UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS RURAIS
PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA VETERINÁRIA

Jéssica Caroline Gomes Noll

CONSTRUÇÃO E CARACTERIZAÇÃO DE UM RECOMBINANTE DO *Alphaherpesvirus caprino 1* COM DELEÇÃO NO GENE DA TIMIDINA KINASE

Santa Maria, RS
2019

Jéssica Caroline Gomes Noll

CONSTRUÇÃO E CARACTERIZAÇÃO DE UM RECOMBINANTE DO *Alphaherpesvirus caprino 1* COM DELEÇÃO NO GENE DA TIMIDINA KINASE

Dissertação apresentada ao Programa de Pós-Graduação em Medicina Veterinária, área de concentração em Medicina Veterinária Preventiva da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção de grau de **Mestre em Medicina Veterinária**.

Orientador: Prof. Dr. Alfredo Quites Antoniazzi

Santa Maria, RS
2019

Noll, Jéssica Caroline Gomes
CONSTRUÇÃO E CARACTERIZAÇÃO DE UM RECOMBINANTE DO Alphaherpesvirus caprino 1 COM DELEÇÃO NO GENE DA TIMIDINA KINASE / Jéssica Caroline Gomes Noll.- 2019.
55 p.; 30 cm

Orientador: Alfredo Quites Antoniazzi
Dissertação (mestrado) - Universidade Federal de Santa Maria, Centro de Ciências Rurais, Programa de Pós Graduação em Medicina Veterinária, RS, 2019

1. CpHV-1 2. vírus de cabras 3. alfaherpesvirus 4. gene tk 5. atenuação I. Quites Antoniazzi, Alfredo II. Título.

Sistema de geração automática de ficha catalográfica da UFSM. Dados fornecidos pelo autor(a). Sob supervisão da Direção da Divisão de Processos Técnicos da Biblioteca Central. Bibliotecária responsável Paula Schoenfeldt Patta CRB 10/1728.

Jéssica Caroline Gomes Noll

CONSTRUÇÃO E CARACTERIZAÇÃO DE UM RECOMBINANTE DO *Alphaherpesvirus caprino 1* COM DELEÇÃO NO GENE DA TIMIDINA KINASE

Dissertação apresentada ao Programa de Pós-Graduação em Medicina Veterinária, área de concentração em Medicina Veterinária Preventiva da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção de grau de **Mestre em Medicina Veterinária**.

Orientador: Prof. Dr. Alfredo Quites Antoniazzi

Aprovado em 19 de agosto de 2019

_____
Alfredo Quites Antoniazzi, PhD (UFSM)

_____
Rudi Weiblen, PhD (UFSM)

_____
Diego Gustavo Diel, PhD (Cornell University) – vídeoconferência

Santa Maria, RS
2019

DEDICATÓRIA

Aos meus pais Marcos e Mara
e à minha irmã Giovana
pelo apoio incondicional
a todas as minhas escolhas.

AGRADECIMENTOS

Primeiramente gostaria de agradecer aos meus pais Marcos e Mara, à minha irmã Giovana e minha cadelinha Frida (*in memoriam*) por todo amor e pelos ensinamentos que nenhuma escola seria capaz de proporcionar, tais como comprometimento, seriedade, dedicação e amor ao que se faz.

Agradeço também às médicas veterinárias Maureen Hoch Vieira Fernandes, Nadine Trinks Fischborn, Marilia Balaiardi Ribeiro e Laura Gusman pelo apoio e conforto em todas horas. Ser rodeada por mulheres fortes e competentes e poder chamá-las de amigas me enche de orgulho.

Aos professores Eduardo Furtado Flores e Diego Diel pela amizade, orientação e principalmente pela confiança em mim.

Aos colegas do Setor de Virologia e Diel Lab e todos os amigos dos Estados Unidos e do Brasil.

À família Brockhoft, principalmente Tom, Mike e Kris, que abriram as portas de sua casa pra mim e que tem sido a minha família longe de casa.

Ao Programa de Pós-Graduação em Medicina Veterinária da Universidade Federal de Santa Maria (PPGMV-UFSM).

Agradeço em especial à UFSM pelos conhecimentos adquiridos durante a graduação e mestrado. Como egressa, é meu dever e minha responsabilidade lutar pela permanência do ensino superior público e de qualidade. A garantia constitucional do direito à educação pública é a única forma de garantirmos uma sociedade mais justa e igualitária para todos.

A todos que de alguma forma contribuíram para realização deste trabalho, muito obrigada.

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – código de financiamento 001.

RESUMO

CONSTRUÇÃO E CARACTERIZAÇÃO DE UM RECOMBINANTE DO *Alphaherpesvirus caprino 1* COM DELEÇÃO NO GENE DA TIMIDINA KINASE

AUTORA: Jéssica Caroline Gomes Noll
ORIENTADOR: Dr. Alfredo Quites Antoniazzi

O *Alphaherpesvirus caprino 1* (CpHV-1) é um patógeno de cabras distribuído mundialmente, associado com infecções sistêmicas e doença respiratória em caprinos jovens e infecção subclínica ou doença reprodutiva e abortos em animais adultos. As perdas associadas com a infecção pelo CpHV-1 poderiam ser prevenidas por vacinação, no entanto não existem vacinas comerciais disponíveis. Assim, o presente trabalho teve como objetivo construir e caracterizar um recombinante do CpHV-1 com deleção no gene da enzima timidina quinase (TK) para potencial uso em vacinas. Um recombinante contendo deleção total no gene *tk* foi construído por recombinação homóloga, introduzindo-se em seu lugar o gene da proteína verde fluorescente (eGFP) para seleção dos recombinantes. A caracterização *in vitro* demonstrou que o recombinante CpHV-$1^{\Delta TK}$ replicou em títulos similares e produziu placas de tamanho semelhante ao vírus parental em cultivos celulares. Com isso, demonstrou-se que a deleção do gene *tk* não afetou a capacidade replicativa *in vitro* do vírus. Após inoculação intranasal em 5 cabritos por vírus (10^7 $TCID_{50}$), o vírus parental replicou de forma mais eficiente e por um período de tempo maior que o vírus recombinante. Além disso, o vírus parental produziu sinais clínicos sistêmicos e respiratórios moderados, enquanto os animais inoculados com o recombinante CpHV-$1^{\Delta TK}$ permaneceram saudáveis e soroconverteram em títulos mais baixos. Administração de dexametasona nos dias 35 a 39 pós-infecção (pi) não resultou em excreção viral em secreções nasais dos animais inoculados, indicando ausência de reativação viral. Entretanto, o DNA viral foi detectado no gânglio trigêmeo de 4 animais do grupo parental (4/5) e em 3 animais do grupo recombinante (3/5), eutanasiados no dia 14 pDx, indicando que ambos os vírus estabeleceram infecção latente. Esses resultados demonstram que o recombinante CpHV-$1^{\Delta TK}$ apresenta um fenótipo atenuado em caprinos jovens em comparação com o vírus parental e, portanto, pode ser uma cepa apropriada para uso em vacina.

Palavras-chave: CpHV-1, vírus de cabras, alfaherpesvirus, gene *tk*, atenuação.

ABSTRACT

CONSTRUCTION AND CHARACTERIZATION OF A RECOMBINANT OF *Caprine alphaherpesvirus* 1 WITH A DELETION IN THE THYMIDINE KINASE GENE

AUTHOR: Jéssica Caroline Gomes Noll
ADVISER: Dr. Alfredo Quites Antoniazzi

Caprine alphaherpesvirus 1 (CpHV-1) is a pathogen of goats distributed worldwide, associated with systemic infections and respiratory disease in kids and subclinical infection or reproductive disease and abortions in adult animals. The losses associated with CpHV-1 infection could be prevented by vaccination, however, commercial vaccines are not available. Thus, the present study aimed to construct and characterize a recombinant of the CpHV-1 with a deletion in the thymidine kinase (TK) gene for potential use in vaccines. A recombinant with a total deletion in the *tk* gene was constructed by homologous recombination, by replacing the *tk* with the enhanced green fluorescent protein (eGFP) gene for the selection of recombinants. *In vitro* characterization showed that the recombinant CpHV-$1^{\Delta TK}$ replicated to similar titers and produced plaques of similar size to the parental virus in cell cultures, demonstrating that the deletion of the *tk* gene did not affect the replicative ability of the virus *in vitro*. After intranasal inoculation of five kids per virus (10^7 $TCID_{50}$), the parental virus replicated more efficiently and for a longer period of time than the recombinant virus. In addition, the parental virus produced moderate systemic and respiratory signs, whereas the kids inoculated with the recombinant virus remained healthy and seroconverted to lower titers. The administration of dexamethasone on days 35 to 39 post-infection (pi) did not result in viral excretion in nasal secretions, indicating the absence of viral reactivation. However, viral DNA was detected in the trigeminal ganglia of 4 animals from the parental group (4/5) and 3 animals from the recombinant group (3/5), euthanized at day 14 pDx, indicating that both viruses established latent infection. These results demonstrated that the recombinant CpHV-$1^{\Delta TK}$ shows an attenuated phenotype in kids compared to the parental virus and, thus, may be a suitable strain for use as a CpHV-1 vaccine.

Keywords: CpHV-1, caprine viruses, alphaherpesvirus, *tk* gene, attenuation

LISTA DE ILUSTRAÇÕES

LISTA DE TABELAS

SUMÁRIO

1. INTRODUÇÃO

A família *Herpesviridae* é composta por vírus que coevoluem com seus hospedeiros há milhares de anos, resultando em grande adaptação entre vírus e hospedeiro. Aproximadamente 200 espécies de herpesvírus são conhecidas, e os hospedeiros são muito diversificados, incluindo moluscos, peixes, répteis, pássaros e mamíferos (DAVISON, 2002). Na natureza, a maioria dos herpesvírus está associada com um determinado hospedeiro e, virtualmente, todas as espécies animais estudadas até o momento são susceptíveis à infecção por pelo menos um herpesvírus (ROIZMANN; PELLETT, 2001).

A família *Herpesviridae* é composta por três subfamílias: Alpha-, Beta- e *Gammaherpesvirinae*. A subfamília *Alphaherpesvirinae* é composta por cinco gêneros: *Simplexvirus* e *Varicellovirus*, cujos hospedeiros são mamíferos, e *Mardivirus* e *Iltovirus* que infectam aves (ROIZMAN; PELLET, 2001). Recentemente, o gênero *Scutavirus* foi adicionado à subfamília, contendo duas espécies de herpesvírus de quelônios (GANDAR et al., 2015). Os membros da subfamília *Alphaherpesvirinae* compartilham características tais como ampla variedade de hospedeiros, curto ciclo replicativo, rápida destruição do tapete celular em culturas *in vitro* e capacidade de estabelecer infecção latente principalmente, mas não exclusivamente, em neurônios (ROIZMANN; PELLETT, 2001).

Os vírions dos membros da família *Herpesviridae* são grandes (120 a 300 nm de diâmetro), envelopados e contém uma fita dupla de DNA com genomas que variam entre 125 a 235 quilopares de base (BOEHMER; NIMONKAR, 2003; MOCARSKI; ROIZMAN, 1982). Uma característica que distingue os herpesvírus de grande parte dos outros vírus é a capacidade de persistir em seus hospedeiros indefinidamente, pelo estabelecimento de latência que pode, ocasionalmente, resultar em recrudescência de doença e retomada de excreção viral (BOEHMER; NIMONKAR, 2003). As manifestações clínicas produzidas por um herpesvírus em particular são determinadas pelo tipo celular onde o vírus replica e estabelece latência. Durante a reativação, o vírus retorna ao local de infecção primária por transporte anterógrado, onde é novamente excretado e produz sinais clínicos condizentes com os tecidos afetados (BOEHMER; NIMONKAR, 2003; KRAMER; ENQUIST, 2013). Os membros da subfamília *Alphaherpesvirinae* tem em seu genoma um segmento único longo (UL) e um segmento único curto (US) flanqueado por duas sequências repetidas invertidas chamadas região repetida interna (IR) e região repetida terminal (TR) (SCHWYZER;

ACKERMANN, 1996). Nas sequências únicas estão presentes genes de cópia única no genoma, enquanto nas regiões repetidas estão presentes genes em mais de uma cópia (MOCARSKI; ROIZMAN, 1982; MUYLKENS et al., 2007). As regiões UL e US podem trocar de posição durante a replicação viral, dando origem a formas isoméricas do genoma viral (ROIZMANN; PELLETT, 2001). A expressão gênica dos alphaherpesvírus ocorre em forma de cascata. O primeiro passo ocorre imediatamente após a liberação do genoma viral, com expressão de genes *alfa* (*immediate early*, ou de transcrição imediata). A síntese de proteínas de transcrição imediata leva à transcrição de genes *beta* (*early*, ou de transcrição inicial) e produção de proteínas iniciais. A transcrição de genes *gama* (*late*, ou de transcrição tardia) ocorre apenas após a síntese de DNA viral (ROIZMAN; KNIPE, 2001).

O *Alphaherpesvirus caprino 1* (CpHV-1), membro da subfamília *Alphaherpesvirinae*, gênero *Varicellovirus*, foi isolado pela primeira vez nos Estados Unidos em 1975 e tem caprinos como hospedeiros naturais (BERRIOS; MCKERCHER; KNIGHT, 1975). Infecções pelo CpHV-1 foram reportadas em diversos países como Nova Zelândia, Austrália, Suécia, Espanha, Itália e França (HORNER; HUNTER; DAY, 1982; KEUSER et al., 2004; METTLER et al., 1979; ROPERTO et al., 2000; THIRY et al., 2008; TISDALL et al., 1984). Atualmente, a infecção por CpHV-1 é prevalente em países europeus e mediterrâneos, onde a criação de caprinos para produção de carne e queijo é intensa. Nesses locais, a soro prevalência chega a alcançar 50% dos rebanhos (SUAVET et al., 2016). Recentemente, um estudo de fatores de risco determinou que o CpHV-1 está associado a rebanhos criados de forma extensiva (BERTOLINI et al., 2018).

A severidade da doença causada pela infecção por CpHV-1 está relacionada com a idade dos animais. Em adultos, a infecção é frequentemente assintomática, porém podem ser observadas falhas reprodutivas e abortos, além de vulvo-vaginite e balanopostite (CAMERO et al., 2015; TEMPESTA et al., 2000). As infecções por CpHV-1 causam elevadas taxas de morbidade e mortalidade em cabritos de uma a duas semanas de idade. Nesses animais, a infecção se apresenta de forma generalizada, provocando severas lesões gastroentéricas. Durante a necropsia de cabritos naturalmente infectados, lesões erosivas, ulcerativas e necróticas foram observadas no trato gastrointestinal, além edema pulmonar, hemorragia na vesícula urinária e focos necróticos no fígado. A presença do vírus foi detectada via PCR no duodeno, jejuno, íleo, ceco, linfonodos mesentéricos, baço, fígado, rins, vesícula urinária, pulmões, coração e timo desses mesmos animais (ROPERTO et al., 2000).

As consequências da infecção também variam de acordo com a rota de infecção. Quando a infecção ocorre via intranasal, há excreção viral tanto em secreções nasais quanto genitais, e os sinais clínicos também são observados em ambos os sistemas (TEMPESTA et al., 1999a). Entretanto, quando a infecção ocorre via genital, excreção viral e sinais clínicos são observados apenas no sistema genital (TEMPESTA et al., 1998, 2000). Diante da reativação viral, o vírus é novamente excretado pelas mesmas vias da infecção aguda (TEMPESTA et al., 1999b, 2000).

A reativação da infecção por CpHV-1 é de difícil obtenção tanto em condições naturais quanto experimentais (BUONAVOGLIA et al., 1996; TEMPESTA et al., 1998, 2000). Porém, em condições naturais, a excreção do vírus em secreções vaginais coincide com o estro em fêmeas, sugerindo que o *status* hormonal do estro tem participação na reativação do ciclo lítico do CpHV-1 (KOPTOPOULOS et al., 1988; TEMPESTA et al., 1998). Além disso, considerando o tropismo seletivo do CpHV-1 para o trato genital (BUONAVOGLIA et al., 1996; TEMPESTA et al., 1999a), a latência estabelecida no gânglio sacral (TEMPESTA et al., 1999b) e a transmissão venérea do vírus (SILVA et al., 2013; TEMPESTA et al., 2000), é possível afirmar que a monta natural pode ser um dos principais fatores responsáveis pela manutenção do vírus nos rebanhos (BERTOLINI et al., 2018; CAMERO et al., 2015; SILVA et al., 2013; TEMPESTA et al., 2000).

Essas características biológicas se assemelham ao *Alphaherpesvirus humano tipo* 2 (HSV-2), que também apresenta tropismo pelo trato genital, produz lesões tópicas vesiculares e ulcerativas, transmissão através de contato próximo e/ou sexual, entre outras. Devido a essas similaridades, o tratamento com antivirais para prevenção e tratamento do CpHV-1 utilizando cabras como modelo experimental vem sido desenvolvido com objetivo de traçar paralelos no tratamento e prevenção do HSV-2 em humanos (CAMERO et al., 2017; ELIA et al., 2015; TEMPESTA et al., 2008).

O CpHV-1 apresenta próxima relação antigênica ao *Alphaherpesvirus bovino 1* (BoHV-1), agente da rinotraqueíte infecciosa bovina (IBR). Estudos moleculares revelaram que há reação cruzada de anticorpos entre os dois vírus, especialmente induzida pela glicoproteína B (gB), mas também gC e gD (BERTOLOTTI et al., 2013; ENGELS et al., 1992). Experimentos realizados com ambos os vírus apontam que tanto bovinos são susceptíveis ao CpHV-1, quanto cabras são susceptíveis ao BoHV-1. Tanto CpHV-1 quanto BoHV-1 são capazes excretar o vírus na infecção aguda e de estabelecer infecção latente na espécie heteróloga. O BoHV-1 é capaz de provocar sinais clínicos leves quando inoculado em cabras. Entretanto, sinais clínicos provocados pelo CpHV-1

parecem ser restritos ao seu hospedeiro natural (ENGELS et al., 1992; GÜR et al., 2019; SIX et al., 2001; THIRY et al., 2006).

Atualmente, não existem vacinas comerciais disponíveis para prevenção das doenças produzidas pelo CpHV-1. Porém, diversos estudos realizados com cepas atenuadas do CpHV-1 com e sem a presença de adjuvantes, e utilizando a glicoproteína E do CpHV-1 inserida em um vetor, obtiveram sucesso na proteção contra a doença genital provocada pelo CpHV-1 (DONOFRIO et al., 2013; MARINARO et al., 2012).

A enzima timidina quinase (TK) está associada a síntese de nucleotídeos e está presente em uma grande variedade de organismos, incluindo bactérias, células procarióticas e eucarióticas e vírus (BLACK; HRUBY, 1991; WEN et al., 2010). A maioria dos herpesvírus e alguns outros vírus DNA como vaccínia vírus (VACV) e o vírus da peste suína africana (ASFV) possuem genes que codificam a sua própria enzima TK (DENG et al., 2017; SANFORD et al., 2016; TENSER; MILLER; RAPP, 1979). O gene *tk* é parte dos genes iniciais da cascata de replicação dos herpesvírus e, portanto, a expressão funcional da TK precisa ser regulada por proteínas de transcrição imediata, além de depender de transcrição pela RNA polimerase celular II (WAGNER; DELUCA, 2013).

Desde a década de 70 tem-se estudado as propriedades biológicas da TK, majoritariamente no *Alphaherpesvirus humano 1* (HSV-1). Esses primeiros estudos concluíram que a expressão da TK não afeta a infecção viral em células *in vitro*, porém, é uma enzima necessária para replicação viral em células não replicativas e células cultivadas em meio de cultura sem soro (JAMIESON; GENTRY; SUBAK-SHARPE, 1974). No entanto, estudos posteriores indicam que mutantes do HSV-1 TK deletados (TK^-) apresentam replicação defectiva *in vivo* (TENSER; MILLER; RAPP, 1979).

Posteriormente, pela realização de estudos com outros herpesvírus defectivos na TK, tais como BoHV-1 (CHOWDHURY, 1996; KIT et al., 1985), *Alphaherpesvirus bovino 5* (BoHV-5) (ANZILIERO et al., 2011; BRUM et al., 2010), *Alphaherpesvirus equino* 1 (EHV-1) (SLATER; GIBSON; FIELD, 1993), *Alphaherpesvirus suíno 1* (PRV) (FERRARI et al., 2000; KIT; KIT; PIRTLE, 1985), entre outros, foi possível observar que a TK viral não é essencial para a replicação em cultivo celular e também no hospedeiro. No entanto, mutantes TK^- geralmente apresentam diminuição da virulência *in vivo*.

A partir desses estudos, pode-se concluir que o gene *tk*, embora não-essencial para replicação viral *in vitro e in vivo*, é fundamental para a expressão da virulência dos vírus nos seus hospedeiros. Devido ao fato de neurônios serem células não replicativas, com baixos níveis de ácido timidílico, a habilidade do vírus em expressar o gene *tk* é crucial para a replicação em tecido nervoso *in vivo*. O baixo nível de ácido timidílico também é observado em cultivos celulares privados de soro e células em repouso (JAMIESON; GENTRY; SUBAK-SHARPE, 1974). Esta pode ser a explicação sobre o porque de mutantes TK^- terem sua neurovirulência reduzida (SUZUTANI et al., 1995). Por essa razão, mutantes TK defectivos tem sido amplamente utilizados para produção de vacinas vivas atenuadas e vetores vacinais (ANZILIERO et al., 2011; KIT et al., 1985; KIT; KIT; PIRTLE, 1985; MCGREGOR et al., 1985; SMITH et al., 1994).

O transcrito associado à latência (LAT) é transcrito a partir das sequências repetidas do genoma viral e é o produto viral mais abundante durante a fase de latência do HSV-1 e HSV-2 (STEVENS et al., 1987). A presença de LAT foi detectada em mutantes HSV-1 TK defectivos após infecção por diversas vias (COEN et al., 1989; LEIST; SANDRI-GOLDIN; STEVENS, 1989; TENSER; HAY; EDRIS, 1989). Além disso, os mutantes foram capazes de estabelecer latência com praticamente a mesma eficiência que o vírus parental (CHEN et al., 2004; LEIST; SANDRI-GOLDIN; STEVENS, 1989), podendo-se concluir com base nesses estudos que a habilidade dos mutantes em estabelecer latência não está relacionada a sua capacidade em expressar o gene *tk* (SEARS; MEIGNIER; ROIZMAN, 1985). Os mutantes HSV-1 TK^- normalmente não reativam, estabelecendo latência incompleta (TENSER, 1991). Estudos realizados com alphaherpesvirus de interesse veterinário tais como BoHV-1 (KIT et al., 1985), BoHV-5 (ANZILIERO et al., 2011; CADORE et al., 2013; SILVA et al., 2010) e PRV (KIT; KIT; PIRTLE, 1985) apresentaram resultados similares no que condiz a inexistência ou redução de reativação viral de mutantes TK^- em diversas espécies.

O presente estudo relata a construção e caracterização *in vitro e in vivo* de um mutante do CpHV-1 com deleção total no gene *tk* (CpHV-$1^{\Delta TK}$) para potencial uso em vacinas.

2. ARTIGO

DELETION OF THE THYMIDINE KINASE GENE ATTENUATES *Caprine alphaherpesvirus 1* IN GOATS

Jéssica Caroline Gomes Noll, Lok Raj Joshi, Gabriela Mansano do Nascimento, Maureen Hoch Vieira Fernandes, Bishwas Sharma, Eduardo Furtado Flores, Diego Gustavo Diel.

(Artigo submetido à revista *Veterinary Microbiology* – 2019)

Deletion of the thymidine kinase gene attenuates *Caprine alphaherpesvirus type 1* in goats

Jéssica Caroline Gomes Noll[1,2,§], Lok Raj Joshi[2,§], Gabriela Mansano do Nascimento[2,§], Maureen Hoch Vieira Fernandes[2,§], Bishwas Sharma[2], Eduardo Furtado Flores[1], Diego Gustavo Diel[2,§*].

[1] Programa de Pós-graduação em Medicina Veterinária, Departamento de Medicina Veterinária Preventiva, Universidade Federal de Santa Maria, Santa Maria, RS 97105-900, Brazil.

[2]Animal Disease Research and Diagnostic Laboratory, Department of Veterinary and Biomedical Sciences, South Dakota state University, Brookings, SD 57007, USA.

[§]Current affiliation: Department of Population Medicine and Diagnostic Sciences, Animal Health Diagnostic Center, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853, USA.

[*]Corresponding author: D.G. Diel. Department of Population Medicine and Diagnostic Sciences, Animal Health Diagnostic Center, College of Veterinary Medicine, Cornell University. 240 Farrier Rd, A3-114 AHDC, Ithaca, NY 14853. E-mail address: dgdiel@cornell.edu

Abstract

Caprine alphaherpesvirus 1 (CpHV-1) is a pathogen associated with systemic infection and respiratory disease in kids and subclinical infection or reproductive failure and abortions in adult goats. The enzyme thymidine kinase (TK) is an important viral product involved in nucleotide synthesis necessary for virus replication. Therefore, it is a common target for herpesvirus attenuation. Here we deleted the *tk* gene of a CpHV-1 isolate and characterized the recombinant CpHV-$1^{\Delta TK}$ *in vitro* and *in vivo*. *In vitro* characterization revealed that the recombinant CpHV-$1^{\Delta TK}$ replicated to similar titers and produced plaques of similar size to the parental CpHV-1 strain in BT and CRIB cell lines. Upon intranasal inoculation, of young goats, the parental virus replicated more efficiently and for a longer period than the recombinant virus. In addition, infection with the parental virus resulted in mild systemic and respiratory signs whereas the kids inoculated with the recombinant CpHV-$1^{\Delta TK}$ virus remained healthy. Goats inoculated with the parental virus also developed higher neutralizing antibody titers when compared to CpHV-$1^{\Delta TK}$ inoculated animals. Dexamethasone (Dx) administration on days 35 to 39 post-inoculation did not result in virus shedding in nasal secretions, indicating lack of reactivation from latency. However, viral DNA was present in trigeminal ganglia of animals euthanized at 14 days post-Dx, indicating that both viruses successfully established latent infection. Our results show that the recombinant CpHV-$1^{\Delta TK}$ presents an attenuated phenotype when compared to the parental virus, and hence may represent a promising strain to be used as a vaccine to prevent CpHV-1 disease in goats.

Keywords: CpHV-1, caprine viruses, animal alphaherpesvirus, *tk* gene, attenuation

Introduction

The family *Herpesviridae* is a large family of viruses comprising multiple viral species such that virtually all animal species host at least one herpesvirus. The members of the subfamily *Alphaherpesvirinae* are classified based on unique biological properties, including short replicative cycle, rapid spread and destruction of cultured cells and the capacity to establish latency primarily in sensory nerve ganglia (Roizman e Pellet, 2001). *Caprine alphaherpesvirus 1* (CpHV-1) is a member of the subfamily *Alphaherpesvirinae* and it is closely related to *Bovine alphaherpesvirus* 1 (BoHV-1), an important pathogen of cattle. Goats and cattle are susceptible to CpHV-1 infection, although the development of overt clinical disease seems to be restricted to goats, the virus' natural host (Engels et al., 1992).

Caprine alphaherpesvirus 1 was first reported in 1975 in California (Berrios and McKercher, 1975) and, subsequently, has been reported in Europe, Australia, New Zeeland and Canada (Keuser et al., 2006). Currently, CpHV-1 is more commonly detected in European and Mediterranean countries, where goat production for meat and cheese is intensive, with seroprevalence rates reaching up to 50% (Suavet et al., 2016). A recent study showed that CpHV-1 infection is more frequently associated with large, extensively reared herds (Bertolini et al., 2018).

Infection by CpHV-1 takes place through the nasal (Tempesta et al., 1999a) or genital routes (Tempesta et al., 2000). In naturally infected kids, CpHV-1 causes hyperthermia, abdominal pain and anorexia, producing lesions in the intestine, lungs, urinary bladder and liver (Roperto et al., 2000). Most adult, immunocompetent animals infected with CpHV-1 present a subclinical infection, yet nonspecific clinical signs may develop, including hyperthermia and leukopenia. Importantly, vulvovaginitis and balanoposthitis characterized by edema, erythema, ulcers and

purulent discharge have been described in goats (Tempesta et al., 1999b, 1999a, 2000). Additionally, CpHV-1 has also been associated with reproductive failures and abortion storms in adult female goats (Chénier et al., 2004; Gonzalez et al., 2017). In kids, CpHV-1 infection is associated with systemic infection, characterized by ulcerative lesions in the gastrointestinal system, frequently leading to high morbidity and mortality (Roperto et al., 2000). Calves experimentally inoculated with CpHV-1 did not develop clinical signs but the virus was re-isolated from nasal swabs during acute infection. All animals seroconverted to CpHV-1 by day 14 post-infection (pi), however, no reactivation was detected in this species after dexamethasone administration (Six et al., 2001).

Thymidine kinase (TK) is an enzyme involved in nucleotide metabolism necessary for DNA synthesis and is encoded by most herpesviruses and by other DNA viruses including, African swine fever virus (ASFV), vaccinia virus (VACV) and other poxviruses (Sanford et al., 2016; Deng et al., 2017). Although TK activity is not essential for herpesvirus replication *in vitro*, studies on *Human alphaherpesvirus* 1 (HSV-1) have shown that *tk*-deleted mutants are usually replication-defective and do not reactivate from latency in neuronal tissue *in vivo* (Tenser, 1991). Thymidine kinase expression is an important factor that has been shown to influence viral properties during primary, acute infection and during establishment and reactivation of latent infection (Tenser et al., 1979). Importantly, deletion of *tk* gene from the genome of different herpesviruses, including BoHV-1 (Kit et al., 1985b; Chowdhury, 1996), *Bovine alphaherpesvirus 5* (BoHV-5) (Brum et al., 2010) and *Suid alphaherpesvirus 1* (PRV) (Ferrari et al., 2000) has been shown to directly attenuate these viruses *in vivo*. Deletion of *tk* gene has been used to study the role of TK during acute and latent herpesvirus infection and for the production of attenuated strains for use in vaccines and vaccine delivery vectors (Kit et al., 1985a, 1985b; McGregor et al., 1985; Smith et al., 1994; Anziliero et al., 2011).

In the present study, we constructed a *tk*-deleted CpHV-1 (CpHV-1$^{\Delta TK}$) and assessed its growth properties *in vitro* and its virulence in goats.

Material and Methods

Cells and viruses

Bovine turbinate cells (BT, ATCC® CRL-1390™) and primary bovine fetal turbinate cells (generated *in house*) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL^{-1}) streptomycin (100 μg/mL^{-1}) and gentamycin (50 μg/mL^{-1}). CRIB cells (Flores e Donis, 1995) (kindly provided by Dr. Clinton Jones, Oklahoma State University) were cultured in minimum essential medium (MEM) supplemented with 5% FBS, L-glutamine (2 mM), penicillin (100 U/mL^{-1}) streptomycin (100 μg/mL^{-1}) and gentamycin (50 μg/mL^{-1}). The cell cultures were maintained at 37ºC with 5% CO_2.

The CpHV-1 isolate WI 13-46 (isolated in North America) was used as parental virus to construct the *tk*-deleted CpHV-1$^{\Delta TK}$ recombinant virus. The isolate CpHV-1 WI 13-46 was amplified and titrated in BT cells. Low passage virus stocks (passage 4) were used in all experiments described below.

Construction of a tk-deleted CpHV-1 (CpHV-1$^{\Delta TK}$)

To generate the *tk*-deleted CpHV-1 (CpHV-1$^{\Delta TK}$), we initially constructed a recombination plasmid by inserting the right and left flanks of the *tk* gene into a pUC57 plasmid backbone containing the enhanced green fluorescent protein gene (eGFP) under control of a cytomegalovirus (CMV) promoter (pUC57-eGFP). The right and left *tk* flanks were amplified by PCR from the parental CpHV-1 genome, using the Q5® Hot Star High-Fidelity 2X Master Mix (New England

BioLabs catalog #M0494L) according to the manufacturer's instructions. The restriction enzymes SpeI and KpnI, and EcoRI and SalI (New England BioLabs catalog #R3133S, #R3142S, #R3101S, #R3138S) were used to clone the right and left flanking sequences into the pUC57-eGFP plasmid, respectively. The ligation reactions were performed using T4 DNA Ligase (New England BioLabs catalog #M0202S) according to the manufacturer's instructions. The resultant recombination plasmid pUC57-tkLF-eGFP-tkRF was used to generate the recombinant.

The recombinant CpHV-1$^{\Delta TK}$ virus was generated by homologous recombination, using infection/transfection, a procedure adapted from poxviruses (DeLange e McFadden, 1986). For this, primary BT cells were infected with CpHV-1 at a 0.5 multiplicity of infection (MOI). At 3 h post-infection, 2.5 µg of the recombination plasmid (pUC57-tkLF-eGFP-tkRF) DNA were transfected in primary BT cells using Lipofectamine® 3000 (Invitrogen by Life Technologies™ catalog #L3000015) according to manufacturer's instructions. The strategy of construction of the recombinant is depicted in Figure 1A.

Approximately 48 h after infection/transfection, GFP expression was monitored under a fluorescence microscope and the cells were subjected to three freeze-thaw cycles. The selection/purification of the recombinant CpHV-1$^{\Delta TK}$ virus was performed through plaque assays, as follows: 2 mL of the infection/transfection supernatant was diluted 1:10 in plain DMEM and 1 mL was inoculated into each well of a 6 well plate containing BT cells prepared 24 h in advance. After 1 h of adsorption at 37^{o}C, the inoculum was removed, and cells were overlaid with 3mL of 2X complete growth media (CGM) + 1% agarose and kept in 37^{o}C. At 72 h of incubation, the plates were screened for viral plaques expressing eGFP. These plaques were marked, picked and transferred into 1.7 ml tubes containing 250 µL MEM and frozen at -80^{o}C. The subsequent plaque assays were performed as described above, by diluting each of the selected clones/plaques at 1:10,

1:100 and 1:1000 in MEM. The deletion of the *tk* gene was confirmed by PCR using *tk*-specific primers (Fw: CTCGTCGTCTGCACCCTTC, Rv: CGACATGTCCAGCGTGAATA). The amplification conditions used were as follows: one cycle of initial denaturation (98°C, 30 sec), followed by 35 cycles of denaturation, annealing and extension (95°C, 10 sec; 59°C, 30 sec; 72°C, 1 min), and a final extension (72°C, 2 min). The identity and integrity of the CpHV-$1^{\Delta TK}$ sequences were confirmed by DNA sequencing. Stocks of the recombinant CpHV-$1^{\Delta TK}$ were thereafter produced in CRIB cells.

In vitro characterization of the* tk*-deleted CpHV-1 (CpHV-*$1^{\Delta TK}$*)

To assess the kinetics of replication of the recombinant CpHV-$1^{\Delta TK}$ virus, CRIB and BT cell monolayers were inoculated with 0.1 and 10 MOI of parental CpHV-1 and recombinant CpHV-$1^{\Delta TK}$ and the cultures (cells plus supernatants) were harvested at 0, 6, 12, 24, 48 and 72 h post infection. Later, titrations were performed in BT cells for each time point.

To investigate differences in plaque size between the parental and recombinant viruses, plaque assays were performed in BT and CRIB cells. After 72 h, the agarose overlay was removed, the cells were fixed with 10% formalin and stained with 0.2% crystal violet. The plaques were measured using the software ImageJ (Schneider et al., 2012). Plaques produced at the 10^{-3} dilution by both the parental CpHV-1 isolate WI 13-46 and the recombinant CpHV-$1^{\Delta TK}$ viruses were measured, and the plaque size compared using unpaired t test ($p < 0.05$).

Animal experiment

The effect of the *tk*-deletion on the virulence and pathogenicity of respiratory CpHV-1 infection was evaluated in young goats. For this, fifteen 4 to 6-month-old goats were randomly allocated into three groups as follows: parental virus group (n = 5), recombinant virus group (n =

5) and negative control group (n = 5). Animals were acclimated for seven days before virus inoculation. The parental and recombinant virus groups were inoculated intranasally (IN) with 10 mL of a virus suspension containing 10^7 $TCID_{50}$ of the parental CpHV-1 WI 13-46 virus or the recombinant CpHV-$1^{\Delta TK}$ virus, respectively. The negative control group was inoculated with 10 mL of MEM. Following virus inoculation all animals were monitored daily and clinical signs and rectal temperature were recorded for 14 days. Whole blood (one tube containing sodium heparin for buffy coat separation and one tube for serum collection), nasal and rectal swabs were collected at 0, 1, 3, 5, 7, 10, 14, 21, 28 and 35 dpi.

To assess the ability of CpHV-$1^{\Delta TK}$ and CpHV-1 WI 13-46 to reactivate from latent infection, animals from both groups and three control animals received daily intramuscular administrations of dexamethasone (Dx, 0.4 mg/kg/day) (Diel et al., 2007) for five consecutive days (days 35 to 39 dpi). Animals were monitored daily for 14 days post dexamethasone (dpDx) and clinical signs and rectal temperatures were recorded. Whole blood, nasal and rectal swabs were collected on days 1, 3, 5, 7, 10 and 14 pDx. All samples collected during acute or latent phase of infection (pDx) were subjected to virus isolation and/or PCR. At 14 dpDx, all animals were euthanized and the trigeminal ganglia (TG) and olfactory bulbs (OB) were collected for DNA extraction and PCR. The animal experiment is illustrated in Figure 2. The mean of rectal temperatures was analyzed using two-way ANOVA ($p < 0.05$). All animal experiments and procedures were reviewed and approved by the South Dakota State University Institutional Animal Use Committee (Approval no. 18-043A).

Sample handling

Immediately after collection, the blood was centrifuged at room temperature for 10 min at 3500 rpm. The buffy coats were purified from 8 mL of blood collected in tubes containing sodium

heparin, using red blood lysis buffer. The buffy coat pellet was resuspended in 500 μL of PBS. Serum was separated from blood collected in red cap tubes by centrifugation at 3500 rpm for 10 min.

Serum, buffy coats, nasal and rectal swabs were stored at -80ºC until virus isolation was performed then transferred to -20ºC for long term storage.

Virus neutralizing assays

Virus neutralizing (VN) assays were performed to assess neutralizing antibody responses in inoculated animals. Briefly, heat inactivated (56ºC for 30 min) serum samples were subjected to two-fold dilutions and incubated with a constant amount of virus (CpHV-$1^{\Delta TK}$ 200 $TCID_{50}$ per well) for 1 h at 37ºC. After the incubation, a suspension of CRIB cells was added to each well and plates incubation at 37ºC for 72 h. Assays were read under a fluorescence microscope and the titer of neutralizing antibodies was considered as the reciprocal of the highest serum dilution that prevented virus replication. Group differences were assessed using two-way ANOVA ($p < 0.05$)

Virus isolation

Virus isolation was performed in BT cells. Nasal swabs, serum, buffy coats, and homogenates of TG and OB were inoculated in semi-confluent monolayers of BT cells, and adsorbed for 1 h at 37ºC. After adsorption, 500 μL of complete culture media was added to each well and cells incubated at 37ºC four days. Samples were considered negative after three four-day passages without the evidence of cytopathic effect.

qPCR and nested PCR

Total DNA was extracted from serum, buffy coats, fecal and nasal swabs using the Cador® Pathogen 96 kit (cat no.54161) and the QIAcube HT (QIAGEN cat no.ID 9001793) automated extractor according to the manufacturer's instructions. The buffy coats underwent pretreatment T2 for enzymatic digestion of tissues described at the cador® Pathogen 96 QIAcube HT handbook. For all extractions, a dilution of 10^4 $TCID_{50}$ of the CpHV-1 virus stock was prepared in plain MEM and used as positive control. A CpHV-1 negative serum sample was used as negative control. DNA extraction from TGs and OBs was performed using TRIzol® reagent (Thermo Fischer Scientific catalog number 15596026) according to the manufacturer's instructions.

Total DNA extracted from nasal secretions and feces, serum and buffy coats were subjected to qPCR for CpHV-1 DNA. The qPCR reaction was performed with SensiFAST™ Probe Lo ROX Mix (Bioline, cat. No. BIO-84050), following the manufacturer's instructions and using 5 µL of DNA as template. For the qPCR, two sets of primers and TaqMan probes were designed and each sample tested independently with each set. The first set targeted CpHV-1 gD (probe: 5'-/56-FAM/CAATAAGCA/ZEN/CTTTGGCTACTGCCGG/3IABkFG/-3', primer 1: 5'-CGCGAACCCAGACAGAAA-3', primer 2: 5'- GTACGTGATGGAGTACCAAGAG-3') and the second set targeted CpHV-1 polymerase (probe: 5'-/56-FAM/CAATAAGCA/ZEN/CTTTGGCTACTGCCGG/3IABkFG/-3', primer 1: 5'-GTAAACTTCGACTGGGCCTAC-3', primer 2: 5'-CTTCACCTTGCTCTGCTTCT-3'). The amplification/detection conditions for these qPCR reactions were the following: one cycle of polymerase activation (95ºC, 5 min) and 40 cycles of denaturation and annealing/extension (95ºC, 10 sec followed by 60ºC, 50 sec). A standard curve was generated with serial 10-fold dilutions of the parental and recombinant viruses to determine the limit of detection of each set of primers and

probe. The dilution series were subjected to DNA extraction and PCR amplification. The detection limit of 10 $TCID_{50}$ per reaction (equivalent to a CT value of 37.2728) was used as a cutoff value for the qPCR. All samples with CT values higher than this cutoff were considered negative. Samples were only considered positive if CpHV-1 amplification was detected with both sets of primers and probes.

DNA extracted from TG and OB was tested through nested PCR (nPCR), performed with Q5® Hot Star High-Fidelity 2X Master Mix, using 0.5 μL of primers for each reaction and 2 μL of DNA as template for each reaction. For the nPCR, we used two sets of primers targeting the CpHV-1 gC (Nes1 Fw: CCGTCACGGTCTTTAGCTG, Nes1 Rv: CACCCCCAACAACTTTGACT – amplicon size 584 bp; Nes2 Fw:CTCGTGGTCGCAGAGCAT, Nes2 Rv: CACCCCCAACAACTTTGACT – amplicon size 220 bp). After optimization of the primers, amplification conditions used in our study were: first reaction – one cycle of initial denaturation (98ºC, 30 sec), followed by 35 cycles of denaturation, annealing and extension (95ºC, 10 sec; 64ºC, 30 sec; 72ºC, 30 sec), and a final extension (72ºC, 2 min); second reaction – one cycle of initial denaturation (98ºC, 30 sec), followed by 35 cycles of denaturation, annealing and extension (95ºC, 10 sec; 59ºC, 30 sec; 72ºC, 30 sec), and a final extension (72ºC, 2 min). The product of the first reaction was purified with GeneJET PCR Purification Kit (Thermo Scientific ref K0702), using 35 μL of ultra-pure water for the final elution and the purified PCR product used as template in the second amplification reaction. The amplicons of the second reaction were analyzed by electrophoresis in a 1% agarose gel stained with GelRed® (Biotium #41003) and visualized under UV light. The limit of detection of the nPCR (10 $TCID_{50}$ per reaction) was determined as described above for the qPCR assays. All amplification included a positive (DNA extracted from the CpHV-1 stock) and negative (ultra-pure water) controls.

Results

Generation of the tk*-deleted CpHV-1$^{\Delta TK}$ virus*

Three independent eGFP positive viral plaques were selected and picked in the first plaque assay following the infection/transfection. A representative plaque expressing eGFP is shown in Fig. 1B. During each plaque assay, three independent clones were selected and used in the next plaque purification round. After five rounds of plaque purification (plaque assay no. 5), 15 clones were picked and amplified in CRIB cells. Inoculated cultures were harvested and subjected to DNA extraction and PCR amplification to confirm the deletion of the *tk* gene. Two clones presented weak bands corresponding to the *tk* amplicon (data not shown). Two more plaque assays were performed, and, at plaque assay no. 7, DNA extraction and PCR amplification were repeated confirming the deletion of *tk* sequences and insertion of the GFP sequences in the recombinant CpHV-1$^{\Delta TK}$ virus (data not shown). One clone was then selected, amplified in CRIB cells and subjected to the *tk*-PCR screening. Results from the PCR amplification confirmed the deletion of *tk*-gene sequences from the *CpHV-1$^{\Delta TK}$* virus genome (Fig.1C). This *tk*-deleted clone was amplified in CRIB cells and used in all experiments described below.

The tk *gene is non-essential for CpHV-1 replication* in vitro

Replication kinetics, plaque size and morphology of the parental and recombinant viruses were investigated in BT and CRIB cells. Multi-step and single-step growth curves demonstrated that both viruses present similar replication kinetics in these cells, but the recombinant CpHV-1$^{\Delta TK}$ produced slightly lower viral yields. Significant differences were only observed at 24 h (0.1 MOI) in BT cells (ANOVA $p < 0.05$) (Fig. 3A). Plaque assays revealed similar plaque morphology between the parental and the recombinant CpHV-1$^{\Delta TK}$ viruses (Fig. 3E). Likewise, no differences

in the size of plaques produced by both viruses were observed. These results indicate that deletion of the *tk* gene from the genome of CpHV-1 did not adversely affect the virus ability to replicate *in vitro*, confirming that the viral TK is nonessential for CpHV-1 replication in BT and CRIB cells.

The recombinant CpHV-1$^{\Delta TK}$ is attenuated in goats

To investigate the effect of *tk* deletion on CpHV-1 virulence and pathogenesis *in vivo*, we performed a study in young goats. For this, fifteen CpHV-1-seronegative goats were inoculated with the parental virus (CpHV-1 WI 13-46; n = 5), the recombinant CpHV-1$^{\Delta TK}$ virus (n = 5) or mock-inoculated with MEM (n = 5). Upon IN inoculation, four animals (4/5) from the parental virus group developed moderate mucous nasal secretion starting at 5 dpi and lasting up to 14 dpi. At 7 dpi, two animals from the parental virus group presented respiratory distress and one animal presented serous ocular secretion at 8 dpi. In contrast, no respiratory or systemic signs of infection were observed in goats from the recombinant virus group nor in the negative control animals. Animals inoculated with the parental virus presented fever between days 2 pi and 7 pi when compared with the animals in recombinant and control groups (Fig. 4A). Serological responses to CpHV-1 were also evaluated after inoculation. Virus neutralizing (VN) assays demonstrated that goats inoculated with the parental virus developed higher neutralizing antibodies (NA) titers than those inoculated with the recombinant virus (Fig. 4B).

The recombinant CpHV-1$^{\Delta TK}$ replicates with lower efficiency* in vivo *when compared to the parental virus

Viremia and virus shedding were assessed by virus isolation and qPCR performed in serum, buffy coats, nasal and rectal swabs during acute infection (Table 1). During acute infection, viral DNA was detected in the serum of two animals from parental group (2/5) at 5 dpi and in none of

the animals from the recombinant group (0/5). Nasal swabs were positive for viral DNA in all five animals from the parental virus group between day 1 and 10 pi (average shedding 5.4 days, range: 1 - 10). Infectious virus was only recovered from nasal swabs of three (3/5) animals in the parental virus group between days 3 and 5 pi. In contrast, only two animals (2/5) from the recombinant virus group were positive for viral DNA between days 3 and 7 pi. Virus shedding in feces was detected in rectal swabs of three animals (3/5) from parental virus group between days 3 and 7 pi, and in one animal (1/5) of the recombinant virus group at 14 dpi. No virus or viral DNA was detected in buffy coats. These findings demonstrate that the recombinant CpHV-1$^{\Delta TK}$ replicated with lower efficiency and was shed for a shorter period than the parental virus. Taken together, these results demonstrated that deletion of the *tk*-gene resulted in attenuation of CpHV-1 in goats.

Dexamethasone (Dx) administration did not result in reactivation of CpHV-1

To assess the ability of the parental and recombinant viruses to reactivate from latency, the inoculated animals were subjected to Dx administration from 35 to 39 dpi and monitored thereafter. No infectious virus was isolated from serum, buffy coats or nasal secretions collected from animals from both parental and recombinant virus groups after Dx administration. The qPCR performed in these samples also resulted negative. Serological testing of animals prior to Dx administration (35 dpi) and on day 14 pDx revealed a ≥ four-fold increase in VN titer in one animal from the parental virus group (1802, 16 to 64) (Table 1). Following Dx administration, four animals (4/5) from the parental virus group presented nasal discharge, lasting from 2 dpDx to 11 dpDx. At 5 dpDx and 7 dpDx, two animals (2/5) from parental virus group presented mild cough which extended up to 8 dpDx in one animal. Only one animal from the recombinant virus group presented fever after Dx administration. None of the animals from the control group presented any clinical signs. Virus

neutralizing antibody titers remained higher in the parental virus group than in the recombinant virus group, resembling what had been observed during acute infection (Fig. 5).

One animal from the recombinant group was found dead in the pen at 6 dpDx. A necropsy performed at South Dakota State University's Animal Disease Research and Diagnostic Laboratory revealed an intestinal mesenteric torsion as the cause of death. From 7 dpDx to 10 dpDx, one animal from the recombinant virus group presented high fever and coughing. This animal was treated with antibiotic (tulathromicin 75 mg) at 10 dpDx and recovered promptly.

CpHV-1 and CpHV-1$^{\Delta TK}$ established latent infection in the trigeminal ganglia

At 14 dpDx, all animals were euthanized for tissue collection (TG, OB) and investigation of latent infection by nested-PCR (nPCR). The nPCR performed in total DNA extracted from TGs was positive in four animals (4/5) from the parental virus group and in three animals (3/5) from the recombinant virus group. No infectious virus was isolated from TG homogenates inoculated in BT cells after three passages. Viral DNA was not detected in the OBs of any animals and the virus was not recovered when OB homogenates when inoculated in BT cells (Table 1). These results indicate that both parental CpHV-1 WI 13-46 and recombinant *CpHV-1$^{\Delta TK}$* did establish latent infection in TGs of inoculated goats, but latency was not reactivated upon Dx administration.

Discussion

Caprine alphaherpesvirus 1 (CpHV-1) has been associated with systemic, respiratory and reproductive disease in goats. The enzyme thymidine kinase (TK) is an important product involved in herpesvirus replication and, consequently, in the virulence of alphaherpesviruses (Tenser et al., 1979; Roizman e Pellet, 2001). Hence, deletion of the *tk* gene has been used for the generation of attenuated virus strains for use as vaccines for several animal alphaherpesviruses (Cornick et al.,

1990; Mengeling, 1991; Anziliero et al., 2011). Here we hypothesized that deletion of the *tk* gene from the CpHV-1 isolate WI 13-46 would result in attenuation of the virus in goats. To assess this hypothesis, we generated a recombinant virus (CpHV-1$^{\Delta TK}$) in which the *tk* gene was deleted from the viral genome and replaced by the eGFP reporter gene by using homologous recombination. The replication properties of recombinant CpHV-1$^{\Delta TK}$ were assessed *in vitro*, and its pathogenicity and virulence were investigated upon intranasal inoculation in young goats.

Caprine alphaherpesvirus 1 represents one of the least studied alphaherpesviruses and the role of TK in CpHV-1 infection and pathogenesis have not yet been investigated. Results presented here demonstrate that deletion of the *tk* gene from the CpHV-1 genome did not significantly affect the ability of the recombinant CpHV-1$^{\Delta TK}$ virus to replicate *in vitro*, as evidenced by similar replication kinetics and plaque characteristics (size and morphology) in CRIB and BT cells when compared to the parental virus (Fig 3A-E). Consistent with our observations, no differences in plaque size and morphology and/or on viral replication kinetics were observed between the parental virus and *tk*-deleted recombinants of BoHV-1 (Chowdhury, 1996), BoHV-5 (Brum et al., 2010), *Equid alphaherpesvirus 1* (EHV-1) (Slater et al., 1993) and *Human alphaherpesvirus 2* (HSV-2) (Da Costa et al., 1999). For other alphaherpesviruses such as HSV-1, however, deletion of the *tk*-gene led to production of smaller plaques and to impaired growth properties in cell cultures *in vitro* (Jacobson et al., 1989). These findings suggest a diverse role of TK during herpesvirus infection and replication. Results here indicate that the TK is non-essential for replication of CpHV-1 *in vitro* and its deletion did not adversely affect the ability of the virus to replicate in BT and CRIB cells.

Upon intranasal inoculation, goats in the parental virus group presented respiratory (nasal secretion, respiratory distress) and systemic clinical signs (increased body temperature) (Fig 4A), while animals inoculated with the recombinant virus did not present overt clinical disease (Fig 4A).

Animals in both groups developed neutralizing antibodies, however, infection with the parental virus resulted in a more robust NA response when compared to antibody levels detected in recombinant CpHV-1$^{\Delta TK}$ inoculated animals (Fig 4B). In addition, virus shedding was more frequently detected in animals in the parental virus group, which also were the only ones to present viremia (Table 1). Together these results demonstrate that, although the recombinant CpHV-1$^{\Delta TK}$ was able to infect all inoculated animals, as evidenced by seroconversion, deletion of the *tk* gene from the virus genome resulted in attenuation of the recombinant virus when compared to the parental virus. Attenuation of *tk*-deleted herpesvirus mutants has been previously reported for other animal alphaherpesviruses, including BoHV-1 (Kit et al., 1985b; Chowdhury, 1996), BoHV-5 (Anziliero et al., 2011), EHV-1 (Slater et al., 1993) and PRV (Kit et al., 1985a; Ferrari et al., 2000). The absence of virus-encoded TK reduces the availability of nucleotides for the synthesis and replication of the viral genome and, thus, impairs the ability of herpesviruses to replicate *in vivo* (Tenser et al., 1983; Tenser, 1991). Our findings with CpHV-1 here confirm and extend previous results showing that the *tk* gene represents a bona fide virulence determinant of animal alphaherpesviruses in their natural hosts.

Reactivation of latent CpHV-1 infection has been difficult to achieve under experimental conditions (Tempesta et al., 1999b) and only high doses of Dx (2.5-4.0 mg/kg) have been shown effective in inducing CpHV-1 reactivation in goats (Buonavoglia et al., 1996). Although in our study a few animals in the parental and recombinant viruses presented an increase in NA titers following Dx administration (which is an indirect suggestion of replication/reactivation) (Table 1), only one animal inoculated with the parental virus (no. 1802) presented a 4-fold increase in VN titers after Dx administration suggesting a re-stimulation of the immune system and potentially virus reactivation. Despite the seroconversion, no infectious virus nor viral DNA was detected in

nasal secretions, feces or serum of this animal and nor in any other inoculated animal following administration of 0.4 mg/kg of Dx for 5 consecutive days. Observations from Buonavoglia and collaborators (Buonavoglia et al., 1996) suggest that higher doses of Dx may be needed in order to effectively reactivate CpHV-1 from latently infected animals. Despite the fact that we were not able to directly detect CpHV-1 reactivation following Dx administration, establishment of latent infection by both parental and recombinant viruses was demonstrated by detection of viral DNA by nPCR in the TGs of inoculated animals. Thus, deletion of the *tk* (and absence of viral encoded TK) apparently did not abolish the ability of CpHV-1 to establish latent infection in the neuronal cells of inoculated goats.

Although *tk*-deleted alphaherpesvirus are able to establish infection in neurons, the lack (or expression at low levels) of host TK in this cell type impairs nucleotide synthesis required for herpesvirus genome replication, events required for virus reactivation (Jamieson et al., 1974). Limited expression of TK in neurons – linked to the inability of these cells to divide – may explain why the expression of viral-encoded TK is critical for virus reactivation (Jamieson et al., 1974; Tenser, 1991). Future studies using higher doses of Dx will be needed, however, to determine whether deletion of *tk* from CpHV-1 virus genome results in a reactivation-defective virus as observed for several other alphaherpesviruses (Jamieson et al., 1974; Tenser et al., 1983; Chen et al., 2004; Anziliero et al., 2011).

In summary, we successfully constructed a *tk*-deleted CpHV-1 and demonstrated that the recombinant virus is attenuated in young goats upon intranasal inoculation. While deletion of the *tk* gene did not adversely affect the ability of the virus to replicate *in vitro,* the recombinant gene-deleted virus presented an attenuated phenotype *in vivo.* Both parental and recombinant viruses were able to establish latent infection in sensory nerve ganglia. Since there are no vaccines

currently available for CpHV-1, the CpHV-1$^{\Delta TK}$ developed here may represent a promising vaccine candidate to prevent and control CpHV-1 infection in the field.

Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgements

We thank the staff of the SDSU Animal Resource Wing (ARW) for proper care of animals. The work was funded by the SD Governor's Office for Economic Development through a grant to the Center for Biologics Research and Commercialization (CBRC) and in part by the USDA National Institute of Food and Agriculture Hatch project SD00H517-14 and Multi-state project SD00R518-14. This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001.

References

Anziliero, D., Santos, C.M.B., Brum, M.C.S., Weiblen, R., Chowdhury, S.I., Flores, E.F., 2011. A recombinant bovine herpesvirus 5 defective in thymidine kinase and glycoprotein E is immunogenic for calves and confers protection upon homologous challenge and BoHV-1 challenge. Vet. Microbiol. 154, 14–22. doi:10.1016/J.VETMIC.2011.03.019

Berrios, P.E., McKercher, D.G., Knight, H.D., 1975. Pathogenicity of a caprine herpesvirus. Am. J. Vet. Res. 36, 1763–9.

Bertolini, S., Rosamilia, A., Caruso, C., Maurella, C., Ingravalle, F., Quasso, A., Acutis, P.L., Pitti, M., Masoero, L., Ru, G., 2018. A cross-sectional study to identify a set of risk factors for caprine herpesvirus 1 infection. BMC Vet. Res. 14. doi:10.1186/s12917-018-1401-8

Brum, M.C.S., Weiblen, R., Flores, E.F., Chowdhury, S.I., 2010. Construction and growth properties of bovine herpesvirus type 5 recombinants defective in the glycoprotein E or thymidine kinase gene or both. Brazilian J. Med. Biol. Res. 43, 217–224. doi:10.1590/S0100-879X2009007500008

Buonavoglia, C., Tempesta, M., Cavalli, A., Voigt, V., Buonavoglia, D., Conserva, A., Corrente, M., 1996. Reactivation of caprine herpesvirus 1 in latently infected goats. Comp. Immunol. Microbiol. Infect. Dis. 19, 275–281. doi:10.1016/0147-9571(96)00014-8

Chen, S.-H., Pearson, A., Coen, D.M., Chen, S.-H., 2004. Failure of Thymidine Kinase-Negative Herpes Simplex Virus To Reactivate from Latency following Efficient Establishment. J. Virol. 78, 520–523. doi:10.1128/jvi.78.1.520-523.2004

Chénier, S., Montpetit, C., Hélie, P., 2004. Caprine herpesvirus- 1 abortion storm in a goat herd in Quebec. Can. Vet. J. = La Rev. Vet. Can. 45, 241–3.

Chowdhury, S.I., 1996. Construction and characterization of an attenuated bovine herpesvirus

type 1 (BHV-1) recombinant virus. Vet. Microbiol. 52, 13–23. doi:10.1016/0378-1135(96)00043-0

Cornick, J., Martens, J., Martens, R., Crandell, R., Mcconnell, S., Kit, S., 1990. Safety and Efficacy of a Thymidine Kinase Negative Equine Herpesvirus-1 Vaccine in Young Horses, Canadian journal of veterinary research = Revue canadienne de recherche veterinaire. Canadian Veterinary Medical Association.

Da Costa, X.J., Jones, C.A., Knipe, D.M., 1999. Immunization against genital herpes with a vaccine virus that has defects in productive and latent infection. Proc. Natl. Acad. Sci. 96, 6994–6998. doi:10.1073/pnas.96.12.6994

DeLange, A.M., McFadden, G., 1986. Sequence-nonspecific replication of transfected plasmid DNA in poxvirus-infected cells. Proc. Natl. Acad. Sci. 83, 614–618. doi:10.1073/pnas.83.3.614

Deng, L., Fan, J., Ding, Y., Zhang, J., Zhou, B., Zhang, Y., Huang, B., 2017. Oncolytic efficacy of thymidine kinase-deleted vaccinia virus strain Guang9. Oncotarget 8, 40533–40543. doi:10.18632/oncotarget.17125

Diel, D.G., Almeida, S.R., Brum, M.C.S., Dezengrini, R., Weiblen, R., Flores, E.F., 2007. Acute and latent infection by bovine herpesvirus type 5 in experimentally infected goats. Vet. Microbiol. 121, 257–267. doi:10.1016/J.VETMIC.2006.12.019

Engels, M., Palatini, M., Metzler, A.E., Probst, U., Kihm, U., Ackermann, M., 1992. Interactions of bovine and caprine herpesviruses with the natural and the foreign hosts. Vet. Microbiol. 33, 69–78. doi:10.1016/0378-1135(92)90036-S

Ferrari, M., Gualandi, G.L., Corradi, A., Monaci, C., Romanelli, M.G., Losio, M.N., Cantoni, A.M., Pratelli, A., 2000. The response of pigs inoculated with a thymidine kinase-negative (TK-) pseudorabies virus to challenge infection with virulent virus. Comp. Immunol.

Microbiol. Infect. Dis. 23, 15–26. doi:10.1016/S0147-9571(99)00019-3

Flores, E.F., Donis, R.O., 1995. Isolation of a Mutant MDBK Cell Line Resistant to Bovine Viral Diarrhea Virus Infection Due to a Block in Viral Entry. Virology 208, 565–575. doi:10.1006/viro.1995.1187

Gonzalez, J., Passantino, G., Esnal, A., Cuesta, N., García Vera, J., Mechelli, L., Saez, A., García Marín, J., Tempesta, M., 2017. Abortion in goats by Caprine alphaherpesvirus 1 in Spain. Reprod. Domest. Anim. 52, 1093–1096. doi:10.1111/rda.13034

Jacobson, J.G., Martin, S.L., Coen3, D.M., 1989. A Conserved Open Reading Frame That Overlaps the Herpes Simplex Virus Thymidine Kinase Gene Is Important for Viral Growth in Cell Culture, J. Virol. 63-4, 1839-1843.

Jamieson, A.T., Gentry, G.A., Subak-Sharpe, J.H., 1974. Induction of Both Thymidine and Deoxycytidine Kinase Activity by Herpes Viruses. J. Gen. Virol. 24, 465–480. doi:10.1099/0022-1317-24-3-465

Keuser, V., Detry, B., Thiry, J., Fays, K. De, Schynts, F., Pastoret, P.-P.P., Vanderplasschen, A., Thiry, E., 2006. Characterization of caprine herpesvirus 1 glycoprotein D gene and its translation product. Virus Res. 115, 112–121. doi:10.1016/j.virusres.2005.07.009

Kit, S., Kit, M., Pirtle, E.C., 1985a. Attenuated properties of thymidine kinase-negative deletion mutant of pseudorabies virus. Am. J. Vet. Res. 46, 1359–67.

Kit, S., Qavi, H., Gaines, J.D., Billingsley, P., McConnell, S., 1985b. Thymidine kinase-negative bovine herpesvirus type 1 mutant is stable and highly attenuated in calves. Arch. Virol. 86, 63–83.

McGregor, S., Easterday, B.C., Kaplan, A.S., Ben-Porat, T., 1985. Vaccination of swine with thymidine kinase-deficient mutants of pseudorabies virus. Am. J. Vet. Res. 46, 1494–7.

Mengeling, W.L., 1991. Virus reactivation in pigs latently infected with a thymidine kinase

negative vaccine strain of pseudorabies virus. Arch. Virol. 120, 57–70.

Roizman, B., Pellet, P.E., 2001. The family Herpesvridae: a brief introduction, in: Knipe, D.M., Howley, P.M. (Orgs.), Fields Virology. Lippincot Williams & Wilkins, Philpadelphia, p. 2381–2398.

Roperto, F., Pratelli, A., Guarino, G., Ambrosio, V., Tempesta, M., Galati, P., Iovane, G., Buonavoglia, C., 2000. Natural caprine herpesvirus 1 (CpHV-1) infection in kids. J. Comp. Pathol. 122, 298–302. doi:10.1053/jcpa.1999.0375

Sanford, B., Holinka, L.G.G., O'Donnell, V., Krug, P.W.W., Carlson, J., Alfano, M., Carrillo, C., Wu, P., Lowe, A., Risatti, G.R.R., Gladue, D.P.P., Borca, M.V. V., 2016. Deletion of the thymidine kinase gene induces complete attenuation of the Georgia isolate of African swine fever virus. Virus Res. 213, 165–171. doi:10.1016/j.virusres.2015.12.002

Schneider, C.A., Rasband, W.S., Eliceiri, K.W., 2012. NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9, 671–5. doi:10.1038/NMETH.2089

Six, A., Banks, M., Engels, M., Bascuñana, C.R., Ackermann, M., 2001. Latency and reactivation of bovine herpesvirus 1 (BHV-1) in goats and of caprine herpesvirus 1 (CapHV-1) in calves. Arch. Virol. 146, 1325–1335. doi:10.1007/s007050170094

Slater, J.D., Gibson, J.S., Field, H.J., 1993. Pathogenicity of a thymidine kinase-deficient mutant of equine herpesvirus 1 in mice and specific pathogen-free foals. J. Gen. Virol. 74, 819–828. doi:10.1099/0022-1317-74-5-819

Smith, G., Young, P., Rodwell, B., Kelly, M., Storie, G., Fsrrah, C., Mattick, J., 1994. Development and trial of a bovine herpesvirus 1 - thymidine kinase deletion virus as a vaccine. Aust. Vet. J. 71, 65–70. doi:10.1111/j.1751-0813.1994.tb03329.x

Suavet, F., Champion, J.-L., Bartolini, L., Bernou, M., Alzieu, J.-P., Brugidou, R., Darnatigues, S., Reynaud, G., Perrin, C., Adam, G., Thiéry, R., Duquesne, V., 2016. First Description of

Infection of Caprine Herpesvirus 1 (CpHV-1) in Goats in Mainland France. Pathogens 5, 17. doi:10.3390/pathogens5010017

Tempesta, M., Pratelli, A., Corrente, M., Buonavoglia, C., 1999a. A preliminary study on the pathogenicity of a strain of caprine herpesvirus-1. Comp. Immunol. Microbiol. Infect. Dis. 22, 137–143. doi:10.1016/S0147-9571(98)00029-0

Tempesta, M., Pratelli, A., Greco, G., Martella, V., Buonavoglia, C., 1999b. Detection of caprine herpesvirus 1 in sacral ganglia of latently infected goats by PCR. J. Clin. Microbiol. 37, 1598–9.

Tempesta, M., Pratelli, A., Normanno, G., Camero, M., Buonavoglia, D., Greco, G., Buonavoglia, C., 2000. Experimental intravaginal infection of goats with caprine herpesvirus 1. J. Vet. Med. Ser. B 47, 197–201. doi:10.1046/j.1439-0450.2000.00334.x

Tenser, R.B., 1991. Role of Herpes simples Virus Thymidine Kinase Expression in Viral Pathogenesis and Latency. Intervirology 32, 76–92. doi:10.1159/000150188

Tenser, R.B., Miller, R.L., Rapp, F., 1979. Trigeminal ganglion infection by thymidine kinase-negative mutants of herpes simplex virus. Science 205, 915–7. doi:10.1126/SCIENCE.224454

Tenser, R.B., Ressel, S.J., Fralish, F.A., Jones, J.C., 1983. The Role of Pseudorabies Virus Thymidine Kinase Expression in Trigeminal Ganglion Infection. J. Gen. Virol. 64, 1369–1373. doi:10.1099/0022-1317-64-6-1369

Figure 1. Construction and characterization of the recombinant CpHV-1$^{\Delta TK}$. A. Schematic representation of the recombination plasmid and the homologous recombination to delete the *tk* gene from the CpHV-1 genome. B. CpHV-1$^{\Delta TK}$ virus plaque showing expression of GFP in BT cells. C. PCR for an internal region of the *tk* gene.

Figure 2. Animal experiment design. Fifteen goats were allocated in three groups: parental virus group, recombinant virus group and negative control (n = 5/group). Acute phase: Animals were monitored daily and rectal temperatures were measured daily for 14 days. Nasal and rectal swabs and blood samples were collected on days 0, 3, 7, 10, 14, 21, 28 and 35 pi and processed for virological assessments. Latent phase: Animals were subjected to Dx administration (0.4 mg/kg IM) for five consecutive days (between days 35 and 39 pi). Nasal and rectal swabs and blood samples were collected on days 0, 3, 5, 7, 10, and 14, pDx and processed for virological assessments. On day 14 pDx, all animals were euthanized for collection of trigeminal ganglia (TG) and olfactory bulb (OB).

Figure 3. *In vitro* characterization of the recombinant CpHV-1$^{\Delta TK}$. A. Multi-step growth curve (MOI = 0.1) comparing replication properties of the parental virus and recombinant virus in BT cells. B. Single-step growth curve (MOI = 10) comparing replication properties of the parental virus and recombinant virus in BT cells. C: Multi-step growth curve (MOI = 0.1) comparing replication properties of the parental virus and recombinant virus in CRIB cells. D. Single-step growth curve (MOI = 10) comparing replication properties of the parental virus and recombinant

virus in CRIB cells. E. Comparison of plaque sizes produced by the parental virus and recombinant in BT and CRIB cells. Statistical differences were assessed by two-way ANOVA (*, $p < 0.05$).

Figure 4. A: Mean rectal temperature of goats inoculated with CpHV-1 (parental) and recombinant CpHV-$1^{\Delta TK}$ during acute infection. B: Neutralizing antibodies developed by inoculated animals during acute infection. Statistical differences were assessed by two-way ANOVA ($p < 0.05$): a – control group vs recombinant group; b – control group vs parental group; c – recombinant group vs parental group.

Figure 5. A: Mean rectal temperature of goats inoculated with CpHV-1 (parental) and recombinant CpHV-$1^{\Delta TK}$ after dexamethasone administration B: Neutralizing antibodies levels during latent infection (after Dx administration). Statistical differences were assessed by two-way ANOVA ($p < 0.05$): a – control group vs recombinant group; b – control group vs parental group; c – recombinant group vs parental group.

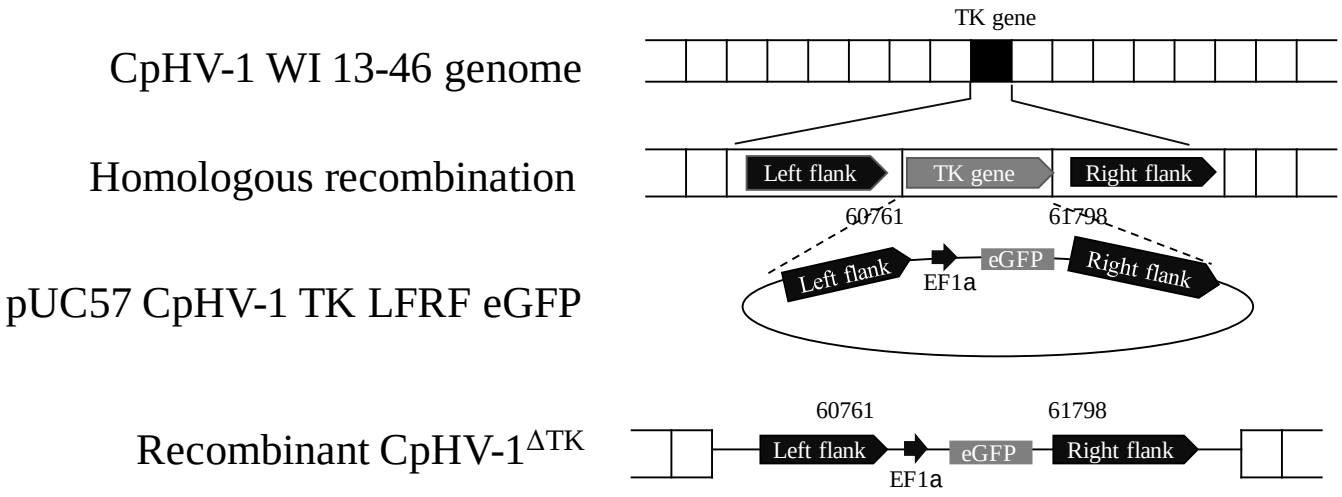
Table 1. Virological and serological findings in kids inoculated intranasally with the parental CpHV-1 and recombinant CpHV-1$^{\Delta TK}$ viruses

Group	Animal ID	Acute infection					Latent infection		
		Viral DNA (dpi)				VN 35	VN 14	Viral DNA	
		Nasal swab	Rectal swab	Serum	Buffy coat	dpi	dpDx	TG	OB
Parental	0011	3-10	-	-	-	32	32	+	-
	0005	1-10	5-7	-	-	32	64	+	-
	1803	5-7	3 and 7	5	-	32	64	+	-
	1599	3-7	7	5	-	16	16	-	-
	1802	3	-	-	-	16	64	+	-
Recombinant	0006	3 and 7	-	-	-	8/16	32	+	-
	35492	-	14	-	-	2	*	+	-
	1874	3-5	-	-	-	4	4	+	-
	1807	-	-	-	-	< 2	16	-	-
	1830	-	-	-	-	8	16	-	-
Control	0010	-	-	-	-	< 2	< 2	-	-
	1827	-	-	-	-	< 2	< 2	-	-
	1817	-	-	-	-	< 2	< 2	-	-
	1824	-	-	-	-	< 2	< 2	-	-
	1875	-	-	-	-	< 2	< 2	-	-

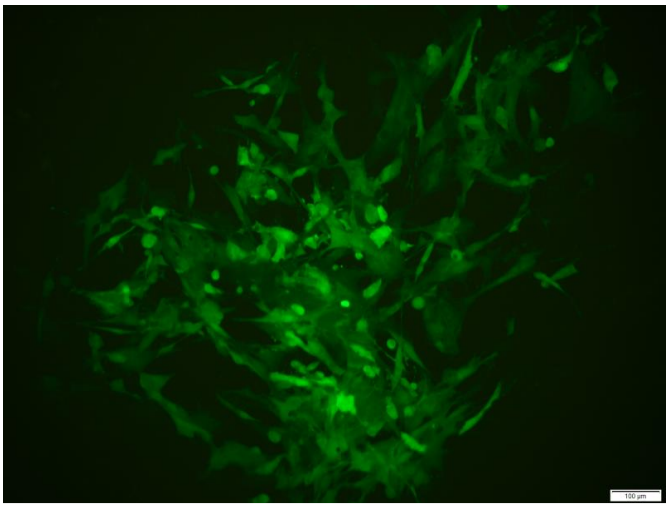
* animal died at 6 dpDx

Figure 1.

A


CpHV-1 WI 13-46 genome
TK gene
Homologous recombination
Left flank
TK gene
Right flank
60761
61798
pUC57 CpHV-1 TK LFRF eGFP
Left flank
EF1a
eGFP
Right flank
Recombinant CpHV-1ΔTK
60761
61798
Left flank
eGFP
Right flank
EF1a

B



C

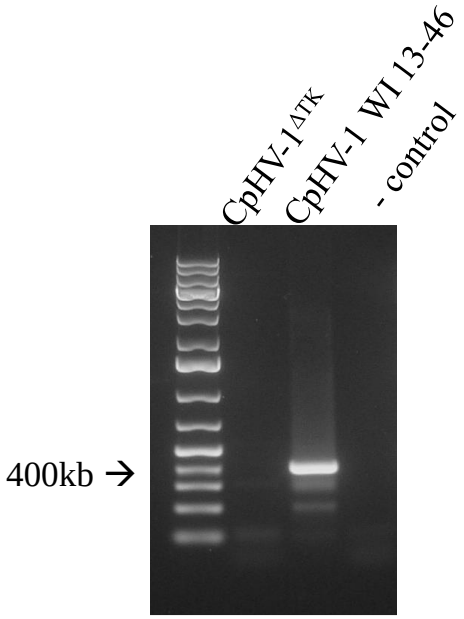

CpHV-1ΔTK
CpHV-1 WI 13-46
- control
400kb →

Figure 2.

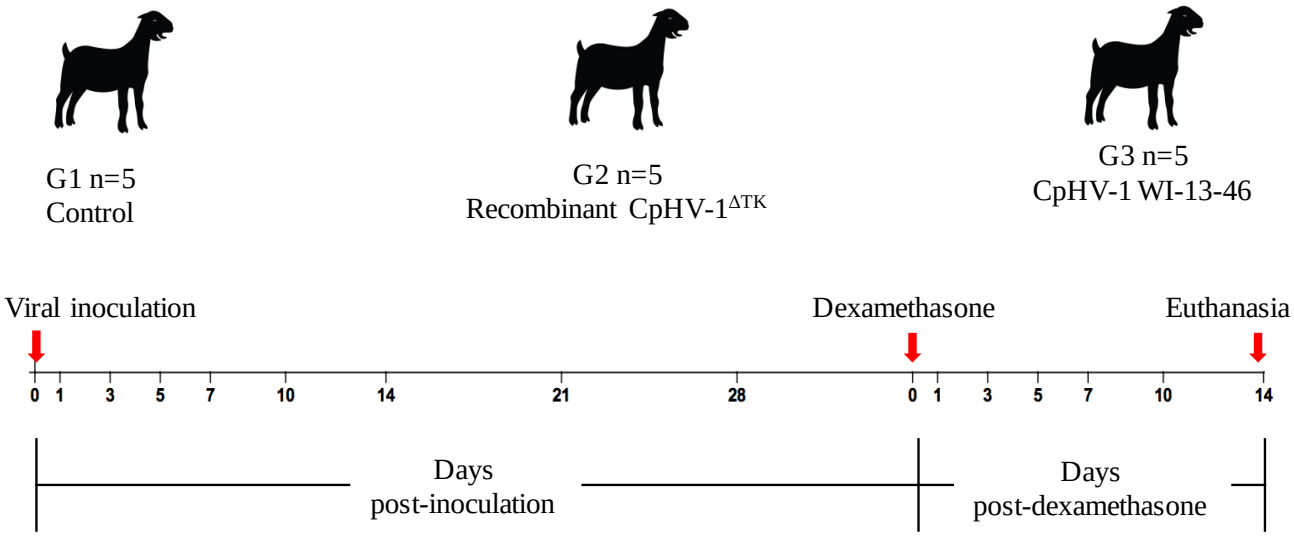

G1 n=5
Control
G2 n=5
Recombinant CpHV-1ΔTK
G3 n=5
CpHV-1 WI-13-46
Viral inoculation
Dexamethasone
Euthanasia
0 1 3 5 7 10 14 21 28 0 1 3 5 7 10 14
Days
post-inoculation
Days
post-dexamethasone

Figure 3.

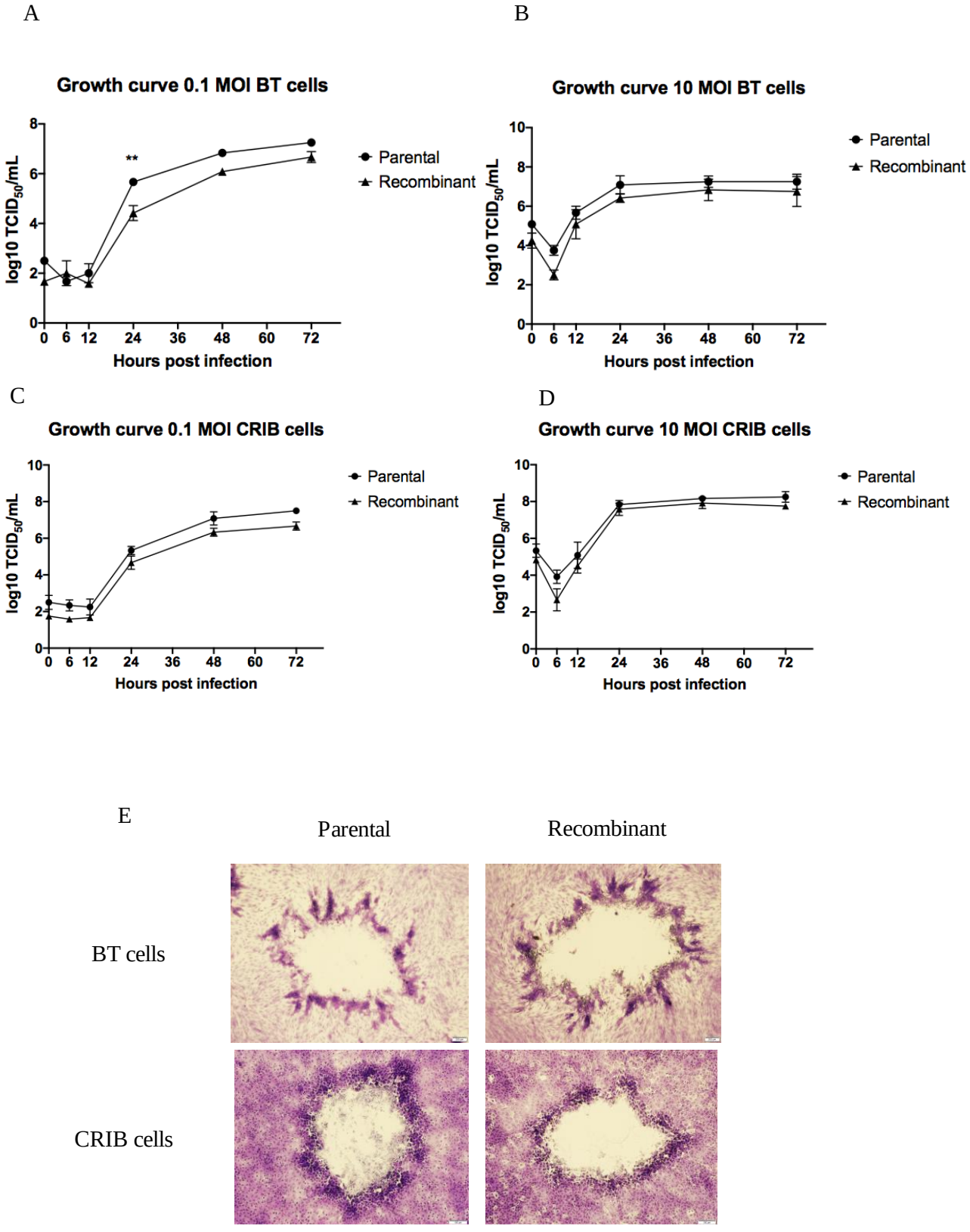
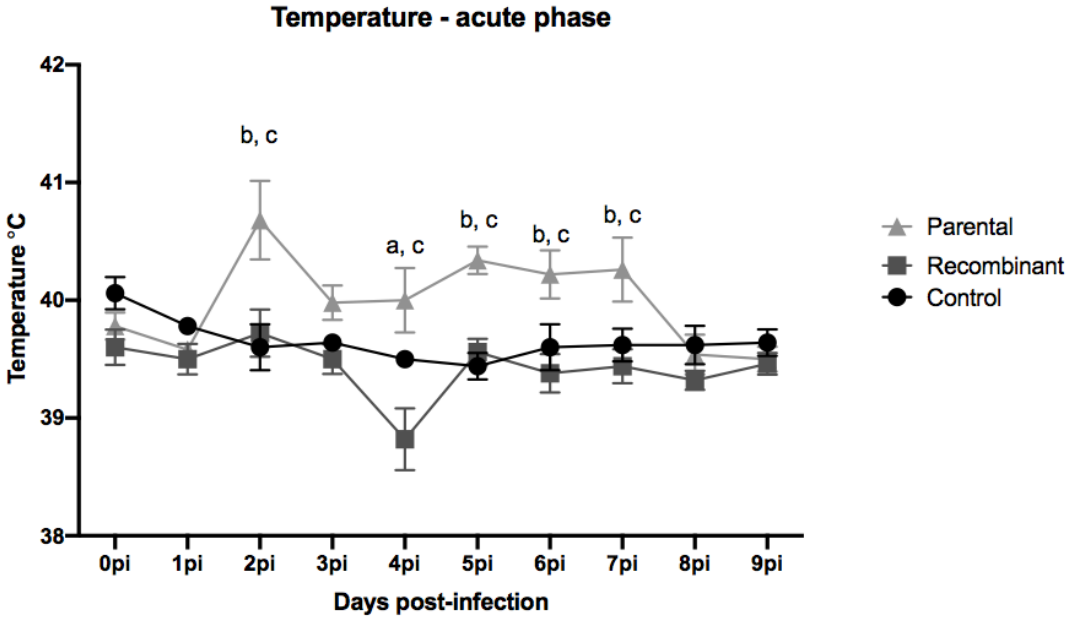

A
Growth curve 0.1 MOI BT cells
log10 TCID50/mL
Hours post infection
Parental
Recombinant
**
B
Growth curve 10 MOI BT cells
log10 TCID50/mL
Hours post infection
Parental
Recombinant
C
Growth curve 0.1 MOI CRIB cells
log10 TCID50/mL
Hours post infection
Parental
Recombinant
D
Growth curve 10 MOI CRIB cells
log10 TCID50/mL
Hours post infection
Parental
Recombinant
E
Parental
Recombinant
BT cells
CRIB cells

Figure 4.

A


Temperature - acute phase
Temperature °C
42
41
40
39
38
b, c
a, c
b, c
b, c
b, c
0pi
1pi
2pi
3pi
4pi
5pi
6pi
7pi
8pi
9pi
Days post-infection
Parental
Recombinant
Control

B

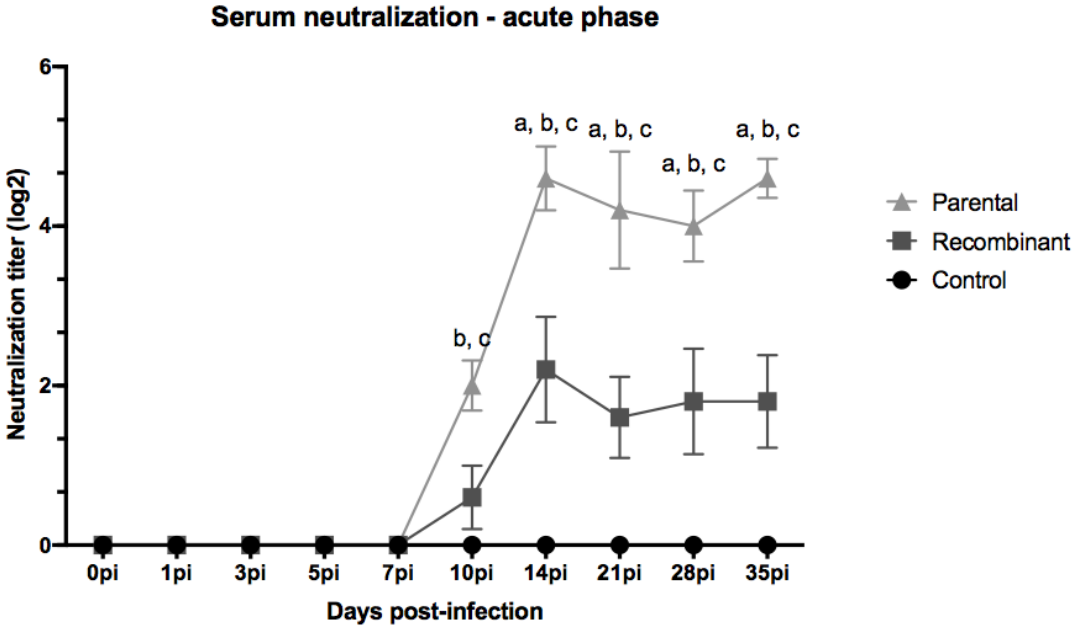
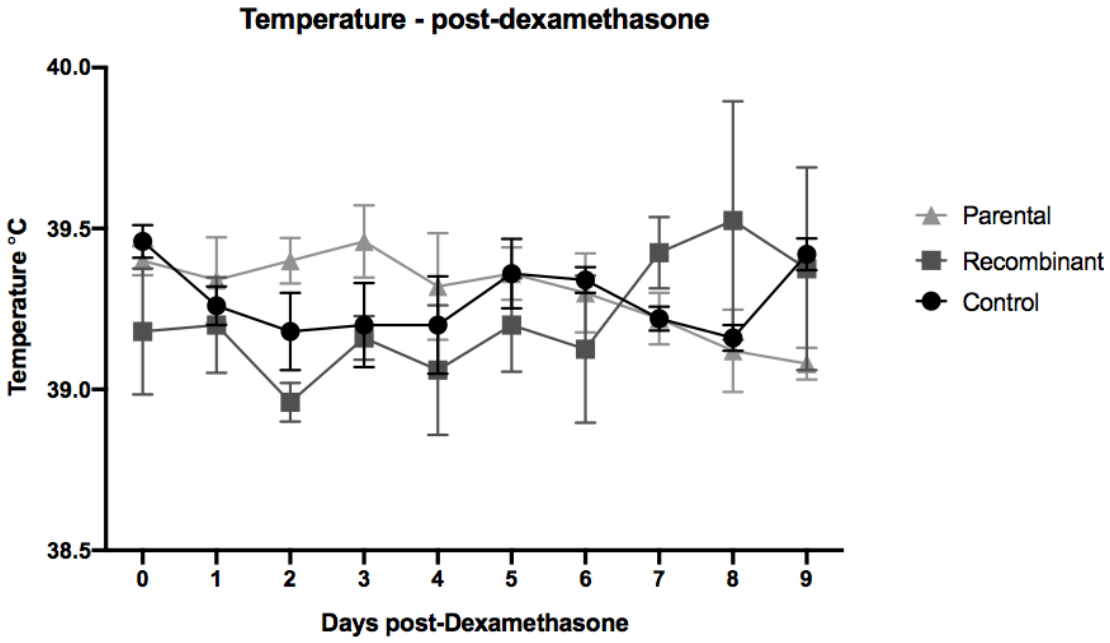
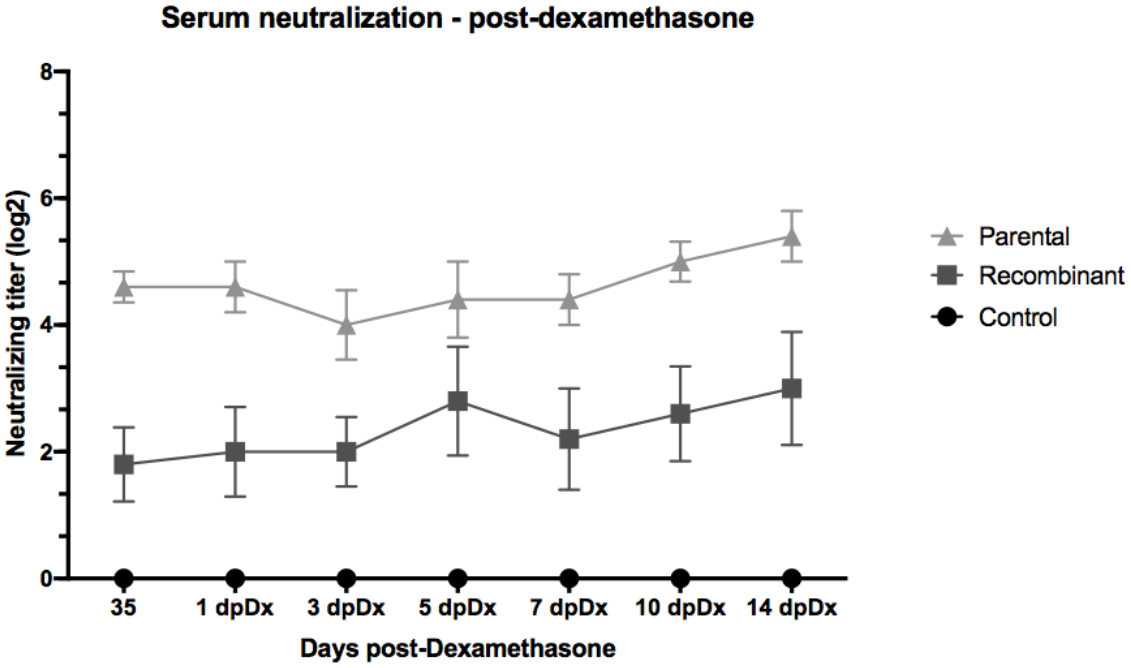

Serum neutralization - acute phase
Neutralization titer (log2)
6
4
2
0
a, b, c
a, b, c
a, b, c
a, b, c
b, c
0pi
1pi
3pi
5pi
7pi
10pi
14pi
21pi
28pi
35pi
Days post-infection
Parental
Recombinant
Control

Figure 5.

A


Temperature - post-dexamethasone
Temperature °C
40.0
39.5
39.0
38.5
0
1
2
3
4
5
6
7
8
9
Days post-Dexamethasone
Parental
Recombinant
Control

B


Serum neutralization - post-dexamethasone
Neutralizing titer (log2)
8
6
4
2
0
35
1 dpDx
3 dpDx
5 dpDx
7 dpDx
10 dpDx
14 dpDx
Days post-Dexamethasone
Parental
Recombinant
Control

3. REFERÊNCIAS

ANZILIERO, D. et al. A recombinant bovine herpesvirus 5 defective in thymidine kinase and glycoprotein E is immunogenic for calves and confers protection upon homologous challenge and BoHV-1 challenge. **Veterinary Microbiology**, v. 154, n. 1–2, p. 14–22, 29 dez. 2011.

BERRIOS, P. E.; MCKERCHER, D. G.; KNIGHT, H. D. Pathogenicity of a caprine herpesvirus. **American journal of veterinary research**, v. 36, n. 12, p. 1763–9, dez. 1975.

BERTOLINI, S. et al. A cross-sectional study to identify a set of risk factors for caprine herpesvirus 1 infection. **BMC Veterinary Research**, v. 14, n. 1, 2018.

BERTOLOTTI, L. et al. Characterization of caprine herpesvirus 1 (CpHV1) glycoprotein E and glycoprotein I ectodomains expressed in mammalian cells. **Veterinary Microbiology**, v. 164, n. 3–4, p. 222–228, 28 jun. 2013.

BLACK, M. E.; HRUBY, D. E. Nucleotide sequence of the Escherichia coli thymidine kinase gene provides evidence for conservation of functional domains and quaternary structure. **Molecular Microbiology**, v. 5, n. 2, p. 373–379, 1 fev. 1991.

BOEHMER, P.; NIMONKAR, A. Herpes Virus Replication. **IUBMB Life (International Union of Biochemistry and Molecular Biology: Life)**, v. 55, n. 1, p. 13–22, 1 jan. 2003.

BRUM, M. C. S. et al. Construction and growth properties of bovine herpesvirus type 5 recombinants defective in the glycoprotein E or thymidine kinase gene or both. **Brazilian Journal of Medical and Biological Research**, v. 43, n. 2, p. 217–224, fev. 2010.

BUONAVOGLIA, C. et al. Reactivation of caprine herpesvirus 1 in latently infected goats. **Comparative Immunology, Microbiology and Infectious Diseases**, v. 19, n. 4, p. 275–281, 1 set. 1996.

CADORE, G. C. et al. A thymidine kinase-deleted bovine herpesvirus 5 establishes latent infection but reactivates poorly in a sheep model. **Pesquisa Veterinaria Brasileira**, v. 33, n. 3, p. 331–338, 2013.

CAMERO, M. et al. Caprine herpesvirus type 1 infection in goat: Not just a problem for females. **Small Ruminant Research**, v. 128, p. 59–62, jul. 2015.

CAMERO, M. et al. Enhancement of the antiviral activity against caprine herpesvirus type 1 of Acyclovir in association with Mizoribine. **Research in Veterinary Science**, v. 111, p. 120–123, 1 abr. 2017.

CHEN, S.-H. et al. Failure of Thymidine Kinase-Negative Herpes Simplex Virus To Reactivate from Latency following Efficient Establishment. **Journal of Virology**, v. 78, n. 1, p. 520–523, 2004.

CHOWDHURY, S. I. Construction and characterization of an attenuated bovine herpesvirus type 1 (BHV-1) recombinant virus. **Veterinary Microbiology**, v. 52, n. 1–2, p. 13–23, 1 set. 1996.

COEN, D. M. et al. Thymidine kinase-negative herpes simplex virus mutants establish latency in mouse trigeminal ganglia but do not reactivate. **Proceedings of the National Academy of Sciences of the United States of America**, v. 86, n. 12, p. 4736–40, 1 jun. 1989.

DAVISON, A. J. Evolution of the herpesviruses. **Veterinary Microbiology**, v. 86, n. 1–2, p. 69–88, abr. 2002.

DENG, L. et al. Oncolytic efficacy of thymidine kinase-deleted vaccinia virus strain Guang9. **Oncotarget**, v. 8, n. 25, p. 40533–40543, 20 jun. 2017.

DONOFRIO, G. et al. Clinical Protection of Goats against CpHV-1 Induced Genital Disease with a BoHV-4-Based Vector Expressing CpHV-1 gD. **PLoS ONE**, v. 8, n. 1, 2013.

ELIA, G. et al. In vitro inhibition of caprine herpesvirus 1 by acyclovir and mizoribine. **Research in Veterinary Science**, v. 99, p. 208–211, 1 abr. 2015.

ENGELS, M. et al. Interactions of bovine and caprine herpesviruses with the natural and the foreign hosts. **Veterinary Microbiology**, v. 33, n. 1–4, p. 69–78, 1 nov. 1992.

FERRARI, M. et al. The response of pigs inoculated with a thymidine kinase-negative (TK-) pseudorabies virus to challenge infection with virulent virus. **Comparative Immunology, Microbiology and Infectious Diseases**, v. 23, n. 1, p. 15–26, 2000.

GANDAR, F. et al. The Genome of a Tortoise Herpesvirus (Testudinid Herpesvirus 3) Has a Novel Structure and Contains a Large Region That Is Not Required for Replication In Vitro or Virulence In Vivo. **Journal of virology**, v. 89, n. 22, p. 11438–56, 15 nov. 2015.

GÜR, S. et al. The role of goats as reservoir hosts for bovine herpes virus 1 under field conditions. **Tropical Animal Health and Production**, v. 51, n. 4, p. 753–758, 22 maio 2019.

HORNER, G. W.; HUNTER, R.; DAY, A. M. An outbreak of vulvovaginitis in goats caused by a caprine herpesvirus. **New Zealand Veterinary Journal**, v. 30, n. 10, p. 150–152, 23 out. 1982.

JAMIESON, A. T.; GENTRY, G. A.; SUBAK-SHARPE, J. H. Induction of Both Thymidine and Deoxycytidine Kinase Activity by Herpes Viruses. **Journal of General Virology**, v. 24, n. 3, p. 465–480, 1 set. 1974.

KEUSER, V. et al. Isolation of caprine herpesvirus type 1 in Spain. **The Veterinary record**, v. 154, n. 13, p. 395–9, 27 mar. 2004.

KIT, S. et al. Thymidine kinase-negative bovine herpesvirus type 1 mutant is stable and highly attenuated in calves. **Archives of virology**, v. 86, n. 1–2, p. 63–83, 1985.

KIT, S.; KIT, M.; PIRTLE, E. C. Attenuated properties of thymidine kinase-negative deletion mutant of pseudorabies virus. **American journal of veterinary research**, v. 46, n. 6, p. 1359–67, jun. 1985.

KOPTOPOULOS, G. et al. The epizootology of caprine herpesvirus (BHV-6) infections in goat populations in Greece. **Comparative Immunology, Microbiology and Infectious Diseases**, v. 11, n. 3–4, p. 199–205, 1 jan. 1988.

KRAMER, T.; ENQUIST, L. W. Directional spread of alphaherpesviruses in the nervous system. **Viruses**, v. 5, n. 2, p. 678–707, 11 fev. 2013.

LEIST, T. P.; SANDRI-GOLDIN, R. M.; STEVENS, J. G. Latent infections in spinal ganglia with thymidine kinase-deficient herpes simplex virus. **Journal of virology**, v. 63, n. 11, p. 4976–8, nov. 1989.

MARINARO, M. et al. A Caprine Herpesvirus 1 Vaccine Adjuvanted with MF59^{TM} Protects against Vaginal Infection and Interferes with the Establishment of Latency in Goats. **PLoS ONE**, v. 7, n. 4, p. e34913, 12 abr. 2012.

MCGREGOR, S. et al. Vaccination of swine with thymidine kinase-deficient mutants of pseudorabies virus. **American journal of veterinary research**, v. 46, n. 7, p. 1494–7, jul. 1985.

METTLER, F. et al. Herpesvirus infection in kids in Switzerland. **Schweizer Archiv fur Tierheilkunde**, v. 121, n. 12, p. 655–62, 1979.

MOCARSKI, E. S.; ROIZMAN, B. Structure and role of the herpes simplex virus DNA termini in inversion, circularization and generation of virion DNA. **Cell**, v. 31, n. 1, p. 89–97, nov. 1982.

MUYLKENS, B. et al. Bovine herpesvirus 1 infection and infectious bovine rhinotracheitis. **Veterinary Research**, v. 38, n. 2, p. 181–209, 9 mar. 2007.

ROIZMAN, B.; PELLET, P. E. The family Herpesvridae: a brief introduction. In: KNIPE, D. M.; HOWLEY, P. M. (Eds.). . **Fields Virology**. 4th. ed. Philpadelphia: Lippincot Williams & Wilkins, 2001. p. 2381–2398.

ROPERTO, F. et al. Natural caprine herpesvirus 1 (CpHV-1) infection in kids. **Journal of Comparative Pathology**, v. 122, n. 4, p. 298–302, abr. 2000.

SANFORD, B. et al. Deletion of the thymidine kinase gene induces complete attenuation of the Georgia isolate of African swine fever virus. **Virus Research**, v. 213, p. 165–171, fev. 2016.

SCHWYZER, M.; ACKERMANN, M. Molecular virology of ruminant herpesviruses. **Veterinary microbiology**, v. 53, n. 1–2, p. 17–29, nov. 1996.

SEARS, A. E.; MEIGNIER, B.; ROIZMAN, B. Establishment of latency in mice by herpes simplex virus 1 recombinants that carry insertions affecting regulation of the thymidine kinase gene. **Journal of virology**, v. 55, n. 2, p. 410–6, ago. 1985.

SILVA, M. L. C. R. et al. Serological evidence and risk factors associated with *Caprine herpesvirus 1* in dairy goat flocks in a semiarid region of northeastern Brazil. **Journal of Veterinary Diagnostic Investigation**, v. 25, n. 1, p. 125–128, 28 jan. 2013.

SILVA, S. C. et al. A bovine herpesvirus 5 recombinant defective in the thymidine kinase (TK) gene and a double mutant lacking TK and the glycoprotein E gene are fully attenuated for rabbits. **Brazilian Journal of Medical and Biological Research**, v. 43, n. 2, p. 150–159, fev. 2010.

SIX, A. et al. Latency and reactivation of bovine herpesvirus 1 (BHV-1) in goats and of caprine herpesvirus 1 (CapHV-1) in calves. **Archives of Virology**, v. 146, n. 7, p. 1325–1335, jul. 2001.

SLATER, J. D.; GIBSON, J. S.; FIELD, H. J. Pathogenicity of a thymidine kinase-deficient

mutant of equine herpesvirus 1 in mice and specific pathogen-free foals. **Journal of General Virology**, v. 74, n. 5, p. 819–828, 1993.

SMITH, G. et al. Development and trial of a bovine herpesvirus 1 - thymidine kinase deletion virus as a vaccine. **Australian Veterinary Journal**, v. 71, n. 3, p. 65–70, 1 mar. 1994.

STEVENS, J. et al. RNA complementary to a herpesvirus alpha gene mRNA is prominent in latently infected neurons. **Science**, v. 235, n. 4792, p. 1056–1059, 27 fev. 1987.

SUAVET, F. et al. First Description of Infection of Caprine Herpesvirus 1 (CpHV-1) in Goats in Mainland France. **Pathogens**, v. 5, n. 1, p. 17, 6 fev. 2016.

SUZUTANI, T. et al. Analysis of the Relationship between Cellular Thymidine Kinase Activity and Virulence of Thymidine Kinase-Negative Herpes Simplex Virus Types 1 and 2. **Microbiology and Immunology**, v. 39, n. 10, p. 787–794, 1 out. 1995.

TEMPESTA, M. et al. Natural reactivation of caprine herpesvirus 1 in latently infected goats. **The Veterinary record**, v. 143, n. 7, p. 200, 15 ago. 1998.

TEMPESTA, M. et al. A preliminary study on the pathogenicity of a strain of caprine herpesvirus-1. **Comparative Immunology, Microbiology and Infectious Diseases**, v. 22, n. 2, p. 137–143, 1 abr. 1999a.

TEMPESTA, M. et al. Detection of caprine herpesvirus 1 in sacral ganglia of latently infected goats by PCR. **Journal of clinical microbiology**, v. 37, n. 5, p. 1598–9, maio 1999b.

TEMPESTA, M. et al. Experimental intravaginal infection of goats with caprine herpesvirus 1. **Journal of Veterinary Medicine, Series B**, v. 47, n. 3, p. 197–201, abr. 2000.

TEMPESTA, M. et al. Assessing the efficacy of cidofovir against herpesvirus-induced genital lesions in goats using different therapeutic regimens. **Antimicrobial agents and chemotherapy**, v. 52, n. 11, p. 4064–8, 1 nov. 2008.

TENSER, R. B. Role of Herpes simples Virus Thymidine Kinase Expression in Viral Pathogenesis and Latency. **Intervirology**, v. 32, n. 2, p. 76–92, 1991.

TENSER, R. B.; HAY, K. A.; EDRIS, W. A. Latency-associated transcript but not reactivatable virus is present in sensory ganglion neurons after inoculation of thymidine kinase-negative

mutants of herpes simplex virus type 1. **Journal of virology**, v. 63, n. 6, p. 2861–5, jun. 1989.

TENSER, R. B.; MILLER, R. L.; RAPP, F. Trigeminal ganglion infection by thymidine kinase-negative mutants of herpes simplex virus. **Science (New York, N.Y.)**, v. 205, n. 4409, p. 915–7, 31 ago. 1979.

THIRY, J. et al. Ruminant alphaherpesviruses related to bovine herpesvirus 1. **Veterinary Research**, v. 37, n. 2, p. 169–190, mar. 2006.

THIRY, J. et al. Serological evidence of caprine herpesvirus 1 infection in Mediterranean France. **Veterinary Microbiology**, v. 128, n. 3–4, p. 261–268, 30 abr. 2008.

TISDALL, D. J. et al. New Zealand caprine herpes virus : Comparison with an Australian isolate and with bovine herpes virus type 1 by restriction endonuclease analysis. **New Zealand Veterinary Journal**, v. 32, n. 6, p. 99–100, 23 jun. 1984.

WAGNER, L. M.; DELUCA, N. A. Temporal Association of Herpes Simplex Virus ICP4 with Cellular Complexes Functioning at Multiple Steps in PolII Transcription. **PLoS ONE**, v. 8, n. 10, p. e78242, 11 out. 2013.

WEN, Y. et al. A Thymidine Kinase recombinant protein-based ELISA for detecting antibodies to Duck Plague Virus. **Virology Journal**, v. 7, n. 1, p. 77, 23 abr. 2010.