UNIVERSIDADE ESTADUAL PAULISTA

FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA

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Didier Quevedo Cagnini

Botucatu-SP

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Tese apresentada junto ao Programa de Pós-Graduação em Medicina Veterinária para obtenção do título de Doutor.

Orientador: Prof. Alexandre Secorun Borges

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RESUMO

Herpesvirus bovino (BoHV-5) é um alfaherpesvirus que causa meningoencefalite não-supurativa preferencialmente em bovinos jovens. Esta enfermidade ocorre naturalmente em surtos ou casos isolados, apresentando baixa morbidade e alta letalidade. A epidemiologia, as características anatomopatológicas e o diagnóstico do BoHV-5 são bem conhecido. No entanto, as interações moleculares entre a células hospedeiras e o BoHV-5 são pobremente compreendidas. Técnicas moleculares como a PCR quantitativa e o sequenciamento de RNA (RNA-seq) são importantes ferramentas para o estudo de interações entre vírus e células hospedeiras. Neste estudo células MDBK foram infectadas pelo BoHV-5, cepa SV507-99 e o mRNA extraído em 0, 6, 12, 18, e 24 horas após a infecção (pi) foi utilizado para avaliar a expressão de genes do vírus e da célula hospedeira por qPCR e o mRNA de 1h, 6h e 24h pi foram utilizado no estudo por RNA-seq. Células MDBK não infectadas foram usadas como controle. A qPCR demonstrou que os três genes do BoHV-5 estudados (*bICP0*, *UL9* e *US4*) apresentaram perfil de expressão semelhante nos diferentes momentos após infecção e que o gene bovino *GAPDH* teve sua expressão diminuída pela infecção viral. Ao menos 900 genes foram diferencialmente expressos para cada momento de infecção na análise por RNA-seq. Estes genes estavam principalmente relacionados a vias celulares de controle de ciclo celular, produção de interleucinas, quimiotaxia para células inflamatórias, reparo e dano ao DNA, resposta a vírus, apoptose, processo oxidativo, e ubiquitização de moléculas. Estes resultados representam novos conhecimentos sobre a interação entre o BoHV-5 e as células bovinas e podem representar um ponto de partida para novas pesquisas e melhor compreensão da patogenia deste vírus.

Palavras-chave: Bovino, sequenciamento de RNA, PCR quantitative, herpesvirus.

CAGNINI, D.Q. *In vitro* mRNA differential expression in Bovine Herpesvirus 5 infection. Botucatu, 2014. 90p. Thesis (PhD) - Faculdade de Medicina Veterinária e Zootecnia, Campus de Botucatu, Universidade Estadual Paulista "Júlio de Mesquita Filho". Botucatu, São Paulo.

ABSTRACT

Bovine herpesvirus 5 (BoHV-5) is an Alphaherpesvirus that causes nonsuppurative meningoencephalitis mainly in young cattle. This disease occurs naturally in either outbreaks or isolated cases, and exhibits low morbidity and high lethality. Besides BoHV-5 epidemiology, pathological findings and diagnosis were well known, the molecular interactions between host cell and BoHV-5 are poorly understood. Molecular biology techniques such as quantitative PCR (qPCR) and RNA sequencing (RNA-seq) are important tools to study virus and host cell interactions. In this study we infected MDBK cells and use the extracted mRNA at 0, 6, 12, 18 and 24h post infection (pi) to analyse BoHV-5 (*bICP0*, *UL9* e *US4*) and host cell gene (GAPDH) expression using qPCR and 1h, 6h and 24h pi in the RNA-seq study. Mock-infected cells were used to control purpouse. The qPCR releveled that the three BoHV-5 genes showed the same expression behavior during the infection and the GAPDH gene were down-regulated in the infected group. At least 900 genes were differentially expressed during BoHV-5 in each moment analysed by RNA-seq. These genes were up- or down-regulated and mainly associated with cell cycle, interleukin production, inflammatory cells chemotaxis, DNA damage and repair, response to virus, apoptosis, oxidation-reduction process, and ubiquitination pathways. The results demonstrate new insights about BoHV-5 and bovine cells interactions and could be a starting point to new researches and to better understand the pathology of this virus.

Key words: Bovine, RNA sequencing, quantitative PCR, Herpesvirus.

Capítulo 1

1. INTRODUÇÃO

O Herpesvirus bovino 5 é um vírus DNA de fita dupla, envelopado, membro da família *Herpesviridae*, da subfamília *Alphaherpesvirinae* e do gênero *Varicellovirus* (Franco e Roehe, 2007; Del Medico Zajac, M. P. *et al.*, 2010). A doença causada pelo BoHV-5 acomete principalmente animais jovens, podendo também afetar animais adultos, ocorrendo em forma de surtos ou de casos isolados (Salvador *et al.*, 1998; Rissi *et al.*, 2006). Geralmente apresenta morbidade baixa e letalidade alta. O aparecimento de casos de meningoencefalite herpética geralmente está associado a situações de estresse, como desmame, transporte, vacinações, castrações, troca de pastos, mudanças na alimentação ou intempéries. Acredita-se que isto diminui a imunidade dos animais e facilita a replicação viral, causando a doença (Barros *et al.*, 2006; Rissi *et al.*, 2006).

No Brasil, depois da raiva, o BoHV-5 é considerado como uma das principais causas virais de encefalopatia nos bovinos e a ocorrência da doença tem aumentado (Lisboa et al. 2009). Há relatos de casos isolados e surtos da doença nos Estados de Mato Grosso do Sul e São Paulo (Salvador *et al.*, 1998), Minas Gerais (Gomes *et al.*, 2002; Aquino Neto *et al.*, 2009), Rio Grande do Sul (Elias, Schild e Riet-Correa, 2004; Rissi *et al.*, 2006; Sá E Silva *et al.*, 2007; Rissi *et al.*, 2008), Mato Grosso (Colodel *et al.*, 2002; Arruda *et al.*, 2010), Goiás (De Paula *et al.*, 2005), Pará (Riet-Correa *et al.*, 2006), Paraíba (Galiza *et al.*, 2010) e Paraná (Lisbôa *et al.*, 2009; Lunardi *et al.*, 2009).

O diagnóstico definitivo de infecção pelo BoHV-5 antes da morte é difícil de ser estabelecido, visto que não existem sinais patognomônicos da enfermidade, a qual pode ser confundida com outras encefalopatias. No entanto, os achados clínicos e epidemiológicos, associados à análise do líquido cefalorraquidiano (LCR) podem auxiliar o diagnóstico de encefalite viral. Apesar da sensibilidade da PCR não ser alta na avaliação do LCR para BoHV-5, esta pode ser uma ferramenta útil na tentativa de realizar o diagnóstico definitivo no animal vivo (Lisbôa *et al.*, 2009; Lunardi *et al.*, 2009; Cunha, 2010).

Por isso, o diagnóstico após a morte, com o auxílio de métodos laboratoriais, é fundamental. À necropsia, as lesões macroscópicas podem ser discretas, como hiperemia de leptomeninges ou mais evidentes, como malacia do córtex cerebral (Rissi *et al.*, 2006). Histologicamente, podem ser encontrados diferentes graus de meningoencefalite não supurativa com presença de manguitos perivasculares composto por células mononucleares, infiltrado inflamatório mononuclear, necrose neuronal,

edema, tumefação de células endoteliais e gliose (Barros *et al.*, 2006; Cagnini, 2011). Em alguns casos pode haver malacia da substância cinzenta do córtex cerebral e corpúsculos de inclusão intranucleares (CI) em astrócitos e neurônios (Rissi *et al.*, 2008; Cagnini, 2011). O diagnóstico histopatológico pode ser considerado conclusivo apenas na presença dos corpúsculos de inclusão (Barros *et al.*, 2006). Porém, a presença do CI é muito variável e a ausência deste não exclui o diagnóstico de doença por BoHV-5 (Rissi *et al.*, 2007). Além disto, o diagnóstico definitivo pode ser realizado pelo isolamento viral associado às técnicas de imunoperoxidase (IPX), imunofluorescência indireta (IFA) ou ao uso de enzimas de restrição para confirmar a infecção pelo BoHV-5 (Barros *et al.*, 2006; Cagnini, 2011). Contudo, muitas vezes as amostras não estão adequadas para o cultivo celular, podendo-se obter resultado falso-negativo. Nestes casos, as técnicas de imunoistoquímica com o uso de anticorpos monoclonais e a reação em cadeia da polimerase (PCR) apresentam-se como melhores alternativas para se obter o diagnóstico positivo para BoHV-5 (Barros *et al.*, 2006; Cunha, 2010).

Ao longo dos anos pesquisas ampliaram muito o conhecimento sobre a epidemiologia, os sinais clínicos, as lesões anatomopatológicas e as vias de acesso do vírus ao SNC. O conhecimento relacionado a métodos diagnósticos para infecção pelo BoHV-5 apresentou grande crescimento nos últimos 20 anos, fazendo relação com isolamento viral, sorologia, imunoistoquímica, hibridização *in situ*, pelo uso de enzimas de restrição, PCR, *nested* PCR e qPCR.

Além disso, estudos foram realizados para elucidar a função de genes e proteínas virais. Estes foram focados principalmente na identificação da função de genes virais responsáveis pela síntese de glicoproteínas. Para isso, as pesquisas geralmente utilizaram cepas mutantes para genes de interesse na infecção de cultivos celulares, coelhos e bovinos e compararam o comportamento daquelas com as cepas virais de campo (Meyer, Bare e Thiry, 1999; Chowdhury, Lee, Onderci*, et al.*, 2000; Chowdhury, Lee, Ozkul*, et al.*, 2000; Chowdhury, S. I. *et al.*, 2002; Al-Mubarak e Chowdhury, 2004; Hübner, Pescador*, et al.*, 2005). Com isso, pode-se determinar a função de alguns genes e proteínas na patogenia do BoHV-5. No entanto, o conhecimento gerado até o momento está longe de cobrir todas as lacunas existentes relacionadas à biologia deste vírus.

Neste contexto, trabalhos relacionados à expressão de genes virais usando técnicas moleculares como a PCR em tempo real (qPCR), microarray ou

sequenciamento de alta capacidade a partir de bibliotecas de cDNA (RNA-seq) ainda não foram descritos na literatura relacionada ao BoHV-5.

Neste, temos por objetivo realizar a infecção de células MDBK com o BoHV-5, em diferentes períodos de tempo de infeção e o estudo da expressão gênica de genes do tipo alfa, beta e gama por meio da técnica de PCR em tempo real. Além disso, realizar o transcriptoma celular e viral usando a técnica de RNA-seq e avaliar a expressão relativa global dos genes celulares e virais pela análise de bioinformática em três momentos distintos de infecção. Com isso, esperamos contribuir para o conhecimento da biologia molecular do BoHV-5 e estudar possíveis padrões de expressão e os possíveis processos biológicos que podem estar alterados na infecção das células infectadas.

2. REVISÃO DE LITERATURA

2.1 Revisão das características dos Herpesvirus durante a infeção celular.

O Herpesvirus bovino 5 possui genoma composto por fita dupla de DNA linear que se circulariza ao penetrar a célula do hospedeiro. Dentro dos 138.390 pares de base que integram o genoma do BoHV-5 estão distribuídos 72 genes, dos quais 68 apresentam apenas uma única cópia, e 2 genes (BICP4 e BICP22) estão presentes duplificados na região repetida invertida do genoma viral. O BoHV-5 e o BoHV-1 apresentam genomas similares em organização e tamanho, com 82% de identidade de aminoácidos e compartilhando quase todas as ORFs, com única exceção da ORF do gene UL0.5 que não está presente no BoHV-5 (Delhon *et al.*, 2003).

Os genes são transcritos pela maquinaria celular de transcrição (RNA polimerase II e fatores de transcrição), possivelmente assistida por fatores virais. A transcrição de cada gene origina um mRNA, que possui um *cap* na extremidade 5'e é poliadenilado na extremidade 3'. Poucos transcritos dos herpesvírus sofrem *splicing* antes de serem exportados para o citoplasma (Franco e Roehe, 2007).

De acordo com a cinética de expressão e com a função de seus produtos, os genes virais são classificados em três grupos principais (Franco e Roehe, 2007):

1. Genes alfa (*immediate early* ou transcrição imediata);
2. Genes beta (*early* ou iniciais);
3. Genes gama (*late* ou tardios).

Os genes alfa e beta são expressos abundantemente antes da replicação do genoma, enquanto os genes gama somente são expressos em quantidades significativas após a replicação do DNA viral (Franco e Roehe, 2007). No entanto, a literatura não apresenta estudos sobre a expressão dos genes do BoHV-5 e o conhecimento de como ocorre à interação entre estes genes e genes hospedeiros dá-se por extrapolação do conhecimento adquirido em estudos de outros *alphaherpesvirus*.

Simultaneamente à expressão das proteínas virais, ocorre a inibição da transcrição de genes do processamento e transporte de mRNA e síntese de proteínas da célula hospedeira. Estes eventos são induzidos por proteínas virais e tem por objetivo subverter a maquinaria celular para o processamento e transporte de mRNA virais e

síntese de proteínas virais. Em células permissivas o ciclo replicativo dos herpesvírus é completo em aproximadamente 18-20 horas (Franco e Roehe, 2007).

De modo geral, os *alphaherpesvirus* inicialmente infectam as células epiteliais da mucosa nasal, mucosa ocular (fase lítica do ciclo) e chegam ao sistema nervoso central via transporte axonal retrógrado, onde podem realizar um ciclo lítico inicial, que logo em seguida é interrompido (latência). Portanto, na infecção aguda (fase lítica do ciclo) há grande expressão de todos os genes virais, com replicação do genoma e produção de progênie viral (Engels e Ackermann, 1996) enquanto que na latência, o vírus interrompe o ciclo replicativo, podendo permanecer inativo ou eventualmente ser reativado em situações de estresse ou tratamento com corticosteróides (Claus, Alfieri e Alfieri, 2002). Durante a latência não há expressão gênica significativa (Engels e Ackermann, 1996; Franco e Roehe, 2007).

A ausência de replicação viral resultará na ausência de sinais clínicos, tornando difícil a caracterização clínica da infecção pelo BoHV-5 (Engels e Ackermann, 1996; Franco e Roehe, 2007). Deste modo, animais infectados podem eventualmente re-excretar vírus e infectar outros animais do rebanho (Engels e Ackermann, 1996; Cascio *et al.*, 1999; Meyer *et al.*, 2001).

É indubitável que latência é uma grande vantagem evolutiva que os vírus da família *Herpesviridae* apresentam, pois permite ao vírus permanecer dentro das células infectadas numa taxa de replicação extremamente baixa, e deste modo, imperceptível ao reconhecimento pelo sistema imunológico. Deste modo, pode ser afirmado que o indivíduo infectado pelo herpesvirus bovino 5 que não morrer devido à infecção pelo herpesvirus permanecerá infectado pelo resto da vida (Cascio *et al.*, 1999; Del Medico Zajac, M. P. *et al.*, 2010). Porém, o conhecimento molecular de como ocorre este evento de entrar em latência e quais os mecanismos envolvidos no momento em que o vírus sai deste estado de latência não foram completamente elucidados.

Os genes e as respectivas proteínas produzidas pelo BoHV-5 podem ser classificadas como enzimas que regulam o metabolismo de ácidos nucléicos, fatores que modulam a resposta imune e como glicoproteínas virais (Del Medico Zajac *et al.*, 2010). Como descrito anteriormente, a maioria dos estudos realizados sobre funções de genes e proteínas do BoHV-5 recai sobre função de glicoproteínas.

As glicoproteínas apresentam funções quanto a adsorção viral, penetração do vírus na célula, egresso do vírus e transmissão viral entre células (Engels e Ackermann, 1996). A glicoproteína gE é importante para a transmissão do BoHV-5 entre neurônios

e neurovirulência do vírus (Chowdhury, Lee, Ozkul*, et al.*, 2000; Al-Mubarak, Zhou e Chowdhury, 2004; Al-Mubarak *et al.*, 2007), mas não é fundamental para a entrada do vírus pela via olfatória em coelhos (Chowdhury, Lee, Ozkul*, et al.*, 2000). A gI parece não ser importante para a neuroinvasividade em coelhos e aparentemente a gE pode manter essa função na ausência da gI (Al-Mubarak e Chowdhury, 2004). Cepas de BoHV-5 com deleção para o gene responsável por codificar a gE apresentaram comportamento atenuado e diminuição na capacidade de infecção, estabelecimento e reativação de latência em bovinos (Santos *et al.*, 2011).

A glicoproteína C apresenta diferença entre o BoHV-5 e BHV-1 (Chowdhury, 1995) o que reflete no fenótipo de ligação a heparina distinto entre os vírus sendo uma possível explicação para a modulação da infecção do SNC pelo BoHV-5 (Liman *et al.*, 2000). Além disso, a glicoproteína C (gC) tem importância no neurotropismo e na neurovirulência do BoHV-5, porém não é essencial para esta última, visto que coelhos inoculados com BoHV-5 com deleção da gC deletada foram capazes de invadir o SNC e causar doença neurológica em coelhos (Chowdhury, Lee, Onderci*, et al.*, 2000).

A glicoproteína US9 é essencial para a neurovirulência, pois está diretamente ligada a capacidade de movimentação anterógrada desde a mucosa olfatória até o bulbo olfatório. Quando o gene US9 foi deletado, o BoHV-5 infectou as células epiteliais, mas não conseguiu chegar ao SNC, não causando doença. No entanto, este mesmo vírus, ao ser injetado diretamente no bulbo olfatório, foi capaz de causar doença de modo semelhante ao vírus selvagem (Chowdhury, S.I. *et al.*, 2002). Entretanto, o US9 não é o fator determinante, ao menos sozinho, das diferenças relacionadas à neurovirulência do BoHV-5 e do BHV-1. Isto ficou claro ao inocular-se coelhos com BoHV-5 mutante contendo o gene US9 do BHV-1 causando doença neurológica nos animais estudados (Chowdhury *et al.*, 2006).

Quando foram deletados concomitantemente os genes que codificam as glicoproteínas gI, gE e US9, os vírus foram menos excretados e causaram menos doença comparados ao vírus selvagem na infecção experimental de coelhos (Silva *et al.*, 2009). A deleção dos mesmos genes alterou a capacidade do vírus em infectar, causar doença e ser reativado do estado de latência em bovinos (Hübner, Oliveira*, et al.*, 2005).

A virulência dos *alphaherpesvirus* depende da expressão adequada da timidina quinase, estando envolvida no metabolismo dos ácidos nucléicos (Silva *et al.*, 2010). A deleção deste gene no BoHV-5 resultou em atenuação do vírus e na

capacidade de estabelecer e reativar do estado de latência de modo eficiente durante infecção de bovinos (Santos *et al.*, 2011). Quando somadas, as deleções da gE e da timidina quinase, os efeitos de atenuação foram ainda mais intensos, não tendo havido estabelecimento e reativação de infeção latente nos bovinos estudados (Santos *et al.*, 2011). Em coelhos, a deleção destes mesmos genes causou resultados semelhantes aos descritos em bovinos (Silva *et al.*, 2010). Em ovinos, a deleção do gene responsável pela timidina quinase não impediu o BoHV-5 de causar infecção latente, mas alterou a capacidade do vírus em ser reativado da latência, pois este não foi encontrado nas secreções dos ovinos após o tratamento com dexametasona (Cadore *et al.*, 2013).

Diferenças moleculares entre a gH do BHV-1 e BoHV-5 foram demonstradas e foi levantada a hipótese de que possam estar relacionadas as diferenças de tropismo para o SNC apresentadas entre estas espécies intimamente relacionadas de herpesvírus bovino (Meyer, Bare e Thiry, 1999). Contudo, não foram realizados estudos *in vivo* para responder a esta hipótese.

Outros fatores de virulência tem sido estudados em outros herpesvirus como polimerase viral no herpesvirus equino 1 (EHV-1) (Nugent *et al.*, 2006). A mutação pontual na polimerase viral do EHV-1 está associada às cepas neurovirulentas (Nugent *et al.*, 2006). Até o momento a maioria dos genes do BoHV-5 não tem suas funções determinadas e geralmente faz-se a extrapolação da função de genes homólogos descritos em outros herpesvírus.

De modo semelhante pouco se sabe sobre os fatores do hospedeiro que estão relacionados à infeção pelo BoHV-5. Sabe-se que fatores como imunidade baixa ou sistema imunológico imaturo são importantes para a ocorrência de doença neurológica (Barros *et al.*, 2006; Del Medico Zajac *et al.*, 2010). No entanto, os mecanismos moleculares envolvidos continuam completamente desconhecidos.

Neste contexto, estudos de expressão de genes virais e de células hospedeiras tornam-se muito importantes para a melhor compreensão da fisiopatologia da infecção e possíveis mecanismos virais e celulares de resistência ou susceptibilidade (Flori *et al.*, 2008). Estudos foram realizados com outros herpesvirus, como o vírus da pseudoraiva (PrV), Herpesvirus humano 1 e 2 e vírus Varicella-zoster e também com Vaccinia vírus e o Poxvirus do macaco, buscando a compreensão global da expressão de genes celulares e virais (Ray e Enquist, 2004; Kennedy *et al.*, 2005; Aguilar *et al.*, 2006; Flori *et al.*, 2008; Rubins *et al.*, 2008; Nagel *et al.*, 2011). Com o advento das técnicas de sequenciamento de última geração tornou-se possível realizar estudos de expressão

global simultânea de genes virais e celulares pela técnica de RNA-seq, neste caso denominada Dual RNA-seq (Westermann, Gorski e Vogel, 2012).

2.2 Perspectivas de pesquisa

Estudos de expressão de genes tem sido realizados por PCR em tempo real, *microarrays*, SAGE, CAGE, ESTs e mais recentemente, pela técnica de RNA-seq. Cerca de uma década atrás, estudos de expressão gênica eram reservados à genética médica em humanos ou a modelos genéticos de estudos como camundongos, mosca da fruta e netamatódeos (Wolf, 2013). Para estes sistemas, *microarrays* e alguns sistemas de análises de expressão de genes eram as únicas ferramentas existentes para estudar as características do transcriptoma ou de padrões de expressão global de genes (Wolf, 2013). Além disso, estudos de expressão gênica eram restritos a análises de PCR apenas em pequena escala de genes candidatos, usando PCR em tempo real, utilizando sobre o uso de ligação cruzada entre espécies por meio de *microarrays* (Naurin *et al.*, 2008). Atualmente, o modo mais moderno de se realizar estudos globais de expressão gênica é o uso do RNA-seq, que consiste na preparação de uma biblioteca de mRNA que serve como molde para a formação do cDNA de fita dupla utilizado para realizar o sequenciamento de última geração. Após o sequenciamento, são formados milhões ou até bilhões de leituras (*reads*). Segundo esta técnica, quanto maior o número de leituras, maior é a expressão do gene naquele momento estudado. Com isso, pode-se ter uma visal global de expressão gênica e realizar estudos de possíveis padrões de expressão relacionados a tratamentos, a presença ou ausência de patógenos, etc. (Wang, Gerstein e Snyder, 2009; Oshlack, Robinson e Young, 2010; Ozsolak e Milos, 2011; Wolf, 2013).

Tendo em vista a carência de estudos de expressão gênica relacionados ao BoHV-5 e o possível impacto destes resultados no conhecimento da biologia molecular desta espécie de herpesvirus acreditamos ser de grande valia a realização do presente estudo. Conhecer melhor o comportamento molecular do vírus e da célula hospedeira infectada pode possibilitar o melhor entendimento de como o vírus causa lesão e morte celular, como a célula permanece infectada com o vírus em sua forma latente e como este percebe qual o momento de sair do estado de latência e entrar no ciclo replicativo novamente, gerando novas partículas virais. Com nosso estudo esperamos dar um primeiro passo para um melhor entendimento das interações vírus-célula em nível molecular e gerar novas perguntas e hipóteses para estudos posteriores.

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3. OBJETIVOS

3.1 Avaliar a expressão *in vitro* de genes celulares e do BoHV-5 por qPCR.

3.2 Avaliar a expressão diferencial *in vitro* de genes celulares e do BoHV-5 usando a técnica de RNA-seq.

CAPÍTULO 2

4. ARTIGO CIENTÍFICO 1

Artigo científico formatado de acordo com as normas do *Virology Journal*.

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

Abstract

Background: *Bovine herpesvirus* 5 (BoHV-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 viral (alpha, beta and gamma genes) and host cells genes (*GAPDH* and *18S*) during BoHV-5 infection.

Findings: three BoHV-5 genes (*bICP0*, *UL9*, *US4*) and one structural bovine cell gene have their expression accessed by qPCR. While BoHV-5 genes expression increased in the time course of infection, the GAPDH decreased, showing the effect of virus genes expression in bovine cells genes expression. 18S gene was constitutively expressed during all moments in the experiment.

Conclusions: our data clearly demonstrates that GAPDH should not be used as a reference gene in studies of BoHV-5 infection because it was influenced by viral replication. However, 18S rRNA was constitutively expressed and is recommended for normalization in BoHV-5-bovine-cells. mRNA expression of viral genes was not altered by multiplicity of infection (MOI). All viral genes demonstrated the same expression pattern and there was no difference of viral genes expression among the time course of infection. Our data show important differences comparing to classical studies regarding herpesvirus alpha, beta and gamma gene expression. However, different virus and techniques were applied in those previous studies and maybe it could explain the differences. More research is necessary to enhance our knowledge concerning the BoHV-5 biology and we believe that a global gene expression using RNA-seq *in vitro* and *in vivo* would be the next step in this concern.

Keywords: *Bovine herpesvirus* 5; quantitative PCR; viral gene expression; molecular biology.

Findings

Introduction

Bovine herpesvirus 5 (BoHV-5) is an Alphaherpesvirus (Franco e Roehe, 2007; Davison *et al.*, 2009; Del Medico Zajac *et al.*, 2010) that causes nonsuppurative meningoencephalitis mainly in young cattle. This disease occurs naturally in either outbreaks or isolated cases, and exhibits low morbidity and high lethality (Salvador *et al.*, 1998; Rissi *et al.*, 2006). BoHV-5 is important by leanding cattle deaths and as a differential diagnosis of as 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 e Roizman, 1974). In permissive cells, the process of *Human herpesvirus 1* (HHV-1) viral replication takes 18 to 20 h (Pellett e Roizman, 2007). The orchestrated gene and protein expression are crucial for viral replication after infection in epithelial cells of the nasal or vaginal mucosa (Bagust, 1972; Bagust e Clark, 1972; Padgett, Bailey e Sheridan, 2007; Van Lint e Knipe, 2009).

The classical herpesvirus cascade of alpha, beta and gamma gene expression was first studied in vitro using HHV-1, former-named Herpes simplex virus 1 (HSV-1), protein expression profile (Honess e Roizman, 1974). The expression peak of α genes occurs within 2 to 4 h post infection (p.i.) encoding proteins that regulate (Honess e Roizman, 1974) subsequent viral gene expression and contribute to evasion of the cellular responses to the infection (Van Lint e 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 e Roizman, 1974). In addition, expression of β genes induces viral DNA replication within the cell, and also downregulates α genes (Van Lint e Knipe, 2009). The expression of γ genes peaks at 6 h p.i. encoding viral structural proteins. However, it can be detectable at low levels at 3h p.i. and reaching maximum expression at 12 h p.i. (Nichol *et al.*, 1996). DNA replication stimulates expression of γ genes at high levels, whereas shut 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 e Knipe, 2009). Nevertheless, the current understanding of the biology underlying the complex life cycle of herpes simplex virus (HSV) is incomplete (Sawtell, Thompson e Haas, 2006).

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

The *UL9* gene is a β gene that encodes an origin-binding protein (Olivo, Nelson e Challberg, 1988). This protein is one of the seven proteins that are essential for the replication of HHV-1 DNA (Boehmer e 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 e Lehman, 1997; Lehman e Boehmer, 1999).

The *US4* is a γ gene that is responsible for transcribe the glycoprotein G (gG) (Engelhardt e 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 to facilitate viral cell-to-cell spread by maintaining the cell-to-cell junctions among the infected cells (Nakamichi, Matsumoto e Otsuka, 2002). Furthermore, this gG can interfere at different distinct stages of chemokine action and therefore constitutes yet another immunoevasion tool used by alphaherpesviruses (Van De Walle, Jarosinski e Osterrieder, 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 spatiotemporal expression patterns of BoHV-5 during viral replication remains poorly explored. In this context, research using molecular techniques to study viral genes expression is very important to elect possible pharmacological targets to eliminate viral replication. Besides that, molecular techniques could help us to figure out which host cell genes are up or down regulated in the presence of infection, and then which molecular functions and biological process are affected, and how the herpesvirus interacts with host cell in latency. In the present study, we used RT-qPCR to characterize the in vitro spatiotemporal expression profile of crucial BoHV-5 and cellular genes.

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 of three BoHV-5 genes (*bICP0*, *UL9*, and *US4*) and two cellular genes (*GAPDH* and *18S*) using RT-qPCR. 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 24 h prior use, and infected at 80-90% confluence. The MDBK monolayers were infected at multiplicity of infection (MOI) of 0.75-1 or 7.5-10. The inoculum was maintained into the culture for one 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 caried 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. 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 *GAPDH* 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 BiosystemsTM), and analyzed using the comparative Ct method (2^{-}

$^{\Delta\Delta Ct}$ method) (Livak e Schmittgen, 2001). In brief, 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 15s and 60 °C for 1 min, followed by a melting curve. Also, negative controls were included in triplicate in each plate. Standard curves were used to test primers' efficiency. Data analysis was performed by normalizing amplification values of the target genes with the endogenous control. We included one sample (6 h p.i.) to serve as calibrator sample to certificate that efficiency was the same among qPCR plaques.

Data were analyzed in a completely randomized design with repeated measures. The gene expression data were 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

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 with 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 *GAPDH* and *β-actin* vary by several orders of magnitude during spatiotemporal the HSV-1 infection whereas *18S rRNA* were constitutively expressed (Nystrom *et al.*, 2004). *GAPDH* mRNA levels were drastically reduced during infection with HHV-1 infection of HeLa and HEL cells due virion host shutoff (VHS) protein, product of *UL41* gene (Hsu, Saffran e Smiley, 2005). The VHS is a 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 spares other viral mRNAs (Weir, 2001; Van Lint e Knipe, 2009; Shu *et al.*, 2013).

Therefore, *18S rRNA* is a reliable constitutive gene for monitoring viral and cellular gene expression in BoHV-5-infected cells while *GAPDH* 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 evaluate 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 higher expressed in all moments when compared to the moment 0 h p.i ($p<0.01$). In our study, Accordingly with classical data (Honess e Roizman, 1974), *ICP0* (bICP0 homologous), *UL9* and *US4* proteins could be detected at 6 h p.i., which corroborates with our mRNA results.

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 12h p.i (Honess e 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 7h 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, they have high level of aminoacid identity in their protein repertoires (average, 82%) (Delhon *et al.*, 2003), which should explain they 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 0h p.i.. Previous studies have demonstrated that *UL9* gene expression in HHV-1 infection begins at 1.5h 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 previously studies (Durmanova *et al.*, 2001; Režuchová *et al.*, 2003) because it was not presented.

US4 transcripts were upregulated from 6 to 24 h p.i. ($p<0.001$) compared with 0h p.i. Late gene expression in 1 h pi. (UL49.5 gene) and 8 to 12h 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 two hours after the infection of MDBK cells by BoHV-5 (Meyer, Bare e Thiry, 1999). The *UL27* gene (Rafield e Knipe, 1984), and *ICP5*, *US6* and *ICP34.5* genes (Van Lint e Knipe, 2009), all late genes, were also expressed early in infection.

Finally, we believe 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 the exactly moment at which gene starts to be differentially expressed (DE). Moreover, in a second moment a RNA-Seq study would also be very informative, mainly regarding to host differential expressed (DE) genes and the molecular and cellular process that are modified by virus replication. In a third moment, we need to perform these DE experiments in an animal model 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. As the *bICP0* gene was expressed constitutively as previously described to BoHV-1 and maybe it could be used in future *in vitro* studies as a reference gene to the virus, but more studies are needed to prove this. The *18S* was a good reference gene to our study while the *GAPDH* clearly was not. The differences observed in the present study between the expression pattern of key BoHV-5 and HHV-1 genes published 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 specie or because different techinques were used. We consider that more research using modern techniques as quantitative PCR, microarrays and RNA-Seq are necessary to amplify the current knowledge about molecular biology of BoHV-5 to better understand how this virus replication affects the host cells and how they communicate with each other.

Abbreviations

BoHV-5 – Bovine herpevirus 5;

MOI - multiplicity of infection.

Competing interests

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

Authors' contributions

All authors read and approved the final manuscript.

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Illustrations and figures

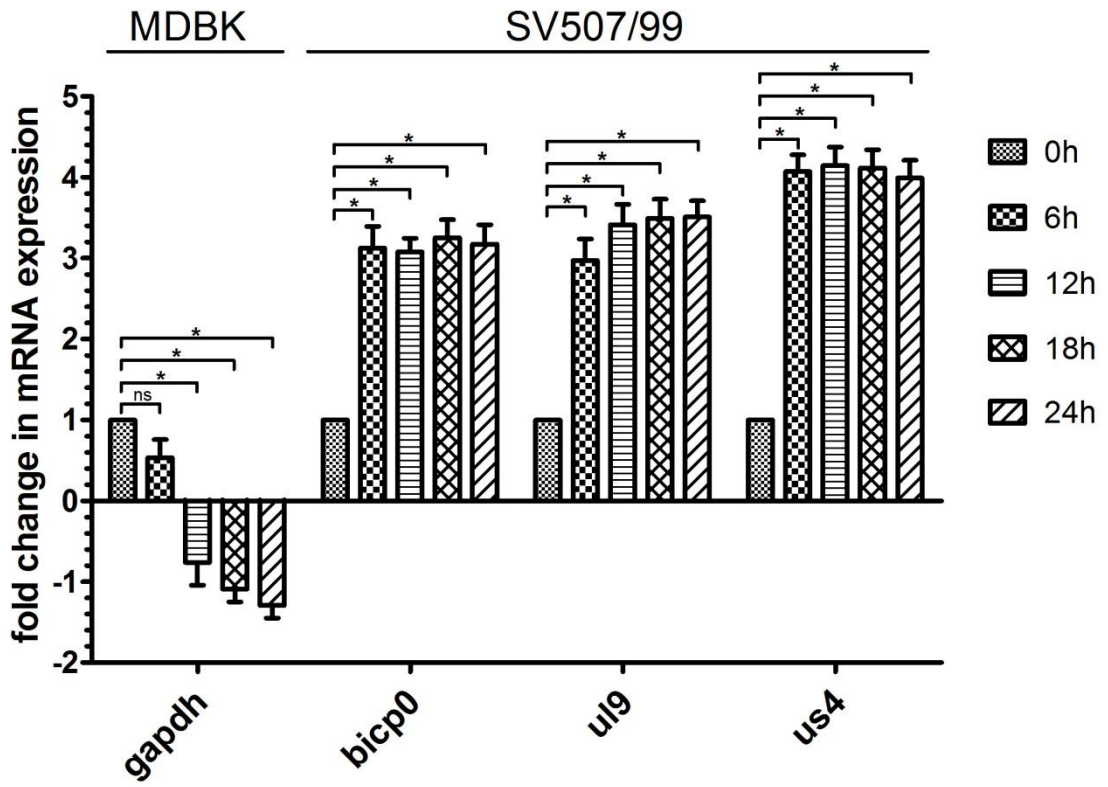

Figure 1. Relative mRNA expression of viral (*bICP0*, *UL9*, and *US4*) and cellular (*GAPDH*) genes during 24 hours of the BoHV-5 (strain SV507/99) infection in MDBK cells. The 18S gene was used as a reference gene to calculated the relative mRNA expression. The mean values with standard deviation are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

Tables and Captions

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

Primer	Sequences (5' - 3')	Produto (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 92% to 95%.

Capítulo 3

5. ARTIGO CIENTÍFICO 2

Artigo científico formatado de acordo com as normas do *BMC genomics.*

Transcriptome analysis of bovine cells infected by BoHV-5

Abstract

Background

Bovine herpesvirus 5 (BoHV-5) is associated to non-suppurative meningoencephalitis, being an important cause of neurological disease of cattle in South America. This DNA virus has been studied for years regarding to epidemiology, pathogenesis, immune response and genes function aspects. However, the molecular mechanisms that underlie the pathogenesis of the disease remain poorly understood. In this study, a deep sequencing of RNA library was used to study gene expression profile of infected MDBK cell at different times after BoHV-5 infection. This study demonstrated the complexity of virus-host cell interactions; adding new data for host cells mRNA responses to viral infection and speculating possible genes as candidates for future studies.

Results

A total of approximately 178 million of reads were generated from Illumina Sequencing comprising both control and infected samples. After trimming for adapter sequences and sequences smaller than 20bp, a total of approximately 143 million of reads were then used for the analysis of differentially expressed genes. The moments 1h, 6h and 24h pi presented 937, 1164, and 969 differentially expressed genes respectively. These up- and down-regulated genes were primarily involved in cell cycle, interleukin production, inflammatory cells chemotaxis, DNA damage and repair, response to virus, apoptosis, oxidation-reduction process, and ubiquitination pathways.

Conclusion

In this study, we demonstrate the data from transcriptome of MDBK cells at 1h, 6h and 24h after BoHV-5 infection. A large number of genes were differentially expressed under BoHV-5 infection and annotated to Biological Process, Molecular Function, and Cellular Component. Moreover, the present data shows host cell gene expression unrecognized until now for BoHV-5 infection *in vitro*.

Background

Bovine herpesvirus 5 (BoHV-5) is an enveloped double-stranded DNA alphaherpesvirus, which is associated to non-suppurative meningoencephalitis in cattle. The disease affects mainly young animals under stressful conditions with no seasonal pattern or predilection for breed or gender (Barros *et al.*, 2006).

Studies investigating the transcriptional modifications on host cells during herpesvirus infection are imperative to better understanding the disease's pathogenesis, susceptibility, and resistance (Flori *et al.*, 2008). Previous studies have used BoHV-5 as a model to evaluate herpesvirus infection either *in vitro* using Madin-Darby Bovine Kidney (MDBK) cells (Meyer, Bare e Thiry, 1999) and Bovine Wharton's jelly-derived multipotent mesenchymal stromal cells (bWJ-MSCs) (Garcia *et al.*, 2013), or *in vivo* using rabbits (Flores *et al.*, 2009), calves (Santos *et al.*, 2011) and sheep (Cadore *et al.*, 2013).

Gene expression studies were traditionally produced using real time PCR (qPCR) and microarrays platforms. A decade ago studies of gene expression used to be more restrict to genetic model organisms, like mouse, fruit fly and nematodes, and human genetics (Wolf, 2013). For these organisms microarray was the best system to study transcriptomes (Wolf, 2013). Deep sequencing platforms have changed this reality and make possible to study non-models organisms (Wolf, 2013). This approach is currently considered the best cost-benefit systems to run a transcriptome analysis. This technique principle is the sequencing of double-strand DNA libraries constructed from total RNA or mRNA libraries. After sequencing, millions or billions of reads are generated, mapped to a reference genome or to *de novo* sequence. After normalization procedures the number of reads mapped to each gene is compared in the analysis of differentially expressed genes (Wolf, 2013). This method of analyze gene expression of transcriptomes is denominate RNA sequencing (RNA-seq) (Wang, Gerstein e Snyder, 2009).

Studies on global gene expression were described for other herpesvirus using microarray data of Pseudorabies virus (PrV), Human herpesvirus 1 and 2 (HHV-1 and HHV-2), (Ray e Enquist, 2004; Kennedy *et al.*, 2005; Aguilar *et al.*, 2006; Flori *et al.*, 2008), or using multiplex reverse transcription (RT)-PCR to Varicella Zoster Virus (VZV) (Nagel *et al.*, 2011). Recently, RNA-seq studies were published for Epstein-Barr virus (EBV) (Lin *et al.*, 2010), murine herpesvirus 68 (MHV68) (Clyde e Glaunsinger,

2011), VZV (Jones *et al.*, 2014), and Spring viraemia of carp virus (SVCV) (Yuan *et al.*, 2014).

Although the prevalence of BoHV-5 infection is important in cattle, especially in South America, studies describing gene expression profile on host cells during BoHV-5 infection have not yet been reported. The present study reports the application of the deep sequencing of cDNA libraries of BoHV-5-infected and non-infected MDBK cells transcriptome with special emphasis on differential expression (DE) analysis.

Results

Transcriptome analysis

A total of approximately 178 million of reads were generated from Illumina Sequencing comprising both control and infected samples. After trimming for adapter sequences and sequences smaller than 20bp, a total of approximately 143 million (Table 1) of reads were then used to DE analysis. From the total of 26,065 transcripts (mRNA) of the bovine genome, we found 937, 1164, and 969 DE genes (DEGs) in the moments 1h, 6h, and 24h pi, respectively (Table 2).

Table 1. Results of RNA-seq analysis using CLC Genomics Workbench*

Features	1h pi	6h pi	24h pi	Control	Total
Total reads	60,408,084	51,333,198	37,063,960	29,938,504	178,743,746,00
After trimming	47,789,043	40,642,347	29,253,562	25,344,925	143,029,877,00
Average length	101	106	115	118	110.00
Average after trim	86	101	106	111	101.00

* bp = base pairs; Removal of low quality sequence. (limit = 0.05). Removal of ambigious nucleotides: maximal 2 nucleotides allowed. Removal of sequences on length: minimum length of 20 nucleotides. Removal of Illumina adaptors.

Table 2. Differentially expressed genes of MDBK cells during the time course of BoHV-5 infection. Values indicate number of DE genes in the Baggerley's test with Bonferroni corretion ($p \leq 0,05$) after passing cutoff of fold change >2.0, False Discovery Rate (FDR) <0.05, RPKM values ≥4.0.

	1h pi	6h pi	24h pi
Up-regulated	506	575	527
Down-regulated	431	589	442
Total	937	1164	969

Kinetics and Functional annotation of the DEGs

Using the CLC Workbench software, we verified the GO terms related to DE genes according to their Biological Process (BP), Molecular function (MF), and Cellular compounds (CC). Crossing GO terms of the down- and up-regulated DE genes during infection, we observed that 246, 256, and 164 genes were exclusively down-regulated, and 401, 383, and 376 genes were exclusively up-regulated at 1h, 6h, and 24h, respectively (Figure 1). There were 82 genes that were still down-regulated between 1h and 6h pi, whereas 175 remained reduced from 6h pi to 24h pi, and 27 genes were reduced at 1h and 24h pi. Seventy-six genes were down-regulated all over the time of course infection (Table 3, supplementary information).

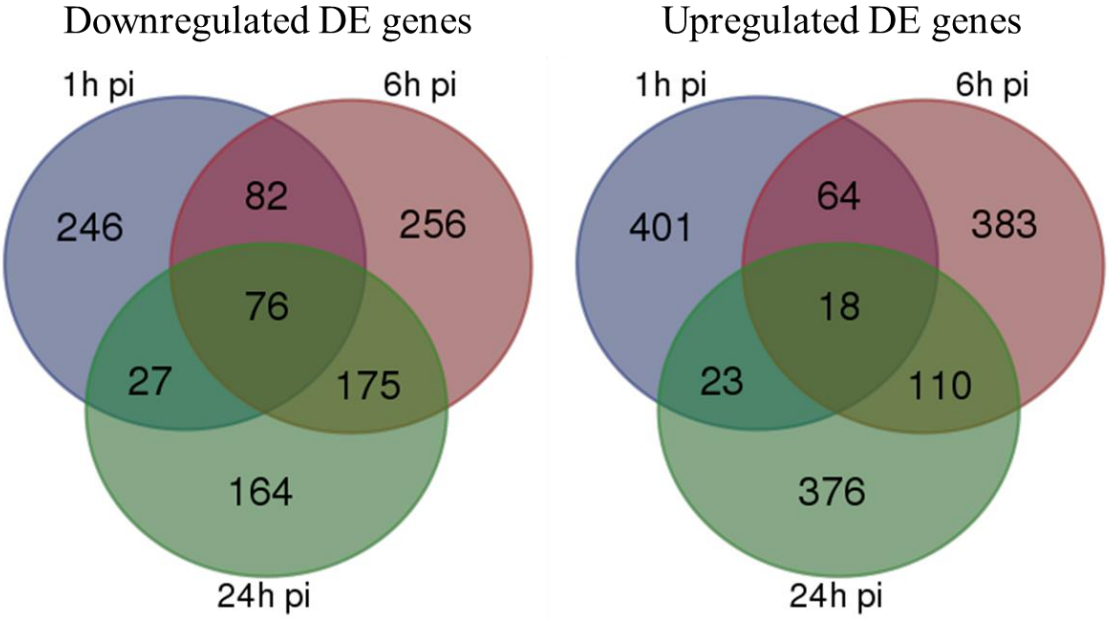

Figure 1. Crossing GO terms of the DE genes during the spatiotemporal expression pattern. We can observe up and down-regulated genes that are exclusive to each time post infection and the number of genes that remained up or down-regulated during the moments.

Among the up-regulated genes, 64 genes were up-regulated at 1h and 6h pi, 110 at 6h to 24h pi, and 23 between 1h and 24h pi. Only 18 genes had increased expression among the evaluated time points of infection (Table 4). The majority of those genes were associated with apoptotic process, protein ubiquitination, cell cycle as poly(A)+ mRNA export from nucleus, transcription from RNA polymerase II promoter, gluconeogenesis, and regulation of transcription and transduction. The expression levels of 300 genes were increased more than 4 times, and 60 genes were increased more than

16 times. Regarding to down-regulated genes, the expression of 222 genes were decreased more than 4 times, and 40 genes were decreased more than 16 times.

We filtered the up- and down-regulated genes by GO terms related to biological functions: apoptosis, viral response, oxidative-reduction process, and immune response. The majority of GO terms were related to the cell cycle, interleukin production, inflammatory cells chemotaxis, DNA damage and repair, response to virus, apoptosis, oxidation-reduction process, and ubiquitination activities.

Apoptosis

Considering the up-regulated DEGs, 108 genes presented GO terms related to apoptosis considering the 3 times of infection while 112 genes were related to apoptosis in the down-regulated DEGs (Table 5 and 6, supplementary information). From these, 5 apoptotic DEGs were down-regulated over the entire course of infection as follow: ASNS, ITGA6, ITGB1, PARK7 and VCP. The CLU and RBM5 genes were the up-regulated genes related to apoptosis during the 24 hours of infection. DIABLO and MCL1 gene, two important apoptotic genes, were up-regulated only at 6h and 24h pi respectively. Thirteen apoptotic DEGs changed their expression during the time course of infection, while 8 genes were up-regulated and became down-regulated and 5 did the opposite (Table 7). Only 4 of these genes were expressed in all moments of infection where the PRDX5, DYNLL1, and CD74 were up-regulated at 1h pi and turn to down-regulated at 6h and 24h pi. The PDCD10 gene was down-regulated at 1h and 6h pi and up-regulated at 24h pi.

Viral response

Besides that, we found 38 genes that were up- or down-regulated regarding to viral response (Table 8, supplementary information). From these, 8 DEGs were also related to apoptosis and 12 to immune response. The BAD, BCL3, C1QBP, FADD, and ITGB1 genes were related to apoptosis, immune response, and viral response. Five genes were down-expressed in all moments of infection as following: DYNLL1, HMGA1, ITGB1, PPIA and PSMA2.

Oxidative-reduction process

A total of 55 DEGs related to oxidative-reduction process were up- or down-regulated (Table 9 supplementary information). Thirty seven genes were also related to apoptosis and 23 to immune response. Six DEGs related to oxidative process were down-regulated all over the time course of infection as follow: LOX, PSMB5, IMPDH2, PARK7, RRM1, and LDHB.

Immune response

Immune response GO terms were associated with 149 DEGs, where 53 were also associated to apoptosis and 16 to oxidation-reduction process. Only PSMA6, PARK7, ITGA6, IL6ST, IMPDH2, and CD74 genes were expressed in all moments of infection. Most part of the genes was associated with inflammatory cells chemotaxis, interleukin production, interferon, immunoglobulin, immune response, regulation of NF-kappaB transcription factor, and inflammatory response.

Discussion

BoHV-5 is an important causative organism of bovine meningoencephalitis in cattle mainly in South America (Rissi *et al.*, 2008). Although the epidemiological and pathological features of the BoHV-5 infection have been studied, there is no study investigating the impact of the virus on bovine cell genes expression during infection using deep sequencing techniques such as Illumina technology (MiSeq and HiSeq). Using this approach, we observed 937, 1,164 and 969 DEGs at the moments 1h, 6h and 24h pi, respectively. This strategy is interesting because allowed the identification of candidate genes modulated on the host during virus-bovine cell interactions more efficiently when compared to qPCR approach. RNA-Seq analysis is a quantitative technique and eliminates the need of qPCR validation (Westermann, Gorski e Vogel, 2012) allowing to study the differential expression of hundreds of host cell genes at the same time using the same infected culture plate and, therefore, reducing bias.

It is important to note that the present study has some limitations and should be carefully interpreted. First, infinite fold change expressions were not presented in the present study because we excluded samples with normalized RPKM lower than 4, avoiding an analytical error. Second, the increase or decrease in interleukins transcripts has no biological significance because MDBK are not inflammatory cells. In the present

study, the L6ST gene was marked down-regulated during herpesviral infection. The protein encoded by IL6ST gene is a signal transducer activated by many cytokines, including interleukin 6 (IL6) (Taga e Kishimoto, 1997). However, unlike other cytokines that mainly trigger hematopoietic and lymphoid cells responses, IL6 interacts with several cells, e.g. cardiomyocytes and neurons, and is ubiquitously expressed in those tissues (Taga e Kishimoto, 1997). The IL6 is a hepatocyte-stimulating factor and induces the expression of several acute phase proteins (Taga e Kishimoto, 1997). Therefore, it is reasonable to think that IL6ST down-regulation during BoHV-5 infection might be an evolutionary advantage for viral survival into the host cells impairing pro-inflammatory response stimulation.

Several genes were down-regulated and remained reduced all over the infection. The ITGA6 gene encodes the integrin alpha chain alpha 6, which is a cell-surface protein. Integrins are receptors used for viral entry into host cells (Franco e Roehe, 2007; Van De Walle *et al.*, 2008) and might be modulated by herpesvirus molecules (Bowles *et al.*, 2012). *In vitro* infection of Varicella Zoster virus (VZV) down-regulates both ITGA6 mRNA and protein expression (Bowles *et al.*, 2012). It might be speculated that the observed down-regulation of ITGA6 is a host cell response to virus entry, because as less membrane receptors the cell presents less would be the chance of new viral particles to enter. On the other hand, it might be the reflection of viral molecules modulating the host cell ITGA6 transcripts. Also, another integrin family member - the ITGB1 gene - was down-regulated throughout the experiment.

PARK7 gene protects cells against oxidative stress and cell death. Previously, defects in this gene were associated with the autosomal recessive early-onset of Parkinson disease (Meulener *et al.*, 2006). This gene was down-regulated at 1h to 24h pi in the present experiment. PARK7 gene may also function as a redox-sensitive chaperone, as a sensor for oxidative stress, and apparently protects neurons against oxidative stress and cell death (Meulener *et al.*, 2006). Therefore, decreased expression of this gene might be associated with neuron death during BoHV-5 infection.

Previously, it was demonstrated that down-regulation of ATF3 gene expression inhibited apoptosis induced by phosphatidylinositol 3 kinase signaling pathway in HCT-116 cells (Yamaguchi *et al.*, 2006), whereas ATF3 upregulation activated p53 by blocking its ubiquitination (Yan *et al.*, 2005). In the present study, ATF3 gene expression was up-regulated in all sampling times and might have contributed to up-

regulate TP53BP2 at 6h pi, a gene that stimulate the apoptotic function of p53 (Samuels-Lev *et al.*, 2001). On the other hand, overexpression of ATF3 in macrophages reduces the induction of IFNβ and increases viral replication (De Nardo *et al.*, 2014). The ATF3 gene was one of the 20 most up-regulated genes in Crandell Rees Feline Kidney (CRFK) cells infected with Feline Infectious Peritonitis virus (FIPV) at 3 h pi (Harun *et al.*, 2013). The RBM5 gene may both positively and negatively regulate apoptosis by regulating the alternative splicing of several genes involved in this process, including FAS and CASP2/caspase-2 (Sutherland *et al.*, 2005). RBM5 was up-regulated in all moments in our study, as it was CASP8, an apoptotic gene.

PRDX5, DYNLL1, and CD74 genes were transiently up-regulated at 1h pi and were down-regulated thereafter. On the other hand, PDCD10 gene was down-regulated at 1h pi and turned out up-regulated at 6h and 24h pi. PRDX5 has been associated to antioxidant protective mechanisms and in signal transduction in cells (Declercq *et al.*, 2001). In humans, PRDX5 alterations have been implicated in many diseases such as cataract formation, ischemic heart diseases, cancers, AIDS, complications of diabetes, and hypertension (Maulik e Das, 2010). PRDX5 down-regulation due to BoHV-5 infection may be associated to the increase in oxidative stress and neurodegeneration as it has been suggested in Alzheimer's disease were the PRDX5 down-regulation may be, in part, involved in this process (Lovell *et al.*, 2000). DYNLL1 is an pro-apoptotic protein and its down-regulation prevent p53 nuclear accumulation (Lo *et al.*, 2005), preventing host cell death, and may be it could represent an advantage to BoHV-5 replication. The CD74 gene is associated with class II major histocompatibility complex (MHC), regulates antigen presentation, act as cell surface receptor for the cytokine macrophage migration inhibitory factor (MI), regulates B-cell differentiation, and have been implicated in several types of cancer (Burton *et al.*, 2004; Starlets *et al.*, 2006). CD74 gene downregulation could be a consequence of BoHV-5 modulation to escape from host immune system response. PDCD10 gene is associated with Cerebral Cavernous Malformation 3 in humans and it presents a pro-apoptotic function in the cell culture models (Chen *et al.*, 2009). May be the PDCD10 up-regulation in BoHV-5 infection could be associated to MDBK cell death *in vitro*. More studies at 36, 48 and 72h pi – when cytopathic effect is present (data not show) – could confirm if PDCD10 gene still up-regulated.

We observed 112 genes associated to GO terms that include apoptosis, but only the ASNS, ITGA6, ITGB1, PARK7, and VCP genes were down-regulated throughout the experiment. ASNS gene negatively regulates apoptosis in pancreatic cancer cells treated with either glucose or cisplatin (Cui *et al.*, 2007). Down-expression of ASNS mRNA transcripts in BoHV-5-infected MDBK cells may be due viral proteins interactions or, alternatively, an attempt of the host cells to promote apoptosis preventing viral replication. VCP gene positively modulates apoptosis and various cellular processes, such as protein degradation, membrane fusion and chaperone activity (Braun e Zischka, 2008). Therefore, VCP gene down-regulation might be an important host cell mechanism promoting cell death during BoHV-5 infection.

A previous study demonstrated that activation of caspase-8 and caspase-9 were implicated in apoptosis during BoHV-1 infection of MDBK cells (Xu *et al.*, 2012). In our study, caspase-8 mRNA expression increased 27.8 fold at 24h pi suggesting it might also be important in BoHV-5-induced apoptosis in MDBK cells. Caspase-1 and caspase-3 were down-regulated, while caspase-7 was up-regulated in porcine epithelial cells infected with Porcine Pseudorabies virus, which is also an Alphaherpesvirus (Flori *et al.*, 2008).

BoHV-1 infection in MDBK cells enhances p53 protein expression resulting in host cell death and virus release (Devireddy e Jones, 1999). In the present study, the TP53BP2 gene was up-regulated at 6h pi suggesting it might contribute to programmed cell death during BoHV-5 infection. The TP53BP2 gene encodes a protein that stimulates the apoptotic function of p53. BoHV-1 infection slightly increase the p53 mRNA expression at 24h pi in MDBK cells (Devireddy e Jones, 1999). Similarly, p53 mRNA was not up-regulated in the present study. BoHV-5 up-regulates BCL2 (anti-apoptotic) and down-regulates BAX (apoptotic) genes 24-48h pi in bWJ-MSCs cells, indicating that BoHV-5 postpones cell death to elevate viral titer. However, this event could not be evaluated in our study due the different times of infection. CSK2 gene, an anti-apoptotic gene, was down-regulated in our study as well as in with CRFK cells infected with FIPV after 3 hours of infection (Harun *et al.*, 2013). In the same study, the CRFK cells down-expressed ID1 gene (anti-apoptotic activity, TGF-beta signaling pathway) while in BoHV-5 infection the MDBK it was up-regulated at 1h hour and became down-regulated at 6h pi.

Interferon-induced GTP-binding protein Mx1 encoded by MX1 gene was up-regulated at 6h pi corroborating with MX1 mRNA in CRFK cells infected with FIPV after 3 hours of infection (Harun *et al.*, 2013). IFN-α stimulates the expression of MX1 mRNA and proteins inhibiting viral replication (Haller, Staeheli e Kochs, 2007; Hoenen *et al.*, 2007). However, virus have also evolved to escape from this IFN system by trying to go undetected, suppressing IFN expression, blocking IFN signaling. (Haller, Kochs e Weber, 2007). HSV-1 infection induces host cells to produce an MxA protein isoform, which in turn stimulates HSV-1 replication (Ku *et al.*, 2011). It is possible that BoHV-5 might also induce MxA protein expression since MX1 mRNA gene is up-regulated. However, only future studies could identify if this phenomena is shared between these two related herpesvirus.

Methods

Cells

MDBK cells were infected with SV-507/99 strain of BoHV-5 (Delhon *et al.*, 2003) to construct the mRNA library. MDBK cells were seeded into flasks 24h prior use, maintained in Minimum Essential Media (MEM) supplemented 10% Fetal Bovine Serum (FBS), and infected (m.o.i of 7.5-10) at approximately 80-90% confluence. The inoculum was maintained in contacted with the cells for one hour to properly adsorb to the cells. Then, the viral particles were washed out from the culture using MEM. This time point was considered 0 hour (h) post-infection (pi). Infected MDBK cells monolayers were harvested at 1h, 6h, and 24h pi. Also, mock infected MDBK monolayers were harvested at 6h and 24h for control purposes. All experiments were performed in triplicates.

Total RNA extraction, library construction, and sequencing

Total RNA was recovered using the Total RNA Purification Kit (NorgenBiotek Corp.) according to the manufacturer's instructions. RNA quality was verified using the Bioanalyzer instrument (RNA 6000 NanoLabChip kit/ 2100 Agilent Technologies), whereas concentration was accessed using Qubit Quantitation Platform (Invitrogen). Samples were immediately frozen at -80 °C until their using. Before cDNA library preparation, mRNA was isolated from total RNA using GenElute™ mRNA Miniprep

Kit (Sigma-Aldrich) according to the manufacture's recommendations. All samples were treated with RQ1 RNAse-Free DNase (Promega) to eliminate any trace of genomic DNA.

Single strand cDNA was synthetized using ImProm-II™ Reverse Transcription System™ (Promega, USA). To confirm positivity of samples to BoHV-5 or MDBK cells gene expression, we used a previously standardized qPCR to amplify BICP0, US4, and UL9 viral genes, and 18S and GAPDH host cell genes (data not showed). Then, double strand cDNA (dsDNA) synthesis was performed according Gubler e Hoffman (1983), with some modifications. The Agencourt AMPure XP kit (Beckmann & Coulter) was used to separate only dsDNA. The first two replicates were submitted to Nextera® XT DNA (Illumina, Inc., San Diego, USA) to library preparation and sequenced with MiSeq® System (Illumina, Inc., San Diego, USA) in the Univ. Estadual Paulista (UNESP), Laboratory of Animal and Human Virology, and Immunology. The last replicate was its transcriptome sequencing performed by LC Sciences as described by Battich, Stoeger e Pelkmans (2013). Briefly, sample preparation was done using the TruSeq RNA Sample Prep Kit v.2 (RS-122-2001, Illumina) as specified by the manufacturer. Enrichment for polyadenylated RNA was done using poly (T) beads, and cDNA was obtained from random primers after RNA fragmentation. Sequencing was done using a HiSeq 2000 sequencer from Illumina.

The total number of reads was imported into the CLC bio Genomic Workbench (GWB). The sequences were trimmed for adapter sequences, low quality base, and only sequences greater than 20bp were accepted (Harun *et al.*, 2013). During mapping, the parameters were set to 95% to align to the reference transcriptome and a maximum of two mismatches were allowed. The total mapped reads results for each transcript were analyzed, and then normalized to Reads Per Kilo base per Million (RPKM). After statistical analysis using Baggerley's test, the data was further normalized using CLC tool to avoid bias related to different number of reads among the samples. Baggerley's test was used to access the differential expressed genes (DEGs) based on False Discovery Rate (FDR) <0.05, RPKM values ≥ 4.0, fold change >2.0 and Bonferroni correction ($p \leq 0,05$). We also prepared a list of GO terms and merge with our bovine gene list. This allowed us to export our lists (xlsx format) of DEGs with the respective GO terms for Biological Process, Molecular Function and Cellular components. Only

significant expressed genes were submitted to pathway analysis using Blast2GO software.

Conclusion

In this study, we demonstrate the data from transcriptome of MDBK cells at 1h, 6h and 24h after BoHV-5 infection. A large number of genes were differentially expressed under BoHV-5 infection and annotated to Biological Process, Molecular Function, and Cellular Component. Moreover, the present data shows host cell gene expression unrecognized until now for BoHV-5 infection *in vitro*. These findings could bring to light new insights about biological processes that are changed by BoHV-5 infection and warrant for further experimentation. In the future, an *in vivo* experiment, using rabbits or cattle, could also generated new ideas about Host cell and BoHV-5 molecular interactions using RNA-Seq. This approach seems particularly interesting to find out how BoHV-5 and host cells interact during latency and reactivation.

Abbreviations

DEGs (differentially expressed genes);

Competing interests

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

Authors' contributions

All authors read and approved the final manuscript.

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Table 3. List of DE genes down-regulated in all 3 moments of infection (1h, 6h, 24h pi) with respective GO Biological process terms and fold change.

Abrr.	Fold change	Biological process
TBCB	-46.69	cell differentiation; nervous system development; post-chaperonin tubulin folding pathway meiosis I;
CKS2	-37.94	regulation of protein kinase activity
ASNS	-29.89	glutamine metabolic process; L-asparagine biosynthetic process; positive regulation of mitotic cell cycle wound healing
DCBLD2	-21.04	cell adhesion; negative regulation of cell growth
IL6ST	-13.31	negative regulation of interleukin-6-mediated signaling pathway; interleukin-11-mediated signaling pathway; positive regulation of T cell proliferation; interleukin-27-mediated signaling pathway; glycogen metabolic process
SRSF1	-11.58	regulation of mRNA stability; regulation of transcription, DNA-dependent; mRNA 5'-splice site recognition ; mRNA transport
ITGA6	-7.71	cell-matrix adhesion; positive regulation of transcription from RNA polymerase II promoter; positive regulation of cell-cell adhesion; integrin-mediated signaling pathway; positive regulation of phosphorylation; positive regulation of apoptotic process; leukocyte migration
BCCIP	-6.14	DNA repair; cell cycle; neuroendocrine cell differentiation
TAF9	-5.91	positive regulation of transcription from RNA polymerase II promoter; DNA-dependent transcription, initiation
IMPDH2	-5.64	cellular response to interleukin-4; oxidation-reduction process; lymphocyte proliferation
SNRPA1	-5.54	associated with sn-RNP U2. It helps the A' protein to bind stem loop IV of U2 snRNA
DNAJC8	-5.12	------
CPXM2	-4.95	cell adhesion; proteolysis
SPP1	-4.61	ossification; response to vitamin D; neutrophil chemotaxis; cell adhesion
ACOT7	-4.56	coenzyme A biosynthetic process; long-chain and medium-chain fatty-acyl-CoA catabolic process
PPA2	-4.18	dephosphorylation
HMGA1	-4.14	base-excision repair; positive regulation of transcription, DNA-dependent; response to virus

SHMT2	-3.91	L-serine metabolic process; tetrahydrofolate interconversion; glycine metabolic process
HDLBP	-3.79	Appears to play a role in cell sterol metabolism. It may function to protect cells from over-accumulation of cholesterol
SAE1	-3.66	protein sumoylation; regulation of mitotic cell cycle; protein sumoylation
FCF1	-3.64	rRNA processing
CDH17	-3.61	oligopeptide transmembrane transport; homophilic cell adhesion; calcium-dependent cell-cell adhesion
GUK1	-3.57	nucleotide phosphorylation; purine nucleotide metabolic process
PTGES3	-3.56	glucocorticoid receptor signaling pathway; chaperone cofactor-dependent protein refolding; prostaglandin biosynthetic process; RNA-dependent DNA replication; glycogen biosynthetic process
PSMA2	-3.52	response to virus; ubiquitin-dependent protein catabolic process
ITGB1	-3.46	positive regulation of establishment of protein localization to plasma membrane; cell migration involved in sprouting angiogenesis; cell-cell adhesion mediated by integrin; viral reproduction; response to virus; leukocyte cell-cell adhesion; positive regulation of apoptotic process; integrin-mediated signaling pathway
RANBP1	-3.3	intracellular transport; positive regulation of GTPase activity
CAPZA1	-3.15	actin cytoskeleton organization; actin filament capping
HSPA8	-3.13	clathrin coat disassembly; protein refolding; RNA splicing; mRNA processing; chaperone mediated protein folding requiring cofactor; negative regulation of transcription, DNA-dependent; response to stress
CCNA2	-3.08	cell cycle; Ras protein signal transduction; regulation of cyclin-dependent protein serine; threonine kinase activity; mitosis
PSMA6	-3.06	positive regulation of NF-kappaB transcription factor activity; ubiquitin-dependent protein catabolic process
TMED10	-3.04	COPI-coated vesicle budding; beta-amyloid formation; regulated secretory pathway; retrograde vesicle-mediated transport, Golgi to ER; intracellular protein transport
SUCLG2	-2.99	tricarboxylic acid cycle
ATP5O	-2.9	ATP synthesis coupled proton transport
TCAM1	-2.9	cell-cell adhesion
DAZAP1	-2.88	maternal placenta development; spermatogenesis; cell proliferation
CNN3	-2.87	actomyosin structure organization

TM4SF1	-2.84	transmembrane activity; Tumor-Associated Antigen L6
SAR1A	-2.83	negative regulation of cargo loading into COPII-coated vesicle; vesicle-mediated transport; intracellular protein transport
MYH9	-2.83	actin filament-based movement; establishment of T cell polarity; monocyte differentiation; regulation of cell shape; cytokinesis; angiogenesis; in utero embryonic development; blood vessel endothelial cell migration; protein transport
GOT2	-2.83	glutamate metabolic process; glutamate catabolic process to aspartate; fatty acid transport; aspartate catabolic, metabolic and biosynthetic process; oxaloacetate metabolic process; response to ethanol
MRPL9	-2.81	translation
ISCA1	-2.79	iron-sulfur cluster assembly
VCP	-2.74	aggresome assembly; positive regulation of protein complex assembly; retrograde protein transport, ER to cytosol; protein N-linked glycosylation via asparagine; protein ubiquitination; ubiquitin-dependent protein catabolic process// translesion synthesis; response to DNA damage stimulus; double-strand break repair; activation of cysteine-type endopeptidase activity involved in apoptotic process
DUT	-2.73	dUTP metabolic process
PSMB7	-2.73	-------
SERBP1	-2.72	May play a role in the regulation of mRNA stability
NDUFA7	-2.7	mitochondrial electron transport, NADH to ubiquinone; ATP synthesis coupled electron transport
TUBA1B	-2.69	structural molecule activity; GTP binding
CCNG1	-2.68	mitosis; regulation of cell cycle
ERH	-2.68	cell cycle; osteoblast differentiation
CDC20	-2.63	positive regulation of synapse maturation; positive regulation of synaptic plasticity; regulation of meiosis
YWHAZ	-2.62	apoptotic process; histamine secretion by mast cell;protein targeting; protein targeting to mitochondrion; signal transduction
PDLIM7	-2.6	cell differentiation; ossification; multicellular organismal development; actin cytoskeleton organization
ANXA3	-2.58	positive regulation of sequence-specific DNA binding transcription factor activity; neutrophil degranulation; negative regulation of catalytic activity; defense response to bacterium; positive regulation of angiogenesis; positive regulation of endothelial cell migration; phagocytosis
PPIA	-2.54	protein folding; regulation of viral genome replication; protein peptidyl-prolyl isomerization

PARK7	-2.49	positive regulation of oxidative phosphorylation uncoupler activity; mitochondrion organization; inflammatory response; negative regulation of cell death; negative regulation of neuron apoptotic process; protein stabilization; regulation of neuron apoptotic process; positive regulation of transcription from RNA polymerase II promoter; positive regulation of interleukin-8 production; cellular response to oxidative stress; regulation of inflammatory response; membrane depolarization; negative regulation of cysteine-type endopeptidase activity involved in apoptotic signaling pathway; negative regulation of extrinsic apoptotic signaling pathway; negative regulation of protein kinase activity; positive regulation of protein localization to nucleus; adult locomotory behavior; dopamine uptake involved in synaptic transmission
RRM1	-2.44	DNA replication; protein heterotetramerization; oxidation-reduction process
FKBP4	-2.42	steroid hormone receptor complex assembly; prostate gland development; embryo implantation; negative regulation of neuron projection development; chaperone-mediated protein folding; protein complex localization; male sex differentiation; androgen receptor signaling pathway
LDHB	-2.42	oxidation-reduction process; cellular carbohydrate metabolic process; glycolysis
FAM96B	-2.4	chromosome segregation
NARS	-2.38	asparaginyl-tRNA aminoacylation
GNG2	-2.36	cellular response to catecholamine stimul
TUBB	-2.35	spindle assembly; protein polymerization; GTP catabolic process
PTMA	-2.35	Prothymosin alpha may mediate immune function by conferring resistance to certain opportunistic infections
HMGN2	-2.34	DNA binding; nucleosomal DNA binding; poly(A) RNA binding
PARP1	-2.32	regulation of transcription, DNA-dependent; protein ADP-ribosylation; double-strand break repair; transcription, DNA-dependent; regulation of growth rate; cellular response to insulin stimulus; telomere maintenance; base-excision repair
SARS	-2.27	seryl-tRNA aminoacylation; selenocysteinyl-tRNA(Sec) biosynthetic process
LOX	-2.2	blood vessel development; oxidation-reduction process; lung development; elastic fiber assembly; collagen fibril organization
MEA1	-2.19	cell differentiation; multicellular organismal development; spermatogenesis
TMED2	-2.17	transport

PSMB5	-2.15	proteasomal ubiquitin-dependent protein catabolic process; response to oxidative stress
VIM	-2.14	intermediate filament organization; lens fiber cell development; Bergmann glial cell differentiation; astrocyte development; negative regulation of neuron projection development; positive regulation of gene expression; in utero embryonic development
SEC61G	-2.12	protein transmembrane transport; protein targeting to ER
ARPC4	-2.07	actin filament polymerization
ENY2	-2.07	mRNA export from nucleus; positive regulation of transcription, DNA-dependent; transcription elongation from RNA polymerase II promoter; histone deubiquitination; protein transport

Table 4. List of DE genes up-regulated in all 3 moments of infection (1h, 6h, 24h pi) with fold change and gene description.

Abbr.	Fold change	Gene description
TMBIM1	20.66	Transmembrane BAX inhibitor motif containing 1
TIMP2	20.64	TIMP metallopeptidase inhibitor 2
PSMG4	17.76	Proteasome assembly chaperone 4
ATF3	14.08	Activating transcription factor 3
RBM5	9.76	RNA binding motif protein 5
RNF114	8.24	Ring finger protein 114
NXF1	7.01	Nuclear RNA export factor 1
PTMA	4.26	Prothymosin, alpha
YIPF3	3.67	Yip1 domain family, member 3
ROGDI	3.13	Protein rogdi homolog
MAP2K3	3.08	Mitogen-activated protein kinase kinase 3
RPS25	2.93	40S ribosomal protein S25
SERTAD1	2.88	SERTA domain containing 1
CLU	2.66	Clusterin
SDC4	2.63	Syndecan 4
GLTSCR2	2.4	Glioma tumor suppressor candidate region gene 2
ID2	2.37	DNA-binding protein inhibitor ID-2
RPL17	2.13	60S ribosomal protein L17

Table 5. Spatiotemporal expression of up-regulated genes with GO terms related to apoptosis in MDBK cells infected with BoHV-5.

Gene name	GO term	1h	6h	24h
AAMDC	negative regulation of apoptotic process	11.6		
ACSL5	regulation of extrinsic apoptotic signaling pathway			5.7
AEN	intrinsic apoptotic signaling pathway in response to DNA damage by p53 class mediator		6.8	
ARAF	negative regulation of apoptotic process			5.4
ATF4	intrinsic apoptotic signaling pathway in response to endoplasmic reticulum stress positive regulation of neuron apoptotic process intrinsic apoptotic signaling pathway in response to endoplasmic reticulum stress			2.6
AVEN	negative regulation of apoptotic process			9.0
AXL	negative regulation of dendritic cell apoptotic process negative regulation of neuron apoptotic process			10.3
BAD	intrinsic apoptotic signaling pathway in response to DNA damage; positive regulation of apoptotic process by virus; suppression by virus of host apoptotic process// extrinsic apoptotic signaling pathway via death domain receptors		5.0	
BCL10	positive regulation of cysteine-type endopeptidase activity involved in apoptotic process; cell death; B cell apoptotic process; positive regulation of extrinsic apoptotic signaling pathway; negative regulation of mature B cell apoptotic process	3.8		
BCL3	regulation of apoptotic process ; intrinsic apoptotic signaling pathway in response to DNA damage by p53 class mediator; negative regulation of apoptotic process		17.9	
BDNF	negative regulation of neuron apoptotic process		5.7	
BIRC6	negative regulation of extrinsic apoptotic signaling pathway; negative regulation of apoptotic process			7.1
BLCAP	apoptotic nuclear changes	9.2		
BMI1	negative regulation of apoptotic signaling pathway			10.7
BNIP3L	negative regulation of apoptotic process; positive regulation of apoptotic process	4.4		
BTG1	positive regulation of fibroblast apoptotic process		7.7	

C1D	apoptotic process	5.3		
C1QBP	positive regulation of apoptotic process; apoptotic process		4.4	14.0
CASP8	regulation of thymocyte apoptotic process; positive regulation of extrinsic apoptotic signaling pathway; hepatocyte apoptotic process; negative regulation of necroptosis; extrinsic apoptotic signaling pathway via death domain receptors; execution phase of apoptosis; apoptotic process			17.8
CD74	negative regulation of mature B cell apoptotic process; negative regulation of DNA damage response, signal transduction by p53 class mediator; negative regulation of DNA damage response, signal transduction by p53 class mediator	3.3		
CDK5	positive regulation of neuron apoptotic process; neuron apoptotic process	12.6		
CDKN1A	intrinsic apoptotic signaling pathway in response to DNA damage by p53 class mediator; positive regulation of programmed cell death		2.6	2.5
CHD8	negative regulation of fibroblast apoptotic process			19.3
CITED2	negative regulation of apoptotic process	6.0		
CLPTM1L	apoptotic process		6.9	
CLU	intrinsic apoptotic signaling pathway; positive regulation of intrinsic apoptotic signaling pathway; positive regulation of apoptotic process	2.7	2.4	2.8
COL18A1	positive regulation of endothelial cell apoptotic process	2.3		
CTSH	negative regulation of apoptotic process; activation of cysteine-type endopeptidase activity involved in apoptotic process		2.5	
DAD1	apoptotic process; negative regulation of apoptotic process	2.1		
DAPK3	regulation of apoptotic process; apoptotic signaling pathway		2.4	
DDIT3	positive regulation of neuron apoptotic process; apoptotic process; intrinsic apoptotic signaling pathway in response to endoplasmic reticulum stress		5.6	
DDX5	positive regulation of DNA damage response, signal transduction by p53 class mediator; intrinsic apoptotic signaling pathway by p53 class mediator		2.3	5.6
DIABLO	intrinsic apoptotic signaling pathway in response to oxidative stress; neuron apoptotic process; positive regulation of apoptotic process; activation of cysteine-type endopeptidase activity involved in apoptotic process by cytochrome c	5.5		

DRAM1	apoptotic nuclear changes		37.5	
DYNLL1	apoptotic process	2.6		
DYRK2	intrinsic apoptotic signaling pathway in response to DNA damage by p53 class mediator		9.7	
EGLN3	regulation of neuron apoptotic process	3.9		
EI24	positive regulation of intrinsic apoptotic signaling pathway; intrinsic apoptotic signaling pathway in response to DNA damage	2.6		
EMC4	apoptotic process	3.9	8.7	
EPHA2	intrinsic apoptotic signaling pathway in response to DNA damage		4.4	4.4
FADD	apoptotic process; extrinsic apoptotic signaling pathway in absence of ligand; motor neuron apoptotic process	37.3		
FIS1	positive regulation of intrinsic apoptotic signaling pathway; positive regulation of cysteine-type endopeptidase activity involved in apoptotic process; mitochondrial fragmentation involved in apoptotic process	2.6		
FLCN	positive regulation of intrinsic apoptotic signaling pathway; positive regulation of apoptotic process			9.1
GADD45A	positive regulation of apoptotic process		5.1	
GATAD2A	programmed cell death		4.7	
GNB2L1	positive regulation of apoptotic process ; positive regulation of intrinsic apoptotic signaling pathway		2.2	
GSTP1	negative regulation of extrinsic apoptotic signaling pathway			3.2
HINT2	apoptotic process	18.2		
HMOX1	apoptotic process		3.1	
HOXA5	positive regulation of apoptotic process		8.9	
ID1	negative regulation of apoptotic process	3.0		
IER3	intrinsic apoptotic signaling pathway in response to DNA damage; negative regulation of apoptotic process		3.8	
JMJD6	recognition of apoptotic cell		5.2	7.0
LAMP1	granzyme-mediated apoptotic signaling pathway	3.2	2.5	

LOC519172	extrinsic apoptotic signaling pathway via death domain receptors	3.0		
LYN	positive regulation of dendritic cell apoptotic process		2.3	
MAPK1	apoptotic process		10.4	
MCL1	negative regulation of intrinsic apoptotic signaling pathway; extrinsic apoptotic signaling pathway in absence of ligand; negative regulation of apoptotic process; intrinsic apoptotic signaling pathway in response to DNA damage			8.2
MDM4	negative regulation of apoptotic process; DNA damage response, signal transduction by p53 class mediator		12.3	18.9
MKL1	negative regulation of apoptotic signaling pathway		22.1	
MLLT11	intrinsic apoptotic signaling pathway; positive regulation of apoptotic process; extrinsic apoptotic signaling pathway			18.5
MYC	apoptotic DNA fragmentation; regulation of apoptotic process; negative regulation of apoptotic process; intrinsic apoptotic signaling pathway		4.6	
NDUFS3	negative regulation of intrinsic apoptotic signaling pathway	7.3		
NES	negative regulation of neuron apoptotic process			2.3
NFE2L2	negative regulation of endothelial cell apoptotic process		3.3	
NLE1	negative regulation of cysteine-type endopeptidase activity involved in apoptotic signaling pathway			12.5
NOC2L	negative regulation of intrinsic apoptotic signaling pathway; apoptotic process; negative regulation of B cell apoptotic process	5.7		
NUAK2	negative regulation of apoptotic process		7.3	
PAK1	apoptotic process			4.3
PAX8	negative regulation of apoptotic process involved in metanephric nephron tubule development		13.7	
PDCD10	negative regulation of apoptotic process			5.0
PLAGL1	apoptotic process			40.6
PLK2	negative regulation of apoptotic process		3.3	
PPARD	apoptotic signaling pathway			6.3

PPP1R13L	apoptotic process		7.8	
PPP1R15A	apoptotic process		7.2	20.6
PPP2R5C	intrinsic apoptotic signaling pathway in response to DNA damage by p53 class mediator; DNA damage response, signal transduction by p53 class mediator resulting in cell cycle arrest			9.2
PRDX5	negative regulation of apoptotic process; regulation of apoptosis involved in tissue homeostasis	2.7		28.9
RBM5	apoptotic process; positive regulation of apoptotic process	9.8	8.3	12.6
RHOB	positive regulation of apoptotic process; transformed cell apoptotic process		18.1	
RIPK1	positive regulation of extrinsic apoptotic signaling pathway; extrinsic apoptotic signaling pathway; negative regulation of extrinsic apoptotic signaling pathway in absence of ligand; T cell apoptotic process; apoptotic process; positive regulation of extrinsic apoptotic signaling pathway	6.3		
RNPS1	positive regulation of apoptotic process			5.9
RPS3	regulation of apoptotic process; positive regulation of apoptotic signaling pathway			2.1
RPS3A	negative regulation of apoptotic process		2.0	
S100A14	apoptotic process	2.1		
SELK	intrinsic apoptotic signaling pathway in response to endoplasmic reticulum stress	17.5		
SERPINE1	negative regulation of endothelial cell apoptotic process		6.3	26.4
SGK1	apoptotic process		2.8	
SGPL1	execution phase of apoptosis			7.3
SIRT2	positive regulation of execution phase of apoptosis	3.9		
SKI	regulation of apoptotic process		12.0	
SLC9A3R1	positive regulation of intrinsic apoptotic signaling pathway		2.3	
SMNDC1	apoptotic process		4.4	3.9
SNX30	apoptotic process			11.0
SOD2	intrinsic apoptotic signaling pathway in response to DNA damage; intrinsic apoptotic signaling pathway in response to oxidative stress; negative regulation of neuron apoptotic process		3.9	
SON	negative regulation of apoptotic process		2.9	

SRSF6	negative regulation of cell death			2.2
TNFRSF12A	extrinsic apoptotic signaling pathway; positive regulation of apoptotic process; positive regulation of extrinsic apoptotic signaling pathway		7.4	
TNFRSF1A	apoptotic process; intrinsic apoptotic signaling pathway in response to DNA damage	5.9	5.2	
TOPORS	intrinsic apoptotic signaling pathway in response to DNA damage by p53 class mediator			14.1
TP53BP2	intrinsic apoptotic signaling pathway by p53 class mediator		6.5	
TRAF7	positive regulation of apoptotic signaling pathway; apoptotic process		4.0	
TRIM35	positive regulation of apoptotic process		21.9	
UBE2D3	apoptotic process			2.0
VEGFB	negative regulation of neuron apoptotic process	16.6		
VIMP	negative regulation of macrophage apoptotic process		8.8	
ZNF259	negative regulation of motor neuron apoptotic process			14.8

Table 6. Spatiotemporal expression of Down-regulated genes with GO terms related to apoptosis in MDBK cells infected with BoHV-5.

Gene name	GO term	1h	6h	24h
ACIN1	positive regulation of apoptotic process; apoptotic chromosome condensation	-5.2		
ACSL5	regulation of extrinsic apoptotic signaling pathway		-3.2	
ANKRD1	positive regulation of apoptotic process		-17.9	
ANP32B	activation of cysteine-type endopeptidase activity involved in apoptotic process		-2.5	-4.4
ANXA1	positive regulation of neutrophil apoptotic process		-38.1	-41.5
ASNS	negative regulation of apoptotic process	-29.9	-7.8	-4.0
ATN1	neuron apoptotic process			-5.6
BAG3	extrinsic apoptotic signaling pathway via death domain receptors extrinsic apoptotic signaling pathway in absence of ligand negative regulation of striated muscle cell apoptotic process	-6.4	-5.4	
BAG6	negative regulation of apoptotic process		-3.7	
BCAP31	positive regulation of intrinsic apoptotic signaling pathway positive regulation of cysteine-type endopeptidase activity involved in apoptotic process		-7.6	-6.8
BCL2L13	regulation of apoptotic process		-10.7	
BCLAF1	positive regulation of apoptotic process positive regulation of intrinsic apoptotic signaling pathway apoptotic process	-4.7		
BIRC5	negative regulation of apoptotic process negative regulation of cysteine-type endopeptidase activity involved in apoptotic process			-6.2
CAV1	positive regulation of extrinsic apoptotic signaling pathway positive regulation of intrinsic apoptotic signaling pathway apoptotic signaling pathway		-7.7	
CD44	negative regulation of apoptotic process			-2.9

CD74	negative regulation of mature B cell apoptotic process		-8.2	-5.1
CIDEB	execution phase of apoptosis; regulation of apoptotic process		-24.6	
CKAP2	apoptotic process	-4.2		
CRYAB	apoptotic process involved in morphogenesis negative regulation of cysteine-type endopeptidase activity involved in apoptotic process		-6.4	-4.4
CSDA	negative regulation of necroptosis; negative regulation of apoptotic process	-3.3		-3.1
CTTN	negative regulation of extrinsic apoptotic signaling pathway		-4.0	
CYR61	negative regulation of apoptotic process; positive regulation of apoptotic process; positive regulation of cysteine-type endopeptidase activity involved in apoptotic process			-14.3
DAD1	apoptotic process; negative regulation of apoptotic process		-3.3	
DHCR24	negative regulation of cysteine-type endopeptidase activity involved in apoptotic process negative regulation of apoptotic process	-3.8		
DNAJA1	positive regulation of apoptotic process	-4.8		
DYNLL1	apoptotic process		-2.7	-5.6
EIF5A	positive regulation of apoptotic process		-3.1	
FAM32A	apoptotic process		-4.5	
FHL2	negative regulation of apoptotic process			-3.1
FIS1	positive regulation of intrinsic apoptotic signaling pathway		-10.0	
FKBP8	negative regulation of apoptotic process			-2.9
GABARAP	extrinsic apoptotic signaling pathway via death domain receptors		-2.6	-11.8
GCLM	negative regulation of extrinsic apoptotic signaling pathway		-11.2	
GSTP1	negative regulation of extrinsic apoptotic signaling pathway		-10.3	
HINT1	intrinsic apoptotic signaling pathway by p53 class mediator	-3.5	-18.4	
HMGB1	positive regulation of apoptotic process		-13.4	

HSP90B1	negative regulation of apoptotic process		-2.5	-3.3
HSPD1	activation of cysteine-type endopeptidase activity involved in apoptotic process negative regulation of apoptotic process positive regulation of apoptotic process		-9.2	-10.4
ID1	negative regulation of apoptotic process		-8.4	
IER3	intrinsic apoptotic signaling pathway in response to DNA damage negative regulation of apoptotic process			-9.7
IGF1R	negative regulation of apoptotic process			-8.0
ITGA6	negative regulation of extrinsic apoptotic signaling pathway positive regulation of apoptotic process	-7.7	-3.4	-7.1
ITGB1	positive regulation of apoptotic process	-3.5	-6.9	-9.6
ITM2B	extrinsic apoptotic signaling pathway in absence of ligand			-3.9
KRT18	extrinsic apoptotic signaling pathway; negative regulation of apoptotic process hepatocyte apoptotic process		-2.9	-6.5
LGALS1	apoptotic process	-4.1	-4.3	
LGALS3	regulation of T cell apoptotic process negative regulation of extrinsic apoptotic signaling pathway		-15.3	-11.1
LMNA	negative regulation of extrinsic apoptotic signaling pathway		-6.0	
LYN	positive regulation of dendritic cell apoptotic process			-8.5
MFGE8	positive regulation of apoptotic cell clearance		-5.9	
MIF	negative regulation of myeloid cell apoptotic process			-6.7
MTDH	negative regulation of apoptotic process	-3.1		
MYBBP1A	intrinsic apoptotic signaling pathway by p53 class mediator		-5.6	-14.6
NDUFA13	apoptotic signaling pathway		-18.6	
NDUFS1	apoptotic mitochondrial changes	-4.7		

NES	negative regulation of neuron apoptotic process	-2.9		
NEXN	regulation of apoptotic process	-3.6		
NPM1	negative regulation of apoptotic process		-2.5	
P2RX4	apoptotic signaling pathway	-14.3		
PARK7	negative regulation of cell death; negative regulation of neuron apoptotic process negative regulation of cysteine-type endopeptidase activity involved in apoptotic signaling pathway negative regulation of extrinsic apoptotic signaling pathway	-2.5	-12.5	-15.7
PDCD10	negative regulation of apoptotic process	-2.6	-3.0	
PDCD5	apoptotic process// positive regulation of apoptotic process		-47.0	
PDIA3	positive regulation of extrinsic apoptotic signaling pathway; positive regulation of apoptotic process		-4.0	-5.8
PLK2	negative regulation of apoptotic process	-2.5		
PPP2CB	apoptotic mitochondrial changes	-14.8		
PPP2R1A	positive regulation of extrinsic apoptotic signaling pathway in absence of ligand		-2.4	
PRDX2	regulation of apoptotic process		-2.5	-7.7
PRDX3	negative regulation of apoptotic process	-2.2	-8.1	
PRDX5	negative regulation of apoptotic process regulation of apoptosis involved in tissue homeostasis		-4.7	
PRKCQ	negative regulation of T cell apoptotic process	-6.0		
PSME3	negative regulation of extrinsic apoptotic signaling pathway	-5.8		
PSMG2	negative regulation of apoptotic process			-3.6
PYGL	necroptosis			-6.2
RHOA	negative regulation of neuron apoptotic process			-5.4
ROCK1	negative regulation of neuron apoptotic process	-12.5		
SHISA5	apoptotic process		-7.7	

SIGMAR1	regulation of neuron apoptotic process	-5.0	-6.4	
TEAD2	negative regulation of cell death	-4.7		
THBS1	negative regulation of cysteine-type endopeptidase activity involved in apoptotic process			-2.7
THOC6	negative regulation of apoptotic process		-13.5	
TMEM109	intrinsic apoptotic signaling pathway in response to DNA damage by p53 class mediator; negative regulation of cell death		-6.8	-10.2
TNFRSF12A	extrinsic apoptotic signaling pathway; positive regulation of apoptotic process; positive regulation of extrinsic apoptotic signaling pathway			-5.4
UBE2B	negative regulation of apoptotic process	-2.1	-2.8	
VCP	activation of cysteine-type endopeptidase activity involved in apoptotic process	-2.7	-2.3	-5.4
VDAC1	apoptotic process			-4.2
VDAC2	negative regulation of intrinsic apoptotic signaling pathway			-2.1
ZFAND6	negative regulation of apoptotic process		-8.1	

Table 7. Apoptotic DEGs that changed their expression during the time course of infection in MDBK cells infected with BoHV-5.*

Genes	1h pi	6h pi	24h pi
PRDX5	2.7	-4.7	28.9
IER3	---	3.8	-9.7
ID1	3.0	-8.4	---
ACSL5	---	-3.2	5.7
DYNLL1	2.6	-2.7	-5.6
CD74	3.3	-8.2	-5.1
PLK2	-2.5	3.3	---
DAD1	2.1	-3.3	---
LYN	---	2.3	-8.5
PDCD10	-2.6	-3.0	5.0
NES	-2.9	---	2.3
GSTP1	---	-10.3	3.2
FIS1	2.6	-10.0	---

* genes up-regulated are positive while genes down-regulated are negative

Table 8. Spatiotemporal expression of DE genes with GO terms related to virus infection in MDBK cells infected with BoHV-5.

Abrr.	1h pi	6h pi	24h pi	Gene description	GO Biological function
ALYREF		-2.7		THO complex subunit 4	intronless viral mRNA export from host nucleus
AP1S1	-3.8			AP-1 complex subunit sigma-1A	response to virus
BAD*		5.0		Uncharacterized protein	positive regulation of apoptotic process by virus; cellular process regulating host cell cycle in response to virus; suppression by virus of host apoptotic process
BCL3*		17.9		Uncharacterized protein	response to virus
BNIP3L*	4.4			BCL2/adenovirus E1B 19 kDa protein-interacting protein 3-like	defense response to virus
C1QBP*		4.4	14.0	Complement component 1 Q subcomponent-binding protein, mitochondrial	negative regulation of defense response to virus
CCNT1		6.1		Cyclin-T1	viral reproduction
CCT5		-4.9		Uncharacterized protein	response to virus
CFL1		-8.5	-9.6	Cofilin-1	response to virus
DAG1	-7.4			Dystroglycan	virus-host interaction
DNAJC14			11.1	DnaJ homolog subfamily C member 14	viral reproduction
DYNLL1*	2.6	-2.7	-5.6	Dynein light chain 1, cytoplasmic	viral reproduction
EXOSC4	2.9			Exosome complex component RRP41	defense response to virus
EXOSC5	10.6			Uncharacterized protein	defense response to virus
FADD*	37.3			FAS-associated death domain protein	defense response to virus
FDPS		-4.3		Farnesyl pyrophosphate synthase	viral reproduction
FDPS			5.0	Farnesyl pyrophosphate synthase	viral reproduction
FURIN			7.2	Furin (Paired basic amino acid cleaving enzyme)	viral infectious cycle
HDAC1			3.7	Histone deacetylase 1	negative regulation by host of viral transcription

HMGA1	-4.1	-2.3	-8.1	Uncharacterized protein	response to virus
HSPB1		-5.6	-3.4	Heat shock protein beta-1	response to virus
IST1			6.5	IST1 homolog	viral release from host cell; viral capsid re-envelopment
ITGB1*	-3.5	-6.9	-9.6	Integrin beta-1	viral reproduction; response to virus
MX1		5.2		Interferon-induced GTP-binding protein Mx1	defense response to virus; negative regulation of viral genome replication
MYD88		7.0		Myeloid differentiation primary response protein	response to virus
NEDD4			18.3	Uncharacterized protein	transmission of virus
NPC2	3.6	2.8		Epididymal secretory protein E1	response to virus
PCBP2		-5.0	-6.2	Uncharacterized protein	negative regulation of defense response to virus
PPIA	-2.5	-26.5	-35.1	Peptidyl-prolyl cis-trans isomerase	regulation of viral genome replication
PPIH	-3.1	-41.0		Peptidyl-prolyl cis-trans isomerase H	positive regulation of viral genome replication
PSMA2	-3.5	-4.5	-12.6	Proteasome subunit alpha type-2	response to virus
RAD23A	2.3			UV excision repair protein RAD23 homolog A	positive regulation of viral genome replication
SMARCB1		-5.8		SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily B member 1	positive regulation by host of viral transcription
TAF11	10.8			TAF11 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 28kDa	positive regulation by host of viral transcription
THOC5			2.9	THO complex subunit 5 homolog	intronless viral mRNA export from host nucleus
THOC6*		-13.5		THO complex 6 homolog	intronless viral mRNA export from host nucleus
TRIM27		9.7		Uncharacterized protein	negative regulation of viral transcription
TRIM8			4.4	Uncharacterized protein	negative regulation of viral entry into host cell; negative regulation of viral transcription
USF1		3.7		Upstream stimulatory factor 1	late viral mRNA transcription

* Genes with are also related to apoptosis.

Table 9. Top 56 Greater DE genes up and down-regulated associated with oxidative response in MDBK cells infected by BoHV-5.

Gene	1h pi	6h pi	24h pi	GO terms	Gene description
ACAD8			23.1	oxidation-reduction process	Isobutyryl-CoA dehydrogenase, mitochondrial
ADI1			16.8	oxidation-reduction process	1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase
AKR1A1		-18.2		oxidation-reduction process	Alcohol dehydrogenase [NADP(+)]
AKR1B1		-6.3		oxidation-reduction process	Aldose reductase
ALDH1A1		-9.3		oxidation-reduction process	Retinal dehydrogenase 1
ALDH2	20.6			oxidation-reduction process	Aldehyde dehydrogenase, mitochondrial
ALDH9A1			13.3	oxidation-reduction process	4-trimethylaminobutyraldehyde dehydrogenase
CBR4	-6.4			oxidation-reduction process	Carbonyl reductase family member 4
CHCHD10	55.4			oxidative phosphorylation	Uncharacterized protein
COQ7	16.8			age-dependent response to oxidative stress; cellular response to oxidative stress	Ubiquinone biosynthesis protein COQ7 homolog
CYB5R1	-12.8			oxidation-reduction process	NADH-cytochrome b5 reductase 1
CYC1		-7.5	-11.8	oxidation-reduction process	Cytochrome c1, heme protein, mitochondrial
CYR61			-14.3	reactive oxygen species metabolic process	Uncharacterized protein
DHRS11			12.6	oxidation-reduction process	Dehydrogenase/reductase SDR family member 11
DUS1L			12.5	oxidation-reduction process	Uncharacterized protein
ERCC1		-5.7		response to oxidative stress	DNA excision repair protein ERCC-1
ETFB	21.6			oxidation-reduction process	Electron transfer flavoprotein subunit beta
EZH2	-11.7			cellular response to hydrogen peroxide	Uncharacterized protein
FADS3		-2.4		oxidation-reduction process	Fatty acid desaturase 3
FDFT1		-4.0		oxidation-reduction process	Squalene synthase
FDX1L	-18.1			oxidation-reduction process	Adrenodoxin-like protein, mitochondrial
FTH1		-2.2		oxidation-reduction process	Ferritin
G6PD		-2.6		cellular response to oxidative stress	Glucose-6-phosphate 1-dehydrogenase
GCLM*		-11.2		response to oxidative stress	Glutamate--cysteine ligase regulatory subunit

GPX2	57.5			response to oxidative stress; oxidation-reduction process	Glutathione peroxidase
GPX4			-9.8	oxidation-reduction process; response to oxidative stress	Glutathione peroxidase
GPX7		16.7		oxidation-reduction process; response to oxidative stress	Glutathione peroxidase 7
IER3			-9.7	regulation of reactive oxygen species metabolic process	Immediate early response 3
IMPDH1	16.1			oxidation-reduction process	Inosine-5'-monophosphate dehydrogenase 1
IMPDH2	-5.6	-4.3	-3.6	oxidation-reduction process	Inosine-5'-monophosphate dehydrogenase 2
IVD		6.0	15.5	oxidation-reduction process	Isovaleryl-CoA dehydrogenase, mitochondrial
KDM5B		10.0		oxidation-reduction process	Uncharacterized protein
KDM5C			10.6	oxidation-reduction process	Uncharacterized protein
LOX			-11.0	oxidation-reduction process	LOX protein
MICAL2		9.2		oxidation-reduction process	Protein-methionine sulfoxide oxidase MICAL2
NDUFA3	16.5	107.1		oxidation-reduction process	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 3
NDUFA6			-8.1	response to oxidative stress; oxidation-reduction process	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 6
NDUFB6	-7.0			oxidation-reduction process	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 6
NDUFS8			-8.9	oxidation-reduction process; response to oxidative stress	NADH dehydrogenase [ubiquinone] iron-sulfur protein 8, mitochondrial
PARK7			-15.7	positive regulation of oxidative phosphorylation uncoupler activity; cellular response to hydrogen peroxide; cellular response to oxidative stress; oxidation-reduction process	Protein DJ-1
PDGFB		10.1		reactive oxygen species metabolic process;	Uncharacterized protein

				positive regulation of reactive oxygen species metabolic process	
PECR	18.7			oxidation-reduction process	Uncharacterized protein
PHGDH			-33.2	oxidation-reduction process	D-3-phosphoglycerate dehydrogenase
PNKP			10.0	response to oxidative stress	Uncharacterized protein
PNPT1			7.2	cellular response to oxidative stress	Uncharacterized protein
PPP2CB*	-14.8			dephosphorylation; response to hydrogen peroxide	Serine/threonine-protein phosphatase 2A catalytic subunit beta isoform
RHOB*		18.1		cellular response to hydrogen peroxide	Rho-related GTP-binding protein RhoB
ROMO1	-5.4			positive regulation of reactive oxygen species metabolic process; cellular response to reactive oxygen species	Reactive oxygen species modulator 1
RRM2		7.8	7.5	oxidation-reduction process	Uncharacterized protein
SELK*	17.5			response to oxidative stress	Selenoprotein K
TMX1	-7.8			oxidation-reduction process	Thioredoxin-related transmembrane protein 1
TXN			-34.8	oxidation-reduction process	Thioredoxin
TXNRD1		5.6		oxidation-reduction process	Thioredoxin reductase 1, cytoplasmic
UGDH	-7.5			oxidation-reduction process	UDP-glucose 6-dehydrogenase
VIMP*		8.8		cellular response to oxidative stress	Selenoprotein S
VKORC1	19.9			oxidation-reduction process	Vitamin K epoxide reductase complex subunit 1

* Genes with are also related to apoptosis

CAPÍTULO 4

6. DISCUSSÃO GERAL

Os resultados apresentados em ambos os trabalhos evidenciam a influencia da infecção do BoHV-5 na expressão de mRNA das células MDBK, causando alteração em genes estruturais como o *GAPDH*, e em centenas de outros genes que foram diferencialmente expressos quando comparados as células não infectadas. Das centenas de genes diferencialmente expressos foram descritas as vias celulares alteradas, e alguns genes foram particularmente discutidos relacionados às vias de apoptose, estresse oxidativo, resposta a vírus e resposta imune.

Estes resultados são importantes por levantarem hipóteses sobre a relação entre o vírus e a célula hospedeira. Estas hipóteses tornam-se possíveis ao relacionar os genes bovinos diferencialmente expressos com a função conhecida de genes homólogos em humanos e organismos-modelo para estudos genéticos como camundongos, mosca-da-fruta e nematódeos. Em nosso estudo, vários genes diferencialmente expressos foram relacionados com o metabolismo e deposição de proteína beta-amilóide em neurônios e ao estresse oxidativo. Sabe-se que o depósito desta proteína no sistema nervoso central leva a degeneração e espessamento dos vasos sanguíneos e morte de neurônios, tendo sido relacionada à degeneração congofílica em cães (Walker, 1997) e a lesão cerebral em casos de Mal de Alzheimer em humanos (Butterfield *et al.*, 2001; Smith, Cappai e Barnham, 2007). A hipótese de o HHV-1 estar relacionado com o mal de Alzheimer foi levantada a mais de 30 anos e evidências foram apontadas (Ball, 1982; Ball, 2006). A infecção pelo HHV-1 de cultivos primários de neurônios humanos denota expressão diminuída do microRNA humano (miRNA146a). O HHV-1 utiliza este mecanismo biológico como ferramenta de evasão do sistema complemento, mas como consequência leva a ativação da cascata do ácido aracdônico, o que sabidamente contribui para as alterações descritas no Mal de Alzheimer (Hill *et al.*, 2009). Neste contexto podemos questionar se este mecanismo de lesão as células do sistema nervoso central de humano causado pelo HHV-1 possam também estar relacionados a infeção pelo BoHV-5 em células bovinas e gerar novos estudos.

Além disto, o BoHV-5 poderia servir de modelo experimental para estudo de alfaherpesvirus, pois mutiplica bem em células de cultivo celular e possui um modelo animal (coelho) de infecção bem instituído (Flores *et al.*, 2009). Neste aspecto, estudos futuros realizados em animais poderão ser fundamentais para elucidar aspectos ainda

desconhecidos relacionados ao estabelecimento de latência e a reativação do BoHV-5 e, com isso, contribuir para elucidar estes aspectos ainda pouco compreendidos na infecção por herpesvirus. Outra consequência do aumento de conhecimento das interações entre herpesvirus e células infectadas seria identificar possíveis alvos para o desenvolvimento de drogas capazes de controlar a infecção viral.

7. CONCLUSÕES GERAIS

De acordo com os resultados obtidos no presente estudo, pode-se concluir:

1. Os genes virais da classe alfa, beta e gama do BoHV-5 apresentaram expressão de mRNA semelhantes na infecção experimental de células MDBK, diferindo da literatura clássica onde genes alfa, beta e gama apresentam diferenças no perfil de expressão na infecção aguda. O gene celular *GAPDH* teve sua expressão de mRNA alterada pela infecção do BoHV-5 e não serve como gene de referência para estudos de expressão gênica viral ou celular no contexto utilizado neste experimento. O gene 18S pode ser considerado um bom gene de referência para estudos de infecção de células MDBK pelo BoHV-5 por não ter sua expressão de mRNA alterada pela infecção viral. As diferenças apresentadas entre o presente estudo e a literatura clássica podem estar relacionadas à espécie de herpesvirus estudado ou as técnicas utilizadas. Novos estudos devem ser realizados para elucidar estas possíveis diferenças e trazer luz ao conhecimento do comportamento molecular do BoHV-5.

2. A qPCR padronizada para os genes virais apresentou-se eficaz para o estudo da expressão de mRNA do BoHV-5, podendo também ser utilizada na rotina laboratorial para o diagnóstico de casos clínicos suspeitos infecção pelo BoHV-5 por meio da detecção do DNA viral.

3. O sequenciamento massivo apresentou bons resultados em número e qualidade das sequencias obtidas (*reads*). A abordagem de bioinformática utilizada foi essencial e possibilitou o estudo concomitante de milhares de genes bovinos, corroborando com a literatura descreve a técnica de RNA-seq como a mais moderna para estudos de expressão gênica global. Os resultados apresentados neste trabalho são ponto de partida para que novas pesquisas e irão possibilitar o conhecimento de inúmeras vias celulares alteradas na infecção pelo BoHV-5.

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