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In vitro amplification of BVDV field strains isolated in Argentina: effect of cell line and culture conditions

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ABSTRACT

The aim of this work was to study the *in vitro* amplification of BVDV (Pestivirus, *Flaviridae*) field isolates from Argentina in MDBK, BoTur and BHK-21 continuous cell lines. Field isolates 99/134 (mucosal disease), 00/693 (mucosal disease), 04P7016 (respiratory disease) and 04/89 (mucosal disease), genotype 1b, were used and compared with the Singer and NADL reference strains, genotype 1a. Additionally, cell lines derived from explants of bovine testis (RD-420), bovine uterus (NCL-1) and porcine kidney (PKZ) were tested as alternative substrates for BVDV propagation *in vitro*. The effect of cell line, harvest time and infection protocol was evaluated. The viral titers observed depended on the virus and harvest time but not on the infection protocol. We found that MDBK and BoTur cell lines were susceptible to the infection whereas BHK-21 and PKZ were not. NADL viral titers, 00/693 and 04/89, increased from 24 to 48 h p.i. in BoTur cells and then reached a *plateau,* whereas those of 99/134 and 04P7016 remained constant between 24 and 72 h p.i. BVDV Singer, on the other hand, presented a maximum titer at 24 h p.i. and then decreased. BVDV-NADL titers increased in MDBK and NCL-1 but not in RD-420 between 24 and 48 h p.i., and then decreased at 72 h p.i. These facts lead us to conclude that neither the subgenotypes (1a, 1b) nor the clinical symptoms of the animal from the virus had been isolated seem to affect the virus cell line kinetics of viral replication *in vitro.* On the other hand, the most homogenous behavior, the most similar replication curves, and highest titers observed in MDBK and NCL-1 seem to indicate that these lines are generally more susceptible to BVDV replication.

Key words: Pestivirus, bovine viral diarrhea virus, BVDV, Argentinean field strains, cell culture, kinetics of viral replication

RESUMEN

Amplificación *in vitro* de cepas de campo de virus de la diarrea viral bovina (VDVB) aisladas en Argentina: efecto de la línea celular y las condicioes de cultivo. Se estudió la interacción de aislamientos de campo de Argentina del VDVB (Pestivirus, *Flaviridae*) en las líneas celulares continuas MDBK, BoTur y BHK-21. Se utilizaron los virus de campo genotipo 1b, 99/134, 00/693 (casos compatibles con enfermedad de las mucosas) y 04P7016 (cuadro respiratorio) y las cepas de referencia genotipo 1a Singer y NADL. Además se evaluó la interacción de VDVB-NADL con las líneas celulares experimentales de bovino RD-420 y NCL-1 y de riñón porcino (PKZ). Se usaron 2 protocolos de infección. Los títulos virales observados dependieron del virus y del tiempo de infección y no así del modo de infección. Mientras que MDBK y BoTur resultaron susceptibles a la infección, BHK-21 y PKZ no lo fueron. Los virus NADL, 00/693 y 04/89 incrementaron su título entre las 24 y las 48 h p.i. en BoTur para mantenerlo posteriormente; los virus 99/134 y 04P7016 no presentaron variaciones y la cepa Singer presentó título máximo a las 24 h p.i para luego descender. La cinética del virus NADL en las células MDBK, RD-420 y NCL-1 tuvo un incremento de título para MDBK y NCL-1 entre las 24 y 48 h p.i que descendió a las 72 h p.i. La interacción virus-línea celular no estaría relacionada con el sub-genotipo del virus (1a o 1b), ni con el cuadro clínico; las células MDBK y NCL-1 serían más susceptibles a la replicación del VDVB.

Palabras clave: Pestivirus, virus de la diarrea viral bovina, VDVB cepas de campo argentinas, cultivos celulares, interacción virus-célula

INTRODUCTION

Bovine viral diarrhea virus (BVDV) is an important pathogen of bovine cattle with a worldwide distribution (16). In cattle, due to its easy transmission, it is possible to detect a high prevalence of antibodies to the virus (17) and although most infections are subclinical, the infection with BVDV can be associated with various clinical manifestations. These include reproductive problems (infertility, abortions, teratogenesis), respiratory disease and diarrhea of variable degree (16). Additionally, the infection at the beginning of gestation can originate the birth of calves that are persistently infected with the virus; these animals tend to suffer a fatal disease known as mucosal disease (16).

BVDV is a Pestivirus from the family *Flaviridae* with a 12-kb single-stranded RNA molecule of positive polarity. According to its biological characteristics during *in vitro* culture, BVDV can be classified as cytopathic (CP) or non-cytopathic (NCP) (16). During replication, the enveloped virions transfer the genomic RNA to the susceptible cells by fusing to endocytic membranes (7). While the NCP biotype does not cause visible alterations, the cells infected with the CP biotype exhibit a characteristic pattern of morphological and structural changes associated with viral replication; these include the generation of cytoplasmic vacuoles, pycnosis, and cell death mediated by induction of apoptosis (12).

BVDV can be propagated either in primary cell cultures or in established lines. For research and industrial purposes continuous cell lines are the most common systems used for viral multiplication (9). BVDV can replicate in cultures of cells obtained from the species it usually infects, e.g. bovines, ovine, goats, etc., as well as in cells from other species such as rabbits, cats, swine, etc. (6) and although several cell lines are susceptible to BVDV infections, the multiplication of the virus *in vitro* can vary due to factors that are not yet well known (19).

Previous studies on the replication of BVDV have shown that although the virus can adsorbed to different cell types, but not all of them are susceptible to viral multiplication (22). On the other hand, the kinetics of viral replication depends not only on the cell line used (22), but also on the phase of cell multiplication in which the infection takes place (4, 13).

Although there is some information on the *in vitro* behavior of some reference strains of BVDV (6), the behavior of many Argentinean field strains has not yet been thoroughly studied. According to the results obtained by Diamond *et al*., (4), there could be *in vitro* dominant mutations that define phenotypes whose kinetics of viral replication in field viruses are different from those in reference strains.

The susceptibility of the substrate to the virus is a relevant issue that can be used for viral isolation for diagnostic purposes, characterization of the biological behavior of a certain strain (e.g., in pathogenicity studies), evaluation of immunogenicity tests and vaccine production.

In the present work, we aimed to analyze the *in vitro* kinetics of reference strains and field isolates of BVDV in Argentina in various continuous cell lines and assessing the capacity of replication in cell cultures according to the protocol of infection *in vitro*.

MATERIALS AND METHODS

Cultures and cell lines

Madin-Darby Bovine Kidney (MDBK), Bovine Turbinate (BoTur) and Baby Hamster Kidney (BHK-21) cell lines from the American Type Cell Collection (ATCC), were provided by the Institute of Virology, National Institute of Agrarian Technology (INTA), Castelar, Argentina. RD-420 (from bovine testicle) (8), NCL-1 (from bovine uterus) (14) cell lines, were kindly provided by Dr. R. Donis, University of Nebraska, Lincoln, USA. PKZ cell line was derived from culture of porcine kidney tissue at the Institute of Virology, INTA, Castelar, Argentina (Dr. O. Zabal, personal communication). All the cell lines were free from viral, bacteriological and mycoplasma contaminants, under standard culture conditions.

Cell lines MDBK, BoTur, RD-420 and NCL-1 were propagated in Minimum Essential Medium(Eagle), with Earle salts (MEM-E) (Sigma Chemicals, U.S.A.) and cell line BHK -21 in Dulbecco´s Modified Eagle Medium (MEM-D) (Sigma Chemicals, U.S.A.), supplemented with 10% controlled bovine fetal serum (BFS) free from virus and antibodies, 100 U/ml penicillin G and100 mg/ml streptomycin sulfate. Cells were incubated at 37 °C in an atmosphere with 5% CO_2.

Virus

All BVDV strains used belonged to the cytopathic (CP) biotype. Reference strains NADL and Singer, genotype 1a, were used. Argentinean field isolates from bovines with clinical-pathological signs compatible with mucosal disease (04/89, 99/134, 00/693) and bovine respiratory disease (04P7016A) typified by RT-PCR of 5'UTR region of the genome as genotype 1b, were used. The viruses were originally isolated in MDBK cells, characterized and provided by the Specialized Veterinary Diagnostic Service from INTA, Balcarce (18). Previous to this study, the field isolates had a maximum of four cells passages. The viruses were amplified in monolayers of MDBK cells prepared in T-*75* flasks (1 x 10^5 cell/ml), for 72 h. The supernatants were harvested and fractionated in 1 ml aliquots and conserved at -80 °C for their titration and use. The virus titer (VT) were obtained by the end-point titration method determined by observation of the cytopathic effect (CPE). The infective titer ($TCID_{50}$) was calculated by the Reed and Müench method.

In certain circumstances, the presence or absence of BVDV was confirmed by a direct immunofluorescence method using a polyclonal antibody against Pestivirus (American BioResearch, USA).

Infection protocol and viral quantification

We initially compared the replication kinetics of four BVDV field isolates from Argentina (04/89, 99/134, 00/693 and 04P7016) and the reference strains Singer and NADL in MDBK, BoTur and BHK21 cell lines. Then we evaluated the kinetics of BVDV viral replication of the reference strain NADL in the continuous bovine cell lines of experimental use RD420, NCL-1 and PKZ, using the MDBK line as a control.

The cells to be infected were grown in 24-well plates (Greiner bio-one, Germany) at a concentration of 1 x 10^5 cell/ml and infected with each of the BVDV reference strains and field isolates at a multiplicity of infection (MOI) of 0.1 $TCID_{50}$/ml of the originally cultured cells. Two infection protocols were assayed: 1) an infection simultaneous to the cell passage, at the beginning of the logarithmic growth phase, and 2) an infection on a preformed confluent monolayer with 24 h of growth, at the end of the logarithmic growth phase. In consequence, the virus replication during the study was evaluated in two different substrates: a suspension of cells and a fully grown monolayer. The cells were incubated at 37 °C with 5% CO_2 and observed daily to detect the presence of CPE. The supernatants corresponding to the different cell lines were harvested at three different times: 24, 48 and 72 hours post-infection (h p.i.) and frozen at -80 °C for further viral quantification. The VT was determined by the end-point titration method and observation of the CPE. The $TCID_{50}$ was calculated with the Reed and Müench method. To establish the kinetics of viral replication in each treatment (virus, cell line, the infection protocol and harvest time) six repetitions were carried out. Appropriate negative controls were included in each treatment.

Statistical analysis

The experimental design consisted of divided plots with repeated measurements over time. The main plot included the effect of the virus with six repetitions, whereas the sub-plot included the effect of the infection protocols. The measurements were repeated at 24, 48 and 72 h p.i. The comparisons of the least-square means were carried out by the Tukey-Kramer's test. The MIXED procedure from the SAS (Ver. 8, 2000. SAS Institute Inc., Cary, NC, USA) was used. The level of significance established was 0.05.

RESULTS

General features

In order to determine the susceptibility of MDBK, BoTur and BHK-21 cells to BVDV infection, cultures of each cell line were infected with both the BVDV reference strains and field isolates from Argentina. While MDBK and BoTur cells were susceptible to infection and viral multiplication, and presented CPE and viral antigen by means of immunofluorescence, BHK-21 and PKZ cells were not. The CPE characteristic of BVDV infection was not observed in the latter, thus confirming the absence of the virus by means of immunofluorescence at 24, 48 and 72 h p.i.

In order to evaluate the effect of different infection protocols on the VT obtained at different times post infection, MDBK and BoTur cells were infected simultaneously to the cell passage or on a monolayer established with more than 90% confluence.

The infection protocol did not have any effect on the final titer obtained ($p < 0.05$) (Table 1), and differences related to the cell line, virus and time of harvest were observed ($p < 0.001$).

Figure 1 shows the VT obtained for different BVDV in MDBK and BoTur cell lines at 24, 48 and 72 h p.i. The VT obtained presented differences depending on the cell line, virus and time of harvest ($p < 0.001$).

Cell line-virus interaction.

Most of the viruses evaluated presented higher titers in the MDBK cell line than in the BoTur cell line($p < 0.05$) (Table 1). In a few instances, the BVDV Singer (at 24 h p.i.) and the field strain 04/89 (at 724 h p.i.) had higher titers in Botur than in MDBK ($p < 0.05$) (Table 1).

Replication kinetics

After the initial infection, independently of the infection protocol assayed, there were no differences in the titers between the viruses analyzed in the MDBK line at 24 h p.i. (Table 2, Figure 1). A variable behavior was observed in the BoTur line at 24 h p.i., with a higher titer for BVDV Singer and a lower one for 04P7016. At 48 h p.i., a higher titer was determined for NADL and a significantly lower one for 04/89 in the MDBK cells ($p < 0.05$). When analyzing viral multiplication at 72 h p.i. and comparing the different BVDV isolates in MDBK cells, we observed that the reference strain NADL kept the highest VT, whereas the 04/89 strain showed the lowest one ($p < 0.05$) at 48 h p.i. However, this virus showed higher titers ($p < 0.05$) than the rest of the viruses in the BoTur line at 72 h p.i. (Table 2).

Table 1. Viral titers ($log_{10}TCID_{50}$/ml) obtained for the NADL and Singer strains and BVDV field isolates 99/134, 04P7016, 04/89 and 00/693 at 24, 48 and 72 h p.i. in the cell lines MDBK and BoTur according to the infection protocol used. The values correspond to the mean of six replicates ± Standard Error.

		Protocol of Infection					
		Preformed confluent monolayer			Simultaneous to cell passage		
Virus	Time Cell Line	24 h	48 h	72 h	24 h	48 h	72 h
NADL	MDBK	3.85(±0.36)	9,14(±0,36)	9,09(±0,38)	4,51(±0,36)	8,24(±0,36)	7,52(±0,38)
	BoTur	3.29(±0.19)	4,71(±0,26)(1)	4,72(±0,16)(1)	3,42(±0,19)	4,43(±0,26)(1)	4,27(±0,16)(1)
Singer	MDBK	4.78(±0.36)	6,88(±0,36)	5,46(±0,38)	5,26(±0,36)	5,71(±0,36)	5,42(±0,38)
	BoTur	6.36(±0.19)(1)	5,46(±0,26)(1)	4,96(±0,16)	6,32(±0,19)(1)	4,49(±0,26)(1)	4,35(±0,16)(1)
00/693	MDBK	5.27(±0.36)	6,46(±0,36)	7,00(±0,38)	6,10(±0,36)	6,80(±0,36)	7,10(±0,38)
	BoTur	5.06(±0.19)	6,38(±0,26)	5,82(±0,16)(1)	4,80(±0,19)(1)	6,42(±0,26)	5,35(±0,16)(1)
99/134	MDBK	4.46(±0.36)	6,15(±0,36)(1)	5,19(±0,38)	4,59(±0,36)	5,92(±0,36)	5,16(±0,38)
	BoTur	4.53(±0.19)	4,17(±0,26)	4,17(±0,16)(1)	4,05(±0,19)	3,18(±0,26)(1)	3,14(±0,16)(1)
04P7016	MDBK	4.43(±0.36)	5,89(±0,36)	6,30(±0,38)	4,48(±0,36)	5,61(±0,36)	6,73(±0,38)
	BoTur	2.68(±0.19)(1)	3,10(±0,26)(1)	2,28(±0,16)(1)	2,51(±0,19)(1)	2,47(±0,26)(1)	2,22(±0,16)(1)
04/89	MDBK	4.15(±0.36)	5,30(±0,36)	5,16(±0,38)	4,52(±0,36)	5,10(±0,36)	5,22(±0,38)
	BoTur	4.69(±0.19)	5,71(±0,26)	6,32(±0,16)(1)	4,18(±0,19)	5,17(±0,26)	6,42(±0,16)(1)

(1): indicate differences between cell lines for each time and virus ($p < 0.05$).

Table 2. Viral titers ($log_{10}TCID_{50}$/ml) obtained for the NADL and Singer strains and the BVDV field isolates 99/134, 04P7016, 04/89 and 00/693 at 24, 48 and 72 h p.i. in the MDBK and BoTur cell lines independently of the infection protocol used. The values correspond to the mean of 12 replicates ± Standard Error.

	MDBK			BoTur		
Virus / t	24 h	48 h	72 h	24 h	48 h	72 h
NADL	4.18(±0.33)a A	8.69(±0.45)b A	8.31(±0.79)b A	3.36(±0.07)a A	4.47(±0.14)b A	4.49(±0.23)b A
Singer	5.02(±0.24)a A	6.30(±0.59)b B	5.44(±0.02)a B	6.34(±0.02)a B	4.98(±0.49)b A	4.66(±0.31)b A
00/693	5.69(±0.42)a A	6.63(±0.17)b B	7.05(±0.05)b B	4.93(±0.13)a A	6.40(±0.02)b B	5.59(±0.24)b B
99/134	4.26(±0.07)a A	6.35(±0.12)b B	5.18(±0.15)a C	4.29(±0.24)a A	3.68(±0.50)a C	3.65(±0.56)a A
04P7016	4.46(±0.03)a A	5.75(±0.14)b B	6.52(±0.22)b B	2.60(±0.09)a C	2.79(±0.32)a C	2.25(±0.03)a C
04/89	4.34(±0.19)a A	5.20(±0.10)a C	5.19(±0.03)a C	4.44(±0.26)a A	5.44(±0.27)b A	6.37(±0.05)b B

Small case letters (a, b) indicate comparisons between means of different viruses for each time within a monolayer ($p < 0.05$) and uppercase letters (A, B) indicate comparisons of different viruses to each time within the monolayer ($p < 0.05$).

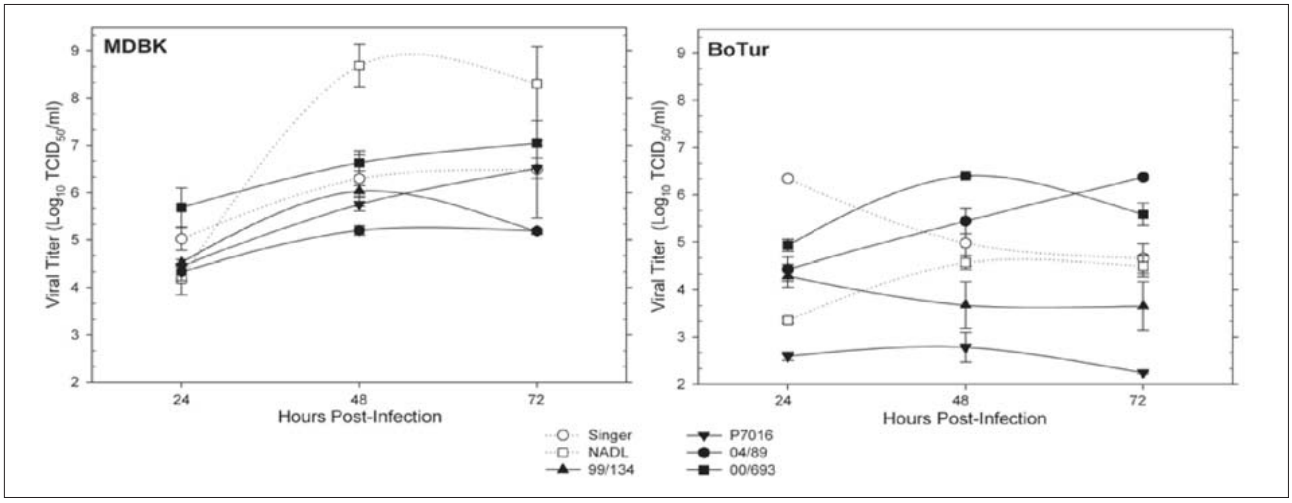

Figure 1. Kinetics of growth of the reference strains NADL and Singer and the BVDV field strains 00/134, 04P7016, 04/89 and 00/693 in the MDBK and BoTur cell lines.
The viral titers correspond to an average between the titers obtained after an infection simultaneous to the cell passage and on a monolayer with 80% of confluence. The points represent the least-square mean and standard error for the cell line-time interaction corresponding to slx replicates in each of the cell line.

When evaluating the kinetics of replication of each virus in the MDBK and BoTur cell lines (Table 2) independently of the infection protocol, we observed that in the MDBK cell line, the viruses NADL, 00/693 and 07P7016 increased their titer from 24 h p.i. to 48 h p.i. and remained the same at 72 h p.i. ($p < 0.05$). On the other hand, the Singer strain and the virus 99/134 presented a maximum titer at 48 h p.i., which decreased at 72 h p.i. The virus 04/89 did not present any relevant variations in its titer at any of the three times assayed ($p < 0.05$) in the MDBK cells. The NADL titers, 00/693 and 04/89 increased from 24 h p.i. to 48 h.p.i. in the BoTur cell line, and remained without significant variations at 72 h p.i., whereas viruses 99/134 and 04P7016 did not present any variations in their titers at any of the three times assayed. Finally, the Singer reference strain presented a maximum titer at 24 h p.i. in the BoTur cell line and decreased both at 48 h p.i. and 72 h p.i. ($p < 0.05$).

BVDV-NADL interaction with different lines.

When the reference strain NADL was analyzed in the experimental lines RD-420 and NCL-1, and then compared with its behavior in the MDBK line, we determined that the cell line-time interaction presented the same pattern as that for the average viral titer reached in the three time post-infection studied, observing higher titers at 48 h.p.i. (Table 3). On the other hand, similarly to that observed in other cell lines, the infection protocol (i.e. either simultaneous to the cell passage or on a preformed monolayer) did not have any effects on the final titer

Table 3: Least-square mean and standard errors of the BVDV infection of the NADL strain in MDBK, NCL-1 and RD-420 cell lines according to the infection protocol used and time evaluated.

	Mean(1)	Standard Error
Cell line		
MDBK	5.85 a	0.08
NCL-1	6.36 b	0.10
RD-420	4.81 c	0.10
Monolayer		
Preformed confluent	5.72 a	0.05
Simultaneous to cell passage	5.63 a	0.07
Time (H)		
24	5.44 a	0.12
48	6.57 b	0.12
72	5.01 c	0.12

(1) Least-square mean. Small case letters (a. b, c) indicate the comparisons between means within the cell line, monolayer and time ($p < 0.05$).

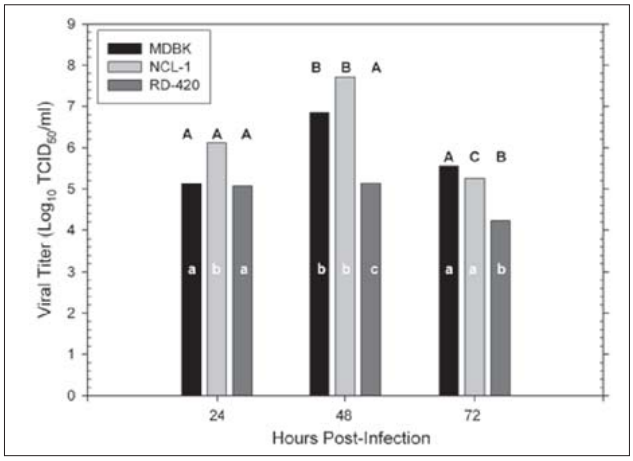

Figure 2. Interaction of the NADL strain with the RD-420, NCL-1 and MDBK cell lines. The viral titers correpsond to the average after the infection simultaneous to the cell passage and on a monolayer with 80% of confluence. The bars represent the least-square means for the cell line-time interaction corresponding to six replicates of each of the two experiments. Uppercase letters (A, B, C) indicates comparisons between means of different viruses for each time within a monolayer (P < 0.05) and lowercase letters (a, b, c) indicate comparisons of each virus to different time within the monolayer (P < 0.05).

obtained in the experimental lines RD-420 and NCL-1 ($p < 0.05$).

The replication of BVDV NADL showed significant higher titers ($p < 0.05$) at 24 h p.i. in the NCL-1 cell line as compared with RD-420 and MDBK (Figure 2). The same tendency was observed at 48 h p.i., with a significantly lower titer in the RD-420 cell line. The highest titer of BVDV NADL in the NCL-1 cell line was 1 x $10^{7.71\ (\pm\ 0.230)}$ $TCID_{50}$/ml at 48 h p.i. vs 1 x $10^{5.14\ (\pm\ 0.230)}$ $TCID_{50}$/ml in RD-420 and MDBK 1 x $10^{6.85\ (\pm\ 0.168)}$ $TCID_{50}$/ml at the same time post-infection. Similar titers were determined in the MDBK and NCL-1 cell lines at 72 h p.i. These titers were higher ($p < 0.05$) than those of the RD-420 cell line at that time.

Finally, when evaluating the kinetics of replication of BVDV NADL in the MDBK, RD-420 and NCL-1 cell lines, we observed a significant increase in the titers for the MDBK and NCL-1 cell lines between 24 and 48 h p.i., which decreased at 72 h p.i. ($p < 0.05$) (Figure 2). In addition, in the RD-420 cells, no increase in the titer was observed at 24 h p.i. or 48 h p.i., whereas the viral concentration decreased significantly at 72 h p.i. ($p < 0.05$).

DISCUSSION

BVDV can be propagated either in primary cell cultures or in established cell lines. No differences have been previously observed among the susceptibility of primary cultures of different kinds of cells, such as kidney cells (pBEK), the established line of bovine fetal trachea (EBTr), the calf testicle (pCT) and the buffalo lungs (IMR-31) (5). The BVDV replication in cells derived from the uterus, kidney, turbinates and bovine testis seems to be associated with the sites of virus multiplication during the evolution of a natural infection (6), whereas the multiplication in tissues of species it can infect seems to be variable, thus preventing the prediction of their susceptibility to viral infection (1).

It has been determined that the stage of cellular multiplication is associated with the efficacy of replication of several viruses (4, 13); this does not seem to be the case of BVDV since the infection protocol in different cell lines (simultaneous to the cell passage or in previously established monolayers) did not generate any differences in the VT obtained. Although in different experimental conditions, a similar situation was also observed when infecting the cell line SW13 (cells from adenocarcinorna of the adrenal cortex) with different strains of the dengue virus (4), which also belong to the *Flaviviridae* family. In the case of the dengue virus, the intracellular viral titers vary according to whether the cell is infected in phase S (DNA synthesis) (13).

The results obtained in the present work show significant differences in the ability of the different BVDV strains to infect various cell types, depending on the cell line and time of infection analyzed. Although the genetic changes between the BVDV reference strains and field isolates may be related to variations in their capacity to infect a certain cell type, they do not seem to explain why the infection of the same virus is variable in different cell lines. One hypothesis that might explain this phenomenon has been proposed by Diamond *et al.* (4), who observed the existence of qualitative and quantitative differences in the receptors of the cells that seem to modulate the suscep-

tibility to BVDV infection. It is well known that these receptors determine the susceptibility of a cell line to a specific viral infection, since they allow the adhesion and internalization of the virus and later interaction with cellular factors that lead to the production of an infective viral progeny (3).

In our work, we confirmed that cell lines MDBK, BoTur, NCL-1 and RD-420 are susceptible to BVDV infection, since they produced infective viral particles when exposed to BVDV field isolates and reference strains, whereas BHK-21 and PKZ cells were not.

Although previous reports have shown the presence of a 50-kDa receptor for BVDV in the cell membrane of cell line BHK-21 (22), no infective viral progeny was obtained in this cell line for any of the viruses analyzed. Similar results have been observed by Bolin *et al.* (1) when inoculating BHK-21 cells with BVDV-TGAN and BVDV-Fc2Lu strains. The works by Xue and Minocha (22) have also shown the presence of a 50-kDa protein in the cell membrane as a possible BVDV receptor in MDBK, EBK, BoTur, PK15, MA1O4 and Vero cell lines, although the latter were not susceptible to the virus. These results indicate the presence of other factors related to the susceptibility of a certain cell line to viral infection such as alterations in the virus receptor (14, 21), the receptor blockage and the expression of glycoproteins in infected cells (6, 15).

The cell lines established from bovine testicle RD-420 and the epithelioid cells from bovine uterus NCL-1 had been developed and assayed with BVDV in previous studies by Flores *et al*. (8) and Gil *et al*. (14). In the work by Flores *et al.* (8), the RD-420 cell line was used to determine the infectivity of BVDV opsonized with monoclonal antibodies (Ab) in cells that either expressed or did not express the murine receptor B2 Fc-γ. These authors postulated that the adhesion of the virus-Ab complex to the FcR results in a productive infection only if the binding of the Ab to the virion does not interfere with the functions that take place after the virus entrance into the cell (8). On the other hand, Gil *et al*. used NCL-1 cells for the evaluation of the action of interferon alpha/beta (IFN-α/β) in BVDV-CP infection, and observed that the virus is inefficient in the induction of IFN-α/β. They postulated that this blockage may be related to the activity of viral protease N^{pro}, independently of the virus replication efficiency (10).

Our results show that the highest titers of BVDV reference strains and field isolates were obtained in MDBK and NCL-1 cells, thus indicating that these cell lines are more susceptible to BVDV replication. On the other hand, a more homogeneous behavior of the viruses evaluated was observed in the MDBK cell line, with maximum VT at 48 h p.i. Although the kinetics of multiplication of the viruses analyzed presented a higher variation in BoTur cells, a *plateau* was observed in BVDV multiplication at 48 h p.i. In addition, in the case of NCL-1 cells, the same behavior was confirmed with the NADL strain, where higher yields of viral progeny were detected at 48 h p.i.

Studies carried out by Xue and Minocha (22) have shown that in MDBK and BoTur bovine cells, the NADL strain presents a similar behavior, with VT that increase gradually until 1.6×10^6 PFU/ml at 48 h p.i. (MOI=1). These results partially agree with those obtained in this work, since although NADL presented similar replication curves in both cell lines, the titers reached in each of them differed significantly, obtaining more than four logarithms of infectious particles in MDBK at 48 h p.i. with a MOI=1.

Furthermore, Onyekaba *et al*. (19) obtained a viral titer of $1 \times 10^{4.13}$ $TCID_{50}$/ml in BoTur at 8 days p.i. with BVDV. In the present work, similar or higher titers were obtained (except for isolate 04P7016) at 48 h p.i. in the same cell line, which seems to indicate a more efficient system for viral multiplication. The causes of this difference need to be elucidated.

Titers of $1 \times 10^{5.50}$ $TCID_{50}$/ml at 48 h p.i. have been obtained in infections carried out with NADL (MOI=1) in the bovine fetal kidney cell line (BFK) (17). Although these were higher than those reached in BoTur, they were three logarithms lower than the viral concentration found in MDBK in this work. In the PK 15 (porcine kidney) cell line, the NADL strain presented a titer of 5×10^5 PFU/ml at 72 h p.i., at a MOI=1 (22), whereas in monocytes and dendritic cells, similar titers were obtained with a BVDV field strain at a MOI=2 (11). In a study aimed at determining the BVDV susceptibility of five cell lines, viral titers of $1 \times 10^{1.13}$, $1 \times 10^{3.25}$, $1 \times 10^{4.13}$, 1×10^{1} and 1×10^{1} $TCID_{50}$/ml were determined in the cell lines of swine testicle (ST), mink lungs (ML), BoTur, porcine kidney, and equine dermis (ED) respectively, eight days p.i. (19). In that work, BoTur and ML were considered the most appropriate cells for BVDV propagation. Based on that information and the results obtained in this work, where higher VT were determined at a lower MOI and with shorter times of incubation, we can infer that maximum viral production can be obtained with a MOI = 0.1 and incubation periods of 48-72 h, thus being unnecessary to use a higher viral multiplicity of infection or longer incubation time. The MDBK and NCL-1 cells appear to be an optimal substrate for BVDV replication, since the infection of these cells generates high VT with a lower multiplicity of infection at times similar or lower to those described by others (12, 19, 22).

The *in vitro* behavior of the BVDV field isolates from Argentina, with a low number of passages in culture, did not differ from that of the reference strains, with a high number of manipulations in the laboratory. However, other studies have shown differences or antigenic heterogeneity between field isolates and the reference strains NADL and Singer (20), which suggests that there might be a relationship between these differences and the different pathogenicity or behavior *in vitro*.

Under the experimental conditions of the present work we were able to determine that the viruses analyzed differed in their capacity to replicate in the cell lines evaluated, and did not observe a differential behavior between the reference strains and the field isolates from Argentina. Besides species specificity, we determined that the virus-cell line interaction may not be related with the viral genotype or the clinical symptoms observed in the animal from which the virus was obtained.

Practical aspects that arise from the result of this study indicate that after virus challenge in cell cultures in S phase of replication, the virus titer peaks at 48 h, being not necessary long incubation periods to obtain the maximum expression of the virus. In addition, the most homogeneous behavior of all BVDVs tested and the highest VT obtained in MDBK and NCL-1 cell lines make these cell lines adequate substrates for BVDV multiplication. Certainly, these observations have implications for disease diagnosis, research and industrial application.

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REFERENCES

1. Bolin S, Ridpath J, Black J, Macy M, Roblin R. Survey of cell lines in the American Type Culture Collection for bovine viral diarrhea virus. J Virol Meth 1994; 48: 211-21.
2. Buckwold V, Beer B, Donis R. Bovine viral diarrhea virus as a surrogate model of hepatitis C virus for the evaluation of antiviral agents. Antiviral Res 2003; 60: 1-15.
3. Carrascosa Al, Bustos Mj, Galindo L, Viñuela E. Virus-specific cell receptors are necessary, but nor sufficient, to confer cell susceptibility to African swine fever virus. Arch Virol 1999; 144: 1309-21.
4. Diamond M, Edgil D, Roberts T, Lu B, Harris E. Infection of human cells by dengue virus is modulated by different cell types and viral strains. J Virol 2000; 74: 7814-23.
5. Ferrari M. Susceptibility of various cell culture systems to bovine viral diarrhea virus. Microbiologica 1985; 8: 17-22.
6. Flores E, Donis R. Isolation of a mutant MDBK cell line resistant to bovine viral diarrhea virus infection due to a block in viral entry. Virology 1995; 208: 565-75.
7. Flores EF, Kreutz LC, Donis RO. Swine and ruminant pestiviruses require the same cellular factor to enter bovine cells. J Gen Virol 1996; 77: 1295-303.
8. Flores EF, Risatti GR, Donis RD. Expression of the mouse Fc receptor B2 in bovine cells rescues the infectivity of conditionally neutralized bovine viral diarrhea virus. Vet Microbiol 2002; 85: 99-109.
9. Freshney IR. Culture of animal cells: a manual of basic technique. In: Willey-Liss Inc. editors, 3º Ed, New York, 1994, p. 486.
10. Gil LH, Ansari IH, Vassilev V, Liang D, Lai VCH, Zhong W, *et al.* The amino-terminal domain of bovine viral diarrhea virus N^{pro} protein is necessary for alpha/beta interferon antagonism. J Virol 2006; 80: 900-11.
11. Glew E, Carr B, Brackenbury L, Hope J, Charleston B, Howard C. Differential effects of bovine viral diarrhoea virus on monocytes and dendritic cells. J Gen Virol 2003; 84: 1771-80.
12. Grummer B, Bendfelt S, Wagner B, Greiser-Wilke I. Induction of the intrinsic apoptotic pathway in cells infected with cytopathic bovine virus diarrhoea virus. Virus Res 2002; 90: 143-53.
13. Helt AM, Harris E. S-phase-dependent enhancement of Dengue virus 2 replication in mosquito cells, but not in human cells. J Virol 2005; 79: 3218-23.
14. Krey T, Himmelreich A, Heimann M, Menge C, Thiel HJ, Maurer K, *et al*. Function of bovine CD46 as a cellular receptor for bovine viral diarrhea virus is determined by complement control protein 1. J Virol 2006; 80: 3912-22.
15. Krey T, Moussay M, Thiel HJ, Rümenapf T. Role of the low-density lipoprotein receptor in entry of bovine viral diarrhea virus. J Virol 2006; 80: 10862-7.
16. Nettleton PE, Entrican G. Ruminant Pestiviruses: a review. Br Vet J 1995; 151: 615-42.
17. Odeón A, Späth EJA, Paloma EJ, Leunda MR, Fernández Sainz IJ, Pérez SE, *et al*. Prevalencia de anticuerpos al virus de diarrea viral bovina, herpesvirus bovino y virus sincicial respiratorio bovino en Argentina. Rev Med Vet (Bs As) 2001; 82: 216-20.
18. Odeón AC, Risatti G, Kaiser GG, Leunda MR, Odriozola E, Campero CM, *et al*. Bovine viral diarrhea virus genomic associations in mucosal disease, enteritis and generalized dermatitis outbreaks in Argentina. Vet Microbiol 2003; 96: 133-44.
19. Onyekaba C, Fahrmann J, Bueon L, King P, Goyal SM. Comparison of five cell lines for the propagation of bovine viral diarrhea and infectious bovine rhinotracheitis viruses. Microbiologica 1987; 10: 211-315.
20. Reddy JR, Xue W, Rivera S, Minocha HC. Antigenic differences between a field isolate and vaccine strains of bovine viral diarrhea virus. J Clin Microbiol 1995; 33: 2159-61.
21. Tscherne DM, Evans MJ, MacDonald MR, Rice CM. Transdominant inhibition of bovine viral diarrhea virus entry. J Virol 2008; 82: 2427-36.
22. Xue W, Minocha HC. Identification of bovine viral diarrhea virus receptor in different cell types. Vet Microbiol 1996; 49: 67-79.

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