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

PRODUÇÃO E CARACTERIZAÇÃO DE CEPAS RECOMBINANTES DO HERPESVÍRUS BOVINO TIPO 5 DEFECTIVAS NA ENZIMA TIMIDINA QUINASE E GLICOPROTEÍNA E

TESE DE DOUTORADO

Mário Celso Sperotto Brum

Santa Maria, RS, Brasil
2009

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por

Mário Celso Sperotto Brum

Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Medicina Veterinária, Área de Concentração em Fisiopatologia da Reprodução da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de
Doutor em Medicina Veterinária

Orientador: Prof. Rudi Weiblen

Santa Maria, RS, Brasil.
2009

Universidade Federal de Santa Maria
Centro de Ciências Rurais
Programa de Pós-Graduação em Medicina Veterinária
Departamento de Medicina Veterinária Preventiva

A Comissão Examinadora, abaixo assinada,
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Elaborada por
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Como requisito parcial para obtenção do grau de
Doutor em Medicina Veterinária

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AGRADECIMENTOS

Aos professores Rudi Weiblen, Eduardo F. Flores e Luciane T. Lovato pela oportunidade, incentivo, orientação e exemplos de dedicação ao trabalho.

Ao prof. Dr. Shafiqul I. Chowdhury, *Departament of Veterinary Medicine Diagnostic/Pathobiology, Kansas State University* pela oportunidade e orientação essencial para desenvolver parte do projeto no seu laboratório. Aos colegas de laboratório Sajid Mahmood, Ying Wang, Zengh Fei Liu e Charles Coats pelo auxílio e momentos de descontração no período da estada no Kansas.

A todos os colegas, bolsistas, mestrandos, doutorandos e funcionários do Setor de Virologia da UFSM pelo auxílio nos experimentos, companheirismo e amizade.

Aos demais professores e laboratórios da UFSM que de alguma maneira auxiliaram na realização do projeto ou que compartilharam reagentes e equipamentos.

Aos meus familiares pelo amor, confiança e compreensão.

À Daniela S. da Silva pelo carinho, compreensão e apoio incondicional.

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) e ao Conselho de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, programa PDEE/UFSM) pelas concessões das bolsas de doutorado e de estágio de doutoramento no exterior.

À Pró-Reitoria de Pós-Graduação e Pesquisa, e especialmente a Sra. Maria Regina Roso pela dedicação e empenho em auxiliar.

À Universidade Federal de Santa Maria e ao Programa de Pós-Graduação em Medicina Veterinária pela educação e formação.

RESUMO
Tese de Doutorado
Programa de Pós-Graduação em Medicina Veterinária
Universidade Federal de Santa Maria

PRODUÇÃO E CARACTERIZAÇÃO DE CEPAS RECOMBINANTES DO HERPESVÍRUS BOVINO TIPO 5 DEFECTIVAS NA ENZIMA TIMIDINA QUINASE E GLICOPROTEÍNA E
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ORIENTADOR: RUDI WEIBLEN
Santa Maria, 2 de março de 2009.

O herpesvírus bovino tipo 5 (BoHV-5) é um alfaherpesvírus associado com meningoencefalite, uma doença de grande importância em bovinos na América do Sul. Vacinas atenuadas por manipulação genética e contendo marcadores antigênicos – que permitem a diferenciação sorológica entre animais vacinados e infectados naturalmente – têm sido utilizadas com sucesso no controle e erradicação de infecções por herpesvírus suínos e bovinos em vários países. Como em outros alfaherpesvirus, a enzima timidina quinase (TK) e glicoproteína E (gE) estão relacionados com a patogenicidade e virulência do BoHV-5. Assim, o presente trabalho teve como objetivo produzir e caracterizar cepas do BoHV-5 defectivas na TK e gE a partir de uma amostra brasileira (SV507/99). Na primeira parte do estudo foram selecionadas variantes resistentes a brivudin (BVDU), um análogo de nucleosídeo que seleciona vírus defectivos na atividade TK. Um clone viral resistente ao BVDU (BoHV-5/R-27) demonstrou ser geneticamente estável *in vitro*, replicou com a mesma cinética e produziu placas com diâmetro e morfologia similares às da cepa parental. A atenuação do variante BoHV-5/R-27 foi confirmada pela inoculação intranasal em coelhos, nos quais não produziu enfermidade. Os resultados demonstram que variantes do BoHV-5 resistentes ao BVDU podem ser selecionados e que mantém a capacidade de replicar em cultivo celular e são atenuados para coelhos. Na segunda parte do trabalho, três cepas recombinantes do BoHV-5 defectivas nos genes da gE, TK e ambos foram construídas a partir da cepa parental SV507/99 e caracterizadas *in vitro*. A técnica de recombinação homóloga foi utilizada para substituir integral ou parcialmente a região codificante dos genes da gE e TK pelos genes da proteína verde fluorescente (GFP) ou betagalactosidase (β-gal), respectivamente. A deleção das sequências da gE e TK foram confirmadas por *immunoblotting* e PCR, respectivamente. A caracterização *in vitro* das cepas recombinantes (BoHV-5 gEΔ; BoHV-5 TKΔ e BoHV-5 gE/TKΔ) demonstrou que as deleções não alteraram a capacidade de replicação em cultivo celular, embora as cepas BoHV-5 gEΔ e BoHV-5

gE/TKΔ produzissem placas menores do que a cepas BoHV-5 TKΔ e parental. Desta maneira, demonstrou-se que os vírus recombinantes são biologicamente viáveis, podendo ser utilizados em estudo de patogenia ou como candidatos a cepas vacinais. Na terceira parte do estudo foi avaliada a imunogenicidade em bovinos de duas formulações vacinais inativadas oleosas, produzidas a partir de cultivos celulares infectados com a cepa parental (SV507/99) ou recombinante BoHV-5 gE/TKΔ. Para isso, quarenta bovinos (20 animais por grupo) foram vacinados nos dias 0 e 22 pós-vacinação (pv) e amostras de soro, coletadas a diferentes intervalos pós-vacinação, foram testadas para anticorpos neutralizantes contra a cepa homóloga e frente a diferentes isolados de BoHV-5 e herpesvírus bovino tipo 1 (BoHV-1). Todos os animais vacinados soroconverteram após a segunda dose da vacina (títulos médio de 17,5 para o grupo SV507/99 e 24,1 para o grupo BoHV-5 gE/TKΔ) e os níveis de anticorpos neutralizantes foram detectados até o final do experimento (dia 116 pv), porém com redução gradual. A sorologia cruzada com amostras heterólogas do BoHV-5 e BoHV-1 indicou que ambos os grupos reagiram em níveis similares frente ao mesmo vírus, no entanto, com maior magnitude contra amostras de BoHV-5. Os resultados demonstram que a vacina experimental composta por antígenos do BoHV-5 gE/TKΔ recombinante induziu resposta sorológica em níveis semelhantes ao induzida pela vacina produzida a partir do vírus parental. Dessa forma, e considerando-se a gE como marcador sorológico negativo, essa cepa recombinante se constitui em uma alternativa para possível vacina diferencial. Em resumo, os resultados apresentam a obtenção e caracterização de cepas recombinantes do BoHV-5 defectivas em dois genes associados com virulência. Essas cepas podem ser utilizadas em estudos de patogênese e, sobretudo, se constituem em candidatos potenciais para a formulação de vacinas diferenciais.

Palavras-chave: herpesvírus recombinantes; vacina diferencial; patogenia.

ABSTRACT

Doctoral Thesis

Programa de Pós-Graduação em Medicina Veterinária

Universidade Federal de Santa Maria

PRODUCTION AND CHARACTERIZATION OF BOVINE HERPESVIRUS TYPE 5 RECOMBINANTS DEFECTIVE IN THE THYMIDINE KINASE AND GLYCOPROTEIN E GENES

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ADVISER: RUDI WEIBLEN

Santa Maria, March 2nd 2009.

Bovine herpesvirus type 5 (BoHV-5) is an alphaherpesvirus associated with meningoencephalitis, an important disease of cattle in South America. Differential modified live vaccines – that allow differentiation between vaccinated and naturally infected animals – have been used in programs for control and eradication of porcine and bovine herpesvirus infections in several countries. Like in other alphaherpesviruses, the enzyme thymidine kinase (TK) and the glycoprotein E (gE) are related with pathogenicity and virulence of BoHV-5. Thus, the present study aimed to produce and characterize BoHV-5 viruses defective in TK and gE genes out of a Brazilian wild-type strain (SV507/99). In the first part of this study, several BoHV-5 clones resistant to brivudin (BVDU), a nucleoside analog which select TK-deficient virus, were selected. One such clone (BoHV-5/R-27) demonstrated to be genetically stable *in vitro*, and displayed the same kinetics of replication, plaque size and morphology in cell monolayers compared to the parental strain. Moreover, BoHV-5/R-27 was shown to be attenuated for rabbits since no clinical signs were observed after intranasal inoculation. These results demonstrate that BoHV-5 is sensitive to BVDU; BVDU-resistant mutants can be selected, and the BoHV-5/R-27 resistant virus retained its ability to replicate in tissue culture and was attenuated for rabbits. In the second part of the study, three recombinants BoHV-5 strains defective in gE, TK and both genes were constructed using the strain SV507/99 as a background and were characterized *in vitro*. Homologous recombination in cell culture was used to delete and substitute the entire or part of the coding regions of gE and TK for the green fluorescent protein (GFP) and betagalactosidase (β-gal) genes, respectively. The absence of gE and TK genes was confirmed by immunoblotting and PCR, respectively. *In vitro* characterization of the recombinant viruses (BoHV-5 gEΔ, BoHV-5 TKΔ and BoHV-5 gE/TKΔ) demonstrated that the deletions did not affect their kinetics of replication when compared to the parental strain. However, recombinants BoHV-5 gEΔ and BoHV-5 gE/TKΔ

produced smaller plaques than BoHV-5 TKΔ and SV507/99. This study demonstrated the viability of the recombinant BoHV-5 viruses, which can be further used in pathogenesis studies and for vaccine development as well. The third part of this study evaluated the immunogenicity in cattle of two inactivated oil-adjuvanted vaccines containing SV507/99 or BoHV-5 gE/TKΔ infected cell cultures. For this, forty calves (20 animals/group) were vaccinated on days 0 and 22 post-vaccination (pv) and serum samples collected at different time points pv were tested for neutralizing antibodies against the homologous BoHV-5 strain and against several heterologous BoHV-5 and bovine herpesvírus type 1 (BoHV-1) isolates. All vaccinated animals seroconverted after the second vaccination (mean titers of 17.5 for the SV507/99 group and 24.1 for the BoHV-5 gE/TKΔ group), and the neutralizing antibody titers remained up to day 116 pv, showing a gradual reduction. Cross-serology with heterologous BoHV-5 and BoHV-1 isolates indicated that both vaccinated groups reacted similarly to the same virus, but with higher magnitude against BoHV-5 isolates. These results demonstrated that the inactivated vaccine using the BoHV-5 gE/TKΔ recombinant induced a satisfactory serological response. Based on the fact that gE can be used as a negative serological marker, this strains can be an alternative as a differential vaccine. In summary, the presented results describe the production and characterization of BoHV-5 strains defective for two genes associated with virulence. The recombinant strains can be used in pathogenesis studies and constitute potential candidate for differential vaccines.

Keywords: recombinants herpesvirus; differential vaccines; pathogenesis.

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1. INTRODUÇÃO

O herpesvírus bovino tipo 5 (BoHV-5) é o agente etiológico da meningoencefalite não-supurativa em bovinos (ENGELS, ACKERMANN, 1996). Esse agente viral também tem sido isolado de amostras de sêmen, de casos de doença respiratória e de infecções generalizadas em bezerros jovens (SILVA et al., 2007b). O BoHV-5 apresenta grande similaridade molecular, biológica e antigênica com o herpesvírus bovino tipo 1 (BoHV-1), agente da rinotraqueíte infecciosa (IBR) e vulvovaginite/balanopostite pustular bovina (IPV/IBP) (BRATANICH et al., 1991; DELHON et al., 2003; SCHWYZER, ACKERMANN, 1996). A infecção e doença neurológica pelo BoHV-5 já foram descritas na Europa, Estados Unidos e Austrália, porém na Argentina, Brasil e Uruguai os relatos têm sido mais freqüentes (STUDDERT, 1989; SUAREZ HEINLEIN et al., 1993; WATT et al., 1981; WEIBLEN et al., 1989). A prevalência da infecção pelo BoHV-5 nestes países não é conhecida, sobretudo pela inexistência de testes sorológicos que o diferenciem do BoHV-1 (BRATANICH et al., 1991; TEIXIERA et al. 1998).

O BoHV-5 está classificado na família *Herpesviridae*, subfamília *Alphaherpesvirinae*, gênero *Varicellovirus* (ROIZMANN et al., 1992). Esse vírus apresenta um ciclo de replicação rápido e lítico *in vitro* e possui capacidade de infectar células epiteliais e nervosas e estabelecer latência em neurônios ganglionares *in vivo* (ROIZMANN et al., 1992; SCHWYZER, ACKERMANN, 1996). Os herpesvírus possuem vírions pleomórficos, com 150 a 200 nm de diâmetro, envelopados, que possuem como genoma uma molécula de DNA de fita dupla de aproximadamente 138 quilobases (Kb) (DELHON et al., 2003; ROIZMANN et al., 1992; SCHWYZER, ACKERMANN, 1996). Apesar das semelhanças genéticas e moleculares, a diferenciação entre o BoHV-1 e BoHV-5 pode ser realizada por análise de restrição genômica, pela reatividade com determinados anticorpos monoclonais e pela reação em cadeia da polimerase (PCR) com *primers* específicos, além de sequenciamento de alguns segmentos genômicos (ENGELS et al., 1986; METZLER et al., 1986).

O genoma do BoHV-5 contém aproximadamente 70 genes (DELHON et al., 2003), cuja expressão – a exemplo do que ocorre nos outros alfaherpesvírus - ocorre sob regulação temporal e pode ser dividida em genes de transcrição imediata (IE), iniciais (E) e tardios (L). O repertório de produtos gênicos inclui proteínas regulatórias da replicação viral, enzimas, proteínas estruturais e glicoproteínas (DELHON et al., 2003; SCHWYZER, ACKERMANN,

1996). No envelope viral encontram-se inseridas aproximadamente 11 proteínas, que são responsáveis pelas interações vírus-célula e são alvos para a resposta imunológica do hospedeiro. As glicoproteínas (g) são denominadas por letras (K, N, C, B, H, M, L, G, D, I e E) (DELHON et al., 2003; SCHWYZER, ACKERMANN, 1996). Aproximadamente a metade dos genes dos alfaherpesvírus pode ser deletada individualmente do genoma sem efeitos deletérios sobre a replicação viral *in vitro* e, por isso, esses genes são denominados não-essenciais. Em contrapartida, vários genes são absolutamente requeridos para a replicação viral em cultivo celular e são denominados genes essenciais (SCHWYZER, ACKERMANN, 1996).

Durante a replicação primária (aguda), o BoHV-5 infecta células epiteliais da mucosa respiratória e, em seguida, invade as terminações nervosas dos nervos sensoriais e autonômicos presentes na cavidade nasal, sobretudo as terminações do nervo trigêmeo e as ramificações do nervo olfatório (LEE et al., 1999; RISSI et al., 2006; SILVA et al., 1999b). A infecção destas terminações, principalmente da via olfatória, resulta no transporte das partículas virais no sentido retrógrado até o sistema nervoso central (SNC), onde a replicação e disseminação viral podem levar ao desenvolvimento de meningoencefalite acompanhada de doença neurológica ou ao estabelecimento da infecção latente (PEREZ et al., 2002; RISSI et al., 2006; VOGEL et al., 2003). A reativação da infecção latente pelo BoHV-5 – de forma similar com o que ocorre no BoHV-1 e outros alfaherpesvírus - ocorre naturalmente em situações de estresse ou por ser induzida artificialmente, pela administração de corticosteróides, e é seguida de excreção viral ocasionalmente acompanhada de recrudescência clínica (CARON et al., 2002; VOGEL et al., 2003). Os animais portadores da infecção latente se constituem nos principais reservatórios do vírus e são responsáveis pela introdução e disseminação do vírus em rebanhos e áreas livres (ACKERMANN, ENGELS, 2006; ENGELS, ACKERMANN, 1996).

Experimentalmente o BoHV-5 possui a capacidade de infectar várias espécies de mamíferos, incluindo bovinos, caprinos, ovinos, coelhos e camundongos (ABRIL et al., 2004; BELTRÃO et al., 2000; DIEL et al., 2007; SILVA et al., 1999a; SILVA et al., 1999b). No entanto, somente os bovinos são considerados os hospedeiros naturais da infecção. Por reproduzirem vários aspectos virológicos e clínicos da infecção aguda e latente, os coelhos têm sido utilizados como modelo experimental (LEE et al., 1999; SILVA et al., 1999b). O mecanismo da produção da doença neurológica parece estar associado com a replicação viral e progressão da infecção no encéfalo (LEE et al., 1999; SILVA et al., 1999b). Os sinais clínicos são comumente observados em animais com até 24 meses e iniciam entre 4 a 7 dias

após a infecção (PEREZ et al., 2002; RISSI et al., 2007). Inicialmente, observa-se depressão e secreção nasal e ocular, que progridem para emagrecimento, dificuldade respiratória, tremores, bruxismo, andar em círculos, incordenação, cegueira, nistagmo e disfagia (RISSI et al., 2006). No estágio mais avançado da enfermidade, os animais permanecem em decúbito, com movimentos de pedalagem, opistótomo, convulsões e morte (PEREZ et al., 2002; RISSI et al., 2007). As lesões macroscópicas nem sempre são evidentes, no entanto quando presentes observam-se principalmente hiperemia difusa dos vasos das leptomeninges e malacia das porções rostrais do telencéfalo (RISSI et al., 2006; RISSI et al., 2007). Histologicamente, os achados são característicos de meningoencefalite não-supurativa necrosante, composta por infiltrado linfoplasmocitário perivascular, necrose neuronal, neuronofagia e presença de corpúsculos de inclusão intranucleares em neurônios e astrócitos (RISSI et al., 2006; RISSI et al., 2007). Pelo uso de imunoistoquímica, pode-se observar a presença de proteínas virais em neurônios, células da glia e macrófagos (CARON et al., 2002; VOGEL et al., 2003).

A prevalência da infecção pelo BoHV-5 é desconhecida, isso deve-se a grande reatividade cruzada e a inexistência de um teste sorológico capaz de diferenciá-lo do BoHV-1 (TEIXIERA et al. 1998). A infecção clínica pelo BoHV-5 afeta principalmente animais jovens (até 24 meses de idade) e menos frequentemente animais adultos, apresenta-se na forma de surto ou em casos isolados (SALVADOR et al., 1998; RISSI et al., 2006). As situações em que ocorrem os surtos geralmente estão relacionadas com aumento da concentração de animais e/ou situações de estresse, como a introdução e movimentação dos animais, desmane, assinalação, vacinação e castração (RISSI et al., 2006; RISSI et al., 2007; SALVADOR et al., 1998). As medidas de controle da infecção pelo BoHV-5 são direcionadas para rebanhos onde ocorra atividade viral e casos clínicos são diagnosticados. Nesse sentido, medidas de manejo que segreguem os animais clinicamente afetados e diminuam a exposição dos animais susceptíveis são aconselhadas (RISSI et al., 2007). Os animais na fase latente são os responsáveis pela manutenção do vírus na propriedade e pela introdução do agente em áreas ou em rebanhos livres da infecção (ACKERMANN, ENGELS, 2006; VAN OIRSCHOT, 1999). Devido a marcante reatividade sorológica cruzada entre o BoHV-1 e BoHV-5, vacinação com vacinas contendo antígenos do BoHV-1 têm sido recomendadas (MUYLKENS et al., 2007; RISSI et al., 2007; VOGEL et al., 2002).

A vacinação é uma medida complementar ao manejo e amplamente utilizada no controle das infecções pelo BoHV-1 e herpesvírus suíno (PRV) (VAN OIRSCHOT, 1999; VAN OIRSCHOT et al., 1996b). Devido à importância que o BoHV-5 possui na América do

Sul e a existência de somente uma vacina específica para o BoHV-5, o emprego de vacinas para o BoHV-1 tem sido indicada no controle da infecção pelo BoHV-5 (RISSI et al., 2007). Os herpesvírus possuem características adequadas para o desenvolvimento de vacinas. Esses vírus apresentam multiplicação satisfatória em cultivos celulares, possuem várias glicoproteínas no envelope viral que são altamente imunogênicas e podem servir como marcadores antigênicos (ACKERMANN, ENGELS, 2006; TIKOO et al., 1995; VAN OIRSCHOT, 1999). Com base nessas características, foram desenvolvidas várias vacinas contra o BoHV-1, PRV e também para o BoHV-5 (FRANCO et al., 2007; VAN DRUNEN LITTEL-VAN DEN HURK, 2007; VAN OIRSCHOT, 1999; VAN OIRSCHOT et al., 1996b). Em geral, as vacinas para os herpesvírus podem ser classificadas em inativadas ou atenuadas, e convencionais ou diferenciais (VAN DRUNEN LITTEL-VAN DEN HURK, 2007; VAN OIRSCHOT et al., 1996b).

As vacinas convencionais (inativadas ou atenuadas) são amplamente utilizadas no controle das infecções pelo BoHV-1 (JONES, CHOWDHURY, 2007; VAN DRUNEN LITTEL-VAN DEN HURK, 2007; VAN OIRSCHOT, 1999). As cepas virais presentes nesse tipo de vacina são usualmente vírus de campo inativados por métodos químicos ou, no caso das vacinas atenuadas, são obtidas pela adaptação em cultivos celulares e/ou exposição a agentes mutagênicos (VAN OIRSCHOT, 1999; VAN OIRSCHOT et al., 1996b). As vacinas convencionais possuem eficácia satisfatória, sendo capazes de reduzir a severidade das infecções clínicas e a excreção viral (JONES, CHOWDHURY, 2007; VAN DRUNEN LITTEL-VAN DEN HURK, 2007). As vacinas inativadas, também denominadas de vacinas mortas, são seguras, não apresentam o risco de reversão à virulência, porém necessitam da adição de adjuvantes na sua formulação (VAN DRUNEN LITTEL-VAN DEN HURK, 2007; VAN OIRSCHOT, 1999). Estas vacinas, no entanto, estimulam preferencialmente resposta humoral e necessitam de reforços periódicos para manter níveis adequados de anticorpos. As vacinas atenuadas ou vivas modificadas possuem a vantagem de induzirem resposta imunológica humoral e celular de maior magnitude e duração (VAN DRUNEN LITTEL-VAN DEN HURK, 2007). Ocasionalmente estas vacinas podem reverter a virulência ou conter vírus adventícios como contaminantes e, por isso, não são recomendadas para aplicação em animais prenhes e imunodeprimidos (ACKERMANN, ENGELS, 2006; JONES, CHOWDHURY, 2007).

No ínicio da década de 90, surgiram as vacinas diferenciais para os herpesvírus (ACKERMANN, ENGELS, 2006; VAN OIRSCHOT, 1999). Estas vacinas são baseadas na deleção de um ou mais genes não-essenciais e são acompanhadas de um teste sorológico

capaz de diferenciar animais vacinados daqueles naturalmente infectados. As vacinas diferenciais podem ser inativadas, vivas ou de subunidades (VAN DRUNEN LITTEL-VAN DEN HURK, 2007). Os genes candidatos à deleção são principalmente enzimas relacionadas com a replicação do DNA e/ou glicoproteínas do envelope viral (VAN DRUNEN LITTEL-VAN DEN HURK, 2007). Desta forma, além da redução da virulência, essas deleções podem servir como marcadores antigênicos (TIKOO et al., 1995; VAN DRUNEN LITTEL-VAN DEN HURK, 2007; VAN OIRSCHOT, 1999). A obtenção de vírus recombinantes com deleção ou inativação de um ou mais genes pode ser realizada pelo uso de drogas (MITTAL, FIELD, 1989; WAGNER et al., 2002; ZYGRAICH et al., 1974) ou pela manipulação do genoma por técnicas de recombinação (CHOWDHURY, 1996; VAN OIRSCHOT, 1999; VAN OIRSCHOT et al., 1996a).

Vários são os genes candidatos para deleção como objetivo de atenuação e de marcação antigênica. A inativação do gene da enzima timidina quinase (TK) reduz a virulência do vírus, pois esta enzima está relacionada com a fosforilação de nucleotídeos necessários à síntese de DNA viral em neurônios (MITTAL, FIELD, 1989). As glicoproteínas não-essenciais do envelope são candidatos adequados para a deleção com o objetivo de produzir um marcador antigênico. Várias glicoproteínas (gG, gI, gC e gE) já foram deletadas e testadas na produção de vacinas (KAASHOEK et al., 1998; VAN OIRSCHOT, 1999). A deleção das glicoproteínas gC e gE, muitas vezes associada com a inativação do gene TK, apresentaram os melhores resultados (FLORES et al., 1993; KAASHOEK et al., 1995; KAASHOEK et al., 1996; VAN OIRSCHOT, 1999).

No mercado brasileiro encontram-se disponíveis oito vacinas para o BoHV-1. Sete destes imunógenos são inativados e um é constituído por um vírus atenuado termo-sensível (SILVA et al., 2007a; VOGEL et al., 2002). No entanto, todas as formulações são constituídas por vacinais convencionais, o que impossibilita a diferenciação entre animais vacinados e infectados. Em uma destas vacinas inativadas, além da amostra de BoHV-1, está presente um isolado de BoHV-5 (FLORES, E. F. comunicação pessoal). A avaliação da imunogenicidade das vacinas comerciais foi testada em bovinos, e foi demonstrado que a maioria das vacinas não induziu níveis satisfatórios de anticorpos neutralizantes contra o BoHV-1 e BoHV-5 (SILVA et al., 2007a; VOGEL et al., 2002). No Brasil, o desenvolvimento de cepas do BoHV-1 e -5 recombinantes para utilização como vacina diferencial foi inicialmente realizado e encontra-se em fase experimental (FRANCO et al., 2002; FRANCO et al., 2007; HUBNER et al., 2005; SILVA et al., 2006).

No presente trabalho são descritos experimentos realizados para a produção e caracterização de cepas do BoHV-5 defectivas nos genes da gE e da TK para uso em vacinas. O Capítulo 1 relata a seleção de uma amostra do BoHV-5 resistente ao brivudin, possivelmente defectiva para o gene da TK, e a sua caracterização *in vitro* e *in vivo*. No Capítulo 2, são descritas a construção por recombinação homóloga e caracterização *in vitro* de três cepas recombinantes do BoHV-5, defectivas nos genes da gE, TK e ambos. O Capítulo 3 relata a avaliação da imunogenicidade de antígenos inativados da cepa de BoHV-5 gE/TKΔ em bovinos, pela mensuração da atividade neutralizante desenvolvida em resposta à vacinação.

2. CAPÍTULO 1

Selection and characterization of brivudin resistant bovine herpesvirus type 5

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(Artigo submetido ao periódico *Brazilian Journal of Microbiology*, novembro de 2008)

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Abstract

Bovine herpesvirus type 5 (BoHV-5) is the agent of meningoencephalitis, an important disease of cattle in South America. The neuropathogenesis of BoHV-5 infection is poorly understood and most previous research focused on the role of envelope glicoproteins in neurovirulence. Thymidine kinase (TK) is a viral enzyme necessary for virus replication in neurons and, therefore, represents a potential target for virus attenuation. The selection and characterization of BoHV-5 variants resistant to the nucleoside analog brivudin (BVDU), which selects TK-defective viruses is here described. Several BVDU-resistant clones were obtained after multiple passages in tissue culture in the presence of BVDU and one clone (BoHV-5/R-27) was further characterized. The selected clone replicated to similar titers and produced plaques with similar size and morphology to those of wild-type virus (SV507/99). The genetic stability of the resistant virus was demonstrated after ten passages in cell culture in the absence of the drug. Moreover, the drug-resistant virus showed reduced virulence in a rabbit model: virus inoculation in four rabbits did not result in disease, in contrast with 75% morbidity (3/4) and 50% mortality (2/2) among rabbits inoculated with the parental virus. These results demonstrate that BoHV-5 is sensitive to BVDU and that drug-resistant mutants can be readily selected upon BVDU treatment. BVDU-resistant mutants, likely defective in TK, retained their ability to replicate in tissue culture yet were attenuated for rabbits. This strategy to obtain TK-defective BoHV-5 may be useful to study the role of TK in BoHV-5 neuropathogenesis and for vaccine development.

Key-words: BoHV-5, thymidine kinase, drug resistant, brivudin

Seleção e caracterização de herpesvírus bovino tipo 5 resistente ao brivudin

Resumo

O herpesvírus bovino tipo 5 (BoHV-5) é o agente de meningoencefalite, uma doença de grande importância em bovinos na América do Sul. A neuropatogênese do BoHV-5 não está completamente definida e grande parte dos trabalhos tem estudado o papel das glicoproteínas do envelope na neurovirulência. A enzima viral timidina quinase (TK) é necessária para a replicação do vírus em neurônios, representando assim, um potencial candidato para atenuação. O presente trabalho descreve a seleção e caracterização de variantes do BoHV-5 resistentes ao brivudin (BVDU), um análogo de nucleosídeo que seleciona vírus deficientes na TK. Clones resistentes foram obtidos após duas passagens do vírus em cultivo na presença de BVDU e um clone (BoHV-5/R-27) foi escolhido para a caracterização. A amostra BoHV-5/R-27 replicou com cinética semelhante e produziu placas de tamanho e morfologia similar quando comparada com a cepa parental (SV507/99). A estabilidade genética da cepa resistente foi constatada após dez passagens em cultivo celular sem a presença do agente químico. A inoculação de coelhos com a cepa BoHV-5/R-27 não produziu doença, contrastando com coelhos inoculados a cepa original, quando observou-se morbidade de 75% (3/4) e mortalidade em 50% (2/4) dos coelhos. Os resultados demonstram que o BoHV-5 é sensível ao BVDU e que amostras resistentes podem ser selecionadas pelo cultivo viral na presença do agente químico. A cepa resistente, provavelmente deficiente na enzima TK, manteve a capacidade de replicar em cultivo celular e foi atenuada em coelhos. Esta estratégia para obtenção de amostras do BoHV-5 deficiente no gene da TK pode ser útil no estudo do papel desta enzima na patogenia do BoHV-5 e também para o desenvolvimento de vacinas.

Palavras-chave: BoHV-5, timidina quinase, resistente, brivudin

Introduction

Bovine herpesvirus type 5 (BoHV-5) is the agent of necrotizing meningoencephalitis, but sporadically has been also isolated from cases of genital disease, abortions and systemic infection in calves (23). Unlike bovine herpesvirus type 1 (BoHV-1), BoHV-5 seems to have a more limited geographic distribution and has been frequently isolated from outbreaks of neurological disease in South American countries such as Brazil, Argentina and Uruguay (22, 25, 30).

BoHV-5 belongs to the subfamily *Alphaherpesvirinae*, genus *Varicellovirus* (20). Its genome consists of a linear double stranded DNA molecule with approximately 138kb in length (9, 20). The viral genome encodes around 70 different polypeptides, which include glycoproteins, structural, regulatory proteins and enzymes (9). Like all known herpesviruses, BoHV-5 encodes its own thymidine kinase (TK), which based on the homology with human herpes simplex virus (HSV) and BoHV-1, is an enzyme involved in the metabolism of deoxyribonucleotides in non-replicating cells, such as neurons (9, 24, 26). The role of TK in the herpesvirus replication is to supply a pool of deoxyribonucleotides for viral DNA synthesis in non dividing cells such as neurons (26, 31). As TK is not required for virus replication in cell culture, its gene has been classified as non-essential. Nonetheless, TK activity is necessary for the full expression of virulence *in vivo* and its deletion from HSV-1, BoHV-1 and pseudorabies virus (PRV) genomes is associated with significant reduction in virulence (1, 3, 16, 27). Therefore, TK deletion has become an attractive target for gene manipulation of herpesviruses aiming to obtain attenuated viruses for vaccine use (3, 10, 17, 26, 28).

The ability to establish latent infection seems not to be affected in TK-negative viruses, but its reactivation and subsequent replication are seriously impaired (26). The substrates for herpesvirus TK are deoxypyrimidine nucleosides and synthetic nucleoside

analogs such as acyclovir (ACV), penciclovir (PCV), ganciclovir (GCV) and brivudin (BVDU) (18, 26, 31). Since BoHV-5 displays a marked neurotropism and its replication in the brain results in severe neurological signs and neuropathology, it is reasonable to assume that TK plays an important role in BoHV-5 neuropathogenesis.

BoHV-5 neuropathogenesis is not completely understood, yet multiple factors - host, viral and environmental - probably influence the production of clinical signs and neuropathology (4, 19, 21, 25). Most of the previous research to elucidate the pathogenesis of BoHV-5 focused on the role of envelope glicoproteins (5-7, 15) and very little has been done to determine the role of other viral products.

Different approaches have been developed to study the role of specific gene products on the biology of viruses. Chemical induced selection or mutagenesis is a very well established mean to produce variants defective in certain gene products, especially TK and DNA polymerase (8, 12, 18). Brivudin (BVDU, E-5-bromovinyl-2'deoxyuridine) was first described in late 70's and it is commercially available in Europe to treat HSV-1 and varicella-zoster infections (8). Viruses expressing TKs resistant to BVDU activity have been selected and used to map TK active sites and to characterize its role in the biology of the virus *in vivo* and *in vitro* (1, 8, 18).

In this study we describe the production and characterization of BoHV-5 mutants resistant to BVDU. The variants were readily obtained after treatment with BVDU, a nucleoside analog, and characterized *in vitro* and *in vivo*. For in vitro characterization, growth properties like plaque morphology, kinetics of replication, resistance to BVDU and stability were assayed. The neurovirulence of the BoHV-5 resistant virus was tested through inoculation of rabbit experimental model. Based on the characteristics of BoHV-1 or HSV BVDU-resistant viruses, our results suggests that our BoHV-5 variants are likely deficient in TK. Our data also indicate that BoHV-5 BVDU resistant variants can be easily obtained upon

exposure to BVDU; the mutation (s) leading to resistant phenotype is (are) stable and possibly present in the TK gene. Furthermore, this (these) mutation (s) confer (s) viral attenuation in the rabbit model.

Material and Methods

Cells, viruses and chemical: CRIB cells (cells resistant to infection with bovine viral diarrhea virus), a cell line derived from MDBK cells (13), were maintained in Minimal Essential Medium (MEM, Nutricell, Brazil), supplemented with 5% fetal bovine serum (Nutricell), and 1x antibiotics and antifungics (Gibco, USA). The virus strains used in this study were: BoHV-1 Cooper (wild-type TK) and IBRV(NG)dltkdlgC (a TK-defective recombinant strain) (14), provided by Dr. Fernando A. Osorio (University of Nebraska, NE, USA); and BoHV-5 SV507/99. The strain SV507/99 is a BoHV-5 isolated from an outbreak of meningoencephalits in cattle in Southern Brazil (9). This virus is highly virulent for calves and rabbits (9, 22, 29), and was used as the parental strain to produce/select the resistant viruses. The brivudin BVDU (E-5-bromovinyl-2'deoxyuridine) was a generous gift from Dr. Erik De Clercq (Rega Institute for Medical Research, Katholieke Universiteit Leuven, Belgium). The chemical was diluted in ultrapure sterile water at a concentration of 2.5mg/mL.

Cell citotoxicity assays: the toxicity of BVDU for CRIB cells was evaluated by using the Trypan Blue assay (2). Briefly, different concentrations of BVDU were prepared in MEM with 0.5% fetal bovine serum and incubated in duplicate during 72 h in 6 well plates containing a preformed monolayer of CRIB cells. Then, the cells were trypsinized and stained with 0.4% of Trypan Blue, counted in a Neubauer chamber and the cell viability was calculated.

Sensitivity of BoHV-5 to BVDU: to demonstrate the activity and specificity of BVDU against BoHV-5 SV507/99 TK, an experiment comparing the activity of the drug against BoHV-1

Cooper strain and IBRV(NG)dltkdlgC was performed. CRIB cells cultivated in 6 well plates were inoculated (2000 tissue culture infectious dose - $TCID_{50}$) in duplicate with one of the three viruses and incubated with 1µg/ml of BVDU (18). Seven two hours post infection, the cells were harvested by freezing at -80°C, clarified by centrifugation and the supernatant was titrated by limiting dilution. The titers were calculated using Reed & Muench methodology and expressed in $TCID_{50}$/mL. To evaluate the sensitivity of BoHV-5 SV507/99 to different BVDU concentrations (0, 0.01, 0.1, 1, 10, 50, 100 and 200µg/mL), a dose-response assay was performed. Six well plates containing monolayers or CRIB cells were inoculated and adsorbed with 2000 $TCID_{50}$ of the virus during 1 h, the monolayers were washed and MEM containing 0.5% of fetal bovine serum and crescent concentrations of BVDU were added in duplicate (two wells per each drug concentration) and incubated for 72 h. Cultures were frozen at -80°C, clarified by centrifugation and the supernatants were titrated as described above.

Isolation of BoHV-5 BVDU-resistant mutants: the methodology described by Mittal & Field (18) was used to obtain BoHV-5 BVDU-resistant viruses. Briefly, a 25 cm^2 flask with a confluent monolayer of CRIB cells was inoculated with a m.o.i. (multiplicity of infection) of 1 and adsorbed for 1 h. After adsorption, the inoculum was removed and the medium was replaced with 0.5% fetal bovine serum added of BVDU to 1µg/mL. The cultures were maintained until the cytophatic effect (CPE) reached 90% of the monolayer. On the second round, the cells were infected with a m.o.i. of 10 as described above, and replaced with medium containing 10µg/mL of BVDU. When the CPE reached 90%, cells were frozen at -80°C, clarified by centrifugation and the supernatant was used to infect cells in a plaque assay. In this assay, individual plaques were subsequently picked, purified and amplified for further analysis. To confirm the resistant phenotype, isolated viruses were grown in the

absence or presence of 10µg/mL of BVDU and the supernatants collected at 72 h were titrated and the titers were compared.

In vitro characterization of BoHV-5 resistant to BVDU: to evaluate the growth characteristics of BoHV-5 BVDU-resistant viruses, experiments to determine plaque morphology, kinetics of replication and stability were performed. Plaque morphology and one step growth curve were performed as described (3). To determine the stability of the resistant phenotype, ten blind passages of the BoHV-5 BVDU resistant clones were performed in CRIB cells, without the presence of BVDU. At the end, the supernatant from the first and tenth passages were used to infected cell cultures (2000 $TCID_{50}$/well) in presence or absence of BVDU. Again, 48 h post-infection, the viruses were harvested and titrated as described above, and the resulting titers were compared.

***Virulence in vivo*:** the virulence of one BoHV-5 BVDU-resistant virus compared to the parental BoHV-5 strain was investigated in the rabbit model (4, 21). Briefly, animals were tranquilized (Zoletil®, Virbac, France) and inoculated with 1mL of virus suspension containing approximately $10^{7.5}$ $TCID_{50}$ through the nephrine openings into the nasal cavity. The animals were monitored thereafter on a daily basis until day 21 post infection (pi). Animals that developed neurological disease were euthanized *in extremis*. 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).

Results

BVDU cell toxicity: the toxicity of BVDU for CRIB cells was evaluated by morphological examination of cultures and by Trypan Blue assay. Concentrations of 1µg/mL of the drug led to rounding and detachment of some cells. These alterations were more frequently observed with concentrations higher than 10µg/mL; doses of 100 and 250µg/mL reduced dramatically the number of surviving cells. Cell viability tested by Trypan Blue assay confirmed the morphological examination and demonstrated that concentrations higher than 10µg/mL were toxic and reduced the number of viable cells in more than 50% (data not shown). Similar results were obtained using the crystal violet assay described by Fernandes et al. (11) (data not shown).

Sensitivity of BoHV-5 to BVDU: the sensitivity of BoHV-5 to BVDU was determined in two experiments. First, the sensitivity of BoHV-5 TK to BVDU (1µg/mL) was demonstrated by testing the SV507/99 strain in parallel with BoHV-1 Copper and IBRV(NG)dltkdlgC strains. Fig. 1A demonstrates the sensitivity of SV507/99 and Cooper strains, known to have wild-type TKs, and the resistance of IBRV(NG)dltkdlgC, a well characterized TK deficient virus (14). When the SV507/99 was grown in presence of BVDU, the virus titer was reduced over two logs. A titer reduction was also observed in the Cooper strain, yet with a lower magnitude. The difference in the degree of reduction in titer (BoHV-1 versus BoHV-5) might indicate that BoHV-5 TK is more sensitive to BVDU than BoHV-1 TK. However more specific and detailed assay would be necessary to confirm the difference in BoHV-5 and BoHV-1 TK sensitivity. On the other hand, the growth of IBRV(NG)dltkdlgC was not affected by the presence of BVDU, demonstrating the specificity of the drug on TK activity.

In a dose-response experiment, it was possible to observe that SV507/99 was sensitive to concentrations higher than 0.01µg/mL (Fig. 1B). Concentrations of 0.1 and 1µg/mL reduced more than 50% of virus yield, and concentrations higher than 10µg/mL resulted in

lack of detection of infectious virus in the cell supernatant. Infectious virus could not be detected in cell supernatants in which the cultures were maintained with concentrations higher of 100µg/mL, yet some positive staining for virus antigens using imunoperoxidase assay was observed (not shown). This indicates that virus replication was not totally inhibited, but reduced to very low levels.

Taken together, these results showed that BoHV-5 replication in tissue culture is sensitive, in a dose-dependent manner, to BVDU. Based on the known mechanism of drug action, these results also indicate that this inhibition is due to the impairment of viral TK activity.

Isolation and characterization of BoHV-5 BVDU-resistant mutants in vitro: isolation of viral plaques that were able to grow after two rounds of treatment with BVDU was readily achieved. At least eighteen individual plaques were selected and picked after agar overlay assay, using the virus harvested from the second round of BVDU treatment. After plaque purification and amplification, the BVDU-resistant phenotype (R) was confirmed by assaying the ability of replication in presence (1µg/mL) or absence of BVDU, followed by virus titration of the supernatant. From 18 viral plaques isolated, at least 11 were shown to have a BVDU-resistant phenotype (not shown) and one, named BoHV-5/R-27, was chosen for further characterization.

Plaque morphology evaluated by plaque assay and staining with 0.35% of crystal violet demonstrated that both SV507/99 and BoHV-5/R-27 produced plaques with similar diameter and morphology (not shown). These results indicate that the resistant phenotype of this variant is not accompanied by altered capacity of cell-to-cell dissemination. The kinetics of replication in cell culture was evaluated using the parental virus (SV507/99) and the BVDU-resistant virus BoHV-5/R-27. Results demonstrated that the efficiency and kinetics of replication were not significantly affected (Fig. 2). Again, these results demonstrate that the

acquisition of BVDU resistant phenotype by BoHV-5/R-27 was not associated with a reduced ability to replicate in tissue culture.

The genetic stability of the BoHV-5/R-27 resistant phenotype was evaluated after ten blind passages in cell culture in the absence of the drug. Viruses harvested at first and tenth passages were titrated and assayed, as described earlier, for the sensitivity to BVDU. Both passages of BoHV-5/R-27 (first and tenth) replicated to similar titers in the presence (1μg/mL) or absence of BVDU, indicating that alterations in TK leading to resistance were stable and did not revert to the wild type phenotype.

Virulence in vivo: The virulence of BoHV-5/R-27 was tested in a rabbit model and compared to the parental virus. Inoculation of rabbits (n=4) with the parental strain was followed of development of classical neurological signs of BoHV-5 infection in three animals, starting at day 6 pi. Two rabbits were euthanized *in extremis* and one recovered by day 12 pi. In contrast, rabbits (n=4) inoculated with the BoHV-5/R-27 virus remained healthy and did not show any sign of neurological disease up to the last day of monitoring (day 21 pi). Virus replication in both groups was confirmed by seroconversion detected at 21 pi. These results demonstrated that the BVDU-resistant BoHV-5 virus displays a reduced virulence for rabbits, while keeping its ability to replicate efficiently in the cell culture.

Discussion

We herein demonstrated that BoHV-5 is sensitive to BVDU, as previously described for other alphaherpesviruses (1, 8, 12, 18). BVDU-resistant BoHV-5 variants were readily obtained by multiplicating the virus in tissue culture during two passages in the presence of the drug. Characterization of a BVDU-resistant viral clone demonstrated that the resistant phenotype is stable and that the acquisition of the resistance did not affect the ability of the virus to replicate in MDBK cells. Our results also demonstrate, for the first time, that TK

activity is essential for the full expression of BoHV-5 neurovirulence: a BVDU-resistant mutant (likely defective in TK activity) was attenuated for rabbits. The methodology used for obtaining the BVDU-resistant virus was the same preconized by Mittal and Field (18). BVDU-resistant viruses obtained by this method – likely presenting an impairment in TK activity – may be used for different purposes, including studies on the role of TK in neuropathogenesis and for vaccine development.

Bovine herpesvirus TK is not essential for virus replication in cell culture; TK-defective viruses do not present significant difference in virus growth *in vitro* but show reduced virulence *in vivo* (3, 17, 27). The reduced virulence *in vivo* makes the TK an attractive target for attenuation towards vaccine production (3, 28). However, only partial deletions are allowed in TK coding region by genetic manipulation, because both flanking, essential genes (UL24 upstream and gH downstream) share coding regions or have regulatory regions inside TK ORF (3, 27, 31). Thus, manipulation of BoHV-5 TK gene should be careful to avoid interfering with the expression of flanking genes. Since the role of TK in the biology and pathogenesis of BoHV-5 has not been studied, and genetic manipulation of the gene faces some technical problems, we took advantage of a well established method to produce BVDU-resistant mutants out of a highly virulent BoHV-5 strain (1, 18).

BVDU is a potent inhibitor of herpesvirus replication and its antiviral activity has been demonstrated against a wide variety of *alpha* and *beta* herpesviruses (8). The potency of antiviral activity is dependent of TK affinity for the drug and varies according to the virus species (8). BVDU is phosphorylated to both 5’ monophosphate and 5’diphosphate forms by virally encoded TK, followed by phosphorylation by cellular kinases producing the active form of BVDU 5’ triphosphate (BVDU-TP) (8, 12). The active form of BVDU has antiviral activity by competing with natural nucleoside substrate or through incorporation into the growing DNA chain, resulting in chain termination (8).

Herpesviruses expressing the enzyme TK resistant to BVDU naturally occur in a low frequency in the virus population (~0.1%) and can be readily selected *in vitro* by multiplying the virus in the presence of the drug (12). The drug resistant viruses usually have: i) a TK enzyme with an altered substrate specificity; ii) lack of expression of TK; or iii) decreased activity or production of small amounts of TK (8, 12). The most frequent mutations leading to a drug resistant phenotype are result of deletions, insertions or base substitutions, often in regions of repeated G, C or occasionally A residues found in the TK coding region (1, 12, 18). Mittal and Field (18) used a similar methodology to map BoHV-1 TK wild-type and demonstrated that mutations leading to a deficient TK phenotype were mostly associated with frameshift and chain termination mutations due to a nucleotide deletion. Also, the same kind of mutations were demonstrated to occur in HSV-1 TK BVDU resistant mutants (1).

Comparison of TK of BoHV-5 and BoHV-1 demonstrated that both genes share an homology of 83% at amino acid (aa) level and that BoHV-5 TK is 3 aa shorter than the BoHV-1 counterpart (9, 24). Most of the nucleotide sequence is conserved between these two viruses, including the putative enzyme active sites and the hot spots that are targets for mutations leading to the TK deficient phenotype (9, 24). We used similar methodology to that described by Mittal & Field (18) and were able to isolate BoHV-5 clones resistant to BVDU. The selected viruses showed a marked resistance to BVDU, as ascertained by the capacity to replicate in presence of high concentrations of the drug (data not shown). The demonstration of reduced TK enzymatic activity or the determination of the genetic basis of the mutant phenotype were not attempted. Nevertheless, based on the similarity with other herpesviruses and already characterized BVDU resistant viruses, it is reasonable to assume that BoHV-5/R-27 has an impairment in TK activity.

Neurological disease associated with BoHV-5 infection results from invasion and widespread replication of the virus in the brain (4, 19, 21, 29). In this sense, it has been

postulated that several gene products and factors may contribute for the neuropathogenesis of BoHV-5 (5-7, 22). The role of TK in the neuropathogenesis of this virus, however, has not been addressed to date. The association of TK function – as ascertained in other viral systems - and BoHV-5 pathogenesis indicate a possible and important role of TK in virus replication and dissemination in nervous tissue. Our results corroborate this hypothesis: inoculation of rabbits with BoHV-5/R-27 did not result in neurological disease, in contrast with neurological infection and disease observed among rabbits inoculated with wild-type virus. Thus, we demonstrated for the first time that BoHV-5 TK plays an essential role in the full expression of the neurovirulent phenotype in the rabbit model.

Functional characterization of herpesvirus genes has been classically performed by genetic manipulation of target genes, followed by *in vivo* and *in vitro* analysis of the resulting phenotype (26, 28). These studies can provide important insights for understanding the molecular basis of viral replication and pathogenesis; and may help the design of new strategies of control, including the development of safer and more effective vaccines. Herpesvirus vaccine candidates should be able to replicate to high titers in tissue culture; must be attenuated, genetically stable and should induce a strong immune response, and would preferentially have a serological marker to discriminate between vaccinated and infected animals (26, 28). In this case, the TK gene represents an attractive target for manipulation towards attenuation. However, an additional deletion would be necessary to introduce a serological marker; and the envelope glycoproteins gE, gC, gI, or Us9 apparently represent adequate candidates for such (15, 16, 26, 28).

In summary, we demonstrated that BoHV-5 is sensitive to BVDU and were able to produce BVDU-resistant mutants. These mutants – likely defective in TK activity – retain their ability to grow in tissue culture, are genetically stable and showed a marked reduction in virulence in a rabbit model. Although these mutants need further genetic characterization

before use as vaccine candidates, the attenuated phenotype is a highly desirable trait towards vaccine development and may be also explored to study the role of TK in the pathogenesis of BoHV-5 neurological infection.

Acknowledgements

This work is part of doctoral thesis of first author and was supported with funds from CNPq and FAPERGS. M.C.S. Brum is recipient of fellowship from CNPq (Brazilian Council for Research). E. F. Flores (301666/04-0) and R. Weiblen (301339/04-0) are recipients of CNPq scholarships. We are grateful to Dr. Erik De Clercq (Rega Institute for Medical Research, Katholieke Universiteit Leuven, Belgium) for providing the brivudin (BVDU) and M. S. e Silva and R. Dezengrini for helping with the animal experiments.

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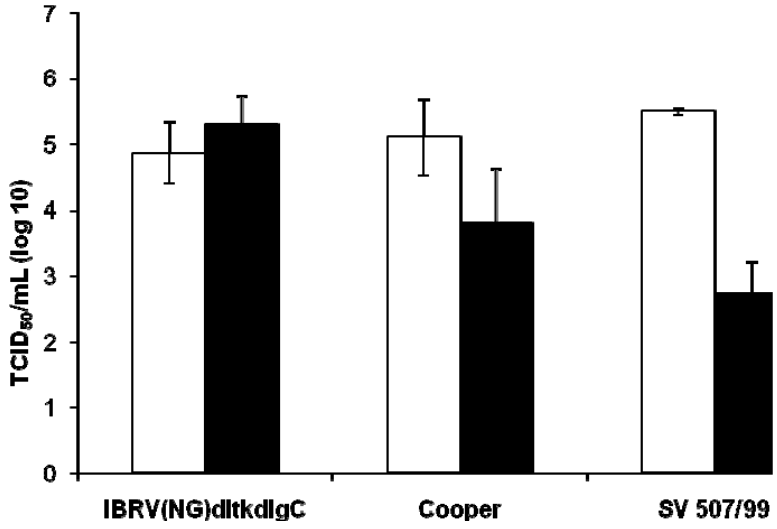
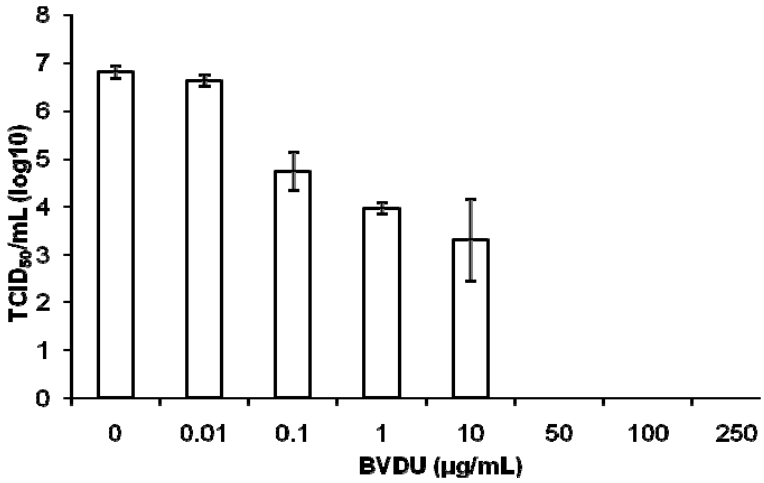


A)



B)

Figure 1 – A) Effect of brivudin (BVDU) for bovine herpesvirus type 1 (BoHV-1 strains – IBRV(NG)dltkdlgC and Cooper) and bovine herpesvirus type 5 (BoHV-5 - SV507/99). All three viruses were grown in the absence (□) or presence (■) of BVDU (1 µg/mL) and, after 72 h, the supernatants were harvested and titrated. Average of duplicates and standard deviations are shown. **B**) Dose-response experiment to evaluate the sensitivity of BoHV-5 to different concentrations of BVDU. SV507/99 wild-type was multiplied in the presence of different concentrations of BVDU (0.001 – 250 µg/mL) and the supernatant collected 72 h was titrated. Bars represent the average of duplicate and error bars represent standard deviation.

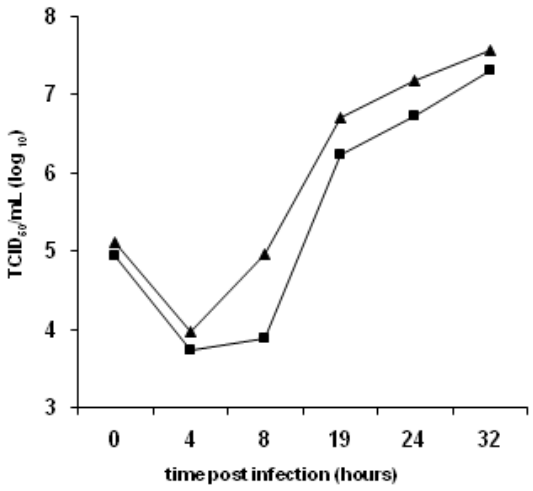


Figure 2 - Kinetics of replication of bovine herpesvirus type 5 (BoHV-5) SV507/99 wild-type and BoHV-5/R-27 in tissue culture. CRIB cell monolayers were inoculated in duplicate with each virus SV507/99 (▲) or BoHV-5/R-27 (■), harvested and titrated at different time-points post-inoculation.

3. CAPÍTULO 3

Virus neutralizing response in cattle induced by an inactivated bovine herpesvirus type 5 strain defective in the thymidine kinase and glycoprotein E

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(artigo a ser submetido ao periódico *Pesquisa Veterinária Brasileira*)

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ABSTRACT. – Brum, M.C.S., Caron, L., Chowdhury, S.I., Weiblen, R. & Flores, E.F. 2009. [**Virus neutralizing response in cattle induce by an inactivated bovine herpesvirus type 5 strain defective in the thymidine kinase and glycoprotein E]** Resposta de anticorpos neutralizante em bovinos induzidos por uma vacina inativada de uma cepa do herpesvírus bovino tipo 5 defectiva na timidina quinase e glicoproteína E. Pesquisa Veterinária Brasileira xx(x):xx-xx. Setor de Virologia, Departamento de Medicina Veterinária Preventiva, e Departamento de Microbiologia e Parasitologia, Centro de Ciências Rurais. Universidade Federal de Santa Maria, Av, Roraima, 1000, Camobi, Santa Maria, RS 97105-900, Brazil. E-mail: flores@ccr.ufsm.br

The immunogenicity of an inactivated vaccine based on a bovine herpesvirus type 5 strain defective in the thymidine kinase and glycoprotein E (BoHV-5 gE/TKΔ) was evaluated in cattle and the results were compared with a vaccine prepared with parental strain SV507/99. To formulate the vaccines, each virus (wt SV507/99 and BoHV-5 gE/TKΔ) was multiplied in cell culture and inactivated with binary bromoethylinimine (BEI). Each vaccine dose contained approximately of $10^{7.5}$ $TCID_{50}$ of inactivated virus mixed with an oil-based adjuvant (46:54). Forty mixed breed cattle, aged between 6 and 9 months, were divided in two groups of 20 animals each and vaccinated twice (days 0 and 22 pv) by the subcutaneous route with either vaccine. Serum samples collected at day 0 pv and at different intervals (22, 56, 78 and 116 pv) after vaccination were tested for virus neutralizing antibodies (VN) against the respective homologous BoHV-5 strains and seven heterologous BoHV-5 and BoHV-1 isolates. The VN assays demonstrated seroconversion to the respective homologous viruses in all vaccinated animals after the second vaccine dose (mean titers of 17.5 for the wt vaccine; 24.1 for the recombinant virus). All animals remained reagents up to day 116 pv, yet showing a gradual reduction in VN titers. Animals from both vaccine groups reacted in similar VN titers to different BoHV-1 and BoHV-5 isolates, yet the magnitude of serological response of

both groups was highest against BoHV-5 isolates. In conclusion, these results indicate that antigens of BoHV-5 gE/TK deleted virus induced an adequate serological response against BoHV-5 and BoHV-1 and thus can be used as an alternative vaccine candidate.

INDEX TERMS: vaccines, DIVA, differential vaccine, recombinant vaccine

RESUMO

A imunogenicidade de uma vacina inativada constituída por uma cepa do herpesvírus bovino tipo 5 defectivo nos genes da timidina quinase e da glicoproteína E (BoHV-5 gE/TKΔ) foi avaliada em bovinos e o resultado foi comparado com o obtido com uma vacina preparada com a cepa parental SV507/99. Para a formulação das vacinas, cultivos de células infectados com cada um dos vírus (SV507/99 ou BoHV-5 gE/TKΔ) foram inativados com bromoetilimina binária. Cada dose de vacina constitui-se de aproximadamente $10^{7,5}$ $TCID_{50}$ de um dos vírus inativados e misturado com adjuvante oleoso (46:54). Quarenta bovinos cruzados, com idade entre seis a nove meses, foram divididos em dois grupos de 20 animais cada e vacinados duas vezes (0 e 22 pv) pela via subcutânea com uma das vacinas. Amostras de soro foram coletadas no dia 0 pv e em diferentes intervalos (22, 56, 78 e 116 pv) após vacinação para pesquisa de anticorpos neutralizantes (VN) frente ao BoHV-5 homólogo ou diferentes isolados de BoHV-5 e BoHV-1. Os testes de soroneutralização demonstraram que todos os animais soroconverteram somente após a administração da segunda dose da vacina (títulos médio de 17,5 para o grupo SV507/99; 24,1 para o grupo BoHV-5 gE/TKΔ). Todos os animais mantiveram níveis de anticorpos neutralizantes até o dia 116 pv, no entanto foi observado uma redução gradual nos títulos. A sorologia cruzada com amostras heterólogas do BoHV-5 e BoHV-1 indicou que ambos os grupos reagiram em níveis similar frente ao mesmo vírus, no entanto a magnitude da resposta sorológica foi maior quando utilizou-se amostras de BoHV-5. Os resultados demonstram que vacina constituída pelo BoHV-5 gE/TKΔ

recombinante induziu resposta sorológica em níveis satisfatórios, constituindo-se uma alternativa a cepa vacinal.

TERMOS DE INDEXAÇÃO**:** vacina, vacina diferencial, DIVA, vírus recombinante

INTRODUCTION

Bovine herpesvirus type 5 (BoHV-5) is the agent of necrotizing meningoencephalitis in cattle and is closely related to bovine herpesvirus type 1 (BoHV-1), the agent of infectious bovine rhinotracheitis (IBR), vulvovaginitis/balanopostitis (IPV/IBP) and abortions (Engels & Ackermann 1996). BoHV-5 and -1 are widespread among South American cattle and cause important losses to the livestock industry (Silva et al. 2007b, Suarez Heinlein et al. 1993). Although, BoHV-5 infection has been described in other continents, it seems to be more prevalent in South American countries like Argentina and Brazil (Silva et al. 2007b)

BoHV-5 is an enveloped virus, containing a linear double stranded DNA molecule as genome (approximately 138kb in length) and belongs to the subfamily *Alphaherpesvirinae*, genus *Varicellovirus* (Delhon et al. 2003, Roizmann et al. 1992). The viral genome encodes around 70 different polypeptides, which include structural proteins, enzymes, regulatory proteins and glycoproteins (usually present in the envelope) (Delhon et al. 2003). Following primary infection, the virus establishes lifelong latency in neurons of the trigeminal ganglia (TG) or central nervous system (CNS) (Vogel et al. 2003). Latently infected animals are the main reservoirs of infection, from which the virus can be periodically reactivated and transmitted to susceptible animals (Engels & Ackermann 1996, Vogel et al. 2003).

Vaccination represents an efficient strategy to control bovine herpesvirus infections in the field (Ackermann & Engels 2006, van Oirschot 1999). The main goal of vaccination is to prevent or reduce the severity of clinical disease and reduce/abolish virus shedding and transmission (Ackermann & Engels 2006, van Oirschot 1999). Nonetheless, prevention of the

establishment of latency by the infecting (challenge) virus has not been achieved so far by BoHV-1 vaccines (van Drunen Littel-van den Hurk 2007).

Most of vaccines available in South America are based on conventional inactivated BoHV-1 strains and, at least one conventional, temperature-sensitive, modified live vaccine (MLV) (Silva et al. 2007a). Based on the antigenic similarity and serological cross-reactivity between BoHV-1 and -5, it has been postulated that vaccines containing either virus could be of value to control infection by the homologous and also by the heterologous virus (Bratanich et al. 1991, Cascio et al. 1999, Del Medico Zajac et al. 2006, Silva et al. 2006). Nonetheless, an important restriction for the use of conventional vaccines is the impossibility of differentiation between vaccinated and naturally infected (latently infected) animals (van Oirschot 1999).

Advances in molecular virology and immunology have led to the development of differential vaccines (DIVA), which are based on the capacity of the vaccine to induce a serological immune response that can be differentiated from the response induced by infection with wild-type virus (van Oirschot 1999). The association of DIVA vaccines with a differential serological test has been widely used to control herpesvirus infections, like pseudorabies virus (PRV) and BoHV-1 in several countries (Ackermann & Engels 2006, van Oirschot 1999). In the case of BoHV-1, several DIVA vaccines are based on subunits (usually recombinant proteins) or gene-deleted virus strains (van Drunen Littel-van den Hurk 2007). DIVA vaccines based on gene-deleted virus can be designed either as inactivated or live virus (van Drunen Littel-van den Hurk 2007). In the case of live virus, besides the deletion of the marker gene (usually the glycorpotein gE or gC), is necessary to associate a deletion of some gene involved in virulence, like thymidine kinase (TK) (Flores et al. 1993, Kaashoek et al. 1996). Several BoHV-1 gene-deleted viruses have been constructed and some have been used in control/eradication programs of BoHV-1 infection in Europe and North America

(Kaashoek et al. 1995, Strube et al. 1996). Recently, gene-deleted BoHV-1 and -5 Brazilian isolates were developed and are currently under testing for a future use in vaccine formulations (Brum et al. 2009, Franco et al. 2002, Franco et al. 2007).

In this study, the immunogenicity of a vaccine formulation containing inactivated antigens of a recombinant Brazilian BoHV-5 isolate deleted in gE and TK genes was tested in cattle. The immunogenicity was assessed by testing post-immunization serum collected at different intervals for virus-neutralizing (VN) antibodies against the homologous (BoHV-5) and heterologous viruses (BoHV-1 and BoHV-5 isolates). The results demonstrated seroconversion in all vaccinated animals, which developed VN antibodies that were detectable up to day 116 post-vaccination. In general, VN titers were higher to BoHV-5 than to BoHV-1 field isolates. Taken together, these results indicate that antigens of BoHV-5 gE/TK deleted virus induced a strong immune response against BoHV-5 and BoHV-1 and thus can be used as an alternative vaccine candidate.

MATERIAL AND METHODS

Virus strains and cells

A Brazilian BoHV-5 virus containing a double deletion in gE and TK genes (SV507 gE/TKΔ), using as background the strain SV507/99 was constructed and previously characterized (Brum et al. 2009). Both viruses used in this study (wild-type and the double deletion mutant) were propagated in CRIB cells (a MDBK-derived cell line, ATCC - CRL 11883) (Flores & Donis 1995) maintained in MEM (minimum essential medium, Invitrogen, USA), supplemented with 10% inactivated fetal bovine serum (Nutricell, Brazil), 100 U/mL of penicillin, 100 µg/mL of streptomycin (Invitrogen, USA). The reference strain of BoHV-1 Cooper was obtained with Dr. Fernando A. Osorio (Center for Virology, UNL, Nebraska, USA). The field isolates used in cross-neutralization assays (BoHV-1: SV56/90, EVI123 and

SV371/05 and BoHV-5: EVI88, SV136/88 and EVI340) have been isolated in Brazil and were previously characterized (Silva et al. 2007b).

Binary ethylenimine (BEI) inactivation and vaccine formulation

BoHV-5 gE/TKΔ and SV507/99 were grown in T-150 flasks (150 cm^2) containing monolayers of CRIB cells. When the cytophatic effect (CPE) reached around 90% of the monolayers, the whole cell-virus-supernatant mixture was inactivated for vaccine production. Before virus inactivation, infectivity was determined by limiting dilution and virus titers were calculated by Reed & Muench method and expressed as median tissue culture infection dose per mililiter ($TCID_{50}$/mL). Inactivation was performed essentially as described by Bahneman (1976) with BEI (1mM) for at least 12 h at 37° C under constant agitation. BEI was obtained after cyclization of BEA with NaOH (100mM) at 37° C for 1 hour. Residual BEI was inactivated by addition of 1% of sodium thiosulfate solution (1M) and finally 0.01% of thimerosal was added as a preservative. Inactivated viral suspensions were kept at 4° C until vaccine preparation. To detect residual virus, samples from inactivated virus before adding sodium thiosulfate were collected and inoculated in preformed monolayers of CRIB cells and the production of CPE was thereafter monitored.

The vaccine was prepared in water-in-oil-in-water emulsions (Montanide ISA 206, Seppic, France) containing approximately $10^{7.5}$ $TCID_{50}$ of either virus per dose. The volume of the dose was adjusted to 3 mL and was composed by 46% antigen and 54% of adjuvant. The inactivated virus suspension was slowly mixed with adjuvant under constant agitation at room temperature. The final mixture was maintained in sterile recipient at 4° C. The sterility of the vaccine formulation was confirmed by the absence of bacterial or fungal growth in routine culture medium after 48 h of inoculation.

Vaccination and sample collection

Forty mixed breed, aged between 6 to 9 months, BoHV-1 and -5 seronegative calves were used. The animals were randomly allocated in two groups of 20 calves each and vaccinated by the subcutaneous route in the right shoulder with either vaccine formulation on day zero post vaccination (pv). A second vaccine dose was administered at day 22 pv. Serum samples were collected at days 0, 22, 56, 78 and 116 dpv, heat inactivated at 56° C for 30 min and stored at -20° C until testing.

Virus neutralizing tests

Virus-neutralizing (VN) tests were performed according to routine procedures (Lovato et al. 2003). Briefly, serial two-fold serum dilutions (starting at 1:2) were incubated with a constant amount of virus (approximately 100 $TCID_{50}$) for 1 h at 37 °C. A suspension of CRIB cells was added at the final of serum-virus incubation period and the plates were incubated at 37°C under a 5% CO_2 atmosphere for 72 h. Test readings were performed and the virus titers were considered the reciprocal of the highest dilutions of sera capable of preventing the production of CPE. Geometric mean titers (G) were calculated according $G = 2^{(\log x1 + \log x2 + \ldots \log xn)/n}$ and only positive serum included (Toma et al. 1999). Serum samples collected from vaccinated animals at different intervals were tested against the homologous viruses (SV507/99) and against several isolates of BoHV-1 (Cooper, SV56/90, EVI123 and SV371/05) and BoHV-5 (EVI88, SV136/88 and EVI340). Statistical analysis of the data was performed by analysis of variance and Tukey's test.

RESULTS

Both viruses replicated at high titers in CRIB cell monolayers (> $10^{7.8}$ $TCID_{50}$/mL) and thus were suitable to formulate an inactivated vaccine. Virus inactivation using the BEI protocol was efficient such residual virus or bacterial contamination were not detected in the

vaccine formulation throughout the process. Vaccine administration was performed by the subcutaneous route in the lateral face of the right shoulder. No adverse reactions were noticed in the days following vaccination.

Serum samples were collected at different intervals after vaccination and submitted to VN tests using the homologous BoHV-5 virus to analyze the immunogenicity of both vaccines. Serological VN response was similar in both groups throughout the serum collections and the differences observed were not statistically significantly ($p>0.05\%$) (Fig.1). At day 22 pv, 12 animals in the SV507/99 group and 12 calves in the BoHV-5 gE/TKΔ group were serologically reagents, and highest titers were 8 and 16 for SV507/99 or BoHV-5 gE/TKΔ, respectively (Table 1). After the second vaccine dose all animals seroconverted and remained positive until the last bleed. The maximum levels of neutralizing antibodies were detected at 56 dpv in both groups (Fig. 1). At this time, the VN titers ranged from 4 to 256 in the SV507/99 and from 2 to 128 in the BoHV-5 gE/TKΔ group. The VN levels at 78 dpv were still high and thereafter demonstrated a tendency to decrease, which was confirmed at 116 pv. The mean neutralizing titers on the last bleed (116 pv) decreased approximately 1 $\log_2$ (Table 1). These results demonstrated that the deletion of gE and TK in the parental BoHV-5 had no adverse effect in the immunogenicity of the viral antigens, since both vaccine groups responded in a similar fashion. Nonetheless, the observed immune response was typical of an inactivated vaccine, in which a boost was necessary to induce an adequate response, and the magnitude of the immunity tended to decrease over time.

Sera collected at 56 dpv from both groups were tested by VN against heterologous isolates of BoHV-5 (EVI88, SV136/88 and EVI340) and BoHV-1 (Cooper strain, SV56/90, EVI123 and SV371/05). In general, the levels of serological cross-neutralization were similar when serum samples from both groups were tested against the same virus, either BoHV-5 or BoHV-1 (Fig. 2A and 2B). However, the neutralizing antibodies titers against BoHV-5

isolates were superior to those to BoHV-1 isolates. These results clearly demonstrated high levels of cross-neutralization between BoHV-5 and BoHV-1, yet the highest titers were observed against heterologous BoHV-5 isolates.

DISCUSSION

The immunogenicity and serological cross-neutralization of two inactivated BoHV-5-based vaccines were tested. A recombinant BoHV-5 lacking the gE and TK genes was used to formulate an inactivated vaccine and the serological response was compared to an analogue vaccine prepared with parental strain SV507/99-based vaccine. The difference between the two viruses used is the absence of gE and TK genes (Brum et al. 2009). Both deleted genes are non essential for virus replication in cell culture and therefore can be deleted without affecting the capacity of the virus to replicate in tissue culture (Ackermann & Engels 2006, van Drunen Littel-van den Hurk 2007). The enzyme TK is a non-structural protein responsible for nucleotide phosphorylation in non dividing cells such neurons (Tikoo et al. 1995). In contrast, glycoprotein E (gE) is a structural protein present in the viral envelope and has been shown to be immunogenic (Ackermann & Engels 2006, Schwyzer & Ackermann 1996). Even that the recombinant BoHV-5 gE/TKΔ presented possibly has all the requirements necessary to be attenuated, it was tested under inactivated form because would be an alternative.

As expected, the inactivated BoHV-5 gE/TKΔ vaccine presented similar immunogenicity to the parental strain, indicating that deletion of the structural gene gE did not affect the immune response induced by viral antigens. However, seroconversion of all animals - in moderate to high neutralizing antibody titers - was obtained only 30 days after the second vaccine dose. Also, the levels of VN antibodies decreased progressively over time, suggesting the need of revaccination every six month or annually. The gradual reduction of

VN titers and the need for periodic revaccination to maintain adequate levels of antibody are typical of BoHV-1 inactivated vaccines (van Drunen Littel-van den Hurk 2007).

Vaccination is the most cost-effective strategy to control BoHV-1 infection in the field and inactivated or modified live virus (MLV) vaccines are available (Ackermann & Engels 2006, van Oirschot 1999). Based on biological properties, alphaherpesviruses are suitable for the development of DIVA vaccines, which can be used either as killed or live vaccines (van Oirschot et al. 1996). Due to cross reactivity between BoHV-1 and -5 and the lack of specific BoHV-5 vaccines, vaccination against BoHV-1 may be indicated to protect against BoHV-5 infection and disease (Cascio et al. 1999, Vogel et al. 2002). Animals immunized with BoHV-1 and challenged with BoHV-5 showed a reduced virus shedding and were partially protected from clinical disease (Cascio et al. 1999, Del Medico Zajac et al. 2006, Silva et al. 2006). In the present study, the results of VN assays testing serum samples from both vaccine groups against different BoHV-5 and BoHV-1 isolates clearly demonstrated cross-reactivity between the two viruses. However, as may be expected, the levels of cross-reactivity were higher when serum samples were tested against BoHV-5 isolates than against BoHV-1 isolates. The serological cross-reactivity between BoHV-1 and BoHV-5 has also been described in others vaccine studies (Petzhold et al. 2001, Silva et al. 2007a, Vogel et al. 2002).

Some Europeans countries controlled and/or eradicated BoHV-1 infection by the use of DIVA vaccines accompanied by serological tests able to differentiate between vaccinated and naturally infected animals (Ackermann & Engels 2006). In this sense, an explanation for the low BoHV-5 prevalence in Europe and United States might be the massive vaccination against BoHV-1 (Ackermann & Engels 2006). Controlling BoHV-1 and -5 infection and disease in South America was not prioritary until recently such massive vaccination is still insipient. Also, commercially available vaccines have been questioned regarding their efficacy (Silva et al. 2007a, Vogel et al. 2002) and DIVA vaccines are not commercially

available nowadays in Brazil (Silva et al. 2007a). Specific control of BoHV-5 infections is restricted to farms where clinical case had been diagnosed. In these cases, management measures and even vaccination with BoHV-1 vaccines have been conducted (Flores, E.F. personal communication, Salvador et al. 1998). Thus, the development of an improved and specific BoHV-5 vaccine become necessary and several efforts to produce such vaccine have been done, including the development of DIVA BoHV-5 vaccines (Fischer et al. 2007, Franco et al. 2007, Halfen et al. 2000). In this sense, the BoHV-5 strain lacking gE and TK used as inactivated vaccine demonstrated to be an adequate alternative for the control of BoHV-5 infection and disease. Albeit the differential capacity was not serologically demonstrated in the BoHV-5 gE/TKΔ vaccinated animals – the main reason being that a companion ELISA gE test is not commercially available in Brazil -, it is assumed the vaccinated animals would not develop anti-gE antibodies since the BoHV-5 gE/TKΔ virus has been conclusively shown not to express this glycoprotein (Brum et al. 2009). Nevertheless, VN tests and titers are used only as a parameter of the magnitude of the immune response and should not be taken as definitive indicators of *in vivo* protection (Tikoo et al. 1995, van Oirschot 1999). To evaluate the protection potentially induced by this vaccine, a vaccination-challenge experiment should be performed. Additionally, challenge with a BoHV-1 virulent isolate would demonstrate the status of protection against this heterologous virus.

The knowledge of the immune response to BoHV-5 is basically limited to investigations of the humoral, virus neutralizing response after experimental infection or vaccination (Cascio et al. 1999, Del Medico Zajac et al. 2006, Vogel et al. 2003). Nevertheless, based on the genetic and biological similarities with BoHV-1, the immunological mechanisms and parameters between these two viruses should be very similar. Humoral and cellular immunity play an important role preventing and controlling BoHV-1 infection (Jones & Chowdhury 2007, van Drunen Littel-van den Hurk 2007), the same likely

being true for BoHV-5. Serum virus-neutralizing (VN) titers have been considered the main immunological indicator to evaluate BoHV-1 vaccine potency (Ackermann & Engels 2006). However, these parameters should not be used as the sole indicators of protection since no definitive correlation between VN titers and protection has been established for this virus (Ackermann & Engels 2006, Galeota et al. 1997).

In the case of BoHV-1, inactivated and MLV vaccines have been shown to clinically protect calves from homologous challenge, to reduce virus shedding, but not to prevent the establishment of latency (Ackermann & Engels, 2006, Strube et al. 1996, van Oirschot 1999). Nonetheless, it has been demonstrated that pre-existing immunity resulting from natural infection or vaccination with BoHV-5 or BoHV-1 can protect or reduce neurological infection upon BoHV-5 challenge (Del Medico Zajac et al. 2006, Silva et al. 2006). An essential experiment to test a candidate BoHV-5 vaccine would be a vaccination-challenge trial in which the validation of the study would rely upon the reproduction of neurological disease in non-vaccinated animals (Silva et al. 2006).

The main goal of the experiment presented herein was to demonstrate that under an inactivated vaccine formulation, the deleted virus BoHV-5 gE/TKΔ has similar immunogenicity to that of the parental strain in cattle, which the advantage of being potentially used as a differential vaccine. In this sense, the development of safe and effective BoHV-1 and BoHV-5 vaccines in Brazil is urgent and necessary, especially upon the results of several studies questioning the efficacy of currently marketed vaccines (Fischer et al. 2007, Franco et al. 2007, Silva et al. 2007a, Vogel et al. 2002).

In conclusion, the data presented demonstrate that a Brazilian BoHV-5 strain defective in the gE and TK genes used in an inactivated vaccine formulation in cattle presented immunogenicity similar to that of the parental wild-type strain. Seroconversion in all animals was detected after the second vaccine dose, the virus-neutralizing titers reached the maximum

titer at day 56 pv and progressively decreased thereafter, yet maintaining moderate levels at day 112 pv. In addition, serum cross-neutralization with different BoHV-5 isolates or heterologous BoHV-1 viruses indicated a broad reactivity. Even though the capacity of serological differentiation was not demonstrated, this property would be a major advantage of the BoHV-5 gE/TK strain to be used in future vaccine formulations.

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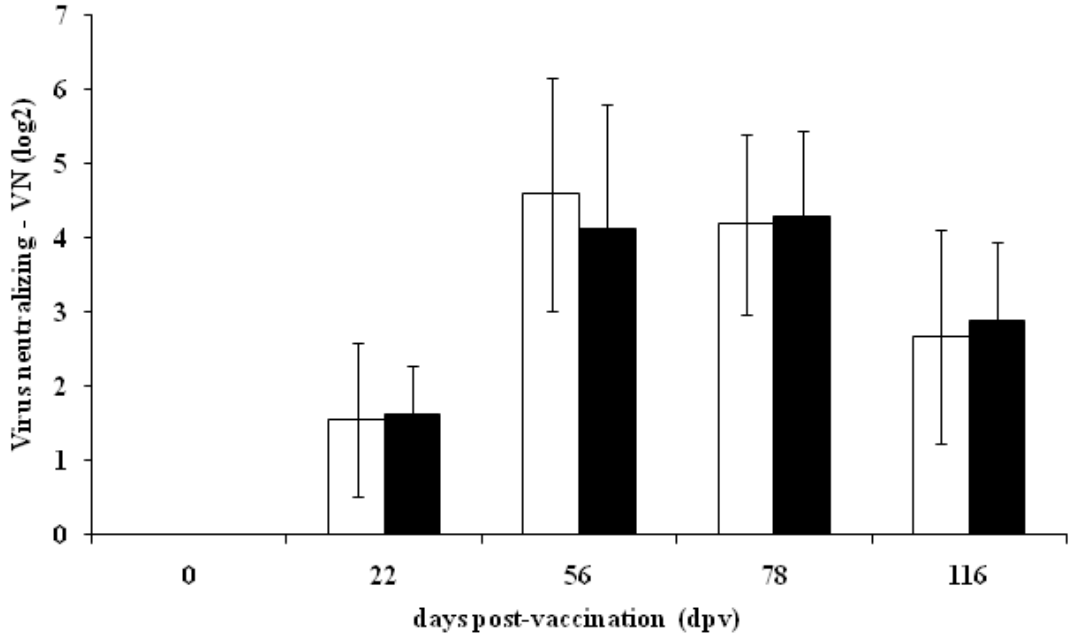

Fig 1. Virus neutralizing (VN) antibodies of inactivated vaccines formulated with bovine herpesvirus type 5 (BoHV-5) SV507/99 (■) or gE/TKΔ (□) viruses. VN titers are expressed as of geometric mean (G) calculated at different days post vaccination (dpv). The vaccines were administered at days 0 and 22 pv.

Table 1 – Distribution of virus neutralizing titers in two groups (20 animals/group) of cattle vaccinated with an inactivated bovine herpesvirus type 5 (BoHV-5) wild-type or gE/TKΔ vaccine.

	SV507/99 vaccine					**BoHV-5 gE/TKΔ vaccine**				
dpv[a]	reagents	lowest titer[b]	highest titer[b]	G[c]	SD[d]	reagents	lowest titer[b]	highest titer[b]	G	SD
0	0%	≤ 2	≤ 2	0	0	0	≤ 2	≤ 2	0	0
22	70%	0 (6)	8 (1)	3.1	0.6	60%	0 (8)	16 (1)	2.9	1.0
56	100%	4 (2)	256 (1)	17.5	1.7	100%	2 (1)	128 (1)	24.1	1.6
78	100%	4 (2)	128 (1)	19.6	1.1	100%	4 (2)	64 (3)	18.2	1.2
116	100%	2 (2)	32 (1)	7.5	1.1	100%	2 (4)	64 (1)	6.4	1.5

[a]dpv - days post vaccination; [b] – VN titer and bracketed number of animals reagents with indicated titer; [c]G - geometric mean titer of positive animals were calculated according to $G = 2^{(\log x1 + \log x2 + \ldots \log xn)/n}$ (Toma et al. 1999). SD - standard deviation of the VN titers.

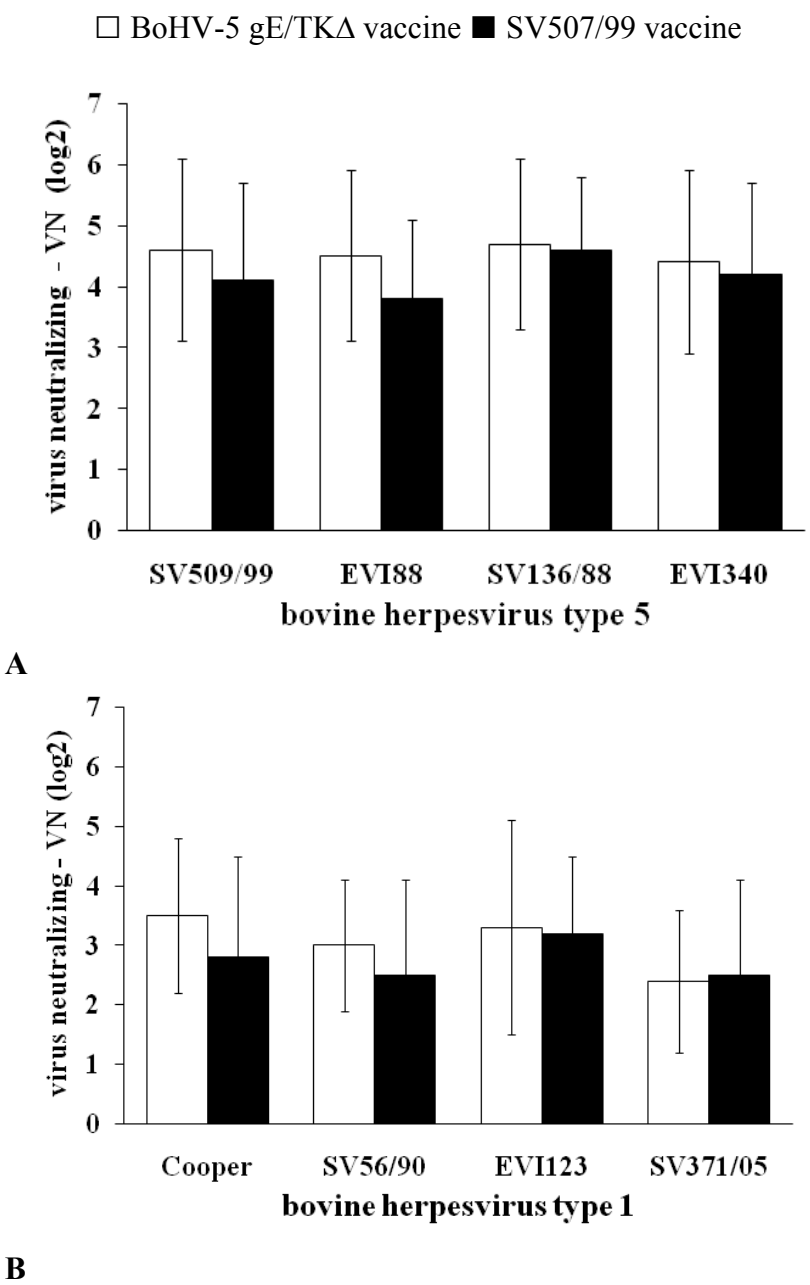

Fig 2. Cross-reactivity of serum samples collected at 56 dpv (day post vaccination) from animals vaccinated with an inactivated bovine herpesvirus type 5 (BoHV-5) SV507/99 or gE/TKΔ-based vaccine (20 animals/group). Columns demonstrate the geometric means of reagents animals and the standard deviation is indicated by error bars. **A)** cross serology of serum samples against homologous (SV507/99) and different field isolates of BoHV-5; **B)** cross serology of serum samples against reference strain Cooper and field isolates of BoHV-1.

4. CONSIDERAÇÕES FINAIS

Os herpesvírus bovinos 1 e 5 (BoHV-1 e BoHV-5) são considerados entre os principais patógenos de bovinos, devido as perdas econômicas resultantes da infecção. A infecção pelo BoHV-1 está distribuída mundialmente podendo estar associada com manifestações clínicas respiratórias (*infectious bovine rhinotracheitis - IBR*), genitais (*infectious pustular vulvovagintis or balonoposthitis – IPV; IPB*), aborto e infecções sistêmicas em animais jovens. A infecção pelo BoHV-5 já foi descrito em vários países, no entanto frequentemente são relatados surtos de doença neurológica na América do Sul, especialmente no Brasil, Argentina e Uruguai. O BoHV-1 e -5 são biológica e geneticamente muito semelhantes, porém uma característica marcante nas amostras do BoHV-5 é a neurovirulência.

Os índices de prevalência pelos BoHV-1 e -5 no Brasil variam conforme o sistema de criação e a região, no entanto a taxa de infecção de cada vírus é desconhecida, devido a inexistência de um teste sorológico capaz de diferenciá-los. Devido a reação sorológica cruzada e a impossibilidade de identificar rebanhos positivos somente para o BoHV-5, o controle da infecção por este vírus está restrita a propriedades onde casos clínicos são diagnosticados. Nestas situações, medidas de manejo que reduzam a disseminação do vírus entre animais e a utilização de vacinas para o BoHV-1 são recomendadas. A avaliação da resposta sorológica das vacinas contra o BoHV-1 comercializadas no Brasil indicou necessidade de melhorá-las na qualidade e potência. Outro aspecto das vacinas comercializadas é a incapacidade de diferenciação entre animais vacinados e infectados naturalmente com os BoHV-1 e -5. Algumas regiões e países da Europa obtiveram sucesso no controle e erradicação do BoHV-1 pela associação de medidas de controle com o uso de vacina diferencial e teste sorológico capaz de identificar animais infectados naturalmente.

O presente estudo teve como objetivo produzir cepas do BoHV-5 recombinantes e possivelmente atenuadas, que possam servir como candidatos a vacinas diferenciais e em estudos de patogenia. Os vírus recombinantes obtidos possuem deleções em dois genes (glicoproteína E - gE e timidina quinase -TK) relacionados com a virulência dos alfaherpesvírus. Além disso, um dos genes deletados codifica uma proteína imunogênica, que pode ser utilizada como marcador sorológico em vacinas diferenciais. A confirmação destes

recombinantes como possíveis cepas vacinais diferenciais constituiriam um avanço no desenvolvimento de vacinas para o herpesvírus bovino e auxiliariam no controle e profilaxia da infecção pelo BoHV-5.

No Capítulo 1 foi descrita a obtenção de uma variante resistente ao brivudin, uma substância capaz de selecionar amostras deficientes para o gene da timidina quinase (TK). Para isso, utilizou-se o cepa virulenta brasileira do BoHV- 5 SV507/99 e realizou-se o cultivo desta na presença de substância. Uma amostra resistente foi selecionada para os testes de caracterização *in vitro*. Esses testes avaliaram a capacidade de replicação, formação de placa e estabilidade genética. Os resultados demonstraram que o SV507/99 é sensível ao brivudin e que amostras resistentes são facilmente selecionadas. Os testes de caracterização indicaram que o caráter resistente ao brivudin não alterou as características de multiplicação em cultivo celular. No entanto, essa amostra resistente demonstrou ser avirulenta quando inoculada em coelhos. Com isso, demonstrou-se que amostras resistentes ao brivudin, possivelmente defectivas para a TK, são facilmente obtidas, não possuem alterações na capacidade de multiplicação em cultivo e são atenuadas *in vivo*.

O Capítulo 2 objetivou a construção de três amostras do BoHV-5 recombinantes para futuro uso em estudos de patogenia e como candidatos a cepas vacinais. Para isso utilizou-se a técnica de recombinação homóloga e como *background* a cepa brasileira SV507/99 do BoHV-5. Os genes da TK e gE foram escolhidos por serem relacionados com a virulência, além de que a gE pode servir como marcador sorológico. Os resultados da caracterização *in vitro* demonstraram que as três cepas recombinantes BoHV-5 gEΔ, BoHV-5 TKΔ e BoHV-5 gE/TKΔ possuem as características de replicação semelhantes ao vírus parental. No entanto, as amostras que possuem deleção do gene da gE produziram placas morfologicamente reduzidas quando comparadas com amostra original, possivelmente como dificuldade na disseminação célula-célula. A ausência da gE em células de cultivo infectadas pelo BoHV-5 gEΔ e confirmada pela técnica de *western blotting*, indica a possibilidade de utilização deste vírus como marcador sorológico em uma vacina diferencial. Os resultados demonstraram a viabilidade das amostras recombinantes obtidas e a possibilidade do emprego destas em estudos de patogenia ou como candidatos a cepas vacinais.

No Capítulo 3 a imunogenicidade para bovinos das amostras BoHV-5 gE/TKΔ e SV507/99 como constituinte de vacinas inativadas foi avaliada. As vacinas foram formuladas com culturas celulares infectadas com a cepa recombinante BoHV-5 gE/TKΔ ou a cepa parental inativadas e misturadas com adjuvante oleoso. A resposta de anticorpos neutralizantes entre as duas formulações de vacinas foi comparada em diferentes intervalos e

os resultados demonstraram que os animais responderam de maneira semelhante. Adicionalmente, testou-se a capacidade de neutralização de amostras heterólogas do BoHV-5 e -1. Os resultados dos testes de sorologia cruzada indicam que não houve diferença entre os grupos vacinais quando testados frente ao mesmo vírus, no entanto a resposta teve maior magnitude frente a amostras de BoHV-5. A principal diferença entre as cepas usadas para a confecção das vacinas é a ausência dos genes da TK e gE na amostra BoHV-5 gE/TKΔ, que baseado nos resultados obtidos não alteraram a imunogenicidade. Mais importante, os resultados demonstram que essa cepa constitui-se em candidato a cepa vacinal e possivelmente com a possibilidade de induzir uma resposta sorológica diferencial.

Em resumo, os resultados apresentados descrevem a produção e caracterização *in vitro* de cepas defectivas para dois genes (TK e gE) envolvidos na virulência dos alfaherpesvírus. Essas cepas serão úteis em estudos futuros de patogenia e como candidatos a inclusão em vacinas. O teste de uma das cepas defectiva para ambos os genes como componente de uma vacina inativada, demonstrou ser possível a sua inclusão na composição de uma vacina específica para o BoHV-5. A diferenciação sorológica dos animais vacinados com a amostra recombinante não foi realizada. No entanto acredita-se que isso seja possível, pois a cepa utilizada possui deleção do gene da gE que é usado como marcador sorológico para vacinas diferenciais do BoHV-1 e de outros alfaherpesvírus. Nesse sentido, estudos que confirmem a capacidade de diferenciação e proteção da doença em estudos de desafio são necessários. Outra possibilidade, seria o emprego destas amostras como vacina viva modificada e diferencial para o BoHV-5. Para isso, deve-se testar a capacidade das cepas recombinantes em replicar em bovinos, bem como determinar a sua atenuação para essa espécie. A confirmação da melhor formulação vacinal deverá ser realizada em um experimento que avalie a proteção clínica de animais vacinados frente ao desafio com amostras virulentas do BoHV-5 e BoHV-1.

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