



Bovine viral diarrhoea virus (BVDV) does not use macropinocytosis or caveolae-mediated endocytic pathway for entry into bovine or ovine cells while brefeldin A inhibits its release in ovine cells

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ABSTRACT

Present knowledge on bovine viral diarrhoea virus (BVDV) entry, morphogenesis and release in host cells is incomplete. This study reports the results of effect of drug cytochalasin D and nystatin on BVDV entry in bovine and ovine cells and effect of Brefeldin-A (BFA), on BVDV release in ovine cells. The bovine (MDBK) and ovine (SFT-R) cells were treated with various concentrations of cytochalasin D or nystatin before infection with BVDV and at 16 h post infection, the number of infected cells was determined by immunocytochemistry. The ovine cells were infected with BVDV before addition of different concentration of BFA at 8 h post infection and the supernatants at 24 hpi were subjected to RT-PCR or immunocytochemistry. The results showed that only a minor inhibitory effect was observed on the entry and infectivity of BVDV in both ovine and bovine cells even at highest concentration of cytochalasin D or nystatin. As revealed by RT-PCR and virus titration, BFA treatment inhibited the BVDV secretion in a dose-dependent manner with complete inhibition obtained by 2 µg/ml of BFA. However, the intracellular virus particles in BFA treated cell pellet were infectious. Taken together, our results showed that entry of BVDV into bovine or ovine cells is not dependent on macropinocytosis or caveolae-mediated endocytosis and provided evidence of existence of a common mechanism of BVDV release in bovine and ovine cells.

Key words: BVDV, Caveolae-mediated endocytosis, Entry, Macropinocytosis, Release

Bovine viral diarrhoea viruses (BVDV) are economically important pathogens of cattle and sheep worldwide. Bovine viral diarrhoea virus 1 (BVDV-1), bovine viral diarrhoea virus 2 (BVDV-2) along with border disease virus (BDV) and classical swine fever virus (CSFV) belong to the genus Pestivirus in the family Flaviviridae (King *et al.* 2011). Under natural conditions cattle and sheep can be infected by BVDV-1, BVDV-2 and BDV and both BVDV-1 and BVDV-2 have been detected in cattle and sheep in India (Mishra *et al.* 2004, Mishra *et al.* 2008). The BVDV genome is approximately 12.3 kb long single-stranded positive-sense RNA containing one large open reading frame that encodes a single poly-protein of about 3900 amino acids which gets cleaved into four structural and 7–8 nonstructural proteins by viral and host cell proteases (Meyers and Thiel 1996). The 5'-end of ORF codes structural proteins comprising the capsid protein (C) and 3 envelope proteins, namely, E^{ms}, E1 and E2. E2 is the determinant of cell culture tropism for BVDV and other ruminant pestiviruses (Liang *et al.* 2003).

Animal viruses use several routes of entry for entering susceptible host cells. Some viruses use fusion with cell

membrane, whereas others use receptor-mediated endocytosis. The endocytic pathways exploited by animal viruses include macropinocytosis, clathrin-dependent endocytosis, and caveolae-dependent endocytosis, as well as poorly characterized routes such as clathrin- and caveolae-independent endocytosis (Pelkmans *et al.* 2003, Sieczkarski *et al.* 2002, Wang *et al.* 2008). Previous studies have shown that BVDV entry into bovine and ovine cells occurs by clathrin-dependent endocytosis and endosomal fusion (Grummer *et al.* 2004, Krey *et al.* 2005, Mathapati *et al.* 2009). However, it is not yet known whether BVDV can enter ovine or bovine cells through other alternate routes of entry such as caveolae-mediated endocytosis or macropinocytosis.

Similarly, scanty information is available on the assembly and release of BVDV from infected cells. Previous studies revealed that in bovine cells, BVDV most likely matures at intracellular membranes such as endoplasmic reticulum (ER) followed by exocytosis (Gray and Nettleton 1987, Gu *et al.* 2006). Later it was shown that BVDV assembly is completed at endoplasmic reticulum (ER) followed by release through exocytosis and trafficking beyond the cis-Golgi is not a prerequisite for BVDV infectivity (Macovei *et al.* 2006). But it is not known, whether a similar mechanism exists for BVDV release from

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ovine cells. Therefore, to expand the knowledge in understanding the processes involved in BVDV invasion, we determined the possible existence of alternate routes of BVDV entry into ovine and bovine cells by using inhibitors of caveolae-mediated endocytosis and macropinocytosis and tried to elucidate mechanism of BVDV assembly and release in ovine cells by using the inhibitor Brefeldin-A (BFA).

MATERIALS AND METHODS

Cells and viruses: The fetal sheep thymus cells SFT-R (RIE43) and Madin-Darby bovine kidney (MDBK) cells obtained from the Cell Culture Collection of Veterinary Medicine, Friedrich-Loeffler Institute, Island of Riems, Germany were proliferated in antibiotic free Eagle's minimum essential medium (EMEM) containing BVDV and BVDV antibody free 10% fetal bovine serum at 37°C in 5% CO₂ atmosphere. Indian cattle BVDV-1 ncp strain Ind S-1449 (Mishra *et al.* 2004) was propagated on MDBK cells and Indian sheep BVDV-1 ncp strain Ind S-17555 (Mishra *et al.* 2012) was propagated on SFT-R cells and stored at -80°C until use. The titers of the stock viruses were determined by immunoperoxidase monolayer assay (IPMA) using anti-BVDV polyclonal antibodies as described earlier (Mishra *et al.* 2008) and were expressed as TCID₅₀/ml.

Cytotoxicity assay: Confluent ovine cells (SFT-R) and bovine cells (MDBK) cultured in 96-well TC plate were treated with various concentrations (10, 5, 2.5, 1, 0.5 µg/ml) of cytochalasin D, an actin filament disrupting drug or nystatin (20, 10, 5, 2.5, 1.25, 0.625 µg/ml), an caveolae disrupting drug and were incubated at 37°C in CO₂ incubator. Similarly, SFT-R cells were treated with various concentrations (16, 8, 4, 2, 1, 0.5, and 0.1 µg/ml) of Brefeldin A. The cells were examined microscopically up to 48 h to determine cytotoxicity of the drugs.

Nystatin and cytochalasin-D treatment and infection with BVDV: The cells grown on 96-well plates were pre-treated with cytochalasin D (concentrations ranging from 0.4 to 0.0125 µg/ml) or nystatin (concentrations ranging from 10 to 0.625 µg/ml) for 2 h at 37 °C. Following washing twice with EMEM, the ovine cells (3.6×10^4) were infected with BVDV-1 strain Ind S-17555 and bovine cells were infected with BVDV-1 strain Ind S-1449 at a multiplicity of infection (MOI) of 1 and the cells were incubated at 37 °C for 1 h in presence or absence of drugs. The cells were washed, fresh EMEM was added and the cells were incubated at 37°C for 16 h.

BVDV infectivity assay: After 16 h, the number of infected cells was determined by IPMA using BVDV-1 E2 specific mAb 157 as described earlier (Mishra *et al.* 2008). The number of infected cells without any drug treatment (prior or during) was set to 100% and susceptibility was calculated as percentage of the control value. All experiments were performed thrice independently in triplicates. The mean and standard errors were calculated using standard statistical methods. To confirm that the effect

of each drug was restricted to the virus entry process, another set of ovine or bovine cultures were infected with BVDV-1 and drugs were added 1 h post infection and were treated similarly.

BVDV infection and BFA treatment: To study the morphogenesis and release of BVDV in ovine cells, confluent SFT-R cells in 6-well TC plates were infected with BVDV-1 strain Ind S-17555 at a MOI of 1 for 1 h. Fresh EMEM was added after washing the cells thrice with EMEM. BFA was added to the medium at various final concentrations (2 µg, 1 µg, 0.5 µg and 0.1 µg / ml) at 8 h post infection and the cells were incubated at 37°C for 24 h. After 24 h, the supernatants were collected following centrifugation at 5,000 g for 5 min and were subjected for detection of BVDV genomic RNA or infectivity assay. The experiments were repeated thrice and suitable controls (infected cells in absence of BFA and uninfected cells) were included in each experiment.

RT-PCR analysis: Viral RNA from infected cell culture supernatants was extracted by QIAamp viral RNA mini kit using manufacturer's protocol. The RNA was recovered in 30 µl of RNase-free water and stored at -80°C. For detection of BVDV E2 gene, cDNA synthesis was carried out in 20 µl volume using random hexamer primers and Superscript II reverse transcriptase followed by PCR using primers 2274F and 3422R (Arnal *et al.* 2004) and standard reagents which amplify a 1160 bp fragment overlapping E2 gene of BVDV-1. Amplified DNA products were detected by electrophoresis on an agarose gel stained with SYBR Safe.

Infectivity assay of extracellular BVDV: To determine the infectivity of the extracellular BVDV released from BFA treated and untreated ovine cells, the supernatant collected at 24 h post infection was used to infect SFT-R cells in 96-well TC plate. Following adsorption, the cells were washed, fresh EMEM was added and the cells were incubated at 37°C. After 72 h of infection, the number of infected cells was determined by IPMA using BVDV-1 E2 specific mAb 157 as described earlier (Mishra *et al.* 2008). Three independent experiments were conducted with suitable controls.

RESULTS AND DISCUSSION

BVDV entry into bovine and ovine cells occurs by clathrin-dependent endocytosis (Grummer *et al.* 2004, Krey *et al.* 2005, Mathapati *et al.* 2009). However, recent studies have shown that some viruses use multiple mechanisms to gain entry into host cells (Suksanpaisan *et al.* 2009, Aleksandrowicz *et al.* 2011). Hence, we explored the possible existence of alternate routes of BVDV entry viz, macropinocytosis and caveolae-mediated endocytosis. To define the role of these routes in BVDV entry in bovine and ovine cells, we used the drugs that affect these pathways, such as cytochalasin-D and nystatin.

Cytochalasin D, is an actin-disrupting drug, specifically affects the actin cytoskeleton by preventing its proper polymerization into microfilaments and promoting microfilament disassembly (Flanagan *et al.* 1980). Only a

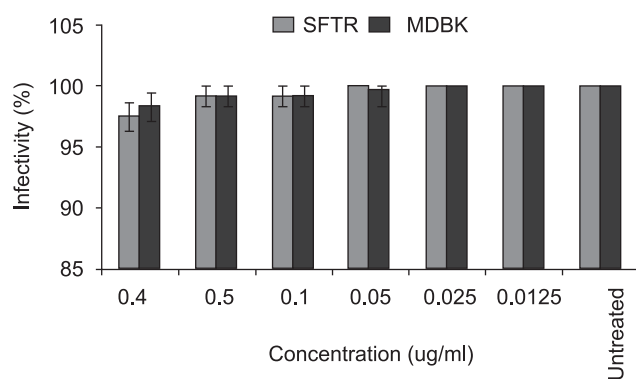


Fig. 1. Effect of cytochalasin-D on BVDV infectivity in bovine (MDBK) and ovine (SFT-R) cells. Columns represent means of triplicate experiments and bars indicate standard errors.

minor inhibitory effect (0–2.5%) was observed on the entry and infectivity of BVDV in both ovine and bovine cells even at highest concentration (0.4 µg/ml) of cytochalasin D (Fig. 1). Almost identical results were obtained when ovine (SFT-R) or bovine (MDBK) cells were treated with 0.4 µg/ml of cytochalasin D at 1 hpi. These results revealed that BVDV entry remained unaffected when macropinocytosis is inhibited, suggesting that BVDV does not use macropinocytosis for entry into ovine or bovine cells. Nystatin acts on caveolae, characterized by high levels of cholesterol and glycosphingolipids and also by the presence of caveolin, an integral membrane protein (Schnitzer *et al.* 1994). The results of BVDV infectivity assay using immunochemistry showed that only a slight inhibitory effect (0–9.4%) was observed on the entry and infectivity of BVDV in both ovine and bovine cells when treated even with the highest concentration (10 µg/ml) of caveolae disrupting drug nystatin (Fig. 2). Treatment of SFT-R and MDBK cells with 10 µg/ml of nystatin at 1 hpi reduced infection to the same extent as pre-treatment, suggesting that the slight effect observed with nystatin is not specific to entry. These results show that BVDV does not use caveolae-mediated endocytosis for ovine or bovine cell entry during infection.

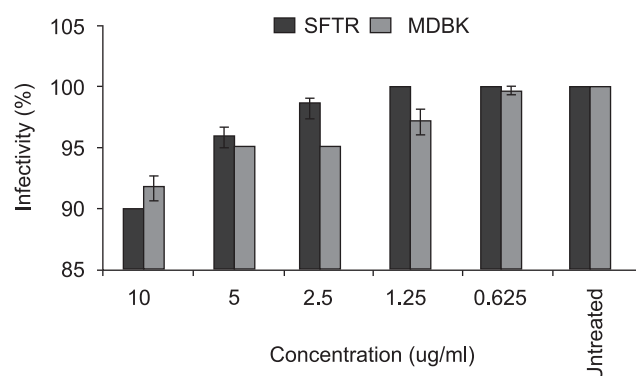


Fig. 2. Effect of nystatin on BVDV-1 infectivity in bovine (MDBK) and ovine (SFT-R) cells. Columns represent means of triplicate experiments and bars indicate standard errors.

Viruses belonging to vaccinia, adeno, picorna and other virus families have been reported to take advantage of macropinocytosis, while SV40, influenza virus and ebola virus have been reported to use caveolae-mediated internalization (Mercer and Helenius 2009). Our results for the first time showed that neither nystatin nor cytochalasin-D was able to block BVDV entry into ovine and bovine cells indicating lipid-raft and membrane actin filaments may not play any role in BVDV entry in these cells. However, it has been shown that the entry of dengue virus, which belongs to the related *Flavivirus* genus in the family *Flaviviridae*, is clathrin and macropinocytosis dependent but not dependent on caveolae (Suksanpaisan *et al.* 2009). It is interesting that viruses within the same family *Flaviviridae* use different mechanisms of entry into cells.

In order to characterize the intracellular route taken by mature BVD virions in ovine cells for secretion, we used brefeldin A (BFA), which blocks the transport of newly synthesized proteins from the ER to the golgi complex

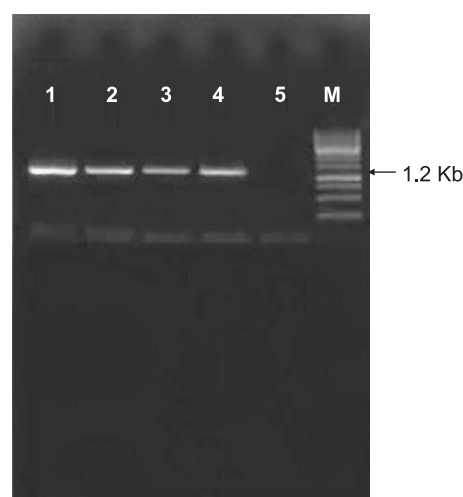


Fig. 3a. Effect of BFA on BVDV secretion from ovine cells as revealed by E2 RT-PCR. Lane 1: Positive control (No BFA), lane 2: 0.1 µg/ml, lane 3: 0.5 µg/ml, lane 4: 1.0 µg/ml, lane 5: 2.0 µg/ml, lane M: 1 Kb DNA ladder.

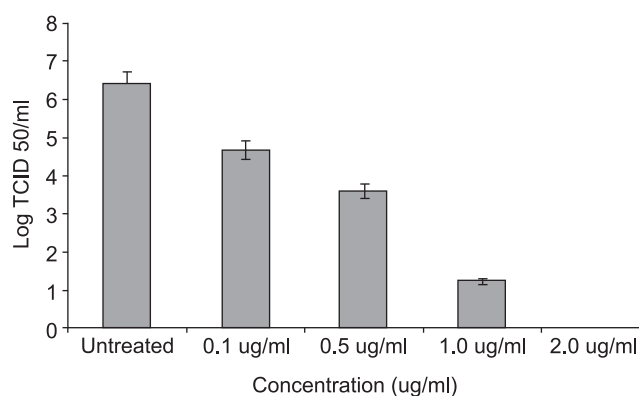


Fig. 3b. BVDV-1 infectivity titre in tissue culture fluids collected at 24 h p.i. from BFA treated and untreated SFTR cells. Columns represent mean values of three independent experiments assayed in duplicate; error bars indicate standard error mean.

(Pelham *et al.* 1991). BFA has been used extensively to study the assembly, morphogenesis and release of many viruses including flaviviruses, such as Kunjin virus and Japanese encephalitis virus (Lad and Gupta 2011, Mackenzie *et al.* 1999). Since a 20 h incubation with >2 µg/ml BFA resulted in visible cytotoxic effects in ovine cells, we used lower (2 or <2 µg/ml) concentrations of BFA and reduced the time of drug exposure (16 h). As revealed by RT-PCR assay, BFA treatment inhibited the BVDV secretion from ovine cells in a dose-dependent manner (Fig. 3a). The highest BFA concentration used (2 µg/ml) completely inhibited ncp BVDV secretion without affecting the SFT-R cell viability, since no infectious BVD virus was detected in extracellular fluid obtained from infected cells treated with 2 µg/ml of BFA by virus titration (Fig. 3b). A previous study has shown that 1 µg/ml of BFA completely inhibited cp BVDV release from bovine (MDBK) cells (Macovei *et al.* 2006) which may be due to the difference in biotypes of BVDV or type of host cells used. However, the intracellular virus particles harvested from BFA treated cell pellet remained unaffected by BFA as revealed by infectivity assay (results not shown). It has been reported that some enveloped viruses fail to assemble into complete virions in presence of BFA (Dasgupta *et al.* 2001). The results in this study suggested that BFA inhibits release of BVDV in ovine cells without affecting BVDV assembly and infectivity. It has been shown that in bovine cells BFA blocks cp BVDV export from ER and causes disruption of golgi without affecting the virus infectivity implying release of virions from ER/cis-golgi followed by exocytosis (Macovei *et al.* 2006). Our results strongly supported a similar mechanism for ncp BVDV assembly and release in ovine cells. It is interesting that both cp and ncp BVDV strains use similar mechanisms of virus release from bovine and ovine cells.

In conclusion, the results of this work showed that entry of BVDV into bovine or ovine cells is not dependent on macropinocytosis or caveolae-mediated endocytosis and provided evidence of existence of a common mechanism of BVD virus release in bovine and ovine cells. Expansion of knowledge with regard to pestivirus entry, morphogenesis and release may lead to development of new antiviral therapeutics in future.

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