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Comparative study on the in vitro replication and genomic variability of Argentinean field isolates of bovine herpesvirus type 4 (BoHV-4)

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Abstract Bovine herpesvirus 4 (BoHV-4) is a gammaherpesvirus, belonging to the Rhadinovirus genus, which is increasingly associated with various problems of the reproductive tract of cattle. In Argentina, analysis of BoHV-4 strains isolated from cervico-vaginal mucus of aborted cows revealed a high genetic divergence among strains, which could be classified in three different groups: Genotype 1 comprises Movar-like strains (European prototype), Genotype 2 includes DN599-like strains (American prototype) and Genotype 3 corresponds to a novel genotype group. Understanding the replication behavior in cell cultures and the molecular characteristics of this pathogen of cattle is critical for the rational design of in vitro experiments. The aim of this work was to quantitatively evaluate the replication properties of different Argentinean BoHV-4 strains and to characterize their phylogenetic relationships. Significant differences were evident among the virus titers of the different BoHV-4 isolates in vitro. The most conserved gene was the major capsid protein (ORF25). The glycoprotein B (gB), glycoprotein H (gH), and thymidine kinsase (TK) genes displayed both synonymous and non-synonymous substitutions, with the highest diversity observed for gB, which displayed amino acid substitutions in 24 out of the 178 positions examined. Strains 09/759, 12/512, and 07/568 presented a deletion encompassing amino acid position 27 to 35, whereas strains 07/435 and 09/227 had a deletion from position 28 to 35. Two strains, 07/435 and 09/227, also displayed the highest divergence compared to the other strains analyzed. This study provides information about the in vitro replication and behavior of nine field isolates of BoHV-4. These findings are relevant since available information on the in vitro growth characteristics of BoHV-4 strains is scarce. The results from this study may also be useful for establishing comparisons with other related viruses.

Keywords Gammaherpesvirus · In vitro replication · Genome variability · Argentinean field strains

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Introduction

Bovine herpesvirus 4 (BoHV-4) belongs to the *Herpesviridae* family, the *Gammaherpesvirinae* subfamily, and the *Rhadinovirus* genus [1]. BoHV-4 has been isolated throughout the world from healthy cattle, as well as from cattle exhibiting a variety of diseases. The biological life cycle of BoHV-4 relies, as for all *Herpesviridae*, on the existence of two types of infection: lytic (or replicative)

and latent infections. The former leads to the production of progeny virions and generally to the lysis of the infected cell. During a lytic infection, the expression of herpesvirus proteins is temporally regulated. The proteins are classified into three classes depending on the order of their synthesis during the infection [2]. These proteins are expressed chronologically as immediate-early (IE), early (E), and late (L) proteins. IE proteins are expressed directly after release of the viral genome from the capsid into the nucleus. Although E protein expression occurs after synthesis of IE proteins, L protein expression depends on the expression of both IE and E proteins and viral DNA synthesis. In comparison, latent infection consists of a dormant state associated with the expression of a limited number of viral genes which might affect the biology of the infected cell. Various stimuli can turn the latent infection into a lytic infection, a phenomenon known as reactivation [3].

The replication of most gammaherpesviruses is restricted to their natural host species. BoHV-4 is one of the few exceptions to this rule. Indeed, it has been shown that BoHV-4 is able to replicate in a broad range of host species both in vivo and in vitro. In addition to cattle, isolates of BoHV-4 have been recovered from other ruminant species such as zebu (*Bos indicus*) [4], American bison (*Bison bison*) [5], African buffalo (*Syncerus caffer*) [6], and sheep [7]. Sporadic isolations were reported from lions, cats, and owl monkeys (*Aotus trivirgatus*) [8, 9]. Experimentally, BoHV-4 was also shown to infect goats [4], guinea pigs, and rabbits [10]. In vitro, BoHV-4 is able to replicate in primary cell cultures or cell lines from various animal species [8–12]. In 2008, [13] reported the isolation of BoHV-4 from cervico-vaginal mucus of aborted cows. Thereafter, the virus was isolated from nasal swabs, brain tissue, granulosa cells, oocytes [14], and bovine semen [15]. In addition, preliminary studies have shown that some human cell lines support BoHV-4 replication, leading to the hypothesis that this virus could represent a risk to human health [12, 16]. The frequent isolation of BoHV-4, alone or in combination with other pathogens, from the genital tract of cows with metritis [17, 18] has suggested a direct or indirect role of the virus in genital disease. In Argentina, analysis of BoHV-4 strains isolated from cervico-vaginal mucus of aborted cows revealed a high genetic divergence among strains, which could be classified in three different groups: Genotype 1 comprises Movar-like strains (European prototype), Genotype 2 includes DN599-like strains (American prototype), and Genotype 3 corresponds to a novel genotype group [19]. 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 infection by BoHV-4 is available [3], the in vitro behavior of this virus has not been thoroughly studied. Understanding the patterns of replication in cell cultures and the molecular characteristics of this pathogen of cattle is critical for the rational design of in vitro experiments and vaccine production.

The aim of the present study was to quantitatively evaluate the replication properties of different Argentinean BoHV-4 strains and to characterize their phylogenetic relationships.

Materials and methods

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. MDBK cells were propagated in Minimum Essential Medium (Eagle), with Earle salts (MEM-E) (Sigma–Aldrich, Saint Louis, MO, USA), supplemented with 10 % fetal bovine serum (Bioser, Buenos Aires, Argentina) free from viruses and antibodies and with antibiotic–antimycotic solution (Gibco, Langley, OK, USA), including 100 U/ml penicillin G, 100 g/ml streptomycin sulfate, and 0.025 g/ml amphotericin B. The cells were incubated at 37 °C in a 5 % CO_2 atmosphere.

Virus strains

Nine BoHV-4 strains, previously described by [19] were used in this study. The strain genotype and the clinical condition of the aborted cows from which the virus was identified in vaginal discharges are detailed in Table 1 and Fig. S1. Virus identification was confirmed by isolation in cell culture followed by direct immunofluorescence using a monoclonal antibody against BoHV-4 (FITC anti-BoHV-4 monoclonal antibody, Bio-X Diagnostics S.P.R.L.U) and by nested PCR, as previously described [19], using primers that amplify the thymidine kinase (TK) gene of BoHV-4.

Viral stocks were propagated in MDBK cells, in T-25 flasks (Greiner Bio-one, Frickenhausen, Germany) (0.5×10^6 cells/ml), for 24 h. Supernatants were harvested and frozen at −80 °C. Virus titers were determined by the endpoint titration method and expressed as $TCID_{50}$, according to the method of [20].

Infection and viral quantitation method for determination of the replication kinetics

MDBK cells were grown in 24-well plates (Greiner Bio-one, Frickenhausen, Germany) at a concentration of 0.05×10^6 cell/ml and infected with BoHV-4 strains at a multiplicity of infection (MOI) of 0.1. Virus replication was evaluated after infection of a preformed, more than 90 % confluent monolayer with 24 h of growth, at the end of the logarithmic growth phase. Cells were incubated at 37 °C with 5 % CO_2

Table 1 Cases in which BoHV-4 strains were isolated in vaginal discharges of aborted cows

No.	Isolate	Genotype	Comments
1	07/435	3	Abortion-causing pathogens were not identified. Circulating antibodies to some Leptospira serovars were detected
2	09/227	3	Negative to common agents associated with abortion
3	09/508	1	Isolation of *Trueperella pyogenes*
4	08/330	2	Negative to common agents associated with abortion
5	09/759	2	Abortion attributed to multiple infectious causes. Seropositive to *N. caninum* (titer: 1/800). Non-cytopathic bovine viral diarrhea virus (BVDV-1) was detected in vaginal discharges. Fescue grass parasited with a high percentage of the endophyte fungi *Neotyphodium*
6	07/568	2	Seroconversion to BVDV and animals seropositive to *N. caninum* were detected. *Histophilus somni* was isolated from the vaginal discharge; titers 1/800 to *Leptospira interrogans* serovar Hardjo
7	10/154	2	Antibody titer to *Leptospira interrogans* serov. Hardjo: 1/200. *Trueperella pyogenes* detected in the vaginal discharge. Presence of neutralizing antibodies to BVDV-1 and BoHV-1 and 5
8	12/512	2	Negative to common agents associated with abortion
9	12/365	2	Negative to common agents associated with abortion

and observed daily for the presence of cytopathic effect (CPE). The supernatants were harvested at 24, 48, 72, 96, and 120 h post-infection (hpi) and frozen at −80 °C for further viral quantitation. At the time of supernatant collection, the percentage of CPE was recorded. Virus titers were determined as previously described. Five replicates were performed in order to establish the kinetics of viral replication for each experiment (virus strain and harvest time-points). Appropriate negative controls (mock-infected cells) were included in each experiment.

Extraction of viral DNA and PCR

DNA from the nine BoHV-4 strains was purified from infected cells using the DNeasy Blood & Tissue Kit (Cat. 69504, Qiagen), according to the manufacturer's instructions. DNA concentration was determined by spectrophotometry at an absorbance of 260 nm. The presence of BoHV-4 DNA was evaluated by PCR assays targeting three ORFs of the viral genome: ORF8 glycoprotein B (ORF8) [21], glycoprotein H (ORF22) [3] and the major capside protein (ORF25) [22]. TK gene was evaluated by a nested PCR modified by (19). For ORF25 a nested PCR cited by (22) was performed. The primers used for the amplifications are described in Table 2. DNA from mock-infected MDBK cells was used as negative control. The amplified products were separated by electrophoresis in a 2 % agarose gel, and DNA bands were visualized by SYBR Safe DNA gel stain (Invitrogen).

Phylogenetic analysis

Sequence alignments were obtained with MAFFT using iterative refinement and weighted sum-of-pair scores and consistency score obtained from local alignments [23]. Phylogenetic trees were inferred using the program FastTree [24]. FastTree automatically explores the tree space by setting up to $4 \times \log_2$ (N) rounds of minimum-evolution NNI, two rounds of SPR moves, and up to $2 \times \log$ (N) rounds of maximum-likelihood NNIs, where N is the number of unique sequences in the dataset. FastTree automatically handles positions with gaps. Genotype designations for strains 08/330, 12/512, and 12/365 were determined by TK tree topology following the procedures described in the original paper describing the BoHV-4 genotypes and the therein used reference sequences [19]. The sequences described here were deposited in GenBank under accession numbers. The herein reported nucleotide sequences have been assigned GenBank accession numbers KU180388-KU180423.

Statistical analysis

The experimental design for the 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. The measurements were repeated at the time-points as indicated in Table 3 and Fig. 1. A comparison of least-square means was carried out using Tukey–Kramer's test. The MIXED procedure [25] was used. As the statistical assumptions were not fulfilled, a logarithmic transformation of the data was performed. The GLM procedure [25] was used to analyze the variance.

Results

BoHV4 strains isolated from aborted cows

As described in Table 1, BoHV-4 isolates analyzed in this study were obtained from vaginal discharge samples of aborted adult cows. Fetuses that were recovered after

Table 2 Primers used in this study

Gene	Reference	Orientation	Primer	Fragment (bp)
gH	Gillet et al. [3]	Forward	5′-CCGGGTGAAACAAGTTCCTG-3′	563
		Reverse	5′- GTCAGAGAACATATGATAACATC-3′	
gB	Wellenberg et al. [21]	Forward	5′- CCCTTCTTTACCACCACCTACA-3′	615
		Reverse	5′-TGCCATAGCAGAGAAACAATGA-3′	
TK	Verna et al. [19]	Forward	5′-GTTGGGCGTCCTGTATGGTAGC-3′	576
		Reverse	5′-TGTATGCCCAAAACTTAATATGACCAG-3′	
TK	Verna et al. [19]	Forward	5′-TTGATAGTGCGTTGTTGGGATGTGGT-3′	216
		Reverse	5′-CACTGCCCGGTGGGAAATAGCA-3′	
ORF25	Fábian and Egyed [22]	Forward	5′-GACTATGAGGAATGGCACAAG-3′	337
		Reverse	5′- TACTCGTAGGCTGGGTCTGG-3′	
ORF25	Fábian and Egyed [22]	Forward	5′-GGTTGGAAGTGAGCGTATGAT-3′	271
		Reverse	5′-GTAGGCGGGGTCTGGAAT-3′	

Table 3 BoHV-4 titers ($log_{10}TCID_{50}$/ml) at 24, 48, 72, 96, and 120 hpi on MDBK cells

Virus	Genotype	24 h	48 h	72 h	96 h	120 h
08/330	2	5.28 ± 0.13^{A}	5.79 ± 0.13^{A}	5.31 ± 0.13^{A}	4.78 ± 0.13^{A}	4.13 ± 0.13^{A}
07/435	3	5.07 ± 0.21^{AB}	5.27 ± 0.21^{B}	5.11 ± 0.21^{A}	4.32 ± 0.21^{B}	3.69 ± 0.21^{B}
09/759	2	3.05 ± 0.12^{C}	3.7 ± 0.21^{D}	3.42 ± 0.13^{C}	3.27 ± 0.12^{D}	2.84 ± 0.13^{DEF}
12/512	2	2.17 ± 0.07^{D}	2.71 ± 0.07^{E}	3.22 ± 0.07^{CD}	3.37 ± 0.07^{D}	3.35 ± 0.07^{C}
12/365	2	0.8 ± 0.12^{F}	0.8 ± 0.12^{G}	1.94 ± 0.13^{E}	2.36 ± 0.13^{E}	2.75 ± 0.13^{EFG}
07/568	2	0.8 ± 0.08^{F}	0.8 ± 0.08^{G}	1.89 ± 0.08^{E}	2.21 ± 0.08^{EF}	2.56 ± 0.07^{FG}
10/154	2	0.8 ± 0.02^{F}	0.8 ± 0.19^{G}	1.84 ± 0.02^{E}	2.03 ± 0.02^{F}	3.03 ± 0.02^{D}
09/508	1	0.8 ± 0.02^{F}	0.8 ± 0.02^{G}	$0.8(\pm 0.03^{F}$	1.5 ± 0.03^{G}	$2,5 \pm 0.03^{F}$
09/227	3	0.8 ± 0.12^{F}	0.8 ± 0.12^{G}	0.8 ± 0.12^{F}	0.8 ± 0.12^{H}	2.38 ± 0.12^{G}

The values correspond to the average titers of five replicates. Means and standard errors are shown. Upper case letters ($^{A, B, C, D, E, F, G, H}$) indicate comparisons between the viruses at each time (≤ 0.05)

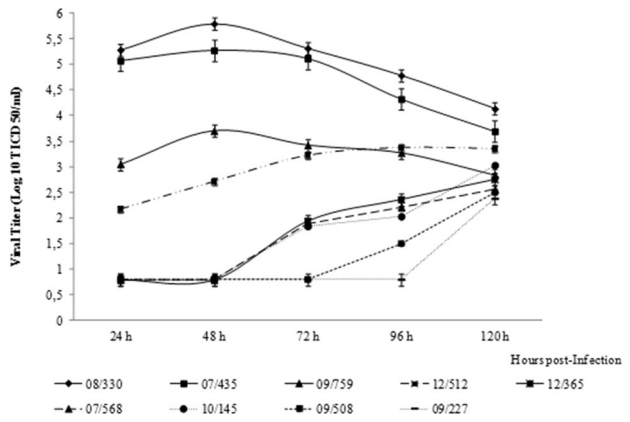

Fig. 1 Growth kinetics of nine BoHV-4 field strains on MDBK cells. Average virus titers obtained at 24, 48, 72, 96, and 120 h after infection of a preformed cell monolayer with 90 % confluence

abortion ranged from 4 to 8 months of gestation. Bovine viral diarrhea virus (BVDV), *Histophilus somni*, and *Trueperella pyogenes* were detected in the vaginal discharges of aborted cows, in combination with BoHV-4. The presence of BoHV-1 was investigated in all vaginal discharge samples. However, this virus was not identified by co-cultivation on MDBK cells and direct immunofluorescence (data not shown).

Serum antibody titers to *Neospora caninum* and *Leptospira* spp. were detected in some aborted animals. In agreement with other reports [26, 27], even in those cases in which BoHV-4 was the only pathogen identified (09/227, 08/330, 12/512, and 12/365), it was not possible to demonstrate that the virus was the only agent responsible for the abortion.

A BoHV-4-specific 260-bp fragment, corresponding to the TK gene, was amplified from all the vaginal discharge samples analyzed. These sequences were used to study the phylogenetic patterns of the viral strains identified in the vaginal discharges of the nine aborted cows (Table 1 and Fig. S1).

Replication kinetics: in vitro characterization

An in vitro comparison of the growth properties among the nine BoHV-4 isolates was performed. The mean virus titers obtained for the field isolates at 24, 48, 72, 96, and 120 hpi

on MDBK cells, are presented in Table 3. Significant differences ($p < 0.05$) were observed when the viral strains and post-infection times were analyzed.

According to the in vitro results presented in Table 3, BoHV-4 strains can be classified in three groups, independently of their prototype. In Group 1, the isolates 08/330 and 07/435 presented the highest titers at 48 hpi. Particularly, for isolate 08/330 the titer at this time-point was significantly higher with respect to the other strains and differences at the other analyzed time-points were not observed ($p \geq 0.05$). For the field isolate 07/435, differences in viral titers were not observed ($p \geq 0.05$) when the supernatant was collected at 48 h, 96 h, and 120 hpi. At 72 hpi, the viral titer between these two strains, which belong to a distinct phylogenetic group, did not differ ($p \geq 0.05$). In Group 2, isolates 09/759 and 12/512 showed the highest titers at 48 and 96 hpi, respectively. For the field isolate 09/759 differences in the virus titers at 24–72 hpi and 48–96 hpi were not observed ($p \geq 0.05$). However, for strain 12/512 a high variability in the virus titers at the different time-points was detected. Particularly, at 96 hpi significant differences were not recorded ($p \geq 0.05$) for both isolates belonging to the same prototype. In Group 3, isolates 07/568, 09/508, 09/227, 10/154, and 12/365 presented the highest titers at 120 hpi. From 72 hpi, differences among strains are detected ($p \leq 0.05$), mainly for isolates 07/568, 10/154, and 12/365, which had significantly higher titers ($p \leq 0.05$) compared to isolates 09/508 and 09/227. Isolates in this group belong to the three phylogenetic prototypes and their in vitro replication was very slow. Isolates 07/435 and 09/227 belong to a new prototype identified in Argentina. In cell culture, these two strains showed a biological behavior which is distinct from each other.

The replication kinetics for BoHV-4 field isolates on MDBK cells at 24, 48, 72, 96, and 120 hpi is shown in Fig. 1. Virus titers for 08/330, 09/759, and 07/435 increased during the first 24 hpi, followed by a gradual decrease from 48 hpi. For the field isolates 07/568, 09/508, 09/227, 10/154, 12/365 it was observed that the virus titers increased at 120 hpi, whereas strain 12/512 increases its replication activity up to 72 hpi and thereafter, the virus titer decreases. Significant differences ($p \leq 0.05$) were evident among the virus titers of the different BoHV-4 isolates in vitro. In general, the statistical interaction of the virus titer vs time was remarkable; not only among isolates but also within the same genotype.

Molecular characterization: nucleotide and amino scid sequences

The sequences for the major capsid protein (ORF25), glycoprotein (B), glycoprotein (H), and thymidine kinase (TK) genes from the nine isolates studied were compared to each other. The TK sequences for isolates 33/63, 10/154, 09/508, 09/759, 07/568, 07/435, and 09/227 were previously published [19]. The ORF25, gB, and TK sequences have been deposited in GenBank. The most conserved gene was ORF25, which displayed three variable sites and only synonymous substitutions. The gB, gH, and TK genes displayed both synonymous and non-synonymous substitutions, with the highest diversity observed for gB, which displayed amino acid substitutions in 24 out of the 178 positions determined (Fig. 2). Nucleotide and amino acid sequence alignments for the major capside protein (ORF25), glycoprotein (H), and thymidine kinase (TK) are depicted in supplementary Figs. S1–S3, respectively. Interestingly, gB sequences also displayed length heterogeneity. Three sequence classes regarding an insertion/deletion event affecting a region encompassing positions 27–35 of the amino acid sequence alignment were determined (Fig. 2). Strains 09/759, 12/512, and 07/568 presented this amino acid stretch completely deleted, whereas strains 07/435 and 09/227 had a deletion from position 28–35. These two strains (07/435 and 09/227) also displayed the highest divergence compared to the other strains analyzed (Fig. 2, S1–S3).

Discussion

The growth kinetics of nine strains representative of the three previously described BoHV-4 genotypes [19] were studied and the corresponding glycoprotein H (gH), glycoprotein B (gB), the major capside protein (ORF25), and thymidine kinase (TK) genes were sequenced and compared to each other. Despite differences in the replication kinetics among the different strains that were detected (discussed below), there was no apparent association between growth kinetics and genotype. Notwithstanding, the genotype was associated with differences at the nucleotide level for the major capside protein (ORF25) gene and at both nucleotide and amino acid levels for glycoprotein B (gB), glycoprotein H (gH), and thymidine kinase (TK) (Fig. 2, S1–S3). Particularly, variations in the glycoprotein B (gB) may have important in vivo consequences on the virus life cycle and for cell adhesion and virus progression into the replicative cycle [28]. The members of the gammaherpesvirus group have a slow replication cycle, a narrow host range, and cytopathogenic effects characteristic of cytomegaloviruses, but a Group B genome is similar to that of lymphotropic herpesvirus saimiri [29]. In contrast to other beta- and gammaherpesviruses, BoHV-4 replicates in a wide variety of cell cultures, i.e., established primary cell cultures of cattle, sheep, goats, dogs, cats, rabbits, pigs, and primary chicken kidneys [11]. Various cell lines of these species are

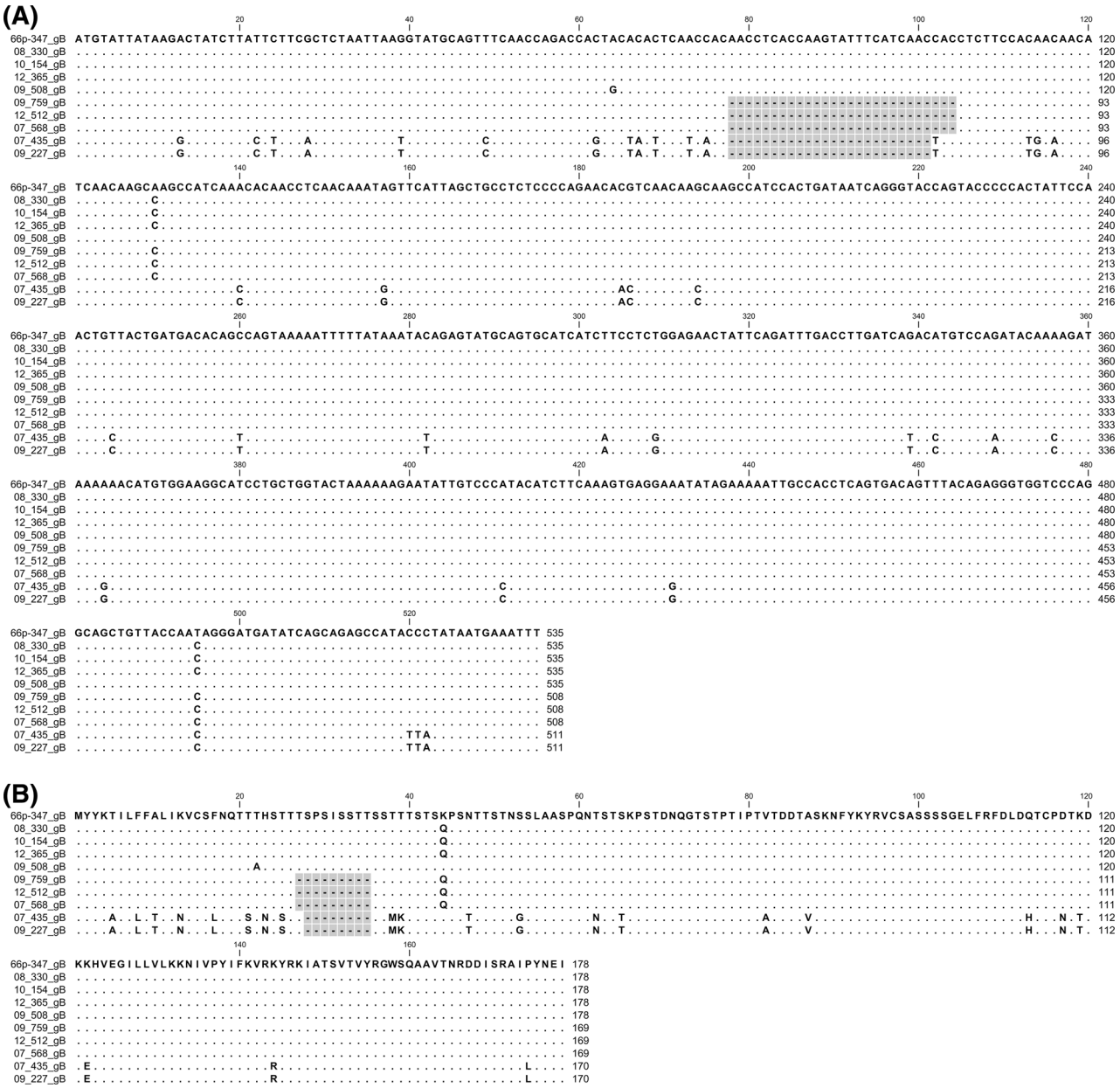

Fig. 2 Multiple nucleotide (**a**) and protein (**b**) sequence alignments for glycoprotein B. Identical residues are indicated with *dots*. Gaps in the sequence are indicated by *dashes*. *Shading* indicates variable positions

also susceptible to BoHV-4. The association between the stages of cell multiplication and the efficacy of replication of different isolates of the bovine alpha-herpesviruses, BoHV-1 and BoHV-5 has been previously studied [30, 31]. According to the results obtained in the present work, it is clear that there is a relationship between the replication of the different BoHV-4 strains, the multiplication stage of MDBK cells, and time post-infection. When the kinetics of viral replication was evaluated, it became evident that the highest virus titers were obtained with the isolates 08/330 and 07/435. This property of particular BoHV-4 isolates has not been reported by other authors. Only three strains had their highest titers at 120 hpi, in agreement with a previous report [29]. Thus, the results from this study demonstrate that the biological behavior on MDBK cells of BoHV-4 field isolates obtained from aborted cows is independent of their genetic classification. This study might contribute to understand the genetic diversity of BoHV-4 isolates, a virus property which has only been reported in Argentina [19]. 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.

The in vitro replication features of a virus may also have in vivo relevance. As a consequence, it is evident that the in vitro characterization (biological and molecular) of a viral strain is essential when aspects of the pathogenesis are being evaluated. This observation might be related to the different potential for the in vivo dissemination and circulation of the viral strains. The question arises whether this could be a difference in virulence between strains, rather than a difference in their in vitro replication on MDBK cells. BoHV-4 field strains analyzed in this study have been isolated from cows with similar clinical conditions. Therefore, correlations between in vitro replication and clinical manifestations cannot be performed at this time. Further studies will be required to determine whether the differences in virus replication in cell culture correlate with in vivo virulence.

This study provides information about the in vitro replication and behavior of nine field isolates of BoHV-4. These findings are relevant since available information on the in vitro growth characteristics of BoHV-4 strains is scarce. During evolution, did some strains become more virulent by acquiring advantageous genetic material over time? To answer these questions it will be ideal to determine the in vitro behavior of a broader range of wild-type bovine gammaherpesvirus strains on different cell lines, at different MOI.

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Compliance with ethical standards

Conflict of Interest None of the authors have any competing interests in the manuscript.

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

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