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CONSTRUÇÃO E CARACTERIZAÇÃO DE RECOMBINANTE DO HERPESVÍRUS BOVINO TIPO 1 COM DELEÇÃO DA GLICOPROTEÍNA *E* PARA USO EM VACINA

TESE DE DOUTORADO

Marcelo Weiss

Santa Maria, RS, Brasil
2015

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Marcelo Weiss

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

Orientador: Prof. Eduardo Furtado Flores

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Universidade Federal de Santa Maria
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como requisito parcial para a obtenção do grau de
Doutor em Medicina Veterinária

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RESUMO

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CONSTRUÇÃO E CARACTERIZAÇÃO DE RECOMBINANTE DO HERPESVÍRUS BOVINO TIPO 1 COM DELEÇÃO DA GLICOPROTEÍNA *E* PARA USO EM VACINA

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Vacinas com marcadores antigênicos – também denominadas vacinas diferenciais – tem sido amplamente utilizadas no controle e prevenção da infecção pelo herpesvírus bovino tipo 1 (BoHV-1). Com este objetivo, uma amostra brasileira de BoHV-1 (SV56/90) foi submetida à deleção do gene da glicoproteína E (gE) para potencial uso em vacinas. A deleção do gene da gE foi realizada por recombinação homóloga, sendo o gene da gE substituído por um marcador para seleção (*green fluorescent protein*, GFP). Após co-transfecção de plasmídio de deleção e DNA genômico viral em células MDBK, três recombinantes expressando a GFP foram obtidos (e denominados BoHV-1ΔgE). Os vírus recombinantes produziram placas menores em células MDBK, porém com cinética e em títulos semelhantes ao vírus parental, demonstrando que a deleção da gE não afetou negativamente a sua capacidade replicativa *in vitro*. Treze bezerros inoculados pela via intramuscular (IM) com o recombinante BoHV-1ΔgE desenvolveram anticorpos neutralizantes (títulos entre 2 e 16), demonstrando a sua capacidade replicativa e imunogênica *in vivo*. Além disso, a resposta sorológica induzida pelo recombinante pode ser diferenciada daquela induzida pelo vírus parental pelo uso de um teste ELISA específico para anticorpos anti-gE. Posteriormente, o vírus recombinante foi submetido a testes de segurança/atenuação, de imunogenicidade e de proteção frente a desafio *in vivo*. No teste de segurança, cinco bezerros de três meses de idade foram inoculados pela via IM com o recombinante em uma dose aproximadamente 10-100 vezes a dose contida em vacinas comerciais ($10^{8,5}TCID_{50}$ por animal). Os animais inoculados permaneceram saudáveis e não excretaram o vírus, confirmado pela ausência de vírus em secreções nasais e pela ausência de soroconversão em bezerros sentinelas mantidos em contato. O vírus recombinante também não foi excretado após administração de dexametasona (dia 42pi), demonstrando a incapacidade de reativar e/ou ser excretado após tentativa de reativação de infecção latente. No teste de imunogenicidade, bezerros com 8 a 10 meses de idade foram vacinados uma vez pela via IM (grupo I, n=8) ou subcutânea (SC, grupo II, n=9) com o recombinante BoHV-ΔgE viável, ou duas vezes (30 dias de intervalo) com o vírus inativado, conjugado com hidróxido de alumínio (grupo IV, n= 13) ou com Montanide™ Gel 1 (Seppic - grupo V, n=14). Como controle, três animais (grupo III) foram vacinados com o vírus parental (uma dose pela via IM). Todos os bezerros vacinados com o vírus viável desenvolveram anticorpos neutralizantes em títulos de 2 a 8 (grupo I, GMT: 2; grupo II, GMT: 1,65; grupo III, GMT: 1,65) no dia 42pv. Os demais animais desenvolveram títulos de 2 a 16 (grupo IV, GMT: 2,45) e de 2 a 128 (grupo V, GMT: 3,9). Todos os animais vacinados com o BoHV-1ΔgE permaneceram negativos no teste ELISA anti-gE. No teste de vacinação com desafio, seis bezerros (3-4 meses de idade) foram vacinados com o recombinante BoHV-1ΔgE viável na dose de $10^{7,3}TCID_{50}$/animal e quatro foram mantidos como controle. No dia 47pv, os animais foram submetidos a desafio com uma cepa heteróloga do BoHV-1 (dose de $10^{7,5}TCID_{50}$/animal) pela via intranasal. Os animais vacinados desenvolveram sinais clínicos mais brandos e por período mais curto e, excretaram vírus em menores títulos e por menos tempo do que os controles. Esses resultados demonstram que o vírus recombinante BoHV-1ΔgE é seguro/atenuado e imunogênico para bezerros, tanto na forma replicativa quanto na forma inativada. Além disso, induz resposta sorológica capaz de ser diferenciada daquela induzida pelo vírus parental. Sendo assim, o BoHV-1ΔgE apresenta propriedades adequadas para ser usado em formulações vacinais.

Palavras-chave: bovinos, vacinação, imunização, vacina diferencial, marcador vacinal.

ABSTRACT

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

CONSTRUCTION AND CHARACTERIZATION OF A RECOMBINANT BOVINE HERPESVIRUS TYPE 1 VIRUS DELETED IN THE GLYCOPROTEIN *E* AS A VACCINE CANDIDATE STRAIN

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Vaccines with antigenic markers – also known as differential vaccines – have been largely used for control and prevention of bovine herpesvirus 1 (BoHV-1) infection. With this purpose, a Brazilian BoHV-1 isolate (SV56/90) was submitted to deletion of the glycoprotein E (gE) gene for a potential use in vaccines. BoHV-1 gE gene deletion was performed by homologous recombination, being the gE gene replaced by the green fluorescent protein (GFP) gene for selection. Upon co-transfection of MDBK cells with genomic viral DNA plus the GFP-bearing gE-deletion plasmid, three fluorescent recombinant clones were obtained (and nominated as BoHV-1ΔgE). The recombinant viruses formed smaller plaques in MDBK cells yet with similar kinetics and grew to similar titers to those of the parental virus, showing that gE deletion had no deleterious effect on the replication efficiency in *vitro*. Thirteen calves inoculated intramuscularly (IM) with the recombinant BoHV-1ΔgE developed virus neutralizing (VN) antibodies at day 42pi (titers from 2 to 16), demonstrating his ability to replicate and to induce a serological response *in vivo*. Furthermore, the serological response induced by the recombinant virus could be differentiated from that induced by wild-type BoHV-1 by the use of an anti-gE antibody ELISA kit. Experiments to determine the safety, immunogenicity and protection were performed with the BoHV-1ΔgE candidate vaccine strain. In the safety test, five three months-old calves were inoculated with approximately 10-100 times the usual vaccine dose ($10^{8.5}TCID_{50}$ per animal). The inoculated animals remained healthy and did not shed virus, confirmed by the absence of virus in nasal secretions and lack of seroconversion by sentinel calves kept in contact. In addition, the recombinant virus was not shed upon dexamethasone administration (at day 42pi) showing the inability of reactivation and/or shedding after attempts of reactivation of latent infection. In the immunogenicity test, calves (8 to 10 months-old) were vaccinated once IM (group I, n=8) or subcutaneously (group II, n=9) with live BoHV-1ΔgE or twice (30 days apart) with inactivated virus plus aluminum hydroxide (group IV, n=13) or Montanide™ Gel 1 (Seppic - group V, n=14). As controls, three animals (group III) were vaccinated once IM with the parental virus. All calves vaccinated with live virus developed VN titers of 2 to 8 (group I, GMT: 2; group II, GMT: 1.65; group III, GMT: 1.65) at day 42pv. Animals of groups IV and V developed VN titers of 2 to 16 (GMT: 2.45) and 2 to 128 (GMT: 3.9), respectively. All calves vaccinated with the BoHV-1ΔgE remained negative in the gE ELISA. In a vaccination-challenge experiment, six calves (three to four-months-old) were vaccinated with live virus ($10^{7.3}TCID_{50}$/animal) and four calves were kept as controls. Forty-seven days after vaccination, the calves were challenged with a heterologous BoHV-1 strain ($10^{7.5}TCID_{50}$/animal) by the intranasal route. Vaccinated animals developed only mild and transient nasal signs comparing with the control calves. Virus shedding by vaccinated animals was also significantly reduced compared to controls. These results demonstrate that the recombinant BoHV-1ΔgE is safe/attenuated, immunogenic for calves both in a live or inactivated, adjuvanted vaccine formulation. Moreover, it induces a humoral response that can be distinguished from that induced by the wild type virus. Thus, the recombinant BoHV-1ΔgE presents suitable properties to be used in vaccine formulations.

Key words: cattle, vaccination, immunization, differential vaccine, marker vaccine.

LISTA DE TABELAS

CAPÍTULO 1

CAPÍTULO 2

LISTA DE FIGURAS

CAPÍTULO 1

CAPÍTULO 2

SUMÁRIO

1. INTRODUÇÃO

O herpesvírus bovino tipo 1 (BoHV-1) é um importante patógeno de bovinos e está distribuído mundialmente, com exceção de alguns países que o erradicaram. A infecção com o BoHV-1 pode resultar em doença respiratória, genital, abortos, infertilidade, conjuntivite, doença multissistêmica, febre dos transportes e encefalite (STRAUB, 2001). Geralmente as taxas de mortalidade são baixas, porém a infecção pode ter um impacto no desempenho do crescimento dos animais, queda na produção de leite, além de oportunizar o desenvolvimento de infecções bacterianas secundárias (BISWAS et al., 2013).

A infecção pelo BoHV-1 leva ao estabelecimento de infecção latente nos gânglios nervosos regionais após a infecção primária na cavidade nasal e/ou oral, ou da conjuntiva ocular, ou dos gânglios sacrais após infecção genital (ACKERMANN; WYLER, 1984; WINKLER et al., 2000). A reativação da infecção latente pode estar associada a fatores estressantes, como o parto (THIRY et al., 1985), transporte (JONES; CHOWDHURY, 2010), mudanças ambientais extremas (VAN DRUNEN LITTEL-VAN DEN HURK, 2006) ou tratamento com corticosteróides (WINKLER et al., 2000). Animais latentemente infectados devem ser considerados como fontes potenciais de infecção. A vacinação dos animais pode reduzir consideravelmente a quantidade viral excretada em secreção nasais e/ou genitais após episódios de reativação da infecção latente (BOSCH et al., 1997; MARS et al., 2001).

A prevenção e controle da infecção pelo BoHV-1 é baseada em diversas medidas, incluindo quarentena, uso de inseminação artificial, vacinação e, identificação e eliminação de animais positivos (OIE, 2012). Para que o controle da infecção utilizando vacinação seja efetiva, é necessário que os animais vacinados possam ser, de alguma forma, diferenciados dos animais naturalmente infectados, o que pode ser obtido pelo uso de vacinas com marcadores antigênicos (BISWAS et al., 2013). Vacinas com marcadores antigênicos – também chamadas de vacinas diferenciais – tem sido amplamente utilizadas no controle da infecção pelo BoHV-1 em vários países, porém ainda não são disponíveis no mercado brasileiro (ANZILEIRO et al., 2015).

Assim, o presente trabalho descreve a construção e caracterização de uma cepa recombinante do BoHV-1 com deleção no gene da glicoproteína E, para potencial uso em vacinas.

2. REVISÃO BIBLIOGRÁFICA

2.1 O agente

O herpesvírus bovino tipo 1 (BoHV-1) é um vírus DNA pertencente à ordem *Herpesvirales*, família *Herpesviridae*, subfamília *Alphaherpesvirinae*, gênero *Varicellovirus* (DAVISON et al., 2009). Os vírions apresentam capsídeo com simetria icosaédrica e envelope lipoprotéico. Entre essas duas estruturas está presente um material amorfo denominado tegumento (MUYLKENS et al., 2007). O capsídeo mede aproximadamente 100 a 110 nm de diâmetro, enquanto que os vírions medem em torno de 120 a 300 nm, dependendo da quantidade de tegumento. Os vírions apresentam formas pleomórficas devido a distribuição irregular do tegumento (ROIZMANN et al., 1992). O genoma do BoHV-1 é constituído de uma fita dupla e linear de DNA, com aproximadamente 135 quilopares de bases (Kb) (GenBank AJ004801). Este genoma é classificado como do tipo D, que compreende duas sequências únicas, uma longa – *unique long*, UL – e outra curta – *unique short*, US – e de duas sequências repetidas, uma interna – *internal repeat*, IR – e a outra terminal – *terminal repeat*, TR – (VAN ENGELENBURG et al., 1994).

Os vírus da subfamília *Alphaherpesvirinae* apresentam uma gama relativamente ampla de hospedeiros, um período curto de replicação e a habilidade de produzir infecção latente principalmente em sítios neurais (ROCK, 1994). Esses vírus codificam um grande número de proteínas, utilizam o núcleo da célula hospedeira para a síntese do DNA e montagem do capsídeo e adquirem o envelope a partir da membrana nuclear e/ou de organelas celulares (METTENLEITER, 2002; METTENLEITER et al., 2009).

Um total de 73 ORFs (*open reading frame*) já foram identificadas no genoma do BoHV-1, sendo que a maioria apresenta sequência homóloga à ORFs de outros membros da subfamília *Alphaherpesvirinae* (MUYLKENS et al., 2007). No entanto, o produto codificado pela ORF UL0.5 parece ser exclusivo deste vírus (DELHON et al., 2003). Alguns genes dos alphaperhesvirus podem ser delatados individualmente do genoma sem efeitos deletérios sobre a replicação viral *in vitro* e, por isso, esses genes são denominados não-essenciais. Da mesma forma, alguns genes são requeridos para a replicação viral em cultivo celular, sendo estes denominados genes essenciais (SCHWYZER, ACKERMANN, 1996). As glicoproteínas

(gps) do BoHV-1 estão localizadas no envelope viral e desempenham várias funções na biologia do vírus, participando também da patogenia e das interações com o sistema imunológico do hospedeiro (TURIN et al., 1999). Os genes de seis gps estão localizados na porção UL do genoma, incluindo a glicoproteína K (gK), que é codificada pelo gene UL53, sendo relacionada com a prevenção da fusão das células infectadas com células adjacentes (ROBINSON et al., 2008). A gC, codificada pelo gene UL44, medeia a ligação dos vírions aos receptores glicosaminoglicanos do heparan sulfato localizados na membrana celular (LI et al., 1995). A gB (codificada pelo gene UL27) está relacionada com a ligação viral ao receptor celular – heparan sulfato (LI et al., 1996). A gH (codificada pelo gene UL22) forma heterodímeros com a gL e atua na fusão do envelope viral com a membrana celular (MEYER et al., 1999). A gM (codificada pelo gene UL10) atua no envelopamento viral e na inibição da fusão de células infectadas (CRUMP et al., 2004) e a gL (codificada pelo gene UL1), forma heterodímeros com a gH, sendo necessária para o processamento e transporte da gH (KHATTAR et al., 1996).

Os genes das outras gps estão localizados na porção US do genoma, incluindo a gG (codificada pelo gene US4), que facilita a disseminação entre células por manter a junção celular entre células infectadas (NAKAMICHI et al., 2002); a gD (codificada pelo gene US6) relacionada com a ligação ao receptor de entrada celular nectin-1 (GABEV et al., 2010) e a disseminação entre células (KALTHOFF et al., 2008); a gI (codificada pelo gene US7) forma heterodímeros com a gE; e a gE (codificada pelo gene US8), que juntamente com a gI, facilita a disseminação da progênie viral entre células (REBORDOSA et al., 1996; YOSHITAKE et al., 1997). A gN (codificada pela ORF do gene UL49.5 e que forma heterodímeros com a gM) é considerada uma falsa glicoproteína, pois no BoHV-1 ela não é glicosilada, ao contrário do que ocorre em outros vírus desta subfamília (MUYLKENS et al., 2007).

2.2 Epidemiologia

O BoHV-1 está presente em praticamente todos os países, sendo que a prevalência varia amplamente, com exceção de alguns países de onde foi erradicado, como a Áustria, Dinamarca, Finlândia, Suécia, Suíça, Noruega e em parte da Itália e Alemanha (OIE, 2012). No Brasil, o primeiro relato sorológico de rinotraqueíte infecciosa bovina (*infectious bovine*

rhinotracheitis - IBR) foi feito na década de 1960, na Bahia (GALVÃO et al., 1963). Após isso, vários estudos comprovaram que a infecção por BoHV-1 estava disseminada no país. A prevalência de anticorpos para o BoHV-1 no Brasil é muito variável entre as regiões e entre os estudos. No nordeste, foram relatadas prevalências variando entre 56 e 96%, dependendo do tipo de criação e do método diagnóstico (SILVA et al., 1995; CERQUEIRA et al., 2000; SOUSA et al., 2011). Na região Centro-Oeste, a soroposividade de rebanhos foi de 83% (VIEIRA et al., 2003) e 51,9% (BARBOSA et al., 2005). No Sudeste do país, a ocorrência de anticorpos contra o BoHV-1 variou de 14,2% a 87,3% em Minas Gerais (MELO et al., 2002) e, na região Sul, foram encontradas variações de 18,8% a 64,41% de animais reagentes para BoHV-1 (LOVATO et al., 1995; VIDOR et al., 1995; MÉDICI et al., 2000; DIAS et al., 2008).

Os bovinos são os principais reservatórios e fontes de infecção para outros animais. A perpetuação do BoHV-1 na população ocorre principalmente pelo contato de animais susceptíveis com animais que estejam excretando o vírus. Animais com sinais clínicos ou reativando a infecção latente são fontes de contaminação (KAHRS, 2001). Caprinos, suínos e búfalos podem ser naturalmente infectados, porém o significado epidemiológico destas espécies é ainda desconhecido (BARNARD; COLLETT, 1994; KAHRS, 2001). Segundo Boelaert e colaboradores (2000), búfalos e outros animais selvagens podem contribuir para a manutenção do vírus nos rebanhos.

2.3 Patogenia

A infecção de bovinos pelo BoHV-1 podem resultar em doença respiratória (rinotraqueíte infecciosa bovina - IBR), vulvovaginite/balanopostite pustular infecciosa (IPV/IBP), infertilidade temporária e abortos (KAHRS, 2001). Isolados virais originários de doença respiratória podem causar doença genital e vice-versa, porém surtos que envolvam concomitantemente casos de doença respiratória e genital são raros (BARNARD; COLLETT, 1994). Existem também relatos da associação do BoHV-1 com doença neurológica, porém essa doença tem sido mais frequentemente atribuída ao BoHV-5 (SILVA et al., 2007c). Pela sua relevância sanitária e econômica, o BoHV-1 é considerado um dos principais patógenos de rebanhos bovinos de leite e corte (NANDI et al., 2009).

Após a infecção, o período de incubação varia de 2 a 6 dias dependendo da dose, rota de inoculação e habilidade de reconhecimento do início dos sinais clínicos. A penetração do vírus ocorre preferencialmente pela mucosa nasal ou genital (KAHRS, 2001; MUYLKENS et al., 2007) e lesões associadas à infecção geralmente se desenvolvem nestes locais (ENGELS; ACKERMANN, 1996). A infecção genital ocorre principalmente pela monta natural, mas pode ser causada pela utilização de equipamentos ou sêmen contaminados (KUPFERSCHMIED et al., 1986). Após replicação inicial nas células da mucosa, o BoHV-1 infecta terminações nervosas e os vírions são transportados até os corpos neuronais dos gânglios nervosos regionais (ENGELS; ACKERMANN, 1996). O vírus pode ser ainda carreado da cavidade nasal, via ductos lacrimais, para tecidos oculares e estabelecer sítios secundários de replicação (BARNARD; COLLETT, 1994). A viremia é rara, porém o vírus pode ser recuperado dos rins, linfonodos mesentéricos e fluído peritoneal. Essa distribuição é associada ao achado de monócitos e linfócitos infectados, que disseminam o vírus para os tecidos, incluindo a placenta e o feto (BARNARD; COLLETT, 1994).

O BoHV-1 atua como um importante agente no complexo respiratório bovino (VAN DRUNEN LITTEL-VAN DEN HURK et al., 1993), sendo que a sua atuação pode favorecer o estabelecimento de outros agentes, principalmente bacterianos, como a *Mannheimia haemolytica, Pasteurella multocida* e *Haemophilus somnus,* podendo levar à quadros de pneumonia nos animais (YATES, 1982). O BoHV-1 pode atuar sinergicamente com estes agentes pelos seguintes mecanismos: dano ao epitélio respiratório e consequente redução da produção de muco e redução da atividade ciliar (TIKOO et al., 1995); diminuição da atividade de macrófagos alveolares e de neutrófilos (BROWN; ANANABA, 1988; LEITE et al., 2005), acompanhado de uma depleção seletiva de linfócitos T $CD4^+$ (WARREN et al., 1996; WINKLER et al., 1999); e pela exposição prévia dos leucócitos à citocinas inflamatórias liberadas pela infecção pelo BoHV-1 (LEITE et al., 2002; 2004).

A severidade da doença é infuenciada por fatores como virulência das amostras virais; fatores ligados ao hospedeiro, como idade; estresse e presença de infecções bacterianas secundárias (MUYLKENS et al., 2007). Após o período de incubação, sinais respiratórios e oculares podem ser observados. Hiperemia das mucosas, descarga nasal e/ou ocular serosa a mucopurulenta, dificuldade de respiração, salivação, hipertermia, inapetência e depressão são alguns dos sinais apresentados (NANDI et al., 2009). A queda da produção de leite e diminuição do peso corporal são outros sinais comumente descritos (BARNARD; COLLETT, 1994).

A infecção genital caracteriza-se pela hiperemia da mucosa com presença de vesículas que tendem a coalescer e erodir associada ou não há secreção fibrinosa (VOGEL et al., 2004; HENZEL et al., 2008; WEISS et al., 2010), porém deve-se levar em conta que a maioria das infecções pelo BoHV-1 apresentam sinais clínicos leves ou até mesmo inaparentes (VAN OIRSCHOT et al., 1993). Abortos podem ser consequências da infecção em vacas soronegativas, sendo geralmente observados após o terceiro mês de gestação. Lesões decorrentes da infecção fetal podem ser observadas no fígado destes, e o período desde a infecção até o aborto pode variar de 15 a 64 dias (MUYLKENS et al., 2007).

O BoHV-1, como todos os vírus pertencentes a família *Herpesviridae*, utiliza a latência como meio para a sua manutenção na população (ROCK, 1994). O vírus persiste em neurônios na forma de DNA episomal (DNA na forma circular que é encontrado no citoplasma destas células). A transcrição de alguns RNA mensageiros (RNAm) pode ser detectada, porém ocorre pouca ou nenhuma tradução de proteínas virais (BARNARD; COLLETT, 1994). Quando da infecção do trato respiratório superior, o principal sítio de latência para o BoHV-1 é o gânglio do nervo trigêmeo, sendo que a neuroinvasão por este vírus é visto esporadicamente em animais com sinais clínicos neurológicos (HORIUCHI et al., 1995; ROELS et al., 2000). Quando há reativação da infecção latente, o BoHV-1 é transportado pela via anterógrada de volta ao tecido de replicação inicial (principalmente nas células da mucosa nasal), ocorrendo excreção, com possível infecção de novos animais (ROCK, 1994; PATEL; DIDLICK, 2009). A reativação da infecção latente não é necessariamente acompanhada da recrudescência da doença clínica, porém pode explicar a ocorrência de surtos em populações onde não há presença conhecida de fontes ativas de infecção (KAHRS, 2001).

2.4 Glicoproteína E

A gE tem sido estudada nos diferentes membros da família *Herpesviridae* há muito tempo, sendo a sua função e os efeitos da sua deleção os focos principais destes estudos. No vírus da pseudoraiva (PRV), a gE foi descrita no início dos anos 80 por Hampl e colaboradores (1984), sendo que no seguinte ano o seu gene foi mapeado e o seu produto identificado (METTENLEITER et al., 1985). O gene que codifica a gE apresenta um grau elevado de homologia entre diferentes membros da família *Herpesviridae*, indicando a

importância desta glicoproteína na biologia destes vírus (JACOBS, 1994). A porção amino terminal da gE do PRV e do HSV é parcialmente conservada, sendo que a deleção de um resíduo de cisteína e de um resíduo de valina, nas posição 126 e 125, respectivamente, tem o mesmo efeito da deleção completa do gene, quando comparado a replicação viral e na virulência destes mutantes, indicando que estes dois resíduos são importantes para a biologia e função da gE no PRV (JACOBS et al., 1993b). Porém estas deleções não afetam a imunogenicidade do vírus (JACOBS et al., 1993a). Na porção carboxi-terminal do gene da gE, há cinco resíduos de cisteína, sendo estes resíduos extremamente conservados, podendo indicar que esta proteína tem função biológica semelhante estre os diferentes herpesvírus (JACOBS, 1994).

O gene que codifica a gE não é essencial para a replicação do BoHV-1 tanto *in vitro* quanto *in vivo*, isto é, a sua deleção não impede a replicação viral (SCHWYZER; ACKERMANN, 1996). A gE é traduzida como uma beta proteína, sendo que a sua presença pode ser detectada a partir de 2-3 horas após a infecção de células de cultivo (VAN DRUNEN LITTEL-VAN DEN HURK; BABIUK, 1986; LUDWIG; LETCHWORTH, 1987).

A gE forma heterodímeros com a gI, sendo que em células infectadas com PRV a formação destes complexos é necessária para eficiente transporte destas proteínas do retículo endoplasmático rugoso para o complexo de Golgi. Esses heterodímeros são visualizados tanto na célula infectada quanto no envelope dos vírions maduros (WHEALY et al., 1993; WHITBECK et al., 1996).

Quanto à função, a gE do PRV induz fusão celular e promove a disseminação viral entre as células (ZSAK et al., 1989), além de participar na liberação dos vírions em alguns tipos celulares (METTENLEITER et al., 1987; ZSAK et al., 1989). Mutantes com deleção no gene da gE produzem placas virais menores *in vitro*, porém essa característica é revertida quando células 3T3 que expressam a gE são infectadas (JACOBS et al., 1993a). No BoHV-1, a função de disseminação entre células foi primeiramente demostrada por Rebordosa e colaboradores (1996).

No HSV-1, a gE facilita a disseminação entre células *in vivo* e o transporte viral entre as junções celulares em células e cultivo (DINGWELL et al., 1994). Os heterodímeros entre a gE e a gI possuem função direta ao se ligarem na porção Fc da imunoglobulina G – IgG (JOHNSON et al., 1988). Essa ligação parece prevenir a lise mediada pelo complemento tanto de células infectadas quanto diretamente no envelope viral, além de poder proteger contra a fagocitose mediada pela porção Fc (BELL et al., 1990). Entretanto, essa função não foi

identificada no PRV ou no BoHV-1 (OHMANN; BABIUK, 1988; ZUCKERMANN et al., 1988).

A deleção do gene da gE do BoHV-1 reduz drasticamente a virulência do vírus quando inoculado em bovinos (VAN ENGELENBURG et al., 1994), porém essa redução da virulência não se reflete na disseminação viral nos tecidos. Van Engelenburg e colaboradores (1995) inocularam animais com o vírus gE deletado ou com o vírus parental constatando uma infecção semelhante quanto aos tecidos infectados, porém de forma menos eficiente, tendo em vista um menor número de células infectadas e menor quantidade de DNA latente nestes tecidos.

A gE não é crucial para o estabelecimento da infecção latente, já que o DNA viral pode ser detectado no glânglio trigêmeo de animais infectados com mutantes deletados na gE (VAN ENGELENBURG et al., 1995). Porém, a excreção do vírus que contém a deleção na gE é menos eficiente do que nos animais com infecção latente pelo vírus parental (KAASHOEK et al., 1998). Brum e colaboradores (2009) mostraram que a gE é necessária para o transporte neuronal anterógrado do nervo trigêmeo até os sítios de replicação primária (mucosas nasal e ocular) em episódios de reativação da infecção latente.

Entre o BoHV-1 e o BoHV-5, há diferenças na composição da gE, visto que há somente 77% de similaridade entre as ORFs que codificam a gE nestes vírus e 72% de identidade de aminoácidos. Essa diferença foi comprovada por Chowdhury e colaboradores (2000) quando da construção de recombinantes de BoHV-5 que continham a deleção no gene da gE, substituição da gE do BoHV-5 pela gE do BoHV-1 e um recombinante de BoHV-1 contendo a gE do BoHV-5. Estes experimentos em animais demostraram que a gE do BoHV-5 é importante para a neurovirulência e para a neuroinvasividade deste vírus no SNC de coelhos, uma vez que os vírus recombinantes foram menos neurovirulentos e neuroinvasivos do que o BoHV-5 parental. A gE do BoHV-5 é ainda associada à neurovirulência, possivelmente pela modulação do transporte viral nas junções transneuronais por dois principais mecanismos: formação de pontos de fusão da membrana ou pela formação de poros na membranas, que facilitariam a passagem de capsídeos ou dos vírions envelopados pelas membranas (AL-MUBARAK et al., 2007).

2.5 Vacinas

A vacinação é uma das medidas de controle que podem ser adotadas na prevenção do aparecimento de doença clínica causada pelo BoHV-1. As vacinas tradicionais (atenuadas ou inativadas) tem o objetivo de prevenir, em níveis variáveis, a produção da doença clínica e, reduzir com isso as consequentes perdas econômicas. Porém a vacinação não impede a infecção dos animais vacinados pelos vírus de campo (VAN OIRSCHOT et al., 1999). Pelo fato do BoHV-1 iniciar a infecção nas mucosas (do trato respiratório superior e do trato genital), a indução de imunidade de mucosa por parte das vacinas é particularmente importante para o controle da infecção (VAN DRUNEN LITTEL-VAN DEN HURK et al., 1993).

Várias tipos de vacinas tem sido desenvolvidas e utilizadas na prevenção das perdas pelo BoHV-1. Essas vacinas apresentam composição, formulação e eficácia variáveis. No Brasil, atualmente existem oito vacinas disponíveis comercialmente (ANZILIERO et al., 2015)

2.5.1 Vacinas com vírus vivo modificado (*modified live virus* – MLV – vaccines)

A primeira vacina para o BoHV-1 foi produzida em 1957, sendo atenuada por passagens em células de cultivo de origem bovina e administrada em bovinos pela via parenteral (SCHWARTZ et al., 1957). Atenuação por adaptação em cultivos celulares de origem suína (SCHWARTZ et al., 1958) e canina (ZUSCHEK; CHOW, 1961), e adaptações em cultivo sob temperaturas mais baixas (30°C) com posterior administração parenteral também foram testadas (INABA, 1975). Vacinas MLV para a administração intranasal foram produzidas por sucessivas passagens do vírus em células de coelhos (TODD, 1974) ou por tratamento com ácido nitroso (HNO_2) com posterior seleção de mutantes termossensíveis (ZYGRAICH et al., 1974). Porém, em nenhum dos casos houve a tentativa de identificação das mutações que levava a atenuação desses vírus (VAN DRUNEN LITTEL-VAN DEN HURK et al., 1993).

De maneira geral, as vacinas MLV tem capacidade limitada de replicação no organismo dos bovinos, porém esta é suficiente para estimular a resposta imune celular e humoral (RUIZ-SÁENZ et al., 2009). Vacinas MLV geralmente induzem tanto resposta humoral quanto celular, pois a multiplicação em células infectadas leva a apresentação de antígenos virais tanto pela via do MHC classe I quanto do MHC classe II (JONES; CHOWDHURY, 2007). Após a vacinação, a proteção sistêmica e de mucosas se estabelece em um período de 2 a 3 semanas, sendo que esta pode ser constatada por períodos prolongados de tempo (RUIZ-SÁENZ et al., 2009), independentemente da via de administração, pois mesmo após inoculação intramuscular, são capazes de promover imunidade local intranasal (VAN DRUNEN LITTEL-VAN DEN HURK et al., 1993). Endsley e colaboradores (2002) relataram que a proteção clínica contra o BoHV-1 inicia-se de 40-96h após a vacinação, sendo este efeito atribuído à produção local de IFN no sítio primário de replicação. É descrito ainda que a aplicação intranasal da vacina leva à indução de IgA secretória e de imunidade celular (CASTRUCCI et al., 2004). A vacinação intranasal pode ser utilizada em surtos em populações previamente vacinadas na tentativa de reduzir o número de novos casos (RUIZ-SÁENZ et al., 2009).

A vantagem da indução de imunidade humoral e celular, desenvolvida por esse tipo de vacinação, esbarra em algumas desvantagens, como: possibilidade de reversão de virulência; risco para fêmeas gestantes pela potencial ocorrência de abortos (MILLER et al., 1991); e o estabelecimento de latência pela cepa vacinal (VAN DRUNEN LITTEL-VAN DEN HURK et al., 1993). Episódios de reativação da latência de cepas vacinais pode levar a transmissão do vírus à animais gestantes e causar episódios de aborto (JONES; CHOWDHURY, 2007).

2.5.2 Vacinas inativadas

As vacinas inativadas são produzidas pela multiplicação do vírus em cultivo celular e com posterior inativação física (RUIZ-SÁENZ et al., 2009), ou química, sendo que os inativantes mais utilizados são o formaldeído, β-propiolactona e a etilenimina binária – BEI (VAN DRUNEN LITTEL-VAN DEN HURK, 2006).

Vacinas inativadas são usualmente mais seguras do que as atenuadas, porém são geralmente menos eficazes, pois produzem apenas imunidade humoral, além de não prevenir

o estabelecimento da infecção latente após a exposição ao vírus de campo (RUIZ-SÁENZ et al., 2009). Outras desvantagens, como a denaturação de antígenos virais pelo processo de inativação, afetando a imunogenicidade da vacina; a necessidade de múltiplas aplicações para atingir níveis adequados de anticorpos neutralizantes e; a necessidade da adição de adjuvantes para melhorar a resposta imune do hospedeiro também são descritas (SCHUDEL et al., 1986). A adição de certos adjuvantes pode causar reação no sítio de inoculação da vacina (JONES; CHOWDHURY, 2007).

Alguns estudos com o objetivo de avaliar a eficácia de vacinas contra o BoHV-1 já foram realizados no Brasil. Vogel e colaboradores (2002) observaram que após três aplicações de uma vacina inativada, os animais desenvolveram títulos variáveis de anticorpos neutralizantes, sendo que alguns animais não soroconverteram. Considerando que Pospisil e colaboradores (1996) relatam que títulos entre 16 e 32 seriam os títulos mínimos protetores, foi demonstrado por Silva e colaboradores (2007a; b) que a grande maioria vacinas inativadas contra o BoHV-1 disponíveis no comércio induzem títulos abaixo ou similares à estes títulos. Em trabalho mais recente, oito vacinas comerciais disponíveis no mercado brasileiro foram testadas. Destas, apenas duas obtiveram índices satisfatório, sendo que em uma, 80% dos animais obtiveram títulos iguais ou superiores aos considerados protetivos e em outra vacina, 60% dos animais obtiveram títulos iguais aos considerados protetivos. Quanto ao restante das vacinas testadas, estas apresentaram resultados de sorologia onde no máximo 20% dos animais apresentaram sorologia igual ao considerado protetivo e, em duas vacinas, nenhum animal atingiu tal resultado, demostrando com isso que no geral, as vacinas comerciais para BoHV-1 estão muito aquém do desejável para que uma vacina seja eficaz (ANZILIERO et al., 2015).

2.5.3 Vacinas de subunidade

As vacinas de subunidade possuem uma ou mais proteínas virais como antígeno. Para o BoHV-1, as glicoproteínas são as proteínas mais imunogênicas, dentro delas, a gB, gC e gD. Animais que foram experimentalmente vacinados com estas glicoproteínas apresentaram altos níveis de anticorpos e se mostraram protegidos perante o desafio experimental, sendo esta resposta melhor do que a obtida com uma vacina inativada (BABIUK et al., 1987). A

exemplo de vacinas inativadas, as de subunidade necessitam da adição de adjuvantes para uma resposta imune eficaz. Vacinas de subunidade utilizando a gD tem-se mostrado bastente eficazes na redução de sinais clínicos e excreção viral após desafio quando adicionadas de adjuvantes como o quitosan e a CpG oligodeoxinucleotídeo (IOANNOU et al., 2002; NICHANI et al., 2006).

2.5.4 Vacinas vetoriais

Vacinas vetoriais utilizam vírus natural ou artificialmente atenuados para carrear um ou mais genes que codificam antígenos virais imunoprotetores de outros vírus (CANAL;VAZ, 2012). Donofrio e colaboradores (2006) utilizaram o BoHV-4 expressando a gD do BoHV-1, sendo que quando esta vacina foi utilizada em coelhos, pode-se observar a produção de anticorpos neutralizantes específicos contra esta proteína, porém sem o desenvolvimento de anticorpos neutralizantes para o vetor viral.

Outros autores utilizaram o adenovírus humano 5 (hAd5) como vetor viral tanto na forma replicativa como na forma não-replicativa deste. Quando estas foram administradas pela via intranasal em modelo murino, a forma replicativa deste vetor foi mais eficiente em induzir resposta de anticorpos tanto no soro quanto no trato respiratório dos animais vacinados (PAPP et al., 1999). Outro estudo utilizando modelo bovino encontraram uma melhor resposta quando utilizado o vetor na sua forma não replicativa expressando a gD do BoHV-1, provendo proteção clínica e redução significativa nos níveis de vírus excretados após desafio. Estes resultados foram melhores que os induzidos pela vacina comercial atenuada (GOGEV et al., 2002). Posteriormente, evidenciou-se ainda que os bovinos produzem títulos muito baixos de anticorpos anti-adenovírus, sendo este um bom candidato para o desenvolvimento de vacinas comerciais (GOGEV et al., 2004).

2.5.5 Vacinas com marcadores antigênicos

Vacinas com marcadores antigênicos tem como principal característica a indução de resposta sorológica passível de ser distinguida da resposta à infecção natural ou à vacinação convencional. Essa característica é obtida principalmente pela deleção de glicoproteínas de superfície viral por manipulação genética (CANAL;VAZ, 2012). Dentre as glicoproteínas presentes no BoHV-1, a gC, gE, gI e gM são classificadas como proteínas não-essenciais e estes podem ser deletados com baixo prejuízo na quantidade de vírus produzida *in vitro* ou *in vivo* (VAN DRUNEN LITTEL-VAN DEN HURK, 2006). Vacinas obtidas com esta estratégia são chamadas de vacinas DIVA (*differentiating infected from vaccinated animals*), sendo as mais utilizadas em planos de vacinação quando se almeja a erradicação do BoHV-1 de um país/região, onde métodos convencionais de segregação e eliminação são impraticáveis (RUIZ-SÁENZ et al., 2009).

A deleção de glicoproteínas para a avaliação do efeito desta sobre o BoHV-1 foi testada com diferentes glicoproteínas com resultados diversos. Denis e colaboradores (1996) realizaram a deleção das glicoproteínas gC, gE, gI e gG (cepa LAM) e estudaram a influência destas deleções na resposta imune mediada por célula. Os resultados obtidos demonstraram que o vírus com a deleção na gC mostrou uma maior proliferação específica de células contra o BoHV-1 duas semanas após a inoculação dos animais. Após desafio com vírus heterólogo (cepa IOWA), foi constatado que a gI, gE ou a gC não parecem ser os antígenos mais importantes na proliferação especifica de células para o BoHV-1, sendo que o mutante deletado na gC obteve a melhor resposta imune mediada por células.

Flores e colaboradores (1993) administraram pelo via intramuscular um vírus duplo deletado (tanto na TK como na gC, proveniente da cepa Los Angeles) e 30 dias após esta inoculação, desafiaram estes animais com cepa heteróloga (cepa Cooper) pela via intranasal. A maioria dos animais vacinados desenvolveram apenas sinais leves de infecção e excretaram vírus por um período menor do que os animais controle. Os animais inoculados se mantiveram ainda negativos quando testados para um ELISA específico para a gC, porém estes eram positivos para demais anticorpos anti-BoHV-1.

Outro estudo avaliou a imunogenicidade e reativação de mutantes deletados da gC, gG, gI, gE e combinação de gI e gE (KAASHOEK et al. 1998). Os resultados obtidos demonstraram que todos os mutantes se monstraram mais atenuados que o vírus parental

(cepa LAM), com exceção do mutante na gC. Os mutantes gI e a combinação entre gI e gE mostraram uma virulência bastante acentuada. Os animais foram desafiados com cepa virulenta heteróloga (cepa IOWA), sendo que todos os animais previamente inoculados não mostraram sinais clínicos após o desafio. A excreção do vírus utilizado no desafio dos animais foi menor no grupo previamente inoculado com a cepa parental LAM e no grupo contendo a deleção da gC, e menos evidente nos grupos contendo a deleção na gI ou duplamente deletados (gI/gE). Seis semanas após o desafio, todos os animais foram submetidos ao protocolo de reativação com dexametasona. Das cepas vacinais deletadas, apenas as que continham deleção na gC e na gG puderam ser isoladas. Este estudo ainda concluiu que o mutante na gC é muito virulento para ser utilizado como vacina sendo sugerido que os melhores candidatos para serem incorporados na vacina diferencial seriam os mutantes na gG e na gE, sendo o mutante na gG menos seguro por manter uma virulência residual e poder ser recuperado em episódios de reativação viral. Mutantes na gE já haviam previamente demostrado algumas características favoráveis como a possibilidade de manter a resposta sorológica por pelo menos três anos (KAASHOEK et al., 1996). Além disso, provavelmente a gE está presente em todos os isolados de BoHV-1.

Basicamente, após estes estudos iniciais, a grande maioria dos trabalhos foram voltados ao uso de mutantes deletados na gE. Estudos avaliando a resposta imunológica e proteção foram conduzidos utilizando mutantes na gE tanto na sua forma inativada (BOSCH et al., 1998; KERKHOFS et al., 2003) quanto na sua forma atenuada (KAASHOEK et al., 1994; KAASHOEK; VAN OIRSCHOT, 1996; MAKOSCHEY et al., 2007). Estudos para o aperfeiçoamento de técnicas diagnósticas para a detecção de anticorpos específicos contra a gE do BoHV-1 também foram realizados (LEHMANN et al., 2002).

No Brasil, Franco e colaboradores (2002b) construíram uma cepa do BoHV-1 contendo a deleção na gE deste vírus (SV 265gE-). A inoculação deste em bovinos, pela via intranasal, não resultou em sinais clínicos significativos e, principalmente, conferiu proteção aos animais frente ao desafio com o vírus homólogo parental (FRANCO et al., 2002a). Foi demonstrado ainda que esta cepa é também atenuada para fêmeas gestantes, não produzindo perdas fetais após desafio com inoculação de vírus homólogo parental (SPILKI et al., 2005). A inoculação deste vírus na mucosa vaginal, seguido por desafio com vírus heterólogo, obteve resultados de proteção clínica parcial e diminuição da excreção viral (WEISS et al. 2010). Porém um problema detectado com este vírus é que este replica em títulos inferiores aos do vírus parental, o que para a indústria de produção de vacinas é indesejável.

Dois estudos sugerem que a probabilidade de que uma vacina viva contendo amostra deletada na gE seja disseminada após possível reativação do vírus latente é muito baixa (MARS et al., 2000a, b). Estudos posteriores comprovaram estas suposições, pois estes demostraram que a gE é necessária para o transporte anterógrado neuronal do trigêmio até as terminações nervosas oculares e nasais frente a episódios de reativação (LIU et al., 2008; BRUM et al., 2009). A presença desta característica reforça a segurança do uso destes mutantes em vacinas comerciais (VAN OIRSCHOT et al., 1999). Além disso, virtualmente todos os isolados de campo do BoHV-1 apresentam o gene codificante da gE e a variabilidade antigênica entre estes é baixa, confirmando a possibilidade da utilização desta glicoproteína como um bom marcador antigênico para vacinas deletadas (RIJSEWIJK et al., 2000). A diferenciação antigênica entre animais vacinados com cepas deletadas na gE e animais infectados com o vírus de campo baseia-se na utilização de testes imunoenzimáticos (ELISA), peças fundamentais em programas de controle e erradicação do BoHV-1 (VAN OIRSCHOT et al., 1996, 1997).

Alguns pontos sobre a utilização de vacinas vivas deletadas no gene da gE devem ser discutidos e ponderados, como a possibilidade de que animais vacinados tornem-se latentemente infectados pelo vírus vacinal, o que poderia levar a formação de animais portadores e soronegativos se estes forem vacinados perante a presença de anticorpos maternos (LEMAIRE et al., 2000a, b). Além disso, a utilização de uma vacina viva é contraditória à erradicação de um agente (VAN DRUNEN LITTEL-VAN DEN HURK, 2006). A ocorrência de resultados falsos, tanto positivos como negativos, no teste de ELISA é um outro fator que pode influenciar na eficiência de programas de controle e erradicação, visto que é relatado a ocorrência de resultados falso-positivos em animais submetidos à múltiplas vacinação (LEHMANN et al., 2002). Quanto a utilização do teste ELISA para a detecção de anticorpos contra a gE, Kramps e colaboradores (2004) descrevem este teste ELISA como sendo menos sensível do que o teste de vírus neutralização e outro teste ELISA (este direcionado para a detecção de anticorpos contra a gB).

Brum e colaboradores (2010a) desenvolveram mutantes do BoHV-5 com deleção individual da gE e da timidina kinase (TK), ou a deleção de ambos genes. Estes mutantes apresentaram características desejáveis de replicação *in vitro*, não havendo diferença quando comparado à cepa parental, sendo o mutante duplamente deletado um possível candidato ao uso em vacinas. Em estudo posterior, utilizando coelhos como modelo experimental foi, observado que mediante inoculação dos mutantes deletados na TK ou duplamente deletados na TK e na gE, os animais não demostraram sinais clínicos compatíveis com a infecção pelo

BoHV-5 na infecção aguda. Também não foi possível a detecção de vírus e/ou incremento no título de anticorpos após administração de dexametasona (SILVA et al., 2010). O mutante deletado na TK falhou em reativar a infecção latente em outro estudo realizado, porém esta deleção não impediu a invasão do SNC (SILVA et al., 2011).

Em bovinos, o mutante duplamente deletado foi primeiramente testado na sua forma inativada, sendo este e a cepa parental administrados pela via subcutânea em formulação oleosa (BRUM et al., 2010b). Soroconversão foi observada até 116 dias pós vacinação em ambos grupos, quando usado teste de soroneutralização, sendo os testes realizados frente ao vírus do BoHV-1 e do BoHV-5. Os animais vacinados com a cepa mutante não demostraram qualquer indício de resposta específica contra a gE, resultado este obtido com o uso de teste ELISA comercial. Quando bovinos foram inoculados pela via intranasal com os vírus mutantes para o BoHV-5, Santos e colaboradores (2011) observaram excreção viral destes durante períodos variáveis de tempo, porém os animais não apresentaram sinais clínicos aparentes ou os sinais clínicos foram menos severos em comparação com os animais que foram inoculados com a cepa parental. Quando da administração de dexametasona, animais inoculados com mutantes que apresentavam deleção na gE ou na TK individualmente, apresentaram reativação do vírus em alguns animais, porém isso não foi observado nos animais inoculados com o vírus contendo a dupla deleção.

Em outro estudo (ANZILIERO et al., 2011a), o mutante duplamente deletado foi inoculado em animais e após 30 dias administrou-se dexametasona, sendo observada excreção de vírus sem a presença de sinais clínicos na fase aguda e ausência de recuperação viral e/ou elevação de títulos de anticorpos após tentativa de reativação viral. Os autores administraram ainda a cepa duplamente deletada pela via intramuscular em dois grupos, um mantido sob condições experimentais sem revacinação e outro mantido em condições de campo e submetidos à revacinação 240 dias após a primeira dose. No primeiro grupo não foi possível recuperação viral na secreção nasal dos animais e todos estes desenvolveram títulos virais que puderam ser diferenciados quando da utilização de kit ELISA comercial para a detecção de resposta para a gE. No segundo grupo, todos os animais, após uma segunda dose aos 240 dias pós vacinação, desenvolveram uma resposta sorológica consistente tanto para o BoHV-5 quanto para o BoHV-1. Finalmente, a utilização do vírus duplamente deletado pela via intramuscular foi capaz de proteger os animais contra desafio tanto com vírus homólogo como com o BoHV-1 (ANZILIERO et al., 2011b).

O presente trabalho teve por objetivo a construção e execução de testes *in vitro* e *in vivo* de um mutante viral de BoHV-1 com deleção na gE visando a sua utilização em formulações vacinais.

3. CAPÍTULO 1

A glycoprotein E gene-deleted bovine herpesvirus 1 as a candidate vaccine strain

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Abstract

A bovine herpesvirus 1 (BoHV-1) defective in glycoprotein E (gE) was constructed out of a Brazilian genital BoHV-1 isolate, by replacing the full gE coding region by the green fluorescent protein (GFP-gene) for selection. Upon co-transfection of MDBK cells with genomic viral DNA plus the GFP-bearing gE-deletion plasmid, three fluorescent recombinant clones were obtained out of approximately 5.000 viral plaques. The deletion of gE gene and presence of GFP marker in the genome of recombinant viruses were confirmed by PCR. Albeit forming smaller plaques, the recombinants BoHV-1ΔgE replicated in MDBK cells with similar kinetics and to similar titers to that of the parental virus (SV56/90), demonstrating that gE deletion had no deleterious effect on the replication efficacy *in vitro*. Thirteen calves inoculated intramuscularly with BoHV-1ΔgE developed virus neutralizing (VN) antibodies at day 42 post-infection (titers from 2 to 16), demonstrating the ability of the recombinant to replicate and to induce a serological response *in vivo.* Furthermore, the serological response induced by the recombinant BoHV-1ΔgE could be differentiated from that induced by a wild-type BoHV-1 by the use of an anti-gE antibody ELISA kit. Taken together the results are promising towards the use of the recombinant BoHV-1 ΔgE in vaccine formulations to prevent the losses caused by BoHV-1 infections while allowing for differentiation of vaccinated from naturally infected animals.

Key words: Cattle pathogen; Immunization; Control; Differential vaccine.

1 – Introduction

Bovine herpesvirus 1 (BoHV-1) is an important pathogen of cattle, associated with a variety of clinical manifestations including respiratory disease (infectious bovine rhinotracheitis – IBR), genital disorders (infectious pustular vulvovaginitis - IPV or infectious pustular balanoposthitis – IPB), transient infertility and abortions in cattle (1). BoHV-1 is an enveloped DNA virus belonging to the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Varicellovirus* (2). BoHV-1 infection is widely distributed around the world, with the exception of a few European countries that have eradicated the infection. A number of studies have demonstrated the wide distribution of BoHV-1 infection and disease in Brazil (3, 4). As other alphaherpesviruses, BoHV-1 establishes lifelong latent infection in sensory nerve ganglia following acute infection, from which it can be periodically reactivated and transmitted. Thus, latency and reactivation provide adequate means for virus perpetuation in nature (5).

Vaccination has been largely used as one of the strategies to prevent and to reduce the losses associated with BoHV-1 infection (6). Traditional vaccines usually contain attenuated or whole inactivated virus and induce a serological response undistinguishable from that induced by natural infection. The impossibility to differentiate vaccinated from naturally infected animals impairs control/eradication efforts based on the identification and segregation and/or culling of seropositive animals (7). In this regard, gene-deleted vaccines that allow for serological differentiation – also called DIVA vaccines ("differentiating infected from vaccinated animals") - have arisen as alternatives for traditional vaccines (8). Such vaccines have long been used in several European and North American countries (2). In particular, this strategy fits well for herds and/or regions undertaking control/eradication

efforts (8). A similar approach was successfully employed to eradicate pseudorabies virus (PRV) in several countries (9).

The BoHV-1 genome is approximately 138kb in length and encodes around 70 products, from which 10 are envelope glycoproteins (gps). Envelope gps play important roles in viral biology, pathogenesis and constitute major targets for the host immune system (10). Interestingly, some envelope gps are non-essential for virus replication in cell culture and *in vivo* as well and, as such, have been deleted towards the production of attenuated and/or antigenically marked vaccine strains (11). The envelope glycoprotein E (gE) has been the choice target for deletion towards the production of antigenically marked vaccines for several herpesviruses such as BoHV-1 (7, 12, 13) and BoHV-5 (14, 15). The choice for gE relies upon the following reasons: i. gE is non-essential for virus replication *in vitro* and *in vivo* and its deletion usually does not reduce significantly the efficiency of virus replication *in vivo* (16); ii. gE deletion usually contributes to viral attenuation (11); iii. gE deletion does not affect viral immunogenicity (7, 11, 13) and, finally, iv. gE is fairly immunogenic, a desirable property for an antigenic marker (12). For these reasons, most BoHV-1 marker vaccines available worldwide contain recombinant gE-negative viral strains (7, 17, 18).

Efforts to produce and make commercially available BoHV-1 marker vaccines have long been reported in Brazil (19, 20). A gE-negative BoHV-1 strain has been constructed and evaluated regarding to safety, immunogenicity and potential serological differentiation (21, 22). More recently, a gE and thymidine kinase (TK) double deletion BoHV-5 recombinant strain was constructed and proposed as a candidate vaccine strain (14). Unfortunately, no BoHV marker vaccine is currently available in Brazil. To fill this gap, we constructed a gE-deleted strain out of a well characterized genital Brazilian BoHV-1 strain (23) intended to be used as a vaccine strain. This article reports its construction, *in vitro* characterization and preliminary characterization regarding to immunogenicity and serologic differential property.

2 - Material and Methods

2.1 – Virus strain, cells and plasmid vectors

The Brazilian BoHV-1 strain SV56/90 - isolated from preputial swabs and semen of bulls with balanoposthitis (23) - was used as the parental virus to construct the recombinant viruses. Madin Darby bovine kidney cells (MDBK, ATCC - 22) maintained in MEM (Minimun essential medium Eagle, HiMedia Laboratories, India), supplemented with 10% inactivated and γ-irradiated fetal bovine serum (Nutricell, Brazil), 100U/mL of penicillin and 100µg/mL of streptomycin (Invitrogen, USA) were used in all procedures.

The plasmid vectors used in the construction/recombination procedures included; i: a deletion plasmid (pBoHV-1ΔgE) to introduce the gE deletion on the BoHV-1 genome and replace by the GFP marker; ii: a plasmid expressing the bovine immediate-early gene ICP0 (bICP0), used as transactivator of the initiation of the transcription of the immediate early genes of the BoHV-1 genome (24); and iii: a plasmid expressing the GFP gene used for construction of the pBoHV-1ΔgE plasmid. The bICP0 plasmid was kindly provided by Dr. Clinton Jones (University of Nebraska at Lincoln, USA).

2.2 – Construction of BoHV-1 gE deletion plasmid

The deletion plasmid pBoHV-1ΔgE was constructed by replacing the entire gE open reading frame (ORF – Figure 1A) by the green fluorescent protein (GFP) gene as a marker for selection. To construct this plasmid, the upstream and downstream sequences of gE gene (gI and US9, respectively) were amplified by PCR, using Platinum® Taq DNA Polymerase High Fidelity (Invitrogen, USA) and cloned into pBlueScriptII KS (+) vector (Stratagene, USA). The gE upstream sequence was PCR amplified using a pair of primers (gI FW 5’-CACAGGATCCGTTTGTACACAGCTTCGG-3’ and gI RW 5’-CACAGAATTCTGCCAAATGCCCTTTTCG-3’), resulting in a product of 933 bases pairs

(bp) which incorporates a BamHI/EcoRI sites in the 5' and 3' ends, respectively. The gE downstream sequence was PCR amplified using a pair of primers (Us9 FW 5'-CACAAAGCTTCTGTGCCGTCTGACGGAA-3' and Us9 RW 5'-CACAGGTACCGCCCGAATCCCCTCCTTC-3') resulting in a product of 888bp, which incorporates the HindIII/KpnI sites at the 5' and 3' ends, respectively (Figure 1B). To introduce the GFP gene between the gI and Us9 fragments, a PCR reaction using a pair of primers (GFP insertion FW 5'- CACAGAATTCTGACTTGAGCGTCGATTT-3'and GFP insertion RW 5'- CACAAAGCTTCCGATTTCGGCCTATTGG-3) was performed using the pEGFP-C1 plasmid (Clontech Lab, USA) as template, resulting in a product with 1.856bp that incorporates EcoRI and HindIII sites. At the ends, PCR products were digested with the respective enzymes and cloned between the gI and Us9 fragments. The deletion plasmid (pBoHV-1ΔgE) contains the GFP gene replacing the gE gene (Figure 1C).

2.3 – DNA extraction and transfection

Extraction of genomic viral DNA was performed essentially as described (13). Briefly, MDBK cells were inoculated with SV56/90 strain at a multiplicity of infection (m.o.i.) of 0.1. When the cytopathic effect (CPE) reached around 90% of the monolayer, the supernatant was collected and clarified by low speed centrifugation. The supernatant was then submitted to ultracentrifugation in a 30% sucrose cushion for 2h at 112.500xg. The resulting pellet was resuspended in 1xTE (10mM Tris-HCl, 5mM EDTA, pH 8.0) and pre-digested in 1% SDS and RNAse A (Invitrogen, USA) for 30 min at room temperature. The digestion was completed by adding 500µg/mL of proteinase K (Sigma-Aldrich, USA) and performing a new incubation at 56°C for 30 min. Following digestion, viral DNA was extracted with phenol:chloroform:isoamyl-alcohol (25:24:1), followed by ethanol precipitation according to standard protocols.

DNA of pBoHV-1ΔgE and BoHV-1 bICP0 gene was extracted using Qiagen Plasmid Midi Kit (Qiagen, USA). Full length viral DNA and plasmids were co-transfected into MDBK cells using Lipofectamine reagent (PolyFect Transfection Reagent, Qiagen, USA) and Opti-MEM I media (Gibco-BRL, USA) as part of the lipofectamine protocol (13, 14).

2.4 – Generation and selection of recombinant viruses

To generate the BoHV-1 gE deleted virus, the linearized pBoHV-1ΔgE plasmid, the full length wild type virus SV56/90 DNA and the bICP0 plasmid were co-transfected into MDBK cells, using Lipofectamine reagent (Invitrogen, USA) as described (13). After 48-72h, cell cultures showing evident CPE were freeze-thawed, low speed centrifuged and the supernatants were submitted to plaque purification in MDBK monolayers using low melting agarose overlay. After 72h, the plates were examined under UV light to search for fluorescent plaques. Fluorescent plaques were picked and amplified in MDBK cells for subsequent characterization.

2.5 – PCR confirmation of gE deletion

To confirm the deletion of gE gene in the fluorescent viruses recovered from transfected cultures, a PCR reaction using a pair of primers that amplify the deleted region was performed. Total DNA from mock-infected MDBK cells, MDBK cells infected with the parental virus (BoHV-1 SV56/90) or MDBK cells infected with viruses amplified from fluorescent plaques was extracted using proteinase K digestion and phenol/chloroform extraction as described. The PCR reaction was carried out in a 50 µL volume containing 1 x PCR buffer, 0.2 mM dNTPs, 0.4 µM of each primer (BoHV-1 gE FW 5'-GCCAGCATCGACTGGTACTT - 3' and BoHV-1 gE RW 5'-GCACAAAGACGTAAAGCCCG - 3'), 1.25 U of Taq DNA polymerase (Invitrogen, USA),

1.5 mM of $MgCl_2$, 10% DMSO and 0.1 µg of DNA as template. The PCR conditions consisted of initial denaturation at 95°C – 10 min; followed by 40 cycles of 95°C – 45 s, 57°C – 45 s and 72°C – 1 min and final extension of 10 min at 72°C. Ten microliters of each reaction were eletrophoresed in 1.5% agarose gel and stained with ethidium bromide. A 325 bp product is expected in DNA samples that contain the gE gene. As controls, PCR reactions for the gB coding gene (25) and for the GFP gene (using the same par of primers used for the construction of the pBoHV-1ΔgE plasmid) were performed.

2.6 –Growth properties of recombinants *in vitro*

A virus growth experiment was performed to analyze the kinetics of replication of BoHV-1ΔgE in comparison with the parental BoHV-1 SV56/90. Cultures of MDBK cells were infected with each virus at an m.o.i. of 0.1 at 4°C for 1h. Cultures were then incubated at 37°C with 5% of CO_2 and harvested at different intervals and frozen at -80°C. The supernatants were titrated and the titers were expressed as $TCID_{50}$/mL (log_{10}). To compare plaque size and morphology, MDBK cells were inoculated and adsorbed 2h with each virus, overlayed with 1.6% carboxymethylcellulose, incubated for 72h, fixed with 10% buffered formalin and stained with 0.35% crystal violet.

2.7 - Animal inoculation and serological testing

Two to four months-old calves, negative to BoHV-1 antibodies, were inoculated with BoHV-1ΔgE#3 by the intramuscular route (IM) at two different doses: eight animals received a viral dose of $10^{7.3}TCID_{50}$/animal and five received $10^{8.5}TCID_{50}$/animal. We chose these titers to meet on the average titers used by other authors (13, 22, 26). The number and age of the animals used for each inoculation followed recommendations by European Pharmacopoeia for tests of BoHV-1 live vaccines. Three calves were inoculated with the

parental virus SV56/90 ($10^{7.3}$$TCID_{50}$/animal). The VN antibodies titers, expressed as the reciprocal of the highest dilution that prevents virus replication, were transformed in geometrical mean titers (GMT – log_2 [27]) for the calculation of the mean antibody titers of each group. After 42 days, sera was tested for virus neutralizing antibodies against BoHV-1 by virus neutralization (VN) assay, according to standard protocols (22). To verify seroconversion to gE, serum samples were tested by a commercial anti-gE antibody ELISA test (Bovine Rhinotracheitis Virus gE Antibody Test – IDEXX – the Netherlands). Sera of calves previously inoculated with a gE-positive virus (28) were tested in parallel by VN and ELISA kit, as additional positive controls in both tests (Table 1).

All procedures of animal handling and experimentation were conducted under veterinary supervision and according to recommendations by the Brazilian Committee of Animal Experimentation (COBEA, law #6.638 of May, 8th, 1979). The experiment was approved by an Institutional Animal Ethics Committee (UFSM, approval # 34/2014).

3 – Results

3.1.Selection of GFP-positive, gE-negative BoHV-1 recombinant viruses

Recombinant BoHV-1 viruses lacking the gE gene were constructed by homologous recombination between genomic DNA of a BoHV-1 strain (SV56/90) and a plasmid containing the gE flanking regions and the GFP gene replacing the gE coding region (Figure 1). After two attempts of co-transfection of MDBK cells with parental virus DNA, deletion plasmid and a bICP0 plasmid and screening of approximately 5.000 plaques, three fluorescent plaques were picked and amplified for further characterization. A representative fluorescent plaque is shown in Figure 2. Initially, viral clones derived from the three plaques were amplified and submitted to three rounds of plaque purification.

Then, DNA extracted from MDBK cells infected with each viral clone was submitted to PCR for GFP and gE genes. One recombinant clone (clone #1) presented double bands in the gE PCR suggesting contamination with a gE-positive virus (data not shown), and was discarded. The remaining clones (#2 and 3) were further characterized. Figure 3 shows that these recombinants were indeed lacking the gE coding region (Figure 3A) and harboring the GFP marker gene (Figure 3C). Thus, the recombination strategy was successful and two pure BoHV-1 clones lacking the gE gene were obtained. These viral clones were further amplified for characterization and will be thereafter called BoHV-1ΔgE#2 and BoHV-1ΔgE#3.

3.2. *In vitro* characterization of BoHV-1ΔgE recombinants

The *in vitro* properties of the recombinant BoHV-1ΔgE#2 and #3 were investigated and compared with the parental virus. The plaque size and morphology produced by the recombinants and parental viruses were monitored in MDBK cell monolayers. In general, the plaques produced by BoHV-1ΔgE#2 and #3 were smaller than those produced by the parental virus (Figure 4). The virus growth curve of the recombinants and parental viruses were assayed in MDBK cells and the results are shown in Figure 5. The results demonstrate that both BoHV-1ΔgE clones replicated with similar kinetics and to similar – even slightly higher - titers as compared with the parental virus. Taken together these results indicate that gE deletion apparently had no major deleterious effects on the ability of the recombinant viruses to replicate efficiently in cell culture. This is a highly desirable property for a virus intended to be used as a vaccine strain.

3.3. Behavior of the recombinant BoHV-1ΔgE *in vivo*: immunogenicity and serological differentiation in calves

As the BoHV-1ΔgE recombinant viruses are intended for vaccine use, we next investigated their ability to replicate and to induce an immune response in calves. For this, two groups of calves (eight and five animals each) were inoculated IM with the BoHV-1ΔgE #3 virus in two doses ($10^{7.3}$TCID$_{50}$ and $10^{8.5}$TCID$_{50}$ per animal) and tested for VN antibodies 42 days after inoculation (pi). All calves inoculated with the BoHV-1ΔgE #3 seroconverted, developing VN titers from 2 to 16 at 42pi. Similar titers were observed in three calves inoculated with the parental virus (Table 1). These data indicate that the recombinant BoHV-1ΔgE#3 is able to replicate efficiently in calves following IM administration and induces a VN response in levels comparable to that induced by the parental virus.

We next investigated the ability of the recombinant BoHV-1ΔgE#3 to induce a serological response that could be differentiated from the immune response induced by a gE positive virus. For comparison, we used serum samples collected from calves inoculated with a wild-type BoHV-1 (28). Serum samples collected at 42pi were tested by an anti-gE ELISA kit. As shown in Table 1, all calves immunized with the recombinant BoHV-1ΔgE#3 remained negative in the gE ELISA, contrasting with the animals immunized with a gE-positive virus. These data demonstrate that the serological response induced by the recombinant BoHV-1ΔgE can be differentiated from that induced by the wild type virus by the use of an anti-gE ELISA test.

4 – Discussion

A recombinant BoHV-1 deleted in gE gene (BoHV-1ΔgE) was constructed with the primary goal to be used as a vaccine strain. Following a long-term trend observed in many European and North American countries, Brazil has also embarked on the development of

antigenically marked BoHV-1 strains for vaccine use (19-21). A gE negative BoHV-1 strain constructed out of a Brazilian isolate has proven to be safe, immunogenic and allow for serological differentiation (19, 21, 22). More recently, a double deletion (TK/gE) BoHV-5 recombinant was constructed and evaluated positively as a candidate vaccine strain (14, 15, 28-30). However, in spite of the fact that BoHV-1 and BoHV-5 are antigenically very similar and that vaccines based on either virus are expected to confer cross-protection (28), no vaccine containing gene-deleted BoHV-1 or BoHV-5 is yet available in Brazil. Thus, to fill this gap we constructed a gE-deleted recombinant BoHV-1 strain. We chose a genital Brazilian BoHV-1 strain (SV56/90) as the parental virus for the following reasons: 1 – SV56/90 is a well characterized BoHV-1 strain; 2 – it replicates to high titers *in vitro*, a desirable property for a vaccine strain; 3 – is highly immunogenic in cattle. 4 – genital and respiratory BoHV-1 are antigenically very similar (sometimes undistinguishable) and are highly cross-reactive serologically. In addition, the strain SV56/90 has been extensively characterized at biological, antigenic and molecular levels (31-33).

The strategy of full gE deletion has also been used to construct recombinant BoHV-1ΔgE virus by other authors (20, 17). In the other hand, some authors chose to perform a partial deletion in the gE gene, keeping the portion next to the Us9 gene, but no significant differences were observed comparing to full deletion (13, 26). In all these strategies, the possibility of serological differentiation by an ELISA test was maintained.

The homologous DNA recombination that results in the generation of recombinant genomes is a rare event and, as consequence, the selection of the recombinant viruses resulting from this event may be a very laborious work. The incorporation of the GFP gene into the BoHV-1ΔgE genome was an easy means to identify and recover gE deleted recombinant viruses after transfection and helped to monitor virus purity after plaque purification as well (13). The bICP0 expressing plasmid was pivotal for the success of the

recombination protocol since bICP0 is an essential transactivator of BoHV-1 immediate early genes (34).

In vitro characterization of the two BoHV-1ΔgE clones showed that their ability to replicate in cell culture was not adversely affected by gE deletion, as they replicated to titers comparable to those of the parental virus (Figure 1). Indeed, previous studies have shown that BoHV-1ΔgE recombinants are able to replicate *in vitro* to similar titers to the parental virus (13, 14). The ability to replicate to high titers in cell culture is an obvious advantage of virus strains intended to be used for vaccine production. On the other hand, the recombinants produced smaller plaques than the parental virus, a property already observed in a gE-defective BoHV-1 (7, 13) and BoHV-5 viruses (14). This phenotype is probably associated with the fact that gE – complexed with gI - is involved in cell-to-cell spread *in vitro* (35, 36). The choice for gE as a target for deletion also relied upon the role of this glycoprotein in anterograde transport of the virus from nerve ganglia to the nose after reactivation of latency (37). Thus, gE-deleted viruses are not transported efficiently back to the nose and, consequently, they are not re-excreted and transmitted upon reactivation (38).

To access whether the BoHV-1ΔgE would retain its replication ability and immunogenicity *in vivo*, groups of calves were inoculated IM with the virus and the serological response was measured at 42pi. The serological response of the animal inoculated with the BoHV-1ΔgE virus was similar in magnitude to that induced in animals inoculated with wild type virus. In general, the antibody titers observed here were similar to those reported in previous studies (13, 26) when live gE-deleted BoHV-1 was inoculated by the IM route, even when younger animals were used. These results show that the gE deleted virus retained its immunogenicity and, thus, presents a potential to be used as a vaccine strain. Early studies have shown that gE deleted herpesviruses generate similar or a slightly lower serological responses when compared with wild-type viruses (13) or vaccines strains (7, 12).

In addition to the immunogenic potential, an important feature of a gene-deleted vaccine is the possibility of differentiation of vaccinated from naturally infected animals (6). In our testing, the animals inoculated with the recombinant BoHV-1ΔgE mounted a serological response that, at 42 days post inoculation, could be differentiated from that mounted by animals inoculated with gE-positive viruses. Although based on a small number of animals, these results demonstrate the differential property of this candidate vaccine strain.

An antigenically marked BoHV-1 vaccine to be used in Brazilian cattle would bring an important contribution for the control of the infection in the country: 1- control and eradication of BoHV-1 have been achieved in some European countries by using a similar strategy; 2- Brazil and other South American coutries have long been planning to use DIVA vaccines against bovine herpesviruses; 3- a commercial anti-gE ELISA kit for differentiation of vaccinated from naturally infected animals is already available. For these reasons, and considering the properties demonstrated by recombinant BoHV-1ΔgE *in vitro* and *in vivo*, we consider that this strain is suitable to be included in either modified-live or inactivated vaccine formulations for the control of BoHV-1 infection in the country.

The *in vivo* data presented here is still preliminary and require further experimentation before the recombinant strain is considered adequate for vaccine use. These studies are underway and include: 1 – safety and immunogenicity tests in different animal categories (including young calves and pregnant cows); 2 – immunogenicity tests using inactivated virus, since the licensing of such vaccines is more feasible and easy in Brazil, comparing with live ones; 3 – vaccination-challenge experiments to investigate the ability of the recombinant to confer protection upon challenge and; 4 – an experiment to investigate whether the recombinant is safe for use in pregnant cows. In any case, the preliminary data presented in this report are very promising regarding to the potential use of the recombinant as a vaccine strain.

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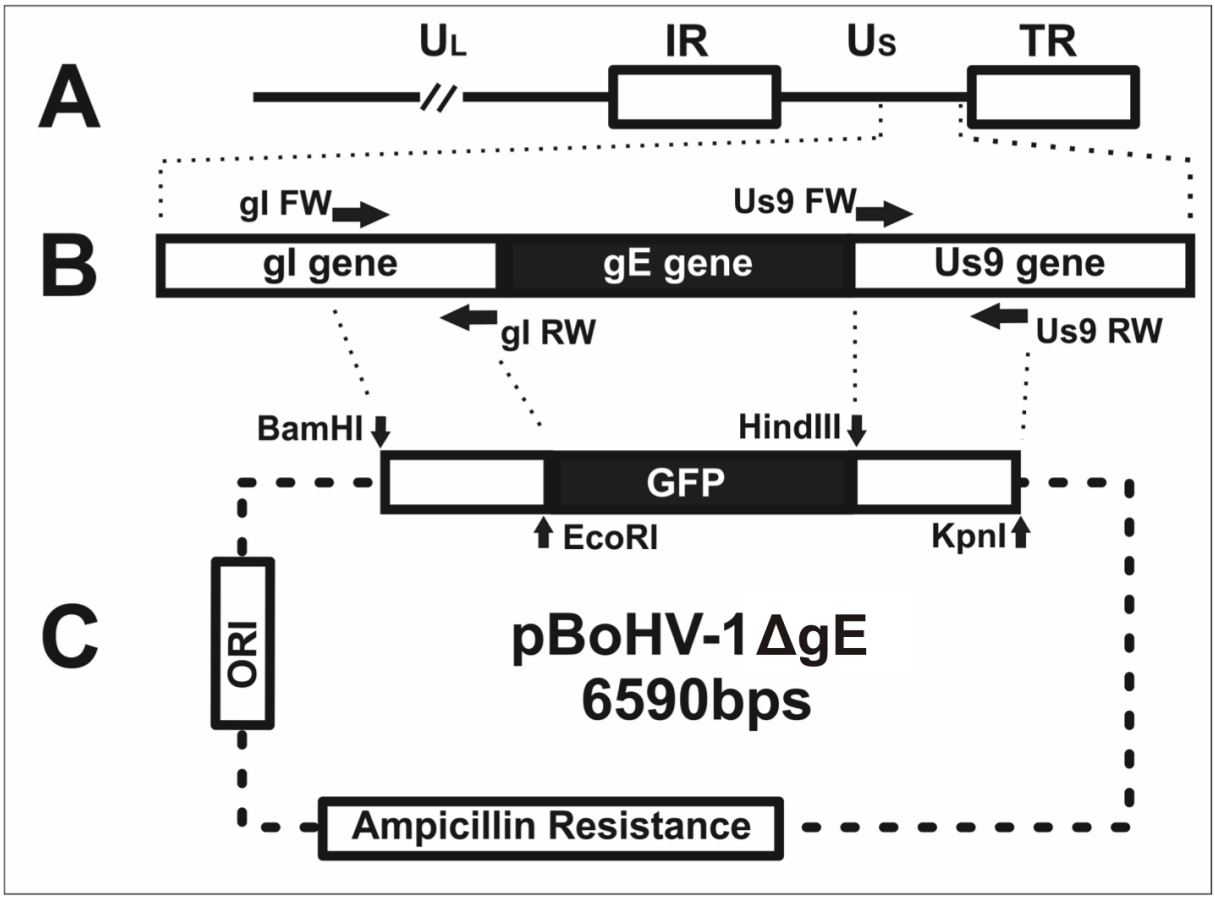

Figure 1. Strategy for construction of the gE deletion plasmid. A. Schematic organization of the BoHV-1 genome and their unique long (U_L) and short (U_S) regions and repeats (internal – IR – and terminal – TR). B. Amplified view of the organization of the BoHV-1 genome on the U_S region correspondent to gE gene. Arrows show the primers used for amplifying the gE flanking regions. C. Schematic organization of the deletion plasmid containing the regions for homologous recombination and the restriction endonuclease sites used in the cloning strategy.

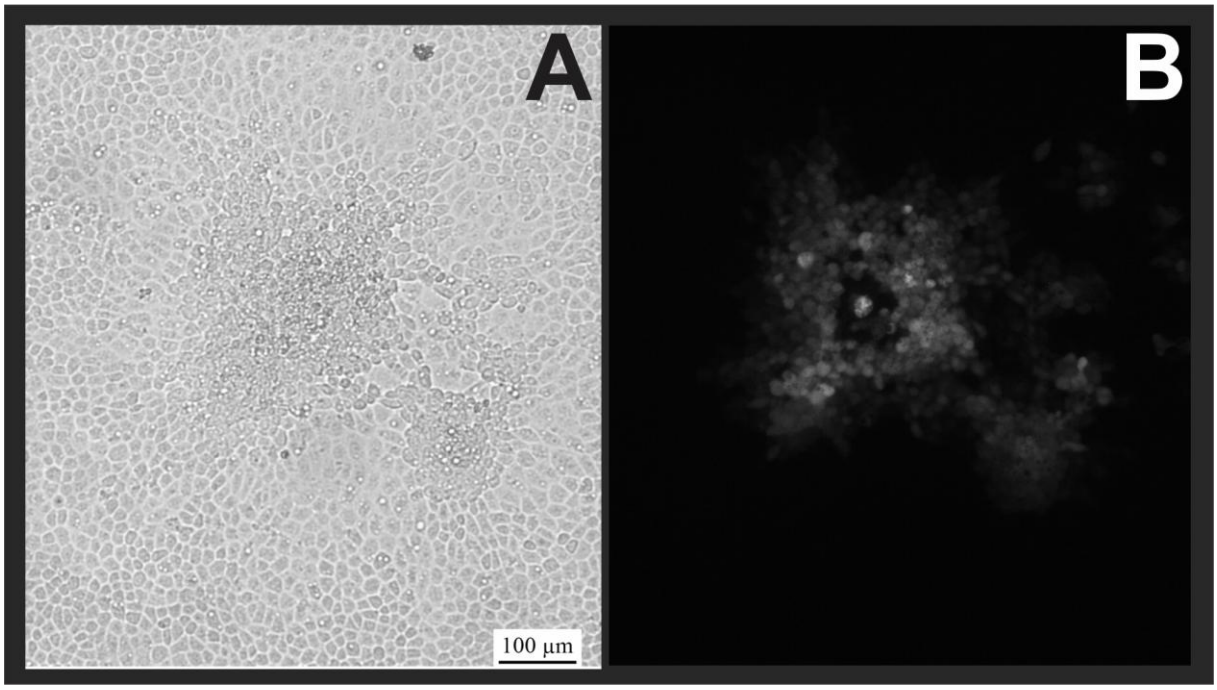

Figure 2. Identification of a recombinant virus expressing the GFP selection marker. Plaques formed in MDBK cell monolayers were visualized under light microcopy (A) and under UV light (B).

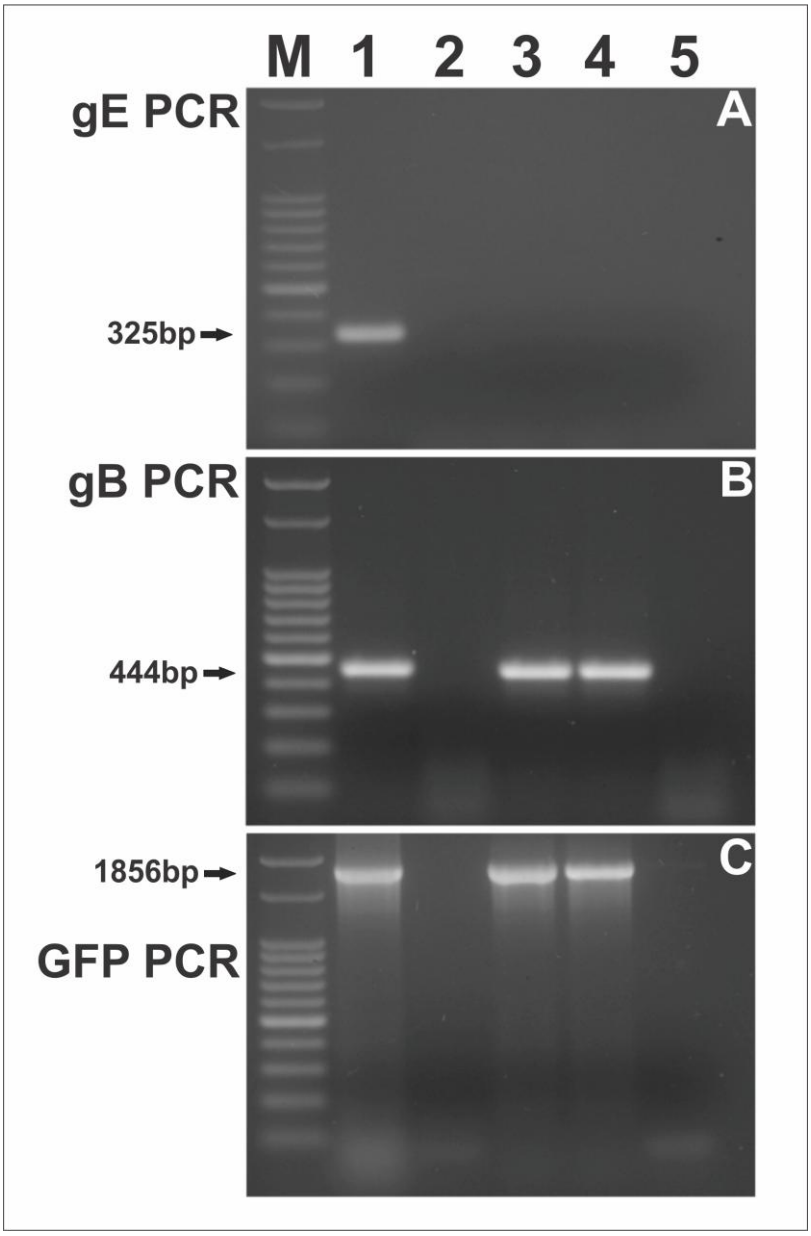

Figure 3. Confirmation of gE gene deletion in the recombinant BoHV-1ΔgE by PCR. Figure 3A: PCR for the gE gene. Figure 3B: PCR for the gB gene. Figure 3C: PCR for the GFP marker gene. Lane M – molecular weight marker; lane 1: parental virus (SV56/90) in A and B or peGFP-C1 plasmid in C; lane 2 –DNA sample from non-infected MDBK cells – negative DNA control; lane 3 – BoHV-1ΔgE #2; lane 4 - BoHV-1ΔgE #3 and lane 5 – negative control (water).

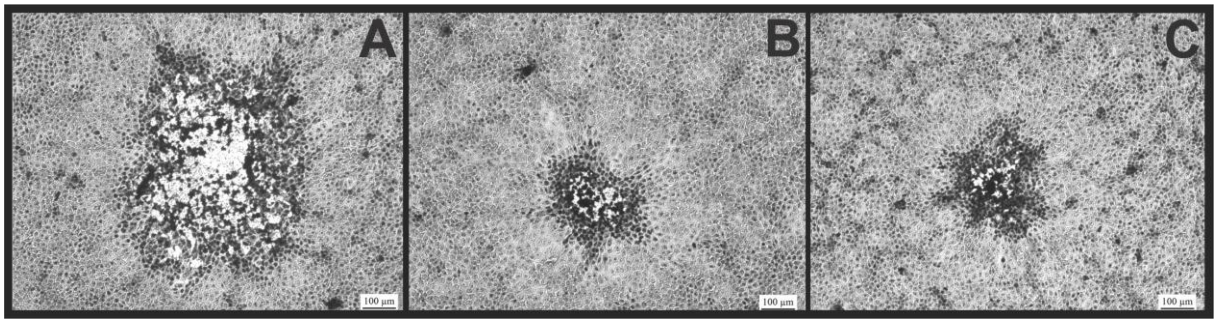

Figure 4. Plaque morphology of parental virus and recombinant BoHV-1ΔgE viruses. Plaque assays were performed in MDBK cell monolayers overlayed with 1.6% carboxymethylcellulose and stained with crystal violet at 72h. A: Parental virus (SV56/90 strain); B: BoHV-1ΔgE#2; C: BoHV-1ΔgE#3.

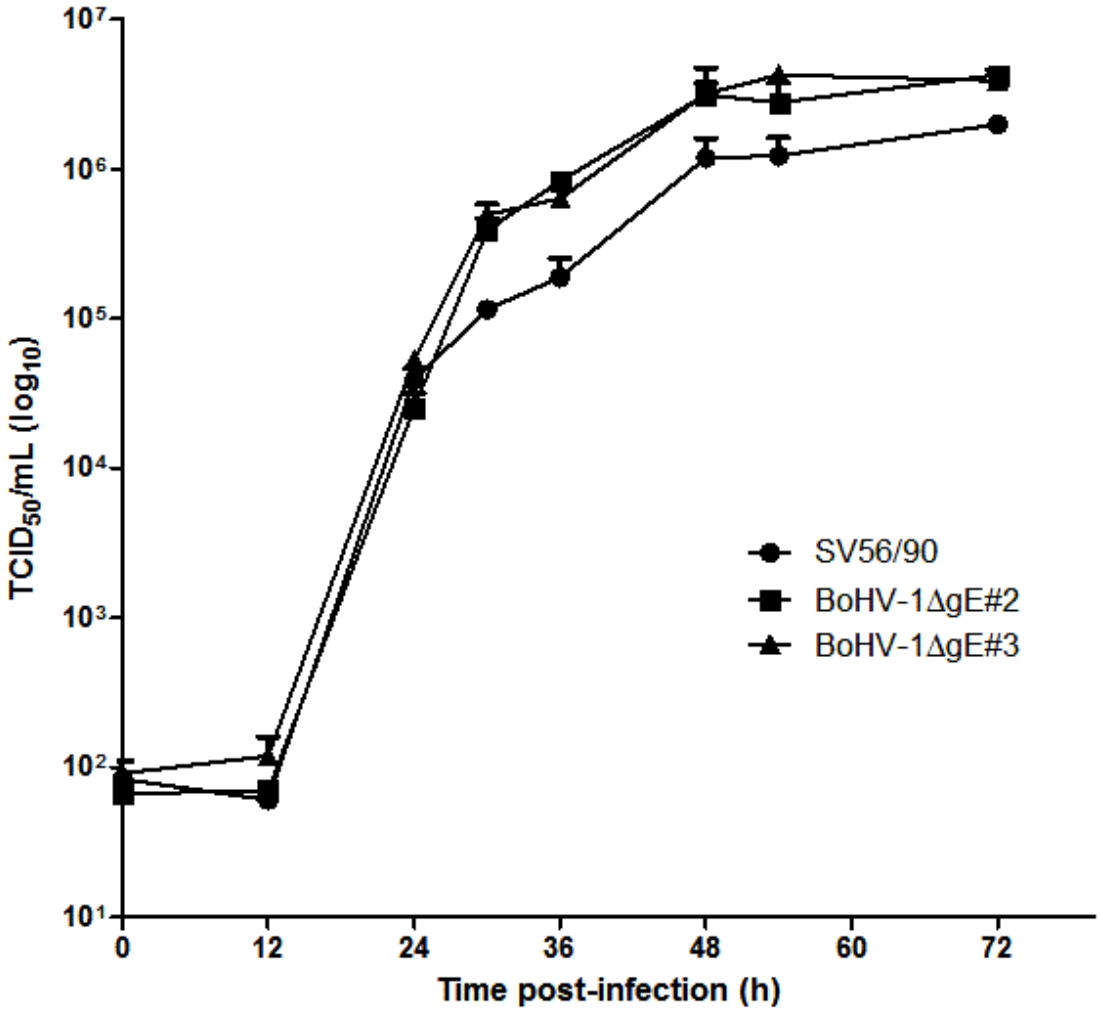

Figure 5. Virus growth curve. MDBK cells were inoculated with the parental virus (BoHV-1 SV56/90) or with the recombinant viruses (BoHV-1ΔgE clones #2 and 3). At different times post-infection cells were freeze-thawed and the viral progeny was quantified. The virus titers are expressed as log_{10} $TCID_{50}$/mL. Bars represent the standard error of mean of three different titrations for each time point.

Table 1. Serological response in calves inoculated with wild-type BoHV-1 or with recombinant BoHV-1ΔgE#3.

Treatment	Animal	Virus neutralizing (VN) antibodies [1]		Anti-gE ELISA[2]
		Day 0	Day 42pi[3]	Day 42
BoHV-1ΔgE#3 $10^{7.3}$TCID$_{50}$/animal Intramuscular	101	<1[4]	3[5]	-[6]
	102	<1	3	-
	104	<1	1	-
	105	<1	2	-
	106	<1	2	-
	107	<1	3	-
	108	<1	1	-
	109	<1	1	-
BoHV-1ΔgE#3 $10^{8.5}$TCID$_{50}$/animal Intramuscular	117	<1	2	-
	127	<1	2	-
	129	<1	3	-
	132	<1	4	-
	136	<1	2	-
BoHV-1 SV56/90 (WT) $10^{7.3}$TCID$_{50}$/animal Intramuscular	112	<1	1	+[7]
	113	<1	2	+
	114	<1	2	+
BoHV-1 EVI123 $10^{7.6}$TCID$_{50}$/animal Intranasal[8]	179	<1	4	+
	192	<1	4	+
	349	<1	3	+
	381	<1	4	+
	4325	<1	2	+

[1]Virus neutralizing antibodies were measured by virus neutralization (VN) assay as described in material and methods.
[2]Sera from day 42 post-inoculation were submitted to a commercial anti-gE antibody ELISA test (Bovine Rhinotracheitis Virus gE Antibody Test – IDEXX – the Netherlands).
[3]pi – post-inoculation.
[4]Sample negative in VN assay at its lower dilution (1:2).
[5]Results from the VN assay are shown as geometrical means titers (GMT – $\log_2$).
[6]Samples negative for gE antibodies.
[7]Samples positive for gE antibodies.
[8]Animals from a previous study. Reference number 28.

4. CAPÍTULO 2

Safety and immunogenicity of a glycoprotein E gene-deleted bovine herpesvirus 1 candidate vaccine strain

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Abstract

A glycoprotein E-deleted Brazilian bovine herpesvirus 1 (BoHV-1ΔgE) was tested regarding to safety and immunogenicity. Intramuscular inoculation of young calves with a high virus dose did not result in clinical signs or virus shedding during acute infection or after dexamethasone administration. Calves vaccinated once IM (group I) or subcutaneously (group II) with live BoHV-1ΔgE or twice with inactivated virus plus aluminum hydroxide (group IV) or Montanide™ (group V) developed VN titers of 2 to 8 (GMT:2); 2 to 4 (GMT:1.65); 2 to 16 (GMT:2.45) and 2 to 128 (GMT:3.9), respectively. All BoHV-1ΔgE vaccinated calves remained negative in an anti-gE ELISA. Lastly, six young calves vaccinated with live BoHV-1ΔgE and subsequently challenged with a virulent BoHV-1 strain shed less virus and developed only mild and transient nasal signs comparing to unvaccinated calves. Thus, the recombinant BoHV-1ΔgE is safe and immunogenic for calves and allows for serological differentiation by a gE-ELISA test.

Key words: BoHV-1; cattle; marker vaccine; differential vaccine.

1. Introduction

Bovine herpesvirus 1 (BoHV-1) is associated with a variety of clinical manifestations in cattle, including respiratory disease (infectious bovine rhinotracheitis – IBR), genital disorders (infectious pustular vulvovaginitis - IPV or infectious pustular balanoposthitis – IPB), transient infertility and abortions (Kahrs, 2001). BoHV-1 is an enveloped DNA virus belonging to the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Varicellovirus* (Muylkens et al., 2007). As other alphaherpesviruses, BoHV-1 establishes lifelong latent infection in sensory nerve ganglia after acute infection, from which it can be periodically reactivated and transmitted (Roizmann et al., 1992).

Vaccination has been largely used to prevent and reduce the losses associated with BoHV-1 infection, but traditional vaccines usually contain live attenuated or whole inactivated virus and induce a serological response undistinguishable from that induced by natural infection (van Drunen Littel-van den Hurk, 2006). In this regard, gene-deleted vaccines that allow serological differentiation – also called DIVA vaccines (differentiating infected from vaccinated animals) - have proven to be attractive alternatives for traditional vaccines (Kaashoek et al., 1994). The envelope glycoprotein E (gE) has been the choice target for deletion towards the production of antigenically marked vaccines for several alphaherpesviruses, including BoHV-1 (Chowdhury et al., 1999; Kaashoek et al., 1994; Kaashoek et al., 1995), BoHV-5 (Brum et al., 2010a; Brum et al., 2010b) and swine herpesvirus 1 or Aujeszky disease virus (Moormann et al., 1990; van Oirschot et al., 1990).

Several efforts have been made to produce and to make commercially available BoHV-1 marker vaccines in Brazil (Franco et al., 2002a; Franco et al., 2002b; Spilki et al., 2005). A gE-negative BoHV-1 strain has been constructed and evaluated regarding to safety, immunogenicity and potential serological differentiation (Spilki et al., 2005; Weiss et al., 2010). A gE and thymidine kinase (TK) double deletion BoHV-5 recombinant strain was

constructed and proposed as a candidate vaccine strain (Brum et al., 2010b). Nonetheless, no BoHV-1 or BoHV-5 marker vaccine is currently available in Brazilian market. To fill this gap, we constructed a gE-deleted strain (BoHV-1ΔgE) out of a well characterized genital Brazilian BoHV-1 strain, intended to be used as a vaccine strain (Weiss et al., 2015).

This article reports an investigation on the safety and immunogenicity of the recombinant BoHV-1ΔgE in calves and on its ability to confer protective immunity against challenge with a heterologous BoHV-1 strain.

2. Material and Methods

2.1. Viruses and cells

The recombinant BoHV-1ΔgE was constructed out of a Brazilian BoHV-1 SV56/90 strain (Weiblen et al., 1992). All procedures of construction and *in vitro* characterization of the recombinant were published recently (Weiss et al., 2015). The experiments described herein used the clone #3, passage # 3 of the recombinant BoHV-1ΔgE. The BoHV-1 strain EVI123 was used in the challenge experiment (virus kindly provided by Dr. Paulo Michel Roehe, Universidade Federal do Rio Grande do Sul, Brazil). Madin Darby bovine kidney cells (MDBK, ATCC – CCL-22) maintained in MEM (Eagle's Minimum Essential Medium, HiMedia Laboratories, India), supplemented with 10% inactivated and γ-irradiated fetal bovine serum (Nutricell, Brazil), 100U/mL of penicillin and 100µg/mL of streptomycin (Invitrogen, USA) were used in all procedures.

2.2. Animals, virological and serological monitoring

All experiments used calves free of BoHV-1 antibodies, as ascertained by two negative VN assays 30 days apart (Weiss et al., 2015). The breed and age of the animals varied according to the experiment (safety, immunogenicity and vaccination-challenge) and

are specified in the respective sections. Animals were maintained in native grass and/or supplemented with alfalfa and given water *ad libitum*.

Nasal swabs were collected and processed for virus isolation in MDBK cells as described previously (Anziliero et al., 2011) and positive samples were quantitated by limiting dilution and virus titers were expressed as $TCID_{50}$/mL. Serum samples obtained at different times after virus inoculation or immunization were tested for virus neutralizing (VN) antibodies against BoHV-1 by virus neutralization assay, using the parental virus as the challenge virus (Weiss et al., 2010). Titers were expressed as the reciprocal of the highest dilution that prevented virus replication, were transformed in geometric mean titers (GMT – log_2) for the calculation of the mean antibody titers of each group (Thrusfield, 1986). Anti-gE antibodies in sera of inoculated-vaccinated calves were tested using a commercial anti-gE antibody ELISA test according to instructions (Bovine Rhinotracheitis Virus gE Antibody Test – IDEXX – the Netherlands).

All procedures of animal handling and experimentation were conducted under veterinary supervision and according to recommendations by the Brazilian Committee of Animal Experimentation (COBEA, law #6.638 of May, 8th, 1979). The experiment was approved by an Institutional Animal Ethics Committee (UFSM, approval # 34/2014).

2.3. Safety test

Five 3 to 4-months-old Holstein calves, seronegative to BoHV-1, were inoculated by the intramuscular route (IM) with the recombinant BoHV-1ΔgE in a dose of $10^{8.5}TCID_{50}$ per animal. Two calves were kept in contact with the inoculated animals. Inoculated and sentinel animals were monitored in a daily basis (clinical signs, body temperature) and submitted to collection of nasal swabs up to day 14 post-inoculation (pi). At day 42pi, inoculated and sentinel animals were submitted to dexamethasone administration (Dx, Decadronal, Achê,

Brazil, 0.1mg.kg.day during 5 days) and monitored for virus shedding and clinical signs up to day 14 post-Dx.

2.4. Immunogenicity tests

The immunogenicity tests were performed in 8 to 10-months-old cross-breed calves maintained in extensive conditions on natural grass. The immunogenicity of BoHV-1ΔgE was evaluated both as a live virus and in an inactivated vaccine preparation. In the first, 17 calves were inoculated IM (group I, n=8) or SC (group II, n=9) with $10^{7.3}TCID_{50}$ of viable BoHV-1ΔgE. As controls, three calves were inoculated IM with the parental virus (SV56/90 strain – group III). Sera collected at the day of vaccination (day zero) and at day 42 post-vaccination (pv) were tested for VN and gE antibodies.

In the second test, a BoHV-1ΔgE virus stock was inactivated with binary ethylenimine according to standard protocols (Brum et al., 2010a). Then, two vaccine formulations were prepared, either using 15% of aluminum hydroxide (Omega, Brazil – group IV) or 50% of Montanide™ gel 1 (high molecular weight polyacrylic polymer – Seppic, USA – group V), both in an aqueous formulation. Each viral dose contained inactivated virus correspondent to $10^{7.3}TCID_{50}$ before inactivation in a final volume of 5mL. Calves were immunized IM twice (30 days apart) and serum for VN tests and ELISA was collected at day 30pv and 30 days later (day 60pv).

2.4. Vaccination-challenge experiments

This experiment used 3 to 4-months-old Holstein calves, seronegative to BoHV-1. Six calves were vaccinated IM with BoHV-1ΔgE in a dose of $10^{7.5}TCID_{50}$ and four were kept as non-vaccinated controls. Forty-seven days pv, vaccinated and controls were challenged by IN instillation of a highly virulent BoHV-1 strain – EVI123 – (Spilki et al., 2004) in a dose of

$10^{7.5}TCID_{50}$ per animal. Clinical signs, body temperature and nasal swabs for virus isolation and quantitation were collected in a daily basis for 14 days. Blood for serology (VN and gE-ELISA) was obtained at the day of vaccination, at the day of challenge (47 days pv) and 14 days after challenge. The clinical monitoring consisted of daily clinical examination by two persons who attributed scores to the following parameters for each animal/day: nasal secretion (0 = absence to 4 = abundant, mucopurulent); ocular secretion (0 = absence or 1 = presence); dyspnea (0 = absence or 1 = presence); conjunctivitis (0 = absence or 1 = presence); nasal signs (0 = normal nasal mucosa to 6 = generalized pustules). The clinical scores were adapted from a previous paper (Anziliero et al., 2011).

2.5. Statistical analysis

The results shown in the text, tables and figures are expressed as the mean standard error (SEM). Differences among the treatments were tested by Student's t-test in one way analysis of variance (ANOVA), using the Assistat program, version 7.7. The least significance difference between groups for $p<0.05$ was calculated to determine whether treatments were statistically different.

3. Results

3.1. Safety of the recombinant BoHV-1ΔgE in calves

Even using such a high virus dose, correspondent to 10- to 100-times the highest commercial vaccine dose, none of the inoculated animals showed any clinical sign suggestive of BoHV-1 infection. Inoculated animals remained healthy and with normal temperature throughout the monitoring period (Table 1). Likewise, no infectious virus was recovered from nasal swabs collected daily following virus inoculation. In addition, the sentinel in-contact calves remained healthy and seronegative, demonstrating lack of transmission of the vaccine

virus. To confirm efficient replication of the vaccine virus, all vaccinated animals developed VN antibodies on day 42pi (4 – 16, GMT: 2.6). As expected, these calves remained negative for gE antibodies (Table 1).

To investigate whether the vaccine virus would reactivate latent infection, vaccinated animals were submitted to Dx treatment at day 42pi and monitored for virus shedding and VN antibodies. Again, none of the vaccinated animals showed clinical signs of BoHV-1 infection, shed virus in nasal secretions or had an increase in VN titers, indicating lack of reactivation. Taken together, these results showed that the recombinant BoHV-1ΔgE is safe for calves after IM administration – even using an excessively high virus titer – and is not excreted in nasal secretions during acute replication or after Dx administration.

3.2. Immunogenicity of the recombinant BoHV-1ΔgE in calves

The immunogenicity of the recombinant BoHV-1ΔgE was evaluated in beef calves (8 to 10-months-old) under field conditions, testing either a live virus vaccine (one dose) or an inactivated, adjuvanted vaccine preparation (two doses). Serological tests (VN and anti-gE ELISA) were performed in sera collected day 42pv (live virus) or after the second vaccine administration, at day 60pv (inactivated preparation). Calves immunized with live virus IM (group I) developed VN titers of 2 – 8 (GMT: 2), whereas calves immunized with live virus SC (group II) or the group immunized with the parental vírus (group III) developed VN titers of 2 – 4 (GMT: 1.65). Both groups (group I and II) remained negative for gE antibodies at day 42pv and the three control calves inoculated with the parental virus developed gE antibodies (Table 2). These results showed that calves immunized with live recombinant virus developed VN titers comparable to those developed by calves immunized with wild type virus, yet remained negative for gE antibodies.

All calves immunized with inactivated virus, regardless the adjuvant, developed VN titers and remained negative for gE antibodies at day 30 post-revaccination (Table 3). Calves of group V (adjuvant Montanide^{TM} gel 1) developed higher titers (2 to 128, GMT: 3.9) than those of group IV (aluminum hydroxide, VN titers 2 to 16, GMT: 2.45). Both groups remained negative for gE antibodies after the two vaccine doses (Table 3). These results demonstrate that the inactivated vaccine preparation was immunogenic for calves and produced an immune response that could be differentiated from that induced by wild type BoHV-1 by the use of a commercial anti-gE ELISA kit.

3.3. Protection conferred by the recombinant BoHV-1ΔgE

The protection conferred by vaccination with the recombinant BoHV-1ΔgE was evaluated in 3 to 4-months-old Holstein calves, challenged IN with a highly virulent BoHV-1 at day 47pv. The body temperature of vaccinated calves remained within normal limits up to day 6 post-challenge (pc), in contrast with the controls, which presented an increase in temperature (Figure 1). From day 7pc to the end of monitoring, both groups presented a drop in body temperature, accompanying a drastic drop in ambient temperature (not shown). Regardless, the temperatures of vaccinated animals remained generally below the temperature of controls.

The duration of virus shedding was significantly reduced ($p<0.05$) in vaccinated animals (6.8 days ± 2.4 days), comparing with controls (11 days ± 2.6 days). Virus shedding was no longer detected after day 9 pc in vaccinated animals whereas it continued up to day 13 pc in the controls. The differences in virus titers in nasal secretions (controls versus vaccinated) were 3 $\log_{10}$ (3 days); 2 $\log_{10}$ (3 days); 1 $\log_{10}$ (3 days) whereas in three days there was no difference (Figure 2).

The vaccinated animals presented milder and transient clinical signs comparing to the control group. The main differences concerned to the amount and aspect of nasal discharge, presence of ocular discharge and conjunctivitis, and lesions (vesicles, pustules, fibrinous membrane) in the nasal mucosa. The animals from the control group presented higher amount of nasal discharge and the secretion evolved to mucopurulent in 3 out of 4 animals. These animals also presented serous ocular discharge and conjunctivitis in five of the 14 days of monitoring; in addition to higher number and size of pustules in the nasal mucosa. Two of the control animals presented coalescent pustules covering most of the nasal mucosa and the other two controls presented localized coalescent pustules. In contrast, the vaccinated group presented only serous nasal discharge, did not show ocular discharge or conjunctivitis and developed few, small and localized pustules in the nasal mucosa. The overall daily clinical score developed by control and vaccinated groups are shown in Figure 3.

All vaccinated animals remained negative to gE antibodies up to the day of challenge (day 47pv), seroconverting to gE thereafter. At day 30pc all vaccinated and control animals were positive in the gE ELISA, remaining positive up to day 90pc (Table 4).

Taken together these results showed that immunization of calves with live BoHV-1ΔgE conferred satisfactory protection against challenge with a heterologous, virulent BoHV-1 strain. Protection was demonstrated by reduction in virus shedding (magnitude and duration) and significant reduction in the intensity and duration of nasal and systemic signs (fever) in vaccinated animals.

4 – Discussion

Our results showed that the recombinant BoHV-1ΔgE - a candidate vaccine strain constructed out of a Brazilian BoHV-1 isolate - is safe and immunogenic for calves and, as expected, induces a serological response that can be differentiated from that induced by wild

type virus. Highly susceptible calves inoculated IM with a viral dose higher than 10 to 100-times an usual vaccine dose remained healthy, did not shed virus following virus inoculation nor after Dx treatment. Immunization of calves with live virus or with inactivated, adjuvanted virus preparations resulted in a VN response of adequate magnitude. In addition, vaccination-challenge experiments demonstrated that the recombinant was able to induce satisfactory protection upon stringent challenge with a heterologous BoHV-1 strain, as measured by reduction in viral shedding and clinical protection. These results are promising towards the use of this recombinant in vaccine preparations. Additional safety tests in pregnant cows, large-scale immunization in field conditions and evaluation of the duration of immunity are currently underway to confirm the adequacy of this recombinant for vaccine use.

DIVA vaccines – most of them based on gE deletion - have long been used for the control/prevention of BoHV-1 infection and disease in several European countries (van Drunen Littel-van den Hurk, 2006) and have been gradually introduced in other continents. In South America, a number of gE-deleted BoHV-1 and BoHV-5 recombinants have been constructed and proposed as candidate vaccine strains (Brum et al., 2010b; Franco et al., 2002a; Romera et al., 2014). Nonetheless, no such vaccine is yet commercially available in South American countries (Anziliero et al., 2015). The delay in making these recombinants commercially available is probably due, in part, to safety and regulatory issues concerning the use of live recombinant vaccines in livestock.

As the recombinant BoHV-1ΔgE is intended to be used either in modified live vaccine (MLV) or in inactivated vaccines, a safety would be required to assess its inocuity. The safety test, performed in highly susceptible young calves and using such a high virus dose (as recommended by the European Pharmacopea for live viral vaccines) confirmed the safety of the recombinant. Upon IM inoculation, no infectious virus was recovered from nasal secretions nor seroconversion of sentinel calves was observed. In this sense, a number of

studies have demonstrated that gE deletion contributes for BoHV-1 attenuation and, thus, reinforces the choice for gE deletion as an antigenic marker for BHV-1 vaccines (Franco et al., 2002b; Kaashoek et al., 1994; Kaashoek et al., 1998; Mars et al., 2000; Romera et al., 2014; Van Engelenburg et al., 1994). On the other hand, the serological response developed by inoculated calves demonstrated that proper vaccine virus replication did ensue upon IM inoculation in these calves.

In addition to the safety during acute infection, the recombinant was not excreted upon Dx treatment, confirming previous findings that gE-deleted BoHV-1 recombinants are deficient in reactivation and/or in shedding upon corticosteroid treatment (Brum et al., 2009; Chowdhury et al., 2010; Kaashoek et al., 1998; Liu et al., 2008; Mars et al., 2000). Regardless the reasons for the lack in virus shedding upon Dx treatment (defficient establishment of latency, deficient reactivation or deficient anterograde transport), this is a highly desirable phenotype in a herpesvirus candidate vaccine strain.

Glicoprotein E-deleted BoHV-1 mutants usually produce smaller plaques in cell culture than wt virus and show a reduced immunogenicity *in vivo* (Chowdhury et al., 1999; Kaashoek et al., 1994). In our experiments, however, immunization of calves with the recombinant induced a VN response equivalent to that induced by the parental virus (Table 2). Nonetheless, as the protection induced by live herpesvirus vaccines also rely upon the cellular response, it would be interesting to assess whether the cellular response induced by gE-deleted virus would differ from that induced by wt virus (Romera et al., 2014).

The immunogenicity of the recombinant was also demonstrated in inactivated vaccine preparations. In this assay, the VN response in calves immunized with inactivated virus plus Montanide^{TM} gel 1 was significantly higher than the VN response to virus combined with aluminum hydroxide (Table 3). As the licensing of recombinant live vaccines for livestock is a time-consuming and laborious process in Brazil, this recombinant would probably be used

first in inactivated formulations. Regardless, in both cases, the serological response induced by vaccination could be differentiated from that induced by parental virus by the use of a commercial ELISA (Tables 2 and 3).

The vaccination-challenge experiments demonstrated a satisfactory degree of protection conferred by vaccination with live recombinant virus, as demonstrated by reduction in virus shedding and clinical protection. It should be emphasized that we performed a highly stringent challenge, using a high dose of a highly virulent virus (Spilki et al., 2004). Even though, vaccinated animals shed virus for a shorter period of time and in much lower titers (in most of the time) and presented only mild signs of nasal/respiratory disease and recovered faster than the controls. It is reasonable to speculate that, under natural conditions, in which challenge would be likely significantly lower/weaker, the vaccine would confer a higher degree of protection.

Bovine herpesvirus 5 (BoHV-5) is a neuropathogenic virus, closely related to BoHV-1 and highly prevalent in South American countries (Del Medico Zajac et al., 2010). The high antigenic similarity and extensive cross-neutralization between BoHV-1 and 5 has led to the concept that proper immunization with either virus would confer cross protection (Anziliero et al., 2011; Del Medico Zajac et al., 2010). Thus, an adequate BoHV-1 vaccine accompanied by a reliable vaccination program would probably confer protection to both viruses in areas where they co-circulate. Regardless these hypothetical considerations, it should be advisable to test this vaccine against BoHV-5 challenge to generate confirmatory data that would allow confirmation of this hypothesis.

Regarding to safety, vaccine tests involving cows in different stages of pregnancy need to be performed to demonstrate that the vaccine strain is safe to be used in these animals. Likewise, vaccination-challenge experiments after immunization with an inactivated virus

formulation still have to be performed in order to ensure the safety and efficacy of this vaccine candidate.

In summary, our experiments demonstrated that the recombinant BoHV-1ΔgE virus is safe (was not shed by inoculated animals nor transmitted to sentinel animals; did not reactivate infection upon Dx treatment) and immunogenic for calves both in live and inactivated preparations. Vaccination-challenge tests demonstrated that immunization of calves with the recombinant conferred partial virological and clinical protection upon challenge. Further, both live and inactivated virus preparations induced a serological response that could be differentiated from that induced by wt virus. Thus, the recombinant BoHV-1ΔgE presents properties that candidates it as a vaccine strain, upon additional experiments to confirm its safety and immunogenicity. The availability of a commercial gE-ELISA test for serological differentiation would favor its use as a vaccine strain in control/eradication programs of BoHV-1/5 infection in Brazil.

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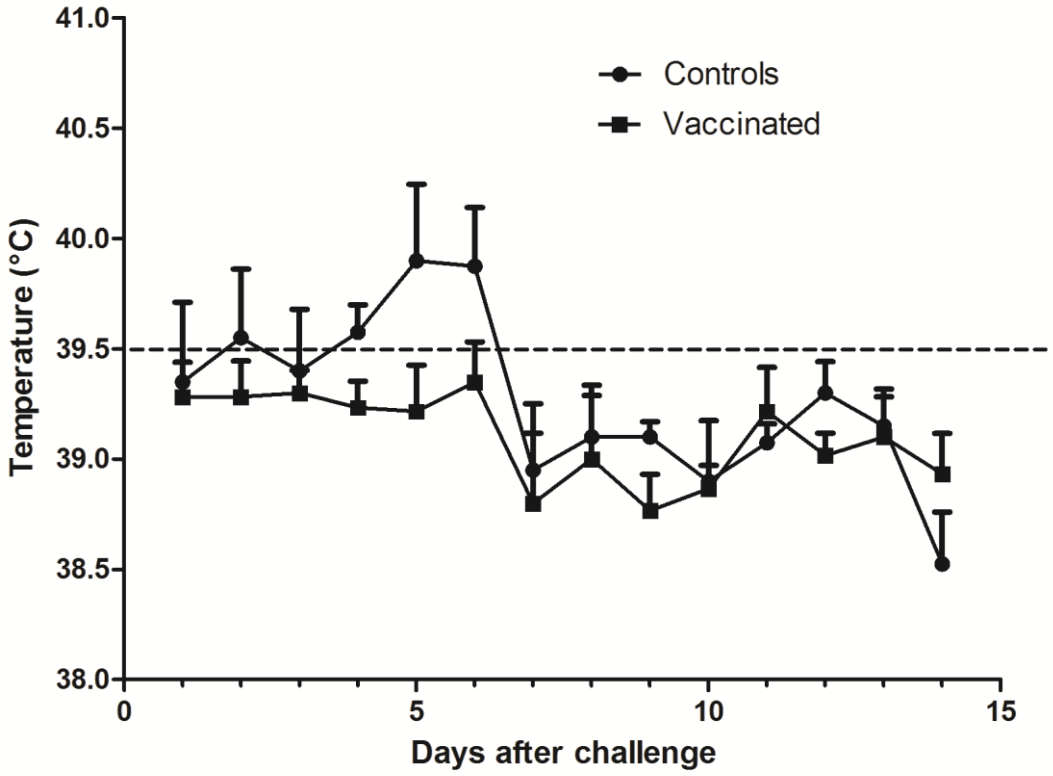

Figure 1. Body temperature of calves of the challenge test. Six animals were vaccinated with a BoHV-1ΔgE virus and 47 days later were challenged with a heterologous virulent BoHV-1 strain. Four calves were kept as controls. Dotted line marks the threshold for fever. Bars represent the mean standard error.

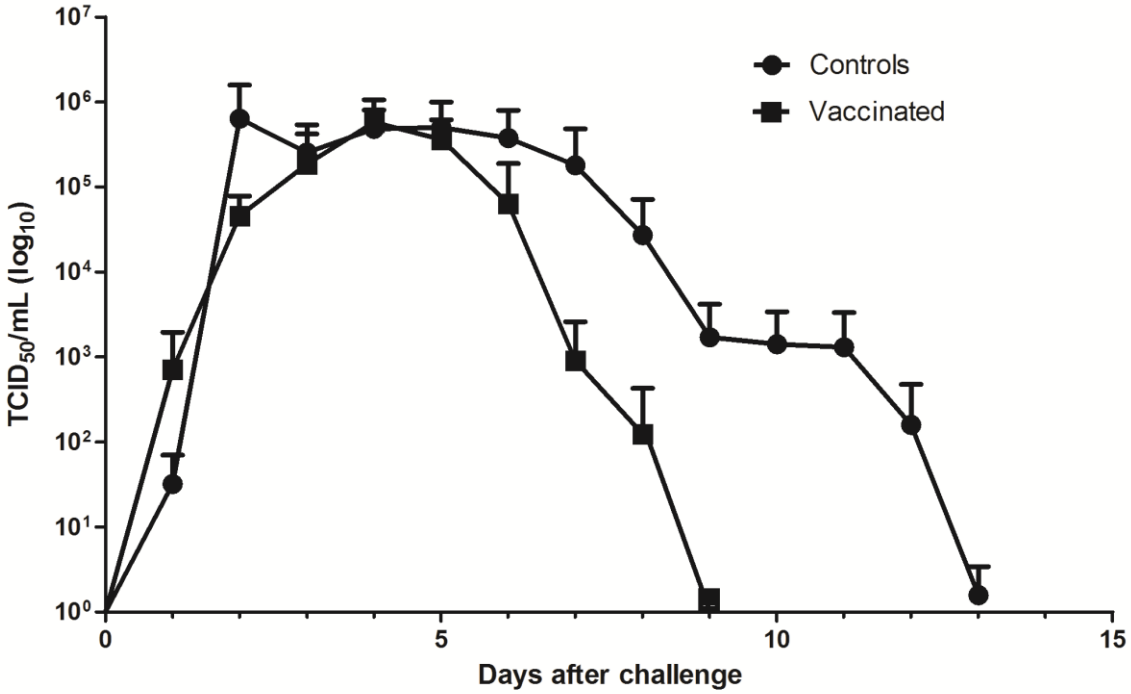

Figure 2. Virus shedding in nasal swabs collected from the calves of the challenge test. Mean virus titers for each group (vaccinated, controls) expressed in $\log_{10}$ $TCID_{50}$/ml. Six animals were vaccinated with a BoHV-1ΔgE virus and 47 days later were challenged with a heterologous virulent BoHV-1 strain. Four calves were kept as controls. Bars represent the mean standard error.

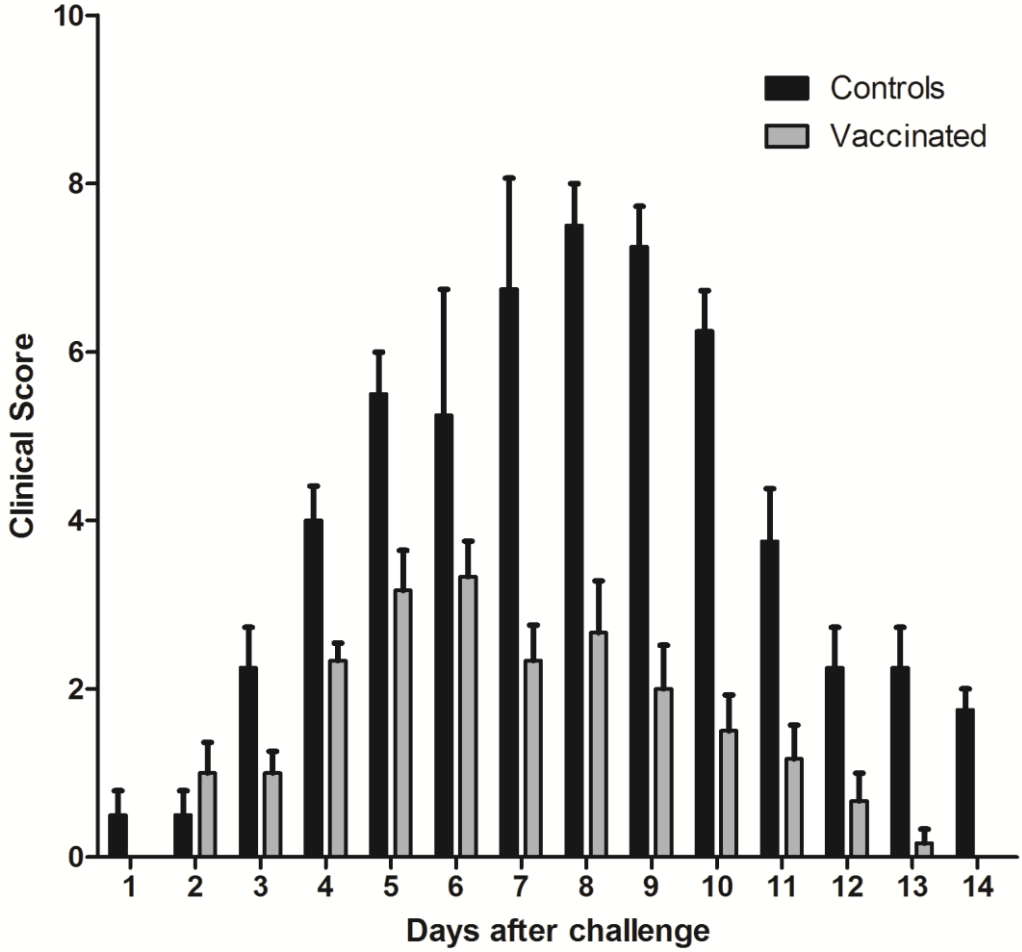

Figure 3. Clinical score of the challenge test. Six calves were vaccinated with BoHV-1ΔgE and 47 days later were challenged with a heterologous virulent BoHV-1 strain. Four calves were kept as controls. Bars represent the mean standard error.

Table 1. Results of the safety test. Clinical, virological and serological findings in calves inoculated intramuscularly with the recombinant BoHV-1ΔgE and monitored during the acute phase and after dexamethasone (Dx) administration.

Treatment	Animal #	Acute infection					Dexametasone treatment		
		Virus shedding/ clinical	VN antibodies[1]		Anti-gE ELISA[2]		Virus shedding/ clinical	VN antibodies D14 pDx[4]	Anti-gE ELISA D14pDx
			D0pi[3]	D47pi	D0pi	D47pi			
BoHV-1gEΔ $10^{8.5}TCID_{50}$/animal Intramuscular	117	-[5]	<2[6]	4	-[7]	-	-	2	-
	127	-	<2	4	-	-	-	8	-
	129	-	<2	8	-	-	-	8	-
	132	-	<2	16	-	-	-	8	-
	136	-	<2	4	-	-	-	8	-
				GMT:2.6[8]				GMT:2.6	
Control animals	118	-	<2	<2	-	-	-	<2	-
	135	-	<2	<2	-	-	-	<2	-

[1]Virus neutralizing antibodies were measured by virus neutralization assay as described in material and methods.
[2]Sera submitted to a commercial anti-gE antibody ELISA test (Bovine Rhinotracheitis Virus gE Antibody Test – IDEXX – the Netherlands).
[3]pi – post-inoculation.
[4]pDx – post-dexamethasone.
[5] No clinical signs or virus shedding.
[6]Sample negative in VN assay at its lower dilution (1:2).
[7]Samples negative in the anti-gE ELISA test.
[8]The average of serology results is expressed as geometric mean titers (GMT – $\log_2$).

Table 2. Results of the immunogenicity test using live virus. Serological response of calves vaccinated with live recombinant BoHV-1ΔgE and tested for virus neutralizing and gE antibodies at day 42 post-vaccination.

Treatment	Animal #	VN antibodies[1]		Anti-gE antibodies[2]
		Day 0	Day 42pv[3]	Day 42pv
Group I BoHV-1gEΔ $10^{7.3}TCID_{50}$/animal Intramuscular	101	<2[4]	8	-[5]
	102	<2	8	-
	104	<2	2	-
	105	<2	4	-
	106	<2	4	-
	107	<2	8	-
	108	<2	2	-
	109	<2	2	-
			GMT: 2a[6]	
Group II BoHV-1gEΔ $10^{7.3}TCID_{50}$/animal Subcutaneous	5	<2	4	-
	76	<2	4	-
	89	<2	2	-
	99	<2	4	-
	152	<2	2	-
	154	<2	4	-
	157	<2	4	-
	158	<2	2	-
	166	<2	4	-
			GMT: 1.65a	
Group III BoHV-1 SV56/90 $10^{7.3}TCID_{50}$/animal Intramuscular	112	<2	2	+[7]
	113	<2	4	+
	114	<2	4	+
			GMT: 1.65a	

[1]Virus neutralizing antibodies measured by virus neutralization assay as described in material and methods.
[2]Sera submitted to a commercial anti-gE antibody ELISA test (Bovine Rhinotracheitis Virus gE Antibody Test – IDEXX – the Netherlands).
[3]pv – post-vaccination.
[4]Sample negative in VN assay at its lower dilution (1:2).
[5]Samples negative in the anti-gE ELISA test.
[6] The average of serology results is expressed as geometric mean titers (GMT – $\log_2$).
[7]Sample positive in the anti-gE ELISA test.
[a]GMT from groups are statistically similar.

Table 3. Results of the immunogenicity test using inactivated vírus. Serological response of calves vaccinated twice with inactivated, adjuvanted BoHV-1ΔgE and tested for virus neutralizing and gE antibodies at day 60 post-vaccination (30 days after the second vaccine dose).

Treatment	Animal #	VN titers[1]		Anti-gE antibodies[2]
		Day 0	Day 60[3]pv[4]	Day 60pv
Group IV BoHV-1gEΔ $10^{7.3}TCID_{50}$/animal Subcutaneous Adjuvant: Aluminum hydroxide Two vaccine doses at 30 days interval	2	<2[5]	4	-[6]
	30	<2	4	-
	50	<2	2	-
	54	<2	4	-
	71	<2	4	-
	74	<2	4	-
	87	<2	8	-
	95	<2	8	-
	96	<2	8	-
	98	<2	16	-
	119	<2	8	-
	121	<2	8	-
	125	<2	4	-
			GMT: 2.45a[7]	
Group V BoHV-1gEΔ $10^{7.3}TCID_{50}$/animal Subcutaneous Adjuvant: Montanide™ Gel 1 Two vaccine doses at 30 days interval	1	<2	16	-
	3	<2	8	-
	8	<2	16	-
	9	<2	16	-
	11	<2	16	-
	22	<2	8	-
	35	<2	4	-
	58	<2	32	-
	61	<2	128	-
	62	<2	16	-
	78	<2	2	-
	85	<2	32	-
	88	<2	32	-
	120	<2	16	-
			GMT: 3.9b	

[1]Virus neutralizing antibodies measured by virus neutralization assay as described in material and methods.
[2]Sera submitted to a commercial anti-gE antibody ELISA test (Bovine Rhinotracheitis Virus gE Antibody Test – IDEXX – the Netherlands).
[3]30 days after the second vaccine dose.
[4]pv – post-vaccination.
[5]Sample negative in VN assay at its lower dilution (1:2).
[6]Samples negative in the anti-gE ELISA test.
[7] The average of serology results is expressed as geometric mean titers (GMT – $\log_2$).
[a and b] GMT from groups differs statistically.

Table 4. Serological response of calves immunized with the recombinant BoHV-1ΔgE and challenged intranasally with a heterologous, virulent BoHV-1 strain at day 47 post-vaccination.

Treatment	Animal #	Post-vaccination (pv)				Post-challenge (pc)			
		VN titer[1]		Anti-gE antibodies[2]		VN titer		Anti-gE antibodies	
		D0	D47pv	D0	D47pv	D30pc	D90pc	D30pc	D90pc
Immunized BoHV-1gEΔ $10^{7.5}TCID_{50}$/animal Intramuscular	109	<2[3]	4	-[4]	-	16	4	+[5]	+
	130	<2	4	-	-	64	8	+	+
	131	<2	8	-	-	128	16	+	+
	134	<2	4	-	-	32	8	+	+
	139	<2	4	-	-	16	2	+	+
	142	<2	2	-	-	128	32	+	+
			GMT: 2[6]			GMT: 5.5	GMT: 3		
Control animals	01	<2	<2	-	-	4	32	+	+
	03	<2	<2	-	-	2	4	+	+
	108	<2	<2	-	-	8	4	+	+
	105	<2	<2	-	-	4	32	+	+
						GMT: 2	GMT:3.5		

[1]Virus neutralizing antibodies measured by virus neutralization assay as described in material and methods.
[2]Sera submitted to a commercial anti-gE antibody ELISA test (Bovine Rhinotracheitis Virus gE Antibody Test – IDEXX – the Netherlands).
[3] Sample negative in VN assay at its lower dilution (1:2).
[4]Samples negative in the anti-gE ELISA test.
[5]Samples positives in the anti-gE ELISA test.
[6] The average of serology results is expressed as geometric mean titers (GMT – $\log_2$).

5. CONCLUSÕES

Os resultados obtidos nos experimentos apresentados nesta Tese permitem concluir que:

1 – A deleção do gene que codifica a gE foi obtida com êxito;

2 – A deleção do gene da gE não afeta a capacidade replicativa do vírus *in vitro*;

3 – O vírus recombinante BoHV-1ΔgE é atenuado para bezerros; não é excretado após administração IM ou re-excretado após administração de dexametasona;

4 – O vírus recombinante BoHV-1ΔgE é imunogênico em bezerros, independente da via de administração (IM ou SC), tanto na forma replicativa quanto inativada;

6 – A imunização com o vírus recombinante BoHV-1ΔgE confere proteção parcial frente à desafio com cepa virulenta e heteróloga do BoHV-1;

7 – A resposta sorológica induzida pelo vírus recombinante BoHV-1ΔgE pode ser diferenciada daquela produzida pelo vírus parental pelo uso de um teste sorológico específico para a gE.

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