In vitro Replication Activity of Bovine Viral Diarrhea Virus in an Epithelial Cell Line and in Bovine Peripheral Blood Mononuclear Cells

Lauretta TURIN1), Barbara LUCCHINI1), Valerio BRONZO1) and Camilla LUZZAGO1) *

1)Department of Veterinary Pathology, Hygiene and Public Health, University of Milan, Via Celoria 10, 20133 Milano, Italy

(Received 12 January 2012/Accepted 29 May 2012/Published online in J-STAGE 12 June 2012)

ABSTRACT. The present study focused on the in vitro infection of Madin-Darby bovine kidney (MDBK) cells and bovine peripheral blood mononuclear cells (PBMCs) from naïve animals with non-cytopathic (ncp, BVDV-1b NY-1) and cytopathic (cp, BVDV-1a NADL) strains. Infections with 0.1 and 1 multiplicity of infections (MOI) and incubation times of 18 and 36 hr were compared. Twelve BVDV naïve heifers were enrolled to collect PBMCs. The viral loads in MDBK cells and in PBMCs after in vitro infections were measured by real-time polymerase chain reaction (PCR) assays. The highest viral loads were measured at 1 MOI and 36 hr post infection in both cell systems and the lowest at 0.1 MOI and 18 hr with the exception of the cp strain NADL in PBMCs, for which the highest viral load was observed at 0.1 MOI and 36 hr. Viral load mean values were higher for the cp strain than the ncp strain irrespective of the extent of the infection period and MOI. The models of infection studied uncovered different replication activities respectively according to the biotype of virus, the cell substrate and the duration of infection. Replication tends to be higher in PBMCs, particularly at low MOIs and for the ncp strain.

KEY WORDS: bovine viral diarrhea virus, in vitro infection, MDBK, PBMC, real-time PCR.


Bovine viral diarrhea virus (BVDV) is an economically important pathogen of cattle belonging to the Pestivirus genus of the Flaviviridae family. Genetic typing of BVDV isolates has distinguished two species, BVDV-1 and BVDV-2, plus a recent putative BVDV-3 species [11]. While the former virus species includes at least 11 subtypes, BVDV-1a to −1k [19], BVDV-2 viruses are classified into two subtypes [15].

A number of studies are available on in vivo experimental infections, while the in vitro models used to investigate the infectivity and replication of different BVDV genotypes and subtypes in permissive cells are limited [2, 16]. Therefore, the development of in vitro systems is a valid support to underlie the interactions of different virus subtypes with cell substrates and correlate them with the in vivo infection. This objective is particularly important because a wide spectrum of virulence has been observed among strains belonging to the same genotype, such as that of the well known BVDV-2 species [8, 10]. Recently, a virulence variation among BVDV-1 subtypes has been reported based on comparison of clinical outcomes following in vivo infection [17].

BVDV infects a wide variety of cell types but has a preferential tropism for immune cells, affecting their functions. BVDV infects both cells of the innate arm, such as neutrophils, monocytes, macrophages and dendritic cells, and cells devoted to the adaptive response, namely T- and B-lymphocytes [7, 18].

Following in vitro and in vivo infections, both non-cytopathic (ncp) and cytopathic (cp) biotypes can affect the activity of alveolar macrophages; alterations of the surface expression of the Fc receptor and the complement receptor result in depressed phagocyte activity, impairment of the microbicidal activity and reduced responsiveness to chemo-tactic factors [4, 14].

The aim of the present study was to investigate the in vitro replication activity of BVDV-1 cp and ncp biotypes in Madin-Darby bovine kidney (MDBK) cells and peripheral blood mononuclear cells (PBMCs) from BVDV naïve animals.

MATERIALS AND METHODS

Animal selection and blood handling: This study involved 12 BVDV naïve Holstein-Friesian heifers (10–14 months old) from three BVDV-free dairy herds that participated in a BVDV control program [12]. None of the animals had received vaccination against BVDV. The experimental protocol was ethically approved by the Italian Ministry of Health (protocol number 2006070977-003).

Blood samples from each animal were collected with and without anticoagulant (lithium-heparin), kept at 4°C during delivery to the laboratory and processed within 2 hr. Sera were isolated from blood samples without anticoagulant by centrifugation at 600 × g for 10 min and stored at −20°C.

Sera from each animal were tested by virus neutralization (VN) test [6] for the presence of BVDV antibodies against different BVDV-1 and BVDV-2 strains.

PBMCs from each animal were separated by density gradient centrifugation from blood with anticoagulant; after centrifugation at 1200 × g for 10 min, theuffy coat was collected, diluted 1:2 with phosphate buffered saline (PBS)
and stratified on Histopaque-1077 (Sigma-Aldrich, Munich, Germany) at a the proportion of 2/3. After centrifugation at 400 × g for 30 min at room temperature, PBMCs were collected and washed three times in PBS. Viable PBMCs were counted (trypan blue exclusion method) and resuspended at 6 × 10^6 cells/ml in RPMI-1640 medium supplemented with 10% BVDV- and antibody-free fetal calf serum (FCS), 1% L-glutamine and 0.01% β-mercaptoethanol.

Viruses and cells: Two viral strains were used for in vitro infections, BVDV-1a cp NADL (ATCC VR-534) and BVDV-1b ncp NY-1 (ATCC VR-524); the BVDV-1a cp Singer strain (NVSL, Ames, IA, U.S.A.) was used as a calibrator for real-time polymerase chain reaction (PCR) quantification. To obtain the stocks, the viruses were grown in MDBC cells (ATCC CCL-22), maintained in minimal essential medium (MEM) with 10% FCS, 1% L-glutamine 200 mM and 100 U/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml fungizone. The MDBC cell line was also used for virus stocks titration, serological tests and in vitro infections.

Virus titration: Tissue culture end point titration in 96-well plates was performed for quantification of each virus stock; tenfold dilutions of each virus stock in quadruplicate virus stocks titration, serological tests and infections. (TCID50/titer was expressed by 50% tissue culture infective dose/titer was expressed by 50% tissue culture infective dose/to the virus). For both ncp and cp strains, the virus staining in at least one cluster of infected cells was indicative of a positive reaction. For both ncp and cp strains, the virus titer was expressed by 50% tissue culture infective dose/µl, according to the Spearman-Karber method.

MDBC and PBMCs infections: MDBC cells, suspended in the amount of 10^5/well in culture medium, were seeded in 24-well plates and allowed to adhere for 2 hr at 37°C in 5% CO2. Infections with each viral strain at 0.1 and at 1 MOI were performed in 4 wells, and an extra well was used as a negative control. Viruses were allowed to adsorb for 1 hr at 37°C in 5% CO2. After this period, non-adsorbed viruses were eliminated by washing MDBC monolayer cells twice with culture medium. The plates were incubated at 37°C in 5% CO2, for 18 and 36 hr. MDBC assays were repeated twice.

PBMCs obtained from each animal as described above, suspended in the amount of 1.2 × 10^6/well in PBMC culture medium, were seeded in 24-well plates. Five replicates were seeded for each assay, the negative control and 4 wells for infection with each viral strain at 0.1 and at 1 MOI, allowing an adsorption time of 1 hr at 37°C in 5% CO2. After this period, non-adsorbed virus was eliminated by cell washing and centrifugation twice in PBMC culture medium. The cell pellets were resuspended in PBMC culture medium, plated and incubated at 37°C in 5% CO2 for 18 and 36 hr.

After incubation, both MDBC and PBMC plates were frozen at −80°C until virus quantification was performed.

Quantification of viral loads by real-time PCR: The total content of each well, MDBC cells plus culture medium and PBMCs plus culture medium, underwent viral RNA extraction with a QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany); the final concentration of RNA was determined using a spectrophotometer at a wavelength of 260 nm. The whole sample, corresponding to about 1 µg of RNA, was reverse transcribed to cDNA using a high-capacity cDNA archive retrotranscription kit with random hexamers (Applied Biosystems, Foster City, CA, U.S.A.). For the calibrator, BVDV-1a Singer strain RNA was extracted with the same protocol from the supernatant of MDBC cells containing 2000 TCID50/µl. The cDNA (9 µl) obtained from the reference virus and from each sample was used as a template for TaqMan real-time PCR assays in an optimized reaction volume of 20 µl on MicroAmp optical 96-well plates in the presence of 2X Master Mix (10 µl) (Applied Biosystems), probe and 300 nM each of the primers (0.3 µl of 10 µM solution) [3]. In addition to cDNA samples, duplicates of a 10-fold dilution of cDNA from the reference virus were included in each plate and used to generate a calibration curve for virus quantification. Preliminarily, 10-fold serial dilutions of reference virus cDNA were used as templates to generate standard curves for determination of the assay sensitivity. A duplicate no-template control was also included in each plate. Real-time quantitative PCR was carried out in an ABI PRISM 7000 Sequence Detection System, and results were analyzed with its software (Applied Biosystems). For each sample tested, the virus quantity was calculated in relationship to the viral strain calibrator and expressed as the TCID50/assay.

RESULTS

The virus quantification in the present study was performed on the total volume of cells and medium in order to make sure to record all of the virus. Indeed, we had performed preliminary assays (data not shown) comparing the cells alone versus the medium alone and versus the cells plus the medium. The results of the preliminary assays showed positive signals for virus in all the samples, with a higher amount of virus detected in cells plus medium than in cells or medium separately. Therefore, we decided to quantify the medium and cells together.

The results obtained after infection of MDBC cells with NADL and NY-1 BVDV strains showed the highest viral loads for both strains at 1 MOI and 36 hr post infection. Higher viral loads were observed for the cp strain than the ncp strain. This difference was observed irrespective of the extent of the infection period and MOI. In particular, NADL and NY-1 reached mean values (±standard error) of 61.94 (±45.28) and 35.03 (±31.90) TCID50/assay respectively at 1 MOI and 36 hr versus 4.69 (±1.97) and 2.84 (±2.83) TCID50/assay at 1 MOI and 18 hr for NY-1 and 36 hr. For NY-1, 0.1 MOI showed slightly positive results both at 18 (0.14 ± 0.03) and 36 (0.49 ± 0.39) hr. The results are summarized in Fig. 1.

PBMCs from all tested animals were infected by the selected BVDV strains at both times of infection and MOIs. All the selected animals were negative for VN antibodies against BVDV-1 and −2 strains at the time of the blood sam-
Infection of PBMCs showed higher viral loads for the cp strain than the ncp strain. This difference was observed at both times of infection and MOIs with the exception of 1 MOI at 36 hr. In this case, the viral load of NY-1 was 28.41 (±8.73) TCID50/assay versus 21.15 (±2.94) TCID50/assay for NADL. The results are summarized in Fig. 2.

When comparing the 2 in vitro models, an opposite trend was observed at 1 MOI for NADL strains in MDBK cells versus PBMCs in terms of the amount of virus, which increased in MDBK cells and decreased in PBMCs.

**DISCUSSION**

The in vitro permissivity of MDBK cells and PBMCs from BVDV naïve animals to infection with 0.1 or 1 MOIs BVDV-1 reference strains was investigated by mean of quantification of viral loads at 18 and 36 hr post infection. MDBK cells and PBMCs were efficiently infected by NADL and NY-1 strains at both times of infection and MOIs. The highest viral loads were measured at 1 MOI and 36 hr post infection in both cell systems with the exception of the cp strain NADL in PBMCs, which allowed an intense replication rate already at 18 hr at both MOIs. Indeed, PBMCs represent, although in an in vitro system, the main target for BVDV proliferation, as observed for both the strains investigated. The opposite trend observed for NADL at 36 hr in both models can be explained by the different cell substrates available for the virus. Indeed, PBMCs do not replicate in vitro, while MDBK cells are characterized by a high replication rate. Therefore, we could hypothesize that the cp virus at the higher post infection time and higher MOI causes damage to all the available substrates necessary for replication, as supported by the efficient growth for the ncp strain, that does not affect the cells. Since the progression of infection up to 36 hr seems to be affected by PBMC vitality, a kinetic study in a narrow range of time post infection would be useful to highlight virus replication properties of different BVDV strains.

Concerning the different replication activities of BVDV biotypes, we observed higher viral loads for cp strains than ncp strains in both cell types. Our results are in accordance with other reports, which showed an increase of virus replication and production of a large amount of dsRNA in cells infected with cp, but not with ncp BVDV strains [1, 9, 20]. This feature may represent a survival strategy of ncp strains, because low production of viral particles can prove to be helpful in escaping the immune system and consequently persist in the host population.

Comparison of cp and ncp homologous biotypes could determine whether such difference is due to the strain itself or to the biotype.

In conclusion, PBMCs, which were efficiently infected in all the tested conditions, were shown to be a fitting substrate mainly for the ncp strain. This preliminary result shows that such an infection model could be useful to investigate the virus-host interactions of different subtypes and to correlate them with in vivo infection without any ethical impact and at lower costs. A wider panel of ncp BVDV strains characterized by a different in vivo virulence profile would be required to confirm the trends that we preliminarily observed.

**ACKNOWLEDGMENTS.** We are very grateful to Prof. Wilma Ponti for the valuable contribution to the experimental design and for helpful discussions. We acknowledge Professor E. J. Dubovi for kindly providing monoclonal antibody. Further, we wish to thank Dr. Michela Frigerio for help in collecting samples. This work was financially supported by a PRIN Grant (2006) from the Italian Ministry of Education, Scientific Research and Health.

**REFERENCES**

cytopathogenic virus and induction of lethal disease. *J. Virol.* **75**: 6256–6264. [Medline] [CrossRef]


