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RELAÇÃO ANTIGÊNICA ENTRE ALFAHERPESVÍRUS CAPRINO 1 (CpHV-1) E ALFAHERPESVÍRUS BOVINO 1 (BoHV-1) E INFECÇÃO EXPERIMENTAL DE CABRITOS E BEZERROS COM O CpHV-1

DISSERTAÇÃO DE MESTRADO

Bruno Martins

Santa Maria, RS, Brasil
2019

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Bruno Martins

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Orientador: Prof. Eduardo Furtado Flores

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Elaborada por
Bruno Martins

como requisito parcial para obtenção do grau de
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RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Medicina Veterinária
Universidade Federal de Santa Maria

RELAÇÃO ANTIGÊNICA ENTRE ALFAHERPESVÍRUS CAPRINO 1 (CpHV-1) E ALFAHERPESVÍRUS BOVINO 1 (BoHV-1) E INFECÇÃO EXPERIMENTAL DE CABRITOS E BEZERROS COM O CpHV-1

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Santa Maria, 19 de dezembro de 2019

O alfaherpesvírus caprino 1 (*Caprine alphaherpesvirus 1*, CpHV-1) pertence à família *Herpesviridae*, gênero *Varicellovirus* e está relacionado genética e antigenicamente com o alfaherpesvírus bovino 1 (*Bovine alphaherpesvirus 1*, BoHV-1). Em caprinos jovens, o CpHV-1 tem sido associado com doença gastroentérica e respiratória, enquanto em animais adultos a infecção é geralmente subclínica ou associada com abortos. Neste estudo, investigou-se as relações antigênicas entre o CpHV-1 e BoHV-1 e a patogênese do CpHV-1 em cabritos e bezerros. A relação antigênica foi analisada pela reatividade com anticorpos monoclonais (AcMs) contra o alfaherpesvírus bovino e por soro-neutralização (SN). A reatividade dos AcMs revelou que o CpHV-1 e BoHV-1 compartilham epítopos nas principais glicoproteínas do envelope: gB, gC e gD. Adicionalmente, a relação antigênica foi demonstrada por ensaios de SN, quando se observou neutralização cruzada entre os vírus. Nesses testes, verificou-se que anti-soro contra o BoHV-1 neutralizou o CpHV-1 com maior eficiência do que o anti-soro do CpHV-1 neutralizou o BoHV-1. A relação antigênica entre a CpHV-1 e BoHV-1 pode ser associada à possível transmissão desses vírus em hospedeiros heterólogos, com um potencial impacto em sua epidemiologia, diagnóstico sorológico e controle. A patogênese do CpHV-1 (isolado WI 13-46) foi estudada em caprinos jovens e bezerros. Para tal, sete caprinos, com idade entre quatro e seis meses, e doze bovinos, com idade de seis a oito meses, foram inoculados pela via intranasal (IN) com dose de $5x10^7$ doses infectantes para 50% dos cultivos celulares ($DICC_{50}$) do isolado WI 13-46. Após a inoculação, os animais foram monitorados nos aspectos clínicos, virológicos e sorológicos. Os sete caprinos inoculados apresentaram secreção nasal entre os dias 3 e 14 pós-infecção (pi) e dificuldade respiratória entre os dias 5 e 8 pi. O vírus foi isolado de secreções nasais dos caprinos inoculados, entre os dias 1 e 9 pi. Com objetivo de investigar a capacidade do CpHV-1 reativar a infecção latente, a partir do dia 36 (pi) os cabritos foram tratados com dexametasona (Dex, 0,4mg/kg/dia) por 5 dias e monitorados nos 15 dias subsequentes. A administração de Dex não resultou em excreção de vírus nas secreções nasais ou em aumento nos títulos dos anticorpos neutralizantes. No entanto, a infecção latente foi estabelecida, como evidenciado pela detecção do DNA do CpHV-1 no gânglio trigêmeo (GT) e bulbos olfatórios (BO) dos caprinos eutanasiados no dia 67 pi. A inoculação de CpHV-1 em bezerros não resultou em infecção produtiva, não havendo replicação ou excreção viral. Em conjunto, esses resultados demonstram que: i) CpHV-1 e BoHV-1 são antigenicamente relacionados; ii) O CpHV-1 (WI 13-46) replica com eficiência em caprinos jovens e pode produzir doença respiratória de leve à moderada após inoculação IN; iii) O CpHV-1 estabelece infecção latente no GT e BO de caprinos, mas não é reativado por protocolos-padrão de reativação de alfaherpesvírus; e iv) Bezerros não foram susceptíveis à infecção por CpHV-1 após inoculação IN. Por fim, esses resultados auxiliam no conhecimento das relações antigênicas entre herpesvírus animais e da patogenia da infecção pelo CpHV-1 em caprinos jovens e bezerros.

Palavras-chave: herpesvírus caprino; herpesvírus bovino; patogênese; relação antigênica; infecção latente.

ABSTRACT

Master's Dissertation
Programa de Pós-Graduação em Medicina Veterinária
Universidade Federal de Santa Maria

ANTIGENIC RELATIONSHIP BETWEEN CAPRINE ALPHAHERPESVIRUS 1 (CpHV-1) AND BOVINE ALPHAHERPESVIRUS 1 (BoHV-1) AND PATHOGENESIS OF CpHV-1 IN KIDS AND CALVES

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Santa Maria, December 19th, 2019

Caprine alphaherpesvirus 1 (CpHV-1), family *Herpesviridae*, genus *Varicellovirus* is genetically and antigenically related to *Bovine alphaherpesvirus 1* (BoHV-1). In kids, CpHV-1 infection has been associated with gastroenteric and respiratory diseases, whereas in adult the infection is usually subclinical or associated with abortions. Here, we investigated further the antigenic relationships of CpHV-1 with BoHV-1 and the pathogenesis of CpHV-1 in goats and calves. The antigenic relationship between CpHV-1 and BoHV-1 was analyzed by reactivity with monoclonal antibodies (MAbs) and by virus-neutralizing assays (VN). Reactivity of MAbs revealed that CpHV-1 and BoHV-1 share epitopes on the major envelope glycoproteins, e.g. gB, gC and gD. Additionally, the antigenic relationship was demonstrated by VN assays, when cross neutralization was observed between the viruses. On these tests, BoHV-1 antisera neutralized CpHV-1 more efficiently than CpHV-1 antisera neutralized BoHV-1. The antigenic relationship between CpHV-1 and BoHV-1 may have impact in serological diagnosis and control. The pathogenesis of CpHV-1 (Isolate WI 13-46) was studied in experimentally infected kids and calves. For this, seven four to six-months-old kids, and twelve calves (six to eight-months-old), were inoculated intranasally (IN) with WI 13-46 isolate ($5x10^{7,6}$ infective doses, $TCID_{50}$). After inoculation, clinical, serological and virological monitoring were performed. The seven kids inoculated with CpHV-1 presented nasal secretion between day 3 and 14 post-infection (pi) and respiratory distress between days 5 and 8 pi. The virus was isolated from the nasal swabs of all kids between day 1 and 9 pi. In order to verify the reactivation of the latent infection, on day 36 pi, the animals were treated with dexamethasone (Dex, 0.4mg/Kg/day) for 5 days and monitored for 15 subsequent days. Administration of Dex did not result in virus excretion in nasal secretions nor in increase in neutralizing antibodies titers. However, latent infection had been established, as evidenced by detection of CpHV-1 DNA in the trigeminal ganglia (TG) and olfactory bulbs (OB) of kids euthanized on day 67 pi. On the other hand, inoculation of CpHV-1 in calves did not result in viral replication/excretion, clinical signs or seroconversion. Overall, these results demonstrate that: i) CpHV-1 and BoHV-1 are antigenically related; ii) CpHV-1 (WI 13-46) replicates efficiently in kids and may produce mild to moderate respiratory disease after IN inoculation; iii) CpHV-1 establishes latent infection in TG and OB of kids but was not reactivated following standard herpesviruses reactivation protocols; and iv) Calves were not susceptible to CpHV-1 infection after IN inoculation. Finally, these results helped in understanding the antigenic relationships among animal herpesviruses and pathogenesis of CpHV-1 infection in kids and calves.

Keywords: caprine alphaherpesvirus; bovine alphaherpesvirus; pathogenesis; antigenicenic relationship; latent infection.

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1. INTRODUÇÃO

O alfaherpesvírus caprino 1 (*Caprine alphaherpesvirus 1*, CpHV-1) pertence à ordem *Herpesvirales*, família *Herpesviridae*, subfamília *Alphaherpesvirinae* e gênero *Varicellovirus* (ICTV, 2019). Os membros da família *Herpesviridae* são vírus envelopados, possuem cerca de 120 a 300 nm de diâmetro e apresentam como genoma uma fita dupla de DNA, com extensão entre 125 a 235 kb (MOCARSKI & ROIZMAN, 1982; BOEHMER & NIMONKAR, 2003). Uma das principais características dos herpesvírus é a capacidade de estabelecer latência e persistir indefinidamente em seus hospedeiros. Essa latência, por sua vez, pode ser reativada por diferentes fatores, resultando na retomada da replicação e excreção viral, com ou sem a recorrência de doença (BOEHMER & NIMONKAR, 2003)**.**

O CpHV-1 foi inicialmente descrito na década de 1970 nos Estados Unidos e na Suíça, em infecções graves e generalizadas de cabritos (WALDVOGEL et al., 1981; UZAL et al., 2004; MCCOY et al., 2007). Em seguida, infecções pelo CpHV-1 foram relatadas em vários países, como Nova Zelândia, Austrália, Suécia, Espanha, Itália e França (METTLER et al., 1979; HORNER; HUNTER; DAY, 1982; TISDALL et al., 1984; ROPERTO et al., 2000; KEUSER et al., 2004; THIRY et al., 2008). A infecção por CpHV-1 é prevalente em países europeus e mediterrâneos, onde a soroprevalência chega a 50% dos rebanhos (SUAVET et al., 2016). Um estudo sobre os fatores de risco envolvendo CpHV-1 demonstrou que a presença do CpHV-1 está associada à criação extensiva dos caprinos (BERTOLINI et al., 2018).

Em infecções naturais, o CpHV-1 geralmente penetra no hospedeiro pela mucosa oral ou respiratória (TEMPESTA et al., 2004). Após a replicação primária nesses locais, os componentes do capsídeo viral migram para o núcleo de células nervosas, onde o genoma permanece inativo (sem expressão gênica e/ou replicação) em neurônios dos gânglios trigêmeos ou sacrais por tempo indefinido, o que caracteriza infecção latente (VOGEL et al., 2003). Os locais de infecção latente dependem da via de infecção e da disseminação do vírus (TEMPESTA et al., 2004). Por outro lado, a latência do CpHV-1 pode ser de difícil reativação. Em condições naturais, a reativação foi observada apenas em animais no cio e com baixos títulos de anticorpos neutralizantes (TEMPESTA et al., 1998; CAMERO et al., 2010).

O CpHV-1 está associado com uma variedade de manifestações clínicas em caprinos jovens, decorrentes de infecções gastroentéricas e respiratórias. Já em animais adultos, embora as infecções por CpHV-1 sejam frequentemente subclínicas, o vírus pode ser responsável por diferentes sinais clínicos, como febre, quadros de doenças respiratórias, vulvovaginite e

balanopostite, podendo também levar ao aborto ou à morte de neonatos (TEMPESTA et al., 1999; SUAVET et al., 2016). Os casos de abortos, natimortos e outras falhas reprodutivas resultam em importantes perdas econômicas, especialmente em países com alta concentração de rebanhos caprinos (ROPERTO et al., 2000). Os animais neonatos podem também apresentar doença grave, caracterizada por febre, conjuntivite, aumento de secreção ocular e nasal, dispneia, lesões ulcerativas e necróticas em todo o trato entérico, as quais podem cursar com alta morbidade e mortalidade (TEMPESTA et al., 2000; CAMERO et al., 2015).

O CpHV-1 é genética e antigenicamente relacionado com outros membros da subfamília *Alphaherpesvirinae* de ruminantes, incluindo o alphaherpesvírus de cervídeo (*Cervid alphaherpesvirus,* CvHV-1), o alphaherpesvírus de búfalo (*Buffalo alphaherpesvirus*, BuHV-1) (DE CARLO et al., 2004) e os alphaherpesvírus 1 e 5 de bovinos (*Bovine alphaherpesvirus* 1 e 5, BoHV-1 e BoHV-5) (THIRY et al., 2006). Especificamente em relação ao BoHV-1, agente da rinotraqueíte infecciosa bovina (*Infectious bovine rhinotracheitis*, IBR), a relação antigênica com o CpHV-1 foi evidenciada por reação cruzada de anticorpos, especialmente com anticorpos direcionados à glicoproteína B (gB), mas também contra a gC e gD (ENGELS et al., 1992; BERTOLOTTI et al., 2013). Essa proximidade antigênica resulta na dificuldade de um diagnóstico específico entre os alphaherpesvírus (BERTOLOTTI et al., 2013). De fato, ainda não foi desenvolvido um teste sorológico específico que seja capaz de detectar o CpHV-1 e que apresente baixa ou limitada reatividade cruzada com outros vírus de ruminantes pertencentes à família dos alphaherpesvírus (BERTOLOTTI et al., 2013). Apesar disso, o diagnóstico sorológico da infecção pelo CpHV-1 pode ser realizado utilizando o teste de soroneutralização (SN) e o teste de ensaio imunoenzimático (*Enzyme-linked immunosorbent assay*, ELISA). O teste SN é considerado o padrão-ouro e exibe alta sensibilidade. A especificidade da SN, por sua vez, pode ser avaliada pelo teste de soroneutralização cruzada, no qual a média dos títulos de anticorpos contra o vírus homólogo deve ser maior que os títulos contra o vírus heterólogo (THIRY et al., 2008). O ELISA indireto provou ser tão sensível quanto o teste de SN, embora tenha sido detectada reação cruzada com caprinos infectados experimentalmente com BoHV-1 (MARINARO et al., 2010).

Experimentos realizados com CpHV-1 e BoHV-1 sugerem que tanto bovinos são susceptíveis ao CpHV-1, quanto caprinos são susceptíveis ao BoHV-1, quando esses são inoculados pela via intranasal. Em espécie heteróloga, o CpHV-1 e o BoHV-1 seriam ainda capazes de serem excretados durante a infecção aguda e de estabelecer infecção latente. O BoHV-1 é capaz de provocar sinais clínicos leves quando inoculado em cabras (SIX, et al., 2001; THIRY et al., 2006). Entretanto, sinais clínicos provocados pelo CpHV-1 parecem ser

restritos ao seu hospedeiro natural, sendo a patogênese da infecção do CpHV-1 em bovinos ainda não totalmente compreendida (TEMPESTA et al., 2001).

Assim, os objetivos do presente estudo foram aprofundar a investigação sobre as relações antigênicas entre CpHV-1 e BoHV-1 e estudar a patogênese do CpHV-1 (isolado WI 13-46) em caprinos e bovinos.

2. CAPÍTULO 1

Antigenic relationship between *Caprine alphaherpesvirus 1* (CpHV-1) and *Bovine alphaherpesvirus 1* (BoHV-1) and experimental CpHV-1 infection of kids and calves

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Antigenic relationship between *Caprine alphaherpesvirus 1* (CpHV-1) and *Bovine alphaherpesvirus 1* (BoHV-1) and experimental CpHV-1 infection of kids and calves

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Highlights

- CpHV-1 and BoHV-1 are antigenically closely related;
- Monoclonal antibodies to BoHV-1 glycoproteins B, C and D recognize CpHV-1 antigens;
- BoHV-1 antisera neutralized CpHV-1 with higher efficiency than CpHV-1 antisera neutralized BoHV-1;
- CpHV-1 is able to produce respiratory disease in kids, but does not replicate to detectable in calves.

Abstract

Caprine alphaherpesvirus 1 (CpHV-1) is a worldwide pathogen of goats and is closely related to *Bovine alphaherpevirus 1* (BoHV-1). We herein studied the antigenic relationships of CpHV-1 with BoHV-1 and investigated the pathogenesis of CpHV-1 in kids and calves. Monoclonal antibody reactivity revealed that CpHV-1 and BoHV-1 share immunogenic epitopes in the major envelope glycoproteins, gB, gC and gD. The antigenic relationship was further demonstrated by virus-neutralizing assays, in which CpHV-1 and BoHV-1 antisera presented varied degrees of cross-neutralization against the respective heterologous viruses. Although cross-neutralization was observed between both viruses and the heterologous antisera, BoHV-1 antisera neutralized CpHV-1 with higher efficiency than CpHV-1 antisera neutralized BoHV-1. Hence, the antigenic cross-reactivity between CpHV-1 and BoHV-1 should be considered upon serologic testing of goats and cattle in regions where the two viruses co-circulate. Intranasal (IN) inoculation of CpHV-1 (WI13-46 isolate) in seven seronegative kids resulted in efficient viral replication in the respiratory tract. Additionally, mild to moderate systemic and respiratory signs were observed, including apathy, hyperthermia, nasal discharge and respiratory distress. Dexamethasone administration to the inoculated kids between days 36 and 40 pi did not result in virus shedding in nasal secretions. However, latent infection had been established, as evidenced by the detection of CpHV-1 DNA in trigeminal ganglia and olfactory bulbs of kids euthanized at day 67 pi. Contrasting with the outcome of infection in kids, IN inoculation of CpHV-1 in calves did not result in productive infection as no virus replication or shedding were detected, and the animals did not develop clinical signs nor seroconverted. The animal experiments demonstrated that CpHV-1 was able to produce respiratory disease in kids, but did not replicate to detectable levels in calves.

Keywords: caprine herpesvirus, bovine herpesvirus, pathogenesis, antigenic relationship, latent infection.

1. Introduction

Caprine alphaherpesvirus 1 (CpHV-1) is a member of the family *Herpesviridae,* subfamily *Alphaherpesvirinae* and genus *Varicellovirus* [1]. Varicelloviruses are viruses ranging from 120 to 300 nm in diameter, enveloped, containing a double-stranded linear DNA genome arranged in a long unique region (UL) and a short unique region (US) flanked by inverted internal- (IR) and terminal repeats (TR) [2]. CpHV-1 is widely distributed in goat populations and is associated with severe systemic disease in young kids and reproductive disease and abortion in adult goats [3–6]. CpHV-1 is genetically and antigenically related to *Bovine alphaherpesvirus 1* (BoHV-1), an important pathogen of cattle involved in respiratory, reproductive disease and abortions [2].

The genetic and antigenic relationship between CpHV-1 and BoHV-1 has potential implications in their epidemiology, diagnostic and control [2]. Data from natural and experimental infections have shown the ability of these viruses to cross-infect the respective heterologous hosts. Upon experimental inoculation, BoHV-1 is able to replicate acutely, to establish and to reactivate from latent infection in goats [7]. In addition, natural cases of BoHV-1 latent infection and positive serology have been reported in goats [8,9]. Similarly, CpHV-1 replicated efficiently and was shed by experimentally infected calves during acute infection and established latent infection, yet did not reactivate from latency upon corticosteroid administration [7,10]. These observations suggest that cross-infections between BoHV-1 and CpHV-1 in their respective hosts may potentially occur.

The similarity in viral encoded structural proteins, including envelope glycoproteins, gB, gC and gD, results in serological cross-reactivity between BoHV-1 and CpHV-1 [11]. In addition, antibodies reacting with BoHV-1 have been detected in other ruminants, including goats, suggesting a potential role of this species as BoHV-1 reservoir [2,9,12]. The serological cross-reactivity between BoHV-1 and CpHV-1 may affect the specificity of serological tests

and, thus, compromise diagnostic and control efforts [2]. A better understanding of the antigenic relationship between BoHV-1 and CpHV-1 and the potential impact of cross-species transmission is critical for the design and implementation of effective and specific diagnostic tools and control measures.

The pathogenesis of acute CpHV-1 infection in goats and of BoHV-1 in calves is similar [2,13]. Natural CpHV-1 infections of adult and immunocompetent goats are frequently subclinical [6,14,15], but have been associated with systemic disease and high mortality in young kids [4,16], reproductive disorders and abortions in adult goats [17–19]. After acute infection, CpHV-1 establishes latent infection in trigeminal or sacral nerve ganglia, depending on the route of entry [20–22]. Latent infection may be naturally reactivated under stressful conditions [20–22], but it seems rather infrequent [23]. Likewise, experimental reactivation is difficult to achieve and usually requires the administration of high doses of corticosteroids [10,20].

CpHV-1 and BoHV-1 are important pathogens in their natural hosts and cross-infection of their respective heterologous hosts might have clinical, epidemiological and diagnosis implications. Due to their clinical relevance, both agents have been targets for vaccine development in the last decades. Importantly, BoHV-1 eradication programs have been initiated in Europe. We are currently prospecting viral agents to serve as new vaccine delivery vectors for cattle. In this sense, CpHV-1 appeared to be an attractive alternative. Here, we investigated the antigenic relationship between CpHV-1 and BoHV-1 and assessed the pathogenesis of CpHV-1 in kids and calves.

2. Materials and methods

2.1 Cells and viruses

Cell lines MDBK (Madin-Darby bovine kidney – ATCC-CL22), CRIB (Madin-Darby bovine kidney BVDV-infection-resistant) and bovine turbinate (BT) (ATCC – CRL - 1390) were used for virus amplification, titrations and growth curves. Cells were cultured in minimum essential medium (MEM) (Vitrocell®, Nova Campinas, Sao Paulo - Brazil), supplemented with 10% fetal bovine serum (FBS) (Vitrocell®), penicillin (100 $IU.mL^{-1}$) and streptomycin (100 $\mu g.mL^{-1}$) (Sigma – Aldrich®, Darmstadt, Hessen, Germany) at 37 °C with 5% CO_2. The viruses used herein were: CpHV-1 WI 13-46 isolated and BoHV-1 SV56/90 isolate. CpHV-1 WI 13-46 was isolated in BT cells from the lungs of sick kids from herd in Wisconsin (2013) with history of weak kids, diarrhea, coughing, eye infections and abortions. The virus was amplified in MDBK cells and used for animal inoculation at passage # 7. SV56/90 strain is a BoHV-1 isolated from preputial swabs and semen of a bull with balanoposthitis in Southern Brazil [24]. This virus was amplified in CRIB cells and used to immunize calves to produce BoHV-1 antisera (passage # 8) and in MAB-binding assay an VN assays.

2.2 Growth curves

The replication properties of CpHV-1 were assessed *in vitro* by single- and multi-step growth curves in MDBK, CRIB and BT cells. Cells cultured in 12-well plates were inoculated with CpHV-1 at multiplicity of infections (MOI) of 0.1 or 10 (multi-step and single-step growth curves, respectively) and harvested at various time points post-infection (6, 12, 24, 48 and 72 h pi). Cells were freeze-thawed three times, centrifuged at 5.000 x *g* and the virus titers were determined by limiting dilution. Virus titers were determined for each time point using end-point dilutions and the Spearman and Karber's method and expressed as mean tissue culture infectious doses per mL ($TCID_{50}.mL^{-1}$).

2.3. Antigenic analysis

Antigenic analysis of CpHV-1 and BoHV-1 was performed by monoclonal antibody (MAb) reactivity and virus-neutralizing (VN) assays. For MAb-reactivity assays, CRIB cells were inoculated with CpHV-1 or BoHV-1 at an MOI of 0.1 and 18 – 24 h later were fixed with cold acetone (5 min), washed in phosphate-buffered saline and distilled water and incubated with individual MAbs produced against BoHV-1 (and BoHV-5) antigen (Table 1), followed by washing and incubation with a FITC-conjugated anti-mouse IgG antibody (1:100) (Sigma, Inc.). Slides were observed under UV light in an epifluorescence microscope (Axiolab ZEISS®).

The serological cross-reactivity between CpHV-1 and BoHV-1 was investigated by VN assays using the following sera: i. Sera of calves inoculated intranasally (IN) with BoHV-1 (SV56/90 isolate) or subcutaneously (SC) with CpHV-1 (WI 13-46 strain); ii. Sera of goats inoculated IN with CpHV-1; iii. Field bovine sera submitted to routine BoHV-1 serology. These sera were tested against CpHV-1 WI 13-46 and BoHV-1 SV56/90 in a standard VN assay. Briefly, VN assays were performed in 96-well plates, by incubating two-fold dilutions of serum against 100-200 $TCID_{50}$ of the respective virus for 2 h. Then, a suspension of CRIB cells was added and the plates were incubated at 37 °C with 5% CO_2. End-point titers were determined by the presence or not of cytopathic effect on infected cell cultures that were read after 96 h of incubation. The neutralizing titers were considered the reciprocal of the highest serum dilution capable of preventing virus replication.

2.4. Animal experiments

This study was approved by the institutional Ethics Committee on Animal Use (CEUA – UFSM - protocol nº 7209040618).

Goat experiment: nine male kids, 4 to 6 months-old, tested seronegative to CpHV-1 by virus neutralizing (VN) test, were allocated in two groups, including one group inoculated with CpHV-1 (n = 7) and the other inoculated with MEM (n = 2). The virus inoculum consisted of a 5 mL suspension containing $10^{7,6}$ $TCID_{50}.mL^{-1}$ of CpHV-1 (WI 13-46 strain) divided in the two nostrils (2.5 mL each). Virus inoculation was performed with the aid of syringe-type sprayers followed by swabbing of the inoculum against the nasal mucosa.

Starting at day 36 pi, inoculated kids were subjected to five daily intramuscular administrations of dexamethasone (0.4mg/kg/day, Decadronal, Aché®, Sao Paulo - Brazil). Clinical monitoring accompanied by nasal/ocular swab collection was conducted during 15 days after Dx treatment. Blood for serology was collected at days 36, 54 and 67 pi. At day 67 pi, inoculated and control kids were euthanized for tissue collection. Trigeminal ganglia (TG), olfactory bulbs (OB) and tonsils were collected for DNA extraction and PCR.

Calf experiment: sixteen Holstein male calves, 6 to 8 months-old, seronegative to BoHV-1 and CpHV-1 by VN, were allocated in two groups, being one inoculated with CpHV-1 (n = 12) and the other with MEM (n = 4). The inoculated animals received the virus by intranasal (IN) route as described above, each animal receiving 5 mL of the viral suspension ($10^{7,6}$ $TCID_{50}$ mL^{-1}) in each nostril. Control calves were inoculated with MEM in the same fashion.

Following inoculation, the animals were monitored in a daily basis for 15 days pi for body temperature, nasal/respiratory signs. Additionally, nasal swabs were collected daily to evaluate virus replication and shedding. Blood for serology was collected on days 0, 15 and 30 pi. Serum samples were submitted to VN assays as described above, using the homologous CpHV-1 as standard virus.

2.5. DNA extraction and PCR

Total DNA extraction and PCR for CpHV-1 DNA were performed in tissues collected at necropsy (day 67 pi). DNA was extracted from TGs, OB and tonsils (~100 mg) using the phenol/chloroform method, according to standard protocols. Approximately 200 ng of each DNA sample were subjected to PCR to amplify an internal region of CpHV-1 thymidine kinase (TK) gene. For this, primers forward 5 '- CTC GTC GTC TGC ACC CTT C-3' and reverse 5 '- CGA CAT GTC CAG CGT GAA TA-3' were used, resulting in the amplification of a 399 bp product. PCR reactions were performed in a volume of 25 μL using 2 μL of template DNA, 12.5 mM of each primer, 2.5 mM $MgCl_2$, 10 mM dNTPs, 1x reaction buffer and 1 unit of Taq DNA polymerase (Fischer™). PCR conditions were: initial denaturation (94 °C for 10 min), followed by 30 cycles of 94 °C for 1 min; 54 °C for 1 min for primer annealing and 72 °C for 1 min for primer extension, and a final extension of 7 min at 72 °C. The products were visualized on 1.5% agarose gel, stained with Gel Red (Biotium®) and visualized under UV light. In all reactions, the DNA extracted from cells infected with CpHV-1 was used as positive control and ultrapure water as negative control.

2.6. Statistical analysis

Statistical analysis was performed using the Prism software (GraphPad; 6th version). Students T-test was performed on all groups. Statistical differences between groups were considered significant at $P<0.01$ (***) or $P<0.05$ (*).

3. Results

3.1. Growth curves

The replication kinetics of CpHV-1 was assessed in BT, MDBK and CRIB cells (MOI = 0.1 [multi-step growth curve], and MOI = 10 [single-step growth curve]). CRIB cells are a

mutant MDBK cell line resistant to infection with bovine viral diarrhea virus (BVDV) whose susceptibility to CpHV-1 was unknown. No differences in replication kinetics were observed comparing the three different cell lines (Fig. 1). Hence, CRIB cells were used thereafter for virus replication, quantitation, virus isolation and VN assays.

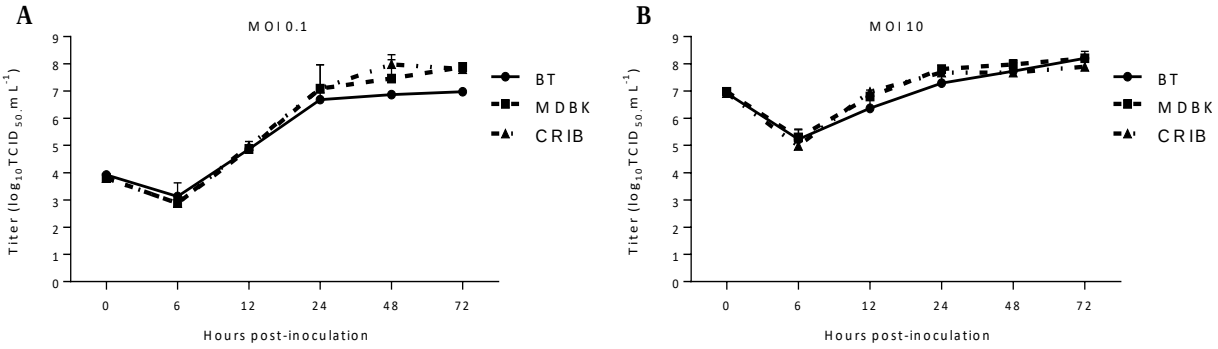

Fig 1. Replication kinetics of *Caprine alphaherpesvirus* (CpHV-1) in three cell lines. (A) Multi-step growth curves of CpHV-1 in bovine turbinate (BT), Madin-Darby bovine kidney (MDBK) and CRIB cells (Madin-Darby bovine kidney BVDV-infection-resistant) at multiplicity of infections (MOI) of 0.1. (B) Single-step growth curve of CpHV-1 in BT, MDBK and CRIB cells at MOI of 10. Virus titers were determined on each time point using end-point dilutions and the Spearman and Karber's method and expressed as mean tissue culture infectious doses per milliliter ($TCID_{50}.mL^{-1}$). Virus titers were calculated based on two independent experiments and the bars represent the standard deviation.

3.2. Antigenic analysis

MAb reactivity assays - The results of indirect fluorescent immunoassays (IFAs) with BoHV-1 and BoHV-5 MAbs in CRIB cells inoculated with CpHV-1 or BoHV-1 are presented in Table 1. Among seven anti-gC MAbs, one antibody (HB24L) showed strong reaction and three (1F3, 2F9 and 4B2) showed weak reaction with CpHV-1 antigens. One anti-gB (420304) and two anti-gD MAbs (MM113 and 110204) recognized both BoHV-1 and CpHV-1 infected cells; whereas an anti-BoHV-1 gE Mab (102102) reacted only with BoHV-1 antigen. One out of eleven BoHV-1 MAbs of undetermined protein specifity (non-gC, gE or gI) was able to recognize BoHV-1 and CpHV-1 antigens as well; ten of these MAbs failed to react with CpHV-1 infected cells. These results demonstrated that CpHV-1 and BoHV-1 share immunogenic epitopes in their major envelope glycoproteins, noticeably gB, gC and gD.

Table 1
Reactivity of a panel of monoclonal antibodies (MAbs) raised against *Bovine alphaherpesviruses* with antigens of *Caprine alphaherpesvirus 1* (CpHV-1) and *Bovine alphaherpesvirus 1* (BoHV-1) in indirect fluorescent assay.

Protein Specificity	gB	gC							gD		gE	Non-gC, gE, gI			Non -gC			Undetermined				
MAb / Virus	420304[a]	HB24L[b]	1F3[b]	2F9[b]	2G10[b]	1D12[b]	2A6[b]	4B2[b]	MM113[c]	110204[a]	102102[e]	2H4[d]	4D7[d]	1F1[d]	2E2[b]	4E4[b]	3D6[b]	2G5	12F2	Mab60	67c2	MabG8
BoHV-1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CpHV-1	+	+	(+)	(+)	-	-	-	(+)	+	+	-	-	-	-	-	-	-	+	-	-	-	-

+ Strong positive reaction, (+) Weak reaction, - No reaction.

[a] [25].
[b] [26].
[c] [27].
[d] [28].
[e] [Marshall et al. unpublished; kindly provided by Dr Geoffrey Lechtworth III].

Virus-neutralizing assays - The results of cross-neutralization assays with bovine and goat sera raised against CpHV-1 and BoHV-1, to the homologous and heterologous viruses, are presented in Tables 2 and 3. Among 11 sera of cattle immunized with CpHV-1 and containing VN titers to the homologous virus of 2 (n = 2), 4 (n = 7) and 8 (n = 2), none showed detectable neutralizing activity against BoHV-1 (titers $\leq$ 2). However, sera of goats inoculated with CpHV-1 containing homologous VN titers of 32 (n = 1), 64 (n = 5) and 256 (n = 1) were able to neutralize BoHV-1. Neutralization of the heterologous virus was observed in samples with titers usually 8 to 32-fold lower than the homologous virus. In contrast, 17/22 (77.2%) serum samples of calves immunized with BoHV-1 (and presenting VN titers to BoHV-1 ranging from 2 to 256) presented neutralizing activity against CpHV-1, including samples containing moderate to low BoHV-1 VN titers of 8 (n = 5), 4 (n = 7) and 2 (n = 3) (Table 2). These results demonstrated cross-neutralization between CpHV-1 and BoHV-1. In addition, they demonstrated that CpHV-1 antisera were able to neutralize BoHV-1 only in the presence of moderate to high CpHV-1 titers; whereas BoHV-1 antisera containing low homologous titers (2 - 8) were able to neutralize CpHV-1.

Table 2

Virus neutralizing activity of sera of cattle and goats immunized/inoculated with *Caprine alphaherpesvirus 1* (CpHV-1) and *Bovine alphaherpesvirus 1* (BoHV-1) against the homologous and heterologous virus.

Serum origin					
Calves immunized with CpHV-1 (SC)[a]		Goats inoculated with CpHV-1 (IN)[b]		Calves immunized with BoHV-1 (SC)[a]	
VN titer to CpHV-1	VN titer to BoHV-1	VN titer to CpHV-1	VN titer to BoHV-1	VN titer to CpHV-1	VN titer to BoHV-1
4	<2	64	8	4	4
2	<2	64	32	<2	4
4	<2	64	16	4	4
8	<2	256	32	4	16
4	<2	64	32	2	2
8	<2	64	16	<2	2
2	<2	64	32	2	8
4	<2			4	8
4	<2			2	8
4	<2			4	32
4	<2			<2	4
				4	8
				2	8
				4	4
				64	256
				64	128
				2	4
				16	32
				<2	4
				<2	2
				8	16
				256	128

[a] Subcutaneous.

[b] Intranasal.

Table 3 presents the results of VN assays performed with bovine sera submitted to routine BoHV-1 serology. These samples were initially tested against BoHV-1 strain Cooper and, the positive samples (titers ≥ 2) were subsequently tested against CpHV-1 (WI 13-46) and BoHV-1 (SV56/90). Among 124 bovine samples presenting VN antibodies to BoHV-1 Cooper strain (titers ≥ 2), 101 (81.4%) showed neutralizing activity against CpHV-1. As expected, the cross-neutralization increased as the VN titers to BoHV-1 increased, but even samples containing low BoHV-1 titers (2-4) were able to neutralize CpHV-1 (Table 3). Considering samples with VN titers of 2-16 to BoHV-1, 52 out of 75 (69.3%) also neutralized CpHV-1. Seven samples presented the same VN titer to both viruses. These results demonstrate that sera of cattle containing VN antibodies to BoHV-1 (and possibly to BoHV-5) due to natural infection or vaccination did cross-neutralize CpHV-1, generally in two to 16-fold lower titers. Taken together, the results of VN assays demonstrated strong cross-neutralization between BoHV-1 and CpHV-1 antisera.

3.2. Animal experiment

3.2.1. Goat experiment

Acute infection. Kids inoculated with CpHV-1 shed virus in nasal secretions up to day 9 pi. Duration of virus shedding ranged from 5 to 9 days (x = 7.8 days). No infectious virus was detected in nasal secretions collected after day 9 pi. Swabs collected from uninfected controls were negative in virus isolation (Table 4).

Table 3
Virus neutralizing (VN) activity of bovine sera submitted to routine serology to *Bovine alphaherpesvirus 1* (BoHV-1).

VN titer[a]	BoHV-1 (SV56/90) positive (n)	CpHV-1 (WI 13-46) positive (n)	% agreement
2	9	3	33.3
4	19	12	63.1
8	20	14	70
16	27	23	85.1
32	23	23	100
64	19	19	100
128	5	5	100
256	2	2	100
Total	124	101	81.4

[a] VN titers to BoHV-1 Cooper strain.

Table 4

Virological, clinical and serological findings during acute infection in kids inoculated intranasally with *Caprine alphaherpesvirus* 1 (CpHV-1).

Animal ID	Virus shedding (days pi)	Clinical signs (days pi[a])			VN[b] titer (day 36 pi)
		Nasal secretion	Ocular secretion	Respiratory distress	
26	3-9	3-12	4-9	5-7	128
30	1-5	3-13	3-8	5-8	128
31	1-9	3-14	3-11	5-8	64
32	1-8	3-14	4-9	5-7	128
33	1-9	3-14	4-9	5-8	128
47	1-8	4-9	4	5-7	64
49	1-9	3-14	4-13	5-8	128
99[c]	-	-	-	-	<2
10[c]	-	-	-	-	<2

[a] post-infection.

[b] virus-neutralizing.

[c] mock-inoculated controls.

Inoculated kids presented an increase in body temperature between days 2 and 7 pi, comparing to controls ($P < 0.01$). The peak in body temperature was observed between days 3 and 5 pi, with the temperature of some animals reaching up to 41.5 °C (day 3 pi). The temperatures of inoculated animals returned to normal levels from day 8 pi onwards (Figure 2A). Control animals did not present changes in body temperature compatible with fever.

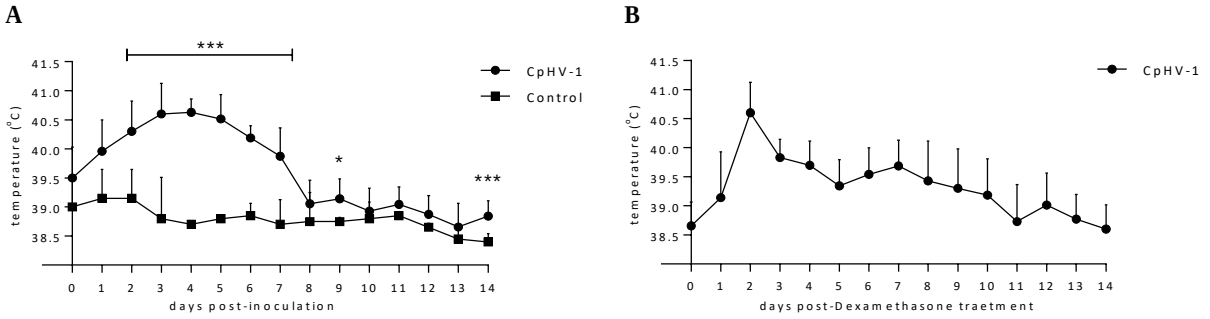

Fig 2. Mean rectal temperatures of kids inoculated with CpHV-1 during acute infection (A) and after administration of dexamethasone (B). Students T-test was performed on all groups. Each point means the group average the bars represent the standard deviation. Statistical differences between groups were considered significant at P < 0.01 (***) and P < 0.05 (*).

Starting at day 3 pi, inoculated kids progressively presented nasal secretion, initially serous, changing to mucous and, lastly, to mucopurulent. Nasal secretion was observed from day 3-14 pi, peaking between days 3 and 5 pi and lasting from 6 to 11 days (x = 9.8 days). Serous ocular secretion was also observed in inoculated kids, between days 3 and 13 in some animals, lasting from 1 to 10 days (x = 6.3 days).

Concomitantly with abundant nasal secretion, inoculated kids developed respiratory distress (difficult and noisy breathing) between days 5 and 8 pi (x = 3.6 days). The peak in severity was observed between days 6 and 7 pi. From day 9 pi onwards breathing returned to normal parameters. Sera collected at day 36 pi from inoculated kids presented anti-CpHV-1 VN antibodies with titers ranging from 64 to 128. Control animals remained healthy and seronegative during the experimental period. These results indicated efficient virus replication in the upper respiratory tract of inoculated kids, accompanied by the development of mild to

moderate respiratory signs. The summary of virological, clinical and serological findings observed in kids inoculated with CpHV-1 is presented in Table 4.

Latent infection. No infectious virus was detected in nasal secretions collected from inoculated kids during days 36-40 pi or after Dx treatment (days 41-54 pi). The animals subjected to Dx treatment presented a transient increase in body temperature, reaching a peak at day 2 after the drug administration (Fig. 2B). No other clinical signs were observed in these animals following Dx treatment. VN titers remained without significant changes after Dx administration, as demonstrated by VN assays performed with sera collected at days 36 and 54 pi. PCR performed in total DNA extracted from TGs and OBs of kids euthanized at day 67 pi revealed the presence of CpHV-1 DNA in both TGs and OB of inoculated animals. Inoculation of TG and OB homogenates followed by three passages in CRIB cells did not result in isolation of CpHV-1, confirming latent infection. Total DNA extracted from tonsils were negative for viral DNA. These results indicated that CpHV-1 did establish latent infection in TGs and OBs after acute infection but was not reactivated upon Dx treatment (Table 5).

3.2.2. Calf experiment

Inoculation of 12 calves with CpHV-1 isolate WI 13-46 did not result in detectable virus replication in the nasal mucosa. No infectious CpHV-1 was isolated from nasal secretions collected after virus inoculation after three passages in CRIB cells. Likewise, CpHV-1 inoculation in calves did not result in evident nasal or systemic signs of infection. Additionally, no neutralizing antibodies were detected in serum collected on day 30 pi. Taken together, these results demonstrated that CpHV-1 did not replicate to detectable levels in calves upon IN inoculation.

Table 5

Virological, clinical and serological findigs after dexamethasone administration (days 36-40 pi) in kids inoculated intranasally with *Caprine alphaherpesvirus 1* (CpHV-1).

Animal ID	Clinical signs	Virus isolation	VN[a] titer		Latent viral DNA (day 67 pi)[f]		
			Day pi[b]		Trigeminal ganglia	Olfactory bulbs	Tonsils
			(36)[c]	(54)[d]			
26	-	-	128	64	+	+	-
30	-	-	128	64	+	+	-
31	-	-	64	64	+	+	-
32	-	-	128	256	+	+	-
33	-	-	128	64	+	+	-
47	-	-	64	32	+	+	-
49	-	-	128	64	+	+	-
99[e]	-	-	<2	<2	-	-	-
10[e]	-	-	<2	<2	-	-	-

[a] Virus-neutralizing.

[b] Post-infection.

[c] Day corresponding to the first day of administration of dexamethasone.

[d] 15 days after administration of dexamethasone.

[e] Mock-inoculated control.

[f] Detected by PCR.

4. Discussion

Our findings add important information on the antigenic relationships between CpHV-1 and BoHV-1 and in the susceptibility of young goats and cattle to CpHV-1. First, a close antigenic relationship between CpHV-1 and BoHV-1 was demonstrated by MAb reactivity and cross-neutralization assays. Second, CpHV-1 was able to produce overt, mild to moderate respiratory disease in kids upon IN inoculation. Third, latent CpHV-1 infection was established in TGs and OBs of kids inoculated intranasally but the virus was not reactivated upon Dx administration, a standard method of alphaherpesvirus reactivation. Fourth, CpHV-1 did not replicate to detectable levels in calves upon IN exposure. Therefore, an occasional transmission of CpHV-1 to cattle in nature would likely have limited - if any - epidemiological relevance.

The antigenic relationship between CpHV-1 and BoHV-1 – associated with possible cross-species transmission of these viruses – may have a potential impact to their epidemiology, serological diagnosis and control [2]. This antigenic relationship has been demonstrated by different means [7,29,30], yet the degree of cross-neutralization *in vitro* seemed to be very low or has been poorly examined to date [7,29]. Our MAb-reactivity assays demonstrated that CpHV-1 and BoHV-1 share immunogenic epitopes, noticeably in the major glycoproteins gB, gC and gD. Engels et al. (1992) [7] showed that CpHV-1/BoHV-1 cross-reacting antibodies present in the sera of goats and cattle were mainly directed to gB, whereas virus-specific antibodies were mostly those reacting with glycoproteins gC and gD. Our data corroborate these findings and add novel information. Indeed, MAb-reactivity assays indicated that CpHV-1/BoHV-1 gC and gD also share immunogenic epitopes which were recognized by BoHV-1-specific MAbs. The gB gene is the most conserved among the major herpesvirus glycoproteins [31]. Although the amino-acid (*aa*) identity of BoHV-1 and CpHV-1 gB is the lowest among ruminant herpesviruses (about 78%) [32], one BoHV-1 MAb against gB also recognized CpHV-1 infected cells (Table 1). Glycoprotein C displays a low *aa* identity among different

herpesviruses and has been associate the attachment of virions to host cell surface receptors [33]. Interestingly, our Mab-binding assays demonstrated that CpHV-1 and BoHV-1 gC share some immunogenic epitopes, revealing some degree of conservation in these regions (Table 1). Likewise, the gD gene presents an homology lower than 70% between BoHV-1 and CpHV-1, but the two MAbs used [27] were able to recognize both BoHV-1 and CpHV-1 (Table 1). The only gE MAb used herein did not recognize CpHV-1 infected cells (Table 1), corroborating a previous study indicating that gE-based assays may be useful to differentiate CpHV-1 from BoHV-1 antibodies in the sera of goats and cattle [11].

The results of VN assays showed that CpHV-1 and BoHV-1 antisera raised in goats and cattle were able to cross-neutralized the heterologous viruses to different extents. Notably, the cross-neutralization was bi-directional, but it was asymmetrical since BoHV-1 antisera neutralized CpHV-1 more efficiently than CpHV-1 antisera neutralizes BoHV-1. This asymmetrical cross-neutralization has been previously suggested by Martin et al. [29] and Engels et al. [7] using a limited number of rabbit-, goat- (n = 2) or calf- (n = 2) sera in VN assays. The *aa* sequence similarities in gC (and perhaps in gB and gD) - as demonstrated by MAb reactivity – likely explain the cross-neutralization between these virus since gC is the most abundant envelope glycoprotein and a major target for virus-neutralizing antibodies [33]. The extent of serological cross-reactivity between CpHV-1 and BoHV-1 may have implications in serological diagnosis, epidemiological studies and control efforts for these viruses as cross-reacting antibodies may led to misinterpretations of serological data [2]. Thus, as CpHV-1 and BoHV-1 may potentially cross-infect cattle and goats in nature [2,8,9,12], positive serology to either virus in herds raising these species together should be interpreted with caution.

Natural CpHV-1 infections in young kids frequently result in mild nasal and respiratory signs [16]. Papanastasopoulou et al. [34] inoculated two 10 - 13 days-old colostrum deprived kids with CpHV-1 intravenously and observed apathy, anorexia, fever, nasal discharge,

abdominal pain and hemorrhagic feces, a clinical presentation that was similar to natural disease in young kids [16]. Additionally, inoculation of a CpHV-1 isolate intranasally into three adult goats resulted in fever, leukopenia and development of pustular lesions in the vulva [36]. On the other hand, attempts to reproduce a severe respiratory disease observed in a field outbreak by inoculation of CpHV-1 resulted in only mild nasal secretion [37].

In the present study, inoculation of CpHV-1 in the nose of kids resulted in efficient virus replication and shedding in nasal secretions. Additionally, CpHV-1 infection led to serous to mucopurulent nasal secretion in inoculated animals. Concomitantly, inoculated animals developed moderate respiratory distress (difficult and noisy breathing) (Table 3). These observations indicated efficient virus replication in the upper respiratory tract, accompanied by the development of mild to moderate respiratory disease.

Latent infection by CpHV-1 has been previously demonstrated in goats after natural and experimental infections [20–22]. In addition, natural reactivation [21] or chemically induced reactivation [38] have been reported in naturally infected goats. In the present study, we used a Dx protocol successfully used for reactivation of BoHV-5 in goats [39]. Nonetheless, attempts to recover infectious virus from nasal secretions after Dx administration failed after three passages in CRIB cells. In addition, VN titers remained unaltered after Dx treatment, reinforcing that reactivation probably did not take place. Nonetheless, detection of CpHV-1 DNA in TGs and OBs of inoculated kids euthanized on day 67 pi demonstrated that the virus did established latent infection in these tissues. The failure to recover infectious virus in nasal secretions after Dx treatment was not a surprising finding since previous studies have shown that latent CpHV-1 infection in goats is not easily reactivated [20,21,36,40]. A cohort study by Tempesta et al. [21] detected natural CpHV-1 reactivation only in goats presenting low VN titers. As the kids in the present study presented moderate to high VN titers (64-128) on the

reactivation day, it should not be discarded that these antibodies might have somehow affected virus reactivation and/or detection.

Intranasal inoculation of CpHV-1 WI 13-46 in calves did not result in detectable virus replication nor in clinical signs or seroconversion. These results contrast with some experimental studies demonstrating the susceptibility of calves to CpHV-1 infection [7,10]. On the other hand, these findings corroborate old studies questioning the susceptibility of cattle to CpHV-1 [41]. In fact, experimental studies addressing the ability to CpHV-1 to infect cattle are scarce and yielded conflicting results [7,10,41]. Inoculation of calves of different ages by different routes with a virulent herpesvirus isolated from goats resulted in early and transient virus detection in nasal secretions of 2/6 inoculated animals, probably representing residual inoculum virus. In the absence of further virological, clinical and serological evidence of infection, the authors concluded that the calves were not susceptible to that caprine herpesvirus [41]. Engels et al. [7] inoculated two calves IN with CpHV-1 and detect only transient virus shedding and seroconversion to low titer in one animal. Six et al. [10] inoculated eight young calves (~two-months-old) with CpHV-1 (Swiss E/CH strain) and demonstrated transient virus shedding (1-5 days) and low titer seroconversion in all animals. The virus was not reactivated upon Dx administration but CpHV-1 DNA was demonstrated by PCR in TGs of 2/8 animals. Taken together, these studies results suggest that the susceptibility of cattle to CpHV-1 infection may vary according to the viral strain/isolate, animal age and route of inoculation. Interestingly, calves inoculated subcutaneously with CpHV-1 for the production of antisera seroconverted in titers of 2-8, indicating an immune response to the input viral antigen or to low level virus replication. Regardless, the results of experimental infection demonstrated that calves were not readily infected by CpHV-1, even under highly favorable experimental conditions. Thus, would cattle be naturally exposed to CpHV-1, this species is very unlikely to play a role in the perpetuation and spread of the virus in nature.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micpath.2019.103663.

Conflict of interest statement

The authors declare no conflict of interest.

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