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ANÁLISE FILOGENÉTICA DE ISOLADOS AUTÓCTONES DO VÍRUS RESPIRATÓRIO SINCICIAL BOVINO (BRSV) E APRIMORAMENTO DE UM MODELO EXPERIMENTAL EM CAMUNDONGOS

Tese apresentada ao Instituto de Biologia da Universidade
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Orientador: Profa. Dra. Clarice Weis Arns

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ÍNDICE

1. RESUMO

O Vírus Respiratório Sincicial Bovino (BRSV) é uma causa importante de doença respiratória em bovinos. O BRSV é um membro da família *Paramyxoviridae*, subfamília *Pneumovirinae*, pertencendo ao gênero *Pneumovirus*. Nos últimos quinze anos, evidências sorológicas e de isolamento do vírus revelaram que o BRSV está circulando no Brasil, causando doença clinicamente evidente ou formas subclínicas da infecção.

Na primeira parte do presente trabalho, seis diferentes linhagens celulares foram examinadas quanto a sua susceptibilidade à infecção pelo BRSV, levando em conta a variabilidade entre diferentes isolados e características de crescimento do vírus. Chicken embryo related cells (CER), e células CRIB (MDBK-resistentes à infecção pelo Vírus da Diarréia Viral Bovina, BVDV) foram as mais apropriadas à multiplicação do vírus. Ambas as linhagens permitiram o cultivo do vírus em títulos de até $10^{5,5}$ $DICC_{50}$ (Doses Infectantes para 50% dos Cultivos Celulares por 100 μL).

Na segunda parte do trabalho o objetivo foi analisar a variação genética dos isolados de BRSV circulantes no Brasil, comparando as seqüências previamente obtidas do gene que codifica para a sua proteína G com seqüências parciais e completas do gene obtidas de isolados de outros países. A análise filogenética realizada permitiu classificar os isolados brasileiros de BRSV como pertencentes ao subgrupo B, o qual não foi relatado no resto do mundo desde a década de 1970. Uma das amostras brasileiras (BRSV-25-BR) apresentou uma importante substituição de aminoácidos entre os resíduos 173 e 178 da proteína, na região hidrofóbica central da mesma, modificando duas cisteínas constituintes do nó de cisteínas que se forma nesta região. A modelagem computacional por homologia da possível estrutura desse domínio da proteína G, com base na estrutura obtida por outros

pesquisadores através de ressonância magnética nuclear, foi conduzida com vistas a determinar possíveis alterações induzidas na conformação da proteína.

Na terceira parte, as relações genéticas entre os isolados de BRSV foram construídas com base em seqüências do gene F, incluindo três isolados brasileiros. A modelagem da proteína F de BRSV foi feita, como uma tentativa de entender melhor as pressões seletivas sobre essa glicoproteína e ainda para determinar um modelo provável da estrutura terciária da proteína. Alguns pontos de seleção positiva puderam ser observados entre nos nucleotídeos 279 e 351 do gene, quando analisamos amostras do grupo genético I, onde se incluem os isolados brasileiros. O modelo de estrutura obtido para a forma não-clivada da proteína conserva os caracteres observados em proteínas de fusão de membros da família *Paramyxoviridae*.

Na quarta parte da tese, é descrita a tentativa de estabelecer um modelo de estudo em camundongos para o estudo de aspectos da patologia e imunologia da infecção por BRSV. O estudo foi conduzido com base no uso de três diferentes linhagens de camundongos (Balb/C, A/J and C57BL6), detentoras de distintos genomas, sendo as mesmas testadas quanto a sua susceptibilidade à infecção pelo BRSV. Os animais das linhagens A/J and C57BL6 revelaram-se mais sensíveis que aqueles da linhagem Balb/C.

A quinta parte do trabalho trata da influência de diferentes adjuvantes na produção de anticorpos da classe IgG anti-BRSV em camundongos. Dentre os adjuvantes testados (emulsão de água-em-óleo, Quil A, hidróxido de alumínio) os melhores resultados foram obtidos com a emulsão de óleo em água, seguidos pela preparação feita com Quil A.

PHYLOGENETIC ANALYSIS OF AUTHOCHTONOUS BOVINE RESPIRATORY SYNCYTIAL VIRUS ISOLATES AND IMPROVEMENT OF AN EXPERIMENTAL MODEL OF INFECTION IN MICE

ABSTRACT

Bovine respiratory syncytial virus (BRSV) is a major cause of respiratory disease in young cattle. The virus is a member of the *Paramyxoviridae* family, *Pneumovirinae* subfamily, belonging to the *Pneumovirus* genus. During the last fifteen years, serological evidence and isolation of the virus revealed that BRSV is circulating in Brazil, causing clinically evident respiratory disease or subclinical forms of the infection.

In the first part of the present work, susceptibility of six different cell lines to BRSV infection in regard to viral isolate variability and growth characteristics of the virus were examined. Chicken embryo related cells (CER), and bovine CRIB cells (a bovine viral diarrhea virus-resistant clone of MDBK cells) showed to be the most appropriate for virus multiplication. Both cells provided infectious virus titres of up to $10^{5.5}$ $TCID_{50}$ (50% tissue culture infective doses per 100 µL).

The second part of the thesis aims to analyze the genetic variation of BRSV circulating in Brazil, comparing previously obtained sequences from the G protein gene of such strains with other partial and complete gene sequences from other countries. The phylogenetic analysis conducted here allowed us to allocate Brazilian strains within the subgroup B, which was no longer found in the world since the 1970s. One of the Brazilian strains (BRSV-25-BR) has a major mutation between amino acid residues 173 and 178, within the central hydrophobic conserved region, exactly on the site of two of the four cysteine-noose forming cysteine residues. Homology modeling with the previously determined NMR structure of this protein domain was made to check whether these mutations altered the conformation of the protein.

On the third part, genetic relationships between BRSV isolates based on F gene, including tree Brazilian isolates, and comparative modeling of BRSV fusion protein were conducted, as an attempt to better understand the selective pressures exerted on this glycoprotein and to determine the putative tertiary structure of the protein. Some positively selected sites between gene positions 279 to 351 were found, analyzing isolates of genetic group I, were Brazilian isolates are located. The modeled structure of F0 presents the same structural constraints described for homolog proteins previously described on others members of the *Paramyxoviridae* family.

On the fourth section of the thesis, it is described an attempt to establish a mice model that could be used further for preliminary studies of pathological and immunological aspects of BRSV infection, three mice inbred lineages (Balb/C, A/J and C57BL6), possessing different genetic backgrounds, were tested about its susceptibility to the inoculation with BRSV. Animals were inoculated through the nasal and ocular routes and were observed after inoculation. A/J and C57BL6 showed to be more susceptible than Balb/C mice.

The fifth part of the present work deals with the influence of different adjuvants on the production of specific anti-Bovine respiratory syncytial virus IgG antibodies in mice. From the adjuvants tested (water-in-oil emulsion, Quil A, Aluminium-hydroxide), the best results were obtained using water-in-oil emulsion adjuvant, followed by Quil A adjuvanted vaccines.

2. INTRODUÇÃO GERAL

2.1. Introdução

O Vírus Respiratório Sincicial Bovino (BRSV) foi isolado pela primeira vez em 1967 (Paccaud & Jacquier, 1970). O BRSV tem distribuição mundial e causa severa doença respiratória em bovinos jovens, caracterizada por bronquiolite e pneumonia intersticial (Larsen, 2000), de forma similar à enfermidade decorrente da infecção pelo Vírus Respiratório Sincicial Humano (HRSV) em crianças (Van der Poel et al., 1994).

O BRSV pertence ao gênero *Pneumovirus*, da família *Paramyxoviridae*, ordem Mononegavirales (Pringle, 1999). Essa ordem compreende vírus dotados de genoma composto de RNA de fita simples ("mono") com sentido negativo ("nega", nsRNA) (Rodhain, 1995). O BRSV está intimamente relacionado ao HRSV (Van der Poel et al., 1994), bem como aos vírus respiratórios sinciciais ovino e caprino (ORSV e CRSV) (Larsen, 2000; Valentova, 2003). O BRSV possui inúmeras similaridades com o HRSV, especialmente no que tange à organização genômica, estrutura viral e antigenicidade (Baker, 1991; Van der Poel et al., 1994; Kovarcik, 1997).

BRSV e HRSV são vírus pleomórficos, envelopados, de tamanho variável: as partículas esféricas medem entre 80 e 350 nm de diâmetro; as partículas filamentosas medem entre 60 e 100 nm de diâmetro com aproximadamente 1 μm de comprimento (Baker et al., 1997; Larsen, 2000).

2.2. Genoma viral

Conforme mencionado anteriormente, as partículas infecciosas do BRSV contém um genoma de RNA fita simples, não segmentado, de polaridade negativa, envolto por um complexo de nucleoproteína que protege o genoma da ação de RNAses (Larsen, 2000).

O genoma do BRSV, com aproximadamente 15.000 nucleotídeos, codifica 10 RNAs mensageiros (mRNA) subgenômicos (Zamora & Samal, 1992; Buchholz et al., 1999). Cada um destes contém uma única fase aberta de leitura (*open reading frame*, ORF), à exceção do gene M2, que contém as ORFs M2-1 e M2-2 (Buchholz et al., 1999). O RNA genômico é utilizado na síntese de mRNAs, que por sua vez são subseqüentemente traduzidos em novas proteínas virais; por outro lado ocorre a síntese de anti-genomas que irão servir como moldes na síntese de novo RNA genômico. O RNA viral é transcrito em 10 mRNAs por uma RNA polimerase RNA-dependente, sendo que cada mRNA codifica para a tradução de uma proteína diferente. A quantidade de cada mRNA está relacionada com a posição do gene no sentido 3'-5', havendo um maior acúmulo dos mRNAs quanto mais próximos os mesmos se encontram da extremidade 3' (Larsen, 2000). As proteínas são nomeadas conforme a ordem de aparecimento e distância da extremidade 3' na seqüência nucleotídica: NS1 (antes chamada 1C), NS2 (antes denominada 1B), N, P, M, SH (1A), G, F, M2 e L (Buchholz et al., 1999; Yunus et al., 2001). Todos os mRNAs formados são dotados de um sítio CAP em sua extremidade 5' e poliadenilados na sua extremidade 3', sendo essas modificações finais do mRNA formado processadas pela polimerase viral. Cada um dos genes tem uma seqüência de iniciação de 10 nucleotídeos, altamente conservada e termina com seqüências de 12 a 13 nucleotídeos que induzem a poliadenilação e liberação do mRNA viral completo (Zamora & Samal, 1992). Regiões

intergênicas estão presentes entre os genes à exceção da junção entre os genes M2 e L, que se sobrepõe em 56 nucleotídeos (Zamora & Samal, 1992).

2.3. Proteínas virais

A proteína de fusão viral F é uma glicoproteína transmembrana inserida no envelope viral, sendo responsável pela penetração viral e formação de sincícios *in vitro* e *in vivo* (Pastey & Samal, 1993). A exemplo da proteína F de outros paramixovírus, estima-se que a proteína F de BRSV forme trímeros (Joshi et al., 1998; Dutch et al., 1999; Mathews et al., 2000; Cianci et al., 2004). A mesma é sintetizada no retículo endoplasmático rugoso como um precursor inativo (F0), formado por 574 resíduos de aminoácidos que, posteriormente é clivado por proteases celulares na rede trans-Golgi, dando origem ao heterodímero F2-F1, que consiste nas subunidades protéicas ligadas por pontes dissulfeto (Dutch et al., 1999; Cianci et al., 2005). Essa clivagem libera o denominado peptídeo de fusão ($_{131}$KKRKRR$_{136}$), um fragmento hidrofóbico localizado na região amino-terminal da subunidade F1, o qual está diretamente envolvido na inserção da proteína na membrana celular (Ellis et al., 1992; Earp et al., 2004; Ruiz-Arguello et al., 2004; Schickli et al., 2005). A proteína de fusão constitui o principal alvo do sistema imune do hospedeiro, sendo que apenas anticorpos direcionados contra a proteína F e a proteína de adesão G são capazes de neutralizar o BRSV (Munoz et al., 1991; Ellis et al., 1995; Ellis et al., 1995; Pastey & Samal, 1998). A subunidade F2 é o determinante de especificidade nas infecções por vírus respiratórios sinciciais (Schlender et al., 2003). Em vírus com deleção induzida da proteína de adesão G, a proteína F é capaz de mediar a adesão da partícula viral à membrana celular (Karger et al., 2001).

A proteína G é responsável pela adesão dos vírions à membrana celular do hospedeiro, já que na subfamília *Pneumovirinae*, ao contrário dos membros da subfamília *Paramyxovirinae*, não estão presentes as proteínas hemaglutinina e neuraminidase (Larsen, 2000). A proteína G é uma glicoproteína transmembrana do tipo II, já que sua região N-terminal tende a expor-se para o citoplasma, enquanto seu fragmento C-terminal deve permanecer no lúmen do compartimento interno à membrana (Doreleijers et al., 1996; Langedijk et al., 1997; Langedijk et al., 1997; Langedijk et al., 1998). O mRNA que codifica a proteína G possui aproximadamente 838 nucleotídeos de extensão, excluindo a cauda de poli-A, e o mesmo codifica para um polipeptídeo formado de 257 resíduos de aminoácidos (Valentova, 2003). A massa molecular estimada é de 28,6 kDa; todavia, a mesma apresenta-se variável quando avaliada quanto à migração em géis de poliacrilamida, dada a intensa glicosilação, semelhante à mucina, existente nas regiões amino e carboxi-terminal da proteína, o que se teoriza seja uma maneira de facilitar a penetração do vírion através do muco para atingir o epitélio respiratório (Mallipeddi & Samal, 1993; Pastey & Samal, 1997). Tais regiões flanqueiam um domínio central, o qual é formado por 32 resíduos de aminoácidos, que apresenta uma estrutura denominada de nó de cisteínas, induzida pela presença de duas pontes dissulfeto formadas entre as quatro cisteínas presentes (Langedijk et al., 1998). O nó de cisteínas parece ser importante na indução de eosinofilia pulmonar nas infecções por HRSV em modelos experimentais (Sparer et al., 1998; Oumouna et al., 2005). A proteína G não é essencial à replicação viral *in vitro*, mas desempenha um importante papel na infecção *in vivo* (Karger et al., 2001).

A proteína SH (do inglês *small hydrofobic*), é uma proteína integral de membrana inserida no envelope viral, cuja função é ainda desconhecida (Samal & Zamora, 1991). A mesma associa-se em oligômeros que se acumulam nas membranas do Complexo de Golgi

que se associam posteriormente a regiões de permeabilidade alterada na membrana, ricas em lipídeos, os "*lipid-rafts*", e sua expressão em sistemas heterólogos altera a permeabilidade sugerindo que a proteína SH forme canais de membrana (Rixon et al., 2004).

A proteína de matriz M é uma proteína não-glicosilada, interna à partícula viral. A proteína M é composta por 256 aminoácidos, com uma massa molecular de 28,7 kDa (Samal & Zamora, 1991). A mesma media a associação do nucleocapsídeo viral ao envelope da partícula (Larsen, 2000). A proteína é dotada de um domínio hidrofóbico na extremidade C-terminal (resíduos 188-204), responsável pela interação da mesma com membranas. A proteína M também interage com a proteína não-estrutural NS1 (Evans et al., 1996; Hengst & Kiefer, 2000).

As proteínas N, P e L podem ser purificadas juntas de suspensões virais na forma de nucleocapsídeos (Krishnamurthy & Samal, 1998; Khattar et al., 2001). A nucleoproteína N se liga fortemente aos RNAs genômicos e anti-genômicos na formação do nucleocapsídeo resistente às RNAses. A proteína N é formada por 391 aminoácidos, sendo que seu peso molecular é de 43 kDa (Krishnamurthy & Samal, 1998). A mesma apresenta altos níveis de identidade entre os isolados de BRSV e destes com outros paramixovírus, sendo altamente conservada (Krishnamurthy & Samal, 1998).

A fosfoproteína P atua como uma chaperonina para a forma solúvel da proteína N (Mallipeddi & Samal, 1992; Shadomy et al., 1997; Khattar et al., 2000; Khattar et al., 2001). A proteína P ainda serve como um co-fator para a polimerase viral (L), após a sua fosforilação (Yunus et al., 1998; Khattar et al., 2001). A fosforilação da proteína P se deve ao alto conteúdo de resíduos de serina e treonina, que constituem aproximadamente 17 % dos 241 aminoácidos da mesma (Khattar et al., 2001). Na ausência de fosforilação da

proteína P, a polimerase produz apenas uma série de oligonucleotídeos a partir da extremidade 3' do genoma, o que sugere que uma proteína P funcional é necessária para converter a polimerase viral nascente em um complexo estável (Yunus et al., 1998; Khattar et al., 2001).

A polimerase viral L é a fração mais importante do complexo RNA polimerase RNA-dependente. Ao longo dos 2161 aminoácidos constituintes da proteína, podem ser encontrados quatro motivos conservados comuns a todas as RNA polimerases.

As proteínas N, P e L são responsáveis e suficientes para a replicação do RNA viral. Associada à replicação do RNA pode ser ainda arrolada a proteína M2, cujo gene correspondente M2 apresenta duas ORFs (Taylor et al., 1997; Yunus et al., 2001). Sabe-se que o polipeptídeo de 186 aminoácidos codificado pela ORF1 (M2-1) funciona conferindo ao complexo RNA polimerase RNA-dependente um nível adequado de processividade ao impedir a terminação abrupta da síntese dos mRNAs virais. A proteína M2-2 (95 aa) regula também a síntese de RNA, já que vírus dotados de deleções artificialmente induzidas da proteína M2-2 apresentam taxas de transcrição aumentadas e baixas taxas de replicação (Yunus et al., 1998).

Ao contrário de outros membros da família *Paramyxoviridae*, os vírus do gênero *Pneumovirus* apresentam ainda outros dois genes que codificam para proteínas não estruturais (NS1 e NS2) (Evans et al., 1996; Schlender et al., 2000; Bossert and Conzelmann, 2002). As proteínas NS1 e NS2 inibem a síntese e ação de interferons (IFN) alfa e beta pela célula hospedeira, através do bloqueio da ativação do fator 3 do IFN e da inibição da expressão de Stat2, diminuindo a responsividade da célula à ação dos IFNs (Pastey & Samal, 1995; Bossert et al., 2003; Valarcher et al., 2003).

2.4. Replicação viral

A adesão dos pneumovírus à célula é mediada pela ligação da proteína G a receptores celulares, os quais ainda não foram determinados; todavia, supõe-se que estes receptores sejam glicosaminoglicanos (GAGs) encontrados na matriz extracelular (Krusat & Streckert, 1997; Hallak et al., 2000; Movlett et al., 2000; Karger et al., 2001; Teng et al., 2001; Teng & Collins, 2002; Schlender et al., 2003; Shields et al., 2003). Essa etapa é provavelmente completada por uma segunda ligação da proteína F e talvez ainda da proteína SH a proteínas de superfície da membrana celular. Conforme mencionado anteriormente, uma amostra de HRSV recombinante com deleção dos genes G e SH pode multiplicar-se de forma eficiente *in vitro*, o que sugere que a proteína F pode mediar sozinha a ligação do vírus à célula hospedeira (Karger et al., 2001). Após a adesão, o BRSV adentra a célula por fusão do envelope viral à membrana celular, mecanismo mediado pela proteína F (Kovarcik, 1997; Larsen, 2000). O processo tem como resultado a entrada do nucleocapsídeo viral no citoplasma, domínio celular no qual irão se suceder todos os próximos passos da replicação do BRSV.

A replicação intracelular inicia com a transcrição do genoma viral em mRNAs por ação do complexo RNA polimerase RNA-dependente. A RNA polimerase viral inicia a síntese de RNA na extremidade 3' do genoma e os genes são transcritos de maneira seqüencial terminando e reiniciando a cada uma das junções intergênicas (Zamora & Samal, 1992; Buchholz et al., 1999; Yunus et al., 2001). Tão logo sucede uma região intergênica não transcrita, segue-se uma região inicial de um gene que determina a adição de um sítio 5'-CAP e a iniciação da transcrição do mRNA. Ocasionalmente, o complexo RNA polimerase RNA-dependente falha em reiniciar a transcrição em genes localizados mais próximos à extremidade 5' do genoma, o que resulta em um gradiente de acúmulo dos

mRNAs mais próximos ao início do genoma (3'). A síntese de anti-genomas (5'-3'), que servirão de molde na síntese de novos genomas virais de polaridade negativa, inicia apenas após a tradução dos primeiros transcritos primários em proteínas virais. A síntese dos anti-genomas é mediada pela mesma polimerase viral; todavia, na síntese destas cópias complementares ao genoma viral, o complexo enzimático ignora todas as junções gênicas (Buchholz et al., 1999). Os anti-genomas são de 10 a 20 vezes menos abundantes que o genoma viral em uma célula infectada; ainda assim podem ser encontrados vírions empacotando essas moléculas intermediárias na síntese de novos genomas virais, pois não há um sinal específico de encapsidação (Buchholz et al., 1999; Yunus et al., 1999; Yunus et al., 2001). Acredita-se que, de modo genérico para os paramixovírus, a concentração de proteína N no meio celular determine a mudança do estado de transcrição para a replicação do RNA viral (Krishnamurthy & Samal, 1998; Valarcher et al., 2000).

A montagem dos nucleocapsídeos se dá no citoplasma e acredita-se que a mesma ocorra em passos distintos. Primeiramente, a proteína N livre se associa aos genomas ou anti-genomas, formando um complexo ribonucleoproteína (RNP) de simetria helicoidal. Na segunda etapa, as proteínas P e L se associam à RNP formando o nucleocapsídeo. A proteína M direciona os nucleocapsídeos às regiões da membrana celular, ricas em proteínas de superfície viral e mais apropriadas ao brotamento da partícula viral. A maturação da partícula viral se dá na superfície da célula hospedeira e os vírions permanecem firmemente aderidos à membrana celular, o que explica por que para a completa liberação destes *in vitro* faz-se necessária a indução de agitação mecânica sobre os cultivos (Wechsler et al., 1985).

2.5. Variabilidade em amostras do BRSV

As diferenças antigênicas entre isolados de BRSV levaram à classificação antigênica dessas amostras em diferentes subgrupos, denominados A, AB (ou Intermediário) e B (Furze et al., 1994; Furze et al., 1997; Stine et al., 1997). A análise de diferentes isolados e amostras de fragmentos de pulmão infectadas colhidas a campo, de animais sadios, utilizando anticorpos monoclonais contra a proteína G, demonstram a divisão das amostras de BRSV em três grandes subgrupos antigenicamente distintos denominados A, AB e B, ocorrendo ainda isolados que não se enquadram nesta classificação (Schrijver et al., 1996; Schrijver, 1998; Schrijver et al., 1998). As implicações práticas dessa diversidade quanto à patogenicidade e imunoprofilaxia ainda não foram devidamente estudadas (Baker & Velicer, 1991; Duncan & Potgieter, 1993).

Em termos genômicos, essa mesma classificação pode se confirmar, conforme o tipo de análise filogenética utilizado e a seqüência gênica utilizada na comparação (Prozzi et al., 1997; Valentova, 2003; Kovarcik & Valentova, 2004; Valentova et al., 2005); todavia, trabalhos mais recentes demonstram a existência de seis grupos genômicos distintos, dada a presença de quatro genogrupos dentre as amostras do subgrupo antigênico AB (Valarcher et al., 2000; Yaegashi et al., 2005).

O isolamento de determinados subgrupos parece estar associado a questões temporais e de distribuição geográfica, sendo o subgrupo antigênico A encontrado nos Estados Unidos, Japão, Reino Unido e alguns países da Europa continental (Stine et al., 1997; Larsen et al., 1998; Larsen et al., 2000; Nettleton et al., 2003; Yaegashi et al., 2005). O subgrupo AB está presente na Europa continental e Japão (Larsen et al., 1998; Larsen et al., 2000; Kovarcik & Valentova, 2004; Yaegashi et al., 2005); já o subgrupo B teve circulação limitada no Reino Unido e Estados Unidos em meados da década de 1970

(Mallipeddi & Samal, 1993; Valarcher et al., 2000; Nettleton et al., 2003), sendo isolado posteriormente apenas no Brasil, sendo que dentre as poucas amostras isoladas até o presente momento neste país, todas foram classificadas no subgrupo B (Arns et al., 2003; Almeida et al., 2005).

2.6. Epidemiologia

A transmissão do BRSV provavelmente ocorre por via aérea ou contato direto entre animais (Larsen, 2000). A infecção primária pelo BRSV induz severa doença respiratória do trato inferior em bovinos suscetíveis, tanto jovens quanto adultos; todavia, a maioria das infecções deve transcorrer de forma assintomática (De Jong et al., 1996; Larsen, 2000). Em áreas endêmicas, a doença clínica é mais aparente nos animais jovens, à semelhança do que ocorre em crianças infectadas pelo HRSV (Van der Poel et al., 1994). Após infecção natural pelo BRSV, a proteção contra reinfecções é de curta duração e as reinfecções são comuns (Collins et al., 1988; Van der Poel et al., 1997). Em humanos, a severidade da enfermidade decresce após reinfecções consecutivas; em bovinos, sinais clínicos evidentes normalmente só são observados na primeira infecção (Van der Poel et al., 1994).

No Brasil, o BRSV foi detectado pela primeira vez em 1992 pela técnica de imunofluorescência em corte de tecido congelado de bovinos (Gonçalves et al., 1993). O BRSV foi posteriormente isolado e caracterizado em 1995, a partir da secreção naso-traqueal de bezerros com sintomas respiratórios procedentes do Rio Grande do Sul (Arns et al., 2003). A doença parece vir ocorrendo sistematicamente na região Sul do Brasil desde a década de 1980 (Flores et al., 2000) e já foram relatados casos nas regiões Sudeste e Norte do país (Driemeier et al., 1997; Peixoto et al., 2000).

2.7. Patogenia e sinais clínicos

Os sinais clínicos após uma infecção natural pelo BRSV incluem pirexia (>39,5°C), descargas nasais, tosse, taquipnéia, respiração bucal e abdominal, enfisema pulmonar e subcutâneo e morte (Van der Poel et al., 1994; Peixoto et al., 2000; Larsen et al., 2001; Tjornehoj et al., 2003). Infecções bacterianas secundárias, especialmente por *Pasteurella multocida* e *Mannheimia (Pasteurella) haemolytica*, são achados comuns em surtos de BRSV, bem como o isolamento concomitante de outros vírus, no que constitui no hemisfério norte no chamado Complexo das Doenças Respiratórias dos Bovinos (CDRB) (Larsen et al., 1999; Larsen, 2000; Larsen et al., 2001). Diarréia também pode ser observada (Driemeier et al., 1997).

Os achados de necropsia incluem pneumonia intersticial multifocal, enfisema alveolar disseminado com focos de atelectasia, sendo notável um aspecto ondulado claro irregular na superfície da pleura visceral (Driemeier et al., 1997; Ordobazari and Steinhagen, 2001; Letesson, 2003). Enfisema intersticial pode estar presente em graus moderados. Característica marcante é o espessamento marcado dos septos interlobulares (Driemeier et al., 1997; Ordobazari & Steinhagen, 2001; Arns et al., 2003; Letesson, 2003). Pequenas franjas conjuntivas evidenciam-se nos bordos do pulmão e dão um aspecto fosco a estas porções. Alguns relatos dão conta de marcada hipertrofia do miocárdio do ventrículo direito (Driemeier et al., 1997).

No exame histopatológico é possível observar células sinciciais em grande quantidade, localizadas principalmente nos bordos dos lóbulos pulmonares, presentes nos alvéolos, bronquíolos e por vezes em vasos linfáticos tal qual observado em casos de HRSV (Driemeier et al., 1997; Sparer et al., 1998; Larsen et al., 2001; Viuff et al., 2002; Corbiere et al., 2003). As células sinciciais apresentam número variável de núcleos

dispostos centralmente. Há presença de enfisema alveolar crônico com bordos de septos alveolares rompidos em forma de clava por vezes intercalada com áreas de atelectasia, hipertrofia da camada muscular peribronquiolar e focos de metaplasia escamosa do epitélio bronquial e bronquiolar. São notáveis ainda alterações inflamatórias mononucleares com áreas focais de infiltração por eosinófilos (Hussell et al., 1998; Sparer et al., 1998; Larsen, 2000; Peixoto et al., 2000).

2.8. Infecções experimentais

Tentativas de induzir os sinais clínicos e patológicos relacionados à infecção pelo BRSV em bovinos têm obtido diferentes graus de sucesso, com base no protocolo experimental adotado (Schrijver et al., 1998; Heegaard et al., 2000; Viuff et al., 2002; Arns et al., 2003; Grell et al., 2005). Alternativas com a inoculação intra-traqueal (Almeida et al., 2005), exposição dos animais à aerossolização lenta em câmara fechada (Taylor et al., 1998; Tjornehoj et al., 2003) ou pré-imunização dos mesmos com vacinas inativadas pela formalina foram relatados com meios de induzir sinais clínicos e lesões pulmonares mais evidentes em bovinos (Antonis et al., 2003; Soethout et al., 2004).

Dado o relativo grau de insucesso na inoculação experimental do BRSV em bovinos, tanto no estudo de diferentes aspectos da imunopatogenia da infecção, bem como no teste de triagem de vacinas para o combate da infecção, seria de interesse a padronização de um protocolo de infecção do BRSV em um modelo experimental (Almeida et al., 2005). Ovinos já foram utilizados para tal fim, apresentando bons resultados (Masot et al., 1995; Masot et al., 2000; Redondo et al., 2003; Meyerholz et al., 2004). Com vistas a reduzir custos o uso de animais de laboratório, tais como camundongos, seria bem vinda, trazendo ainda a vantagem adicional de poder se utilizar linhagens isogênicas conferindo maior

repetibilidade e uniformidade aos resultados. Tal estratégia é amplamente utilizada no estudo das infecções pelo HRSV (Piazza et al., 1993; Easton et al., 2004). No entanto, experimentos anteriores utilizando a inoculação de BRSV em camundongos da linhagem Balb/C demonstraram níveis moderados de replicação viral (Almeida et al., 2004), na presença de pouco ou nenhum sinal clínico ou patológico evidente (Matumoto et al., 1974; Taylor et al., 1984). Estes mesmos trabalhos sugeriram a possibilidade do uso de diferentes amostras virais ou linhagens celulares na preparação dos inóculos a serem utilizados nos experimentos como meio de reproduzir melhor as características da infecção pelo BRSV em bovinos (Taylor et al., 1984; Almeida et al., 2004).

2.9. Imunidade

A proteína F é considerada a mais imunogênica do BRSV (Baker et al., 1997; Schrijver et al., 1997; Taylor et al., 1997; Letesson, 2003). A proteína F é superior à proteína G na indução de anticorpos neutralizantes e imunidade mediada por células T citotóxicas (Sharma et al., 1991; Thomas et al., 1996; Werling et al., 2002; Gaddum et al., 2003). A proteína F ainda induz à formação de anticorpos inibidores da fusão, relacionados à proteção frente à infecção (Ellis et al., 1992; Ellis et al., 1995; West & Ellis, 1997; Pastey and Samal, 1998). Anticorpos contra as proteínas P, M e M2 também estão presentes em infecções naturais.

Ainda que anticorpos maternos (AMs), majoritariamente direcionados contra as proteínas F, G e N, estejam comumente presente nos bezerros, estes não conferem proteção frente à infecção pelo BRSV (Belknap et al., 1991). Por outro lado, a incidência e severidade da doença é inversamente proporcional ao título de AMs. Os AMs causam um decréscimo na replicação viral nos pulmões após o desafio; a presença dos AMs suprime

ainda a resposta imune humoral local e sistêmica à infecção (Belknap et al., 1991). Deste modo, a vacinação de animais jovens pode ser prejudicada pela presença de AMs (Ellis et al., 1996; Hagglund et al., 2004; Patel, 2004).

Estudos em bovinos têm demonstrado que após a infecção por BRSV ocorre no hospedeiro um intenso direcionamento da resposta imune no sentido linfócito T auxiliar tipo 2. Respostas do tipo Th2 são caracterizadas pela expressão aumentada das citocinas IL-4 e IL-10, as quais provocam incremento na síntese de anticorpos, incluindo a classe IgE, e estimulam o recrutamento de eosinófilos para o parênquima pulmonar. Tal tipo de resposta é semelhante àquela induzida pelas infecções helmínticas e deve explicar em parte o quadro de intensa bronquiolite evidenciado nas infecções pelo BRSV (Antonis et al., 2003; Kalina et al., 2004; Oumouna et al., 2005).

2.10. Diagnóstico

O diagnóstico da infecção pelo BRSV é baseado na detecção do antígeno em amostras clínicas ou na sorologia dos rebanhos. Os métodos de escolha na detecção de antígenos do BRSV em amostras de pulmão são as técnicas de imunofluorescência e imunoperoxidase. Amostras de secreção nasal têm sido descritas como alternativas para o diagnóstico no animal vivo. O lavado broncoalveolar (BAL) pode ter mais sucesso que os suabes nasais na detecção de antígenos ou no isolamento viral. A fragilidade do vírion do BRSV torna o isolamento do vírus em cultivo celular trabalhoso e muitas vezes infrutífero (Larsen, 2000). A alternativa de detecção de fragmentos do genoma em espécimes clínicos utilizando a técnica de RT-PCR é hoje amplamente utilizada (Oberst et al., 1993; Vilcek et al., 1994; Almeida et al., 2005; Boxus et al., 2005; Valentova et al., 2005). A técnica de ELISA, assim como a soroneutralização, tem sido também muito utilizadas no diagnóstico

sorológico das infecções pelo BRSV (Florent & Wiseman, 1990; Vilcek et al., 1994; Graham et al., 1998; Graham et al., 1999; Alkan et al., 2000; Schreiber et al., 2000; Domingues et al., 2002).

2.11. Cultivo do vírus

O BRSV pode replicar em uma grande variedade de células de cultivo primário, preparadas a partir de órgãos de bovinos e ovinos. O vírus pode ainda ser adaptado a cultivos celulares de origem humana e de outras espécies (Larsen, 2000). Poucos trabalhos foram feitos quanto à determinação das características de multiplicação viral e títulos virais alcançados pelo BRSV em diferentes cultivos primários e de linhagem (Matumoto et al., 1974).

2.12. Controle

O desenvolvimento de vacinas contra as infecções por BRSV ou HRSV foi em parte prejudicado por um fato inusitado acontecido na década de 60: o uso de uma vacina inativada pela formalina contra o HRSV induziu a um nível mais grave da enfermidade induzida pelo HRSV de campo e mortes em um grande número de crianças vacinadas (Brandenburg et al., 2001; Openshaw et al., 2001). Tal fato foi relacionado a alterações conformacionais sofridas pelos antígenos vacinais em contato com a formalina; tais alterações levariam a formação de imunocomplexos no hospedeiro e uma conseqüente reação de hipersensibilidade do tipo III (Hancock et al., 1996; Waris et al., 1996; Brandenburg et al., 2001). Em bovinos, imunização contra o BRSV com vacinas inativadas em formalina levaram a lesões pulmonares mais graves em um estudo; todavia, essa exacerbação nas lesões pelo tipo de vacina citado não foi encontrado em outros

experimentos (West et al., 1999; Antonis et al., 2003; Kalina et al., 2004; Kalina et al., 2005).

Além destes problemas, a imunidade de curta duração conferida após infecção natural coloca dúvidas sobre a durabilidade da proteção conferida pelas vacinas (Larsen et al., 2001). Outra exigência de difícil resolução é a necessidade de que a vacina proteja contra as diferentes variantes antigênicas encontradas a campo (Duncan & Potgieter, 1993; Valarcher et al., 2000; Larsen et al., 2001; Nettleton et al., 2003), pois ainda que exista imunidade sorológica cruzada, não se tem certeza sobre os graus de proteção cruzada entre os subgrupos.

Inúmeras vacinas inativadas e vivas estão disponíveis comercialmente para o controle das infecções pelo BRSV. Estudos utilizando o desafio experimental e a campo têm levado a resultados pouco conclusivos quanto à eficácia das vacinas (Frankena et al., 1994; Peinhopf et al., 1996; Schreiber et al., 2000; Norstrom et al., 2001; Ellsworth et al., 2003).

O uso de diferentes adjuvantes em vacinas inativadas, buscando uma melhor resposta imune humoral e celular assim como uma apropriada modulação dos ramos Th1 e Th2 do sistema imune têm se revelado uma estratégia promissora no desenvolvimento de vacinas mais seguras e eficazes no combate a infecções pelo BRSV (Nelson et al., 1992; Walravens et al., 1996; Simmons et al., 2001; Morein et al., 2004; Ellis et al., 2005). Assim, especificamente no caso do BRSV, onde um balanço adequado da resposta imune é necessário pra proteger o animal dos efeitos clínicos da infecção, fazem-se necessárias mais pesquisas sobre diferentes adjuvantes para o uso em vacinas inativadas.

Vacinas utilizando outros vírus como vetores, a exemplo de uma vacina utilizando o Herpesvírus Bovino (BHV-1) como vetor para a proteína G do BRSV diminuíram os sinais

clínicos e excreção viral após o desafio por BRSV (Schrijver et al., 1997; Kuhnle et al., 1998). Outra estratégia no desenvolvimento de novas vacinas contra o BRSV é o uso de vacinas de DNA, já experimentalmente testadas com relativo sucesso (Schrijver et al., 1997; Schrijver et al., 1998). É necessário levar em conta que resultados adversos também foram demonstrados quanto à imunização de animais com vacinas de DNA contra infecções pelo HRSV, na dependência do tipo protocolo utilizado na imunização (Bartholdy et al., 2004; Tree et al., 2004).

As infecções pelo BRSV, dadas as suas características únicas em relação à imunopatogenia da enfermidade e à ubiqüidade da infecção, representam um desafio ao desenvolvimento de vacinas eficazes e seguras. De todo modo, são necessários esforços mais aprofundados visando elucidar a real importância desta infecção no rebanho bovino brasileiro.

3. OBJETIVOS

3.1. Objetivos gerais

Contribuir no estudo dos aspectos de classificação, patogenicidade e imunogenicidade de isolados brasileiros de BRSV, através do aprimoramento de técnicas de cultivo do vírus *in vitro*, análises filogenéticas, modelagem estrutural de glicoproteínas de superfície e de inoculação experimental em camundongos de diferentes linhagens.

3.2. Objetivos específicos

- Determinar a linhagem celular mais adequada ao cultivo do BRSV *in vitro*, investigando os títulos de vírus infecciosos obtidos após várias passagens em diferentes células e o estudo das curvas de multiplicação viral do mesmo em diferentes linhagens celulares;

- Com base em seqüências anteriormente obtidas do gene G de amostras brasileiras de BRSV, realizar estudos sobre os aspectos evolutivos e estruturais relativos ao gene e à proteína G desses isolados;

- Com base em seqüências anteriormente obtidas do gene F de amostras brasileiras de BRSV, estudar os aspectos evolutivos e estruturais relativos ao gene e à proteína de fusão desses isolados;

- Padronizar um modelo murino de infecção pelo BRSV, tendo por base o uso de diferentes linhagens de camundongos isogênicos e isolados virais geneticamente distintos;

- Avaliar o uso de diferentes adjuvantes quanto à produção de imunoglobulinas G (IgG) anti-BRSV em camundongos.

Artigo 1

Susceptibility of different cell lines to infection with bovine respiratory syncytial virus

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Susceptibility of different cell lines to infection with bovine respiratory syncytial virus

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Abstract

The growth of bovine respiratory syncytial virus (BRSV) was evaluated in six different cell lines. Chicken embryo related cells (CER), a chicken embryo fibroblast/baby hamster kidney hybrid and bovine CRIB cells (a bovine viral diarrhea virus-resistant clone of MDBK cells) showed to be the most appropriate for virus multiplication. Both cells provided infectious virus titres of up to $10^{5.5}$ 50% tissue culture infective doses per 100 μl ($TCID_{50}$/100 l). One-step growth curves revealed no significant differences in the growth of BRSV in these two cell lines. Furthermore, they proved to be susceptible to infection with three different BRSV strains. It was concluded that both CER and CRIB cells may be used for laboratory multiplication of BRSV with optimal results.

Keywords: BRSV; Bovine respiratory syncytial virus; Cell line susceptibility

1. Introduction

Bovine respiratory syncytial virus (BRSV), a member of the subfamily *Pneumovirinae*, family *Paramyxoviridae*, has often been reported as a major cause of respiratory disorders in young cattle (Castleman et al., 1985; Kimman et al., 1989; Schreiber et al., 2000; Scott et al., 1996; Van der Poel et al., 1994). Virus isolation from clinical specimens in cell culture is the gold standard for the diagnosis of infection (Larsen, 2000). However, due to the lability of the virus particle, the efficacy of such procedure is critically dependent on the time spent between the moment when the clinical samples are taken and the laboratory attempt to isolate the virus (Larsen, 2000). As reported for other paramyxoviruses (Ferrari et al., 1981; Lednicky et al., 2004; Zhang et al., 2002, 2005), the maintenance of stocks of BRSV strains with appropriate high titers in the laboratories is often impaired by the characteristic fragility of the viral particle, as well as the production of defective interfering particles, which may impair efficient viral replication. Such drawbacks are especially relevant to vaccine production, and also to a number of other laboratory procedures that require high virus titres, such as virus neutralization, antigen production for diagnostic tests and animal inoculation experiments. In the past, chemical treatments and other techniques have been tried to enhance viral infectivity (Rossi and Kiesel, 1978). An option to circumvent such problems may be the use of different cell lines in order to increase the sensitivity of virus isolation and enhance the number of viable viral particles achieved after virus passage in cell culture. Previous reports have shown that primary cell cultures derived from bovine and ovine respiratory tracts, as well as other cell lines, may be more appropriate for the multiplication of BRSV in vitro (Bostandzhieva, 1986; Liebermann and Riebe, 1981; Uzunova, 1968; Uzunova and Atzev, 1968; Uzunova and Gagov, 1968).

In the present study, a set of experiments was carried out to compare the susceptibility of different readily available cell lines to BRSV infection in regard to viral isolate variability and growth characteristics of the virus.

2. Materials and methods

2.1. Susceptibility of different cells to BRSV infection

The following cell lines were used: chicken embryo related cells (CER), a cell hybrid derived from chicken embryo

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fibroblasts and BHK-21 (Baby hamster kidney cells, clone 21, ATCC CCL-10); Vero (African green monkey kidney cells); MDBK (Madin-Darby bovine kidney cells, ATCC CCL-22) and CRIB cells, which are derived from MDBK (Flores and Donis, 1995; Flores et al., 1996). Besides, it was also used two primary cell cultures of chicken embryo fibroblasts (CEF) and bovine embryonic lung (BEL). CRIB cells were kindly provided by Dr. Eduardo F. Flores (Universidade Federal de Santa Maria, RS, Brazil). All cell lines used in the present study were proven to be free of BVDV by an immunoperoxidase monolayer assay using monoclonal antibodies against BVDV (Kreutz et al., 2000). Furthermore, attempts to infect CRIB cells used in this study with BVDV prototype strain Singer were unsuccessful (data not shown).

The cells were grown in flasks of 75 cm^2 at 37 °C, using an initial concentration of 1.5×10^5 cells/ml in Eagle's minimal essential medium (E-MEM) free of antibiotics and supplemented with 10% BRSV-free fetal calf serum (FCS). Monolayers were grown to 85% of cell confluence and cells were then inoculated with 100 $TCID_{50}$ (50% tissue culture infective dose) of each viral isolate for virus production. Adsorption was allowed for 1 h at 37 °C. Subsequently, the medium was replaced and the flasks were incubated at 37 °C. The cells were monitored daily for the development of viral cytopathic effect (CPE) using an inverted microscope (Axiovert 100, Carl Zeiss, Oberkochen, Germany). Cells and supernatants containing viruses were harvested using a cell scraper when 75% CPE was observed. The mixtures of cells and supernatants were vigorously shaken (Wechsler et al., 1985) and stored at −70 °C in small aliquots until analysis. After three passages in each cell line, aliquots were titrated in 96-well plates following standard procedures. Titers were calculated by the Spearmann–Kärber method and expressed as the log_{10} tissue culture infective dose per 50 μl ($TCID_{50}$/50 μl).

2.2. Susceptibility of CER and CRIB cells to different isolates of BRSV

Three different viral strains representing diverse antigenic subgroups of BRSV were used in the experiments: strain 375 (Mallipeddi and Samal, 1993), strain 88 and strain BRSV-25-BR (Arns et al., 2003). The trials were conducted essentially as described above. The viruses were passaged three times in each cell line. Titration results were confirmed in an immunoperoxidase monolayer assay performed as described previously (Schrijver et al., 1995), except that the primary antibody was an anti-BRSV polyclonal sera prepared in our laboratory by repeated immunizations of rabbits with the isolate BRSV-25-BR.

2.3. Growth characteristics in different cell lines

Growth characteristics of the strain BRSV-25-BR in CER and CRIB cells were assayed in a multiplication kinetics experiment. One-step growth curves were assessed following infection of preformed CER or CRIB cell monolayers at an multiplicity of infection of 1, which had been previously determined for both cells. Adsorption was allowed for 1 h at 37 °C, the inoculum was removed and extracellular virus was inactivated with 1 M sodium citrate (pH 3.0). The monolayers were then washed with fresh medium and incubated for different time intervals (0, 2, 4, 6, 8, 24, 36, 48 and 72 h post-infection, p.i.). After incubation, the supernatants were harvested and assayed for the presence of the virus. All experiments were performed in triplicates. Infectious virus titres were calculated according to the Spearmann–Kärber method and expressed as log_{10} $TCID_{50}$/50 l.

2.4. Statistical analysis

Statistical analysis was performed using the Student's *t*-test and analysis of variance (ANOVA); the least significant difference at $p = 0.05$ was determined. 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 $p \leq 0.05$.

3. Results

3.1. Susceptibility of different cell lines to infection with BRSV

Mammalian cell lines (CER, Vero, MDBK, CRIB and BEL) were susceptible to infection with BRSV, in contrast to avian cells (CEF), which did not produce detectable infectious virus after one passage (Table 1).

3.2. Susceptibility of CER and CRIB cells to different strains of BRSV

Both cell lines are permissive to infection with different strains BRSV, giving rise to efficient replication and increases in the infectious virus titres after three passages in each cell (Table 2). The only noticeable difference was that the CPE predominantly observed in CER was an initial rounding of the cells followed by formation of syncytia. In both CER and CRIB cells, BRSV-25-BR caused 75% CPE in the monolayers within 3–4 days post-infection (dpi), whereas strains 375 and 88 multiplied slowly, and 75% CPE was observed on cell monolayers only after 7–10 days.

Table 1
Titres of infectious virus recovered after three passages of BRSV in different mammalian and avian cell lines

Cell line	Virus titre
CER	5.5
VERO	3.5[a]
MDBK	5.2
CRIB	5.5
CEF	–[a]
BEL	5.0

Titres are expressed in log_{10} $TCID_{50}$/100 μl. There was no detectable viral yield in CEF cells.

[a] Significant minor at $p < 0.05$.

Table 2
Titres of infectious virus recovered in each of the three passages of three antigenically distinct BRSV strains in CER and CRIB cells

Viral strain	Cell lines	Number of passages		
		1	2	3
375	CER	1.75	2.5	3.0
	CRIB	2.5	4.0	4.5
BRSV-25-BR	CER	3.5	3.75	4.0
	CRIB	3.75	4.5	4.75
88	CER	1.0	1.75	2.0
	CRIB	1.75	2.0	2.5

Titres are expressed in $\log_{10}$ $TCID_{50}/100$ l.

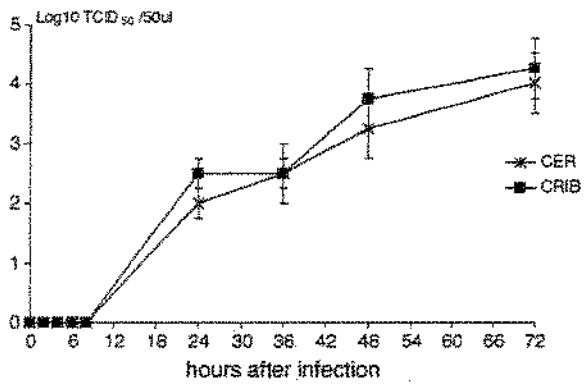

Fig. 1. Comparison of BRSV-25-BR strain growth characteristics in CER and CRIB cell lines. Each value is the mean titre of three infected wells with the respective standard deviation bars, at different time intervals (hours post-infection).

3.3. Growth characteristics in CER and CRIB

One-step growth curves of the strain BRSV-25-BR in CER and CRIB revealed no significant differences ($p = 0.8661$) in the infectious virus titres from 0 to 72 h after infection, evidencing that these cell lines are equally permissive to BRSV infection (Fig. 1).

4. Discussion

BRSV multiplied only in the mammalian cell lines that were tested. The cell line CER, which is susceptible to viral infection with a wide spectrum of different pathogens, has often been used for multiplication of rabies virus (Cardoso et al., 2004; Sinibaldi et al., 1990; Smith et al., 1977) and other viruses from mammals (El Karamany et al., 1979), as well as a number of avian viruses (Cardoso et al., 2000). Despite of what the name "chicken embryo related" may tell about the cell line origin, most characteristics of CER cells are acquired from the parental hamster cells (Urmanova et al., 1989; Urmanova and Tsareva, 1996a,b,c).

Considering that both CER and CRIB cells were the most permissive cell lines for multiplication of BRSV in the first experiments described here (Table 1), the multiplication of three BRSV isolates was assayed using such cells. It was not possible to observe significant differences in BRSV infectious titres from these three strains multiplied in either CER or CRIB cells. Some differences could be observed in the characteristics of the CPE induced by BRSV in each cell type, which is in agreement with previous descriptions of CPE in MDBK and CER cells (Arns et al., 2003) or in different bovine cells (Bostandzhieva, 1986).

One-step growth curves of the strain BRSV-25-BR were performed using both CER and CRIB cells. No significant differences were observed between the results obtained for each cell type up to 72 h after infection. It was concluded that both cell lines may be useful as targets for BRSV cultivation, with no adverse effects on the viral growth characteristics in vitro.

CER cells may be easily maintained in the laboratory and are susceptible to a wide spectrum of different viral infections. Therefore, this cell line may constitute an interesting alternative to the diagnosis of respiratory syncytial virus infection in cattle, as well to the detection of other bovine respiratory viruses. The susceptibility of CRIB cells to BRSV infection has been tested before (Flores et al., 1996), but this is the first report that compares the viral multiplication of BRSV in both CRIB and its parental cell line MDBK and shows that both cells are highly susceptible to BRSV infection. CRIB cells are not susceptible to infection with BVDV (Flores and Donis, 1995), which is a major contaminant of cell lines (Bolin et al., 1991; Levings and Wessman, 1991; Makoschey et al., 2003; Rossi et al., 1980; Studer et al., 2002; Zabal et al., 2000). Therefore, CRIB cells may be highly recommended for growing BRSV virus stocks to be used in animal experiments and specially in vaccine production, avoiding the undesirable events induced by BVDV contamination (Antonis et al., 2001; Audet et al., 2000; Falcone et al., 2003).

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Artigo 2

Phylogenetic relationships of Brazilian bovine respiratory syncytial virus isolates and molecular homology modeling of attachment glycoprotein

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Phylogenetic relationships of Brazilian bovine respiratory syncytial virus isolates and molecular homology modeling of attachment glycoprotein

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Abstract

Bovine respiratory syncytial virus (BRSV) causes lower respiratory tract disease in young cattle. Recently, it was possible to determine the sequence of the G protein gene, which plays a role in the attachment of BRSV particles to the cells, from three distinct Brazilian isolates. The phylogenetic analysis conducted here using those sequences compared to other worldwide distributed isolates of BRSV allow us to allocate Brazilian strains within the subgroup B, which was no longer found in the world since the 1970s. One of the Brazilian strains has a major mutation between amino acid residues 173 and 178, within the central hydrophobic conserved region, exactly on the site of two of the four cysteine-noose forming cysteine residues. Homology modeling with the previously determined NMR structure of this protein domain was made to check whether these mutations altered the three-dimensional conformation of this immunodominant region. Possible consequences on the biological effects induced by such mutation on the G protein are discussed.

Keywords: Bovine respiratory syncytial virus; Attachment glycoprotein G; BRSV; Brazilian isolates

1. Introduction

Bovine respiratory syncytial virus (BRSV), a member of the genus *Pneumovirus*, from the family *Paramyxoviridae*, is a major cause of lower respiratory tract illness in young cattle, as the closely related Human respiratory syncytial (HRSV) is a common threat for young infants (Van der Poel et al., 1994).

The viral envelope glycoprotein (G protein) is responsible for binding to the cell receptor in HRS viruses (Levine et al., 1987), playing an important role in the immune response against BRSV infection (Bastien et al., 1997; Buchholz et al., 2000; Ellis et al., 1995; Samal and Pastey, 1997; Schrijver et al., 1997). It is structurally different from its counterparts present in other paramyxoviruses, like hemagglutinin and neuraminidase-hemagglutinin (Langedijk et al., 1997a). The ectodomain of RSV G protein has a modular architecture that comprises a central conserved folded region and two bounding regions which are highly glycosilated, polymeric mucin-like regions (Doreleijers et al., 1996; Langedijk et al., 1997b, 1998; Lerch et al., 1990). The C-terminal half of the central conserved region is composed of two helices, connected within by a turn and linked by two disulfide bridges (Langedijk et al., 1997b). This double linked cystine-noose folds into a flat disc with a hydrophobic pocket formed by relatively high conserved residues (Doreleijers et al., 1996), corresponding to the immunodominant region of BRSV G protein (Langedijk et al., 1997b), as well as demonstrated in the HRSV homologous region (Gorman et al., 2001).

HRSV isolates have been divided into two subgroups, named A and B, based on its reactions with monoclonal antibodies against both fusion protein (F protein) and G protein (Duncan and Potgieter, 1993; Van der Poel et al., 1994; Woelk and Holmes, 2001; Zheng et al., 1999), but the major antigenic differences are reported in the G protein (Duncan and Potgieter, 1993; Kao et al., 1984; Lukic-Grlic et al., 1998; Mlinaric-Galinovi et al., 1994; Storch et al., 1991, 1993; Sullender, 2000; Zheng et al., 1999). Antigenic diversity in HRSV is marked by an

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extensive degree of amino acid heterogeneity, and also phylogenetic analyses confirm such distinction in subgroups (Woelk and Holmes, 2001). BRSV subgroups were also identified (A, B and AB or Intermediary) based on monoclonal antibody and polyclonal sera analyses against F (Schrijver et al., 1996a) and G proteins (Furze et al., 1994, 1997; Lerch et al., 1989; Prozzi et al., 1997); however, detailed phylogenetic analyses and comparisons with the homologous genes on HRSV revealed that BRSV has a smaller degree of variation on its nucleotide and amino acid sequences when compared to the variability found on its human counterpart (Furze et al., 1997; Woelk and Holmes, 2001), forming a continuum rather than the three subgroups defined on antigenic analyses. Valarcher et al. (2000) proposed that six genetics subgroups may be found on BRSV strains, when F, G and nucleoprotein sequences are phylogenetically analyzed by maximum-likelihood algorithms.

A major concern related with the presence of those antigenic subgroups in cattle is whether infection or vaccination with a BRSV strain of one subgroup protects against heterologous reinfection or challenge (Duncan and Potgieter, 1993; Schrijver et al., 1998; Schrijver et al., 1996b; Van der Poel et al., 1994; Woelk and Holmes, 2001; Zheng et al., 1999). Differing from the situation found for HRSV, vaccines are already commercially available to prevent BRSV infections. In Europe and the United States, vaccination against BRSV is common practice (Valarcher et al., 2000); however, in Brazil and South America, for practical and economical reasons, vaccinations against BRSV are not often performed. There are reports attributing site changes on conserved regions of the G protein as a response to the selective pressure exerted by BRSV vaccination (Valarcher et al., 2000), which are similar to those already described for neutralizing antibody escape mutants of HRSV (Martinez et al., 1997; Melero et al., 1997; Walsh et al., 1998).

A growing number of BRSV infections have been reported principally on the Southern region of Brazil during the last decade (Arns et al., 2003; Driemeier et al., 1997; Flores et al., 2000) but there is also evidence of infection on the Northeastern region (Peixoto et al., 2000) and the virus may be causing clinically evidenced disease in Brazilian cattle for at least the last 20 years (Flores et al., 2000). The present study aims to analyze the genetic variation of BRS viruses circulating in Brazil, comparing the partial sequences from the G protein of such strains obtained by RT-PCR with the other partial and complete gene sequences from other countries. Molecular modeling of the immunodominant region of those viruses was also performed to better characterize the structural importance on any mutations present on the cystine-noose of G protein.

2. Materials and methods

2.1. Sequences

In the Genbank, we identified 106 nucleotide sequences of the G protein gene for use in this study. They were retrieved searching on the GenBank Database through the Nucleotide sequences search page (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide&itool=toolbar). We specified "BRSV" and "attachment" as the search words; an additional search was performed searching for "BRSV" and "G". The same procedure was made to collect the deduced amino acid sequences on the NCBI Protein Database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Protein&itool=toolbar), and 110 sequences were collected. Two Brazilian sequences, from the Southern region of the country, BRSV-25-BR (Arns et al., 2003) and BRSV-108-BR (Almeida et al., 2005), that are under submission to the GenBank at the time of this search, were also introduced, and a third Brazilian virus was included (BRSV-4-BR), from which partial sequence of the gene was achieved from the RT-PCR products from nasal swabs samples collected in São Paulo state, at the Southeastern region The PCR product forms an amplicon that included the nucleotide positions 353–680. A phylogenetic tree was constructed using 39 representative sequences and the respective access numbers are shown for each taxon accompanied by the name of the strain in brackets (Fig. 1). The nucleotide sequence of the G protein of ovine RSV (Alansari and Potgieter, 1993) was included as an out-group for phylogenetic analysis.

2.2. Sequence analysis

The BioEdit software, version 7.01 (Hall, 1999), was used to manipulated the nucleotide and amino acid retrieved sequences. The alignment of the sequences was performed using the ClustalW software, version 1.83 (Thompson et al., 1994), using full alignment and a number of 2000 total replications on the bootstrap, in order to ensure a higher level of confidence to our analysis (Efron et al., 1996).

For nucleotide sequences phylogenetic analyses were performed using the neighbor-joining algorithm as implemented in MEGA version 2.1 software package (Kumar et al., 1994), based on the Kimura two-parameter distance estimation method of the proportion of differences (p distance). Bootstrap re-sampling was performed again for each analysis (2000 replications). An additional analysis of non-synonymous and synonymous nucleotide substitutions (those which change or do not change the amino acid, respectively) was also performed on the same computer program. After that, they were also calculated and plotted for individual codons with the help of SNAP program (http://hiv-web.lanl.gov/content/hiv-db/SNAP/WEBSNAP/SNAP.html). Estimation of the both synonymous (ds) and non-synonymous (dn) distances was based on the Nei-Gojobori method (Kumar et al., 1994); the ratios of non-synonymous to synonymous substitutions (dn/ds) were calculated.

2.3. Modeling of secondary and tertiary structures of the G protein

Protein sequences of Brazilian strains and the prototype subgroup B strain BovX were used to predict secondary structure using Garnier-Robinson and Chou-Fasman parameters, Kyte-Doolittle hydrophilicity, surface probability and Jameson-Wolf and Antigenic Index (AI) analysis using Protean 5.0 inside the DNAStar software package (DNAStar,

Inc.). The MODELLER 7 package (Fiser and Sali, 2003) was used to build models of G protein sequences with basis on the available NMR G protein immunodominant derived structure code 1BRV (Langedijk et al., 1997b) at the RCSB Protein Databank (http://www.rcsb.org/pdb/). This program was used to build a model by satisfaction of spatial restraints, applying the default model building routine. To test the stereochemical evaluation and theoretical validation of the 3D profile of the models, programs PROCHECK was used, using the web interface of Parmodel (http://laboheme.df.ibilce.unesp.br/cluster/parmodel_mpi/) (Uchoa et al., 2004). These homology modeling analysis were also performed using SDPMOD web-based feature (Kong et al., 2004) and EsyPred3D (Lambert et al., 2002). The outputs of MODELLER and the other cited web based modeling tools were graphically visualized and prepared using PyMol (http://pymol.sourceforge.net/; DeLano Scientific, San Carlos, CA, USA).

3. Results

3.1. Nucleotide and deduced amino acid sequences

The phylogenetic relatedness of Brazilian BRS viruses according to the sequences taken from the G protein gene is shown in Figs. 1 and 2. The analysis conducted here, using Kimura two parameters, allowed us to allocate BRSV strains in within the six genetic groups proposed by (Valarcher et al., 2000) and additionally to identify those groups in the subgroups A (group III), AB (intermediary; groups II, IV, V and VI) and B (group I). It is clear when analyzing the tree, that Brazilian strains are located on the B lineage (group II). Those Brazilian sequences have also demonstrated the higher degree of homology (96.7–100%) with strains within the B subgroup (Table 1). The nucleotide composition of the G protein gene sequenced from Brazilian BRSV strains (A = 36.76–38.96%;

Fig. 1. Phylogenetic tree prepared with the G protein gene nucleotide sequences (from positions 335–604, 39 sequences) of Brazilian and worldwide distributed strains of BRSV. For the parameters used to measure genetic relationships please refer to the text. GenBank accession numbers are given in each taxon and the correspondent name of the strain is shown in brackets. Only the bootstrap values higher than 50% of 2000 replicates are shown. The roman numbers are related to the genetic groups proposed by Valarcher et al. (2000). The Brazilian strains are narrowed with a triangle; the tree was rooted using an ORSV sequence.

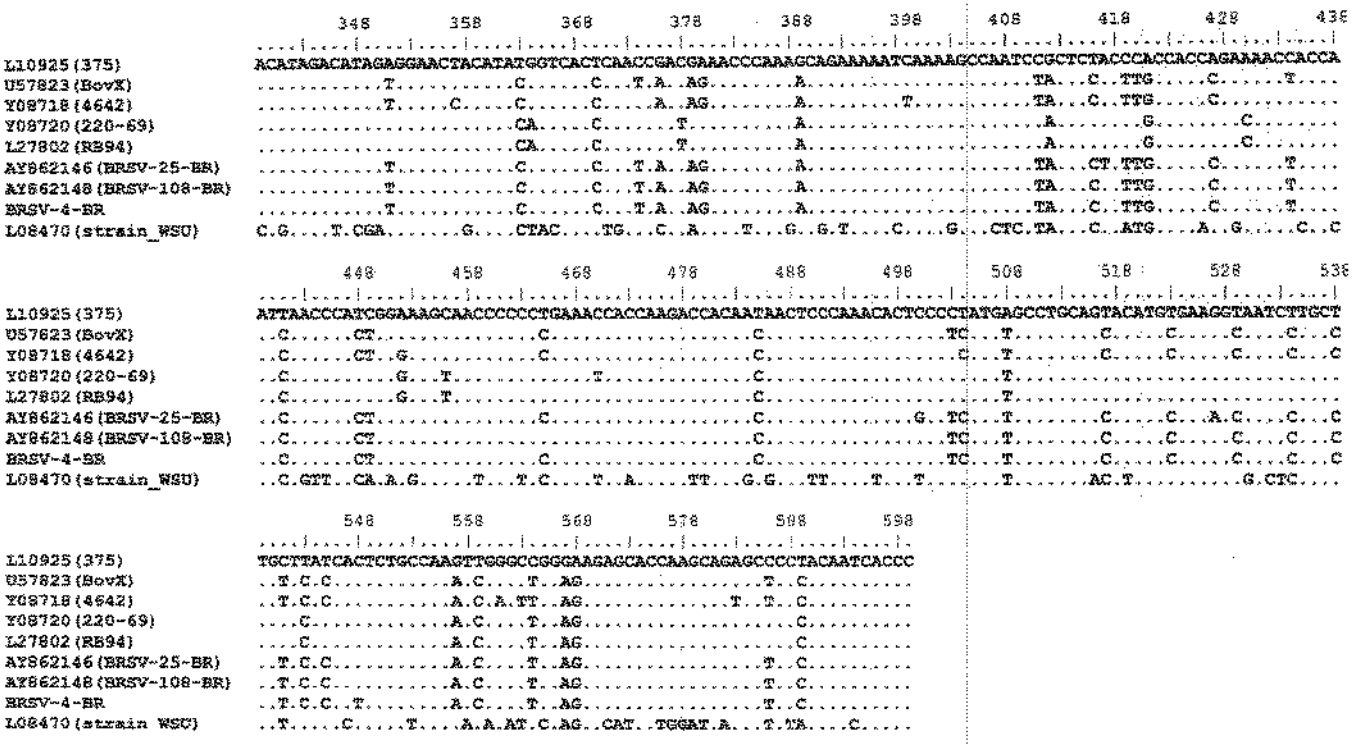

Fig. 2. G protein gene nucleotide sequences (positions 338–599) of Brazilian and worldwide distributed strains of BRSV. NCBI Protein Data Bank accession numbers are given for each sequence and the correspondent name of the strain is shown in brackets.

C = 36.2–36.47; G = 12.35–12.92%; T = 12.35–12.62%) was also very similar to the composition of strains from its same genetic group (A = 36.6–38.8%; C = 33.2–37.8; G = 11–12.2%; T = 14.7–15.89%), but no remarkable differences may be found when comparing to strains belonging to A (A = 37.6–38.9%; C = 31.5–38.5; G = 11.42–15.7%; T = 12.72–16.81%) and AB subgroups (A = 38.64–39.14%; C = 32.67–37.96; G = 12.56–17.3%; T = 10.72–19.93%). The parental strains of most commonly used vaccines against BRSV (RB94; 220–69) were clearly aligned within the AB (intermediary) subgroup.

Amino acid composition of the deduced amino sequences obtained also remained similar to those deposited at the NCBI Protein Data Bank. However, some differences were observed at the amino acid level on the immunodominant region of the strain BRSV-25-BR (Fig. 3), and other remarkable divergences were present on the variable mucin-like regions of the sequence derived from BRSV-4-BR, which may explain the overall low percentages of identity when analyzing BRSV-4-BR amino acid

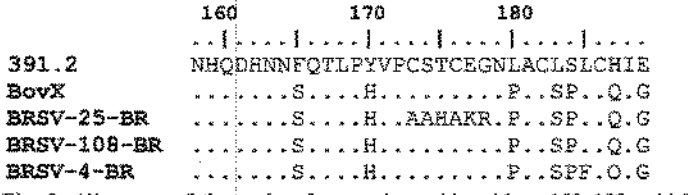

Fig. 3. Alignment of the region from amino acid residues 158–189, which comprises the cystine-noose component residues showing the differences when comparing Brazilian strains with the subgroup A strain 391–2 and prototype subgroup B strain BovX.

sequence and the other strains analyzed. The two cysteines in positions 173 and 176 of the amino acid sequence were replaced by alanine residues in BRSV-25-BR; there were also other substitutions at this site: Ser^{174} was replaced by another alanine; Thr^{175} was substituted by an hystidine and Glu^{177} and Gly^{178} were replaced by lysine and arginine residues, respectively (Fig. 3). The strain BRSV-4-BR has also a mutation within this region (Leu^{185} → Phe).

Table 1
Homology percentages of pairwise distances between G protein nucleotide and deduced amino acid sequences Brazilian strains and selected representative prototype strains of BRSV

	391–2	220–69	RB94	BovX	BRSV-25-BR	BRSV-108-BR	BRSV-4-BR
391–2	–	*87.7*	*94.1*	*84.5*	*72*	*76.4*	*79.5*
220–69	92.3	–	*88.9*	*86.5*	*77.6*	*81.8*	*90.9*
RB94	94.6	99.9	–	*82.2*	*77.8*	*80.9*	*90.9*
BovX	89.1	92.1	92.2	–	*92.5*	*100*	*97.7*
BRSV-25-BR	82.6	87	87.3	97.8	–	*92.6*	*84.0*
BRSV-108-BR	82.9	87.2	87.6	100	97.9	–	*97.7*
BRSV-4-BR	83.7	88.8	89.2	98.8	98.2	99.6	–

Nucleotide and amino acid values are given in normal and italic letters, respectively.

3.2. Rates of evolution between Brazilian G protein gene sequences and genetic groups of BRSV

The ratio of synonymous and non-synonymous substitutions (dn/ds) between Brazilian G protein gene sequences and its own genetic group I, as well as genetic group III, and a set of genetic groups II, IV, V and IV were calculated. When comparing Brazilian strains with those on the genetic group I, dn/ds gives a mean number of 1.333 showing that positive selection is underwent within this group, were the non-synonymous substitutions are higher; when comparing with strains inside the genetic group III (subgroup A) the mean values where estimated as 0.656, forming the far distant group; and for the AB subgroup (genetic groups II, IV, V and VI) the mean value of dn/ds ratio was 1.485.

3.3. Molecular modeling of the immunodominant region of BRSV attachment protein

As mentioned before, we detected mutations in the amino acid sequences of the Brazilian strain BRSV-25-BR on the conserved immunodominant region of the G protein. The amino acid changes (^{173}CSTCEG^{178} → ^{173}AAHAKR^{178}) were examine for putative structural changes on the central domain of the protein. Based on the structural coordinates and parameters deposited on the model available at the PDB of Brookhaven National Laboratory (PDB code 1BRV), we examined the modification that these mutations may induce at the three-dimensional level on the structure of the immunodominant region of such viral strain. As the initial resolution of the structure was made using the subgroup A strain 391–2 (Langedijk et al., 1997b), we firstly developed a model of the homologous structure on BovX strain, which belongs to the same subgroup B as the Brazilian isolates. Despite of the differences between subgroup A and B (Phe^{165} → Ser; Tyr^{170} → His; Leu^{180} → Pro; Leu^{183} → Ser; Ser^{184} → Pro) there are no alterations on the structure of BovX cysteines-noose, when comparing to the model determined with basis on the 392-1 strain; the two conserved disulfide bridges Cys^{173}–Cys^{186} and Cys^{176}–Cys^{182} are present and the two anti parallel helices are preserved as well as the whole structure of the cystine-noose remained stable (Fig. 4). The same may be observed when the putative structures of BRSV-108-BR and BRSV-4-BR were resolved; however, on the BRSV-25-BR derived structure, them was replacement of two of the four cysteines by alanine residues, which did not disrupt the first α helix, where those mutations are located, but destroyed the disulfide bridges that exist in other strains between cysteines residues (Fig. 4).

4. Discussion

From the data presented here, it is confirmed that from phylogenetic analysis of the G protein gene and its deduced amino acid sequences, the BRS viruses circulating in Brazil fall within the previously proposed B subgroup (Nettleton et al., 2003; Prozzi et al., 1997; Schrijver et al., 1996a; Valarcher et al., 2000; Yaegashi et al., 2005), as suggested by Almeida et al. (submitted paper). The virus was firstly referred as a monotypic genetic group (Mallipeddi and Samal, 1993; Stine et al., 1997). However, subsequent analysis showed some correlation between the subgroups determined by monoclonal antibody analysis and genetic analysis (Prozzi et al., 1997), but in a much lower degree of genetic variability than is presented by HRSV (Furze et al., 1997; Prozzi et al., 1997; Valarcher et al., 2000; Woelk and Holmes, 2001), and BRSV seems to form at least six different genetic groups, which are not so diverse from each other (Valarcher et al., 2000). On the present study, an analysis of the nucleotide sequences including the Brazilian strains confirms the last hypothesis, but it seems to be a correlation between the antigenic subgroups and genetic groups, when using the parameters applied on the present study.

The calculated pairwise distances and the relatively ratios of synonymous and non-synonymous substitutions for codons position at the G protein gene confirm that Brazilian BRSV strains are evolving from subgroup B strains, being more distant from those strains in the subgroup A and B, with local differentiation.

The most striking finding of the phylogenetic analyses of Brazilian strains is that, belonging to the subgroup B, these are first strains classified within this subgroup since the 1970s. Unfortunately, it is not possible to trace the exact source from where these viruses came to our country and this effort can only be speculative. This is in agreement with the previous observations that the circulation of BRSV genetic and antigenic groups have a temporal and geographical well defined distribution (Larsen et al., 1998, 2000; Nettleton et al., 2003; Schrijver et al., 1996a; Valarcher et al., 2000; Yaegashi et al., 2005) in an epidemiological situation that is found similar for HRSV (Lukic-Grlic et al., 1998; Mlinaric-Galinovi et al., 1994; Sullender, 2000; Van der Poel et al., 1994; Zlateva et al., 2004).

Another interesting finding is that a major substitution occurred in the cystine-noose coding region of the Brazilian strain BRSV-25-BR. This is a highly conserved region located within the central hydrophobic region of the protein, which may interact with the cellular receptor (Valarcher et al., 2000) and plays an important role on the induction of antiviral host immunity (Langedijk et al., 1997b). In BRSV, mutations in this region were firstly described for three French isolates (Valarcher et al., 2000), where the four cysteines residues that forms disulfide bonds within the cysteine-noose were replaced by arginines, and consequently the second α helix of such structure was disrupted. On the case of BRSV-25-BR, there was a complete substitution of the amino acid residues between position 173 and 178, and the cysteines inside this frame were replaced by alanines. The three-dimensional structure of the cystine-noose remained similar to that proposed by (Langedijk et al., 1997b), however, there are no more disulfide bridges to connect with the other two-mutated cysteines. This region was shown to be the immunodominant portion in the G protein (Langedijk et al., 1997b, 1998); others demonstrated that this central conserved region triggers eosinophilia and augmentation of pulmonary pathology in a RSV mice model, depending on the genetic background of the host (Hussell et al., 1998; Sparer et al., 1998).

In the case of French isolates, it was proposed that immune-driven selection exerted by vaccination with commercial vac-

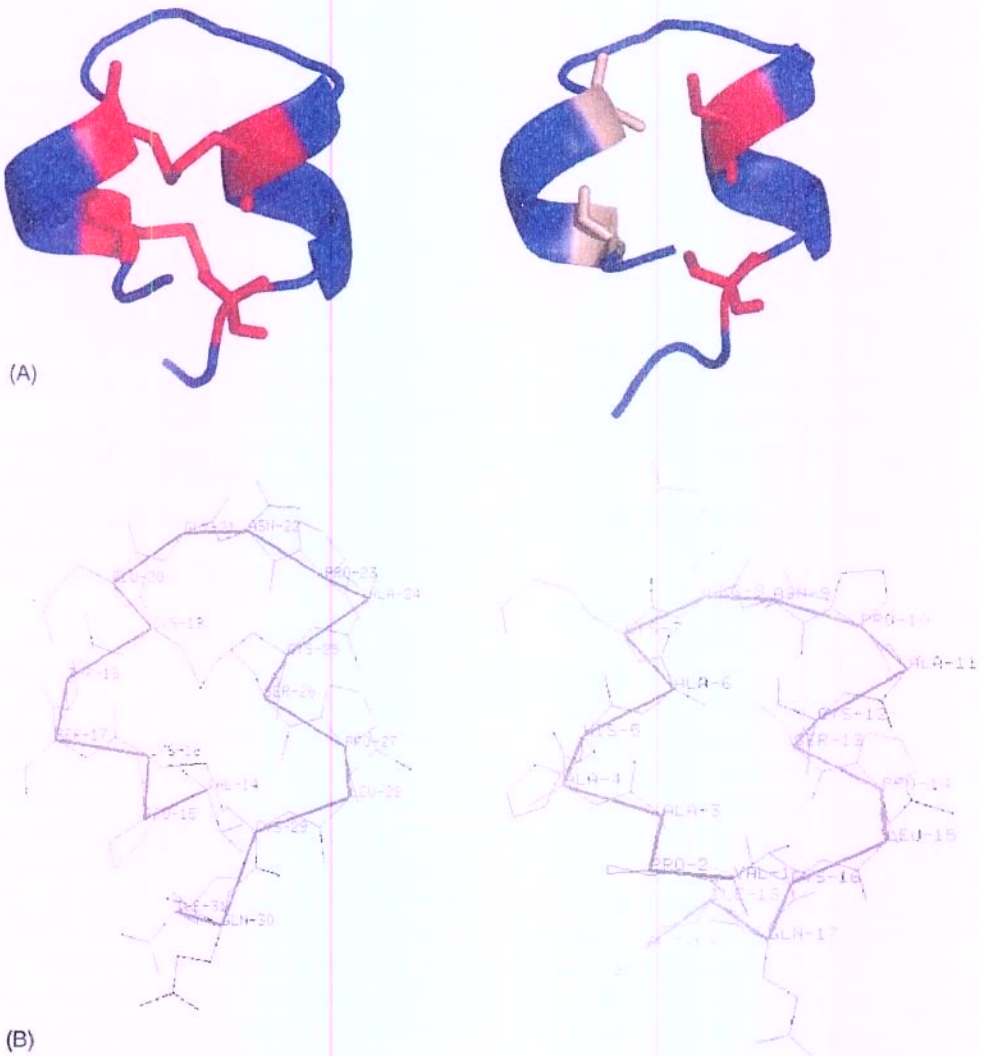

Fig. 4. Schematic drawing (A) of the homology modeling deduced conformation of the upper immunodominant region of the prototype subgroup B strain BovX (left) and the impact of the mutations present on the strain BRSV-25-BR (right). Cysteine residues and dissulfide bridges are shown in red; yellow residues are the Ala mutation sites presented on BRSV-25-BR protein. Technical schemes (B) of the amino acid sequence shown in the lower part of the figure, for BovX (left) and BRSV-25-BR (right) strains. The amino acid residue numbers are only illustrative.

cines may have induced these mutations in such strains (Valarcher et al., 2000). This hypothesis is not applicable to the specific case of BRSV-25-BR strain, however. Despite the presence of commercially available vaccines (that may be allocated within the AB subgroup, until the present moment), vaccination against BRSV infection is minimally used in our territory, and this particular strain was isolated from a farm that never has used immunization to prevent BRSV infections. It may be speculated that the mutations presented by BRSV-25-BR may have been induced by the presence of active and passive naturally acquired immune response against viral infection itself, and the virus may has been selected by infection in animals possessing antibodies to the virus, that selects antibody escape mutants, as demonstrated in vitro for HRSV (Martinez et al., 1997; Melero et al., 1997).

Despite of these alterations on the central hydrophobic region of the attachment protein, this virus infects calves efficiently under experimental conditions, inducing moderate lesions on the lungs of infected animals (Almeida et al., 2005) and the pathogenicity of this strain is now being compared by our group with other BRSV strains in a mouse model. However, more detailed studies have to be conducted with this particular strain, in order to evaluate the possible consequences of viral pathogenicity, induction of immunity and antibody recognition, since this region showed to be involved for both virulence (Sparer et al., 1998) and induction of protective immunity (Schrijver, 1998) in RSVs; another aspect to be further examined is whether such mutation may influence the biological effects induced by the homology of G protein to TNF (Langedijk et al., 1998) and CX3C chemokine (Tripp et al., 2001).

Another practical concern is whether vaccination using an immunogen made with a strain of a particular subgroup may protect against heterologous challenge. It was shown that the whole virus particles of one given viral subgroup may at least partially protects against challenge with a strain presenting a dissimilar G protein sequence (Schrijver et al., 1998). However, there is also some evidence that this partial protection may gave rise to the generation of new BRS viral genetic groups (Valarcher

et al., 2000). Considering that only A and AB subgroup strains are included in vaccines marketed in Brazil at this moment, and only B subgroup were found circulating in our country so far, we are currently making an effort to test whether these vaccines may protects animals against infections with those Brazilian strains and consequently the need and validity to include autochthonous isolates in Brazilian vaccines.

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Artigo 3

Genetic diversity and predicted structure of Bovine respiratory syncytial virus fusion protein

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Manuscrito em preparação

Genetic diversity and predicted structure of Bovine respiratory syncytial virus fusion protein

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Running title: BRSV fusion protein

Keywords: Bovine respiratory syncytial virus; Fusion protein; genetic diversity; comparative modeling; fold recognition

Abstract

Bovine respiratory syncytial virus (BRSV) is a major cause of respiratory disease in young cattle worldwide. The BRSV fusion protein (F) mediates fusion between viral envelope and cell membrane, being essential for virus growth. The protein is secreted as an inactive precursor (F0) and is subsequently cleaved in two subunits, F1 and F2, which are associated by disulfide bonds. On the present study genetic relationships between BRSV isolates based on F gene, including tree new Brazilian isolates, and comparative modeling of the uncleaved form of BRSV fusion protein (F0) were conducted, as an attempt to better understand the selective pressures exerted on this glycoprotein and to determine the putative tertiary structure of the protein. No positively selected sites were found on F gene when non-synonymous per synonymous (Ka/Ks) amino acid substitution rations from different genetic groups were calculated. However, some positively selected sites between gene positions 279 to 351, between isolates of genetic group I, were Brazilian isolates are located. The modeled structure of F0 presents the same structural constraints described for homolog proteins previously described on others members of the *Paramyxoviridae* family.

1. INTRODUCTION

Bovine respiratory syncytial virus (BRSV) is widely recognized as an important cause of respiratory illness in young cattle (Van der Poel et al., 1994; Larsen, 2000). The virus, a negative-sense single stranded RNA virus, belongs to the *Paramyxoviridae* family, being classified in the genus *Pneumovirus*, from the sub-family *Pneumovirinae* (Pringle, 1999) as its close relative, the human respiratory syncytial virus (HRSV). The viral genome, 15,140 nucleotides long (Buchholz et al., 1999), encodes 10 proteins; three of those are glycosylated peptides which are presented on the viral membrane. Glycoprotein G, a mucin-like glycosylated protein, is involved in the attachment of the virion to the cell membrane (Langedijk et al., 1997; Langedijk et al., 1998). G protein, as well as the membrane glycoprotein SH (small hydrophobic) are non-essential for the growth of BRSV in cell cultures (Karger et al., 2001). Protein G and its coding gene (G gene) are often used as a source of information for genetic characterization of BRSV strains and studies on the evolution of the virus (Elvander et al., 1998; Larsen et al., 1998; Valarcher et al., 2000; Kovarcik and Valentova, 2004; Valentova et al., 2005; Spilki et al., 2006) and the structure of such protein is fairly well known (Langedijk et al., 1997; Langedijk et al., 1998).

The third envelope protein, protein F, mediates the fusion of the viral envelope to the cell membrane (Pastey and Samal, 1993; Pastey and Samal, 1997; Schrijver et al., 1997; Pastey and Samal, 1998) being an important target for the immune system of cattle infected or vaccinated with BRSV (Nelson et al., 1992; Ellis et al., 1995; Schrijver et al., 1997; Gaddum et al., 2003; Taylor et al., 2005). Fusion proteins from paramyxoviruses are found in a homotrimer form. They are secreted as an inactive precursor (F0), and proteolytical cleavage of this polypeptide in two subunits (F1 and F2) is required for protein function

(Gonzalez-Reyes et al., 2001; Zimmer et al., 2001; Zimmer et al., 2002; Rixon et al., 2004; Ruiz-Arguello et al., 2004). F2 subunit determines host specificity of infection for both bovine and human respiratory syncytial viruses (Schlender et al., 2003) and it is associated to the anchor F1 subunit by disulphide bonds. The F protein structure of BRSV, like its HRSV counterpart, must conserve many of the secondary and tertiary features found on other paramyxoviruses fusion proteins (Walter et al., 2002; Xu et al., 2004; Yin et al., 2005). Differences on the F protein structure of BRSV and HRSV and within genetically divergent BRSV were found (Mallipeddi et al., 1990; Mallipeddi and Samal, 1993; Pastey and Samal, 1993; Pastey and Samal, 1997; Pastey and Samal, 1997).

BRSV infections do occur worldwide (Van der Poel et al., 1994; Furze et al., 1997; Costa et al., 2000; Larsen, 2000; Arns et al., 2003; Nettleton et al., 2003; Almeida et al., 2005; Yaegashi et al., 2005). Different viral antigenic and genetic subgroups were found (Baker et al., 1992; Duncan and Potgieter, 1993; Pastey and Samal, 1997; Prozzi et al., 1997), resembling the situation for HRSV (Duncan and Potgieter, 1993; Van der Poel et al., 1994; Zlateva et al., 2004). Antigenic subgroups were named as A, AB and B (Baker et al., 1992; Schrijver et al., 1996; Schrijver et al., 1998). Later, six distinct genomic subgroups were proposed (Valarcher et al., 2000; Yaegashi et al., 2005) and adopted by other authors (Yaegashi et al., 2005; Spilki et al., 2006). Phylogenetic studies are often conducted using the G protein for classification, since it is more variable than F protein (Valentova, 2003). However, BRSV subtyping is also possible using antigenic methods (Furze et al., 1994; Pastey and Samal, 1997) and F gene sequences for phylogenetic characterization (Valarcher et al., 2000). Three BRSV subgroups were identified so far (A, B and AB or Intermediary) with basis on monoclonal antibody and polyclonal sera analyses against F (Schrijver et al., 1996) and G proteins (Lerch et al., 1989; Furze et al., 1994; Furze et al., 1997).

On the present study genetic relationships between BRSV isolates based on F gene, including tree new Brazilian isolates, and comparative modeling of BRSV fusion protein were conducted, as an attempt to better understand the selective pressures exerted on this glycoprotein and to determine the putative tertiary structure of the protein.

2. MATERIALS AND METHODS

2.1. Nucleotide and amino acid sequences

Thirty-one nucleotide sequences of the F protein gene available at the Genbank were used this study. They were retrieved by search on GenBank Database through the Nucleotide sequences search page (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide&itool=toolbar). We specified "BRSV" and "fusion" or "F" as the search words; A51908 F gene was taken from the full genome available at the same database. Similar procedure was made to collect the F protein deduced amino acid sequences on NCBI Protein Database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Protein), and 30 sequences were found. A new Brazilian virus was included (BRSV-4-BR), from which tha partial sequence of the gene was obtained from RT-PCR products (Almeida et al., 2004; Almeida et al., 2005). A phylogenetic tree was constructed using gene sequences and the respective access numbers are shown for each taxon accompanied by the name of the strain in brackets (Figure 2). The nucleotide sequence from ovine RSV and HRSV F proteins were included on the analyses for comparison.

2.2. Sequence analysis

The BioEdit software, version 7.0.5.2 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) (Hall, 1999) was used to manipulate the nucleotide and amino acid sequences. Alignment of the sequences was performed using the ClustalW software, version 1.83 (Thompson et al., 1994). Two thousand total replications were used as the bootstrap value, in order to ensure a higher level of confidence to the analysis (Efron et al., 1996). The multiple alignments were checked and corrected through visual inspection and manipulation using BioEdit 7.0.5.2.

Phylogenetic analyses were performed on the MEGA version 3.1 computer software (Kumar et al., 2004). Distance methods were used for determination of phylogenetic relatedness with Kimura two-parameters distance estimation method of the proportion of differences, using the neighbor-joining algorithm. Bootstrap re-sampling was performed again for each analysis (500 replications). An additional analysis of non-synonymous and synonymous nucleotide substitutions (those which change or do not change the amino acid, respectively) and evolutionary hypothesis (neutral evolution; positive selection or purifying selection) were also performed on MEGA 3.1. Estimation of the synonymous (Ks) and non-synonymous (Ka) distances was based on the Nei-Gojobori method (Kumar et al., 1994) the ratios of non-synonymous to synonymous substitutions (Ka/Ks) were calculated and plotted as well as other complementary analyses (rates of transitions and transversions; identity and similarity) using the SWAAP 1.0.2 software (http://www.bacteriamuseum.org/SWAAP/SwaapPage.htm).

2.3. Comparative modeling of BRSV F0 protein

Fusion protein amino acid sequences of 391-2, RB-94 and A51908 strains were submitted to analysis of transmembrane regions prediction based on the putative secondary structure

using TMpred (http://www.ch.embnet.org/software/TMPRED_form.html) (Heckert and Hofmann, 1993). Fold recognition was used to generate the putative three-dimensional structures using the 3D-PSSM web server (http://www.sbg.bio.ic.ac.uk/~3dpssm/index2.html) (Kelley et al., 2000) and its substitute PHYRE (http://www.sbg.bio.ic.ac.uk/~phyre/). A set of analysis showed that the structure with the PDB ID:1ZTM at the RCSB Protein Databank (http://www.rcsb.org/pdb/), regarding the 3.05 Å resolution X-ray diffraction solved structure of the human parainfluenza type 3 fusion protein (Yin et al., 2005) was the template that best fits for fold recognition with the BRSV sequences. The resulted models were refined subsequently (Deliyannis et al., 1998). To test the stereochemical evaluation and theoretical validation of the 3D profile of the models programs PROCHECK, WHAT_CHECK and Verify3D were used, using the web interface of Parmodel (http://laboheme.df.ibilce.unesp.br/cluster/parmodel_mpi/)(Uchoa et al., 2004).

3. RESULTS

3.1. Analysis of nucleotide and amino acid sequences

F gene of BRSV, similarly to other genes and whole genomes of *Paramyxoviridae* family is A-T rich: A, from 35.13% to 37% of the gene; T, 27.29-28.3%; C, 14.80-17.77%; *G*, 17.18-24.47%. No remarkable differences may be found within different genetic groups of BRSV for these parameters with the exception of a lower level of identity between groups IV, V and VI and the other groups considering both nucleotide and amino acid sequences. In deduced amino acid sequences of full length fusion protein, Serine (10.54 %), Isoleucine and Leucine (both 9.14 %) are the most abundant amino acid residues found.

Nucleotide and amino acid identity is high among BRSV F genes from the different isolates analyzed and its products (Table 1). Percentual identities between 96.5 % and 100 % within BRSV gene sequences and 91.1-100 % of identity were observed for deduced amino acid sequences. As expected, the level of identity for both gene and protein sequences was higher between BRSV and ORSV derived sequences than the identity that may be found between those cited and HRSV F sequences (Table 1).

Phylogenetic relationships within BRSV isolates and other mammalian pneumoviruses with basis on the F gene nucleotide sequences are shown in Figure 1.

Graphical plotting of the identities between both gene and amino acid sequences (Figure 2) showed that the major numbers of differences among BRSV isolates may be found at the level of 3' extremity of the gene and the corresponding F2 region of the protein.

Rates of non-synonymous (Ka) and synonymous (Ks) substitutions, as well as transitions and transversions along the F gene were calculated and plotted (Figure 3). Levels of positive selection are low for the gene, but analysis using a sliding window revealed that Ka/Ks ratios are not constant along the F gene. Such analysis showed that the majority of

nucleotide substitutions (mostly synonymous) are located on the portion of the gene codifying for F1. Positively selected sites were observed when comparing only isolates within the genetic group I, from nucleotide positions 279 to 351.

3.2. Comparative modeling

The three-dimensional model obtained by fold recognition for the BRSV fusion protein may be seen at the Figure 4. The model was divided for inspection in three regions (head, neck and core). The predicted base consisted of an elongated core, formed by an alpha-helix. On the lower part of this core, transmembrane regions were predicted with basis on the analysis performed in TMPred. This core region was followed by the neck region that is on the base of the head region, which may be described as an inverted triangular prism containing four channels, one on the presumably internal face of the protein and three located the external face of the protein.

At least 83% of the amino acid residues present on these models was located in most favored regions of the Ramachandran plot. It was not possible to obtain models with higher levels of precision due to limitations of the 1ZTM template itself, were many residues also fall on disallowed regions of the Ramachandran plot. In BRSV F0 structures modeled here, Glu31, Thr37, Asn338, Ile410, Ala412, and Asn 454 fall in generously allowed and disallowed regions of the Ramachandran plot. No remarkable differences may be seen at visual inspection among models obtained for F proteins from different genetic groups. Considering the BRSV F0 model obtained, the protein possesses the conserved features observed for the uncleaved form of fusion proteins of other paramyxoviruses.

4. DISCUSSION

Genetic differences between BRSV F gene and the corresponding fusion protein were analyzed. As reported in previous works (Larsen et al., 1998; Valarcher et al., 2000), F gene is highly conserved between BRSV strains, and a few number of differences are present when analyzing fusion protein deduced amino acid sequences.

Phylogenetic analysis based BRSV F gene sequences, as conducted on the present work, results in lower levels of bootstrap and genetic differentiation between strain within cluster when compared to those often reported for phylogenetic relatedness using G gene (Furze et al., 1997; Elvander et al., 1998; Valarcher et al., 2000; Yaegashi et al., 2005; Spilki et al., 2006) similarly to the findings for HRSV (Zlateva et al., 2004) and other pneumoviruses (Bastien et al., 2003; Ishiguro et al., 2004; van den Hoogen et al., 2004). Characterization of BRSV in different clusters may be performed with basis on the F gene (Figure 1) and it was also reported by others (Valarcher et al., 2000). F gene amplification and sequencing may be also considered for the preliminary characterization of BRSV field isolates; however, the results obtained using such strategy tends to be poor in terms of separation between clusters when compared to those obtained using gene sequencing and phylogenetic analysis of BRSV G gene, as observed on the present work and previously published studies (Valarcher et al., 2000).

These low levels of differences in F gene and fusion protein sequences when compared to those observed for G gene and its translation product may be consequent to the essential role of fusion protein on virus growth (Karger et al., 2001; Zimmer et al., 2001; Schlender et al., 2003; Zimmer et al., 2005), allowing little or no room for strong variation of such peptide. This phenomenon also may be the cause for the low levels of transversions and non-synonymous substitutions observed on the present work and by other authors

(Valarcher et al., 2000). However, the levels of transitions and transversions and nucleotide substitutions, as well as the identity between different sequences, are not equal even along F gene (Figure 2). The second third fragment of the gene sequence is suffering a higher ratio of evolution than others, accumulating both synonymous substitutions and transversions (Figure 2). This gene fragment corresponds to an antigenic domain located at the neck region of the protein (Figure 3). Then, it may be concluded that the selective pressures exerted by the immune system of cattle are of minor effect for the generation of genetic diversity observed when analyzing the BRSV F gene. Thus, these silent mutations are present on the F gene not because it is subjected to selective pressure by the immune system, but probably because viruses carrying replacements at this domain must be subjected to negative selection, in an effort to maintain the structural constraints of the protein. These regions also presented most the variability between BRSV F gene sequences; however, as mentioned before (Valarcher et al., 2000), no antigenic sites were experimentally identified in this region. On the other hand, looking at the Ka/Ks ratios considering only BRSV B subgroup strains, some non-synonymous substitutions may be found on nucleotide sequence at the beginning of the gene fragment coding for the first heptad and adjacent regions of the F1 protein domain, at the core region of the F0 model obtained here. Neutralization of the virus by monoclonal antibodies binding to amino acid residues $_{173}$STNKAVVSLS$_{182}$, located in this same region was reported (Langedijk et al., 1998). At least for the BRSV subgroup B, positive selection due to host's immune response may be present for the gene F. The same phenomenon was not observed for subgroup AB, and it was not possible to test such assumptions for the subgroup A, since only 2 sequences from BRSV A (genetic group 1) isolates are available. It is difficult to explain why positive selection may be found only on such BRSV subgroup B strains. It is interesting to observe

that Brazilian strains were included on the analysis and vaccination of cattle against BRSV is not widely practiced until now in our country, differing from the situation that may be observed in many of the European countries. Thus, immune responses of cattle in the absence of vaccination may theoretically induce mutations on BRSV F protein, as hypothesized before for BRSV G protein (Spilki et al., 2006).

Previous studies have shown that some differences may be found at the structural level among fusion proteins from antigenically and genetically divergent BRSV strains (Mallipeddi and Samal, 1993; Pastey and Samal, 1993; Pastey and Samal, 1997; Pastey and Samal, 1998) and this attributed to different patterns of N-glycosylation (Mallipeddi and Samal, 1993). Effectively, considering the three-dimensional models obtained by fold recognition on the present study, where no remarkable differences on the predicted tertiary structure of F0 peptide may be observed among genetically divergent BRSV isolates. It is expected that those reported structural differences may be accounted for post-transcriptional processing and specially glycosylation of such peptides. The models obtained here were based on fold recognition, since low degrees of identity were observed for BRSV F and HPI3V fusion protein (17 %). The model is based only on the HPI3V previously reported structure, considering no other experimental support, then one have to take in account that some of the structural constraints of the protein may be lost. But preliminary assumptions, such as the extreme structural similarity of the BRSV F0 model obtained here and those previously described for its homolog in HRSV (Melero et al., 1997; Smith et al., 2002; Walter et al., 2002; Xu et al., 2004) and the fusion protein of other paramyxoviruses (Joshi et al., 1998; Dutch et al., 1999; Morrison, 2003).

Diagnostic tools and subunit vaccines were developed previously based on the expression of BRSV fusion protein and its immunogenic properties (Nelson et al., 1992; Schrijver et

al., 1997; Pastey and Samal, 1998; Gaddum et al., 2003; Taylor et al., 2005). We believe analysis of the BRSV F protein structure taken together with evolutionary analysis of this protein and its gene may shed some light on the further design of safer and more effective subunit vaccines using BRSV F as immunogen.

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Table 1. Identity percentages of pairwise distances among F protein nucleotide (BLOSUM62) and deduced amino acid sequences (PAM120) BRSV isolates. GenBank accession numbers are given together with the name of the strains in brackets. Nucleotide and amino acid values are given in normal and italic letters, respectively.

	BRSV Genetic groups*									
	IV, V and VI			I		III	II	Untyped		
	AF188564(F2)	AF188570(5761) AF188569(V347) AF188568(88P) AF188567(P8) AF188566(P3) AF188565(W6) AF188563(L1) AF188562(90504) AF188561(K1) AF188560(J1) AF188559(G2) AF188555(A1) AF188554(394)	AF188558(C2) AF188557(B2) AF188556(B1)	AF188576(4642)	AY862148 (BRSV-25-BR) BRSV-108-BR BRSV-4-BR	AF188571 BAYOVAC-375) M58350(391-2)	AF188575(22069) AF188574(FV160) AF188573(LELYSTAD) AF188572(W2) D00953(RB94)	AF295544(A51908)	AF013254 (HRSV-B1)	AF334398(ORSV)
AF188564(F2)		*98.7*	*99.5*	*98.3*	*98.3*	*98.7*	*98.3*	*97.5*	*91.1*	*96.7*
AF188570(5761) AF188569(V347) AF188568(88P) AF188567(P8) AF188566(P3) AF188565(W6) AF188563(L1) AF188562(90504) AF188561(K1) AF188560(J1) AF188559(G2) AF188555(A1) AF188554(394)	98.9		*99.1-99.5*	*99.79-99.83*	*99.79 - 99.83*	*98.3-99.1*	*97.5-97.9*	*97.1-97.9*	*91.1-91.5*	*96.3-97.1*
AF188558(C2) AF188557(B2) AF188556(B1)	97.8	98,9		*98.3-98.7*	*98.3-98.7*	*99.5-100*	*98.3-98.7*	*97.5-97.9*	*91.1-91.5*	*96.7-97.1*
AF188576(4642)	95.6	96.7	95.6		*100*	*97.9*	*98.7*	*98.7*	*90.7*	*97.5*
AY862148(BRSV-25-BR) BRSV-108-BR BRSV-4-BR	95.6	96.7	95.6	95,6		*97.9*	*98.7*	*98.6-98.7*	*90.7*	*97.5*
AF188571(BAYOVAC-375) M58350(391-2)	97.8	98.9	97.8	96.7	97.8		*97.9-100*	*97.9*	*91.5*	*97.1*
AF188575(22069) AF188574(FV160) AF188573(LELYSTAD) AF188572(W2) D00953(RB94)	96.7	97.8	96.7	96.7	95.6	98.9		*97.9*	*90.7*	*96.3*
AF295544(A51908)	95.6	95.6	95.6	98.9	100	97.8	98.9		*89.9*	*96.7*
AF013254(HRSV-B1)	77.4	78.4	78.4	76.3	76.3	78.4	79.5	76.3		*90.7*
AF334398(ORSV)	87.0	88.1	89.2	88.1	89.2	89.2	89.2	89.2	80.6	

*** based on Valarcher et al., 2000**

Figure captions

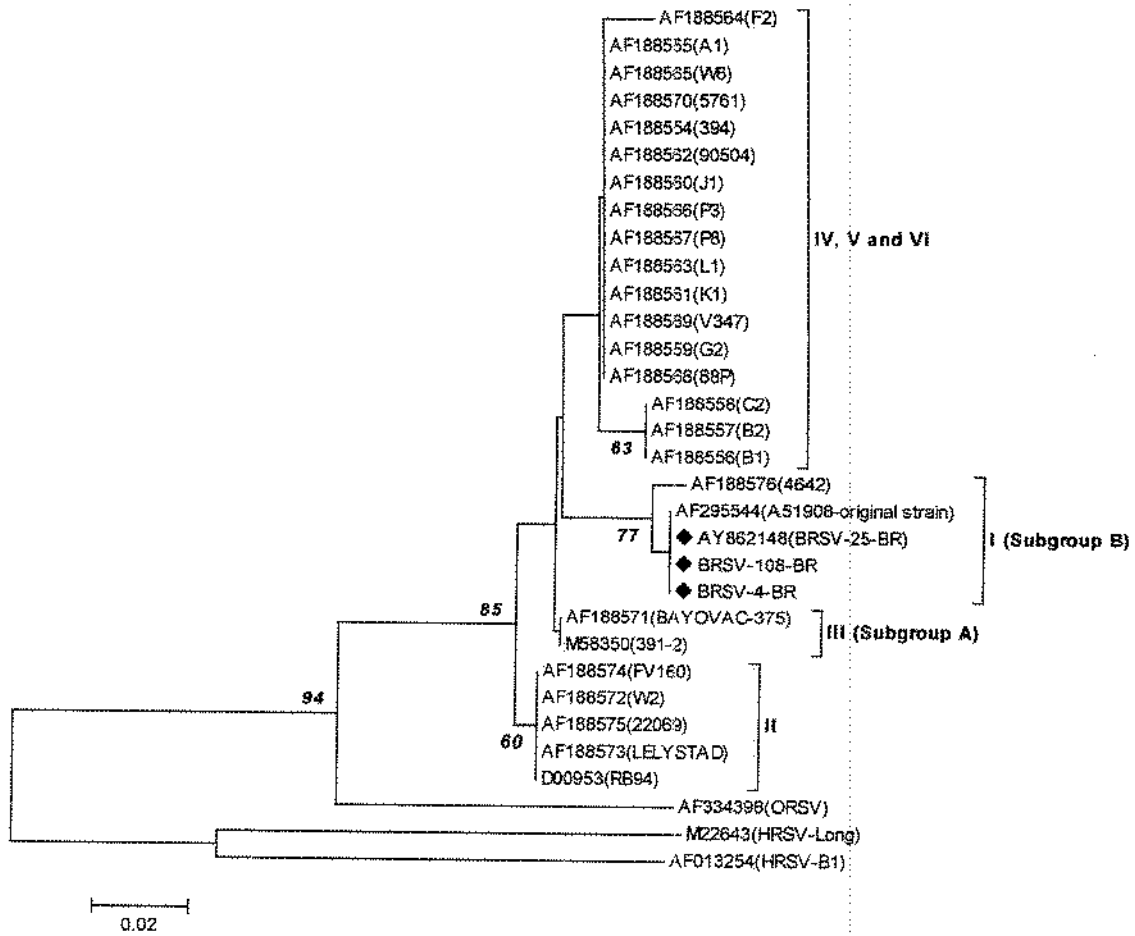
Figure1. Phylogenetic tree prepared with the F protein gene nucleotide sequences (positions 512-609) from different isolates of BRSV. Neighbour-joining using Kimura-2 parameters were used for phylogenetic reconstruction. GenBank accession numbers are given in each taxon and the correspondent name of the isolates are shown in brackets. Only the bootstrap values higher than 60% of 250 replicates are shown. The roman numbers are related to the genetic groups proposed by Valarcher et al. (2000). The Brazilian strains are assigned with a triangle; the tree was rooted using HRSVB and ORSV sequences.

Figure 2. Nucleotide (above) and amino acid (below) percentual identities for the F gene and F0 protein comparing strains of BRSV. A higher level of divergence may me found at the region corresponding for the F2 fragment of the protein.

Figure 3. Levels of synonymous (Ks) and non-synonymous (Ka) substitutions along the BRSV F gene (above). Levels of transitions (Ts) and tranversions (Tv) for this same gene using the plotting resulted from sliding windows calculation.

Figure 4. Three-dimensional models predicted for the uncleaved form of BRSV fusion protein. Predicted tertiary structures for monomers from three genetically divergent strains are shown.

Figure 1.


AF188564(F2)
AF188555(A1)
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AF188554(394)
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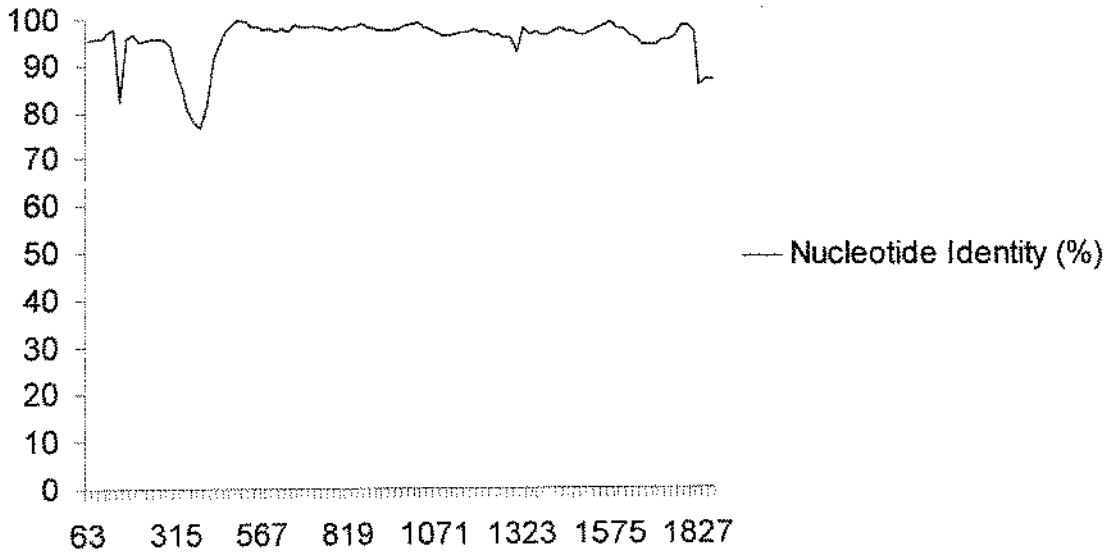
AF188556(P3)
AF188557(P8)
AF188563(L1)
AF188561(K1)
AF188569(V347)
AF188559(G2)
AF188568(88P)
AF188558(C2)
AF188557(B2)
AF188556(B1)
IV, V and VI
63
AF188576(4642)
AF295544(A51908-original strain)
AY862148(BRSV-25-BR)
BRSV-108-BR
BRSV-4-BR
I (Subgroup B)
77
85
AF188571(BAYOVAC-375)
M58350(391-2)
III (Subgroup A)
AF188574(FV160)
AF188572(W2)
AF188575(22069)
AF188573(LELYSTAD)
D00953(RB94)
II
94
60
AF334398(ORSV)
M22643(HRSV-Long)
AF013254(HRSV-B1)
0.02

Figure 2.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80
70
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20
10
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63
315
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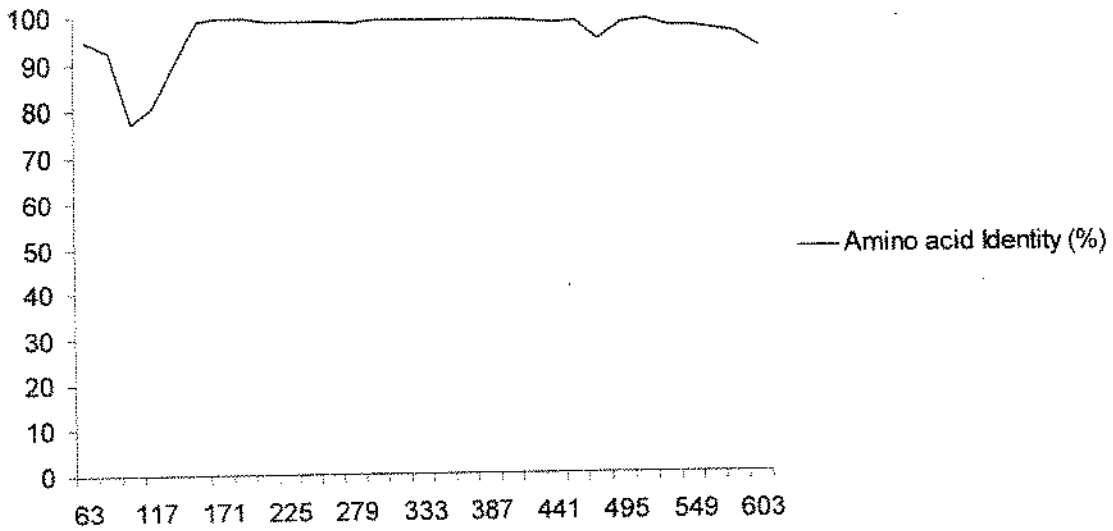
1071
1323
1575
1827
Nucleotide Identity (%)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80
70
60
50
40
30
20
10
0
63
117
171
225
279
333
387
441
495
549
603
Amino acid Identity (%)

Figure 3.

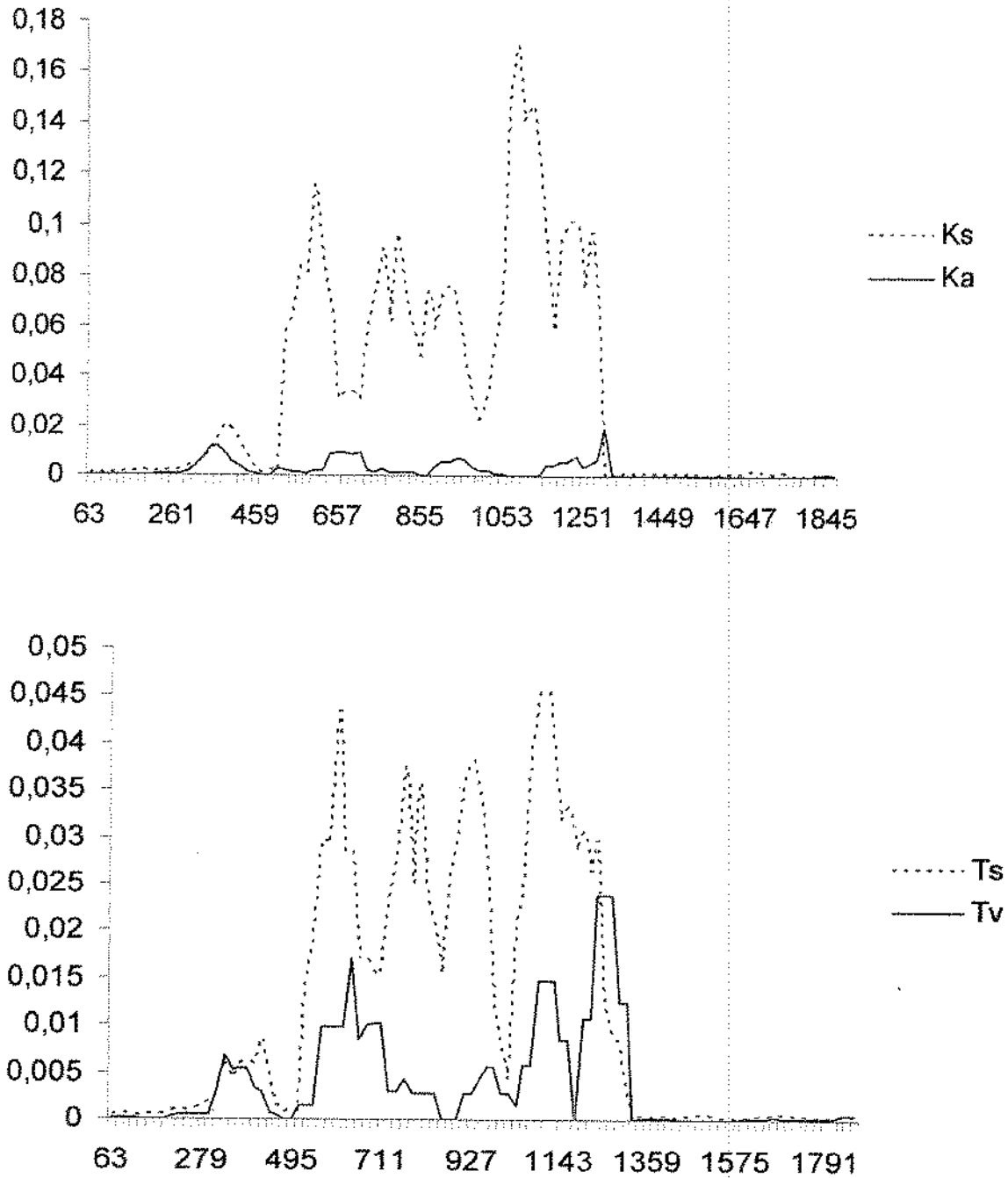
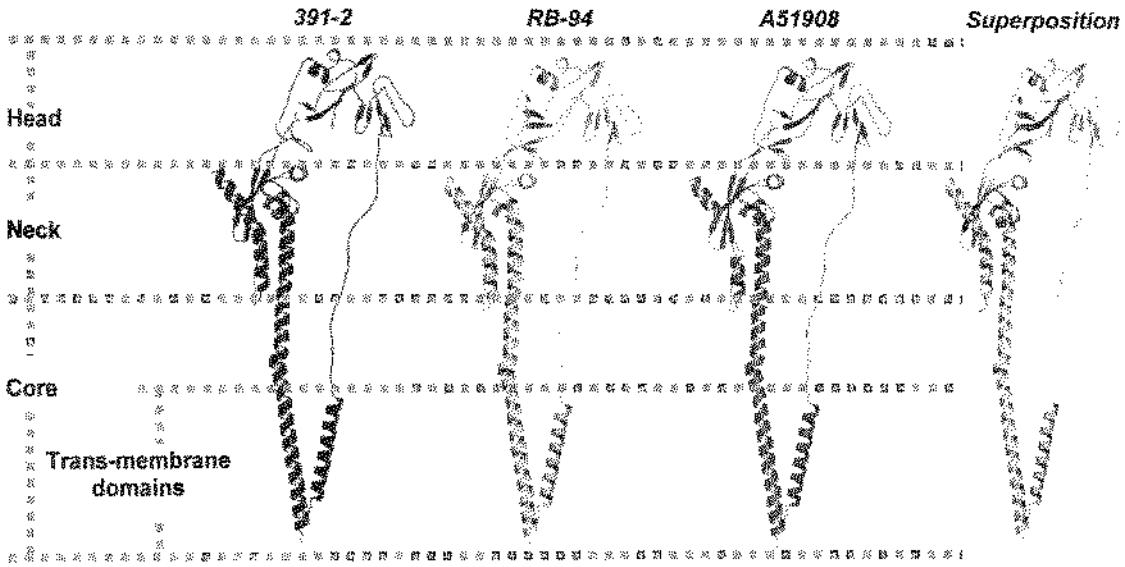

0,18
0,16
0,14
0,12
0,1
0,08
0,06
0,04
0,02
0
63
261
459
657
855
1053
1251
1449
1647
1845
Ks
Ka
0,05
0,045
0,04
0,035
0,03
0,025
0,02
0,015
0,01
0,005
0
63
279
495
711
927
1143
1359
1575
1791
Ts
Tv

Figure 3.


391-2
RB-94
A51908
Superposition
Head
Neck
Core
Trans-membrane domains

Artigo 4

Effects of experimental inoculation of bovine respiratory syncytial virus in different inbred mice lineages: Establishment of a murine model for BRSV infection

Fernando Rosado Spilki, Renata Servan Almeida, Helena Lage Ferreira, Jacy Gameiro, Liana Verinaud, Clarice Weis Arns

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Effects of experimental inoculation of bovine respiratory syncytial virus in different inbred mice lineages: Establishment of a murine model for BRSV infection

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Abstract

Bovine respiratory syncytial virus (BRSV), a member of the subfamily *Pneumovirinae*, family *Paramyxoviridae*, is a major cause of respiratory disorders in young cattle. A number of studies were conducted to validate a reliable animal model for the infection, since BRSV inoculation on the natural host is costly and often unsuccessful. Unfortunately, after inoculation of BRSV in Balb/C mice, viral replication may be detected; however, evident pathological alterations are absent on the experimentally infected animals. In order to establish a mice model that could be used further for preliminary studies of pathological and immunological aspects of BRSV infection, three mice inbred lineages (Balb/C, A/J and C57BL6), possessing different genetic backgrounds, were tested about its susceptibility to the inoculation with BRSV. Animals were inoculated through the nasal and ocular routes and were observed after inoculation. At 7 days post-inoculation (dpi) animals were necropsied and virological (virus isolation and viral nucleic acid amplification) as well as histopathological examinations were performed. A/J and C57BL6 showed interstitial pneumonia, when compared to the Balb/C group. These findings shows that mice may constitute a suitable model for the study of BRSV infections, depending on the mice strain used for experimental inoculations.

Keywords: Bovine respiratory syncytial virus; BRSV; Inbred mice; Murine model; Experimental infection; RT-Nested-PCR

1. Introduction

Bovine respiratory syncytial virus (BRSV) is a member of the *Paramyxoviridae* family classified within the *Pneumovirus* genus, a section of the *Pneumovirinae* subfamily. All members of such genus

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are involved in respiratory disease, and BRSV is one of the major causes of lower respiratory tract illness in young cattle (Larsen, 2000) as well as its human counterpart, Human respiratory syncytial virus (HRSV) remains the principal cause of respiratory disease in young children (Easton et al., 2004).

Animal models are useful tools for the study of immunology, pathology and vaccine development for a number of reasons. In human diseases, like HRSV, the study of many aspects following infection by the virus may be only investigated properly using animals, due to obvious ethical reasons. Thus, for immunopathological studies, preclinical development of new treatments and vaccines for HRSV, animal experiments are required (Easton et al., 2004), and a number of animal models, including monkey (Takimoto et al., 2004; Weltzin et al., 1996), ferrets (Byrd and Prince, 1997; Colasurdo et al., 1998), chinchilla (Doyle and Alper, 2003; Gitiban et al., 2005), mice (Easton et al., 2004) and other animals (Byrd and Prince, 1997) were already used for this purpose.

For studies of BRSV infection in large animals, other advantages may be obtained by the use of animal models: the substitution of cattle for smaller species, at least on preliminary studies, may be highly cost-effective, since maintaining of bovine under experimental conditions following animal welfare guidelines is often expensive, requiring appropriate rooms and trained personnel. Other ruminants, specially sheep, were used for study of immunopathological aspects regarding BRSV infection (Meehan et al., 1994; Meyerholz et al., 2004; Sharma et al., 1991). Another limitation is that both cattle and sheep are not inbred raised, being difficult to avoid individual variations under experimental conditions. BRSV can replicate in a number of mammalian cell lines and primary cell cultures including cell lines derived from small rodents (Spilki et al., 2006); however, attempts to inoculate mice with BRSV were unsuccessful on reproduce efficiently pathological and immunological aspects of the disease on the original host (Almeida et al., 2004; Taylor et al., 1984), although virus isolation (Taylor et al., 1984) and detection of viral nucleic acids using RT-nested-PCR was reported (Almeida et al., 2004). These previous inoculations attempts of BRSV in a murine model used only Balb/C mice lineage (Almeida et al., 2004; Taylor et al., 1984). For HRSV, there is a plethora of published information on different levels of susceptibility for different mice strains to the viral infection (Prince et al., 1979; Stark et al., 2002).

On the present work, inbred mice from lineages possessing different haplotypes were used for inoculation of BRSV, as an attempt to develop a better protocol for the study of such infection in a murine model. Animals were monitored for clinical signs, pathological alterations and virus detection, using virus isolation and viral nucleic acid detection.

2. Materials and methods

2.1. Viruses and cells

The Brazilian strain BRSV-25-BR (Arns et al., 2003) and the prototype strain 375 (Stine et al., 1997) were used on the present study. CRIB cells (Flores and Donis, 1995), a clone of MDBK cells resistant to the infection with BVDV, which is susceptible to the infection with BRSV (Flores and Donis, 1995; Spilki et al., 2006) were used throughout. Cells were cultivated in Eagle's minimal essential medium (E-MEM) supplemented with 100% foetal calf serum (FCS), free of antibiotics, following routine protocols.

2.2. Production of challenge virus

For the production of challenge inoculum used for mice infection, 150 cm^2 bottles containing CRIB monolayers were infected with a multiplicity of infection of 1 (1 infectious particle per cell) of one of the viral strains. Bottles were kept at 37 8C and microscopically monitored until presence of 60–70% of the typical cytopathic effect (syncytium formation), usually 72 h after infection. The infected monolayers were then scrapped and the resulting suspension was vigorously shaken. Cell debris were centrifuged (700 X g, 10 min, and 4 °C) and the supernatants stored at -70 °C until use.

2.3. Animal inoculation

Three mice inbred lineages: Balb/C (haplotype: H-2^d), A/J (H-2^a) and C57BL6 (H-2^b), were used on the experiments. The animals, 8–10-week old specific-pathogen-free males, were purchased from CEMIB (Multidisciplinary Centre for Biological Investigation,

at UNICAMP, Brazil). The animals were kept behind barriers. At the day of arrival on the isolation units at the Departamento de Microbiologia e Imunologia, UNICAMP, the animals were divided in groups, in separate cages. Each viral strain (BRSV-25-BR or 375) was inoculated in a group containing 6 animals; a third mock infected group (n = 4) was kept as control for each mice lineage. Animals were kept for acclimatizing 12 days before the inoculation and maintained with food and water ad libitum throughout the experiment.

Animals on the inoculated groups were slowly aerosolized in a closed cage, using a viral suspension containing $10^{6.0}$ $TCID_{50}$/50 μL (50% tissue culture infective doses per 50 μL) added by 5% FCS. Controls animals were mock infected using CRIB cells supernatants with 5% FCS. Animals were monitored twice a day until day 7 post-inoculation (dpi) when they were deeply anesthetized using a o of xylazine (Rompun™, Bayer) and ketamine (Vetanarcol™, König), and exsanguinated through the femoral vein. Necropsy was performed; lung fragments were collected for histopathological and virological examination. Animals were killed 7 dpi based on previous experience of our group for enhanced virus detection and histopathological findings (Almeida et al., 2004).

Animals' experiments were ethically conducted following Brazilian Laws concerning Animal Welfare (Law 6638, May 8th 1979) and the guidelines for animal experimentation published by Brazilian College for Animal Experimentation (COBEA, http://www.cobea.org.br/).

2.4. Histopathology

Lung samples were fixed in 4% paraformaldehyde (Sigma–Aldrich Co., Missouri, USA) for 24 h, washed in deionized and water left in 70 °GL alcohol until embedding in paraffin wax. Fragments were cut in 5 mm thick sections, and then mounted on poly-L-lysine coated microscopic slides. For histopathological examination, sections were stained with haematoxylin and eosin (H & E), following standard protocols.

2.5. Virus isolation

Lung fragments (approximately 100 mg) were macerated in 1 mL of sterile E-MEM supplemented with 200 U/mL penicillin, 200 mg/mL streptomycin and 5 mg/mL amphoterycin B. Samples were stored at -70 °C until processing. These lung homogenates were submitted to virus isolation in 96-well microplates in 10-fold dilutions of the homogenate supernatants. Recovered viruses were titrated and its identity confirmed by an immunoperoxidase monolayer assay (IPMA) as described previously (Spilki et al., 2006). Infectious titres were calculated according to Spearmann and Kärber and expressed as the log10 tissue culture infective dose per 50 μL ($TCID_{50}$/50 μL) of lung tissue homogenate.

2.6. Viral nucleic acid detection

For BRSV nucleic acid detection in mice lung samples, the RNA was extracted from 200 mL the same supernatants prepared for virus isolation. RNA extraction was performed using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics, Manheim, Germany) following manufacturer instructions. Positive controls were processed from the supernatant of CRIB monolayers infected with BRSV.

The RT-nested-PCR technique was standardized to amplify a fragment of 449 bp of the G gene of BRSV, using G1 (5⁰-CAGCCATTATTTACAT-3⁰) and G2 (5⁰-CTCCACAACAAGGCATCC-3⁰) as outer primers and G3 (5⁰- AAGCCCACATCCAAACCA-3⁰) and G4 (5⁰- CCAACCAAGACAACAATC -3⁰) as inner primers. Different RT-nested-PCR steps were performed in separate laminar flows to avoid contamination.

For the synthesis of cDNA the SuperScriptIII FirstStrand Synthesis Super MIX™ (Invitrogen Ltd., California, USA) following kit instructions for use with random hexamers.

PCR reactions were performed as following: for a final volume of 50 μL, 5 μL 10X amplification buffer (200 mM Tris–HCl, pH 8.4; 500 mM KCl); 2 μL 10 mM dNTP MIX; 2.5 μL PCR enhancer; 1 μL primer G1; 1 μL primer G2 (both primers 20 pmoles); 0.6 μL Platinum™ Pfx DNA Polymerase (Invitrogen Ltd., California, USA); 1 μL Mg_2SO_4 (50 mM); 2 mL cDNA added by 34.9 μL RNAse/DNAse-free water (Millipore water system; Millipore Corp., Massachu- setts, USA). Mixture was pre-heated for 2 min. at 94 °C for denaturation. Amplifications were carried out in a thermal cycler PCR System 9700 (Gene Amp,

Applied Biosystems, Perkin-Elmer, California, USA). The cycling program consisted of denaturation at 94 8C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 1 min. Thirty-five cycles of amplification were made. The second PCR product was done using the same reagents and the quantities of the first step, with first step PCR product as the template and substitutions of the primers for G3 and G4. The cycling was performed essentially as described for the first step; both PCR reactions were prolonged by a final cycle of elongation 7 min at 72 °C.

PCR products were analyzed in 1% agarose gel added by 0.5 mg ethidium bromide/mL run at 90 V for 45 min. Molecular sizes of the products were compared with those of a 100 bp DNA ladder (Invitrogen Ltd., California, USA). Ethidium-bromide stained bands were visualized under UV light and photographed in Image Master VDS equipment (Amershan Biosciences, Buckinghamshire, England).

2.7. Statistical analysis

Statistical analysis was performed using the chi-square test, with $p = 0.05$ for the presence of pathological findings between different groups. The data on viral excretion and serological status was evaluated by analysis of variance (ANOVA). The least significant difference for $p = 0.05$ was determined. Comparisons were made from day to day within the

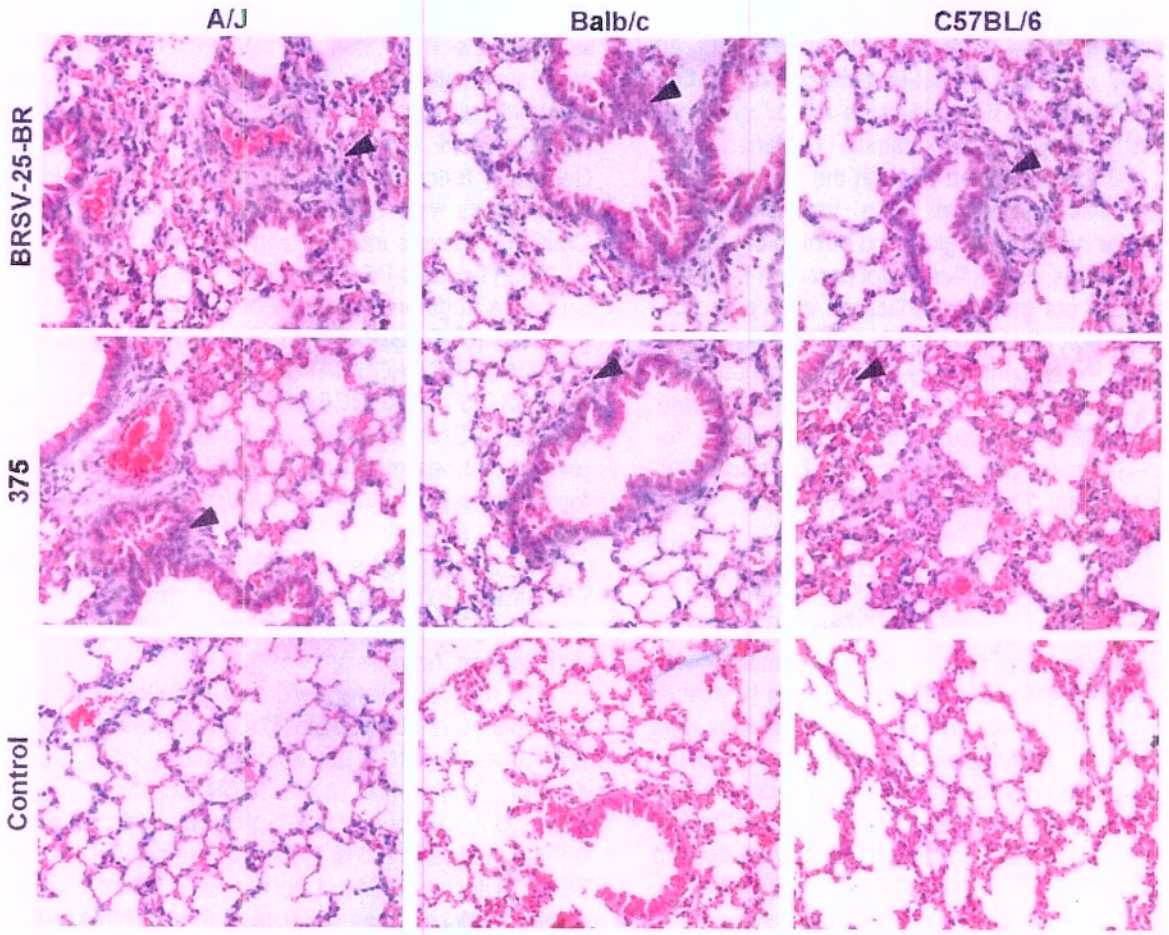

Fig. 1. Microscopical lesions in BRSV inoculated mice of lineages A/J, Balb/C and C57BL/6, at 7 days after infection. Interstitial pneumonia, diffuse oedema and peribronchiolar mononuclear infiltration (arrow heads) was present in different degrees, according to mice lineage and BRSV strain used (BRSV-25-BR or 375). (Haematoxylin and eosin stained, magnified 40X).

groups and between groups. Statistical analysis was performed with Data Analysis Supplement for Excel™ (Office System 2003 for Windows™, Microsoft Corp., Seattle, USA). The term "significant" (statistically significant) in the text means $p \leq 0.05$.

3. Results

3.1. Clinical signs

Moderate apathy and light dispnoea was observed on the A/J inoculated mice; those clinical signs were seen from days 3 to 7 pi. The signs for the strain BRSV-25-BR were more evident but started later, on day 4 pi. Animals of the C57BL/6 showed mild signs of respiratory distress between days 4 and 5 pi; no noticeable clinical signs were seen in Balb/C mice. Control mock infected animals remained healthy throughout the experiment.

3.2. Pathological findings

Few areas of bulbous emphysema and pulmonary consolidation were found at necropsy in animals of all lineages (virus strain BRSV-25-BR: A/J, 4 animals in 6; C57BL/6, 4/6; Balb/C, 2/6; virus strain 375: A/J, 4 animals in 6; C57BL/6, 1/6; Balb/C, 1/6).

Different degrees of severity were found for the lung lesions at microscopical examination, depending on mice strain used (Fig. 1). Lesions induced by BRSV-25-BR were slightly more pronounced than those observed for strain 375. Interstitial pneumonia, focal pulmonary and perivascular oedema, mononuclear infiltration around bronquioles, mild bronchiolitis and thickening of alveolar walls were observed; in some animals, especially A/J and C57BL/6 mice, swollen of epithelia covering bronchioles and alveoli may be found. Eosinophilia was present in very low levels, in A/J and C57BL/6 mice infected with the strain 375. No pathological alterations were found on mock infected mice.

3.3. Virus isolation and nucleic acid detection

Infectious virus was recovered at first passage from animals of all mice lineages (virus strain BRSV-25- BR: A/J, 3 animals in

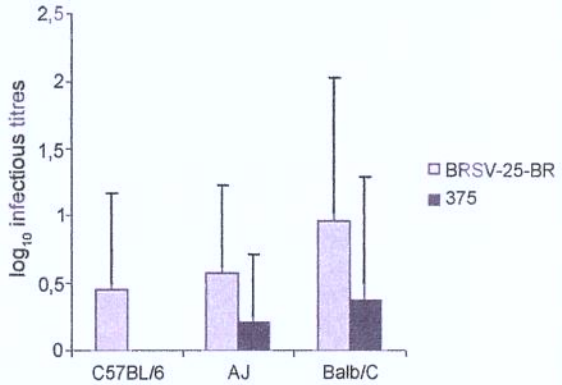

Fig. 2. Mean infectious virus titres in lung homogenates from BRSV experimentally infected mice according to mice lineage and BRSV virus strain used. Bars are indicating maximal titres. For information about the number of positive animals for viral isolations see Section 3.

virus strain 375: A/J, 1 animal in 6; C57BL/6, 0/6; Balb/C, 1/6). The geometric means of infectious titres were plotted for each group and the excretion for Balb/C mice was slightly higher than to other mice lineages (Fig. 2). These results were confirmed by RT-nested-PCR gene G amplification; BRSV nucleic acid was also amplified from one C57BL/6 individual that was negative for isolation (Fig. 3).

4. Discussion

Small rodents are widely recognized as useful for the study of HRSV infections (Easton et al., 2004; Harrod et al., 2003; Klinguer-Hamour et al., 2003; Piazza et al., 1993; Rosenberg et al., 2005). However, previous studies from our group (Almeida et al., 2004) and other researchers (Taylor et al., 1984) found that BRSV infections in mice, despite of the recovery of genetic material or infectious virus, are characterized by absence of clinical signs and evident pulmonary pathology. These previous efforts on establishment of a BRSV infection mice model were made on Balb/C mice and no other mice strains were tested. On the present work we tested other H-2 haplotype mice variants (A/J and C57BL/6) to compare the virological and pathological findings for these animals in comparison to Balb/C.

Similarly to what was observed for HRSV (Byrd and Prince, 1997; Prince et al., 1979; Stark et al.,

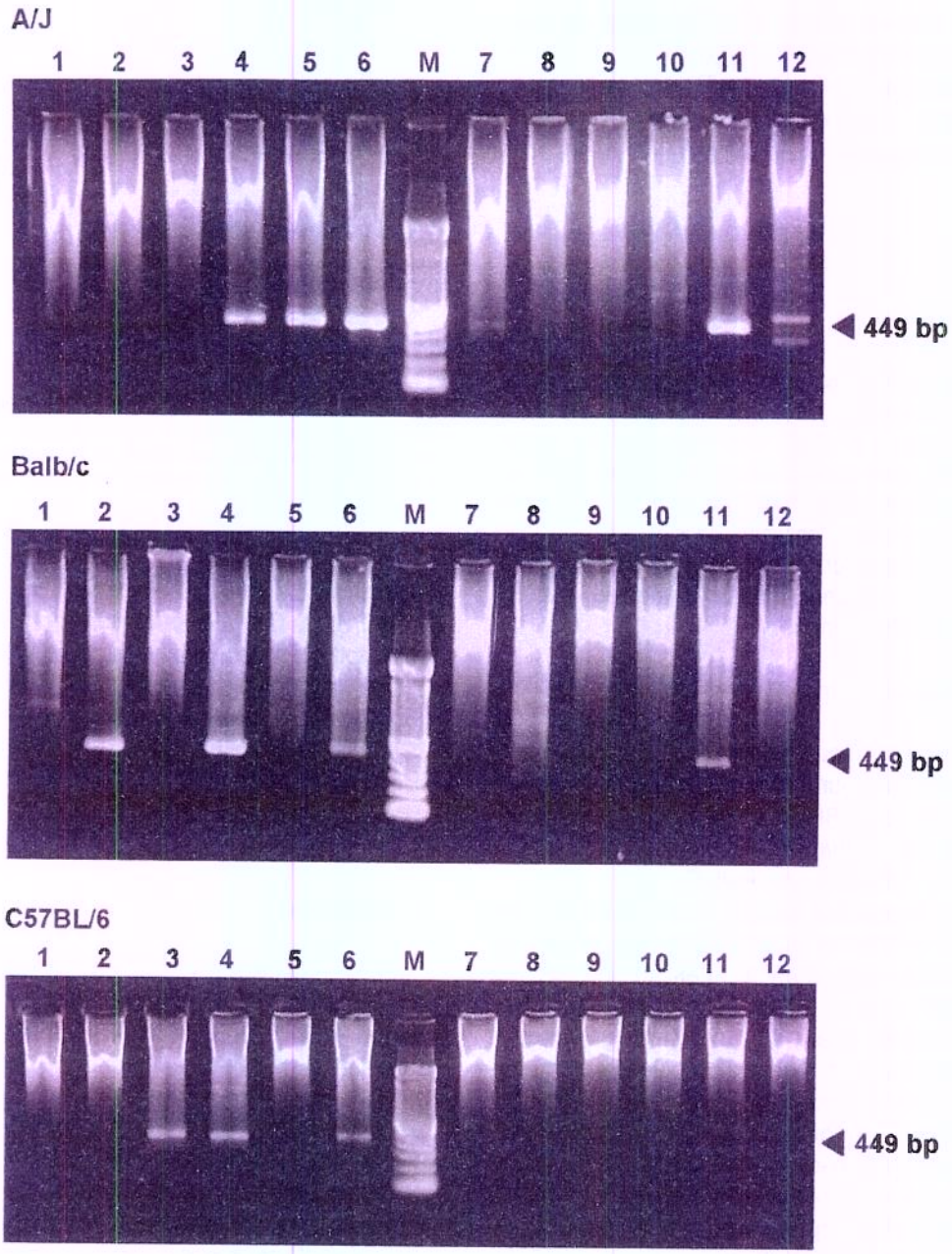

Fig. 3. RT-nested-PCR for the detection of a fragment of the G gene in mice of different lineages infected with BRSV. Lanes 1–6 are showing the results for animals infected with BRSV-25-BR strain. Lanes 7–12 are showing the results obtained from lungs of animals inoculated with the strain 375. M is 100 bp molecular weight marker.

2002) on the present work different mice lineages showed different levels of lung pathology when inoculated with BRSV, as well as variation in viral titres recovered from infected animal was observed as mentioned before (Stark et al., 2002). A/J and C57BL/6 showed more prominent microscopical pathological findings than those observed for Balb/C mice. There were also more animals presenting both gross and microscopical lesions on these lineages. Paradoxally, some animals on the Balb/C group presented higher

titres of infectious virus recovered from lungs, when compared to mice of other strains. However, these data was not statistically significant.

Virus isolation may be important as a marker during BRSV experimental infection since virus clearance may be hampered in animals presenting more lesions induced by BRSV (Meyerholz et al., 2004; Viuff et al., 2002; Woolums et al., 1999). Some authors reported that BRSV isolation may be difficulty from tissues of experimentally inoculated mice (Matumoto et al., 1974; Taylor et al., 1984); however, from the results obtained here, the proper conservation of the material may be of great value, and the confirmation by RT-nested-PCR may also report more positive animals in a group. The titre of virus inoculum used here, at least 10-fold higher than those used in previous works, probably facilitated infections.

The differences of susceptibility between different mice lineages may be attributed to several reasons: differences in haplotype (and consequent recognition of viral epitopes by MHC-I); patterns of viral chemokines expressed as well as other factors like weight and body size at the time of inoculation (Balb/C mice are heavier than C57BL/6 and A/J mice at the age of inoculation); diameter of bronchioles and other immune system related and anatomical differences among such mice lineages. In order to investigate in what extent these differences may be genetically determined, further studies using cross-bred mice resulted from the mating among the mice lineages studied here have to be done. This may be of interest also to study quantitative trait locus analysis in order to determine the genome regions associated with the susceptibility for BRSV infection in mice. Afterwards, this would be useful for identification of bovine homolog corresponding for these mice chromosomal regions, then providing a tool for prediction of what bovine breeds or individuals may be at higher risk for BRSV infection.

There were some differences between the viral strains used. C57BL/6 mice were efficiently infected by BRSV-25-BR virus strains; however, despite of some microscopical findings no infectious virus or viral nucleic acid of strain 375 may be detected on such mice lineage. The titres of infectious virus recovered from mice of all lineages infected with the strain BRSV-25-BR were statistically significant higher than those observed for 375 virus strain. Other authors have hypothesized that some strains of BRSV may be more feasible for mice inoculation than others (Taylor et al., 1984), and it was noted on the present work.

Our results demonstrate that it is possible to obtain a suitable murine model for BRSV, using mice lineages other than Balb/C, a high titre of infectious virus on the inoculum and it is worthy to test different virus strains for this purpose. Further work may even enhance the clinical and pathological scores obtained on such murine model, by the use of improved techniques allowing more evident disease reproduction, such as previous immunization of mice with formalin-inactivated BRSV (Antonis et al., 2003; Kalina et al., 2004, 2005; West et al., 1999) and killing of inoculated animals at different intervals for determination of viral load on lung parenchyma, since inoculation of BRSV in mice may results in a transient infecion. A possible improvement for the model may be also the use of daily measurements of body weight, as a marker for the general health status of the inoculated animals. This mice model will be of interest for the study of immunopathological aspects of BRSV infection, as well as for preliminary tests on the development of new BRSV vaccines.

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Artigo 5

Influence of different adjuvants on the production of specific anti-Bovine respiratory syncytial virus IgG antibodies in mice

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Manuscrito em preparação

Influence of different adjuvants on the production of specific anti-Bovine respiratory syncytial virus IgG antibodies in mice

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1 (one) Figure

Running title: Effects of adjuvants on BRSV immunization

Keywords: Bovine respiratory syncytial virus, BRSV, adjuvants, murine model, inactivated vaccines, ELISA

Summary

Bovine respiratory syncytial virus (BRSV) causes pneumonia in young cattle, resembling respiratory disease caused by Human RSV in infants. Modified live virus (MLV) and inactivated vaccines are currently used for the control of clinical effects of BRSV in cattle. On the present work, we investigated the stimulation of specific anti-BRSV IgG class antibodies, through the use of different "classical" adjuvants (Water-in-oil emulsion, Quil A, Aluminium-hydroxide) in inbred mice (Balb/C and C57BL/6). BRSV antibodies were measured using an enzyme-linked immunosorbent assay (ELISA) and the results were compared to the IgG levels induced by immunization of animals using live BRSV virus. The best results were obtained using water-in-oil emulsion as adjuvant, followed by Quil A prepared vaccines. Differences were observed on the levels of IgG antibodies to BRSV induced by Quil A and live virus inoculation in mice from Balb/C and C57BL/6 lineages.

Introduction

Bovine respiratory syncytial virus (BRSV) is a member of the *Paramyxoviridae* family. BRSV is included, together with human respiratory syncytial virus (HRSV), pneumonia virus of mice (PVM) and other pneumoviruses from ruminants (Ovine repisratory syncytial virus and caprine respiratory syncytial virus), within the *Pneumovirus* genus of the *Pneumovirinae* subfamily (Larsen, 2000). These viruses are responsible for disease of the lower respiratory tract on its respective hosts (Van der Poel et al., 1994).

There are a number of BRSV commercially available vaccines (Ellis et al., 2001; Ellis et al., 1995; Nettleton et al., 2003; Valarcher et al., 2000); its use is controversial since its efficacy and potential enhancement of disease in vaccinated animals are not well documented under field conditions (Larsen et al., 2001; Letesson, 2003). Experimental assays using modified live virus (MLV) or inactivated vaccines (Ellis et al., 1995) showed different degrees of protection against pulmonary pathology induced by the challenge as well as production of neutralizing and non-neutralizing antibodies to BRSV, depending on the type of vaccine and adjuvant used (Ellis et al., 2001; Ellis et al., 1995; Ellis et al., 2005; West et al., 1999).

It was reported that cattle vaccinated using MLV vaccines may be clinically protected against BRSV challenge and develop high titres of virus neutralizing antibodies, in the presence of low levels of total anti-BRSV specific IgG antibodies (Ellsworth et al., 2003; West et al., 1999). On the other hand, animals vaccinated with inactivated BRSV virus as immunogen produced high levels of total anti-BRSV IgG antibodies and lower titres of BRSV-neutralizing antibodies (Ellis et al., 1995; Ellis et al., 2005; Mawhinney and Burrows, 2005; Patel and Didlick, 2004). Another important issue regarding the use of

inactivated BRSV vaccines is the shift of the immune response to the stimulation of T helper 2 (Th2) immune responses. This was well demonstrated in humans and cattle vaccinated using formalin-inactivated vaccines (Antonis et al., 2003; Dudas and Karron, 1998; Kalina et al., 2004; Kim et al., 1969; Woolums et al., 2004). However, virus-inactivated vaccines may be highly efficacious for the control of viral diseases, and are generally safer than MLV vaccines (Tizard, 1990). The efficacy of inactivated BRSV vaccines was experimentally assayed in cattle before (Ellis et al., 2001; Ellis et al., 1995; Ellis et al., 2005; Kerkhofs et al., 2004; Mawhinney and Burrows, 2005). The efficacy of any virus-inactivated vaccine depends on the use of appropriate methods of inactivation of the virus and the use of a suitable adjuvant (Cox and Coulter, 1997). The choice of the adjuvant have to follow determined parameters: i) induction of specific antibodies; ii) stimulation of cellular immune responses, which are often lower using inactivated vaccines, since poor MHC class I presentation of antigen is observed on the absence of viral replication); iii) appropriate immunomodulation (Th1/Th2 balance), avoiding side effects and exacerbation of the disease on post-vaccination challenged animals; iv) tolerability of the hosts to the components of the adjuvant or adjuvant combinations used.

In the present work, we investigated the stimulation of specific anti-BRSV IgG class antibodies, through the use of different "classical" adjuvants in inbred mice. BRSV antibodies were measured using an enzyme-linked immunosorbent assay (ELISA). The results were compared to the IgG levels induced by immunization of animals using live-BRSV-virus.

Materials and methods

Viruses and cells

The Brazilian strain BRSV-25-BR (Arns et al., 2003; Spilki et al., 2006) was used for the preparation of inactivated vaccines and ELISA antigen. CRIB cells, a clone of MDBK cells resistant to the infection with BVDV (Flores and Donis, 1995; Flores et al., 1996), which is fully susceptible to the infection with BRSV (Flores and Donis, 1995; Spilki et al., 2006) were used throughout. Cells were cultivated in Eagle's minimal essential medium (E-MEM) supplemented with 10 % Foetal calf serum (FCS), free of antibiotics, following routine protocols (Spilki et al., 2006). Viral titres were calculated according to Spearman and Kärber and expressed as the tissue culture infectious doses per 50 μL (TCID50/50 μL).

Vaccine production

Twenty-four hours after seeding of the cells in 150 cm^2 flasks, the medium was removed and bottles infected with $10^{5.5}$ $TCID_{50}$/50 μL of BRSV-25-BR viral strain. After one hour left for adsorption at 37 °C, the bottles were replenished with E-MEM without foetal calf serum and incubated for 72 hours at 37 °C, when cytopathic effect was evident in about 90 % of the monolayers. Bottles were then vigorously shaken to remove attached cells and stored at 4°C for 24 hours. The virus-cells mixture was centrifuged (5000 X g, 10 minutes). The resulting pellet was then discarded and the infectious titre of the bulk suspension was determined ($10^{6.0}$ $TCID_{50}$/50 μL). The viral suspension was inactivated with

binary ethylenimine (BEI). BEI was prepared as a 0.1 mol/L solution by cyclization of 0.1 mol/L 2-bromoethylamine hydrobromide (Sigma) in 0.175 mol/L NaOH solution at 37°C for one hour following the method of Bahnemann (1990). The reaction was controlled by following the drop in pH due to the formation of BEI. The BEI preparation was added to the virus suspensions at a final concentration of 0.001 mol/L. A control without addition of BEI was included. Virus suspensions were incubated at 37 °C for 36 h. The residual BEI was hydrolysed in samples by the addition of 1 mol/L sterile Sodium-thiosulfate (Merck) solution at 10% of the volume of the BEI used. The vaccine was prepared from aliquots of the BEI inactivated virus suspensions, using different adjuvants, namely: water-in-oil type emulsion (W/O; Biovet, Brazil), Quil A saponin (Biovet, Brazil), Aluminium hydroxide ($Al_2(OH)_3$; Biovet, Brazil), or no adjuvant (N/A). Non-adjuvanted vaccines were also prepared using live BRSV virus (LV; infectious titre $10^{6.0}$ $TCID_{50}$/50 µL) and other animals were mock vaccinated (C-) using the supernatant of non-infected CRIB cells, collected as mentioned for virus suspensions.

Animal vaccination

Thirty-six Balb/C (haplotype: H-2d) and 36 C57BL6 (H-2b) mice, which presented different levels of pulmonary pathology after BRSV inoculation were used on the present experiment. The results for inbred mice susceptibility to BRSV will be described elsewhere. The animals, 8 week old specific-pathogen-free males, were purchased from CEMIB (Multidisciplinary Centre for Biological Investigation, at UNICAMP). The animals were kept behind barriers. At the day of arrival on the isolation units at the Departamento de Microbiologia e Imunologia – UNICAMP, the animals were divided in six groups (n=6)

per mice lineage, in separate cages. Animals were kept for acclimatizing for 12 days before the inoculation and maintained with food and water ad libitum throughout the experiment. Each virus inactivated vaccine, live-BRSV-virus or mock-prepared vaccine was administered subcutaneously (100 µL per animal) on day 0 post-vaccination (DPV). Animals were re-vaccinated on 30 DPV. Blood samples were collected on days 0, 30, 60 and 90 DPV and after separation and aliquoting of the serum; the sera were stored at -20 °C until use. At 90 DPV, animals were deeply anesthetized using a combination of xylazine (Rompun, Bayer) and ketamine (Vetanarcol, König), and exsanguinated through the jugular vein.

Animals' experiments were ethically conducted following Brazilian Laws concerning Animal Welfare (Law 6638, May 8th 1979) and the guidelines for animal experimentation published by Brazilian College for Animal Experimentation (COBEA, http://www.cobea.org.br/).

Determination of levels of BRSV-specific IgG antibodies

For the preparation of BRSV antigen, the BRSV-25-BR strain was inoculated onto nearly confluent monolayers of CRIB cells at a multiplicity of infection between 0.1 and 1, following standard procedures. When cytopathic effect (CPE) was evident in about 90% of the monolayers, cells and supernatants were frozen at - 70 °C, thawed, clarified by low speed centrifugation and used as virus stocks. Titres obtained were of $10^{6.0}$ $TCID_{50}$/50 µL.

Cell culture flasks (150 cm2) were infected the BRSV-25-BR virus at a multiplicity of infection between 0.1 to 1 and left 1h for adsorption at 37 °C. After adsorption, 50 mL E-MEM and left at 37 °C until CPE was evident in about 60 % of the monolayer, the medium was removed and cells overlayed with 0.2% OGP (n-octyl-β-d-glucopyranoside) in phosphate buffered saline (NaCl 8.5g, $Na_2HPO_4.2H_2O$ 1.55g, $NaH_2PO_4.H2O$ 0.23g, distilled water q.s.p. 1000 mL, pH 7.2) for 2 h at 4°C. Next, the cells were scraped off the flasks, mixed with the supernatant and centrifuged at

1500 x g to remove cell debris. The crude antigen obtained was aliquoted and stored at -70 °C until use.

The variables within the test were optimized (antigen concentration, serum, secondary antibody dilutions and reduction of background noise) by testing pooled control positive and negative mice sera. ELISA plates were coated with an appropriate dilution of the antigen (1:100) 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 pooled mice sera from each group were diluted 1:2 in PBST-20 and added to duplicate wells. After 1 h incubation at 37 °C, the plates were washed three times with PBST-20. Following washing, anti-mouse IgG/peroxidase conjugate (Bethyl Laboratories, Montgomery TX, USA) was added as secondary antibody (diluted 1:1000) and plates incubated for another hour at 37 °C. After three washings with PBST-20, 100 µL of the substrate 3,3',5,5'-teramethylbenzidine (TMB, Sigma) was added and allowed to react at room temperature for 30 minutes. The reaction was stopped by the addition of 50 µL of 0.5N H_2SO_4. The optical densities (OD) were determined at 450 nm in a Labsystem Multiskan Bichromatic (Titertek) ELISA reader. ODs were directly plotted in computer spreadsheet for graphical analysis (Excel™, Office System 2003 for Windows™, Microsoft Corp., Seattle, USA).

Statistical analysis

Statistical analyses were performed through the analysis of variance (ANOVA), compared each group to another group of same mice lineage and the corresponding on to the opposite mice lineage, and to the mock-vaccinated control values. The least significant difference for $p < 0.05$ was determined. Statistical analysis was performed with Data Analysis Supplement for Excel™ (Office System 2003 for Windows™, Microsoft Corp., Seattle, USA). The term "significant" (statistically significant) in the text means $p \leq 0.05$.

Results

The levels of measured specific anti-BRSV antibodies measured by ELISA are shown in Figure 1. Water-in-oil (W/O) adjuvant gave highest values for both Balb/C and C57BL/6 mice, followed by Quil A and Aluminium hydroxide $Al_2(OH)_3$.

Statistical analysis showed that levels of BRSV-specific IgG induced by W/O were significantly higher ($p< 0.05$) than those obtained for all other treatments used, including Live-virus (LV). No significant differences were observed for W/O induced IgG responses between the two mice lineages ($p= 0.6362$).

$Al_2(OH)_3$ induced moderately good levels of anti-BRSV IgG antibodies in both inbred mice lineages when compared to N/A and C- treatments; however, there was no difference between $Al_2(OH)_3$-adjuvant-BRSV-inactivated vaccine and LV in Balb/C mice ($p=0.6624$), differing from the situation for C57BL/6 mice, were no differences between those treatments may be found ($p= 0.002$). Despite of this, no differences were found comparing the $Al_2(OH)_3$ results obtained for both mice lineages used ($p=0.8302$)

Results for LV were only statistically significant different from the N/A and C- groups, considering the samples taken after re-vaccination, at 30 DPV. It was also observed that LV treatment induced higher levels of BRSV-IgG antibodies in Balb/C than in C57BL/6 mice ($p=0.0143$).

Non-adjuvanted vaccine treatment showed no statistical differences from mock vaccinated controls.

Discussion

Vaccines been used at least for the last 30 years attempting the control of BRSV infections (Ellis et al., 2001; Ellis et al., 1995; Ellis et al., 2005; Fulton et al., 2004; Kerkhofs et al., 2004). However, to date, vaccine failure events are reported both in scientific literature (Larsen, 2000; Larsen et al., 2001) and by reports from field veterinarians. In order to determine the levels of BRSV-specific IgG induced by different commonly used adjuvants in a experimental BRSV-inactivated vaccine, and to compare those results with those obtained when use live-virus immunization, we tested such variables in mice. Mice from Balb/C and C57BL/6 lineages were used for repetition of the treatments, since we have detected recently that those inbred mice lineages showed different levels of pulmonary pathology when intranasally inoculated with virulent BRSV. These results are beyond the scope of the present article and will be published elsewhere (Spilki et al., *in prep.*). This was an attempt to compare the results for BRSV-resistant mice (Balb/C), and the results for a BRSV-susceptible lineage (C57BL/6).

Water-in-mineral-oil emulsions (W/O) showed the best results when compared to any other treatments used, and it was included on the present work to serve as a "gold standard" for comparison with the other treatments. W/O is widely recognized as a potent adjuvant for use in farm animals (Barnard et al., 2005; Barteling and Vreeswijk, 1991; Filgueira et al., 1999; Iyer et al., 2000). However, W/O may persist for long periods at the site of injection, being related with adverse reactions and carcinogenesis (Filgueira et al., 1999; Gupta and Siber, 1995; Spickler and Roth, 2003). Therefore, consumer markets may claim for the substitution of W/O for other adjuvants (Gupta and Siber, 1995; Spickler and Roth, 2003). One have also to consider that despite of high levels of both neutralizing and non-neutralizing antibodies may be often present when W/O is used (as measured on the present study), low levels of cellular mediated immune responses are present after vaccination using such adjuvant (Cox and Coulter, 1997).

Aluminium hydroxide showed moderately good results on the present work. However, aluminium based adjuvants are often associated to high levels of IgE and Th2 like responses (Cox

and Coulter, 1997), which may be deleterious for immunized animals, enhancing BRSV disease after challenge (Graham et al., 2000; Kalina et al., 2004; Srikiatkhachorn and Braciale, 1997). In fact, together with the modifications on epitopes related to HRSV formalin-inactivated vaccines in children, we have to remember that these vaccines were also alum-adjuvanted (Godefroy et al., 2003; Kim et al., 1969), which have influenced the fatal outcome of the HRSV infections in vaccinated individuals.

The levels of IgG obtained for Quil A in both Balb/C and C57BL/6 mice were better than those obtained for $Al_2(OH)_3$ and LV; Quil A gave higher levels of IgG antibodies to BRSV in Balb/C mice ($p= 0.0193$). Saponins are also largely used as adjuvants for farm animal vaccines (Barteling and Vreeswijk, 1991; Ellis et al., 2005; Katayama et al., 2000; Spickler and Roth, 2003), showing good results for clinical protection against BRSV infections under experimental conditions (Ellis et al., 2005). They are considered relatively low-cost for vaccine production. Additional advantages are the good levels of both humoral (as observed here) and cellular immune responses (Spickler and Roth, 2003). Good levels of Th1 and Th2 responses are elicited by the use Quil A as an adjuvant (Barr et al., 1998; Cox and Coulter, 1997; Sheikh et al., 2000). From the results obtained here and those previously reported in the literature for the immunization using BRSV-saponin-adjuvanted vaccines, this may be consider as a proper choice for the formulation of new BRSV-inactivated immunogens.

It is interesting that differences on the levels of IgG antibodies to BRSV were found for Balb/C and C57BL/6 mice when using both Quil A and live virus vaccine preparations. We may hypothesize that this may be a result from the differences on the haplotypes of these mice lineages, since presentations of antigens in the context of MHC-I plays a role on the elicitation of immune responses by both saponin adjuvant and live-virus immune responses (Bomford, 1998; May et al., 1999; Sjolander et al., 1997). This may be indirectly reflected on the levels of IgG antibodies. This may be relevant, if similar further experimental evidence may be observed for cattle possessing different haplotypes.

Further studies in cattle have to be conducted, comparing the immunogenicity of those better preparations (W/O and Quil A). Adjuvant combinations, like Quil A and another adjuvant must also to be tested in the near future. This may be useful for the development of more efficacious BRSV-inactivated vaccines.

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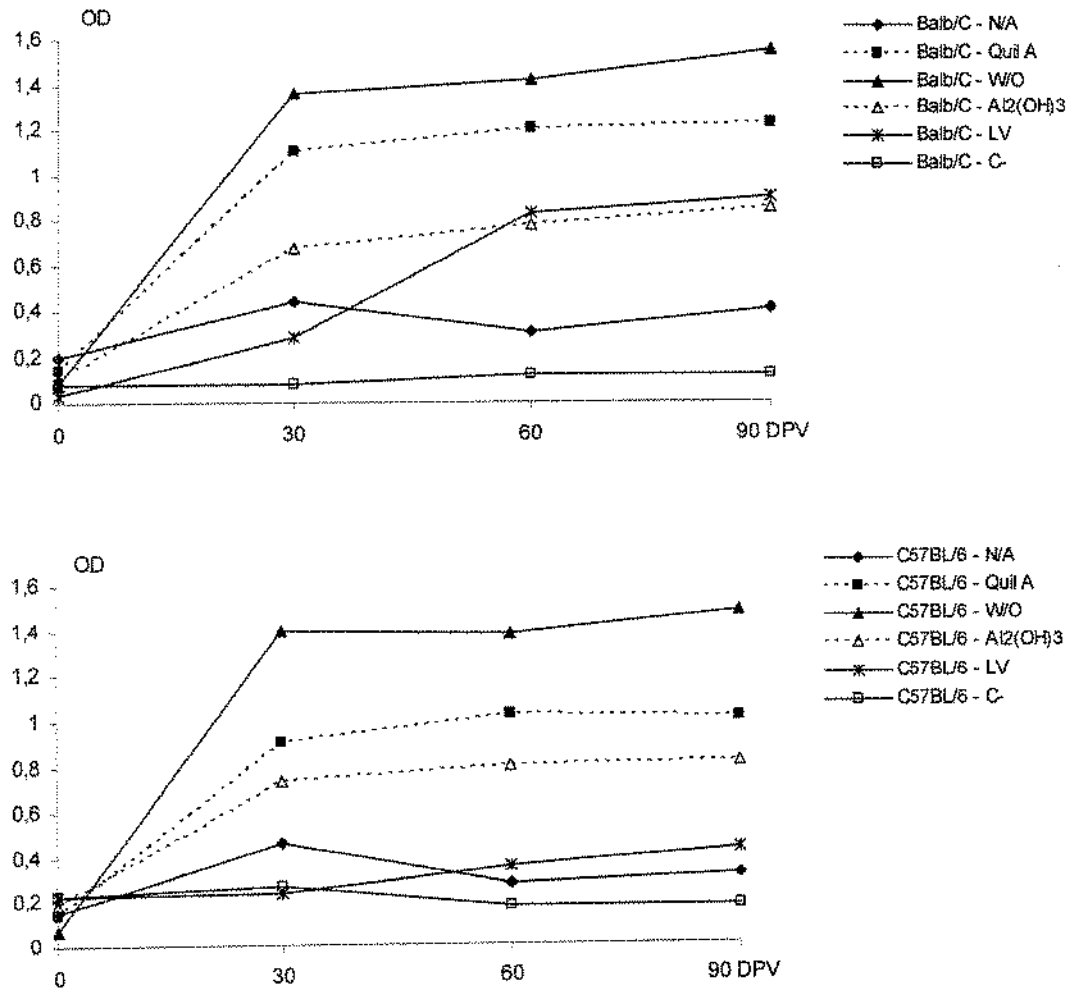
Figure captions

Figure 1. Levels of anti-BRSV IgG specific antibodies elicited by experimental inactivated vaccines, using different adjuvants (N/A, no-adjuvant; Quil A; W/O, water-in-oil emulsion; $Al_2(OH)_3$, aluminium-hydroxide) compared to live-virus (LV) and mock-vaccinated mice (C-). Animals were re-vaccinated at day 30 DPV. The first graph describes the levels of BRSV-specific IgG through the experiment in Balb/C mice; the second, in C57BL/6 mice. See text for statistical analysis.

Figure 1.


OD
1,6
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0
30
60
90 DPV
Balb/C - N/A
Balb/C - Qui A
Balb/C - W/O
Balb/C - Al2(OH)3
Balb/C - LV
Balb/C - C-
OD
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
0
30
60
90 DPV
C57BL/6 - N/A
C57BL/6 - Qui A
C57BL/6 - W/O
C57BL/6 - Al2(OH)3
C57BL/6 - LV
C57BL/6 - C-

4. DISCUSSÃO GERAL

4.1. Cultivo do BRSV em diferentes tipos celulares

Dentre as linhagens celulares e cultivos primários testados por nosso grupo, o BRSV só replicou nas células de origem mamífera (CER, Vero, CRIB, MDBK, BEL) não sendo detectado após passagens em células de cultivo primário de fibroblasto de embrião de galinha (FEG) (Spilki et al., 2006). O conhecimento futuro do receptor celular associado à adsorção do BRSV, provavelmente um glicosaminoglicano (GAG), será válido em explicar se as células de origem aviária não foram infectadas por BRSV por diferenças nesse receptor entre aves e mamíferos ou se o vírus não replica adequadamente em células aviárias por diferenças encontradas no microambiente celular.

Com base nos dados de multiplicação do vírus nas diferentes células de linhagem, a célula CRIB, por constituir uma célula de origem bovina e ainda conferir a vantagem adicional de evitar a contaminação dos estoques virais pelo vírus da Diarréia viral bovina (BVDV), foi utilizada em todas as outras etapas do trabalho que exigiram a multiplicação do vírus. Tal estratégia seria de grande valia na confecção de futuras vacinas de vírus vivo modificado (MLV), como um meio de evitar a contaminação dos lotes de vacina pelo BVDV e os eventuais prejuízos que são causados aos animais nesta situação. A célula CER também apresentou bons resultados quanto ao crescimento do vírus, e a facilidade de manutenção dos estoques dessa célula é uma vantagem que a mesma pode trazer para o cultivo do BRSV em laboratório.

4.2. Classificação filogenética dos isolados brasileiros de BRSV e a importância de mutações na conformação estrutural da proteína G da amostra BRSV-25-BR

Com base nos dados de seqüências nucleotídicas dos genes G e F de BRSV gerados neste trabalho a partir da amplificação dos respectivos fragmentos do genoma de isolados de BRSV brasileiros, foram determinadas as relações filogenéticas entre esses isolados e outros oriundos de várias partes do mundo. Claramente, tanto na caracterização dos mesmos, baseada tanto no gene G quanto no gene F, tais isolados pertencem ao subgrupo B (grupo genômico I) do BRSV. Isso é relevante do ponto de vista epidemiológico, já que amostras do subgrupo B não circulam em outras partes do mundo já há 30 anos; tal fato sugere que a introdução do BRSV em nosso território deu-se em meados dos anos 1970, confirmando dados anteriores obtidos a partir da pesquisa de antígenos de BRSV em tecidos pulmonares de animais com achados histopatológicos compatíveis coletados na década de 1980 (Flores et al., 2000). Outro aspecto a ressaltar é o fato das vacinas comercialmente disponíveis no mercado brasileiro para o controle de infecções pelo BRSV, trazerem em sua formulação isolados pertencentes ao subgrupo A. Testes devem ser conduzidos futuramente visando determinar o grau de proteção clínica e mais que isso, a diminuição na replicação, com a conseqüente redução na circulação de vírus nos rebanhos, através da imunização utilizando tais produtos. Relatos anteriores dão conta de que o uso de vacinas cujos vírus vacinais apresentam baixos graus de identidade com os isolados circulantes na região não só confere proteção clínica insatisfatória, como poderia levar ainda à seleção de mutantes. Esse fenômeno constituiria em um possível mecanismo de escape desses novos vírus ao sistema imune do hospedeiro, o que acontece quando a proteção dada pelas vacinas é apenas parcial.

Foi determinada ainda, com base nas seqüências deduzidas de aminoácidos da proteína G, a presença de mutações na região central desta glicoproteína, presentes no isolado BRSV-25-BR, afetando entre outros resíduos de aminoácidos, duas cisteínas do nó

de cisteínas existente nesse domínio da proteína (Spilki et al., 2006). Ao contrário de mutantes naturais de BRSV com substituições nestes mesmos aminoácidos relatados em rebanhos vacinados, esse isolado é oriundo de um rebanho não vacinado. A fixação desse mutante pode ter se dado pela pressão seletiva exercida pela imunidade adquirida por estes animais apenas pelas reinfecções com BRSV, não sendo portanto necessária a vacinação para a ocorrência desse fenômeno como mencionado anteriormente. Tais eventos devem de fato estar envolvidos como estratégia de evolução dos vírus na natureza. A análise estrutural feita por meio de modelagem por homologia não revelou alterações na possível estrutura tridimensional dessa região da proteína. Essa mesma região é considerada importante no HRSV para a indução de migração de eosinófilos para o parênquima pulmonar, bem como pelo direcionamento da resposta imune do hospedeiro. Será necessário realizar estudos mais aprofundados utilizando o modelo experimental aprimorado no presente estudo e estudos em bovinos que possam elucidar se tais funções foram alteradas no isolado BRSV-25-BR.

Em outra etapa foi feita a modelagem estrutural da proteína F do BRSV. A comparação da mesma aos modelos propostos para a proteína de fusão de outros paramixovírus permite afirmar que a proteína F de BRSV mantém o arcabouço estrutural presente nas proteínas homólogas dos vírus dessa família. Tal estrutura não parece variar mesmo quando ocorrem variações de aminoácidos entre os subgrupos de BRSV, o que aponta para a necessidade de conservação dos elementos básicos estruturais da proteína F para a realização de suas funções na replicação viral.

4.3 Aprimoramento de um modelo experimental para infecções pelo BRSV

Ainda que o uso de pequenos ruminantes mostre-se promissor no estudo das infecções pelo BRSV, um modelo murino como foi proposto no presente estudo, baseado na determinação de linhagens mais suscetíveis à infecção viral pode ser de grande interesse prático na pesquisa de diferentes aspectos relativos à infecção pelo BRSV. Os animais da linhagem Balb/C revelaram permitir níveis mais elevados de replicação viral, todavia os animais das linhagens C57BL6 e A/J demonstraram lesões histopatológicas evidentes. Isto pode ser de grande interesse especialmente na compreensão de mecanismos ligados à imunobiologia da infecção pelo BRSV, bem como em testes de triagem de vacinas e drogas terapêuticas. Um refinamento possível do modelo obtido aqui seria a co-infecção dos animais com o BRSV e bactérias comumente associadas nos casos clínicos do vírus nos rebanhos bovinos, como uma tentativa de aproximar ainda mais os resultados clínicos e de patogenia observados em camundongos daqueles notados no hospedeiro natural. Outra estratégia seria a pré-imunização dos animais com vacinas inativadas utilizando formalina e alumínio como adjuvante, tal qual sugerido para bovinos, como uma tentativa de exacerbar ainda mais os sinais clínicos e patológicos observados.

4.4 Uso de diferentes adjuvantes associados a uma vacina inativada de BRSV

A imunização de camundongos com diferentes graus de susceptibilidade ao BRSV permitiu demonstrar que o adjuvante oleoso induz níveis mais elevados de IgGs específicas após um período de acompanhamento de 90 dias. A saponina também apresentou resultados bastante satisfatórios, e no presente momento está sendo conduzido um teste em bovinos com desenho experimental semelhante ao descrito em camundongos neste trabalho, com vistas a averiguar o efeito imunogênico do BRSV inativado associado ao

Quil A, testando este último como um futuro adjuvante na formulação de vacinas para o controle de infecções pelo BRSV.

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