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INFECÇÃO EXPERIMENTAL DE COELHOS COM RECOMBINANTES DO HERPESVÍRUS BOVINO TIPO 5 DEFECTIVOS NOS GENES DA TIMIDINA QUINASE E DA GLICOPROTEÍNA E

DISSERTAÇÃO DE MESTRADO

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Santa Maria, RS, Brasil
2009

INFECÇÃO EXPERIMENTAL DE COELHOS COM RECOMBINANTES DO HERPESVÍRUS BOVINO TIPO 5 DEFECTIVOS NOS GENES DA TIMIDINA QUINASE E DA GLICOPROTEÍNA E

por

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RESUMO

Dissertação de Mestrado
Programa de Pós-graduação em Medicina Veterinária
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INFECÇÃO EXPERIMENTAL DE COELHOS COM RECOMBINANTES DO HERPESVÍRUS BOVINO TIPO 5 DEFECTIVOS NOS GENES DA TIMIDINA QUINASE E DA GLICOPROTEÍNA E

AUTORA: SARA CAMPOS DA SILVA
ORIENTADOR: RUDI WEIBLEN
Santa Maria, 9 de dezembro de 2009.

O herpesvírus bovino tipo 5 (BoHV-5), agente da meningoencefalite herpética bovina, possui grande importância na América do Sul e tem motivado pesquisas para o desenvolvimento de vacinas eficazes e seguras. Essa dissertação relata a investigação da virulência em coelhos de três recombinantes do BoHV-5, candidatos vacinais, contendo deleções nos genes da glicoproteína E (gE) (BoHV-5gEΔ), da enzima timidina quinase (TK) (BoHV-5TKΔ) e deleção dupla nos genes da gE e TK (BoHV-5gEΔTKΔ). Para isso, quatro grupos de oito coelhos cada foram inoculados pela via intranasal com um dos recombinantes ou com a cepa parental (SV507/99) e monitorados nos dias seguintes à inoculação. No dia 42 pós-inoculação (pi) realizou-se a administração de dexametasona (Dx) para reativar a infecção latente e no dia 70 pi a eutanásia para a coleta do encéfalo para a pesquisa de DNA latente por PCR. Os coelhos inoculados com o vírus parental (SV507/99) excretaram vírus nas secreções nasais entre os dias 2 e 8 pós-inoculação (pi), e todos (n=8) desenvolveram doença neurológica e morreram ou foram submetidos à eutanásia *in extremis* entre os dias 7 e 13 pi. Excreção viral entre os dias 2 e 10 pi também foi detectada em 7 de 8 coelhos inoculados com o BoHV-5gEΔ; 6 de 8 inoculados com BoHV-5TKΔ e 3 de 8 inoculados com BoHV-5gEΔTKΔ. Apesar dos níveis variáveis de excreção viral, os animais inoculados com os três recombinantes soroconverteram, apresentando anticorpos neutralizantes em títulos entre 2 e 256 no dia 42 pi. Nos animais inoculados com o vírus parental, o vírus foi detectado amplamente disseminado no encéfalo, incluindo o bulbo olfatório, córtices, bulbo, ponte, mesencéfalo e tálamo. Dentre os animais inoculados com o recombinante BoHV-5gEΔ, três desenvolveram doença neurológica, nos dias 10 e 15 pi. Uma distribuição viral restrita aos córtices e tálamo foi detectada no encéfalo desses animais. Os coelhos inoculados com os recombinantes BoHV-5TKΔ (n=8) e BoHV-5gEΔTKΔ (n=8) permaneceram saudáveis. A administração de dexametasona (Dx) no dia 42 pi não resultou em reativação da infecção por nenhum dos recombinantes, demonstrada por ausência de soroconversão ou excreção viral em secreções. Entretanto, o DNA viral latente foi detectado no gânglio trigêmeo ou no bulbo olfatório de todos esses animais no dia 28 pDx (70dpi), demonstrando o estabelecimento da infecção latente. Esses resultados demonstram que os recombinantes são capazes de estabelecer a infecção latente, mas não são facilmente reativados pela administração de Dx. Em resumo, os recombinantes BoHV-5TKΔ e BoHV-5gEΔTKΔ são atenuados para coelhos constituindo-se, assim, em candidatos vacinais em potencial.

Palavras-chave: BoHV-5; recombinantes; patogenia; virulência, candidatos vacinais.

ABSTRACT

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

EXPERIMENTAL INFECTION OF RABBITS WITH BOVINE HERPESVIRUS 5 RECOMBINANTS DEFECTIVE IN THYMIDINE KINASE AND GLYCOPROTEIN E GENES

AUTHOR: Sara Campos da Silva
ADVISER: Rudi Weiblen
Santa Maria, December 9th, 2009.

Bovine herpesvirus 5 (BoHV-5) – the agent of meningoencephalitis in cattle - is an important pathogen of cattle in South America and efforts have been made to produce safer and more effective vaccines. This dissertation relates an investigation of the virulence in rabbits of three BoHV-5 recombinants, vaccine candidates, defective in the glycoprotein E (BoHV-5gEΔ), thymidine kinase (BoHV-5TKΔ) and both genes (BoHV-5gEΔTKΔ). To this, four groups of eight rabbits each were inoculated intranasally with each recombinant or the parental strain (SV507/99) and monitored thereafter. At day 42 post inoculation (pi) the inoculated animals were submitted to dexamethasone (Dx) administration to reactivate latent infection. At day 70 pi, all animals were euthanized and the brain was collected for investigation of latent viral DNA by PCR. Rabbits inoculated with the parental virus shed virus between days 2 and 8 pi and all rabbits (n=8) developed neurological disease and died or were euthanized *in extremis*, between days 7 and 13 pi. Among the animals inoculated with the recombinants, viral shedding was detected between days 2 and 10 pi, in 7 out of 8 rabbits of the BoHV-5gEΔ group, in 6 out of 8 rabbits of the BoHV-5TKΔ group and in 3 out of 8 of the BoHV-5gEΔTKΔ group. In spite of variable levels of virus shedding, all rabbits inoculated with the recombinants seroconverted, developing virus-neutralizing antibodies in titers from 2 to 256 at day 42 pi. The rabbits inoculated with the parental virus showed a wide distribution of the virus in their brains, including the olfactory bulbs, cortices, medulla oblongata, pons, midbrain and thalamus. Three out of eight rabbits inoculated with the recombinant BoHV-5gEΔ developed neurological signs at days 10 and 15pi. A more restricted virus distribution, confined mainly to cerebral cortices and thalamus was detected in the brain of these animals. Rabbits inoculated with the recombinants BoHV-5TKΔ (n=8) or BoHV-5gEΔTKΔ (n=8) remained healthy during the experiment. Dx administration to rabbits inoculated with the three recombinants at day 42 pi did not result in viral reactivation, as demonstrated by lack of seroconversion or virus shedding. Nevertheless, viral DNA was detected in the trigeminal ganglia or olfactory bulbs of all these animals at day 28pDx, demonstrating they were latently infected. These results showed that the three recombinants were able to establish latent infection yet they were not easily reactivated by Dx administration. In summary, the recombinants BoHV-5TKΔ and BoHV-5gEΔTKΔ are attenuated for rabbits and constitute potential vaccine candidates.

Keywords: BoHV-5; recombinants; pathogenesis; virulence; vaccine candidate.

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SUMÁRIO

1. INTRODUÇÃO

A infecção pelo herpesvírus bovino tipo 5 (BoHV-5) está associada com meningoencefalite de curso geralmente fatal em bovinos jovens (ROIZMAN et al., 1992). A enfermidade causada pelo BoHV-5 possui maior importância na América do Sul, onde tem sido relatada com freqüência no Brasil e na Argentina (CARRILLO et al., 1983; SALVADOR et al., 1998, RISSI et al., 2007). O BoHV-5 apresenta grande similaridade genética e antigênica com o herpesvírus bovino tipo 1 (BoHV-1), agente da rinotraqueíte infecciosa (IBR) e da vulvovaginite/balanopostite pustular bovina (IPV/IBP) (SCHWYZER; ACKERMANN, 1996; DELHON et al., 2003). Devido a reatividade sorológica cruzada entre estes agentes, e a inexistência de um teste que os diferencie sorologicamente, a prevalência da infecção pelo BoHV-5 ainda permanece desconhecida (FLORES et al., 2009).

O BoHV-5 pertence à família *Herpesviridae*, subfamília *Alphaherpesvirinae*, gênero *Varicellovirus* (ROIZMAN et al., 1992). Os vírions são pleomórficos, com 150 a 200 nm de diâmetro, envelopados e possuem como genoma uma molécula de DNA de fita dupla com aproximadamente 138 quilobases (Kb) (ROIZMAN et al., 1992; DELHON et al., 2003). Os alfaherpesvírus são reconhecidos por infectar várias espécies animais, replicar de forma rápida e lítica em células de cultivo celular, possuir neurotropismo e estabelecer infecções latentes em gânglios do sistema nervoso periférico e em outros tecidos neurais (SCHWYZER; ACKERMANN, 1996). Mesmo sendo semelhantes genética e antigenicamente, e estabelecerem infecções latentes em gânglios sensoriais, o BoHV-1 e o BoHV-5 diferem na habilidade de invadir o sistema nervoso central (SNC) e causar doença neurológica (METZLER et al., 1986; BELKNAP et al., 1994; DELHON et al., 2003). Embora o BoHV-1 tenha sido ocasionalmente isolado de casos de meningoencefalite, a maioria dos casos de doença neurológica são associados com o BoHV-5 (SILVA et al., 2007).

Os bovinos são os hospedeiros naturais do BoHV-5, embora experimentalmente a infecção já tenha sido reproduzida em outras espécies de mamíferos (SILVA et al., 1999; BELTRÃO et al., 2000; ABRIL et al., 2004; DIEL et al., 2007). Nesse sentido, coelhos jovens têm constituído um modelo experimental adequado para o estudo da infecção pelo BoHV-5, podendo desenvolver doença aguda e fatal, semelhante à observada em bovinos; ou ainda estabelecer infecção latente (MEYER et al., 1996; LEE et al., 1999; BELTRÃO et al., 2000, CARON et al., 2002). A doença neurológica desenvolvida pelos animais infectados com o BoHV-5 é consequência da replicação e invasão viral progressiva no encéfalo (LEE et

al., 1999). Inicialmente, observa-se depressão e secreção nasal e ocular, que progridem para emagrecimento, dificuldade respiratória, tremores, bruxismo, andar em círculos, incoordenação, cegueira, nistagmo e disfagia, chegando a convulsão e morte em estágios mais avançados (CHOWDHURY et al., 1997; PEREZ et al., 2002). Embora classicamente o BoHV-5 esteja associado à doença neurológica, este agente já foi isolado de amostras de sêmen, de casos de doença respiratória e de infecções generalizadas em bezerros jovens (SILVA et al., 2007; KIRKLAND et al., 2009).

Durante a infecção aguda, o BoHV-5 replica nas células da mucosa nasal, invadindo as terminações dos nervos autonômicos e sensoriais que inervam a mucosa, sobretudo as terminações dos nervos olfatório e ramo maxilar do nervo trigêmeo (LEE et al., 1999). A seguir, os vírions invadem o SNC, principalmente pela via olfatória, no sentido retrógrado a partir da mucosa nasal (CHOWDHURY et al., 1997; LEE et al., 1999; DIEL et al., 2005). A disseminação viral no encéfalo pode ter como consequência o desenvolvimento de doença neurológica ou o estabelecimento de infecção latente (LEE et al., 1999; PEREZ et al., 2002; VOGEL et al., 2003). A infecção latente pode ser reativada naturalmente, durante episódios de estresse, ou artificialmente, pela administração de corticóides, sendo geralmente acompanhada de excreção viral e, ocasionalmente, de recrudescência clínica (CARON et al., 2002; VOGEL et al., 2003). Animais portadores da infecção latente são epidemiologicamente importantes, pois representam a principal fonte para introdução, disseminação e manutenção do vírus nos rebanhos (ACKERMANN; ENGELS, 2006).

A vacinação é uma medida complementar ao manejo e amplamente utilizada no controle das infecções pelos herpesvírus (VAN OIRSCHOT, 1999). Devido à importância que o BoHV-5 possui na América do Sul e a existência de apenas uma vacina específica para este agente no Brasil, vacinas para o BoHV-1 tem sido utilizadas no controle da infecção pelo BoHV-5 (RISSI et al., 2007). A grande maioria das vacinas disponíveis comercialmente no Brasil são inativadas. Apesar destas vacinas apresentarem eficácia satisfatória reduzindo a severidade da doença e a excreção viral, serem seguras e não apresentarem riscos de reversão à virulência, elas estimulam preferencialmente a resposta humoral e necessitam de reforços periódicos para manter níveis adequados de anticorpos (VAN OIRSCHOT, 1999; VAN DRUNEN LITTEL-VAN DEN HURK, 2007). Além disso, estas vacinas são classificadas como convencionais, não permitindo a diferenciação sorológica entre animais naturalmente infectados e vacinados, aspecto importante para o controle e erradicação da infecção do rebanho (VAN OIRSCHOT, 1996; VAN OIRSCHOT, 1999). Como alternativa, esforços têm sido empregados no desenvolvimento de vacinas diferenciais, baseadas na deleção de um ou

mais genes não-essenciais que além de resultar em redução da virulência podem ser utilizados como marcadores antigênicos (ACKERMANN; ENGELS, 2006).

O genoma do BoHV-5 possui aproximadamente 70 genes, cujos produtos podem ser classificados em essenciais e não-essenciais, de acordo com a sua necessidade para a replicação viral em cultivo celular. Entretanto, todos os genes provavelmente possuem papel importante na infecção viral *in vivo* (SCHWYZER; ACKERMANN, 1996; DELHON et al., 2003; METTENLEITER, 2003). A deleção de alguns genes não-essenciais dos herpesvírus pode resultar em redução ou incapacidade do vírus causar doença, sem afetar a capacidade de replicação *in vitro* (ENQUIST et al., 1998). Proteínas estruturais, glicoproteínas e enzimas têm sido alvo de deleção para a produção de cepas defectivas com o objetivo de desenvolver vacinas atenuadas e diferenciais ou, ainda, para estudos de patogenia (ENQUIST et al., 1998; METTENLEITER, 2003).

A glicoproteína E (gE) é uma proteína estrutural do envelope do BoHV-5, conservada entre os isolados de campo, e não-essencial para a replicação viral em células de cultivo (ENQUIST et al., 1998). Além disso, a gE está envolvida na neuropatogênese da infecção, desempenhando um importante papel na neuroinvasidade e neurovirulência (METTENLEITER, 2003). Estas características fazem da gE um adequado alvo para deleção e produção de cepas atenuadas de herpesvírus neurovirulentos, como o vírus do herpes simplex tipo 1 (HSV-1), vírus da doença de Aujeszky (PRV) e BoHV-5 (MULDER et al., 1994; DINGWELL et al., 1995; CHOWDHURY et al., 2000). Um recombinante do BoHV-5 com deleção no gene da gE manteve a capacidade de penetrar no SNC pela via olfatória, porém apresentou uma redução significativa na replicação, disseminação e conseqüente neurovirulência (CHOWDHURY et al., 2000). As causas para a deficiente disseminação transneuronal do BoHV-5 na ausência da gE ainda não foram investigadas. Entretanto, acredita-se que, assim como proposto para o HSV-1 e o PRV, o transporte axonal das glicoproteínas de envelope e o capsídeo viral tegumentado é realizado separadamente (TOMISHIMA; ENQUIST, 2001; SNYDER et al., 2008). Na ausência da gE, a vesícula contendo as glicoproteínas não seria transportada ao longo do axônio, não ocorrendo a maturação final dos vírions junto à sinapse e a sua transmissão trans-sináptica (TOMISHIMA; ENQUIST, 2001; SNYDER et al., 2008).

Os vírus da subfamília *Alphaherpesvirinae* codificam a sua própria enzima timidina quinase (TK), cuja função é fornecer deoxiribonucleotídeos para a síntese de DNA viral em células pós-mitóticas, como os neurônios (ENQUIST et al., 1998; ROIZMAN; KNIPE, 2001). Nestes vírus, a TK é absolutamente necessária para a expressão completa da virulência *in vivo*

(ENQUIST et al., 1998). Assim, a deleção da TK do BoHV-1, PRV e HSV-1 está associada com uma significativa redução da virulência (CHOWDHURY, 1996; FERRARI et al., 2000; CHEN et al., 2004). Entretanto, o envolvimento da TK na neuropatogênese do BoHV-5 ainda não foi totalmente elucidado, pois cepas do BoHV-5 defectivas nesta enzima ainda não foram avaliadas *in vivo*. Estudos demonstram que variantes do BoHV-5 resistentes à brivudina e, provavelmente, deficientes na atividade da enzima TK foram atenuados quando inoculados em coelhos (BRUM, 2009).

A capacidade de estabelecer a infecção latente parece não ser afetada nas cepas contendo deleções na gE ou na TK (CHOWDHURY et al., 2000; FERRARI et al., 2000; CHEN et al., 2004; LIU et al., 2008). Entretanto, após administração de corticóides em animais inoculados com cepas gE deletadas, não é possível detectar indícios de reativação, como excreção viral ou sinais clínicos (VAN ENGELENBURG et al., 1995; KAASHOEK et al., 1998). Um estudo utilizando uma cepa do BoHV-1 deletada na gE demonstrou que o vírus reativa a infecção latente no gânglio trigêmeo, porém não é transportado no sentido anterógrado em direção as terminações nervosas na mucosa olfatória e ocular (LIU et al., 2008; BRUM et al., 2009). Os dados de reativação viral de cepas TK deletadas são bastante controversos, indicando provavelmente as diferentes condições em que foram realizados os experimentos (MENGELING, 1991; WHETSTONE et al., 1992; CHEN et al., 2004).

Neste trabalho são relatados os aspectos clínicos e virológicos da infecção aguda e latente em coelhos inoculados com três recombinantes do BoHV-5, individualmente, defectivos no gene da gE, da TK e ambos gE e TK. Estes recombinantes poderão ser utilizados para a produção de vacinas diferenciais para o BoHV-5, além da possibilidade da realização de estudos sobre importantes aspectos da patogenia deste agente.

2. CAPÍTULO 1

A bovine herpesvirus 5 recombinant defective in thymidine kinase (TK) gene and a double mutant lacking TK and glycoprotein E gene are fully attenuated for rabbits

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A bovine herpesvirus 5 recombinant defective in thymidine kinase (TK) gene and a double mutant lacking TK and glycoprotein E gene are fully attenuated for rabbits

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Running title: Attenuation of BoHV-5 recombinants in rabbits

Abstract

Bovine herpesvirus 5 (BoHV-5) – the agent of herpetic meningoencephalitis in cattle - is an important pathogen of cattle in South America and efforts have been made to produce safer and more effective vaccines. In this article, we investigated the virulence in rabbits of three recombinant viruses constructed out of a neurovirulent Brazilian BoHV-5 strain (SV507/99). The recombinants are defective in the glycoprotein E (BoHV-5gEΔ), enzyme thymidine kinase (BoHV-5TKΔ) and both proteins (BoHV-5gEΔTKΔ). Rabbits inoculated with the parental virus (n=8) developed neurological disease and died or were euthanized *in extremis* between days 7 and 13 pi (post infection). Infectivity was detected in several areas of their brains. Three out of eight rabbits inoculated with the recombinant BoHV-5gEΔ

developed neurological signs between days 10 and 15 pi and were also euthanized. A more restricted virus distribution was detected in the brain of these animals. Rabbits inoculated with the recombinants BoHV-5TKΔ (n=8) or BoHV-5gEΔTKΔ (n=8) remained healthy throughout the experiment, in spite of variable levels of virus replication in the nose. Dexamethasone (Dx) administration to rabbits inoculated with the three recombinants at day 42 pi did not result in viral reactivation, as ascertained by absence of virus shedding and/or increase in VN titers. Nevertheless, viral DNA was detected in the trigeminal ganglia or olfactory bulbs of all animals at day 28 pDx (post Dx), demonstrating they were latently infected. These results show that recombinants BoHV-5TKΔ and BoHV-5gEΔTKΔ are attenuated for rabbits and constitute potential vaccine candidates upon the confirmation of this phenotype in cattle.

Key words: BoHV-5, gE and TK deletion mutants, pathogenesis, virulence, vaccine candidate.

Introduction

Bovine herpesvirus type 5 (BoHV-5) is a neurovirulent alphaherpesvirus associated with meningoencephalitis generally fatal in cattle (1). BoHV-5 infection and disease have been occasionally described in several countries, yet the disease is noticeably more frequent in Brazil and Argentina where outbreaks are reported every year (2-6). BoHV-5 is related antigenically and genetically to bovine herpesvirus 1 (BoHV-1), the agent of respiratory (*infectious bovine rhinotracheitis, IBR*) and genital disease in cattle (*infectious pustular vulvovaginits/balanopostitis,* IPV/IBP) (1). These viruses belong to the family *Herpesviridae,* subfamily *Alphaherpesvirinae*, genus *Varicellovirus* (7, 8). BoHV-1 and BoHV-5 genomes display the same genomic organization, share a nucleotide similarity 85% and 82% at the

nucleotide and protein level, respectively (8). The major biological difference between these viruses seems to be the neurovirulent potential: BoHV-5 is highly neurovirulent for the natural hosts and for experimental animals (9), whereas BoHV-1 is far less neurovirulent and only occasionally has been associated with neurological infection (2). As other alphaherpesviruses, BoHV-5 and BoHV-1 establish lifelong latent infection in sensory nerve ganglia (9, 10).

The BoHV-5 genome consists of a linear, double stranded DNA molecule of approximately 138kb in length and encodes at least 70 gene products (8). Alphaherpesviruses (and other herpesviruses as well) encode a number of gene products that are non-essential (NE) for virus replication in tissue culture, although they are probably required for virus maintenance in nature (7, 11). Deletions of such genes have been used to study the role of individual proteins in virus replication *in vitro* and *in vivo* (12, 13) and for the production of recombinant strains for vaccine use (14, 15). In this sense, the genes encoding the envelope glycoprotein E (gE) and the enzyme thymidine kinase (TK) are among the most frequent targets for deletion towards vaccine production (16).

The envelope gE has been shown to be important for invasion and dissemination of BoHV-5 within the brain and for anterograde transport of BoHV-1 from the trigeminal ganglia to peripheric sites after reactivation (12, 17, 18). Furthermore, deletion of gE gene from BoHV-1 and BoHV-5 genomes contributes for virus attenuation in calves and rabbits (16-18). In addition, the deletion of gE from vaccine strains provides an antigenic marker which would allow serological differentiation of vaccinated from naturally infected animals (19). Vaccines with antigenic markers (also called DIVA vaccines – for *differentiating infected from vaccinated animals*) have constituted the basis for eradication of BoHV-1 and pseudorabies virus (PRV) from European countries and US (1) and are beginning to be used in South America (20).

The full expression of virulence of neurovirulent alphaherpesviruses depends upon the function of the enzyme TK (12). Herpesvirus-encoded TK is an enzyme involved in the metabolism of deoxyribonucleotides (dNTPs), which is necessary for viral DNA synthesis and genome replication in non-dividing cells such as neurons (12, 21). Deletion of TK gene in neurovirulent alphaherpesviruses, namely human herpes simplex virus (HSV) and pseudorabies virus (PRV), has been associated with deficient replication in neurons and reduced neurovirulence (22-25). For these reasons, BoHV-1 mutants lacking TK activity are also attenuated, albeit to a lesser extent (16, 26). For this reason, the TK gene has been an attractive target for gene deletion towards the production of attenuated strains for vaccine use (16).

Reactivation of latent infection by TK-defective alphaherpesviruses is also significantly impaired or even abolished, especially for HSV in mice (22, 23). On the other hand, attempts to reactivate latent infection by TK-negative BoHV-1 and PRV strains have yielded conflicting results, probably reflecting differences in the viruses, animals and experimental procedures (16, 27-30). Despite these conflicting results, it is generally accepted that TK-defective alphaherpesviruses replicate poorly (if so) in neurons and, therefore, are not expected to reactivate efficiently from latency.

The vaccines marketed in South America contain either inactivated or modified live BoHV-1 strains, none of them containing antigenic markers (31). Following the trend of European countries and predicting future sanitary restrictions for trading of animals and products, some South American countries are now embarking on the development and use of DIVA vaccines (20). Our group recently described the construction and *in vitro* characterization of three recombinants (BoHV-5gEΔ, BoHV-5TKΔ and BoHV-5gEΔTKΔ) out of a highly neurovirulent Brazilian BoHV-5 strain (31). These recombinants were constructed for vaccine purposes yet they may also be useful for pathogenesis studies since

they are defective in specific gene products involved in different stages of virus infection. The TK deletion, in particular, would provide an interesting means of attenuation for such neurovirulent agent.

The present article describes studies on the pathogenesis of acute and latent infections by these recombinants in a well characterized rabbit model (32). Our results show that the recombinants BoHV-5TKΔ and BoHV-5gEΔTKΔ are fully attenuated for rabbits upon intranasal inoculation and induce a virus-neutralizing antibody response. Although these recombinants retained the ability to establish latent infection, they did not reactivate readily upon Dx treatment. Thus, these results are promising towards the use of these recombinants as vaccine strains, depending on equivalent phenotype in cattle.

Material and Methods

Experimental design

Groups of weanling rabbits were inoculated intranasally (IN) with each BoHV-5 recombinant or the parental virus and submitted to clinical, virological and serological monitoring during acute infection. The identity of viruses shed in nasal secretions by rabbits of each group was confirmed by PCR. Rabbits dying of neurological disease during acute infection had sections of their brains examined for infectivity. At day 42 post-infection (pi), rabbits were submitted to administration of dexamethasone (Dx) for reactivating latent infection and were monitored thereafter as described for acute infection. Twenty eight days after Dx treatment, the rabbits were euthanized and trigeminal ganglia (TG) and olfactory bulbs (OB) were examined for viral DNA by PCR.

Viruses and cells

The Brazilian BoHV-5 strain SV507/99 - isolated from a cow with neurological disease in southern Brazil and submitted to sequencing of the entire genome (8) was used as

the parental virus to construct the recombinants. The construction and characterization *in vitro* of recombinants defective in the glycoprotein E (BoHV-5gEΔ), thymidine kinase (BoHV-5TKΔ) and both genes (BoHV-5gEΔTKΔ) was described previously (31). The viruses were propagated in a MDBK-derived cell line named CRIB (ATCC-CRL 11883) maintained in MEM (minimum essential medium, Gibco, Brazil), supplemented with 10% fetal bovine serum (Cultilab, Brazil) and 100 U/mL of penicillin, 100 µg/mL of streptomycin (Nutricell, Brazil).

In vitro growth kinetics

Before rabbit inoculation, the recombinants were evaluated in their ability to replicate in rabbit kidney cells **(**RK-13; ATCC-CCL 37), in an one step growth curve according to Brum et al. (31). RK-13 cell monolayers were inoculated with each recombinant at multiplicity of infection of 5. At intervals after virus inoculation (0, 4, 10, 18 and 24 hours) aliquots of culture supernatants were harvested and submitted to virus quantitation by limiting dilution. Virus titers were calculated according to Reed & Muench (33) and expressed as $\log_{10}$ median tissue culture infectious dose per mililiter ($TCID_{50}$/mL).

Animals, virus inoculation, monitoring and dexamethasone treatment

Thirty-six New Zealand rabbits (28-30 days-old) weighting 300-400 g were used for virus inoculation. The animals were randomly divided in four groups of eight rabbits each plus a control group of four animals. Each group was maintained in separated cages, without contact with each other, receiving water and food *ad libitum*. Rabbits of each experimental group (n=8) were inoculated with one virus: parental SV-507/99; gE negative (BoHV-5gEΔ), TK-deleted (BoHV-5TKΔ) and defective on both genes (BoHV-5gEΔTKΔ). The inoculum consisted of supernatants of CRIB cells infected with each virus, containing approximately $10^{7.5}$ $TCID_{50}$/mL. The animals were inoculated IN with 1 mL of virus suspension into the

paranasal sinuses (0.5 mL in each side) through nephrine openings (34) after tranquilization with tiletamine/zolazepan (30 mg/kg IM; Zoletil, Virbac, Brazil). Controls (n=4) were inoculated IN with culture medium.

Following virus inoculation the rabbits were monitored twice a day for clinical signs. Nasal swabs for virus isolation were collected every two days up to day 14 pi (acute infection) and up to day 10 pDx (after Dx treatment). Swabs were vortexed, drained and the supernatant was inoculated onto CRIB cell monolayers and submitted to three passages of five days each with the cultures being monitored for cytopathic effect (CPE). Pools of nasal secretions of each group were subsequently submitted to PCR to confirm the identity of the inoculated viruses. Rabbits developing severe neurological signs during acute infection were euthanized *in extremis*; brain sections were aseptically collected and tissue homogenates (10% w/v) were submitted to virus isolation in CRIB cells. Serum samples were collected at days 0 and 42 pi and submitted to a standard virus neutralizing assay (VN) for neutralizing antibodies, using two-fold dilutions of sera against 100-200 $TCID_{50}$ of the parental virus (SV507/99), 2h of incubation of the virus-serum mixture and 72h of incubation before to reading. CRIB cells were used as indicators of virus replication. Beginning at day 42 pi, the rabbits were submitted to five daily administrations of dexamethasone (2.6 mg/kg/day, Decadronal, Achè, Brazil) by the intramuscular route. In the days following Dx treatment the animals were monitored as described for acute infection. Serum samples collected at the day of the first Dx administration (42 pi/0 pDx) and 28 days later (day 28 pDx) were submitted to a VN assay as described above. At day 28 pDx, inoculated and control animals were euthanized, the olfactory bulbs (OB) and trigeminal ganglia (TGs) were collected for detection of viral DNA by PCR.

All procedures of animal handling and experimentation were performed according to recommendations by the Brazilian Committee on Animal Experimentation (COBEA; law

#6.638 of 8th May 1979). The animal experiments were approved by an Institutional Ethics and Animal Welfare Committee (Comitê de Ética e Bem Estar Animal, Universidade Federal de Santa Maria, UFSM, approval #44/2008; process #23081.010078/2008-41).

Identity of the viruses shed by inoculated rabbits

In order to confirm the identity of each recombinant virus being shed for the respective groups, nasal secretions collected daily from the animals of each group were polled and submitted to DNA extraction. Total DNA extracted from secretions was submitted to PCR for detection of TK and gE deletions. A PCR for the gene of glycoprotein B was used as control (35). Detection of gE gene (or its absence), was performed by using the following primers: forward 5'-ACGAGACGTGCATCTTCC-3' (position 124.888 on the BoHV-5 genome) and reverse – 5'-CAGCACGAAGACGTAGAG-3' (position 125.156), giving rise to a 269 bp product. The TK gene (or its absence) was detected by using the primers: forward 5'-GACGTCGTGACCCTCGTGTTTG-3' (position 64.971) and reverse 5'-TAGGAAGGCGCACGTGTTCG-3' (position 65.255), which amplify a 286 bp product. PCR reactions were performed essentially as described by Brum et al. (31). Total DNA extracted from CRIB cells infected with BoHV-5TKΔ or BoHV-5gEΔ and SV-507/99 were used as negative and positive controls, respectively.

DNA extraction from tissues and PCR

Neural tissues collected at day 28 pDx (TGs, BOs) were submitted to total DNA extraction using DNAzol reagent (Invitrogen, Carlsbad, CA, USA). The extracted DNA was solubilized in buffer Tris-EDTA (0.1 – 0.2 mL) and stored at -20°C until testing. DNA concentration was determined in a UV spectrophotometer at 260 nm.

Total DNA was submitted to a nested PCR using two set of primers corresponding to positions 57.338 and 57.782 (primers 1 and 2) and 57.372 and 57.666 (primers 3 and 4) of the

glycoprotein B gene coding region of the BoHV-5 strain SV-507 (8, 35). The external primers (primers 1 and 2) used in the first reaction were -forward: 5’ CTC GAA AGC CGA GTA CCT GCG 3’ and reverse: 5’-5’ CCA GTC CCA GGC AAC CGT CAC 3’. The internal primers (primers 3 and 4) used in the second reaction were - forward: 5’ GTG GTG GCC TTT GAC CGC GAC 3’ and reverse: 5’5’ GCT CCG GCG AGT AGC TGG TGT G 3’. The first PCR reaction amplifies a 444 bp DNA fragment and the second reaction results in a 295 bp amplicon. The PCR reactions were performed in 25 µL, using 2 µL template DNA (corresponding to approximately 1 µg of total DNA), 100 ng of each primer, 2 mM $MgCl_2$, 10 mM dNTPs, 10% DMSO, 1x reaction buffer, and 2.5 units of Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA). The PCR conditions were: initial denaturation at 94°C for 5 min, following by 35 cycles at 94°C for 45 sec for DNA denaturation, 56°C for 45 sec for primer annealing and 72°C for 45 sec for primer extension, and final extension at 72°C for 10 min. Total DNA extracted from the brain of a control non-infected rabbit, and from a rabbit with acute BoHV-5 infection were used as negative and positive controls, respectively. PCR products were analysed under UV light after electrophoresis in an 1.5% agarose gel stained with ethidium bromide. To determine the sensitivity of the PCR, 10-fold dilutions of strain SV-507 DNA were prepared with DNA (1 µg/µl) extracted from the brain of a BoHV-5-seronegative cow and used as templates for PCR. Based on the average size of the BHV-5 genome (137 kb), an estimate of the number of genome copies detected in the PCR was calculated (18).

Results

Kinetics of virus replication in rabbit cells

As the recombinants have been amplified and characterized in bovine cells (MDBK) and BoHV-5gEΔ and BoHV-5gEΔTKΔ produced slightly smaller plaques, before inoculation into rabbits we investigated their ability to replicate in cultured rabbit cells. An one step

growth curve experiment performed in RK-13 cells demonstrated that the recombinants BoHV-5TKΔ and BoHV-5gEΔ replicated with similar kinetics and to equivalent titers to those of the parental virus in rabbit cells (not shown). The double deletion mutant (BoHV-5gEΔTKΔ) replicated with a slightly lower kinetics and to a lower titer. In a previous experiment in MDBK cells, the three recombinants were shown to retain their ability to replicate with about the same efficiency as the parental virus (31). Thus, a possible impairment in replicating in rabbits *in vivo* would not be attributable to a gross defect in replicating in rabbit cells.

Animal experiment – acute infection

All rabbits inoculated with the parental virus SV507/99 shed virus in nasal secretions (between days 2 and 8 pi) and developed typical neurological signs (Table 1). The neurological disease started from day 7 to 13 pi, depending on the animal, and was characterized by depression/excitation, ptialism, bruxism, opisthotonus, seizures and blindness in some cases. Animals showing severe neurological signs were euthanized.

Rabbits inoculated with the recombinant BoHV-5gEΔ shed virus between days 2 and 8 and three (# 1, 2 and 7) developed neurological signs similar to those of the rabbits inoculated with the parental virus. These rabbits were euthanized *in extremis* at days 10 and 15 pi. Infectious virus was not recovered from nasal swabs collected from rabbit # 7, yet infectivity was detected in several areas of the brain of this animal (Table 2). The rabbits surviving acute infection developed VN titers from 8 to 256 at day 42 pi.

The group inoculated with the recombinant BoHV-5TKΔ remained healthy throughout acute infection. Virus shedding was detected in 6/8 rabbits and lasted from one to eight days (Table 1). These rabbits, including those from which virus shedding was not detected (# 1 and 4) developed VN titers from 2 to 64 in sera collected at day 42 pi, indicating they were efficiently infected.

None of the rabbits inoculated with the double mutant (BoHV-5gEΔTKΔ) developed clinical signs during the period of monitoring. Virus shedding (in swabs collected every two days) was detected in 3 out of 8 animals. All inoculated animals seroconverted by day 42 pi (titers ranging from 2 to 32), indicating that virus replication took place.

These results showed that the recombinants BoHV-5TKΔ and BoHV-5gEΔTKΔ were fully attenuated for rabbits upon IN inoculation. Apparently, these viruses (especially the double mutant) replicated with a lower efficiency in the nasal mucosa. Nevertheless, data of serology demonstrated that the viruses replicated in all animals in levels that sufficed to stimulate the immune response. In contrast, the recombinant BoHV-5gEΔ retained part of its neurovirulence, producing neurological disease in 3 out of 8 rabbits. The duration of virus shedding by this recombinant in most animals was similar to that of the parental virus and virus replication resulted in moderate to high VN titers (Table 1).

Confirming the identity of virus shed by inoculated rabbits

The experimental groups were maintained in separate cages without direct or indirect contact among them to avoid possible cross-infection. Nonetheless, in order to confirm the identity of each recombinant virus being shed for their respective groups, nasal secretions collected daily from the animals of each group were pooled, submitted to DNA extraction and to a specific PCR designed to detect each and both gene deletions (31). This procedure was repeated from day 2 to 8 pi, the period of virus shedding by most animals. A representative result of such PCRs is shown on Figure 1. Nasal secretions of parental, SV507/99 strain-inoculated rabbits contained virus with both deleted genes; individual (gEΔ, TKΔ) and double deletions (gEΔTKΔ) were detected in nasal secretions collected from the respective groups. These results showed that each group excreted the respective virus and discarded any possible cross-infection among the groups.

Distribution of parental virus and recombinant BoHV-5gEΔ in the brain

Three out of eight rabbits inoculated with the recombinant BoHV-5gEΔ developed neurological disease clinically undistinguishable to that developed by rabbits inoculated with the parental virus. In order to determine the extent of dissemination and distribution of each virus, brain sections of rabbits from both groups (SV507/99 and BoHV-5gEΔ) were submitted to virus isolation. As shown in Table 2, the parental virus was more widely distributed in the brain than the recombinant virus. It reached deeper areas of the brain, considering the olfactory pathway of virus invasion, being detected in all frontal structures, cortices, thalamus and also in the brain stem. In contrast, the recombinant virus was detected in the anterior, median sections and barely reached the posterior areas. These results demonstrated that the recombinant BoHV-5gEΔ invaded and replicated within the brain yet showed a relatively restricted distribution compared to the parental virus.

Latent infection – attempts of reactivation and molecular detection

At day 42 pi, all rabbits that survived the acute infection with the recombinants BoHV-5gEΔ (n=5), BoHV-5TKΔ (n=8) and BoHV-5gEΔTKΔ (n=8) were submitted to Dx treatment trying to reactivate a putative latent infection. At that day, all inoculated animals had VN antibodies, in titers ranging from 2 to 256, indicating they harbored virus replication in the nose during acute infection. However, five daily administrations of Dx to these animals did not result in detectable virus shedding or in increase in VN titers (Table 3). Likewise, none of the animals showed clinical signs indicative of clinical recrudescence. Thus, these results demonstrated the recombinants were not reactivated upon Dx treatment. Since all rabbits inoculated with the parental virus died during acute infection, reactivation attempts were not possible. Nevertheless, previous studies have achieved virus reactivation by Dx treatment ranging from 56.8 to 100% of the BoHV-5 inoculated rabbits, even those inoculated with relatively low virus titers ($10^{6.5-7.0}$ $TCID_{50}$) (36, 37).

The BoHV-5TKΔ recombinant, and to a lesser extent the BoHV-5gEΔTKΔ virus, replicated with an apparent low efficiency in the nose during acute infection – in spite of the VN titers developed by these animals at day 42 pi – and were not reactivated upon Dx treatment. Then, we argued whether acute replication by these viruses – and by recombinant BoHV-5gEΔ as well, was sufficient to assure the establishment of latency. Then, a nested PCR (the test sensitivity was estimated to be around 10 to 100 genome copies per reaction) performed on total DNA extracted from the TGs of rabbits from groups inoculated with each single mutant (gEΔ and TKΔ) confirmed they all harbored latent viral DNA, albeit it failed in detecting viral DNA in the TGs of 7 out of 8 rabbits of the group inoculated with the double mutant (Table 3). A second round of PCR was then performed on total DNA extracted from the olfactory bulbs (OB) of this last group. This PCR confirmed the presence of latent viral DNA in the OB of all rabbits inoculated with the double mutant (Table 3). Previous studies have demonstrated the presence of BoHV-5 latent DNA in the BOs of experimentally infected calves (9) and rabbits (36). Taken together these results showed that all three recombinants were able to establish latent infection in the TGs and/or in the OB of inoculated rabbits, yet they were not reactivated by Dx treatment.

Discussion

We herein investigated the virulence of three BoHV-5 recombinants in rabbits. The recombinants defective in the glycoprotein E (BoHV-5gEΔ), enzyme TK (BoHV-5TKΔ) and both TK and gE (BoHV-5gEΔTKΔ) were constructed out of a neurovirulent Brazilian BoHV-5 strain (SV507/99) as part of a strategy to produce an attenuated, differential vaccine for use in South America. The recombinant BoHV-5TKΔ and the double deletion mutant (BoHV-5gEΔTKΔ) were fully attenuated for rabbits after IN inoculation. These viruses – especially the double mutant -replicated less efficiently than the parental virus in the nasal cavity yet

were able to induce a VN antibody response in the inoculated rabbits. In contrast, the recombinant defective in gE (BoHV-5gEΔ) retained partially its neurovirulence for rabbits. All three recombinants conserved their ability to establish latent infection in neural tissues (TGs and/or OBs) albeit were not capable of reactivating from latency upon Dx administration. These results open the way for further testing the recombinants BoHV-5TKΔ and BoHV-5gEΔTKΔ in cattle towards a potential use of these viruses as vaccine strains. These recombinants may also be useful to study the role of each deleted gene product in the biology of BoHV-5 infection and its interaction with the host.

The pathogenesis of BoHV-5 neurological disease in cattle is still poorly understood and several aspects of acute and latent infection have been studied in a rabbit model (32, 34). The neurological infection involves an initial viral replication in the site of entry (nasal epithelium), axonal transport to and replication in second, third and fourth-order neurons in several areas of the brain (38). Regardless the biological and molecular mechanisms underlying the development of neurological disease – which are merely hypothetical at this time -, massive virus replication in several areas of the brain is a consistent finding during acute BoHV-5 infection in rabbits and calves (34, 38, 39). Thus, the full expression of BoHV-5 neurovirulence likely depends upon the ability of the virus to reach the brain from peripheric sites (neuroinvasiveness) and to replicate to high titers in neuronal cells (neurogrowth).

Based on pathogenesis studies with other neuropathogenic alphaherpesviruses - HSV, PRV, BoHV-1 and BoHV-5 as well - the envelope glycoprotein E (gE) plays an important role in virus transport along circuits of synaptically connected neurons and, consequently, influence virus invasion of the brain (17). Glycoprotein E-defective BoHV-5 strains show reduced neuroinvasiveness and neurovirulence in rabbits (17); while a BoHV-1 gE-negative recombinant is poorly transported anterogradely from the TGs to the nose after virus

reactivation (18, 40). Deletion of gE in BoHV-1 strains is associated with significant – yet partial - attenuation for calves and has been also used as the antigenic marker in differential vaccines (16). Our recombinant BoHV-5gEΔ replicated efficiently in the nose and was partially neurovirulent for rabbits, producing neurological disease in 3/8 animals. Although the recombinant was less widely distributed in the brain than the parental virus, it was able to reach and replicate in the anterior areas of the CNS. A similar phenotype has been observed for other BoHV-5 gE negative recombinant (17). Virus replication in these anterior areas has been implicated in the production of seizures, a hallmark of BoHV-5 neurological infection (38). Thus, gE deletion in the strain SV507/99 reduced the virulence but did not suffice to abolish the ability of the virus to invade and replicate in the brain of rabbits producing neurological disease. This recombinant established latent infection in TG/OB albeit it was not reactivated upon Dx treatment. The lack of excretion of this virus after Dx treatment – taken as indicative of virus reactivation - may be related to deficient anterograde axonal transport as demonstrated for a gE-negative BoHV-1 strain in calves (18, 40). Alternatively, this virus was not reactivated in the ganglia by corticosteroid treatment. Further studies will determine the impact of gE deletion on the phenotype of this virus in cattle and whether a single gE- mutant is sufficiently attenuated for use as a live vaccine.

The alphaherpesvirus TK is an enzyme involved in the metabolism of deoxyribonucleotides (dNTPs) and is absolutely necessary for efficient viral replication in non-dividing cells such as neurons (28). Consequently, TK activity is required for efficient replication in neurons during acute infection and reactivation from latency but is not necessary for virus replication in epithelial cells or in cell culture. PRV, HSV - and to a lesser extent BoHV-1 - defective in TK gene product display reduced virulence and a relative inability to reactivate from latent infection (12, 25, 28, 30). As BoHV-5 neuropathogenesis is associated which virus replication and dissemination within the brain, we assumed that

functional TK would be necessary for the full expression of virus neurovirulence *in vivo*. Our results confirmed this prediction: deletion of TK gene in SV507/99 resulted in a dramatic reduction in neurovirulence for rabbits. In a previous study, our group reported the production of a brivudin-resistant BoHV-5 variant – likely defective in TK activity – that displayed a similar, attenuated phenotype in rabbits (31). Studies to determine whether these mutants are impaired in invasion, dissemination or productive replication in the brain are underway and may shed light on the exact role of TK in the biology of BoHV-5 and the effects of its deletion on the viral phenotype. Thus, our BoHV-5TKΔ recombinant was shown to be fully attenuated for rabbits after IN inoculation and would be worthwhile to investigate whether it presents the same phenotype in cattle.

On the other hand, a search for BoHV-5TKΔ DNA by PCR at day 28 pDx demonstrated the presence of latent viral DNA in TGs, demonstrating that the recombinant virus was capable of reaching these sites during acute infection. However, the TK-negative virus was not reactivated from latent infection, confirming findings observed for other TK-defective alphaherpesviruses (22, 23, 25). The lack of reactivation by TK-defective alphaherpesvirus is probably related to their inability to replicate productively in neurons, a condition necessary for virus reactivation and excretion (21).

The double deletion mutant BoHV-5gEΔTKΔ was also fully attenuated for rabbits. The replication of this recombinant in the nose was noticeably reduced what could partially explain its inability to produce neurological disease. Nevertheless, the relatively low replication levels in the nose did not abolish the ability of the virus to induce a VN response. Whether this magnitude of VN response would protect upon challenge is not known and would deserve investigation. Virus replication at the site of entry also sufficed to assure virus transport to the OB and/or TGs where latent infection was established. Again, as demonstrated for the other two recombinants, the double mutant was not capable of

reactivating the latent infection, probably reflecting a combined deleterious effect of lacking both TK and gE. Thus, this recombinant would be a candidate to be used as an attenuated, antigenically marked vaccine strain for cattle. Subsequent studies are needed to determine the replication efficiency, attenuation and immunogenicity of this recombinant in cattle.

In summary, the BoHV-5 recombinants defective in TK alone or in combination with gE are attenuated for rabbits and constitute potential vaccine candidates, depending upon the confirmation of this phenotype in cattle. The single mutant gE- retained part of its virulence for rabbits yet its phenotype awaits confirmation in cattle before discarding it for vaccine use. Regardless their potential use in vaccine formulations, the recombinants may represent useful tools to study the function of each gene product in the biology of BoHV-5 infection.

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Table 1- Virological, clinical and serological findings during acute infection in rabbits inoculated with parental virus and recombinants of bovine herpesvírus 5 (BoHV-5).

Strain	Animal #	Viral shedding[a] (day pi)	Neurological disease (day pi)	VN antibodies[b] (42 pi)
SV-507/99	1	2-8	+[c] (8)[d]	nt[e]
	2	2-8	+ (8)	nt
	3	2-6	+ (13)	nt
	4	2-6	+ (13)	nt
	5	2-6	+ (7)	nt
	6	2-6	+ (8)	nt
	7	2-8	+ (10)	nt
	8	2-8	+ (9)	nt
gEΔ	1	2-4	+ (15)	nt
	2	2-6	+ (10)	nt
	3	2-6	-[f]	256
	4	2-8	-	256
	5	4-6	-	256
	6	2-6	-	32
	7	-	+ (10)	nt
	8	4-8	-	8
TKΔ	1	-	-	32
	2	8	-	64
	3	6	-	16
	4	-	-	32
	5	4-8	-	64
	6	4-8	-	32
	7	4	-	32
	8	2-10	-	2
gEΔTKΔ	1	6	-	2
	2	4	-	2
	3	-	-	16
	4	-	-	16
	5	6	-	8
	6	-	-	2
	7	-	-	4
	8	-	-	32

[a] Period of virus shedding in nasal secretions;
[b] VN titers expressed as the reciprocal of the highest serum dilution capable of previning virus replication;
[c] Development of neurological signs;
[d] Day post-inoculation in which the animals died or were submitted to euthanasia;
[e] Not tested;
[f] Absence of virus shedding or neurological signs.

Table 2- Distribution of infectivity in the brain of rabbits inoculated with the parental virus or with the recombinant BoHV-5gEΔ.

Virus	Animal #	Brain area[a]									
		OB/OC	AC	VLC	DLC	PC	Th	TG	MO	PO	MB
SV 507/99	1	+[b]	+	+	+	+	+	-[c]	+	+	+
	2	+	+	+	-	-	-	-	-	+	-
	3	+	-	+	-	-	+	-	-	-	-
	4	-	+	+	+	+	-	-	+	-	+
	5	+	+	+	-	+	+	-	+	-	+
	6	-	+	-	+	-	+	-	-	+	+
	7	-	-	+	-	-	-	-	-	-	-
	8	+	+	+	+	+	+	-	+	+	+
gEΔ	1	-	-	+	-	-	-	-	-	-	-
	2	+	+	+	+	+	+	-	-	-	-
	7	+	+	+	+	+	+	-	-	-	-

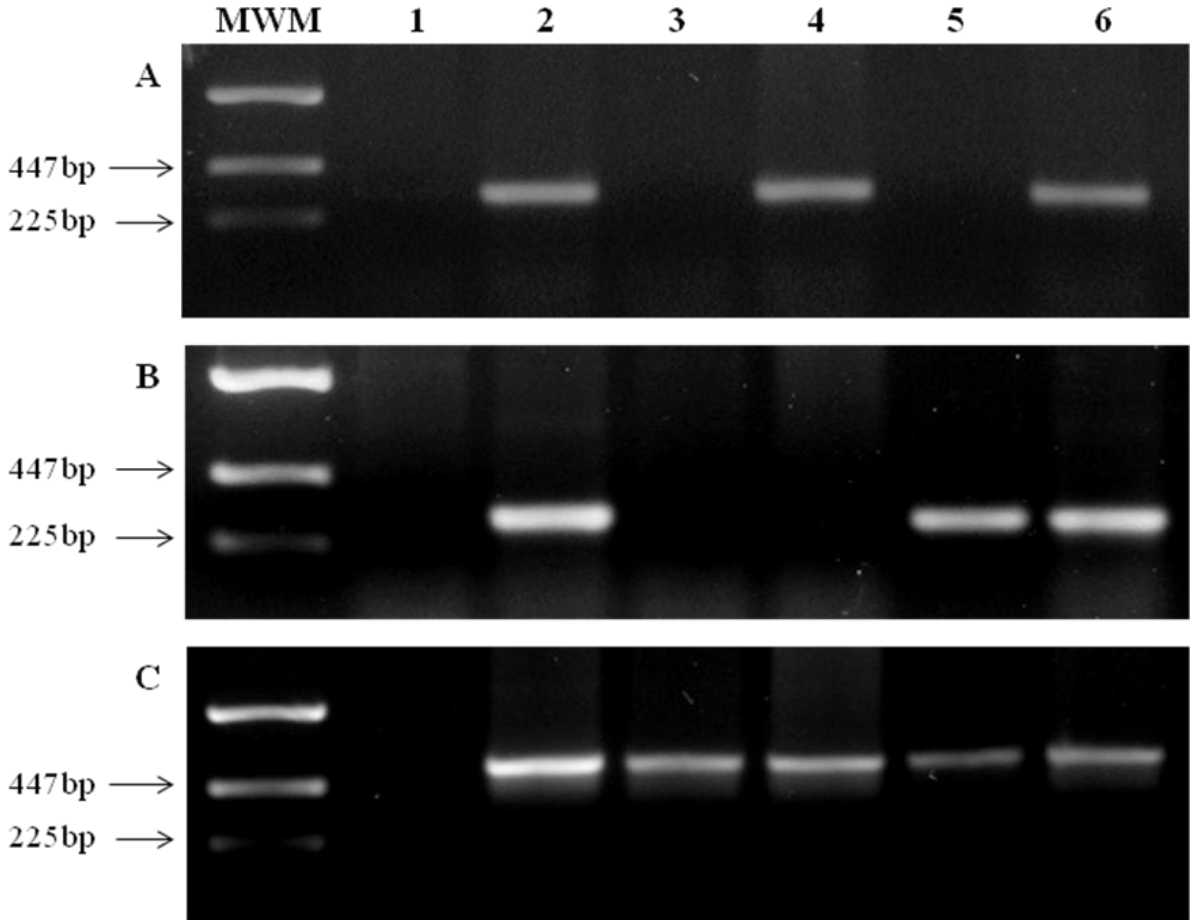
[a] OB/OC: olfactory bulb/olfactory cortex; AC: anterior cortex; VLC: ventrolateral cortex; DLC: dorsolateral cortex; PC: posterior cortex; Th: thalamus; TG: trigeminal ganglia; MO: medula oblongata; PO: pons; MB: midbrain.
[b] Positive for infectivity;
[c] Negative for infectivity.

Table 3- Findings after dexamethasone (Dx) administration in rabbits inoculated with the parental virus and recombinants of bovine herpesvirus 5 (BoHV-5).

Strain	Animal	Viral shedding (pDx)	Neurological disease	VN antibodies[a]		Viral DNA (28 pDx)	
				42 pi/0 pDx	28 pDx	TG[b]	OB[c]
gEΔ	3	-[d]	-	> 256	>256	+[e]	nt[f]
	4	-	-	>256	64	+	nt
	5	-	-	> 256	128	+	nt
	6	-	-	32	16	+	nt
	8	-	-	8	2		
TKΔ	1	-	-	32	64	+	nt
	2	-	-	64	32	+	nt
	3	-	-	16	32	+	nt
	4	-	-	32	8	+	nt
	5	-	-	64	4	+	nt
	6	-	-	32	4	+	nt
	7	-	-	32	16	+	nt
	8	-	-	2	2	+	nt
gEΔTKΔ	2	-	-	2	-	-	+
	3	-	-	16	2	-	+
	4	-	-	16	2	-	+
	5	-	-	8	2	-	+
	6	-	-	2	2	-	+
	7	-	-	4	-	+	nt
	8	-	-	32	2	-	+

[a] VN titers expressed as the reciprocal of the highest serum dilution capable of previning virus replication;
[b] Trigeminal ganglia;
[c] Olfactory bulb (tested only in animals whose TG was negative for viral DNA by PCR);
[d] Negative in the respective test;
[e] Positive for viral DNA by PCR;
[f] Not tested.

Figure 1. Illustrative results of a PCR performed to confirm the identity of viruses shed in nasal secretions by rabbits of the respective groups. Panel A. PCR for the TK gene (285 bp). Panel B. PCR for the glycoprotein E gene (269 bp). Panel C. PCR for the glycoprotein B gene (control) (444 bp). MWM: molecular weight marker. Lane 1: negative control (DNA template from the brain of a BoHV-5 seronegative cow); lane 2: positive control, DNA extracted from the supernatant CRIB cells infected with the parental virus; lane 3-6: pool of nasal secretions of rabbits inoculated with recombinants - double mutant BoHV-5gEΔTKΔ (lane 3); BoHV-5gEΔ (lane 4); BoHV-5TKΔ (lane 5); parental virus (SV507/99; lane 6). Ethidium bromide stained 1.5% agarose gel; the sizes of the corresponding markers are indicated



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