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

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

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

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

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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. This property makes the *tk* gene 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 35–39 post-inoculation did not result in virus shedding in nasal secretions, indicating lack of reactivation from latency. However, viral DNA was detected in the 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 vaccine candidate to prevent CpHV-1 disease in goats.

1. 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 their short replicative cycle, rapid spread and destruction of cultured cells and their ability to establish latency primarily in sensory nerve ganglia (Roizman and 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, USA (Berrios and McKercher, 1975) and, subsequently, has been reported in Europe, Australia, New Zealand 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 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

* Corresponding author at: 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, USA.

E-mail address: dgdiel@cornell.edu (D.G. Diel).

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

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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 several 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.

2. Material and methods

2.1. 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 and 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 37 °C 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.

2.2. 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) and flanked by multiple cloning sites compatible with herpesvirus recombination. The right and left flanking regions of *tk* were amplified by PCR from the parental CpHV-1 genome, using the Q5® Hot Star High-Fidelity 2X Master Mix (New England BioLabs catalog #M0494 L) 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 virus.

The recombinant CpHV-$1^{\Delta TK}$ virus was generated by homologous recombination, using infection/transfection, a procedure adapted from poxviruses (DeLange and McFadden, 1986). For this, primary BT cells were infected with CpHV-1 at a multiplicity of infection (MOI) of 0.5. 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 virus is depicted in Fig. 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 °C, the inoculum was removed, and cells were overlaid with 3 mL of complete growth media (CGM) + 1% agarose and kept in 37 °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 °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: one cycle of initial denaturation (98 °C, 30 s), followed by 35 cycles of denaturation, annealing and extension (95 °C, 10 s; 59 °C, 30 s; 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 produced in CRIB cells.

2.3. 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 the parental CpHV-1 and recombinant CpHV-$1^{\Delta TK}$ viruses and the cultures (cells plus supernatants) were harvested at 0, 6, 12, 24, 48 and 72 h post infection. Viral yields were determined on each time point by limiting dilutions and viral titers were calculated based on the Spearman-Karber method and expressed as tissue culture infectious dose 50 ($TCID_{50}$/ml).

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 size (mm^2) was determined 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 their sizes compared using unpaired *t*-test ($p < 0.05$).

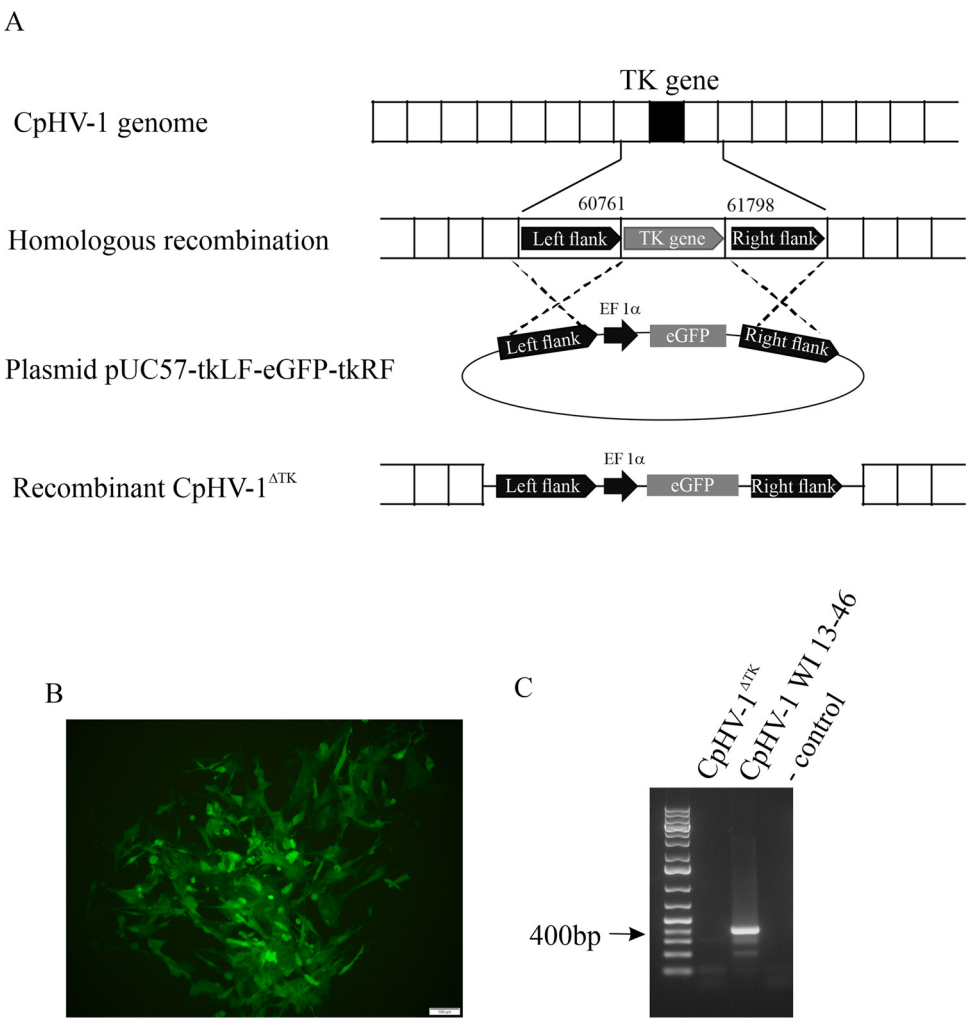

Fig. 1. Construction and characterization of the recombinant CpHV-1$^{\Delta TK}$. A. Schematic representation of the recombination plasmid and the homologous recombination to delete the *tk* gene from the CpHV-1 genome. Numbers indicated in the figure represent the nucleotide position of the *tk* gene start (60761) and stop (61798) codons. B. CpHV-1$^{\Delta TK}$ virus plaque showing expression of GFP in BT cells. C. PCR for an internal region of the *tk* gene.

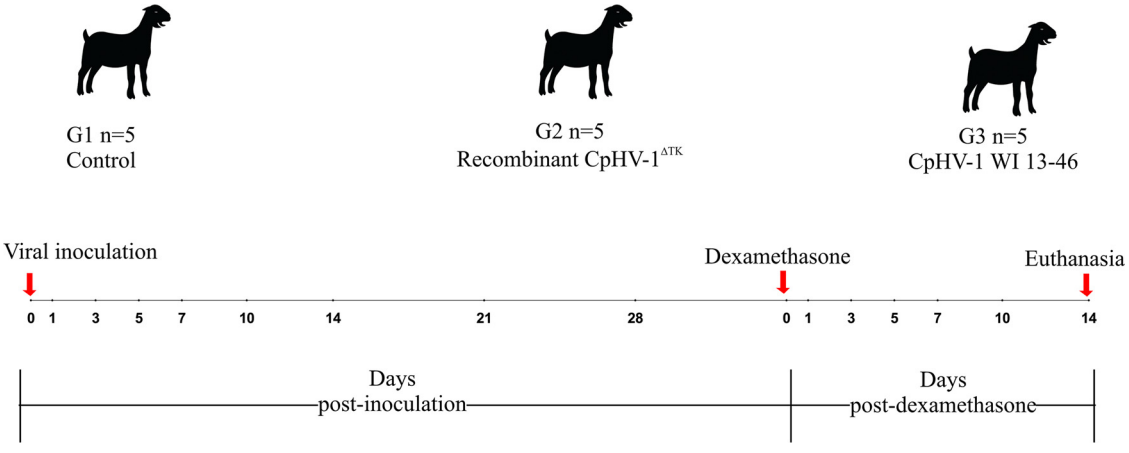

Fig. 2. Animal experiment design. Fifteen goats were allocated in three groups: parental virus group, recombinant virus group and negative control (n = 5/group). Acute phase: Animals were monitored daily and rectal temperatures were taken daily for 14 days. Nasal and rectal swabs and blood samples were 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).

2.4. 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 Fig. 2. The mean daily 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).

2.5. 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 and transferred to Red blood cells were lysed. 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 −80 °C until virus isolation was performed then transferred to −20 °C for long term storage.

2.6. Virus neutralization assays

Virus neutralization (VN) assays were performed to assess neutralizing antibody responses in inoculated animals. Briefly, heat inactivated (56 °C 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 37 °C. After the incubation, a suspension of CRIB cells was added to each well and plates incubated at 37 °C for 72 h. Assays were read under a fluorescence microscope (GFP expression by the CpHV-$1^{\Delta TK}$ virus) 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 ($p < 0.05$)

2.7. 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 37 °C. After adsorption, 500 µL of complete culture media was added to each well and cells incubated at 37 °C for four days. Samples were considered negative after three four-day passages without the evidence of cytopathic effect.

2.8. qPCR and nested PCR

Total DNA was extracted from serum, buffy coats, fecal and nasal swabs using the Cador® Pathogen 96 kit and the QIAcube HT (QIAGEN) automated extractor according to the manufacturer's instructions. The buffy coats underwent pretreatment T2 for enzymatic digestion of tissues described in 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) according to the manufacturer's instructions.

Total DNA extracted from nasal secretions, 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), 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 at 95 °C for 5 min, followed by 40 cycles of denaturation and annealing/extension (95 °C, 10 s followed by 60 °C, 50 s). 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 37.2) 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. 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 at 98 °C for 30 s, followed by 35 cycles of denaturation, annealing and extension (95 °C, 10 s; 64 °C, 30 s; 72 °C, 30 s), and a final extension (72 °C, 2 min); second reaction – one cycle at 98 °C for 30 s, followed by 35 cycles of denaturation, annealing and extension (95 °C, 10 s; 59 °C, 30 s; 72 °C, 30 s), and a final extension (72 °C, 2 min). The product of the first reaction was purified with GeneJET PCR Purification Kit (Thermo Fisher Scientific), using ultrapure 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) 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 (ultrapure water) controls.

3. Results

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

Three independent eGFP positive viral plaques were selected and

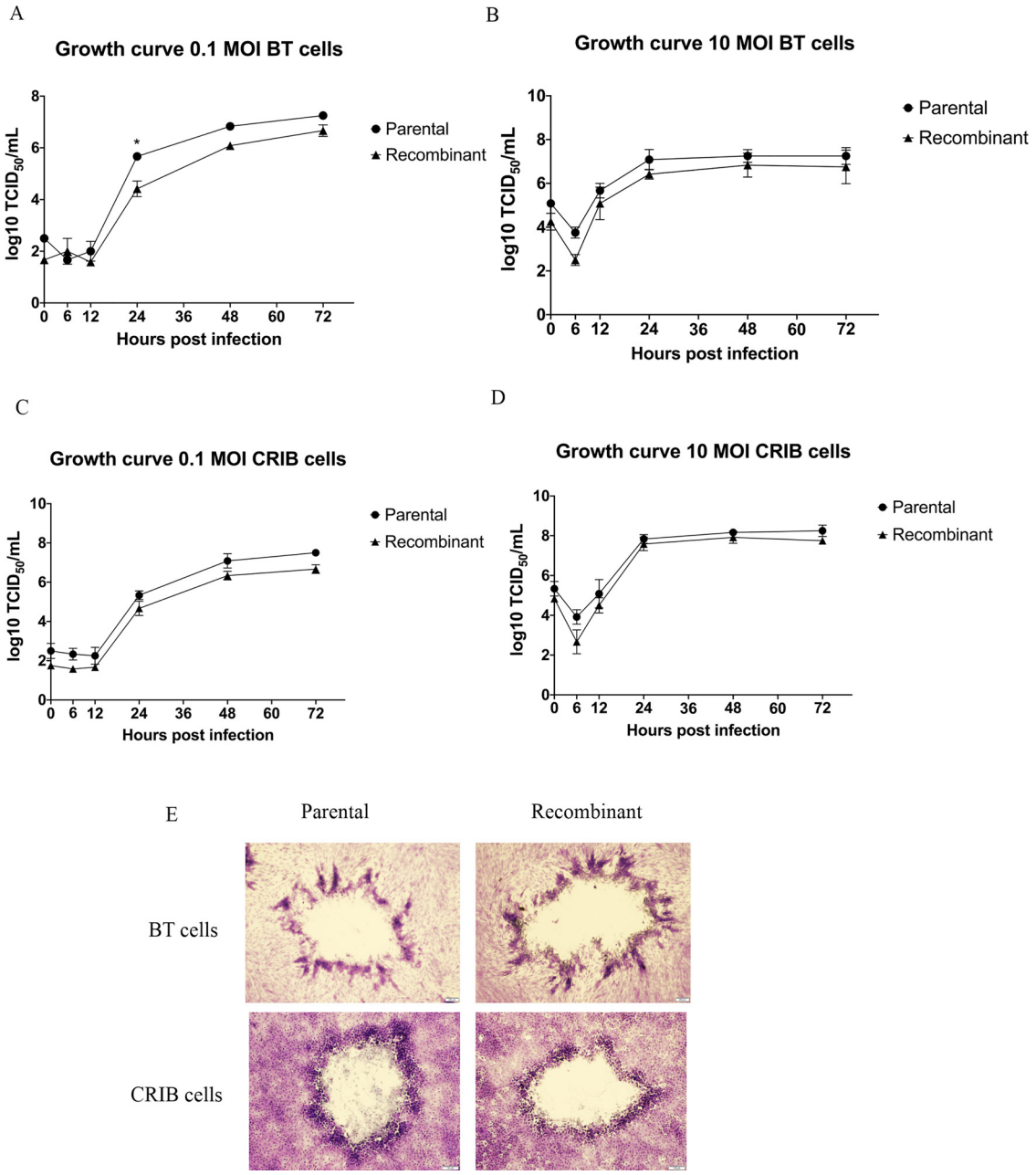

Fig. 3. *In vitro* characterization of the recombinant CpHV-1$^{\Delta TK}$virus. A. Multi-step growth curve (MOI = 0.1) comparing replication properties of the parental virus and recombinant virus in BT cells. B. Single-step growth curve (MOI = 10) comparing replication properties of the parental virus 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 and recombinant viruses in BT and CRIB cells. Statistical differences were assessed by two-way ANOVA (*, $p < 0.05$).

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 here.

3.2. 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 assessed 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 $p < 0.05$)

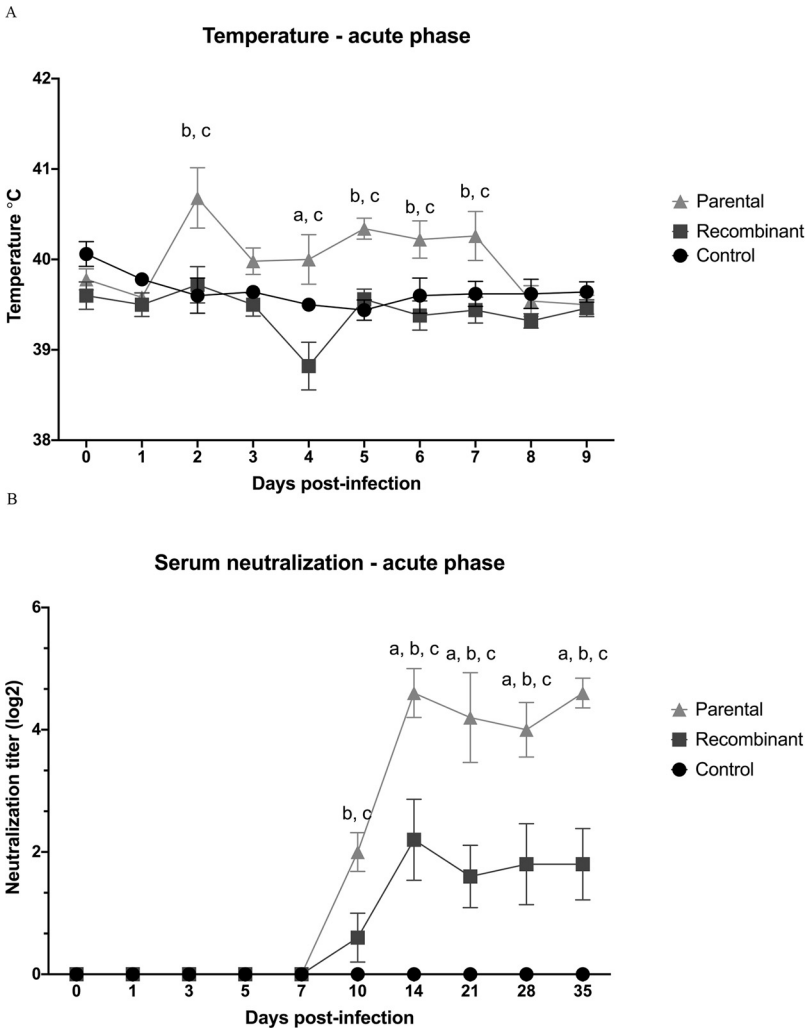

Fig. 4. A: Mean rectal temperature of goats inoculated with CpHV-1 (parental) and recombinant CpHV-1$^{\Delta TK}$ during acute infection. B: Neutralizing antibodies developed by inoculated animals during acute infection. Statistical differences were assessed by two-way ANOVA ($p < 0.05$): a – control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

(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 (Fig. 3A–D).

3.3. 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 alocated into three experimental groups and 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 augmented body temperature 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 neutralization (VN) assays demonstrated that goats inoculated with the parental virus developed higher neutralizing antibody (NA) titers than those inoculated with the recombinant CpHV-1$^{\Delta TK}$ virus (Fig. 4B).

3.4. 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). 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

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

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

[a] Viral DNA not detected.
[b] Viral DNA detected by PCR.
[c] Sample not available as the animal died of unrelated causes prior to the end of the experiment.

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.

3.5. 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 augmented body temperature after Dx administration; however, no differences between the treatment groups were noted (Fig. 5A). 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.

3.6. CpHV-1and 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$^{\Delta TK}$ established latent infection in TGs of inoculated goats, but latency was not reactivated upon Dx administration.

3.7. 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 and 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) (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

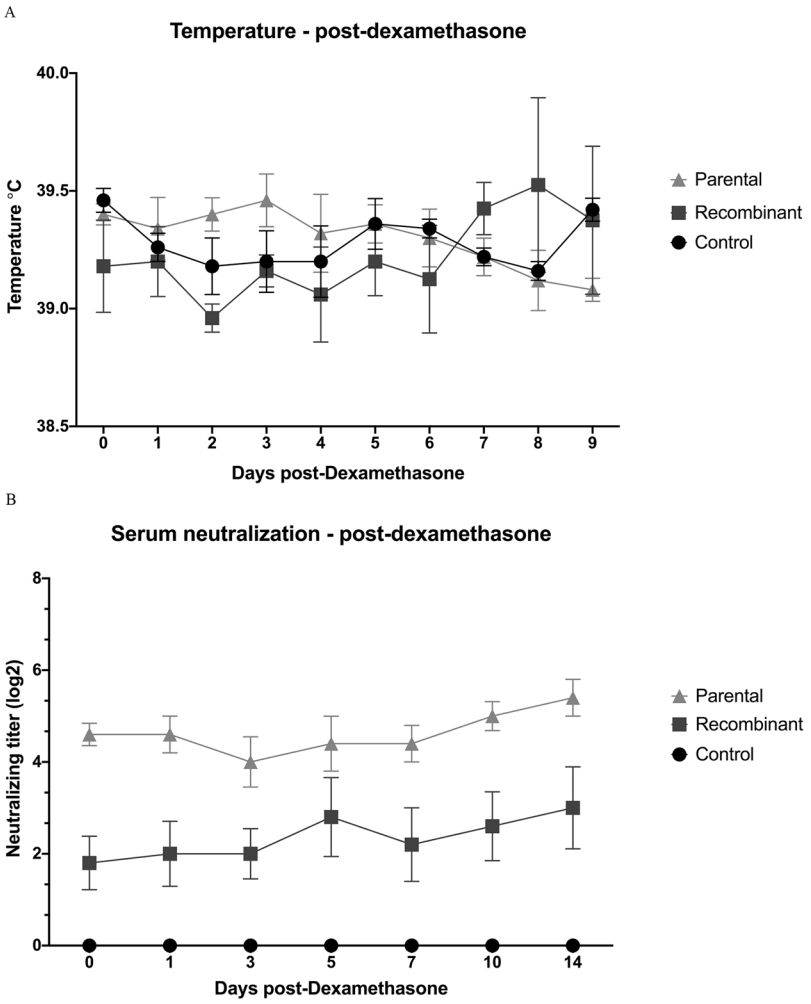

Fig. 5. A: Mean rectal temperature of goats inoculated with CpHV-1 (parental) and recombinant CpHV-1$^{\Delta TK}$ after dexamethasone administration B: Neutralizing antibodies levels during latent infection (after Dx administration).

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 were 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.

Declaration of Competing Interest

The authors declare no conflict of interest.

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