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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 (IMPs) 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.

1. Introduction

Orf virus (ORFV) is the prototype of the genus *Parapoxvirus*, subfamily *Chordopoxvirinae,* family *Poxviridae* (ICTV, 2015). ORFV is ubiquitous and 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 (VIR; *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 determined (Deane et al., 2000; Diel et al., 2011a, 2011b, 2010; Fleming et al., 1997; McInnes et al., 1998; Seet et al., 2003; Wise

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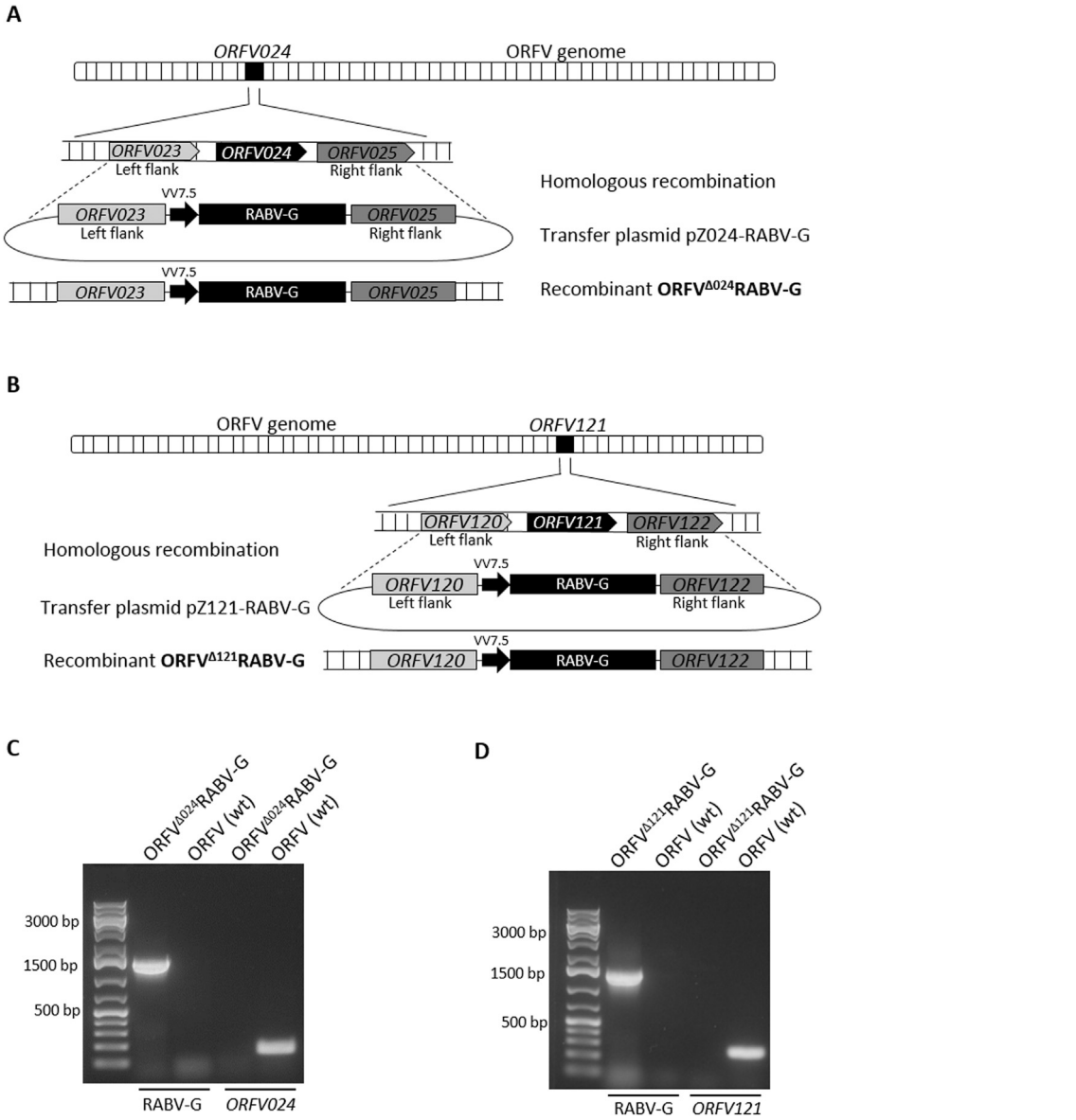

Fig. 1. Generation of recombinant ORFV-RABV-G viruses. (A) Schematic representation of the ORFV genome depicting *ORFV024* insertion site and flanking regions (*ORFV023* and *ORFV025*) used to generate the recombinant ORFV$^{\Delta 024}$RABV-G. (B) Schematic representation of the ORFV genome depicting *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 virus 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 virus and absence of *ORFV121* gene sequences on the recombinant virus genome. 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.

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., 2003; 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 glycoprotein (RABV-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, 2015). 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 canarypox virus) 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 good immunogenicity in mice, cats and 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.

2. Results

2.1. 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 RABV-G under the control of the early/late VV7.5 poxvirus promoter (Figs. 1A and 1B). 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 (Figs. 1C and 1D). 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 (Figs. 1C and 1D). 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 primary swine turbinate (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 OFTu cells (Fig. 2B).

2.2. 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 post-infection (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 virus 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. 3C). 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 (Fig. 3C), 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 (Figs. 4C and 4D).

2.3. 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 2). 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 p.i. (Table 2). Following the booster immunization on day 21, all immunized animals presented anamnestic serological responses, as evidenced by a marked increase in

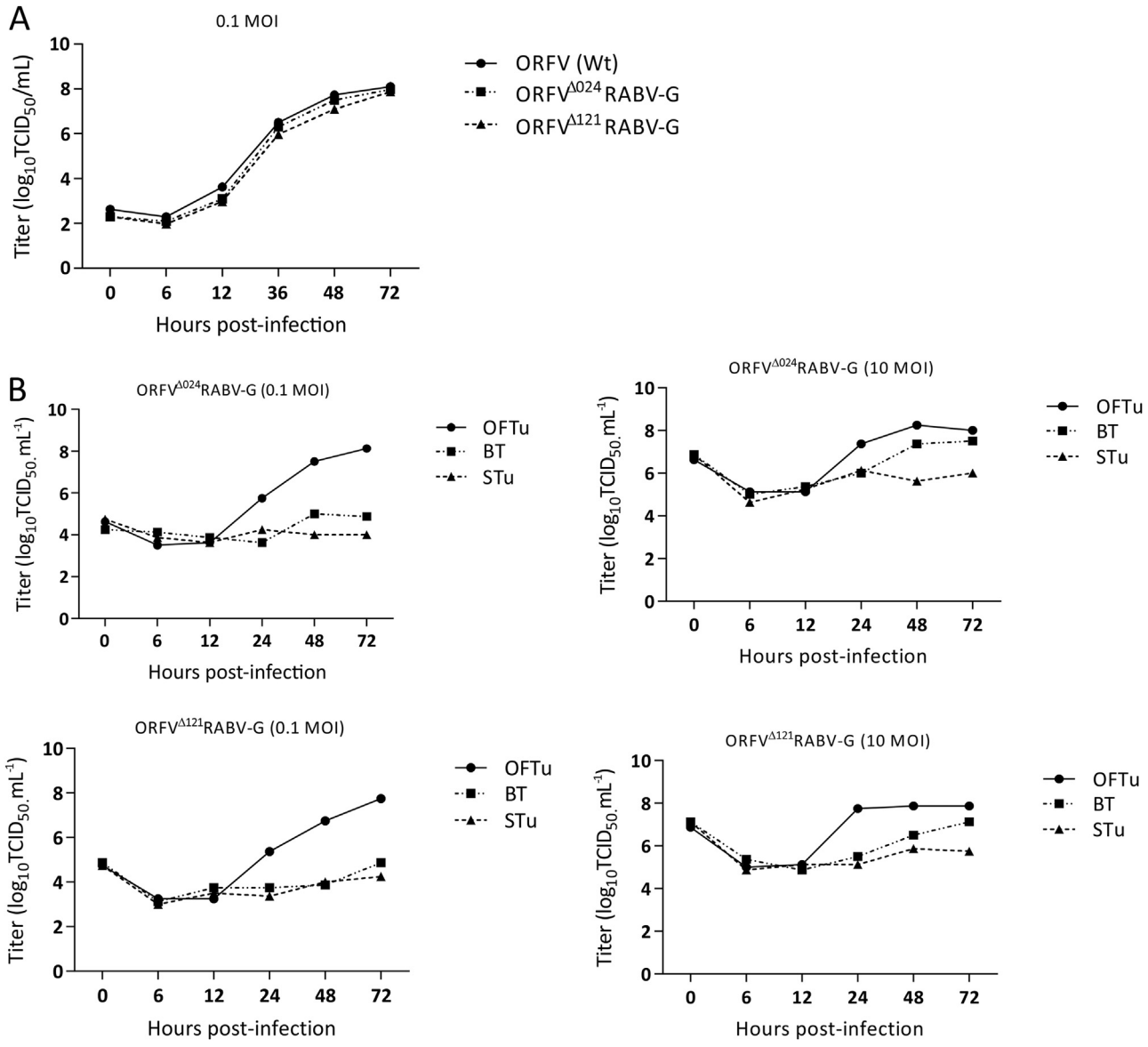

Fig. 2. Replication kinetics of the recombinant ORFV-RABV-G viruses. (A) Multi-step growth curve of the recombinants ORFV$^{\Delta024}$RABV-G, ORFV$^{\Delta121}$RABV-G and wild type virus in primary OFTu cells. Results were calculated based on two independent experiments. (B) Multi- and single-step growth curve of the recombinant ORFV$^{\Delta024}$RABV-G and ORFV$^{\Delta121}$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.

the levels of RABV neutralizing antibodies on day 42 p.i. (Table 2). In fact, the geometric mean titers (GMT) of RABV neutralizing antibodies were significantly higher in animals immunized with ORFV$^{\Delta121}$RABV-G recombinant virus (GMT = 905) when compared to animals immunized with ORFV$^{\Delta024}$RABV-G recombinant virus (GMT = 160; $P < 0.01$; Fig. 5A).

2.4. *Immunogenicity of ORFV$^{\Delta024}$RABV-G and ORFV$^{\Delta121}$RABV-G recombinant viruses in cattle*

The immunogenicity of ORFV$^{\Delta024}$RABV-G and ORFV$^{\Delta121}$RABV-G recombinant viruses was also evaluated in cattle. All animals immunized with ORFV$^{\Delta024}$RABV-G and ORFV$^{\Delta121}$RABV-G recombinant viruses developed neutralizing antibodies to RABV after the primary immunization (titers ranging between 40 and 160 for Group 1, or 40 and 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$^{\Delta121}$RABV-G recombinant virus resulted in higher RABV neutralizing antibody titers (GMT = 970), when compared to the titers detected in animals immunized with ORFV$^{\Delta024}$RABV-G (GMT = 538.2) ($P < 0.05$; Fig. 5B). Together, these results indicate that ORFV$^{\Delta121}$RABV-G recombinant virus is more immunogenic and elicits robust antibody responses in swine and cattle.

2.5. *Memory responses following immunization of cattle with ORFV$^{\Delta024}$RABV-G and ORFV$^{\Delta121}$RABV-G recombinant viruses*

The immunogenicity of ORFV$^{\Delta024}$RABV-G and ORFV$^{\Delta121}$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$^{\Delta024}$RABV-G or ORFV$^{\Delta121}$RABV-G recombinant virus. All animals immunized with ORFV$^{\Delta121}$RABV-G recombinant virus presented RABV neutralizing antibodies (titers from 10 to 320), whereas only 7 of

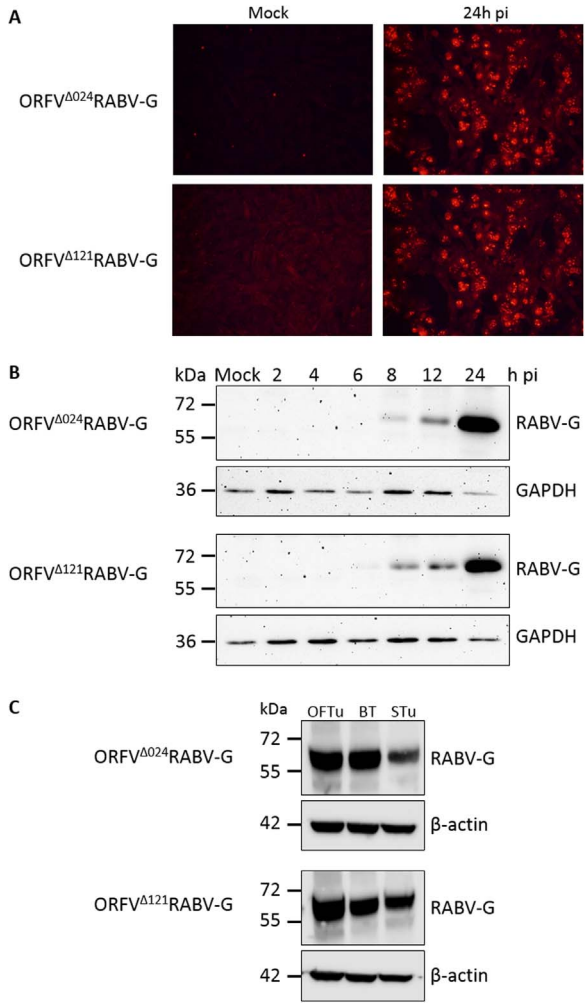

Fig. 3. Expression of RABV glycoprotein by the recombinants ORFV-RABV-G. (A) Immunofluorescence assay performed in primary OFTu cells infected (MOI = 1) with the recombinant ORFV$^{\Delta 024}$RABV-G or ORFV$^{\Delta 121}$RABV-G virus. Cells were fixed at 24 h post-infection (pi) and incubated with mouse IgG anti-FLAG antibody followed by incubation with a goat anti-mouse IgG secondary antibody (Alexa Fluor® 594 conjugate) and visualized under an UV microscope. (B) Western blot assay performed in OFTu cells inoculated with ORFV$^{\Delta 024}$RABV-G or ORFV$^{\Delta 121}$RABV-G recombinant virus (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. (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 pi. 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.

15 animals immunized with ORFV$^{\Delta 024}$RABV-G recombinant virus presented RABV neutralizing antibodies on day 30 p.i. (titers from 10 to 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 p.i. (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 390 p.i. (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.

3. 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., 2003), 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 protective antigen of RABV, the 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 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

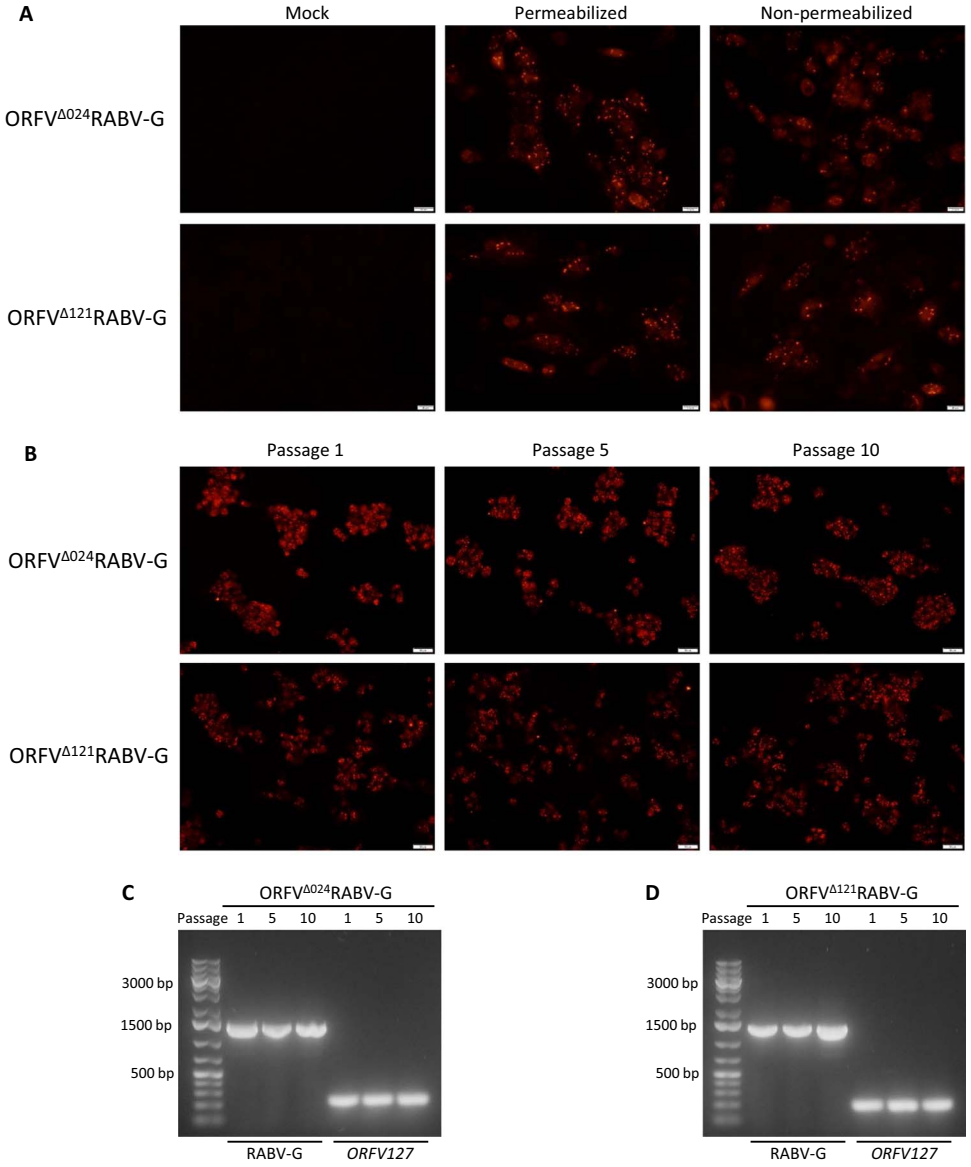

Fig. 4. Expression of RABV-G by the ORFV$^{\Delta 024}$RABV-G and ORFV$^{\Delta 121}$RABV-G recombinant viruses assessed by immunofluorescence assay. (A) OFTu cells were infected with the ORFV$^{\Delta 024}$RABV-G or ORFV$^{\Delta 121}$RABV-G recombinant virus (MOI = 1) and fixed with 3.7% formaldehyde at 24 h post-infection (pi). 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 an UV 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 recombinant ORFV$^{\Delta 024}$RABV-G or ORFV$^{\Delta 121}$RABV-G virus (MOI ~ 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 MOI (~ 1) on each passage and viruses were harvested at 48 pi 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* (IL10 homologue; available upon request) were used as controls to confirm that similar amounts of virus DNA were used in the PCR amplifications.

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 (Figs. 3A, 3B and 3C). 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).

Table 1
Experimental design of animal immunization studies.

Specie	*n*	Recombinant virus	Dose ($TCID_{50}$·ml^{-1})	Route of immunization	Immunization days	Serum collection days
Swine	5	$ORFV^{\Delta 024}$RABV-G	$10^{7.8}$	IM[a]	0, 21	0, 21, 42
	4	$ORFV^{\Delta 121}$RABV-G	$10^{7.8}$	IM	0, 21	0, 21, 42
Bovine exp#1[b]	4	$ORFV^{\Delta 024}$RABV-G	$10^{7.9}$	IM	0, 30	0, 30, 60
	5	$ORFV^{\Delta 121}$RABV-G	$10^{7.9}$	IM	0, 30	0, 30, 60
Bovine exp#2[c]	15	$ORFV^{\Delta 024}$RABV-G	$10^{7.9}$	IM	0, 30, 390	0, 30, 60, 390, 420
	15	$ORFV^{\Delta 121}$RABV-G	$10^{7.9}$	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	nAb[a] titer		
	ID	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.

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 (Figs. 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. 3C). Results from the immunization experiments in pigs and cattle demonstrating neutralizing antibody responses in both species, however, confirmed that the recombinant vectors effectively expressed/delivered 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

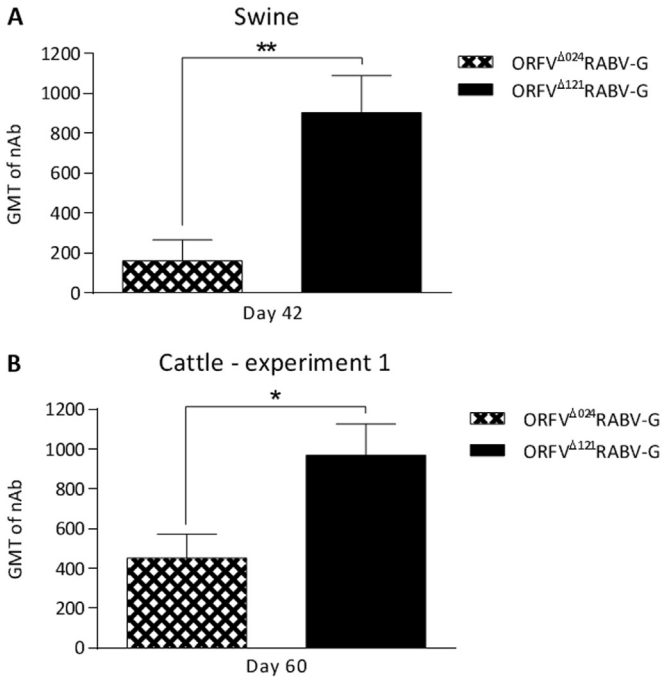

Fig. 5. Immunogenicity of recombinant ORFV-RABV-G viruses. (A) Immune responses elicited by immunization with $ORFV^{\Delta 024}$RABV-G and $ORFV^{\Delta 121}$RABV-G recombinant viruses in pigs at day 42 p.i. (21 days post-booster). Geometric mean titer (GMT) of individual titers obtained through rapid fluorescent focus inhibition test (RFFIT), which detects RABV neutralizing antibodies (nAb; 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 p.i. (30 days post-booster) (experiment 1). GMT of individual titers obtained through RFFIT (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 ($P < 0.05$), *.

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., 2003; Henkel et al., 2005; Rohde et al., 2011; Voigt et al., 2007), no studies have reported the efficacy of the vector in cattle. Here 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 (Tables 3, 4), and a booster immunization on day 30

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$^{\Delta024}$RABV-G	280	< 10	40	160
	284	< 10	80	640
	293	< 10	160	640
	299	< 10	80	640
ORFV$^{\Delta121}$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.

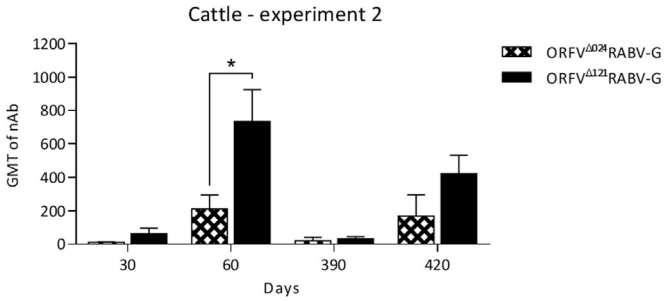

Fig. 6. Serological response following immunization with ORFV$^{\Delta024}$RABV-G and ORFV$^{\Delta121}$RABV-G recombinant viruses in cattle (experiment 2). Immune responses elicited by immunization with ORFV$^{\Delta024}$RABV-G and ORFV$^{\Delta121}$RABV-G recombinant viruses in heifers at days 30, 60, 390 and 420 p.i. Geometric mean titer (GMT) of individual titers obtained through rapid fluorescent focus inhibition test (RFFIT), which detects RABV neutralizing antibodies (nAb; group ORFV$^{\Delta024}$RABV-G $n = 15$ and group ORFV$^{\Delta121}$RABV-G $n = 15$). Error bars represent the standard error of the median (SEM). Immunizations were performed on days 0, 30 and 390. Statistical differences were determined using the students T-test ($P < 0.05$), *.

p.i. led to anamnestic serological responses (Tables 3, 4). Interestingly, similar to the results observed in pigs, immunization with ORFV$^{\Delta121}$RABV-G recombinant virus resulted in higher neutralizing antibody responses when compared to those detected in ORFV$^{\Delta024}$RABV-G-immunized animals. Together these observations suggest that ORFV$^{\Delta121}$-based vectors present an enhanced immunogenicity when compared to ORFV$^{\Delta024}$-based vectors in both swine and cattle, which results in higher levels of neutralizing antibodies against RABV-G. Although the mechanism(s) underlying this phenotype was/were 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.

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

Group	Animal	nAb[a] titers				
	ID	0 d.p.i.[b]	30 d.p.i.	60 d.p.i.	390 d.p.i.	420 d.p.i.
ORFV$^{\Delta024}$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$^{\Delta121}$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.

Although this study focused on comparing the immunogenicity between ORFV$^{\Delta024}$RABV-G and ORFV$^{\Delta121}$RABV-G in target animal species, the results with ORFV$^{\Delta121}$RABV-G in cattle are encouraging if considered in the context of the current strategies to control RABV in this species. Rabies is one of the most important diseases of livestock species and humans. In the Americas, for example, rabies causes yearly losses of ~ $30 million (US dollars) due to mortality of livestock, with cattle being the most commonly affected species (WHO, 2005). The results obtained in the present study demonstrating the superior immunogenicity of ORFV$^{\Delta121}$RABV-G recombinant vector in cattle, indicate that this recombinant virus represents an attractive alternative to the current inactivated RABV vaccines. Although the exact levels of RVNA required for protection are not known, titers of ~ 0.5 international units (IU)·ml^{-1} (serum neutralization titer of ~ 1:50) are considered sufficient to demonstrate a robust immune response to vaccination (Moore and Hanlon, 2010). Results here with ORFV-based vectors are consistent with this, highlighting the potential of the ORFV$^{\Delta121}$RABV-G recombinant virus as a vaccine candidate for use in cattle. Additional studies, however, are needed to compare the protective efficacy of ORFV$^{\Delta121}$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$^{\Delta024}$RABV-G and ORFV$^{\Delta121}$RABV-G) in potential target animal species for ORFV-vectored vaccines. As evidenced by the higher neutralizing antibody responses in pigs and cattle, ORFV$^{\Delta121}$-based vector presented an enhanced immunogenicity, when compared to its counterpart ORFV$^{\Delta024}$-vector. Given the immunogenicity of ORFV$^{\Delta121}$-vector in both swine and cattle, this vector represents an excellent candidate for novel vaccine designs for use in these animal species.

4. Methods

4.1. Cells and viruses

Primary ovine fetal turbinate (OFTu, provided by Dr. D.L. Rock, University of Illinois or generated in house), primary bovine fetal turbinate (BT, generated in house), primary swine turbinate cells (STu, generated in house) 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^{-1}) streptomycin (100 µg·ml^{-1}) and gentamycin (50 µg·ml^{-1}). Cell cultures were maintained at 37 °C 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.

4.2. 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.

4.3. 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 °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 plates 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′- GCGGCGGAT CCATGAAATTCCCCATCTACACAATACCAG-3′ and RabVGEx-Rv (NotI)-5′-ATAATGCGGCCGCGTATTTCCCCCAACTCGGGAGACCAAGG-3′; 024int-Fw-5′- ACTTGGATCTGTCCGACGAC-3′ and 024int-Rv-5′-AGCTGTTCCACGTCCCTCT-3′ and 121int-Fw-5′-GGCGGACTACCAGA GACATC-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.

4.4. 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 pi (6, 12, 24, 48, and 72 h pi) 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 and the Spearman and Karber's method and expressed as tissue culture infectious dose 50 ($TCID_{50}$) per 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.

4.5. 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 pi. After fixation cells were washed three times with phosphate buffer saline (PBS) and permeabilized with 0.2% PBS-Triton ×100 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 under an UV microscope.

4.6. 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 virus (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). One 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 4 °C. 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).

4.7. 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 $10^{7.8}$ $TCID_{50}.ml^{-1}$. 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 4–6-months-old calves were randomly allocated in two experimental groups as follows: Group 1, ORFV$^{\Delta 024}$RABV-G-immunized (n = 4) and Group 2, ORFV$^{\Delta 121}$RABV-G-immunized (n = 5). 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.. 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 56 °C for 30 min for complement inactivation and stored at − 20 °C 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 no. 035/2014).

4.8. 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 37 °C with 5% of CO_2 for 48 h. At 48 h, 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 1 h at 37 °C 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 under an UV microscope. 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 GMT (Perkins, 1958).

4.9. Statistical analysis

Statistical analysis was performed using the Prism software (GraphPad; 6th version). 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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