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Effect of bovine viral diarrhea virus on subsequent infectivity of bovine gammaherpesvirus 4 in endometrial cells in primary culture: an *in vitro* model of viral co-infection

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Highlights

- Previous infection with BVDV affects *in vitro* assays for bovine gammaherpesvirus 4 (BoHV-4).

- BoHV-4 gene expression kinetics is affected by the presence of BVDV.
- BoHV-4 titers in primary cultures of bovine endometrial cells decrease in the presence of BVDV.

Abstract

Bovine viral diarrhea virus (BVDV) and bovine gammaherpesvirus 4 (BoHV-4) infect the uterus of cattle, being responsible for huge economic losses. Most of the pathogenesis of BoHV-4 in the bovine reproductive tract has been elucidated by conducting tests on primary cultures. Thus, it is important to have optimal *in vitro* conditions, avoiding the presence of other pathogens that can alter the results. BVDV is one of the most frequent viral contaminants of cell cultures. Considering that non-cytopathic (NCP) BVDV biotype can generate persistently infected (PI) cattle, which are the major source for virus transmission in susceptible herds, it is important to check products derived from cattle that are intended to be used in research laboratories. The aim of this work was to evaluate how the natural infection of bovine endometrial cells (BEC) with a NCP BVDV strain (BEC+BVDV) affects BoHV-4 replication. We have demonstrated a delay in BoHV-4 gene expression and a decrease in viral load in the extracellular environment in BEC+BDVD cells compared to BEC (BVDV-free) cells. These results confirm that replication of BoHV-4 in BEC primary cultures is affected by previous infection with BVDV. This finding highlights the importance of ruling out BVDV infection in bovine primary cell cultures to avoid biological interference or misinterpretation of results at the time of performing *in vitro* studies with BoHV-4.

Key words: Bovine viral diarrhea virus; Bovine gammaherpesvirus 4; Endometrial cells; Replication kinetics; Gene expression.

Pestiviruses and herpesviruses are the major viral pathogens contributing to abortion in cattle, sheep, and goats (Anderson, 2007), either alone or in co-infections (Reed et al., 1979). Bovine viral diarrhea virus (BVDV) and bovine gammaherpesvirus 4 (BoHV-4) belong to the genus *Pestivirus* and *Rhadinovirus*, respectively. Both viral pathogens infect the uterus of cattle and are responsible for huge economic losses in livestock production (Lanyon et al., 2014).

Bovine viral diarrhea is one of the most important bovine diseases worldwide with most herds being at risk of infection (Uzal et al., 2016). BVDV can be categorized into cytopathic (CP) and non-cytopathic (NCP) biotypes in cell cultures (Ridpath et al., 2010), but genetically it is highly diverse (Yeşilbağ et al., 2017). The NCP biotype is associated with the majority of BVDV transient infections (> 90%) and generates persistently infected (PI) cattle (Birk et al., 2008). PI animals are the major source of virus transmission in susceptible herds. Therefore, it is important not only to carry out control measures to eliminate PI animals but also to check products derived from cattle that are intended to be used in research laboratories, considering that BVDV is one of the most frequent viral contaminants of cell cultures (Uryvaev et al., 2012).

BoHV-4 experimental disease has rarely been successfully reproduced. Hence, the main advances in the knowledge of the role of BoHV-4 infections in the bovine endometrium have been achieved through trials in primary cultures (Donofrio et al., 2010; Jacca et al., 2014; Chanrot et al., 2017). For this purpose, it is important to have optimal *in vitro* conditions for the study of BoHV-4 pathogenesis, avoiding the presence of other pathogens, which can alter the results. The objective of this work was to evaluate how the natural infection of bovine endometrial cells with a NCP-BVDV strain affects BoHV-4 replication. This finding is particularly relevant when these types of cultures are used as substrate for BoHV-4 *in vitro* tests.

To study the effect of BoHV-4 infection on the bovine endometrium, primary cultures of bovine endometrial cells (BEC) were performed. Uteri from cattle without evidence of genital disease were

collected at a local abattoir immediately after slaughter and kept on ice until further processing in the laboratory, using a method described previously (Tebaldi et al., 2016). The cells obtained for primary culture and the fetal bovine serum (FBS) used for their growth were tested for bovine alphaherpesvirus 1 (BoHV-1) and BVDV antigens by direct immunofluorescence (DIF) test using fluorescein isothiocyanate (FITC) labeled porcine polyclonal antiserum (VMRD, Inc., Pullman, WA, USA). Briefly, BEC were attached to multi-well slides by incubation (37°C) for 35 min in an atmosphere of 5% CO_2, followed by fixation in acetone for 20 min. After this step, incubation with FITC antiserum, washings, and a counterstain with Evans blue were performed. Cytopathic effect (CPE) was not observed in the primary culture of BEC, but the presence of BVDV antigen was confirmed (Figure 1-A). Additionally, RNA was extracted from these naturally infected BEC cells to determine the BVDV species [BVDV-1 (*Pestivirus A*) and BVDV-2 (*Pestivirus B*)]. A nested multiplex RT-PCR with modifications (Spetter et al., 2018) was performed to amplify specific fragments of the NS5B genomic region. The results revealed an amplicon of 369 bp indicating the presence of BVDV-1 (Figure 1-B). Based on the phylogeny of the 5´UTR sequence, this strain could be potentially allocated into one of 21 subgenotypes (subtypes). However, most Argentinean strains belong to BVDV-1b and -1a according to the results obtained by our research group (Odeón et al., 2003, Pecora et al., 2014, Spetter et al., 2020).

Primary cultures of BEC cells (control, BVDV negative) and BEC naturally infected with BVDV (BEC+BVDV) were grown in 24-well plates (Greiner Bio-One, Germany) at a concentration of 2.2 $\times 10^5$ cells/ml. Confluent monolayers were inoculated with BoHV-4 strain 10/154 at a multiplicity of infection (MOI) of 0.5. This strain was selected because it belongs to the most prevalent genotype circulating in Argentina (Verna et al., 2012). Cultures were maintained for 96 hours post-inoculation (hpi) and cells were observed daily for the appearance of CPE. At different post-inoculation times (24, 48, 72, and 96 hpi), the cells and their respective uninfected controls were harvested and stored in Trizol (Invitrogen, USA) at -80°C for subsequent RNA extraction. The synthesis of cDNA and amplification of the genes encoding for immediately early transcript 2 (IE2)

and glycoproteins B (gB), H (gH), and l (gL) were performed by RT-PCR, as previously described by Romeo et al. (2020).

Cells cultures (BEC and BEC+BVDV) inoculated with BoHV-4 strain 10/154 were also evaluated for viral replication kinetics. For this purpose, the supernatants were harvested (24, 48, 72, and 96 hpi) and frozen at -80 °C. The end point titration method was conducted using MDBK (as the gold standard line for viral titration), BEC (as control cells free of BVDV), and BEC+BVDV cells seeded in 96-well microtiter plates. Viral titers were expressed as $TCID_{50}$/ml (Reed and Muench., 1938). All assays were performed by triplicate, whereas virus titers were estimated by three independent observers and the obtained values were averaged. Appropriate negative controls (mock-infected cells) were included in each experiment.

Hypotheses for the effect of cell cultures (MDBK, BEC, and BEC+BVDV) and BoHV-4 post-inoculation time (24, 48, 72, and 96 hpi) interactions were tested through analysis of variance. Then, the multiple comparisons of means were carried out using Fisher's LSD (minimum significant difference). Viral titer curves were adjusted by multiple linear regression models, depending on whether the addition of a term plus model was significant or not (alpha = 0.05). All data analyses were performed in the R version 3.6.3 (R Core Team, 2020).

Both primary BEC cultures were readily infected by BoHV-4 strain 10/154. The CPE was observed similarly at 48 hpi in BEC and BEC+BVDV cells, with increasing numbers of scattered cells showing roundness, which is characteristic of BoHV-4. (Figure 2, A-B).

RT-PCR data showed that IE2 gene expression took place as early as 4 hpi (BEC cells) and 6 hpi (BEC+BVDV cells), remaining detectable until 8 hpi in both primary cell cultures. Differences in the time course expression kinetics of gH and gL in both BEC cells were not observed, being detected at 48 hpi and 30 hpi, respectively. However, the expression of gB was observed earlier in BEC (10 hpi) than in BEC+BVDV (24 hpi) cells (Table 1).

The viral titers of strain 10/154 at all time-points revealed significant differences ($p < 0.05$) between cell cultures. Titers obtained when BEC cells (BVDV-free) were used as substrate were always higher. The increase of BoHV-4 titer was gradual, reaching its maximum at 72 hpi (7.28 $\log_{10}$ $TCID_{50}$/ml) in BEC cells, while the highest titer in BEC+BVDV cells was observed at 96 hpi (3.92 log $\log_{10}$ $TCID_{50}$/ml), which was the last time-point evaluated (Table 2). Replication kinetics shows that the behavior of BoHV-4 in BEC primary culture fits a quadratic regression model; it reached a maximum titer and then began to decrease. However, in BEC+BVDV cells the growth curve adjusts to a simple linear regression, with a steady increase, without reaching a maximum in the evaluated times (Figure 3). The lowest viral titers were obtained in BEC+BVDV cells at each time point. Significant differences ($p < 0.05$) in the viral titers of BoHV-4 between BEC and BEC+BVDV cultures could indicate that natural BVDV infection would temporarily affect its replication kinetics (Table 2).

In the present study, we demonstrated how the natural infection of BEC with a NCP-BVDV strain affects the gene expression of gB and replication kinetics of BoHV-4. It is important to mention that the quantitative aspect of these experiments may differ when other BoHV-4 strains are used due to their genetic diversity (Verna et al., 2016; Pérez et al., 2020).

BEC primary cultures are highly susceptible to BoHV-4 replication as evidenced by the strong CPE reported (Donofrio et al., 2010). IE2 is the molecular master switch for BoHV-4 replication and is expressed immediately during cell infection, without requiring prior viral protein synthesis for its expression (Zimmermann et al., 2001). gB, gH, and gL are three highly conserved BoHV-4 glycoproteins that constitute the core machinery for herpesvirus entry, along with one or more accessory glycoproteins necessary for binding to cell surface receptors (Heldwein et al., 2008). The delay in gene expression in BEC+BVDV cells can be attributed to alterations produced by BVDV in the host cell. Therefore, BoHV-4 would not have the necessary cellular factors to carry out its replicative cycle efficiently in cells infected with BVDV.

Interestingly, the comparison of BoHV-4 titers in cell cultures showed that viral load in extracellular medium is affected by previous infection with BVDV. This observation could be related to the strategy of NCP-BVDV strains of avoiding the induction of cell death. Therefore, in case of concomitant infection, BoHV-4 replication may be altered in cells primarily infected with BVDV.

Our results confirm that the expression of gB and replication of BoHV-4 in BEC cells is affected by previous infection with BVDV. Thus, it is important to rule out the presence of BVDV in bovine primary cell cultures to avoid biological interference or misinterpreted results at the time of performing *in vitro* studies with BoHV-4.

Author Statement

The manuscript entitled: "Effect of bovine viral diarrhea virus on subsequent infectivity of bovine gammaherpesvirus 4 in endometrial cells in primary culture: an *in vitro* model of viral co-infection" is being submitted for consideration for publication as Short Communication in Journal of Virological Methods.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Conflict of interest

There are no potential conflicts of interest.

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Fig. 1 Characterization of the BVDV strain in BEC cells naturally infected

(A) Detection of bovine viral diarrhea virus by direct immunofluorescence. A cytoplasmic fluorescence (green color) is observed in BEC+BVDV cells monolayer. Uninfected BEC cells and BVDV-free MDBK cell monolayers are stained in red (negative controls). (B) Detection of amplified products after 2% agarose gel electrophoresis and ethidium bromide staining: 369-bp fragment corresponds to BVDV-1 amplified ARN; 615-bp fragment corresponds to BVDV-2 amplified ARN. Lane 1 and 6: Standard 100 bp DNA Ladder; Lane 2: BEC+BVDV cells; Lane 3: Strain NADL (reference strain of genotype-1); Lane 4: Strain Vs 253 (reference strain of genotype-2) and Lane 5: Negative control.

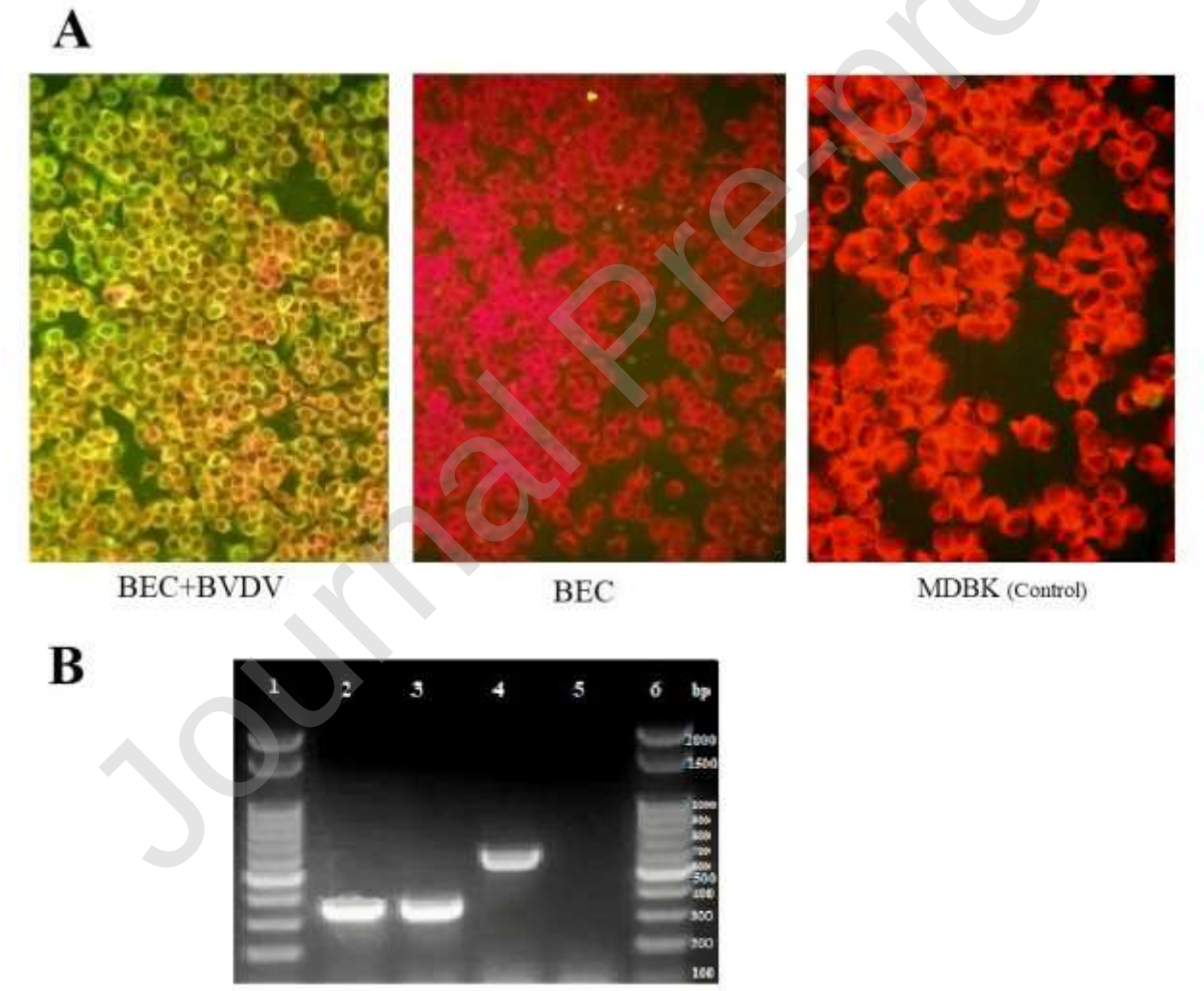

Fig. 2 Cytopathic effect of BoHV-4

Cytopathic effect of BoHV-4 after 48 hpi in BEC (A) and BEC+BVDV (B) cell monolayers. Uninfected monolayers (controls) of each cell culture are shown in the second column, 40x.

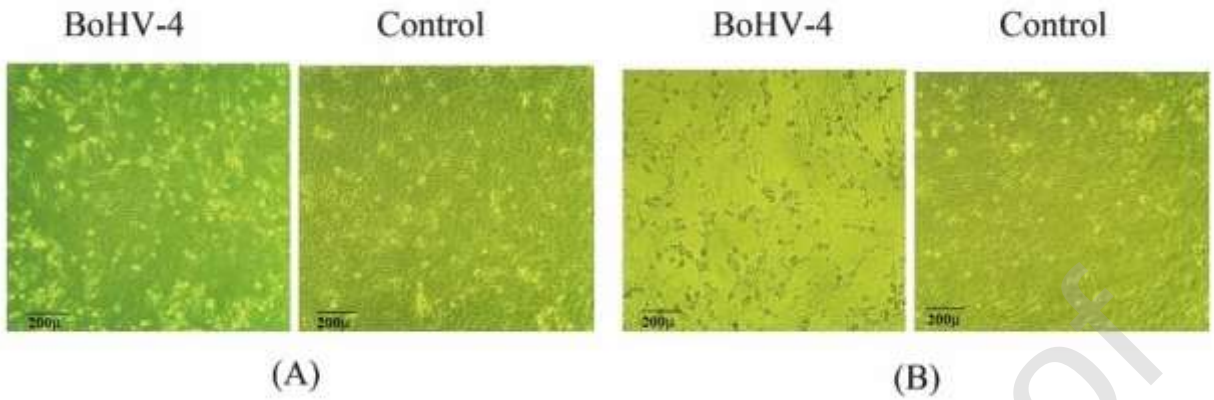

Fig. 3 Growth kinetics of BoHV-4 on MDBK, BEC and BVDV+BEC cell monolayers.

The graph shows the average titers and standard deviations from three replicates (log_{10} $TCID_{50}$/ml) at 24, 48, 72, and 96 hours after infection (hpi). In addition, the estimated quadratic model of each function is detailed, the R2 adjusted to the number of estimated parameters and the p-value resulting from the hypothesis test of the complete model.

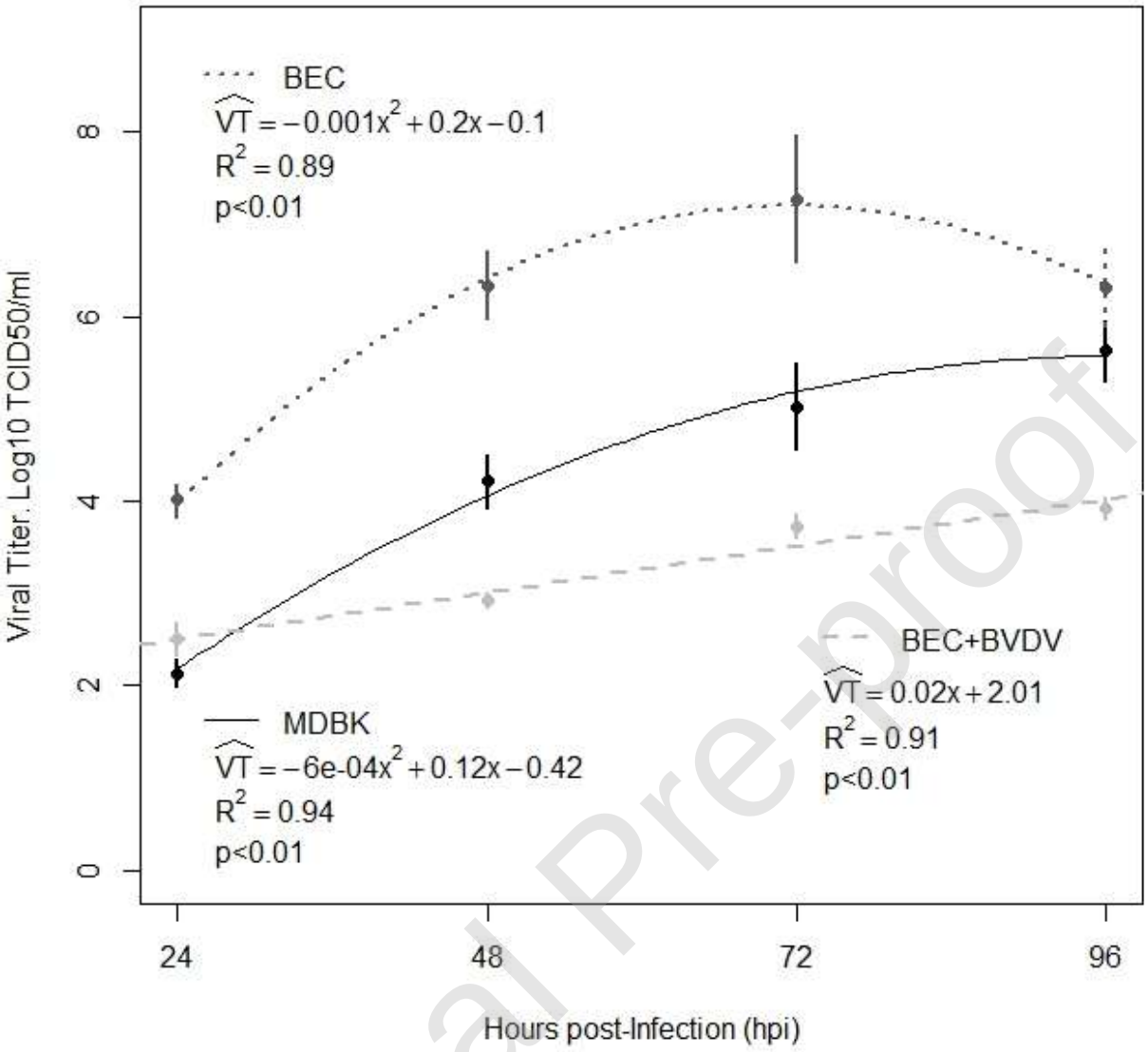

Table 1. Time course expression of BoHV-4 genes after inoculation of primary cell cultures from bovine endometrium.

Cell cultures	BoHV-4 **IE2** gene expression (hpi*)									
	2	4	6	8	10	24	30	48	72	96
BEC[a]	-	+	+	+	-	-	-	-	-	-
BEC+BVDV[b]	-	-	+	+	-	-	-	-	-	-
	BoHV-4 **gB** expression (hpi)									
	2	4	6	8	10	24	30	48	72	96
BEC	-	-	-	-	+	+	+	+	+	+
BEC +BVDV	-	-	-	-	-	+	+	+	+	+
	BoHV-4 **gH** expression (hpi)									
	2	4	6	8	10	24	30	48	72	96
BEC	-	-	-	-	-	-	-	+	+	+
BEC +BVDV	-	-	-	-	-	-	-	+	+	+
	BoHV-4 **gL** expression (hpi)									
	2	4	6	8	10	24	30	48	72	96
BEC	-	-	-	-	-	-	+	+	+	+
BEC +BVDV	-	-	-	-	-	-	+	+	+	+

* hpi: hours post-inoculation; - ausense of expression ; + presense of expression ; [a]bovine endometrial cells; [b] bovine endometrial cells naturally infected with BVDV.

Table 2. Replication kinetics of BoHV-4 in primary cultures of bovine endometrial cells and in MDBK cell line.

Cell cultures	Virus titers expressed as log_{10} $TCID_{50}$/ml at indicated times post-inoculation (hpi*)			
	24	48	72	96
BEC[a]	4.01# a	6.34 a	7.28 a	6.32 a
BEC+BVDV[b]	2.51 b	2.92 c	3.73 c	3.92 c
MDBK	2.13 b	4.22 b	5.03 b	5.63 b

* hpi: hours post-inoculation, # the log10 values correspond to the average values of three replicates; [a] bovine endometrial cells; [b] bovine endometrial cells naturally infected with BVDV. Means accompanied by the same letter indicate non-significant differences ($\alpha = 0.05$) within each cell culture for each hpi.