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Mathias Martins

ORF VIRUS COMO PLATAFORMA PARA VACINAS VETORIAIS PARA SUÍNOS E BOVINOS

Santa Maria, RS
2018

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Tese apresentada ao Programa de Pós-Graduação em Medicina Veterinária da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do título de **Doutor em Medicina Veterinária.**

Orientador: Prof. Dr. Rudi Weiblen

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RESUMO

ORF VIRUS COMO PLATAFORMA PARA VACINAS VETORIAIS PARA SUÍNOS E BOVINOS

AUTOR: Mathias Martins
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O *Parapoxvirus ovis* (PPVO) ou vírus da *orf* (ORFV) é o agente do ectima contagioso (ou *orf*), uma enfermidade mucocutânea que afeta principalmente ovinos e caprinos. O ORFV pertence à família *Poxviridae*, subfamília *Chordopoxvirinae* e gênero *Parapoxvirus*. Os vírions possuem envelope e o genoma consiste de uma molécula de DNA linear e de fita dupla, com aproximadamente 138 quilobases (kb) e contém pelo menos 131 genes. O ORFV possui várias propriedades biológicas e genômicas que o tornam um atraente candidato a vetor vacinal. Por isso, tem sido proposto como plataforma vacinal para vetorar genes heterólogos de interesse veterinário. Em um primeiro estudo, descreve-se a construção e caracterização de dois recombinantes do ORFV, a partir da cepa parental ORFV IA82, que expressam a glicoproteína G do vírus da raiva (RABV-G) nos loci dos genes *ORFV024* ou *ORFV121*, respectivamente, e a avaliação da sua imunogenicidade em suínos e bovinos. A caracterização *in vitro* demonstrou que os recombinantes ORFV$^{\Delta 024}$RABV-G e ORFV$^{\Delta 121}$RABV-G não apresentaram alterações na capacidade replicativa em cultivo celular, comparando-se ao vírus parental. Foi demonstrado que os recombinantes expressam a RABV-G com eficiência, mesmo após 10 passagens em cultivo celular, demonstrando a estabilidade dessas inserções. A imunização de suínos e bovinos com o ORFV$^{\Delta 024}$RABV-G e ORFV$^{\Delta 121}$RABV-G resultou em resposta robusta de anticorpos neutralizantes contra o vírus da raiva (RABV). Os títulos de anticorpos induzidos pelo recombinante ORFV$^{\Delta 121}$RABV-G em suínos e bovinos foram superiores aos induzidos pelo ORFV$^{\Delta 024}$RABV-G, indicando uma maior eficiência do recombinante ORFV$^{\Delta 121}$ como vetor nessas espécies. Em um segundo estudo, mutantes do ORFV com deleções individuais nos genes *ORFV112*, *ORFV117* ou *ORFV127* foram construídos, caracterizados *in vitro* e inoculados experimentalmente em cordeiros. Quando caracterizados *in vitro*, os mutantes de deleção replicaram em células de corneto etmoidal ovino (OFTu) com a mesma eficiência do vírus parental, sem alterações na cinética de replicação, tamanho e morfologia de placas virais. A inoculação experimental de cordeiros na junção mucocutânea da comissura oral demonstrou que as deleções individuais dos genes *ORFV112*, *ORFV117* ou *ORFV127* não resultaram em alterações evidentes de virulência, pois os mutantes produziram lesões tão severas quanto as induzidas pelo vírus parental. No entanto, a resolução das lesões aparentemente ocorreu de forma mais rápida nos animais inoculados com os vírus mutantes do que naqueles inoculados com o vírus parental. Além disso, os vírus mutantes foram excretados das lesões em títulos inferiores aos do vírus parental. Estes resultados demonstraram que os genes *ORFV112*, *ORFV117* e *ORFV127* não são essenciais para a viabilidade do ORFV *in vitro* ou *in vivo* e interferem apenas parcialmente na virulência em ovinos. Um terceiro estudo foi conduzido para comparar a magnitude e duração da resposta sorológica induzida em bovinos por quatro vacinas comerciais contra o RABV. Após duas vacinações com intervalo de 30 dias, os animais receberam reforço vacinal um ano após, sendo submetidos a pesquisa e quantificação de anticorpos neutralizantes a diferentes intervalos após a vacinação e reforço. Verificou-se que as quatro vacinas são adequadamente imunogênicas, ou seja, induziram altos títulos de anticorpos neutralizantes nos animais após a primovacinação. Porém, verificou-se um decréscimo acentuado nos níveis de anticorpos antes do reforço, de forma que vários animais já não apresentavam níveis adequados de anticorpos por ocasião do reforço. Assim, recomenda-se que os protocolos de revacinação anual sejam revistos, antecipando-se a data prevista para reforçar a vacinação. Em resumo, os experimentos apresentados na presente tese demonstram que o ORFV representa uma plataforma promissora para vetorar antígenos vacinais em suínos e bovinos e que, além dos genes *ORFV024* e *ORFV121*, os loci *ORFV112*, *ORFV117* e *ORFV127* também podem ser utilizados para a inserção de genes heterólogos de interesse.

Palavras-chave: Orf vírus recombinante. Vacina recombinante. Patogenicidade. Raiva.

ABSTRACT

ORF VIRUS AS A PLATFORM FOR VECTOR VACCINES FOR SWINE AND CATTLE

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Parapoxvirus ovis (PPVO) or *orf* virus (ORFV) is the causative agent of contagious ecthyma (or *orf*), a mucocutaneous disease that affects mainly sheep and goats. The ORFV belongs to the family *Poxviridae*, subfamily *Chordopoxvirinae* and genus *Parapoxvirus*. The virions are enveloped and their genome consist of a linear, double stranded DNA molecule of approximately 138 kb and contains at least 131 genes. ORFV has several biological and genomic properties that make it an attractive candidate for a vaccine vector. Therefore, it has been proposed as a vaccine platform to carry heterologous genes of veterinary interest. The first study reports the construction and characterization of two ORFV recombinants out of the parental strain ORFV IA82, expressing the rabies virus glycoprotein G (RABV-G) at the loci of *ORFV024* or *ORFV121* genes, respectively, and an evaluation of their immunogenicity in swine and cattle. The *in vitro* characterization showed that the recombinants ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G retained their replicative capacity in cell culture, compared to the parental virus. The recombinant viruses expressed RABV-G efficiently even after 10 passages in cell culture, demonstrating the stability of the inserts. Immunization of swine and cattle with ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G resulted in a robust neutralizing antibody response against the rabies virus (RABV). The neutralizing antibodies titers induced by ORFV$^{\Delta 121}$RABV-G in swine and cattle were higher than those induced ORFV$^{\Delta 024}$RABV-G, indicating a higher efficiency of ORFV$^{\Delta 121}$ as a vector in these species. In a second study, ORFV mutants with individual deletions of genes *ORFV112*, *ORFV117* or *ORFV127* were constructed, characterized *in vitro* and inoculated experimentally in lambs. When characterized *in vitro*, the deletion mutants replicated in ovine fetal turbinate (OFTu) cells with the same efficiency of the parental virus, with no changes in replication kinetics, plaque size and morphology. Experimental inoculation of lambs at the mucocutaneous junction of the oral commissure demonstrated that individual deletions of *ORFV112*, *ORFV117* or *ORFV127* genes did not result in obvious changes in virulence, since the mutants produced lesions as severe as those induced by the parental virus. However, resolution of the lesions apparently occurred more rapidly in the animals inoculated with the mutant virus than in those inoculated with the parental virus. In addition, the mutant viruses were excreted from the lesions in lower titers than those of the parental virus. These results demonstrated that genes *ORFV112*, *ORFV117* and *ORFV127* are not essential for viability of ORFV *in vitro* and interfere only partially in ORFV virulence in lambs. A third study was conducted to compare the magnitude and duration of the serological response induced in cattle by four commercial vaccines against RABV. After two vaccinations 30-days apart, the animals received a booster vaccination one year later, and were subjected to serological tests for virus neutralizing (VN) antibodies at different intervals after vaccination and booster. The four vaccines were found to be suitably immunogenic, e.g., induced high titers of VN antibodies in the animals after primary vaccination. However, a marked decrease in antibody levels was observed prior to the annual booster. Therefore, it is recommended that annual revaccination protocols be reviewed, since part of the vaccinated animals no longer have adequate antibodies levels one year after vaccination. In summary, the experiments presented in this thesis demonstrate that ORFV represents a promising platform for vectoring vaccine antigens in swine and cattle and that, in addition to the *ORFV024* and *ORFV121* genes, loci *ORFV112*, *ORF117* and *ORFV127* can also be used for heterologous gene insertion.

Keywords: Recombinant orf virus. Recombinant vaccine. Pathogenicity. Rabies.

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1. INTRODUÇÃO

O *Parapoxvirus ovis* (PPVO) ou vírus da *orf* (ORFV) é o agente do ectima contagioso, uma enfermidade que afeta principalmente ovinos e caprinos e, ocasionalmente, humanos (MOSS, 2013). A doença é caracterizada por lesões mucocutâneas que, na ausência de infecções secundárias, são autolimitantes e desaparecem em 3 a 6 semanas (HAIG et al., 1997). O ORFV pertence à família *Poxviridae*, subfamília *Chordopoxvirinae* e gênero *Parapoxvirus* (ICTV, 2017). Os vírions possuem envelope e o genoma consiste de uma fita dupla de DNA linear, com aproximadamente 138 kb e contém pelo menos 131 genes (DELHON et al., 2004). A organização do genoma consiste em uma região central, conservada entre os poxvírus, e em regiões terminais que são regiões altamente variáveis (MOSS, 2013). Vários genes presentes principalmente nas regiões terminais do genoma são únicos do ORFV e codificam proteínas envolvidas na evasão da resposta imune do hospedeiro (DELHON et al., 2004). A função de alguns destes genes já foi demostrada e muitos deles não são essenciais para a replicação do ORFV *in vitro* (DEANE et al., 2000; DIEL et al., 2011a, 2011b, 2010; HAIG, 2006; HAIG & FLEMING, 1999a; KHATIWADA et al., 2017).

Devido à sua habilidade em modular a resposta imune do hospedeiro, o ORFV inativado tem sido utilizado como imunoestimulante em Medicina Veterinária, para o tratamento e/ou prevenção de diversas enfermidades em animais de produção e de companhia (ANZILIERO et al., 2014; FRIEBE et al., 2011; WEBER et al., 2013, 2007). Além disso, o ORFV também tem sido proposto como candidato a vetor vacinal (RZIHA et al., 2000) e tem sido utilizado com sucesso como plataforma vetorial para vacinas de uso veterinário em espécies não-permissivas (AMANN et al., 2013; FISCHER et al., 2003b; HAIN et al., 2016).

O ORFV possui uma série de propriedades biológicas e moleculares que fazem deste vírus um atraente candidato a vetor vacinal, incluindo: 1) o ORFV possui um estreito espectro de hospedeiros *in vivo*, viabilizando o seu uso em várias espécies por não possuírem imunidade prévia por infecção natural; 2) a infecção natural em ovinos e caprinos, ou mesmo em outras espécies infectadas experimentalmente, é autolimitante, localizada e restringe-se a pele e junção mucocutânea; 3) a infecção pelo ORFV não induz anticorpos com atividade neutralizante; 4) o genoma do ORFV é extenso e comporta a inserção de grandes segmentos de DNA, permitindo a inserção de vários genes de interesse, inclusive possibilitando o desenvolvimento de vacinas multivalentes; 5) o genoma possui vários genes não-essenciais para replicação viral, que podem ser deletados/substituídos sem prejuízos à replicação viral; 6) além de ser naturalmente pouco virulento, o ORFV pode ser ainda mais atenuado por deleção de genes não-essenciais; 7) o

genoma do ORFV já foi inteiramente sequenciado e é facilmente manipulável; 8) na natureza, o ORFV raramente infecta outras espécies que não o seus hospedeiros naturais, por isso é considerado de baixo risco biológico. Por essas propriedades, o ORFV representa um promissor candidato a plataforma vetorial vacinal para várias espécies animais (MOSS, 2013; RZIHA et al., 2000).

O presente estudo descreve a construção e caracterização de dois recombinantes do ORFV que expressam a glicoproteína G do vírus da raiva (RABV) no loci dos genes *ORFV024* ou *ORFV121* e a avaliação da sua imunogenicidade em suínos e bovinos. Além disso, outros três mutantes do ORFV com deleções nos genes *ORFV112*, *ORFV117* ou *ORFV127* foram construídos, caracterizados *in vitro* e utilizados para infecção experimental de cordeiros, visando avaliar a sua virulência/atenuação. Um estudo adicional envolveu a avaliação da imunogenicidade de quatro vacinas comerciais contra a raiva em bovinos.

2. REVISÃO BIBLIOGRÁFICA

2.1 Ectima contagioso

A *orf* (do inglês: *rough*, ou rugoso) ou ectima contagioso, é uma enfermidade mucocutânea que acomete principalmente ovinos, caprinos e, eventualmente, humanos (FLEMING; MERCER, 2007). O ectima contagioso, também chamado de dermatite pustular contagiosa, estomatite pustular contagioso, ou dermatite labial infecciosa é causado pelo *Parapoxvirus ovino (*PPVO) ou vírus da *Orf* (ORFV). O ORFV pertencente ao gênero *Parapoxvirus*, subfamília *Chordopoxvirinae* da família *Poxviridae* (ICTV, 2017). Outros *Parapoxvirus* de importância veterinária incluem o vírus da estomatite papular bovina (BPSV), o vírus da pseudovaríola (PCPV) e o parapoxvírus do veado vermelho da Nova Zelândia (ICTV, 2017). O ORFV possui distribuição mundial e está amplamente difundido em países que possuem rebanhos ovinos e caprinos (HOSAMANI et al., 2009). No Brasil, o ectima contagioso é endêmico e foi descrito em várias regiões (DE OLIVEIRA et al., 2012; DE SANT'ANA et al., 2013; MARTINS et al., 2014; NOBREGA et al., 2008; SCHMIDT et al., 2013).

A infecção pelo ORFV ocorre em locais com soluções de continuidade, na pele ou mucosas e o vírus replica em queratinócitos da epiderme (HAIG et al., 1997a). As lesões são localizadas ao redor da boca e das narinas e, menos frequentemente, no úbere, coroa dos cascos e região interdigital (HAIG, 2006). A doença afeta principalmente animais jovens (menos de um ano de idade), causando uma condição debilitante como consequência da diminuição da ingestão de alimentos. No entanto, animais adultos também podem ser acometidos (FLEMING; MERCER, 2007). A mortalidade resultante da infecção é baixa, porém a morbidade geralmente é alta, podendo atingir mais de 90% dos animais do rebanho. A disseminação do vírus entre animais é rápida e pode ocorrer pelo contato direto com animais infectados, pelo contato com as crostas das lesões eliminadas no solo ou pelo contato com alimentos e pasto contaminados (BUTTNER; RZIHA, 2002; FLEMING; MERCER, 2007; HAIG, 2006). Animais lactentes também podem transmitir o vírus para os tetos e úberes das mães durante a amamentação (FLEMING; MERCER, 2007).

O período de incubação da doença varia entre dois e seis dias e as lesões progridem pelos estádios de eritema, vesícula, pústula, pápula e crostas (CARGNELUTTI et al., 2011; FLEMING; MERCER, 2007; HAIG, 2006). O curso clínico inicia com hiperemia e edema em torno do sítio de infecção e desenvolvimento de pequenas vesículas em aproximadamente 48-72 h. As vesículas progridem para os estádios de pústulas e pápulas. Lesões adjacentes podem

coalescer e, com a progressão da doença, culminam com a formação de crostas (BUTTNER; RZIHA, 2002; FLEMING; MERCER, 2007). Em infecções primárias, as lesões regridem em três a seis semanas, porém em reinfecções a resolução geralmente é mais rápida, geralmente duas a quatro semanas (FLEMING; MERCER, 2007; HAIG, 2006; HAIG et al., 1997). Outra característica da infecção pelo ORFV é ausência de disseminação sistêmica do vírus (HAIG, 2006). O exame histológico das lesões revela lesões proliferativas na epiderme, caracterizada por proliferação, vacuolização e edema de queratinócitos do estrato espinhoso, degeneração reticular, acúmulo de crostas e presença de micro abcessos (CARGNELUTTI et al., 2011; HAIG et al., 1997). A derme apresenta lesões como edema, dilatação dos capilares e infiltração de células inflamatórias. Além disso, lesões papilomatosas que consistem de hiperplasia e formação de granulomas são frequentemente observadas e, em alguns casos, podem ser extensas (FLEMING; MERCER, 2007).

2.2 O vírus da *orf* (ORFV)

Os vírions do ORFV são partículas grandes, 300nm aproximadamente, que possuem um envelope proteico. O genoma consiste de uma molécula de DNA linear de fita dupla com aproximadamente 138 Kb (BUTTNER; RZIHA, 2002; DELHON et al., 2004; FLEMING; MERCER, 2007). O conteúdo aproximado de G + C no genoma é de 64%, e contém pelo menos 131 genes (DELHON et al., 2004). As duas cadeias de DNA que compõe o genoma são unidas por *loops* nas extremidades (FLEMING; MERCER, 2007). Semelhante aos outros poxvírus, o genoma do ORFV é composto por regiões terminais variáveis que representam aproximadamente 20% do genoma e codificam produtos potencialmente envolvidos na patogenia, espectro de hospedeiros e evasão da resposta imune, flanqueada por duas regiões de sequências repetidas invertidas (ITRs), que são idênticas entre si, mas orientadas opostamente nas duas extremidades do genoma (DELHON et al., 2004). A região central do genoma contém genes conservados entre os poxvírus e os produtos desses genes participam de mecanismos básicos de replicação, estrutura e morfogênese (DELHON et al., 2004; MERCER et al., 2006).

Os poxvírus codificam as enzimas necessárias para a transcrição e replicação do genoma viral, e empacotam nos vírions as enzimas essenciais para a produção e modificação dos RNAs mensageiros (mRNA) para a síntese de suas proteínas no início do ciclo replicativo (BALASSU; ROBINSON, 1987; FLEMING; MERCER, 2007). O processo de expressão gênica dos poxvírus é caracterizado, classicamente, pela transcrição temporal de três classes de genes: *early*, *intermediate* e *late* (FLEMING; MERCER, 2007). A transcrição de cada classe de genes requer a fatores de transcrição específicos, que são produzidos pela expressão dos

genes da classe precedente (FLEMING; MERCER, 2007). Após a fusão do envelope do vírion com a membrana plasmática ou após a endocitose, o núcleo viral é liberado no citoplasma e a expressão gênica é iniciada com a transcrição dos genes da classe *early* ainda no interior do núcleo viral (FLEMING; MERCER, 2007). Os mRNAs transcritos no interior do núcleo viral são liberados no citoplasma para serem traduzidos. A maioria dos genes transcritos na fase *early* participa dos processos de desnudamento e replicação do DNA, virulência, evasão do sistema imune e na transcrição dos genes das classes *intermediate* e *late* (ASSARSSON et al., 2008; SCHRAMM; LOCKER, 2005). Em contrapartida, grande parte dos genes pertencentes às classes *intermediate* e *late* codificam proteínas que serão empacotados nos vírions como componentes do envelope viral (ASSARSSON et al., 2008). A replicação do genoma do vírus da vaccinia (VACV), um orthopoxvirus, é iniciada aproximadamente 1 a 2h após a infecção, enquanto que nos parapoxvírus o inicio da replicação é mais tardio, ocorrendo em torno de 4-6 h após a infecção (BALASSU; ROBINSON, 1987; FLEMING; MERCER, 2007).

O mecanismo de replicação do genoma viral ainda não foi completamente elucidado. No entanto, o modelo do *auto-priming* é o mais aceito, e baseia-se na observação de junções concateméricas no DNA em replicação e na ausência de uma origem de replicação (FLEMING; MERCER, 2007). Este modelo sugere que o início da replicação ocorre pela clivagem do DNA em uma ou nas duas extremidades, resultando em uma extremidade 3' livre que atua como primer para a DNA polimerase viral iniciar a replicação. A molécula de DNA que está sendo replicada assume a conformação original e o genoma é copiado por deslocamento da cadeia complementar (FLEMING; MERCER, 2007). Ao final do processo replicativo longos concatâmeros (moléculas do DNA genômicos unidos pelas extremidades) são formados. Estas formas concateméricas serão separadas por clivagem, após o início da transcrição dos genes *late*, dando origem a cópias únicas do genoma, nas quais, as extremidades em *loop* são regeneradas. A replicação do genoma dos poxvírus ocorre em locais específicos do citoplasma, denominados viroplasmas ou fábricas virais, que são acopladas ao retículo endoplasmático rugoso (KATSAFANAS; MOSS, 2007), e com a progressão do ciclo tornam-se dispersas pelo citoplasma. Além de servirem como sítio de replicação do genoma e montagem dos vírions, as fábricas virais também suportam a transcrição de genes das classes *intermediate* e *late* e ainda atuam como local de tradução de proteínas virais (KATSAFANAS; MOSS, 2007). Após o início da replicação, ocorre uma alteração na expressão gênica, quando os produtos dos genes *early* atuam como fatores de transcrição para os genes *intermediate* e *late*. Por sua vez, esses serão transcritos e traduzidos originando as proteínas que irão participar da morfogênese, bem

como os fatores de transcrição dos genes *early* que serão empacotados com os vírions para atuarem no próximo ciclo de infecção (FLEMING; MERCER, 2007).

2.3 O ORFV como vetor

O advento da tecnologia de manipulação genética tem possibilitado a construção e utilização de vários vírus como vetores vacinais (PASTORET; VANDERPLASSCHEN, 2003; RZIHA et al., 2000). Os poxvírus, em especial o VACV, têm sido amplamente utilizados como vetores de genes heterólogos com fins vacinais (PASTORET; VANDERPLASSCHEN, 2003; WEYER; RUPPRECHT; NEL, 2009). Esses vírus apresentam uma série de propriedades que viabilizam esse uso, incluindo: 1) um genoma grande (130 kbp no ORFV a 375 kbp nos *Avipoxvirus*) e facilmente manipulável que comporta a inserção e/ou deleção de grandes segmentos de DNA; 2) possuem múltiplos sítios para a inserção de genes heterólogos e sequências regulatórias; 3) possuem vários genes não-essenciais, que podem ser substituídos pelos genes heterólogos; 4) induzem robusta resposta humoral e celular contra antígenos heterólogos; 5) são, em geral, pouco patogênicos para humanos; 6) podem ser usados em várias espécies animais; 7) são relativamente estáveis a liofilização, uma propriedade altamente desejável para um vírus vacinal (FLEMING; MERCER, 2007; PASTORET; VANDERPLASSCHEN, 2003; RZIHA et al., 2000; WEYER; RUPPRECHT; NEL, 2009).

Desde as primeiras descrições de seu uso para carrear genes heterólogos em 1982, vários poxvírus recombinantes foram licenciados para uso vacinal, incluindo o poxvírus do canário com antígenos do vírus da cinomose para cães e furões; com antígenos do vírus do Nilo Ocidental (WNV) para equinos; com antígenos do RABV para gatos; do vírus da influenza para equinos; o poxvírus aviário com antígenos do vírus da doença de Newcastle e influenza H5 para galinhas; e o VACV com antígenos do RABV para a imunização de canídeos silvestres (NAYAK et al., 2009; POULET et al., 2003, 2007; WEYER; RUPPRECHT; NEL, 2009; WIKTOR et al., 1984).

O primeiro poxvírus licenciado como vetor vacinal foi o VACV Copenhagen expressando a RABV-G no sítio da enzima timidina quinase (TK) (WIKTOR et al., 1984). Esse vírus vacinal tem sido amplamente usado para vacinação oral de raposas, furões e coiotes na Europa e América do Norte. No Brasil, a ampla circulação do VACV em bovinos e espécies de roedores silvestres (DE SANT'ANA et al., 2013), e o constante risco de infeção natural por este vírus em outras espécies animais, incluindo equinos contraindicam a sua utilização como vetor vacinal. Estas restrições têm levado a esforços visando o aperfeiçoamento dessa

plataforma e também a busca de poxvírus alternativos como vetores (HICKS; FOOKS; JOHNSON, 2012; WEYER; RUPPRECHT; NEL, 2009).

A construção de um recombinante do ORFV como vetor para a glicoproteína G do RABV foi realizada (AMANN et al., 2013). O recombinante D1701-V-RabG expressa o gene heterólogo em quantidades adequadas, mesmo sem replicar produtivamente em espécies não-permissivas como cães, gatos e camundongos. Uma administração única do recombinante a essas espécies resultou na produção de anticorpos com atividade neutralizante contra o RABV e conferiu proteção contra desafio em camundongos (AMANN et al., 2013). Uma das principais restrições apresentadas pelo recombinante D1701-V-RabG, é a cepa viral utilizada como vetor. A cepa D1701 do ORFV foi adaptada em células de macaco e apresenta múltiplas alterações genéticas (incluindo deleções, inserções, e duplicações de genes) que ocorreram aleatoriamente no genoma do vírus durante as mais de 200 passagens em células Vero (COTTONE et al., 1998). O grande número de genes afetados por alterações genéticas também impossibilita a optimização deste vetor afim de melhorar a resposta imune induzida contra antígenos heterólogos.

Não obstante, estes estudos demonstram que o ORFV se constitui em um agente com grande potencial para vetorar genes heterólogos, podendo ser utilizado como plataforma vacinal em espécies permissivas e não-permissivas (PASTORET; VANDERPLASSCHEN, 2003; ROHDE; AMANN; RZIHA, 2013; RZIHA et al., 2000; WEYER; RUPPRECHT; NEL, 2009). A geração de um recombinante do ORFV expressando a glicoproteína S do PEDV (*porcine epidemic diarrhea virus*) utilizando a cepa IA82 do ORFV (DELHON et al., 2004), imunização e desafio de leitões, demonstrou a sua capacidade de induzir resposta imune robusta contra antígenos heterólogos em suínos e proteção frente ao desafio com o PEDV (HAIN et al., 2016).

2.4 ORFV: genes não-essenciais

A função de vários genes do ORFV ainda é desconhecida (FLEMING; MERCER, 2007; FRIEBE et al., 2011). No entanto, muitos têm sido caracterizados ou possuem homologia com genes com funções conhecidas em outros poxvírus (DELHON et al., 2004). O ORFV apresenta diversos genes não-essenciais, passíveis de deleção e inserção de sequências de nucleotídeos (RZIHA et al., 1999). De especial interesse, o ORFV codifica várias proteínas imunomodulatórias (IMP) que modulam a resposta imune do hospedeiro (HAIG, 2006; HAIG et al., 2002a; WEBER et al., 2013). Estas IMPs incluem um homólogo da interleucina 10 (vIL-10; *ORFV127*) (FLEMING et al., 2007a), uma proteína ligante da quimiocina (CBP; *ORFV112*) (SEET et al., 2003b), um inibidor do fator estimulador de colônia granulacítica-monocítica

(GMC-CSF) e interleucina-2 (IL-2) (GIF, *ORFV117*) (DEANE et al., 2000), um fator de resistência ao interferon (VIR; *ORFV020*) (MCINNES; WOOD; MERCER, 1998), um homólogo do fator de crescimento endotelial e vascular (VEGF; *ORFV132*) (WISE et al., 1999), um inibidor de apoptose (*ORFV125*) (WESTPHAL et al., 2007), e ao menos quatro inibidores da via de sinalização nuclear do NF-kappa-B (NF-κB) (*ORFV002*, *ORFV024*, *ORFV073* e *ORFV121*) (DIEL et al., 2010, 2011a, 2011b; KHATIWADA et al., 2017). As funções e os mecanismos de ação dos produtos destes genes tem sido caracterizados (DEANE et al., 2000; DIEL et al., 2010, 2011a, 2011b; FLEMING et al., 1997; MCINNES; WOOD; MERCER, 1998; SEET et al., 2003b; WISE et al., 1999).

Embora vários genes presentes no genoma dos *Parapoxvirus* estejam presentes em outros poxvírus (DELHON et al., 2004), foram identificados até o momento 15 genes que não possuem homologia com nenhum outro gene descrito em vírus ou qualquer outro organismo. Entre estes, os genes *ORFV024* e *ORFV121*, únicos dos parapoxvirus, atuam inibindo a ativação da via de sinalização NF-κB (DIEL et al., 2010, 2011b). O NF-κB é um importante modulador da resposta imune inicial contra infecções virais (BONIZZI; KARIN, 2004). Foi demonstrado que a deleção dos genes *ORFV024* e *ORFV121* do genoma do ORFV resulta no aumento da expressão de quimiocinas e citocinas pró-inflamatórias reguladas pelo NF-κB em células infectadas pelo ORFV (DIEL et al., 2010, 2011b).

O *ORFV112* é um dos genes não-essenciais para replicação do ORFV *in vitro*. Porém, diferentemente dos genes *ORFV024* e *ORFV121*, não é exclusivo do *Parapoxvirus* e já foi descrito em *Orthopoxvirus* e *Leporipoxvirus* (SEET et al., 2003a, 2003b). O gene *ORFV112* codifica uma proteína ligante a quimiocina, um produto com função similar as proteínas CBP-II com habilidade de ligar-se e inibir quimiocinas com alta afinidade (SEET et al., 2003b). As quimiocinas são uma família ampla de proteínas secretadas que ativam e regulam a inflamação e recrutamento de leucócitos nos sítios de infecção (SEET et al., 2003b). A CPB do ORFV também liga com alta afinidade a linfotactin, um membro da família das C-quimiocinas. As sequências do gene da CPB e GIF (produto do gene *ORFV117*) do ORFV compartilham alto nível de similaridade (DEANE et al., 2009). Estes resultados providenciam um *link* entre uma família de CC-CPB e o gene que codifica o GIF. Utilizando a cepa NZ7, foi demostrado que o gene *ORFV112* interfere na patogenia do ORFV em cordeiros (FLEMING et al., 2017).

O *ORFV117* codifica um inibidor do fator estimulador de colônia granulacítica-monocítica (GMC-CSF) e interleucina-2 (IL-2) (GIF, *ORFV117*) (DEANE et al., 2000). O GIF é uma proteína solúvel que forma homodímeros e tetrâmeros em solução e se liga ao GM-CSF e IL-2 ovino. Como GM-CSF e IL-2 são importantes na resposta imune antiviral, a expressão

do GIF pelo ORFV é um meio de manipular a resposta imune do hospedeiro durante a infecção. O GIF se liga e inibe a atividade biológica do GM-CSF e IL-2 (DEANE et al., 2000). Embora a função do gene *ORFV117* tenha sido descrita, até o momento não há na literatura informação sobre a viabilidade do ORFV mutante deletado no *ORFV117 in vitro*. Assim, carece também de estudos da função deste gene na patogenia do vírus mutante infectando ovinos.

Outro gene não-essencial para replicação *in vitro* presente no genoma do ORFV é o *ORFV127*. Este gene codifica a vIL-10, uma interleucina com papel importante na resposta imune do hospedeiro (FLEMING et al., 1997; HAIG et al., 2002b). A sequência de aminoácidos da vIL-10 tem 80% de identidade com a IL-10 ovina, sendo a região C-terminal, idêntica entre ovinos e o ORFV, sugerindo que o gene tenha sido adquirido pelo ORFV a partir do hospedeiro (FLEMING et al., 1997). A infecção de cordeiros com a cepa NZ2 do ORFV com deleção no gene *ORFV127* demonstrou que este gene interfere parcialmente na virulência do ORFV. Comparado ao vírus parental, a infecção com o ORFV mutante vIL-10 deletado reduziu a extensão e duração das lesões em cordeiros infectados experimentalmente (FLEMING et al., 2007a).

2.5 Vacinas contra a raiva

A raiva se constitui em uma das principais zoonoses de distribuição mundial. É uma doença neurológica letal de evolução aguda, que afeta diversas espécies de mamíferos, inclusive o homem (WHO, 2005). A raiva é causada pelo vírus rábico (RABV), um vírus envelopado que pertence à família *Rhabdoviridae* e gênero *Lyssavirus* (ICTV, 2017). O genoma do RABV constitui-se de uma molécula de RNA fita simples sentido negativo, não segmentado, de aproximadamente 12 kpb (SMITH, 1996), que codifica cinco proteínas principais, incluindo a polimerase de RNA dependente de RNA (L), a glicoproteína de superfície (G), a nucleoproteína (N), uma fosfoproteína (NS ou M1) e uma proteína de matriz (M ou M2) (SMITH, 1996). Com base em análise genômica e filogenética, doze genótipos e três filogrupos já foram identificados dentre os lyssavirus, sendo que o RABV pertence ao genótipo 1. Além disso, seis variantes do RABV já foram identificadas, de acordo com o hospedeiro e o perfil antigênico, podendo indicar a origem e distribuição geográfica (BEATRIZ; RUTHNER; ROEHE, 2007).

A glicoproteína G (RABV-G) é a proteína mais importante para a virulência do RABV e também o principal antígeno responsável pela indução de imunidade protetora (DIETZSCHOLD et al., 1983; SEIF et al., 1985). Os anticorpos neutralizantes produzidos contra a RABV-G são os principais mecanismos efetores de proteção (COX; DIETZSCHOLD;

SCHNEIDER, 1977). Assim, vacinas recombinantes que expressam a RABV-G têm sido utilizadas com sucesso na prevenção da raiva animal na Europa e Estados Unidos (BROCHIER et al., 1996; FABER; DIETZSCHOLD; LI, 2009).

A vacinação de pessoas e animais tem sido amplamente utilizada no controle da raiva, principalmente a partir da segunda metade do século XX. O histórico de vacinas contra a raiva abrange desde os experimentos pioneiros de Pasteur, no final do século XIX – que envolveram imunização com macerados de cérebro de coelhos – até as vacinas recombinantes atuais, obtidas por manipulação genética e utilizadas no controle da raiva silvestre na Europa e Estados Unidos (BRIGGS, 2012; KIENY et al., 1984). Nesse sentido, importantes passos incluíram a vacina desenvolvida por Fuenzalida e Palacios, em 1955, com vírus produzido no cérebro de camundongos lactentes e inativado (SCHNEIDER; SANTOS-BURGOA, 1995), o desenvolvimento dos cultivos celulares nos anos 40 e a capacidade de produzir grandes quantidades de vírus rábico em biorreatores nos anos 70 (WHO, 1992). Esses avanços contribuíram para o gradual abandono do uso de animais para a produção do vírus rábico para ser usado nas vacinas, mas ainda hoje alguns países asiáticos e sul-americanos utilizam esse método (BRIGGS, 2012).

Várias alternativas têm sido propostas para desenvolver vacinas antirrábicas mais seguras, efetivas e de uso universal, a maioria delas envolvendo manipulação genética do RABV (HICKS; FOOKS; JOHNSON, 2012). Como a RABV-G se constitui no principal antígeno do RABV, e anticorpos contra ela conferem proteção, a expressão da RABV-G em uma variedade de vetores tem sido testada com sucesso e sugerida como alternativa, incluindo-se a expressão da RABV-G pelo VACV (WIKTOR et al., 1984), canaripox (CADOZ et al., 1992), adenovírus canino (YAROSH et al., 1996) e pelo ORFV (AMANN et al., 2013). Para animais silvestres já estão disponíveis nos Estados Unidos e Europa vacinas recombinantes que utilizam o adenovírus ou o VACV como vetores para a expressão da RABV-G (BROCHIER et al., 1996). Resultados muito satisfatórios de proteção tem sido descritos com o uso dessa vacina, pela indução tanto da resposta imune humoral quanto celular (BROCHIER et al., 1996). A estratégia de vacinação oral com vacinas recombinantes tem sido expandida e proposta também para cães de rua, silvestres ou mesmo urbanos em vários continentes (FABER; DIETZSCHOLD; LI, 2009; HAMMAMI et al., 1999; KNOBEL; DU TOIT; BINGHAM, 2002).

No Brasil, as vacinas antirrábicas disponíveis para animais de produção (bovinos, ovinos e equinos) são inativadas, com adjuvante, e uma proteção adequada depende de revacinações anuais. Uma cobertura vacinal ampla e duradoura nem sempre é obtida, sobretudo

em comunidades remotas e carentes. Vacinas contra a raiva de herbívoros estão disponíveis há mais de meio século e são utilizadas com relativo sucesso no Brasil. A vacinação de bovinos e equinos, e em alguns casos, ovinos e suínos, tem sido utilizada para reduzir as perdas em áreas endêmicas. No entanto, a raiva continua representando uma ameaça para a saúde pública e para animais de produção, provocando muitas perdas econômicas (VIGILATO et al., 2013).

As vacinas contra a raiva têm experimentado uma evolução tecnológica lenta e gradual, desde o final do século 19 até as vacinas vetoriais recombinantes utilizadas no controle e erradicação da raiva em animais silvestres na Europa e América do Norte no final dos anos 1980. Não obstante, o Brasil e países da América Latina seguem utilizando as clássicas vacinas inativadas com adjuvante (VIGILATO et al., 2013; WHO, 2005). Essas vacinas parecem conferir boa proteção, pelo menos em caninos, de acordo com a redução de incidência de raiva canina urbana (VIGILATO et al., 2013). No entanto, apresentam restrições importantes que deixam margem para melhorias que poderiam decisivamente impulsionar os programas de controle e erradicação.

A presente tese é composta por três estudos apresentados sob a forma de artigos científicos, a seguir:

- ARTIGO 1: Immunogenicity of *ORFV*-based vectors expressing the rabies virus glycoprotein in livestock species;

- ARTIGO 2: Deletion of putative immunomodulatory genes *ORFV112*, *ORFV117* or *ORFV127* from the ORFV genome does not result in marked reduction in virulence in lambs;

- ARTIGO 3: Serological response to rabies virus induced by commercial vaccines in cattle.

3. ARTIGO 1

Immunogenicity of *ORFV*-based vectors expressing the rabies virus glycoprotein in livestock species

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Abstract

The parapoxvirus *Orf* virus (ORFV) encodes several immunomodulatory proteins that modulate host-innate and pro-inflammatory responses and has been proposed as a vaccine delivery vector for use in animal species. Here we describe the construction and characterization of two recombinant ORFV vectors expressing the rabies virus (RABV) glycoprotein (G). The RABV-G gene was inserted in the *ORFV024* or *ORFV121* gene loci, which encode for IMPs that are unique to parapoxviruses and inhibit activation of the NF-κB signaling pathway. The immunogenicity of the resultant recombinant viruses (ORFV$^{\Delta 024}$RABV-G or ORFV$^{\Delta 121}$RABV-G, respectively) was evaluated in pigs and cattle. Immunization of the target species with ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G elicited robust neutralizing antibody responses against RABV. Notably, neutralizing antibody titers induced in ORFV$^{\Delta 121}$RABV-G-immunized pigs and cattle were significantly higher than those detected in ORFV$^{\Delta 024}$RABV-G-immunized animals, indicating a higher immunogenicity of ORFV$^{\Delta 121}$-based vectors in these animal species.

Keywords: Orf virus, ORFV, Parapoxvirus, vector, Rabies, Glycoprotein.

Introduction

Orf virus (ORFV) is the prototype of the genus *Parapoxvirus*, subfamily *Chordopoxvirinae,* family *Poxviridae* (ICTV, 2017). ORFV is an ubiquitous virus which causes a self-limiting mucocutaneous infection in sheep and goats, known as *orf* or contagious ecthyma (Haig and Mercer, 1998). The ORFV genome consists of a double-stranded DNA molecule of approximately 138 kbp in length and contains 131 putative open reading frames (ORFs) (Delhon et al., 2004). Notably, ORFV encodes several immunomodulatory proteins (IMPs) that modulate host-innate and pro-inflammatory responses to infection (Haig et al., 2002; Weber et al., 2013). These IMPs include an interleukin 10 homologue (vIL-10; *ORFV127*) (Fleming et al., 2007), a chemokine binding protein (CBP; *ORFV112*) (Seet et al., 2003), an inhibitor of granulocyte-monocyte colony-stimulating factor (GMC-CSF) and IL-2 (GIF, *ORFV117*) (Deane et al., 2000), an interferon (IFN)-resistance gene (*ORFV020*) (McInnes et al., 1998), a homologue of vascular endothelial growth factor (VEGF; *ORFV132*) (Wise et al., 1999) an inhibitor of apoptosis (*ORFV125*) (Westphal et al., 2007), and at least three inhibitors of the nuclear factor-kappa (NF-κB) signaling pathway (*ORFV002*, *ORFV024*, and *ORFV121*) (Diel et al., 2011a, 2011b, 2010). The function(s) and/or mechanism(s) of action of these IMPs have been recently determined (Deane et al., 2000; Diel et al., 2011a, 2011b, 2010; Fleming et al., 1997; McInnes et al., 1998; Seet et al., 2003; Wise et al., 1999). Most importantly, while these genes are non-essential for ORFV replication *in vitro*, the viral homologues of IL-10 (*ORFV127*) and VEGF (*ORFV132*), the CBP (*ORFV112*) and the NF-κB inhibitor *ORFV121* are virulence factors that contribute to ORFV pathogenesis in the natural host (Diel et al., 2011b; Fleming et al., 2017, 2007; Meyer et al., 1999).

Given its immunomodulatory and biological properties, ORFV has been proposed as a vaccine delivery vector for use in animal species (Rziha et al., 2000). The unique features that make ORFV an attractive vector for vaccine delivery include: 1) its restricted host range (sheep

and goats); 2) its tropism for skin keratinocytes or their counterparts in the oral mucosa; 3) the absence of systemic dissemination and 4) the low or absent neutralizing activity of ORFV-induced antibodies (Amann et al., 2013; Fischer et al., 2003b; Hain et al., 2016; Henkel et al., 2005; Rohde et al., 2011; Rziha et al., 2000). Additionally, the presence of well characterized IMPs in the ORFV genome provides a unique opportunity for rational engineering of a safe and highly immunogenic ORFV-based vector platform. Recently, we have shown that immunization of pigs with a recombinant ORFV with a deletion of *ORFV121* (IMP that contributes to ORFV virulence) and expressing the porcine epidemic diarrhea virus (PEDV) spike (S) glycoprotein induced neutralizing antibody responses and protected pigs from clinical signs of PED (Hain et al., 2016). Here the immunogenicity of two ORFV-based recombinant viruses with single gene deletions on NF-κB-inhibitors *ORFV024* or *ORFV121* was investigated in pigs and cattle. The rabies virus (RABV) glycoprotein (G) was used as a model antigen to evaluate the immunogenicity of the recombinant vector candidates in the target animal species.

Rabies virus (RABV) is an enveloped RNA virus of to the genus *Lyssavirus*, family *Rhabdoviridae* (ICTV, 2017). The RABV genome consists of a single-stranded, negative sense RNA molecule with approximately 12 kb in length (Smith, 1996), which encodes five major proteins (nucleocapsid, N; phosphoprotein, P; matrix, M, glycoprotein, G; and polymerase, L) (Lytle et al., 2013). The RABV-G is the surface glycoprotein that plays an important role in virus virulence and is the main target of neutralizing antibodies against RABV (Dietzschold et al., 1983) (Seif et al., 1985). Notably, neutralizing antibodies against RABV-G are the main correlates of protection against RABV and they are known to play a critical role in protection against RABV infection and disease (Cox et al., 1977). Recombinant vectored vaccines (including poxviral vectors such as vaccinia virus and canary poxvirus) expressing the RABV-G have been successfully used for control of rabies in wild and domestic animals (Faber et al.,

2009; Pastoret and Brochier, 1996). Recently, an experimental recombinant ORFV (strain D1701) expressing the RABV-G demonstrated promising results in mice, cats in dogs against RABV (Amann et al., 2013).

Herein we describe the construction and characterization of two recombinant ORFV vectors expressing the RABV glycoprotein (ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G). The RABV-G was inserted either into the *ORFV024* or *ORFV121* gene loci and the immunogenicity of the resultant recombinant viruses (ORFV$^{\Delta 024}$RABV-G or ORFV$^{\Delta 121}$RABV-G, respectively) was evaluated in pigs and cattle. Immunization of the target species with ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G elicited robust neutralizing responses against RABV. Notably, neutralizing antibody titers detected in ORFV$^{\Delta 121}$RABV-G-immunized animals (pigs and cattle) were significantly higher than those detected in ORFV$^{\Delta 024}$RABV-G-immunized animals, indicating a higher immunogenicity of ORFV$^{\Delta 121}$-based vector on these species.

Results

Construction of ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G recombinant viruses. The RABV-G gene was inserted into the *ORFV024* or *ORFV121* gene loci of the ORFV genome by homologous recombination. *ORFV024* or *ORFV121* were deleted from the ORFV genome and replaced by a DNA fragment encoding the glycoprotein of RABV under the control of the early/late VV7.5 poxvirus promoter (Fig. 1A and B). RABV-G sequences were detected in the ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G recombinant viruses but not in the wild type ORFV genome (Fig. 1C and D). Deleted *ORFV024* or *ORFV121* gene sequences were not detected in the purified recombinant viruses ORFV$^{\Delta 024}$RABV-G or ORFV$^{\Delta 121}$RABV-G, respectively (Fig. 1C and D). Sequencing of the complete genomes of ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G recombinant viruses confirmed the integrity of RABV-G and ORFV sequences, with no nucleotide changes other than the deletion of ORFV024 or ORFV121

coding sequences being detected across the entire genome of the recombinant viruses (data not shown).

The replication kinetics of ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G recombinant viruses was assessed *in vitro*. No differences in replication kinetics and viral yields were observed comparing the recombinant viruses with the wild-type virus (ORFV IA82) in primary ovine fetal turbinate (OFTu) cells (Fig. 2A). Replication kinetics of ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G were also assessed in primary bovine fetal turbinate (BT) and swine turbinated (STu) cells (MOI = 0.1 [multi-step growth curve], and MOI = 10 [single-step growth curve]). Notably, a marked growth defect characterized by altered replication kinetics and lower viral yields of both ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G recombinant viruses were observed in BT and STu cells, when compared to their replication in ORFV natural host cells (OFTu; Fig. 2B).

Recombinant ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G viruses stably express the RABV-G protein *in vitro*. Expression of RABV-G by the ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G recombinant viruses was assessed by indirect immunofluorescence assays (IFA) and western blot (WB) analysis. Expression of RABV-G by the recombinant viruses during infection in OFTu cells was assessed by using an anti-FLAG antibody in an indirect IFA assay. High levels of RABV-G were detected in ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G infected cells at 24 h pi (Fig. 3A). Similarly, RABV-G was also detected in ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G infected cells by WB. Low levels of expression of RABV-G was detected as early as 8 h pi, with increasing levels of the protein accumulating in infected cells up to 24 h pi (Fig. 3B). Expression of RABV-G by the recombinant ORFV$^{\Delta 024}$RABV-G or ORFV$^{\Delta 121}$RABV-G was also assessed in cells derived from target animal species (cattle, BT; and swine, STu cells). Despite the lower replication efficacy in BT and STu cells (Fig. 2B), both recombinant viruses efficiently expressed the RABV-G in cells derived from target animal

species (Fig. 3). As expected, the levels of expression of RABV-G by the recombinant viruses in BT and STu cells were lower than those detected in OFTu cells, which are fully permissive to ORFV infection and replication (Fig. 2B).

The localization of RABV-G expressed by the recombinant ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G viruses was assessed by IFA assays. Abundant RABV-G expression was detected in both permeabilized and non-permeabilized cells, indicating expression of RABV-G on the surface and intracellular compartments of cells infected with ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G recombinant viruses (Fig. 4A).

The stability of RABV-G gene inserted into the *ORFV024* and *ORFV121* locus of the ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G genome, respectively, was assessed by IFA and PCR assays following serial passages of the recombinant viruses in cell culture *in vitro*. Expression of RABV-G was consistently detected in ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G infected cells after 1, 5 and 10 passages of the recombinant viruses in cell cultures (Fig. 4B). Additionally, PCR amplification of the RABV-G from the genome of passage 1, 5 and 10 ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G recombinant viruses confirmed the stability of RABV-G gene inserted into the *ORFV024* or *ORFV121* genome loci (Fig. 4C and 4D).

Immunogenicity of ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G recombinant viruses in pigs. Recently, we have shown that an ORFV recombinant virus expressing the PEDV S protein and lacking the NF-κB inhibitor *ORFV121* induces neutralizing antibody responses that led to protection from clinical disease and reduced virus shedding after challenge in pigs (Hain et al., 2016). Here the immune responses elicited by immunization with ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G recombinant viruses were evaluated and the immunogenicity of each vector candidate was compared in pigs. For this, nine piglets were immunized with ORFV-RABV-G recombinant viruses and received a booster on day 21 post-immunization (p.i.) (Table

1). The serological responses elicited against RABV were monitored by rapid fluorescent focus inhibition test (RFFIT) to detect RABV neutralizing antibodies (Smith et al., 1973).

All pigs immunized with ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G viruses developed neutralizing antibodies to RABV by day 42 p.i. (Table 1). While 3 of 4 animals from Group 2 (ORFV$^{\Delta 121}$RABV-G-immunized group) presented low levels of neutralizing antibodies after the primary immunization, none of the animals from Group 1 (ORFV$^{\Delta 024}$RABV-G-immunized group) presented detectable levels of RABV neutralizing antibodies on day 21 (Table 2). Following the booster immunization on day 21, all immunized animals presented anamnestic serological responses, as evidenced by a marked increase in the levels of RABV neutralizing antibodies on day 42 (Table 2). In fact, the geometric mean titers (GMT) of RABV neutralizing antibodies were significantly higher in animals immunized with ORFV$^{\Delta 121}$RABV-G recombinant virus (GMT = 905) when compared to animals immunized with ORFV$^{\Delta 024}$RABV-G recombinant virus (GMT = 160; $P < 0.01$; Fig. 5A).

Immunogenicity of ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G recombinant viruses in cattle. The immunogenicity of ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G recombinant viruses was also evaluated in cattle. All animals immunized with ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G recombinant viruses developed neutralizing antibodies to RABV after the primary immunization (titers ranging between 40-160 for Group 1, or 40-320 for Group 2 on day 30 p.i.) (Table 3). After the booster immunization on day 30, animals presented anamnestic serological responses as evidenced by a marked increase in the levels of RABV neutralizing antibodies on day 60 p.i. (Table 3). Similar to the results observed in pigs, immunization with ORFV$^{\Delta 121}$RABV-G recombinant virus resulted in higher RABV neutralizing antibody titers (GMT = 970), when compared to the titers detected in animals immunized with ORFV$^{\Delta 024}$RABV-G (GMT = 538.2) ($P < 0.05$; Fig. 5B). Together, these results indicate that

ORFV$^{\Delta 121}$RABV-G recombinant virus is more immunogenic and elicits robust antibody responses in swine and cattle.

Memory responses following immunization of cattle with ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G recombinant viruses. The immunogenicity of ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G recombinant viruses in cattle was confirmed following a second immunization experiment in this species (experiment 2). Thirty heifers were randomly allocated into two experimental groups (n = 15) and subjected to a prime-boost immunization regimen with the ORFV$^{\Delta 024}$RABV-G or ORFV$^{\Delta 121}$RABV-G recombinant viruses. All animals immunized with ORFV$^{\Delta 121}$RABV-G recombinant virus presented RABV neutralizing antibodies (titers from 10-320), whereas only 7 of 15 animals immunized with ORFV$^{\Delta 024}$RABV-G recombinant virus presented RABV neutralizing antibodies on day 30 p.i. (titers from 10-40) (Table 4). Notably, anamnestic neutralizing antibody responses were observed on day 60 p.i. in all immunized animals after the booster immunization on day 30 (Table 4). Most importantly, similar to results from experiment 1, animals immunized with ORFV$^{\Delta 121}$RABV-G recombinant virus presented significantly higher neutralizing antibody titers (GMT = 735.2), when compared to animals immunized with ORFV$^{\Delta 024}$RABV-G recombinant virus (GMT = 211.1) on day 60 p.i.. These results confirmed the immunogenicity of ORFV-based vectors in cattle and further indicate that ORFV$^{\Delta 121}$ is more immunogenic than ORFV$^{\Delta 024}$-based vector in swine and cattle.

To assess the duration of immunity and induction of B-cell memory responses following immunization with ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G recombinant viruses, animals from experiment 2 were monitored for 420 days post-primary immunization. Serum samples collected on days 390 and 420 p.i. (following a booster immunization on day 390 p.i), were tested for the presence of RABV neutralizing antibodies. Most animals immunized with either ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G recombinant viruses presented low levels of

RABV neutralizing antibodies on day 320 pi (Table 4). However, following the booster immunization on day 390 p.i., all animals rapidly responded to the immunization and developed anamnestic antibody responses to RABV (Fig. 6, Table 4). Animals from ORFV$^{\Delta 121}$RABV-G-immunized group developed higher levels of neutralizing antibodies against RABV. These results indicate that immunization of cattle with ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G recombinant viruses elicits immunological B-cell memory in immunized animals and further confirmed the higher immunogenicity of ORFV$^{\Delta 121}$-based vectors in target animal species.

Discussion

The parapoxvirus ORFV has been long used in veterinary medicine as an immunomodulator. Given its unique biological and immunomodulatory properties the virus has been proposed as a vaccine delivery vector for use in animals (Rziha et al., 2000). Several studies using the cell culture adapted and highly attenuated ORFV strain D1701 have demonstrated the efficiency of ORFV as a vaccine delivery platform in non-permissive species, including mice (Fischer et al., 2003a), rats (Henkel et al., 2005), rabbits (Rohde et al., 2011), cats, dogs (Amann et al., 2013) and swine (Dory et al., 2006; Voigt et al., 2007). Recently, we have shown that ORFV strain IA82 carrying the PEDV S protein gene in the locus of the NF-κB inhibitor *ORFV121* induced neutralizing antibody responses in swine and conferred protection against clinical disease after oral challenge with PEDV (Hain et al., 2016). Here we assessed and compare the immunogenicity of two ORFV-based recombinant viruses containing individual deletions of NF-κB inhibitors *ORFV024* or *ORFV121* in swine and cattle. The well-characterized RABV protective antigen glycoprotein G, which has known correlates of protection (neutralizing antibodies) (Cox et al., 1977; Wiktor et al., 1973), was used as the model antigen to assess the immunogenicity of the candidate vectors in the target animal species.

ORFV *ORFV024* and *ORFV121* encode for IMPs that are unique to parapoxviruses and were shown to inhibit activation of the NF-κB signaling pathway (Diel et al., 2011b, 2010). The NF-κB is an important modulator of early immune responses against viral infections (Bonizzi and Karin, 2004), and deletion of *ORFV024* and *ORFV121* from the ORFV genome has been shown to result in an increased expression of NF-κB-regulated pro-inflammatory chemokines and cytokines in ORFV infected cells (Diel et al., 2011b, 2010). Given the immunomodulatory properties of ORFV024 and ORFV121 and the fact that ORFV121 contributes to ORFV virulence in the natural host (Diel et al., 2011b, 2010), we hypothesized that deletion of these genes from the ORFV genome would result in safe and immunogenic vaccine delivery platforms. Our previous study with the ORFV-PEDV-S (in which *ORFV121* was replaced with the PEDV S) has shown that *ORFV121* is a suitable insertion site for heterologous genes in the ORFV genome (Hain et al., 2016). The results presented here confirmed these findings and further demonstrated that, in addition to *ORFV121*, *ORFV024* can also serve as an insertion site for stable expression of heterologous genes in ORFV-based vectors. In addition, the present study also expands the species range of ORFV-based vectors by demonstrating that cattle immunized with ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G recombinant viruses developed robust neutralizing antibody responses against RABV-G.

After generating the recombinant ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G viruses and confirming that no changes were present in their genome other than the deletion of *ORFV024* or *ORFV121* coding sequences through complete genome sequencing, the viruses were characterized *in vitro* and used in immunization studies in target animal species. No differences in replication kinetics and viral yields were observed when growth curves of the recombinant ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G viruses were compared to those of the wild-type virus and in OFTu cells, demonstrating that insertion of RABV-G did not alter ORFV replication properties in natural host cells. Similar results were observed with the

ORFV-PEDV-S, containing the spike protein in the *ORFV121* gene locus (Hain et al., 2016). When the replication kinetics of the recombinant viruses was assessed in primary cells derived from target animal species (STu and BT) and compared to natural host cells (OFTu), a marked growth defect was observed for both recombinant viruses. These results suggest that BT and STu cells likely pose a restriction to the replication of ORFV-based recombinant viruses. Notably, in spite of the altered replication kinetics and reduced viral yields in these cells, IFA and WB assays revealed that both ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G recombinant viruses efficiently expressed high levels of RABV-G in cells from target animal species (Fig. 3A and B). This is likely due to the early transcription of RABV-G driven by the VV7.5 early/late promoter during recombinant virus infection in non-permissive STu and BT cells. This is one of the unique features of poxvirus vectors that allows for efficient antigen expression/delivery in non-permissive animal species (Rziha et al., 2000).

The stability of RABV-G gene inserted into the *ORFV024* and *ORFV121* gene loci was investigated by IFA and PCR. Similar to our previous results with ORFV-PEDV-S (Hain et al., 2016), both recombinant ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G viruses stably expressed the RABV-G after serial passages in cell culture, as evidenced by abundant expression of RABV-G in passages 1, 5 and 10 (Fig. 4B). Additionally, PCR amplification of RABV-G from passage 1, 5 and 10 confirmed the stable insertion of RABV-G in the *ORFV024* and *ORFV121* gene loci (Fig. 4C and 4D). These observations suggest the genetic stability of the inserted RABV-G over multiple virus generations, which is one of the requirements for a viral vector (Liniger et al., 2007). The kinetics of expression of RABV-G by ORFV$^{\Delta 024}$RABV-G or ORFV$^{\Delta 121}$RABV-G recombinant viruses was assessed in OFTu cells. At 8 h pi RABV-G was first detected at low levels in ORFV$^{\Delta 024}$RABV-G- or ORFV$^{\Delta 121}$RABV-G-infected cells, with increasing levels of the protein being detected up to 24 h pi. This expression kinetics is consistent with the early/late activity of the VV7.5 promoter (Chakrabarti et al., 1997). As

expected, when the levels of expression of RABV-G in OFTu cells (permissive) were compared to those in BT and STu cells, a decreased expression was observed in cells infected with both ORFV$^{\Delta 024}$RABV-G or ORFV$^{\Delta 121}$RABV-G recombinant viruses (Fig. 5). Results from the immunization experiments in pigs and cattle demonstrating neutralizing antibody responses in both species, however, confirmed that the recombinant vectors effectively expressed RABV-G in the heterologous species.

The efficacy of ORFV-based vectors for vaccine delivery in pigs has been demonstrated in several studies, with recombinant ORFV-vectors expressing the protective antigens of pseudorabies virus (PRV), classical swine fever virus (CSFV), and PEDV (Dory et al., 2006; Hain et al., 2016; Voigt et al., 2007) inducing protective immune responses against the respective viruses. The results showing that immunization of pigs with ORFV$^{\Delta 024}$RABV-G- or ORFV$^{\Delta 121}$RABV-G recombinant viruses resulted in serological responses against the model RABV-G antigen confirm the potential of ORFV-based vectors in swine. Most important are the results showing that immunization of pigs with ORFV$^{\Delta 121}$RABV-G recombinant virus induced significantly higher levels of neutralizing antibodies when compared to those induced by ORFV$^{\Delta 024}$RABV-G recombinant virus (GMT = 905 and 160, respectively) ($P < 0.01$). These results indicate that ORFV$^{\Delta 121}$-based vectors elicit robust immune responses in pigs.

Despite of the long use of ORFV as an experimental vaccine delivery vector in several animal species (Amann et al., 2013; Dory et al., 2006; Fischer et al., 2003b; Henkel et al., 2005; Rohde et al., 2011; Voigt et al., 2007), no studies have reported the efficacy of the vector in cattle. He we first show that ORFV-based vectors can efficiently deliver foreign viral antigens in this important livestock species. Immunization of cattle with recombinant ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G viruses resulted in high levels of neutralizing antibodies in all immunized animals (Table 3 and 4), and a booster immunization on day 30 pi led to anamnestic responses in all immunized animals (Table 3 and 4). Interestingly, similar to the results

observed in pigs, immunization with ORFV$^{\Delta 121}$RABV-G recombinant virus resulted in higher neutralizing antibody responses when compared to those detected in ORFV$^{\Delta 024}$RABV-G-immunized animals. Together these observations suggest that ORFV$^{\Delta 121}$-based vectors present an enhanced immunogenicity when compared to ORFV$^{\Delta 024}$-based vectors in both swine and cattle, which, as shown here, results in higher levels of neutralizing antibodies against RABV-G. Although the mechanism underlying this phenotype was not investigated in our study, a recent study has shown that vaccinia virus (VACV) NF-κB inhibitors A52, B15 and K7 differentially regulate innate and adaptive immune responses (specifically CD8 and IgG antibodies) against human immunodeficiency 1 (HIV-1) antigens vectored by VACV in mice (Di Pilato et al., 2017). In this study, the authors showed that deletion of A52R from the VACV-vector led to higher immune responses against the heterologous antigen when compared to the other two NF-κB inhibitors (B15 and K7) (Di Pilato et al., 2017). Taken together these findings indicate that poxviral NF-κB inhibitors offer excellent options for rational design of poxvirus-based vectors leading to enhanced immunogenicity in select animal species.

Although this study focused on comparing the immunogenicity between ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G in target animal species, the results with ORFV$^{\Delta 121}$RABV-G in cattle are encouraging if considered in the context of strategies to control RABV in this species. Rabies is one of the most important diseases of livestock species and human health. In the Americas, for example, rabies causes yearly losses of ~$30 million (US dollars) due to mortality of livestock, with cattle being the most commonly affected species (WHO, 2005). The results obtained in the present study demonstrating the superior immunogenicity of ORFV$^{\Delta 121}$RABV-G recombinant vector in cattle, indicate that this recombinant virus represents an attractive alternative to the current inactivated RABV vaccines. Although the exact levels of RVNA required for protection are not known, titers of ~0.5 international units (IU)/ml (serum neutralization titer of ~1:50) are considered sufficient to

demonstrate a robust immune response to vaccination (Moore and Hanlon, 2010). Results here with ORFV-based vectors are consistent with this, highlighting the potential of the ORFV$^{\Delta 121}$RABV-G recombinant virus as a vaccine candidate for use in cattle. Additional studies would, however, be needed to compare the protective efficacy of ORFV$^{\Delta 121}$RABV-G recombinant virus with currently available inactivated rabies virus vaccines.

In summary, the present study defined the immunogenicity of two ORFV-based recombinant viruses (ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G) in potential target animal species for ORFV-vectored vaccines. As evidenced by the higher neutralizing antibody responses in pigs and cattle, ORFV$^{\Delta 121}$-based vector presented an enhanced immunogenicity, when compared to its counterpart ORFV$^{\Delta 024}$-vector. Given the immunogenicity of ORFV$^{\Delta 121}$-vector in both swine and cattle, this vector represents an excellent candidate for novel vaccine designs for use in these animal species.

Methods

Cells and viruses. Primary ovine fetal turbinate (OFTu, provided by Dr. D.L. Rock, University of Illinois or generated in house), bovine fetal turbinate (BT, generated in house), swine turbinate (STu, generated in house) cells and Baby Hamster Kidney cells (BHK-21 - [C-13 ATCC® CCL-10™]) were cultured in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), L-glutamine (2 mM), penicillin (100 U/mL), streptomycin (100 µg/mL) and gentamycin (50 µg/mL). Cell cultures were maintained at 37ºC with 5% CO_2.

The ORFV strain IA82 (Delhon et al., 2004, provided by Dr. D.L. Rock) was used as the parental virus to construct the recombinant viruses expressing the RABV glycoprotein (G). Parental and recombinant ORFV viruses expressing the RABV-G were amplified and titrated in primary OFTu cells. RABV strain Challenge Virus Standard (CVS) (CVS132-11A) was amplified and titrated in BHK-21 cells. RABV strain CVS was used in rapid fluorescent focus

inhibition test (RFFIT) to determine the levels of neutralizing antibodies in the sera of immunized animals.

Construction of recombination plasmids. The full-length coding sequence of the G gene of RABV isolate BRBv39 (GenBank accession no. AB110666) was analyzed, and restriction endonuclease sites required for insertion into the ORFV genome [*ORFV024* locus (Diel et al., 2010) or *ORFV121* locus (Diel et al., 2011b)] were removed through silent nucleotide substitutions. Coding sequences of the FLAG-Tag epitope (GACTACAAAGACGATGACGACAAG) were added to the 3'end of the G coding sequence. Additionally, EcoRI and NotI restriction sites were added to the 5' and 3' ends of the RABV-G construct, respectively. A DNA fragment containing the full-length RABV-G coding sequences was chemically synthesized (Epoch Life Science Inc, Sugar Land, TX) and subcloned into the poxviral transfer vector pZippy-Neo/Gus (Dvoracek and Shors, 2003) using EcoRI and NotI restriction enzymes (pZ-RABV-G). This resulted in the cloning of RABV-G under the control of the early/late poxviral promoter VV7.5 (Chakrabarti et al., 1997).

To insert the RABV-G coding sequences into the *ORFV024* or *ORFV121* genome loci, two recombination plasmids were constructed. *ORFV024* left (LF, 856 bp) and right (RF, 866 bp) flanking regions were PCR amplified from the ORFV strain IA82 genome [primers previously described (Diel et al., 2010)] and cloned into the vector pZ-RABV-G resulting in the recombination vector pZ024-RABV-G. *ORFV121* left (LF, 1016 bp) and right (RF, 853 bp) flanking regions were PCR amplified from the ORFV IA82 genome [primers previously described (Hain et al., 2016)] and cloned into the vector pZ-RABV-G resulting in the recombination vector pZ121-RABV-G. Cloning of *ORFV024* and *ORFV121* LF and RF were confirmed by restriction enzyme analysis.

Generation and characterization of the ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G recombinant viruses. The coding sequence of the RABV-G was inserted into the *ORFV024*

or *ORFV121* gene loci of the ORFV genome by homologous recombination between the parental ORFV IA82 and the recombination cassette pZ024-RABV-G or pZ121-RABV-G as previously described (Hain et al., 2016). Cell cultures were harvested at 72 h post-infection/transfection, subjected to three freeze-and-thaw cycles and cell lysates were used for selection of recombinant viruses by limiting dilution followed by plaque assays. OFTu cells cultured in 96-well plates were infected with 10-fold serial dilutions of the cell lysates (10^{-1} to 10^{-3}) and incubated at 37^{o}C for 72 h. Total DNA was extracted using the Quick-DNA™ 96 kit (Zymo Research, Irvine, CA) and screened with a RABV-G-specific real time PCR assay (qRT-PCR; PrimeTime® qPCR assay, IDT). Wells that were qRT-PCR positive were subjected to additional rounds of limiting dilution and qRT-PCR screening (7-10 rounds). Final purification/selection was performed by plaque assays. qRT-PCR positive cell cultures were diluted (10-fold; 10^{-1} to 10^{-3}), inoculated in OFTu cells cultured in 6-well plated and overlaid with culture medium containing 0.5% agarose (SeaKem GTC agarose, Lonza Inc., Alpharetta, GA). Individual plaques were picked and amplified in OFTu cells cultured in 96-well plates. At 72 h pi, total DNA was extracted and screened by qRT-PCR for RABV-G as above. After three rounds of plaque assays, individual clones were amplified in OFTu cells cultured in 12-well plates and screened for the presence of RABV-G and absence of *ORFV024* or *ORFV121* sequences by conventional PCR. Primers used for PCR amplification of the RABV-G insert or deleted *ORFV024* or *ORFV121* sequences were RabVGEx-Fw(BamHI)-5'-GCGGCGGATCCATGAAATTCCCCATCTACACAATACCAG-3' and RabVGEx-Rv(NotI)-5'-ATAATGCGGCCGCGTATTTCCCCCAACTCGGGAGACCAAGG-3'; 024int-Fw-5'- ACTTGGATCTGTCCGACGAC-3' and 024int-Rv-5'-AGCTGTTCCACGTCCCTCT-3'and 121int-Fw-5'- GGCGGACTACCAGAGACATC-3' and 121int-Rv-5'-GTCTTCCGGGATGTCGTAGA-3', respectively. PCR amplicons were analyzed by electrophoresis in 1% agarose gels. Integrity of the RABV-G and ORFV IA82

sequences as well as deletion of *ORFV024* or *ORFV121* sequences was confirmed by whole genome sequencing using the Nextera XT DNA library preparation kit (Illumina, San Diego, CA) followed by sequencing on the Illumina MiSeq sequencing platform (Illumina, San Diego, CA). A clone of each recombinant virus ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G without any change in the ORFV genome other than the deletion of *ORFV024* or *ORFV121* were used in all experiments described below.

Growth curves and passage experiments. Replication properties of ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G recombinant virus were assessed *in vitro.* OFTu cells were cultured in 6-well plates, inoculated with ORFV$^{\Delta 024}$RABV-G, ORFV$^{\Delta 121}$RABV-G or wild type virus (ORFV IA82) (MOI = 0.1 [multi-step growth curve]) and harvested at various time points post-infection (6, 12, 36, 48, and 72 h pi). OFTu, BT and STu cells were culture in 6-well plates, inoculated with ORFV$^{\Delta 024}$RABV-G or ORFV$^{\Delta 121}$RABV-G (MOI = 0.1 [multi-step growth curve], and MOI = 10 [single-step growth curve]), and harvested at various time points post-infection (6, 12, 24, 48, and 72 h p.i.) to compare the replication kinetics of recombinant viruses in cells of target animal species. Virus titers were determined on each time point using end-point dilutions, the Spearman and Karber's method and expressed as tissue culture infectious dose 50 ($TCID_{50}$)/mL. To assess the stability of RABV-G inserted in the genome of ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G recombinant viruses, serial passages of the viruses were conducted in OFTu cells. Low MOI = 1 infections were performed with both viruses and cells were harvested at 48 h pi. Passages 1, 5 and 10 viruses were subjected to IFA and PCR assays to assess expression and presence of RABV-G in the recombinant virus genomes.

Immunofluorescence. Expression of RABV-G by the ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G recombinant viruses was assessed by indirect fluorescent antibody assay (IFA). OFTu cells were infected with the ORFV$^{\Delta 024}$RABV-G or ORFV$^{\Delta 121}$RABV-G recombinant virus (MOI = 1) and fixed with 3.7% formaldehyde at 24 h post-infection. After

fixation cells were washed three times with phosphate buffer saline (PBS) and permeabilized with 0.2% PBS-Triton X100 for 10 min at room temperature (RT). Unpermeabilized cells were used as controls to assess expression of RABV-G on the membrane of ORFV$^{\Delta 024}$RABV-G or ORFV$^{\Delta 121}$RABV-G infected cells. Cells were washed three times with PBS, incubated with anti-FLAG antibody and for 1 h at RT. After primary antibody incubation, cells were washed as above and incubated with goat anti-mouse IgG (H+L) secondary antibody (Alexa Fluor® 594 conjugate; Life Technologies, Carlsbad, CA) for 1 h at RT. Cells were washed three times with PBS, the slides were mounted and visualized in an UV microscope.

Western blot. Expression of RABV-G by the ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G recombinant viruses was assessed by Western blot (WB). OFTu cells cultured in 6-well plates were inoculated with ORFV$^{\Delta 024}$RABV-G or ORFV$^{\Delta 121}$RABV-G recombinant viruses (MOI = 10) and harvested at 2, 4, 6, 8, 12 and 24 h pi to assess the expression kinetics of RABV-G. Mock-infected cells were used as controls. OFTu, BT and STu cells cultured in 6-well plates were inoculated with ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G recombinant viruses (MOI = 10) and harvested at 24 h pi. Cells were lysed with M-PER mammalian extraction reagent (Thermo Scientific, Waltham, MA) containing protease inhibitors (RPI, Mount Prospect, IL). Hundred micrograms of whole cell protein extracts were resolved by SDS-PAGE in 10% acrylamide gels and transferred to nitrocellulose membranes. Blots were incubated with 5% non-fat-dry-milk TBS-Tween 20 (0.1%; TBS-T) solution for 1 h at RT and probed with the FLAG-tag epitope antibody (THE™ DYKDDDDK Tag antibody, GenScript, Piscataway, NJ) or loading control antibodies against β-actin (C4; Biotechnology, Dallaz, TX) overnight at 4ºC. Blots were washed three times with TBS-T for 10 min at RT and incubated with a goat anti-mouse IgG-HRP conjugate secondary antibody for 2 h at RT. Blots were washed three times with TBS-T for 10 min and developed by using a chemiluminescent substrate (Clarity, ECL; Bio-Rad, Hercules, CA).

Animal immunizations. The immunogenicity of ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G recombinant viruses was compared in swine and cattle. Nine eight-week-old piglets were randomly allocated in two experimental groups as follows: Group 1, ORFV$^{\Delta 024}$RABV-G-immunized (n=5) and Group 2, ORFV$^{\Delta 121}$RABV-G-immunized (n=4). Immunization was performed by intramuscular (IM) injection of 2 ml of a virus suspension containing $10^{7.8}$ tissue culture infectious dose 50 ($TCID_{50}$) ml^{-1}. Animals were immunized on day 0 and received a booster immunization on day 21 post-primary vaccination. Serum samples were collected on days 0 (day of first immunization), 21 (day of booster) and 42 p.i..

Two experiments were performed in cattle. In the first experiment (Exp.#1), nine 4 to 6-months-old calves were randomly allocated in two experimental groups as follows: Group 1, ORFV$^{\Delta 024}$RABV-G-immunized (n=5) and Group 2, ORFV$^{\Delta 121}$RABV-G-immunized (n=4). Immunization was performed by intramuscular (IM) injection of 2 ml of a virus suspension containing $10^{7.9}$ $TCID_{50}.ml^{-1}$. Animals were immunized on day 0 and received a booster immunization on day 30 p.i.. Serum samples were collected on days 0, 30, and 60 p.i.. The second experiment (Exp.#2) was performed to confirm the immunogenicity of ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G recombinant viruses and to assess the duration of the serological response to RABV induced by the recombinant viruses in cattle. For this, 30 two-year-old heifers were randomly allocated in two experimental groups (Group 1, n=15, and Group 2, n=15). Immunization was performed by IM injection of 2 ml of a virus suspension containing $10^{7.9}$ $TCID_{50}.ml^{-1}$. Animals were immunized on day 0 and received a booster immunization on day 30 p.i.. A second booster immunization was administered at day 390 p.i.. Serum samples were collected on days 0, 30, 60, 390 and 420 p.i.. All serum samples were incubated at 56ºC for 30 min for complement inactivation and stored at -20ºC until testing. All animal experiments were conducted following the guidelines and protocols approved by the

UFSM institutional ethics committee (Ethics Committee on the Use of Animals [CEUA/UFSM]; protocol approval n° 035/2014).

Rapid fluorescent focus neutralization test. The presence of neutralizing antibodies against RABV in the sera of immunized animals was investigated by a modified RIFFT (rapid inhibition fluorescent focus test) (Smith et al., 1973). Briefly, 10-fold dilutions of sera were incubated with 100-200 $TCID_{50}$ of RABV strain CVS for 90 min, followed by addition of a suspension of BHK-21 cells and incubation at 37ºC with 5% of CO_2 for 48h. At 48h, indicator cells were individualized by trypsin, resuspended in culture medium and allowed to attach to multispot glass slides for FA. Slides containing attached cells were fixed in cold acetone for 5 min, air dried and incubated with an anti-RABV FITC-conjugate (Instituto Pasteur, Sao Paulo, Brazil) for 1h at 37ºC in a humid chamber. Mock-infected BHK-21 cells and cells infected with RABV strain CVS were used as negative and positive controls. Slides were examined in an epifluorescence microscope (Axiolab ZEISS®). The virus neutralizing (VN) titer was considered the highest dilution of serum capable to completely preventing virus infection/replication, as indicated by the absence of virus antigen in indicator cells. A reference serum (provided by Instituto Pasteur, Sao Paulo, Brazil) was used as positive control in all tests. Neutralizing titers were transformed to group geometric mean titers (GMT) (Perkins, 1958).

Statistical analysis. Statistical analysis was performed using the Prism software (GraphPad; 6th version) software. Students T-test was performed on all groups. Statistical differences between groups were considered significant at $P < 0.05$.

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The authors D. G. D. and E. F. F. declare that there is a patent pending related to this work (US patent pending, P11703US00).

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Table 1. Experimental design of animal immunization studies.

Specie	*n*	Recombinant virus	Dose ($TCID_{50}$)/mL	Route of immunization	Immunization days	Serum collection days
Swine	5	$ORFV^{\Delta 024}$RABV-G	$2x10^{7.8}$	IM[a]	0, 21	0, 21, 42
	4	$ORFV^{\Delta 121}$RABV-G	$2x10^{7.8}$	IM	0, 21	0, 21, 42
Bovine exp#1[b]	4	$ORFV^{\Delta 024}$RABV-G	$2x10^{7.9}$	IM	0, 30	0, 30, 60
	5	$ORFV^{\Delta 121}$RABV-G	$2x10^{7.9}$	IM	0, 30	0, 30, 60
Bovine exp#2[c]	15	$ORFV^{\Delta 024}$RABV-G	$2x10^{7.9}$	IM	0, 30, 390	0, 30, 60, 390, 420
	15	$ORFV^{\Delta 121}$RABV-G	$2x10^{7.9}$	IM	0, 30, 390	0, 30, 60, 390, 420

[a] intramuscular;
[b] experiment 1;
[c] experiment 2.

Table 2. Serological responses of piglets against RABV detected by rapid fluorescent focus inhibition test (RFFIT).

Group	Animal ID	nAb[a] titer		
		0 d p.i.[b]	21 d p.i.	42 d p.i.
ORFV$^{\Delta 024}$RABV-G	34	<10	<10	160
	61	<10	<10	160
	69	<10	<10	640
	74	<10	<10	80
	97	<10	<10	80
ORFV$^{\Delta 121}$RABV-G	25	<10	10	1280
	33	<10	10	1280
	67	<10	<10	640
	72	<10	10	640

[a] neutralizing antibodies;
[b] days post-immunization.

Table 3. Serological responses of cattle (experiment 1) against RABV detected by rapid fluorescent focus inhibition test (RFFIT).

Group	Animal	nAb[a] titer		
	ID	0 d p.i.[b]	30 d p.i.	60 d p.i.
ORFV$^{\Delta 024}$RABV-G	280	<10	40	160
	284	<10	80	640
	293	<10	160	640
	299	<10	80	640
ORFV$^{\Delta 121}$RABV-G	271	<10	40	640
	276	<10	320	1280
	282	<10	40	320
	287	<10	40	1280
	294	<10	320	1280

[a] neutralizing antibodies;
[b] days post-immunization.

Table 4. Serological responses of cattle (experiment 2) against RABV detected by rapid fluorescent focus inhibition test (RFFIT).

Group	Animal ID	nAb[a] titers				
		0 d p.i.[b]	30 d p.i.	60 d p.i.	390 d p.i.	420 d p.i.
ORFV$^{\Delta 024}$RABV-G	12	<10	40	1280	320	2560
	29	<10	20	80	<10	80
	33	<10	10	320	10	160
	48	<10	10	160	10	320
	57	<10	10	320	10	320
	75	<10	<10	80	20	na
	83	<10	10	640	40	320
	90	<10	<10	320	40	1280
	162	<10	<10	80	10	40
	163	<10	<10	160	40	40
	164	<10	<10	80	<10	20
	165	<10	10	80	<10	20
	167	<10	<10	160	10	40
	168	<10	<10	640	10	1280
	169	<10	<10	320	40	640
ORFV$^{\Delta 121}$RABV-G	38	<10	320	640	10	640
	42	<10	320	320	160	320
	43	<10	320	2560	40	1280
	49	<10	20	640	10	80
	56	<10	20	320	20	640
	65	<10	320	640	na[c]	na
	77	<10	80	640	20	na
	79	<10	40	640	80	320
	123	<10	80	320	40	80
	159	<10	40	1280	na	na
	160	<10	40	320	na	na
	161	<10	40	640	na	na
	170	<10	40	1280	40	1280
	171	<10	10	1280	40	640
	172	<10	40	2560	40	640

[a] neutralizing antibodies;
[b] days post-immunization;
[c] not available.

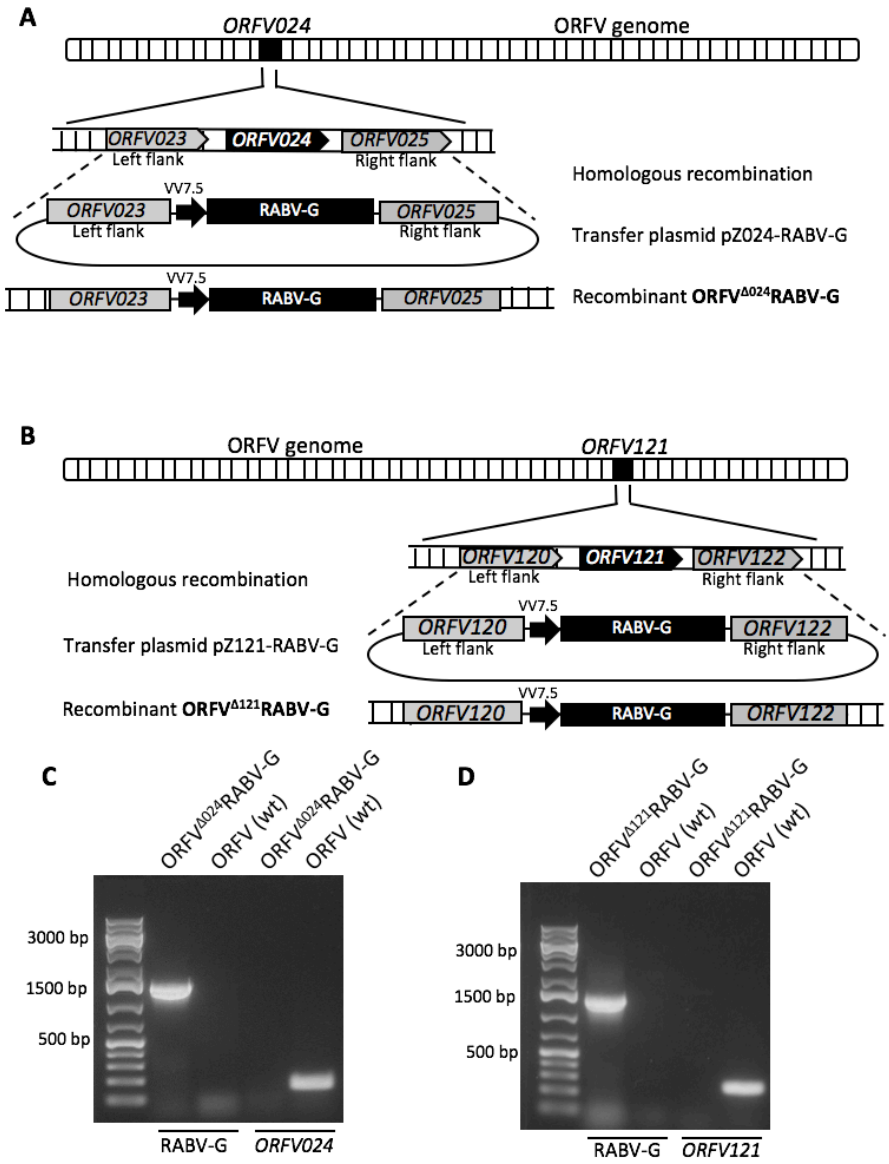

Fig. 1. Generation of recombinant ORFV-RABV-G viruses. (A) Schematic representation of the ORFV genome depicting ORFV024 insertion site and flanking regions (ORFV023 and ORFV025) used to generate the recombinant ORFV$^{\Delta 024}$RABV-G. (B) Schematic representation of the ORFV genome depicting ORFV121 insertion site and flanking regions (ORFV120 and ORFV122) used to generate the recombinant ORFV$^{\Delta 121}$RABV-G. The coding sequence of the RABV G was inserted into the ORFV024 or ORFV121 gene loci of the ORFV genome by homologous recombination between the parental ORFV IA82 and the recombination cassette pZ024-RABV-G or pZ121-RABV-G. The pZ024-RABV-G and pZ121-RABV-G transfer plasmids containing the full-length Glycoprotein gene under the control of the early/late VV7.5 poxviral promoter. (C) Agarose gel demonstrating PCR amplification of an internal region of the glycoprotein gene from the genome of the recombinant ORFV$^{\Delta 024}$RABV-G gene and absence of ORFV024 gene sequences on the recombinant virus genome. (D) Agarose gel demonstrating PCR amplification of an internal region of the glycoprotein gene from the genome of the recombinant ORFV$^{\Delta 121}$RABV-G gene and absence of ORFV121 gene sequences on the recombinant virus. Wild type ORFV DNA was used as a negative and positive control on the PCR amplifications with glycoprotein specific and ORFV024 or ORFV121 specific primers, respectively.

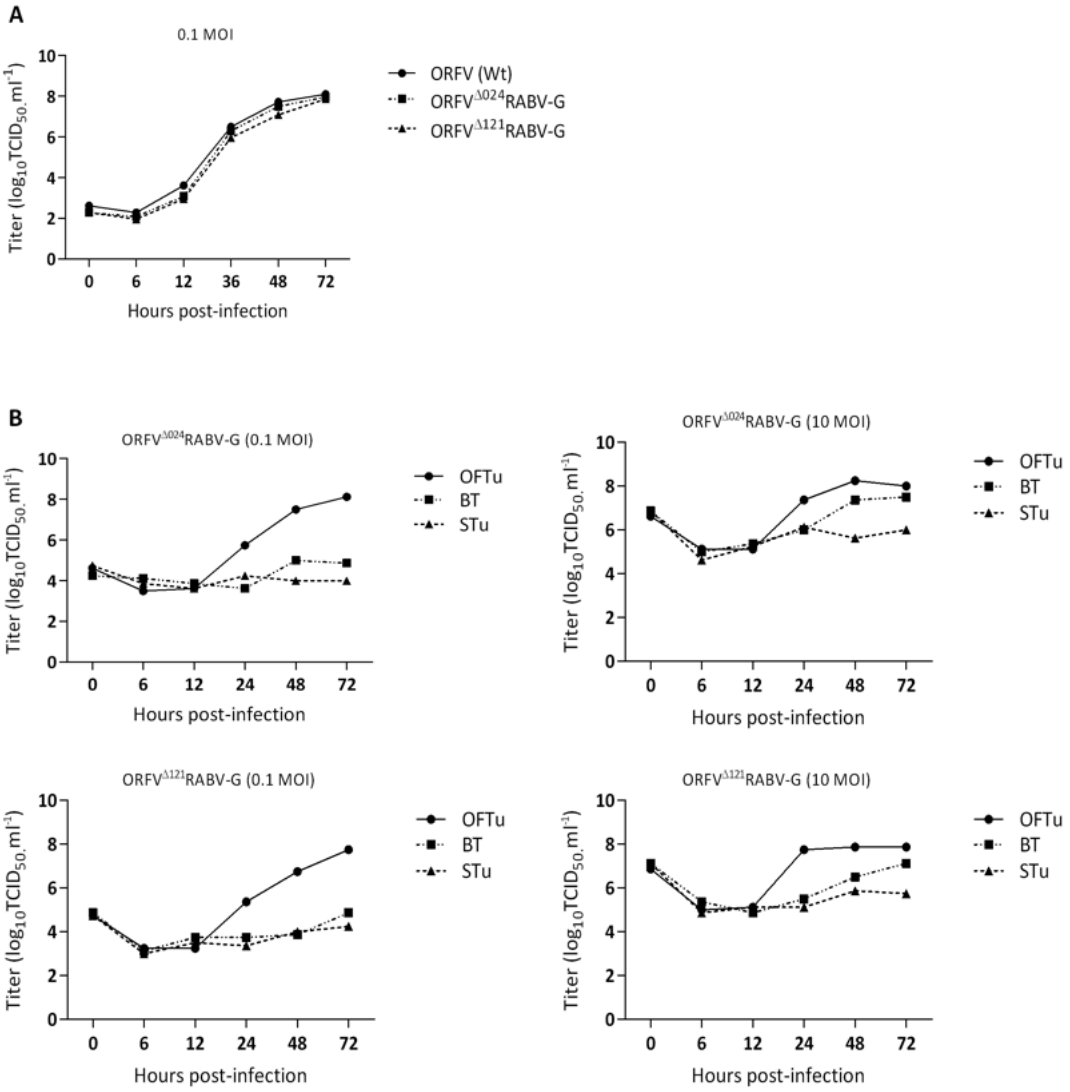

Fig. 2. Replication kinetics of the recombinant ORFV-RABV-G viruses. (A) Multi-step growth curve of the recombinants ORFV$^{\Delta 024}$RABV-G, ORFV$^{\Delta 121}$RABV-G and wild type virus in primary OFTu cells. Results were calculated based on two independent experiments. (B) Multi- and single-step growth curve of the recombinant ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G viruses in primary OFTu, BT and STu cells. Results represent the average of three independent experiments. The virus titers were determined by the Spearman and Karber's method and expressed as tissue culture infections dose 50 ($TCID_{50}$) per mL.

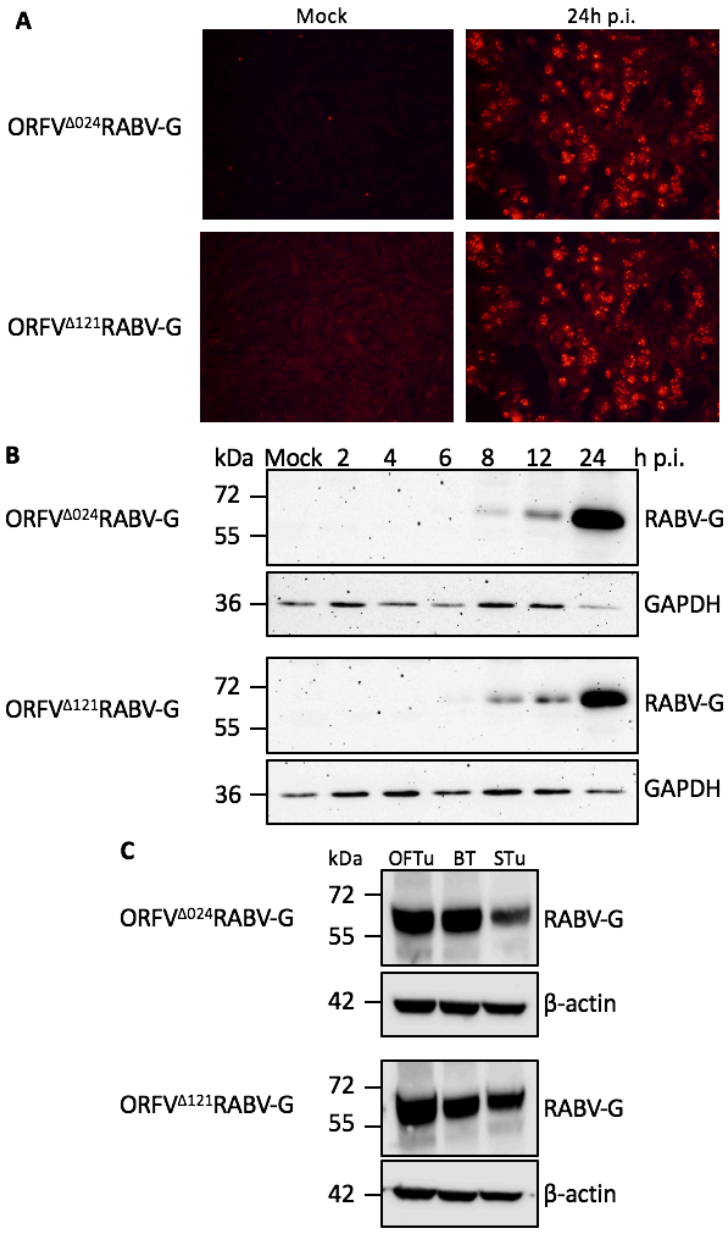

Fig. 3. Expression of RABV glycoprotein by the recombinants ORFV-RABV-G. (A) Immunofluorescence assay performed in primary OFTu cells infected (MOI = 1) with the recombinants ORFV$^{\Delta 024}$RABV-G or ORFV$^{\Delta 121}$RABV-G. Cells were fixed at 24 h post-infection (h p.i.) and incubated with mouse IgG anti-FLAG antibody followed by incubation with a goat anti-mouse IgG secondary antibody (Alexa Fluor® 594 conjugate) and visualized under a fluorescence microscope. (B) Western blot assay performed in OFTu cells inoculated with ORFV$^{\Delta 024}$RABV-G or ORFV$^{\Delta 121}$RABV-G recombinant viruses (MOI = 10) and harvested at 2, 4, 6, 8, 12 and 24 h p.i. to assess the expression kinetics of RABV-G. Mock-infected cells were used as controls. (C) Western blot assay performed in OFTu, BT and STu cells inoculated with ORFV$^{\Delta 024}$RABV-G or ORFV$^{\Delta 121}$RABV-G (MOI = 10) and harvested at 24 h p.i.. One hundred micrograms of whole cell protein extracts were resolved by SDS-PAGE in 10% acrylamide gels, transferred to nitrocellulose membranes and probed with the FLAG-tag epitope antibody or loading control antibodies against GAPDH or β-actin. A goat anti-mouse IgG-HRP conjugate secondary antibody was used and developed by using a chemiluminescent substrate.

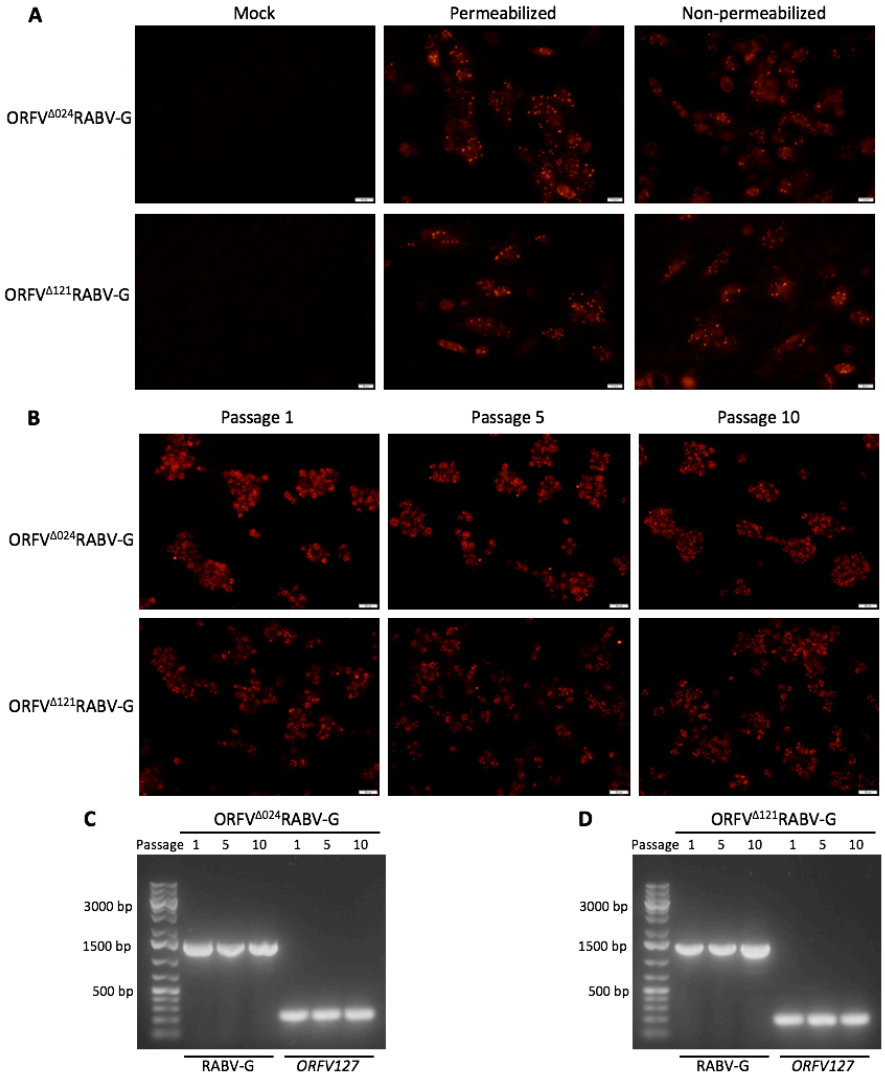

Fig. 4. Expression of RABV-G by the ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G recombinant viruses assessed by immunofluorescence assay. (A) OFTu cells were infected with the ORFV$^{\Delta 024}$RABV-G or ORFV$^{\Delta 121}$RABV-G recombinant virus (MOI = 1) and fixed with 3.7% formaldehyde at 24 hours post-infection (h p.i.). After fixation, cells were permeabilized with Triton X-100 or maintained non-permeabilized to assess expression of RABV-G on the membrane of ORFV$^{\Delta 024}$RABV-G or ORFV$^{\Delta 121}$RABV-G infected cells. Cells were incubated with a mouse IgG anti-FLAG antibody, incubated with a goat anti-mouse IgG secondary antibody (Alexa Fluor® 594 conjugate) and visualized under a fluorescence microscope. (B) Immunofluorescence assay showing expression of RABV-G by the recombinants ORFV$^{\Delta 024}$RABV-G or ORFV$^{\Delta 121}$RABV-G after serial passages in cell culture. Primary OFTu cells were infected with passages 1, 5 or 10 of ORFV$^{\Delta 024}$RABV-G or ORFV$^{\Delta 121}$RABV-G (MOI of ~1) and fixed, stained and permeabilized as described in (A). Serial passages (P1-P10) of each recombinant virus were performed in OFTu cells using low multiplicity of infection (~1) on each passage and viruses were harvested at 48 h p.i. to allow for multiple rounds of recombinant virus replication on each passage. (C-D) Agarose gel demonstrating PCR amplification of an internal region of the glycoprotein gene from the genome of passages 1, 5 and 10 of ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G recombinant viruses. Ten ng of total DNA extracted from P1, P5 and P10 infected cells were used on each PCR reaction. Primers specific for ORFV127 (IL-10 homologue; available upon request) were used as controls to confirm that similar amounts of virus DNA were used in the PCR amplifications.

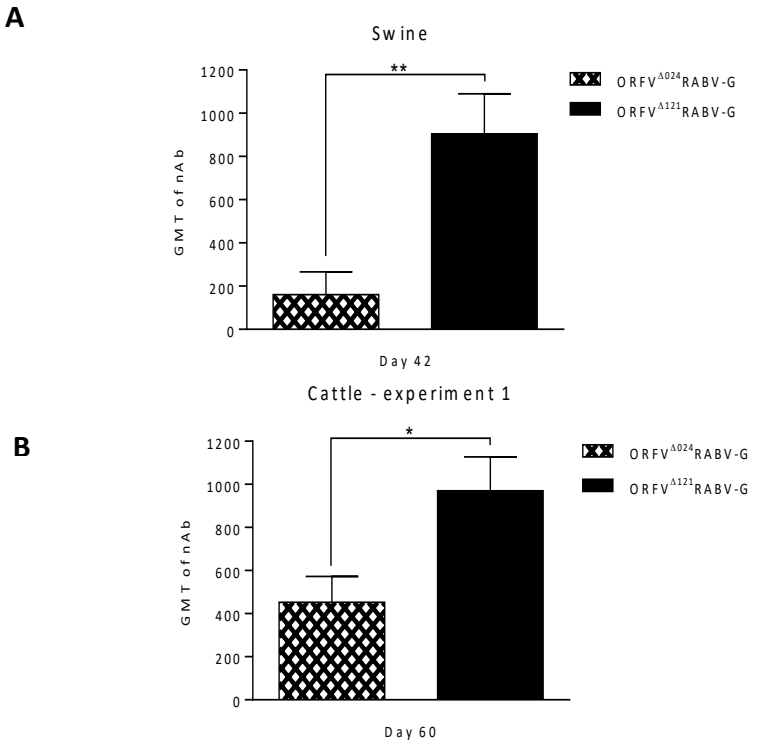

Fig. 5. Immunogenicity of recombinant ORFV-RABV-G viruses. (A) Immune responses elicited by immunization with ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G recombinant viruses in pigs at day 42 (21 days post-booster). Geometric mean titer (GMT) of individual titers obtained through rapid fluorescent focus inhibition test (RFFIT), which detects RABV neutralizing antibodies (group ORFV$^{\Delta 024}$RABV-G n = 5 and group ORFV$^{\Delta 121}$RABV-G n = 4). Statistical differences (**) were determined using the students T-test ($P < 0.01$). (B) Immune responses elicited by immunization with ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G recombinant viruses in cattle at day 60 (30 days post-booster) (experiment 1). Geometric mean titer (GMT) of individual titers obtained through rapid fluorescent focus inhibition test (RFFIT), which detects RABV neutralizing antibodies (group ORFV$^{\Delta 024}$RABV-G n = 4 and group ORFV$^{\Delta 121}$RABV-G n = 5). Error bars represent the standard error of the median (SEM). Statistical differences (*) were determined using the students T-test ($P < 0.05$).

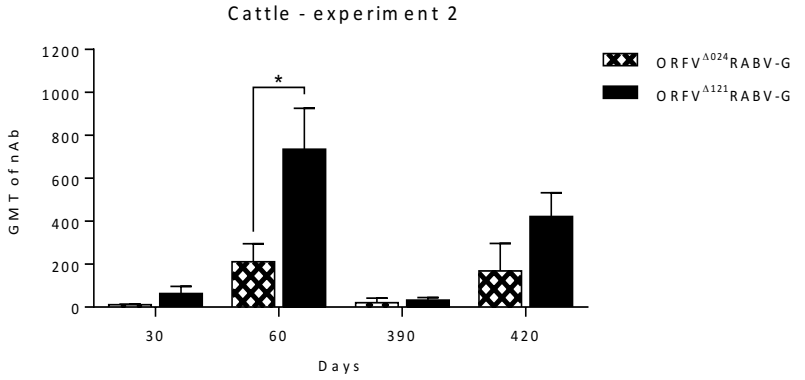

Fig. 6. Serological response following immunization with ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G recombinant viruses in cattle (experiment 2). Immune responses elicited by immunization with ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G recombinant viruses in heifers at days 30, 60, 390 and 420 post-immunization (p.i.). Geometric mean titer (GMT) of individual titers obtained through rapid fluorescent focus inhibition test (RFFIT), which detects RABV neutralizing antibodies (group ORFV$^{\Delta 024}$RABV-G n = 15 and group ORFV$^{\Delta 121}$RABV-G n = 15). Error bars represent the standard error of the median (SEM). Statistical differences (*) were determined using the students T-test ($P < 0.05$).

4. ARTIGO 2

Deletion of putative immunomodulatory genes *ORFV112*, *ORFV117* or *ORFV127* from the ORFV genome does not result in marked reduction in virulence in lambs

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Abstract

Parapoxvirus ovis (PPVO), also known as *orf* virus (ORFV), encodes several immunomodulatory proteins (IMPs) that modulate host-innate and pro-inflammatory responses to infection. Using the ORFV IA82 strain as parental virus, recombinant viruses with individual deletions in the proposed immunomodulatory genes chemokine binding protein (CBP; *ORFV112*), inhibitor of granulocyte-monocyte colony-stimulating factor and IL-2 (GIF, *ORFV117*) and interleukin 10 homologue (vIL-10; *ORFV127*) were generated and characterized *in vitro* and *in vivo*. Deletion mutant viruses for each gene were successfully generated by homologous recombination in which the respective coding regions were replaced by the gene encoding the green fluorescent protein (GFP). Genome manipulation including gene deletion/GFP insertion and nine-to-eleven rounds of purification in ovine fetal turbinate cells (OFTu) did not result in additional nucleotide changes in the genome of recombinant viruses, as ascertained by complete genome sequencing. Likewise, the replication ability of the deletion mutants in cell culture was not affected, as ascertained by plaque assays and growth kinetic experiments. To access possible impacts of the deletions in the virus biology and pathogenesis, groups of four to six-months-old lambs were inoculated with each virus in the oral commissure and the internal face of the hindlimbs. Lambs inoculated with either recombinant or with the parental ORFV IA82 developed classical lesions of contagious ecthyma, a progression of hyperemia-vesicles-pustules and proliferative scabs from days 1-3 to day 17-21 post-infection (p.i.). The course, nature and severity of the lesions in the oral commissure were similar in all inoculated groups from the onset (3 days p.i.) to the peak of clinical lesions (days 11 to 13 p.i.). Nonetheless, from the peak of clinical course onwards, the oral lesions in the lambs inoculated with the recombinant viruses regressed faster than the lesions of lambs inoculated with the parental virus. Likewise, the titers of virus shedding were similar among the groups up to day 15 p.i. but they were higher in the parental virus group from day 16 to 21 p.i. In conclusion, individual deletion of these putatively IMP genes from the ORFV genome resulted only in mild reduction in virulence *in vivo*, reflected mainly by a slight reduction in the duration of the clinical disease.

Keywords: Orf virus, recombinant virus, pathogenesis, virulence.

Introduction

Contagious ecthyma, or *Orf*, is a contagious disease of sheep, goats and, occasionally, humans [1]. The disease, also known as contagious pustular dermatitis or scabby mouth, is caused for the *Parapoxvirus ovis* (PPVO), also called *Orf* virus (ORFV) [2]. The infection usually initiates through abrasions in the lips, around the mouth or nostrils, oral mucosa and teats and lesions are typically characterized by maculo-papular and proliferative scabby lesions [1, 2]. In the absence of secondary infections, lesions are usually self-limiting and resolve within 4 to 6 weeks. Herd morbidity is generally high and the mortality is usually low [3].

ORFV is the prototype member of the genus *Parapoxvirus*, subfamily *Chordopoxvirinae,* family *Poxviridae,* along with bovine papular stomatitis virus (BPSV), pseudocowpox virus (PCPV) and parapoxvirus of New Zealand red deer [4]. The ORFV genome is a double stranded, linear DNA molecule of approximately 138 kbp and contains 131 putative genes [5]. Notably, ORFV encodes several immunomodulatory proteins (IMPs) that modulate host-innate and pro-inflammatory responses to infection, whose genes are located near the genome termini and, in general, lack similarity with other poxvirus or cellular proteins [5–7]. Among the IMPs described to date, a chemokine binding protein (CBP; *ORFV112*) [8], the soluble protein inhibitor of ovine granulocyte-monocyte colony-stimulating factor and interleukin-2 (GIF, *ORFV117*) [9] and a gene encoding an orthologue of the ovine interleukin-10 (vIL-10; *ORFV127*) [10] have been characterized based on ORFV strain NZ2, a New Zealand extensively studied ORFV strain [11]. The impact of deletion of *ORFV112* in the virulence in lambs was investigated [12] and the authors concluded that deletion of the CPB gene resulted in the attenuation of the ORFV. However, although the function of *ORFV117* has been described [9], the result in *in vivo* pathogenesis has not been investigated. The *ORFV127* gene is other that has already been deleted from the ORFV and whose deletion has been shown to have a partial effect on virulence in lambs [13].

Based on its restricted host range, absence of virus-neutralizing antibodies, large and editable genome, besides the immunomodulatory properties, ORFV has been proposed as a vaccine vector [14]. In this sense, several studies have demonstrated the efficiency of ORFV as a vaccine delivery platform [15–22]

Our group has investigated the efficacy of ORFV strain IA82 as vaccine vector in swine [18]; and swine and cattle [20], using de *ORFV024* or *ORFV121* gene loci, two inhibitors of the nuclear factor-kappa-Beta (NF-κB) signaling pathway [23, 24], for heterologous gene insertion. As a first step for recombinant virus generation, we have deleted genes supposedly

involved in ORFV immunomodulatory properties and investigated the impact/interference of these mutations in virus viability and biology/pathogenesis in lambs.

Herein we describe the generation of three ORFV mutants: ORFVΔ112, ORFVΔ117 and ORFVΔ127, from which the genes encoding CBP, GIF and vIL-10, respectively, have been deleted. The mutant viruses were characterized *in vitro* and their ability to replicate and cause disease was evaluated *in vivo*, upon experimental infection of lambs.

Materials and methods

Based on the parental ORFV IA82 strain, three ORFV deleted in immunomodulatory genes (ORFVΔ112, ORFVΔ117 and ORFVΔ127) were generated and characterized *in vitro*. Subsequently, the biology and pathogenesis of these deletion mutants were investigated in lambs. For this, twenty lambs were allocated in five groups and inoculated in the labial commissures with each of the mutant or with the parental virus. Inoculated lambs were submitted to clinical, pathological and virological monitoring during 21 days post-infection (d p.i.).

Cells and viruses

Primary ovine fetal turbinate (OFTu) were cultured in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) at 37ºC with 5% CO_2, and 2 mM L-glutamine (2 mM), penicillin (100 U/mL), streptomycin (100 µg/mL) and gentamicin (50 µg/mL). ORFV strain IA82 [5] was used as the parental virus to construct the deletion mutants of *ORFV112*, *ORFV117* and *ORFV127* genes.

Plasmid construction

Plasmid pZ-GFP was used for construction of recombination plasmid, in which the green fluorescent protein (GFP) coding sequence is under the control of the early/late VV7.5 poxvirus promoter [25]. To insert the GFP gene into the *ORFV112, ORFV127* or *ORFV127* genome loci, three recombination plasmids were constructed. *ORFV112* left and right flanking regions were PCR amplified and cloned into the vector pZ-GFP resulting in the recombination vector pZ112LR-GFP. *ORFV117* left and right flanking regions were PCR amplified and cloned into the vector pZ-GFP resulting in the recombination vector pZ117LR-GFP. *ORFV127* left and right flanking regions were PCR amplified and cloned into the vector pZ-GFP resulting in the recombination vector pZ127LR-GFP. All flanking regions were PCR amplified from the

ORFV strain IA82 genome. Cloning of *ORFV112, ORFV117* and *ORFV127* left and right flanks was confirmed by restriction enzyme analysis. The primers used in this study for PCR amplification are shown in table 1.

Deletions in immunomodulatory genes

The GFP coding sequence was inserted into the *ORFV112, ORFV117* or *ORFV127* gene loci of the ORFV genome by homologous recombination between the parental ORFV IA82 and the recombination cassette pZ112LR-GFP, pZ117LR-GFP or pZ127LR-GFP. OFTu cells cultured in 6-well plates were infected with ORFV IA82 (MOI = 1) and 3 h later were transfected with 2 µg of pZ112-GFP, pZ117-GFP or pZ127-GFP DNA using Lipofectamine 3000 (Life Technologies) according to the manufacturer's instructions. At 72 h post-infection/transfection cell cultures were harvested, subjected to three freeze-and-thaw cycles and cell lysates were used for selection of recombinant viruses by plaque assay. OFTu cells cultured in 6-well plates were infected with 10-fold serial dilutions (10^{-1} to 10^{-3}) of cell lysates from GFP positive viral plates (obtained during the first plaque assay selection), and overlaid with culture medium containing 0.5% agarose (SeaKem GTC agarose, Lonza Inc., Alpharetta, GA). Fluorescent plaques were subjected to additional rounds of plaque assay for purification of recombinant viruses. The presence of GFP and absence of *ORFV112*, *ORFV117* and *ORFV127* sequences in the purified recombinant viruses was confirmed by PCR using primers that amplify an internal region of each of the genes. The sequences of the primers are shown in table 1. PCR amplicons were analyzed by electrophoresis in 1% agarose gels. The deletions and GFP insertions were confirmed by whole genome sequencing using the Nextera XT DNA library preparation kit (Illumina, San Diego, CA) followed by sequencing on the Illumina Mi-Seq sequencing platform (Illumina, San Diego, CA).

Growth curves

Replication properties of ORFVΔ112, ORFVΔ117 and ORFVΔ127 viruses were assessed *in vitro*. OFTu cells cultured in 6-well plates were inoculated with ORFVΔ112, ORFVΔ117 and ORFVΔ127 (MOI = 0.1 [multi-step growth curve], and MOI = 10 [single-step growth curve]) and harvested at various time points post-infection (6, 12, 24, 48, and 72 hours post-infection [h p.i.]). Virus titers were determined on each time point using the Spearman and Karber's method and expressed as tissue culture infectious dose 50 for mL ($TCID_{50}$/mL).

Animals and virus inoculation

Twenty 4 to 6-months-old ORFV naïve lambs were allocated in five groups of four lambs each and inoculated with each of three ORFV-mutant (ORFVΔ112, ORFVΔ117 and ORFVΔ127) or parental virus (ORFV IA82). The fifth control group received minimal essential medium (MEM). The lambs were inoculated in the labial commissures, with 200µL of supernatant of inoculated cells containing 10^7 $TCID_{50}$/mL of infectivity. Inoculation was performed with the help of cotton swabs after scarification with a hypodermic needle. Control lambs were inoculated with MEM, also after scarification. For colletion of biopsy, 50µL of supernatant of inoculated cells containing 10^7 $TCID_{50}$/mL of infectivity were inoculated in four different sites in the hindlimb internal face. The experimental design of animal study is showed in the figure 1.

Clinical and virological monitoring

Following virus inoculation, the animals were monitored daily for 21 days p.i.. Clinical examination, photographs were taken and clinical scoring were performed every two days by two examiners who were not aware of the experimental groups. For clinical monitoring, three indicators were evaluated: hyperemia, vesicles and/or pustules, and scabs. Each indicator was scored from 0 – (absence) at 5 – (severe or high number). Taking these scores, a clinical score was calculated for each animal and a mean clinical score was established for each group. The mean clinical score and the standard error of the mean were calculated (mean ± standard error of mean [SEM]). Swabs collected from the lesions every two days up to 21 d p.i. were submitted to titration for limiting dilution in OFTu cells and virus titers were determined using the Spearman and Karber's method and expressed as $TCID_{50}$/mL. Skin biopsies of on the inner side of the leg inoculation sites were performed in all animals at days 3, 5, 7 and 11 p.i.. Punched 5mm tissue fragments were coleted and fixed in 10% buffered formalin, embedded in paraffin, stained with hematoxilin and eosin (H&E) and submitted to microscopic examination according to routine protocols.

Statistical analysis

Statistical analysis was performed using the Prism software (GraphPad; 6th version) software. Students T-test was performed on all groups. Statistical differences between groups were considered significant at $P < 0.05$.

Results

Virus generation and *in vitro* characterization

The GFP gene was inserted into the *ORFV112, ORFV117* or *ORFV127* gene loci of the ORFV genome by homologous recombination. *ORFV112, ORFV117* or *ORFV127* were deleted from the ORFV genome and replaced by a DNA fragment encoding the GFP under the control of the early/late VV7.5 poxvirus promoter (Fig. 2A). GFP sequences were detected in the ORFVΔ112, ORFVΔ117 and ORFVΔ127 viruses but not in the original ORFV genome (Fig. 2B, C and D). Deleted *ORFV112, ORFV117* or *ORFV127* gene sequences were not detected in the purified viruses ORFVΔ112, ORFVΔ117 and ORFVΔ127, respectively, but were detected in parenteral virus (Fig. 2B, C and D). Sequencing of the complete genomes of ORFVΔ112, ORFVΔ117 and ORFVΔ127 viruses confirmed the integrity ORFV sequences, with no nucleotide changes other than the deletions of *ORFV112, ORFV117* or *ORFV127* coding sequences being detected across the entire genome of the viruses (data not shown). Nine, eleven or ten additional rounds of plaque purification were necessary for purification of ORFVΔ112, ORFVΔ117 and ORFVΔ127, respectively. Plaque morphology of ORFVΔ112, ORFVΔ117, ORFVΔ127, parental virus (ORFV IA82) showed no apparent differences (Fig. 3). The replication kinetics of ORFVΔ112, ORFVΔ117 and ORFVΔ127 recombinant viruses was assessed *in vitro*. No differences in replication kinetics and viral yields were observed comparing the recombinant viruses with the parental virus (ORFV IA82) in primary ovine fetal turbinate (OFTu) cells. Replication kinetics were assessed in MOI = 0.1 [multi-step growth curve] (Fig. 4A), and MOI = 10 [single-step growth curve]) (Fig. 4B).

Pathogenesis in lambs

All inoculated lambs developed lesions progressing through the stages of hyperemia, macules/papules, vesicles, pustules and proliferative scabs, characteristics of contagious ecthyma (Fig. 5). The signs started around 1-3 d p.i. and resolved between 17–21 d p.i.. Control animals (inoculated with MEM) developed a mild local hyperemia with duration of 1 at 3 days. The duration of lesions differed slighly among the groups. In lambs inoculated with mutant viruses ORFVΔ112, ORFVΔ117 and ORFVΔ127, the course of lesions/disease was shorter (17 d p.i.) than lesions observed in lambs inoculated with parental virus (19-21 d p.i.). Lambs inoculated with ORFV IA82 presented scabs firmly attached up to day 19 p.i. (Fig. 5). The clinical score revealed differences between lambs inoculated with mutant viruses and ORFV

IA82 from 15 d p.i (P<0.05) (Fig. 6). The mean clinical scores at day 15 p.i. were 5.75±0.25, 6.75±0.63 and 8±0.4 (mean ± standard error of the mean [SEM]) (ORFVΔ112, ORFVΔ117 and ORFVΔ127, respectively), while the mean clinical score lambs inoculated with parental virus was 9±0.4. This difference in clinical scores between mutant groups and ORFV IA82 was maintained until the end of the experiment (21 d p.i.) (P<0.05) (Fig. 6).

To investigate the changes of the ORFV infection in the skin of the infected lambs, biopsies were performed at days 3, 5, 7 and 11 p.i.. Skin infiltration with inflammatory cells, pustules, and the accumulation of scabby tissue, were observed in variable degree in samples from lambs inoculated with both deleted and parental virus, no significant differences were observed in the time to appearance and magnitude of changes between. Characteristics histological changes associated with ORFV infection, including epidermal hyperplasia, hydrophobic degeneration of keratinocytes was observed (Fig. 7).

Viral shedding through the lesions was detected from all inoculated lambs for at least 17 d p.i., only in the lambs inoculated with the parental virus shedding was detected in all animals for 21 d p.i. or more (end of experiment). The mean duration of viral shedding was different between the lambs inoculated with mutant virus and ORFV IA82 (ORFVΔ112: 18 days ±0.6; ORFVΔ117: 19 days ±0.8; ORFVΔ127: 18 days ±0.6; ORFV IA82: ≥21 days) (P<0.05) (Table 2). Interestingly, the mean viral titers shed by all groups were similar until 15 d p.i. (Fig. 8). From day 17 p.i., the animals of group ORFV IA82 shed higher titers compared to the mutant viruses groups and the mean of titers remained higher until the end of experiment (21 d p.i.) (P<0.05).

Discussion

The ORFV genome contains several genes encoding products that interact with the host immune response [26, 27]. Some of these genes modulate the host response to viral infection and favor virus replication either by inhibiting or boosting the inflammatory response and, as result, perpetuating viral replication and progeny virus production [27–29]. The immunomodulatory proteins (IMPs) already described included interferon (IFN)-resistance gene (VIR; *ORFV020*) [30], an inhibitor of apoptosis (*ORFV125*) [31], a homologue of vascular endothelial growth factor (VEGF; *ORFV132*) [32] and, at least, four inhibitors of the nuclear factor-kappa (NF-κB) signaling pathway (*ORFV002*, *ORFV024*, *ORFV073* and *ORFV121*) [23, 24, 33]. In addition, three other genes coding for IMPs are studied here, e.g. *ORFV112* (a chemokine binding protein - CBP); *ORFV117* (inhibitor of granulocyte-monocyte colony-

stimulating factor (GM-CSF) and IL-2 - GIF); and *ORFV127* (interleukin 10 homologue - vIL-10) (*ORFV112* [8]; *ORFV117* [9]; *ORFV127* [31]; respectively). In this study, we deleted individually these three genes from the ORFV IA82 strain genome and studied the impact of each deletion in the ORFV biology *in vitro* and *in vivo*.

The three deletion mutant viruses were successfully generated through homologous recombination in OFTu cells. The recombinant viruses ORFVΔ112, ORFVΔ117 and ORFVΔ127 were submitted to nine, eleven or ten rounds of plaque purification, respectively. Complete genome sequencing at the end of rounds of purification revealed no additional changes in the viral genome, other than the respective gene deletions. Although mutations in the ORFV genome are commonly observed in cell cultured viruses [1], such changes were not observed upon genome sequencing of these viruses after 9 to 11 passages. *In vitro* characterization of recombinants ORFVΔ112, ORFVΔ117 and ORFVΔ127 revealed no evident changes in plaque morphology and the replication kinetics comparing to the parental virus. Thus, respective gene deletions – and GFP insertion - had no apparent impact on their ability to replicate in natural host cells *in vitro* (Fig. 3 and 4).

In order to investigate potential effects of the deletions of genes *ORFV112*, *ORFV117* and *ORFV127* on ORFV biology and pathogenesis *in vivo*, groups of lambs were inoculated with each recombinant or with the parental virus. Following virus inoculation, inoculated lambs underwent a progressive course of lesions typical of ORFV infection, from hyperemia at days 1-3 p.i. to complete resolution of lesions by days 17–21 p.i.. Interestingly, the nature, progression and severity of lesions induced by the three recombinant viruses were undistinguishable from those associated with the parental virus, with peaks in severity observed around day 11 p.i. (Fig. 5). Thus, individual gene deletions had no apparent effect on the ability of the viruses to produce mucocutaneous lesions in inoculated lambs. Nevertheless, a discrete difference was evident in the duration of the disease/lesions. Lambs inoculated with parental ORFV presented scabs firmly attached up to days 19-21 p.i. while lambs inoculated with the recombinant viruses ORFVΔ112, ORFVΔ117 and ORFVΔ127, the course of disease was shorter and the lesions resolver earlier (15-17 d p.i.). This difference became more evident when comparing the clinical scores from day 15 p.i. up to the end of the experiment (Fig. 6) ($P<0.05$). This difference was more easily observed at day 19 p.i. (Fig. 6).

Histopathological changes associated with replication of mutants and parental ORFV were investigated through biopsies collected days 3, 5, 7 and 11. Skin infiltration with inflammatory cells, pustules, and the accumulation of scabby tissue, were observed in variable

degrees in samples collected from lambs inoculated with either recombinant or parental ORFV, with no apparent differences in timing, qualitative and/or quantitative histological changes. Characteristics histological changes associated with ORFV infection, including epidermal hyperplasia, hydrophobic degeneration of keratinocytes were observed throughout the lesions (Fig. 7).

The *ORFV112* gene encodes the chemokine-binding protein (CBP), a product functionally similar to the CBP-II proteins in its ability to bind and inhibit many CC-chemokines with high affinity [8]. Chemokines are a large family of secreted chemotactic proteins that activate and regulate inflammation induced leukocyte recruitment to sites of infection [8, 27, 34]. ORFV CBP also binds with high affinity to lymphotactin, a member of the C-chemokine family. This finding also provides an unexpected familial link between a family of poxvirus CC-CBP and a previously described ORF virus (ORFV) granulocyte–macrophage colony-stimulating factor (GM-CSF) IL-2 inhibitory factor (GIF) protein. CBP shares sequence similarities to the ORFV protein GIF [35]. The effect of *ORFV112* deletion from the ORFV genome in experimentally infected sheep has been recently demonstrated [12]. The authors concluded that CBP gene deletion from the ORFV strain NZ7 severely attenuated the virus. In contrast, our study showed ORFVΔ112 deletion from the ORFV IA82 genome had no evident impact on virus virulence in lambs (Fig. 4). Slight differences in clinical score were only observed from day 11 p.i. ($P<0.05$), when the mean clinical scores of the ORFVΔ112 group were lower than the parental virus group (Fig. 6). The same occurred with viral shedding, with similar titers observed up to day 11 p.i. and differences observed from day 13 p.i. to the end of the experiment (21 d p.i.) ($P<0.05$) (Fig. 8).

The *ORFV117* gene encodes a protein inhibitor of ovine granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-2 (IL-2), the GM-CSF- and IL-2-inhibitory factor (GIF) [9]. GIF is a soluble protein that forms homodimers and tetramers in solution and binds to ovine GM-CSF and IL-2 [27]. As GM-CSF and IL-2 are important in host antiviral immunity, GIF expression by ORFV is a means of handling the host immune response during infection. GIF binds to and inhibits the biological activity of the GM-CSF and IL-2 [9]. Although the function of the *ORFV117* gene product (GIF) has been described, the effects of its deletion from the ORFV genome have not been investigated. Here, we successfully generated an ORFV deleted in the *ORFV117* gene (Fig. 2A and C) and described the virological and clinico-pathological outcome of experimental infection of lambs with the deleted virus. Notably, no major changes were observed *in vitro* when comparing plaque morphology and replication kinetics with the parental ORFV (Fig. 3 and 4, respectively). In addition, GIF

deletion had no apparent influence in ORFV biology *in vivo,* as the nature, course and severity of lesions were similar to those associated with the parental virus (Fig. 5) ($P<0.05$). The difference between the ORFVΔ117 and parental ORFV group was restricted to a lower clinical score from day 13 p.i. to the end of the experiment (21 d p.i.) (Fig. 6) ($P<0.05$). In addition, viral excretion from animals inoculated with ORFVΔ117 at day 13 p.i. was lower (Fig. 8).

The *ORFV127* gene encoding a homologous to interleukin-10 (IL-10) has been described in the genome of orf virus (OV) strain NZ2 [10]. The amino acid sequence of viral IL-10 of ORFV has 80% of identity with ovine IL-10 and the C-terminal region is identical to ovine IL-10, suggesting that this gene has been captured by ORFV from its host. The IL-10 is a multifunctional cytokine presenting inhibitory effects on nonspecific immunity and Th1 effector function [10]. The effects of *ORFV127* deletion from the ORFV genome has been demonstrated in sheep experimentally infected (Fleming et al., 2007). The animals inoculated with the mutant virus lacking vIL-10 gene developed milder lesions than those developed by animals inoculated with the parental ORFV. Thus, the authors concluded that ORFV IL-10 is a virulence factor but have only a partial effect on virulence. In our study, after generation and *in vitro* characterization of ORFVΔ127 (Fig. 2A and D, 3 and 4), the infection of lambs with the ORFVΔ127 (vIL-10 gene lacking) resulted in similar disease in lambs compared to disease caused by the parental ORFV. The only noticeably difference between ORFVΔ127 and parental ORFV inoculated groups was in clinical score at day 15 p.i. ($P<0.05$) (Fig. 5). The results of infection with ORFVΔ127 in lambs corroborate with results observed previously with other *ORFV127* deleted strain (ORFV NZ2) (Fleming et al., 2007), in which an only partial influence for vil-10 virulence in sheep was observed.

In summary, the present study reports the successful generation and characterization *in vitro* of three ORFV mutants: ORFVΔ112, ORFVΔ117 and ORFVΔ127, from which the genes encoding CBP, GIF and vIL-10 were deleted. Individual gene deletion apparently did not affect the replication ability of recombinants *in vitro*. Experimental infection of lambs with the recombinant viruses revealed that individual deletions resulted in mild and partial reduction in virulence, reflected mainly by a shortened clinical course after a peak in disease severity.

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Conflict of interst

The authors declare that they have no conflict of interest.

Ethical approval

All experimental procedures were performed according to recommendations by the Brazilian College of Animal Ethics and Experimentation and were approved by an Institutional Ethics Committee (CEUA/UFSM protocol number 035/2014).

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Table 1. Primer sequences used in this study

description	sequence 5'-3'	product (bp)
112LF-Fw(SpeI)	cgaatcactagtcgtggtatgcatgaaggagatgc	851
112LF-Rv(HindIII)	agatcaagcttcttggttgttgcaagattctag	
112RF-Fw(NotI)	cgaatcgcggccgccctcagccctggccattgagctg	855
112RF-Rv(BglII)	cgaatcagatctgagtcagacattccttgaccag	
117LF-Fw(SpeI)	ctgcagctatactagtcatccctgctcctcccatgactc	884
117LF-Rv(HindIII)	taaggcctctaagcttctgaggcacgccatctttcctaga	
117RF-Fw(NotI)	catatcatgcggccgcatctccaggaagtgcagcatgcaga	804
117RF-Rv(BglII)	cgtcgcacgcagatctcgaaggagagggtgatttacgtgc	
127RF-Fw2(KpnI)	cgaatcaggcctcgctgcactaccactgcgagtc	925
127LF-Rv(HindIII)	agatcaagctttccaattgtaagtacactaact	
127RF-Fw(BamHI)	agatcggatccctacatagaatcatacatgactac	856
127RF-Rv(NheI)	cgaatcgctagcgcagcatgcgcaccagctctgc	
i112LF-Fw	cataggccttggattcaacc	335
i112LF-Rv	cgatcagcgagaagtcaatg	
i117LF-Fw	caacatgcgccaagtagaga	169
i117LF-Rv	ctcgctagctccagaacctc	
i127RF-Fw	aaggctacctcgggtgtcag	203
i127RF-Rv	gacttgctccacggccttac	

Table 2. Viral shedding through lesions in lambs inoculated with orf virus at the oral commissure

Group	ID[a]	Days post-infection		
		1-17[b]	19	21
ORFVΔ112	1	+[c]		
	2	+	+	
	3	+	+	
	4	+		
ORFVΔ117	5	+	+	+
	6	+	+	
	7	+		
	8	+	+	
ORFVΔ127	9	+	+	
	10	+		
	11	+		
	12	+	+	
Parental ORFV	13	+	+	+
	14	+	+	+
	15	+	+	+
	16	+	+	+

a = animal identification;
b = from day 1 to day 17 post-infection all the samples were positive;
c = viral isolation in cells culture of ovine fetal turbinate (OFTu).

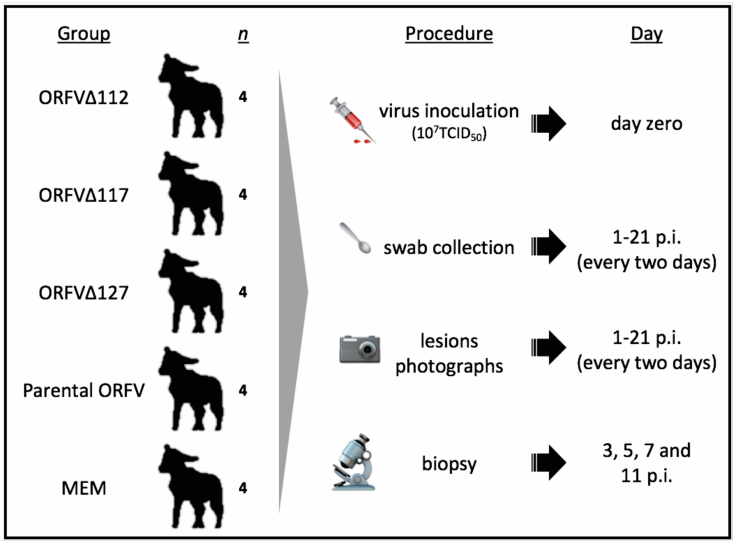

Fig. 1. Experimental design of animal study. Twenty lambs were allocated in five groups of four lambs each and inoculated with each of three ORFV-mutant, with the parental virus (ORFV IA82) or with minimal essential medium (MEM). The lambs were inoculated in the oral commissure for clinical monitoring and swab collection. For biopsies, lambs were inoculated in the internal face of the hindlimb. Virus inoculation was performed after scarification with a hypodermic needle. $TCID_{50}$ = tissue culture infectious dose 50; p.i. = post-infection.

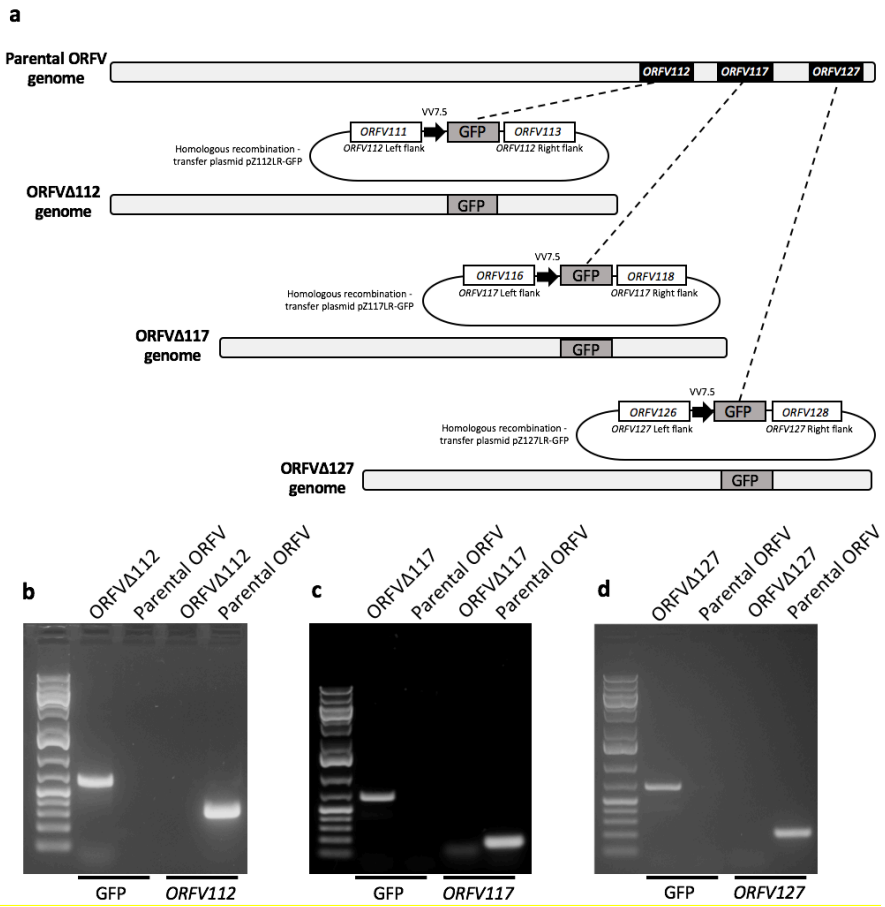

Fig. 2. Construction of deletion mutant viruses. a) Schematic representation of genomes and homologous recombination between plasmid DNA and genome of parental virus (ORFV IA82). b) Agarose gel demonstrating PCR amplification of an internal region of the GFP from the genome of the recombinant ORFVΔ112 virus and absence of ORFV112 gene sequences on the recombinant virus genome. These same defaults of agarose gel were follow for to show the others two recombinants viruses: ORFVΔ117 (c) and ORFVΔ127 (d).

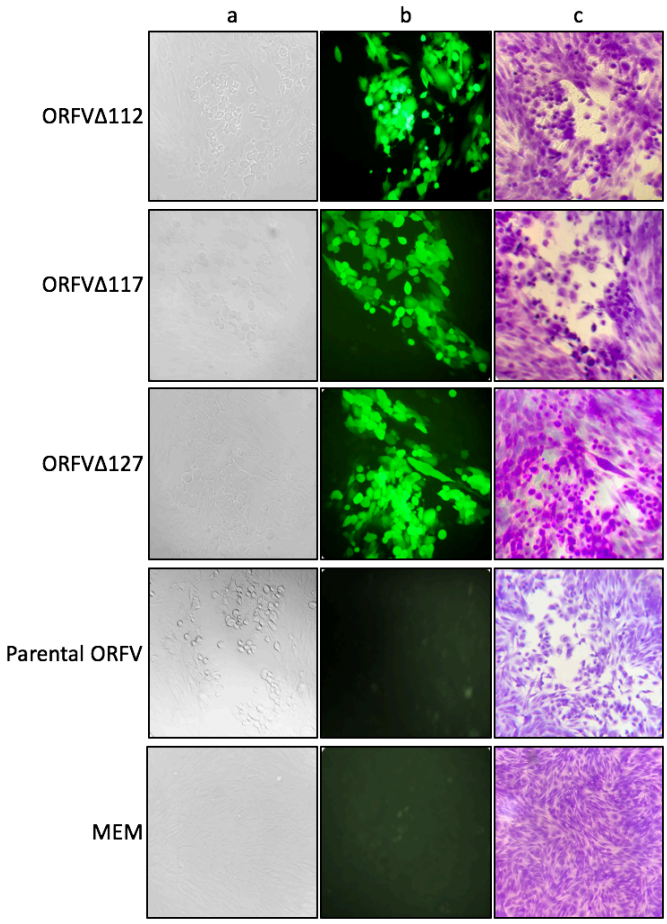

Fig. 3. Plaque morphology of ORFVΔ112, ORFVΔ117, ORFVΔ127, parental virus (ORFV IA82) or cell control (minimum essential medium [MEM]). Plaque assays were performed in OFTu cell infected with a multiplicity of infection = 0.1. Cells monolayers were overlaid with 0.5% agarose and visualized 72 h post-infection under light microscopy (a), under UV light (b), and under light microscopy after stained with crystal violet (c).

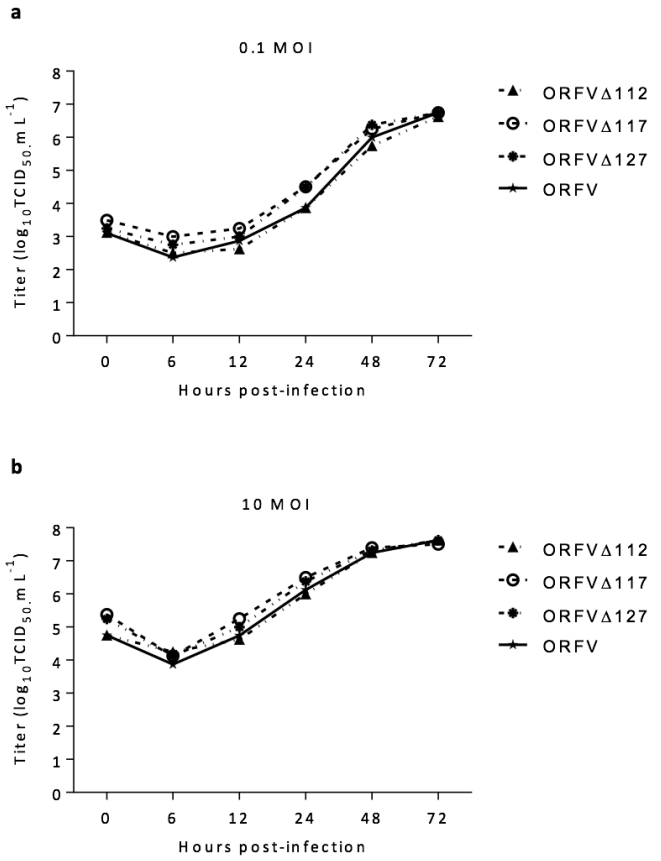

Fig. 4. Replication kinetics of the deletion mutant viruses. (a) Multi-step growth curve (multiplicity of infection [MOI]=0.1) of the ORFVΔ112, ORFVΔ117, ORFVΔ127 and parental virus (ORFV IA82) virus in primary OFTu cells. (b) Single-step growth curve (MOI=10). The virus titers were determined by the Spearman and Karber's method and expressed as tissue culture infections dose 50 ($TCID_{50}$) for ml. Results were calculated based on two independent experiments.

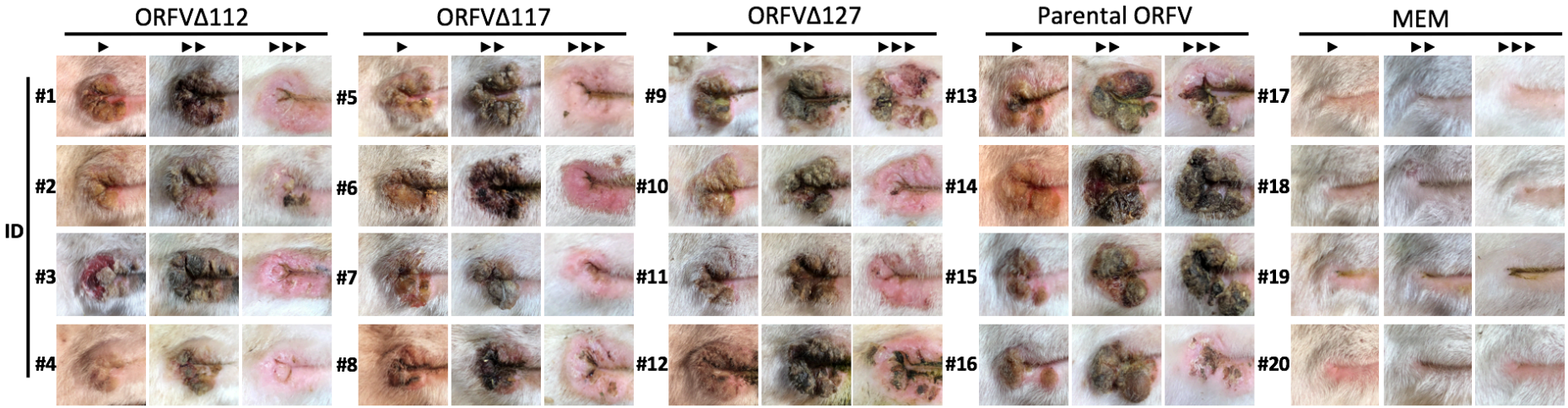

Fig. 5. Clinical course of orf virus in lambs inoculated with ORFVΔ112, ORFVΔ117 ORFVΔ127 or parental (ORFV IA82) at the oral commissure. Virulence in the natural host is maintained, but the length of clinical course is slightly shorter in the animals infected with the mutant viruses than in the parental ORFV. MEM= minimum essential medium; ID = animal identification; ►= 7 days post-infection (d p.i.); ►►= 11 d p.i.; ►►►= 19 d p.i..

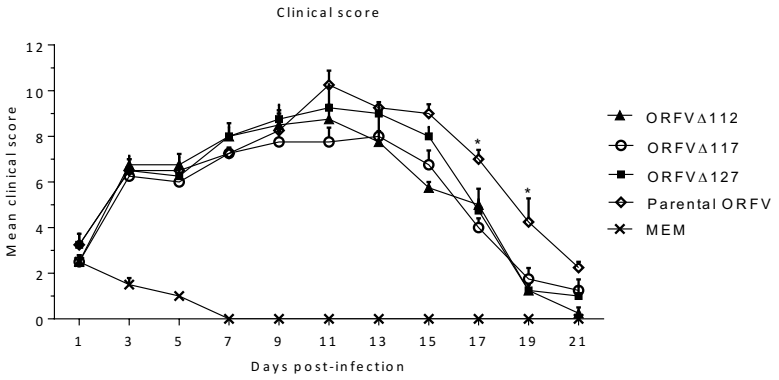

Fig. 6. Clinical course of orf virus in experimentally infected lambs. Mean of clinical scores of lambs inoculated with ORFVΔ112, ORFVΔ117 ORFVΔ127, parental virus (ORFV IA82) or MEM (no virus control). Clinical scores are shown for each group every two days (from 1 at 21 days post-infection). Each time point is the average of group (n=4). The bars represent the standard error of the mean. Students T-test was performed and statistical differences between mean of clinical scores of parental ORFV IA82 group and recombinant virus groups were considered significant at *=P <0.05.

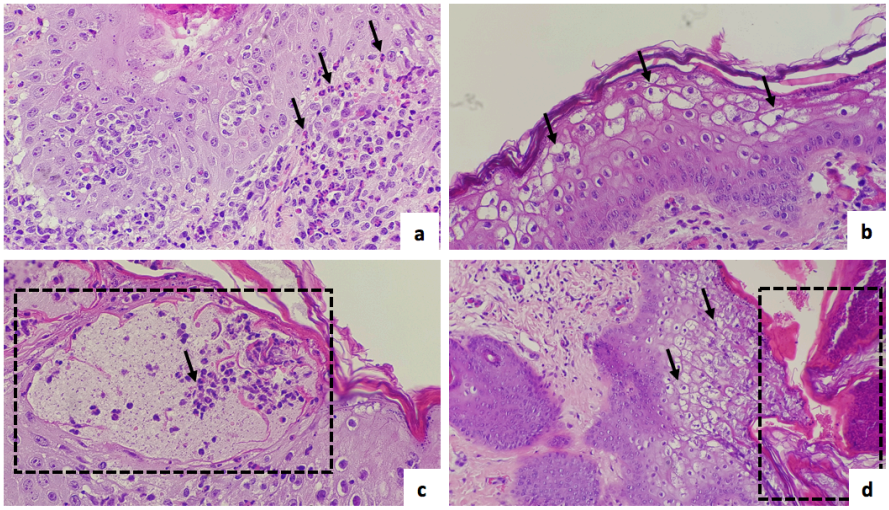

Fig. 7. Skin biopsies of lambs following infection with ORFV. (A) Lamb #3 (ORFVΔ112 group) at day 3 post-infection (p.i.) with epidermal hyperplasia and inflammatory cell infiltration arrows stand out. (B) Lamb #6 (ORFVΔ117 group) at day 5 p.i. with severe hydropic and ballooning degeneration highlighted by arrows. (C) Lamb #11 (ORFVΔ127 group) day 5 p.i. with pustule formation (dotted rectangle), neutrophilic exocytosis and inflammatory cell infiltration arrows stand out. (D) Lamb #16 (parental ORFV IA82) at day 5 p.i. with severe acanthosis with an extensive subcorneal pustule and severe ballooning degeneration of keratinocytes (dotted rectangle). Routine standard technical coloring haematoxylin and eosin (H&E) (400x).

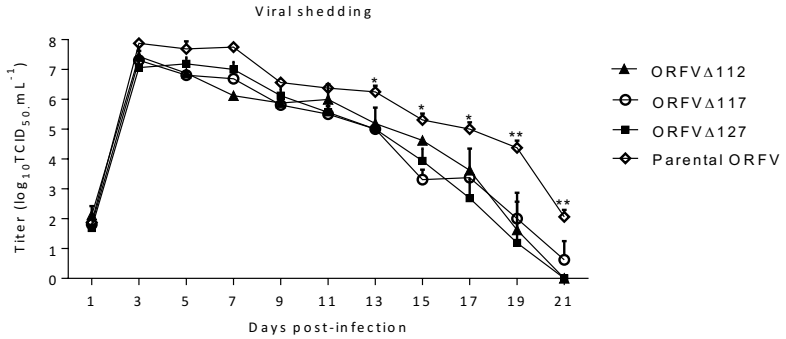

Fig. 8. Viral shedding from lesions of lambs inoculated with ORFVΔ112, ORFVΔ117 ORFVΔ127 or parental virus (ORFV IA82). Virus titers were determined in swabs collected from the inoculation sites every two days following virus inoculation. The dots in each time point represent the average of viral titers of group (n=4). The bars represent the standard error of the mean. Students T-test was performed and statistical differences between mean of titers viral shedding of parental ORFV IA82 group and recombinant ORFV groups were considered significant at *=P <0.05 or **=P <0.01.

5. ARTIGO 3

Serological response to rabies virus induced by commercial vaccines in cattle

Resposta sorológica ao vírus da raiva induzida por vacinas comerciais em bovinos

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ABSTRACT

The antibody response to rabies virus (RABV) induced by commercial vaccines in heifers was investigated. For this, 84 heifers were vaccinated twice (30 days interval) with each of four vaccines (G1 = 14 animals; G2 = 24; G3 = 22 and G4 = 24) and received a booster vaccination 360 days later. Serum samples collected at different intervals after vaccination and 30 days after booster were submitted to a virus neutralizing (VN) assay for RABV antibodies. Thirty days after the second vaccine dose, 92% of the immunized animals presented VN titers ≥0.5IU/mL (geometric mean titers [GMT] 1.7 to 3.8IU/mL). However, at the day of the booster (360 days post-vaccination) the percentage of animals harboring antibody titers ≥0.5IU/mL had dropped to 31% (0-80% of the animals, depending on the vaccine), resulting in lower GMT (0.1 to 0.6IU/mL). Booster vaccination at day 360 resulted in a detectable anamnestic response in all groups, resulting in 83% of animals (65 to 100%) harboring VN titers ≥0.5IU/mL thirty days later (GMT 0.6 to 4.3IU/mL). These results indicated that these vaccines were able to induce an adequate anti-RABV response in all animals after prime vaccination (and after booster as well). However, the titers decreased, reaching titers <0.5IU/mL in approximately 70% of animals within the interval before the recommended booster. Thus, booster vaccination for rabies in cattle using the current vaccines should be performed before the recommended one-year interval, as to maintain neutralizing antibodies levels in most vaccinated animals.

Key words: rabies, cattle, vaccines, neutralizing antibodies.

RESUMO

A resposta sorológica contra o vírus da raiva (RABV) induzida por vacinas comerciais foi investigada em bovinos. Para isso, 84 novilhas foram vacinadas duas vezes (30 dias de intervalo) com cada vacina (G1 = 14 animais; G2 = 24; G3 = 22 e G4 = 24) e receberam uma vacinação de reforço 360 dias depois. Amostras de soro coletadas em diferentes momentos após a vacinação e após o reforço vacinal foram submetidas ao teste de vírus neutralização (VN) para detecção de anticorpos contra o RABV. Trinta dias após a segunda dose vacinal, 92% dos animais apresentaram títulos neutralizantes ≥0,5UI/mL (título médio geométrico [TGM] de 1,7 a 3,8UI/mL). Porém, no dia do reforço (360 dias pós-vacinação), a porcentagem de animais que ainda apresentava títulos ≥0,5UI/mL havia se reduzido a 31% dos animais (0 a 80%, dependendo da vacina), resultando em baixos TMGs (0,1 a 0,6UI/mL). A vacinação de reforço no dia 360 resultou em resposta anamnéstica em todos os grupos, resultando em 83% (65 a 100%) de animais com títulos VN ≥0,5UI/mL trinta dias após (GMT 0,6 a 4,3UI/mL). Esses resultados indicam que as vacinas avaliadas induzem uma resposta adequada de anticorpos anti-RABV após a vacinação (e também após o reforço). No entanto, os títulos reduzem-se,

atingindo níveis <0,5UI/mL em 70% dos animais durante o intervalo antes do reforço recomendado. Assim, vacinação de reforço contra a raiva em bovinos, utilizando-se as vacinas atuais, deve ser realizada em intervalo inferior a um ano, de forma a manter os níveis de anticorpos neutralizantes na maioria dos animais.

Palavras-chave: raiva, bovinos, vacinas, anticorpos neutralizantes.

INTRODUCTION

Rabies is an acute and generally fatal neurological disease of mammals, including humans. Annually, an estimated number of 60.000 people die of rabies worldwide, manly in African and Asian countries (OIE, 2016). The disease is caused by rabies virus (RABV), an enveloped RNA virus belonging to the family *Rhabdoviridae,* genus *Lyssavirus* (ICTV, 2017). RABV is maintained in nature in cycles involving wild and domestic animals, noticeably carnivores and bats (CONDORI-CONDORI et al., 2013). In South America, the bat ***Desmodus rotundus*** is the main reservoir of RABV in nature, with frequent transmission to livestock, mainly cattle, horses and sheep (SCHNEIDER et al., 2009).

The economic losses associated with bovine rabies in Latin America may reach 15 million dollars, due to approximately 100 at 500 thousand deaths every year (HEINEMANN et al., 2002). Bovine rabies is endemic in most Brazilian regions represents an important sanitary and economic problem (HEINEMANN et al., 2002). Although, rabies is considered endemic in Brazil, the index varies between different regions of the country. Estimates of deaths reach up to 30.000 to 40.000 cattle annually (RODRIGUES DA SILVA et al., 2000; HEINEMANN et al., 2002).

Rabies vaccination is widely used in Brazilian regions where RABV infection is endemic, frequently associated with control of bat populations ***Desmodus rotundus*** (JOHNSON et al., 2014). Unfortunately, vaccination seems not to confer complete protection since bovine rabies has been reported even in vaccinated animals (LIMA et al., 2005). Indeed, some studies revealed a fast drop in neutralizing antibodies after vaccination, what could partially explain some vaccine failures (RIBEIRO NETTO et al., 1973; ALBAS et al., 1998; QUEIROZ DA SILVA et al., 2003). In addition, experimental data has demonstrated that booster vaccinations at somewhat short intervals are required for maintain adequate VN antibody titers (ITO et al., 1991; CÔRTES et al., 1993; RODRIGUES DA SILVA et al., 2000). Protection to RABV infection induced by vaccination is based mainly on neutralizing antibodies directed to the envelope glycoprotein G (WIKTOR et al., 1973; WUNDERLI et al., 1991). According to OIE, immunized animals must have levels of neutralizing antibodies of ≥0.5IU/mL.

Several inactivated, adjuvanted vaccines are available commercially and are widely used in Brazilian regions endemic for rabies. Licensed vaccines are subjected to an official quality control (MAPA, 2009). In spite of the manufacturer's instructions (two 30-days apart initial doses followed by annual boosters), some producers perform only a single prime vaccination followed by occasional boosters, usually when cases of rabies are reported nearby (LIMA et al., 2005). The absence or incomplete vaccination protocols performed in most herds certainly contributes for the reported cases of rabies in vaccinated animals (FILHO et al., 2010; JOHNSON et al., 2014).

In Rio Grande do Sul (RS), the southernmost Brazil State, bovine rabies was historically endemic in well defined, restricted regions (FLORES – verbal report). Hence, vaccination was usually restricted to the affected and nearby herds. Beginning in 2011, an unprecedented rabies outbreak is occurring in the state, with estimates reaching up to 40.000 deaths by 2013 (SEAPA, 2013). In addition to the dramatic increase in the number of cases, the distribution of the disease also changed, with cases/outbreaks occurring in otherwise free areas. As a consequence of the increase in the number of cases and expansion of the affected areas, rabies vaccination has been gradually implemented in many RS regions (FLORES – verbal report). Thus, the objective of this study was to evaluate the serological response of cattle to four commercial rabies vaccines, used according to the manufacturer's recommendations.

MATERIALS AND METHODS

Eighty-four heifers (12 to 24 months-old) belonging to herds with no historic of vaccination against rabies in the central region of RS were used. Heifers were randomly allocated in four groups, each group receiving one commercial rabies vaccine, as follows: G1 = 14 animals; G2 = 24; G3 = 22 and G4 = 24. The four vaccines have been purchased in veterinary stores, kept refrigerated and used before the expiration date. All vaccines contain the RABV strain *Pasteur virus* (PV) inactivated and aluminum hydroxide as adjuvant. Animals were vaccinated according to the manufacturer's instructions, receiving two doses subcutaneously (2mL) with a 30 day-interval, followed by a booster approximately 360 days later. Serum samples were collected at days 0 (first vaccine dose), 30 (second dose), 60, 360 (day of the booster) and 390 (30 days after booster). Serum samples were submitted to a modified RFFIT (rapid fluorescent focus inhibition test) for neutralizing antibodies to RABV, according to SMITH et al. (1973), with minor modifications. Briefly, 10-fold dilutions of serum were incubated with approximately 100-200 $TCID_{50}$ (50% tissue culture infective dose) of CVS (Challenge Virus Standard - CVS132-11A), kindly provided by Instituto Pasteur, São Paulo, Brazil) for 90min, followed by addition of a suspension of Baby Hamster Kidney cells (BHK-

21 - C-13 ATCC® CCL-10™) and incubation at 37ºC - 5% of CO_2 for 48 h. At the end of this period, the indicator cells were submitted to a fluorescent antibody (FA) assay, using an anti-RABV FITC-conjugate (Instituto Pasteur, São Paulo, Brazil). Mock-infected BHK-21 cells and cells infected with CVS were used as controls. Slides were observed in an UV epifluorescence microscope (Axiolab ZEISS®). The virus neutralizing (VN) titer was considered the highest dilution of serum able to prevent virus replication, as indicated by the absence of viral antigens in indicator cells. A reference serum (containing 0.5IU/mL, provided by Instituto Pasteur, São Paulo, Brazil) was used as control in all tests. The neutralizing titer of this serum was used to convert the VN titers of the samples to UI/mL. Neutralizing titers were converted to GMT according to PERKINS (1958). The GMT for each vaccine group at different intervals were submitted to statistical analysis, using the ANOVA and test of the Tukey in *software* Assistat version 7.7 beta.

RESULTS AND DISCUSSION

The results of RFFIT assays for RABV neutralizing antibodies in the sera of heifers immunized with commercial vaccines are presented in table 1 and figure 1. Table 1 presents the number and percentage of seropositive cattle (titers ≥0.5IU/mL) and the GMT of the vaccinated animals after vaccination and booster; figure 1 shows the evolution of VN titers (expressed as GMT) at these time points.

None of the vaccinated animals had VN antibodies to RABV at the day of first vaccination, as verified by the RFFIT (not shown). Thirty days after the first vaccine dose, 57% of the animals presented VN titers ≥0.5IU/^{mL} (Table 1). The percentage of seropositive cattle varied among the groups, from 25% (G2) to 86% (G1). The GMT at this day ranged from 0.3 (G2) to 1.4IU/mL (G1). In naïve animals, a single dose of inactivated rabies vaccine has been considered insufficient for adequate immunization (ALBAS et al., 1998; FILHO et al., 2010). ALBAS et al. (2005) compared different vaccination protocols with a commercial vaccine containing inactivated RABV (strain PV) and aluminum hydroxide as adjuvant. When evaluating the neutralizing antibody titers 30 days after the first vaccination, only 30% of the animals had developed neutralizing antibodies in titers ≥0.5IU/mL. Our results corroborated these findings, demonstrating that a single dose of the current inactivated vaccines is insufficient to induce suitable antibody levels. However, it should be emphasized that seroconversion at day 30pv (post-vaccination) should not be considered a definitive indicator of vaccine immunogenicity since the vaccine protocols recommend two initial doses 30 days apart. Unfortunately, many Brazilian farmers do not perform the complete vaccination protocol, applying only a single dose. According to our study and previous results, this simplified

protocol results in low antibody titers and/or in a low percentage of seropositive cattle, leaving unprotected a considerable part of the herd (ALBAS et al., 2005).

At day 60 (30 days after the second vaccine dose), the heifers had seroconverted to RABV in titers ≥0.5IU/mL in percentages of 100% (G1), 95% (G2 and G3) and 76% (G4), respectively. The GMT ranged from 1.0 (G4) to 3.8IU/mL (G1). Again, G1 heifers developed the highest VN titers comparing to the other groups ($P<0.05$). Thus, considering the recommended protocol of two initial doses, three out of four vaccines were able to induce VN titers above the cut-off value recommended by OIE (≥0.5IU/mL) against RABV in at least 95% of heifers. Surprisingly, only 76% of the animals of one vaccine group (G4) developed antibody titers higher than 0.5IU/mL after the second dose. The reasons for this low performance are unclear and somewhat surprising since these vaccines are expected to fulfill the official requirements that include innocuity, sterility and potency before are made available for commercial use (MAPA, 2009).

In general, the VN titers at day 60 were well above the reference value, in at least three vaccine groups (in some cases they reached up to 8IU/mL). The magnitude of VN titers, as indicated by GMT, was highly variable among the groups, indicating important differences in the immunogenicity among the vaccines. PIZA et al. (2002) verified that the quantification of virus attached rabies glycoprotein present in vaccines, has a strong correlation with VNA elicited in the target species. This could explain the observed differences between the vaccines we tested. However, we did not assess the virus attached rabies glycoprotein, nor total glycoprotein nor free soluble glycoprotein. Thus, it is not possible to attribute the observed differences to this factor. As mentioned before, licensed rabies vaccines are subjected to official control by the Brazilian Ministry of Agriculture Livestock and Supply (MAPA). Our results confirmed the adequate immunogenicity of at least three of these vaccines, as ascertained by VN titers developed in >95% animals at day 60pv.

At the day of the booster, the percentage of animals with titers ≥0.5IU/mL had dropped dramatically comparing to day 60. Percentage of animals with titers ≥0.5IU/mL ranged from 0 (G4) to 80% (G1). These results indicated that approximately 31% (20 to 100%, depending on the vaccine) of the vaccinated animals would become unprotected to rabies (antibody VN titers lower then 0.5IU/mL) before the time recommended for booster. At this day, the GMT were also significantly lower (0.1 to 0.6IU/mL), illustrating the VN very low antibody levels after the one year-interval. The fast decline in VN titers induced by inactivated RABV vaccines has also been observed in other studies. ALBAS et al. (2005) evaluated the neutralizing antibody titers 360 after vaccination and observed that none of the nine vaccinated animals was able to

maintain adequate antibody titers. In other study, ALBAS et al. (1998) investigated the importance of the booster in the duration of immune response, observing that only 19% of the animals receiving two vaccine doses, 30 days apart, were able to maintain antibody titers >0.5IU/mL at day 360. Our results corroborated these findings, indicating an early decrease of neutralizing antibody titers in most vaccinated animals.

Following the manufacturer's recommendations, we performed a booster vaccination approximately 360 days after the initial vaccination. Sera of vaccinated animals were tested for RABV neutralizing antibodies at the day of the booster and 30 days later. Analyzing the individual vaccines, only G1 was able to maintain adequate antibody levels in a high proportion of animals (80%) during the one-year interval. Considering that the vaccination protocols recommend a booster vaccination one year after the prime vaccination, a high percentage of animals (69%) would be unprotected before receiving the booster. This window of susceptibility may partially explain some cases of vaccine failure occasionally reported (LIMA et al., 2005; FILHO et al., 2010). Conversely, these results indicated that shortening the interval between vaccination and booster may be necessary as to eliminate this window of susceptibility, reducing the number of animals susceptible to RABV before booster, as observed in other studies (OLIVEIRA et al., 2000; ALBAS et al., 2005), mainly in young cattle (UMEHARA et al., 2002; LIMA et al., 2005). In hiperendemic areas, where the animals are exposed to a high infection pressure or risk, booster vaccinations at every six or eight months may be required for some vaccines, mainly in the first year after the prime vaccination. A 180 days interval between initial vaccination and booster has been proposed by ALBAS et al. (2005) upon evaluating different vaccination protocols.

Booster vaccination at day 360 resulted in a detectable anamnestic response in all groups as demonstrated by 83% of animals harboring VN titers ≥0.5IU/mL at day 390. The GMT also increased significantly (0.6 to 4.3 ≥0.5IU/mL) (Table 1, Figure 1). Considering the GMT post-booster; however, booster immunization had variable effects among the groups. Only animals in G1 presented a strong anamnestic response, developing GMT (4.3IU/mL) higher that those observed after the complete prime vaccination (3.8UI/mL). The other vaccines provided a boost sufficient to increase the VN titers of all animals above the value of the 0.5IU/mL. However, the GMT of groups G2, G3 and G4 post-booster were significantly lower than those measured at day 60pv (Figure 1). As the sera was collected 30 days post-booster and the peak of antibody titers may be observed earlier (RODRIGUES DA SILVA et al., 2005), it is possible that slightly higher titers were indeed reached soon after booster. Interestingly, vaccine G4 had the worst performance at day 60pv, yet provided an adequate immune boost in 90% of the animals upon

revaccination. Unfortunately, we could not follow the animals longer as to ascertain the duration of the post-booster antibodies and whether a shortened interval should also be necessary in the following boosters.

CONCLUSION

Our results showed that the tested vaccines fulfilled the minimum requirements of immunogenicity, e.g. conferring adequate VN levels in the vaccinated animals after completion of the prime vaccination protocol. Booster immunization revealed an anamnestic response in all vaccine groups. The significant differences in GMT; however, indicated an important variation in the immunogenicity among the vaccines. The most important finding was that adequate VN levels were not maintained over the period of one year in 69% of the animals, indicating the need of shortening the interval between vaccination and booster, mainly in regions of high infection pressure.

INFORMAL INFORMATION

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ETHICS COMMITTEE AND BIOSAFETY

All procedures were approved by an institutional committee of animal use (Comissão de Ética no Uso de Animais (CEUA/UFSM) (approval protocol n° 147/2014).

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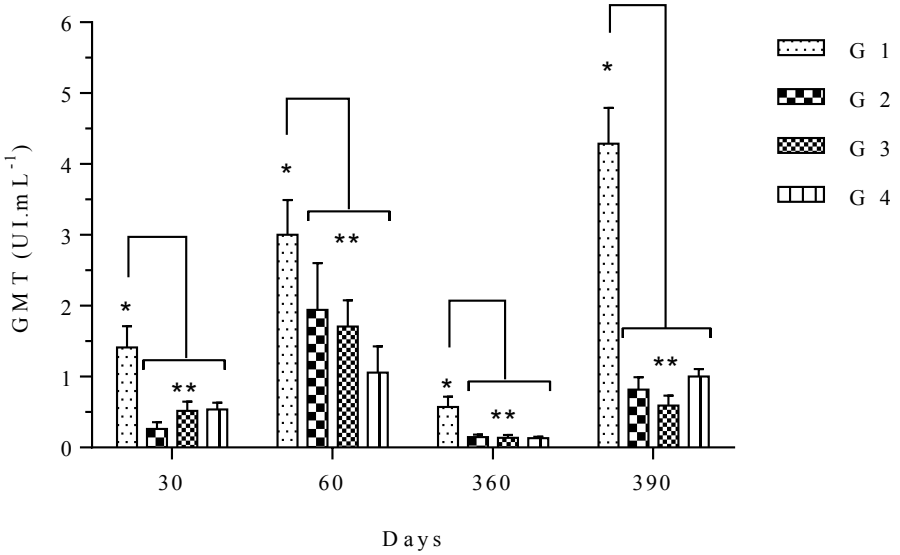

Figure 1 - Evolution of the geometric mean titers (GMT) of virus-neutralizing antibodies in the sera of heifers vaccinated with each of four commercial rabies vaccines. G1-G4: vaccine groups. * and ** are different ($P<0.05$).

Table 1 - Serological response of heifers to rabies virus following vaccination and booster with each of four commercial rabies vaccines.

Day		30pv[1]		60pv			360pv		390pv	
Group	*n*	% seropositive cattle [2]	GMT[3]	% seropositive cattle	GMT	*n*	% seropositive cattle	GMT	% seropositive cattle	GMT
G 1	14	86 (12/14)	1.4	100 (14/14)	3.8	10	80 (8/10)	0.6	100 (10/10)	4.3
G 2	24	25 (6/24)	0.3	95 (23/24)	1.9	17	23 (4/17)	0.2	65 (11/17)	0.8
G 3	22	73 (16/22)	0.5	95 (21/22)	1.7	15	27 (4/15)	0.1	87 (13/15)	0.6
G 4	24	58 (14/24)	0.5	76 (19/24)	1.0	10	0 (0/10)	0.1	90 (9/10)	1.0
Total[4]	84	57 (48/84)		92 (77/84)		52	31 (16/52)		83 (43/52)	

[1] Post-vaccination;
[2] Determined by a VN assay. Seropositive cattle were the animals with VN titers of ≥0.5IU/mL;
[3] Geometric mean titer;
[4] Between vaccination and the booster, number of animals decreased from 84 to 52 for reasons unrelated to the experiment.

6. DISCUSSÃO

Nesta tese são apresentados os resultados de três estudos, sob a forma de artigos científicos. Dois deles são referentes a construção e caracterização de recombinantes do ORFV candidatos a vetores vacinais e um artigo descreve a avaliação da imunogenicidade de quatro vacinas antirrábicas comerciais disponíveis no Brasil.

O ORFV tem sido proposto como plataforma vetorial por apresentar diversas características que o tornam um atrativo candidato a vetor (RZIHA et al., 1999, 2000). Os estudos apresentados nesta tese que abordam o ORFV como vetor vacinal fazem parte de um projeto de colaboração desenvolvido em conjunto pelo grupo do Dr. Diego G. Diel (*South Dakota State University*, USA) e Setor de Virologia-UFSM. Esse projeto propõe o desenvolvimento de uma plataforma vetorial vacinal para animais domésticos, utilizando-se o ORFV como vetor e inclui construção e caracterização de vírus recombinantes, caracterização básica do vírus (função de produtos gênicos e estudos de patogenia)(CARGNELUTTI et al., 2011; DIEL et al., 2010, 2011a, 2011b; MARTINS et al., 2014; SCHMIDT et al., 2013) e a utilização de recombinantes do ORFV como vetor vacinal (HAIN et al., 2016).

O primeiro artigo desta tese (Artigo 1) apresenta a construção e caracterização *in vitro* de dois recombinantes do ORFV com deleções em genes não essenciais. Foram removidos, de forma individual, dois genes que atuam na via de sinalização do fator de transcrição NF-kappa-beta: *ORFV024* e *ORVF121* (DIEL, 2010; DIEL et al., 2011b). No *loci* destes genes, foi inserida a sequência de nucleotídeos que codifica a glicoproteína G do vírus da raiva sob controle do promotor VV7.5 do VACV gerando os vírus recombinantes: ORFV$^{\Delta 024}$RABV-G e ORFV$^{\Delta 121}$RABV-G, respectivamente. A cinética de replicação demonstrou que os vírus construídos replicam de forma tão eficiente quanto o vírus parental em cultivos celulares. Os vírus recombinantes gerados expressaram a glicoproteína G em grande quantidade após replicar em cultivos celulares de células OFTu, STu ou BT. Quando utilizados para imunizar suínos e bovinos, estes vírus induziram uma robusta resposta de anticorpos neutralizantes antirrábicos. Em ambas as espécies, os vírus recombinantes induziram níveis altos de anticorpos e a resposta promovida pelo ORFV$^{\Delta 121}$RABV-G foi significativamente mais intensa quando comparada a resposta imune induzida pelo ORFV$^{\Delta 024}$RABV-G. Em suínos, o ORFV tem demonstrado ser uma excelente opção de plataforma vetorial (FISCHER et al., 2003b; HAIN et al., 2016a; VOIGT et al., 2007). No entanto, pela primeira vez foi testado o ORFV como vetor em bovinos. Demonstrou-se que os vírus recombinantes expressam a proteína heteróloga (RABV-G) em

quantidade suficiente para induzir elevados níveis de anticorpos. Portanto, além de suínos, o ORFV é uma alternativa promissora para vetorar genes heterólogos de interesse para imunização de bovinos.

O segundo artigo desta tese (Artigo 2) descreve a geração de ORFV mutantes com deleção individual de três genes: *ORFV112*, *ORFV117* e *ORFV127*. O gene *ORFV112* codifica uma proteína ligante a quimiocina (CBP) (SEET et al., 2003), o gene *ORFV117* codifica um inibidor do fator estimulador de colônia granulacítica-monocítica (GMC-CSF) e interleucina-2 (IL-2) (GIF) (DEANE et al., 2000) e o *ORFV127* um homólogo da interleucina-10 (vIL-10) (FLEMING et al., 1997). Foram gerados os vírus ORFVΔ112, ORFVΔ117 e ORFVΔ127 com objetivo de identificar novos loci para inserção de genes heterólogos. Os experimentos de caracterização *in vitro* demonstraram que as deleções não interferem na cinética de replicação viral, demostrando que o *loci* dos genes investigados são opções para inserções de genes heterólogos. Além dos estudos *in* vitro, um estudo de patogenia foi conduzido para investigar o impacto destas deleções na virulência do ORFV em cordeiros. Verificou-se que os mutantes de deleção induziram lesões tão graves quanto as lesões induzidas pelo vírus parental. No entanto, a regressão das lesões nos animais infectados com os vírus mutantes ocorreu em menor tempo, excretam vírus em níveis mais baixos e por período mais curto. Assim, conclui-se que os genes estudados atuam de forma parcial na virulência do ORFV em seu hospedeiro natural. Deste modo, os genes *ORFV112*, *ORFV117* e *ORFV127* são opções para inserção de genes heterólogos. A possibilidade de deleções duplas e triplas deve ser considerada.

O terceiro artigo (Artigo 3) descreve um estudo da imunogenicidade de quatro vacinas inativadas contra raiva dos herbívoros disponíveis no Brasil. As vacinas foram administradas de acordo com o protocolo recomendado pelos fabricantes. O estudo foi conduzido durante um ano para verificar a magnitude e duração da resposta sorológica induzida pelas vacinas. Verificou-se que as vacinas avaliadas são imunogênicas, porém a manutenção de níveis adequados de anticorpos provavelmente requer a aplicação de reforço a intervalos menores do que um ano, como recomendado pelos fabricantes.

7. CONCLUSÃO

Baseando-se nos resultados apresentados nesta tese conclui-se que o ORFV se constitui em um promissor vetor vacinal para utilização em suínos e bovinos. Os recombinantes construídos com a deleção dos genes *ORFV024* e *ORFV121* e a inserção do gene da glicoproteína G do RABV induziram níveis elevados de anticorpos neutralizantes antirrábicos nessas espécies.

A construção e caracterização *in vitro* e *in vivo* dos mutantes de deleção nos genes *ORFV112*, *ORFV117* e *ORFV127* confirmou o loci destes genes como sítios adequados para inserção de genes heterólogos. O estudo de patogenia em cordeiros demonstrou que estes genes atuam de forma parcial na virulência do ORFV em ovinos.

O estudo da imunogenicidade de vacinas antirrábicas possibilitou verificar que as quatro vacinas avaliadas são imunogênicas, porém requerem reforços a intervalos inferiores a um ano para a manutenção de níveis adequados de anticorpos neutralizantes.

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