



## Transcriptomic analysis of differential gene expression reveals an increase in COX2 levels during *in vitro* canine herpesvirus infection

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**ABSTRACT:** *Canine herpesvirus (CaHV-1) affects canids worldwide, causing death in neonates and immunosuppressed hosts. Acute infection by CaHV-1 can cause reproductive, respiratory, and neurological problems in adult animals. Viral pathogenesis and host genes expressions during of CaHV-1 infection are not clearly understood. In the present study, the transcriptome of canine kidney cell Madin-Darby (MDCK) infected in vitro with canine herpesvirus was explored. For this, RNA sequencing (RNA-seq) of the samples in different moments during infection was carried out. Subsequently, the transcriptomic analysis genes related to cell activities and process involved to viral cycle infection were evaluated until 32h post-inoculation (pi). Among evaluated genes, was verified a significant and gradative increase of the prostaglandin-endoperoxide synthase 2 (PTGS2) or cyclooxygenase 2 (COX2) gene expression, throughout of infection, though differential gene expression analysis and validated by quantitative reverse transcription PCR (RT-qPCR). High COX2 expression is usually induced in response to inflammation, pathogens or activation of the immune system but can be a viral mechanism to favor viral replication. Thus, COX2 level increase can be a favorable factor for viral infection with Cahv-1 virus and the use of selective COX2 inhibitors may be beneficial for limiting the infection or clinical signs by causing interruption of the viral replication cycle during active infection. Additionally, the regulation genes expression differential verified in this study can contribute to determining important targets for inhibiting canine herpesvirus infection either by cellular or viral mechanisms.*

**Key words:** transcriptome, herpesvirus, CaHV-1, PTGS2, COX2, canine pathogen.

### Análise transcriptômica da expressão gênica diferencial revela um aumento nos níveis de COX2 durante a infecção *in vitro* por herpesvirus canino

**RESUMO:** *O herpesvírus canino (CaHV-1) afeta os canídeos em todo o mundo, causando morte em neonatos e hospedeiros imunossuprimidos. A infecção aguda por CaHV-1 pode causar problemas reprodutivos, respiratórios e neurológicos em animais adultos. A patogênese viral e expressão de genes hospedeiros durante a infecção por CaHV-1 ainda não são bem compreendidos. No presente estudo, o transcriptoma de células de rim canino Madin-Darby (MDCK) infectadas in vitro com herpesvírus canino foi explorado. Para isso, foi realizado o sequenciamento de RNA (RNA-seq) de amostras coletadas em diferentes momentos durante a infecção. Subsequentemente, a análise transcriptômica dos genes relacionados à atividade celular e aos processos envolvidos no ciclo de infecção do vírus foram avaliadas até 32 horas após a inoculação (pi). Dentre os genes avaliados, constatamos uma elevação significativa e gradativa da expressão da Prostaglandina-endoperoxide sintase 2 (PTGS2) ou ciclooxigenase 2 (COX2), ao longo da infecção viral, foi verificada por análise de expressão gênica diferencial e validada por resultados de transcrição reversa por PCR quantitativo (RT-qPCR). O aumento da expressão de COX2 geralmente é induzida em resposta a inflamação, patógenos ou ativação do sistema imune, mas também pode ser um mecanismo para favorecer a replicação viral. Assim, o aumento do nível de COX2 pode ser um fator favorável à infecção viral pelo vírus CaHV-1 e o uso de inibidores seletivos da COX2 pode ser benéfico para limitar a infecção ou os sinais clínicos, causando a interrupção do ciclo de replicação viral durante a infecção ativa. Além disso, a regulação diferencial da expressão dos genes verificados neste estudo podem contribuir para determinar alvos importantes para inibir a infecção por herpesvírus canino, seja por mecanismos celulares ou virais.*

**Palavras-chave:** transcriptome, RNA-seq, herpesvírus, CaHV-1, COX2, patógeno.

## INTRODUCTION

The canine herpesvirus causes a contagious viral infection among canids in many countries (ROBINSON et al., 2005; RONSSE et al., 2005). Etiological agent of canine herpesvirus belongs to the species Canine alphaherpesvirus 1 (CaHV-1),

genus Varicellovirus, subfamily *Alphaherpesvirinae*, and family *Herpesviridae* (ICTV 2017). It is a virus in which double-stranded DNA is contained in a nucleocapsid, immersed in the tegument, and enclosed into a lipoprotein envelope (DAVISON et al., 2009). Naturally infected dogs with CaHV-1 represent an economic loss to breeding kennels due to

reproductive, respiratory, and neurologic problems, and death by systemic disease in immunosuppressed adults and neonates (MALONE et al., 2010; EVERMANN et al., 2011; LARSEN et al., 2015). However, the pathogenesis of canine herpesvirus infection is not well known. The inactivated subunit vaccine against CaHV-1 is available in Europe, where it is used on breeding females, conferring short-duration passive immunity (CARMICHAEL & GREENE, 2012).

Herpesviruses are ubiquitous pathogens, well adapted to their hosts through many regulation mechanisms related to viral replication and cellular process domains (HOULDCROFT & BREUER, 2015; WEI et al., 2016). During productive infection, herpesviruses establish a complex and robust standard for viral genic expression (JURAK et al., 2011). Additionally, host response to viral infections involves intricate sets of regulatory processes (LAN et al., 2018). Therefore, the herpesviruses develop a mechanism to ensure the efficient translation of both viral and cell mRNA (GALE JR et al., 2000). The expression of the gene encoding prostaglandin-endoperoxide synthase 2 (PTGS2), better known as cyclooxygenase 2 (COX2), is induced by inflammatory response and infections, including those caused by viruses, in which COX products and derivatives may play a role in host response (KIRKBY et al., 2011; KUNZMANN et al., 2013). COX2 is involved in the production of prostanoids, which contribute to vasodilation, leukocyte chemotaxis, fever, and potential nociception (KIRKBY et al., 2011). However, during herpesvirus infection, the transcription of COX2 is essential for viral replication and virus-host interaction (RAY & ENQUIST, 2004). Under resting conditions, COX2 mRNA is not expressed by most cells (PABLOS et al., 1999).

Transcriptomic analysis is essential to study functional genome elements, subjacent mechanisms and disease triggers (YANG & KIM, 2015). RNA-sequencing (RNA-seq) using the next generation sequencing (NGS) platform has enabled the analysis of millions of transcripts from biological samples (MUTZ et al., 2014). Furthermore, RNA-seq is considered the gold standard for whole transcriptome gene expression quantification (EVERAERT et al., 2017). Thus, through transcriptional profile analysis, information on the biology of cells during certain conditions, as well as mechanisms and cell response to viral infection, can be obtained (WANG et al., 2009). Therefore, the purpose of this study was to analyze differential cellular gene expression during CaHV-1 infection *in vitro*. Additionally, the levels

of COX2 expression in response to viral replication were evaluated. To our knowledge, this is the first transcriptomic analysis of CaHV-1 infection *in vitro* using RNA-seq.

## MATERIALS AND METHODS

### *Cell culture and viral infection*

For viral infection, a viral suspension of CaHV-1 strain BTU-1 ( $10^{6.1}$  TCID<sub>50</sub>) was obtained from the kidney of an infected neonatal puppy that died of a systemic infection. The genomic sequence of CaHV-1 strain BTU-1 was deposited in GenBank (KT828242). Subsequently, MDCK cells were subcultured in a 24-well plate in Dulbecco's modified Eagle medium (DMEM) and 10% fetal bovine serum (FBS), and maintained at 37°C in the presence of 5% CO<sub>2</sub> and 80% humidity. After incubation for 24h, when 90% monolayer confluence was achieved, containing approximately  $9.24 \times 10^5$  cells/well. Cells were infected with CaHV-1 at a multiplicity of infection (MOI) of 3. Next, the cell culture plate and viral suspension were maintained at 4°C for 1h to allow the adsorption and synchronization of the infection, as described by FLORES & KREUTZ, 2012. Next, the cell monolayer was washed three consecutive times with sterile phosphate-buffered saline (PBS) solution (0.01M PO<sub>4</sub><sup>-4</sup>, 0.14M NaCl, pH 7.4). Finally, DMEM containing 1% FBS was added, and the cells were incubated at 37°C, under conditions of 5% CO<sub>2</sub> and 80% humidity atmosphere. Nucleic acid extraction from cells was performed at different time periods after infection in duplicate.

### *Isolation of nucleic acids*

For RNA extraction, the Total RNA Purification Kit (Norgen Biotek Corp.) was used according to the manufacturer's instructions. RNA samples were evaluated for purity by spectrophotometry using a Nanodrop ND1000 (Thermo Scientific), and the concentration was measured by fluorimetry using a Qubit 2.0 Fluorometer (Invitrogen Corp.). The integrity and quality of the RNA were analyzed using a Bioanalyzer 2100 (Agilent Technologies); a RNA integrity number (RIN) above 8.0 was considered acceptable. DNA extraction was performed using a Thermo Scientific GeneJET Genomic DNA Purification Kit (Thermo Fischer Scientific Inc.), following the manufacturer's instructions.

### *Sequencing libraries*

Samples for sequencing libraries were prepared using the Sure Select Strand-specific RNA

Library Prep reagents of the Illumina® Multiplexed Sequencing mRNA library kit (Agilent Technologies), following the manufacturer's instructions. Specifically, duplicate samples collected at pre-inoculation, 0h (post-adsorption), 1h post-inoculation (pi), 4h pi, 16h pi, 20h pi, 24h pi, 28h pi, and 32h pi were used. Quantification of the sample libraries was performed based on the absolute standard curve of the quantitative polymerase chain reaction (qPCR), using the Kapa Library Quantification Kit Illumina® platform (Kapa Biosystem Inc.), following the manufacturer's instructions. Amplification reaction of the library fragments was performed using an AB 7500 Fast thermal cycler (Applied Biosystem). After quantification, each sample library was normalized to 0.5nM to prepare a library pool. Capillary electrophoresis was performed using a Qiaxcel Advanced System (Qiagen Company) to evaluate the presence of dimers and estimate the fragment length of the libraries. Additionally, the qPCR products were loaded onto an agarose gel (1.5%), stained with Neotaq Brilliant Plus DNA Stain (NeoBio) at 0.5µL/mL, and electrophoresed. Gel bands were observed under ultraviolet light using a Fluor Chem FC2 Imaging system (Alpha Innotech Corp.) and analyzed using the Alpha View Program version 3.2.2 (Cell Biosciences Inc.).

#### *Clustering and sequencing*

For sequencing of mRNA libraries, the High Output Kit for up to 400M cluster - V2 75 cycles (Illumina) was used; sequencing was performed on the Illumina Next seq 500/550 platform (Illumina) for single-end and 75-bp sequences.

#### *Differential expression analysis*

The sequences obtained by RNA-seq were analyzed using the CLC Genomics Workbench 7.0.4 software (CLC Bio, Qiagen Company). Sequences were cleaned with a quality index limit of 0.08 and maximum limit of 2 ambiguous nucleotides and fragments above 40bp. In addition, the analysis of the components of differentially expressed genes (DEGs) reported in each library was performed. Sequence reads were analyzed with the RNA-seq software on the CLC platform, and mapped onto the mRNAs of the *Canis lupus familiaris* genome. Expression levels were assessed using the RPKM (reads per kilobase million mapped reads) as a method of analysis, and submitted to quantile normalization (ROBINSON & SMYTH, 2008). Next, transformed into log<sub>2</sub> expression values for subsequent statistical analysis. The Kal test (Z-Test) was employed to identify differentially expressed transcripts (KAL et al., 1999). In DEG analysis, data

filtering at least two fold-change (FC) values (>2 and <-2), P-values <0.05, a false positive rate (FPR) <0.05, and RPKM values ≥2 were used.

#### *Functional enrichment analysis based on gene ontology*

Enrichment analysis was performed for differentially expressed transcripts using an FC value greater than 2 across the Gene Ontology Database released by the Gene Ontology Consortium (GOC; <<http://www.geneontology.org>>).

#### *Reverse transcription (RT)-qPCR for COX2 gene expression validation*

Total RNAs were isolated from cells at various times after viral infection (pre-inoculation, 0h, 1h, 4h, 16h, 20h, 24h, 28h, and 32h) using the Total RNA Purification Kit (NorgenBiotek Corp.). The samples were incubated with deoxyribonuclease I (Invitrogen Corp.) to remove residual DNA. RT-qPCR experiments were carried out on a 7500 Fast Real-Time PCR System (Applied Biosystem) using Go Script Reverse Transcriptase reagents (Promega), 0.2µL of each primer (10µM), and nuclease-free water q.s.p. to achieve a final reaction volume of 20µL. Reverse transcription was performed at 45°C for 10min, followed by reverse transcriptase inactivation and Taq polymerase activation at 95°C for 2min, 40 cycles at 95°C for 10s, and 60°C for 30s. Primers used for COX2 target amplification were based on CLEMENTE et al. (2013). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference gene, and the sequence primers were based on those used in a study by PARK et al. (2013). Normalization of the COX2 expression data was performed to canine GAPDH, according to a previously published method (LARIONOV et al., 2005). The expression FC value was calculated using the 2<sup>-ΔΔCt</sup> method, according to LIVAK & SCHMITTGEN (2001).

#### *Construction of the plasmid standards for the qPCR assay*

A fragment of the TK gene (494bp) was used as the CaHV-1 standard DNA, with an external primer set described by SCHULZE and BAUMGARTNER (1998). This fragment sequence was cloned into the pGEM-T Easy Vector plasmid (Promega), according to the manufacturer's instructions. *Escherichia coli* JM109 was used as the cloning strain. The recombinant plasmids were purified using E.Z.N.A Plasmid Mini Kit (Omega Bio-tek), and quantified by fluorimetry using the Qubit® Quantification System (Invitrogen



Corp.). For preparing the absolute standard curve, the recombinant plasmid DNA containing CaHV-1TK region was serially diluted 10-fold, i.e. dilutions ranging from  $10^{-1}$  to  $10^{-10}$  were prepared in nuclease-free water. Aliquots (2 $\mu$ L) of the diluted plasmid were used in duplicate in the qPCR for the amplification of TK using the internal primer set, as described by SCHULZE & BAUMGARTNER (1998).

#### *Estimation of the viral load during in vitro infection by qPCR*

Samples of the infected cells were collected in duplicate to perform DNA extraction for the determination of the viral load at the following infection times: 0h, 1h pi, 4h pi, 16h pi, 20h pi, 24h pi, 28h pi, and 32h pi. The mixture for the qPCR was prepared using the master mix qPCR Gotaq Sybr Green (Promega), 0.4 $\mu$ L of each primer (0.01 $\mu$ M), 2 $\mu$ L of sample DNA, and nuclease-free water q.s.p, to achieve a final reaction volume of 20 $\mu$ L. Amplification of TK was conducted using the thermocycler AB 7500 Fast (Applied Biosystem) under the following conditions: 95°C for 2min, 40 cycles at 95°C for 15s, 57°C for 10s, and 72°C for 30s. The amplification run contained control reactions. The negative control contained DNA extracted from uninfected MDCK cells, whereas the positive control contained the DNA extracted from CaHV-1 stocks. Nuclease-free water was used instead of DNA as the reaction control.

## RESULTS AND DISCUSSION

#### *Analysis of differentially expressed genes*

After filtering and treatment of the sequences for the analysis of DEGs, RNA-seq generated 164.81 million reads, which were mapped to the canine genome to yield 124.02 million reads, which were analyzed by CLC Genomics Workbench 7.0.4 software. Of the 2,999 DEGs identified 1,662 were positively regulated and 1,335 were negatively regulated.

The collection times were initially selected to evaluate the cycle of infection of the virus in the cell, being 1h and 4h pi to evaluate the virus entry into the cell. Viral replication was evaluated at 16h pi. At 20h pi, it was evaluated the onset of cytopathic effects in infected cells and then monitored until 32h to evaluate the possible effects of cytolysis and apoptosis.

Cell genes expressed most up-regulated verified during *in vitro* CaHV-1 infection are shown in table 1. Among them, were verified genes associated with apoptosis or cell cycle arrest, cell proliferation and immune response. The genes with

higher expression levels throughout the course of the infection were EGR-1 (1h pi, FC=35.30 $\pm$ 5.52), FOS (1h pi, FC=33.38 $\pm$ 0.89), and COX2 (32h pi; FC=26.41 $\pm$ 0.74). Thus, EGR-1 and FOS well as well DUSP-1, are important in response to the immune system to infection, including virus (BAHRAMI & DRABLØS, 2016). However, EGR-1 can increase viral replication by activating promoters of viral productive cycle genes and suppressing the promoter of the latent gene (CHEN et al., 2009). In a previous transcriptomic analysis, PAULUS et al. (2016) verified that the expression of FOS and DUSP-1 was activated by immediate stress response in cells infected with Suid alphaherpesvirus 1 (more commonly known as pseudorabies virus or Aujeszky's disease virus). Therefore, in the early stages of the viral infection, the highest expression of cellular immediate early genes (IEGs) was verified. IEGs are genes that encoding the first phase of mRNAs expressed from the viral genome of pre-existing host cell proteins (TINDALL and CLERK, 2014). Additionally, IEGs are expressed rapidly and transiently, induced in cells by mitogenic factors due to changes in the physiological state, stress and pathogens (RAHAUS & WOLFF, 2003; PRADO & DEL BEL, 1998). The ATF3 (activating transcription factor) is an IEG and designated as a stress-inducible gene (LU et al., 2006). The induction transcriptional mechanism of ATF3 by DNA damage response is rapid and transient (FAN et al., 2002). Thus, at 1h pi the ATF3 was up-regulated (FC=8.86 $\pm$ 0.81), but at 32h pi it expression was noted as being 2.3 times higher (FC=20.37 $\pm$ 2.24) than at 1h pi. Thus, ATF3 late expression may play a role as pro-apoptotic gene being induced by pro inflammatory cytokines and hypoxia (ZHANGA et al., 2002; LU et al., 2006). Table 2 contains the 20 major inhibited cell genes during CaHV-1 infection in MDCK cell. Most of the genes were associated with inhibition of maintenance or structure cell while the remainder were associated with cell regulation cycle or apoptosis and immune. Thus, the virus interact with cell cycle regulatory and rapidly shuts off cellular protein synthesis, dampening host responses to infection and allowing viral mRNAs to dominate translational machinery (FLEMINGTON, 2001; SMILEY, 2004). The most inhibited gene, ENSCAFG00000004590 (PTHR11143) which encodes the 60S ribosomal protein L26 (RPL26) family, showed the maximum inhibition at time 24h pi (FC=-236.25). In cells subjected to DNA damage or stress, RPL26 binds to the mRNA responsible for inducing the translation of the p53 protein, thus leading to cellular halt response or apoptosis (KASTAN & BARTEK, 2004). However,

Table 1 - List of 20 most up-regulated genes during *in vitro* CaHV-1 infection as a function of time (H) and quantity (FC).

Gene Symbol	Description	Gene product function	Time	FC
1. EGR-1	Early growth response protein 1	Transcriptional regulator	1h	35.30
2. FOS	Proto-oncogene c-Fos	Essential for cell proliferation and differentiation	1h	33.38
3. COX2	Prostaglandin g/h synthase 2	Increase cell adhesion, phenotypic changes, resistance to apoptosis and tumor angiogenesis	32h	26.41
4. CHAC1	Cha C glutathione specific gamma-glutamylcyclotransferase 1	Pro-apoptotic component	32h	24.74
5. DDIT4	DNA damage-inducible transcript 4 protein	Responses to cellular energy levels and cellular stress, including responses to hypoxia and DNA damage.	32h	20.83
6. ATF3	Cyclic amp-dependent transcription factor ATF-3	This protein binds the camp response element (CRE), present in many viral and cellular promoters, repressor of inflammatory response	32h	20.37
7. ISG20	Interferon stimulated exonuclease gene 20	Interferon-induced antiviral exoribonuclease	32h	12.75
8. BTG2	BTG anti-proliferation factor2	Anti-proliferative protein	32h	11.44
9. RPL4	RPL4 ribosomalprotein L4	Compose a large ribonucleoprotein complex responsible for the synthesis of proteins in the cell	1h	11.13
10. DUSP1	Dual specificity protein phosphatase 1	Cellular response to environmental stress	1h	10.79
11. MARCKSL1	MARCKS like1	Controls cell movement by regulating actin cytoskeleton homeostasis	32h	9.79
12. TNFAIP3	TNF alpha inducedprotein3	Involved in the cytokine-mediated immune and inflammatory responses.	32h	9.52
13. SON	Protein SON	DNA-binding, tumorigenesis and apoptosis, but functional significance of this protein had not been recognized.	16h	9.42
14. MRPL33_1	Mitochondrial ribosomal protein L33	Large mitoribosomal subunit protein, possible involvement in mitochondrial translation.	16h	9.14
15. YPEL3	Yippee like 3	Inhibitory effects in normal and tumor cell lines	32h	9.08
16. SMIM10L1	Small integral membrane protein 10 like 1	The function SMIM10L1 is not clear	16h	8.13
17. MYH14	Myosin heavy chain 14	Regulate the cytokinesis, motility and polarity cell	28h	7.76
18. EIF4A1	Eukaryotic translation initiation factor 4A1	Proliferation and growth cell	16h	7.38
19. RPL37	Ribosomal protein L37	Component of the 60S subunit	16h	6.61
20. UBALD1	UBA like domain containing 1	No characterized	0h	6.6

in cells with DNA damage and RPL26 inhibition, p53 protein levels do not increase (TAKAGI et al., 2005; CHEN & KASTAN, 2010). During infection, alphaherpesviruses use anti-apoptotic strategies to maintain their replication and propagation (YOU et al., 2017). Therefore, it is possible that CaHV-1 uses the mechanism of RPL26 expression inhibition to complete its life cycle. Considering that, alpha herpes virus infections occurs lytic replication in epithelial cells before becoming latent in the trigeminal ganglion (COHRS & GILDEN, 2016; LUECKE & PALUDAN, 2015). Therefore, in MDCK cell, inhibitory process of RPL26 genic expression could ensure viral multiplication and complete viral cycle.

The ATP5G2 and MRPS17 genes down-regulation can be related to compromising mitochondrial function. BRÜGGEMANN et al. (2017),

verified that ATP5G2 down-regulation reduced activity of the mitochondrial electron chain and this leads to reduced oxidative phosphorylation. The MRPS17 is required for the maintenance of respiratory-proficient mitochondria (HANLON et al., 2004). Some studies related the damage in this organelle by herpesvirus that induce shutoff of host functions, and contribute to the cell death and tissue damage (SAFFRAN et al., 2010; MURATA et al., 2000).

#### COX2 expression validation by RT-qPCR

Figure 1 shows the temporal evolution of PTGS2 (COX2) expression levels during infection, based on the RPKM values and relative quantification by RT-qPCR. Interestingly, both showed similar performance and a peak elevation at 32h pi. Additionally, expression of COX2 during

Table 2 - List of 20 most down-regulated genes during *in vitro* CaHV-1 infection as a function of time (H) and quantity (FC).

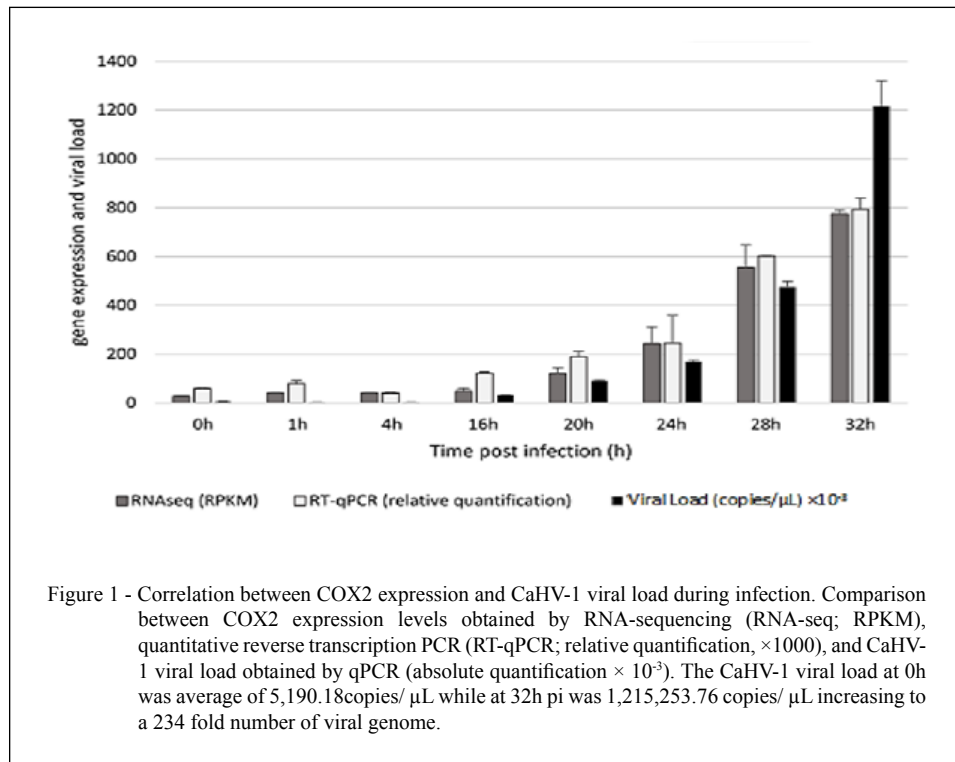
Gene Symbol	Description	Encodes function protein	Time	FC
1. RPL26	Ribosomal protein L26	Cell proliferation	24h	-236.25
2. ATP5G2	ATP synthase, H <sup>+</sup> transporting, mitochondrial F0 complex, subunitc-2	Mitochondrial ATP synthesis coupled proton transport	16h	-99.11
3. ATP5MC2	ATP synthase membrane subunit c locus 2	Lipid binding and proton transmembrane transporter activity	4h	-46.36
4. TMED9	Trans membrane p24 trafficking protein 9	Transport proteins located in the endoplasmic reticulum and Golgi complex	16h	-38.05
5. RPL23A	Ribosomal protein L23a	Cell growth and regulation	0h	-35.08
6. RPS27	Ribosomal protein S27 or metalloproteinase 1 (MPS-1)	Extra-ribosomal functions, regulate growth and survival cell as well as DNA repair and transcription	0h	-26.26
7. ARPC3	Actin related protein 2/3 complex subunit 3	Control of actin polymerization in cells	4h	-24.84
8. RPL29	RPL29 ribosomal protein L29	Cell proliferation	16h	-24.54
9. S100A2	S100 calcium binding protein A2	Cell regulation	16h	-19.79
10. CRYAB	Crystallin alpha B	Apoptosis inhibition	0h	-15.40
11. EEF1A1	Eukaryotic elongation factor 1 alpha	Instead of upregulation, the down regulation of eEF1A1, is required for the apoptosis	4h	-14.12
12. TMEM97	Transmembrane protein 97	Cell proliferation	16h	-12.94
13. DLADRA	MHC class II DR alpha chain	Cellular immunity	28h	-12.26
14. MRPS17	Mitochondrial ribosomal protein S17	Mitochondrial proteins synthesis	4h	-11.17
15. DHRS2	Dehydrogenase/reductase2	Cycle cell regulation and apoptosis	28h	-10.95
16. MMP13	Matrix metalloproteinase 13	Cell proliferation	4h	-10.68
17. KRT14	Keratin 14	Structural molecule activity and keratin filament binding	0h	-10.65
18. SMIM7	Small integral membrane protein 7	Integral component membrane	4h	-10.39
19. TMEM179B	Transmembrane protein 179B	Membrane protein transporter	0h	-9.71
20. CCL17	C-C motif chemokine ligand 17	Mediating by chemotaxis the infiltration of Th2 cells in inflammatory reaction	4h	-9.69

CaHV-1 infection has been analyzed by comparing the FC values obtained with RNA-seq and RT-qPCR (Figure 2). The comparison performed between RNA-seq and RT-qPCR reveals a correlation between both techniques used for the quantification of COX2 expression level. Thus, it was verified that the RNA-seq technique can be reliable for the quantification of gene expression, and it was possible to confirm the increase in COX2 expression level.

In cells infected with PRV, the highest level of COX2 transcription occurred at 8h pi, showing a 11-fold elevation (RAY et al., 2004). In HSV-1-infected cells, the transcription of COX2 mRNA reached a fold-change peak of 14.5 at 8h pi (LIU et al., 2014). However, in CaHV-1 infection, the onset of COX2 elevation occurred 16h pi with FC=2.54±0.16 (RT-qPCR) and 1.51±0.32 (RNA-seq). The peak of COX2 expression in CaHV-1 infection was observed later in relation to PRV and HSV-1

infection, reaching maximum levels 32h pi with FC values of 25.40±2.37 (RT-qPCR) and 26.41±3.27 (RNA-seq). These different expression patterns might occur due to infection cycle of CaHV-1 be longer than those exhibited by PRV and HSV-1. During *in vitro* PRV infection, the cytopathic effects occurs at 16h pi (PASDELOUP et al., 2013). In this study, virus isolate presents onset of cytopathic effect at 20h post inoculation and evidenced at 24h pi (data not shown). Additionally, LARA and colleagues (2016) observed late cytopathic effects during CaHV-1 infection after 48h pi, while DE PALMA et al. (2010) at 72h pi.

Hypoxia Response (GO: 0001666) was among the biological processes related to COX2 expression. Hypoxia inducible factors (HIFs) modulate the gene expression of DNA viruses, such as herpesviruses, due to the presence of elements responsive to hypoxia in their promoters, which increase viral replication (TOMASKOVA



et al., 2011). High COX2 expression levels occur during inflammation, and oxygen levels decrease, thus leading to the activation of adaptive responses related to the expression of HIFs via nitric oxide (CARIA et al., 2014). In addition, hypoxia promotes vessel growth through the positive regulation of several pro-angiogenic pathways (KROCK et al., 2013). Interestingly, angiogenesis has been linked to elevated COX2 levels due to the increase of the levels of associated pro-angiogenic growth factors (YAO et al., 2011). Our gene ontology analysis confirmed the presence of the biological process Regulation of angiogenesis (GO: 0045765), among the up-regulated genes. Therefore, hypoxia, DNA damage response, and apoptosis may indicate the occurrence of viral replication and consequent increase in COX2 levels. Gene expression levels associated with hypoxia-related genes, with the exception of COX2, remained constant after their detection (24h pi). However, levels of other genes related to COX2 increased 32h pi, indicating that COX2 possibly interacts or activates other genes that promote other cellular biological processes. Thus, gene ontology analysis of the positively regulated genes revealed that when the FC value of COX2 increased, the number of biological processes that were involved with the gene increased as well (Figure 3).

#### *Absolute viral load quantification and COX2 gene expression*

Viral load during infection was measured by qPCR and correlated to COX2 expression by RT-qPCR (relative quantification) and RNA-seq (RPKM). Interestingly, both viral load and COX2 expression increased during the evolution of the infection, with the onset occurring 20h pi. Considering pre-inoculation COX2 levels as basal values, it may be suggested that viral replication is involved in the induction of COX2 expression. Moreover, the highest viral load (~1.62×10<sup>6</sup>copies/μL) was reached 32h pi, which is when the expression level of COX2 also reached its maximum (Figure 1). The increase in both COX2 expression and viral load throughout the viral infection time can indicate a correlation between the two parameters.

COX2 transcripts are important for viral replication and virus-host interaction (RAY et al., 2004; REINOLDS & ENQUIST, 2006). Thus, herpesviruses exploit the induction of the COX2 cellular pathway for viral replication (CHENG et al., 2015). Therefore, the increase in COX2 transcription reported in this study was verified in infections by other herpesviruses, including bovine alphaherpesvirus 4 (BoHV-4), human herpesvirus 4 [HHV-4, also known as Epstein-



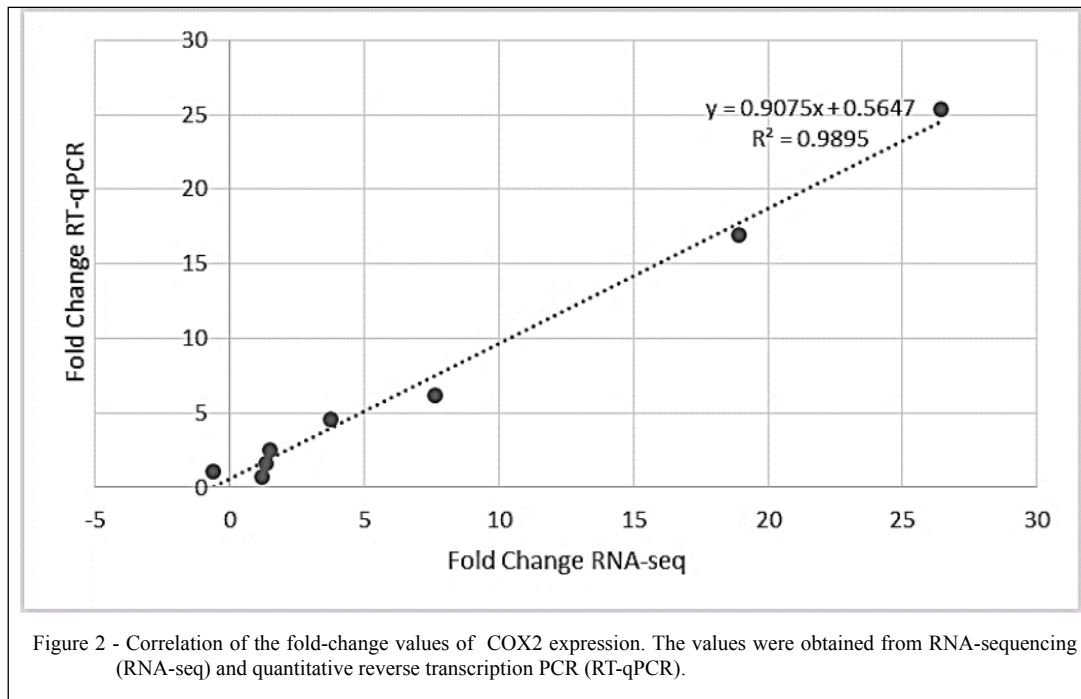


Figure 2 - Correlation of the fold-change values of COX2 expression. The values were obtained from RNA-sequencing (RNA-seq) and quantitative reverse transcription PCR (RT-qPCR).

Barr virus (EBV)], human herpesvirus 6 (HHV-6), HSV-1, and Kaposi's sarcoma-associated herpesvirus (KSHV) (JANELLE et al., 2002; DONOFRIO et al., 2007; SHARMA-WALIA et al., 2010; AL-SALAM et al. 2013; LIU et al., 2014). The role of COX2 in the viral replication cycle is unclear (REINOLDS & ENQUIST, 2006). However, in HHV-6, viral gene transcription is required for COX2 promoter activation (JANELLE et al., 2002). In cytomegalovirus infections, blockage of COX2 inhibited the viral mRNA by interrupting protein and DNA synthesis (ZHU et al., 2002). The increase in COX2 expression can lead to the enhanced production of prostaglandin (PG), which is an important factor in the inflammatory cascade reaction (LIU et al., 2014). The rise in prostaglandin levels was associated to latent herpesvirus activation (BAKER et al., 1985). In contrast, previous studies indicated that the PGs act as inhibitors of replication in some negative-strand RNA viruses such as the Sendai (SeV), Mayaro (MAYV) and poliovirus (SANTORO et al., 1983; CONTI et al., 1996; BARBOSA & RABELLO, 1998). In these viruses, the PGs can affect an early and a late phase of viral production, including the synthesis and/or maturation of viral proteins (SANTORO et al., 1994).

COX2 inhibitors have been tested for the treatment of the infection and, interestingly, have proven efficient in reducing the frequency and severity of the lesions (CHENG et al., 2015). Moreover, use

of selective COX2 inhibitors may favor interferon responses during infections, and increase antiviral immunity (KIRKBY et al., 2011). Considering the availability of drugs that selectively inhibit the COX2 pathway, further studies to assess their therapeutic potential for use against CaHV-1 infections are needed. However, these results may serve for as a basis for future studies on the use of selective COX2 inhibitors for the suppression of CaHV-1 replication in active infection states.

## CONCLUSION

In summary, RNA-seq allowed for the transcriptomic analysis of differentially expressed genes in host cell *in vitro*, during CaHV-1 infection. COX2 (i.e., PTGS2) expression from infected cells was among the most induced. High expression levels of this gene were related to replication and viral load enhancement during CaHV-1 infection. In contrast, RPL26 was the most inhibited gene, possibly because of apoptosis inhibition, which contributed to viral infection in the host cells.

Therefore, the findings verified in this study can contribute to determining important targets for inhibiting or preventing canine herpesvirus infection either by cellular or viral mechanisms and lessening clinical signs and mortality during disease outbreaks.





Figure 3 - Gene ontology of the genes regulated positively related to COX2 expression. The values are represented as fold-change (cutoff >2) involving biological process of cellular genes. RNA-seq analyses did not show the differential expression of PTGS2 at the time periods before 16h pi.

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## BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

The experimental design was approved by the local Ethics Committee for the Use of Animals (CEUA) of the UNESP, under the protocol number 141/2016, in accordance with the standards of the National Council for the Control of Animal Experimentation (CONCEA).

## DECLARATION OF CONFLICTING INTERESTS

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

## AUTHORS' CONTRIBUTIONS

The authors contributed equally to manuscript.

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