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Bruna Portolan Amaral

# PATOGÊNESE E TERAPIA EXPERIMENTAL DA INFECÇÃO PELO *ALFAHERPESVÍRUS BOVINO 2*

Santa Maria, RS

2020

**Bruna Portolan Amaral**

# PATOGÊNESE E TERAPIA EXPERIMENTAL DA INFECÇÃO PELO *ALFAHERPESVÍRUS BOVINO 2*

Tese apresentada ao Programa de Pós-Graduação em Medicina Veterinária, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do título de **Doutora em Medicina Veterinária.**

Orientador: Prof. Dr. Eduardo Furtado Flores

Santa Maria, RS

2020

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**Aprovado em 25 de agosto de 2020:**

**Eduardo Furtado Flores, Dr. (UFSM)**
(Presidente/Orientador)

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Santa Maria, RS

2020

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## RESUMO

### PATOGÊNESE E TERAPIA EXPERIMENTAL DA INFECÇÃO PELO *ALFAHERPESVÍRUS BOVINO 2*

AUTORA: Bruna Portolan Amaral
ORIENTADOR: Eduardo Furtado Flores

O *Alfaherpesvírus bovino 2* – agente da mamilite herpética bovina (BHM) – é relacionado aos *Alfaherpesvírus humanos* 1 e 2 (HHV-1, HHV-2) e, como tal, tem sido sugerido como modelo para testes de vacinas e drogas antivirais. Além disso, pouco é conhecido sobre a patogenia do BoHV-2. Neste trabalho foi estudada a patogenia da infecção pelo BoHV-2 em bezerros, após diferentes rotas de inoculação e investigada a atividade de diferentes fármacos antivirais *in vitro e in vivo* contra o BoHV-2. No primeiro estudo, bezerros soronegativos de três a quatro meses foram inoculados com BoHV-2 ($10^7 TCID_{50}.mL^{-1}$) por via intramuscular (IM, n=4), por via intravenosa (IV, n=4) ou transdérmica (TD, n=4) após escarificação leve e submetidos a monitoramento. Os bezerros inoculados pela via IV apresentaram aumento leve da temperatura corporal entre os dias 6 a 9 pós-inoculação (pi). A inoculação do vírus pela via TD resultou em lesões inflamatórias leves nos locais de inoculação, caracterizadas por hiperemia, pequenas vesículas, exsudação leve e formação de crostas, entre os dias 2 e 8pi. O vírus ou DNA viral foi detectado por PCR nas crostas/swabs coletados de lesões de 3 de 4 animais inoculados TD do dia 2 ao 8pi. Viremia foi detectada em 3/4 dos animais do grupo IM (do dia 4 ao 8pi); em 2/4 animais do grupo IV (dias 6 e 8pi), mas não no grupo TD. Administração de dexametasona (Dex) nos bezerros inoculados no dia 48pi, não resultou em reativação do vírus. No segundo estudo, investigou-se a atividade anti-viral *in vitro* contra o BoHV-2 de três drogas anti-herpéticas: Cidofovir (CDV), Famciclovir (FAM), Foscarnet (PFA), e do disseleneto de difenila $(PhSe)_2$ pelo teste de redução de placas (PRA). Redução significativa do número de placas virais foi observada pelo tratamento dos tapetes celulares com $(PhSe)_2$ (redução de 79.7%, $p<0.05$) ou com CDV (62.8%, $p<0.05$). Tratamento com FAM resultou em redução menor (22.9%, $p<0.05$) e o PFA não demonstrou atividade antiviral. A seguir, investigou-se o efeito do $(PhSe)_2$ e CDV, isoladamente ou combinados, sobre a infecção e doença produzida pelo BoHV-2 em ovelhas inoculadas pela via transdérmica e submetidas a tratamento tópico com um veículo cremoso contendo $(PhSe)_2$, CDV, e $(PhSe)_2$ + CDV associados. Treze de 14 ovelhas (92.8%) inoculadas desenvolveram lesões locais que progrediram pelas fases de hiperemia (dias 2 – 6pi), grandes pápulas ou áreas planas achatadas/escuras (dias 4 a 12pi), acompanhadas pela formação de crostas que duraram além do dia 15pi nos animais não-tratados. Tratamento com $(PhSe)_2$ resultou em redução importante no escore clínico a partir do dia 8 pi ($p<0.05$), em redução do período clínico e na duração da excreção viral ($p<0.05$) comparado aos controles. Tratamento combinado ($(PhSe)_2$+CDV) e CDV sozinho, também resultaram em melhoria clínica ($p<0.05$), mas menos pronunciada e mais tardia (após dias 11 pi e 13 pi, respectivamente), mas não reduziram significativamente o tempo de excreção viral. Em conjunto, esses resultados contribuem para o conhecimento da patogenia da infecção pelo BoHV-2 e são promissores no sentido do uso disseleneto de difenila $(PhSe)_2$, isolado ou em combinação com drogas anti-herpéticas, no tratamento de lesões ocasionadas pelo BoHV-2 no úbere de vacas leiteiras.


**Palavras-chave**: *Alfaherpesvírus bovino 2*. Bovinos. Patogênese. Mamilite. Tratamento. Ovelhas.

## ABSTRACT

## EXPERIMENTAL PATHOGENESIS AND THERAPY OF *BOVINE ALPHAHERPESVIRUS* 2 INFECTION

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*Bovine alphaherpesvirus 2* (BoHV-2) - the agent of bovine herpetic mamillitis (BHM) – is related to *Human alphaherpesviruses* 1 and 2 (HHV-1, HHV-2) and has been proposed as a model for vaccine and drug testing. In addition, very little is known about the pathogenesis of BoHV-2 infection. Herein we studied the pathogenesis of BoHV-2 in calves after inoculation through different routes and investigated the activity of candidate anti-viral drugs *in vitro* and *in vivo* against BoHV-2. In the first study, 3 to 4-months-old calves were inoculated with BoHV-2 ($10^7$$TCID_{50}$.mL-1) intramuscularly (IM, n=4), intravenously (IV, n=4) or transdermally (TD) after mild scarification (n=4) and monitored thereafter. Calves inoculated by the IV route presented as light increase in body temperature between days 6 to 9 post-inoculation (pi). Virus inoculation by the TD route resulted in mild inflammatory lesions at the sites of inoculation, characterized by hyperemia, small vesicles, mild exudation and scab formation, between days 2 and 8pi.Virus or viral DNA was detected by PCR in the crusts/swabs collected from lesions of 3 out of 4 animals inoculated TD from day 2 to 8pi. Viremia was detected in 3/4 animals of the IM group (from day 4 to 8pi); in 2/4 animals of the IV group (days 6 and 8pi) but not in the TD group. Administration of dexamethasone (Dex) to the inoculated calves at day 48pi, did not result in virus reactivation. In the second study, we investigated the anti-viral activity in vitro against BoHV-2 of three anti-herpetic drugs: Cidofovir (CDV), Fanciclovir (FAM), Foscarnet (PFA), and diphenyl disselenide (PhSe)2 by plaque reduction assays (PRA). A significant reduction in the number of viral plaques was observed by treating the monolayers with (PhSe)2 (79.7% reduction, $p<0.05$) or CDV (62.8%, $p<0.05$). FAM treatment resulted in a slight decrease in plaque number (22.9%, $p<0.05$) and PFA showed no anti-viral activity. Next, we investigated the effects of (PhSe)2 and CDV, alone or in combination, in the infection and disease produced by BoHV-2 in ewes inoculated transdermally and submitted to topic treatment with a vehicle gel containing (PhSe)2, CDV, and combined (PhSe)2 + CDV. Thirteen out of 14 (92.8%) inoculated ewes developed local lesions that typically progressed through the stages of hyperemia (days 2 – 6pi), large papules or depressed/flat dark areas (day 4 to 12pi), accompanied/followed by scab/crust formation that lasted beyond day 15pi in untreated ewes. Treatment with (PhSe)2 resulted in an important reduction in clinical score from day 8 pi onwards ($p<0.05$), shortening of clinical course and reduction in duration of virus shedding ($p<0.05$) compared to untreated controls. Combined ((PhSe)2+CDV) treatment and CDV alone, also led to clinical improvement ($p<0.05$), yet less pronounced and delayed (after day 11 pi and 13 pi, respectively), but no significant reduction in virus shedding. Taken together, these findings contribute to the knowledge of BoHV-2 pathogenesis and are promising towards the use of diphenyl disselenide (PhSe)2, alone or in combination with anti-herpetic drugs, in the treatment of lesions induced by BoHV-2 in the udder and teats of dairy cows.



**Keywords**: *Bovine alphaherpesvirus 2*. Cattle. Pathogenesis. Mammillitis. Therapy. Ewes.

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# SUMÁRIO

## 1 INTRODUÇÃO

O *Alfaherpesvírus bovino 2* (BoHV-2) é o agente da mamilite herpética (BHM), uma doença vesicular, erosiva e necrótica do úbere e tetos de vacas. O BoHV-2 pertence a família *Herpesviridae,* subfamília *Alphaherpesvirinae,* gênero *Simplexvirus. Os* vírus dessa família possuem como importante característica a capacidade de produzir infecção latente em seus hospedeiros (ICTV, 2019). Os vírions dos herpesvírus possuem diâmetro que varia entre 120 a 300 nm, capsídeo icosaédrico (aproximadamente 100 a 110 nm), e uma molécula de DNA de fita dupla linear conjugada com algumas proteínas. Em torno do capsídeo existe uma camada de susbstâcia amorfa denominada tegumento, sendo envolto por um envelope lipoproteico com espículas glicoproteicas na superfície (ROIZMAN et al., 1992). No mesmo gênero do BoHV-2 estão também *os Alfaherpesvírus humano 1 e 2* (HHV-1 e HHV-2), com os quais possuem alto grau de similaridade com BoHV-2 (ICTV, 2019, ROIZMAN et al., 1992). Os *alphaherpesvírus* apresentam um amplo espectro de hospedeiros, seu ciclo replicativo rápido e se disseminam rapidamente em células de cultivo, resultado em destruição celular, além de estabelecerem infecções latentes em neurônios de gânglios sensoriais e autonômicos (ROIZMAN et al., 1992).

A infecção pelo BoHV-2 possui maior repercussão sanitária no gado leiteiro, e ocorre principalmente em regiões de clima temperado. Infecções causadas pelo BoHV-2 são benignas, auto limitantes e acometem principalmente novilhas e primíparas. Possuem duas formas clínicas distintas e bem definidas, na forma localizada as lesões manifestam-se nos tetos ou disseminadas pelo úbere (mamilite herpética). Já na forma generalizada, evidenciam-se lesões na pele generalizadas, principalmente na cabeça, dorso e períneo (*pseudolumpy skin disease* - PLSD*)*. Em geral, a infecção por BoHV-2 provoca lesões cutâneas doloridas, caracterizadas por eritema, edema, formação de vesículas e ulcerações, podendo variar de pequenas placas ou até mesmo extensas vesículas e úlceras na pele (LECHTWORTH & LADUE, 1982). Lesões vesiculares também podem se desenvolver no focinho de bezerros lactentes filhos de vacas afetadas pela doença (WESTBURY, 1981).

A BHM frequentemente é uma doença auto limitante, no entanto, dependendo do grau das lesões nos tetos e úberes, ocorre redução na produção leiteira devido aos sinais clínicos e as lesões que dificultarem a ordenha, bem como, favorece ao desenvolvimento de mastites bacterianas, gerando impactos econômicos e ao bem estar das vacas acometidas (ALMEIDA et al., 2008).

A penetração do vírus na pele é facilitada por lesões abrasivas e escarificações. O vírus replica-se nas camadas mais profundas da derme e epiderme, e o período de incubação varia de

quatro a nove dias (MARTIN et al. 1969; FRANCO et al., 2017). As lesões ocorrem na maior parte dos casos no úbere, tetos e, ocasionalmente, no períneo. Lesões vesiculares também podem se desenvolver no focinho de bezerros lactentes filhos de vacas afetadas pela doença (WESTBURY, 1981).

Na maior parte dos casos de BHM as lesões se apresentam de forma localizada e se caracterizam pelo aparecimento de uma região arredondada, hiperêmica, irregular e que posteriormente passa a se apresentar mais saliente, com bordas bem definidas. Geralmente são observadas vesículas que após romperem-se formam úlceras que podem favorecer a ocorrência de infeções secundárias. As bordas das lesões são bem definidas e por vezes necróticas, com aspecto enegrecido e com aparência de placa (FRANCO et al., 2017). Com menor frequência ocorre a forma generalizada, onde evidenciam-se lesões na pele por todo o corpo, principalmente, na cabeça, dorso e períneo (*pseudolumpy skin disease* - PLSD*)* (GIBBS; RWEYEMAMU, 1977). Em geral, a infecção por BoHV-2 provoca lesões cutâneas doloridas, caracterizadas por eritema, edema, formação de vesículas e ulcerações, podendo variar de pequenas placas ou até mesmo extensas vesículas e úlceras na pele (LECHTWORTH & LADUE, 1982).

A latência é um mecanismo que favorece ao vírus a sua permanência em seus hospedeiros sem que haja multiplicação viral. Esta é considerada uma das mais importantes características dos herpesvírus (JONES, 2003). MARTIN & SCOOT (1979) conseguiram estabelecer infecções latentes do BoHV-2, porém a patogenia desta infecção não foi suficientemente esclarecida. Está hipótese é reforçada pelo desenvolvimento frequente de lesões nos tetos após o parto, sem fontes externas de infecção. As alterações imunológicas e fisiológicas que ocorrem nos períodos próximos ao parto promoveriam a reativação viral. No entanto, mais estudos acerca da latência do BoHV-2 são essenciais para determinar parte da patogenia da doença (WESTBURY, 1981; ALMEIDA et al., 2008).

A doença foi identificada primeiramente na África em 1957 e a infecção tem se mostrado presente em rebanhos bovinos de vários países. Dados sorológicos e virológicos têm demonstrado a ocorrência do vírus em vários países, incluindo o Quênia (MARTIN & GWYNNE, 1968), Reino Unido (JOHNSTON et al., 1971), Estados Unidos (DARDIRI & STONE 1972), Austrália (TURNER et al., 1974), Brasil (ALICE, 1977) e Japão (IMAI, et al., 2005). Sorologia positiva também foi detectada em ruminantes selvagens, mas a participação dessas espécies na epidemiologia da doença ainda é incerta (PLOWRIGHT & JESSET, 1971).

A infecção por BoHV-2 já foi reproduzida experimentalmente em ovinos, caprinos (WESTBURY, 1981; ALMEIDA et al., 2008), camundongos, coelhos e cobaias (PEPPER et

al., 1966; SMEE & LEONHARD, 1994). Ovinos inoculados com o vírus podem desenvolver lesões com as mesmas características das lesões observadas em bovinos (ALMEIDA et al., 2008).

Não há tratamento específico para a doença causada pelo BoHV-2, mas recomenda-se a utilização tópica de antibióticos para combater possíveis infecções secundárias. O objetivo deve ser impedir ou minimizar a formação de crostas, que dificultam a ordenha mecânica, com a loção de solução adstringente, pós-ordenha, e pomadas antissépticas. (FLORES & WEIBLEN, 2018). Por outro lado, a utilização de antivirais para o tratamento de infecções pelo HHV-1 torna promissora a utilização de antivirais para tratamento de lesões causadas pelo BoHV-2, bem como o BoHV-2 um modelo promissor para estudo tanto para HHV-1 e HHV-2 (STERZ, et al., 1973, EHLERS, et al., 1999).

Fármacos antivirais são pouco empregados na medicina veterinária, sendo que essas drogas são mais utilizadas em humanos e o foco de pesquisas da área também são direcionadas ao tratamento de doenças virícas humanas. Os antivirais mais utilizados para inibir a replicação dos herpesvírus são: Aciclovir, Ganciclovir, Cidofovir, Fanciclovir e a Vidarabina. Esses antivirais impedem que a DNA polimerase dos herpesvírus complete o ciclo catalitíco e, por isso, são denominados análogos de nucleosídeos, pois incorporam-se nas novas cadeias de DNA viral (COEN & RICHMAN, 2007).

O Cidofovir (CDV) é um fármaco de amplo espectro antiviral, sendo prescrito para o tratamento de diversas infecções causadas por vírus, tais como HHV-1, vírus da Varicela-Zoster, vírus Epstein-Barr, HHV-6-8, poxvírus, poliomavírus e adenovírus (BONIATTI et al., 2007). Ao contrário do aciclovir e ganciclovir, o CDV não necessita passar pela etapa inicial de fosforilação para ser ativado (CUNDY, LYNCH & LEE, 1997). Um estudo utilizando o CDV no combate ao citomegalovírus, verificou que o fármaco se incorpora à porção terminal da cadeia de DNA viral (TORO et al., 2003). A ação prolongada do CDV ocorre devido à meia-vida de seus metabólitos ativos ser longa, necessitando assim de doses menos frequentes (DE CLERCQ, 2003).

No entanto, a molécula do CDV possui risco elevado de causar danos hepáticos e renais quando administrado pela via intravenosa, dessa forma tem sido recomendado para tratamento de lesões muco-cutâneas para reduzir assim os efeitos colaterais sistêmicos do fármaco (TORO et al., 2003).

O selênio (Se) é fundamental no sistema nervoso central, devido a sua função antioxidante, mantem o estado redox celular e melhora a dinâmica mitocondrial, além de participar na regulação dos canais de cálcio ($Ca^{2+}$) e na moduação da neurogênese (PAPP et al.,

2010). Tendo em vista a sua importância, diversos compostos orgânicos de selênio vêm sendo estudados, dentre eles destaca-se o disseleneto de difenila $(PhSe)_2$, o qual descobriu-se propriedades famacológicas de neuroproteção (GHISLENI et al., 2003; NOGUEIRA et al., 2004; DOBRACHINSKI et al., 2014), antiviral (SARTORI et al., 2016), antifúngica (WOJTOWICZ et al., 2004; LORETO et al., 2011), antioxidante e anti-inflamatória (NOGUEIRA et al., 2003; PETRONILHO et al., 2016; SARTORI et al., 2016).

SARTORI et al. (2016) demonstraram a atividade antiviral do $(PhSe)_2$ contra o HHV-2 *in vitro* e *in vivo. In vivo,* verificaram redução do dano histológico, tamanho da lesão e carga viral da lesão causada pelo HHV-2 em cobaias tratadas com o $(PhSe)_2$. A ação antiviral de $(PhSe)_2$ contra a infecção por HHV-2 foi relacionada às suas propriedades imunomoduladoras, antioxidantes e anti-inflamatórias (SARTORI et al., 2016). Outros trabalhos também verificaram que o efeito imunomodulador do $(PhSe)_2$ pode ser benéfico em outras doenças víricas, como HIV (SCHRAUZER & SACHER, 1994) e vírus da hepatite B (HBV) (YU, 1997).

A similaridade genética e biológica entre BoHV-2 e HHV-1/HHV-2 possibilita seu uso como modelo para estudos de patogenia, estudos imunológicos, eficácia de fármacos e vacinas. Nesse sentido, o BoHV-2 tem sido incluído como um dos vírus a serem usados para estudos de terapias antivirais (FIELD, BISWAS & MOHAMMAD, 2006).

Assim, os objetivos dessa tese foram estudar a patogênese do BoHV-2 em bezerros, bem como propor alternativas terapêuticas para o tratamento de lesões cutâneas causadas pelo BoHV-2. Os estudos desenvolvidos durante o Doutorado e apresentados nessa Tese são apresentados sob a forma de artigos científicos:

**Artigo 1**: Pathogenesis of *Bovine alphaherpesvirus 2* in calves following different routes of inoculation.

**Artigo 2**: Diphenyl diselenide and cidofovir present anti-viral activity against *Bovine alphaherpesvirus 2* in vitro and in a sheep model

## 2. ARTIGO 1

# Pathogenesis of *Bovine alphaherpesvirus 2* in calves following different routes of inoculation[1]

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**ABSTRACT**

*Bovine alphaherpesvirus* 2 (BoHV-2) is the agent of herpetic mammilitis (BHM), a cutaneous and self-limiting disease affecting the udder and teats of cows. Very little is known about the pathogenesis of BoHV-2, hampering the development of therapeutic drugs, vaccines and other control measures. This study investigated the pathogenesis of BoHV-2 in calves after inoculation through different routes. Three- to four-months seronegative calves were inoculated with BoHV-2 ($10^7 TCID_{50}.mL^{-1}$) intramuscular (IM, n=4), intravenous (IV, n=4) or transdermal (TD) after mild scarification (n=4) and submitted to virological, clinical and serological monitoring. Calves inoculated by the IV route presented as light increase in body temperature between days 6 to 9 post-inoculation (pi). Virus inoculation by the TD route resulted in mild inflammatory lesions at the sites of inoculation, characterized by hyperemia, small vesicles, mild exudation and scab formation, between days 2 and 8pi. Virus or viral DNA was detected by PCR in the crusts/swabs collected from lesions of 3 out of 4 animals inoculated TD from day 2 to 8pi. Viremia was detected in 3/4 animals of the IM group (from day 4 to 8pi); in 2/4 animals of the IV group (days 6 and 8pi) but not in the TD group. Calves from all inoculated groups seroconverted to BoHV-2 in titers from 4 to 64, as indicated by virus-neutralizing (VN) assays performed in sera collected at day 15pi. Administration of dexamethasone (Dex) to the inoculated calves at day 48pi, did not result in virus reactivation as indicated by lack of virus detection in the blood and/or in inoculation sites and no increase in VN antibody titers. These results demonstrated that BoHV-2 was able to replicate efficiently in calves following different routes of exposure, produced viremia after IM and IV inoculation and was not reactivated by Dex treatment.

**INDEX TERMS:** Pathogenesis, *Bovine alphaherpesvirus 2*, calves, cattle, acute infection, latency

## INTRODUCTION

*Bovine alphaherpesvirus* 2 - formerly bovine herpesvirus type 2 (BoHV-2) - is an enveloped, double-stranded DNA virus belonging to the family Herpesviridae, subfamily Alphaherpesvirinae, genus *Simplexvirus* (ICTV 2018). BoHV-2 is genetically and antigenically related to *Human alphaherpesviruses* 1 and 2 (HHV-1, HHV-2) (Sterz et al. 1974, Borchers et al. 1990, Ehlers et al. 1999). BoHV-2 is the agent of bovine herpetic mammillitis (BHM), a vesicular, erosive and necrotic cutaneous disease of the udder and teats of cows. The distribution and prevalence of BoHV-2 infection are poorly understood, but serologic data indicate that the virus is widespread in cattle worldwide (Rweyemamu et al. 1966, Dardiri & Stone 1972, Gibbs & Rweyemamu 1977).

Historically, BoHV-2infection has been described in several countries, including the United States (US) (Dardiri & Stone 1972), Great Britain (Johnston & Scott 1971), Brazil (Alice 1977, Campos et al. 2014), Kenya (Martin & Gwynne 1968), Australia and Japan (Turner et al. 1974, Imai et al. 2005). In addition to the classic BHM, BoHV-2 has also been associated with a generalized nodular skin condition called pseudo-lumpy skin disease (PLSD) (Gibbs & Rweyemamu 1977). Antibodies to BoHV-2 have been detected in wild ruminants (Plowright & Jesset 1971) and the infection has been experimentally reproduced in goats (Westbury 1981) and sheep (Torres et al. 2009b). However, a potential role of these species in the epidemiology of BoHV-2 remains uncertain. Likewise, there is no evidence of human infection, in spite of attempts to reproduce the infection upon inoculation of volunteers (Martin et al. 1966). Large outbreaks of BoHV-2-associated cutaneous disease occurring in a 10-year-period were described in dairy breed calves in California, US (Watanabe et al. 2017). These findings suggest that, like to other simplex viruses, BoHV-2 may circulate silently in susceptible host populations, occasionally producing clinical cases or outbreaks. This behavior may also explain findings of positive BoHV-2 serology in cattle populations with no evident, or scarce, history of compatible clinical disease (Almeida et al. 2008, Torres et al. 2009b).

In spite of its long story and several decades of scattered studies, several aspects of BoHV-2 biology and pathogenesis remain obscure. The pathogenesis of acute and latent infection remains poorly understood. The few pathogenesis studies in cattle were performed two or three decades ago, using old and nowadays obsolete techniques and resources. Experimental reactivation of the infection has not been consistently demonstrated (Probert & Povey 1975, Turner et al. 1976, Castrucci et al. 1980) and attempts to recover the virus upon explant cultures of sensory nerve ganglia have failed (Letchworth & Carmichael 1982). In some

of the studies demonstrating latency and reactivation, biases may have been introduced by inoculating the virus intravenously (Castrucci et al. 1972, Scott & Martin 1978). Likewise, the sites in which the virus may remain latent remain unknown (Letchworth & Carmichael 1982). Pathogenesis studies were performed in animal models, with their inherent pitfalls and restrictions (Almeida et al. 2008, Torres et al. 2009a). Thus, a better understanding of BoHV-2 pathogenesis in their natural hosts is needed.

In addition, bovine herpesviruses have been proposed as viral vector for vaccine antigen delivery (Kanekiyo et al. 2019). As such, BoHV-2 may be also a candidate viral vector for vaccine use. Therefore, the present study investigated selected aspects of the pathogenesis of BoHV-2 in calves.

## MATERIALS AND METHODS

Cell culture and virus. Bovine alphaherpesvirus 2 (BoHV-2) strain New York 01 (NY01) was kindly provided by Dr. Fernando A. Osorio (Department of Veterinary and Biomedical Sciences, University of Nebraska, Lincoln, USA). All procedures of virus replication, quantitation and virus neutralizing assays (VN) were performed in CRIB cells (a MDBK-derived cell line resistant to BVDV). Cells were maintained in Eagle's minimum essential medium (MEM) (Vitrocell®, Nova Campinas, São Paulo, Brazil) supplemented with 10% fetal calf serum (Vitrocell®), penicillin (10,000 IU.mL-1), streptomycin (10mg/mL-1) and amphotericin B (250μg.mL-1) (Sigma - Aldrich®, Darmstadt, Hessen, Germany). The virus inoculum consisted of the supernatant of CRIB cells infected with BoHV-2 at passage # 12, containing 107 TCID50.mL-1 (median tissue culture infectious doses).

Animal experiment. Sixteen holstein with ages ranging from 90 to 120 days, tested negative for BoHV-2 antibodies by virus neutralizing (VN) assays were used in the experiment. The animals were randomly allocated in four groups of four animals each and submitted to the following inoculation protocols: four calves were inoculated by the intramuscular route (IM group); four calves were inoculated by intravenous route (IV group); four calves were inoculated in the skin of the internal face of the left hind limb, after scarification with a needle (TD group) and application of the virus inoculum with the help of a cotton swab. Each animal was inoculated with 107 TCID50.mL-1 of the virus. The four remaining animals (Control group) were inoculated with minimal essential médium (MEM) by the IM route (n=1), IV route (n=1) and by scarification of the skin, as described by the TD group (n=2). The experimental groups were maintained in separated barns and given food and water ad libitum.

Experimental inoculated animals were monitored daily for local and systemic clinical

signs; blood, nasal secretions and exudates of the inoculation sites were collected for virus detection. Clinical monitoring consisted of visual inspection and photographic registration of local signs (TD group); measurements of rectal temperatures, recording of appetite and alertness (all groups). Blood samples and nasal swabs were collected every two days from calves of the IM and IV groups. Nasal secretions and buffy coats were submitted to DNA extraction and PCR for the BoHV-2 genome. Swabs collected from the sites of virus inoculation (TD group) were collected daily and also submitted to PCR for BoHV-2 DNA. Serum samples were collected at days 0 and 14 pi and submitted to VN assays for BoHV-2 antibodies. Forty-eigtht days after virus inoculation, inoculated animals were submitted to dexamethasone (Dex) treatment (Azium–Merck Sharp and Dohme®, Kenilworth, Nova Jersey, USA), 0.1mg/kg/day for 5 consecutive days, and monitored thereafter for viremia, virus shedding, clinical signs and seroconversion. All procedures involving animals were approved by an institutional committee on ethics and animal welfare (CEUA-UFSM, approval number 6173221215).

Virus neutralizing assays. Virus-neutralizing (VN) assays were performed in duplicates in 96-well plates, incubating two-fold dilutions of the sera against 100-200 TCID50.50µL-1 of the virus for 1h, followed by addition of a suspension of CRIB cells and incubation of the plates at 37oC in a CO2 incubator. Readings were performed at 96h, by visualization of typical BoHV-2 cytopathic effect (cpe) in indicator cells. VN titers were considered as the reciprocal of the highest serum dilution that prevented the production of cpe. Sera positive and negative for BoHV-2 antibodies were used as controls in all assays.

Nucleic acid extraction and PCR. Total DNA was extracted from buffy coats, nasal secretions or cutaneous scabs/exudates by thephenol-chloroform protocol and submitted to final elution in 50µl of Tris-EDTA solution. A semi-nested PCR targeting a sequence within the BoHV-2 glycoprotein B genewas used for viral DNA detection. The first round targeted a 624bp sequence and used the primers F (forward) - CTCCAGCGACGATCCTAATTTC (position 6528) and R (reverse) TATGCGTTGTGCTCTGAGTG (position 7151). The second reaction targeted a 347pb sequence and used the same forward primers and the reverse primer CGGTGGTCTCAAGGTTGTTC (position 6874). PCR reactions were performed in a 25µl volume, using 3µl of DNA template, 0.4µM of each primer, 2mM MgCl2, 8mM of dNTPs, 1 × reaction buffer, 10% of reaction dimetilsulfoxide and 1.5 unit of Taq polymerase (Life Technologies®, Carlsbad, Califórnia, USA). PCR conditions were: initial denaturation (95oC for 5min), followed by 30 cycles of 95oC for 45s; 56oC for 30s for primer annealing and 72oC for 45s for primer extension; and a final extension of 7 min at 72oC. Products were visualized in a 2% agarose gel, stained with Gel Red (Biotium®, Fremont, California, EUA) and

visualized under UV light. Control included total DNA extracted from mock-infected and BoHV-2 infected CRIB cells.

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 $P<0.01$.

## RESULTS

### Acute infection

No overt systemic signs were observed in inoculated calves in the days following virus inoculation. Food intake and alertness remained unaltered upon detailed observation. The body temperature of animals of the IV group presented an increase between days 4 and 8-9pi; groups IM and TD presented a transient increase in body temperature at day 11pi. The temperature of control calves remained within normal limits (Fig.1). Comparing to the basal temperatures, animals from group IV presented the higher variation ($P<0.01$). Temperatures returned to pre-inoculation levels at day 11-12pi and remained at those levels up to the end of the monitoring period.

Virus inoculation after skin scarification (TD) resulted in mild inflammatory lesions within the inoculation sites. The observed changes were hyperemia, small vesicles, mild exudation and scab formation, recorded between days 2 and 8pi (Fig.2). At day 8pi, only residual and slight scabs were still present. Animals inoculated with MEM presented transient hyperemia (1-2 days) and very thin scab formation along the lines of scarification (Fig.2).
Table 1 presents the results of PCR performed in total DNA extracted from swabs/scabs of cutaneous lesions (or sites of virus inoculation) of the TD group. Viral DNA was detected in material collected from lesions of 3 out of 4 animals (75%). Virus shedding/presence was detected from day 2 to 8pi in two animals and from day 2 to 6pi in one calf (Table 1). No viral DNA was detected after day 10pi up to day 14pi, when the collection was discontinued. These results showed a transitory virus replication/detection (lasting 4 to 6 days) in the lesions associated with skin inoculation.

The presence of viral DNA as indicator of viremic spread was investigated in the blood of inoculated calves in the days following inoculation. For this, total DNA extracted from buffy coats was submitted to the same PCR described above. The results are presented in table 2. Viral DNA was detected in the buffy coats of 3/4 animals (75%) of the IM group, from day 4 to 8pi; and in 2/4 animals (50%) of the IV group, at days 6 and 8pi. No viral DNA was detected

in the buffy coats of TD group nor in control calves (Table 2). These results demonstrated a transient viremia in calves inoculated by the IM and IV routes and no detectable viremia in the TD group.

Calves from all inoculated groups seroconverted to BoHV-2 in titers from 4 to 64, as indicated by VN assays performed in serum samples collected at day 15pi. The mean VN titer developed by the IV group (GMT=38.05) was statistically higher than those from the IM (GMT=8) and TD (GMT=4.75) groups ($P<0.01$). Control animals remained seronegative throughout the experimental period (Table 2). The results of VN tests confirmed, indirectly, an efficient virus replication in calves of the three inoculated groups.

**Latent infection**

In attempts to reactivate latent infection, inoculated calves were submitted to a protocol of Dex administration beginning at day 48pi (five daily IM doses, following by daily examination and specimen collection). Again, food intake and alertness remained unchanged. A slight and transient increase in body temperature was observed in groups TD and IM between days 6 and 9 post-Dex (pDex) and at day 7 pDex in group IV (Fig.3). Control calves presented a slight increase in body temperature at day 8 pDex.

No evidence of virus reactivation was observed in the inoculated calves upon Dex administration. PCRs of the buffy coats (IM, IV groups) and from swabs collected from the inoculation sites (ID group) were negative for viral DNA. Likewise, the VN titers pre- (day 48pi) and post-Dex (day 60pi) remained unaltered in most animals, or with a single dilution difference (higher or lower), demonstrating absence of re-stimulation of the immune system upon Dex treatment. Taken together, these results demonstrated lack of detectable virus reactivation.

**DISCUSSION**

*Bovine alphaherpesvirus 2* (BoHV-2) has been a neglected virus, probably due to its limited sanitary, economic importance and rare occurrence. The scarce knowledge about its biology, molecular biology and pathogenesis has somehow hampered its use as a model for human simplex viruses and as a vector for vaccine delivery, among others. Our group has long been interested in BoHV-2 epidemiology and pathogenesis (Almeida et al. 2008, Torres et al. 2010). More recently, we have focused our interest in the use of BoHV-2 for anti-viral drug testing and for development of vaccine platforms for antigen delivery. In this sense, a better

understanding of the biology of BoHV-2 in its natural host would help future developments.

Early studies on BoHV-2 focused on clinic-pathological description of clinical cases/outbreaks (Weaver et al. 1972, Castrucci et al. 1978, Castrucci et al. 1979, Woods et al. 1996, Kemp et al. 2008) and/or experimental reproduction of clinical disease in cattle (Weaver et al. 1972, Turner et al. 1974, Castrucci et al. 1978, Woods et al. 1996, Kemp et al. 2008) and animal models (Westbury 1981, Almeida et al. 2008, Smee & Leonhardt 1994, Torres et al. 2009a, 2010). A few experimental studies focusing on acute disease (Castrucci et al. 1977, Castrucci et al. 1982) or latent infection Probert & Povey (1975), Scott & Martin (1978), Letchworth & Carmichael (1982) introduced probably biases on data interpretation due to IV virus inoculation, an unlikely natural route of exposure. For instance, the virus tropism and systemic dissemination were investigated after IV inoculation (Castrucci et al. 1977, 1978). Against this hypothesis, several lines of evidence point out for virus penetration/transmission by direct/indirect contact (Castrucci et al. 1982). Thus, the results of some early studies should be interpreted with caution since they may not reflect the natural route of inoculation. Likewise, results from studies in animal models might not necessarily reflect the events occurring in the natural hosts.

We performed BoHV-2 inoculation though different routes for the following reasons. The TD route was chosen because it would more closely resemble the natural route of infection. Using this protocol of virus inoculation, we have successfully reproduced udder and teat lesions typical of BHM in ewes (Almeida et al. 2008). The IV route was employed trying to reproduce the virological findings of early studies which used this route (Castrucci et al.1977, Castrucci et al. 1978, Martin & Scott 1979). Finally, the IM route aimed at stimulating the events following IM administration of BoHV-2 as a vaccine vector.

In our study, viremic dissemination was observed after IM inoculation and, to a lesser extent, after IV administration. In both groups, detection of viral DNA in the blood was transient, lasting 1 to 4 days. In contrast, virus inoculation after skin scarification did not result in viremic spread in spite of virus replication and detection in local sites for 4 to 6 days (Table 1). The localized nature of cutaneous BoHV-2 replication and pathology has been previously attributed to the route of virus penetration/exposure, specific virus tropism and, additionally, to the surface temperature which may favor virus replication (Letchworth et al. 1982a). In fact, early studies have demonstrated that BoHV-2 replicates more efficiently at lower temperatures (30-3$^{o}$C), comparing to replication at physiological bovine temperatures (38-39$^{o}$C) (Letchworth et al. 1982a, Letchworth & Carmichael 1984). The preference for replication at low temperatures might also explain the higher incidence and distribution of BoHV-2 in regions of temperate

climate (Letchworth & Carmichael 1984). On the other hand, systemic viral spread has been demonstrated only after IV inoculation (Castrucci et al. 1977, 1978). In this sense, our results demonstrated that virus replication after IM inoculation results in virus replication and viremic spread. These findings might be a safety concern towards the potential use of BoHV-2 as a vaccine vector for IM use. Thus, the possible consequences of BoHV-2 viremic spread upon IM administration should be properly addressed.

A major route of BoHV-2 penetration in natural infections seems to be throughout micro-lesions/abrasions in the epithelium of the udder and teats. As such, our protocol of TD inoculation aimed at mimetizing the natural events leading to virus penetration and replication. Virus inoculation after skin scarification resulted in mild inflammatory lesions characterized by hyperemia, small vesicles, mild exudation and scab formation between days 2 and 8pi (Fig.2). Contrasting with some previous studies (Gibbs et al. 1973, Letchworth et al. 1982b), no classical BHM lesions were reproduced upon skin inoculation. In a previous study, we reproduced similar BHM lesions in the udder and teats of ewes experimentally inoculated (Almeida et al 2008). The differences in the clinical outcome may attributed to the nature of the skin epithelium of the hind limbs versus udder/teats and/or to the procedure of virus inoculation. In any case, virus replication and mild, transient cutaneous lesions were produced upon TD virus inoculation. Virus replication seemed to be restricted to the cutaneous surface of the inoculation sites since viremic spread was not detected.

Although BoHV-2 is known to establish latent infections, recovery of infectious virus and/or demonstration of virus reactivation after corticosteroid treatment has not been consistently achieved either in natural or experimental infections (Probert & Povey 1975, Castrucci et al 1980, Letchworth & Carmichael 1982). A few attempts were successful in recovering virus and/or demonstrating reactivation upon corticosteroid treatment, yet mainly in animals inoculated by the IV route (Scott & Martin 1978, Martin & Scott 1979, Castrucci et al.1982), which is unlikely to be the natural route of infection. Otherwise, latency, reactivation and colonizing of sensory nerve ganglia with latent DNA have been demonstrated in experimental models (Almeida et al. 2008, Torres et al. 2009a). In our study, no virus shedding or seroconversion were observed upon Dex administration, in spite of virus replication in the sites of virus inoculation and presence of the virus in blood during acute infection. These results corroborate previous studies which failed in demonstrating virus reactivation in BoHV-2-infected cattle (Probert & Povey 1975, Castrucci et al 1980, Letchworth & Carmichael 1982). From the vaccine perspective, the failure/lack of reactivation would be a highly desirable property of a candidate vaccine viral vector. Unfortunately, we did not keep the calves for

investigating the sites of latent infection upon the different routes of exposure.

Nearly 50 years after the initial identification of BoHV-2 as the agent of BHM (Gibbs et al. 1973) and, subsequently, the demonstration of its relationship with human simplex viruses (Snowden et al 1985, Castrucci et al. 1990, Ehlers et al. 1999), the pathogenesis of BoHV-2 in cattle is still poorly understood. Thus, our data contributes for the overall knowledge on the biology and pathogenesis of this simplex virus. Regardless its restricted sanitary/economic relevance, this bovine virus should be looked at from different perspectives. First, BoHV-2 infection offers a unique opportunity to study selected aspects the molecular biology, pathogenesis and immunology of a simplex virus in their natural host. HHV-1 and HHV-2 are among the most studied human viruses, yet the studies *in vivo* are largely restricted to animal models (Kollias et al. 2015). Thus, studying the biology of a simplex virus in its natural host may particularly attractive/fascinating since it may provide new and more realistic insights in the virus-host interactions. Second, the biological and genetic similarity of BoHV-2 with human simplex viruses may be exploited for the research and development of pharmaceuticals and vaccines as well. In this sense, BoHV-2 has been proposed as a potential target/model for the development of anti-herpetic drugs (Field et al. 2006, Dezengrini et al. 2010, Torres et al. 2010). Third, animal herpesviruses have been long proposed as potential platforms for vaccine delivery and a number of current animal vaccines are based on herpesvirus vectors (Verma & Weitzman 2005, Kanekiyo et al. 2019). Thus, more than a cattle pathogen of questionable clinical relevance, BoHV-2 may represent an attractive alternative to study the interactions of simplex viruses with their hosts, for research/development of anti-herpetic drugs and veterinary vaccines.

## CONCLUSION

Inoculation of BoHV-2 in calves by different routes results in virus replication, yet viremic spread occurs after intramuscular or intravenous inoculation but not after transdermal inoculation.

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**Table 1.** Results of PCR performed on DNA extracted from material obtained from swabs and lesions of calves inoculated transdermally with *Bovine alphaherpesvirus* 2 (BoHV-2)

| Animal ID | Days post-inoculation | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 2 | 4 | 6 | 8 | 10 | 12 | 14 |
| 276 | - | + | + | + | + | - | - | - |
| 280 | - | + | + | + | + | - | - | - |
| 284 | - | - | - | - | - | - | - | - |
| 294 | - | + | + | + | - | - | - | - |

**Table 2.** Virological and serological findings in calves inoculated with *Bovine alphaherpesvirus 2* (BoHV-2) by different routes

| Group | Animal ID | PCR days post inoculation | | | | | | | | VN assays - days post inoculation | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 2 | 4 | 6 | 8 | 10 | 12 | 14 | 0 | 15 | 48 | 60 |
| Intramuscular | 274 | - | - | + | + | - | - | - | - | < 2 | < 2 | < 2 | < 2 |
| | 277 | - | - | - | - | - | - | - | - | < 2 | 4 | < 2 | 2 |
| | 281 | - | - | + | + | + | - | - | - | < 2 | 16 | 16 | 8 |
| | 282 | - | - | - | - | + | - | - | - | < 2 | 8 | 8 | 16 |
| Intravenous | 283 | - | - | - | + | + | - | - | - | < 2 | 32 | 64 | 32 |
| | 287 | - | - | - | + | + | - | - | - | < 2 | 32 | 64 | 32 |
| | 295 | - | - | - | - | - | - | - | - | < 2 | 64 | 64 | 32 |
| | 299 | - | - | - | - | - | - | - | - | < 2 | 16 | 32 | 32 |
| Transdermal | 276 | - | - | - | - | - | - | - | - | < 2 | 8 | 4 | 8 |
| | 280 | - | - | - | - | - | - | - | - | < 2 | 4 | 4 | 2 |
| | 284 | - | - | - | - | - | - | - | - | < 2 | 4 | 2 | <2 |
| | 294 | - | - | - | - | - | - | - | - | < 2 | 4 | 4 | 4 |
| Control | 286 | - | - | - | - | - | - | - | - | < 2 | < 2 | < 2 | < 2 |
| | 289 | - | - | - | - | - | - | - | - | < 2 | < 2 | < 2 | < 2 |
| | 292 | - | - | - | - | - | - | - | - | < 2 | < 2 | < 2 | < 2 |
| | 293 | - | - | - | - | - | - | - | - | <2 | < 2 | < 2 | < 2 |

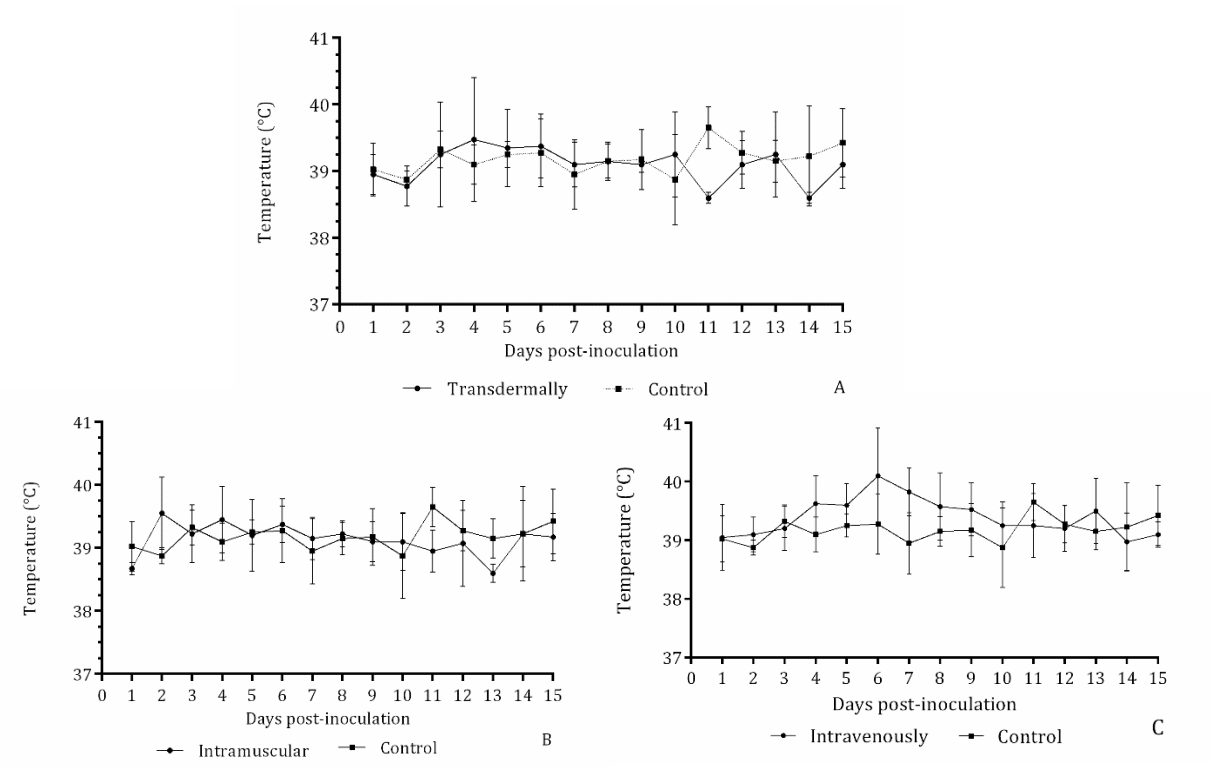


Fig.1. Mean body temperature (T°C) of calves inoculated with *Bovine alphaherpesvirus 2* (BoHV-2) by different routes: (**A**) transdermal, (**B**) intramuscular or (**C**) intravenous.

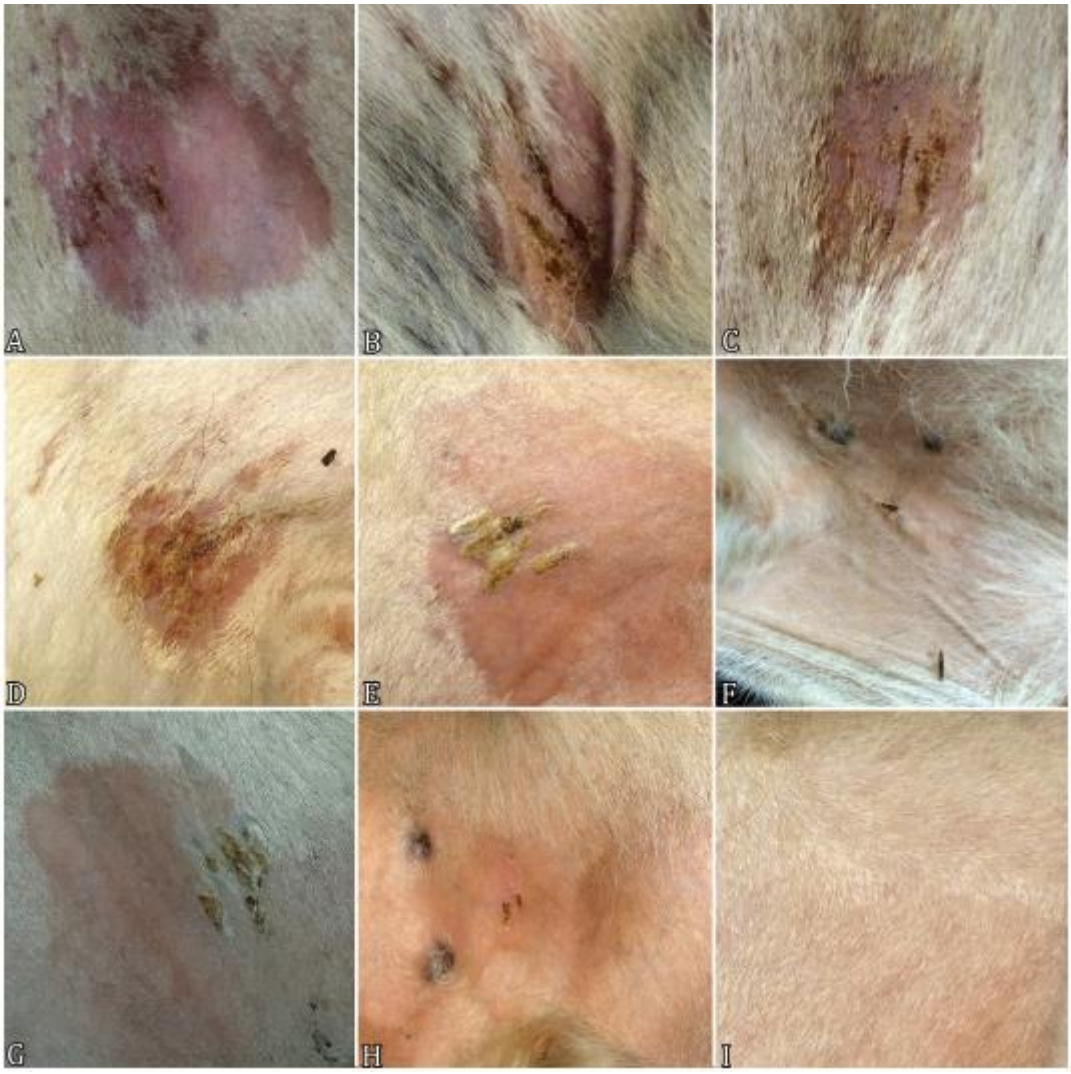


Fig 2. Skin lesions developed by calf 276 inoculated with (**A**, **B**, **D**, **E**, **G**, **H**) *Bovine alphaherpesvirus 2* (BoHV-2) transdermally and (**C**, **F**, **I**) by a control calf inoculated with culture medium, at different days after virus inoculation. (**A**-**B**) 276 and (**C**) control. Days 2 and 4pi, respectively, thin scabs and skin retraction derived from scarification. (**D**-**E**) Days 6 and 8pi. Thick, yellowish scabs and small disrupted vesicles. (**F**) Control. Day 8pi. A thin scab, advanced stage of healing. (**G**-**H**) 276. Days 10 and 12pi. Crust remnants, advanced stage of healing. (**I**) Skin completely healed (control).

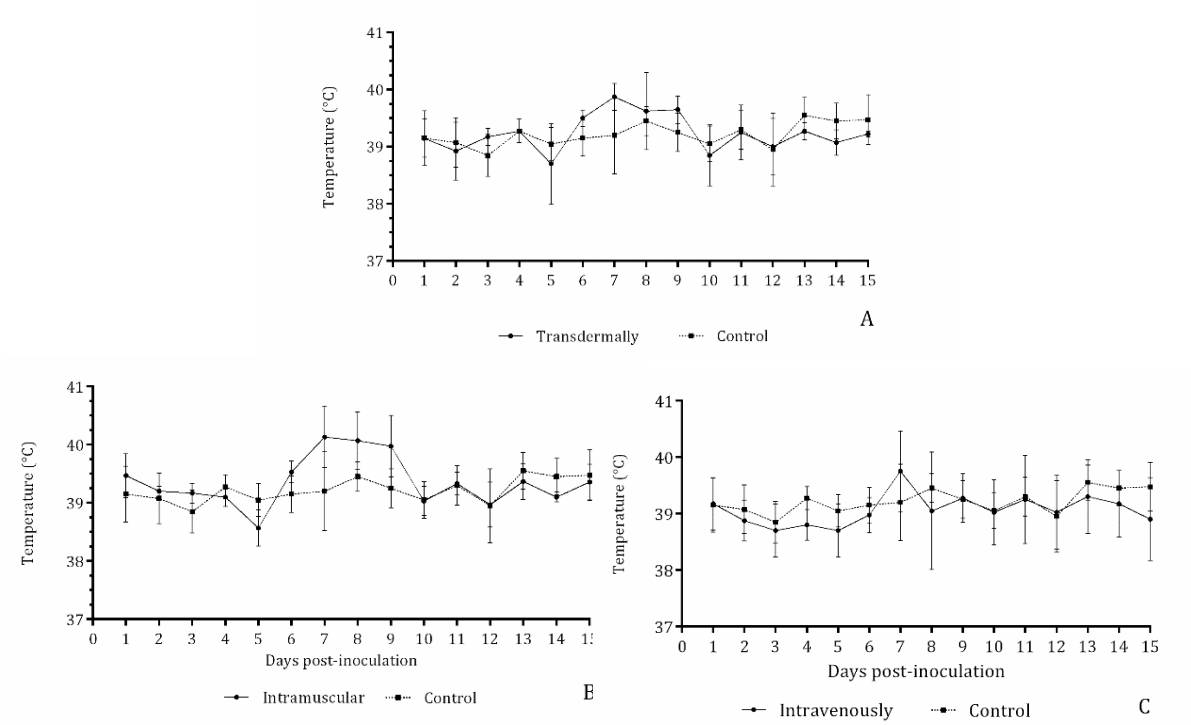


Fig.3. Mean body temperature (TºC) of calves inoculated with *Bovine alphaherpesvirus 2* (BoHV-2) by different routes: (**A**) transdermal, (**B**) intramuscular and (**C**) intravenous and submitted to dexamethasone administration at day 40pi.

## 3. ARTIGO 2

# Diphenyl diselenide and cidofovir present anti-viral activity against bovine alphaherpesvirus 2 in vitro and in a sheep model[1]

*Running title: Activity of disseleneto de difenila and cidofovir against bovine alphaherpevirus 2.*

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**Abstract**

*Bovine alphaherpesvirus* 2 (BoHV-2) - the agent of bovine herpetic mamillitis (BHM) – is related to *Human alphaherpesviruses* 1 and 2 (HHV-1, HHV-2) and, as such, has been proposed as a model for vaccine and drug testing. We herein investigated the anti-viral activity *in vitro* against BoHV-2 of three anti-herpetic drugs: Cidofovir (CDV), Famciclovir (FAM), Foscarnet (PFA), and diphenyl diselenide $(PhSe)_2$, a compound that showed activity against HHV-2. Plaque reduction assays (PRA) revealed a significant reduction in viral plaques ($p<0.05$) in cells treated with $(PhSe)_2$ (79.7% reduction) or CDV (62.8%). FAM treatment resulted in a slight decrease in plaque number (22.9%, $p<0.05$); PFA showed no activity. The effects of $(PhSe)_2$ and CDV, alone or in combination, were investigated in ewes inoculated with BoHV-2 transdermally and submitted to daily topic treatment. Virus inoculated ewes developed lesions progressing through the stages of hyperemia, large papules or depressed dark areas, followed by scab formation. Treatment with $(PhSe)_2$ resulted in reduction in clinical score from day 10 pi onwards ($p<0.05$), shortening of clinical course and reduction in duration of virus shedding ($p<0.05$) compared to untreated controls. Combined treatment ($(PhSe)_2$+CDV) and CDV alone, also led to clinical improvement ($p<0.05$), yet less pronounced and delayed. These results are promising towards the use of $(PhSe)_2$, alone or in combination with anti-herpetic drugs, in the treatment of udder and teat lesions produced by BoHV-2 in dairy cows.

**Keywords:** bovine herpesvirus 2, herpetic mammilitis, anti-virals, therapy.

## 1. Introduction

*Bovine alphaherpesvirus* 2(BoHV-2) is an enveloped, double-stranded DNA virus belonging to the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Simplexvirus (*ICTV, 2019). BoHV-2 is one of the few non-primate simplexviruses, genetically and antigenically related to *Human alphaherpesviruses* 1 and 2 (HHV-1, HHV-2) (Sterz et al., 1974; Castrucci et al., 1990; Ehlers et al., 1999). BoHV-2 infection has been associated with a vesicular, erosive and necrotic cutaneous disease of the udder and teats of cows known as bovine herpetic mammillitis [BHM] (Martin et al. 1966; Johnston et al. 1971; Gibbs and Rweyemamu, 1977). The virus has also been sporadically associated with a cutaneous nodular disease called pseudo-lumpy skin disease (Gibbs and Rweyemamu, 1977; Woods et al., 1996). Epidemiological data indicates the circulation of BoHV-2 in commercial cattle herds in all

continents (Turner et al., 1974; Alice, 1977; Scott and Martin, 1978; Imai et al., 2005; Kemp et al., 2008; Watanabe et al., 2017).

In the last decades, BoHV-2 has received limited attention as a cattle pathogen, mainly due to its rare occurrence and the mild nature of most infections. Rather, the main interest in BoHV-2 relies upon its close genetic and antigenic similarity with human simplexviruses. Early studies have shown that the structure of the BoHV-2 genome mimics the four isomers of HHV rather than the two isomers of other animal alphaherpesviruses, as *Bovine alphaherpesvirus 1* (BoHV-1) and *Porcine alphaherpesvirus 1* (pseudorabies virus, PRV) (Buchman and Roizman1978). Likewise, the sequences of BoHV-2 thymidine kinase (UL23), glycoprotein B (UL27), ICP 18.5 (UL28) and ICP 8 (UL29) genes are more closely related to HHV-1 and HHV-2 than to other bovine alphaherpesviruses (Hammerschmidt *et al*. 1988; Sheppard and May 1989; McGeoch and Cook 1994). In addition, the similarity between BoHV-2 and HHV envelope glycoproteins is such that results in neutralization of BoHV-2 by HHV-1 antisera (Sterz et al., 1974) and has led to the speculation that BoHV-2 may have diverged from primate alphaherpesviruses (McGeogh and Cook, 1994). Regardless, there is no evidence of human infection by BoHV-2, in spite of attempts to reproduce the infection upon inoculation of volunteers (Pepper et al., 1966).

The close relationship between BoHV-2 and human alpha herpesviruses has offered interesting opportunities for comparative genetic/molecular and immunologic studies (Buchman and Roizman, 1978; Hammerschmidt et al., 1988; Sheppard and May, 1989; Castrucci et al., 1990; McGeoch and Cook, 1994), experimental vaccine testing (Skinner et al., 1987, 1991; Maragos et al., 1988; Castrucci et al., 1990) and anti-viral therapy as well (Field et al. 2006). Human simplex viruses, e.g. HHV-1 and HHV-2, have long represented candidate targets for antiviral therapy, and animal models play an important role towards the development and refinement of therapeutic drugs and protocols (Field et al., 2006).

We herein report an investigation on the activity in vitro and in vivo of selected drugs against BoHV-2. Our main interest was to study the anti-viral activity of diphenyl diselenide $(PhSe)_2$, a promising compound that showed anti-viral activity against HHV-2 in vitro and in female Balb/c mice (Sartori et al., 2016). In addition, we investigated the anti-viral activity of three commercial anti-herpetic drugs (Cidofovir [CDV], Famciclovir [FAM], Foscarnet [PFA]), intended to be used alone or in combination with $(PhSe)_2$ in an eventual anti-herpetic protocol. The drugs that presented anti-viral activity in vitro (CDV and $(PhSe)_2$) were tested in vivo, in a sheep model for BoHV-2 disease, previously described by our group (Almeida et al., 2008; Torres et al., 2009).

## 2. Materials and methods

### 2.1 Study design

The sensitivity of BoHV-2 to four drugs was investigated *in vitro*, by plaque reduction assay (PRA). The drugs displaying anti-viral activity *in vitro* were then tested *in vivo*, in a sheep model. For this, ewes were inoculated with BoHV-2 transdermally on the side of the udder and, upon the onset of local signs/lesions, were treated topically on a daily basis with each drug individually or in combination. The severity, duration of lesions and virus shedding were compared to those of untreated ewes inoculated with the virus. The experiments were approved by an Institutional Committee on Ethics and Animal Welfare and Experimentation (UFSM, Comitê de Ética e Experimentação Animal: protocol number 3603180520).

### 2.2 Virus and cells

*Bovine alphaherpesvirus 2* (BoHV-2) strain New York (NY01) was kindly provided by Dr. Fernando A. Osorio (Department of Veterinary and Biomedical Sciences, University of Nebraska, Lincoln, USA). All procedures of virus amplification, quantitation, anti-viral assays and virus neutralizing tests (VN) were performed in CRIB cells (a MDBK-derived cell line resistant to BVDV). Cells were maintained in Eagle's minimum essential medium (MEM) (Vitrocell®, Nova Campinas, São Paulo – Brazil) supplemented with 10% fetal calf serum (Vitrocell®), penicillin (10,000 IU.mL-1), streptomycin (10mg/mL-1) and amphotericin B (250μg.mL-1) (Sigma – Aldrich®, Darmstadt, Hessen, Germany).

### 2.3 Antiviral drugs

Three commercial anti-herpetic drugs of human use were used: Cidofovir (CDV, MW 279.1; Sigma-Aldrich, St Louis, MO, USA), Famciclovir (FAM, MW 321.3; Sigma-Aldrich, St Louis, MO, USA), and Foscarnet (PFA, MW 300.04; Sigma-Aldrich, St Louis, MO, USA). A fourth compound, diphenyl diselenide $(PhSe)_2$ is an organoselenium compound that has *in vitro* and *in vivo* activity against HHV-2 (Sartori *et al*.,2016). $(PhSe)_2$ was synthesized and characterized based on the method previously described by Paulmier (1986). All commercial drugs were diluted to 1 mg/mL in ultrapure water (PFA and CDV) or dimethyl sulfoxide (DMSO). The $(PhSe)_2$ was initially diluted to 31.4 mg/mL in DMSO and subsequently diluted to 12 μM (or 3.76 μg /ml) in culture medium. Next, different drug concentrations were submitted to cellular toxicity assays (MTT) in CRIB cells, adapted by Oliveira *et al*. (2018). Then, the highest non-toxic concentration for CRIB cells was used in antiviral assays by plaque

reduction assays (PRA). The *in vivo* assays were performed using 3.76 μg/ml of $(PhSe)_2$ and 100 μg/ml of CDV in a dermatological cream formulation (auto-emulsifying wax, disodium acid, glycerol, methylparaben, propylene glycol, liquid vaseline, sodium hydroxide, hydrochloric acid and purified water).

**2.4 Plaque reduction assays (PRA)**

The activity of the drugs against BoHV-2 was determined by PRA, using CRIB cells seeded in six-well polystyrene plates ($0.3x10^6$ cells/well). Two conditions/treatments were performed: pre- and post- virus inoculation (pre and post-treatment). To perform the "pre-treatment assay", three wells of CRIB cells monolayers received 1mL of MEM containing different doses of each drug (100 μg/mL to CDV and PFA, 10 μg/mL to FAM and 3.76 μg/mL to $(PhSe)_2$) and incubated for 1h at 37ºC in a $CO_2$ incubator at 5%. Two other wells were maintained as viral and cellular controls, receiving 1 mL of MEM. Next, the supernatant was removed, and 1mL of virus suspension containing, approximately, 100 to 200 plaque forming units (PFU) of BoHV-2 was added. After 2h of adsorption, the inoculum was removed and cell monolayers were covered with a semi-solid gel overlay (MEM, 10% fetal bovine serum and 1% low melting point agar). To perform the "post-treatment assay", three wells of CRIB cells monolayers were inoculated with 1 mL of virus suspension containing approximately 100 to 200 PFU of BoHV-2, and after 2h of adsorption, the inoculum was removed and cell monolayers were covered with 3mL of solid medium culture, containing MEM, 10% fetal bovine serum and 1% low melting point agar besides the respective concentrations (μg/mL) of each drug. The plates were incubated at 37ºC in a $CO_2$ incubator at 5%. After 72h, the gel overlay was removed, the monolayers were fixed and stained with a crystal violet solution (0.65 g violet crystal; 62.5 mL formalin and 500 mL water q.s.p.). Finally, viral plaques were counted in both treated and control cells. Each experiment was performed in triplicate. The tests were conducted in triplicate for each experiment. The data obtained was statistically analyzed by the analysis of variance (ANOVA) and Tukey's multiple comparison test through the program GraphPad Prism 6. Statistical significance was considered when $p<0.05$.

**2.5 Animal experiment**

The animal experiment is illustrated in Figure 1. Sixteen 3 to 4-years-old Hampshire Down ewes were used in the experiment. The animals were originated from a breeding farm and were maintained in the farm during the experiment, receiving water and pasture ad libitum,

and commercial feed once a day. Prior to the experiment, the animals were in a good condition, healthy and treated for parasitic infection.

Fourteen ewes were inoculated with BoHV-2 and two with culture medium (MEM) and served as mock-infected controls. Ewes from control and virus inoculated group were housed in separate paddocks. The animals were inoculated transdermally (TD) in the skin on the left side of the udder, after mild scarification with a hard sponge. After scarification, the virus inoculum was distributed over the scarified area (approximately 1.5 $cm^2$) with the help of a cotton swab. The virus inoculum consisted of the supernatant of CRIB cells infected with BoHV-2 at passage # 12, containing $10^7$ $TCID_{50}.mL^{-1}$ (median tissue culture infectious doses).

At the time of inoculation, the inoculated animals were randomly allocated into four groups, designed to receive the respective treatments: control (CV, no treatment – received only the raw dermatological cream), CDV (treated with CDV), $(PhSe)_2$ (treated with $(PhSe)_2$), CDV + $(PhSe)_2$ (treated with CDV +$(PhSe)_2$). The respective treatments were initiated upon the onset of lesions (hyperemia/papules) observed during the daily examination – typically at day 5 pi. The treatment consisted of daily topic application of the dermatologic cream containing the respective drug over the lesion. A thin layer of the cream was applied over the lesions once a day, extending up to day 15 pi. Virus control (CV) ewes received treatment with the raw cream. From day 0 (day of inoculation) onwards, up to day 15 post-inoculation (pi), the animals were monitored clinically concerning systemic signs (body temperature, alertness, appetite) and local signs/lesions.

A clinical score adapted from Almeida *et al*. (2008) was used to quantify the severity of local lesions (Table 1). Briefly, the individual clinical score consisted of a sum of the values attributed to each lesion: hyperemia – light (1), mild (2), severe (3); papule – light (2), mild (3), severe (4); scab – few (2), mild (4) and severe (6); no lesion (0). Light or few corresponded to a small area of lesion (around 1 $cm^2$) or presenting just one small papule; mild corresponded to lesions with 2 to 3 $cm^2$; and severe corresponded to lesions presenting above 3 $cm^2$. Daily individual scores were converted into a mean group score; daily mean scores of treated groups were compared with CV group. For careful examination and clinical scoring, several digital photographs were taken daily from each lesion with a proper light incidence and distance. The photographs were subsequently examined by a veterinarian who was unaware of the respective treatment group (blind examination).

Swabs for virus isolation were collected daily from lesions/sites of inoculation, blood for serology was collected at days 0 and 20 pi. The material/medium from swabs was submitted to three passages of 72h each in CRIB cells for virus isolation (VI); sera was submitted to a

standard VN assay, testing two-fold dilutions of sera against a fixed dose of virus (100-200$TCID_{50}$). VI and VN protocols have been described in details by Almeida *et al*. (2008). Data of clinical score and duration of virus shedding were statistically analyzed by the analysis of variance (ANOVA) and Tukey's multiple comparison test through the program GraphPad Prism 6. Statistical significance was considered when $p<0.05$.

## 3. Results

### 3.1 Plaque reduction assays

Plaque reduction assays (PRA) were performed to investigate the ability of the respective drugs to inhibit/reduce plaque formation by BoHV-2, as indicators of virus replication. PRA were performed in two conditions: i. Upon the drug addition during 1h, before virus adsorption to cell cultures (*pre-treatment*); ii. Upon removing the inoculum, adding the drug to the gel covering the monolayers (*post-treatment*). In both cases, the drug concentrations were determined by MTT. Among the drugs tested, $(PhSe)_2$ displayed the highest effect on reducing viral replication, reducing plaque formation by 63.5% ($p<0.05$) when added prior to virus adsorption and by 79.7% ($p<0.05$) when added to the gel overlay (Figure 2). Among the anti-herpetic drugs, CDV showed the strongest effect, reducing by 29.4% ($p<0.05$) and 62.8% ($p<0.05$) plaque formation, in each condition, respectively. PFA addition to cell cultures either pre- or post-adsorption did not result in plaque reduction and FAM showed a low decrease (22.9%) in plaque formation when added to the gel overlay (post-treatment). These results showed that $(PhSe)_2$ and, secondly CDV, were able to reduce plaque formation by BoHV-2 when added prior to and after virus adsorption. Hence, $(PhSe)_2$ and CDV were used in anti-viral assays *in vivo*, alone or in combination.

### 3.2 Animal experiment

To investigate the effectiveness of $(PhSe)_2$ and CDV in the course of viral-induced lesions/disease *in vivo*, ewes were inoculated transdermally with BoHV-2 and, upon the onset of virus-induced lesions (day 5pi), were submitted to daily local treatment with a cream containing the above drugs alone ($(PhSe)_2$ or CDV) or in combination ($(PhSe)_2$+ CDV). Clinical and virological parameters observed in treated groups were compared with ewes inoculated and treated with the raw cream/lotion (CV).

Virus inoculation was not followed by systemic signs as fever, apathy, anorexia, etc., regardless the treatment group. Thirteen out of 14 (92.8%) inoculated ewes developed lesions

in and around the site of virus inoculation (Table 1). Animal #101 allocated to the $(PhSe)_2$ + CDV group did not present local lesions. In this animal, the scarified area regressed to the normal appearance, similar to the mock-infected control (MEM) group by day 2 pi.

The lesions developed by inoculated animals progressed typically through the stages of hyperemia (days 2 – 6pi), development of a large papule or depressed/flat dark area (day 4 to 12 pi), accompanied/followed by scab/crust formation. Scab formation was observed as early as day 4 pi and flat scabs/crusts were a prominent finding in all stages of the clinical course, lasting at least up to day 15 pi in many animals (Table 1). The respective treatment was established as soon as the virus-induced lesions became evident (and distinct from the lesions due to inoculation/scarification), typically by day 5 pi ($(PhSe)_2$ +CDV, and two animals of CDV group) or 6 pi (two animals of CDV). In MEM-inoculated animals (C), scarification lesions regressed promptly and disappeared after day 3 pi (Table 1).

In general, the nature and progression of lesions were similar during early disease (up to day 6pi) in treated and untreated ewes (CV). Nonetheless, from day 7 onwards, the severity of lesions began to differ among the groups, most notably between the CV and $(PhSe)_2$ groups. Whereas the clinical score remained high and even increased in the CV group after day 7 pi, a significant and progressive decline in disease severity was observed in the $(PhSe)_2$ group (Figure 3). Animals of this group presented a progressive decline in clinical scoring from day 8 onwards, reaching significant lower levels at day 10 pi ($<0.05$) and maintaining at minimal levels between days 12-15 pi (Figure 3). From day 11 pi onwards, only reminiscent flat scabs were still present in the inoculation sites of some animals, with most resolving it by day 15 pi. The clinical scores in $(PhSe)_2$+ CDV and CDV groups started to decline approximately three to five days later (days 11pi and 13 pi, respectively), and stabilized at low scores by day 13 pi, when reminiscent scabs were present in some animals (Figure 3). In contrast, the CV group still presented pronounced lesions (typically large flat scabs) by day 15 pi, when the clinical monitoring was discontinued. In summary, these results demonstrated that $(PhSe)_2$ treatment, secondly $(PhSe)_2$ + CDV and, to a lesser extent CDV treatment, showed a beneficial role in disease progression, resulting in a reduction in clinical scores, faster improvement of lesions and shortening of lesions produced by BoHV-2.

Table 2 summarizes the clinical observations, virus shedding, and serological findings of the animal experiment. Duration of virus shedding was significantly reduced in the $(PhSe)_2$ group (5.25 days, $p<0.05$) and showed a slight reduction in $(PhSe)_2$ + CDV (6.0) and CDV (8.25) groups comparing to CV (9.5 days). $(PhSe)_2$ treated ewes presented a mean clinical course of 10.25 days; $(PhSe)_2$+ CDV group (11.7 days) and CDV group (13 days). The mean

duration of local signs was apparently reduced in $(PhSe)_2$ group (10.25 days) comparing to CV group (13 days), with two animals presenting a marked reduction in the clinical course (#17 and 23). However, as the ewes of the CV group still presented firmly attached scabs/crusts by day 15 pi, when the clinical monitoring was discontinued, no statistical analysis of the duration of clinical disease has been performed.

Ewes of all groups seroconverted to BoHV-2 at day 20 pi, presenting VN titers from 2 to 16.

## 4. Discussion

Our results reveal that diphenyl diselenide $(PhSe)_2$ and Cidofovir (CDV) have anti-viral activity against BoHV-2 *in vitro*, as demonstrated by a significant reduction in viral plaques in cell monolayers treated with the respective drugs. In addition, the animal experiment demonstrated that topic $(PhSe)_2$ treatment and, secondly, combined $(PhSe)_2$ + CDV treatment of cutaneous lesions induced by BoHV-2 in ewes had a beneficial role in disease progression. $(PhSe)_2$ treatment resulted in significant reduction in virus replication and, more importantly, in a faster clinical improvement and resolution of lesions in most animals. Combined treatment also resulted in clinical improvement, yet less pronounced. These findings are promising towards the development of therapeutic options for BoHV-2 disease, mainly in dairy cows, and for the use of BoHV-2 as a model for the development and refining of human anti-herpetic therapies/protocols.

We have long been studying the effects of commercial and alternative drugs against herpetic infections of domestic animals (Dezengrini et al., 2010a, 2010b; Oliveira et al., 2018; Basso et al., 2019; Mortari et al., 2020). In this scenario, the BoHV-2 system offers a unique opportunity for testing drugs of potential veterinary interest and for human simplexvirus therapy as well. In particular, the antiviral activity of $(PhSe)_2$ against HHV-2 *in vitro* and *in vivo* (Sartori et al., 2016) prompted us to investigate its activity against BoHV-2, a potential target for antiviral therapy (Field et al., 2006). In addition, we searched for another anti-herpetic drug to complement potential therapy, investigating two drugs with yet unknown activity against animal herpesviruses (e.g. FAM and CDV) and PFA, a drug that displayed anti-BoHV-2 activity *in vitro* (Dezengrini et al., 2010a).

The organoselenium compound $(PhSe)_2$ has pharmacological actions mostly related to antioxidant (Rossato et al. 2002) and anti-inflammatory properties (Nogueira et al., 2003). Moreover, an in vitro antifungal activity of $(PhSe)_2$ has been also reported either alone or in combination with commercial drugs (Poester et al., 2018; Rossato et al., 2019, Kubica et al.,

2019). Besides antifungal activity (Denardi et al., 2013), $(PhSe)_2$ has previously shown to present virucidal and antiviral action against HHV-2 (Sartori et al., 2016). Although the mechanisms behind these effects remain unclear, we can infer that its molecular pharmacology is linked to an antioxidant mechanism, where the transient formation of the benzeneselenol plays a central role (Nogueira and Rocha, 2010).

The virucidal activity could be due to binding of the drug to viral particles leading to changes in viral envelope structures necessary for virus binding and penetration. The antiviral activity, observed upon addition of the drug after virus penetration into cultured cells, could be due to interference with some intracellular steps of virus replication. In addition, $(PhSe)_2$ administered orally to female Balb/c mice infected intravaginally with HHV-2 resulted in significant reduction in viral load, clinical score and tissue damage. The antiviral action of $(PhSe)_2$ against HSV-2 infection *in vivo* was likely related to its immunomodulatory, antioxidant, and anti-inflammatory properties. In any case, $(PhSe)_2$ showed antiviral activity against HHV-2 both *in vitro* and *in vivo* (Sartori et al., 2016).

In the present study, the antiviral effect of $(PhSe)_2$ *in vitro* should not be attributed to virucidal activity since the drug was added prior to (*pre-treatment*) or after virus adsorption (*post-treatment*) (Fig. 1). Hence, there was no direct exposure of the virus inoculum to the drug as to attribute direct effects of $(PhSe)_2$ on the virus particles as the cause of plaque reduction. Thus, the antiviral effect was probably derived from drug interference with intracellular steps of virus replication. As the molecular mechanisms of $(PhSe)_2$ action intracellularly are largely unknown, its effects on virus replication are difficult to ascertain.

The beneficial effects of $(PhSe)_2$ on the improvement of clinical signs/lesions may be due, in part, to the antiviral activity, since the treatment resulted in reduction in virus replication and shedding. In addition, and perhaps more importantly, the effects of $(PhSe)_2$ on pronounced lesion regression may be related to its immunomodulatory, antioxidant and anti-inflammatory properties (Sartori et al., 2016; Doleski et al., 2017a, 2017b). In fact, it is conceivable that the beneficial role of $(PhSe)_2$ on the prompt regression of cutaneous lesions induced by BoHV-2 in sheep would reflect a combination of both effects (antiviral plus immunomodulatory /antioxidant /anti-inflammatory).

Cidofovir (CDV) is a cytosine analog that display anti-viral activity through an active metabolite (cidofovir diphosphate) produced intracellularly. Cidofovir diphosphate acts as a competitor-inhibitor substrate for viral DNA polymerases, blocking further viral DNA synthesis and, thus, inhibiting virus replication (Xiong et al., 1997). The antiviral activity of CDV has been exploited to treat infections by human alphaherpesviruses (HHV-1, HHV-2),

adenovirus, cytomegalovirus and human papillomavirus (HPV). Due to hepatotoxicity and nephrotoxicity upon intravenous administration, CDV has been mainly recommended fortopic treatment of muco-cutaneous lesions produced by HHVs and HPVs (Toro et al., 2003). In our study, pre-treatment of cultured cells with CDV resulted in a significant reduction in viral plaques (62.8% reduction), noticeably upon addition of the drug on the agar overlay, after virus adsorption. As cidofovir diphosphate (a CDV metabolite) acts as a nucleoside competitor and inhibitor of herpesvirus polymerases (Xiong et al., 1997), this mechanism was likely responsible for the partial inhibition of BoHV-2 replication/plaque formation. Despite of this anti-viral activity *in vitro*, CDV treatment *in vivo* resulted in a slight reduction in the duration of virus shedding and a moderate and delayed effect in disease regression after day 12pi. In other systems, CDV has been to treat muco-cutaneous lesions caused by human DNA viruses, including HPV and HHV, and has shown a special use in infections caused by drug-resistant strains of HHV, mainly HSV strains that are resistant to acyclovir, ganciclovir, or foscarnet (Mendell et al., 1995).

Surprisingly, the combined treatment ($(PhSe)_2$ + CDV) resulted in a weaker effect than that of $(PhSe)_2$ alone, both in virological and clinical parameters. A methodological pitfall may have caused the inability to compare its effects. Unfortunately, data analysis from the $(PhSe)_2$ + CDV group was based on three animals since the fourth one (#101) did not develop lesions. In any case, the clinical course of this group differed from the CV group. An important improvement in lesions was observed in one animal at day 9 pi and in another at day 11 pi. Further studies are worth to investigate the effect of $(PhSe)_2$ combined with CDV or another antiviral drug against BoHV-2.

**5. Conclusion**

The results are promising towards the topic use of $(PhSe)_2$, alone or in combination with anti-herpetic drugs, in the treatment of lesions induced by BoHV-2 in dairy cows. On the other hand, the BoHV-2 system – e.g. infection and disease in animal models - offers an attractive opportunity for the development and refinement of therapeutic protocols for simplexviruses.

**Highlights**

- The topic use of (PhSe)2, alone or in combination with anti-herpetic drugs, in the treatment of lesions induced by BoHV-2 in dairy cows are promising towards.

- The BoHV-2 system – e.g. infection and disease in animal models - offers an attractive

opportunity for the development and refinement of therapeutic protocols for simplexviruses.

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**Conflict of Interest**

The authors declare no conflict of interests.

**Author Contribution**

B. P. Amaral: Study conception and design, acquisition of data, analysis and interpretation of data, drafting of manuscript;
A.P.G. Mortari, L.M. Feio and I. Merchioratto: help in the in vitro and in vivo experiments/acquisition of data;
J. F. Cargnelutti: analysis and interpretation of data, help in drafting of manuscript;
R. Weiblen: Study conception and design, analysis and interpretation of data and critical revision;
E. F. Flores: supervised the project, study conception and design,acquisition of data, analysis and interpretation of data, drafting of manuscript and critical/final revision.

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Table 1 – Duration of lesions, virus shedding, and virus-neutralizing antibodies in ewes inoculated transdermally with *Bovine alphaherpesvirus 2* (BoHV-2) and submitted to different treatments.

| Group | Lesions (duration -dpi) | Virus shedding | Virus-neutralizing antibodies | |
|---|---|---|---|---|
| | | | Day 0 | Day 20 |
| Control(MEM) | | | | |
| # 25 | - | - | < 2 | < 2 |
| # 50 | - | - | < 2 | < 2 |
| CV (vírus) | | | | |
| # 8 | 2 - 15 | 1 - 10 | < 2 | 4 |
| # 10 | 2 - 15 | 1 - 9 | < 2 | 4 |
| | $\overline{x}$: 14 | $\overline{x}$: 8.5 | | |
| $(PhSe)_2$ | | | | |
| # 14 | 2 - 15 | 4 - 7 | < 2 | 8 |
| # 17 | 2 - 8 | 1 - 6 | < 2 | 2 |
| # 23 | 2 - 9 | 1 - 5 | < 2 | 4 |
| # 24 | 2 - 15 | 5 -10 | < 2 | 8 |
| | $\overline{x}$: 10.75 | $\overline{x}$: 5.25 | | |
| CDV | | | | |
| # 7 | 2 - 15 | 1 - 8 | < 2 | 8 |
| # 12 | 2 - 15 | 1 - 9 | < 2 | 2 |
| # 39 | 2 - 15 | 6 - 10 | < 2 | 4 |
| # 46 | 2 - 15 | 1 - 11 | < 2 | 8 |
| | $\overline{x}$: 14 | $\overline{x}$: 8.25 | | |
| $(PhSe)_2$ +CDV | | | | |
| # 34 | 2 -12 | 1 - 9 | < 2 | 2 |
| # 36 | 2 - 14 | 1 - 9 | < 2 | 8 |
| # 74 | 2 - 15 | 5 - 9 | < 2 | 16 |
| # 101 | - | 1 | < 2 | 4 |
| | $\overline{x}$: 12.67 | $\overline{x}$: 7.67 | | |

dpi: days post-inoculation; - : negative; CV: control vírus; $(PhSe)_2$: Diphenyl diselenide; CDV: Cidofovir; $(PhSe)_2$ +CDV: Diphenyl diselenide + Cidofovi

Table 2 – Local signs observed in lesions developed by ewes inoculated with *Bovine alphaherpesvirus 2*(BoHV-2) and submitted to different topic treatments.

| Group | Day post-inoculation | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 -1 | 2 - 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
| Control (MEM) | | | | | | | | | | | | | | |
| # 25 | H+ | H+, S+ | S+ | S+ | - | - | - | - | - | - | - | - | - | - |
| # 50 | H+ | H+, S+ | S+ | S+ | - | - | - | - | - | - | - | - | - | - |
| CV (virus) | | | | | | | | | | | | | | |
| # 8 | H+ | H+, P+ | H++, P++ | H++, P++ | H++, P++ | P++, S+ | P++, S+ | P+, S++ | P+, S++ | P+++, S++ | P++, S++ | P++, S++ | P++, S++ | P++, S++ |
| # 10 | H+ | H+, P+ | H++, P+, S+ | H+++, S+ | H+++, S+ | H+, P++, S++ | H+, P++, S++ | P++, S++ | P++, S++ | P++, S++ | P++, S++ | P++, S++ | P++, S++ | P++, S++ |
| $(PhSe)_2$ | | | | | | | | | | | | | | |
| # 24 | H+ | H+, P+ | H++, P+, S+ | **H++, S++** | H++, S++ | H+, S+ | P+, S+ | P+, S+ | P+, S+ | P+, S+ | S+ | S+ | S+ | S+ |
| # 14 | H+ | H+, P+ | H+, P+, S+ | **H+, P+, S+** | H+, P+, S+ | H+, S+ | P+, S+ | P+, S+ | P+, S+ | P+, S+ | P+, S+ | S+ | S+ | S+ |
| # 17 | H+ | H+, P+ | H+, P+, S+ | **H+, S+** | H+, S+ | P+, S+ | P+, S+ | - | - | - | - | - | - | - |
| # 23 | H+ | H+, P+ | H++, P++, S+ | **P++, S++** | P++, S++ | P++, S++ | P++, S+ | P+, S+ | - | - | - | - | - | - |
| CDV | | | | | | | | | | | | | | |
| # 7 | H+ | H+, P+ | P++, S++ | **S++** | S++ | P+, S+ | P+, S+ | P++, S+ | P++, S+ | P++, S+ | P+, S+ | S+ | S+ | H+ |
| # 12 | H+ | H+, P+ | P+, S+ | H+, P+, S+ | **H+, P+, S+** | H+, P++, S+ | P++, S++ | P++, S+ | P++, S+ | P++, S+ | P+, S+ | S+ | S+ | S+ |
| # 39 | H+ | H+, P+ | H++, P+, S+ | H++, P+ | **H++, P+** | P+, S+ | P+, S++ | P+, S++ | P+, S+ | P+, S+ | P+, S++ | S++ | S++ | S++ |
| # 46 | H+ | H+, P+ | H++, S+ | **H++, P+, S+** | H++, P++, S++ | H+, P+, S+ | P++, S+ | P+, S++ | P+, S++ | P+, S+ | P+, S+ | S+ | S+ | S+ |
| $(PhSe)_2$ + CDV | | | | | | | | | | | | | | |
| # 101 | H+ | - | - | - | - | - | - | - | - | - | - | - | - | - |
| # 34 | H+ | H+, P+ | H+, P+, S+ | **P++, S++** | P++, S++ | H+, P+, S+ | P++, S+ | P++, S+ | P+, S+ | S++ | S+ | - | - | - |
| # 36 | H+ | H+, P+ | P+, S++ | **P++, S++** | P++, S++ | P++, S++ | P+, S++ | P+, S++ | P+, S++ | S++ | S++ | S++ | S++ | - |
| # 74 | H+ | H+, P+ | H+++, P+, S+ | **H+++, P+, S+** | H+++, P+, S+ | H+, P++, S++ | P+, S++ | P+, S++ | P+, S++ | S++ | S++ | S++ | H+, S+ | S+ |

C: negative control; CV: virus control, $(PhSe)_2$: Diphenyl diselenide; CDV: cidofovir; $(PhSe)_2$ +CDV: Diphenyl diselenide + Cidofovir; H: hiperemia; P: papule; S: scab; +: mild; ++: moderate;

+++ severe; - : no lesions; bold: start of treatment

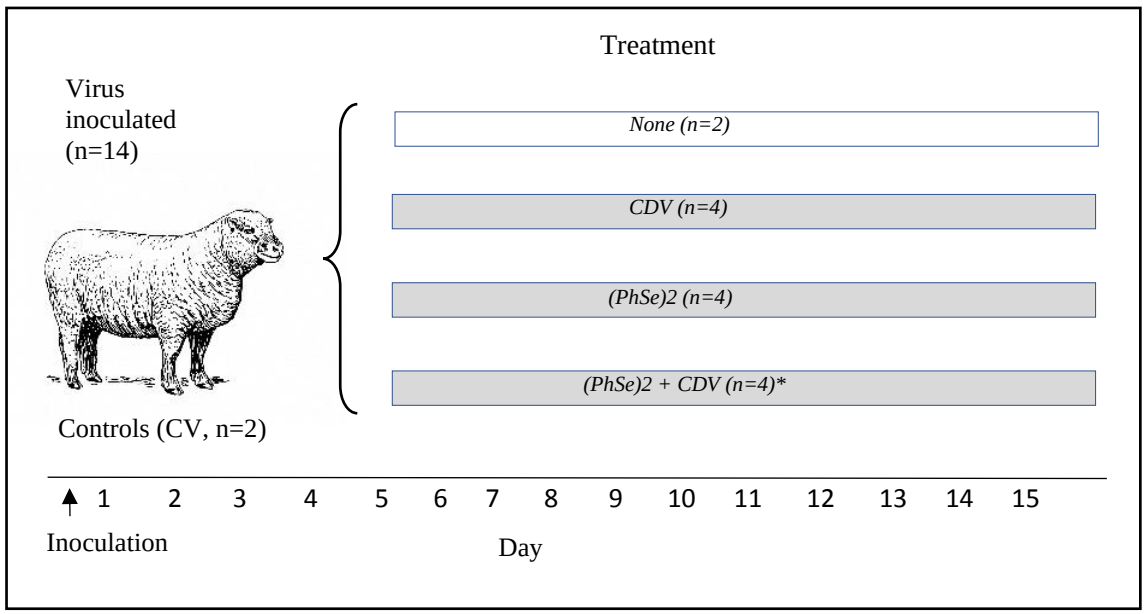
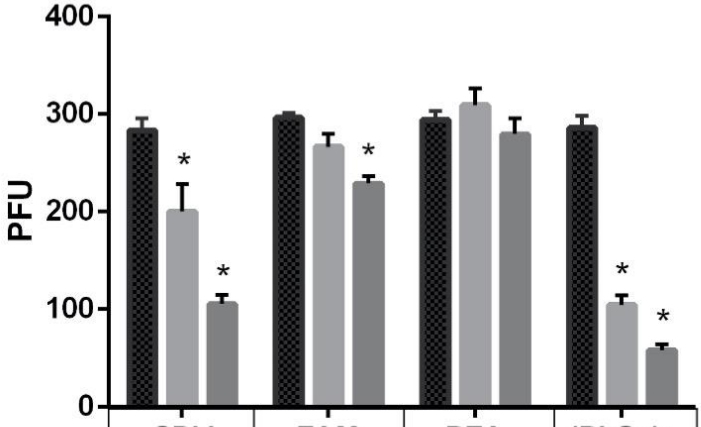



Figure 1. Animal experiment. Fourteen ewes were inoculated with *Bovine alphaherpesvirus 2* (BoHV-2) transdermally and two were inoculated with MEM (controls). Virus-inoculated ewes were randomly assigned to four groups and submitted to topic treatment, beginning ad day 5pi, as follows: untreated controls (CV, n=2), $(PhSe)_2$ (n=4), CDV (n=4), $(PhSe)_2$ + CDV (n=4). Inoculated animals were monitored clinically up to day 15pi. *One animal of the $(PhSe)_2$ + CDV had no virus replication nor developed lesions and was, therefore, excluded from the clinical/virological analyses.




| | CDV | FAM | PFA | $(PhSe)_2$ |
|---|---|---|---|---|
| Control | 283.3 | 296.7 | 294.0 | 286.0 |
| Pre-inoculation | 200.0 (29.4%) | 266.3 (10.2%) | 309.0 (0%) | 104.3 (63.5%) |
| Post-inoculation | 105.3 (62.8%) | 228.7 (22.9%) | 279.7 (4.9%) | 58.0 (79.7%) |

Figure 2. Mean number of plaques produced by *Bovine alphaherpesvirus 2* (BoHV-2) in cell monolayers treated with the respective drugs prior to (*pre-inoculation*) and after virus adsorption (*post-inoculation*). Control: untreated cell monolayers; pre-inoculation: drug addiction prior to virus adsorption; post-inoculation: drug addiction post-adsorption. The respective drug concentration was determined by cytotoxicity assay (MTT). An (*) means statistic difference from the control ($p<0.05$). PFU: plaque-forming unit.

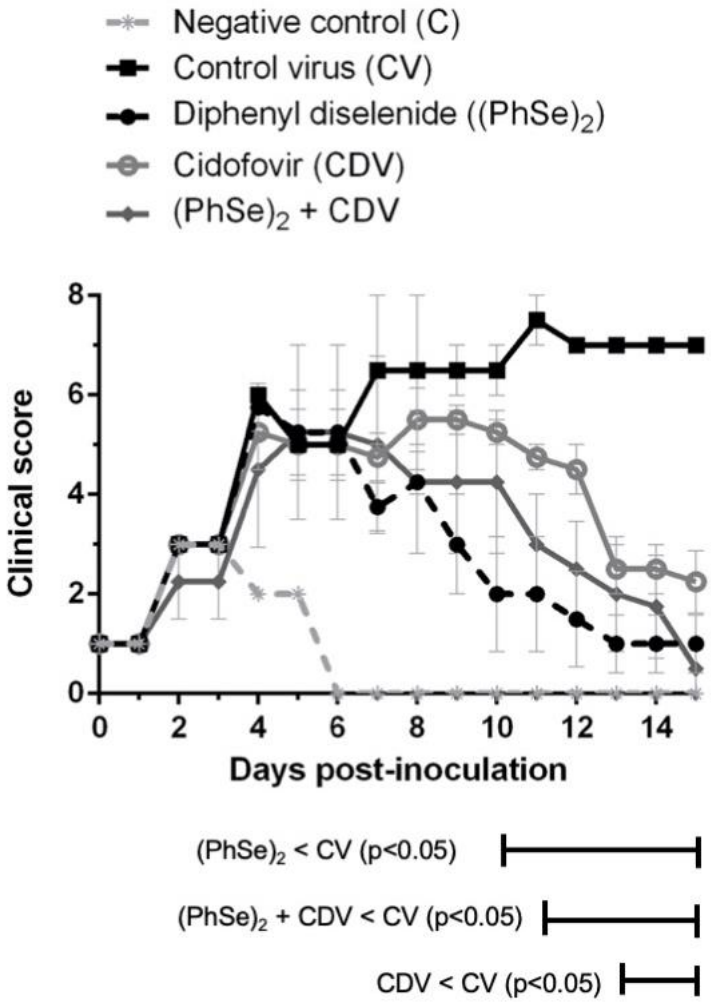



Figure 3. Mean clinical scores recorded in ewes inoculated transdermally with *Bovine alphaherpesvirus 2* (BoHV-2) and submitted to different topic treatments, beginning at day 5 pi. C: control ewes, inoculated with MEM; CV: control virus; $(PhSe)_2$ diphenyl diselenide; CDV: cidofovir; $(PhSe)_2$ + CDV. Bars below the graphic shows the periods in which the differences between treatments and virus control (CV) were statistically different ($p<0.05$).

## 4. DISCUSSÃO

A presente Tese apresenta dois estudos em forma de artigos científicos. O Artigo 1 relata o estudo da patogênese do BoHV-2 em bezerros, após diferentes rotas de inoculação; o Artigo 2 investigou a atividade de fármacos anti-virais *in vitro* e a atividade do Cidofovir (CDV) e disseleneto de difenila $(PhSe)_2$ na infecção e lesões causadas pelo BoHV-2 em ovelhas inoculadas experimentalmente. Os dois estudos justificam-se por: i. Escasso conhecimento acerca da patogênese do BoHV-2 e necessidade de aprofundar-se o conhecimento sobre os mecanismos envolvidos na infecção e doença. Acrescente-se a isso, a necessidade de se obter informações adicionais acerca da capacidade do BoHV-2 reativar a infecção latente, pelo seu potencial uso como vetor vacinal. ii. Falta de terapia específica para a doença causada pelo BoHV-2 e, concomitantemente, potencial uso do BoHV-2 como modelo para desenvolvimento e refinamento de terapias anti-herpéticas para humanos.

No primeiro estudo (Artigo 1) foi investigada a patogênese do BoHV-2 em bezerros após a inoculação por diferentes vias (intramuscular, intravenosa ou transdérmica). O estudo da patogênese do BoHV-2 justifica-se pelo fato de, por mais que já esteja estabelecida a capacidade de infecção latente do BoHV-2 (MARTIN & SCOOT, 1979), vários aspectos em relação a esta infecção não foram esclarecidos (FRANCO et al., 2017) e estudos para determinar a patogenia podem contribuir significativamente tanto para desenvolvimento de modelos terapêuticos, quanto para explorar a capacidade do vírus de ser utilizado como modelo de estudo para herpesvírus similares, como o HHV-1 e 2, assim como potencial vetor vacinal. Segundo, a semelhança biológica e genética do BoHV-2 com vírus simplex humanos pode ser explorada para a pesquisa e desenvolvimento de produtos farmacêuticos e vacinas. Nesse sentido, o BoHV-2 foi proposto como um potencial modelo para o desenvolvimento de medicamentos anti-herpéticos (FIELD, BISWAS & MOHAMMAD, 2006; DEZENGRINI et al., 2010; TORRES et al., 2009). E, portanto, a determinação da patogenia da infecção frente a diferentes vias de inoculação, bem como a capacidade de reativação de infecções latentes precisam ser bem estabelecidas.

Bezerros inoculados pela via transdérmica apresentaram lesões cutâneas, das quais o vírus pode ser isolado. Os resultados dos testes realizados com as amostras coletadas pós-inoculação demonstraram que o BoHV-2 foi capaz de replicar eficientemente em bezerros seguindo diferentes vias de inoculação, produziu viremia após a inoculação IM e IV. No entanto a administração de dexametasona nos bezerros inoculados no dia 48pi, não resultou em reativação do vírus, como indicado pela falta de detecção de vírus no sangue e/ou nos locais de

inoculação e pela ausência de aumento nos títulos de anticorpos. Esses resultados confirmam achados anteriores, nos quais a reativação do BoHV-2 não é facilmente induzida. Do ponto de vista vacinal, vislumbrando-se o BoHV-2 como possível vetor vacinal, essa propriedade é altamente desejável, já que o vírus vacinal não seria facilmente reativado sob condições artificiais de estresse.

Já no segundo estudo (Artigo 2) investigou-se a atividade *in vitro* do Cidofovir, Famciclovir, Foscarnet e disseleneto de difenila $(PhSe)_2$ contra o BoHV-2. Os resultados demonstraram que o $(PhSe)_2$ e o CDV apresentaram atividade antiviral, como demonstrado por uma redução significativa nas placas virais nas monocamadas celulares tratadas com os respectivos fármacos. Posteriormente, testou-se *in vivo,* formulações de pomadas dermatológicas de $(PhSe)_2$ e do CDV, tanto isolados quanto associados, em lesões de pele de ovelhas inoculadas com BoHV-2. O experimento em animais demonstrou que o tratamento tópico $(PhSe)_2$ e, em segundo lugar, o tratamento combinado de $(PhSe)_2$ + CDV em lesões cutâneas induzidas por BoHV-2 em ovelhas, tiveram um papel benéfico na regressão da doença. O tratamento com $(PhSe)_2$ resultou em redução significativa na replicação do vírus e, mais importante, em uma melhoria clínica mais rápida e resolução das lesões na maioria dos animais. O tratamento combinado também resultou em melhora clínica, ainda menos pronunciada. Esses achados são promissores para o desenvolvimento de opções terapêuticas para a doença do BoHV-2, principalmente em vacas leiteiras, e para o uso do BoHV-2 como modelo para o desenvolvimento e refinamento de terapias/protocolos anti-herpéticos humanos.

O CDV é um análogo da citosina que exibe atividade antiviral por meio de um metabólito ativo (difosfato de cidofovir) produzido intracelularmente (XIONG et al., 1997). A atividade antiviral do CDV foi explorada para tratar infecções por vírus alfaherpesvírus humano (HHV-1, HHV-2), adenovírus, citomegalovírus e papilomavírus humano (HPV). Devido à hepatotoxicidade e nefrotoxicidade na administração intravenosa, o cidofovir tem sido recomendado para tratamento de lesões muco-cutâneas para reduzir assim os efeitos colaterais sistêmicos do fármaco (TORO et al., 2003).

O efeito antiviral do $(PhSe)_2$ *in vitro* não pode ser classificada como virucida, tendo em vista que o fármaco era adicionado antes e depois da adsorção, portanto sem exposição direta do inóculo ao $(PhSe)_2$. Possivelmente, o efeito antiviral foi devido ao seu efeito nas etapas intracelulares da replicação do vírus. A regressão mais rápida dos sinais clínicos verificados nos animais tratados com pomadas a base de $(PhSe)_2$, possivelmente foi devido à atividade antiviral, verificada também *in vitro,* quando o uso da droga resultou em redução da replicação viral. Além disso, o $(PhSe)_2$ possui comprovadas propriedades imunomoduladoras,

antioxidantes e anti-inflamatórias (SARTORI et al., 2016; DOLESKI et al., 2017a; 2017b). Portanto, a combinação dos efeitos antiviral, imunomodulador, antioxidante e antiinflamatório provavelmente resultou na regressão mais rápida das lesões herpéicas tratadas com $(PhSe)_2$.

## 5. CONCLUSÃO

Com base nos resultados apresentados nesta Tese conclui-se que a inoculação do BoHV-2 em bezerros por diferentes vias resulta em infecção e replicação viral, no entanto, a disseminação virêmica ocorre somente após inoculação intramuscular ou intravenosa, e não pela via transdérmica. A replicação viral após inoculação transdérmica resultou em lesões cutâneas nos locais de inoculação, lembrando as lesões causadas pelo BoHV-2 em infecções naturais. Por outro lado, reativação viral não foi detectada após tratamento com dexametasona, indicando que o BoHV-2 não é facilmente reativável por meios artificiais.

Dentre os fármacos testados, o CDV e $(PhSe)_2$ apresentaram atividade anti-viral *in vitro*. Já no que se refere ao tratamento tópico de lesões dérmicas causadas pelo BoHV-2, o $(PhSe)_2$, isoladamente ou em combinação com o CDV, mostrou-se promissor como terapia antiviral. Da mesma forma, a infecção e doença causadas pelo BoHV-2 parecem ser modelos adequados para estudar a eficácia e para o aperfeiçoamento de terapias anti-herpéticas para humanos. Esse modelo oferece uma oportunidade atraente para o desenvolvimento e aprimoramento de protocolos terapêuticos para herpesvírus de humanos.

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