells (Chase *et al.,* 2004). Of these, the B cells population seems to make only minor contribution (Ohmann *et al*.,1987), while infection of antigen presenting cells with BVDV perturbs the normal pathway of immune stimulation thereby leading to immune evasion and chronic infection (Glew *et al*., 2003).

Generally, following an acute infection, BVDV can be isolated or detected in PBMCs up to 14 days post infection (p.i.) when neutralizing antibodies become detectable. However, BVDV has been detected in circulating PBMCs from seropositive cattle (Gogorza *et al*., 2005). Recently, it has also been demonstrated that prolonged BVDV infection can occur in PBMCs in acutely infected calves even in presence of neutralizing antibodies (Collins *et al*., 2009). But the detail mechanism of persistence of BVDV in BVDV-immune animals has not yet been elucidated and it is not known, whether PBMCs from BVDV-immune cattle in field following natural infection can be re-infected with BVDVs. Hence, the aim of this study was to determine the *in vitro* permissivity of PBMCs from BVDV-immune field cattle to homologous and heterologous BVDVs.

Materials and Methods

Viruses and cells. Indian cattle BVDV-1b strain, Ind S-1449 (Mishra *et al*., 2004) and BVDV-2a strain, Ind 141353 (Behera *et al*., 2011) were used in this study. MDBK cells obtained from Collection of cell lines in veterinary medicine, Riems, Germany (kind gift by Roland Riebe) were used for virus propagation and virus neutralization tests. The cells were grown in Eagle′s MEM (Sigma Aldrich) containing 10% horse serum (Life Tech, USA) and maintained at 37°C with 5% CO_2 atmosphere. The cells were tested for presence of any adventitious pestivirus contamination by RT-PCR using panpestivirus specific primers.

Infectious virus titration. Following propagation in MDBK cells, the titre of the stock viruses was determined by immunoperoxidase monolayer assay (IPMA) using BVDV polyclonal antibodies (VMRD, USA) as described earlier (Mishra *et al*., 2008).

Animals selection and blood handling. Whole blood samples collected from cattle of different geographic areas during October 2012 and April 2013 were used in the study. Blood leukocytes were tested for BVDV by virus isolation or RT-PCR using standard methods.

Virus neutralization test. The sera separated from blood were tested for the presence of BVDV antibodies by using BVDV-1 and BVDV-2, MDBK cells and immunoperoxidase monolayer assay as described earlier (Mishra *et al*., 2008). Seventeen BVDV-naïve animals were selected from a BVDV-free and BVDV-antibody-free dairy herd while sixteen BVDV-immune animals were selected from four different dairy herds. Serum samples from sixteen BVDV-immune cows were further subjected to end point virus neutralizing antibody titration for ascertaining BVDV-1 or BVDV-2 infection present in the field.

Isolation and cultivation of PBMCs. PBMCs were separated from 2 ml of blood from BVDV-immune (n = 16) and BVDV-naïve animals (n = 17) by density gradient centrifugation with Histopaque 1078 (Sigma-Aldrich) following the earlier reported protocol (Collins *et al*. 2009) with some modifications. Cells were counted and checked for viability by dye exclusion method using 0.4% trypan blue. Appropriate volume of RPMI-1640 (Sigma-Aldrich) supplemented with 10% FCS (Sigma-Aldrich), 200 mmol/l glutamine, 100 IU/ml penicillin and 100 µg/ml of streptomycin was added to obtain a concentration of 2 x 10^6 cells/ml of PBMC culture medium. PBMCs suspended in 0.25 ml of PBMC culture medium containing 5 x 10^5 cells were seeded in each well of 24-well TC plate and were incubated at 37°C in 5% CO_2 atmosphere.

Infection of PBMCs cultures with BVDVs. After 24 hr of culture, old PBMC culture medium was removed and PBMCs were infected with BVDV-1 or BVDV-2 at MOI = 1 in duplicate wells. Uninfected PBMCs in a well served as negative control. Infected PBMCs were incubated for 90 min at 37°C for virus adsorption before washing with plain RPMI-1640 medium and adding fresh PBMC culture medium. The infected PBMCs were incubated for 24 hr at 37°C in 5% CO_2 atmosphere and then kept frozen at -80°C until tested for viral load by real time RT-PCR.

Real-time RT-PCR assay of viral load. Total RNA was extracted from frozen and thawed cell culture containing PBMCs including culture medium (0.25 ml) using TRI Reagent (Ambion, USA) following the manufacturer's recommendations. Pelleted RNA was resuspended in 30 µl of nuclease free water and was stored at -80°C until use. Extracted RNA from infected and uninfected PBMCs was subjected to quantification of viral load by one-step TaqMan real time RT-PCR using Light Cycler 480 (Roche), SuperScript III Platinum One-Step Quantitative RT-PCR system (Invitrogen, USA), primers 190F (Hoffmann *et al*., 2006), V326 (Vilcek *et al*., 1994) and probe TQPESTI (Gaede *et al*., 2005) according to the protocol described earlier (Hoffmann *et al*., 2006). The viral load in PBMCs from each BVDV-naïve and BVDV-immune cattle was calculated in relationship to the reference virus calibrator (BVDV-1 or BVDV-2) and expressed as $TCID_{50}$/assay. For preparation of standard curve, 10-fold dilutions of RNA from the reference virus corresponding to a virus titer of 10^5 $TCID_{50}$/ml were made and tested by real time RT-PCR in triplicate. Adequate precautions were taken to avoid cross contamination by including positive control, negative control (uninfected PBMCs), no template control and extraction control. Data on virus loads were analyzed by standard statistical procedures and Kruskal-Wallis test.

Table 1. BVDV load in PBMCs from BVDV-naïve cattle

Animal No.	No. of PBMCs/ml	Cell (%) viability	BVDV-1 load ($TCID_{50}$/assay)	BVDV-2 load ($TCID_{50}$/assay)
296806	$1.3x10^6$	91	0.680	0.387
296774	$3.2x10^6$	98	0.378	0.3195
296805	$1.7x10^6$	95	0.359	0.251
296775	$2.6x10^6$	92	0.746	0.260
296777	$1.4x10^6$	97	0.693	0.2466
296813	$1.3x10^6$	93	0.531	0.3168
296810	$1x10^7$	94	0.288	0.2367
296788	$1.7x10^6$	90	0.282	0.497
295971	$8.4x10^6$	98	0.171	0.1426
295975	$1.1x10^7$	98	0.1746	0.1426
295967	$1.1x10^7$	98	0.6682	0.188
296905	$2.5x10^4$	98	0.655	0.5539
296900	$9x10^5$	97	0.8226	0.5165
296912	$7.5x10^5$	92	0.6493	0.4023
296921	$4x10^5$	95	0.4927	0.6453
296864	$1.2x10^6$	93	0.4837	0.5165
296860	$6x10^5$	96	0.4023	0.4023
Mean viral load (n = 17)			0.498 ± 0.203	0.354 ± 0.151

Table 2. BVDV load in PBMCs from BVDV-1-immune cattle

Animal No.	No. of PBMCs/ml	Cell (%) viability	BVDV-1 antibody titer	BVDV-2 antibody titer	BVDV-1 load ($TCID_{50}$/assay)	BVDV-2 load ($TCID_{50}$/assay)
130213	$1.5x10^5$	94	1:80	1:40	0.210	0.252
296614	$1.66x10^6$	98	1:80	1:40	0.162	0.344
296616	$3.2x10^6$	92	1:160	1:40	0.124	0.306
296627	$3.6x10^6$	95	1:40	1:10	0.247	0.411
296638	$1.4x10^6$	97	1:80	1:20	0.198	0.273
296641	$3x10^5$	91	1:160	1:20	0.214	0.291
296613	$2x10^6$	93	1:40	1:10	0.234	0.375
296619	$2.5x10^5$	94	1:160	1:10	0.236	0.380
296623	$1.5x10^5$	95	1:160	1:40	0.222	0.360
296631	$5x10^5$	95	1:40	1:20	0.2358	0.2664
Mean viral load (n = 10)					0.208 ± 0.04	0.325 ± 0.06

Table 3. BVDV load in PBMCs from BVDV-2-immune cattle

Animal No.	No. of PBMCs/ml	Cell (%) viability	BVDV-1 antibody titer	BVDV-2 antibody titer	BVDV-1 load ($TCID_{50}$/assay)	BVDV-2 load ($TCID_{50}$/assay)
130213	$2.5x10^5$	92	1:80	1:160	0.269	0.150
130213	$3.5x10^5$	94	1:80	1:160	0.339	0.136
296608	$3x10^6$	98	1:160	1:320	0.264	0.173
296612	$5x10^6$	91	1:40	1.80	0.368	0.146
296863	$5.5x10^5$	97	N	1:10	0.410	0.213
296864	$4x10^5$	95	1:20	1:40	0.421	0.237
Mean viral load (n = 6)					0.345 ± 0.06	0.175 ± 0.04

Results

The results of viral load by real-time RT-PCR showed that in PBMCs from BVDV-naïve cattle, the mean (± SD) viral load of BVDV-1 was 0.498 ± 0.203 $TCID_{50}$/assay, while the BVDV-2 viral load was 0.354 ± 0.151 $TCID_{50}$/assay (Table 1).

BVDV-naïve animals were seronegative to both BVDV-1 and BVDV-2 and on the basis of neutralizing antibody titers, from 16 BVDV-immune cows ten were classified as BVDV-1 -immune and six cows as BVDV-2-immune (Table 2 and 3). Since vaccination against BVDV has never been used in India, BVDV-immune cattle were supposed to be infected naturally with BVDV in the field.

In PBMCs from BVDV-1-immune cattle (n = 10), the mean BVDV-1 viral load was 0.208 ± 0.04 $TCID_{50}$/assay and the mean BVDV-2 viral load was 0.325 ± 0.06 $TCID_{50}$/assay (Table 2). Similarly, in PBMCs from BVDV-2-immune cattle, the mean BVDV-1 viral load was 0.345 ± 0.067 $TCID_{50}$/assay and the mean BVDV-2 viral load was 0.175 ± 0.040 $TCID_{50}$/assay (Table 3). Taken together, the results revealed that PBMCs of both BVDV-naïve and BVDV-immune cattle could be infected by both BVDV-1 and BVDV-2 although the viral load was significantly higher in PBMCs from BVDV-naïve cattle ($P > 0.05$) than in PBMCs from BVDV-immune cattle in the field (Table 2 and 3, Fig. 1).

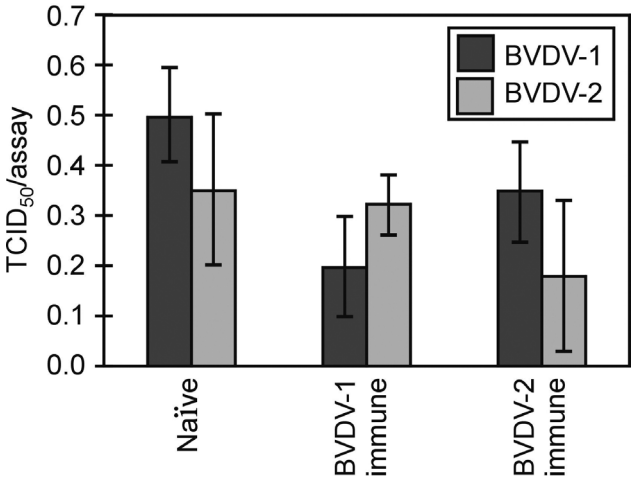

Fig. 1
Viral loads in PBMCs from BVDV-naïve and BVDV-immune cattle
Mean values (± standard error) of viral load in PBMCs are shown.

Discussion

BVDV-1 and BVDV-2 ncp viruses were used for infecting PBMCs from BVDV-naïve and BVDV-immune field cattle in this study, since ncp biotype of BVDV is the major infecting biotype, BVDV-1 and BVDV-2 are prevalent in cattle in most parts of the world including India (Mishra *et al.*, 2004; Ridpath *et al.*, 2010; Behera *et al.*, 2011). In a previous study, the viral load of BVDV-1 in PBMCs from BVDV-naïve cattle has been reported as 1.24 ± 2.12 $TCID_{50}$/assay and that of BVDV-2 as 2 $TCID_{50}$/assay (Lucchini *et al.*, 2012). Another study showed that the viral load of BVDV-1 in PBMCs from BVDV-naïve cattle was 2.84 ± 2.83 $TCID_{50}$/assay 18 hr p.i. and 35.03 ± 31.90 $TCID_{50}$/assay 36 hr p.i. (Turin *et al.*, 2013). A lower viral load for both BVDV-1 and BVDV-2 was evident in our study compared to the results of Lucchini *et al.* (2012), while the BVDV-1 load was almost similar to that found by Turin *et al.*, 2013. Since the assays for viral load in PBMCs were performed 24 hr p.i., it is likely that the replication cycle was complete at the time of assay, as BVDV eclipse period has been reported to be completed at about 12 hr in bovine cells (Gong *et al.*, 1996) and although one-step growth curve has yet to be determined in PBMCs, highest viral load of BVDV-1 in PBMCs from BVDV-naïve cattle has been found 36 hr p.i. (Turin *et al.*, 2013). Hence, viral load in PBMCs may be strain dependent due to difference in efficiency in adsorption and/or replication of BVDVs.

Difference in efficiency of replication of BVDV-1 or BVDV-2 in PBMCs has been reported earlier (Lucchini *et al.*, 2012; Turin *et al.*, 2013). However, in contrast to a previous study (Lucchini *et al.*, 2012), our results showed that the viral load of BVDV-1 was higher than BVDV-2 in PBMCs from BVDV-naïve cattle which may be due to the difference in BVDV strains used. Low viral load of ncp BVDVs in cattle is however not unusual, since it provides an efficient mechanism for virus survival in nature.

A previous study has shown that lymphocytes from BVDV-naïve cattle were more efficiently infected *in vitro* than those of BVDV vaccinated cattle (Beer *et al.*, 1997). Recently, a study involving infection of PBMCs obtained from BVDV-naïve and BVDV-1b experimentally immune cattle with viruses belonging to BVDV-1a, BVDV-1b and BVDV-2a subtypes showed that BVDV-naïve cattle had higher viral load than that of BVDV experimentally immune cattle (Lucchini *et al.*, 2012). Here we also demonstrated that BVDV-naïve cattle had higher viral load than BVDV-immune field cattle.

BVDV has earlier been detected in PBMCs from BVDV-immune animals (Gogorza *et al.*, 2005) and a long term persistent infection has been demonstrated in PBMCs following acute infection where BVDV could be transferred to naïve calves via blood transfusion (Collins *et al.*, 2009). Hence, humoral response against BVDV alone may not be adequate for efficient clearance of BVDV from PBMCs of BVDV infected animals. It has been shown previously that infection of antigen presenting cells in PBMCs with BVDV

can adversely affect their ability to interact with and stimulate T-cell responses which may be a critical viral strategy for immune evasion (Glew *et al.*, 2003). Our results revealed that in PBMCs from BVDV-1 and BVDV-2-immune cattle, the viral load of heterologous virus was more than the homologous virus indicating that cell-mediated immune response is involved in determination of viral load. Although the time of exposure of field cattle with BVDV was not determined, this study for the first time demonstrated that PBMCs from BVDV-immune field cattle are susceptible to re-infection with BVDV.

In conclusion, our results showed that PBMCs from BVDV-immune cattle naturally infected in the field are susceptible to re-infection with both homologous and heterologous BVDVs, although to a lower extent with the former case. Re-infection of PBMCs of BVDV-immune cattle can have epidemiological significance for control of BVD.

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