UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE VETERINÁRIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS VETERINÁRIAS

PATOGENICIDADE E VACINOLOGIA DE AMOSTRAS BRASILEIRAS DE HERPESVÍRUS BOVINO TIPO 1

Fernando Rosado Spilki

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Dissertação apresentada como requisito para obtenção do grau de Mestre em Ciências Veterinárias na área de Medicina Veterinária Preventiva – Virologia Veterinária

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PATOGENICIDADE E VACINOLOGIA DE AMOSTRAS BRASILEIRAS DE HERPESVÍRUS BOVINO TIPO 1

RESUMO

O herpesvírus bovino tipo 1 (BHV-1) está amplamente disseminado no rebanho bovino brasileiro. Visando aprofundar o conhecimento sobre a patogenicidade para o trato respiratório de amostras dos subtipos deste vírus mais freqüentemente encontrados no Brasil (BHV-1.1 e BHV-1.a), isolados autóctones oriundos de casos de doença respiratória foram inoculados em bovinos suscetíveis. As amostras de ambos os subtipos foram capazes de induzir doença respiratória após inoculação pela via intranasal, em intensidade que variou de moderada a grave, independentemente do subtipo de vírus inoculado. Foi ainda caracterizada a resposta imune humoral induzida por tais amostras quanto ao perfil de classes e subclasses de imunoglobulinas, sendo esta igualmente indistinguível com relação aos vírus inoculados. O perfil de classes de imunoglobulinas apresentadas pelos animais permitiu a determinação do status da infecção nos animais inoculados através da análise da resposta sorológica. Na segunda etapa deste trabalho foram testadas as propriedades vacinais de um vírus recombinante, do qual foi deletada a glicoproteína E (gE; 265gE-), gerado a partir de uma amostra brasileira de BHV-1.2a. Experimentos de inoculação do recombinante 265gE- em animais suscetíveis e o desafio destes animais com a amostra parental virulenta demonstraram a segurança e eficácia desta amostra na prevenção de sinais clínicos da infecção pelo BHV-1. Posteriormente, o recombinante 265gE- foi inoculado em vacas em diferentes estágios da gestação. Não foram observadas quaisquer anormalidades nestas gestações e as 22 vacas vacinadas deram à luz a animais saudáveis. Conclui-se que o recombinante é imunogênico e capaz de conferir significativa proteção frente ao desafio com a amostra parental virulenta do vírus. O recombinante também não causou enfermidade nas vacas inoculadas nem tampouco aos fetos quando inoculado em diferentes estágios da gestação.

PATHOGENICITY AND VACCINOLOGY OF BOVINE HERPESVIRUS TYPE 1 BRAZILIAN ISOLATES

ABSTRACT

Bovine herpesvirus type 1 infections are disseminated among Brazilian herds. Aiming a better understanding of the pathogenicity of the most common viral subtypes present in Brazil (BHV-1.1 and BHV-1.2a), field isolates from cases of respiratory disease of both subtypes were inoculated into susceptible calves. Both were capable of inducing similar clinical illness in calves. The intensity of the observed clinical signs on the respiratory tract varied from mild to severe. However, there was no correlation with severity of disease and the viral subtype inoculated. The class and subclass profiles of the humoral immune responses induced by each of these strains were also characterized. No significant differences were detected on the immune response in respect to the virus subtype inoculated. The analysis of the patterns of immune responses allowed the determination of the approximate stage of infection the infected animals were undergoing. On the second part of this work, some of the properties of a glycoprotein E (gE) deleted recombinant virus (265gE-), constructed from a Brazilian BHV-1.2a autochtonous isolate, were examined in order to determine its potential as a vaccine virus. Susceptible calves were inoculated with the recombinant and subsequently challenged with the wild type parental virus. No clinical signs were observed after the inoculation with the 265gE-; after challenge with the virulent wild type strain, minimal clinical signs and reduced viral excretion was observed. The safety of the recombinant for pregnant cows was also investigated. After inoculation of the recombinant strain, 22 cows were observed for abnormalities until the terminus of pregnancy. All inoculated cows gave birth to normal calves. It was concluded that the recombinant virus is immunogenic and capable of conferring significant protection against challenge with the virulent parental virus. Moreover, within the conditions of the present study, when inoculated in cows at different stages of gestation the recombinant did not cause disease in the cows nor was it pathogenic for their foetuses.

SUMÁRIO

1. REVISÃO BIBLIOGRÁFICA

1.1 Herpesvírus bovino tipo 1

O herpesvírus bovino tipo 1 (BHV-1) é um membro da família *Herpesviridae*, subfamília *Alphaherpesvirinae* (Turin et al., 1999). Tal como outros alfaherpesvírus, o BHV-1 possui um genoma composto de DNA de dupla fita linear, circundado por um capsídeo icosaédrico, que por sua vez é recoberto pelo tegumento viral, ao qual se sobrepõe um envelope lipoprotéico (Schwyzer e Ackermann, 1996). O genoma codifica em torno de 70 diferentes proteínas, incluindo proteínas reguladoras, enzimas virais e proteínas estruturais. Dentre as últimas, ocupam especial importância as glicoproteínas (Van Oirschot, 1999). Estas inserem-se no envelope viral e são responsáveis por várias das características biológicas do vírion, seja na interação com membranas celulares, promovendo os mecanismos/ eventos de adsorção, penetração, disseminação célula-a-célula ou mesmo interagindo com diferentes moléculas do sistema imune (Denis et al., 1996; Rebordosa et al., 1996). Algumas destas glicoproteínas são essenciais à multiplicação viral (glicoproteínas gB, gD, gH, gK e gL), enquanto outras são dispensáveis à sua multiplicação, ditas não-essenciais (glicoproteínas gC, gG, gM, gI e gE) ainda que exerçam importantes funções de interação com a célula hospedeira (Dasika e Letchworth, 1999; Denis et al., 1996; Rebordosa et al., 1996; Shaw et al., 2000).

1.2 Subtipos genômicos

De acordo com os perfis de restrição enzimática dos genomas virais, as amostras de BHV-1 foram agrupadas em dois subtipos denominados: BHV-1.1 e BHV-1.2. O último subtipo foi ainda subdivido em BHV-1.2a e BHV-1.2b (Metzler et al., 1985). Os genomas

de amostras padrão de BHV-1.1 (Cooper) e BHV-1.2 (K22) apresentam 95% de similaridade. Todavia, a despeito dessa alta similaridade no conjunto do genoma, determinados genes, como aquele que codifica para 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., 1995; Rijsewijk et al., 1999).

1.3 Manifestações clínicas

A grande maioria das infecções pelo BHV-1 provavelmente ocorre de forma subclínica (Van Oirschot et al., 1993). Quando presentes, os sinais clínicos associados ao BHV-1 parecem estar intimamente ligados à via de infecção/inoculação do hospedeiro (Kaashoek et al., 1996a; Kit et al., 1986; Miller e Van der Maaten, 1987; Narita et al., 2000). Assim, infecções naturais ou experimentais das vias nasais resultam em inflamação aguda da membrana mucosa do trato respiratório superior (Edwards et al., 1991; Kaashoek et al., 1996), quadro que caracteriza a forma clássica da rinotraqueíte infecciosa bovina (*infectious bovine rhinotracheitis*, IBR). A infecção aguda se carateriza por febre (muitas vezes acima de 41°C), apatia, anorexia, hiperemia da mucosa que também pode apresentar pequenas vesículas que tendem a coalescer e formar nódulos necróticos com a evolução da doença, sinais estes acompanhados por secreção nasal inicialmente serosa que muitas vezes pode tornar-se muco-purulenta ou mesmo muco-sanguinolenta (Kaashoek et al., 1996a). Não são incomuns as co-infecções com bactérias do trato respiratório, em especial por *Pasteurella multocida* (Jericho et al., 1982). De forma semelhante, as infecções do trato genital externo resultam em inflamação da mucosa, sendo tal manifestação denominada vulvovaginite pustular infecciosa (*infectious pustular vulvovaginitis*, IPV) na fêmea e balanopostite pustular infecciosa (*infectious pustular balanoposthitis*, IPB; Kit et al., 1986) no macho.

A infecção de animais gestantes, especialmente no terceiro trimestre da gestação pode resultar em abortos (Miller et al., 1988; Miller et al, 1991). Outras manifestações, tais

como reabsorção embrionária, infecção neonatal generalizada (Higgins e Edwards, 1986), conjuntivite (Cook, 1998), queda na produção leiteira (Hage et al., 1998), enterites, mastites e encefalites também são citadas (Roels et al., 2000). De modo geral, a literatura afirma que infecções pelo BHV-1.1 são mais severas para o trato respiratório do que aquelas provocadas pelo subtipo 1.2 (Edwards et al., 1991); todavia, vírus de ambos os genotipos podem ser isolados em casos de IBR. Ainda que alguns experimentos de inoculação tenham sido realizados com o subtipo 1.2b, e menor patogenicidade seja associada a este genótipo (Edwards et al., 1991; Msolla et al., 1983a), isolados do BHV-1.2a não são comuns no Velho Mundo, diferindo do Brasil, onde têm sido isolados com relativa freqüência do trato respiratório (D'Arce et al., 2002).

1.4 Latência

Uma característica marcante das infecções pelo BHV-1, a exemplo de outros alfaherpesvírus, é a capacidade de estabelecer infecções latentes em neurônios dos gânglios que inervam a região associada à primoinfecção (Pastoret e Thiry, 1985). Os principais sítios de latência são o gânglio trigêmio (quando da ocorrência de infecções respiratórias) e os gânglios sacrais (em infecções genitais; Jones, 2003). A reativação da infecção pode ser acompanhada ou não de sinais clínicos (recrudescência), mas geralmente estará associada a uma fase de disseminação do vírus ao ambiente (Kaashoek et al., 1996; Pastoret e Thiry, 1985). Dentre as situações que podem induzir à reativação de infecções latentes, encontram destaque especial as situações estressantes, incluindo os tratamentos com corticosteróides (Castrucci et al., 1983), outras infecções (Edwards e Roeder, 1983), parasitismo (Msolla et al., 1983b), parto e transporte (Pastoret et al., 1987). A excreção de partículas virais a partir da reativação pode ser intensa o suficiente para perpetuar a infecção nos rebanhos, ainda que em menores títulos do que aqueles excretados durante a infecção aguda (Pastoret e Thiry, 1985). Devido ao fenômeno de latência, uma vez infectado, um bovino torna-se portador da infecção por toda a vida (Pastoret e Thiry, 1985; Van Oirschot et al., 1993).

1.5 Imunidade associada à infecção pelo BHV-1

A exposição a antígenos do BHV-1, seja por infecção ou vacinação, resulta em respostas específicas que visam a neutralização da infectividade viral e morte das células infectadas (Babiuk et al., 1996). A resposta imune celular pode ser detectada 5 dias após a infecção (pi) e se torna mais evidente entre 8 e 10 dias pi (Madic et al., 1995). Anticorpos neutralizantes são detectados entre 7 e 10 dias pi (Babiuk et al., 1996; Hofman, 1989). A resposta imune localizada nas mucosas é evidente, podendo ser detectada pela presença de IgA específica (Madic et al., 1995). Ainda que inúmeros elementos do sistema imune estejam envolvidos na recuperação de um quadro clínico, bem como na prevenção de sinais clínicos (Woolums et al., 2003), os anticorpos neutralizantes merecem destaque na inibição da disseminação do vírus pelo organismo e mesmo na indução da própria imunidade celular (Madic et al., 1995). Anticorpos neutralizantes específicos estão presentes na circulação por, no mínimo, três anos após uma infecção pelo BHV-1 (Kaashoek et al., 1996b).

1.6 Controle das infecções pelo BHV-1

A disseminação das infecções pelo BHV-1 se dá primordialmente pelo contato direto, por fômites, pela inseminação artificial e por via aérea a curtas distâncias (Mars et al., 1999; 2000). Dados os prejuízos induzidos pelas infecções pelo BHV-1, produtores isolados ou mesmo países, têm adotado, nos últimos anos, programas de controle ou erradicação da enfermidade (Ackermann et al., 1990a; 1990b; Van Oirschot et al., 1996).

Para atingir tais objetivos diferentes estratégias de manejo e vacinação têm sido adaptadas a cada situação epidemiológica.

1.6.1 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 (Ackerman et al., 1990a; 1990b). Tal método tem como principal vantagem o baixo custo e tem se mostrado bastante eficiente, desde que tomadas medidas restritivas severas, visando evitar a introdução de novos animais infectados e controle das possíveis fontes de material contaminado (em especial sêmen; Van Oirschot et al., 1993). Um dos pontos controversos que repousa sobre tais estratégias é a possibilidade da existência de animais soronegativos latentemente infectados (Lemaire et al., 2000).

1.6.2 Controle com vacinação

Em países ou regiões com alta prevalência de infecções pelo BHV-1, políticas de controle sem vacinação podem tornar-se inviáveis (Van Oirschot et al., 1996). Deste modo, pode ser utilizada neste caso 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 BHV-1 atualmente (Castrucci et al., 2002), cujos princípios são descritos sucintamente a seguir.

Vacinas inativadas

Vacinas com vírus inativado, nas quais o agente é inativado por compostos químicos específicos ou métodos físicos, também têm sido descritas (Halfen, 1996; Petzhold et al., 2001). Diferentes adjuvantes têm sido associados a tais vacinas (Castrucci et al., 2002; Romera et al., 2000). As mesmas são consideradas, em geral, menos efetivas que

as vacinas vivas; todavia, é consenso que são mais seguras devido a não ocorrer replicação do vírus vacinal no hospedeiro (Frerichs et al., 1982).

Vacinas vivas convencionais

Vacinas vivas atenuadas também são comercializadas praticamente desde o primeiro isolamento do BHV-1 (Kendrick et al., 1956). A atenuação é obtida, usualmente, por passagens sucessivas em cultivos celulares ou pela indução de mutações. Desde amostras padrões do vírus naturalmente atenuadas até mutantes termo-sensíveis estão disponíveis no mercado (Zygraich et al., 1974). Tais vacinas são eficazes na prevenção de sinais clínicos associados à infecção. Desvantagens pós-vacinação comumente relatadas são a indução de abortos em vacas prenhes (Lomba et al., 1976), excreção do vírus vacinal e reversão à virulência (Bryan et al., 1994), além da dificuldade em caracterizar-se adequadamente as deleções/ inserções genômicas associadas aos métodos de atenuação convencional (Jones et al., 2000; Thiry et al., 1985). Apresentações das mesmas para utilização pela via intramuscular (IM) ou intranasal (IN) estão disponíveis. O maior empecilho associado às vacinas vivas e mortas convencionais é a impossibilidade de diferenciar-se a resposta imune vacinal daquela induzida pela infecção, o que compromete a realização de programas eficazes de controle e erradicação das infecções com vírus de campo (Turin et al, 1999).

Vacinas de subunidade e vacinas de DNA

Uma das alternativas para contornar o problema da impossibilidade de discriminação da resposta induzida pela infecção ou vacinação com vacinas convencionais, é a imunização utilizando proteínas selecionadas do genoma viral (vacinas de subunidade; Van den Hurk et al., 1990; 1997) que induzam resposta imune protetora e, mais recentemente, a vacinação com o próprio DNA que codifica os genes de tais proteínas (vacinas de DNA;). Merece especial atenção em tais processos a glicoproteína D (gD; Babiuk et al., 2003; Zhu e Letchworth, 1996). Em especial a vacinação com o gene que

codifica a gD tem se mostrado eficaz em promover respostas imunes protetoras seja no que se refere à indução de anticorpos neutralizantes quanto à imunidade de mucosa contra o BHV-1 (Babiuk et al., 2003). Entretanto, em experimentos comparando sua eficácia com vacinas de vírus inativado ou modificadas a mesma apresenta resultados não satisfatórios no que tange à proteção contra os sinais clínicos induzidos pela infecção e excreção viral após o desafio (Bosch et al., 1997; 1998). Visando melhorar este quadro, genes de diferentes citocinas têm sido associados a vacinas de DNA (Hughes et al., 1992). Entretanto, estudos recentes demonstraram que a presença de genes de diferentes citocinas na construção de um vírus recombinante vacinal não melhorou a eficácia da vacina (König et al., 2003).

Vacinas diferenciais

Recentes avanços no entendimento das funções de diferentes glicoproteínas e enzimas do BHV-1 têm permitido o desenho de uma nova geração de imunógenos para tal infecção (Turin et al., 1999). Assim, a deleção de genes estruturais não essenciais (gE e outras; Kaashoek et al., 1994, Belknap et al., 1996; Flores et al, 1993) ou de enzimas associadas à replicação do DNA viral (timidina-quinase viral, tk; Chowdhury, 1996) têm proporcionado a confecção de novos vírus vacinais que, além de mais seguros, ainda permitem, com o auxílio de testes diagnósticos adequados, a identificação dos animais infectados com amostras de campo em meio aos animais vacinados, o que confere a estas vacinas a denominação de vacinas diferenciais (*differential vaccines*, DIVA; Van Oirschot, 1999). Assim, são 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. Deste modo, animais que tenham anticorpos contra a proteína ausente no vírus vacinal são considerados infectados e estratégias de controle adequadas podem ser tomadas, mesmo utilizando-se a vacinação para reduzir a excreção viral e conseqüente disseminação do BHV-1 no rebanho.

2. Objetivos

Ainda que a infecção pelo BHV-1 esteja disseminada no rebanho brasileiro (Melo et al., 2002), pouco ou nada se sabe sobre a patogenicidade das amostras isoladas em nosso território. Do mesmo modo, são pouco conhecidas possíveis diferenças em patogenicidade associadas a distintos subtipos do BHV-1. O primeiro capítulo desta dissertação visa comparar alguns aspectos da patogenicidade de duas amostras autóctones de BHV-1, sendo uma pertencente ao 1 (BHV-1.1) e a outra ao subtipo 2a (BHV-1.2a). O segundo capítulo traz uma comparação do perfil de isotipos de imunoglobulinas específicas induzidas por tais amostras, visando caracterizar a resposta imune humoral induzida pelas mesmas.

No terceiro e quarto capítulos, são abordados testes de segurança e eficácia realizados com a amostra de vírus recombinante diferencial gE- (Franco et al., 2002a), desenvolvida recentemente por nosso grupo a partir de um isolado brasileiro de BHV-1, frente ao desafio com a amostra virulenta parental de BHV-1.2a, assim como a avaliação da segurança e imunogenicidade deste mesmo recombinante para vacas gestantes e seus fetos.

Capítulo 1

Comparative pathogenicity of bovine herpesvirus 1 (BHV-1) subtypes 1 (BHV-1.1) and 2a (BHV-1.2a)[1]

Autores:

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ABSTRACT – The present study aimed to examine the capacity of two bovine herpesvirus type 1 (BHV-1) isolates of different subtypes (EVI 123/96, BHV-1.1; SV265/98, BHV-1.2a) to induce respiratory disease in calves. These two isolates are representative of the BHV-1 subtypes prevalent in Brazil. Viral subtypes were confirmed by monoclonal antibody analysis and by restriction enzyme digestion of viral genomes. The viruses were inoculated intranasally into seven, three months old calves (four with BHV-1.1, three with BHV-1.2a). Three other calves of identical age and condition were kept as uninfected controls. In both groups of infected calves, the clinical signs observed were consistent with typical infectious bovine rhinothracheitis (IBR), including pyrexia, apathy, anorexia, nasal and ocular mucopurulent discharges, erosions on the nasal mucosa, conjunctivitis, lachrymation, redness of nasal mucosa, dyspnoea, coughing, tracheal stridor and enlargement of retropharingeal, submandibular and cervical lymphnodes. No significant differences were observed between the clinical scores attributed to both groups. Virus shedding in nasal and ocular ocular secretions were also similar, apart from a significant difference in nasal virus shedding on days 1 to 3 post-inoculation, which was higher for BHV-1.1 than for BHV-1.2a. Following corticosteroid induced reactivation of the latent infection, recrudescence of clinical signs was also observed, with no significant differences on both groups. It was concluded that both subtypes BHV-1.1 and BHV-1.2a were able to induce clinically undistinguishable respiratory disease in calves, either subsequent to a primary infection or following reactivation.

INDEX TERMS: infectious bovine rinotracheitis, IBR, BHV-1.1, BHV-1.2a; subtypes, pathogenicity

RESUMO – O presente estudo teve como objetivo examinar a capacidade de duas amostras de herpesvírus bovino tipo 1 (BHV-1) de diferentes subtipos (amostra EVI 123/96: BHV-1.1; amostra SV265/98: BHV-1.2a) de induzir doença respiratória em bovinos. Estas duas amostras são representativas de subtipos de BHV-1 prevalentes no Brasil. Os subtipos das amostras foram confirmados por análises com anticorpos monoclonais e com enzimas de restrição. As amostras foram inoculadas por via intranasal em sete bezerros de três meses de idade (quatro com BHV-1.1, três com BHV-1.2a), soronegativos para BHV-1, sendo outros três animais mantidos como controles não infectados. Nos dois grupos de animais inoculados, os sinais clínicos observados foram consistentes com o quadro de rinotraqueíte infecciosa bovina (IBR), incluindo febre, apatia, anorexia, descargas mucopurulentas nasais e oculares, conjuntivite, erosões e hiperemia na mucosa nasal, dispnéia, tosse, estridor traqueal e aumento dos linfonodos retrofaríngeos, submandibulares e cervicais. Não foram observadas diferenças significativas entre os escores clínicos atribuídos aos animais nos dois grupos. Igualmente, foram similares as quantidades de vírus re-isoladas dos animais infectados, à exceção de uma diferença significativa na disseminação de vírus pelas secreções nasais, a qual foi maior nos animais infectados com BHV-1.1 nos dias 1 a 3 pós-inoculação. Após reativação induzida por corticosteróides, foi observado recrudescimento dos sinais clínicos, os quais foram também similares em ambos os grupos. Em conclusão, as amostras de BHV-1 dos subtipos 1 e 2a não apresentaram diferenças significativas em sua patogenicidade sobre o trato respiratório nos animais inoculados, tanto após a infecção primária como após a reativação.

TERMOS DE INDEXAÇÃO: rinotraqueíte infecciosa bovina, IBR, BHV-1.1, BHV-1.2a; subtipos; patogenicidade.

INTRODUCTION

Bovine herpesvirus type 1 (BHV-1), an alphaherpesvirus, is an important pathogen for cattle, causing severe economical losses to cattle industry (Turin et al. 1999). Three BHV-1 subtypes, 1 (BHV-1.1), 2a (BHV-1.2a) and 2b (BHV-1.2b) have been recognized with basis on antigenic and genomic analyses (Metzler et al. 1985). Subtypes 1 and 2b have been associated to different signs of disease or different degrees of pathogenicity. BHV-1.1 refers to the "classical" viruses which have been associated to infectious bovine rhinotracheitis (IBR). This subtype has often been isolated from the respiratory tract as well as from abortions, and is prevalent in many countries in Europe as well as in the Americas (Edwards et al. 1990; Souza et al. 2002). BHV-1.2a, on its turn, has been associated to a broad range of clinical manifestations on both the respiratory and genital tracts such as IBR, infectious pustular vulvovaginitis (IPV) or balanopostitis (IPB), as well as abortions (Van Oirschot 1995). BHV-1.2a is highly prevalent in Brazil; it was present in Europe before the decade of 1970; after that, it became rare in that continent (Van Oirschot 1995, D'Arce et al. 2002). BHV-1.2b, on its turn, has been associated to respiratory disease as well as to IPV/IPB. Therefore, subtype 2b has not been related to abortion (Van Oirschot 1995) and has been considered less pathogenic for the respiratory tract than BHV-1.1 (Edwards et al. 1990). Subtype 2b has been frequently isolated in Australia and Europe (Edwards et al. 1990), but is uncommon in Brazil where, to date, only one virus of this subtype has been so far isolated.

Differences in virulence among BHV-1 subtypes that affect the respiratory tract have also been reported. BHV-1.1 isolates are more pathogenic than BHV-1.2b (Msolla et al. 1983a; Edwards et al. 1990). However, no comparative evaluation of BHV-1.2a pathogenicity on the respiratory tract has been made to date, probably in view of the low prevalence of this subtype in Europe and North America. As BHV-1.1 and BHV-1.2a have often been associated to respiratory disease and are highly prevalent in Brazil, the present study was carried out to examine the virulence of two recent Brazilian isolates of BHV-1.1 and BHV-1.2a following intranasal inoculation in calves.

MATERIALS AND METHODS

Viruses and cells

Madin Darby bovine kidney cells (MDBK; ATCC CCL22) were used throughout. Cells were cultured in Eagle's Minimal Essential Medium (E-MEM) supplemented with 6% foetal bovine serum (FBS) and enrofloxacin (Baytril, final concentration 10 mg/L). The BHV-1.1 strain EVI 123/98 and BHV-1.2a SV 265/96 were used for comparative inoculations. These two viruses were isolated from outbreaks of IBR in Southern Brazil in 1998 and 1996, respectively (D'Arce et al. 2002). Both were multiplied in MDBK cells to prepare stocks with approximately $10^{8.3}$ 50 % tissue culture infective doses per mL ($TCID_{50}$). Virus stocks were clarified by centrifugation (2000 x g) and stored at –70 °C until use. Viruses and cells were proven free of bovine viral diarrhoea virus contamination by immunoperoxidase (Roehe et al, 1991).

Genomic and antigenic analyses

Genomic analyses were performed by restriction endonuclease digestion. Viral DNA was cleaved with enzymes *Bam*HI*, BstE*II, *Eco*RI, *Hind* III and *Pst*I, as described (D'Arce et al. 2002). Antigenic analyses with monoclonal antibodies (Mabs) were carried out by immunoperoxidase monolayer assays (IPMAs) on virus multiplied in microtitre plates, as described elsewhere (Souza et al. 2002). In addition, a differential IPMA was performed with a Mab (Mab 71) that recognizes a specific epitope on BHV-1 glycoprotein C (gC) and does not react with BHV-1.2a (Rijsewijk et al. 1999). The differential IPMA followed essentially the method described previously (Souza et al. 2002), except that Mab 71 was used as primary antibody, followed by an anti-mouse peroxidase/IgG conjugate (DAKO).

Inoculation of calves

Ten, three to four months old calves, seronegative for BHV-1 (as determined by serum neutralization tests and ELISAs), were purchased from a local beef farm. Calves

were randomly alloted into three groups (BHV-1.1, four calves; BHV-1.2a, three calves; three control calves) and kept in identical isolation units with controlled environmental temperature and air supply. The calves were fed with forage and water *ad libitum* and supplemented with concentrate. After twelve days for adaptation in the isolation units, each calf was inoculated with 10^9 $TCID_{50}$ of either BHV-1.1 or BHV-1.2a, in a volume of 8 mL, applied with the aid of an intranasal spray, divided (2 x 4 ml) and inoculated into each nostril. The control group was mock infected with sterile culture medium.

Clinical assessment

The calves were examined daily from day 12 before inoculation until day 14 post-infection (pi). Respiratory rates and rectal temperatures were recorded. Clinical scores were obtained by a scoring method based on that described by Collie (1992), modified by attributing arbitrary points to different signs of disease, based on our previous experience with BHV-1 inoculations. Signs considered more indicative of the calve's well-being were recorded on a higher scale. Thus, apathy, anorexia, dyspnoea and unusual stridors at auscultation were scaled up to 100, whereas nasal and ocular secretions, conjuntivitis, presence of erosions on the nasal mucosae, enlargement of lymph nodes and cough were scaled up to 10. Temperature rates (TR) were included on individual scores using the formula:

TR = 100 x (calf's temperature on each day – calf's mean temperature on the 12 days prior to inoculation).

A mean clinical score was calculated daily for each group.

Reactivation

Starting from day 168 pi, new clinical scores were determined for 12 days, as above. Aiming virus reactivation, from day 180 pi, calves were treated with 0.1 mg per kg of body weight of dexamethasone for 5 consecutive days. Clinical examinations and scoring were performed again from the beginning of the corticosteroid administration up to day 194 pi.

Virological and serological examinations

Samples for virus isolation were collected on day 12 previous to inoculation, on the day of virus inoculation (day 0) and daily from days 1 to 14 post-inoculation (pi); samples were also collected on day 5 before to 14 days after the administration of dexamethasone. Nasal and ocular swabs were eluted in 2 mL of sample medium (E-MEM supplemented with 10 times the usual concentration of antibiotics and 2 % FBS for one hour at room temperature. The samples were vigorously shaken, the swabs removed, drained, the medium clarified by low speed centrifugation and stored at –70 °C until use. Tissues were processed as described below. Virus isolation and titrations of recovered viruses were performed following standard protocols (Roehe 1991). Infectious titres were calculated and expressed as $\log_{10}$ $TCID_{50}$ per mL of nasal or ocular fluid or per grams of tissue. Serum samples were collected by jugular venipuncture on days 0,7, 14 and 30 days pi and 0, 7 and 14 post-reactivation (pr). Sera were tested in serial twofold dilutions in a standard BHV-1 neutralizing antibody test with 1 hour incubation of the serum/virus mixture followed by incubation at 37 ^{o}C for five days (House & Baker 1971), against 100 $TCID_{50}$ of each of the two virus subtypes.

Post-mortem examinations

Calves that died were submitted to necropsy and fragments of tissues for virus isolation were collected from nasal mucosae, nasal hornets, trachea, tonsils, cervical and mediastinic lymph nodes, lung, brain, trigeminal ganglia, liver, spleen, adrenal glands and kidneys. Ten per cent (w/v) suspensions were prepared in MEM, filtered through 22 nm filters and inoculated onto MDBK monolayers following standard procedures (Roehe 1991). Routine bacteriological examinations were also performed on all specimens. Histopathological analysis was performed on buffered formalin paraffin embedded tissues; immuno-histochemical analyses were also performed following routine protocols.

Confirmation of the identity of viruses recovered from calves

To confirm the identity of the viruses recovered from inoculated calves and to ensure that no cross-contamination has taken place, the differential IPMA described above was carried out on all successful virus recovery attempts.

Statistical analysis

The results were statistically examined by analysis of variance (ANOVA); the least significance difference for $p = 0.05$ was determined. Comparisons were made within the groups from day to day and between groups. Statistical analysis was performed with Minitab® for Windows Release 11.1 (Minitab Inc., State College, PA, USA). The term "significant" (statistically significant) in the text means $p \leq 0.05$.

RESULTS

Genomic and antigenic analyses

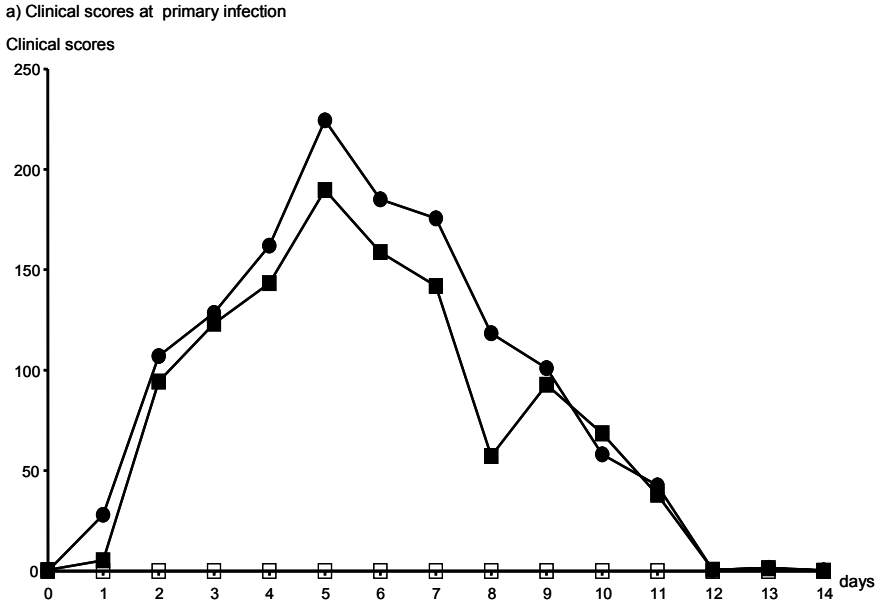
Restriction enzyme analyses confirmed the subtyping of EVI 123/98 (BHV-1.1) and SV 265/96 (BHV-1.2a; D'Arce et al., 2002). Antigenic analyses with anti-BHV-1 Mabs (Souza et al. 2002) as well as with Mab 71 (Rijsewijk et al. 1999) again confirmed the antigenic differences between the two isolates of distinct subtypes (data not shown).

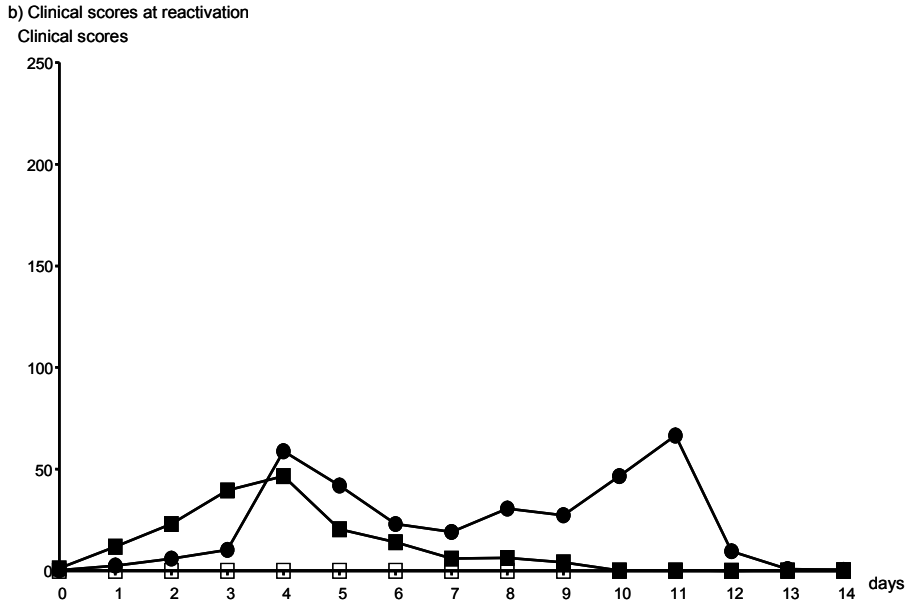
Clinical findings following primary infection and reactivation

The calves infected either with EVI 123/ 98 or SV 265/ 96 showed severe signs of illness (Figure 1a). The respiratory rates were significantly elevated from days 2 to 11 pi. On day 2, the rectal temperatures of the infected calves started to rise (Figure 2a). The highest temperatures were recorded on day 5. Fever ($\geq 39{,}5\ ^{o}C$) was recorded from day 2 to 7 in calves from both groups and respiratory rates accompanied such elevation (Figure 3a). Apathy, anorexia, rhinitis with redness of the nasal mucosae, nasal and ocular discharges, formation of vesicles with tendency to coalesce and form erosions on the nasal mucosae,

conjuntivitis, nasal stridor, sneezing and spontaneous coughing were often observed in all calves. Enlargement of the retropharyngeal, submandibular and cervical lymphnodes were recorded from day 4 to day 12 after infection. Dyspnoea and tracheal stridors were observed in all calves although in different degrees. Despite severely ill on days 5 to 7 pi, all calves completely recovered from primary infection 10 days post-infection without noticeable sequels.

Figure 1. Mean clinical scores attributed to calves after inoculation. a) clinical scores during acute infection b) upon reactivation. Full squares: EVI123/98; black circles: SV265/96 empty squares: control calves. Clinical scores attributed as defined in methods.





At reactivation, clearly noticeable signs of respiratory disease were again observed on both groups of infected calves (Figures 1b, 2b and 3b). However, such signs were less intense than those observed during primary infection. Calves were pirexic from days 1 to six pr (Figure 2b). One of the BHV-1.2a-infected calves died on day 11 pr. This calf had low titres of infectious virus (10 to $10^{2.5}$ $TCID_{50}$) in the nasal hornets, trachea, lung, mediastinic and periportal lymphnodes, liver and spleen. Mild ganglioneuritis was evident in the trigeminal ganglia. Post-mortem examination revealed mild pneumonia and focal hepatic necrosis. In such lesions, BHV-1 antigen was detected by immunohistochemistry. *Escherichia coli* and *Klebsiella sp.* were isolated from the lungs.

Figure 2. Mean rectal temperatures of calves after inoculation. a) during acute infection; b) upon reactivation. Full squares: EVI123/98; black circles: SV265/96 empty squares: control calves.

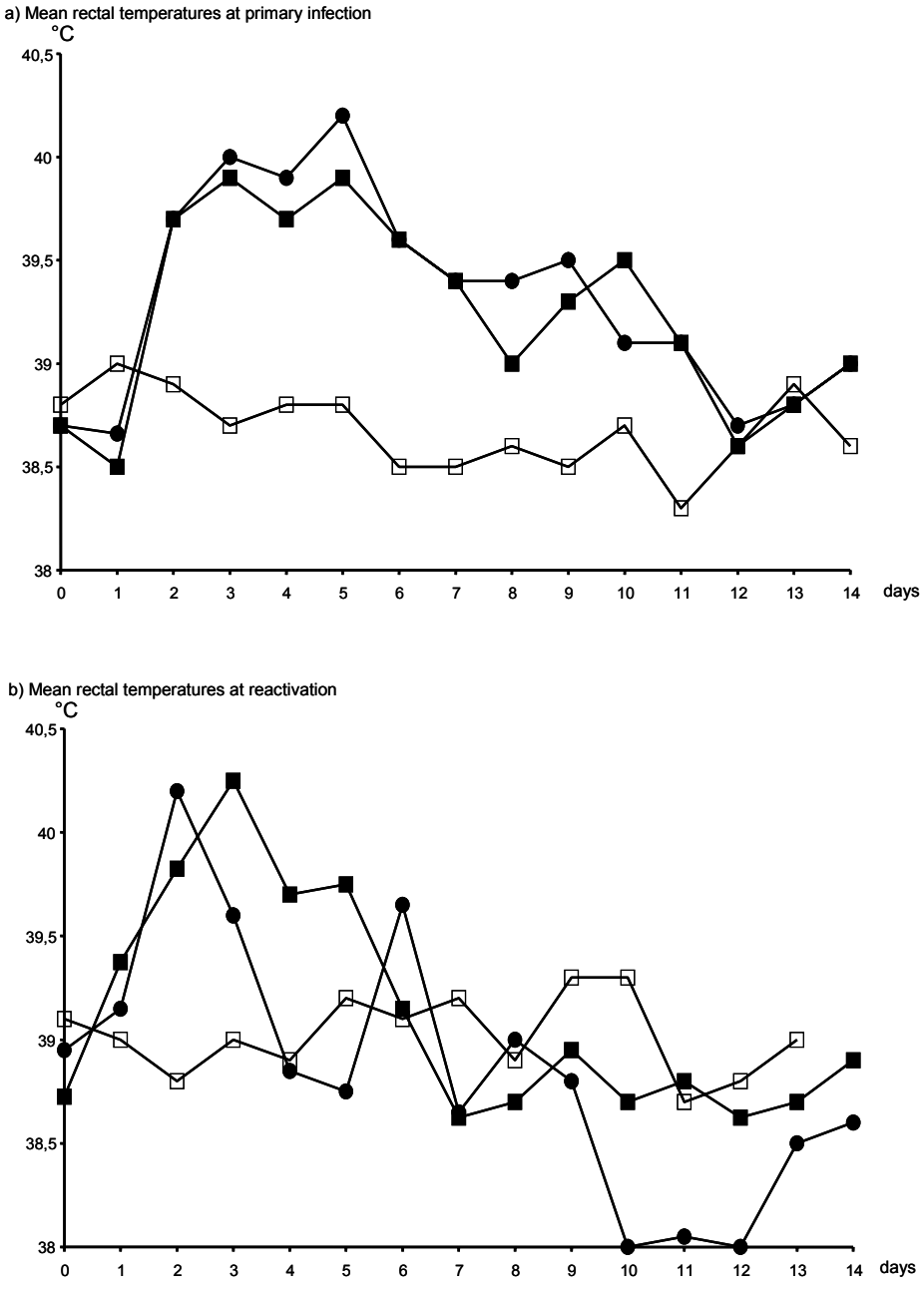
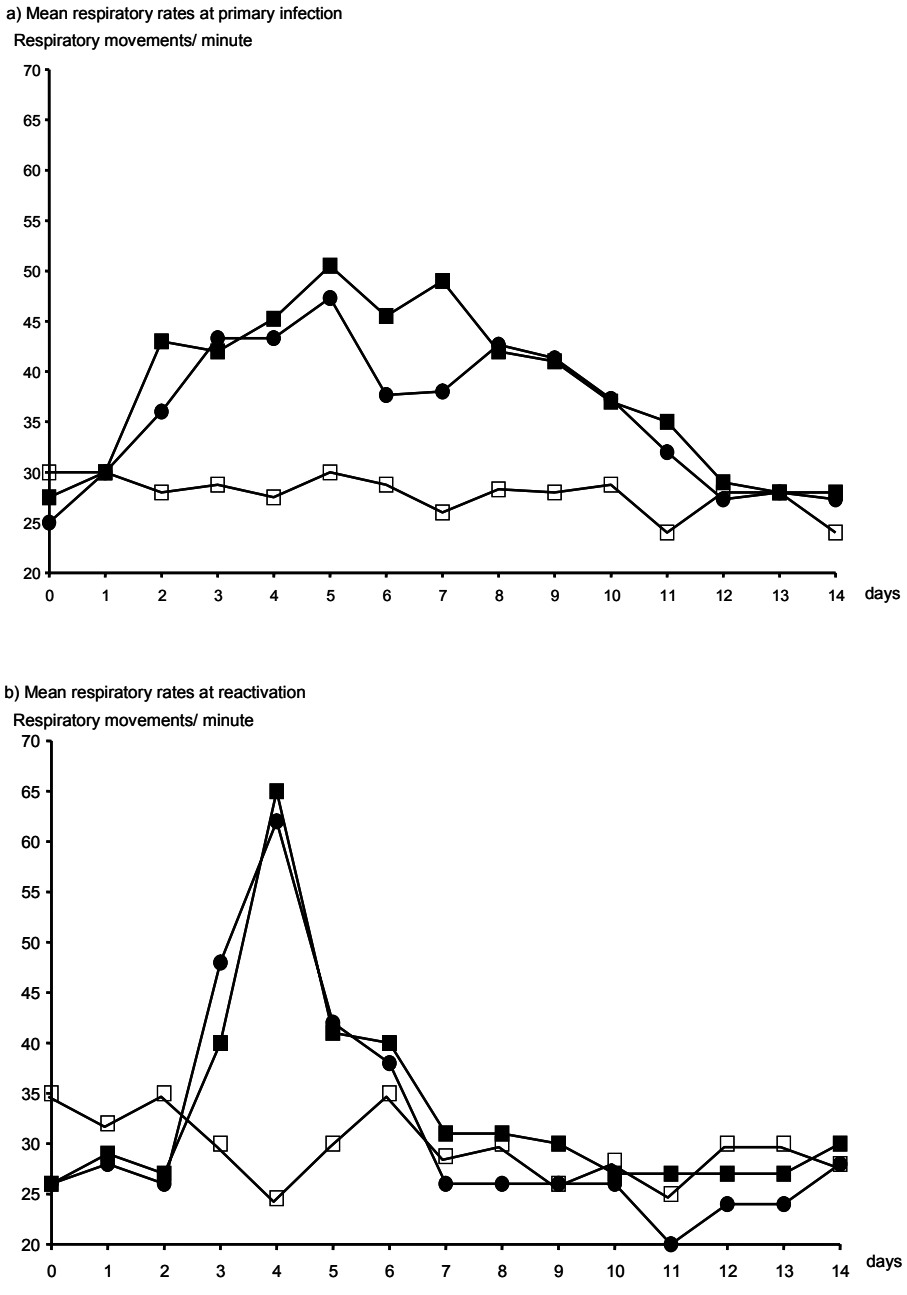


Figure 3. Mean respiratory rates of calves after inoculation. a) during acute infection; b) upon reactivation. Full squares: EVI123/98; black circles: SV265/96 empty squares: control calves.



Virus shedding following primary infection and reactivation

Virus of the expected subtype was isolated from nasal swabs from all infected calves from day 1 until day 14 pi, from calves in both groups (Figure 4a). No cross-contamination was detected, neither after primary infection nor at reactivation, as evidenced by the differential IPMA (data not shown). The amounts of infectious virus shed by calves infected with BHV-1.1 were significantly higher than those from BHV-1.2a-infected calves in samples from days 2 and 3 pi; after that period, both BHV-1.1 and BHV-1.2a were recovered to similar titres, up to day 14 pi. Ocular virus shedding was initially detected on day 1 pi on calf 127 (infected with BHV-1.2a), which shed a large amount of infectious virus ($10^{6.3}$ $TCID_{50}$/mL). However, all other calves started to shed virus by day 4 pi (Figure 4b). Virus shedding was detected at least until day 14 pi, when sampling was discontinued (Figure 4b).

At reactivation, nasal virus shedding was detected from days 2 to 9 pr (Figure 4b). Ocular virus shedding was detected from days 3 to 9 pr (Figure 5b). Control calves were negative at virus isolation attempts throughout the experiment.

Figure 4. Virus shedding during acute infection. a) nasal b) ocular. Infectious virus titres expressed in $\log_{10}$ of the reciprocal of 50% tissue culture infective doses/ 50 µl ($TCID_{50}$) Full squares: EVI123/98; black circles: SV265/96 empty squares: control calves.

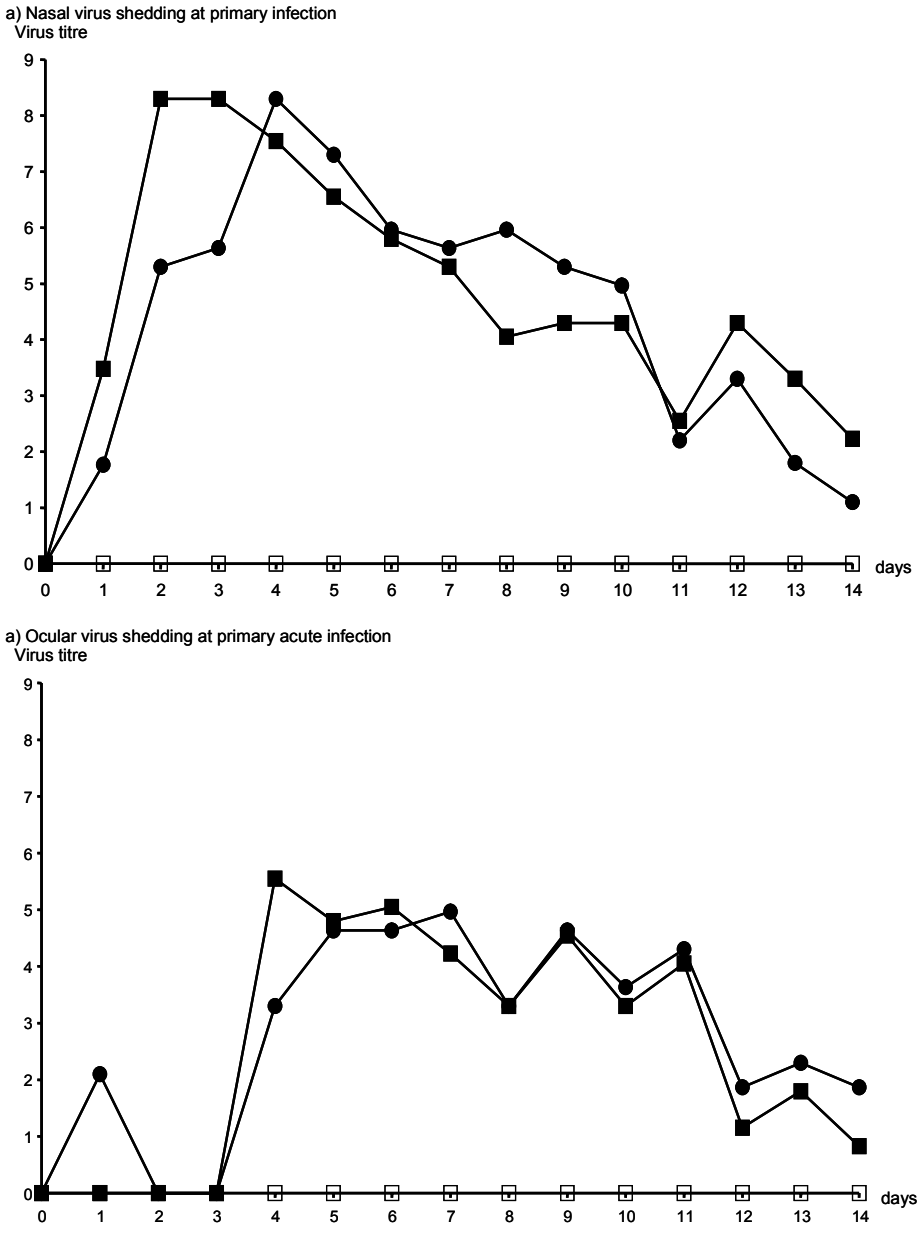
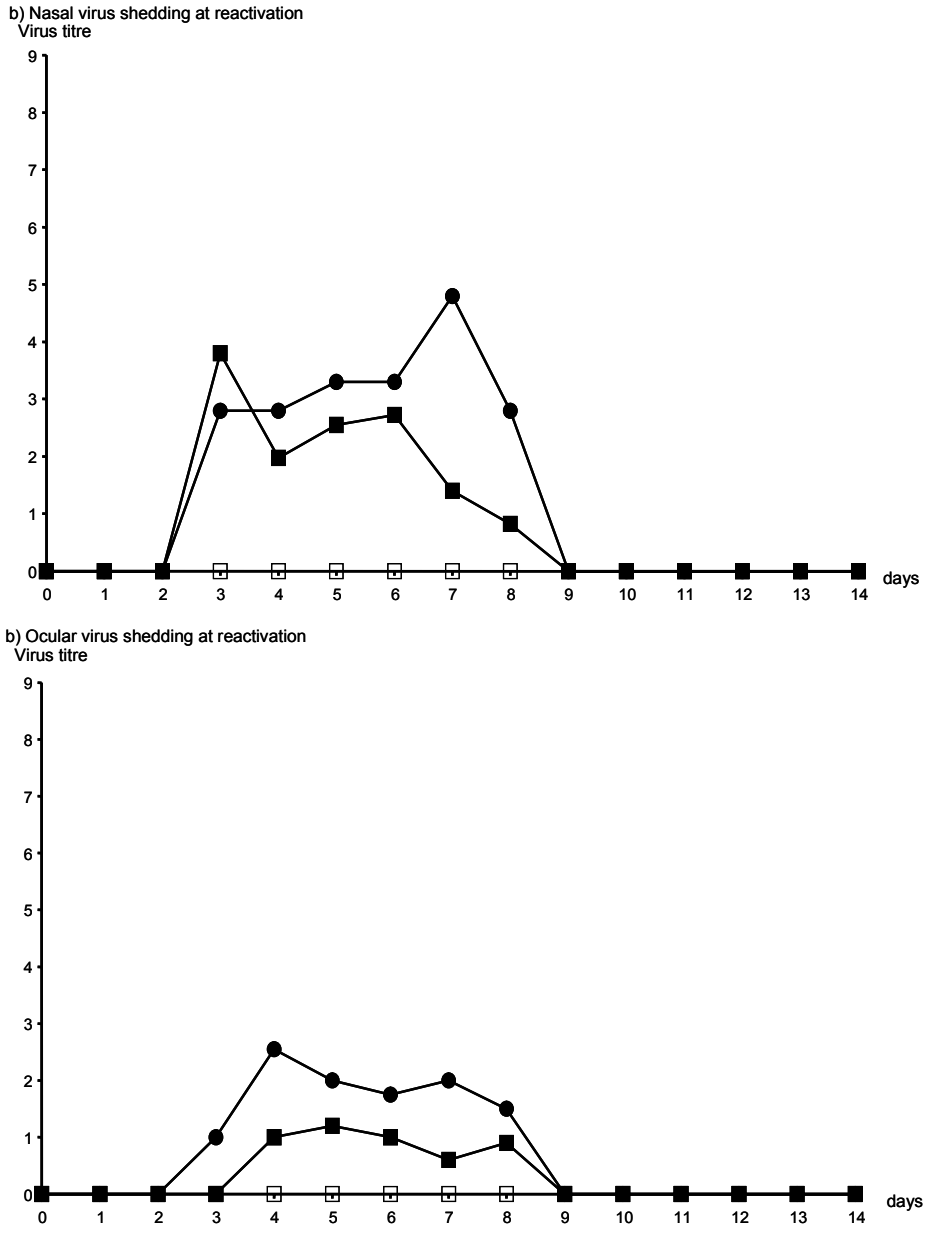


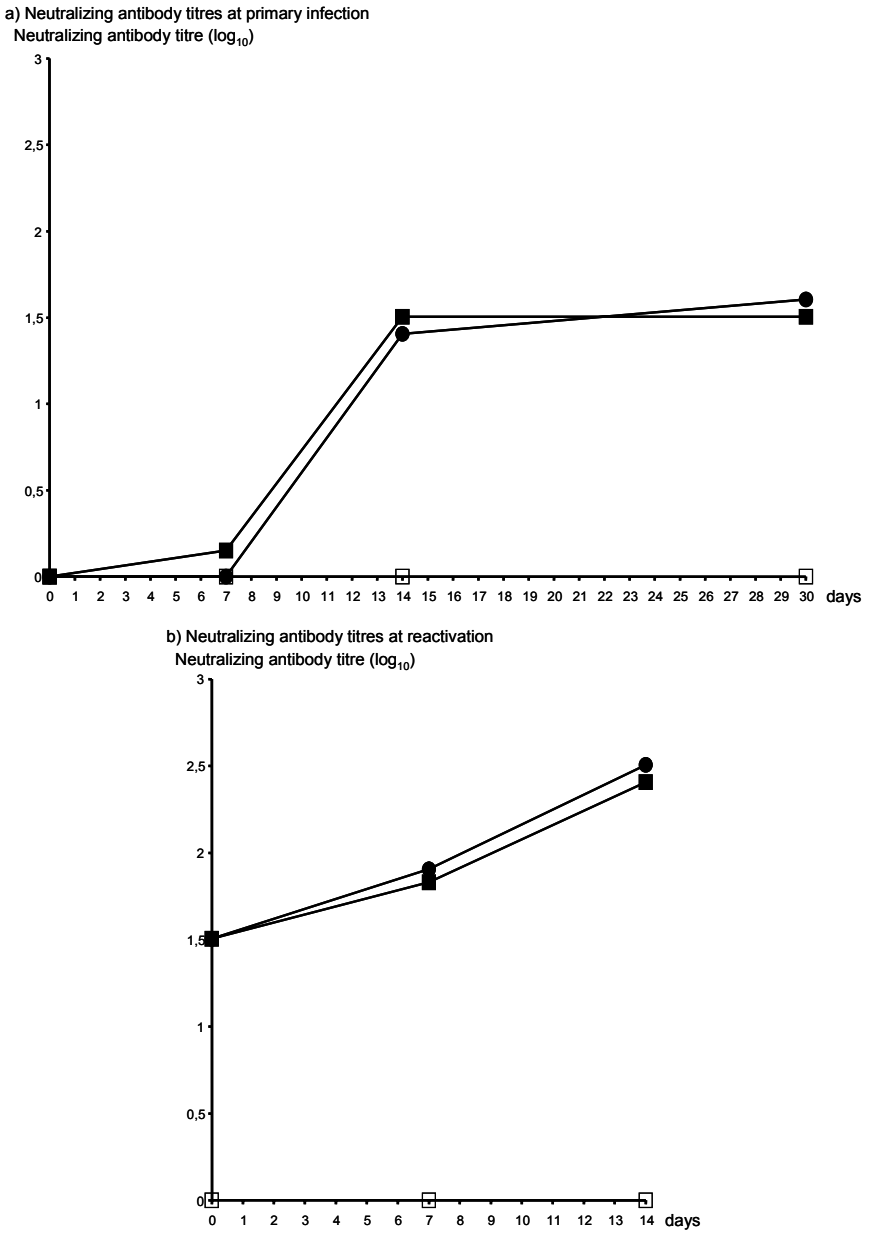
Figure 5. Virus shedding upon reactivation a) nasal b) ocular. Infectious virus titres expressed in $\log_{10}$ of the reciprocal of 50% tissue culture infective doses/ 50 µl ($TCID_{50}$). Full squares: EVI123/98; black circles: SV265/96 empty squares: control calves.



Neutralizing antibody responses

None of the calves had BHV-1 neutralizing antibodies previous to the experiments. On day 7 pi, low titres of neutralizing antibodies were present in 3 of the calves infected with BHV-1.1 and in two of the calves infected with BHV-1.2a. By day 14 pi, seroconversion was evident in all inoculated calves. Neutralizing antibody titres remained at low to moderate levels (above 1:16 or 1:1.2 $\log_{10}$) at least until day 30 pi, when sampling was discontinued (Figure 6a). At reactivation, at 180 days pi, calves still had low to moderate levels of neutralizing antibodies, whose titres increased significantly on day 194 pi (Figure 6b). Control calves remained negative for neutralizing antibodies throughout the experiment.

Figure 6. Neutralizing antibodies a) after inoculation and b) upon reactivation. Full squares: EVI123/98; black circles: SV265/96 empty squares: control calves. Control calves remained seronegative throughout the experiment. Titres expressed in $\log_{10}$ of the reciprocal of the antibody titre.



DISCUSSION

Both viruses inoculated in the presnt study (BHV-1.1 and BHV-1.2a) were highly virulent for three months old calves. Other researchers have met difficulties in experimentally reproducing BHV-1.1 clinical signs (Kaashoek et al. 1995). Here, no difficulties were found in demonstrating the pathogenicity of both virus subtypes. It has been observed previously that the overall responsiveness of younger calves (2 weeks old or younger) to BHV-1 experimental infections may be highly variable (Kaashoek 1994). On the other hand, calves older than 5 months seem less susceptible to infection (Msolla et al. 1983b). In view of that, and based on our own previous experience with BHV-1 inoculations, an option was made for conducting the experiments here described on three months old calves. This lead to more consistent and fairly reproducible findings, as revealed by the analysis of the outcome of the inoculations.

Viruses of both subtypes were shed with no significant differences in infectious titres and for similar lengths of time, except for a short period on the first 3 days pi. Virus shedding was also very similar to the results of Edwards et al. (1991) with BHV-1.1 and BHV-1.2b. The duration of virus shedding was slightly longer than observed by others when studying other bovine herpesvirus subtypes (Msolla et al. 1983b; Miller et al. 1984; Kaashoek et al. 1994; Meyer et al. 2001).

Regarding reactivation, both virus subtypes were reactivated with relative ease following dexamethasone administration, leading to the development of apparent signs of respiratory disease. at reactivation. Such as with primary acute infections, reactivation of BHV-1 latency have sometimes been difficult to reproduce experimentally (Edwards et al. 1990; Meyer et al. 2001). BHV-1 strains that apparently reactivate "more easily than others" have been documented (Kaashoek 1995). The viruses used in the present study may be more easily reactivated than those used in previous studies. As such viruses were chosen as representative of Brazilian BHV-1 isolates, it is possible that the viruses circulating in the country are more easily reativated than those used in previous studies. However, it is also possible that other factors such as the age of calves at inoculation and the amount of virus inoculated may have had some influence on the pathogenicity following reactivation.

Others have used either younger (Edwards et al. 1990) or older animals (Kaashoek 1995), while inoculating similar amounts of virus, both with varying levels of succes at reactivation. In the present study, both virus subtypes inoculated were similarly reactivated and caused no significantly different pathogenic effects on calves at reactivation. Likewise, viral excretion during reactivation was again similar in both groups. Nevertheless, despite the overall similar clinical scores, it is worth highlighting that one calf on the group infected with BHV-1.2a died on day 11 after reactivation. Histopathology and immuno-histochemistry revealed that the most striking finding was the BHV-1-associated hepatic necrosis. Although such kind of lesion has often been reported on aborted foetuses and generalized BHV-1 infections in newborns (Gibbs & Rweyemamu, 1977), this is actually the first mention to such lesions in a 11 months old calf. However, it was beyond the scope of the present study to examine the pathogenesis of such lesion in more detail. It is possible that individual variability may have brought up such unexpected outcome, since all calves were kept under similar conditions throughout.

The present work demonstrated that a BHV-1.2a isolate was capable of inducing as intense respiratory illness for the respiratory tract as BHV-1.1. Both subtypes (BHV-1.1 and BHV-1.2a) were able to induce clinically undistinguishable respiratory disease in calves, either subsequent to primary infection or following reactivation. At least with the viruses examined here, different subtypes were not paralleled by differences in pathogenicity for the respiratory tract.

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Capítulo 2

Analysis of isotype-specific antibody responses to bovine herpesviruses 1.1 and 1.2a allows an estimate on the stage of infection[1]

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ABSTRACT – Specific IgM, IgA, IgG1, IgG2, as well as neutralizing antibody responses were evaluated in sera of calves experimentally infected with two isolates of bovine herpesvirus type 1 (BHV-1) of distinct subtypes (subtype 1, BHV-1.1; subtype 2a, BHV-1.2a). For that purpose, class and subclass-specific indirect enzyme immunoassays (ELISAs) were developed and used along with standard neutralizing antibody assays. No significant differences were observed on the antibody responses induced by either BHV-1 subtype. The antibody responses subsequent to primary acute infection were characterized by a rise in IgM and IgA between days 2 and 14 post inoculation (pi). Neutralizing antibodies were detected since day 14 pi. IgG1 was detected from days 11 to 30 pi. IgG2 was only detected on day 30 pi. Reactivation of infection induced by dexamethasone administration induced a significant rise in IgA levels, whereas IgG1 and IgG2 levels, which were already elevated at the beginning of the reactivation process, showed no significant rise. IgM levels remained at trough levels following reactivation. Neutralizing antibodies were significantly augmented by day 194 pi. The combined analysis of class and subclass-specific antibody responses allowed an estimate on the phases of infection calves were undergoing. Thus, during recent acute infection (from days 2-3 to 14 days pi) only IgM and IgA antibodies could be detected; from days 14 pi to 30 pi, low to moderate levels of neutralizing antibodies (range 1:16 to 1:64), plus IgM, IgA and IgG1, but not IgG2 could be detected; during late acute infection (30 days pi), neutralizing antibodies remained at low to moderate levels, IgM was low or in significant decline and IgA, IgG1 and IgG2 were elevated; finally, after reactivation, neutralizing antibodies were significantly boosted, IgM was at trough levels, IgA was significantly risen and IgG1 and IgG2 remained at high levels. These results suggest that it is possible to make an estimate on the dynamics of

BHV-1 infections based on the analysis of class and subclass-specific antibody responses. Such information might be particularly useful for the control of BHV-1 infections in herds.

INDEX TERMS: BHV-1, bovine herpesvirus type 1, infectious bovine rhinotracheitis, immunoglobulin subclasses, ELISA

INTRODUCTION

Bovine herpesvirus type 1 (BHV-1) is a member of the *Herpesviridae* family, subfamily *Alphaherpesvirinae* (Turin et al., 1999). BHV-1 is a widespread pathogen of cattle, recognized as the causative agent of a number of clinical conditions including infectious bovine rinotracheitis (IBR), infectious pustular vulvovaginitis/balanopostitis (IPV/IPB). In addition, BHV-1 is a major cause of abortion in cattle (Turin et al., 1999). BHV-1 has been subdivided into subtypes 1, 2a and 2b with basis on restriction fragment length positioning (RFLP) of viral DNA. Distinct subtypes have also been related to somewhat different clinical syndromes. Thus, typical or classical BHV-1 strains, presently classified as subtype 1 (BHV-1.1), have been associated to respiratory and genital disease as well as abortions (Turin et al., 1999), subtype 1.2a has been associated to respiratory disease and abortions, whereas BHV-1.2b has been associated to genital disease but to date never linked to abortions (Miller, 1991; Van Oirschot, 1995).

Primary infection with BHV-1 induces a strong humoral and cell-mediated immune response in the host. Class and subclass-specific immunoglobulin levels were also studied following BHV-1 infection, reinfection and reactivation (Bradshaw and Edwards, 1996; Edwards et al., 1991; Madic et al., 1995a; Madic et al., 1995b). After a primary experimental infection, calves develop an immune response revealed by transient IgM and IgA antibodies, followed by persistent IgG1 and IgG2 responses (Edwards et al., 1991; Madic et al., 1995a,b). BHV-1.2b induced a lower level of humoral immune response compared to BHV-1.1 (Edwards et al., 1991). BHV-1.2a immune responses have not so far been studied. As BHV-1.1 and BHV-1.2a are highly prevalent in Brazil (D'Arce et al.,

2002), the present study was undertaken to examine IgM, IgA, IgG1 and IgG2 as well as neutralizing humoral responses following experimental infections with BHV-1.1 and BHV-1.2a in cattle during acute disease, latency and reactivation. In addition, we examined whether the profile of antibody responses could provide information about the stages of infection (acute, latent or reactivation) the calves were undergoing.

2. Materials and Methods

2.1.Viruses and cells

Madin Darby bovine kidney cells (MDBK; ATCC CCL22) free of bovine herpesviruses and of bovine viral diarrhea virus (BVDV) were cultured in Eagle's minimal essential medium (E-MEM) supplemented with 6 % fetal calf serum and enrofloxacin (Baytril, final concentration 10 mg/L). For virus multiplication, BHV-1.1 strain EVI 123/98 (D'Arce et al., 2002) and BHV-1.2a strain SV 265 (Souza et al., 2002) were inoculated onto nearly confluent monolayers of MDBK cells at a multiplicity of infection (m.o.i.) between 0.1 and 1, following standard procedures (Teixeira et al., 2001). When cytopathic effect (CPE) was evident in about 90 % of the monolayers, cells and supernatants were frozen at -70 ^{o}C, thawed, clarified by low speed centrifugation and used as virus stocks. Titres obtained were typically between of $10^{8.3}$ 50 % tissue culture infective doses per mL ($TCID_{50}$). Stocks were used for serum neutralisation assays as well as for the preparation of the ELISA antigen.

2.2. Inoculation and sampling of calves

Nine, three to four months old calves, seronegative for BHV-1 and BHV-5, were kept in isolation units. Four calves were infected intranasally with 8 mL of cell culture medium containing $10^{8.3}$ $TCID_{50}$/mL of BHV-1.1 strain EVI 123/98 (D'Arce et al., 2002). Three other calves were infected by the same route with the same amount of the BHV-1.2a strain SV 265. Two remaining calves were mock infected with virus-free culture medium. Six months after challenge, all calves were subjected to intravenous administration of dexamethasone (0.1 mg per kg of body weight) for 5 consecutive days, aiming to reactivate latent infection. Blood samples were collected by jugular venipuncture on days 0, 2, 4, 7, 9, 11, 14, 30, 180, 187 and 194 days post inoculation (pi). Animals were monitored daily after infection or reactivation for clinical signs, which were scored on severity, and virological findings, using sampling of nasal and ocular swabs. A more detailed description of the clinical and virological findings will be provided elsewhere (Spilki et al., 2004).

2.3. Serum neutralisation assay

The serum neutralisation assay (SN) was performed by the constant virus-variable serum method as described by House and Baker (1971), modified as follows: dilutions (1:2 to 1:2048) of sera to be tested were prepared in quadruplicate in 96 well microtitre plates. An equal volume of medium (50 μl/well) containing 100 $TCID_{50}$ of virus (BHV-1 EVI 123/96) was added to each well. The mixtures were incubated at 37 ^{o}C for one hour. Fifty microlitres of an MDBK cell suspension containing 1.5 to 3 x 10^4 cells were added to each well and plates incubated at 37^{o}C under a 5 % CO_2 atmosphere. Readings were performed daily at 24, 48, 72, 96 and 120 hours pi, in search for the characteristic CPE. Neutralizing

antibody titres were calculated by the method of Spearman and Kärber (Lorenz and Bögel, 1973).

2.4. Preparation of ELISA antigen.

Tissue culture flasks (125 cm^2) were infected with EVI 123/98 virus at a low m.o.i. as above. When CPE was evident in about 60 % of the monolayer, the medium was removed and cells overlaid with 0.2 % OGP (n-octyl-β-D-glucopyranoside, Sigma) in phosphate buffered saline (NaCl 8.5 g, $Na_2HPO_42H_2O$ 1.55 g, $NaH_2PO_4H_2O$ 0.23 g, distilled H_2O q.s.p. 1000 mL, pH 7.2) for 2 h at 4 °C. Next, the cells were scraped off the flasks and centrifuged at 1,500 x g to remove cell debris. The antigen so prepared was aliquoted and stored at -70 °C.

2.5. Class and subclass-specific ELISAs

For the detection of IgA, IgM, IgG1 and IgG2, class or subclass-specific indirect ELISAs were developed. The variables within the tests were optimized (antigen concentration, serum and secondary antibody dilutions) by testing pooled control positive and negative sera. For each ELISA, an appropriate anti-bovine class or subclass-specific peroxidase conjugate, prepared in sheep (Serotec, UK) was used. ELISA plates were coated/ with an appropriate dilution of the antigen (1:3200) in bicarbonate buffer overnight at 4 °C. After adsorption of the antigen, plates were washed once with 100 μL of PBST-20 (0.5 % Tween 20 in PBS), filled with another 100 μL of PBST-20 and left to stand for 1 h at room temperature. The sera under test were diluted 1:5 in PBST-20 and added to

duplicate wells in plates. After 1 h incubation at 37 °C, the plates were washed three times with PBST-20 and incubated with the appropriate class or subclass-specific peroxidase conjugate (diluted in PBS) for 1 h at 37 °C. After three other washings with PBST-20, 100 µL of the substrate ortho-phenylenediamine (OPD; Sigma) with 0.03 % H_2O_2 were added. After 5 minutes of incubation at 37 °C, the reaction was stopped by the addition of 2M H_2SO_4. The optical density (OD) was determined at 492 nm in a Multiskan (Titertek) ELISA reader. Levels of antibodies were recorded and plotted as the percentual mean optical density (%OD) for each group of calves. The %OD was calculated using the formula:

$$\%OD = \frac{\text{OD of serum under test}}{\text{Mean OD of negative control sera in each plate}} \text{ X } 100$$

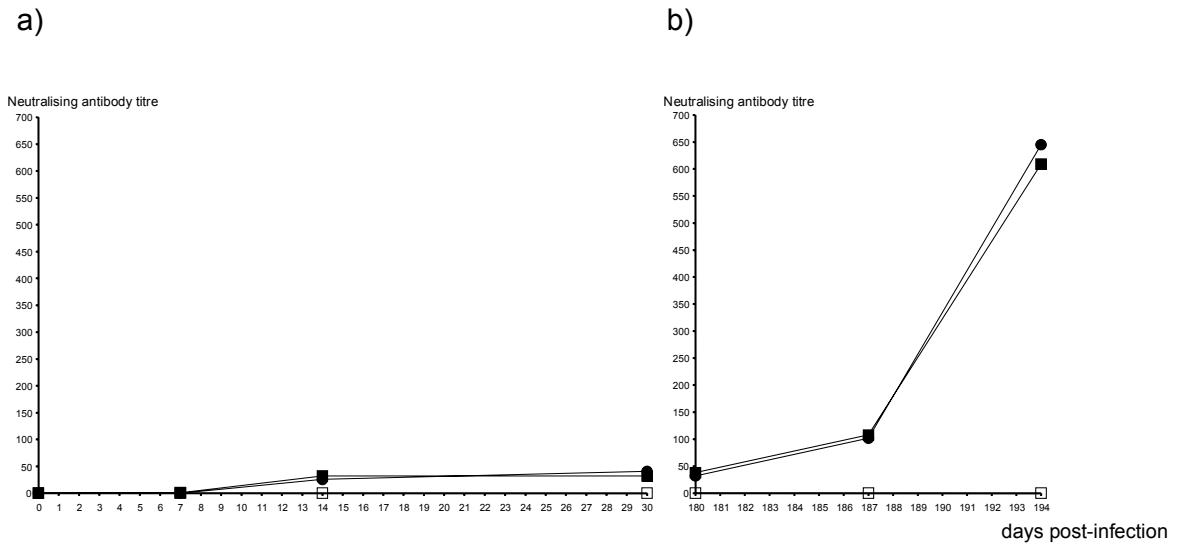
Differences between infected and control groups were analysed with the program Minitab® Release 11.1 for Windows (Minitab Inc., USA). Throughout this study, when applied to ELISAs, the term "significant" refers to %OD equal to or greater than twice the %OD under comparison. When applied to neutralization assays, "significant" means equal to or greater than four times the titre under comparison.

3. Results

Both groups of calves inoculated with either BHV-1.1 or BHV-1.2a showed severe signs of infectious bovine rinotracheitis 3 days after inoculation, with fever, apathy, anorexia, respiratory distress, coughing, sneezing, redness and erosion on the nasal mucosae, mucous nasal discharge, conjunctivitis and lachrymation. No significant differences were detected between the severity of clinical disease or virus shedding from nasal and ocular secretions in calves infected with either virus.

Neutralizing antibodies were present to low titres on day 14 pi and remained at low to moderate levels (range 1:16 to 1:64) until day 30 pi (Figure 1a). After reactivation, a significant rise (range 1:256 to 1:1024) in neutralizing antibody titres was observed (Figure 1b). Uninfected control calves remained seronegative throughout.

Figure 1. Serum neutralizing antibodies to EVI123/98 infected calves (full squares), SV265/96 infected calves (full triangles) and control uninfected calves (empty squares). a) after primary infection; b) at reactivation. Neutralizing antibody titres expressed as the reciprocal of the geometric mean of the neutralizing antibodies for each group.



IgM, IgA, IgG1 and IgG2 responses measured by the ELISAs were not significantly different in both groups of calves infected with either virus subtype. IgM titres started to rise on day 2 pi, reaching peaks between days 7 and 14 pi and displaying a tendency to decrease significantly towards day 30 pi (Figure 2a). IgA responses were detectable from day 2 pi and remained at high levels (as judged by %OD above 450) at least until day 30 pi, when sampling was discontinued (Figure 3a). IgG1 was initially detected at day 11 pi, rising to maximum levels on day 30 post inoculation (Figure 4a). IgG2 levels were detected only on samples collected on day 30 pi (Figure 5a).

Figure 2. ELISA analysis of IgM specific antibody in sera of calves experimentally infected with BHV-1.1 or BHV1.2a. Data expressed as mean percentual optical densities (%OD; see text for methods) for each group of calves. a) at primary acute infection; b) at reactivation. Full squares: BHV-1.1 infected calves; full triangles: BHV-1.2a infected calves. Empty squares: control uninfected calves.

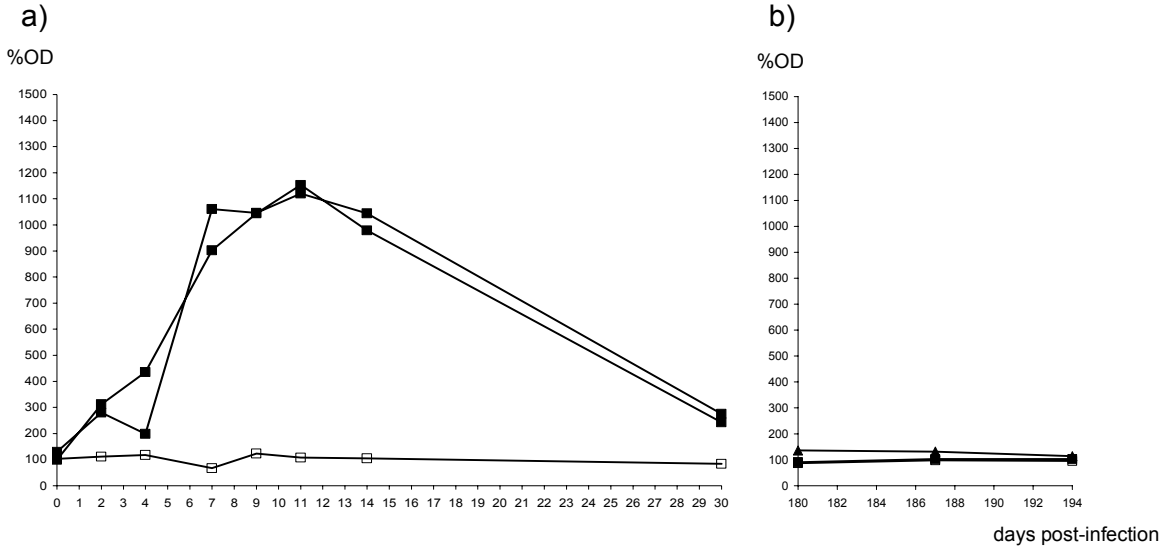


Figure 3. ELISA analysis of IgA specific antibody in sera of calves experimentally infected with BHV-1.1 or BHV1.2a. Data expressed as mean percentual optical densities (%OD; see text for methods) for each group of calves. a) at primary acute infection; b) at reactivation. Full squares: BHV-1.1 infected calves; full triangles: BHV-1.2a infected calves. Empty squares: control uninfected calves.

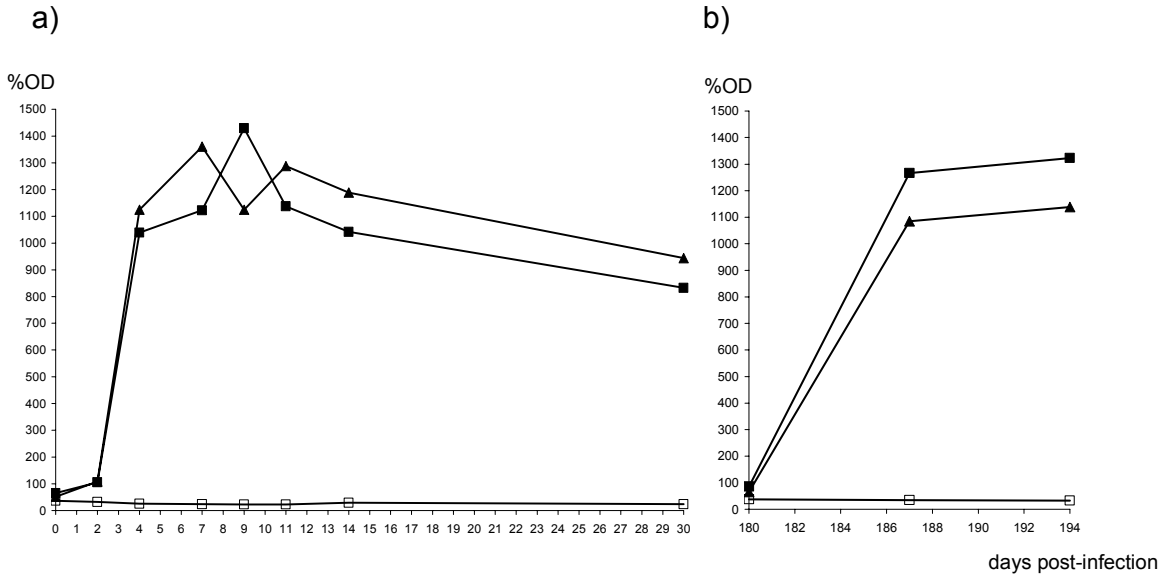


Figure 4. ELISA analysis of IgG1 specific antibody in sera of calves experimentally infected with BHV-1.1 or BHV1.2a. Data expressed as mean percentual optical densities (%OD; see text for methods) for each group of calves. a) at primary acute infection; b) at reactivation. Full squares: BHV-1.1 infected calves; full triangles: BHV-1.2a infected calves. Empty squares: control uninfected calves.

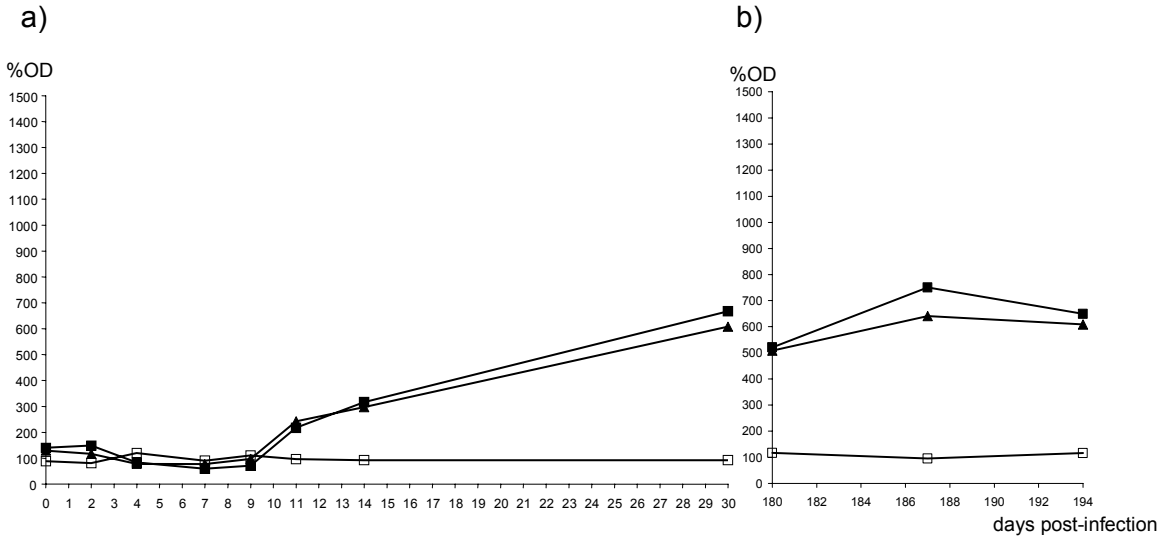
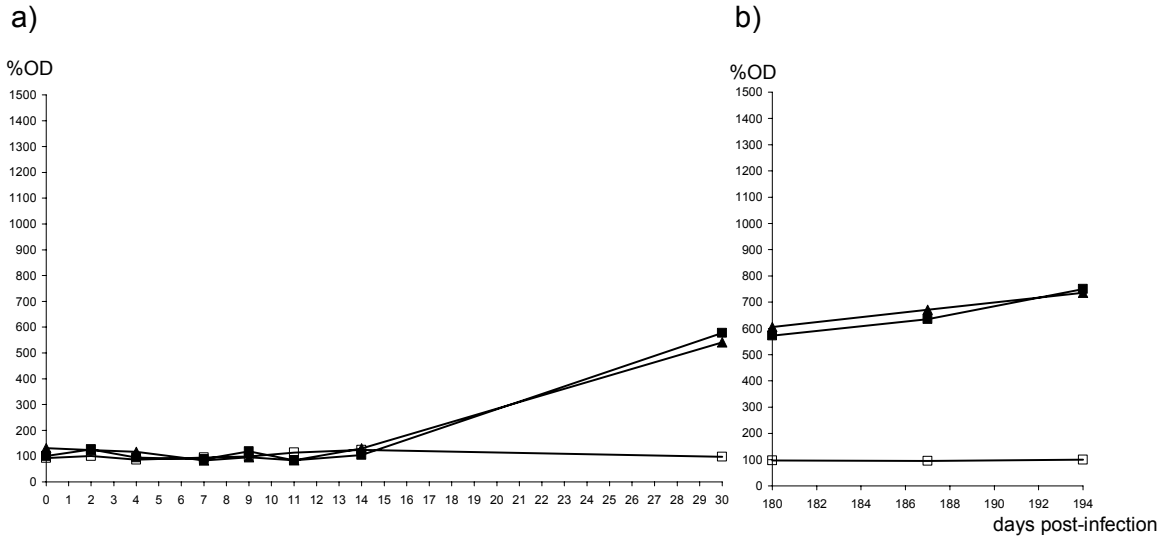


Figure 5. ELISA analysis of IgG2 specific antibody responses in sera of calves experimentally infected with BHV-1.1 or BHV1.2a. Data expressed as mean percentual optical densities (%OD; see text for methods) for each group of calves. a) at primary acute infection; b) at reactivation. Full squares: BHV-1.1 infected calves; full triangles: BHV-1.2a infected calves. Empty squares: control uninfected calves.



At the beginning of the corticosteroid administration, at 180 days pi, IgM was at trough levels and remained so for the next 14 days, when sampling was discontinued (Figure 2b). IgA (Figure 3b) was at trough levels, whereas IgG1 (Figure 4b) and IgG2 (Figure 5b) remained relatively high. Seven days later, IgA peaked with %OD values similar to those observed following primary acute infection. In addition, a slight though not significant increase in IgG1 and IgG2 levels was detected.

By comparing the patterns of antibody response, it was possible to estimate the stage of infection the calves were undergoing (Table 1). After primary infection, from day 2 to 3 pi up to day 15 pi, only IgM and IgA antibodies could be detected. At days 11 to 14 pi, IgA levels remained elevated and IgG1 antibodies became detectable. Neutralizing antibodies were initially detected on day 14 pi. IgG2 antibodies were only detected on day 30 pi (Figure 5a). Thus, as day 30 pi, calves had IgA, IgG1, IgG2, and significantly decaying levels of IgM. On day 180 pi., when dexamethasone administration began, IgG1 and IgG2 were still at levels similar to those on day 30 pi; IgM and IgA were at trough levels. On day 186, IgG1 and IgG2 remained at high levels, though not significantly elevated. However, IgA was significantly risen on day 186 pi. Neutralizing antibodies were significantly elevated on day 194 pi. These results allowed the formulation of the scheme proposed on table 1, which may be useful in estimating the stages of infection.

Table 1. Estimate of the status of infection with basis on the patterns of isotype and subclass specific anti-bovine herpesvirus 1.1 (BHV-1.1) or 1.2a (BHV-1.2a) antibodies.

Status of infection	Isotype-specific profile
Uninfected cattle	**IgM- IgA- IgG1- IgG2- SNA[a]-**
Up to day 15 post-infection	**IgM+ IgA+ IgG1- IgG2- SNA-or+**
Between days 15 and 30 post- infection	**IgM+ IgA+ IgG1+ IgG2+ SNA+**
During latency (after day 30 post infection)	**IgM- IgA- IgG1+ IgG2+ SNA+**
After reactivation	**IgM- IgA+ IgG1+ IgG2+ SNA+**

+ = present ; - = absent ; [a] = serum neutralizing antibodies

4. Discussion

It was demonstrated here that both BHV1.1 and BHV-1.2a were able to elicit similar patterns of humoral antibody responses of all classes and subclasses under study. For BHV-1.1 and BHV-1.2b, other authors reported marked differences in the kinetics of subclass profiles of IgG antibodies. In addition, others reported that lower levels of neutralizing antibodies, IgG1 and IgG2, were induced by BHV-1.2b than by BHV-1.1 (Edwards et al.,

1991). Here, not only the patterns of classes and subclasses of antibodies were similar for both virus subtypes, but also the levels of antibodies detected were not statistically significant.

The initial stages of the immune response detected in the present study following inoculation of both virus subtypes revealed a typical primary antibody response. Primary humoral immune responses are characterized by the proliferation and differentiation of IgM-expressing B lymphocytes into either antibody-secreting effector cells or memory cells (Abbas et al., 1994). This process is accompanied by maturation of antibody affinity and also by T helper cell-mediated heavy chain class switching to other isotypes, including IgG and IgA, so that these predominate in secondary and late responses, when IgM antibodies are usually not detected (Graham et al., 1999). So was the antibody profile detected here, as will be discussed below.

Regarding the neutralizing antibody profile detected here, it did not allow for detailed estimates on the stages of infection. Neutralizing antibodies would, as expected, reveal the primary acute infection, provided that paired serum samples (the first collected at the initial stages of the acute infection, and the second collected at least to two to three weeks later) could be titrated in parallel. Other than that, neutralizing antibodies could not aid in estimating the stages of infection. Antibodies of all subclasses may be capable of inducing neutralization of the virus *in vitro* (Edwards et al., 1991; Engels and Ackerman, 1996; Madic et al., 1995a). However, no neutralizing antibodies were detected only on day 14 after inoculation, suggesting that early IgM antibodies played a minor role - if any at all - in neutralization. At reactivation, neutralizing antibodies were significantly elevated; however, such elevation was not accompanied by a corresponding significant rise in IgG1

or IgG2. On the other hand, IgA levels were risen at reactivation; such rise was only matched by increased neutralization one week later, at 194 days pi. Therefore, it difficult to correlate neutralization directly with the classes and subclasses of antibodies studied here. It is possible that changes in antibody affinity, such as those detected following a second antigenic stimulus (Graham et al., 1999), may have been responsible for the neutralizing antibody responses detected here. It is of interest to note that, after reactivation, neutralizing antibody titres were elevated as if calves had been subjected to such a second antigenic stimulus. Corticosteroid treatment apparently had no inhibitory effect on neutralizing antibody titres, which reached significantly higher titres than those obtained after primary infection. Thus, corticosteroi induced reactivation induced a booster effect on neutralizing antibodies, rather than inhibiting its production.

On day 2 pi, IgM antibodies were initially detected and gradually rose up to day 14 pi; a significant tendency to decay was detected on day 30 pi, when testing was discontinued and IgM was close to trough levels. During reactivation, no rise in IgM levels was detected. In some other herpesvirus infections, such as with human herpes simplex type 1, IgM levels can be elevated upon reactivation (Madic et al., 1995b). For the BHV-1 subtypes examined here, IgM response was only elevated after the primary infection, thus following the usually expected profile of IgM detection (Graham et al., 1999). This may be of use for distinguishing the phases of infection, in that the analysis of the IgM profile can provide a means of differentiating acute infection from reactivation.

IgG1 levels were firts detected by day 11 pi whereas IgG2 was only evidenced by day 30 pi; from then on, during the subsequent phases of infection examined, both IgG1 and IgG2 were present concomitantly and to similar titres. As the presence of IgG1 only, but

not IgG2, is indicative of a recent primary infection, the determination of IgG1 and IgG2 levels may be used to estimate such early acute infection.

Six months after infection, IgG1 and IgG2 levels were still similar to those detected on day 30 pi. Following corticosteroid induced reactivation, small though not significant rises in IgG1 and IgG2 levels were found. IgA levels were low at 180 days pi, but reactivation led to a new IgA peak. IgA was also found by others to rise following reactivation (Madic et al., 1995b). Therefore, the presence of elevated levels of IgA, concomitant with elevated IgG1 and IgG2 and absence of IgM may provide additional evidence to indicate that calves have been through a recent reactivation process.

In the present study, it was shown that it is possible to estimate the status of BHV-1 infection with basis on the serological analysis of the antibody responses induced in calves, provided that levels of IgM, IgA, IgG1 and IgG2 as well as neutralizing antibodies can be measured and compared (Table 1). It has been a hallmark in serology to request paired serum samples to identify the causes of acute infections. Here, in infections with both BHV-1 subtypes tested, it was shown that with simple class and subclass-specific ELISAs, it was possible to estimate with fair accuracy the stages of infection the animals were undergoing. The proposed scheme seem to fit adequately under the conditions of the present study, with a small number of animals and controlled conditions of infection. Although not evaluated here, on a herd basis, it may be possible to make estimates on the stages of infection within flocks, what could be particularly useful for the control of BHV-1 infections in herds, thus allowing appropriate control measures to be taken. Such possibility is under study at the present moment.

The experiments in the present work were performed with subtypes BHV-1.1 and BHV-1.2a; these induced virtually identical profiles of antibody responses. Other authors have also found similar profiles for BHV-1.2b (Bradshaw and Edwards, 1996; Edwards et al., 1991; Madic et al., 1995a; Madic et al., 1995b). Therefore, we believe it is quite likely that the scheme proposed here for the identification of the stages of infection with BHV-1.1 and BHV-1.2a would also be applicable to BHV-1.2b infections. Nevertheless, its validity in examining the responses to bovine herpesviruses other than those tested here must await further experimental evidence.

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Capítulo 3

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[1]

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ABSTRACT - We have previously reported the construction of a glycoprotein E-deleted (gE^-) mutant of bovine herpesvirus type 1.2a (BHV-1.2a). This mutant, $265gE^-$, was designed as a vaccinal strain for differential vaccines, allowing the distinction between vaccinated and naturally infected cattle. In order to determine the safety and efficacy of this candidate vaccine virus, a group of calves was inoculated with $265gE^-$. The virus was detected in secretions of inoculated calves to lower titres and for a shorter period than the parental virus inoculated in control calves. Twenty one days after inoculation, the calves were challenged with the wild type parental virus. Only mild signs of infection were detected on vaccinated calves, whereas non-vaccinated controls displayed intense rhinotracheitis and shed virus for longer and to higher titres than vaccinated calves. Six months after vaccination, both vaccinated and control groups were subjected to reactivation of potentially latent virus. The mutant $265gE^-$ could not be reactivated from vaccinated calves. The clinical signs observed, consequent to reactivation of the parental virus, were again much milder on vaccinated than on non-vaccinated calves. Moreover, parental virus shedding was considerably reduced on vaccinated calves at reactivation. In view of its attenuation, immunogenicity and protective effect upon challenge and reactivation with a virulent BHV-1, the mutant $265gE^-$ was shown to be suitable for use as a BHV-1 differential vaccine virus.

INDEX TERMS: BHV-1, differential vaccine, gE deletion, IBR.

RESUMO - Em estudo prévio relatamos a construção de um mutante do Vírus da Rinotraqueíte Infecciosa Bovina (IBR) ou Herpesvírus Bovino tipo 1.2a (BHV-1.2a), do qual foi deletado o gene que codifica a glicoproteina E. Esse mutante (265gE$^-$) foi construido a partir de uma amostra autóctone do vírus, tendo como objetivo seu uso como amostra vacinal em vacinas diferenciais, capazes de permitir a diferenciação entre animais vacinados e infectados com vírus de campo. Para determinar a atenuação e eficácia do 265gE$^-$ como imunógeno, bezerros foram inoculados por via intranasal com $10^{6,9}$ $DICC_{50}$ do mesmo. O vírus foi detectado em secreções dos animais inoculados em títulos mais baixos e por um período mais curto do que a amostra virulenta parental, inoculada em animais controle. Vinte e um dias após, os animais inoculados com o vírus mutante foram desafiados com a amostra parental, apresentando somente sinais leves de infecção. Os animais controle apresentaram intensa rinotraqueíte e excretaram vírus em títulos mais elevados e por mais tempo do que os vacinados. Seis meses após a vacinação, foi examinada a capacidade de reativação da infecção nos bezerros, através da administração de corticosteróides. O mutante 265gE$^-$ não foi reativado dos animais vacinados. Os sinais clínicos conseqüentes à reativação do vírus parental foram muito atenuados nos animais vacinados, em comparação com os não vacinados. Além disso, a excreção de vírus de campo foi consideravelmente reduzida nestes últimos. Em vista de sua atenuação, imunogenicidade e efeito protetivo frente ao desafio com uma amostra virulenta de BHV-1 e subseqüente reativação, o mutante 265gE$^-$ demonstrou apresentar grande potencial para ser utilizado como vírus vacinal em vacinas diferenciais contra o BHV-1.

TERMOS DE INDEXAÇÃO: BHV-1, IBR, vacina diferencial, gE

INTRODUCTION

Bovine herpesvirus type 1 (BHV-1), a major pathogen of cattle, is the agent of infectious bovine rhinotracheitis/infectious pustular vulvovaginitis (IBR/IPV). The virus is associated with a number of other clinical syndromes, including pustular balanopostitis, conjunctivitis, infertility and abortion (Gibbs & Rweyemamu 1977). BHV-1 strains have been subdivided into three distinct genotypes, BHV-1.1, BHV-1.2a and BHV-1.2b (Metzler et al. 1985). BHV-1.1 and 1.2a seem to be the most pathogenic, being associated with respiratory disease and abortion, while BHV-1.2b usually displays moderate to low pathogenicity and has not so far been associated to abortion (Metzler et al. 1985).

The BHV-1 genome encodes several glycoproteins (gps) that are expressed on the viral envelope and membranes of infected cells. While some some of these are essential for virus replication and mediate different biological functions (Rebordosa et al. 1996), other gps are not essential for virus multiplication, thus representing potential targets for deletions aiming the development of differential vaccines. Glycoprotein E (gE), is one of such non-essential gps (Balan et al. 1994, Dingwell et al. 1994, Dingwell et al. 1995, Dingwell & Jonhson 1998, Maresova et al. 2001, Mettenleiter et al. 1987, Olson & Groose 1997). The gE gene is located in the unique short (U_S) region along the BHV-1 genome, consisting of a 1800 base pair (bp) fragment which codes for a polypeptide of 575 amino acids (Balan et al. 1994). BHV-1.1 gE^- mutants reported so far displayed reduced pathogenicity in calves and were excreted for shorter periods than wild type virus (Chowdhury et al. 1999, Van Engelenburg et al. 1994). In view of those findings, such mutants have been chosen for the development of differential BHV-1 vaccines (Chowdhury et al. 1999, Van Engelenburg et al. 1994). As the mutant viruses do not express gE, vaccinated animals do not develop antibodies to this protein, thus, making it possible to differentiate the immune responses of naturally infected from vaccinated animals with serological tests based on the detection of gE antibodies.

The differential BHV-1 vaccines available to date have been prepared with BHV-1.1 strains, the most common subtype associated with IBR and abortions in Europe and North America (Metzler et al. 1985). In Brazil, such vaccines have not become

available yet. In addition, the differential vaccine viruses prepared in the northern hemisfere (Kaashoek et al. 1994; Chowdhury et al. 1999) have not been tested to determine their protective potential against viruses circulating within Brazil, where most BHV-1 isolates examined so far are of the 1.2a genotype (Souza et al, 2002).

Focusing on the development of a differential vaccine virus to be used within the country, a gE^- BHV-1.2a mutant virus was constructed from an autochtonous virus (Franco et al. 2001). In the present study, the *in vivo* behaviour of such mutant was examined in experimentally inoculated calves, in order to determine its attenuation and its potential use as a differential BHV-1 vaccine.

MATERIAL AND METHODS

Experimental design

A group of four calves was inoculated with the mutant virus $265gE^-$. These will be referred to as "vaccinated" calves. A group of three calves was kept as non-vaccinated controls. Twenty one days after vaccination, vaccinated and control calves were challenged with the parental (wild type) virus by intranasal inoculation and monitored for fourteen days. Six months later, all calves under study were subjected to dexamethasone administration in attempting to reactivate latent viruses.

Cells and viruses

The wildtype BHV-1 strain SV265 (SV265 wt) was isolated from a heifer with signs of respiratory infection in São Borja county, Rio Grande do Sul, Brazil. The virus was used for the construction of the mutant virus ($265gE^-$) as described previously (Franco et al. 2001). Madin Darby bovine kidney (MDBK) cells 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 μg/ml streptomycin).

Animal inoculation

Four calves of mixed European breeds, three to four months old, seronegative to BHV-1 and BHV-5, were inoculated (vaccinated) with $10^{6.9}$ fifty percent tissue culture infective doses ($TCID_{50}$) of the 265gE$^-$ mutant, in 2 mL of virus suspension, administered intranasally into the right nostril. Three calves were kept as non-vaccinated controls for subsequent challenge. Three additional calves were kept as non-vaccinated, non-challenged controls throughout. Clinical signs and virus specimens were collected as described below. Twenty one days later, vaccinated and non-vaccinated calves were challenged with 10^9 $TCID_{50}$ of SV265wt, in 5 mL of suspension administered intranasally, 2.5 mL into each nostril. Six months after challenge, all calves were subjected to corticosteroid administration to reactivate the latent infection. Dexamethasone (0.1 mg per kg of body weight) was administered intravenously for 5 consecutive days. Calves were kept under observation and samples collected as below.

Clinical examination

Clinical examinations were performed daily from day 12 prior to vaccination up to day 14-post challenge (pc). The same examination protocol was followed after dexamethasone administration. Signs recorded were rectal temperature, respiratory rate, cough, congestion of the nasal mucosa, conjunctivitis, ocular and nasal discharges, lesions on the nasal and oral mucosa and changes in behaviour and appetite. The clinical scoring method was adapted from Collie (1992), with modifications based on our previous experience with BHV-1 experimental inoculations. Thus, scores were determined by attributing different weights to different signs of disease. Apathy, anorexia, dyspnoea and alterations at auscultation were scored on an arbitrary scale up to 100. Nasal and ocular secretions, conjunctivitis, erosions of the nasal mucosae, enlargement of lymphnodes and cough were scaled up to 10. Body temperature (TR) was included in individual scores using the following formula:

Scored temperature rate (TR): = 100 X (body temperature – mean of calf's body temperature on the 12 days before inoculation).

Mean clinical scores were calculated daily for each group.

Six months later, during reactivation attempts, clinical and virological examinations were recorded as described above. In view of the aging of calves, a new record for standard body temperatures was obtained by measuring temperatures for 12 days before reactivation.

Virological examination

Samples for virological examination were collected on day 12 prior to inoculation, day 0 (date of inoculation) and daily from days 1 to 14 post vaccination (pv), as well as on days 1 to 14 pc and post reactivation (pr). Nasal and ocular swabs were eluted in 2 mL of sample medium (EMEM supplemented with 10 times the usual concentration of antibiotics and 2 % FBS) for one hour at room temperature. The samples were vigorously shaken, the swabs removed, drained, the medium clarified by low speed centrifugation and stored at –70 °C. Virus titrations were performed on microtitre plates. Infectious titres were calculated and expressed as $\log_{10}$ $TCID_{50}$ per 50 μL of nasal or ocular fluid. Serum samples were collected by jugular venipuncture on days 0, 7, 14 pv, pc and pr. Sera were tested in serial twofold dilutions in a standard BHV-1 neutralizing antibody test against SV265wt (House & Baker, 1971).

Identification of the recovered virus

To confirm the identity of the viruses recovered from inoculated calves, an immunoperoxidase monolayer assay (IPMA) was used, with either a monoclonal antibody (Mab) directed to gE as primary antibody, or an anti-BHV-1 Mab that recognizes both SV265wt and $265gE^-$ (Mab 2G5). The IPMA followed essentially the method described previously (Souza et al, 2002), using initially the anti-gE Mab (Kaashoek et al. 1995) followed by an anti-mouse peroxidase/IgG conjugate. In case uncoloured viral

plaques were observed, a second reaction was performed with the Mab 2G5 (Roehe et al, 1997), following the same protocol.

Statistical analysis

Statistical analysis was performed using the Student *t*-test or the analysis of variance (ANOVA) where the least significance difference for $p \leq 0.05$ was determined. Comparisons were made daily within the groups. Statistical analysis was performed with Minitab® for Windows Release 11.1 (Minitab Inc., State College, PA, USA). The term "significant" (statistically significant) in the text means $p \leq 0.05$.

RESULTS

Clinical signs and virus shedding after vaccination

Clinical signs after inoculation of the vaccine virus were very mild. Serous nasal discharges were detected from day 2 to 5 pv, especially on the right nostril, where the virus had been inoculated. Rectal temperatures of vaccinated calves did not exceed 39.5 °C, except for one calf, which presented pyrexia (39.8 °C) on day 6 pv. The vaccinated calves shed virus for up to 8 days in their nasal secretions The highest virus titre was recorded in one calf on day 2 pv (10^5 $TCID_{50}$/50 µl). In ocular secretions, the 265gE^- mutant was only isolated from one calf on day 4 pv.

Clinical signs after challenge

After challenge, all non-vaccinated calves developed severe clinical signs, which included apathy, anorexia, rhinitis, serous to mucopurulent nasal discharges and serous to mucous ocular discharges, starting from day 3 pc (Figure 1). In the nostrils, formation of vesicles which tended to coalesce and eventually gave rise to erosions of the mucosae

were detected. Conjuntivitis, nasal stridor, sneezing and spontaneous coughing were also recorded. Enlargement of the retropharyngeal, submandibular and cervical lymphnodes were observed from days 7 to 12 pc. Dyspnoea and tracheal stridor were detected in all non-vaccinated calves. Pyrexia (>39.5 °C) was also a consistent finding (Figure 2).

Figure 1. a) Mean clinical scores attributed to calves vaccinated (265gE$^-$) and non-vaccinated (SV265 wt) after challenge with wild type virus (SV265 wt); b) clinical scores after reactivation. Black triangles: wild type virus (SV265 wt); black circles: mutant virus (265gE-); empty squares: control calves.

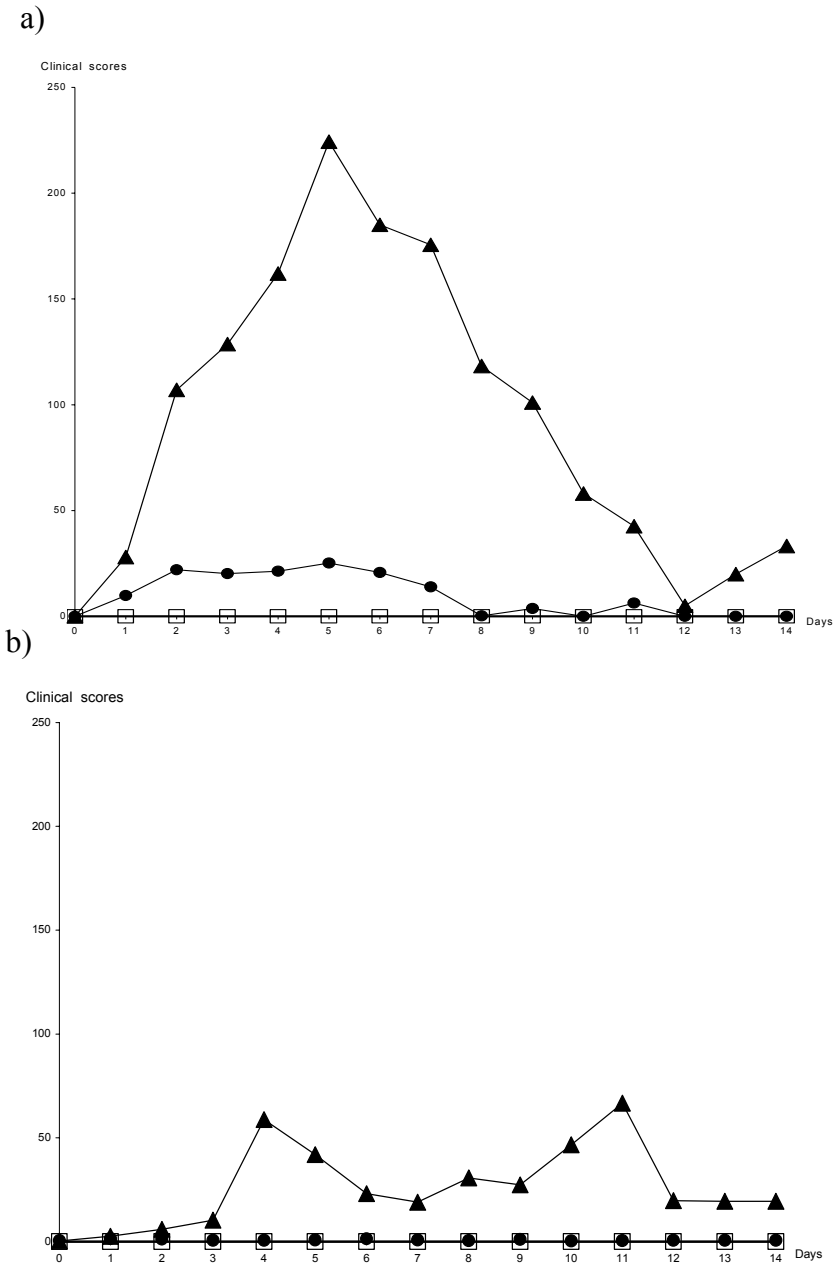
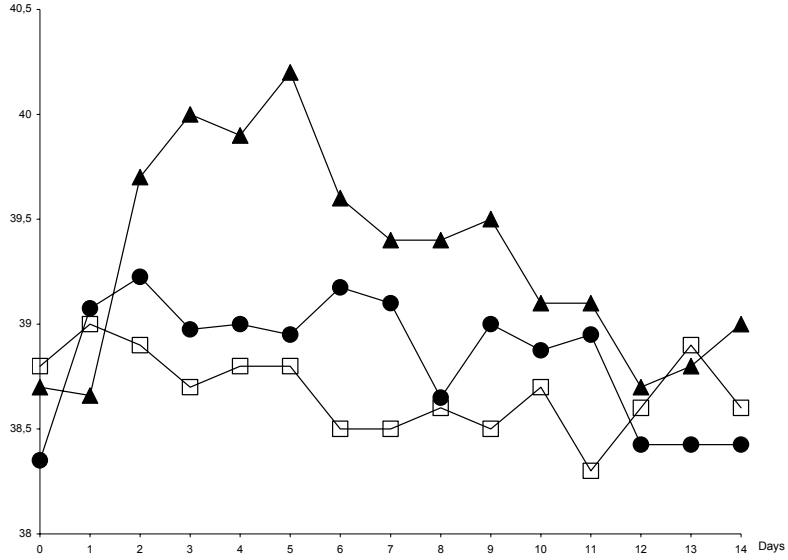


Figure 2: Mean rectal temperatures (^{o}C) a) after challenge of vaccinated and non-vaccinated calves with wild type virus (SV265 wt); b) upon reactivation. Black triangles: wild type virus (SV265 wt); black circles: mutant virus (265gE-); empty squares: control calves.

a)



b)

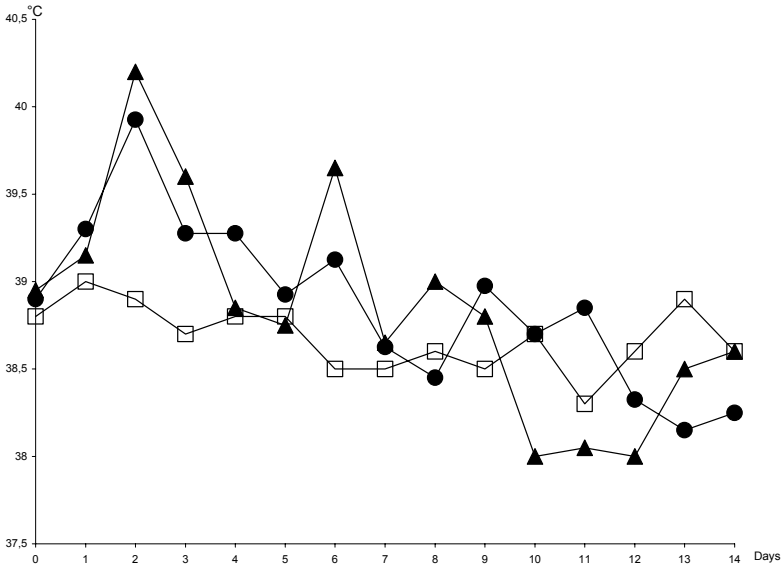
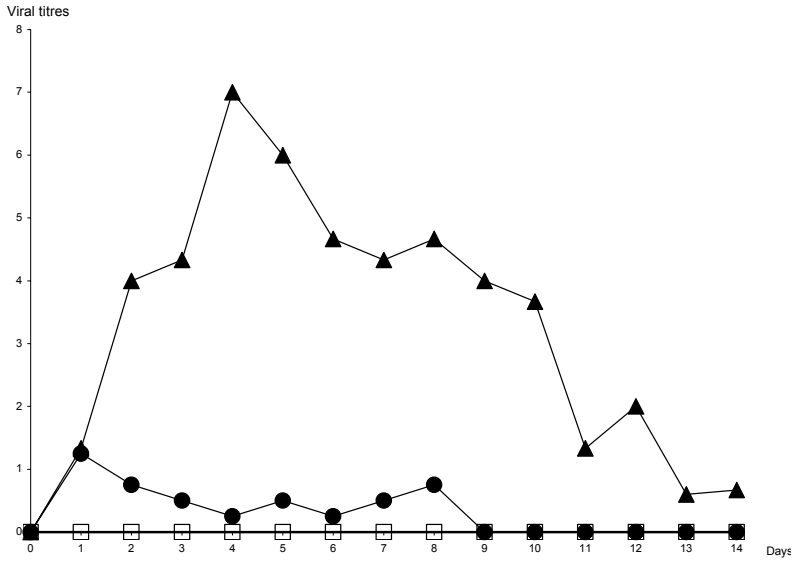


Figure 3: Nasal virus shedding a) after challenge of vaccinated and non-vaccinated calves with wild type virus (SV265wt), and b) upon reactivation. Infectious virus titres expressed in $\log_{10}$ of 50% tissue culture infective doses per 50 µl ($TCID_{50}$). Black triangles: wild type virus (SV265wt); black circles: mutant virus (265gE-); empty squares: control uninfected calves.

a)



b)

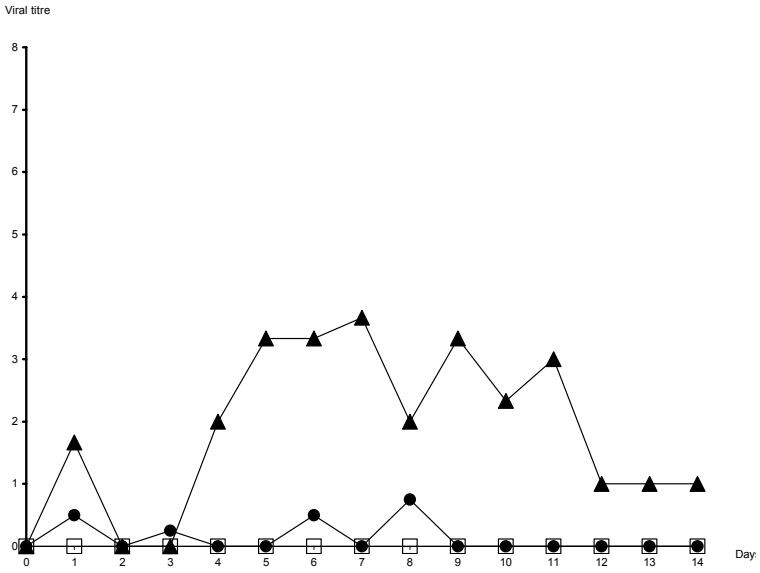
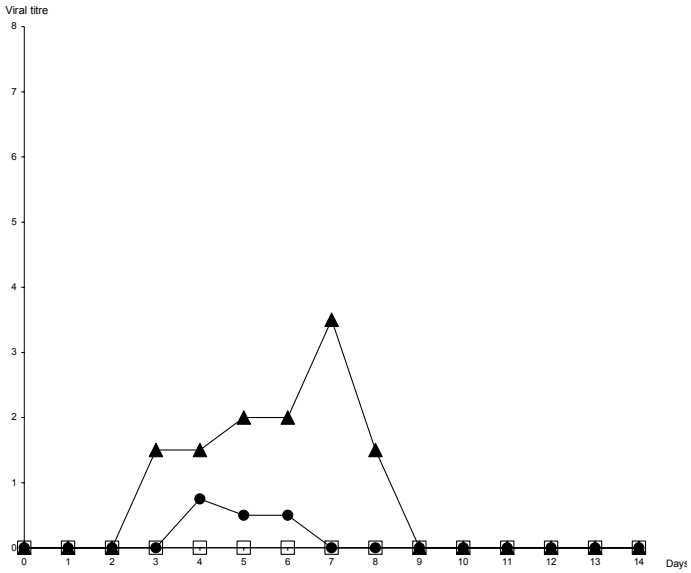


Figure 4: Ocular virus shedding: a) after challenge of vaccinated and non-vaccinated calves with wild type virus (SV265wt); b) upon reactivation. Infectious virus titres expressed in $\log_{10}$ of 50% tissue culture infective doses per 50 µl ($TCID_{50}$). Black triangles: wild type virus (SV265wt); black circles: mutant virus (265gE-); empty squares: control calves.

a)



b)

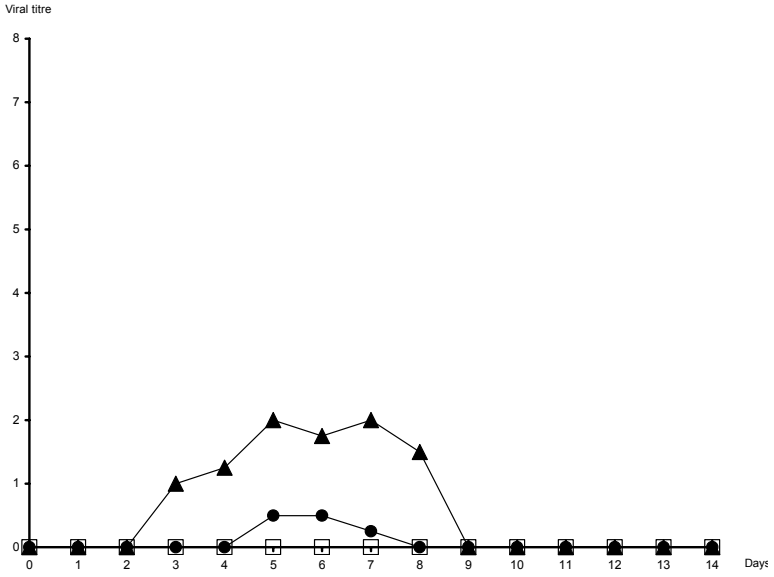
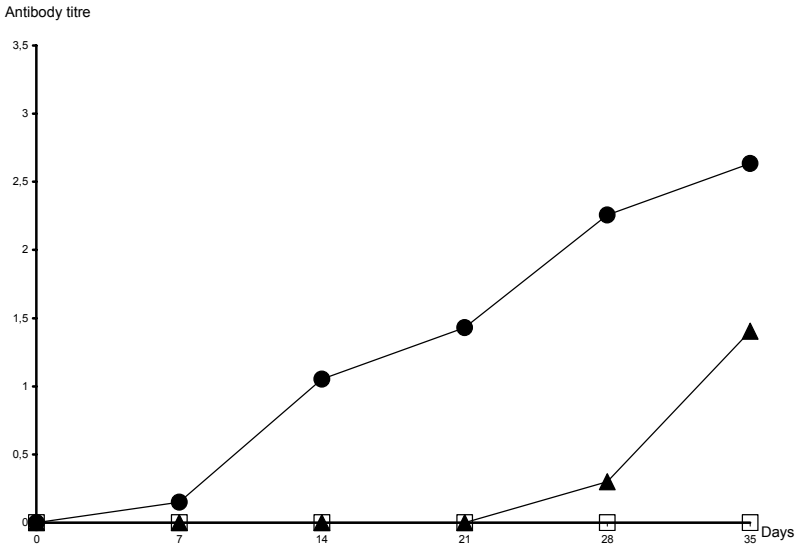
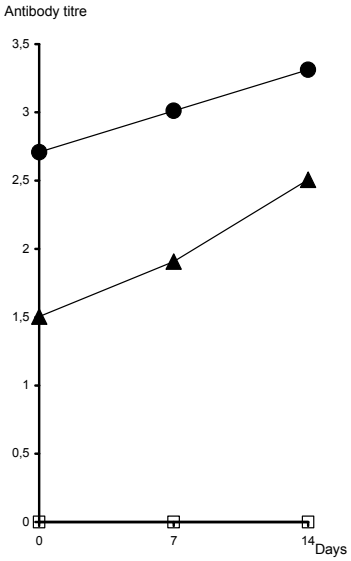


Figure 5: a) Neutralizing antibodies after inoculation of gE^- virus ($265gE^-$) in calves (day 0) and challenge (day 21) with the wild type virus SV265wt; b) Neutralizing antibody titres after dexamethasone administration (180 days after the initial 265 gE^- inoculation). Black circles: calves inoculated with $265gE^-$; Black triangles: calves inoculated with SV265wt only. White squares: Uninfected control calves. Titres expressed in $\log_{10}$ of the reciprocal of the neutralizing antibody titre (refer to text for methods).

a)



b)



In the vaccinated group, all calves had some serous nasal discharge and a few small erosions on the nasal mucosae. Clinical scores on the vaccinated group were very low, in comparison to the non-vaccinated group. Pyrexia was not observed on calves from the vaccinated group.

Both vaccinated and control calves shed virus in nasal secretions. However, the amount of virus shed by the group of vaccinated calves was reduced to a maximum of 10^2 $TCID_{50}$, whereas on the non-vaccinated group virus was excreted for longer and to higher titres (up to 10^9 $TCID_{50}$; Figure 3). Non-vaccinated calves shed virus in ocular secretions for 12 days, with peaks of infectious titres at day 4 post challenge ($10^{4.5}$ $TCID_{50}$/50 µl; Figure 4), whereas only two of the vaccinated calves shed SV265wt virus in their ocular secretions, albeit intermittently and to low titres on days 1, 3, 6 and 8 pc.

Clinical signs after reactivation

Vaccinated calves showed only very mild clinical signs at reactivation, whereas clearly noticeable respiratory signs of disease were observed in non-vaccinated calves (Figure 1 b). These signs were less intense than those observed during acute infection following challenge. However, one of the non-vaccinated calves died on day 11 after reactivation. This calf had low titres of virus in the nasal hornets, trachea, lung, mediastinic and periportal lymphnodes, liver and spleen (data not shown). Post mortem examination of this calf revealed mild pneumonia and hepatic focal necrosis. In such lesions, BHV-1 antigen was detected by immunohistochemistry (not shown). Nasal virus shedding in the group of non-vaccinated calves was detected from day 2 to 9 after reactivation, and to higher titres than on vaccinated calves (Fig. 3).

In the group of vaccinated calves, reactivation was followed by a shorter period of virus shedding, which lasted three days (from day 4 to 6 pr). Ocular shedding was detected from day 5 to 7 pr. All virus samples recovered from secretions obtained from vaccinated calves were confirmed as SV265wt, as evidenced by IPMA with the anti-gE monoclonal antibody (Souza et al, 2002). The mutant $265gE^-$ was not recovered from any of the calves after dexamethasone administration.

The neutralizing antibody profile of calves was similar in both vaccinated and non-vaccinated calves (Figure 5). Differences in antibody titres were never greater than fourfold between vaccinated and non-vaccinated animals, either after challenge or after reactivation.

Analysis of virus shed during the experiments

After vaccination, only gE-negative plaques were detected by IPMA. After challenge, no gE-negative viral plaques were detected. Following dexamethasone induced reactivation, only gE-positive viral plaques were recovered from nasal and ocular samples.

DISCUSSION

The 265gE$^-$ vaccine virus candidate evaluated in the present study was shown to be attenuated when inoculated intranasally into three to four months old calves. After inoculation, the calves showed only very mild clinical signs, as evidenced by the low clinical scores recorded. The virus was excreted in lower titres and for a significantly shorter period of time than the SV265wt virus. Other studies have also shown reduction in virulence and viral excretion with BHV-1.1 gE$^-$ mutants (Kaashoek et al. 1994; Van Engelenburg et al. 1994; Kaashoek et al. 1998; Chowdhury et al. 1999). Likewise, in the present study, the deletion of gE from SV265wt appeared to be responsible for the reduced pathogenicity of the mutant when inoculated into seronegative calves, since calves at the same age inoculated with SV265wt were severely ill. We have not been able to detect any other significant changes along the genome of 265gE$^-$, except for the absence of the gE gene (Franco et al. 2001). Thus, it is very likely that the attenuated phenotype was a consequence of the gE deletion.

Challenge of vaccinated calves with wild type virus gave rise to very mild clinical signs of infection, whereas unvaccinated calves developed severe rhinotracheitis. Although nasal and ocular virus shedding post challenge could not be prevented by the vaccination, infectious titres and the duration of virus shedding were significantly reduced on vaccinated calves. Both aspects are significant for pathogen eradication and disease control programs (Hage et al., 1994), as pointed out by others with similar immunogens (Kaashoek et al. 1994; Chowdhury et al. 1999).

Clinical sighns and virus shedding were also greatly reduced on the group of vaccinated calves after reactivation. Besides, only the wild type virus was recovered after reactivation. Although a 265gE$^-$ latent infection may have been present, BHV-1 gE$^-$ viruses have been reported not to reactivate readily (Van Engelenburg et al., 1995). This has been regarded as a contributing factor to the safety of gE$^-$ vaccines. Moreover, the reduction of wild type virus excretion in vaccinated animals is also of interest, and probably reflects a reduced colonization of the trigeminal ganglia by wild type virus, as pointed out by Galeota et al. (1997).

In the present study, it was demonstrated that the 265gE$^-$ mutant virus is attenuated for calves, induces protection upon challenge and reactivation with a large infectious dose of the parental wild type BHV-1.2a. Therefore, 265gE$^-$ behaved as a suitable candidate for a vaccine virus. Additional experiments are in progress to further examine its immunogenic potential in cattle and to evaluate its protective effect upon challenge, not only against BHV-1.2a, but also against other herpesviruses of cattle.

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Capítulo 4

Safety and immunogenicity of a gE-deleted BHV-1.2a live attenuated vaccine for pregnant cows

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1. manuscrito em preparação

ABSTRACT - Bovine herpesvirus type 1 (BHV-1) is recognized as a major cause of abortion in cattle. Vaccination is widely applied to minimize the losses induced by BHV-1 abortigenic infection; however, vaccination of dams during pregnancy with modified live virus (MLV) vaccines may induce abortions. In order to evaluate the safety of a recombinant BHV-1.2a glycoprotein E (gE) deleted vaccine virus (265 gE- strain) to pregnant cows, were inoculated 22 pregnant cows (14 BHV-1 seronegative; 8 seropositive) in different stages of gestation with 10^7 tissue culture 50% infective doses ($TCID_{50}$) of 265 gE- virus by the intramuscular route. Other 15 pregnant cows were kept as non vaccinated controls. No abortions, stillbirths or fetal abnormalities were seen after vaccination in any group. Seroconversion was observed in both groups of previously seronegative vaccinated animals, but the titers remained similar in previously seropositive cows. Therefore, under the conditions of this study, all cows inoculated with the recombinant 265 gE- had normal gestations and gave birth to apparently healthy offspring.

INTRODUCTION

Bovine herpesvirus type 1 (BHV-1) has been associated to a number of different clinical conditions of cattle, such as infectious bovine rhinotracheitis (IBR) and infectious pustular vulvovaginitis/ infectious pustular balanopostitis (IPV/ IPB). However, perhaps the an important feature of BHV-1 infections is the virus' ability to interfere with pregnancy, often leading to abortions , most often observed during the third trimester of pregnancy (Miller et al., 1991, Siebert et al., 1995a, Turin et al., 1999). Vaccination of the dams before the breeding season is a widely employed in trying to avoid such losses (Whetstone et al., 1986; Lomba et al., 1976). However, the risk of abortions implies in that pregnant animals must not to be vaccinated. Because BHV-1 modified-live-virus (MLV) vaccines may lead to embryonic death or abortions (Miller et al, 1989; McFelly et al., 1968; Mitchell, 1974; Whetsone et al., 1986). Nevertheless, field practices usually lead to a number of pregnant animals being inadvertently vaccinated. Therefore, it is of interest to know whether such vaccines may lead to undesired side effects when applied during gestation. Recently, a new generation of BHV-1 vaccines, the so called differential vaccines, have been developed; such vaccines allow serological differentiation between vaccinated and infected animals (Flores et al., 1993; Van Drunen Littel-van den Hurk et al, 1993; Kaashoek, 1995, Belknap et al., 1999). Our group has developed one of such differential vaccines by deleting the gE gene from the genome of a Brazilian strain of BHV-1 (Franco et al, 2002). The deleted recombinant (265 gE-) was shown to be safe and immunogenic for young calves. In addition it has also shown to protect calves against challenge with autochtonous virus. In the present study, we tested the safety and immunogenicity of the recombinant 265 gE- virus to pregnant cows.

MATERIALS AND METHODS

Recombinant virus – The construction of the recombinant virus (265gE$^-$) has been described previously (Franco et al., 2002a). The virus was multiplied in CRIB-1 cells, a

clone of Madin Darby bovine kidney (MDBK) cells that is resistant to Bovine Viral Diarrhea Virus (BVDV). BHV-1 strain EVI 123/98, a typical BHV-1.1 subtype strain (D'Arce et al., 2002), was multiplied in MDBK cells, and used for serum neutralization assays and for ELISA antigen production. Cell cultures were kept 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 μg/ml streptomycin).

Cows and immunization– Thirty seven European cross-bred pregnant beef cows were used on the present experiments. Twenty two were vaccinated by the intramuscular route on the side of the neck, using 3 mL of a suspension containing $10^{5.3}$ $TCID_{50}$/ 50 μL of the 265gE- recombinant virus. Fourteen pregnant cows (four on the first trimester; 5 on the second and 5 on the third trimester of pregnancy) were seronegative for BHV-1 at the start of the experiment. Another group consisted of eight BHV-1 seropositive pregnant cows (3 on the first trimester; two on the second and three on the third trimester of gestation). Another 15 pregnant cows were kept as non-vaccinated controls. At the start of the experiment seven were BHV-1 seronegative (1 on the 1st trimester, 3, 2nd trimester and 3, 3rd trimester) and 8 were seropositive (2 1st trimester; 2nd trimester; 4, 3rd trimester) seropositive for BHV-1. The stage of pregnancy was determined by rectal palpation and confirmed by the date of calves' birth.

Serology - Serum samples were collected from the dams by caudal or jugular venipuncture on days 0, 40 and 80 post-vaccination (DPV). Samples were also taken from the calves born from dams under test for the first 2 weeks of life. Sera were tested in serial two-fold dilutions in a standard BHV-1 neutralizing antibody test against strain EVI 123/ 98 (Franco et al., 2002). For the detection of both IgG1 and IgG2, an indirect ELISA was designed. The variables within the tests were optimized (antigen concentration, serum and secondary antibody dilutions as well as reduction of background noise) by testing pooled control positive and negative sera. ELISA plates were coated overnight at 4 °C with an appropriate dilution of antigen (1:3200) prepared in carbonate buffer (Na_2CO_2 1.59g;

$NaHCO_3$ 2.93g, distilled water 1L). After adsorption of the antigen, plates were washed once with 100 μL of PBST-20 (0.5% Tween 20 in PBS), filled with another 100 μL of PBST-20 and left to stand for 1 h at room temperature. The sera under test were diluted 1:5 in PBST-20 and added to duplicate wells in plates. After 1 h incubation at 37 °C, the plates were washed three times with PBST-20 and incubated with a mixture of anti-IgG1 and anti-IgG2 peroxidase conjugate (Serotec, UK) properly diluted in PBST-20 for 1 h at 37 °C. After three other washings with PBST-20, 100 μL of the substrate ortho-phenylenediamine (OPD; Sigma, USA) with 0.03% H_2O_2 were added. After 5 minutes of incubation at 37 °C, the reaction was stopped by the addition of 2 M H_2SO_4. The optical density (OD) was determined at 492 nm in a Multiskan (Titertek) ELISA reader. The mean percentual optical density (%DO) was calculated as follows:

$$\%OD = \frac{\text{OD for an individual serum}}{\text{mean OD for negative control sera on different samples and plates}} \text{ X } 100$$

The OD% was taken as a measure of IgG levels for each individual calf.

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 Excel^{tm} (Office XP for Windowstm, Microsoft Corp., USA). The term “significant” (statistically significant) in the text means $p \leq 0.05$.

RESULTS

No embryonic deaths, abortions and stillbirths were detected in any vaccinated cow throughout the present experiment. Likewise, no reproductive abnormalities were detected on the group of non-vaccinated cows during the experiment.

Seroconversion was observed in those vaccinated cows that were seronegative at the start of the experiment , as shown by both serum neutralization and ELISA (Figures 1 and 2). Previously seropositive cows had no significant alterations in serum antibody titres (Figures 1 and 2).

Figure 1. Titres of neutralizing antibodies (expressed in Log10) in previously seronegative gE-negative vaccinated cows (black squares), previously seropositive vaccinated cows (black lozenges), seropositive non-vaccinated cows (blank lozenges), seronegative non vaccinated cows (blank circles).

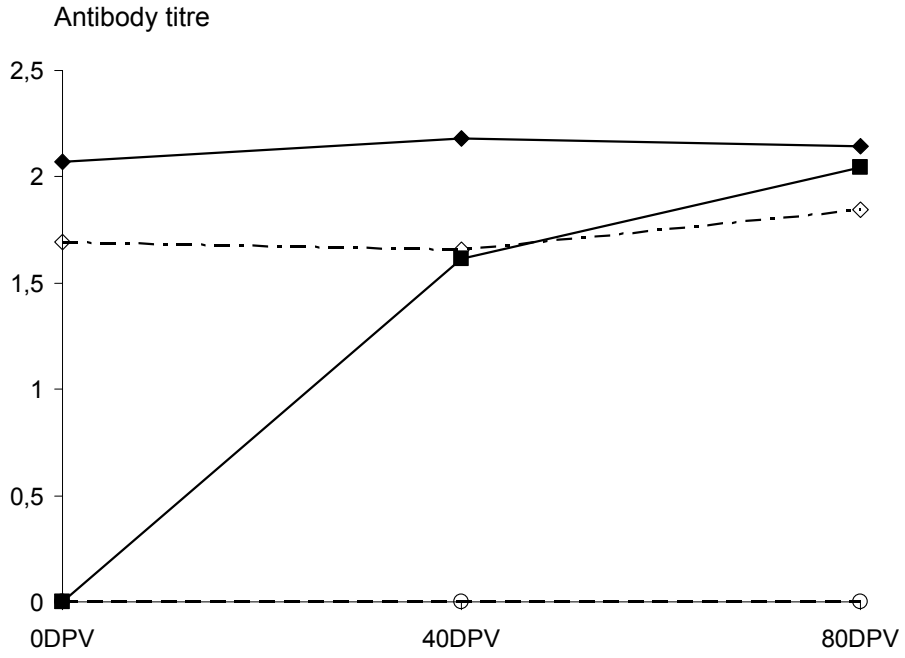
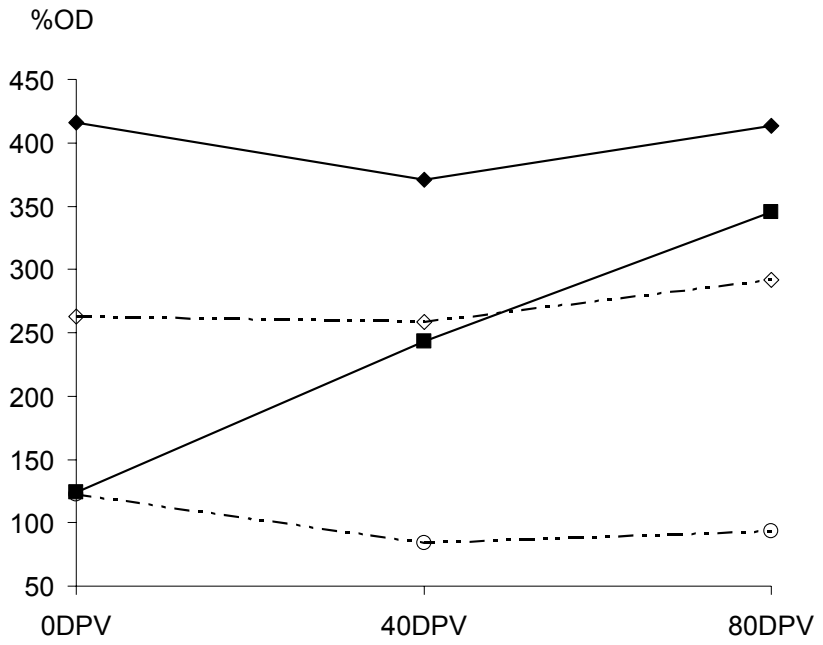


Figure 2. Percentual optical density measured by indirect IgG ELISA in previously seronegative gE-negative vaccinated cows (black squares), previously seropositive vaccinated cows (black lozenges), seropositive non-vaccinated cows (blank lozenges), seronegative non vaccinated cows (blank circles).



Antibody levels on newborn calves ranging from 32 to 64, from either seropositive vaccinated or seronegative dams, were higher than those found on calves born from previously seropositive non-vaccinated cows (GMT = 16). Some calves born to vaccinated, previously seronegative cows, had higher antibody titres in serum than their own dams. No antibodies were found on calves borned to seronegative non-vaccinated dams, except for one calf that seroconverted with a titre of 1:4. Seroconversion was observed in only one animal on the seronegative non-vaccinated group, but that cow gave birth to a healthy calf.

Discussion

Although vaccination with MLV vaccines for IBR virus is well recognized as an efficient way to improve herd immunity against BHV-1 infection (Siebert et al., 1995a; Wentik et al., 1993), the use of these products during pregnancy may result in early embryonic deaths, abortions and stillbirths (Whetstone et al., 1986; Lomba et al; 1976).

On the present experiment, seronegative and seropositive dams were vaccinated intramuscularly with a recombinant strain of subtype BHV-1.2a obtained by our group, that possesses a deletion of gE gene. Previous reports have shown these strain is safe and efficacious for vaccination of calves (Franco et al., 2002b). No embryonic or fetal losses were observed after application of the vaccine. From these data we may conclude that this vaccine candidate virus is also safe for application during pregnancy. Seroconversion obtained, as measured on days 40 and 80 DPV, was also satisfactory and good levels of antibody transfer were found on the newborn calves. That results, obtained in a beef cattle herd, are in agreement with the previously results obtained for another BHV-1.1 gE-deleted vaccine when that was applied to dairy cows during pregnancy (Siebert et al., 1995b), showing that gE deletion abolishes the abortifacient properties of both subtypes BHV-1.2a and BHV-1.1. This is in contrast to the results obtained for deletion of thymidine-kinase (TK) gene, when abortion was observed in 2 of 5 vaccinated dams (Miller et al., 1995).

One cow on the previously seronegative non-vaccinated group has seroconverted during the experiment. It was not probably due to the vaccine virus, as we known form previous experiments that the strain used is not excreted after intramuscular inoculation (data not shown). However, the cow gave birth to a normal calf. In view if the fact that no pregnancy failures were observed also on the non-vaccinated cows on the present experiment, any alterations, if they were observed, would be considered related to the vaccination.

Once the immunogen is shown to be safe for use during pregnancy, vaccination on later stages of gestation may be an efficacious way to transfer higher titres of antibodies to sucklings via colostrum, thus increasing the level of passive protection conferred to newborns. Some calves presented higher levels of neutralizing antibody titres than their dams on the group of previously seronegative, vaccinated cows. That was observed in

previous reports and has been associated to intrauterine infection with the vaccine virus (Lomba et al., 1976) differing from the calves borned from previously seropositive cows, were theoretically present antibodies at the time of vaccination may prevent higher levels of replication of vaccine virus and consequent infection of the fetus. Other very likely and more simple explanation is that there a concentration of dam's serum immunoglobulins on the colostrums at the time of birth.

The group of animal vaccinated animals that were previously seropositive to BHV-1 showed no seroconversion after immunization. A similar situation was found for another herpesvirus (Pseudorabies virus, PrV) in pigs and the use of inactivated vaccines was demonstrated as a solution to induce strong humoral and cellular immunity in such previously seropositive animals that were submitted to vaccination (Zuckermann et al, 1998). That strategy is now under study by our group.

Further studies should be conducted in order to evaluate the efficacy of this strain in preventing abortions by challenge with wild type viruses; on the other hand we have also to test the abortigenic properties of the parental strain SV265/96 in order to clearly define if the gE deletion reduces such pathogenic properties. Another aspect to be considered is the possibility that infection of the fetus *in utero* with these gE-negative viruses may provide the birth of gE-deleted-latently infected animals and we also plan to do these experiments in the near future.

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3. DISCUSSÃO E CONCLUSÕES

O presente estudo foi desenvolvido visando fornecer subsídios sobre os efeitos *in vivo* de isolados brasileiros de BHV-1 já satisfatoriamente caracterizados *in vitro* em trabalhos anteriores (D'Arce et al., 2002; Souza et al., 2002).

A observação de doença respiratória e abortos provocados pelo subtipo BHV-1.2a em nosso meio e os relatos anteriores quanto às características *in vivo* do subtipo 1.2b, muitas vezes intempestivamente generalizadas para todo o genotipo 1.2 (Edwards et al., 1991; Msolla et al., 1983), fizeram necessária a comparação da patogenicidade de um isolado do subtipo 1.2a a partir de um caso de IBR com um vírus de subtipo 1.1, igualmente isolado de doença respiratória. Os resultados revelaram não haverem diferenças marcantes entre ambos os grupos inoculados, seja em aspectos clínicos e virológicos ligados à infecção aguda, seja nas fases de latência ou após a reativação induzida por corticosteróides. Tais evidências diferem daquelas observadas quando outros pesquisadores compararam a virulência da amostras do subtipo 1.1 com amostras do subtipo 1.2b (Edwards et al., 1991; Msolla et al., 1983). Obviamente, diferenças entre animais, ambiente e amostars de vírus utilizadas podem influenciar tais resultados. No entanto, dados prévios obtidos pela inoculação do útero com os subtipos 1.1, 1.2a e 1.2b (Miller et al., 1991), também sugeriram uma virulência mais alta dos vírus 1.2a em relação a isolados de 1.2b. O trabalho aqui apresentado constitui o primeiro relato dos efeitos da inoculação intranasal de um isolado de BHV-1.2a. Dada a clareza na observação de sinais clínicos evidentes de IBR, pôde-se ainda determinar que os isolados utilizados podem ser utilizados como vírus de desafio em testes de vacinas contra as infecções pelo BHV-1.

De modo similar, os trabalhos comparando amostras de BHV-1.1 e 1.2b quanto ao perfil de isotipos de imunoglobulinas secretadas após a infecção inferem uma menor imunogenicidade do genotipo 1.2 como um todo (Edwards et al. 1991; Bradshaw e Edwards, 1996), não considerando possíveis diferenças quanto aos vírus 1.2a. No entanto, os resultados aqui apresentados demonstram não haverem diferenças significativas entre os perfis de resposta imune humoral após uma infecção com BHV-1.1 ou 1.2a, pelo menos comparando os isolados utilizados no presente trabalho. Tal achado tem especial interesse

quando se considera que o isolado utilizado para a construção do recombinante gE-negativo (amostra 265 gE-) elaborado por nosso grupo com vistas a utilizá-lo como amostra vacinal pertence ao subtipo BHV-1.2a.

Com respeito às propriedades vacinais da amostra 265 gE- pôde-se observar adequada atenuação e eficácia em proteger bovinos contra desafio com o vírus parental de campo. Além de ser excretado em títulos baixos após a vacinação o recombinante ainda induziu uma queda acentuada nos títulos do vírus de desafio excretados seja na infecção aguda ou após a latência. A amostra provou ainda ser segura para aplicação em vacas gestantes, induzindo resposta sorológica de satisfatória nesses animais. Estudos futuros devem ser feitos visando complementar tais achados; permanece ainda necessário determinar por exemplo, se a amostra parental SV265/96 é ou não indutora de abortos sob inoculação experimental para então podermos afirmar com certeza que a deleção do gene que codifica a gE reduz a habilidade do vírus em provocar perdas na gestação.

A partir destes dados podemos concluir que amostra 265 gE- atende as condições necessárias para a implementação da mesma como uma alternativa no controle das infecções pelo BHV-1. Dadas as condições geográficas amplas do nosso território e à grande diversidade de métodos de manejo e estágios de avanço tecnológico, bem como igual diversificação nos próprios objetivos propostos para cada propriedade ou região, políticas baseadas no uso de vacinas diferenciais poderiam constituir uma alternativa eficiente de agregar valor aos animais oriundos de cabanhas destinadas à produção de touros por exemplo, o mesmo podendo acontecer com associações de criadores específicas ou regiões geográficas onde o comércio de sêmen e animais vivos é mais intenso.

Outros testes envolvendo o uso do recombinante 265 gE- como vacina vêm sendo ainda realizados, incluindo experimentos de disseminação do vírus a campo, a utilização de diferentes vias de vacinação e a avaliação da existência de proteção cruzada com o herpesvírus bovino tipo (BHV-5). Tais experimentos encontram-se em fase final de análise dos dados.

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ANEXOS

Artigos publicados como primeiro autor durante o mestrado