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## Comparison of viral replication and IFN response in alpaca and bovine cells following bovine viral diarrhoea virus infection

Holly C. Samson, Christina L. Topliff, Ruben O. Donis<sup>1</sup>, Clayton L. Kelling\*

School of Veterinary Medicine and Biomedical Sciences, University of Nebraska-Lincoln, East Campus Loop and Fair Street, Lincoln, NE, 68583–0905, USA

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### ABSTRACT

Alpacas develop diminished disease following bovine viral diarrhoea virus (BVDV) infection compared to cattle. We hypothesized that alpaca and bovine cells have differential permissiveness and responses to BVDV infection. To characterize alpaca testicular (AT) and bovine turbinate (BT) cells BVDV infection permissiveness, viral replication and interferon (IFN) synthesis was evaluated. BVDV replicated 3–4 logs lower in AT cells with diminished antigen deposition compared to BT cells. BVDV infection inhibited IFN response in both AT and BT cells. Compared to BT cells, BVDV-infected AT cells had a 2–5 fold increase in IFN synthesis following dsRNA stimulation. The greater IFN response of AT cells compared to BT cells following poly I:C stimulation with or without ncp BVDV infection, may be the basis for the decreased BVDV permissiveness of AT cells and may contribute to the clinical differences following BVDV infection of alpacas and cattle.

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### Introduction

Bovine viral diarrhoea virus (BVDV) infects members of the order *Artiodactyla* (Wengler, 1991) and is an important bovine pathogen having negative impacts on beef and dairy production (Booker et al., 2008; Fourichon et al., 2005; Houe, 2003). BVDV causes gastroenteritis, immunosuppression and abortion in infected cattle (Galav et al., 2007; Janzen and Clark, 1995; Kelling et al., 1990). The most insidious effect of BVDV infection is the causation of persistently-infected (PI) calves and crias, due to *in utero* infection and immunotolerance of the fetus to the infecting BVDV strain. PI animals shed virus throughout their lifetime and provide a reservoir of BVDV infection in herds (McClurkin et al., 1985; Topliff et al., 2009).

BVDV is an enveloped *Pestivirus* with a positive-sense, single-stranded RNA genome approximately 12.3 kb in length, encoding eleven or twelve proteins in one long open reading frame (Wengler, 1991). Based on comparison of genetic sequences, BVDV isolates can be grouped into BVDV1 and BVDV2 (Ridpath et al., 1994; Thiel et al., 2005) with isolates varying in virulence (Kelling et al., 2002; Topliff and Kelling, 1998).

After infection with BVDV, the first line of cellular defense is interferon (IFN) synthesis. Interferons are inducible cytokines which defend the host against infection by activating cellular factors to shut down viral mRNA synthesis and degrade viral RNA (Castelli et al., 1997) and to promote the activity of natural killer cells (Sato et al., 2001), B cells (Jego et al., 2003; Litniskiy et al., 2002) and T cells (Foster et al., 2004; Le Bon et al., 2003; Rogge et al., 1998) for systemic protection. Evasion of this host immune response is crucial for both acute and persistent infections with BVDV. BVDV has been shown to inhibit the synthesis of IFN (Schweizer et al., 2006) and can also inhibit production of molecules stimulated by IFN synthesis such as Mx protein (Schweizer and Peterhans, 2001).

While BVDV is well-recognized as a cause of both acute and persistent infections in cattle, it was previously thought that New World camelids, such as alpacas, were resistant to infection with BVDV (Wentz et al., 2003). However, occurrences of PI alpacas have recently been reported (Carman et al., 2005; Foster et al., 2007; Goyal et al., 2002; Mattson et al., 2006; Topliff et al., 2009). Previous work indicates that clinical effects of post-natal BVDV infections of New World camelids are diminished in comparison to infections of cattle (Wentz et al., 2003).

Reports of BVDV prevalence (Topliff et al., 2009) and infection in alpacas have prompted investigations into mechanisms of BVDV infection of non-bovine host species. In this study, we characterized the differences in permissivity and IFN response of alpaca and bovine cells to BVDV infection to elucidate mechanisms underlying the differences in clinical disease signs and infections between cattle and alpacas. Results showed that alpaca cells have limited permissiveness to BVDV infection with corresponding elevated levels of IFN synthesis

\* Corresponding author at: School of Veterinary Medicine and Biomedical Sciences, University of Nebraska-Lincoln, Veterinary Basic Science, VBS 210, East Campus Loop and Fair Street, Lincoln, NE 68583–0905, USA. Fax: +1 402 472 9690.

E-mail address: [ckelling1@unl.edu](mailto:ckelling1@unl.edu) (C.L. Kelling).

<sup>1</sup> Current Address: Influenza Division, National Center for Immunization and Respiratory Diseases, Coordinating Center for Infectious Diseases, Center for Disease Control and Prevention, 1600 Clifton Road, Mail Stop G-16, Atlanta, GA, 30333, USA.

which may account for differences in clinical outcomes following BVDV infection of alpacas and cattle.

## Results

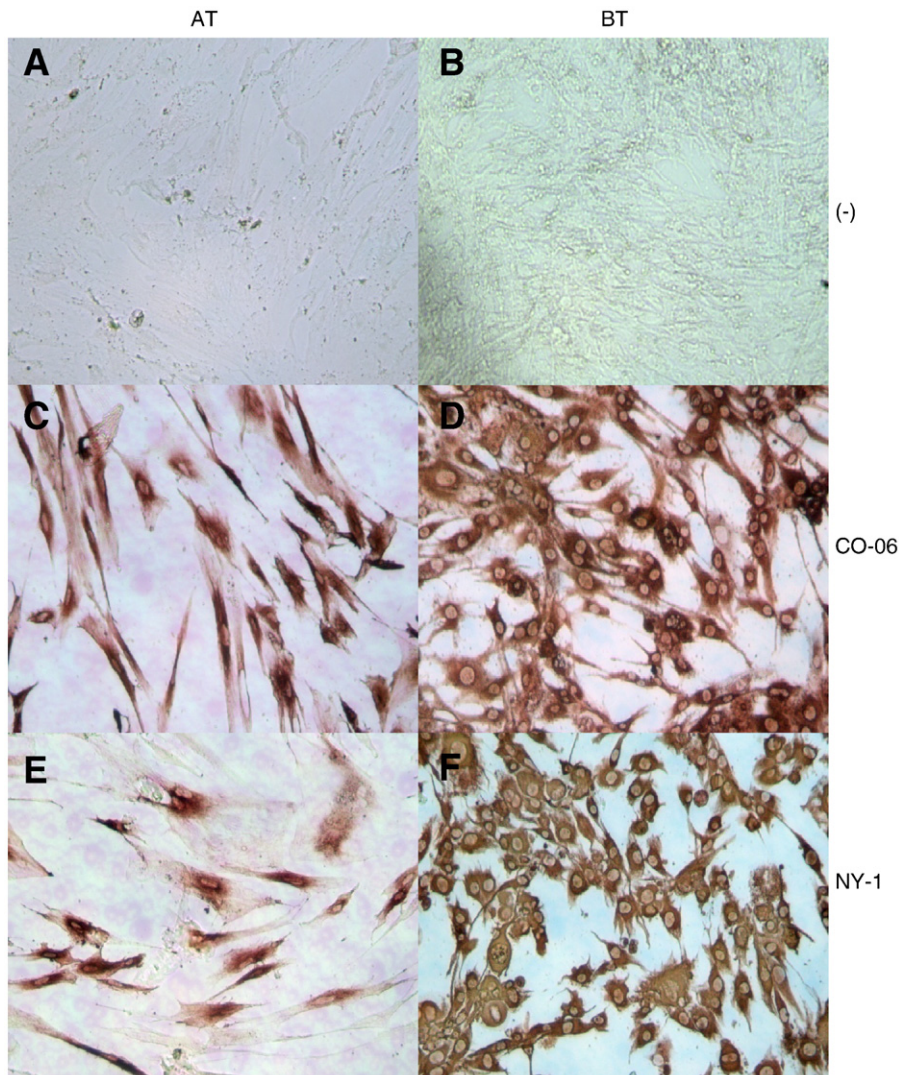
### Viral replication in ruminant cells

Viral replication was characterized for each BVDV isolate in alpaca testicular (AT) and bovine turbinate (BT) cells. BVDV-specific cytoplasmic antigen in alpaca cells stained less intensely and less frequently compared to bovine cells (Figs. 1 and 2). AT cells infected with the BVDV1 alpaca origin isolate CO-06 had a greater frequency of viral antigen staining, with the majority of cells stained, as opposed to isolated clusters of stained cells seen in AT cells infected with the other BVDV isolates, NY-1, 890 and 7937. The reduction in cytoplasmic viral antigen staining was most apparent in AT cells infected with the BVDV2 isolates, 890 and 7937 wherein specific cytoplasmic staining of viral antigen occurred in isolated clusters of cells with less intense staining than observed in infected BT cells (Fig. 2). The greater frequency and intensity of BVDV1 CO-06 viral antigen staining in AT cells when compared to BVDV2 isolates correlates with calculated titer means (Figs. 3 and 4). BVDV1, CO-06, replicated 1 log TCID<sub>50</sub> greater than BVDV2, 890 or 7937 in AT cells.

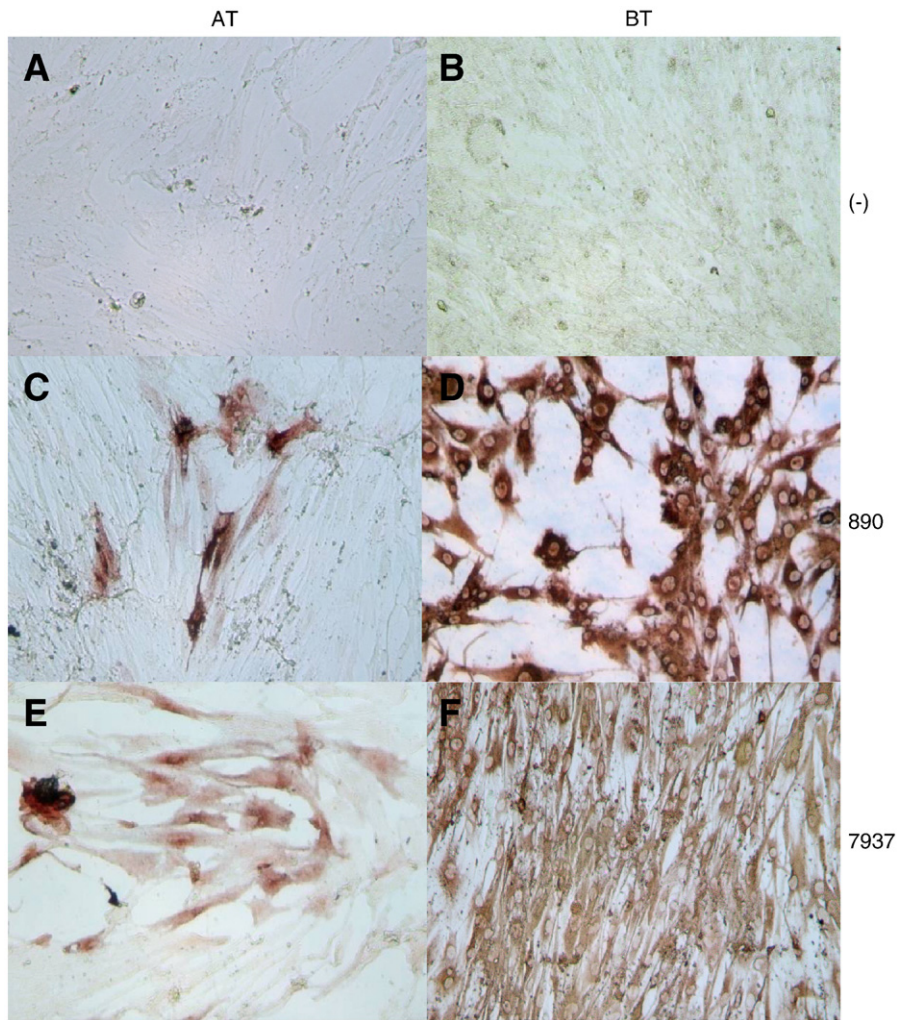
The BVDV1, NY-1, titer mean was similar to the BVDV2 isolates in AT cells.

Differences in the rate of viral replication in AT and BT cells were observed (Figs. 3 and 4), 12 hour post-infection for three of the four viral isolates. BVDV1, NY-1, had a 2.2 log TCID<sub>50</sub> greater titer in BT than AT cells (Fig. 3), as did BVDV2, 7937 and 890 (Fig. 4), with titers 1.24 and 2.87 log TCID<sub>50</sub> greater in BT than AT cells, respectively. At 24 hour post-infection, mean titers in AT cells infected with BVDV NY-1 or CO-06 were statistically ( $p = 0.0372$ ) lower than infected BT cells (Fig. 3). Mean titers of BVDV 7937 infected BT cells were significantly ( $p = 0.0209$ ) greater than AT cells beginning 48 hour post-infection. At 72 hour post-infection, titers in BT cells were 3 to 5 log TCID<sub>50</sub> greater than AT cells.

BVDV total mean viral titer, the mean titer of the time points for each BVDV strain in each cell type was determined. Significant differences were found between the total mean viral titers in alpaca and bovine cells infected with the BVDV1 viruses, CO-06 and NY-1, with significantly higher mean titers observed in BT cells compared to AT cells,  $p = 0.0011$  and  $p < 0.001$ , respectively. Similarly, significant differences were also found between alpaca and bovine cells infected with the BVDV2 viruses, 890 ( $p < 0.001$ ) and 7937 ( $p = 0.001$ ), with a significantly greater mean titer in BT cells compared to AT cells. At 72 hour post-infection, the end titer of BVDV1 viruses in AT cells was significantly ( $p = 0.0238$ ) greater than the end titers of BVDV2 viruses



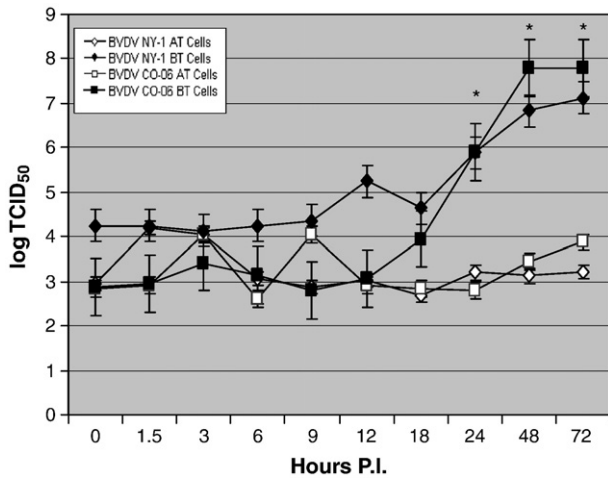
**Fig. 1.** Enzyme-linked immunoassay of alpaca testicular (AT) and bovine turbinate (BT) cells infected with BVDV1 viruses NY-1 (bovine origin) and CO-06 (alpaca origin). (A) Mock-infected AT cells; (B) Mock-infected BT cells; (C) BVDV CO-06-infected AT cells; (D) BVDV CO-06-infected BT cells; (E) BVDV NY-1-infected AT cells; (F) BVDV NY-1-infected BT cells.



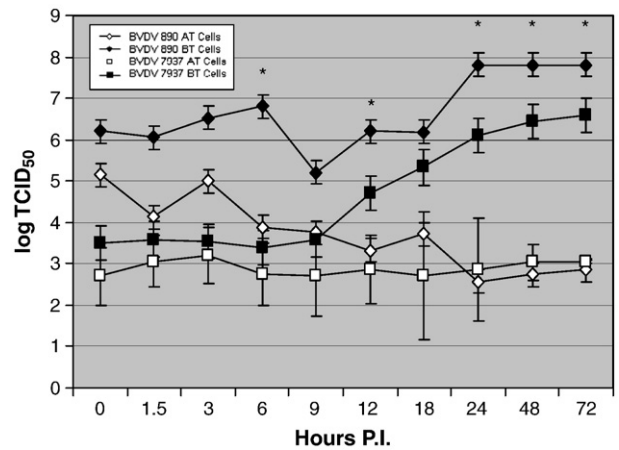
**Fig. 2.** Enzyme-linked immunoassay of alpaca testicular (AT) and bovine turbinate (BT) cells infected with BVDV2 viruses 890 (high virulence) and 7937 (low virulence). (A) Mock-infected AT cells; (B) Mock-infected BT cells; (C) BVDV 890-infected AT cells; (D) BVDV 890-infected BT cells; (E) BVDV 7937-infected AT cells; (F) BVDV 7937-infected BT cells.

in AT cells. The end titer of the alpaca origin isolate BVDV CO-06 in AT cells was significantly ( $p = 0.0307$ ) greater than that of the bovine origin BVDV NY-1, as well as the BVDV2 isolates BVDV 890 and 7937 ( $p = 0.0344$ ) in AT cells. This difference between final titers of BVDV1

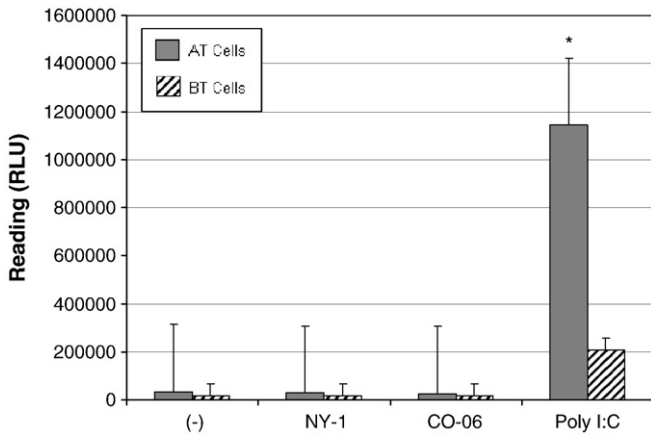
and BVDV2 viruses was not significant ( $p = 0.1340$ ) in BT cells, although BVDV CO-06 replicated to a significantly higher ( $p = 0.0140$ ) end titer than the low virulence BVDV2 isolate 7937 but not the high virulence isolate BVDV 890 ( $p = 0.4226$ ). There was no significant difference in viral replication between the alpaca origin isolate CO-06



**Fig. 3.** Viral replication of BVDV1 isolates NY-1 and CO-06 in alpaca testicular (AT) and bovine turbinate (BT) cells. Cell lysates were harvested at 0, 1.5, 3, 6, 9, 12, 18, 24, 48, and 72 hour post-infection and assayed for viral infectivity. \*Statistically significant between cell types at  $p \leq 0.05$ .



**Fig. 4.** Viral replication of BVDV2 isolates 890 (high virulence) and 7937 (low virulence) in alpaca testicular (AT) and bovine turbinate (BT) cells. Cell lysates were harvested at 0, 1.5, 3, 6, 9, 12, 18, 24, 48, and 72 hour post-infection and assayed for viral infectivity. \*Statistically significant between cell types at  $p \leq 0.05$ .

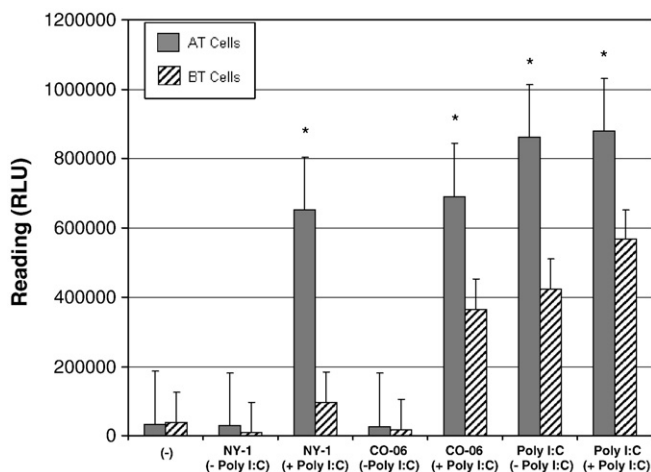


**Fig. 5.** Type I interferon (IFN) responses of alpaca testicular (AT) and bovine turbinate (BT) cells infected with BVDV CO-06 or NY-1 at an m.o.i. of 10. Cell lysates were harvested 24 hour post-infection, clarified by centrifugation and virus inactivated. Test samples were added to NCL1-Luc-ISRE-Hygro cells, incubated for 8 hours and cell lysates analyzed for luciferase activity. \*Differences from other groups are statistically significant at  $p \leq 0.05$ .

and the bovine origin isolate NY-1 ( $p = 0.4480$ ). At 72 hour post-infection, significant differences were not observed between BVDV 890 and 7937 in AT cells ( $p = 0.5044$ ). However, the titer of BVDV 890 was significantly higher ( $p = 0.0006$ ) than BVDV 7937 in BT cells.

#### IFN response of ruminant cells to BVDV infection

IFN responses in AT and BT cells were measured following BVDV infection (Fig. 5). BVDV NY-1 and CO-06 were poor inducers of IFN responses in AT and BT cells. Following stimulation with poly I:C, AT cells had a significantly greater IFN response, synthesizing up to 5 times more IFN, than that of BT cells ( $p = 0.0006$ ) (Fig. 5). AT and BT cells infected with BVDV NY-1 or CO-06 and then stimulated with poly I:C 48 hour post-infection (Fig. 6) showed significant differences in the inhibition of IFN activity. AT cells infected with either BVDV NY-1 or CO-06, then stimulated with poly I:C synthesized significantly greater IFN than AT cells following infection alone ( $p < 0.0001$  and  $p = 0.0002$ , respectively). Similarly, BT cells infected with BVDV CO-



**Fig. 6.** Inhibition of IFN synthesis by BVDV infection of alpaca testicular (AT) and bovine turbinate (BT) cells. Subconfluent AT or BT cells were infected with BVDV1 NY-1 or CO-06 at an m.o.i. of 1, incubated for 48 hours, then treated with growth media without (-) or with poly I:C (+) at a concentration of 50  $\mu\text{g}/\text{ml}$ . Following incubation for 20 hours, cell lysates were harvested and virus inactivated. Test samples were added to NCL1-Luc-ISRE-Hygro cells, incubated for 8 hours, cells lysed and luciferase activity measured. \*Differences from other groups are statistically significant at  $p \leq 0.05$ .

06 synthesized significantly ( $p = 0.0339$ ) greater IFN following poly I:C stimulation than BT cells following infection alone. BT cells infected with NY-1 had no significant ( $p = 0.5899$ ) increase in IFN production following poly I:C stimulation compared to non-stimulated, NY-1 infected BT cells.

There were significant differences in IFN inhibition between the AT and BT cells. AT cells infected with BVDV CO-06 or NY-1 synthesized significantly greater (2–5 times) IFN ( $p < 0.0001$  and  $p = 0.0009$ , respectively) following poly I:C stimulation compared to BVDV-infected BT cells. Significant differences in IFN activity were also evident between AT and BT cells stimulated with poly I:C in lieu of viral infection and later restimulated with additional poly I:C ( $p = 0.0019$ ). AT cells stimulated with poly I:C, either once or twice, synthesized significantly greater ( $p = 0.0020$ ) IFN than unstimulated AT cells, and nearly twice the IFN concentration synthesized by BT cells stimulated once or twice with poly I:C (Fig. 6).

#### BVDV RNA electroporation

To determine whether restricted viral replication in AT cells was due to differences in the intracellular environment, BVDV RNA was electroporated into AT and BT cells and assayed for levels of viral replication by titrating viruses in cell culture. BVDV viral RNA replicated in both AT and BT cells following electroporation (data not shown). AT cells electroporated with BVDV NY-1 RNA had significantly less ( $p = 0.0273$ ) viral replication with an average titer of  $2.18 \pm 0.259$  TCID<sub>50</sub>, in comparison to BT cells which had an average titer of  $4.28 \pm 1.26$  TCID<sub>50</sub>. AT and BT cells electroporated with BVDV CO-06 RNA did not show any significant difference ( $p = 0.5093$ ) in viral replication, with a mean titer of  $2.06 \pm 0.277$  TCID<sub>50</sub> in AT cells and  $2.74 \pm 0.652$  TCID<sub>50</sub> in BT cells.

#### Discussion

Results of the present study indicate that, compared to bovine cells, alpaca cells have a limited permissiveness to BVDV infection. Both alpaca and bovine origin noncytopathic BVDV isolates exhibited reduced growth rates in alpaca cells when compared to bovine cells, with up to 3–5 log TCID<sub>50</sub> difference in infectivity at 72 hour post-infection. This correlated with decreased intensity and frequency of BVDV-specific antigen staining in alpaca cells compared to bovine cells infected with the BVDV isolates. The lower viral titers and diminished BVDV antigen staining in the alpaca cells compared to the bovine cells suggests that alpaca cells are less permissive than BT cells to BVDV infection. While the cells used in the present study had different tissue origins, this did not influence the interpretation of results since BVDV replicates efficiently in both testicular and turbinate cells (Gil et al., 2006b; Topliff and Kelling, 1998).

Restricted replication of BVDV in AT cells corresponds to previous results observed in camel fibroblast cells in which BVDV and border disease virus infected the camel cells but only to a limited extent (Klopries et al., 1995). BVDV isolates, noncytopathic (ncp) Iowa and cytopathic (cp) Ug59 did not replicate in camel fibroblast cells, although infection of different passage levels of camel fibroblast cells with different pestivirus isolates showed that some BVDV and border disease virus isolates did replicate. Only a few camel fibroblast cells showed evidence of viral antigen deposition (Klopries et al., 1995), which correlates with the diminished BVDV antigen staining of AT cells compared to BT cells in the present study.

Restricted replication of pestiviruses in cells outside of the natural host species as observed in the present study is consistent with previous reports. Wieringa-Jelsma et al. (2006) showed that BVDV was able to infect feeder pigs but transmission was limited, with only one out of ten animals in contact with BVDV-inoculated pigs becoming infected with BVDV based on isolation of virus from leukocytes or presence of antibody. None of the infected animals

exhibited clinical signs of BVDV infection (Wieringa-Jelsma et al., 2006).

The reduced permissiveness of alpaca cells to BVDV infection reported in this study and the diminished clinical disease following BVDV infection in alpacas is similar to the report that pigs are less permissive to BVDV infection and did not exhibit clinical signs of disease following infection with BVDV1 or BVDV2 (Walz et al., 1999). BVDV1 was isolated from serum and tissue samples collected from infected pigs; however, BVDV2 was isolated only from tissue samples (Walz et al., 1999). The differences seen between BVDV1 and BVDV2 infections in pigs correlates with our observations in alpaca and bovine cell culture. Alpaca cells were more permissive to BVDV1 infections with BVDV1 replicating to a significantly ( $p=0.0238$ ) higher titer compared to BVDV2 in alpaca cells, with the BVDV1 alpaca isolate CO-06 having a significantly greater titer than BVDV1 bovine isolate NY-1 ( $p=0.0307$ ) and the BVDV2 isolates 890 and 7937 ( $p=0.0344$ ). To date, BVDV isolates from alpacas have been type 1b (Carman et al., 2005; Foster et al., 2007; Goyal et al., 2002; Kim et al., 2009; Mattson et al., 2006; Topliff et al., 2009). This suggests that alpaca cells are more permissive and better adapted for infection with BVDV1 than BVDV2. The predominance of BVDV1b in alpacas could also be a reflection of the regional predominance of BVDV1b in nature.

It may be possible that increased interaction between New World camelids and cattle has allowed for the expansion in host range of BVDV. Other viruses such as influenza virus can mutate and infect a wide range of hosts. Avian influenza viruses with mutations in the hemagglutinin protein at a specific arginine residue (Arg 193) were shown to have an increased affinity for human cellular receptors (Stevens et al., 2008). Infection of human epithelial cells with infectious severe acute respiratory syndrome (SARS) coronavirus clones carrying mutations found in the original zoonotic isolate showed increased growth in the human epithelial cells in comparison to cells from civets, the original host of the zoonotic strain (Sheahan et al., 2008). In both the case of influenza virus and SARS virus, differing species (human, avian, and civet) were living in close proximity previous to the virus acquiring expanded host specificity.

The limited permissiveness of AT cells to BVDV infection may be a result of the greater IFN expression in AT cells acting to inhibit BVDV replication. In our study, ncp BVDV viruses were poor inducers of type I IFN with no significant difference in IFN induction between alpaca and bovine cells following infection, which corresponds to previous work (Gil et al., 2006a; Schweizer et al., 2006). However, alpaca cells synthesized a significantly greater level of IFN following stimulation with poly I:C compared to bovine cells. Similarly, BVDV-infected alpaca cells responded to poly I:C stimulation with significantly greater levels of IFN synthesis than bovine cells. IFN responses are an essential component of the host innate immune defense against viral infections. The more robust IFN response of alpaca cells compared to bovine cells following poly I:C stimulation alone and following infection with ncp BVDV may be the basis for decreased permissiveness of AT cells compared to BT cells. Replication of classical swine fever virus, a pestivirus, was inhibited by recombinant type I IFN (Xia et al., 2005). Suppression of IFN synthesis in BVDV-infected cells has been associated with BVDV proteins N<sup>pro</sup> and E<sup>ns</sup>. Lymphoid cells have been recovered from cattle that are able to produce IFN after infection with ncp BVDV. These bovine lymphoid cells synthesize type I IFN without expressing NS3 antigens (Brackenbury et al., 2005). Similarly, the elevated IFN response in alpaca cells when stimulated with dsRNA, in addition to the reduced inhibition of IFN synthesis following post-infection stimulation with dsRNA in cell culture, suggests that alpaca cells may be able to produce IFN effectively following infection in the host. The reduced or lack of antigen expression in IFN producing bovine lymphoid cells closely parallels the reduced antigen expression seen in our study with alpaca cells.

To characterize post-entry intracellular resistance to viral replication, AT and BT cells were transfected with RNA from BVDV NY-1 or BVDV CO-06 and progeny virus titered to determine levels of replication. NY-1 BVDV replicated to significantly higher levels in BT cells than in AT cells, while BVDV CO-06 replicated to comparable levels in both cell types. These results correlate with results of the IFN inhibition assay. Both alpaca and bovine cells infected with BVDV CO-06, followed by stimulation with poly I:C, were able to synthesize high levels of IFN, which could interfere with the replication of the virus following both infection and electroporation of BVDV RNA. BVDV NY-1 significantly inhibited IFN synthesis in bovine cells, but not in alpaca cells, also correlating with the increased replication seen in bovine but not alpaca cells following transfection. The E<sup>ns</sup> protein of BVDV has been shown to inhibit activation of IFN synthesis by extracellular dsRNA but not intracellular RNA, which may also explain the reduced replication of BVDV CO-06 following transfection (Magkouras et al., 2008). Additional genomic studies directed toward defining genomic differences between CO-06 and NY-1 may help to elucidate this phenomenon.

Results of the present study indicate limited permissiveness of alpaca cells to BVDV infection. Previously, alpacas and other New World camelids had been shown to be resistant to a number of viral diseases, including foot and mouth disease (Wemery and Kaaden, 2004) and acute BVDV infection (Wentz et al., 2003). Acute BVDV-infected alpacas exhibit diminished clinical parameters of disease, such as antigen deposition in lymphoid tissues, when compared to clinical signs in acute BVDV-infected calves (Kelling et al., 1990, 2002; Wentz et al., 2003). The reduced replication of BVDV in alpaca cell culture compared to bovine cell culture and the increased IFN response following poly I:C stimulation suggests that reduced clinical expression of BVDV infection in alpacas may be due to limited permissiveness of alpaca cells to BVDV infection compared to bovine cells.

## Materials and methods

### Cells and viruses

Bovine turbinate (BT, National Veterinary Services Laboratory, USDA, Ames, IA) and Madin–Darby bovine kidney (MDBK, CCL-22, American Type Culture Collection, Manassas, VA) cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% equine serum (Hyclone, Logan, UT). Primary alpaca testicular (AT) cells were harvested from testicular tissue of healthy adult alpacas collected at the time of castration and were grown in DMEM supplemented with 10% fetal bovine serum (FBS) (Hyclone), 0.5 µg/ml fungizone (Invitrogen) and 100 µg/ml gentamycin (Sigma-Aldrich, St. Louis, MO). NCL1-ISRE-Luc-Hygro cells, a modified bovine uterine cell line constitutively expressing an IFN response element gene coupled with firefly luciferase, were maintained in DMEM supplemented with 10% equine serum and 300 µg/ml hygromycin (Cellgro, Manassas, VA). Bovine cell lines were free of adventitious BVDV and fetal bovine serum free of BVDV antibodies. BVDV 890 (Animal, Plant, and Health Inspection Service, Center for Veterinary Biologics, Ames, IA) and BVDV 7937 (Kelling et al., 1990) were originally isolated from acute BVDV-infected and PI calves, respectively, and were determined to be BVDV2 ncp isolates. BVDV NY-1 (National Veterinary Services Laboratory), a prototypical BVDV1 ncp virus, was isolated from an acute BVDV-infected calf. BVDV CO-06 is an alpaca origin BVDV1 isolate recovered from a PI alpaca at the University of Nebraska. BT cell monolayers at 90% confluency were individually infected with each BVDV isolate at a multiplicity of infection (m.o.i.) of 1 in 75 cm<sup>2</sup> tissue culture flasks and incubated at 37 °C in a humidified incubator with 5% CO<sub>2</sub> for 4 days. Following a single freeze–thaw cycle, 1 ml aliquots of mock- and viral-infected BT cell lysates were stored at –80 °C.

### dsRNA stock

Polyribinosinic polyribocytidylic acid (poly I:C) (Amersham Biosciences, Piscataway, NJ) was reconstituted in phosphate buffered saline (PBS) to a stock concentration of 2 mg/ml, passed through a 21 gauge needle to shear the RNA, and stored in aliquots at  $-80^{\circ}\text{C}$ .

### BVDV replication

BT or AT cells were seeded at a density of  $1 \times 10^5$  cells/well into 6 well culture plates and incubated at  $37^{\circ}\text{C}$  in a humidified incubator with 5%  $\text{CO}_2$  until 90% confluent. Cells were then infected with virus at an m.o.i. of 0.3 and incubated at  $37^{\circ}\text{C}$  for 1.5 hours with rocking. Following adsorption, cells were washed with DMEM and incubated in fresh media supplemented with 5% equine serum for BT cells or 5% FBS for AT cells. Cells were monitored by microscopic examination and frozen at  $-80^{\circ}\text{C}$  0, 1.5, 3, 6, 9, 12, 24, 48, or 72 hour post-infection. Cell lysates were thawed on ice and virus titers determined at each time point (Brodersen and Kelling, 1999).

### Virus preparation for IFN assay

Virus preparation for the IFN assay as been previously described (Gil et al., 2006a). Briefly, BT or MDBK cells were inoculated with BVDV NY-1 or CO-06 virus stock at an m.o.i. of 1 and incubated at  $37^{\circ}\text{C}$  for 4 days, then frozen at  $-80^{\circ}\text{C}$ . Flasks were thawed; cell lysates harvested and centrifuged at 2000g for 30 minutes at  $4^{\circ}\text{C}$  to clarify the supernatant. The supernatant was then transferred to ultracentrifuge tubes and centrifuged at 100,000g for 2 hours at  $4^{\circ}\text{C}$ . The resulting viral pellet was resuspended in 500  $\mu\text{l}$  DMEM and stored in aliquots at  $-80^{\circ}\text{C}$ .

### IFN reporter gene assay

BT or AT cells were seeded onto 6 well plates at a cell density of  $1 \times 10^5$  and grown to 90% confluency. Cells were then infected with pelleted virus isolates at an m.o.i. of 10 and incubated at  $37^{\circ}\text{C}$  for 24 hours, then frozen at  $-80^{\circ}\text{C}$ . The plates were thawed and the cell lysate supernatant clarified by centrifugation at 2000g for 30 minutes at  $4^{\circ}\text{C}$ . The pH of the clarified supernatant was adjusted to pH 2 with 2 M HCl. After incubation at  $4^{\circ}\text{C}$  for 24 hours, the pH of the supernatant was adjusted to pH 7 using 2 M NaOH. Test samples (500  $\mu\text{l}$ ) were added to 12 well plates of NCL1-ISRE-Luc-Hygro cells (Gil et al., 2006a) prepared 12 hours earlier by adding  $1.5 \times 10^5$  cells/well and incubating at  $37^{\circ}\text{C}$ . After addition of the test sample, the NCL1-ISRE-Luc-Hygro cells were incubated for 8 hours, followed by cell lysis in 100  $\mu\text{l}$  of passive lysis buffer (Promega, Madison, WI) (Gil et al., 2006a). The luciferase assay was performed using the firefly luciferase assay system (Promega) according to the manufacturer's instructions, with sample luminescence measured by a FLUOStar luminometer (BMG Labtech, Offenburg, Germany).

### IFN inhibition assay

BT or AT cells were seeded onto 6 well plates at a cell density of  $1 \times 10^5$  and grown to 90% confluency. Cells were then infected with BVDV1 NY-1 or CO-06 at an m.o.i. of 1 and incubated for 48 hours at  $37^{\circ}\text{C}$ , at which time poly I:C was added to the cells at a concentration of 50  $\mu\text{g}/\text{ml}$  (Baigent et al., 2002; Gil et al., 2006a). Following incubation for 20 hours at  $37^{\circ}\text{C}$ , cells were harvested by a freeze-thaw cycle at  $-80^{\circ}\text{C}$  and pH adjusted to pH 2 for 24 hours at  $4^{\circ}\text{C}$  to inactivate virus. The pH of the cell lysates was then adjusted to pH 7 and added to reporter cell plates containing NCL1-ISRE-Luc-Hygro cells and assayed as described previously (Gil et al., 2006a).

### RNA electroporation

Purified viral RNA was extracted from pelleted virus stocks using TRIzol LS (Invitrogen) according to the manufacturer's instructions. The concentration and purity of the RNA was determined by measuring the UV absorbance at 260 and 280 nm (Model DU-64, Beckman Coulter, Fullerton, CA). Confluent monolayer flasks of AT and BT cells were washed with 10 ml CMF-PBS, trypsinized with 5 ml 0.05% trypsin-EDTA, resuspended in 5 ml DMEM, and centrifuged at 1000 rpm at  $4^{\circ}\text{C}$  for 10 minutes. The resulting cell pellet was resuspended in 5 ml "E" buffer (DMEM with 1.25% dimethylsulfoxide). Cells ( $1 \times 10^5$ ) were resuspended in 400  $\mu\text{l}$  of "E" buffer and placed into a 0.4 cm gap electroporation cuvette, then electroporated with 2  $\mu\text{g}$  of the purified viral RNA using a Bio-Rad GenePulser electroporator with an exponential curve pulse at 250 V, 950  $\mu\text{F}$ ,  $\infty$  capacitance. After electroporation, cells were recovered in 2 ml of recovery medium ("E" buffer with 5% serum) and placed into a 6 well plate. After ensuring even cell distribution in the well, an aliquot of cell suspension was removed and placed into wells of a 12 well plate with 1 ml of recovery buffer. Cell plates were incubated for 4 days at  $37^{\circ}\text{C}$  in a humidified incubator with 5%  $\text{CO}_2$ . The 12 well plates were fixed and viral antigen detected using an enzyme-linked immunoassay as previously described (Brodersen and Kelling, 1999) using mAb 348 (VMRD, Pullman, WA), specific for BVDV E2 protein, while cells in the 6 well plates were frozen at  $-80^{\circ}\text{C}$  and cell lysates assayed for viral titers (Gil et al., 2006a).

### Statistical analyses

Statistical analyses were carried out with the SAS statistical software program (Cary, NC) using the Student *t* test and least square means test. The level of significance was set at 0.05.

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