FULL ARTICLE

ISSN Online 1678-4456

Spatiotemporal expression pattern of alpha, beta, and gamma genes during BoHV-5 infection

Expressão temporal de genes alfa, beta e gama durante a infecção pelo BoHV-5

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ABSTRACT

Bovine herpesvirus 5 is an alphaherpesvirus that causes nonsuppurative meningoencephalitis in cattle. This disease occurs naturally in either outbreaks or isolated cases, and exhibits low morbidity and high lethality. Although previous studies elucidated crucial aspects involved in the pathogenesis of the disease, there is a paucity of information regarding the molecular events contributing to infection and replication of BoHV-5. The objective of the present study was to determine the *in vitro* gene expression pattern of BoHV-5 (e.g., alpha, beta, and gamma genes) and host cells genes (*GAPDH* and *18S*) over time utilizing different quantities of inoculated virus. Three BoHV-5 genes (*bICP0*, *UL9*, *US4*) and one structural bovine cell gene had their expression accessed by real-time PCR. While the expression of BoHV-5 genes increased during the course of infection, *GAPDH* gene expression decreased in the host cells, evidencing the effect of viral infection on the expression of bovine cell genes. The 18S ribosomal RNA (rRNA) gene was constitutively expressed throughout BoHV-5 infection. Our data clearly demonstrates that *GAPDH* gene should not be used as a reference gene in studies of BoHV-5 infection because it was influenced by viral infection. However, 18S rRNA was constitutively expressed and, therefore, is recommended for normalization of BoHV-5 infection studies in bovine cells. The expression of viral genes transcripts was not altered by increasing number of viral particles added to the culture. All viral genes included here demonstrated the same expression pattern over time and there was no difference in the expression of viral genes among the various time points. Our data show important differences comparing to classical studies regarding herpesvirus alpha, beta, and gamma genes expression. More research is necessary to improve our understanding about the BoHV-5 biology during infection. Studies employing next-generation sequencing (i.e., RNA-seq), using both *in vitro* and *in vivo* models, would be the next logical step to grasp the virus and host cell's transcriptome changes over the course of infection.

Keywords: *Bovine herpesvirus* 5. Quantitative PCR. Viral gene expression. Molecular biology.

RESUMO

Herpesvirus bovino 5 é um alfaherpesvírus causador de meningoencefalite não supurativa em bovinos. Esta doença possui ocorrência natural em surtos ou casos isolados, associadas a baixa morbidade e alta letalidade. Embora estudos anteriores tenham elucidado aspectos relacionados a patogenia da doença, há uma lacuna de conhecimento relacionado aos eventos moleculares que contribuem para a infecção e replicação do BoHV-5. O objetivo do presente estudo foi determinar a expressão gênica *in vitro* de genes virais (i.e., alfa, beta e gama) e das células hospedeiras (*GAPDH* e *18S*) durante a infecção considerando diferentes momentos de infecção e quantidade de vírus utilizado. Três genes do BoHV-5 (*bICP0*, *UL9*, *US4*), um gene estrutural (GAPDH) e um gene constitutivo (18S) da célula bovina tiveram suas expressões avaliadas por PCR quantitativa (qPCR). Enquanto os genes virais tiveram sua expressão aumentada ao longo do tempo de infecção, o gene hospedeiro teve sua expressão diminuída, demonstrando a ação do vírus na expressão gênica de células bovinas *in vitro*. O gene constitutivo 18S teve sua expressão mantida durante todos os momentos do experimento. Nossos resultados claramente demonstraram que o GAPDH não deve ser usado como gene de referência em estudos com infecção por BoHV-5 pois é influenciado pela infecção viral. Entretanto, o 18S rRNA foi constitutivamente expresso e pode ser recomendado para normalização em células bovinas infectadas pelo vírus. A expressão de mRNA

viral não foi alterada pela quantidade de vírus usada. Todos os genes virais demonstraram o mesmo padrão de expressão ao longo do tempo de infecção. Nossos resultados trazem importantes diferenças comparando aos estudos clássicos que avaliaram a expressão de genes alfa, beta e gama. Mais estudos são necessários para aumentar o conhecimento da biologia molecular do BoHV-5. Estudo utilizando sequenciamento de última geração (i.e., RNA-seq), usando modelos *in vitro* e *in vivo*, aparentam ser o próximo passo lógico para acessar as alterações do transcriptoma do hospedeiro e viral ao longo do curso da infecção.

Palavras-chave: *Herpesvirus bovino* 5. PCR quantitativa. Expressão gênica viral. Biologia molecular.

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Submited: October 09, 2018
Approved: February 02, 2019

How to cite: Cagnini DQ, Badial PR, Oliveira Filho JP, Borges AS, Araújo Jr. JP. Spatiotemporal expression pattern of alpha, beta, and gamma genes during BoHV-5 infection. Braz J Vet Res Anim Sci. 2019;56(1):e150972. https://doi.org/10.11606/issn.1678-4456.bjvras.2019.150972.

Introduction

Bovine herpesvirus 5[1] is an alphaherpesvirus (Davison et al., 2009; Del Médico Zajac et al., 2010; Franco & Roehe, 2007) that causes nonsuppurative meningoencephalitis, mainly in young cattle. This disease naturally occurs in either outbreaks or isolated cases, and exhibits low morbidity and high lethality (Rissi et al., 2006; Salvador et al., 1998). BoHV-5 infection, besides leading to cattle death, is an important differential diagnosis of other neurological diseases (e.g., rabies and bovine spongiform encephalopathy). The herpesvirus genome comprises three classes of genes known as immediate early (IE) or alpha (α), early (E) or beta (β), and late (L) or gamma (γ) (Honess & Roizman, 1974). In permissive cells, the process of *Human herpesvirus 1* (HHV-1) viral replication takes 18 to 20 h (Pellett & Roizman, 2007). Orchestrated gene and protein expressions are crucial for viral replication after infection in epithelial cells of the nasal or vaginal mucosa (Bagust, 1972; Bagust & Clark, 1972; Padgett et al., 2007; Van Lint & Knipe, 2009).

The classical herpesvirus cascade of alpha, beta, and gamma gene expression was first studied *in vitro* using the protein expression profile of HHV-1, formerly named herpes simplex virus 1 (HSV-1) (Honess & Roizman, 1974). The expression peak of α genes occurs within 2 to 4 h post-infection (p.i.), encoding proteins that regulate the expression of subsequent viral genes (Honess & Roizman, 1974) and contribute to evasion of the cellular responses to the infection (Van Lint & Knipe, 2009). Although the expression of β genes peaks at 5 to 7 h p.i., it ranges from 3 to 15 h p.i. (Honess & Roizman, 1974). In addition, expression of β genes induce viral DNA replication within the cell, and also downregulates α genes (Van Lint & Knipe, 2009). The expression of γ genes peaks at 6 h p.i. encoding viral structural proteins. However, those viral genes can be detected at low levels at 3h p.i. and reach maximum expression at 12 h p.i. (Nichol et al., 1996). DNA viral replication stimulates expression of γ genes at high levels, whereas it shuts off expression of α genes (Weir, 2001).

Herpesvirus genetic complexity, evolutionary diversity, and widely differing biological properties have generated a considerable research effort worldwide (Van Lint & Knipe, 2009). Nevertheless, the current understanding of the biology underlying the complex life cycle of herpes simplex virus (HSV) is still incomplete (Sawtell et al., 2006).

The *ICP0* is a very important α gene that can stimulate the transcription of others α, β, and γ genes (Engels & Ackermann, 1996). It has also been implicated in the establishment and reactivation of the virus from latency (Du et al., 2011; Roizman, 2011; Sandri-Goldin, 2003). Thus, induction of the expression of *ICP0* gene or its homologs, as bICP0 in BoHV-5, may be sufficient to initiate reactivation of latent herpesviruses (Engels & Ackermann, 1996).

The *UL9* gene is a β gene that encodes an origin-binding protein (Olivo et al., 1988). This protein is one of the seven proteins that are essential for the replication of HHV-1 DNA (Boehmer & Lehman, 1997). The UL9 protein functions to initiate HSV-1 DNA replication by binding and unwinding the three origins of HSV-1 DNA replication, and by recruiting the replication machinery to these origins (Lee & Lehman, 1997; Lehman & Boehmer, 1999).

The *US4* is a γ gene that is responsible for the transcription of the glycoprotein G (gG) (Engelhardt & Keil, 1996). The BoHV-1 homologous gG is involved in cell-to-cell dissemination (Nakamichi et al., 2000), stabilizing the cell structure, postponing apoptotic process (Nakamichi et al., 2001), and facilitating viral cell-to-cell spread by maintaining

[1] BoHV-5

the cell-to-cell junctions among the infected cells (Nakamichi et al., 2002). Furthermore, this gG can interfere at different distinct stages of chemokine action and therefore constitutes yet another mechanism of alphaherpesviruses to evade the immune system (Van De Walle et al., 2008). The BoHV-1 gG has the potential to interfere with acute inflammatory responses mediated by polymorphonuclear leukocytes (Liu et al., 2013). The BoHV-5 gG function as broad-spectrum chemokine binding proteins (Bryant et al., 2003).

The understanding of the spatiotemporal expression patterns of BoHV-5 during viral replication remains poorly explored. The study of the expression of herpesviruses genes contributes to the identification of potential viral target molecules that could be pharmacologically exploited to eliminate viral replication. Such studies could also assist in the better understanding of transcriptional profile changes in host cells after viral infection, identification of altered molecular functions and biological processes, and the role of herpesvirus in inducing host cell latency. In the present study, we used real-time PCR to characterize the *in vitro* spatiotemporal expression profile of essential BoHV-5 and host cell's genes.

Materials and Methods

Madin-Darby Bovine Kidney (MDBK) cells were inoculated with SV-507/99 strain of BoHV-5 (Delhon et al., 2003) to evaluate the spatiotemporal mRNA expression profile of three BoHV-5 genes (*bICP0*, *UL9*, and *US4*) and two cellular genes (*GAPDH* and *18S*) using real-time PCR. MDBK cells were maintained with Dulbecco's modified Eagle medium (DMEM) supplemented with non-essential amino acids (Gibco®) and 10% fetal bovine serum (FBS) in a humidified 5% CO_2 atmosphere at 37°C. Experiments were performed in triplicate.

MDBK cells were seeded into culture flasks (25cm^2) 24 h prior to use, and infected at 80-90% confluence. The MDBK monolayers were infected at multiplicity of infection[2] of 0.75-1 or 7.5-10. The inoculum was maintained into the culture for 1 hour to properly adsorb to the cells, and then removed and washed with fresh DMEM. This time point was considered moment 0 h p.i. Monolayers were harvested at 0 h, 6 h, 12 h, or 24 h p.i. Also, mock-infected MDBK controls were obtained at the same time points. Total RNA was purified using Total RNA Purification Kit (Norgen Biotek Corp.). RNA was frozen to -80°C until cDNA synthesis. Cell counting was compared between 0 h and 24 h p.i to evaluate possible dissimilarities in the number of harvested cells along the experiments.

RNA purity and concentration were accessed using a NanoDrop™ spectrophotometer (Nanodrop 1000 Spectrophotometer, Thermo Scientific™). Further quality analysis was carried out using Bioanalyzer (RNA 6000 NanoLabChip kit/ 2100 Agilent Technologies). Total RNA was treated with RQ1 RNAse-Free DNase (Promega) to eliminate genomic DNA contamination. After that, samples were submitted to PCR according to Cagnini et al. (2015) and to real-time PCR using the primers presented in Table 1 to ensure the absence of DNA. Then, 900 ng of the RNA samples was primed with random hexamers and used to synthesize first-strand cDNA using the ImProm-II™ Reverse Transcription System (Promega).

Primers were designed using the Primer Express 3.0 software (Applied Biosystems) (Table 1) and directed against partial sequences of viral (*bICP0*, *UL9* and *US4*) and cellular genes (*GAPDH* and *18S*) of the BoHV-5 (AY261359.1) and bovine genomes (GCA_000003055.4), respectively. We used the *GAPDH* gene to evaluate the effect of viral infection in the expression of a structural host cell gene, and *18S* as a reference gene.

Relative mRNA expression was quantified using a 7500 Fast Real-Time PCR System (Applied Biosystems™), and analyzed using the comparative Ct method ($2^{-\Delta\Delta Ct}$ method) (Livak & Schmittgen, 2001). Briefly, each 20 µL qPCR reaction was carried out in triplicate, containing 0.3 µM of each primer, 2 µL of cDNA template, 10 µL of GoTaq® qPCR Master Mix (Promega), and 6.8 µL of nuclease-free water. The following qPCR amplification conditions were 95°C for 2 min, 40 cycles at 95°C for 15 sec and 60°C for 1 min, followed by a melting curve. Also, negative controls were included in triplicate in each plate. Five-fold standard curves were used to test primers' efficiency. Data analysis was performed by normalizing

Table 1 – Primers used to evaluate BoHV-5 and MDBK cells gene expression*

Primer	Sequence (5' - 3')	Product size (bp)
bICP0 F	CACACCACCGCGTATTTGC	85
bICP0 R	TTACTTTTGGTTTGGGGATGACA	
US4 F	AGCGGGACCTACGTCTACTT	91
US4 R	ATTTGTGGATGTCGGCACCT	
UL9 F	GCTGGTGCAGGTGGAAA	116
UL9 R	CCATCGTCGGCGAATACAA	
GAPDH *Bos taurus* F	TGACCCCTTCATTGACCTTC	120
GAPDH *Bos taurus* R	ATGGCCTTTCCATTGATGAC	
18S bos taurus F	GAGAAACGGCTACCACATCCA	170
18S bos taurus R	CACCAGACTTGCCCTCCAAT	

* F = forward; R = reverse; R 2 > 0.99; Efficiency varied from 92% to 95%

[2] MOI

amplification values of the target genes with the endogenous control. We included one sample (6 h p.i.) to serve as calibrator sample to certify that efficiency was the same among real-time PCR plaques.

Data were analyzed in a completely randomized design with repeated measures. Relative gene expression data was transformed to the $\log_{10}$ scale to achieve normality. The data is presented as the geometric mean with 95% confidence intervals. A linear mixed model (PROC MIXED; SAS 9.4; SAS Institute Inc., Cary, NC, USA) was used to compare the mean expression values among time points, genes, and their interactions. A first-order autoregressive covariance structure resulted in the best fit based on the Bayesian information criterion and was used to model the correlation among the expression values of each gene in each MOI level. The Tukey test was used to adjust the *p* values resulting of the multiple comparisons. Analyses were conducted using a level of significance of P = 0.05.

Results and discussion

The Relative mRNA expression of viral (*bICP0*, *UL9*, and *US4*) and cellular (*GAPDH*) genes during the course of BoHV-5 (strain SV507/99) infection in MDBK cells is presented in Figure 1. Mock-infected cells exhibited stable *GAPDH* mRNA expression throughout the study. However, *GAPDH* mRNA expression in the BoHV-5 infected cells decreased continuously from 12 h to 24 h p.i. ($p < 0.001$) compared to 0 h and 6 h p.i. On the other hand, *18S rRNA* maintained constant expression levels during the 24 h period of BoHV-5 infection. Similar results were observed in human embryonic lung fibroblasts infected with HHV-1 where the expression of host cell's proteins such as *GAPDH* and β-actin genes decreased by several orders of magnitude during HSV-1 infection, but *18S rRNA* was constitutively expressed (Nystrom et al., 2004). Another study demonstrated that *GAPDH* mRNA levels were drastically reduced during infection with HHV-1 infection of HeLa and HEL cells due to virion host shutoff (VHS) protein expression, product of the *UL41* gene (Hsu et al., 2005). The viral VHS protein is an mRNA-specific RNase linked to the shutoff of host gene expression early in infection and presents a highly selective activity by targeting stable host mRNAs and α mRNAs, while sparing other viral mRNAs (Shu et al., 2013; Van Lint & Knipe, 2009; Weir, 2001). Previous studies have demonstrated that the translation of mRNA encoding ribosomal proteins (e.g., 18s) persists and is even favored over other mRNAs during HHV-1 infection (Greco et al., 1997; Simonin et al., 1997) by a mechanism that redirects the polyribosome complex to mRNAs of ribosomal proteins (Greco et al., 1997). It is very likely that the BoHV-5 infection induced downregulation of *GAPDH* mRNA, while not interfering with 18s mRNA expression, using a similar mechanism.

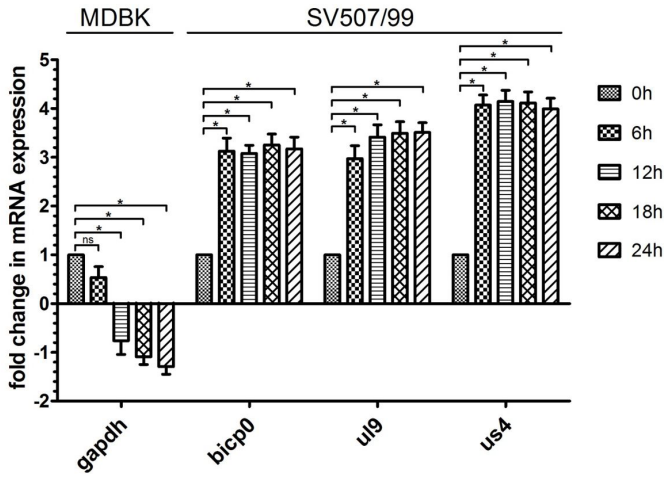

Figure 1 – Relative mRNA expression of viral (*bICP0*, *UL9*, and *US4*) and cellular (*GAPDH*) genes during 24 h of the BoHV-5 (strain SV507/99) infection in MDBK cells. The 18S gene was used as a reference gene to calculate the relative mRNA expression. The mean relative expression values with standard deviation are shown. Asterisks indicate significant ($P < 0.001$) differences between indicated sampling times

Therefore, *18S rRNA* is a reliable constitutive gene for monitoring viral and cellular gene expression in BoHV-5-infected cells, while *GAPDH* gene should not be used for this purpose. However, *18S rRNA* was the least reliable reference gene in a list of 10 commonly used reference genes that were studied in a variety of cell types infected with *Human immunodeficiency virus* 1 (HIV-1), HHV-1, Varicela-Zoster virus and Cytomegalovirus (Watson et al., 2007). These findings highlight the importance of evaluating the reference gene before using it in an experiment.

When individually compared, all viral genes demonstrated the same expression pattern and there was no difference of viral genes expression among the time course of infection. Viral genes were highly expressed in all moments when compared to the moment 0 h p.i ($p<0.01$). According to classical herpesvirus protein expression data (Honess & Roizman, 1974), ICP0 (bICP0 homologous), UL9, and US4 proteins can be detected at 6 h p.i., which corroborates with the mRNA expression data in this study.

bICP0 transcripts were upregulated from 6 h to 24 h p.i. ($P < 0.001$) compared with 0 h p.i. However, the α proteins

should decrease at 12 h p.i (Honess & Roizman, 1974). The expression of *ICP0* gene in HSV-1, which is orthologous to *bICP0* in cattle, peaks at 3 h p.i. and decreases after 7 h p.i (Nichol et al., 1996; Režuchová et al., 2003). However, BoHV-1 infection induces constitutive mRNA expression of *bICP0* gene because it contains immediate early and early promoters (Fraefel et al., 1994). Therefore, BoHV-5 may induce a similar *bICP0* gene expression pattern than BoHV-1, suggesting a similar viral behavior/biology during infection/replication/pathogenesis. BHV-5 is very similar to BHV-1, since they have high level of amino acid identity in their protein repertoires (average, 82%) (Delhon et al., 2003), which should explain their similar *bICP0* gene expression pattern.

UL9 mRNA expression in the BoHV-5 infected cells increased from 6 h to 24 h p.i. ($p < 0.001$) compared with 0 h p.i. Previous studies have demonstrated that *UL9* gene expression in HHV-1 infection begins at 1.5 h p.i. and increases abundantly from 3 to 12 h p.i., peaking at 8 h p.i. (Durmanova et al., 2001; Režuchová et al., 2003). *UL9* gene was differentially expressed 8 to 12 h p.i. compared to 1 h p.i. in cultured cells infected with pseudorabies virus (Flori et al., 2008). In the present study, we observed high expression level of *UL9* transcripts 6 h p.i., which increased at 12 h p.i. and remained high thereafter. The moment 24 h p.i. was not compared to previous studies because those studies did not have that time point (Durmanova et al., 2001; Režuchová et al., 2003).

US4 mRNA transcripts were upregulated from 6 to 24 h p.i. ($p < 0.001$) compared with 0 h p.i. Expression of late viral genes at 1 h pi. (UL49.5 gene) and 8 to 12 h p.i (*UL36* and *UL41* genes) were described in the microarray of pseudorabies virus infection in cultured cells (Flori et al., 2008). The *UL22* gene, a late gene that encodes glycoprotein H, was expressed just 2 hours after the infection of MDBK cells by BoHV-5 (Meyer et al., 1999). The *UL27* gene (Rafield & Knipe, 1984), and *ICP5*, *US6*, and *ICP34.5* genes (Van Lint & Knipe, 2009), all late genes, were also expressed early in infection.

Finally, we consider that a next step in our attempt to understand BoHV-5 molecular biology could be to study the expression of the *bICP0*, *UL9*, *US4*, and *GAPDH* at 1 h, 2 h, 3 h, 4 h, 5 h and 6 h p.i. to determine exactly the moment at which viral and host cell genes start to be differentially expressed. Moreover, in a second moment RNA-Seq, it would also be very informative, mainly regarding to host differential expressed genes and the molecular and cellular processes modified by viral replication. In a third moment, we would expand our approach and perform the above studies using animal models to better understand the molecular biology interaction between BoHV-5 and host cells in a more complex system.

Conclusion

BoHV-5 alpha (*bICP0*), beta (*UL9*), and gamma (*US4*) genes presented a similar expression pattern (upregulated) and the cellular gene (*GAPDH*) was markedly downregulated by viral replication during the time course of infection. The *bICP0* gene was constitutively expressed as previously described in BoHV-1 infection. This viral gene could be used in future *in vitro* studies as a reference gene to the virus, but more studies are needed to prove this claim. The *18S* was a good reference gene for our study, while the *GAPDH* was clearly not. The differences observed in the present study between the expression pattern of key BoHV-5 and HHV-1 published gene expression data might be related to the viral species differences (e.g., tropism, target cell, genetic material, or genomic structure), distinct molecular mechanisms involved in the pathogenesis to infect the host species, or because different techniques were used to compare gene expression in those studies. We consider that more research using modern techniques such as quantitative PCR, microarrays, and RNA-Seq are necessary to expand the current knowledge about BoHV-5 molecular biology to better understand how the replication of this virus affects host cells and how virus and host cells communicate with each other.

Conflict of interest

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article

Ethics Statement

This material has not been published in whole or in part elsewhere. All authors have been personally and actively involved in substantive word leading to the manuscript. This research project was submitted and approved by Research Ethics Committee of FMVZ-Unesp, Botucatu, protocol number 146/2011-CEUA.

Acknowledgements

We would like to thank the Virology Laboratory of the Federal University of Santa Maria, Santa Maria, RS, Brazil, for providing the BoHV-5 SV507/99 strain. The present research was supported by FAPESP 14/13532-3.

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Authors Contributions: All authors read and approved the final manuscript.