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# Comparative analysis of replicative properties of phylogenetically divergent, Argentinean BoHV-4 strains in cell lines from different origins

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Highlights

- The six Argentinean BoHV4 strains analyzed infect cells of human origin
- The lytic capacity of each strain has not direct association with viral titers
- The dynamics of replication is independent of the genetic classification
- The biological behavior would be mainly conditioned by the nature of the cells

**ABSTRACT**

Bovine gammaherpesvirus 4 (BoHV4) is a member of the family *Herpesviridae*. In Argentina, BoHV4 was isolated and characterized in 2007 from samples of aborted cows. Argentinean isolates are highly divergent and are classified as: Genotype 1(Movar-like), Genotype 2 (DN599-like) and Genotype 3 (a novel group). The aim of this study was to comparatively evaluate the biological characteristics of six Argentinean BoHV4 field isolates in cell lines from different origins. All strains induced productive infection in the cell lines used, with different degrees of permissiveness. A direct relationship among the times of appearance of cytopathic effect, the growth kinetics, the size of the lysis plaques and the virulent-like behaviour *in vitro* could not be established. However, although slight, there are differences in the biological behaviour of the BoHV4 fields isolates analyzed. This variability is independent of their genetic classification but would be conditioned by the nature of the infected cells.

*Keywords:* BoHV4, Replicative properties, *In vitro* characterization, Argentinean field isolates

## 1. Introduction

Bovine gammaherpesvirus type 4 (BoHV4) is a member of the family *Herpesviridae* (order *Herpesvirales*), subfamily *Gammaherpesvirinae*. Initially considered as a cytomegalovirus, it was later classified within the genus *Rhadinovirus* according to the activity of the thymidine kinase and to genomic similarities with other viruses of this subgroup, as saimiriine herpesvirus 2

(SaHV2) [1], human herpesvirus 8 (HHV8 or Kaposi's sarcoma virus) and murid herpesvirus 4 (MuHV4).

BoHV4 has been isolated from cattle with respiratory infections, vulvovaginitis, mastitis, abortions, endometritis and apparently healthy animals throughout the world [2-8]. This virus has not yet been established as causal agent of a particular disease entity. However, it is primarily associated with reproductive disorders of cattle, particularly in the postpartum period [9].
Unlike other gammaherpesviruses, BoHV4 can infect a wide range of domestic and wild ruminant species [10-16]. Likewise, this virus infects a variety of cells, including cell lines and primary cell cultures of bovine, ovine, caprine, canine, feline, porcine, rabbit and chicken, as well as several human cell lines [12, 17-19]. Furthermore, it has been shown that human cell lines from lymphoid and myeloid origins were resistant to BoHV4, and epithelial and carcinoma cells were sensitive [20].

It has been previously reported that BoHV4 presents genomic variations. By genetic studies of American (DN599 and 66-p-347) and European (V test, Movar and LVR140) reference strains groups, significant differences were identified in at least nine open reading frames (ORFs) [10, 21, 22], including the presence or absence of sites for EcoRI and BamHI restriction enzymes and variations in the length of polyrepetitive DNA sequences (prDNA) [23]. In addition, variability among isolates from different regions has been observed, as well as emerging new strains, which restriction patterns do not fully match to the previously mentioned prototypes. This variability would be the result of recombination events among strains and within the same strain, mainly in prDNA sequences located on both sides of the long unique region (LUR) of the genome, during viral replication [7, 24-26].

In Argentina, BoHV4 was isolated and characterized in 2007 from vaginal discharge samples of aborted cows [27]. Presently, more than 70 isolates have been identified in our country, mainly associated with abortions in bovine females. Genomic analyses of these Argentinean strains demonstrated that they are highly divergent and can be classified in three different groups: Genotype 1 comprises Movar-like strains (European group), Genotype 2 includes DN599-like strains (American group) and Genotype 3 corresponds to a novel genotype group [26]. Thereafter, the virus was isolated from nasal swabs, brain tissue, granulosa cells, oocytes [28] and bovine semen [29, 30].

Determining the kinetics of viral replication is relevant for the *in vitro* characterization of viruses. Although information on the susceptibility of several cell lines to the infection by BoHV4 is available [20], the *in vitro* behaviour of some Argentinean strains has been only analyzed on one cell lines from bovine origin [35]. Therefore, the aim of the present study was to comparatively evaluate the biological characteristics of six Argentinean BoHV4 field isolates in four cell lines from different origins.

Understanding the *in vitro* biological behaviour of these strains will contribute to characterize the BoHV4 field isolates in Argentina, and to improve the diagnosis of this virus from clinical specimens.

## 2. Materials and methods

### *2.1 Cell lines and culture conditions*

A bovine (Madin-Darby Bovine Kidney, MDBK, [CCL-22]), two human (HeLa [CCL-2] and Hep2 [CCL-23]) and one simian (Vero [CCL-81]) cell lines from the American Type

Culture collection (ATCC, Rockville, MD, USA) were used in this study. The cell cultures were propagated in Minimum Essential Medium (MEM, Sigma–Aldrich), supplemented with 10% inactivated and gamma irradiated fetal bovine serum (FBS) (Natocor - Córdoba, Argentina) free from viruses and antibodies, and with antibiotic solution including 100 µg/l penicillin (penicillin G sodium salt, Sigma-Aldrich) and 200 µg/l streptomycin (streptomycin sulphate, Sigma-Aldrich). Cells were incubated at 37 ◦C in a 5% $CO_2$ atmosphere.

*2.2 Virus strains*

Six BoHV4 strains, previously described by [26] were used in this study: 09/508 (genotype 1), 08/330, 08/263, 12/365 (genotype 2), 07/435 and 09/227 (genotype 3).

All virus stocks were propagated in MDBK cells, in T-25 flasks (Greiner Bio-One, Germany) ($2 \times 10^5$ cells/ml), for 48 h. Supernatants were harvested and frozen at −80 ◦C. Virus titers were determined by the endpoint titration method on MDBK cells in 96-well microtiter plates (Greiner Bio-One, Germany) and expressed as $\log_{10}$ $TCID_{50}$/ml [31].

*2.3 Cell cultures infection and viral quantitation*

Cell cultures were grown in 24-well plates (Greiner Bio-One, Germany) at a concentration of $2 \times 10^5$ cell/ml and infected with BoHV-4 strains at a multiplicity of infection (MOI) of 0.1. Virus was adsorbed for 1 h in the absence of serum, then monolayers were washed and fresh media (MEM, 10% FBS) was added. Virus replication was evaluated after infection of a preformed, confluent monolayer with 24 h of growth. Cells were incubated at 37 ◦C with 5% $CO_2$ and observed daily for the presence of cytopathic effect (CPE). The supernatants were harvested at 24, 48, 72, 96 and 120 hours post-infection (hpi) and frozen at −80 ◦C for further viral quantitation of extracellular

fractions. At the time of supernatant collection, the evidence of CPE was recorded; the cells were harvested with trypsin-EDTA and stored at −80 ˚C for subsequent DNA extraction and viral genome detection. Virus titers were determined as previously described, in 96-well plates (Greiner Bio-One, Germany). All assays were performed by triplicate. Appropriate negative controls (mock-infected cells) were included in each experiment.

*2.4 Detection of BoHV4 DNA by nested PCR*

BoHV4-infected- and mock-infected cells were harvested and DNA was extracted by the phenol-chloroform method. DNA concentration was determined by spectrophotometry in a Nano Drop 2000 (Thermo Scientific - USA) at an absorbance of 260 nm.

The presence of BoHV4 DNA was evaluated by a nested PCR assay adapted from [33] targeting ORF 25 (major capside protein) of the viral genome. The PCR amplifications were carried out in 25 µl reaction mixture containing 1 µl of 5 mM deoxynucleotide triphosphates (Promega), 1µl of 15 pmol primers, 1.25 µl of 50 mM $MgCl_2$ ( PB-L, Argentina); 0.25 µl of 5U/µl *Taq* DNA polymerase (PB-L, Argentina), 2.5 µl of 10 x buffer (PB-L, Argentina) and water. For the second amplification round, 2 µl of the first round PCR product were used. The primers used for the amplifications were BoG3 5'GACTATGAGGAATGGCACAAG 3' and BoGe 5'TACTCGTAGGCTGGGTCTGG3' in the first round, and B4up 5'GGTTGGAAGTGAGCGTATGAT3' and B4 low 5'GTAGGCGGGGTCTGGAAT 3' in the second (Invitrogen). The PCR products were 737 bp and 271 bp for the first and the second round, respectively. The amplifications were carried out in a Veriti Thermal Cycler (Applied Biosystem - USA) as follows: first round consisted of 94 ˚C 15 min; [94˚C 45 s; 56˚C 45 s; 72˚C 90 s] for 25

cycles. The second amplification round was [94 ˚C 45 s; 56˚C 45 s; 72˚C 60 s] for 30 cycles and one extension cycle at 72˚C for 10 min.

As negative control DNA from each mock-infected cell line was used. DNA from the cell lines infected with each viral strain was used as positive control. The PCR products were separated by electrophoresis in 1.2% agarose gels stained with SYBR® Safe DNA gel stain (Thermofisher, Argentina) and visualized under ultraviolet light.

*2.5 Lysis plaques assay*

Cell cultures were grown in triplicate in 6-well plates (Greiner Bio-One, Germany) at a concentration of $2 \times 10^5$ cell/ml, infected with the BoHV4 strains at an MOI of 0.1 and incubated for 1 h. The virus inoculum was removed and 2 ml of the overlay medium (0.6% low-melting point agarose, MEM and 10% FBS) pre-warmed at 60 ºC, was added to each well. The infected- and mock-infected- cell monolayers were incubated at 37 ºC in 5% $CO_2$ for 72 h. After removing the solidified overlay, the monolayers were fixed and stained with 1% crystal violet solution and recorded. Then, 10 plaques per well were randomly selected and measured. The average size of the plaques (mm) was calculated and data were statistically analyzed.

*2.6 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 3 replicates. The measurements were repeated at the previously indicated time-points. A comparison of least-square means was carried out using Tukey–Kramer's test. The MIXED procedure of SAS was used. The GLM procedure of SAS was used to analyze the variance [32].

## 3. Results

### *3.1 Kinetics of replication of Argentinean BoHV4 field isolates*

As a first step to determine if there are differences in virus infectivity among the six BoHV4 strains, virus titers in cell cultures were evaluated at different time-points (Table 1).

In general, the analysis of the kinetics of growth of BoHV4 strains in cell cultures of different origin revealed that strain 08/330 replicated better in all cell lines. Virus titers were significantly higher ($p \leq 0.05$) (5 to 9 log) for 08/330 strain when compared to the remaining strains, with the highest titers detected at 48 hpi (Fig. 1b). Growth kinetics was highly variable for the other five strains in Hela and Hep2 cells when compared to their replication in MDBK and Vero cell lines. It was observed a greater homogeneity in the growth kinetics of 09/508 and 08/330 strains in the four cell lines, maintaining virus titers ranging from 2 to 4.5 and 5 to 9 log, respectively (Figs. 1a-b). Virus production in the extracellular fraction was not detected at 24 hpi for strain 12/365 in any of the cell lines (Fig. 1d). Similar results were obtained for strains 08/263 and 07/435 in Vero and HeLa cells, and for 09/227 in HeLa cells (Figs. 2c-d).

In MDBK cells, strains 12/365 and 09/227 showed similar dynamics of replication, reaching the highest titers at 96 hpi. Strains 07/435 and 08/263 displayed the same growth kinetics, with the highest titers detected between 48 and 72 hpi. Strain 09/508 showed a constant decline from 24 hpi to 120 hpi (Fig. 2a).

In Hep2 cells, average titers for all strains were 4 to 4.5 log, with non-significant differences detected for strains 09/508, 08/263 and 07/435 ($p > 0.05$). For strains 12/365 and 09/227, viral titers in this cell line were significantly lower (2 to 3 log) with respect to the other strains ($p < 0.05$) (Fig. 2b).

Viral titers in Vero cells were 2 to 4 log and there were no significant differences for strains 07/435, 08/263 and 12/365 ($p > 0.05$) and for strains 09/508 and 09/227 ($p > 0.05$) (Fig. 2c).

In HeLa cells, medium values (4 to 4.5 log) were recorded for strains 09/508 and 08/263 at 48 hpi, gradually declining until 120 hpi. The lowest values, 2 to 2.5 log, were observed for strains 07/435, 09/227 and 12/365 (Fig. 2d).

*3.2 Detection of the genome of BoHV4 strains and cytopathic effect in the different cell cultures*

Viral DNA for all strains was detected from 24 hpi in the four cell lines, except for strain 08/330 in MDBK and 09/508 in Hep2 cells. Viral genome was not detected after 72 hpi in HeLa and Vero cells for strains 07/435 and 08/330, respectively.

In MDBK and Hep2 cultures most of the strains produced CPE between 24 and 48 hpi, whereas in HeLa and Vero CPE was evidenced at 72 hpi, except for 09/508 in HeLa and 08/263 in Vero (Table 2).

*3.3 Plaque phenotype of BoHV4 strains*

Plaques of lysis of BoHV4 strains were measured and they were classified according to the size as large (≥ 3 mm), medium (1-3 mm) and minimum (< 1 mm). Large plaques were not evident in any of the cell types. In general, the size of plaques in the infected cell lines varied from 0.4 mm to 1.5 mm (Table 3).

Strains 09/227, 08/263 and 07/435 produced medium-sized plaques in MDBK cells (Fig. 3a) and the size of these plaques was significantly greater ($p <0.05$) with respect to the plaques produced by strains 08/330 and 12/365. On the contrary, these strains produced significantly greater plaques ($p <0.05$) than the remaining four strains in HeLa cells (Fig. 3b).

In Hep2 cells, all the strains produced minimum plaques. In this cell line, strain 09/508 produced significantly smaller plaques ($p < 0.05$) (Fig. 3c). In Vero cells, the six strains produced minute plaques with significant difference ($p < 0.05$) only detected for strains 08/330 and 07/435, which produced minute plaques of larger size than the other strains (Fig. 3d).

Plaque size for strain 09/508 in MDBK and 09/227 in Hep2 cells could not be recorded since virus infection produced a total detachment of the cell monolayer at 72 hpi.

## 4. Discussion

In contrast to other gammaherpesviruses, BoHV4 grows efficiently in different cell cultures [17, 18, 20, 34]. However, on the basis of the information provided by the literature it can be inferred that there is a wide range of differences in the biological properties of BoHV4; coincidently, a significant variation in the kinetics of replication of different BoHV4 local isolates has been previously reported by our research group [35].

In this work, it was evaluated the *in vitro* biological activity of six local field isolates of BoHV4. The study of the dynamics of replication has allowed us to obtain information about the moment at which the extracellular virus is detected and to estimate the probable time in which each strain completes its replicative cycle.

The comparison of the kinetics of replication in each cell line showed that the production and viral detection in the extracellular medium occurred early (24 hpi) for all the strains in MDBK and Hep2 cells, except for strain 12/365. In Vero, extracellular viral detection at 24 hpi was only possible for 50% of the strains (09/508, 08/330 and 09/227), while in HeLa the detection of extracellular virus was not achieved before 48 hpi for 70% of the strains (08/263, 12/365, 09/227

and 07/435). This could indicate that the viral replication cycle would be slower in HeLa and Vero cells than in MDBK and Hep2 for most of the strains.

Direct association was not observed between the time in which each strain completes its cycle of replication, and releases new viral particles to the extracellular medium, with the appearance of CPE. These parameters could only be associated for strain 12/365, since extracellular virions of this strain were not detected before 48 hpi and the CPE was observed at 72 hpi in the four cell lines.

Regarding the susceptibility of the cell lines, the results show that the four lines are susceptible to the infection by strains of different genotype. Nevertheless, different degrees of permissiveness are evidenced depending on the strain.

According to the viral titers obtained in the extracellular fraction, viral production was markedly higher for strain 08/330 in the four cell lines. This data is in agreement with the results of a previous work [35]. Apparently, the four cell lines are highly permissive for strain 08/330, and the lines of human origin showed a variable degree of permissiveness, (intermediate to low), when compared with the five remaining strains. Coincidently, data from the literature indicate that HeLa cells are poorly permissive to the infection by BoHV4 [20].

To study the phenotypic characteristics of these strains a lysis plaque assay was carried out. This is an important parameter for the *in vitro* characterization of viruses since it is a direct measure of the pathogenic potential of a virus, as well as an indirect measure of the greater or lesser release of virions to the extracellular medium and the production of infective viral particles [36].

The lysis plaque assay was performed at a low MOI and using a semi-solid medium. Under these conditions, the release and dissemination by adsorption of the virions to the neighboring

cells, are difficult. Therefore, this technique allows the evaluation of another viral dissemination mechanism, such as cell-to-cell virus diffusion.

The observation and measurement of the lysis plaques produced by these strains in the four cell cultures showed wide variability, mainly among the cell lines. The lytic activity of the strains was lower in Hep2 and Vero cells, in which all strains produced lysis plaques smaller than 1 mm. In MDBK cells, the largest plaque size and the greatest variability among the strains was observed; while in Hela cells the behaviour of the strains was opposite to that observed in MDBK cells. The data obtained by this assay verify that there is not a direct association between the viral titers detected and the lytic capacity of each strain.

The six Argentinean BoHV4 strains analyzed had the capacity to induce productive infection in the cell lines used in this assay. There were differences in the times of appearance of CPE among the strains in relation to the cell cultures. However, it was not possible to establish a direct relationship among this parameter, the growth curves (viral titers), the size of the lysis plaques and the virulent-like behaviour *in vitro*, unlike characterization studies made with field isolates of other bovine herpesviruses [36-38]. Therefore, although viral isolation in cell culture is the gold standard method for diagnosis of a virus infection, the variability in the rate of replication and the presentation of CPE of the different viral strains could make this methodology difficult to perform. Then, the use of highly sensitive techniques, such as nested PCR, provides greater efficacy for the detection of the virus.

Because of the aforementioned and considering the importance of BoHV4 in Argentina in relation to reproductive losses, it is necessary to deepen the biological studies of this viral agent; the characterization of the field isolates will give rise to fundamental knowledge about the molecular aspects of the virus. The high genetic variability among the strains isolated in Argentina

highlights the significance of studying the role of this virus. Furthermore, although the genomic characterization of these strains is currently being expanded, in previous studies, synonymous and non-synonymous substitutions have been observed in the gB, gH and Tk genes of the strains analyzed in this work [26, 35].

## 5. Conclusions

The findings of this study suggest that, although slight, there are differences in the behavioural patterns of the BoHV4 strains used in this *in vitro* study and this variability in the dynamics of replication and biological behaviour is independent of their genetic classification but would be conditioned mainly by the nature of the infected cells. These findings provide an initial step to understand the biology of the Argentinean isolates of BoHV4.

Considering that BoHV4 has been detected, as an emerging and concomitant agent in reproductive infections of cattle in our country, characterizing the *in vitro* replication kinetics and determining the time point after infection at which a viral strain has its maximal potential of replication is a relevant fact to establish differences among the behaviour of BoHV4 isolates in cell cultures, since it might contribute to the understanding of the pathogenic role of each strain in their natural host.

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## Ethical approval

This article does not contain any studies with human participants or animals performed by any of the of the authors.

**Conflict of interest statement**

The authors declare no potential conflicts of interest with respect to the research, authorship, publication of this article and/or financial or personal relationships that could inappropriately influence this work.

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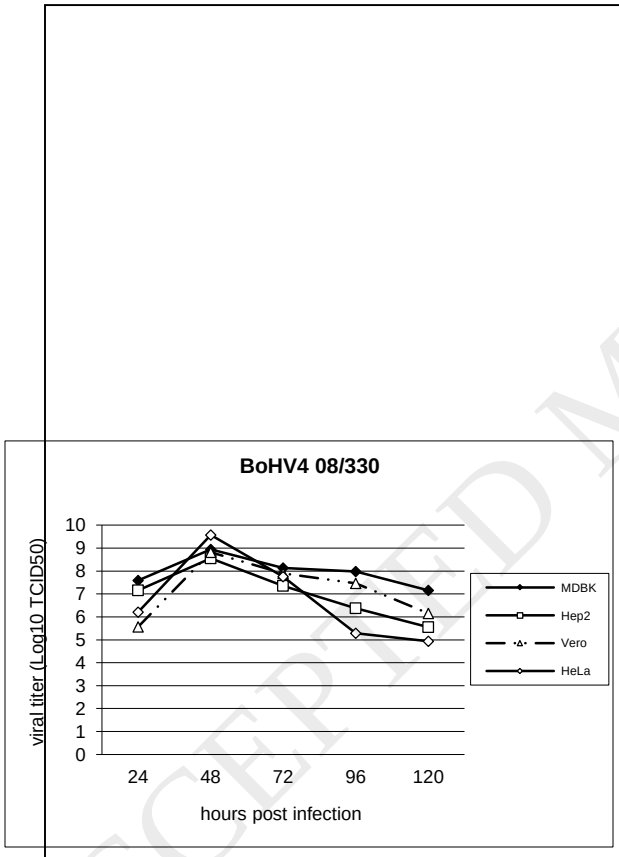
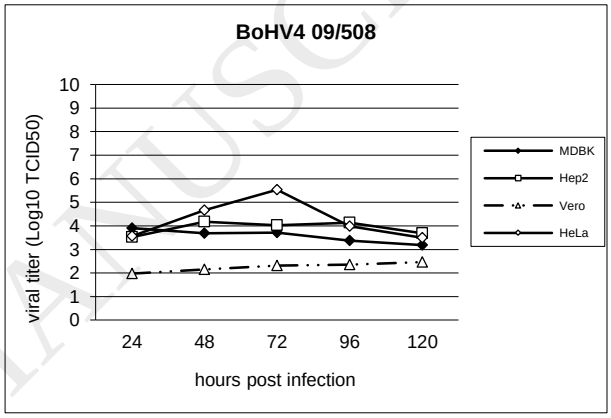
**Figure legends**

**Fig. 1** Growth kinetics of six Argentinean BoHV4 field isolates on MDBK, Hep2,Vero and HeLa cells. Average virus titers ($log_{10}$ $TCID_{50}$), of each strain in the four challenged cell lines, obtained at 24, 48, 72, 96 and 120 h after infection




a

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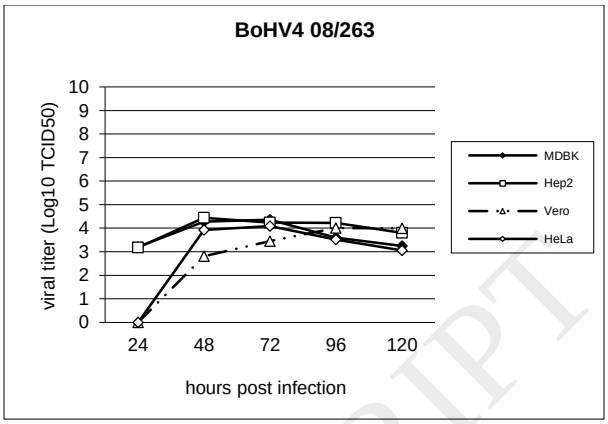


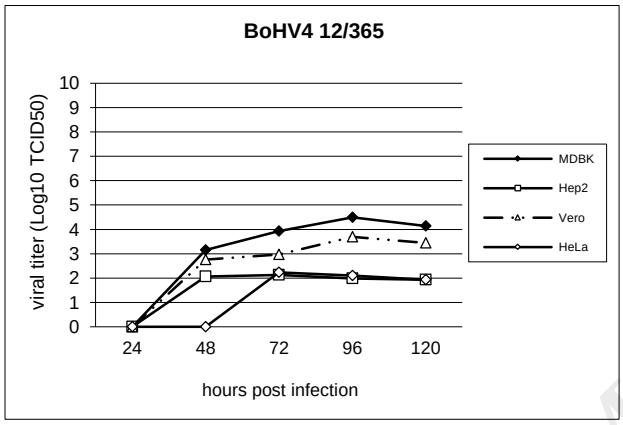


c

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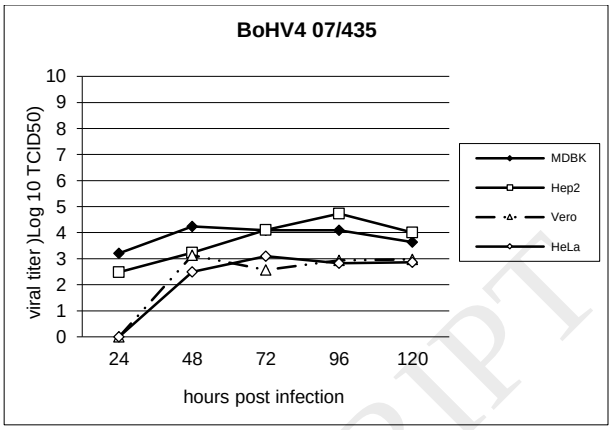


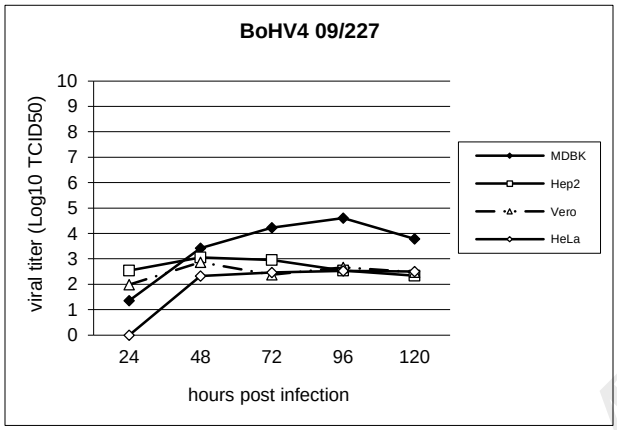


e f

**Fig. 1**

**Fig. 2** Growth kinetics of six Argentinean BoHV4 field isolates on MDBK, Hep2,Vero and HeLa cells. Average virus titers ($log_{10}$ $TCID_{50}$), of the six strains in each cell line, obtained at 24, 48, 72, 96 and 120 h after infection

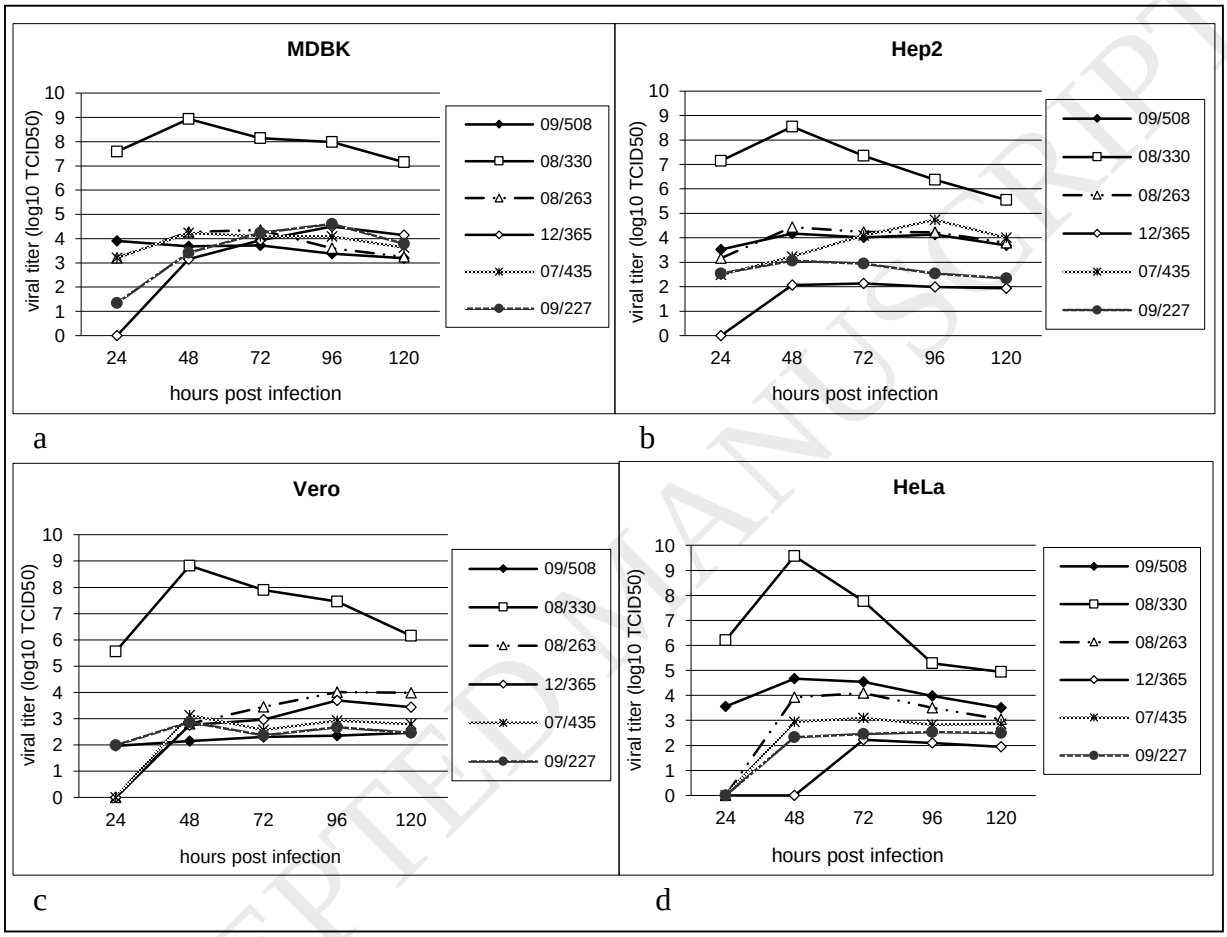


**Fig. 2**

**Fig. 3** Lysis plaque assay for six Argentinean BoHV4 field isolates on (a) MDBK, (b) HeLa, (c) Hep2 and (d) Vero cells 72h after infection. Different uppercase letters indicate statistically significant differences ($p \leq 0.05$) between virus strains within each cell line

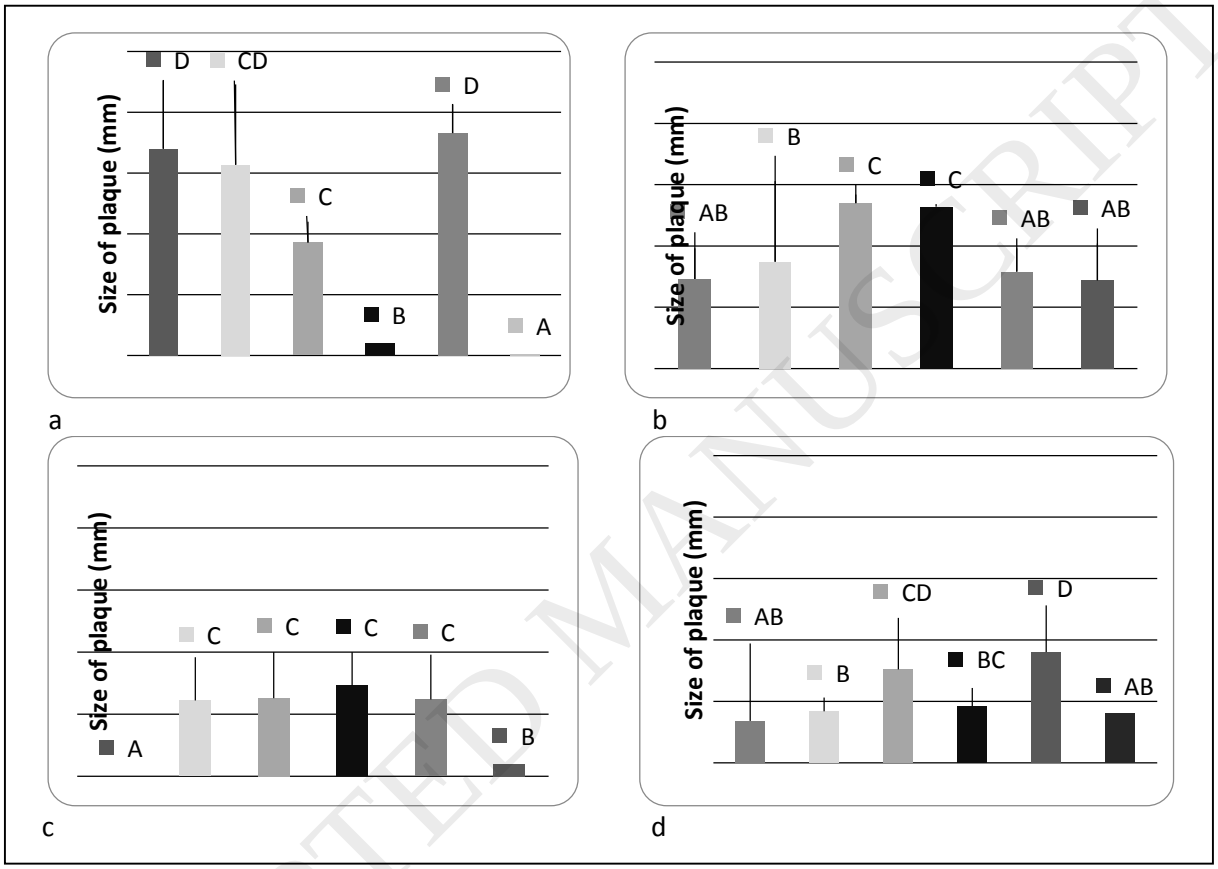


**Fig. 3**

**Table 1.** BoHV4 titers ($log_{10}$ $TCID_{50}$/ml) at 24, 48, 72, 96 and 120 hpi on MDBK, Hep2, Vero and HeLa cell lines. The values correspond to the average of three replicates. Means and standard errors are shown. Upper case letters (A, B, C, D) indicate significant differences among strains at each time ($p < 0.05$).

| Strain | Cell line | hpi 24h | 48h | 72h | 96h | 120h |
|---|---|---|---|---|---|---|
| **09/508** | **MDBK** | 3.90 (+ 0.27)A | 3.68 (+ 0.47)B | 3.72 (+ 0.54)B | 3.38 (+ 0.46)B | 3.19 (+ 0.45)AB |
| | **Hep2** | 3.52 (+ 0.19)A | 4.17 (+ 0.64)AB | 4.02 (+ 0.51)AB | 4.13 (+ 0.36)A | 3.69 (+ 0.29)A |
| | **Vero** | 1.97 (+ 0.06)B | 2.15 (+ 0.08)C | 2.31 (+ 0.19)C | 2.35 (+ 0.48)C | 2.46 (+ 0.25)B |
| | **Hela** | 3.56 (+ 0.35)A | 4.67 (+ 0.15)A | 4.54 (+ 0.07)A | 3.98 (+ 0.37)AB | 3.50 (+ 0.36)A |
| **08/330** | **MDBK** | 7.59 (+ 0.77)A | 8.94 (+ 0.33)AB | 8.14 (+ 0.53)A | 7.98 (+ 0.06)A | 7.16(+ 0.27)A |
| | **Hep2** | 7.15 (+ 0.68)A | 8.55 (+ 0.44)B | 7.35 (+ 0.32)B | 6.37(+ 0.59)B | 5.55 (+ 0.61)BC |
| | **Vero** | 5.56 (+ 0.44)B | 8.82 (+ 0.54)B | 7.90 (+ 0.70)AB | 7.46 (+ 0.55)A | 6.15 (+ 0.44)B |
| | **HeLa** | 6.21 (+ 1.29)B | 9.57 (+ 1.67)A | 7.76 (+ 1.16)AB | 5.29 (+ 0.51)C | 4.94 (+ 0.68)C |
| **08/263** | **MDBK** | 3.21 (+ 0.47)A | 4.28 (+ 0.47)A | 4.36 (+ 0.20)A | 3.60 (+ 0.09)A | 3.25 (+ 0.39)AB |
| | **Hep2** | 3.17 (+ 0.10)A | 4.44 (+ 0.65)A | 4.24 (+ 0.40)A | 4.23 (+ 0.30)A | 3.80 (+ 0.17)A |
| | **Vero** | 0.00 (+ 0.00)B | 2.81 (+ 0.24)B | 3.45 (+ 0.32)B | 4.01 (+ 0.13)A | 3.99 (+ 0.00)AB |
| | **HeLa** | 0.00 (+ 0.00)B | 3.93 (+ 0.31)A | 4.09 (+ 0.36)AB | 3.51 (+ 0.48)A | 3.05 (+ 0.31)B |
| **12/365** | **MDBK** | 0.00 (+0.00)A | 3.16 (+ 0.66)A | 3.93 (+ 0.18)A | 4.50 (+ 0.33)A | 4.14 (+ 0.42)A |
| | **Hep2** | 0.00 (+ 0.00)A | 2.07 (+ 0.98)B | 2.13 (+ 0.14)C | 1.99 (+ 0.05)C | 1.94 (+ 0.01)B |
| | **Vero** | 0.00 (+ 0.00)A | 2.76 (+ 0.78)AB | 2.97 (+ 1.05)B | 3.69 (+ 0.99)B | 3.44 (+ 0.29)A |
| | **HeLa** | 0.00 (+ 0.00)A | 0.00 (+ 0.00)C | 2.23 (+ 0.24)C | 2.10 (+ 0.17)C | 1.94 (+ 0.06)B |
| **07/435** | **MDBK** | 3.21 (+ 0.33)A | 4.24 (+ 0.33)A | 4.09 (+ 0.18)A | 4.09 (+ 0.44)A | 3.63 (+ 0.15)A |
| | **Hep2** | 2.48 (+ 0.50)B | 3.23 (+ 0.40)B | 4.10 (+ 0.53)A | 4.73 (+ 0.13)A | 4.00 (+ 0.10)A |
| | **Vero** | 0.00 (+ 0.00)C | 3.13 (+ 0.35)B | 2.57 (+ 0.20)B | 2.93 (+ 0.19)B | 2.79 (+ 0.45)B |
| | **HeLa** | 0.00 (+ 0.00)C | 2.49 (+ 0.17)B | 3.09 (+ 0.18)B | 2.83 (+ 0.07)B | 2.86 (+ 0.14)B |
| **09/227** | **MDBK** | 1.35 (+ 1.17)B | 3.42 (+ 0.51)A | 4.23 (+ 0.45)A | 4.60 (+ 0.45)A | 3.79 (+ 0.14)A |
| | **Hep2** | 2.54 (+ 0.50)A | 3.06 (+ 0.05)A | 2.95 (+ 0.15)B | 2.54 (+ 0.50)B | 2.34 (+ 0.41)B |
| | **Vero** | 1.99 (+ 0.051)AB | 2.87 (+ 0.58)A | 2.37 (+ 0.26)B | 2.67 (+ 0.67)B | 2.47 (+ 0.40)B |
| | **HeLa** | 0.00 (+ 0.00)C | 2.33 (+ 0.30)D | 2.46 (+ 0.37)B | 2.53 (+ 0.30)B | 2.50 (+ 0.19)B |

**Table 2.** Appearance of CPE in MDBK, HeLa, Hep and Vero cell lines according to the time after infection (hpi) with Argentinean BoHV4 field isolates.

| Cell line | hpi | Strain 09/508 | 08/330 | 08/263 | 12/365 | 07/435 | 09/227 |
|---|---|---|---|---|---|---|---|
| **MDBK** | 24 hpi | + | | + | | + | |
| | 48 hpi | | + | | | | + |
| | 72 hpi | | | | + | | |
| **HeLa** | 24 hpi | | | | | | |
| | 48 hpi | + | | | | | |
| | 72 hpi | | + | + | + | + | + |
| **Hep2** | 24 hpi | + | | + | | | |
| | 48 hpi | | + | | | + | |
| | 72 hpi | | | | + | | + |
| **Vero** | 24 hpi | | | | | | |
| | 48 hpi | | | + | | | |
| | 72 hpi | + | + | | + | + | + |

**Table 3.** Plaque phenotypes for six Argentinean BoHV4 field isolates on MDBK , HeLa, Hep2 and Vero cells 72h after infection: Large (≥ 3 mm), Medium (1-3 mm), Minute (<1 mm).

| **Cell line** | **Strain** | | | | | |
|---|---|---|---|---|---|---|
| | 09/508 | 08/330 | 08/263 | 12/365 | 07/435 | 09/227 |
| **MDBK** | * | 0.93 + 0.17<br>C | 1.57 + 0.66<br>CD | 0.1 + 0<br>B | 1.83 + 0.24<br>D | 1.70 + 0.56<br>D |
| **HeLa** | 0.72 + 0.3<br>AB | 1.35 + 0.07<br>C | 0.87 + 0.66<br>B | 1.32 + 0.02<br>C | 0.79 + 0.19<br>AB | 0.73 + 0.25<br>AB |
| **Hep2** | 0.1 + 0<br>B | 0.63 + 0.37<br>C | 0.61 + 0.35<br>C | 0.73 + 0.27<br>C | 0.62 + 0.36<br>C | * |
| **Vero** | 0.4 + 0<br>AB | 0.76 + 0.42<br>CD | 0.42 + 0.11<br>B | 0.46 + 0.15<br>BC | 0.9 + 0.38<br>D | 0.34 + 0.63<br>AB |

Values are average mm of the lysis plaque + SD (standard deviation).

Different uppercase letters in the same line indicate statistically significant differences ($p \leq 0.05$) between virus strains within each cell line.

* Detachment of monolayer