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In vitro replication of bovine herpesvirus types 1 and 5

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A B S T R A C T

The aim of this work was to study the *in vitro* replication of bovine herpesvirus types 1 and 5 (BoHV-1 and 5) at the beginning and end of the logarithmic growth phase of Madin-Darby Bovine Kidney (MDBK) cells. The replication kinetics and size of lysis and infection plaques of the field isolates 09/210 (BoHV-1) and 97/613 (BoHV-5) and the reference strains BoHV-1.1 Los Angeles 38 (LA38), BoHV-1.1 Cooper, BoHV-5a N569 and BoHV-5b A663 were evaluated. The highest mean virus titre was recorded for N569, followed by LA38 and 97/613. For most of the viruses, the virus titre values increased from 24 h post-infection (hpi) up to 48 hpi and then, they remained unchanged up to 72 hpi. However, the virus titre for 09/210 was significantly lower and a slight, steady increase was observed from 24 to 72 hpi. Furthermore, the largest lysis and infection plaques were recorded for 97/613 and LA38, respectively. According to this work, it is evident that there is a relationship between the replication of BoHV and the multiplication stage of MDBK cells. The results of this study contribute to the understanding of the replication behaviour in cell cultures of several strains of BoHV, which is critical for the rational design of *in vitro* experiments and vaccine production.

1. Introduction

Bovine herpesviruses types 1 (BoHV-1) and 5 (BoHV-5) are two closely related alphaherpesviruses that infect cattle. The distribution of BoHV-1 infection is worldwide. However, BoHV-5 is restricted to South America, mainly Brazil and Argentina (Del Médico Zajac et al., 2010). These viruses belong to the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Varicellovirus*. Members of this subfamily are recognized for their wide host range, rapid growth rate in culture and their ability to destroy infected cells (Roizman and Pellett, 2001). Alphaherpesviruses are characterized by the establishment of latent infections, mainly in sensory ganglionic neurons of the peripheral nervous system (Muylkens et al., 2007; Rock et al., 1992). Bovine herpesvirus type 1 (BoHV-1) is one of the most important pathogens of cattle, causing significant economic losses to the livestock industry (Takiuchi et al., 2005). It causes a variety of clinical syndromes, including respiratory disease, conjunctivitis, abortion (BoHV-1 subtype 1) and genital infections (BoHV-1 subtype 2). Bovine herpesvirus type 5 (BoHV-5) can cause subclinical infection or a disease of moderate severity in adult cattle (Ashbaugh et al., 1997; Cascio et al., 1999; Del Médico Zajac et al., 2006). However, lethal encephalitis was observed in young animals (Bartha et al., 1969; Carrillo et al., 1983; Meyer et al., 2001; Pérez et al., 2002). The strains of BoHV-5 were classified into three subtypes (a, b and non-a-non-b) based on viral DNA analysis by restriction endonuclease fingerprinting (D'Arce et al., 2002). The Australian strain N569, the Argentinean strain A663 and the Brazilian strains belong to subtypes a, b and non-a-non-b, respectively. According to Maidana et al. (2008), the viral subtypes BoHV-5a and BoHV-5b are present in Argentina, where the former is the most prevalent viral subtype in the country.

Several strategies have been developed for rapid virus detection. However, classic cell culture is still "the gold standard" method (Bleotu et al., 2006). The susceptibility of the substrate to the virus is a relevant issue to be considered in viral isolation for diagnostic purposes, for the characterization of the biological behaviour of a certain strain (e.g., in pathogenicity studies), and for immunogenicity tests and vaccine production (Odeón et al., 2009). Continuous cell lines are the most common systems used for viral multiplication for research and industrial purposes (Freshney, 1994).

The kinetics of viral replication depends not only on the cell line used (Xue and Minocha, 1996) but also on the phase of the cell cycle in which the infection takes place. Determining the kinetics of viral replication is relevant for the *in vitro* characterization of viruses. The size of lysis and infection plaques is another important feature to consider. This represents a direct measure of the lytic potential and ability for cell-to-cell spreading of viruses (Ladelfa, 2010). Although information is available on the susceptibility of several cell lines to

Abbreviations: BoHV, bovine herpesvirus; hpi, hours post-infection; LA38, Los Angeles 38; MDBK, Madin-Darby Bovine Kidney cells; MOI, multiplicity of infection.

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infection by BoHV-1 and 5 (Ladelfa et al., 2006; Onyekaba et al., 1987; Peterson and Goyal, 1988), the *in vitro* behaviour of these viruses has not been thoroughly studied.

In this study, the *in vitro* kinetics of reference strains and the Argentinean field isolates of BoHV-1 and 5 on Madin-Darby Bovine Kidney (MDBK) cells and the ability of these viruses to replicate in this cell line was assessed according to two different methods of cell infection. In addition, the size of the lysis and infection plaques produced by the viruses on MDBK cells was compared. Understanding the replication behaviour in cell cultures of these important pathogens of cattle is critical for the rational design of *in vitro* experiments and vaccine production.

2. Materials and methods

2.1. Cell line and culture conditions

Madin-Darby Bovine Kidney (MDBK) cells from the American Type Culture Collection (ATCC, Rockville, MD, USA) were used in this study. The MDBK cells were propagated in Minimum Essential Medium (Eagle), with Earle salts (MEM-E) (Sigma–Aldrich, Saint Louis, MO, USA), supplemented with 10% foetal bovine serum (Bioser, Buenos Aires, Argentina) free from viruses and antibodies and with antibiotic-antimycotic liquid (Gibco, Langley, OK, USA), including 100 U/ml penicillin G, 100 μg/ml streptomycin sulphate and 0.025 μg/ml amphotericin B. The cells were incubated at 37 °C in a 5% CO_2 atmosphere.

2.2. Virus strains

Reference strains Los Angeles 38 (LA38) (BoHV-1.1), Cooper (BoHV-1.1), N569 (BoHV-5a) and A663 (BoHV-5b) were used in this study. The Argentinean field strains BoHV-1 (identified as 09/210) and BoHV-5a (identified as 97/613) were isolated from cattle with clinical-pathological signs compatible with BoHV infection. Virus identification was confirmed by isolation in cell culture followed by direct immunofluorescence using a polyclonal antibody against BoHV (American BioResearch, Sevierville, TN, USA) and multiplex-PCR (Claus et al., 2005). The viral strains were characterized and provided by the Specialized Veterinary Diagnostic Service, INTA Balcarce (Argentina) and the Institute of Virology, INTA Castelar, Argentina (BoHV-5 strains N569 and A663). Viral stocks were replicated in MDBK cells, in T-25 flasks (Greiner Bio-one, Frickenhausen, Germany) (1×10^5 cells/ml), for 24 h. Supernatants were harvested and frozen at −80 °C. The virus titre was determined by the end-point titration method and expressed as $TCID_{50}$, according to the method of Reed and Müench (1938).

2.3. Infection and viral quantitation method for determination of the replication kinetics

The MDBK cells were grown in 24-well plates (Greiner Bio-one, Frickenhausen, Germany) at a concentration of 1×10^5 cell/ml and infected with the reference strains (BoHV-1 LA38, BoHV-1 Cooper, BoHV-5 N569 and BoHV-5 A663) or the field isolates (BoHV-1 09/210 and BoHV-5 97/613) at a multiplicity of infection (MOI) of 0.1. Virus replication was evaluated under two conditions: (1) infection concurrent with cell passage at the beginning of the logarithmic growth phase and (2) infection on a preformed confluent monolayer with 24 h of growth, at the end of the logarithmic growth phase. The cells were incubated at 37 °C with 5% CO_2 and observed daily for the presence of any cytopathic effects. The supernatants were harvested at three different time points: 24, 48 and 72 hpi and frozen at −80 °C for further viral quantitation. The virus titre was determined as described previously. Six replicates were performed in order to establish the kinetics of viral replication for each experiment (virus, the infection method and harvest time points). Appropriate negative controls (mock-infected cells) were included in each experiment.

2.4. Method for the infection and detection of lysis and infection plaques

A preformed confluent monolayer of MDBK cells with 24 h of growth on chamber slides (Nunc, Naperville, IL, USA) was infected with the reference strains (BoHV-1 LA38, BoHV-1 Cooper, BoHV-5 N569 and BoHV-5 A663) or the field isolates (BoHV-1 09/210 and BoHV-5 97/613) at a MOI of 0.05 and incubated at 37 °C with 5% CO_2. At 24 hpi, the supernatants were harvested for virus titre determination and the cells were fixed with acetone for 20 min at −20 °C and incubated with a polyclonal antibody against BoHV conjugated with fluorescein isothiocyanate (American BioResearch, Sevierville, TN, USA) for 35 min at 37 °C. The lysis and infection plaques (80 plaques for each virus) were photographed with a digital camera (Olympus Mod C5500) coupled to a fluorescence microscope. The area of the plaques was determined using the public domain software *ImageJ* (http://rsb.info.nih.gov/ij) and the values were expressed in pixels.

2.5. Statistical analysis

The experimental design for determination of the replication kinetics consisted of divided plots with repeated measurements over time. The main plot included the effect of the virus with six replicates, whereas the sub-plot included the effect of the infection methods. The measurements were repeated at 24, 48 and 72 hpi. A comparison of least-square means was carried out using Tukey–Kramer's test. The MIXED procedure (SAS, 2002) was used.

A completely randomized design with four viral strains was used to analyse the size of the lysis and infection plaques. As the statistical assumptions were not fulfilled, a logarithmic transformation of the data was performed. The GLM procedure (SAS, 2002) was used to analyse the variance. The response variable was the $\log_{10}$ of lysis and infection plaque areas as a function of each strain. In all cases, the level of significance was 0.05.

3. Results

3.1. Replication kinetics

The mean virus titres obtained for LA38, Cooper, N569 and A663 and the field isolates 09/210 and 97/613 at 24, 48 and 72 hpi on MDBK cells, according to the infection method used, are presented in Table 1. Significant differences ($p < 0.05$) were observed when the viral strains, post-infection times and infection methods were evaluated.

The highest virus titre in the cell culture was recorded for BoHV-5 reference strain N569, followed by BoHV-1 reference strain LA38, BoHV-5 field strain 97/613 and BoHV-1 reference strain Cooper (Table 2).

In order to evaluate the effect of the different infection methods on the virus titres obtained at different times post-infection, MDBK cells were infected at either the time of cell passage or at more than 90% confluence of the cell monolayer. The infection conditions tested had effect on virus titre at certain time points for the viruses studied. Significant differences ($p < 0.05$) between the virus titres for the different infection conditions were observed at 24 and 48 hpi. Most of the viruses evaluated presented higher titres at 24 and 48 hpi when infection was investigated concurrently with cell passage. For the field isolate BoHV-5 97/613 this difference was evident only at 24 hpi and BoHV-1 reference strain Cooper presented higher titres at 24 hpi when infection was performed at the time

Table 1
Virus titres (log_{10} $TCID_{50}$/ml) for the BoHV reference strains LA38, Cooper, N569 and A663 and field isolates 09/210 and 97/613 at 24, 48 and 72 hpi on MDBK cells, according to the infection method. The values correspond to the average of six replicates. Means and standard errors are shown.

Virus	Infection method					
	Preformed confluent monolayer			Concurrent with cell passage		
	24 h	48 h	72 h	24 h	48 h	72 h
BoHV-1 LA38	4.48 (±0.14)$^{(1)}$aAB	7.1 (±0.14)$^{(1)}$bA	7.52 (±0.14)bA	6.05 (±0.14)aAB	7.64 (±0.14)bA	7.67 (±0.14)bA
BoHV-1 Cooper	4.12 (±0.14)$^{(1)}$aA	7.21 (±0.14)$^{(1)}$bA	7.31 (±0.14)bA	5.35 (±0.14)aA	6.67 (±0.14)bB	7.2 (±0.14)bA
BoHV-1 09/210	4.48 (±0.14)$^{(1)}$aAB	5.18 (±0.14)$^{(1)}$abB	5.84 (±0.14)bB	5.46 (±0.14)aA	5.75 (±0.14)aC	5.91 (±0.14)aB
BoHV-5 N569	6.1 (±0.14)$^{(1)}$aC	7.09 (±0.14)$^{(1)}$bA	7.53 (±0.14)bA	6.91 (±0.14)aC	7.51 (±0.14)abA	7.67 (±0.14)bA
BoHV-5 A663	4.12 (±0.14)aA	5.77 (±0.14)$^{(1)}$bB	6.04 (±0.14)bBC	4.36 (±0.14)aD	5.15 (±0.14)bC	5.67 (±0.14)bB
BoHV-5 97/613	5.1 (±0.14)$^{(1)}$aB	6.94 (±0.14)bA	6.75 (±0.14)bAC	6.32 (±0.14)aBC	7.14 (±0.14)abAB	6.95 (±0.14)bA

(1) indicate differences between infection methods for each time and virus ($p > 0.05$). Lower case letters (a, b, c) indicate comparisons between the means of virus titres at different time points for each virus and infection method ($p > 0.05$) and uppercase letters (A, B, C, D) indicate comparisons between the viruses at each time point infection method ($p > 0.05$).

of cell passage and at 48 hpi on preformed MDBK cell monolayers. For BoHV-5 reference strain A663, the virus titre was higher at 48 hpi when confluent MDBK cells were infected. However, the infection condition did not have any effect on the virus titre at 72 hpi (Table 1).

When considering the harvest time points, both infection methods yielded the highest virus titre after 48 h for the different viruses, with the exception of the field isolate 09/210. No significant differences were found between the virus titres obtained at 48 and 72 hpi ($p > 0.05$). However, the virus titres at 48 and 72 hpi were significantly higher ($p < 0.05$) when compared to those recorded at 24 hpi. The virus titres for 09/210 remained unchanged from 24 to 72 hpi when the infection was performed at the time of cell passage ($p > 0.05$).

The replication kinetics for BoHV reference strains and field isolates on MDBK cells at 24, 48 and 72 hpi are shown in Fig. 1. When evaluating the replication kinetics of each virus on MDBK cells, independently of the cell infection method used, it was observed that the virus titres for LA38, Cooper, N569, A663 and 97/613 increased from 24 to 48 hpi. The virus titres for these viruses remained unchanged up to 72 hpi. The virus titres for A663 and 09/210 were significantly lower ($p < 0.05$), with a slight, gradual increase from 24 to 72 hpi ($p < 0.05$) for the BoHV-1 isolate 09/210.

After the initial infection of preformed confluent monolayers, differences in virus titres of 97/613 when compared to Cooper and A663 and between N569 and the remaining viral strains ($p < 0.05$) were detected. No differences were recorded between BoHV-1 strains and A663 and between BoHV-1 reference strains and the field isolate 97/613 ($p > 0.05$). When cell infection concurrent with cell passage was evaluated, at 24 hpi, the reference strain of BoHV-5, A663, presented the lowest titre and BoHV-5 N569 and 97/613 showed the highest titres, which did not differ between themselves. At 48 hpi, both infection methods showed different patterns of viral replication for the reference strain A663 and the field isolate 09/210, which presented lower titres when compared to BoHV-1 reference strains and BoHV-5 strains N569 and 97/613. Analysis of viral replication at 72 hpi revealed that this pattern of viral growth did not change and no differences were detected ($p > 0.05$) between viral titres of A663 and 09/210 and between LA38, Cooper, N569 and 97/613. At this time point, reference strains LA38 and N569 reached the highest virus titre (7.67).

Table 2
Least-square means (LSM) and standard errors of the virus titres (log_{10} $TCID_{50}$/ml) on MDBK cells for the BoHV reference strains and field isolates, independently of the infection method used and the supernatant harvest time points.

Virus	LSM	Standard error
BoHV-1 LA38	6.74	0.07
BoHV-1 Cooper	6.31	0.07
BoHV-1 09/210	5.44	0.07
BoHV-5 N569	7.13	0.07
BoHV-5 A663	5.18	0.07
BoHV-5 97/613	6.53	0.07

Virus titres are expressed as the average values of the titres obtained at 24, 48 and 72 hpi, regardless of the infection method used. LSMs correspond to 36 replicates.

3.2. Lysis and infection plaques

The mean size of the lysis and infection plaques produced by the BoHV-1 and BoHV-5 reference strains and field isolates are presented in Table 3. The largest lysis plaques at 24 hpi were recorded for the BoHV-5 field isolate (strain 97/613), with a mean size that significantly differed from the average size recorded for the other viral strains ($p > 0.05$). Significant differences ($p > 0.05$) were also detected between the plaque size of the BoHV-5 reference strain N569 and the BoHV-1 field isolate 09/210. However, the plaque size of these strains was not different ($p > 0.05$) from that observed for the BoHV-1 reference strains (LA38 and Cooper). The smaller lysis plaques were recorded for the BoHV-5 reference strain A663, with a mean size that significantly differed from the size observed for the other reference strains and the field isolate 97/613 ($p > 0.05$) but not from the field BoHV-1 isolate 09/210 ($p > 0.05$). The values for strain 97/613 were set at 100% and the lysis plaque areas recorded for the other viruses were expressed relative to this value (Fig. 2). The average size area for N569 at 24 hpi was 51.8% of the lysis plaque size for 97/613; for LA38 it was 37.1%; for Cooper it was 36.9%; for 09/210 it was 36.6% and for A663 it was 15%.

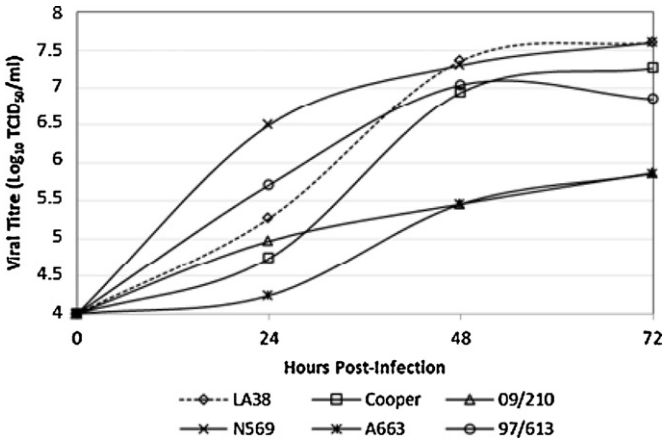

Fig. 1. Growth kinetics of BoHV-1 and BoHV-5 reference and field strains on MDBK cells. The virus titres correspond to the average titres obtained at 24, 48 and 72 h after infection concurrent with cell passage and the infection of a preformed monolayer with 90% confluence. The points represent the least-square means corresponding to 36 replicates. LA38, Cooper, N569 and A663 are BoHV-1 and BoHV-5 reference strains and 09/210 and 97/613 are BoHV-1 and BoHV-5 field isolates, respectively.

Table 3
Least-square means (LSM) and LSM of $\log_{10} \pm$ standard errors (SE) of the size of lysis and infection plaques produced by BoHV-1 and BoHV-5 reference strains and field isolates on MDBK cells at 24 hpi.

Variable		Virus					
		BoHV-1	BoHV-1	BoHV-1	BoHV-5	BoHV-5	BoHV-5
		LA38	Cooper	09/210	N569	A663	97/613
Lysis plaque	LSM	22,803.20(1)ab	22,679.89 ab	22,456.43 ac	31,790.79 b	9,226.35 c	61,403.43 d
	LSM $\log_{10} \pm$ SE	4.06 ± 0.27	4.05 ± 0.27	3.93 ± 0.27	4.15 ± 0.27	3.79 ± 0.27	4.42 ± 0.27
Infection plaque	LSM	221,145.45A	105,370.40B	104,572.31 C	79,343.71 D	30,396.29 E	103,723.6 C
	LSM $\log_{10} \pm$ SE	5.14 ± 0.17	4.92 ± 0.17	4.78 ± 0.17	4.63 ± 0.17	4.36 ± 0.17	4.77 ± 0.17

(1) LSMs are expressed in pixels. Lower case letters (a, b, c) indicate comparisons between the mean size of lysis plaques ($p > 0.05$) and uppercase letters (A, B, C) indicate comparisons between the mean size of infection plaques ($p > 0.05$). LA38, Cooper, N569 and A663 are BoHV-1 and BoHV-5 reference strains and 09/210 and 97/613 are BoHV-1 and BoHV-5 field isolates, respectively.

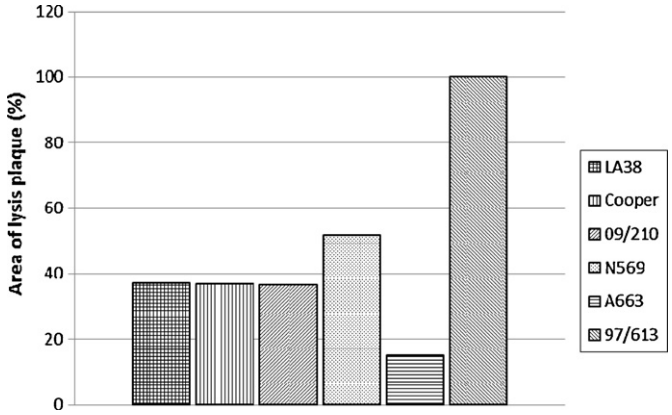

Fig. 2. Size of lysis plaques produced on MDBK cells by the BoHV-1 reference strains (LA38 and Cooper), BoHV-1 field isolate (09/210), BoHV-5 reference strains (N569 and A663) and the BoHV-5 field isolate (97/613). The relative sizes of lysis plaques were calculated and compared to those of strain 97/613. The sizes of the lysis plaques of 97/613 were set at 100%.

The infection plaques for LA38 were significantly larger ($p > 0.05$) than the infection plaques of the remaining strains. Significant differences were not detected ($p > 0.05$) between the size of the lysis plaques of the BoHV-1 and BoHV-5 field isolates (09/210 and 97/613 strains). However, the plaque sizes of these strains and other viruses were significantly different ($p > 0.05$). Values for LA38 were set at 100% and the infection plaques formed by the other strains were expressed relative to this value (Fig. 3). The infection plaques for strains Cooper, 09/210, 97/613, N569 and A663 were 47.6, 47.3, 46.4, 35.9 and 13.7%, respectively, when compared to the mean infection area of the BoHV-1 reference strain.

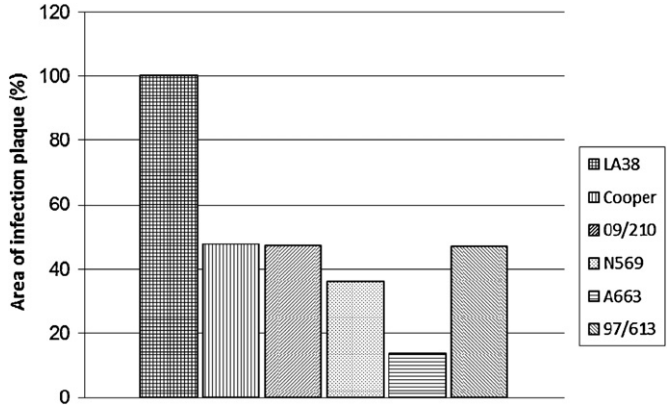

Fig. 3. Relative size of the infection plaques produced on MDBK cells by the BoHV-1 reference strains (LA38 and Cooper), BoHV-1 field isolate (09/210), BoHV-5 reference strains (N569 and A663) and the BoHV-5 field isolate (97/613). The mean area of the infection plaques produced by LA38 was set at 100%.

The virus titres recorded at a MOI of 0.05 for LA38, Cooper, N569 and A663 and the field isolates 09/210 and 97/613 at 24 hpi on MDBK cells were 4.9, 5.1, 5.5, 3.6, 3.5 and 4.8, respectively.

4. Discussion

Bovine herpesvirus (BoHV) can be propagated in primary cell cultures or in established cell lines. Although primary cell culture is the best culture system available for supporting the replication of a large number of viruses, it is expensive and difficult to obtain. Therefore, cell lines, which are easier to manipulate, are commonly used for virus isolation and multiplication (Bleotu et al., 2006). Some alphaherpesviruses, such as pseudorabies virus (PrV) and herpes simplex virus (HSV), are able to grow in a wide range of cell types, while others, like BoHV-1, have a relatively narrow *in vitro* host range. Ladelfa et al. (2006) demonstrated that several cell lines routinely used in the diagnostic laboratory can support the growth of the Argentinean BoHV-5 strain, A663.

The association between the stages of cell multiplication and the efficacy of replication of different isolates of bovine viral diarrhoea virus (BVDV) has been studied previously (Odeón et al., 2009). According to the results obtained in the present work, it is clear that there is a relationship between the replication of BoHV-1 and 5 and the multiplication stage of MDBK cells. When the kinetics of viral replication were evaluated, it became noticeably that higher virus titres were obtained when viral infection of the cell culture occurred simultaneously to cell passage, mainly when virus titres were measured at early time points (24 and 48 hpi). However, the time point at which a virus reached its highest titre did not change with the stage of the cell cycle at the time of infection. Furthermore, the efficiency of BoHV replication at 72 hpi was the same when the infection of the cell culture was performed at the beginning or at the end of the logarithmic growth phase. This issue is particularly relevant for research purposes, as well as for industry, since obtaining the maximum potential of virus replication is critical for several procedures, for example the optimization of vaccine production. In contrast, the highest viral yield obtained with the reference strains BoHV-1 and 5 might reflect an adaptation to *in vitro* culture conditions.

Ladelfa et al. (2006) analysed the replication kinetics for the BoHV-5 strains A663 and N569. Both strains reached similar final virus titres when evaluated at the MOI of 0.1 and 5. According to this finding, it was shown that the replication kinetics of LA38, N569 and A663 had similar profiles (Ladelfa et al., 2008), with virus titres of 6.7, 6.8 and 6.7, respectively, at MOI 5. At MOI 0.1 the virus titres were 7.4, 7 and 7.2 for LA38, N569 and A663, respectively. The highest virus titres were obtained at 18, 24 and 12 hpi at MOI 5 and 48, 36 and 48 hpi at MOI 0.1 for LA38, N569 and A663, respectively. Although A663 and N569 have similar growth

kinetics, significant differences between the extracellular fraction titres were observed at a low MOI, whereas the behavioural pattern of the strain LA38 was similar to that of N569. The results obtained by these authors, who analysed BoHV reference strains under similar experimental conditions, are comparable to the data obtained in the present study in which both, reference and field isolates, were evaluated. In addition, similar patterns in the replication kinetics of the different BoHV-1 and 5 strains were observed in this study. This was clearly evident for LA38, Cooper, N569 and 97/613. Although strain A663 has a replication kinetics profile similar to these strains, it showed lower titres at all time points analysed. Similarly, low viral titres were recorded for the field isolate 09/210 (BoHV-1), at all times evaluated in this study. Therefore, our results confirm previous findings by Ladelfa et al. (2008) on BoHV reference strains and additional information on the replication of circulating virus field isolates is provided. Thus, it is particularly important to remark that data obtained from studies with reference isolates are not representative of the viral behavior under field conditions.

Brum et al. (2010) evaluated the replication kinetics of a Brazilian BoHV-5 strain (SV507/99) isolated from an outbreak of meningoencephalitis in cattle. The strain was analysed on CRIB cells (a cell line derived from MDBK cells, which is resistant to BVDV infection). They showed that the virus titre for this strain started increasing from 4 hpi and continued rising until 32 hpi, the maximum time point evaluated. These data are in agreement with the results obtained in this study at 24 hpi. Determining the time point after infection at which a viral strain has its maximal *in vitro* replication potential is critical when designing *in vitro* studies. *In vitro* replication cannot always be correlated with *in vivo* viral behaviour. Nevertheless, characterizing the *in vitro* replication kinetics of a specific viral strain is an essential start point in the planning of experimental animal inoculations.

For a complete study of the parameters related to the *in vitro* replication of several BoHV strains, the size of the lysis and infection plaques after the infection of MDBK monolayers was also analysed. A MOI of 0.05 was used in this study since higher MOI caused widespread effect on the cell monolayer making plaque evaluation difficult to assess after 24 hpi. It was demonstrated that A663 and the BoHV-1 09/210 have a low lytic potential, which is in agreement with the results of viral replication kinetics. Thus, it is evident that the release of viral particles by strains A663 and 09/210 to the extracellular medium is less efficient compared to the remaining isolates. In contrast, the BoHV-5 field strain 97/613 showed the highest lytic activity. The lytic ability of a virus can be reflected in the damage caused to the host tissues and, as a consequence, it can be related to the development of clinical signs in the animal (Ladelfa, 2010). In agreement with this observation, Pérez et al. (2002) only detected necrosis of neural tissue in animals with neurological signs after experimental inoculation with the BoHV-5a field strain 97/613. This was attributed to the damage caused by viral replication in the central nervous system. Thus, the *in vitro* and *in vivo* replication capability of a viral strain may be directly correlated with the severity of the disease they are able to cause. Similarly, in a recent work, Lobanov et al. (2010) reported that a BoHV-1 U_L47 deletion mutant exhibited a reduced plaque size and more than a 100-fold decrease in the virus titre in cultured cells when compared to the wild-type strain. The product of this gene was required for *in vivo* BoHV-1 replication, since no clinical manifestations or viral shedding were detected in calves infected with this mutant. Therefore, the *in vitro* replication features of a virus might have *in vivo* relevance. As a consequence, it is evident that the *in vitro* characterization of a viral strain is essential when aspects of herpesvirus pathogenesis are being evaluated. In the present report, according to the data obtained from the infection plaques, BoHV-1 LA38 had the highest capacity for cell-to-cell spreading. This observation might be related to a broader potential for the *in vivo* dissemination of this virus.

In a previous work, Marin et al. (2010) studied the effects of acute infection by LA38 in two calves that were euthanized 7 days post-infection. The virus was successfully isolated from all of the encephalic areas analysed (medulla oblongata, brain and cerebellum) and the trigeminal ganglion, showing that the neuroinvasive potential of BoHV-1 is frequently underestimated. It is also evident that neuroinvasiveness is dependent on the properties inherent to the strain, as previously suggested by other authors (Piedrahita et al., 2010; Rissi et al., 2008; Silva et al., 2007).

This study provides significant information about the *in vitro* replication and behaviour of the reference strains and field isolates of BoHV-1 and BoHV-5. These findings are relevant since available information on the *in vitro* growth characteristics of these viral strains is scarce. The results from this study may also be useful for establishing comparisons with other related viruses. Besides this, these results constitute an important tool for future *in vitro* experiments. They also provide additional information that should be considered when designing experimental animal inoculations. Further studies will be necessary to determine the *in vitro* behaviour of a broader range of wild type and reference bovine alphaherpesvirus strains on different cell lines and at different MOI.

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