UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE VETERINÁRIA

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS VETERINÁRIAS

VACINOLOGIA E PATOGENICIDADE DE AMOSTRAS RECOMBINANTES DE HERPESVÍRUS BOVINO TIPO 1 (BoHV-1)

Alessandra D'Avila da Silva

Porto Alegre

2007

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE VETERINÁRIA

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS VETERINÁRIAS

VACINOLOGIA E PATOGENICIDADE DE AMOSTRAS RECOMBINANTES DE HERPESVÍRUS BOVINO TIPO 1 (BoHV-1)

Alessandra D'Avila da Silva*

Tese apresentada como requisito ao grau de Doutor em Ciências Veterinárias na área de Medicina Preventiva Veterinária – Virologia Veterinária

Orientador: Prof. Dr. Paulo Michel Roehe

Co-orientador: Prof. Dra. Ana Claúdia Franco

Porto Alegre

2007

*** Médica Veterinária, MsC**

Alessandra D'Avila da Silva

TÍTULO DO TRABALHO: VACINOLOGIA E PATOGENICIDADE DE AMOSTRAS RECOMBINANTES DE HERPESVÍRUS BOVINO TIPO 1 (BoHV-1)

Aprovada em fevereiro de 2007

APROVADO POR:

_____

Prof. Dr. Paulo Michel Roehe
Orientador e Presidente da Comissão

_____

Prof. Dr. Amauri Simonetti
Membro da Comissão

_____

Prof. Dr. Claudio Wageck Canal
Membro da Comissão

_____

Prof. Dra. Sílvia de Oliveira Hübner
Membro da Comissão

AGRADECIMENTOS

Aos meus familiares: meus pais (Clovis e Mariones), irmãos (Giuliano e Marcelo), sogros (Luís Carlos e Carmem), cunhados e cunhadas (Franciele, Luís Antônio, Jacqueline, Luciane e Alexandre) e sobrinhos (Gabriel, Tamara, Fernando e Julia). Aos meus amados Paulinho e Mari, minha pequena família, muito obrigada pela compreensão e principalmente paciência que tiveram comigo em muitos momentos difíceis. Obrigada Paulinho por sua ajuda incansável e novamente desde o mestrado, sabes que muito deste trabalho devo a ti. O apoio, amor, amizade e carinho de vocês foi, simplesmente, fundamental.

Ao Paulo Roehe, eterno orientador, agradeço infinitamente a oportunidade de desenvolver esse e outros trabalhos e apreender tanto sobre virologia principalmente sobre herpesvírus. Obrigada pelos seus ensinamentos, confiança, amizade, exemplo de seriedade, entusiasmo e de dedicação à pesquisa. À Ana Claúdia, minha co-orientadora e amiga, obrigada pelos ensinamentos, pela ajuda na redação destes artigos, por seu exemplo de dedicação inesgotável a pesquisa. Ao Frans, pela amizade e pelos muitos ensinamentos em biologia molecular.

A todos os amigos e colegas: Tamir, Fernando (Valesca), Marjorie (Laura), Rejane (Marcus e Olívia), Sílvia (Dino e Andressa), Fabrício, Diógenes,Tamahine, Anna Paula, Cíntia, Marco Donini, Helena,Thaís, Samuel muito obrigada pelos mates, churrascos, festas mas principalmente pelo apoio e amizade de todos. Ao funcionário Nilton dos Santos, sem ele seriam muito difícies e demorados os experimentos com animais.

Agradeço ao Programa de Pós graduação da Faculdade de Veterinária da UFGRS, Profa. Dra. Ana Paula Ravazzolo, Maria, Vera, Josiane e todos os professores pelos ensinamentos e oportunidade.

Ao Conselho nacional de desenvolvimento científico e tecnológico (Cnpq) e Coordenação de aperfeiçoamento de pessoal de nível superior (Capes), por ter financiado estes projetos e minha bolsa de doutorado.

À direção e todos os integrantes do Centro de Pesquisas Veterinárias Desidério Finamor (CPVDF), em especial: Alexandre, Júlio, Eduardo, Fernando, João Ricardo, Dercílio.

Todos vocês foram de absoluta importância no desenrolar deste trabalho.

Á Deus, por ter me dado à oportunidade de encontrar todos vocês.

VACINOLOGIA E PATOGENICIDADE DE AMOSTRAS RECOMBINANTES DE HERPESVÍRUS BOVINO TIPO 1 (BoHV-1)

RESUMO

O herpesvírus bovino tipo 1 (BoHV-1) é um dos principais agentes causadores de prejuízos econômicos em criações de bovinos. A vacinação tem sido amplamente utilizada para minimizar as perdas causadas por infecções pelo BoHV-1. Dentre as vacinas disponíveis, as vacinas desenvolvidas a partir de amostras virais recombinantes apresentam a vantagem de permitirem a diferenciação entre animais infectados e imunizados. Anteriormente, foi desenvolvida uma vacina recombinante diferencial com uma amostra de BoHV-1 brasileira baseada na deleção do gene que codifica a glicoproteína E (gE). No primeiro capítulo do presente trabalho, a segurança e imunogenicidade desta vacina recombinante inativada foi avaliada. Os experimentos de imunização, desafio e reativação da vacina diferencial em animais experimentalmente inoculados demonstraram que a vacina recombinante foi segura e eficiente ao minimizar ou mesmo prevenir os efeitos da infecção pelo BoHV-1. No segundo capítulo, a segurança da vacina gE- foi avaliada, através da imunização intramuscular (IM) de 22 vacas (14 BoHV-1 soronegativas e 8 soropositivas) prenhes. Foi observada soroconverão, mas não abortos e nem anormalidades fetais nos animais imunizados. Na segunda parte do mesmo estudo foi analizada a capacidade do vírus recombinante difundir-se em um rebanho bovino. Quatro terneiros foram inoculados pela rota intranasal (IN) com a amostra recombinante gE- e, posteriormente, adicionados a outros 16 animais com mesma idade e semelhante condição corporal durante 180 dias. Todos os animais foram monitorados diariamente em busca de sintomatologia clínica. Foi observada soroconversão apenas nos animais imunizados. Estes resultados indicam que, nas condições deste estudo, a amostra recombinante não causou nenhum dano nas vacas prenhes ou em seus terneiros e não foi capaz de difundir-se horizontalmente no rebanho. No terceiro capítulo foi avaliada a patogenicidade de uma amostra recombinante de BoHV-1 com deleção no gene Us9, utilizando coelhos como modelo experimental. Coelhos com quatro semanas de idade foram divididos em quatro grupos (A, B, C, D). Dois grupos (A e B) foram infectados via intranasal (IN) e dois (C e D) infectados via intraocular (IO). Em cada via de infecção, um grupo foi infectado com o vírus recombinante e o outro com o vírus selvagem (wt). Após a infecção IO, todos os animais, de ambos os grupos, desenvolveram intensa conjuntivite entre os dias 3 a 10 pós-inoculação (pi). Vírus infeccioso foi consistentemente isolado a partir dos suabes oculares entre os dias 1 a 10 pi chegando a um título máximo de $10^{3,05}$ $TCID_{50}$/mL. Nos grupos infectados pela via IN com BoHV-1 wt, 4/4 coelhos apresentaram sintomatologia característica da doença, tais como: pirexia, apatia, anorexia, tosse, secreção nasal severa (entre os dias 2 e 8 pi). Animais inoculados com o recombinante apresentaram apatia, anorexia e descarga nasal (entre os dias 3 e 7 pi). Vírus infeccioso foi isolado em diversos tecidos tanto nos animais inoculados com o vírus wt como recombinante. Ambos os vírus foram capazes de replicar nas mucosas. Análises histológicas dos tecidos dos animais demonstraram lesões em ambos os grupos. Este estudo apresentou que a proteína Us9 não tem um papel significante na patogenicidade *in vivo.*

PALAVRAS-CHAVE: herpesvírus bovino tipo 1, proteína gE, proteína US9, patogenicidade, vacina inativada, vírus recombinate.

VACCINOLOGY AND PATHOGENICITY OF RECOMBINANTS STRAINS OF THE BOVINE HERPESVIRUS TYPE 1 (BoHV-1)

ABSTRACT

Bovine herpesvirus type 1 (BoHV-1) is an the major cause of losses in cattle. Vaccination has been widely applied to minimize losses induced by BoHV-1 infections. Vacines developed from recombinant strains have the advantage of allow the differentiation between immunized and infected animals. Previously, a recombinant differential BoHV-1 vaccine based on a glycoprotein E deleted (gE) virus was developed. In the first chapter of the present work, the safety and immunogenicity of such recombinant, as a inactivate vaccine, was evaluated. The experiments showed that the DIVA vaccinne was safe and efficient in order to minimize or even prevent the clinical signs of the infection by BoHV-1. In the second chapter of the present study, the safety of the gE- vaccine during pregnancy was evaluated by the intramuscular inoculation into 22 pregnant dams (14 BoHV-1 seronegative; 8 seropositive). Seroconversion was detected but no abortions, stillbirths or fetal abnormalities were seen after vaccination. In the second part of the same study, the potential of the gE- vaccine virus to spread among beef cattle under field conditions was examined. Four heifers were inoculated intranasally (IN) with the gE- vaccine and mixed with other 16 animals at the same age and body conditions, for 180 days. All animals were daily monitored for clinical signs.. Seroconversion was observed only in vaccinated heifers. These results indicate that, under the conditions of the present study, the gE vaccine virus did not cause any noticeable harmful effect on pregnant dams and on its offspring and did not spread horizontally among cattle. In the third chapter the pathogenicity of a US9 negative recombinant strain BoHV-1 using rabbits as an experimental model was avaluated. Rabbits four weeks old were divided in four groups (A, B, C, D) within four rabbits per group. Two groups were infected IN route and two via intraocular (IO). In each route, one group was infected by recombinant virus and the other infected by wild type (wt) virus. After IO infection, all rabbits developed intense conjunctivitis between days 3 to 10 pos infection (pi). Infective virus was consistently isolated from ocular swabs on days 1 to 10, reaching a maximum of $10^{3.05}$ $TCID_{50}$/mL. Animals infected in the IN rote with BoHV-1 wt, 4/4 rabbits showed characteristic signs of disease, which included pyrexia, apathy, anorexia, cough, severe nasal secretion between days 2 to 8. Rabbits inoculated with recombinant virus showed apathy, anorexia, nasal secretion (between days 3 and 7pi). Infectious virus was isolated in differents tissues as much as animals inoculated with wt and recombinant virus. Both virus were capable of replication in the mucosa nasal and ocular of the inoculated rabbits. Histopatological lesions were evident in both groups. In the present study showed which the US9 protein have not significantly in the pathogenicity in vivo.

INDEX TERMS: bovine herpesvirus type 1, gE protein, US9 protein, pathogenicity, inactivated vaccine, recombinant virus.

SUMÁRIO

1. INTRODUÇÃO E REVISÃO BIBLIOGRÁFICA

1.1 Herpesvírus

Os herpesvírus pertencem à família *Herpesviridae* e dividem-se em três sub-famílias: *Alphaherpesvirinae*, *Betaherpesvirinae* e *Gammaherpesvirinae* (KNIPE et al., 2001). Os membros da sub-família *Alphaherpesvirinae* possuem um amplo espectro de hospedeiros, ciclo de replicação curto, uma rápida disseminação em cultivo celular e destruição eficiente das células. Estabelecem latência principalmente (mas não exclusivamente), em neurônios dos gânglios sensoriais, como o gânglio trigêmeo (VAN ENGELENBURG et al., 1994; WINCKLER et al., 1998; VOGEL et al., 2004). Os membros da sub-família *Betaherpesvirinae* replicam lentamente, induzindo um aumento de volume das células infectadas (citomegalia). Possuem um número restrito de hospedeiros. Estabelecem latência em células linforeticulares e outros órgãos (KNIPE et al., 2001). Já os membros da sub-família *Gammaherpesvirinae* são capazes de replicar ou estabelecer latência em células linfóides e causar infecção lítica em células epiteliais e fibroblásticas (FIELDS et al., 1996; KNIPE et al., 2001).

1.2 Herpesvírus Bovino

A espécie bovina é hospedeira primária de diversos herpesvírus, distribuídos entre as diferentes subfamílias. Dentre elas estão o herpesvírus bovino tipo 1 (BoHV-1), herpesvírus bovino tipo 2 (BoHV-2), herpesvírus bovino tipo 4 (BoHV-4), o herpesvírus bovino tipo 5 (BoHV-5) e o herpevírus alcelafino tipo 1 ou vírus da febre catarral maligna (AIHV-1; MURPHY et al., 1999; ROIZMANN e PELLETT, 2001). O BoHV-1 é o mais amplamente reconhecido devido aos prejuízos causados, sendo relacionado principalmente com quadros clínicos de rinotraqueíte, ceratoconjuntivite, vulvovaginite, balanopostite e aborto (TIKOO et al., 1995).

1.3 Herpesvírus Bovino tipo 1 (BoHV-1)

1.3.1 Descrição do agente

O herpesvírus bovino tipo 1 (BoHV-1) pertence à família *Herpesviridade*, subfamília *Alphaherpesvirinae*, gênero *Varicellovirus* (ROIZMAN e PELLETT, 2001; MURPHY et al., 1999). Tal como outros alfaherpesvírus, o BoHV-1 apresenta genoma constituído por uma molécula de DNA linear dupla fita com cerca de 130.000 a 150.000 pares de bases. O genoma viral pode ser dividido em uma região longa (UL) e uma região curta (US) cercada por duas regiões repetidas e invertidas, sendo uma interna (IR) e outra terminal (TR), arranjo típico do grupo "D" do gênero *Varicellovirus* (SCHWYZER e ACKERMANN, 1996).

1.3.2 Proteínas Virais

O genoma codifica em torno de 70 proteínas, incluindo proteínas reguladoras, enzimas virais e proteínas estruturais (Tabela 1; VAN OIRSCHOT, 1999). Dentre as proteínas estruturais, devem ser mencionadas, com destaque especial, as glicoproteínas inseridas no envelope viral.

O BoHV-1 expressa cerca de dez glicoproteínas que, por apresentarem homologia com relação os genes do vírus do herpes simples humano tipo 1, foram nomeadas conforme os genes homólogos do HSV-1 (SCHWYZER e ACKERMANN, 1996; ROIZMAN e KNIPE, 2001). As glicoproteínas podem ser divididas em essenciais (gB, gD, gH, gL e gK) ou não essenciais (gC, gE, gI, gM e gN) à replicação viral. Algumas destas desempenham funções vitais no processo de infecção, mediando os processos de adsorção às células alvo, penetração do vírion, fusão e disseminação do vírus célula-a-célula (TIKOO et al., 1995; BRIDEAU et al., 2000; TOMISHIMA e ENQUIST, 2001; ROIZMAN e PELLETT, 2001; METTENLEITER, 2003). É possível que determinadas proteínas não essenciais tenham papel importante na infecção *in vivo*. Estas proteínas não essenciais poderiam, por exemplo, favorecer a disseminação viral em tecidos ou proteção contra a resposta imune do hospedeiro (DINGWELL et al., 1994; ROIZMAN, 1996; SCHWYZER, 1996).

Dentre as proteínas não essenciais codificadas pelo BoHV-1 a glicoproteína E (gE) e a US9, ambas proteínas de membrana do BoHV-1, foram escolhidas para serem objeto deste trabalho.

Tabela 1- Proteínas codificadas pelo BoHV-1

Função/ Localização	Nome
Glicoproteínas	gB, gC, gD, gE, gI, gG, gH, gK, gL, gM
Envelope	UL20, UL34, UL43, UL49.5
Tegumento	UL11, UL36, UL37, UL41, UL46, UL47, UL48, UL49, US9
Capsído	UL18, UL19, UL26,/26.5,UL35,UL38
Outros	UL4, UL21, UL24
Clivagem/ Empacotamento	UL6, UL15, UL25, UL28, UL32, UL33
Replicação de DNA	UL29, UL30, UL42, UL5, UL8, UL52, UL9
Enzimas	UL23, UL39, UL40, UL2, UL50, UL13, US3, UL12
Regulatórias	BICP0, BICP4, BICP22, BICP27
Desconhecida	UL3, UL7, UL14, UL16, UL17, UL31, UL51, US2
Proteínas BoHV-1 específicas	UL0.5, UL3.5, *CIRC*, US1.5

Fonte: SCHWYZER E ACKERMANN, 1996

1.3.3 Glicoproteína E

O gene da glicoproteína E (gE) do BoHV-1 tem um total de 2300 pares de bases (bp) e codifica uma proteína com aproximadamente 575 aminoácidos, a qual apresenta uma alta homologia com a gE do herpesvírus suíno tipo 1 (SHV-1) e do vírus Varicela-Zoster (VZV; REBORDOSA et al, 1994). A gE tem sido associada à virulência e difusão de herpesvírus em diversos modelos animais testados (BABIC et al., 1996; KASHOEK, 1995; FRANCO et al., 2002; METTENLEITER, 2003). A gE do BoHV-1 encontra-se associada à gI, sendo ambas não essenciais para a multiplicação viral (ZUCKERMANN et al., 1988; WHEALy et al., 1993). O complexo gI/gE parece estar envolvido nos processos de disseminação célula-a-célula em cultivos celulares (REBORDOSA et al., 1996; METTENLEITER, 2003). Amostras de alfaherpesvírus com deleções no gene que codifica gE ou gE/gI originam placas de menor tamanho em cultivos celulares (BALAN et al., 1994; DINGWELL et al., 1994; REBORDOSA et al., 1996; METTENLEITER, 2003; SPILKI et al., 2004). *In vivo*, vírus gE negativos (gE-) apresentam virulência reduzida, o que provavelmente está relacionado com a dificuldade que estas amostras têm de realizar a disseminação no organismo animal (VAN ENGELENBURG et al., 1994; CHOWDHURY et al., 1999; FRANCO et al., 2002).

1.3.4 Proteína Us9

O gene que codifica a proteína Us9 se localiza na região US do genoma do BoHV-1, logo após o gene que codifica a gE. Este gene possui 900 pares de bases e codifica uma proteína que contém aproximadamente 15 kilodaltons (kDa). É uma proteína não essencial no herpes simples humano tipo 1 (HSV-1), no SHV-1, no BoHV-1 e no BoHV-5 (BRIDEAU et al., 2000; CHOWDHURY et al., 2002).

Sua função no BoHV-1 ainda não é totalmente conhecida, mas no HSV-1 ela é ubiquitinilada e se associa de forma estável em proteossomos de células infectadas (BRANDIMARTI e ROIZMAN, 1997). Suspeita-se que esta interação possa trazer algum prejuízo à resposta imune do hospedeiro, já que é nesta organela que são gerados os peptídeos virais responsáveis pela ativação da resposta imune celular (BRANDIMARTI e ROIZMAN, 1997).

No SHV-1, esta proteína parece estar envolvida no transporte de novas partículas virais e/ou no transporte de vesículas contendo as glicoproteínas do envelope (TOMISHIMA e ENQUIST, 2001). Segundo CHOWDHURY (2002), em BoHV-5 a proteína Us9 parece ter um importante papel no transporte viral dos neurônios de primeira ordem para os neurônios de segunda ordem no bulbo olfatório de coelhos. Como conseqüência, o recombinante Us9 foi avirulento e falhou em invadir o SNC (CHOWDHURY et al., 2002).

1.4 Latência

Uma característica marcante das infecções causadas pelo BoHV-1, comum também a outros alfaherpesvírus, é o estabelecimento de infecções latentes em neurônios dos gânglios que inervam a região associada à primoinfecção (PASTORET e THIRY, 1985; ROCK et al., 1986; JONES, 2003). O termo “latência" refere-se à persistência do vírus no organismo do animal de forma silenciosa, não detectável por procedimentos virológicos usuais (PASTORET E THIRY, 1985). Durante o período de latência não ocorre à expressão de genes virais requeridos para uma infecção produtiva, não ocorrendo assim à produção de vírus infeccioso (PASTORET E THIRY, 1985). Durante esta fase, ocorre somente a expressão limitada de certos RNAs mensageiros, chamados “transcritos associados à latência” (LAT; ROCK et al., 1986; JONES, 2003). Desta forma, o vírus pode permanecer latente por longos períodos, provavelmente por toda a vida do animal (PASTORET e THIRY, 1985; VAN OIRSCHOT et al., 1993).

1.5 Subtipos gênomicos

Os diferentes isolados de BoHV-1 têm sido subdivididos de acordo com perfis de restrição enzimática dos genomas virais, ou pela análise de perfis de reatividade frente a anticorpos monoclonais. Assim, as amostras de BoHV-1 foram subdivididas em três diferentes genótipos: BoHV-1.1, BoHV-1.2a, BoHV-1.2b (METZLER et al, 1986; RIJSEWIJK et al., 1999, D'ARCE et al., 2002; SOUZA et al., 2002). Os genomas de amostras padrão de BoHV-1.1 (Cooper) e BoHV-1.2 (K22) apresentam 95% de similaridade (MAYFIELD et al., 1983). Todavia, a despeito dessa alta identidade genômica determinados genes, como aqueles que codificam a glicoproteína C (gC), parecem diferir substancialmente entre amostras dos dois subtipos (RIJSEWIJK et al.; 1999). É possível que esta variabilidade no gene da gC contribua para as diferentes manifestações clínicas associadas a estes subtipos (METZLER et al., 1996; RIJSEWIJK et al.; 1999).

As diferenças biológicas entre os subtipos de BoHV-1 também podem estar associadas a adaptações a diferentes vias de infecção (EDWARDS et al., 1991). Entretanto, amostras isoladas da região genital podem causar infecções respiratórias quando inoculados experimentalmente, o que sugere uma maior associação do tipo de enfermidade causada pelo vírus com a rota de infecção e práticas de manejo, do que propriamente por características inerentes ao genótipo de BoHV-1 (SPILKI et al., 2004).

1.6 Transmissão

As infecções pelo BoHV-1 são transmitidas através de aerossóis ou por contato direto ou indireto entre animais ou fômites. O vírus é disseminado através das secreções nasais e oculares, nas quais é eliminado em quantidades variadas por vários dias após uma infecção aguda clinicamente aparente podendo, ainda, se disseminar sem o aparecimento de sinais clínicos (MARS et al., 2000; PEREZ et al., 2002). A excreção de vírus, durante a reativação, parece não ocorrer em tanta quantidade e tanto tempo de duração, como ocorre na forma aguda da infecção. Entretanto, animais que estão reativando o vírus são certamente uma fonte de infecção para animais suscetíveis (BRATANICH et al.,1991; CASCIO et al., 1999; MEYER et al., 2001; PEREZ et al., 2002).

O vírus pode ainda estar presente no sêmen de machos infectados, podendo ser disseminado tanto pela monta natural quanto por inseminação artificial (VAN OIRSCHOT, 1995). A presença de vírus infeccioso foi também detectada em leite de vacas, chamando a atenção como fonte de infecção para outros animais (ROBERTS et al., 1974). Bovinos são os principais reservatórios do BoHV-1, mas inquéritos sorológicos demonstraram a presença de anticorpos em diversas espécies de ruminantes silvestres, bem como em ovinos e caprinos (WYLER et al., 1989). Além disso, tanto ovinos como caprinos desenvolvem infecções agudas e latentes e são capazes de excretar o vírus após tratamento com dexametazona (HAGE et al., 1997; ENGELS et al., 2000). Além das espécies domésticas citadas, bubalinos também são considerados como potenciais reservatórios do BoHV-1, pois inquéritos sorológicos demonstraram que estes animais podem apresentar anticorpos neutralizantes para este vírus (YADAV E PATHAK, 1995).

1.7 Patogenia

Após o contato com a mucosa nasofaríngea ou genital de um animal suscetível, o BoHV-1 estabelece uma replicação inicial nas células epiteliais locais, causando a lise destas, o que leva ao surgimento dos primeiros sinais clínicos da infecção que consistem em congestão local, presença de secreção e lesões vesiculares a erosivas na mucosa (WYLER et al., 1989; VOGEL et al., 2004; SPILKI et al., 2004). Durante esta fase da enfermidade, títulos virais elevados são produzidos e eliminados pelas secreções, podendo o animal enfermo ser uma fonte de infecção para outros animais (WYLER et al., 1989). Após a replicação primária, o vírus invade terminações nervosas e é transportado, através de fluxo axônico retrógrado, até os corpos neuronais situados nos gânglios sensoriais ou autonômicos (ENQUIST et al., 1999). Nestes locais, o vírus é capaz de causar uma infecção latente, durante a qual não há replicação viral ou expressão de antígenos virais (WYLER et al., 1989; PASTORET et al., 1982). Existem também evidências de que, após a infecção primária, o vírus possa causar uma viremia, provavelmente associada a monócitos e linfócitos, através da qual o vírus poderia se disseminar no organismo animal (PASTORET et al., 1985; SCHYNTS et al., 1999; FUCHS et al., 1999).

Eventualmente, sob a influência de fatores externos, como estresse ou tratamento com corticóides, pode ocorrer a reativação da infecção latente, quando ocorre síntese de novas partículas virais infecciosas na célula nervosa e transporte destas via axonal anterógrada até o

sítio inicial da infecção. Neste, se estabelece novamente um ciclo lítico de infecção, com manifestação de sinais clínicos e re-excreção viral (VAN OIRSCHOT, 1995).

1.8 Manifestações Clínicas

O BoHV-1 é o agente etiológico da rinotraqueíte infecciosa bovina (IBR), da vulvovaginite pustular infecciosa (IPV) e da balanopostite infecciosa dos bovinos (IPB) (ROIZMAN e PELLETT, 2001), podendo levar a conjuntivites, diarréias e quedas na produção leiteira (GIBBS e RWEYEMAMU, 1977). O vírus pode estar associado a abortos e, provavelmente, a outras perdas reprodutivas ainda não quantificadas (SILVA et al., 2000).

O período de incubação varia entre 2 e 6 dias, dependendo da dose e rota de inoculação (KAHRS, 1977). Disseminação viral em secreções nasais foi encontrada num período entre 12 e 18 dias, embora haja relatos de isolamento viral intermitente num período de até 578 dias (STUDERT, 1989; SPILKI et al., 2004; VOGEL et al., 2004). Os maiores títulos virais são produzidos e excretados no estágio agudo da infecção, no entanto, a eficiência da transmissão viral está relacionada com o local anatômico desta (ENGELS e ACKERMANN, 1996).

Devido a uma pronunciada resposta imune, estas infecções são geralmente auto-limitantes, sendo comum a recuperação entre 1 e 2 semanas. Entretanto, as lesões associadas a uma imunodepressão provocada pela infecção, podem facilitar a infecção bacteriana secundária, podendo levar a quadros de pneumonias (ENGELS e ACKERMANN, 1996; PITONE et al., 1999). Casos de enterites, mastites, metrites, dermatites, tonsilites e infecções sistêmicas em animais jovens (BLOOD et al., 1988) têm sido relatadas como algumas complicações causadas por BoHV-1. Embora viremia dificilmente possa ser demonstrada nestas infecções, alguns experimentos *in vitro* demonstraram a capacidade de adsorsão de BoHV-1 a linfócitos, quando na ausência de anticorpos neutralizantes, e a possibilidade de infecção de monócitos presentes no sangue (NYAGA e MCKERCHER, 1980).

1.8.1 Rinotraqueíte Infecciosa Bovina

A infecção de bovinos susceptíveis com amostras de BoHV-1.1 e BoHV-1.2a pode desencadear sinais clínicos associados a rinotraqueíte e a problemas reprodutivos como abortos e reabsorção embrionária (EDWARDS et al., 1991; MILLER et al., 1991; SPILKI et al., 2004). As manifestações clínicas observadas durante a infecção respiratória primária incluem febre,

anorexia, dispnéia, taquipnéia, tosse e, em animais em lactação, queda da produção leiteira. Pode ser observada secreção serosa um ou dois dias pós-infecção, a qual pode tornar-se muco-purulenta com a progressão da enfermidade e ocorrência de infecções bacterianas secundárias. A mucosa nasal pode apresentar congestão e lesões vesiculares a erosivas. Animais afetados podem ainda apresentar conjuntivite uni e bilateral, a qual pode ser a única manifestação clínica da doença (GIBBS E RWEYEMANU, 1977; WYLER et al., 1989).

O contato de animais em gestação com amostras de vírus de alta virulência (BoHV-1.1 e BoHV-1.2a) pode ainda levar ao aborto, o que ocorre principalmente entre o quinto e oitavo mês de gestação. O aborto ocorre, normalmente, após um período de incubação de 3 a 6 semanas, quando o vírus alcança o feto através da viremia. Durante um surto da infecção, até 25% das fêmeas em gestação podem abortar, sendo este tipo de manifestação uma das principais causas de perdas econômicas nas criações de bovinos (WYLER et al., 1989).

1.8.2 Vulvovaginite pustular infecciosa (IPV) e Balanopostite pustular infecciosa (IPB)

As manifestações genitais em bovinos estão associadas com o contato da mucosa genital com amostras de BoHV-1.2b (EDWARDS et al., 1991, MILLER et al., 1991). A IPV aguda se desenvolve após o contato do vírus com mucosa genital da fêmea, o que ocorre na cobertura ou inseminação artificial pelo contato com epitélio ou secreções contaminadas com o vírus. Após um curto período de incubação (1 a 3 dias) a vulva se apresenta inflamada e com vesículas espalhadas pela mucosa. As vesículas evoluem para pústulas, que podem estar presentes e podem ser agravados por infecções bacterianas secundárias (VAN OIRSCHOT, 1995).

Na mucosa genital dos machos também se desenvolvem lesões vesiculares e erosivas, um a três dias pós-infecção (VAN OIRSCHOT, 1995). Na ausência de infecções secundárias os sinais de infecção aguda desaparecem 10 a 14 dias, mas seqüelas, como a formação de aderências na mucosa, podem chegar a comprometer a realização de coberturas. Formas subclínicas da infecção podem ocorrer, o que dificulta o diagnóstico e o controle (VAN OIRSCHOT, 1995).

1.9 Modelos experimentais para estudo de infecções por herpesvírus bovino

A elucidação de questões importantes referentes à biologia da infecção aguda e latente causadas por alfaherpesvírus tem determinado a utilização de animais de laboratório como modelo experimental. Camundongos e coelhos têm sido utilizados como modelos em diversos estudos, tal

como: HSV (ROCK et al., 1987; STROOP et al., 1987), pelo SHV-1 em suínos (ENQUISt et al., 1999; ENQUIST et al., 2002; METTENLEITTER et al., 2003), para o BoHV-1 (MEYER et al., 1996) e para o BHV-5 (BROWN E FIELD, 1990; MEYER et al., 1996; CHOWDHURY et al., 1997; SILVA et al, 1999b; BELTRÃO et al, 2000; CARON et al., 2002; SPILKI et al, 2002).

A enfermidade respiratória aguda pode ser reproduzida em coelhos infectados com BoHV-1. Nessa espécie, o BoHV-1, quando inoculado pelas vias nasal ou conjuntival, causa uma enfermidade respiratória comparável aquela observada em bovinos (BELKNAP et al., 1994; MEYER et al., 1996; SPILKI et al., 2002). A excreção viral por secreções nasais e oculares, assim como a indução de anticorpos neutralizantes específicos tem sido evidenciados após a inoculação de coelhos com BoHV-1 (SILVA et al., 1999b; BELTRÃO et al., 2000). Portanto, a utilização desta espécie animal como modelo para infecções pelo BoHV-1 tem sido satisfatoriamente documentada.

1.10 Aspectos Imunológicos

1.10.1 Resposta Imune Inespecífica

Uma vez estabelecida a infecção primária, reações inflamatórias e celulares inespecíficas são a primeira resposta à infecção. IFN α/β são induzidos no início da síntese viral, a partir de 5h pós-infecção, e persistem até que a replicação viral cesse (VAN DRUNEN LITTEL-VAN DEN HURK et al., 1993). Sua produção imediata pode ser importante para a rápida proteção local em estágios iniciais da infecção. A liberação de IFN e outras modificações celulares resultam na modulação do transporte de leucócitos e recrutamento de várias células efetoras, tais como neutrófilos, macrófagos e NK ao local da infecção (BIELEFELDT OHMAN et al., 1991) favorecendo, desta forma, a resposta à infecção primária.

Citocinas proinflamatórias induzem a expressão de moléculas de adesão intracelular tipo I (ICAM-I) em células endoteliais promovendo a aderência de leucócitos. Com o aumento da permeabilidade vascular e adesão, as células migram para o local da infecção liberando espécies reativas de oxigênio, metabólitos do ácido aracdônico e enzimas para eliminar as partículas virais e as células infectadas (SHAPEL et al., 1989). Interleucinas 6 (IL-6) e 10 (IL-10) também induzem a produção do fator estimulante de colônias de granulócito e macrófagos (GM-CSF),

por células parenquimais e linfócitos, permitindo a diferenciação e a liberação do fator de necrose tumoral-α (TNF-α) pelos macrófagos.

IFN-α influencia o tráfego de linfócitos com depleção seletiva de linfócitos T CD8+. NK e macrófagos eliminam células infectadas por BoHV de modo independente do complexo principal de histocompatibilidade tipo I (MHC-I), estimuladas por citocinas tipo IFN-γ produzidas por linfócitos T (CAMPOS et al., 1989; JENSEN & SCHULTZ, 1990). O reconhecimento de células infectadas por células NK parece envolver glicoproteínas expressas precocemente na superfície celular, tais como gB e gD (PALMER et al., 1990). NK e macrófagos estimulam ainda a produção de citocinas que influenciam o desenvolvimento de resposta imune específica. Desta forma, o papel das citocinas é fundamental para a diferenciação, amplificação e regulação da resposta imune celular, induzida por linfócito T auxiliar 1 (Th1) e humoral, por Th2, às infecções por BoHV.

1.10.2 Resposta imune específica

Os anticorpos neutralizantes dirigidos contra as glicoproteínas do envelope viral possuem importância na imunidade à longo prazo. O papel da resposta humoral é questionável com relação à prevenção da disseminação do vírus, devido à sua capacidade de escape por junções celulares e ramos nervosos, bem como devido à persistência da infecção viral na presença de anticorpos neutralizantes. O complexo gE-gI de HSV-1 pode atuar como receptor para a porção Fc das imunoglobulinas , que tem sido relacionado com a redução na eficiência da resposta imune mediada por anticorpos, tanto *in vitro* quanto *in vivo* (DUBIN et al., 1992; NAGASHUNMUGAM et al., 1998). Assim, mecanismos dependentes de gE parecem estar envolvidos no estímulo da transmissão viral de célula-à-célula (resistentes à anticorpos) em resposta à anticorpos extracelulares (RIZVI & RAGHAVAN, 2003).

A detecção de anticorpos é possível entre 8-12 dias após a infecção, persistindo por até 5 anos, ainda que necessite ser reativado (WYLER et al., 1989). A atividade neutralizante pode também ser detectada em secreções nasais e genitais devido a presença de imunoglobulina A (IgA) (WYLER et al., 1989). Bovinos recém-nascidos adquirem anticorpos principalmente via colostro, nas primeiras 12 h após o parto (IgG1), e embora não confiram uma proteção absoluta, diminuem a agressividade da infecção. A lise celular mediada por complemento participa no estágio inicial da infecção e pode também ser importante na fase tardia de recuperação ou durante a reativação de infecções latentes (PIDONE et al., 1999). A citotoxicidade celular dependente de

anticorpos (ADCC) que também participam do processo de destruição de células infectadas. Estes mecanismos são efetivos *in vitro*, porém sua funcionalidade *in vivo* ainda é especulativa (WYLER et al., 1989).

1.10.3 Imunidade mediada por células e imunoreguladores

A resposta imune mediada por células tem sido detectada pela proliferação de linfócitos, citotoxicidade direta e produção de citocinas. O pico da atividade celular ocorre entre 7-10 dias pós-infecção, antes que a resposta por anticorpos tenha sido detectada, podendo ser correlacionada com a recuperação de uma infecção primária. No entanto, a ação da resposta imune humoral em conjunto com a celular permitem uma eliminação mais rápida e eficaz da infecção viral. As glicoproteínas B, C e D possuem grande importância no estímulo de resposta proliferativa, atuando como alvos para a imunidade celular. Epítopos específicos nestas glicoproteínas estimulam linfócitos T CD4+ e CD8+ (LEARY e SPLITTER, 1990).

A eliminação do vírus depende em grande parte dos fatores solúveis produzidos por linfócitos sensibilizados, tais como citocinas, linfotoxinas, fator quimiotático e prostaglandinas. No entanto, características próprias do BoHV impedem que o sistema imune o elimine por completo do organismo (WINCKLER et al., 2001).

A imunodepressão causada pela infecção viral parece estar associada com a diminuição da resposta imune mediada por células (BIELEFELDT OHMANN e BABIUK, 1985; CARTER et al., 1989). O reconhecimento de células infectadas por BoHV, pelos linfócitos T CD8+ é prejudicado devido à inibição da expressão do MHC-I e consequentemente a apresentação de antígenos (HINKLEY et al., 1998; NATARAJ et al., 1997). Mais de um produto gênico viral parece estar envolvido na interferência da expressão de MHC-I na superfície celular (HINKLEY et al., 1998). Esta estratégia, ocorrendo logo no início da infecção viral, impede a eliminação de células infectadas, e permite uma eficiente expressão gênica viral.

Além disso, BoHV-1 induz apoptose em neutrófilos e linfócitos T CD4+, inibindo a resposta proliferativa ao estímulo antigênico (HANON, et al., 1996; WINKLER et al., 1999), bem como inibe a migração de neutrófilos polimorfonucleares, a citotoxicidade mediada por células, a resposta mitótica dos linfócitos sangüíneos periféricos e algumas atividades funcionais do macrófago alveolar (WYLER et al., 1989).

1.11 Epidemiologia

1.11.1 Distribuição geográfica

Em 1956, foi feito o primeiro isolamento do vírus, a partir de um caso de IBR (Madin et al., 1956). A partir de então, o BoHV-1 vem sendo encontrado em todos os continentes (TIKOO et al., 1995). Alguns países europeus, como a Dinamarca e Suiça, são livres da infecção, tendo obtido esta condição através da identificação e eliminação de animais soropositivos dos rebanhos (HAGE et al., 1997; ACKERMANN et al., 1990). Outros países, como Alemanha e Holanda, tem implementado programas de erradicação da infecção através da vacinação compulsória dos rebanhos e identificação e eliminação gradual de animais infectados (HAGE et al., 1997; ACKERMANN et al., 1990).

No Brasil, o BoHV-1 foi isolado pela primeira vez por ALICE (1978), a partir de um caso de vulvovaginite ocorrido no Estado da Bahia. Enquetes sorológicas demonstram que a média de prevalência de infecções por BoHV-1 em rebanhos brasileiros situa-se ao redor de 30%, com a maioria das propriedades apresentando animais positivos. Isto equivaleria a cerca de 57 milhões de bovinos infectados, para um rebanho presentemente estimado em 190 milhões de cabeças (RAVAZZOLO et al., 1989; LOVATO et al., 1995; VIDOR et al., 1995).

1.12 Controle das infecções pelo BoHV-1

As medidas de controle em relação ao BoHV-1 estão diretamente relacionadas com a severidade da infecção em um rebanho, práticas de manejo dos animais e com a prevalência da infecção em uma determinada região ou propriedade (ACKERMANN et al., 1990). Em países ou regiões em que ocorrem perdas econômicas graves ou onde a prevalência da infecção é alta, o controle das infecções se baseia em programas de vacinação (WYLER et al., 1989). Nestas situações, a vacinação sistemática reduz a circulação de vírus em uma população, diminuindo as perdas econômicas (VAN OIRSCHOT et al., 1996a; VAN OIRSCHOT et al., 1999).

1.12.1 Controle sem vacinação

Naquelas propriedades rurais ou países com níveis mínimos de soropositividade no rebanho, estratégias cujo foco central é a identificação, segregação e abate de animais soropositivos podem ser aplicadas (ACKERMANN et al., 1990). Tal método tem como principal vantagem o baixo custo e tem se mostrado bastante eficiente, desde que tomadas medidas severas, visando evitar a introdução de novos animais infectados e controle das possíveis fontes de material contaminado, em especial o sêmem (VAN OIRSCHOT et al., 1993). Um dos pontos controversos que repousa sobre tais estratégicas é a possibilidade da existência de animais soronegativos latentemente infectados (LEMAIRE et al., 2000).

1.12.2 Controle com vacinação

Em países ou regiões com alta prevalência das infecções pelo BoHV-1, políticas de controle sem vacinação podem tornar-se inviáveis (VAN OIRSCHOT et al., 1996b). Deste modo, pode ser utilizada a vacinação visando reduzir os sinais clínicos e diminuir a disseminação do vírus nos rebanhos. Entretanto, ainda que possa ocorrer uma redução do número de novas infecções, nenhuma vacina disponível, até o presente momento, previne satisfatoriamente contra a infecção e/ou indução de latência (GALEOTA et al., 1997). Não obstante, estão disponíveis diferentes tipos de vacinas contra o BoHV-1 (CASTRUCCI et al., 2002), cujos princípios são descritos a seguir.

Vacinas vivas convencionais

As vacinas vivas atenuadas convencionais baseiam-se em amostras natural ou artificialmente atenuadas do agente, o qual mantém a capacidade de replicar-se no hospedeiro (MÄKELÄ, 2000). A atenuação é obtida, usualmente, por passagens sucessivas em animais, ovos embrionados, cultivos celulares ou pela indução de mutações (VAN OIRSCHOT et al., 1996b). As vacinas vivas atenuadas têm reputação de maior eficácia, uma vez que a multiplicação do vírus no organismo leva a um estímulo antigênico bastante semelhante ao provocado pela infecção. Entretanto, algumas delas, ocasionalmente, podem induzir sinais clínicos e até levar a abortos e morte (TURIN et al., 1999). Além disso, no caso das vacinas vivas atenuadas contra herpesvírus disponíveis até o presente, uma desvantagem adicional é o estabelecimento de

infecções latentes no hospedeiro que, tal qual o vírus selvagem, estão sujeitas à reativação (MÄKELÄ, 2000).

Vacinas inativadas convencionais

Nas vacinas inativadas os vírus não são capazes de replicar-se no hospedeiro (MÄKELA, 2000) e por isso, para a indução de resposta imune necessita-se de uma grande massa antigênica, a qual deve ser inativada por tratamentos químicos ou físicos (TURIN et al., 1999). As vacinas inativadas induzem um estímulo antigênico usualmente de menor amplitude, sendo necessária normalmente a adição de adjuvantes à formulação, a fim de proporcionar um maior estímulo à resposta imune (VAN OIRSCHOT et al., 1996b). As mesmas são consideradas, em geral, menos efetivas do que as vacinas vivas; todavia, são mais seguras, em função de que a replicação do vírus vacinal no hospedeiro não ocorre (TURIN et al., 1999).

Uma grande desvantagem associada às vacinas vivas e mortas convencionais é a dificuldade de diferenciar a resposta imune vacinal daquela induzida por infecções com vírus de campo (ou selvagem), o que pode comprometer a realização de programas eficazes de controle através de vacinações e subsequente erradicação da infecção (TURIN et al., 1999).

Vacinas de subunidades

As vacinas de subunidades são preparadas com determinadas proteínas do agente infeccioso em questão, não contendo o agente íntegro (VAN OIRSCHOT, 1999). Um exemplo clássico de uma vacina de subunidade é a vacina contra a gripe que, atualmente, é produzida somente com a hemaglutinina do vírus da influenza. No preparo desta, o vírus é purificado a partir de seu cultivo em ovos embrionados e, posteriormente, a hemaglutinina é extraída com detergentes (STEPHENSON et al., 2004; SUBBARAO e KATZ, 2004). Outro exemplo de sucesso nesse campo é a vacina contra hepatite B humana, a qual contém somente o antígeno de superfície do vírus (HBSAg), o que permite que testes laboratoriais dirigidos contra outros componentes do vírion sejam utilizados de maneira a distinguir pessoas vacinadas de infectadas (YOSHIDA et al., 1990; LAI et al., 1992).

No caso dos herpesvírus, vários experimentos foram realizados com imunógenos baseados em glicoproteínas virais isoladas, como a gB e gD (VAN DRUNEN LITTEL-VAN

DEN HURK et al., 1993; GAO et al., 1994). Dentre estas, a gD se mostrou capaz de induzir nos animais inoculados altos níveis de anticorpos neutralizantes e melhores níveis de proteção após o desafio com vírus de campo (VAN DRUNEN LITTEL-VAN DEN HURK et al., 1993). Entretanto, até o presente, não foram lançadas vacinas comerciais baseadas na gD como imunógeno.

Vacinas diferenciais

As vacinas diferenciais visam à distinção entre a resposta imune induzida pela vacinação daquela induzida por infecções com vírus selvagem ou de campo (VAN OIRSCHOT et al., 1996a; VAN OIRSCHOT et al., 1999). As vacinas diferenciais disponíveis, são utilizadas em conjunto com testes sorológicos que detectam anticorpos específicos contra uma determinada proteína ou marcador, que pode estar presente ou ausente na amostra vacinal. Portanto, podem ser alvos potenciais de tal estratégia quaisquer proteínas que, quando ausentes do vírion, reduzam a virulência do vírus vacinal em relação à amostra parental e que por outro lado, sejam alvo dos anticorpos do hospedeiro após infecções naturais (VAN OIRSCHOT et al., 1999).

A primeira vacina diferencial desenvolvida contra herpesvírus de animais foi a vacina contra o herpesvírus suíno tipo 1 (SHV-1; VAN OIRSCHOT et al., 1986; QUINT et al., 1987; DE JONG e KIMMAN, 1994), causador da pseudo-raiva. Nesse caso, a amostra vacinal contém uma deleção no gene que codifica a gE do SHV-1. Sua utilização em suínos reduziu significantemente a circulação de vírus de campo e, conseqüentemente, as perdas econômicas decorrentes da infecção (VANNIER et al., 1991; KIMMAN et al., 1992).

Após o desenvolvimento dessa vacina, foram desenvolvidos vários experimentos visando a produção de vacinas diferenciais contra infecções pelo BoHV-1. A primeira delas foi desenvolvida por KIT E KIT (1990). A amostra vacinal foi obtida através de deleções nos genes que codificam a enzima timidina quinase (TK) e a glicoproteína C (gC). Animais vacinados intramuscularmente com esta amostra não apresentaram sinais clínicos da enfermidade, não excretaram vírus e foram satisfatoriamente protegidos da enfermidade clínica quando subseqüentemente infectados com uma amostra altamente patogênica (FLORES et al., 1993).

Posteriormente, foram desenvolvidas e avaliadas vacinas diferenciais com amostras de BoHV-1 com deleções na glicoproteína G (gG), glicoproteína I (gI), glicoproteína E (gE) e gI/gE e no gene que codifica a TK (RIJSEWIJK et al., 1994; VAN ENGELENBURG et al., 1994; GALEOTA et al., 1997; CHOWDHURY et al., 1999). A comparação das características de

virulência e imunogenicidade de todos estes vírus recombinantes revelou que amostras gE negativas induzem sinais clínicos em menor intensidade retendo, no entanto, características de imunogenicidade (VAN ENGELENBURG et al., 1994; KAASHOEK et al., 1995).

O uso de vacinas diferenciais para BoHV-1 é capaz de reduzir a transmissão de vírus de campo em populações de bovinos, tanto em experimentos controlados como em experimentos a campo (KAASHOEK et al., 1995; DE JONG et al., 1998, BOSCH et al., 1998; FRANCO et al., 2002). Quando a transmissibilidade de um vírus é suficientemente reduzida pela vacinação, existe a possibilidade de que, além do controle, possa se obter a erradicação do vírus de uma determinada população de animais (VAN OIRSCHOT et al., 1999). Assim, além de possibilitar a diferenciação de respostas imunes de animais infectados e vacinados, o uso de vacinas diferenciais têm ainda a vantagem de reduzir a circulação de vírus de campo, facilitando o controle das infecções virais (KAASHOEK et al., 1995; VAN OIRSCHOT et al., 1999).

2. OBJETIVOS

O presente estudo teve como objetivos:

- Avaliar a proteção de bovinos vacinados com uma vacina de BoHV-1 recombinante inativada contra o desafio por BoHV-1;

- Avaliar a campo a segurança para vacas prenhes de uma vacina diferencial recombinante para BoHV-1 e sua capacidade de disseminação horizontal;

- Avaliar a patogenicidade de um recombinante de herpesvírus bovino tipo 1 (BoHV-1) da qual foi deletada a proteína US9.

Capítulo 1

Efficacy of a gE-deleted, bovine herpesvirus 1 (BoHV-1) inactivated vaccine[1]

Alessandra D. Silva[2, 3], Paulo A. Esteves[2, 4], Diogenes Dezen[2, 3], Anna P. Oliveira[2,5], Fernando R. Spilki[6], Fabrício S. Campos[5], Ana C. Franco[5]and Paulo M. Roehe[2,3, 5*]

ABSTRACT.- Silva A.D., Esteves P.A., Dezen D., Oliveira A.P., Spilki F.R., Campos F.S., Franco A.C.& Roehe P.M. 2009. **[Efficacy of a gE-deleted, bovine herpesvirus 1 (BoHV-1) inactivated vaccine.] Eficácia de uma vacina inativada, gE-deletada, contra o herpesvírus bovino tipo 1 (BoHV-1).** *Pesquisa Veterinária Brasileira 29(0):00-00.* Instituto de Pesquisas Veterinárias Desidério Finamor, Fepagro Saúde Animal, Estrada do Conde 6000, Cx. Postal 47, Eldorado do Sul, RS, 92990-000, Brazil. E-mail: proehe@gmail.com

Bovine herpesvirus type 1 (BoHV-1) is recognized as a major cause of economic losses in cattle. Vaccination has been widely applied to minimize losses induced by BoHV-1 infections. We have previously reported the development of a differential BoHV-1 vaccine, based on a recombinant glycoprotein E (gE)-deleted virus (265gE$^-$). In present paper the efficacy of such recombinant was evaluated as an inactivated vaccine. Five BoHV-1 seronegative calves were vaccinated intramuscularly on day 0 and boostered 30 days later with an inactivated, oil adjuvanted vaccine containing an antigenic mass equivalent to $10^{7.0}$ fifty per cent cell culture infectious doses ($CCID_{50}$) of 265gE$^-$. Three calves were kept as non vaccinated controls. On day 60 post vaccination both vaccinated and controls were challenged with the virulent parental strain. No clinical signs or adverse effects were seen after or during vaccination. After challenge, 2/5 vaccinated calves showed mild clinical signs of infection, whereas all non vaccinated controls displayed intense rhinotracheitis and shed virus for longer and to higher titres than vaccinated calves. Serological responses were detected in all vaccinated animals after the second dose of vaccine, but not on control calves. Following corticosteroid administration in attempting to induce reactivation of the latent infection, no clinical signs were observed in vaccinated calves, whereas non vaccinated controls showed clinical signs of respiratory disease. In view of its

immunogenicity and protective effect upon challenge with a virulent BoHV-1, the oil adjuvanted preparation with the inactivated 265gE$^-$ recombinant was shown to be suitable for use as a vaccine.

INDEX TERMS: Bovine herpesvirus type 1, BoHV-1, recombinant vaccine, inactivated vaccine.

---

[1] Received on December 9, 2008.

Accepted for publication on April 24, 2009, in Pesquisa Veterinária Brasileira, vol 29.

[2] Equipe de Virologia, Instituto de Pesquisas Veterinárias Desidério Finamor (CPVDF), Fepagro Saúde Animal, Estrada do Conde 6000, Cx.Postal 47, Eldorado do Sul, RS 92990-000, Brazil. *Corresponding author: proehe@gmail.com

[3] Programa de Pós-Graduação em Ciências Veterinárias (PPGCV), Universidade Federal do Rio Grande do Sul (UFRGS), Av. Bento Gonçalves 9090, Porto Alegre, RS 91540-000, Brazil.

[4] Embrapa Suínos e Aves, BR153, Km 110, Vila Tamanduá, Cx.Postal 21, Concórdia, SC 89700-000, Brazil.

[5] Instituto de Ciências Básicas da Saúde, UFRGS, Rua Sarmento Leite 500, Porto Alegre, RS 90050-170.

[6] Instituto de Ciências da Saúde, Centro Universitário Feevale, Campus II, RS 239 no.2755, Novo Hamburgo, RS 93352-000, Brazil.

RESUMO.- [Eficácia de uma vacina inativada, gE-deletada, contra o herpesvírus bovino tipo 1 (BoHV-1)]. O Herpesvírus bovino tipo 1 (BoHV-1) é reconhecido como um importante agente de perdas econômicas em bovinos. Vacinação tem sido amplamente empregada para minimizar as perdas conseqüentes a infecções com o BoHV-1. Reportamos previamente o desenvolvimento de uma vacina diferencial para BoHV-1, baseada em um recombinante do qual a glicoproteína gE (gE) foi deletada (265gE$^-$). No presente trabalho foi realizada a avaliação da eficácia de tal recombinante como vacina inativada. Cinco bovinos soronegativos para BoHV-1 foram vacinadas por via intramuscular no dia 0 e revacinadas 30 dias após com uma vacina inativada com adjuvante oleoso, contendo massa antigênica equivalente a $10^{7.0}$ doses infectantes para 50% dos cultivos celulares ($DICC_{50}$) de 265gE$^-$. Três animais foram mantidos como controles não vacinados. No dia 60 pós-vacinação, os animais vacinados e controles foram desafiados com a amostra virulenta parental. Nenhum sinal clínico ou efeito adverso foi observado após ou durante a vacinação. Após o desafio, 2 dos 5 animais vacinados apresentaram sinais leves de infecção, enquanto que todos os animais não vacinados apresentaram intensa rinotraqueíte e disseminaram vírus por mais tempo e em títulos mais elevados do que os animais vacinados. Respostas sorológicas foram detectadas em todos os animais vacinados depois da segunda dose de vacina, mas não nos animais do grupo controle. Após a administração de corticosteróide visando a reativação de infecções latentes, não foram observados sinais clínicos em nenhum dos 5 animais vacinados, enquanto os animais não vacinados apresentaram sinais leves de doença respiratória. Em vista de sua imunogenicidade e efeito protetor frente ao desafio com BoHV-1 virulento, a preparação oleosa com o recombinante 265gE$^-$ inativado foi demonstrada ser adequada para uso como vacina.

TERMOS DE INDEXAÇÃO: Herpesvírus bovino tipo 1, BoHV-1, vacina recombinante, vacina inativada.

INTRODUCTION

Bovine herpesvirus 1 (BoHV-1), a member of the *Alphaherpesvirinae* subfamily, commonly known as infectious bovine rhinotracheits (IBR) virus, is an important cause of losses to cattle industry worldwide. BoHV-1 has been associated to a number of clinical syndromes, including vulvovaginitis, balanopostitis, conjunctivitis, infertility and abortions (Gibbs & Rweyemamu 1977). The BoHV-1 genome consists of a linear dsDNA molecule of about 140Kb (Schwyzer & Ackermann 1996). It encodes several glycoproteins that are expressed on the viral envelope and membranes of infected cells. While some of these are essential for virus replication (Rebordosa et al. 1996), other glycoproteins are not essential for virus replication and represent potential targets for deletions aiming the development of differential vaccines. These vaccines have an advantage over the conventional vaccines because the serological response induced by vaccination can be differentiated from that induced by the wild type virus, allowing identification of wild type virus-infected animals in a herd. Glycoprotein E (gE) is one of such non-essential proteins (Rebordosa et al. 1996, Franco et al. 2002a, Spilki et al. 2004). Although it is conserved among other members of the *Herpesviridae* family, the role of gE in the *in vitro* growth characteristics may vary in function of the virus species and the host cell (Balan et al. 1994). In human herpesvirus type 1 (HHV-1) and varicella-zoster virus (HHV-3), gE can be found non-covalently linked to gI, forming a Fc binding site for immunoglobulins, which has been proposed as a mechanism to protect virus-infected cells from lysis by the immune system (Olson et al. 1997). The gE complex in HHV-1 and HHV-3 is also important for *in vitro* cell-to-cell spread (Balan et al. 1994, Dingwell et al. 1995). In comparison to wild type viruses, gE negative recombinants (gE^-) produce smaller plaque sizes *in vitro*, although the growth kinetics or penetration process of the virus seem not to be dependent on the presence of gE (Rebordosa et al. 1996, Chowdhury et al. 1999, Spilki et al. 2004)

In previous studies, we described the construction of a recombinant vaccine, based on a Brazilian BoHV-1 isolate, from which the gE gene was deleted. This virus was named 265gE$^-$ and evaluated in vaccination/challenge experiments (Franco et al. 2002a). The live 265gE$^-$ virus based vaccine significantly reduced clinical signs of disease and virus shedding from vaccinated/challenged calves (Franco et al. 2002b). However, inactivated vaccine preparations may be considered safer in some instances, and may be favoured in relation to modified live attenuated virus vaccines. In order to check whether vaccination with inactivated 265gE$^-$ would be safe and induce protection to BoHV-1 infections, in the present study calves were vaccinated with an inactivated 265gE$^-$ based vaccine (Franco et al. 2002a,b), and challenged with the parental BoHV-1 wild type strain. Subsequently, vaccinated calves were submitted to corticosteroid administration to examine the capacity of the vaccine to avoid the establishment and the reactivation of a latent infection.

MATERIALS AND METHODS

Experimental design

Five calves were vaccinated twice with the recombinant-based inactivated vaccine on days 0 and 30. Three others calves were kept as non-vaccinated controls for subsequent challenge and comparative analyses. Two additional calves were kept as non-vaccinated, non-challenged controls throughout the experiment. On day 60 post vaccination, vaccinated and non-vaccinated calves were challenged with the wild type parental virus. Two months after challenge, all calves were subjected to the corticosteroid administration in attempting to reactivate latent virus. Throughout the experiments the clinical scores were evaluated; swabs (ocular/ nasal) and serum

samples were collected for further analysis of viral excretion and neutralizing antibodies production.

Cells and viruses

The wild type BoHV-1 strain SV 265 (SV 265wt), was isolated from a heifer with signs of respiratory infection in São Borja, Rio Grande do Sul, Brazil (Weiblen et al. 1996). The virus was used for the construction of the recombinant virus (265gE$^-$) as described previously (Franco et al. 2002a). The SV 265 was also used in the challenge experiments. Virus multiplication, titration and isolation from tissues as well as neutralization assays were performed on Madin Darby bovine kidney cells (MDBK, ATCC CCL-22). Cells were routinely maintained in Eagle's minimal essential medium (E-MEM) supplemented with 10 % fetal calf serum (FCS, Nutricell) and 2mg/L enrofloxacin (Baytril, Bayer).

Vaccine production

About 16-24 hours after seeding of the cells, the medium was removed and bottles infected with recombinant virus (265gE$^-$). After one hour adsorption at 37ºC, the inoculum was removed, the bottles replenished with E-EMEM without fetal calf serum and incubated for 16-24 hours at 37ºC, when cytopathic effect was evident in 90 % of the monolayers. Bottles were then shaken to remove attached cells and stored at 4ºC for 24 hours. Infectious titres of the supernatants of infected cultures were determined following standard procedures (House & Baker 1971). The viral suspension was inactivated with binary ethylenimine (BEI) as described previously (Bahnemann et al. 1974). The vaccine was prepared as a water-in-oil type emulsion and subjected to usual controls, as recommended (Petzhold et al. 2001) and the suspension was stored at 4 °C until use. The inactivation process was evaluated by titration of the inactivated

suspension in a 96 wells plate, followed by the inoculation of serial tenfold dilutions of the suspension in 25 cm^2 cell culture flasks, according to usual methods. As positive control, an aliquot of the same viral suspension previous to inactivation was used in both procedures.

Vaccination, challenge and viral reactivation of calves

Five BoHV-1 seronegative calves, three to four months old, were vaccinated with an inactivated vaccine preparation containing (before inactivation) $10^{7.0}$ fifty percent cell culture infectious doses ($CCID_{50}$) of the recombinant 265gE$^-$. The vaccine dose was 3 mL, which were administered intramuscularly. After 30 days the vaccination procedure was repeated. Three other calves were kept as non vaccinated controls for subsequent challenge. Two additional calves were kept as non vaccinated, non challenged controls throughout the experiment. On day 60 post vaccination, vaccinated and non vaccinated calves were challenged with $10^{7.0}$ $CCID_{50}$ of the parental virus SV265wt, in a 2mL administered intranasally, 1mL into each nostril. Two months after challenge, all calves were subjected to corticosteroid administration in attempting to reactivate latent virus. Dexamethasone (0.26mg per kg of body weigh) was administered intramuscularly for 5 consecutive days as described previously (Caron et al. 2002). Calves were kept under observation and samples collected as described below.

All procedures involving animal care, handling and experiments were performed under veterinary supervision and according to the recommendations of the Brazilian Committee on Animal Experimentation (COBEA; law no. 6.638 of May 8, 1979).

Clinical examination

Clinical examinations were performed daily from day 10 prior to challenge and reactivation up to days 21 post-challenge (pc) and as well as on days 1 to 21 post-reactivation

(pr). Rectal temperature (fever was defined as a rectal temperature of more than 39.5ºC), respiratory rates, clinical signs (coughing, congestion of the nasal mucosa, conjunctivitis, ocular and nasal discharges, changes in behaviour and appetite) as well as the presence of lesions on the nasal and oral mucosa were carefully examined.

Virological examination

Nasal and ocular swabs were collected daily from days 0 to 21 pc, 10 days before the beginning of dexamethasone administration and on days 1 to 21 pr. Swabs were immersed in 2mL sterile E-MEM supplemented with 200 U/mL penicillin, 200 µg/mL streptomycin and 5 µg/mL Amphotericin B. Samples were stored at -70ºC until processing. Infectious titres of positive samples were determined, calculated and expressed as $\log_{10}$ $CCID_{50}$ per mL after 72 hours of incubation at 37^{o}C.

Immunoperoxidase monolayer assay (IPMA)

To confirm the identify of the viruses recovered from nasal and ocular secretions, an immunoperoxidade monolayer assay was performed. The supernatants were inoculated on 96 well plates containing pre-formed MDBK monolayers, fixed in 4 % paraformaldehyde and stained in an immunoperoxidase monolayer assay (IPMA) as previously described (Kramps et al. 1996), with an anti-BoHV-1 monoclonal antibody (MAb) as primary antibody (MAb 11H6; Souza et al. 2002).

Virus neutralization tests

Serum samples were collected on days 0, 30, 60 post-vaccination (pv), and on days 0, 7, 14 and 21 pc and pr. Neutralizing antibodies in serum samples were determined in a varying serum-constant virus neutralization (VN) assay (House & Baker 1971), with twofold dilutions of serum against 100 $CCID_{50}$ of BoHV-1 SV265 wt. Antibody titres were expressed as the reciprocal of the highest serum dilution that prevented the development of cytopathic effect (CPE) after 72 hours of incubation at 37^{o}C.

Statistical analysis

Statistical analysis was performed using the Student *t*-test or the analysis of variance (ANOVA). The least significant difference for $p = 0.05$ was determined. Comparisons were made from day to day within the groups and between groups. Statistical analysis was performed with Data Analysis Supplement for Excel^{tm} (Office System 2003 for Windows^{tm}, Microsoft Corp., Seattle, USA). The term "significant" (statistically significant) means $p \leq 0.05$.

RESULTS

Vaccination

No clinical signs or adverse effects were seen after or during vaccination. All animals were clinically healthy and no signs of respiratory disease were observed before challenge.

Clinical and virological findings following the challenge

Among the vaccinated calves, between days 4 and 10 pc, mild clinical signs of respiratory disease (mild nasal lesions, nasal discharge, sneezing, coughing) were evident in 2/5 calves. The other three vaccinated animals showed no clinical signs of respiratory disease throughout the experiment. Pirexia was not observed among vaccinated calves (Fig.1a). All vaccinated animals eliminated virus from nasal secretions from days 2 to 6 pc. The virus titres varied from $10^{0.75}$ to $10^{5.75}$ $CCID_{50}$/mL (Fig 2a, Table 1). Ocular virus shedding was detected from day 4 on in the vaccinated group. The maximum virus titre was obtained at days 6 and 8 pc ($10^{0.75}$ $CCID_{50}$/mL and $10^{4.0}$ $CCID_{50}$/mL, Fig.3a, Table 1).

All non vaccinated calves (3/3) developed severe clinical signs of illness. Apathy, anorexia, rhinitis with redness of the nasal mucosa, nasal and ocular discharges, formation of vesicles with tendency to coalescence and erosions on the nasal mucosa, conjunctivitis, nasal stridor, sneezing and spontaneous coughing were observed in all non vaccinated calves. Dyspnea and tracheal stridors were observed in different degrees. On day 2 pc, the rectal temperatures of the infected calves started to rise. Fever (≥39.5ºC) was recorded from days 3 to 5 (Fig.1a). Despite severely ill on days 3 to 8 pc, all calves completely recovered from primary infection 13 dpc without noticeable sequels. Virus shedding was detected in nasal secretions from day 2 pc on all non vaccinated calves. However, the period of viral excretion was longer and the amount of virus shed among the non vaccinated group virus was to higher titres in comparison with the vaccinated calves (up to $10^{5.75}$ $CCID_{50}$/mL; Fig. 2a and Table 1). Ocular virus shedding was initially detected on day 2 pc (an animal) in the group of non vaccinated animals and the maximum virus titres were detected at days 5 and 8 pc ($10^{2.75}$ $CCID_{50}$/mL and $10^{5.75}$ $CCID_{50}$/mL respectively). From then on the titres tended to decrease until day 21 pc when sampling was

discontinued (Fig.3a and Table1). Calves of the negative control group remained healthy and did not shed virus in nasal secretions during the experiment.

Clinical and virological findings following reactivation attempts

Following dexamethasone administration, vaccinated calves did not develop clearly noticeable signs of respiratory disease, whereas only mild clinical signs of disease were observed in non vaccinated calves. However, such signs were less intense than those observed during primary infection. All non vaccinated calves were pyrexic from days 5 to 8 pr. (Fig.1b), whereas only two vaccinated calves presented fever for two days. Nasal virus shedding in the group of non vaccinated calves was detected from days 1 to 9 pr, and to higher titres than on vaccinated calves, which excreted virus from days 2-8 pr (until $10^{2.75}$ $TCID_{50}$/mL; Fig.2b and Table1). Ocular virus shedding was detected from day 2 to 9 pr in the non vaccinated calves and day 3 to 8 pr in the vaccinated calves (Fig.3b and Table 1).

Neutralizing antibody responses

None of the calves had detectable BoHV-1 neutralizing antibodies previous to the experiments. Unvaccinated animals remained BoHV-1 seronegative until challenge. After the first vaccination, 4/5 vaccinated animals started to produce neutralizing antibodies. After the booster administration, all calves seroconverted. The mean antibody titre obtained after the second dose of vaccine in all vaccinated animals showed a marked increase in comparison to the titres obtained after the first dose of vaccine. After challenge, again a boost in BoHV-1 neutralizing antibody titres was detected in all vaccinated calves, which revealed higher antibody titres after challenge than non vaccinated calves (Fig.4 and Table 2). Control calves remained negative for neutralizing antibodies throughout the experiment.

DISCUSSION

A gE$^-$ BoHV-1 was previously developed and tested as a live vaccine (Franco et al. 2002a). As inactivated vaccines may be considered safer to use - particularly because these pose virtually no risk of virus reversion - our aim here was to evaluate the clinical, virological and serological findings on animals inoculated with an inactivated vaccine prepared with the same 265gE$^-$ recombinant, in a vaccination/challenge experiment.

Vaccination of calves with the inactivated vaccine preparation was considered safe for use in calves since it did not cause any noticeable adverse effects on inoculated animals. After intranasal challenge with the wild type virus, only two vaccinated calves had a mild increase in body temperature and mild local signs of infection (few small erosions, nasal secretion and a slight increase in the respiratory frequency) were observed in two out of five calves. On the other hand, non vaccinated control calves developed severe signs of infection at the site of wild type virus inoculation (nostrils) and systemic signs of disease after challenge. This indicates that the challenge virus was still able to infect the nasal epithelium of vaccinated animals but was not detected in the lower respiratory system, nor did it cause systemic signs of infection (fever, anorexia, apathy). These results confirm that a significant protection to clinical disease was attained after vaccination. The clinical protection against challenge with wild type virus was comparable to the protection obtained with the attenuated vaccine (Franco et al. 2002b). These results are also comparable with those of previous workers with another, similarly built, inactivated gE$^-$ virus vaccine (Kaashoek et al. 1995).

In the present experiment, after challenge, vaccinated calves shed wild type virus in nasal and ocular secretions to lower titres and for a shorter period of time than did non vaccinated calves (Fig.2a, Fig.3a and Table 1). Thus, the inactivated preparation was as efficacious as the attenuated recombinant vaccine formulation in reducing virus shedding in nasal and ocular

secretions (Franco et al. 2002b). Reduction in challenge virus shedding, though not always linked to clinical protection, is important for the reduction of virus circulation within a herd, or between herds (Van Oirschot 1999).

Although the differential properties of this viral vaccine could not yet be shown because of the lack of commercial serologic tests in Brazil that can differentiate the immune responses of vaccinated and naturally infected animals, the immune responses during this experiment were measured using the virus neutralization test. Although the presence of neutralizing antibodies in serum does not necessarily correlate with reduction of virus shedding or clinical protection in BoHV-1 vaccinated animals, induction of virus neutralizing antibodies is very often used to access immune responses to vaccination (Kramps et al. 2004, Patel et al. 2005, Silva et al. 2007). The neutralizing antibody titres obtained after two doses of the inactivated 265gE$^-$ vaccine were higher than those detected after vaccination with one dose of the attenuated vaccine prepared with the same recombinant (Franco et al. 2002a). Similar observations were reported previously with another gE$^-$ vaccine (Kaashoek et al. 1995). Such differences were probably derived from the fact that the calves immunized with the attenuated virus received a single vaccine dose, whereas the inactivated vaccine was administered twice.

After the reactivation attempt with dexametasone, vaccinated calves did not show noticeable clinical signs. Latency was, nevertheless, established, since vaccinated calves did shed virus in their secretions, albeit for a shorter period and to lower titres than non vaccinated calves. These results indicate that the vaccine did not block the establishment of latent infection by the wild type challenge virus. Consonant with that, previous studies have shown that live, inactivated, conventional or subunit BoHV-1 vaccines, did not prevent the establishment of latency (Ackermann & Engels 2006). However, virus reactivation was less prominent in

vaccinated animals than in controls, a desirable effect of vaccination, since it will reduce virus circulation in the herd.

Analysis of the data presented here show that the inactivated, oil adjuvanted vaccine prepared with the 265gE$^-$ recombinant is safe and efficacious in minimizing clinical signs when vaccinated calves were challenged with an expressive amount ($10^{7.0}$ $CCID_{50}$) of virulent wild type BoHV-1. Such preparation must be evaluated further in order to determine its applicability in controlling BoHV-1 infections in the field.

Acknowledgements.- A.D. Silva and P.A. Esteves were students from the Programa de Pós-Graduação em Ciências Veterinárias da Faculdade de Veterinária (PPGCV/UFRGS/Brazil) grantees of CAPES. P.M. Roehe is a CNPq 1C research fellow. This work was supported by CNPq, PRONEX, FAPERGS and CAPES.

REFERENCES

Ackermann M. & Engels M. 2006. Pro and contra IBR-eradication. Vet. Microbiol. 113(3/4): 293-302.

Bahnemann H., Augé De Mello P., Abaracon D. & Goe S I. 1974. Immunogenicity in cattle of foot and mouth disease vaccines inactivated with binary ethylenimine. Bull. Off. Int. Epiz. 81:1335-1343.

Balan P., Davis-Poynter N., Bell S., Atkinson H., Browne H. & Minson T. 1994. An analysis of the in vitro and in vivo phenotypes of mutants of herpes simplex virus type 1 lacking lycoproteins gG, gE, gI or the putative gJ. J. Gen. Virol. 75 (Pt 6):1245-58.

Caron L., Flores E.F., Weiblen R., Scherer C.F., Irigoyen L.F., Roehe P.M., Odeon A., Sur J.H. 2002. Latent infection by bovine herpesvirus type-5 in experimentally infected rabbits: virus reactivation, shedding and recrudescence of neurological disease. Vet. Microbiol. 84(4):285-95.

Chowdhury S.I., Ross C.S., Lee B.J., Hall V. & Chu H.J. 1999. Construction and characterization of a glycoprotein E gene-deleted bovine herpesvirus type 1 recombinant. Am. J. Vet. Res. 60(2):227-32.

Dingwell K.S., Doering L.C.& Johnson D.C. 1995. Glycoproteins E and I facilitate neuron-to-neuron spread of herpes simplex virus. J. Virol. 69 (11):7087-98.

Gibbs E.P.J. & Rweyemamu M.M. 1977. Bovine herpesvirus. Part I. Bovine herpesvirus 1. Vet. Bull. 47:317-343.

House J.A. & Baker J.A. 1971. Bovine herpesvirus IBR-IPV: The antibody virus neutralization reaction. Cornell Vet. 61:320-335.

Kaashoek, M.J., Moerman A., Madic, J., Weerdmeester K., Maris-Veldhuis, M., Rijsewijk F. A. & van Oirschot J. T. (1995). An inactivated vaccine based on a glycoprotein E-negative strain of bovine herpesvirus 1 induces protective immunity and allows serological differentiation. Vaccine 13(4):342-346.

Kramps J.A., Perrin B., Edwards S. & van Oirschot J.T. 1996. A European inter-laboratory trial to evaluate the reliability of serological diagnosis of bovine herpesvirus 1 infections. Vet. Microbiol. 53(1/2):153-161.

Kramps J.A., Banks M., Beer M., Kerkhofs P., Perrin M., Wellenberg G.J. & Oirschot J.T. 2004. Evaluation of tests for antibodies against bovine herpesvirus 1 performed in national reference laboratories in Europe. Vet. Microbiol. 102(3-4):169-81.

Franco A.C., Rijsewijk F.A.M., Flores E.F., Weiblen R. & Roehe P.M. 2002a. Construction and characterization of a glycoprotein E deletion mutant of bovine herpesvirus type 1.2 strain isolated in Brazil. Braz. J. Microbiol. 33:274-278.

Franco A.C., Spilki F.R., Esteves, F.R., Lima M., Weiblen R., Flores E.F., Rijsewijk F.A.M. & Roehe P.M. 2002b. A Brazilian glycoprotein E negative bovine herpesvirus type 1.2a (BHV-1.2a) mutant is attenuated for cattle and induces protection against wild-type virus challenge. Pesq. Vet. Bras. 22:135-140.

Murphy F.A., Gibbs E.P., Horzinek M.C. & Studdert M.J. 1999. Veterinary Virology. 3rd ed. Academic Press, New York.

Olson J.K., Bishop G.A. & Grose C. 1997.Varicella-zoster virus Fc receptor gE glycoprotein: serine/threonine and tyrosine phosphorylation of monomeric and dimeric forms. J. Virol. 71(1):110-119.

Petzhold S.A., Reckziegel P.E., Prado, J. A.,Teixeira J. C., Wald, V. B., Esteves, P.A.; Spilki F. R. & Roehe, P. M. 2001. Neutralizing antibodies to bovine herpesviruses types 1 (BHV-1) and 5 (BHV-5) induced by an inactivated vaccine to BHV-1. Braz. J. Vet. Res. Anim. Sci. 38(3):184-187.

Rebordosa X., Pinol J., Perez-Pons J. A., Lloberas J., Naval J., Serra-Hartmann X., Espuna E. & Querol E. 1996. Glycoprotein E of bovine herpesvirus type 1 is involved in virus transmission by direct cell-to-cell spread. Virus Res. 45(1):59-68.

Schwyzer M. & Ackermann M. 1996. Molecular virology of ruminant herpesviruses. Vet. Microbiol. 53:17-29.

Spilki F.R., Franco A.C., Rijsewijk F.A.M., Weiblen R., Flores E.F. & Roehe P.M. 2004. In vitro characterization of gE negative Bovine Herpesvirus Types 1.1 (BHV-1.1) and 1.2 (BHV-1.2). Braz. J. Microbiol. 35:264-268.

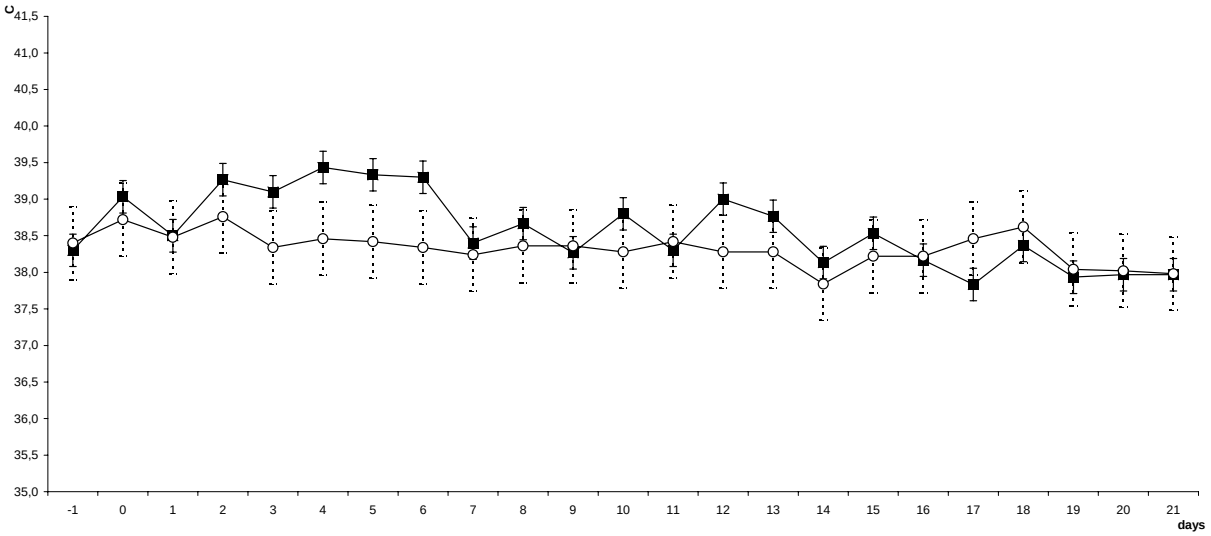
Silva L.F., Weiblen R. & Flores E.F. 2007. Imunogenicidade de vacinas comerciais inativadas contra Herpesvirus Bovino tipo 1. Ciência Rural 37(5): 471-1474.

Souza V.F., Melo S.V., Esteves P.A., Schmidt C.S.R., Gonçalves D.A., Schaefer R., Silva T.C., Almeida R.S., Vicentini F., Franco A.C., Oliveira E.A., Spilki F.R., Weiblen R., Flores E.F., Lemos R.A., Alfieri A.A., Pituco, E.M. & Roehe P.M. 2002. Caracterização de herpesvírus bovinos tipos 1 (BHV-1) e 5 (BHV-5) com anticorpos monoclonais. Pesq. Vet. Bras. 22(1): 13-18.

van Oirschot J. T., Kaashoek, M. J., Maris-Veldhuis M. A.; & Rijsewijk F.A. 1999. Strains of bovine herpesvirus 1 that do not express an epitope on glycoprotein E in cell culture still induce antibodies that can be detected in a gE-blocking ELISA.Vet..Microbiol. 65(2):103-113.

Weiblen R., Moraes M.P., Rebelatto M.C., Lovato L.T. & Canabarro T.F. 1996. Bovine herpesvirus isolates. Revta Microbiol. 27(3):87-90.

a)



b)

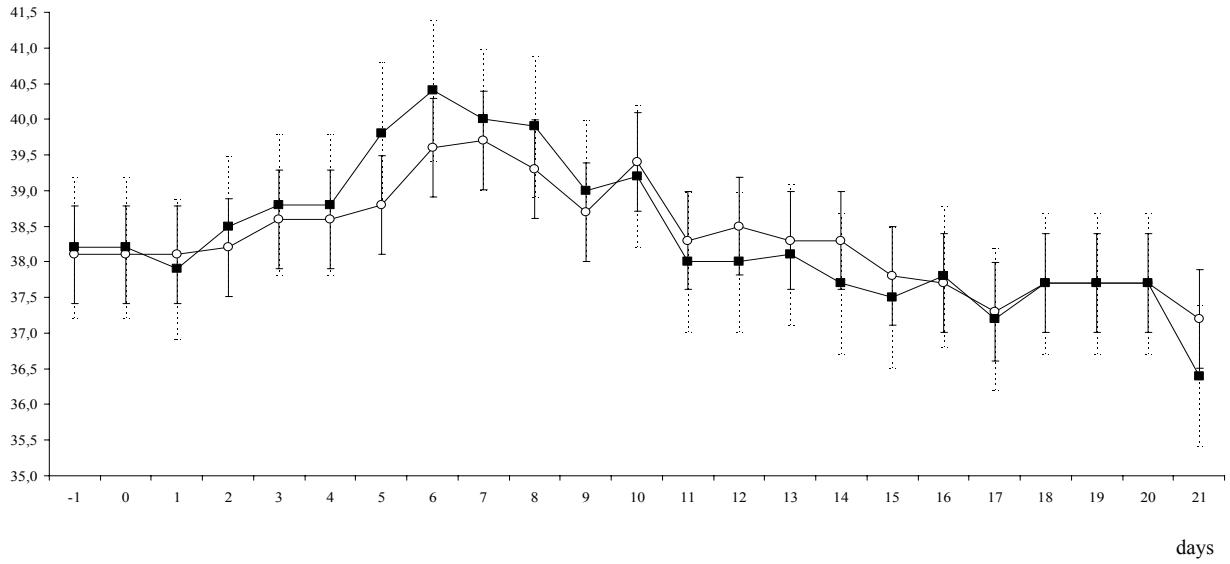
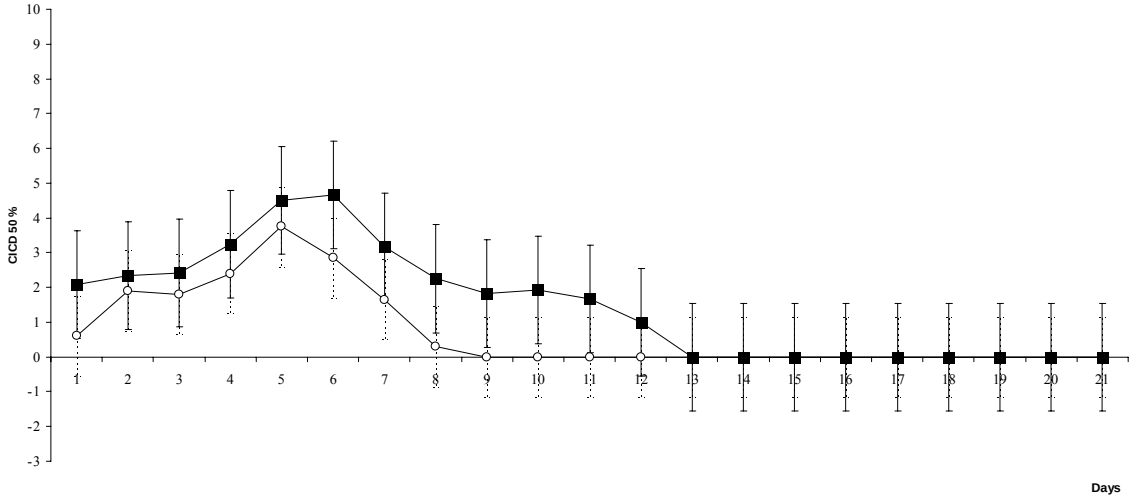


Figure 1. Mean rectal temperatures (ºC) (a) after challenge and (b) after dexamethasone administration of vaccinated BoHV-1 gE- inactivated (empty circles) and non vaccinated calves (black squares). "Days" refers to days after challenge (a) or following dexamethasone administration (b). Vertical bars = standard deviation.

a)



b)

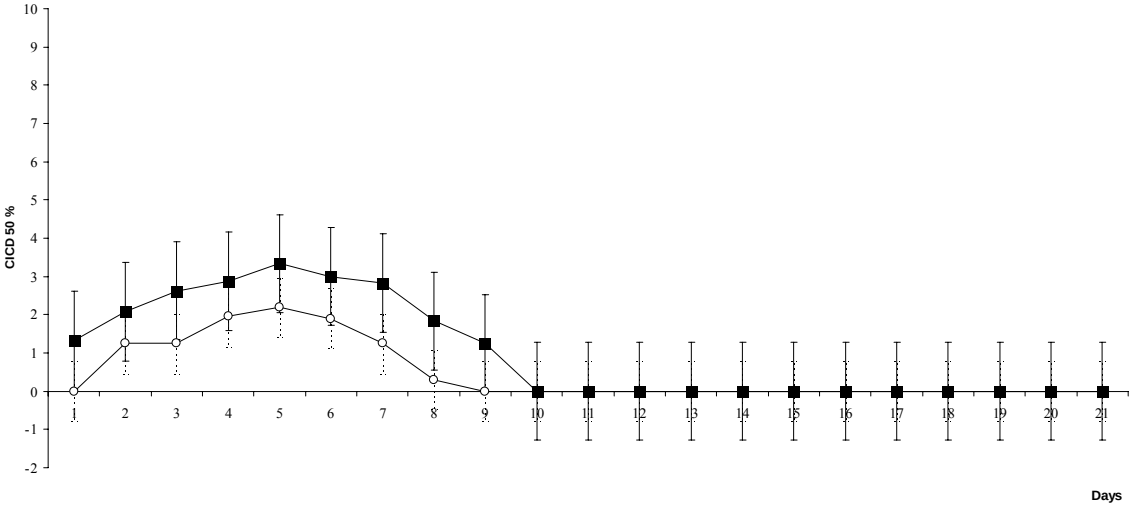
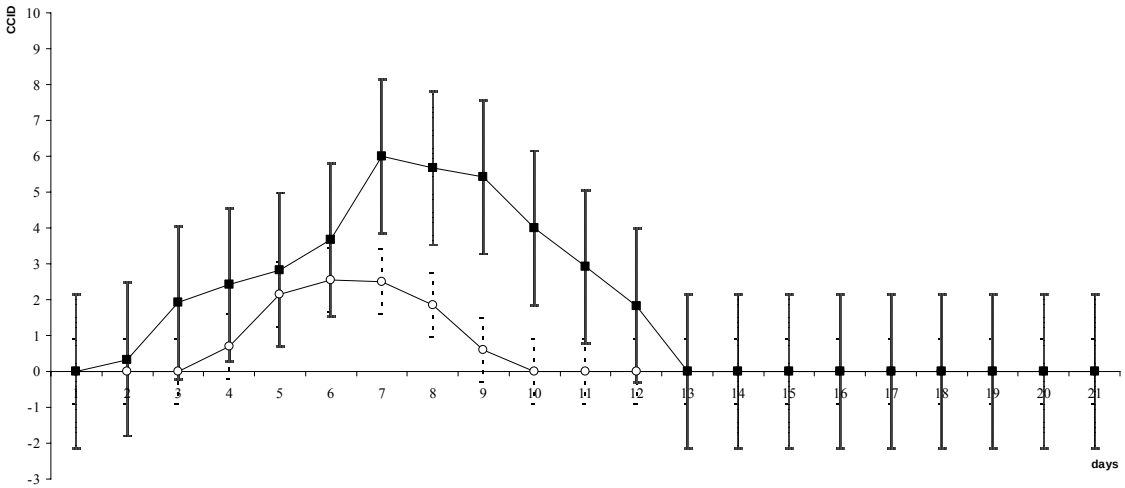


Fig.2. Nasal virus shedding: a) after challenge with wild type virus (SV265wt) challenge; b) after dexamethasone administration. Infectious virus titres expressed in log $_{10}$ of 50 % cell culture infectious doses per mL ($CCID_{50}$). Empty circles: vaccinated calves; black squares: non vaccinated calves. Vertical bars = standard deviation.

a)



b)

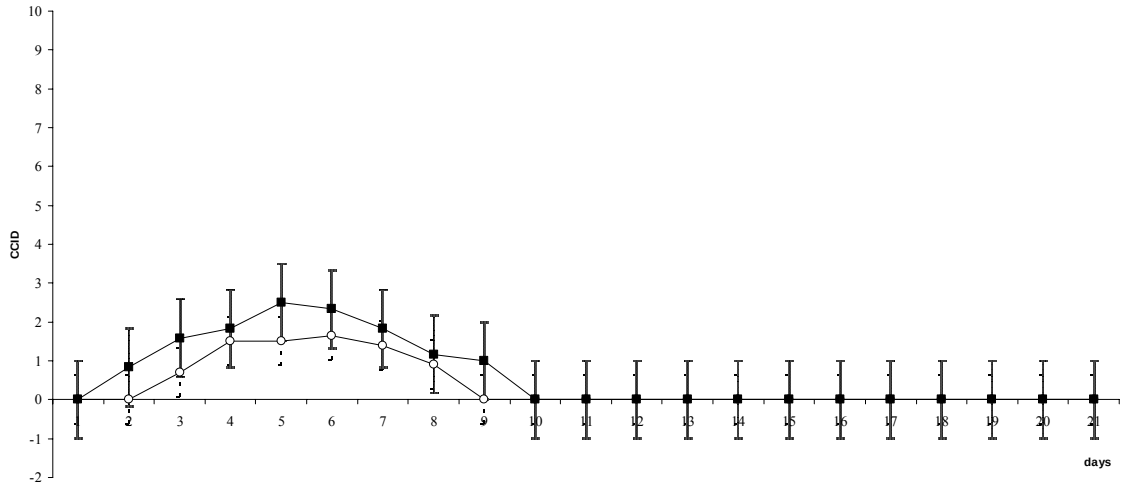


Fig.3. Ocular virus shedding: a) after challenge with wild type virus (SV265wt) challenge, and b) after dexamethasone administration. Infectious virus titres expressed in log $_{10}$ of 50 % cell culture infectious doses per mL ($CCID_{50}$). Empty circles: vaccinated calves; black squares: non vaccinated calves. Vertical bars = standard deviation.

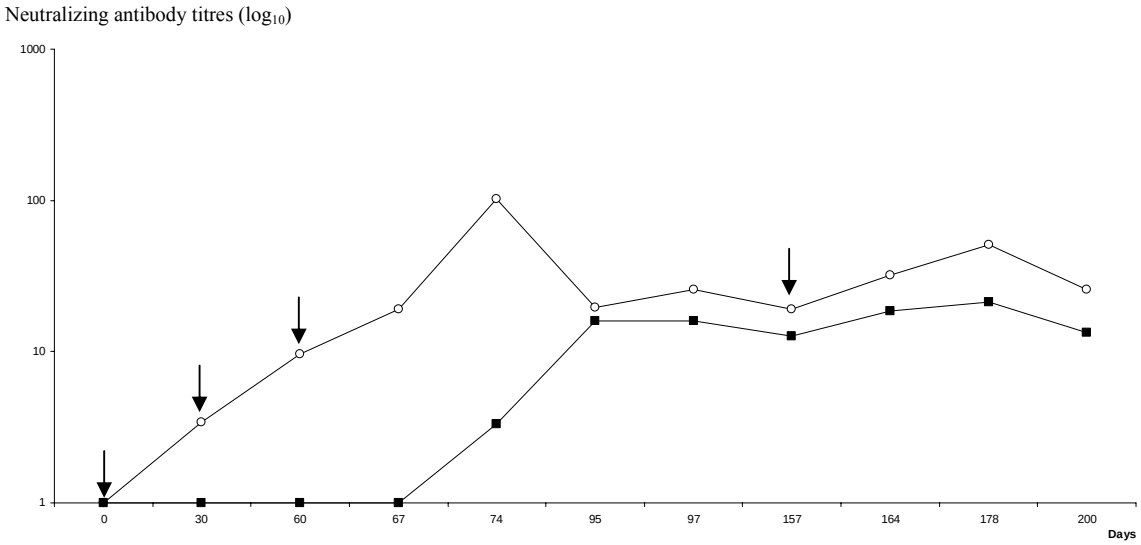


Fig.4. Neutralizing antibody titres (geometric mean titres in each group) after immunization with an inactivated BoHV-1 265gE- vaccine, challenge with wild type virus (SV265 wt) and during reactivation attempts with dexametasone administration. Empty circles: vaccinated calves; black squares: non vaccinated calves. Arrows points to date of immunization (day 0 first dose and day 30 second dose); challenge (60 day post vaccination) and date of the beginning of dexametasone administration (157 days post vaccination) respectively. Titres expressed as $\log_{10}$ of the reciprocal of the neutralizing antibody titres.

Table 1: Viral shedding in nasal and ocular secretions from vaccinated and non vaccinated calves following challenge with wild type parental virus.

			Viral shedding												
Group			Day post challenge												
	Animal	Site	1	2	3	4	5	6	7	8	9	10	11	12	13-21
Vaccinated animals	57	N	-	+	+	++	++	++	+	-	-	-	-	-	-
		O	-	-	-	-	++	++	++	++	+	-	-	-	-
	65	N	-	++	++	++	+++	++	+	+	-	-	-	-	-
		O	-	-	-	+	++	++	+	+	-	-	-	-	-
	64	N	++	+	++	+	++	++	++	-	-	-	-	-	-
		O	-	-	-	-	+	+	++	+	-	-	-	-	-
	60	N	-	+	+	++	+++	+++	++	-	-	-	-	-	-
		O	-	-	-	+	++	++	++	+	+	-	-	-	-
	61	N	-	-	+	+	+	+	-	+	-	-	-	-	-
		O	-	-	-	-	+	+	+	+	-	-	-	-	-
Non vaccinated Animals	67	N	++	+	+	+	+++	+++	+++	++	+	+	+	+	-
		O	-	-	++	++	++	++	++++	+++	+++	+++	++	+	-
	63	N	+++	+++	+++	+++	+++	+++	+++	++	++	+	+	+	-
		O	-	+	+	+	+	++	++++	+++	+++	+++	+	+	-
	59	N	-	+	++	+++	+++	+++	+	+	+	+	+	+	-
		O	-	-	+	++	++	++	+++	+++	+++	++	++	+	-

+: $10^{0.75}$-10^{2} $CCID_{50}$/mL
++: !$10^{2.25}$-10^{4} $CCID_{50}$/mL
+++: $10^{4.25}$-10^{6} $CCID_{50}$/mL
++++: >=$10^{6.25}$ $CCID_{50}$/mL
-: negative sample
N: nasal secretion
O: ocular secretion

Table 2. Virus neutralizing antibodies in vaccinated and non vaccinated calves following vaccination, challenge and dexametasone (Dx) administration. Titres expressed as the reciprocal of the neutralizing antibody titre.

Groups	Animals	Virus neutralizing titres					
		Days post vaccination		Days post challenge[a]		Days post Dx treatment[b]	
		0	60	14	37	7	43
Vaccinated animals	57	<2	8	64	32	16	16
	60	<2	8	128	16	64	32
	61	<2	8	128	16	16	32
	64	<2	16	128	32	32	16
	65	<2	8	64	32	32	32
Non vaccinated animals	67	<2	<2	<2	8	8	16
	63	<2	<2	8	32	32	8
	59	<2	<2	<2	8	16	16

[a] challenge occurred on day 60 post vaccination
[b:] dexametasone treatment started on day 157 post vaccination

Capítulo 2

Field evaluation of safety during gestation and horizontal spread of a recombinant differential bovine herpesvirus 1 (BoHV-1) vaccine

Fernando R. Spilki [2,3*], Alessandra D. Silva[3], Helena Beatriz C. Ruthner Batista[3], Anna P. Oliveira[3], Evandro Winkelmann[4], Ana C. Franco[3], Jorge A. Porciúncula[5] and Paulo Michel Roehe [2,3**]

[2]Departamento de Microbiologia, Laboratório de Virologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS 90050-170, Brazil.

[3]Equipe de Virologia, Instituto de Pesquisas Veterinárias Desidério Finamor (IPVDF), Fundação Estadual de Pesquisa Agropecuária (Fepagro), Saúde Animal, Estrada do Conde 6000, Eldorado do Sul, RS 92990-000, Brazil.

[4]Laboratório de Virologia, Centro de Ciências Rurais, Universidade Federal de Santa Maria (UFSM), Santa Maria, RS 97119-900, Brazil.

[5]Fundação Estadual de Pesquisa Agropecuária, Estação Experimental de São Gabriel, São Gabriel, RS, Brazil.

** Corresponding author. Email: proehe@ufrgs.br

Publicado em Pesquisa Veterinária Brasileira 2005 25(1):54-58.

ABSTRACT

Bovine herpesvirus type 1 (BoHV-1) is recognized as a major cause of respiratory, reproductive disease and abortion in cattle. Vaccination is widely applied to minimize losses induced by BoHV-1 infections; however, vaccination of dams during pregnancy with modified live virus (MLV) vaccines has been occasionally associated to abortions. We have previously reported the development of a BoHV-1 recombinant virus, constructed with basis on a Brazilian BoHV-1 (Franco et al. 2002a) from which the gene coding for glycoprotein E (gE) was deleted (gE-) by genetic manipulation. Such recombinant has been previously evaluated in its potential as a differential vaccine (gE- vaccine) that allows differentiation between vaccinated and infected animals. Here, in the first part of the present study, the safety of the gE- vaccine during pregnancy was evaluated by the intramuscular inoculation of $10^{7.4}$ tissue culture 50 % infective doses ($TCID_{50}$) of the virus into 22 pregnant dams (14 BoHV-1 seronegative; 8 seropositive), at different stages of gestation. Other 15 pregnant dams were kept as non-vaccinated controls. No abortions, stillbirths or fetal abnormalities were seen after vaccination. Seroconversion was observed in group of previously seronegative vaccinated animals. In the second part of the study, the potential of the gE- vaccine virus to spread among beef cattle under field conditions was examined. Four heifers were inoculated intranasally with a larger amount ($10^{7,6}$ $TCID_{50}$) of the gE- vaccine (to increase chances of transmission) and mixed with other sixteen animals at the same age and body condition, in the same grazing area, at a population density equal to the average cattle farming density within the region (one cattle head per 10,000 m^2), for 180 days. All animals were monitored daily for clinical signs. Serum samples were collected on days 0, 30, 60 and 180 post-vaccination. Seroconversion was observed only in vaccinated heifers. These results indicate that, under the conditions of the present study, the gE vaccine virus did not

cause any noticeable harmful effect on pregnant dams and on its offspring and did not spread horizontally among cattle.

INDEX TERMS: Bovine herpesvirus 1, BoHV-1 recombinant gE- vaccine.

INTRODUCTION

Bovine herpesvirus type 1 (BoHV-1) has been associated with a number of different clinical manifestations in cattle, such as infectious bovine rhinotracheitis (IBR) and infectious pustular vulvovaginitis/ infectious pustular balanoposthitis (IPV/ IPB). The most striking effect of BoHV-1 infection is its capacity to interfere in gestation, often leading to termination of pregnancy, with serious economical consequences (Guy & Potgieter, 1985; Miller et al. 1991, Siebert et al. 1995a, Turin et al. 1999). To minimize such losses, both conventional modified live or inactivated as well as recombinant vaccines have been widely used (Kleiboeker et al. 2003, Turin et al. 1999).

One of the recent strategies for the development of BoHV-1 vaccines relies on the deletion of non-essential genes from the viral genome. Such deletions allow the distinction between wild type virus-infected and vaccinated animals, provided that a serological test capable of recognizing antibodies to the deleted protein is available (Belknap et al. 1999, Flores et al. 1993, Franco et al. 2002a). Such vaccines are often referred to as “differential vaccines” (Wentink et al. 1993). Recently, we constructed a glycoprotein E (gE)-negative BoHV-1 recombinant, based on an autochthonous Brazilian strain of BoHV-1. Such recombinant is intended for use as an attenuated, differential vaccine (Franco et al. 2002b) and, as such, was shown to be safe and efficacious for calves (Franco et al. 2002a), yet allowing differentiation

between vaccinated and infected animals. An important drawback occasionally found on other MLV is that those may also lead to embryonic, fetal death and abortions (Miller et al. 1989, McFelly et al. 1968, Mitchell 1974, Whetstone et al. 1986). Therefore, it is essential to investigate whether any new vaccine candidate would bring undesirable side effects if eventually administered during gestation. Another important issue on MLVs is its potential to spread within the herd (Pastoret et al. 1980). This is an undesirable side effect, since the vaccine virus may perpetuate within herds (Hage et al. 1996). Therefore, it would be of interest to examine whether the differential vaccine virus might spread within a herd. In the present study, it was initially aimed to determine the safety of the gE- vaccine for pregnant dams. Subsequently, the potential of the gE- vaccine to spread within a herd under typical beef cattle field conditions was examined.

MATERIALS AND METHODS

Multiplication of the gE- vaccine virus

The construction of the recombinant vaccine virus (265gE-), which gave rise to the gE-negative vaccine (gE- vaccine), was described previously (Franco et al. 2002a). The virus was multiplied in CRIB-1 cells (Flores & Donis 1995). BoHV-1 strain EVI 123/98, a typical representative of BoHV-1.1 isolated in Brasil (D'Arce et al. 2002), was multiplied in CRIB-1 cells and used for serum neutralization (SN) assays. Cell cultures were maintained in Eagle's minimal essential medium (EMEM) supplemented with 5 % to 10 % fetal bovine serum (FBS; Nutricell), 2 mM glutamine and antibiotics (100 IU/ml penicillin and 100 mg/ml streptomycin) following standard procedures.

Safety for pregnant dams and immunization

Thirty seven pregnant dams of mixed European beef breeds were used in the experiment. Twenty two, 2 to 4 years-old dams, were vaccinated intramuscularly (IM) on the side of the neck with 3 mL of a suspension containing 107.4 TCID50 of the gE- vaccine virus in EMEM. Fourteen pregnant dams in different stages of gestation were seronegative for BoHV-1 at the start of the experiment. Another group consisted of eight BoHV-1-seropositive pregnant dams an additional group of 15 pregnant dams were kept as non-vaccinated controls. From the control group, at the start of the experiment, seven dams were BoHV-1 seronegative and 8 were seropositive for BoHV-1. The stage of pregnancy was determined by rectal palpation and confirmed by the date of parturition. Table 1 shows the stages of pregnancy of dams within different groups.

Vaccine virus spread in a seronegative herd

Animals and vaccine virus inoculation

Twenty Aberdeen Angus heifers, aged 18 months, all seronegative for BoHV-1, were selected from the stock of the institution of origin of the authors. Four heifers were inoculated by nasal instillation (IN) of 3 mL of a viral suspension containing $10^{7.6}$ $TCID_{50}$ of the gE- vaccine virus. The animals were observed daily for clinical signs. Serum samples were collected on days 0, 30, 60 and 180 days post-vaccination (DPV).

Seroneutralization assays (SN) were performed as described below

Any seronconversion to BoHV-1 during this period was assumed as induced by the vaccine virus. The animals were kept under field conditions throughout the experiment, in a

grazing area of 10.000 m2, at a density of 1 animal per 10.000 m2 for six months. Serum samples were collected from the dams by caudal or jugular venipuncture on days 0, 40 and 80 post-vaccination (PV). Samples were also taken from the calves born from the dams under study, during the first 2 weeks of life. Sera were tested in serial twofold dilutions in a standard BoHV-1 neutralizing antibody test against strain EVI 123/ 98 (Franco et al. 2002).

Statistical analysis

The results were statistically evaluated by analysis of variance (ANOVA); the least significance difference for $p = 0.05$ was determined. Statistical analysis was performed with Data Analysis Supplement for Exceltm (Office XP for Windowstm, Microsoft Corp., USA). The term "significant" (statistically significant) in the text means $p= 0.05$.

RESULTS

Safety for pregnant dams

No embryonic deaths, abortions and stillbirths were detected in any vaccinated dam throughout the experiment. Likewise, no reproductive abnormalities were detected on the group of nonvaccinated dams. Seroconversion was observed in vaccinated dams that were seronegative at the start of the experiment, as demonstrated by SN (Fig.1). On the other hand, previously seropositive dams had no significant alterations in their serum neutralizing antibody titres (Fig.1).

Vaccine virus spread in a seronegative herd

All four animals vaccinated IN developed a strong immune response against BoHV-1, as measured by SN assays. Only mild clinical signs, characterized by light serous discharges from days 1 to 7 PV, were observed on vaccinated heifers. In contrast, no seroconversion was detected on "in contact" cattle. These results demonstrate that the vaccine virus was not capable of spreading from vaccinated to contact animals.

DISCUSSION

Although vaccination with modificated live vaccine for IBR virus is recognized as, an efficient way to improve herd immunity to BoHV-1 infections (Wentik et al. 1993, Siebert et al. 1995a), the use of this kind of vaccines during pregnancy may result in fertility problems such as early embryonic deaths, abortions and stillbirths (Lomba et al. 1976, Whetstone et al. 1986).

In order to examine the effect of the gE- vaccine during pregnancy, in the first experiment of the present study seronegative and seropositive dams were vaccinated intramuscularly with a gE- vaccine. Vaccination was performed via IM in order to increase the chances of the virus reaching the conceptuses. Despite the inoculation of a large dose of vaccine virus ($10^{7.4}$ $TCID_{50}$) no detectable harmful effect was observed, neither on pregnant dams nor on its offspring, demonstrating its safety for application during pregnancy, at least under the conditions of the present study. Another gE- vaccine (Siebert et al. 1995b) had been also evaluated on pregnant cattle, with similar results. However, other recombinants with a functional gE gene retained its abortigenic capacity (Miller et al. 1995). Such studies, when examined comparatively with the one here reported, suggest that there may be a link between the apparent lack of ability to reach and/or cause fetal damage might be specifically linked to

the removal of the gE gene. Further studies should be able to determine whether gE in fact plays a significant role leading to abnormalities during gestation.

In addition to being apparently safe for pregnant dams, the gE- vaccine was capable of inducing high levels of neutralizing antibodies on vaccinated dams. This is beneficial for the passive transfer of antibodies to the newborn, as shown for other herpesviruses (Casal et al. 2004) and also in response to other viruses (Roehe 1991). In fact, some of the calves born to vaccinated dams in the present study had higher levels of neutralizing antibodies than their own dams. Others have speculated that higher antibody titres in newborns were associated to intrauterine infection with the vaccine virus (Lomba et al. 1976). In our view, a more likely possibility is that a physiological concentration of immunoglobulins in the colostrum would allow more effective transfer of these to the newborn, as also pointed out by others (Odde 1988, Roehe 1991, Ellis et al. 1996).

Interestingly, vaccination of previously seropositive dams led to no significant rise in antibody levels after immunization. In fact, neutralizing antibody levels in such animals showed a tendency to decline at 80 DPV. As neutralizing antibody levels in such dams were already relatively high, it is possible that the vaccine virus could have been inactivated by the host's defense mechanisms, such as shown for pseudorabies virus (PrV) in swine (Zuckermann et al. 1998).

The route of inoculation might also play a role in nasal virus spread. In the experiment designed to detect nasal virus spread, the inoculation was performed via IN and with a larger amount of virus, since this could increase the possibility of shedding. Transmission following IM inoculation is much less likely to occur (Siebert et al. 1995b, Mars et al. 2000). Despite IN inoculation, no transmission of the vaccine virus to herd was detected. The sixteen "in contact" animals kept as sentinels did not seroconvert to BoHV-1 up to six months after vaccination.

This was probably a result of the poor replication of the gE- virus in the host. Viral spread within a herd is not dependent on the herd size, but is directly related to the agent's ability to replicate efficiently in the host and be shed to contacts (Bouma et al. 1995, Hage et al. 1996). We have previously demonstrated (Franco et al. 2002a), that the gE- virus evaluated here replicates to very low titres in calves, as has also been shown for another gE strain (Kaashoek, 1995, Strube et al. 1995, Mars et al. 2000). Such poor replication does not favor efficient transmission, as apparent in the experiment here described. Therefore, at the cattle density employed here, it seems that the gE- vaccine would not spread within the herd.

The experiments reported here suggest that the gE- vaccine was not hazardous to dams vaccinated during gestation. In addition, it did not spread horizontally to herdmates under usual beef cattle farming conditions usually employed for this region. These studies will be extended in the future to evaluate the efficacy of the gE-deleted vaccine in preventing abortions following challenge of pregnant dams with wild type BoHV-1.

REFERENCES

Belknap E.B., Walters L.M., Kelling C., Ayers V.K., Norris J., McMillend J., Hayhowe C., Cochranf M., Reddyd D.N., Wright J. & Collins J.K. 1999. Immunogenicity and protective efficacy of a gE, gG and US2 genedeleted bovine herpesvirus-1 (BOHV) vaccine. Vaccine 17:2297-2305.

Bouma A., De Jong M.C.M. & Kimman T.G. 1995. Transmission of pseudorabies virus within pig populations is independent of the size of the population. Prev. Vet. Med. 23: 163-172.

Casal J., Planasdemunt L., Varo J.A. & Martín M. 2004. The use of different vaccination schedules for sows to protect piglets against Aujeszky's disease. Vet. Med. B 51:8-1

D'Arce R.C.F., Almeida R.S. Silva T.C., Franco A.C., Spilki F., Roehe P.M. & Arns C.W. 2002. Restriction endonuclease and monoclonal antibody characterization of Brazilian isolates of bovine herpesviruses types 1 and 5. Vet. Microbiol. 88:315-324.

Ellis J.A., Hassard L.E., Cortese V.S. & Morley P.S. 1996. Effects of perinatal vaccination on humoral and cellular immune responses in dams and young calves. J. Am. Vet. Med. Assoc. 208:393-400.

Flores E.F. & Donis R.O. 1995. Isolation of a mutant MDBK cell line resistant to bovine viral diarrhea virus infection due to a block in viral entry. Virology 208:565-575.

Flores E.F., Osorio F.A., Zanella E.L., Kit S. & Kit M. 1993. Efficacy of a deletion mutant bovine herpesvirus-1 (BOHV) vaccine that allows serologic differentiation of vaccinated from naturally infected animals. J. Vet. Diagn. Invest. 5:534-540.

Franco A.C., Rijsewijk F.A.M., Flores E.F., Weiblen. R. & Roehe P.M. 2002a. Construction and characterization of a glycoprotein E deletion of bovine herpesvirus type 1.2 strain isolated in Brazil. Braz. J. Microbiol. 33:274-278.

Franco A.C., Spilki F.R., Esteves P.A., Lima M., Weiblen R., Flores E.F., Rijsewijk F.A.M. & Roehe P.M. 2002b. A Brazilian glycoprotein E-negative bovine herpesvirus type 1.2a (BOHV.2a) mutant is attenuated for cattle and induces protection against wild-type virus challenge. Pesq. Vet. Bras. 22:135-140.

Hage J.J., Schukken Y.H., Barkema H.W., Benedictus G., Rijsewijk F.A.M. & Wentink G.H. 1996. Population dynamics of bovine herpesvirus infection a dairy herd. Vet. Microbiol. 53:169-180.

Guy J.S. & Potgieter L.N. 1985. Bovine herpesvirus-1 infection of cattle: kinetics of antibody formation after intranasal exposure and abortion induced by the virus. Am. J. Vet. Res. 46:893-898.

Kaashoek M. 1995. Marker vaccines against bovine herpesvirus 1 infections. Ph.D. Thesis, Utrecht University, Netherlands. 155p.

Kleiboeker, S.B., Lee, S.M., Jones, C.A. & Estes, D.M. 2003. Evaluation of shedding of bovine herpesvirus 1, bovine viral diarrhea virus 1, and bovine viral diarrhea virus 2 after vaccination of calves with a multivalent modified-live virus vaccine. J. Am. Vet. Med Assoc. 222:1399-1403.

Lomba F., Vascoboinic E. & Zygraich N. 1976. Immunization of pregnant dams with a temperature-sensitive mutant of the IBR Virus. 6th Int. Congr. Diseases of Cattle, Paris, p.395-399.

Mars M.H., de Jong M.C.M. & Van Oirschot J.T. 2000. A gE-negative BHV- 1 vaccine virus strain cannot perpetuate in cattle populations. Vaccine 18:2120-2124.

McFelly R.A., Merrit A.M. & Stearly E.L. 1964. Abortion in a dairy herd vaccinated for infectious bovine rhinotracheitis. Vet. Path. 1:7-17.

Miller J.M., Whetstone C.A. & Van der Maaten, M.J. 1991. Abortfacient property of bovine herpesvirus type 1 isolates that represent three subtypes determined by restriction endonuclease analysis of viral DNA. Am. J. Vet. Res., 52:458-461.

Miller J.M., Whetstone C.A., Bello L.J., Lawrence W.C. & Whitbeck J.C. 1995. Abortions in heifers inoculated with a thymidine kinase-negative recombinant of bovine herpesvirus 1. Am. J. Vet. Res. 56:870-874.

Mitchell D. 1964. An outbreak of abortion in a dairy herd following inoculation with an intramuscular infectious bovine rhinotracheitis virus.Can.Vet. J. 26:8-14.

Odde K.G. 1988. Survival of the neonatal calf. Factors influencing colostral and calf serum immunoglobulin levels. Vet. Clin. North Am. Food Anim. Pract. 4:501-508.

Pastoret P.P., Babiuk L.A., Misra V. & Griebel P. 1980. Reactivation of temperature sensitive and non-temperature-sensitive infectious bovine rhinotracheitis vaccine virus with dexamethasone. Infect. Immun. 29:483-488.

Roehe P.M. 1991. Studies on the comparative virology of pestiviruses. Ph.D. thesis. University of Surrey, Guildford, UK. 361p.

Siebert S., Auer S., Heinem E., Kretzdom D. & Strube W. 1995a. Marker vaccines - opportunities for IBR control. Part I: BOHV infections – The problem. Tierärztl. Umschau 50:530-533.

Siebert S., Auer S., Heinem E., Kretzdom D. & Strube W. 1995b. Marker vaccines – opportunities for IBR control. Part II: Safety and efficacy of the gE-deleted Bayovac IBR marker vaccines. Tierärztl. Umschau 50:582-584.

Strube W., Abar B. & Bergle R.D. 1995. Safety aspects in the development of an infectious bovine rhinotracheitis marker vaccine. Non-target effects of live vaccines. Dev. Biol. Stand. 84:75-81.

Turin L., Russo S. & Poli, G. 1999. BOHV: new molecular approaches to control a common and widespread infection. Mol. Med. 5:261-284.

Van Drunen Littel-van den Hurk S., Parker M.D., Massie B., van den Hurk J.V., Harland R., Babiuk L.A. & Zamb T.J. 1993. Protection of cattle from BOHV infection by immunization with recombinant glycoprotein gIV. Vaccine 11:25-35.

Van Engelenburg F.A.C., Kaashoek M.J., Van Oirschot J.T. & Rijsewijk F.A.M. 1995. A glycoprotein E deletion mutant of bovine herpesvirus 1 infects the same limited number of tissues in calves as wild-type virus, but for a shorter period. J. Gen.Virol. 76:2387-2392.

Wentink G.H., Van Oirschot J.T. & Verhoeff J. 1993. Risk of infection with bovine herpes virus 1 (BOHV): a review. Vet. Quarterly 15: 30-33.

Whetstone C.A., Wheeler J.G. & Reed D.E. 1986. Investigation of possible vaccine-induced epizootics of infectious bovine rhinotracheitis, using restriction endonuclease analysis of viral DNA. Am. J. Vet. Res. 47:1789- 1795.

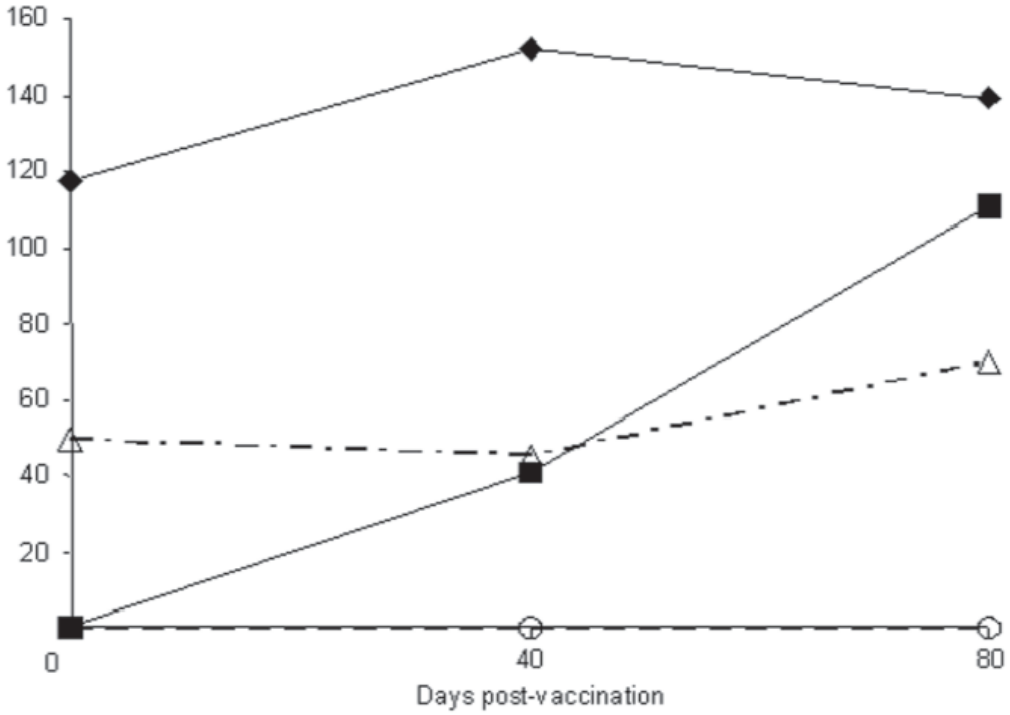
Zuckermann F.A., Husmann R.J., Schwartz R., Brandt J., Mateu de Antonio E. & Martin S. 1998. Interleukin-12 enhances the virus-specific interferon gamma response of pigs to an inactivated pseudorabies virus vaccine. Vet. Immunol. Immunopath. 63:57-67.

Table 1. Serological status to bovine herpesvirus type 1 (BoHV-1) and approximate stage of pregnancy of dams vaccinated (or not) with the recombinant gE-negative vaccine

Group	Serological at day 0 status	1st trimester	2nd trimester	3rd trimester
Vaccinated	Seronegative	4[a]	5	5
	Seropositive	3	2	3
Non-vaccinated	Seronegative	1	3	3
	Seropositive	2	2	4

[a] Number of pregnant dams in that stage of gestation.

Fig.1. Neutralizing antibody titres (geometric mean) in dams vaccinated (or not) with the bovine herpesvirus type 1 (BoHV-1) gE-negative vaccine. Black losenges: vaccinated, previously seropositive animals; White triangles: non-vaccinated, previously seropositive animals; black squares: vaccinated, previously seronegative animals; blank circles: non-vaccinated, seronegative animals.



Capítulo 3

US9 OF BOVINE HERPESVIRUS TYPE 1 (BoHV-1) DOES NOT PLAY A MAJOR ROLE DURING ACUTE INFECTIONS OF RABBITS

A.D. Silva[1,2], P.A. Esteves[2,3], F.R. Spilki [4], A.P. Oliveira [1], A.C Franco[5]; P.M. Roehe[1,2,5]

[1] Instituto de Pesquisas Veterinárias Desidério Finamor (IPVDF), Estrada do Conde 6000, CEP 92990-000, Eldorado do Sul, RS, Brazil. [2] Programa de Pós-Graduação em Ciências Veterinárias (PPGCV), Universidade Federal do Rio Grande do Sul (UFRGS), Av. Bento Gonçalves 9090, CEP 91540-000, Porto Alegre, RS, Brazil. [3] Empresa Brasileira de Pesquisa Agropecuária (Embrapa Aves e Suínos), BR 153, Km 110, Vila Tamanduá, CEP 89700-000, Concórdia, SC, Brazil. [4] Instituto de Ciências da Saúde, Centro Universitário Feevale, RS-239, 2755, CEP 93352-000, Novo Hamburgo, RS, Brazil. [5] Instituto de Ciências Básicas da Saúde, UFRGS, Rua Sarmento Leite, 500, CEP: 90050-170, Porto Alegre, RS, Brazil.

*Correspondence to: Instituto de Ciências Básicas da Saúde, UFRGS, Rua Sarmento Leite, 500, CEP: 90050-170, Porto Alegre, RS, Brazil. FONE/ FAX: + 55 51 33084111. E-mail: anafranco.ufrgs@gmail.com

Running title: Role of BoHV-1 US9 in acute infections

Key words: Bovine herpesvirus type 1, US9 negative, experimental infection, viral pathogenesis

Enviado para pulicação na Pesquisa Veterinária Brasileira

ABSTRACT

Bovine herpesvirus type 1 (BoHV-1), the etiologic agent of bovine rhinotracheitis and genital infections, expresses a number of proteins which are not essential for replication but are frequently associated to viral pathogenesis. To determine the role of the non essential membrane protein of BoHV-1 US9 in viral pathogenesis during acute infections, a BoHV-1 US9 negative (BoHV-1 US9$^-$) was constructed and evaluated in comparison to the wild type strain (BoHV-1 Lam). The experiment was performed in four groups of rabbits, which were inoculated with either the BoHV-1 US9$^-$ or wild type BoHV-1 through the intranasal (IN) or the intraconjunctival (IC) route. All the rabbits infected IN with the wild type or with the US9$^-$ virus displayed signs of BoHV-1-associated disease. Infectious virus was detected in nasal secretions and in tissues to similar titres in rabbits inoculated with either the US9$^-$ or wild type viruses. In addition, similar histological alterations were detected in both groups. All rabbits infected IC either with the US9$^-$ or wild type virus developed intense conjunctivitis with lymphocytic infiltrations in the corneas between days 3 to 10 post infection (pi). Moreover, both viruses were consistently isolated from ocular swabs and tissues from days 1 to 10 pi. In view of the results obtained here, it is concluded that the US9 protein does not play a significant role in BoHV-1 virulence *in vivo* during the acute infection.

INTRODUCTION

Bovine herpesvirus type 1 (BoHV-1) is an important pathogen of cattle, with world wide distribution. The virus has been implicated in a number of diseases of cattle and economic losses (1). Like other herpesviruses, BoHV-1 establishes a lifelong latent infection in the sensory ganglia of animals that survive acute infection (1).

BoHV-1 is a member of the family *Herpesviridae*, subfamily *Alphaherpesvirinae*. It is an enveloped virus, with a linear DNA which encodes approximately 70 proteins (1). Some of these proteins are not essential for viral replication, but are important in the virus-host interactions and influence the outcome of the disease. US9 is a type II tail-anchored membrane protein which is mainly localized in the viral tegument (68 amino acids) and viral envelope (26 amino acids). It is a non essential protein of human herpesvirus type 1 (HHV-1), swine herpesvirus type I (SuHV-1), bovine herpesvirus type 5 (BoHV-5) and BoHV-1 (2, 3). Previous studies demonstrated that the US9 of BoHV-1 activates the apoptotic process and facilitates virus release from the BoHV-1 infected cells (4). Following intranasal infection of calves with a BoHV-1 US9$^-$ it was shown that US9 is essential for reactivation of the virus in the trigeminal ganglia (TG) and anterograde axonal transport from TG to nose and eye (5). The role in axonal transport mediated by US9 was also demonstrated in *in vivo* infections of a BoHV-5 US9$^-$ virus (3). In *in vitro* experiments it was shown that US9 is implicated in the transport of viral glycoproteins over axons (6).

Many of these *in vivo* experiments were performed using rabbits, which have been frequently used for studies on the pathogenesis of BoHV-1 infections (7, 8, 9). In these studies it has been shown that different routes of inoculation can be used to reproduce the disease, however, the severity of the clinical signs may vary according to the route of infection (7, 8, 9). Based on these data, and to determine the role of the BoHV-1 US9 protein in BoHV-1

virulence, during an acute infection, a BoHV-1 US9$^-$ was constructed. Both the recombinant and the wild type viruses were inoculated through the intraconjunctival and intranasal route, in order to investigate possible differences in viral pathogenesis according to the presence or absence of US9 and to the route of infection in rabbits.

MATERIALS AND METHODS

Cells and viruses

The parental Lam BoHV-1 was isolated from an animal with infectious bovine rhinotracheitis (IBR) in The Netherlands in 1972 (10). The recombinant Lam TM$^-$, which expresses a truncated glycoprotein E from which the transmembrane and cytoplasmic domain was removed (11), was used to construct the Lam US9$^-$ virus, as described below.

All viruses were propagated in MDBK, which were kept in Eagle's minimal essential medium (EMEM) supplemented with 5 to 10 % fetal calf serum, 2 mM glutamine and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin). When appropriate, one percent low melting point agarose (Sigma) was added to EMEM to obtain semi-solid medium.

Construction of the BoHV-1 US9$^-$

A schematic representation of the construction of the US9$^-$ virus is described in Figure 1. The US9 flanking regions were co-transfected with purified DNA of the Lam gE TM$^-$ virus. Following the co-transfection the viruses obtained by the recombination should show the TM$^+$/US9$^-$ phenotype. To identify these recombinants, the supernatants of transfection were seeded on monolayers of Madin Darby Bovine Kidney (MDBK) cells and the cells were submitted to an immunoperoxidase monolayer assay (IPMA) using monoclonal antibody 75,

which allows the differentiation of gE TM^- and gE TM^+ (or gE wild type) viruses (11). Few gE $TM^+/US9^-$ viral candidates were identified and after three rounds of viral purification under semi-solid medium we isolated six viruses. These viruses were multiplied and their purified DNA was extracted and submitted to a restriction endonuclease assay (REA) with *Eco*RI and *Bam*HI in order to perform a genomic characterization of the recombinants. To demonstrate the lack of US9 expression these viruses were also submitted to a second IPMA using an anti-US9 rabbit polyclonal sera as described bellow. Following the molecular and antigenic characterization one of the $US9^-$ viruses was selected to be used in the further experiments.

Immunoperoxidase monolayer assay (IPMA)

Cell monolayers prepared on 96 well plates were infected with the six $US9^-$ candidates in order to confirm the lack of expression of US9 in these viruses. After visualization of cytopathic effect (CPE) on MDBK cells, plates were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS; 8.5g NaCl, 1.55g Na_2HPO_4, 0.23g NaH_2PO_4, pH 7.2 per liter) and the IPMA was performed as described (12), using a rabbit anti-US9 polyclonal serum as the primary antibody.

Plaque size assays

Confluent MDBK monolayers prepared on 6 well plates were infected with 50 plaque forming units (p.f.u.) of either the wild type or the $US9^-$ viruses. After two hours of adsorption at 37 °C, the *inoculum* was removed and the cells were overlaid with semi-solid medium. Four days post inoculation (pi) the cells were covered with fixative solution (10 % formalin, 1 % crystal violet) for 4 hours. The agarose was removed and monolayers dried at 37 °C overnight. The diameters of 50 viral plaques were measured.

Design of the animal experiments

Rabbits of a mixed breed were kept in isolation cages supplied with food and water *ad libitum*. For infection procedures, rabbits were previously anaesthetized with 2 mg of tiletamine/zolazepam (Zoletil, Virbac).

To examine the virulence of the recombinant BoHV-1 US9$^-$ and compare it with the BoHV-1 wild type, four weeks old rabbits were inoculated with these viruses. The BoHV-1 wild type and the BoHV-1 US9$^-$ virus were administered to rabbits by inoculation of 0.2 mL in each conjunctival sac or 0.5 mL in each paranasal sinus. The rabbits were divided in four inoculated groups (A, B, C, D) with five rabbits each (in all groups, one animal was kept as non inoculated negative control). In group A and B, rabbits were inoculated with $10^{7.3}$ 50% cell culture infective doses/ml ($CCID_{50}$/mL) of the recombinant BoHV-1 US9$^-$ in each paranasal sinus or each conjunctival sac respectively. In group C and D, rabbits were inoculated with $10^{7.3}$ $CCID_{50}$/mL of BoHV-1 wild type in each paranasal sinus or each conjunctival sac respectively. On day 10 pi, all animals were killed and samples collected for further analysis and processing.

All procedures of animal care, handling and experimentation were performed under veterinary supervision and according to recommendations of the Brazilian Committee on Animal Experimentation (COBEA; law no. 6.638 of May 8, 1979).

Clinical evaluation

The rabbits were observed three times a day in search for clinical alterations throughout the experiments. Clinical examinations included one daily measurement of weigh and rectal

temperature, detection of sneezing, coughing, nasal and/or ocular secretions and signs of depression (reduced appetite, apathy).

Virus excretion

Nasal and ocular swabs were collected daily from days 0 to 10 pi. The swabs were immersed in 1 mL of sterile EMEM supplemented with 200 U/mL penicillin, 200 µg/mL streptomycin and 5 µg/mL amphotericin B. Samples were stored at -70 ºC until processing. Infectious virus titres were determined on MDBK cells by end-point dilution.

Virus isolation

Fragments of brain, conjunctive, lung, kidney, spleen, adrenal glands, liver and intestine were submitted to virus isolation. The tissues were homogenized with sterile sand to 10 % with EMEM supplemented with antibiotics and clarified at 2500 x g for 20 minutes at 4 ºC. Two hundred micro liters of each supernatant were used to inoculate monolayers of MDBK cells prepared on 24 well plates. Infected cultures were checked for the presence of virus for up to 7 days after inoculation. All negative cultures were frozen at -70 °C, thawed and passaged once more on fresh MDBK monolayers. When viral CPE was detected, tenfold dilutions of the original tissue suspensions were titrated on MDBK cells.

Virus Neutralization

Serum samples were collected at days 0, 7 and 10 pi. Neutralizing antibodies in inactivated serum samples were detected in a varying serum-constant virus-neutralization (VN)

assay on MDBK monolayers, with twofold dilutions of serum against 100 $CCID_{50}$ of Lam strain of BoHV-1. Antibody titres were expressed as the reciprocal of the highest serum dilution that prevented the induction of CPE after 5 days of incubation (13).

Histopathology

Tissues for histological examination were fixed in 10 % buffered formalin, embedded in paraffin, sectioned at 6 μm and stained with haematoxylin-eosin (H&E) following routine protocols (14).

Statistical analysis

Statistical analyses were carried out using ANOVA by comparing the mean results for each group, taken daily. The calculation was made using Data Supplemental Analysis for Excel (Microsoft Office XP, Microsoft, USA). Statistical differences were analysed taking the $p<0.05$ as the criterion.

RESULTS

Construction of the BoHV-1 US9$^-$

REA was performed with purified DNA extracted from the BoHV-1 wild type and six US9$^-$ candidates using *Eco*RI and *Bam*HI. The additional DNA fragments obtained after REA from the candidates are compatible with the insertion of recognition sites for both of them in the deletion fragment of US9 (data not shown). No other major changes were observed in the viral genome of the six US9$^-$ candidates.

Immunoperoxidase monolayer assay (IPMA)

The results showed the lack of specific staining of the US9$^-$ candidates, while the parental Lam strain was strongly positive (Figure 2). In addition, all six US9$^-$ candidates and the wild type virus showed a positive reaction when the IPMA was performed with the anti-gE MAb 75 (data not shown).

Plaque size assays

Plaque sizes assays of both the BoHV-1 wild type and the US9$^-$ viruses were not significantly different ($p= 0.494$). The mean plaque size obtained after four of infection with the recombinant was 0.18 mm and with the BoHV-1 wild type was 0.19 mm (data not shown).

Acute infection after intranasal infection

All rabbits inoculated with either the BoHV-1 US9$^-$ or the wild type virus showed apathy, loss of appetite, rhinitis and nasal discharge between days 2 to 8 pi. Body temperatures of rabbits infected with either the recombinant or the wild type viruses increased between days 1 and 10 pi (data not shown). The highest mean body temperature of rabbits inoculated with the BoHV-1 US9$^-$ was 38.7 °C (10 days pi) while among the animals inoculated with wild type virus it was 39.0 °C (10 days pi).

Infectious virus was detected in nasal secretions at titres reaching $10^{1.55}$ to $10^{2.05}$ $CCID_{50}$/mL (BoHV-1 US9$^-$ infected animals) between days 1 and 10 pi (Table 1). In BoHV-1 wild type infected animals virus shedding was detected from day 1 to 10 pi reaching viral titres between $10^{1.55}$ and $10^{3.05}$ $CCID_{50}$/mL.

Tissues (brains, lungs, kidneys, spleen, adrenal glands and liver) of the rabbits were submitted to virus isolation. BoHV-1 US9^{-} was recovered from the infected animals from different tissues as lungs, adrenal glands, spleen and liver (data not shown). In the group inoculated with wild type BoHV-1, infectious virus was found in the kidneys, spleen, adrenal glands, liver, and lungs. Histological alterations in both inoculated groups were observed. Adrenal glands revealed with multiple foci of co-agulative necrosis in the *zona fasciculata* and *zona reticularis*, the liver showed multiple focal to diffuse areas of cogulative necrosis and mild pneumonia was detected in the lungs. Histological lesions were not observed in other tissues. Infectious virus was detected in lungs, spleen, adrenal glands and liver of animals infected with either the recombinant or the wild type virus (Table 3 3a). The highest virus titre was found in one animal in the wild type virus group, in the lungs (>$10^{2.1}$ $CCID_{50}$/mL). Infectious virus was also detected in the kidneys of wild type infected rabbits. The brains of animals in both groups were negative in virus isolation.

Neutralizing antibodies were not detected during the experiment in any of the infected animals.

Acute infection after intraconjunctival inoculation

Animals from both groups developed severe conjunctivitis, anorexia and depression. Ocular lesions were characterized by hyperaemia and oedema of the conjunctiva, injected scleral vessels and muco-purulent discharge. The rabbits exhibited photophobia and blepharospasm. Conjunctivitis persisted for 3 to 10 days. In the first day pi, the rectal temperatures of the animals of both groups started to increase. Highest temperatures were recorded on day 7 pi in both groups (data not shown).

Infectious virus was detected in ocular secretions reaching $10^{1.55}$ to $10^{3.05}$ $CCID_{50}$/mL in rabbits inoculated with both the BoHV-1 US9$^-$ and wild type BoHV-1, between 1 to 10 pi (Table 2).

Histological examination of the tissues of both groups revealed conjunctivitis with infiltration of lymphocytes. Ulceration of the conjunctival epilthelium was evident. Both viruses induced indistinguishable alterations from lesions observed in other tissues (lungs, adrenal glands, liver) of rabbits inoculated intranasally. Infectious virus was found in all the tissues examined, except for the brains of both groups and the kidneys of animals infected with the recombinant virus (Table 3b). The highest virus titre was found in one animal of wild type virus infected group, in the lungs (>$10^{2.0}$ $CCID_{50}$/mL).

Neutralizing antibodies were not detected during the experiment in any of the infected animals.

The uninfected control rabbits remained clinically normal and showed no macro- or microscopic lesions during necropsy or histopathological analysis. They also did not develop neutralizing antibodies against BoHV-1.

DISCUSSION

To determine the role of US9 in acute infections of BoHV-1, a deletion in the US9 gene was introduced in the viral genome and its biological properties were evaluated and compared to wild type virus when they were inoculated through the conjunctiva or the paranasal sinuses of rabbits.

The BoHV-1 US9$^-$ virus did not show additional major genomic changes, as it could be demonstrated by REA. The recombinant virus was able to multiply *in vitro* to similar titres as the BoHV-1 wild type, and its mean plaque size on MDBK monolayers was also comparable to the plaques formed by the wild type virus. These data indicate that the deletion of the US9 gene did not interfere with the virus replication and the cell-to-cell spread *in vitro*, as was demonstrated with previously described US9 recombinants of SuHV-1, BoHV-1 and 5 (2, 3, 5). These results were not expected because it has been demonstrated that the US9 of BoHV-1 activates the apoptotic process and facilitates virus release from the BoHV-1 infected cells (4)

The recombinant and the wild type virus were inoculated in rabbits. Throughout the experiment there was no clear difference in the clinical signs presented by the groups infected IN or IC with the BoHV-1 US9$^-$ or wild type viruses. Animals infected IN showed clinical respiratory signs like coughing, sneezing and severe nasal secretion. Macro- and microscopic lesions were comparable between the groups. Both the recombinant and the wild type virus were able to replicate at the site of inoculation, as detected by virus isolation and titration. In addition, both viruses were equally able to spread from the site of inoculation to other organs, like lungs and liver. Rabbits inoculated with both viruses through the IC route showed severe conjunctivitis and eliminated nasal and ocular secretions. Both viruses were also able to spread to other organs and were excreted in ocular secretions to similar titres, indicating that they replicated at the site of inoculation with a similar efficiency. These results indicate that the lack of US9 did not interfere with the virus replication and dissemination *in vivo* in acute infections after IN or IC inoculation.

Recently it was demonstrated that the association of US9 with lipid rafts is essential for efficient targeting of structural viral proteins to axons and, as a consequence, for directional spread of SuHV-1 in neurons (15). Consequently, a US9 negative SuHV-1 is defective in anterograde

transmission *in vivo* (3, 6), which is essential for reactivation of latent viruses from sensory ganglia to the primary site of infection. In fact, although the exact function of US9 of BoHV-1 is not known, it was shown that US9 of BoHV-1 is also essential for virus reactivation of latently infected bovines (5). However, as demonstrated here, it is not important to the pathogenesis of acute infections of BoHV-1. This, however, is not obvious because it has been established that the US9 of BoHV-1 activates apoptotic processes and facilitates virus release from the BoHV-1 infected cells (4) and might have some effect also during the acute phase.

In conclusion, in view of the results obtained here, the US9 protein does not play a significant role in BoHV-1 virulence in acute infections either when the infection occurs from the intraconjunctival or intranasal sites, since its removal does not affect significantly the outcome of the disease in comparison to the inoculation of BoHV-1 wild type.

Acknowledgements

A. D. Silva and P. A. Esteves were students from the Programa de Pós-Graduação em Ciências Veterinárias da Faculdade de Veterinária (PPGCV/UFRGS/Brazil) and grantees of CAPES. P.M. Roehe is a CNPq research fellow. This study was supported by CNPq, PRONEX, FAPERGS and CAPES.

REFERENCES

1. Jones C, Chowdhury S. A review of the biology of bovine herpesvirus type 1 (BHV-1), its role as a cofactor in the bovine respiratory disease complex and development of improved vaccines. *Anim Health Res Rev* 2007; 8(2):187-205.

2. Brideau AD, Eldridge MG, Enquist LW. Directional transneuronal infection by pseudorabies virus is dependent on an acidic internalization motif in the Us9 cytoplasmic tail. *J Virol* 2000; 74: 4549-4561.

3. Chowdhury SI., Onderci M, Bhattacharjee PS, Al-Murabarak A, Weiss ML, Zhou Y. Bovine herpesvirus 5 (BHV-5) Us9 is essential for BHV-5 neuropathogenesis. *J Virol* 2002; 76: 3839-51.

4. Nakamichi K, Matsumoto Y, Otsuka H. Bovine herpesvirus 1 U(S) ORF8 protein induces apoptosis in infected cells and facilitates virus egress. *Virology* 2002; 304:24-32.

5. Butchi NB, Jones C, Perez S, Doster A, Chowdhury SI. Envelope protein Us9 is required for the anterograde transport of bovine herpesvirus type 1 from trigeminal ganglia to nose and eye upon reactivation. *J Neurovirol* 2007;13(4):384-8.

6. Tomishima MJ, Enquist LW. A conserved alpha-herpesvirus protein necessary for axonal localization of viral membrane proteins. *J Cell Biol* 2001;154(4):741-52.

7. Kelly DF. Experimental infection of rabbits with the virus of infectious bovine rhinotracheitis. *Br J Exp Pathol* 1977; 58:168-76.

8. Lupton HW, Barnes HJ, Reed DE. Evaluation of the rabbit as a laboratory model for infectious bovine rhinotracheitis virus infection. *Cornell Veterinary* 1979; 70: 77-95.

9. Brown GA, Field HJ. A reliable method for establishing viral infection in the rabbit by intranasal inoculation. *J Virol Methods* 1990; 27:341-5.

10. Kaashoek MJ, Rijsewijk FA, Oirschot JT. Persistence of antibodies against bovine herpesvirus 1 and virus reactivation two to three years after infection. *Vet Microbiol* 1996; 53: 103-10.

11. Tyborowska J, Bieńkowska-Szewczyk K, Rychłowski M, Van Oirschot JT, Rijsewijk FA. The extracellular part of glycoprotein E of bovine herpesvirus 1 is sufficient for complex formation with glycoprotein I but not for cell-to-cell spread. *Arch Virol* 2000;145:333-51.

12. Kramps JA, Magdalena J, Quak J, Weerdmeester K, Kaashoek MJ, Maris-Veldhuis MA et al. A simple, specific, and highly sensitive blocking enzyme-linked immunosorbent assay for detection of antibodies to bovine herpesvirus 1. *J Clin Microbiol* 1994; 32: 2175-81.

13. House, JA, Baker JA. Bovine herpesvirus IBR-IPV. The antibody virus neutralization reaction. *Cornell Vet* 1971; 61: 320-35.

14. Prophet, C.M. Patient discharge referral: interdisciplinary collaboration. Proc Annu Symp Comput Appl Med Care, 1992; 332-336.

15. Lyman MG, Curanovic D, Enquist LW. Targeting of pseudorabies virus structural proteins to axons requires association of the viral Us9 protein with lipid rafts. *PLoS Pathog* 2008;4(5):e1000065

Table and figure captions

Tables

Table 1. Viral shedding of nasal secretions from the rabbits inoculated by the intranasal route. Titres are expressed as $\log_{10}$ of the 50 % cell culture infective dose ($CCID_{50}$) per 50µL.

Table 2. Viral shedding in the ocular secretions of the rabbits inoculated through the intraconjunctival route. Titres are expressed as $\log_{10}$ of the 50 % cell culture infective dose ($CCID_{50}$) per 50µL.

Table 3, Virus isolation from the tissues of four weeks old rabbits inoculated a) intranasally and b) intraconjunctivally with the recombinant BoHV-1 US9$^-$ or with wild type BoHV-1 (strain Lam).

Figures

Figure 1 – Schematic representation of the Lam US9$^-$ construction.

A) Represents the complete genome of BoHV-1. B) Flanking regions of US9. C) The 5´US9 region was obtained by amplification using primers P1 and P2 and 3´US9 region was obtained by digestion of p105, a plasmid with a region that encompasses the US9, with *Bsa* I and *Pst* I. D) Both flanking regions were cloned in pUC18. The enzyme used to liberate the deletion fragment is indicated (*Pst*I). This fragment was used in a co-transfection experiment with

purified DNA from gE TM$^-$ to obtain the Lam US9$^-$. Recombinant virus was detected using an immunoperoxidase monolayer assay (Fig. 2) and a restriction endonuclease assay (not shown).

Figure 2 Immunoperoxidase monolayer assay using a rabbit polyclonal anti-US9 serum on a monolayer of MDBK cells infected with wild type BoHV-1 (a) or with US9$^-$ BoHV-1 (b). Magnification: 100 X.

Tables

Table 1

Viral shedding (dpi)												
Groups		Rabbit nº	0	1	2	3	4	5	6	7	8	9	10
BoHV-1 US9^{-}		1	-	++	++	+++	++	+++	++	+++	+++	++	++
		2	-	++	++	++	++	+++	++	++	++	+++	++
		3	-	++	++	++	++	++	++	++	++	++	++
		4	-	++	++	+++	++	++	++	++	++	++	++
BoHV-1 wild type		1	-	+++	+++	++	+++	+++	+++	+++	++	++	++
		2	-	+++	++	+++	++	+++	+++	+++	++	+++	++
		3	-	++	++	++	++	++	++	++	++	+++	++
		4	-	++	++	++	+++	++	++	++	++	++	++

+: $10^{0.5\text{-}1}$ $CCID_{50}$/50µL; ++: $10^{1.25\text{-}2.0}$ $CCID_{50}$/50µL; +++: $\geq 10^{2.25}$ $CCID_{50}$/50µL

Table 2

Viral shedding												
		Days post inoculation										
Groups	Rabbit nº	0	1	2	3	4	5	6	7	8	9	10
Recombinant US9$^-$	1	-	++	++	++	+++	++	+++	++	++	++	++
	2	-	++	++	++	+++	++	+++	++	++	++	++
	3	-	++	++	++	+++	++	++	++	++	++	++
	4	-	++	++	++	+++	+++	+++	+++	+++	+++	++
BoHV-1 wild type	1	-	++	++	++	+++	++	+++	+++	+++	+++	+++
	2	-	++	++	++	++	++	++	++	++	++	++
	3	-	++	++	++	++	++	++	++	++	++	++
	4	-	++	++	++	++	++	++	++	++	++	++

+: $10^{0.5\text{-}1}$ $CCID_{50}$/50µL; ++: $10^{1.25\text{-}2.0}$ $CCID_{50}$/µL; +++: $\geq 10^{2.25}$ $CCID_{50}$/µL

Table 3

a)

	Rabbit no.	Tissues					
		brain	lung	kidney	spleen	adrenal glands	liver
Recombinant US9$^-$	1	-	++	-	++	+++	+
	2	-	++	-	+	++	+++
	3	-	+	-	++	++	++
	4	-	+	-	+++	+	+
BoHV-1 wild type	1	-	+++	+++	+	+	+
	2	-	++++	+++	++	++	++
	3	-	++	++	+	+++	+++
	4	-	+	++	+++	++	++

-: no virus detected; +: $10^{0.5}$-$10^{1.0}$ $CCID_{50}$/mL; ++: $10^{1.1}$-$10^{1.5}$ $CCID_{50}$/mL; +++: $10^{1.6}$-$10^{2.0}$ $CCID_{50}$/mL; ++++: > $10^{2.1}$ $CCID_{50}$/mL.

b)

	Rabbit no.	Tissues					
		brain	lung	kidney	spleen	adrenal glands	liver
Recombinant BoHV-1 Us9-	1	-	++	-	+++	+++	+
	2	-	++	-	+	++	+++
	3	-	+	-	++	++	++
	4	-	+	-	+++	+++	++
BoHV-1 wild type	1	-	+++	+++	++	+	++
	2	-	++++	+++	++	++	++
	3	-	++	++	+++	+++	+++
	4	-	++	++	+++	++	++

-: no virus detected; +: $10^{0.5}$-$10^{1.0}$ $CCID_{50}$/mL; ++: $10^{1.0}$-$10^{1.5}$ $CCID_{50}$/mL; +++: $10^{1.5}$-$10^{2.0}$ $CCID_{50}$/mL; ++++: > $10^{2.0}$ $CCID_{50}$/mL.

Figures

Figure 1

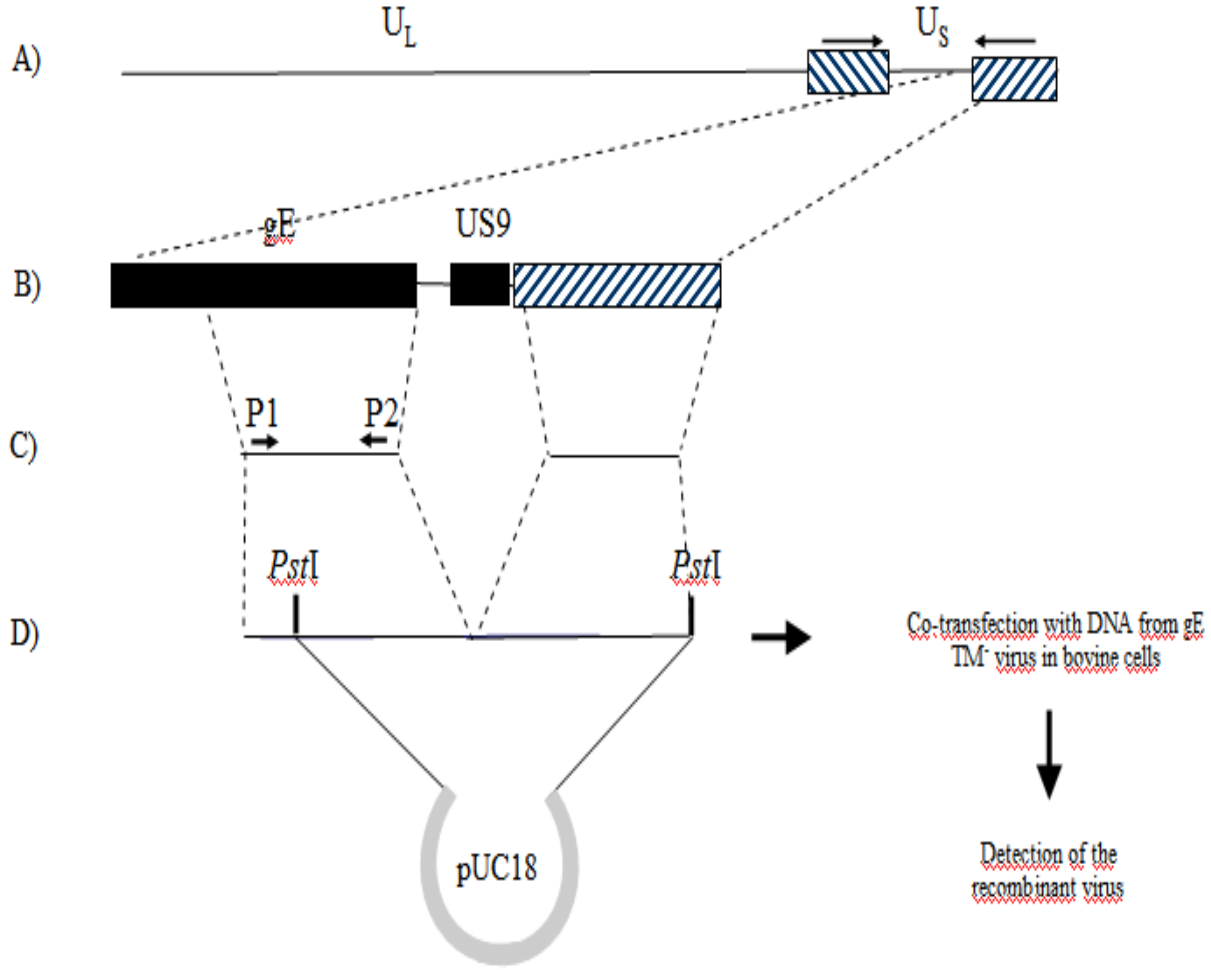
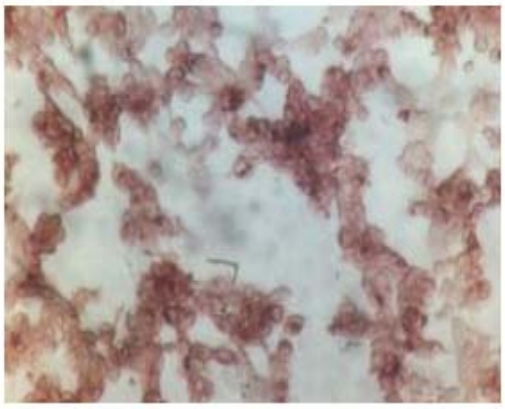
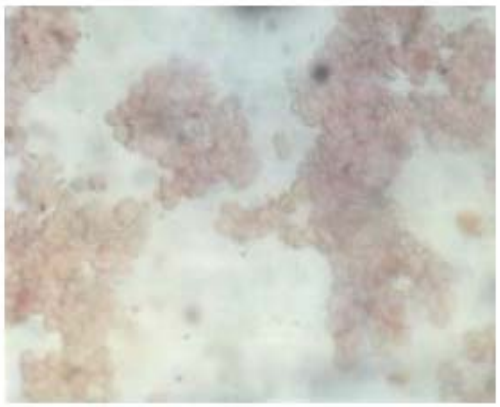


Figure 2

A)



B)



DISCUSSÃO E CONCLUSÕES GERAIS

3- DISCUSSÃO E CONCLUSÕES GERAIS

Vacinas no controle de infecções causadas por BoHV-1

Vários países mantém programas de controle de infecções pelo BoHV-1 com base na vacinação de rebanhos de bovinos. Em países, como Dinamarca, Suíça e Aústria onde a prevalência da infecção é baixa, a erradicação do BoHV-1 foi conseguida devido à eliminação de todos animais que apresentavam anticorpos para BoHV-1. Um país ou região onde exista o estado sanitário livre do agente infeccioso em questão, tem a vantagem de não possuir nenhuma restrição no caso de exportação de animais, carnes e venda de sêmem. Contudo, em muitos países, como é o caso do Brasil, onde a prevalência da infecção causada pelo BoHV-1 é alta, a eliminação de animais positivos é economicamente inviável. No caso de um país com alta prevalência de BoHV-1, bovinos são muitas vezes vacinados. O uso de vacinas contra o BoHV-1 pode levar a um melhor "status" sanitário do rebanho, pois pode reduzir a quantidade de vírus necessária para provocar a infecção em animais, diminuir o tempo e os níveis de excreção viral nos animais infectados e, com isso, diminuir a circulação de vírus em uma população (KAASHOEK, 1995). Contudo, o uso de vacinas convencionais induz uma resposta imune indistinguível da resposta imune produzida após uma infecção. Assim, o uso de vacinas convencionais contra BoHV-1 levou ao aumento do número de animais sorologicamente positivos nos rebanhos e ao interesse de desenvolver vacinas diferenciais, para que animais infectados pudessem ser facilmente detectados e eliminados do rebanho (VAN OIRSCHOT, 1999a). As vacinas diferenciais ou vacinas DIVA são vacinas que induzem uma resposta sorológica que pode ser diferenciada da resposta sorológica após uma infecção com vírus de campo, devido à falta de uma ou mais proteínas antigênicas que estão presentes no vírus selvagem. Testes sorológicos podem ser desenvolvidos para detectar os anticorpos induzidos após uma infecção com vírus de campo, mas que não são sintetizados após uma vacinação. Assim, as vacinas diferenciais podem ser um ferramenta útil para o controle e erradição do BoHV-1 em países onde a prevalência da infecção é alta.

Segurança e Imunogenicidade de uma vacina diferencial BoHV-1 gE- inativada

A primeira etapa do presente trabalho foi desenvolvida visando aprofundar os conhecimentos sobre aspectos de proteção de uma vacina recombinante inativada BoHV-1 gE-

em infecções causadas pelo BoHV-1. Em regiões onde existem perdas econômicas graves ou onde a prevalência da infecção é alta a vacinação contra BoHV-1 é preconizada. Esta prática visa minimizar ou impedir a severidade das manifestações clínicas induzidas pela infecção por BoHV-1 (VAN OIRSCHT et al., 1999a). No estudo aqui descrito, foi avaliada a eficácia de uma vacina recombinante inativada, construída a partir da deleção do gene da glicoproteína E de uma amostra brasileira de BoHV-1 (265 gE-; FRANCO et al., 2002). Durante o período de vacinação, os bovinos não apresentaram nenhum sinal clínico ou efeito adverso. Após o desafio, 2/5 bovinos vacinados, apresentaram brandos sinais respiratórios como secreção nasal, espirros e tosse entre os dias 4 e 10 pc, enquanto, bovinos não vacinados apresentaram graves sinais clínicos de doença como apatia, anorexia, rinite, descarga nasal e ocular, formação de vesículas e erosões na mucosa nasal, conjuntivite, espirros e tosse espontânea. Após a tentativa de reativação da infecção pela administração de corticosteróide, brandos sinais clínicos foram evidenciados novamente nos animais vacinados quando comparado com os animais não vacinados. Contudo, tais sinais clínicos foram menos intensos do que na infecção aguda. Portanto, evidências clínicas e os resultados de isolamento viral das secreções nasais e oculares demonstraram que a vacinação com a vacina recombinante inativada foi capaz de minimizar os sinais clínicos decorrentes da infecção causada por BoHV-1.

Trabalhos realizados anteriormente também procuraram avaliar a capacidade de proteção da vacinação com vacinas recombinantes atenuadas, inativadas e vacinas de sub-unidades em bovinos (BOSCH et al., 1996; BOSCH et al., 1998). Em tais estudos, após a imunização de bovinos com vacina recombinante inativada e atenuada, a vacina atenuada induziu uma melhor proteção após o desafio, sendo evidenciado pela ausência total de sinais clínicos e febre, e ainda redução da eliminação viral pelas secreções nasais quando comparado com a vacina inativada. No mesmo estudo, a vacina recombinante inativada induziu uma melhor proteção clínica em animais vacinados do que no grupo de animais vacinados com uma vacina de sub-unidade-gD (BOSH et al., 1996; BOSCH et al., 1998). Os resultados obtidos no presente trabalho (capítulo 1) evidenciaram adequada inativação e eficácia em proteger bovinos contra desafio com o vírus de campo semelhante à vacina viva atenuada.

Avaliação da segurança durante a gestação e disseminação horizontal de uma vacina recombinante para BoHV-1

A vacinação de fêmeas em gestação com vacinas recombinantes atenuadas têm sido ocasionalmente associada a aborto (PASTORET et al., 1980). Com a finalidade de avaliar a imunogenicidade e segurança induzida em fêmeas gestantes (Capítulo 2), por uma vacina recombinante previamente desenvolvida, a partir de uma amostra brasileira de BoHV-1, fêmeas em diferentes estágios de gestação (14 soronegativas, 8 seropositivas) foram vacinadas intramuscularmente. Nenhum aborto ou alteração fetal foi noticiado após a vacinação. Soroconversão foi observada em animais previamente soronegativas. O uso deste tipo de vacinas durante a gestação pode resultar em problemas tais como morte embrionária, aborto e fetos natimortos (LOMBA et al., 1976; WHETSTONE et al. 1986). A vacina recombinante gE- utilizada neste experimentou provou ser segura para aplicação em vacas gestantes e induziu uma boa resposta sorológica nos animais.

Na segunda etapa deste trabalho, o potencial de disseminação viral a campo da vacina recombinante gE- em bovinos foi avaliado. Novilhos foram inoculados intranasalmente com a vacina recombinante gE- e colocados em contato com outros animais na mesma área durante 180 dias. Brandos sinais clínicos e soroconversão foram observados nos animais previamente vacinados. Estes resultados demonstraram que o vírus vacinal não foi capaz de disseminar dos animais previamente vacinados para os animais em contato.

Apartir dos resultados obtidos aqui e em estudos previamente realizados (FRANCO et al., 2002; SILVA et al., 2006) podemos concluir que a vacina recombinante gE-, produzida a partir de uma amostra brasileira de BoHV-1, possui as condições necessárias para ser uma alternativa no controle das infecções causadas por BoHV-1.

Patogenia de um recombinante BoHV-1 US9 negativo

Estudos vêm sendo realizados no sentido de identificar a função da proteína Us9 em herpesvírus de suíno (SHV-1, BRIDEAU et al., 2000a; BRIDEAU et al., 2000b; METTENLEITER, 2003), herpesvírus bovino tipo 5 (BoHV-5; CHOWDHURY et al., 2002;

CHOWDHURY et al., 2006), herpes simples humano tipo 1 (HSV-1; BRANDIMARTI & ROIZMAN, 1997).

Com o objetivo de determinar a importância da proteína Us9 na disseminação viral do BoHV-1, foi realizada a deleção do gene que codifica esta proteína no BoHV-1 (FRANCO, 2001). No estudo aqui descrito (Capítulo 3), foram inoculados coelhos como modelo experimental, utilizando as rotas nasal e ocular. A deleção do gene que codifica a proteína Us9 no genoma viral do BoHV-1 não apresentou uma diferença significativa na patogenia dos animais quando comparado ao grupo de animais inoculados com o vírus selvagem. Os animais inoculados pela rota intranasal com o vírus recombinante BoHV-1 Us9- apresentaram sinais clínicos e excreção viral semelhantes ao grupo inoculado com o vírus parental. Animais inoculados pela rota ocular apresentaram intensa conjuntivite e título de excreção viral pelas secreções oculares semelhantes ao vírus parental.

Em estudos previamente realizados, utilizando a deleção da proteína Us9 em BoHV-5 e PRV, verificaram que o vírus recombinante replicou com similar eficiência na mucosa olfatória quando comparado com o vírus selvagem (BRIDEAU et al., 1999; BRIDEAU et al., 2000; CHOWDHURY et al., 2002; CHOWDHURY et al., 2006).

Em conclusão, este estudo demonstrou que a proteína Us9 não tem um papel importante na patogenicidade viral *in vivo,* quando comparado com o vírus parental em coelhos como modelo experimental. Estudos futuros deverão se concentrar na continuação da caracterização *in vivo* desta amostra recombinante Us9-. De particular interesse é a interação da proteína Us9 com o sistema nervoso de bovinos infectados, como já foi demonstrado em SHV-1 (BRIDEAU et al., 2000a).

4-REFERÊNCIAS BIBLIOGRÁFICAS

ACKERMANN, M. (2005). [Virus in sheep's skin]. Schweiz Archives Tierheilkd 147(4): 155-64.

ACKERMANN, M., MULLER, H. K., BRUCKNER, L. e KIHM, U. (1990). Eradication of infectious bovine rhinotracheitis in Switzerland: review and prospects. Veterinary Microbiology 23(1-4): 365-70.

BABIC, N., KLUPP, B. G., MAKOSCHEY, B., KARGER, A., FLAMAND, A. e METTENLEITER, T. C. (1996). Glycoprotein gH of pseudorabies virus is essential for penetration and propagation in cell culture and in the nervous system of mice. Journal of General Virology 77 (Pt 9): 2277-85.

BELKNAP, E. B., COLLINS, J. K., AYERS, V. K. e SCHULTHEISS, P. C. (1994). Experimental infection of neonatal calves with neurovirulent bovine herpesvirus type 1.3. Veterinary Pathology 31(3): 358-65.

BIELEFELDT OHMANN, H. e BABIUK, L. A. (1985). Viral-bacterial pneumonia in calves: effect of bovine herpesvirus-1 on immunologic functions. Journal of Infectious Diseases 151(5): 937-47.

BIELEFELDT OHMANN, H., BABIUK, L. A. e HARLAND, R. (1991). Cytokine synergy with viral cytopathic effects and bacterial products during the pathogenesis of respiratory tract infection. Clinical Immunology, Immunopathology and Infectious Diseases 60(2): 153-70.

BOSCH, J. C., DE JONG, M. C., FRANKEN, P., FRANKENA, K., HAGE, J. J., KAASHOEK, M. J., MARIS-VELDHUIS, M. A., NOORDHUIZEN, J. P., VAN DER POEL, W. H., VERHOEFF, J., WEERDMEESTER, K., ZIMMER, G. M. e VAN OIRSCHOT, J. T. (1998). An inactivated gE-negative marker vaccine and an experimental gD-subunit vaccine reduce the incidence of bovine herpesvirus 1 infections in the field. Vaccine 16(2-3): 265-71.

BOSCH, J. C., KAASHOEK, M. J., KROESE, A. H. e VAN OIRSCHOT, J. T. (1996). An attenuated bovine herpesvirus 1 marker vaccine induces a better protection than two inactivated marker vaccines. Veterinary Microbiology 52(3-4): 223-34.

BOSCH, J. C., KAASHOEK, M. J. e VAN OIRSCHOT, J. T. (1997). Inactivated bovine herpesvirus 1 marker vaccines are more efficacious in reducing virus excretion after reactivation than a live marker vaccine. Vaccine 15(14): 1512-7.

BRANDIMARTI, R. e ROIZMAN, B. (1997). US9, a stable lysine-less herpes simplex virus 1 protein, is ubiquitinated before packaging into virions and associates with proteasomes. Proccedings of the National Academy of Science of the United States of America 94: 13973-13978.

BRATANICH, A. C., SARDI, S. I., SMITSAART, E. N. e SCHUDEL, A. A. (1991). Comparative studies of BHV-1 variants by in vivo--in vitro tests. Zentralbl Veterinarmed B 38(1): 41-8.

BRIDEAU, A. D., CARD, J. P. e ENQUIST, L. W. (2000a). Role of pseudorabies virus Us9, a type II membrane protein, in infection of tissue culture cells and the rat nervous system. Journal of Virology 74(2): 834-45.

BRIDEAU, A. D.; ELDRIDGE, M. G.; ENQUIST, L. W. (2000b) Directional transneuronal infection by pseudorabies virus is dependent on an acidic internalization motif in the Us9 cytoplasmic tail. Journal of Virology 74(10): 4549-4561.

BROWN, G. A. e FIELD, H. J. (1990). A reliable method for establishing viral infection in the rabbit by intranasal inoculation. Journal of Virologycal Methods 27(3): 341-5.

CAMPOS, M., OHMANN, H. B., HUTCHINGS, D., RAPIN, N., BABIUK, L. A. e LAWMAN, M. J. (1989). Role of interferon-gamma in inducing cytotoxicity of peripheral

blood mononuclear leukocytes to bovine herpesvirus type 1 (BHV-1)-infected cells. Cellular Immunology 120(1): 259-69.

CARTER, J. J., WEINBERG, A. D., POLLARD, A., REEVES, R., MAGNUSON, J. A. e MAGNUSON, N. S. (1989). Inhibition of T-lymphocyte mitogenic responses and effects on cell functions by bovine herpesvirus 1. Journal of Virology 63(4): 1525-30.

CASCIO, K. E., BELKNAP, E. B., SCHULTHEISS, P. C., AMES, A. D. e COLLINS, J. K. (1999). Encephalitis induced by bovine herpesvirus 5 and protection by prior vaccination or infection with bovine herpesvirus 1. Journal of Veterinary Diagnostic Investigation 11(2): 134-9.

CASTRUCCI, G., FRIGERI, F., SALVATORI, D., FERRARI, M., SARDONINI, Q., CASSAI, E., LO, D. M., ROTOLA, A. e ANGELINI, R. (2002). Vaccination of calves against bovine herpesvirus-1: assessment of the protective value of eight vaccines. Comparative Immunology and Microbiology of Infectious Diseases 25(1): 29-41.

CHOWDHURY, S. I., LEE, B. J., MOSIER, D., SUR, J. H., OSORIO, F. A., KENNEDY, G. e WEISS, M. L. (1997). Neuropathology of bovine herpesvirus type 5 (BHV-5) meningo-encephalitis in a rabbit seizure model. Journal of Comparative Pathology 117(4): 295-310.

CHOWDHURY, S. I., ROSS, C. S., LEE, B. J., HALL, V. e CHU, H. J. (1999). Construction and characterization of a glycoprotein E gene-deleted bovine herpesvirus type 1 recombinant. American Journal of Veterinary Research 60(2): 227-32.

CHOWDHURY, S. I., LEE, B. J., OZKUL, A. e WEISS, M. L. (2000). Bovine herpesvirus 5 glycoprotein E is important for neuroinvasiveness and neurovirulence in the olfactory pathway of the rabbit. Journal of Virology 74(5): 2094-106.

CHOWDHURY, S. I., ONDERCI, M., BHATTACHARJEE, P. S., AL-MUBARAK, A., WEISS, M. L. e ZHOU, Y. (2002). Bovine herpesvirus 5 (BHV-5) Us9 is essential for BHV-5 neuropathogenesis. Journal of Virology 76(8): 3839-51.

CHOWDHURY, S. I., MAHMOOD, S., SIMON, J., AL-MUBARAK, A. e ZHOU, Y. (2006). The Us9 gene of bovine herpesvirus 1 (BHV-1) effectively complements a Us9-null strain of BHV-5 for anterograde transport, neurovirulence, and neuroinvasiveness in a rabbit model. Journal of Virology 80(9): 4396-405.

D'ARCE, R. C., ALMEIDA, R. S., SILVA, T. C., FRANCO, A. C., SPILKI, F., ROEHE, P. M. e ARNS, C. W. (2002). Restriction endonuclease and monoclonal antibody analysis of Brazilian isolates of bovine herpesviruses types 1 and 5. Veterinary Microbiology 88(4): 315-24.

DE JONG, M.C.M. e KIMMAN, T.G. (1994). Experimental quantification of vaccine- induced reduction in virus transmission. Vaccine, v. 12 (8), p. 761-766.

EDWARDS, S., NEWMAN, R. H. e WHITE, H. (1991). The virulence of British isolates of bovid herpesvirus 1 in relationship to viral genotype. British Veterinary Journal 147(3): 216-31.

ENGELS, M. e ACKERMANN, M. (1996). Pathogenesis of ruminant herpesvirus infections. Veterinary Microbiology 53(1-2): 3-15.

ENQUIST, L.W.; HUSAK, P.J.; BANFIELD, B.W.; SMITH, G.A. (1999). Infection and spread of alphaherpesviruses in the nervous system. Advances in Virus Research, v. 51, p. 237-347.

ENQUIST, L.W.; TOMISHIMA, M.J.; GROSS, S.; SMITH, G.A. (2002). Directional spread of an alphaherpesvirus in the nervous system. Veterinary Microbiology, v. 86, p. 5-16.

FLORES, E. F., OSORIO, F. A., ZANELLA, E. L., KIT, S. e KIT, M. (1993). Efficacy of a deletion mutant bovine herpesvirus-1 (BHV-1) vaccine that allows serologic differentiation of vaccinated from naturally infected animals. Journal of Veterinary Diagnostic Investigation 5(4): 534-40.

FRANCO A.C. (2001). Recombinantes de Herpesvirus bovino tipo 1 com deleções nos genes gE e Us9. Tese de doutorado – Porto Alegre: UFRGS,. 193pp

FRANCO, A.C., SPILKI, F.R., ESTEVES, P. A., LIMA, M., WEIBLEN, R., FLORES, E. F., RIJSEWIJK, F.A.M. e ROEHE, P. M. (2002). A Brazilian glycoprotein E-negative bovine herpesvirus type 1.2a (BHV-1.2a) mutant is attenuated for cattle and induces protection against wild-type virus challenge. Pesquisa Veterinária Brasileira 22(4): 135-140.

FUCHS, W., RZIHA, H. J., LUKACS, N., BRAUNSCHWEIGER, I., VISSER, N., LUTTICKEN, D., SCHREURS, C. S., THIEL, H. J. e METTENLEITER, T. C. (1990). Pseudorabies virus glycoprotein gI: in vitro and in vivo analysis of immunorelevant epitopes. Journal of General Virology 71 (Pt 5): 1141-51.

GALEOTA, J. A., FLORES, E. F., KIT, S., KIT, M. e OSORIO, F. A. (1997). A quantitative study of the efficacy of a deletion mutant bovine herpesvirus-1 differential vaccine in reducing the establishment of latency by wildtype virus. Vaccine 15(2): 123-8.

GAO, Y., LEARY, T. P., ESKRA, L. e SPLITTER, G. A. (1994). Truncated bovine herpesvirus-1 glycoprotein I (gpI) initiates a protective local immune response in its natural host. Vaccine 12(2): 145-52.

GIBBS, E.P.J. e RWEYEMAMU, M.M. (1977). Bovine herpesvirus. Part I. bovine herpesvirus 1. The Veterinary Bull 47: 317-343.

HAGE, J. J., VELLEMA, P., SCHUKKEN, Y. H., BARKEMA, H. W., RIJSEWIJK, F. A., VAN OIRSCHOT, J. T. e WENTINK, G. H. (1997). Sheep do not have a major role in bovine herpesvirus 1 transmission. Veterinary Microbiology 57(1): 41-54.

HANON, E., VANDERPLASSCHEN, A., LYAKU, S., KEIL, G., DENIS, M. e PASTORET, P. P. (1996). Inactivated bovine herpesvirus 1 induces apoptotic cell death of mitogen-stimulated bovine peripheral blood mononuclear cells. Journal of Virology 70(6): 4116-20.

HINKLEY, S., HILL, A. B. e SRIKUMARAN, S. (1998). Bovine herpesvirus-1 infection affects the peptide transport activity in bovine cells. Virus Research 53(1): 91-6.

JONES, C. (2003). Herpes simplex virus type 1 and bovine herpesvirus 1 latency. Clinical Microbiology Review 16(1): 79-95.

KAASHOEK, M. J., MOERMAN, A., MADIC, J., WEERDMEESTER, K., MARIS-VELDHUIS, M., RIJSEWIJK, F. A. e VAN OIRSCHOT, J. T. (1995). An inactivated vaccine based on a glycoprotein E-negative strain of bovine herpesvirus 1 induces protective immunity and allows serological differentiation. Vaccine 13(4): 342-6.

KAHRS, R. F. (1977). Infectious bovine rhinotracheitis: a review and update. Journal of American Veterinary Medical Association 171(10): 1055-64.

KIT, S., OTSUKA, H. e KIT, M. (1990). Gene-deleted IBRV marker vaccine. Veterinary Records 127(14): 363-4.

KIMMAN, T.; DE WIND, N.; OEI-LIE, N. (1992). Contribution of single genes within the unique short region of Aujeszky's disease virus (Suid Herpesvirus Type 1) to virulence, pathogenesis, and immunogenicity. Journal of General Virology, v. 73, p. 243-251.

LAI, C.L.; LAU, J.Y.; YEOH, E.K.; CHANG, W.K.; LIN, H.J. (1992). Significance of isolated anti-HBc seropositive by ELISA: implications and the role of radioimmunoassay. Journal of Medical Virology, v. 36, p. 180-183.

LEARY, T. P. e SPLITTER, G. A. (1990). Recombinant herpesviral proteins produced by cell-free translation provide a novel approach for the mapping of T lymphocyte epitopes. Journal of Immunology 145(2): 718-23.

LEMAIRE, M., WEYNANTS, V., GODFROID, J., SCHYNTS, F., MEYER, G., LETESSON, J. J. e THIRY, E. (2000). Effects of bovine herpesvirus type 1 infection in calves with maternal antibodies on immune response and virus latency. Journal of Clinical Microbiology 38(5): 1885-94.

LOMBA, F., BIENFET, V. e WELLEMANS, G. (1976). IBR virus and occurrence of metritis at parturition in the bovine Belgian Blue White Breed. British Veterinary Journal 132(2): 178-81.

LOVATO, L.T., WEIBLEN, R., TOBIAS, F.L. e MORAES, M.P. (1995). Herpesvírus bovino tipo 1 (HVB 1): Inquérito soro-epidemiológico no rebanho leiteiro do estado do Rio Grande do Sul. Ciência Rural 25: 425-430.

MADIN, S.H., YORK, C.J. e MCKERCHER, D.G. (1956). Isolation of the infectious bovine rhinotracheitis virus. Science 124: 721-722.

MARS, M. H., RIJSEWIJK, F. A., MARIS-VELDHUIS, M. A., HAGE, J. J. e VAN OIRSCHOT, J. T. (2000). Presence of bovine herpesvirus 1 gB-seropositive but gE-seronegative Dutch cattle with no apparent virus exposure. Veterinary Records 147(12): 328-31.

MÄKELÄ, P.H. (2000). Vaccines, coming of age after 200 year. FEMS Microbiology Reviews, v. 24, p. 9-20.

MAYFIELD, J. E., GOOD, P. J., VANOORT, H. J., CAMPBELL, A. R. e REED, D. E. (1983). Cloning and cleavage site mapping of DNA from bovine herpesvirus 1 (Cooper strain). Journal of Virology 47(1): 259-64.

METTENLEITER, T. C. (2002). Herpesvirus assembly and egress. Journal of Virology 76(4): 1537-1547.

METTENLEITER, T. C. (2003). Pathogenesis of neurotropic herpesviruses: role of viral glycoproteins in neuroinvasion and transneuronal spread Virus Research 92: 197-206.

METZLER, A. E., SCHUDEL, A. A. e ENGELS, M. (1986). Bovine herpesvirus 1: molecular and antigenic characteristics of variant viruses isolated from calves with neurological disease. Archives of Virology 87(3-4): 205-17.

MEYER, G., LEMAIRE, M., ROS, C., BELAK, K., GABRIEL, A., CASSART, D., COIGNOUL, F., BELAK, S. e THIRY, E. (2001). Comparative pathogenesis of acute and latent infections of calves with bovine herpesvirus types 1 and 5. Archives of Virology 146(4): 633-52.

MILLER, J. M., WHETSTONE, C. A. e VAN DER MAATEN, M. J. (1991). Abortifacient property of bovine herpesvirus type 1 isolates that represent three subtypes determined by restriction endonuclease analysis of viral DNA. American Journal of Veterinary Research 52(3): 458-61.

NATARAJ, C., EIDMANN, S., HARIHARAN, M. J., SUR, J. H., PERRY, G. A. e SRIKUMARAN, S. (1997). Bovine herpesvirus 1 downregulates the expression of bovine MHC class I molecules. Viral Immunology 10(1): 21-34.

PALMER, L. D., LEARY, T. P., WILSON, D. M. e SPLITTER, G. A. (1990). Bovine natural killer-like cell responses against cell lines expressing recombinant bovine herpesvirus type 1 glycoproteins. Journal of Immunology 145(3): 1009-14.

PASTORET, P. P., BABIUK, L. A., MISRA, V. e GRIEBEL, P. (1980). Reactivation of temperature-sensitive and non-temperature-sensitive infectious bovine rhinotracheitis vaccine virus with dexamethasone. Infectious Immunology 29(2): 483-8.

PASTORET, P. P. e THIRY, E. (1985). Diagnosis and prophylaxis of infectious bovine rhinotracheitis: the role of virus latency. Comparative Immunology, Microbiology and Infectious Diseases 8(1): 35-42.

PEREZ, S. E., BRETSCHNEIDER, G., LEUNDA, M. R., OSORIO, E. A., FLORES, E. F. e ODEON, A. C. (2002). Primary infection, latency, and reactivation of bovine herpesvirus type 5 in the bovine nervous system. Veterinary Pathology 39(4): 437-44.

PIDONE, C. L., GALOSI, C. M., ECHEVERRIA, M. G., NOSETTO, E. O. e ETCHEVERRIGARAY, M. E. (1999). Restriction endonuclease analysis of BHV-1 and BHV-5 strains isolated in Argentina. Zentralbl Veterinarmed B 46(7): 453-6.

QUINT, W.; GIELKENS,A.; VAN OIRSCHOTT, J.; BERNS, A.; CUYPERS, HT. Construction and characterization of deletion mutants of pseudorabies virus: a new generation of live vaccines. Journal of General Virology, Feb; 68 (Pt 2): 523-534. 1987

RAVAZZOLO, A.P., DAL PIZZOL, M. e MOOJEN, V. (1989). Evidência da presença de anticorpos para o vírus da rinotraqueíte infecciosa dos bovinos em alguns municípios do Estado do Rio Grande do Sul. Arquivos da Faculdade de Veterinária UFRGS 17: 95-98.

REBORDOSA, X., PINOL, J., PEREZ-PONS, J. A., LLOBERAS, J., NAVAL, J. e QUEROL, E. (1994). Mapping, cloning and sequencing of a glycoprotein-encoding gene from bovine herpesvirus type 1 homologous to the gE gene from HSV-1. Gene 149(2): 203-9.

REBORDOSA, X., PINOL, J., PEREZ-PONS, J. A., LLOBERAS, J., NAVAL, J., SERRA-HARTMANN, X., ESPUNA, E. e QUEROL, E. (1996). Glycoprotein E of bovine herpesvirus type 1 is involved in virus transmission by direct cell-to-cell spread. Virus Research 45(1): 59-68.

RIJSEWIJK, F. A., KAASHOEK, M. J., LANGEVELD, J. P., MELOEN, R., JUDEK, J., BIENKOWSKA-SZEWCZYK, K., MARIS-VELDHUIS, M. A. e VAN OIRSCHOT, J. T. (1999). Epitopes on glycoprotein C of bovine herpesvirus-1 (BHV-1) that allow differentiation between BHV-1.1 and BHV-1.2 strains. Journal of General Virology 80 (Pt 6): 1477-83.

ROCK, D. L., BEAM, S. L. e MAYFIELD, J. E. (1987). Mapping bovine herpesvirus type 1 latency-related RNA in trigeminal ganglia of latently infected rabbits. Journal Virology 61(12): 3827-31.

ROCK, D. L., HAGEMOSER, W. A., OSORIO, F. A. e REED, D. E. (1986). Detection of bovine herpesvirus type 1 RNA in trigeminal ganglia of latently infected rabbits by in situ hybridization. Journal of General Virology 67 (Pt 11): 2515-20.

ROIZMAN, B. e KNIPE, M.D. (2001). Herpes simplex virus and their replication. Philadelphia, Lippincott Williams and Wilkins publishers.

ROIZMAN, B. e PELLETT, P.E. (2001). The family Herpesviridae: A brief introduction. Philadelphia, Lippincott Williams and Wilkins.

SCHWYZER, M. e ACKERMANN, M. (1996). Molecular virology of ruminant herpesviruses. Veterinary Microbiology 53(1-2): 17-29.

SCHYNTS, F., BARANOWSKI, E., LEMAIRE, M. e THIRY, E. (1999). A specific PCR to differentiate between gE negative vaccine and wildtype bovine herpesvirus type 1 strains. Veterinary Microbiology 66(3): 187-95.

SILVA, A. D., SPILKI, F. R., FRANCO, A. C., ESTEVES, P. A., HUBNER, S. O., DRIEMEIER, D., OLIVEIRA, A. P., RIJSEWIJK, F. e ROEHE, P. M. (2006). Vaccination with a gE-negative bovine herpesvirus type 1 vaccine confers insufficient protection to a bovine herpesvirus type 5 challenge. Vaccine 24(16): 3313-20.

SILVA, A. M., WEIBLEN, R., IRIGOYEN, L. F., ROEHE, P. M., SUR, H. J., OSORIO, F. A. e FLORES, E. F. (1999). Experimental infection of sheep with bovine herpesvirus type-5 (BHV-5): acute and latent infection. Veterinary Microbiology 66(2): 89-99.

SOUZA, V.F., MELO, S.V., ESTEVES, P.A., SCHMIDT, C.S.R., GONÇALVES, D., SCHAEFER, R., SILVA, T.C., ALMEIDA, R.S., VICENTINI, F.K., FRANCO, A.C., OLIVEIRA, E.A.S., SPILKI, F.R., WEIBLEN, R., FLORES, E.F., LEMOS, R.A., ALFIERI, A.A., PITUCO, E.M. e ROEHE, P.M. (2002). Monoclonal antibody characterization of bovine herpesviruses types 1 (BHV-1) and 5 (BHV-5). Pesquisa Veterinária Brasileira 22: 13-18. .

SPILKI, F. R., SILVA, A. D., HUBNER, S., ESTEVES, P. A., FRANCO, A. C., DRIEMEIER, D. e ROEHE, P. M. (2004). Partial protection induced by a BHV-1 recombinant vaccine against challenge with BHV-5. Annual New York Academy of Science 1026: 247-50.

SPILKI, F.R., ESTEVES, P. A., LIMA, M., FRANCO, A. C., CHIMINAZZO, C., FLORES, E. F., WEIBLEN, R., DRIEMEIER, D. e ROEHE, P.M. (2004). Comparative pathogenicity of bovine herpesvirus 1 (BHV-1) subtypes 1 (BHV-1.1) and 2a (BHV-1.2a). Pesquisa Veterinária Brasileira 24(1): 43-49.

STEPHENSON, I.; NICHOLSON, K.G.; WOOD, J.M.; ZAMBON, M.C.; KATZ, J.M. (2004) Confronting the avian influenza threat: vaccine development for a potential pandemic. The Lancet Infectious Diseases, v. 4, p. 499-509.

STUDDERT, M. J. (1989). Bovine encephalitis herpesvirus. Veterinary Records 125(23): 584.

SUBBARAO, K.; KATZ, J.M. (2004). Influenza vaccines generated by reverse genetics. Current Topics in Microbiology and Immunology, v. 283, p. 313-342.

TIKOO, S. K., CAMPOS, M. e BABIUK, L. A. (1995). Bovine herpesvirus 1 (BHV-1): biology, pathogenesis, and control. Advanced Virus Research 45: 191-223.

TOMISHIMA, M.J. e ENQUIST, L. W. (2001). A conserved herpesvirus protein necessary for axonal localization of viral membrane proteins. The Journal of Cell Biology 154(4): 741-752.

TURIN, L., RUSSO, S. e POLI, G. (1999). BHV-1: new molecular approaches to control a common and widespread infection. Molecular Medical 5(5): 261-84.

VAN DRUNEN LITTEL-VAN DEN HURK, S., PARKER, M. D., MASSIE, B., VAN DEN HURK, J. V., HARLAND, R., BABIUK, L. A. e ZAMB, T. J. (1993). Protection of cattle from BHV-1 infection by immunization with recombinant glycoprotein gIV. Vaccine 11(1): 25-35.

VAN DRUNEN LITTEL-VAN DEN HURK, S., TIKOO, S. K., LIANG, X. e BABIUK, L. A. (1993). Bovine herpesvirus-1 vaccines. Immunology and Cellular Biology 71 (Pt 5): 405-20.

VAN ENGELENBURG, F. A., KAASHOEK, M. J., RIJSEWIJK, F. A., VAN DEN BURG, L., MOERMAN, A., GIELKENS, A. L. e VAN OIRSCHOT, J. T. (1994). A glycoprotein E deletion mutant of bovine herpesvirus 1 is avirulent in calves. Journal of General Virology 75 (Pt 9): 2311-8.

VAN OIRSCHOT, J. T. (1995). Bovine herpesvirus 1 in semen of bulls and the risk of transmission: a brief review. Veterinary Microbiology 17(1): 29-33.

VAN OIRSCHOT, J. T. (1999a). Diva vaccines that reduce virus transmission. Journal of Biotechnology 73(2-3): 195-205.

VAN OIRSCHOT, J. T., KAASHOEK, M. J., MARIS-VELDHUIS, M. A. e RIJSEWIJK, F. A. (1999). Strains of bovine herpesvirus 1 that do not express an epitope on glycoprotein E in cell culture still induce antibodies that can be detected in a gE-blocking ELISA. Veterinary Microbiology 65(2): 103-13.

VAN OIRSCHOT, J. T., KAASHOEK, M. J. e RIJSEWIJK, F. A. (1996). Advances in the development and evaluation of bovine herpesvirus 1 vaccines. Veterinary Microbiology 53(1-2): 43-54.

VAN OIRSCHOT, J. T., KAASHOEK, M. J., RIJSEWIJK, F. A. e STEGEMAN, J. A. (1996). The use of marker vaccines in eradication of herpesviruses. Journal of Biotechnology 44(1-3): 75-81.

VAN OIRSCHOT, J. T., STRAVER, P. J., VAN LIESHOUT, J. A., QUAK, J., WESTENBRINK, F. e VAN EXSEL, A. C. (1993). A subclinical infection of bulls with bovine herpesvirus type 1 at an artificial insemination centre. Veterinary Records 132(2): 32-5.

VANNIER, P.; HUTET,E.; BOURGUEIL, E.; CARIOLET, R. (1991). Level of virulent virus excreted by infected pigs previously vaccinated wit different glycoprotein deleted Aujeszky's disease vaccines. Veterinary Microbiology, v. 29, p. 213-223.

VIDOR, T., HALFEN, D.C., LEITE, T.E. e COSWIG, L.T. (1995). Herpes bovino tipo 1 (BHV 1): Sorologia de rebanhos com problemas reprodutivos. Ciência Rural 25: 421-424.

VOGEL, F. S., FLORES, E. F., WEIBLEN, R., WINKELMANN, E. R., MORAES, M. P. e BRAGANCA, J. F. (2004). Intrapreputial infection of young bulls with bovine herpesvirus type 1.2 (BHV-1.2): acute balanoposthitis, latent infection and detection of viral DNA in regional neural and non-neural tissues 50 days after experimental reactivation. Veterinary Microbiology 98(3-4): 185-96.

ZUCKERMANN F.A., HUSMANN R.J., SCHWARTZ R., BRANDT J., MATEU DE ANTONIO E. & MARTIN S. 1998. Interleukin-12 enhances the virus-specific interferon gamma response of pigs to an inactivated pseudorabies virus vaccine. Veterinary Immunology Immunopathology, 63:57-67.

WHETSTONE, C. A., WHEELER, J. G. e REED, D. E. (1986). Investigation of possible vaccine-induced epizootics of infectious bovine rhinotracheitis, using restriction endonuclease analysis of viral DNA. American Journal of Veterinary Research 47(8): 1789-95.

WINKLER, M. T., DOSTER, A. e JONES, C. (1999). Bovine herpesvirus 1 can infect CD4(+) T lymphocytes and induce programmed cell death during acute infection of cattle. Journal of Virology 73(10): 8657-68.

WYLER, R., ENGELS, M. e SCHWYZER, M. (1989). Infectious bovine rhinotracheitis/vulvovaginitis London, Kluwer academic publishers, Boston,.

5- ANEXOS

Artigos publicados durante o doutorado

1. **A.D. Silva**, F.R. Spilki, A.C. Franco, P.A. Esteves, S.O. Hubner, D. Driemeier, A.P. Oliveira, F. Rijsewijk, P.M. Roehe. Vaccination with a gE negative bovine herpesvirus type 1 vaccine confers insufficient protection to a bovine herpesvirus type 5 challenge. Vaccine (2006) 24 3313-3320.

2. Esteves PA, Dellagostin OA, Pinto LS, **Silva AD**, Spilki FR, Ciacci-Zanella JR, Hübner SO, Puentes R, Maisonnave J, Franco AC, Rijsewijk FA, Batista HB, Teixeira TF, Dezen D, Oliveira AP, David C, Arns CW, Roehe PM. Phylogenetic comparison of the carboxy-terminal region of glycoprotein C (gC) of bovine herpesvirus (BoHV) 1.1, 1.2 and 5. Virus Research (2008) 131 (1):16-22.

3. **A.D. Silva**, V.A. Sortica, A.C. Braga, F.R. Spilki, A.C. Franco, P.A. Esteves, F. Rijsewijk, J.C.A. Rosa, H.B.C.R. Batista, A.P. Oliveira, P.M. Roehe. Caracterização antigênica e molecular de oito amostras do vírus da doença de Aujeszky isoladas no estado do estado do Rio Grande do Sul em 2003. Pesquisa Veterinária Brasileira (2005) 25 (1): 21-24.

4. F.R.Spilki, P.A.Esteves, **A.D.Silva**, A.C. Franco, F.A.M. Rijsewijk, P.M. Roehe. A monoclonal antibody-based ELISA allows discrimination between respondes induced by bovine herpesvirus subtypes 1 (BoHV-1.1) and 2 (BoHV-1.2). Journal of Virological Methods (2005) 129 191-193.

5. S.O. Hubner, A.P. Oliveira, A.C.Franco, P.A. Esteves, **A.D.Silva**, F.R. Spilki, F.A.M. Rijsewijk, P.M. Roehe. Experimental infection of calves with a gI, gE, US9 negative bovine herpesvirus type 5. Comparative Immunology, Microbiology & Infectious Disease (2005) 28, 187-196.

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Vaccine 24 (2006) 3313–3320
Vaccine
www.elsevier.com/locate/vaccine

Vaccination with a gE-negative bovine herpesvirus type 1 vaccine confers insufficient protection to a bovine herpesvirus type 5 challenge

Alessandra D. Silva[a,b,*], Fernando R. Spilki[a], Ana Cláudia Franco[a], Paulo A. Esteves[b,c], Silvia O. Hübner[a], David Driemeier[d], Anna Paula Oliveira[a,e], Frans Rijsewijk[f], Paulo M. Roehe[a,e]

[a] *Equipe de Virologia, FEPAGRO Saúde Animal – Instituto de Pesquisas Veterinárias Desidério Finamor (CPVDF), Estrada do Conde 6000, Eldorado do Sul, RS, Brazil*
[b] *Programa de Pós-Graduação em Ciências Veterinárias (PPGCV), Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil*
[c] *Empresa Brasileira de Pesquisa Agropecuária (Embrapa Aves e Suínos), BR 153, Km 110, Vila Tamanduá, Concórdia, SC, Brazil*
[d] *Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul (UFRGS), Av. Bento Gonçalves 9090, Porto Alegre, RS, Brazil*
[e] *Instituto de Ciências Básicas da Saúde, UFRGS, Rua Sarmento Leite, 500, CEP: 90050-170, Porto Alegre, RS, Brazil*
[f] *Animal Sciences Group, Postbus 65, 8200 AB, Lelystad, The Netherlands*

Received 6 July 2005; received in revised form 6 January 2006; accepted 10 January 2006
Available online 25 January 2006

Available online at www.sciencedirect.com
ScienceDirect
Virus Research 131 (2008) 16–22
Virus Research
www.elsevier.com/locate/virusres

Phylogenetic comparison of the carboxy-terminal region of glycoprotein C (gC) of bovine herpesviruses (BoHV) 1.1, 1.2 and 5 from South America (SA)

P.A. Esteves[a,b,*], O.A. Dellagostin[c], L.S. Pinto[c], A.D. Silva[b], F.R. Spilki[d], J.R. Ciacci-Zanella[a], S.O. Hübner[c], R. Puentes[e], J. Maisonnave[e], A.C. Franco[f], F.A.M. Rijsewijk[f], H.B.C.R. Batista[b], T.F. Teixeira[b], D. Dezen[b], A.P. Oliveira[f], C. David[f], C.W. Arns[d], P.M. Roehe[b,f]

[a] *Embrapa Suínos e Aves, BR 153, Km 110, CEP: 89700-000, Concórdia, SC, Brazil*
[b] *Centro de Pesquisas Veterinárias Desidério Finamor (Fepagro-Saúde Animal), Estrada do Conde 6000 CEP: 90001-970, Eldorado do Sul, RS, Brazil*
[c] *Universidade Federal de Pelotas, Centro de Biotecnologia, CEP: 99010-900, Pelotas, RS, Brazil*
[d] *Laboratório de Virologia Animal, Departamento de Microbiologia e Imunologia, IB/UNICAMP, P.O. Box 6109, Campinas, CEP: 13083970, SP, Brazil*
[e] *Universidad de la Republica, Facultad de Veterinaria, Area de Inmunología, Lasplaces 1550, Pocitos, Codigo Postal 11600, Montevideo, Uruguay*
[f] *Departamento de Microbiologia, Laboratório de Virologia, UFRGS, Porto Alegre 90050-170, RS, Brazil*

Received 18 June 2007; received in revised form 3 August 2007; accepted 3 August 2007
Available online 24 September 2007

Available online at www.sciencedirect.com
SCIENCE DIRECT
ELSEVIER
Journal of Virological Methods 129 (2005) 191–193
Journal of Virological Methods
www.elsevier.com/locate/jviromet

Short communication

A monoclonal antibody-based ELISA allows discrimination between responses induced by bovine herpesvirus subtypes 1 (BoHV-1.1) and 2 (BoHV-1.2)

Fernando Rosado Spilki[a,b,*], Paulo Augusto Esteves[a,b], Alessandra D'Ávila da Silva[a,b], Ana Cláudia Franco[a,b], Frans A.M. Rijsewijk[c], Paulo Michel Roehe[a,b]

[a] *Centro de Pesquisas Veterinárias Desidério Finamor (CPVDF/FEPAGRO), Estrada do Conde 6000, Eldorado do Sul, RS 90001-970, Brazil*
[b] *Departamento de Microbiologia, Laboratório de Virologia, UFRGS, Porto Alegre, RS 90050-170, Brazil*
[c] *ID-DLO, Lelystad, The Netherlands*

Received 8 April 2005; received in revised form 8 June 2005; accepted 14 June 2005
Available online 19 July 2005

Available online at www.sciencedirect.com
SCIENCE DIRECT
ELSEVIER
Comparative Immunology, Microbiology & Infectious Diseases 28 (2005) 187–196
COMPARATIVE IMMUNOLOGY MICROBIOLOGY & INFECTIOUS DISEASES
www.elsevier.com/locate/cimid

Experimental infection of calves with a gI, gE, US9 negative bovine herpesvirus type 5

S.O. Hübner[a,*], A.P. Oliveira[c], A.C. Franco[c], P.A. Esteves[c], A.D. Silva[c], F.R. Spilki[c], F.A.M. Rijsewijk[b], P.M. Roehe[c,d]

[a] *Laboratório de Virologia, Faculdade de Veterinária, Universidade Federal de Pelotas (UFPel), Campus Universitário, Caixa Postal 356, CEP 96010-900 Pelotas, RS, Brazil*
[b] *Wageningen University and Research Centre, Animal Sciences Group, Postbus 65, 8200 AB Lelystad, The Netherlands*
[c] *Centro de Pesquisa Veterinária Desidério Finamor, Estrada do Conde 6000, CEP 92990-000 Eldorado do Sul, Brazil*
[d] *Instituto de Ciências Básicas da Saúde, UFRGS, Rua Sarmento Leite 500, CEP 91540-000 Porto Alegre, RS, Brazil*

Accepted 5 January 2005

Pesq. Vet. Bras. 25(1):21-24, jan./mar. 2005

Caracterização antigênica e molecular de oito amostras do vírus da doença de Aujeszky isoladas no estado do Rio Grande do Sul em 2003[1]

Alessandra D'Ávila da Silva[2,3], Vinícius A. Sortica[2,4], Alexandre C. Braga[2], Fernando R. Spilki[2,4], Ana C. Franco[2], Paulo A. Esteves[2,3], Frans Rijsewijk[5], Júlio César A. Rosa[2], Helena Beatriz C.R. Batista[2], Anna P. Oliveira[2,4] e Paulo M. Roehe[2,4*]

ABSTRACT.- D'Ávila da Silva A., Sortica V.A., Braga A.C., Spilki F.R., Franco A.C., Esteves P.A., Rijsewijk F., Rosa J.C.A., Batista H.B.C.R., Oliveira A.P. & Roehe P.M. 2005. **[Antigenic and molecular characterization of eight samples of Aujeszky's disease virus isolated in the state of Rio Grande do Sul, Brazil, in 2003.]** Caracterização antigênica e molecular de oito amostras do virus da doença de Aujeszky isoladas no estado do Rio Grande do Sul em 2003. *Pesquisa Veterinária Brasileira 25(1):21-24.* Instituto de Pesquisas Veterinárias Desidério Finamor (IPVDF), Fepagro Saúde Animal, Estrada do Conde 6000, Cx. Postal 47, Eldorado do Sul, RS 92990-000, Brazil. E-mail: proehe@ufrgs.br

Pseudorabies or Aujeszky's disease (AD), caused by pseudorabies virus (PRV) is a major concern in swine production. In the state of Rio Grande do Sul, Brazil, AD was only detected in 1954, in cattle. In 2003 two outbreaks of encephalitis occurred on the northern region of the state, close to the border with the state of Santa Catarina. Pseudorabies virus (PRV) was isolated from distinct farms within the region and subjected to antigenic and genomic analyses. These isolates were compared with prototype strains NIA-3 and NP. Antigenic characterization with a panel of monoclonal antibodies (Mabs) directed to viral glycoproteins (gB, gC, gD and gE-) was performed by an imunoperoxidase monolayer assay (IPMA) on infected cell monolayers. Genomic characterization was carried out by restriction enzyme analysis (REA) of the whole DNA viral genome with *Bam* HI. The antigenic profile of the eight isolates from Rio Grande do Sul as well as strains NIA-3 and NP were similar. REA analysis revealed that all isolates from Rio Grande do Sul displayed a genomic type II arrangement, a genotype often found in other outbreaks of AD previously reported in other Brazilian states. The results obtained suggest that the eight isolates examined here were similar.