RESEARCHREPORT
SWINE HEALTH

**Title** Development of novel foot and mouth disease virus with multiple mutations for evaluation as live attenuated DIVA vaccine candidates – **NPB #12-206**

revised

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**Industry Summary:** Foot-and-mouth disease (FMD) is an extremely contagious viral disease of a variety of wild and domestic cloven-hoofed animals, including pigs. The disease is distributed worldwide and has great negative economic impact not only on livestock health and production but also on international trade. The constant presence of outbreaks in many regions of the world highlights the potential of accidental or deliberate introductions of FMD in the U.S. The current FMD vaccine is a formulation of inactivated whole virus and adjuvant that requires 7 days to induce protection, a time during which vaccinated animals are still susceptible to disease. Therefore, it is essential to develop new control strategies that could confer very early protection and stop disease spread. Attenuated vaccines are expected to elicit more rapid and a long lived immunity and at the same time could be an excellent tool to study the interactions between viruses and the host in detail. Indeed, some viral diseases have been eradicated using live-attenuated vaccines (i.e. Rinderpest and Smallpox). Our goal is to develop new FMD control strategies including novel attenuated vaccine candidates.

In the last couple of years with the support of NPB we have derived a mutant strain of FMDV A12 that did not cause disease in swine (so-called FMDV-SAP mutant) and induced a strong immune response protecting animals from challenge, as early as two days post vaccination. However in rare occasions, revertants with increased virulence spontaneously arose. Here we report the characterization of new FMDV mutants where we have incorporated additional changes in the Leader and other viral proteins to address reversion to virulence and at the same time to add genetic markers for differentiating infected from vaccinated animals. These mutant strains have the potential for further development into novel live attenuated vaccine candidates to improve immunity against FMD.

**Keywords:** Foot-and-Mouth Disease, FMDV, attenuated FMDV strains, leader protein, SAP-mutant, Leader mutant, DIVA markers.

**Scientific Abstract:** Foot-and-mouth disease virus (FMDV) leader proteinase ($L^{pro}$) is a virulence factor. Viruses with deletions of $L^{pro}$ coding region (leaderless) are viable and display an attenuated

phenotype in vivo in swine and cattle. Attempts to use the leaderless virus as a vaccine have shown promising results but with limitations. In some instances the virus was virulent and in others adaptive immunity fell short of inducing protection against challenge. Recently, we have found that viruses with mutations in $L^{pro}$ SAP domain (SAP mutant) are viable and can mount a strong adaptive immunity in swine. Remarkably, SAP mutant virus inoculated animals developed a strong neutralizing antibody response and were completely protected against challenge with WT FMDV as early as 2 and for at least 21 days post inoculation. However, in rare occasions, SAP mutant virus reverted to virulence. We have also reported that mutations to create negative antigenic markers in the 3D polymerase (3Dpol) and 3B nonstructural proteins generated slightly attenuated mutant viruses. Here we have evaluated the possibility of combining mutations in $L^{pro}$ and other non structural viral protein coding regions to increase stability and safety as well as to be able to differentiate between vaccinated and infected animals (DIVA). We observed that mutation of the SAP domain in A24 FMDV $L^{pro}$ was maintained in vivo but virus was only mildly attenuated. Attenuation increased when SAP mutations were combined with mutations in 3B and 3D that conferred DIVA properties, however mild clinical signs of disease were detected. These results suggest that the level of attenuation could be further manipulated to obtain FMDV strains with potential for live attenuated vaccine development as a novel strategy to control FMD.

**Introduction:** Foot-and-mouth disease (FMD) is one of the most contagious diseases of animals (Grubman 2004). The causative agent, FMD virus (FMDV), is the type species of the Aphthovirus genus within the Picornaviridae family (Grubman and Baxt, 2004). The antigenically variable virus, consisting of 7 serotypes [A, O, C, Asia-1, South African Territories 1-3 (SAT1-3)] and numerous subtypes, affects over 70 species of domestic and wild cloven-hoofed animals including swine). The disease generally causes fever, lameness and vesicular lesions on the tongue, snout, feet, and teats of susceptible animals often with sloughing off of the epithelium and rupture of vesicles releasing copious amounts of virus, resulting in high morbidity but low mortality, except in young animals. FMD is currently present in many areas of the world including Africa, Asia, the Middle East and a few countries in South America, while North America, Central America, Western Europe, Australia and New Zealand are FMD-free. The presence of the disease has a significant effect on the international trade of susceptible animals and their products. The ability to block the spread of FMD to disease-free countries has become more difficult because of the globalization of trade and the rapid movement of goods and people worldwide, as demonstrated in 2001 when outbreaks in the United Kingdom (U.K.) spread to several European countries resulting in losses to the agricultural industry surpassing $15B with more than 10M animals slaughtered. In August of 2007 a new outbreak affected the U.K. but in this instance the cause was attributed to accidental viral escape from the Pirbright campus which houses a commercial FMD vaccine production. More recently, in April 2010, an outbreak in Japan resulted in the slaughter of 300,000 animals and over a $2B loss. The U.S. is the world's No. 2 pork producer and exporter, and the No. 3 importer. An outbreak in our country would result in devastating economic losses considering the $100B/year livestock industry. Furthermore, the events of September 11, 2001 have demonstrated that the U.S. is vulnerable to the deliberate introduction of FMDV. Thus it is imperative to develop novel strategies to effectively control this disease, not only in case of an outbreak but also to reduce the number of cases in enzootic countries, resulting in less risk for FMDV-free countries.

The World Organization of Animal Health (OIE) includes FMD on the notifiable list of diseases and requires the immediate official report of confirmed FMD cases with cessation of trading of susceptible animals including their products. In case of an FMD outbreak, OIE demands restriction of animal/animal-product movement, slaughtering of infected/in contact animals, disinfection of premises and optional ring vaccination with an inactivated vaccine. To regain FMD-free status OIE regulations require that a country demonstrate the absence of disease for 3 months, when slaughtering and disinfection are used as control methods, or for 6 months when animals are vaccinated but not slaughtered (Grubman and Baxt, 2004).

Countries endemic for FMD use an inactivated vaccine to control the disease, but FMD-free countries are reluctant to use it, even in case of an outbreak, for several reasons: the vaccine does not allow for differentiation between vaccinated and infected animals (DIVA), there is a potential risk of deriving asymptomatic disease carriers upon exposure of vaccinated animals to infectious virus, and affected countries need more time to regain FMD-free status when vaccine is used. To overcome some of these limitations, scientists at Plum Island (Moraes et al., 2002) have constructed a recombinant vaccine delivered by a replication-defective human adenovirus type 5 (Ad5) vector. However, either the commercial or the new generation Ad5 vaccines need 7 days to induce protection.
In order to get protection prior 7 days induction of the innate immune response is necessary. Interferons (IFNs) are the first line of the host innate immune defense against viral infection in mammals (Ank et al., 2006; Basler, and Garcia-Sastre, 2002). Chinsangaram et al (2003) demonstrated that FMDV is very susceptible to IFN. Furthermore, at adequate doses, swine can be completely protected when challenged one day after treatment, and protection can last for 3-4 days (Chinsagaram et al 2003, Dias et al 2012). Since inoculation of an inactivated FMDV vaccine or a recombinant Ad5-FMD vaccine induces an adaptive immune response in approximately 7 days, even when IFN and vaccine are used in combination, there is a window of opportunity, between the 5$^{th}$ and 7$^{th}$ day, when the virus can successfully grow and spread. These observations, however, suggested that an attainable goal should be to develop a vaccine able to induce innate responses and adaptive responses within the first week, soon after inoculation. Examples of such strategies include Yellow Fever vaccines for humans (YF-Vax, Sanofi Pasteur) or CSF vaccines for swine (Terpstra, 1991). Indeed, using attenuated viral vaccines, both, smallpox and very recently rinderpest, have been eradicated. So far, no attenuated vaccine has been successfully used against FMDV. A candidate for such a vaccine was previously developed at PIADC, ARS, USDA, by deletion of the coding region for the nonstructural viral protein L$^{pro}$ (leaderless virus) (Mason et al., 1997). Despite the reduced pathogenicity of this virus in swine and cattle, the induced protection against viral challenge was incomplete probably due to the very slow and limited replication of the mutant strain. More recently, we have derived other attenuated strains of FMDV containing mutations in a conserved protein motif within the L$^{pro}$ coding region SAP (SAF-A/B, Acinus and PIAS). Inoculation of swine with the SAP mutant virus did not cause clinical signs of disease, viremia or virus shedding even when inoculated at doses 100-fold higher than those required to cause disease with WT virus. Remarkably, SAP mutant virus inoculated animals developed a strong neutralizing antibody response and were completely protected against challenge with WT FMDV as early as 2 and for at least 21 days post inoculation (Diaz-San Segundo et al., 2012). However, SAP mutant virus could revert to virulence and this virus did not allow for differentiation between infected or vaccinated animals (DIVA). Introducing mutations in the 3B or 3D region of FMDV genome is known to support DIVA diagnostic test (Uddowla et al., 2012). We hypothesized that combination of SAP mutations with mutations in 3B and 3D region that conferred DIVA capabilities would prevent reversion to virulence and further attenuate the virus to be evaluated as a DIVA attenuated vaccine candidate. Here we report that, in the context of FMDV serotype A24, SAP mutations resulted in a partially attenuated virus in swine. Initial attempts to obtain a virus with SAP mutation in combination with deletion of two 3B coding regions did not result in a viable virus. However, the use of other mutations in 3B and 3D region gave a viable virus, resulting in increased attenuation in vitro and in vivo, although animals still showed very mild disease. These results suggest that manipulation of the L$^{pro}$ coding region in combination with other FMDV genome regions to derive live attenuated strains with DIVA capability is a viable strategy that deserves further development. However careful considerations and exhaustive experimental testing is required to develop effective vaccines candidates against FMD.

**Objectives:**

1. Test the virulence in swine of A24 FMDV L$^{SAP}$.
2. Construct and characterize in vitro, FMDV strains with mutations in the L$^{SAP}$ in combination with deletions/mutations in the 3B coding regions.
3. Test the virulence in swine of mutant FMDV L$^{SAP}$ 3Bmut strains.

**Materials & Methods:**

**Cells and viruses:** Porcine kidney (IBRS-2) cell lines were obtained from the Foreign Animal Disease Diagnostic Laboratory (FADDL) at the PIADC. These cells were maintained in minimal essential medium (MEM, GIBCO BRL, Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS) and supplemented with 1% antibiotics and non-essential amino acids. BHK-21 cells (baby hamster kidney cells strain 21, clone 13, ATCC CL10), obtained from the American Type Culture Collection (ATCC, Rockville, MD) were used to propagate virus stocks and to measure virus titers. BHK-21 cells were maintained in MEM containing 10% calf serum and 10% tryptose phosphate broth supplemented with 1% antibiotics and non-essential amino acids. Cell cultures were incubated at 37°C in 5% CO2.

FMDV A24 WT was generated from the full-length serotype A24Cru infectious clone, pA24IC, as described below. A24-SAP mutant virus was constructed by introducing mutations in pA24IC using a site directed mutagenesis QuikChange® kit (Qiagen, Valencia, CA) and following the manufacturer's directions. A24-SAP virus with deletions in 3B, was constructed by replacing a DNA fragment of pA24IC-SAP with a synthetic fragment containing a deletion of 2 of the 3B coding regions. In addition, A24-SAP 3B3D mutant virus was derived by partial deletion of 3B and point mutations in the 3D coding regions by site directed mutagenesis of pA24IC using also QuikChange® kit (Qiagen ) and following the manufacturer's directions.

**Virus derivation using full length A24 FMDV clones containing combined mutations in the SAP domains of the FMDV Lpro and 3B or 3D regions:** Plasmids containing the full-length A24Cruzeiro genome with mutations in conserved residues of the SAP with or without mutations in 3B and 3D regions were linearized and then transcribed into RNA using Mega using a MegaScript T7 Kit (Ambion, Inc., Austin, TX) using standard protocols. Following electroporation in BHK21 cells the transfections were incubated at 37°C and at 24h were freeze-thaw and used for five further passages.

All viable viruses were propagated in BHK-21 cells, concentrated by polyethylene glycol precipitation, titrated on the same cells and stored at -70°C.

**Animal experiments:** Two animal experiments were performed in the high-containment facilities of the Plum Island Animal Disease Center following a protocol approved by the Institutional Animal Use and Care Committee. In the first experiment 5 groups of 3 animals each (Yorkshire guilts, five weeks old and weighing approximately 40 lbs) were intradermally (ID) inoculated in the heel bulb of the right rear foot with two doses of FMDV A24-WT ($1x10^4$ or $1x10^5$pfu/animal) or three different doses of FMDV A24-SAP mutant ($1x10^5$, $1x10^6$ and $1x10^7$ pfu/animal). Rectal temperatures and clinical signs, including lameness and vesicular lesions, were monitored daily during the first week and samples of serum and nasal swabs were collected on a daily bases. Serum samples were also collected at days 4 and 21 days post inoculation (dpi) to check for the presence of neutralizing antibodies. Clinical scores were determined by the number of toes presenting FMD lesions plus the presence of lesions in the snout and/or mouth. The maximum score was 17, and lesions restricted to the site of inoculation were not counted. In the second experiment 3 groups of 3 Yorkshire guilts each (five week old and weighing approximately 40 lbs) were used. Each group was ID inoculated with $1x10^5$ pfu/animal of each of three different FMDV: A24-WT, A24-SAP or A24-$SAP_{3B3D}$. As in the previous experiment, rectal temperatures and clinical signs were monitored daily during the first week and samples of serum and nasal swabs were collected on a daily bases. Serum samples were also collected at days 4, 7, 14 and 21 days post inoculation (dpi) to check for the presence of neutralizing antibodies.

**Virus titration in serum and nasal swabs:** Serum and nasal swabs were assayed for the presence of virus by plaque titration on BHK-21 cells (passage levels 60-70). Serial ten-fold dilutions of the samples were allowed to adsorb on monolayers of BHK-21 cells grown in 6-well plates. Following 1 h adsorption, the inoculum was removed and 2 ml of MEM containing antibiotics, essential amino acids and 0.6 % gum tragacanth was added to each well. The plates were incubated for 24 h at 37°C in a humidified atmosphere containing 5% CO2 and then stained with a crystal violet-formalin solution to visualize the plaques. Virus titers were expressed as log10 pfu per ml of serum or nasal swab. The detection level of this assay is 5 pfu/ml.

**Determination of neutralizing antibody titer:** Sera samples were tested for the presence of FMDV-specific neutralizing antibodies by a plaque reduction neutralization assay as previously described (Diaz- San Segundo et al., 2012). Neutralizing titers were reported as the serum dilution yielding a 50% reduction in FMDV A24-WT $TCID_{50}$ in BHK-21 cells.

**Results:**

**Virulence of SAP mutation in the context of FMDV field strain A24.**
To this end all our studies had been performed using FMDV strain A12. However, there is interest in developing FMDV vaccines against serotype A24, a field strain with potential of circulating in South America. Utilizing an FMDV A24 infectious clone recently obtained in Plum Island (Rieder et al., 2005) we prepared new FMDV strain with mutations in $L^{pro}$ SAP, I55A and L58A, identical to the SAP mutant in serotype A12 since this region is conserved in both subtypes.
To compare the virulence of FMDV WT, A24-WT, with an FMD mutant virus containing mutations in the SAP domain of $L^{pro}$ (A24-SAP) groups of three pigs were inoculated intradermally (ID) in the rear heel bulb with different doses of FMDV. Animals were inoculated with $10^4$ and $10^5$ plaque forming units (pfu) per animal of A24-WT, a dose previously shown to cause clinical disease in swine, and with $10^5$, $10^6$ and $10^7$ pfu/animal of mutant A24-SAP. One extra animal was housed in each of the rooms containing the groups inoculated with A24-SAP FMDV to examine transmission by direct contact. All animals inoculated with A24-WT virus developed clinical signs of FMD by 2-3 days post inoculation (Fig. 1). Animals inoculated with the highest dose of A24-SAP also developed clinical signs by 2-3 days postinoculation although the severity of disease was reduced as compared to the WT inoculated groups. Disease also developed in pigs inoculated with lower doses of A24-SAP mutant virus but later and less severe than the in the other groups. All contact animals also showed clinical FMD signs with slight delay as compared with the animals within the same group (Fig. 1).

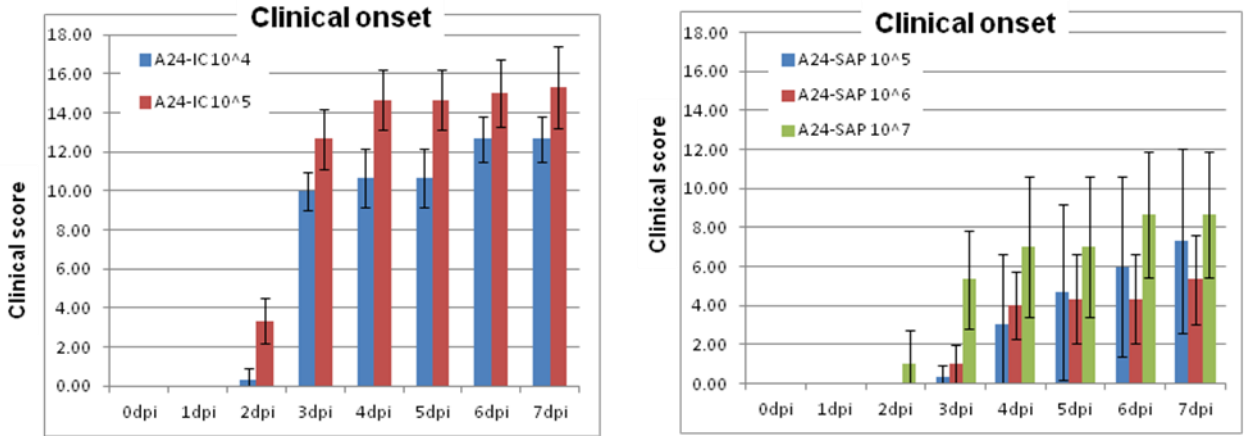


**Figure 1: Clinical outcome after FMDV A24-WT or A24-SAP inoculation.** Different doses of A24-WT (A24-IC) ($10^4$ or $10^5$ pfu/animal) or A24-SAP mutant ($10^5$, $10^6$ or $10^7$ pfu/animal) FMDV were inoculated in groups of swine. Clinical signs were monitored daily during 7 days post inoculation (dpi). Clinical score is expressed as number of toes with lesions plus one more point when lesions were present in the mouth/snout (maximum score is 17). Each data point represents the mean (± SD) of each group.

In accordance with clinical data, virus was detected in all animals in serum and in nasal secretion. However, the level of virus detected was lower in those animals inoculated with the lower doses of mutant virus as compared to WT-inoculated animals (Fig. 2).

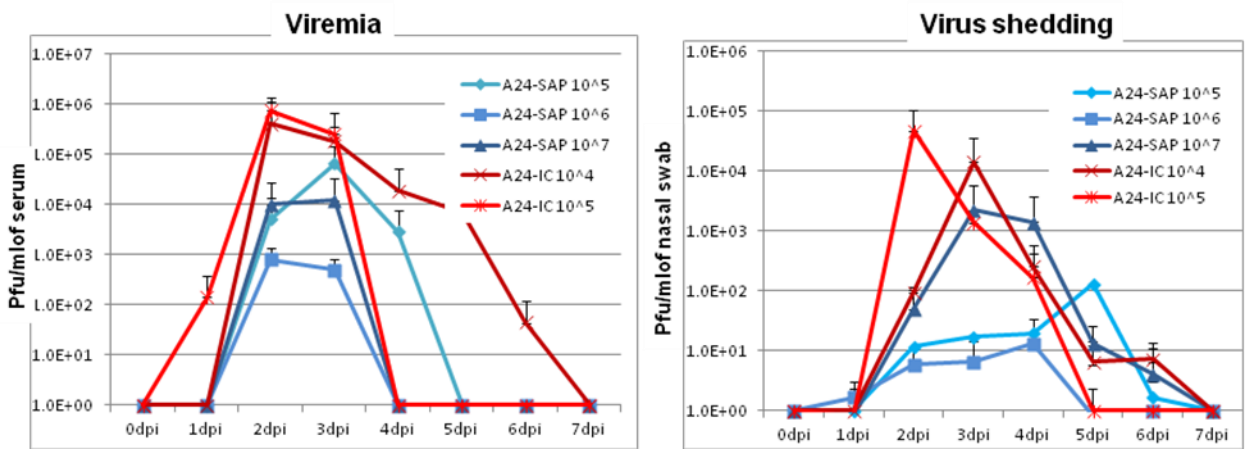


**Figure 2: Detection of viremia and virus shedding in swine after FMDV A24-WT or A24-SAP inoculation.** Virus titers were determined in blood and nasal swabs collected daily from animals inoculated with FMDV A24-WT or A24-SAP from 0 to 7 days post infection. Each data point represents the mean (± SD) of each group.

The levels of neutralizing antibodies were determined in all inoculated animals. As expected, all animals inoculated with A24-WT virus developed high antibody titers starting at 4 days and reaching a maximum titer by 14 days post inoculation (Fig. 3). All animals inoculated with A24-SAP developed significant levels of FMDV specific neutralizing antibodies (Fig. 3), including the contact animals (data not shown). Interestingly, at 21 dpi the levels neutralizing antibodies were slightly higher in the groups inoculated with A24-SAP mutant than in the groups inoculated with WT virus, regardless of the used dose.

Virus recovered from these animals showed no additional mutations or reversions acquired during pig infection. These results indicated that mutation of the SAP domain in A24 FMDV was maintained in vivo but results resulted in mild attenuation, although at lower degree of pathogenicity than seen in the previously tested A12-SAP serotype mutant virus.

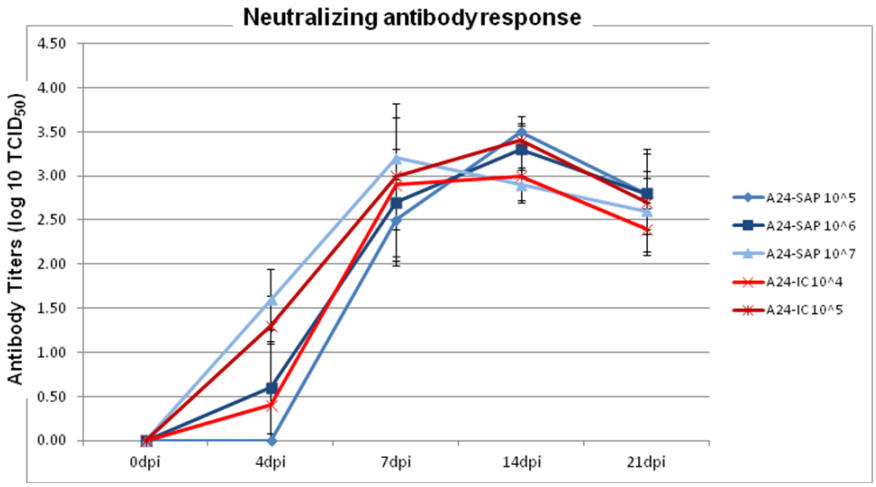


**Figure 3. FMDV A24-WT or A24-SAP serum neutralization titers.** Titers are expressed as the $\log_{10}$ of the inverse dilution of serum yielding a 50% reduction in the number of $TCID_{50}$/ml. Each data point represents the mean (± SD) of each group.

**Construction and characterization in vitro of FMDV strains with $L^{pro}$ SAP mutations in combination with mutations/deletions in the 3B and 3D coding regions.**

In a first approach to introduce antigenic marker suitable for tracking the origin of the virus in animal immunization experiments (DIVA), we first attempted to introduce a deletion of two of the three 3B peptides encoded in the FMDV genome. These deletions eliminate a dominant epitope encoded in the 3B1-3 non-structural proteins and could result in further attenuation of the virus. Furthermore, these deletions have the potential for DIVA capabilities since they eliminate relevant antigenic sites contained in these peptides. Out of three cDNA constructs only the A24-3B3 mutant produced viable progeny. Unexpectedly, the construct containing the combined modification A24-SAP-3B3 did not render viable virus. Analysis of the full genome transcript RNA by in vitro translations using cell-free rabbit reticulocyte lysates with mutant and WT transcript RNA suggested that the lack of viability of the double mutant could have been the result of a translational defect (data not shown). This was surprising considering that each of the two independent modifications built into the A24 Cruzeiro backbone (A24-SAP, -and A24-3B3) had independently produced viable virus.

As a contingency plan, we constructed another mutant virus, A24-$SAP_{3B3D,}$ carrying two of the three encoded 3Bs and the 3D polymerase with mutations that eliminated conserved antigenic sites as previously reported (Uddowla et al., 2012). Transfection of the pA24-$SAP_{3B3D}$ infectious clone resulted in virus progenie that was characterized in vitro. Interestingly FMDV A24-$SAP_{3B3D}$ showed an attenuated phenotype in primary cells which have a competent interferon system but grew to similar levels as the WT virus in an established cell line under no IFN selective pressure (Fig.4).

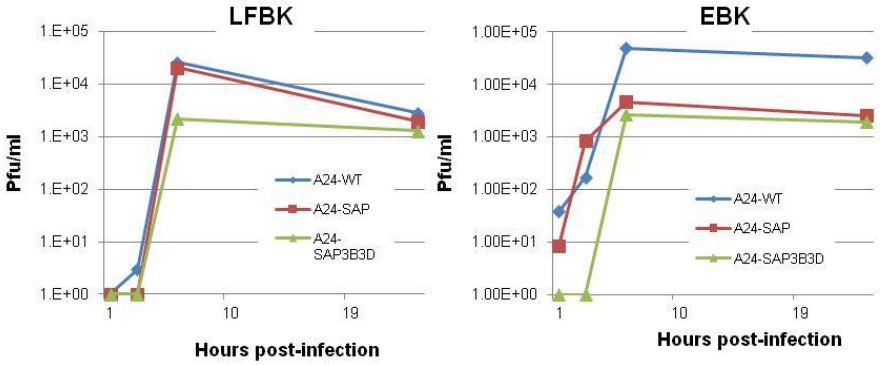


**Figure 4: Kinetics of growth.** LF-BK or EBK cells were infected with the indicated viruses and after 1h, unabsorbed virus was removed by washing with 150 mM NaCl, 20 mM MES (pH=6.0) followed by addition of complete media. Samples were taken at 1, 3, 6 and 24 hpi and virus titers were determined by plaque assay on BHK-21 cells.

The new mutant virus A24-$SAP_{3B3D}$ was able to process cellular proteins during infection as well as the A24-SAP mutant, although processing was slightly delayed as compared to WT virus (Fig. 5).

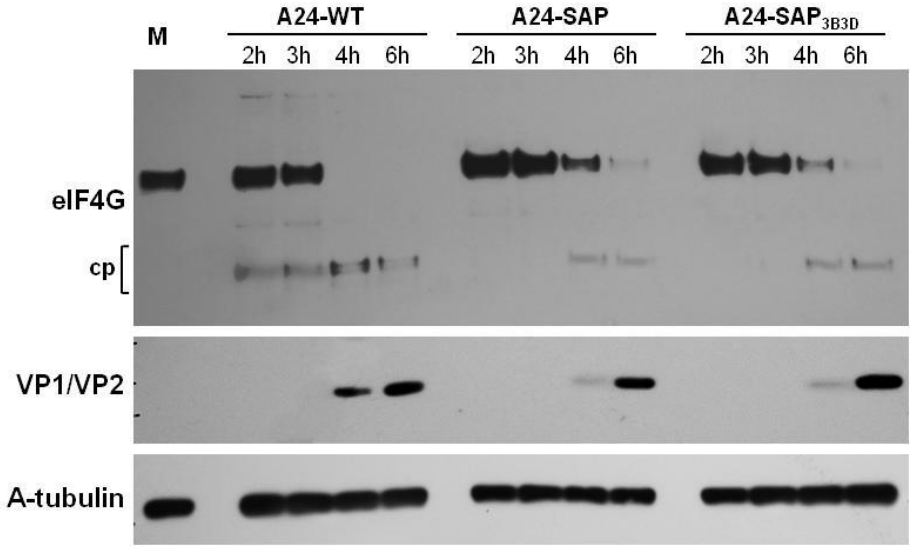


**Figure 5: Processing of cellular proteins during infection.** LF-BK cells were infected with A24-WT, A24-SAP or A24-$SAP_{3B3D}$ at MOI=10 for up to 6 h and samples were collected at the indicated timepoints. Total cell extracts were analyzed by western blotting using anti eIF-4G (p220), anti VP1 and VP2 and anti tubulin-α Abs. M stands for Mock infected cells. CP: p220 cleavage products.

We previously described that SAP mutation altered $L^{pro}$ sub-cellular localization during the course of infection. Interestingly, although its capacity for inhibiting translation was not affected, infection with SAP mutant virus in vitro did not induce degradation of NF-κB and the levels of several transcripts including cytokines, chemokines and other IFN stimulated genes (ISGs) were significantly higher than those induced by WT virus infection (de los Santos et al, 2009). Immunofluorescence analysis of the newly generated A24 viruses showed that subcellular localization of the $L^{pro}$ of A24 –SAP and A24-$SAP_{3B3D}$ was similar to the previously reported A12-SAP $L^{pro}$ mutant (Diaz-San Segundo et al., 2012). Early during infection A24-SAP $L^{pro}$ localized to the cytoplasm of the cells to later migrate to the nucleus of the infected cell. However, at later time points, A24 SAP $L^{pro}$ nuclear staining disappeared suggesting the mutant protein was not retained in the nucleus (data not shown). Similarly to A12-SAP $L^{pro}$, A24-SAP and A24-$SAP_{3B3D}$ did not induce degradation of NF-κB at any time during infection (Fig. 6).

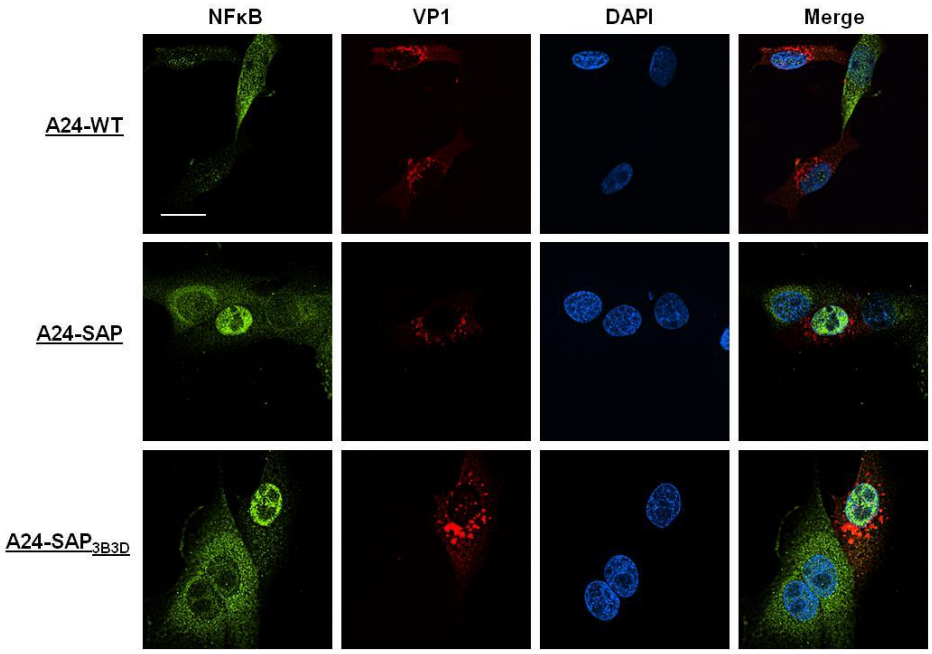


**Figure 6: Nuclear p65 NF-κB subunit signal does not disappear from the nucleus in A24-SAP or A24-SAP$_{3B3D}$ infection**. LF-BK cells were infected with A24-WT, A24-SAP or A24-SAP$_{3B3D}$ FMDV at MOI=10. Six hours post infection cells were fixed and p65/RelA (NF-κB) and viral protein VP1 were detected by indirect immunofluorescence. Bar=20μm

These data indicated that A24-SAP$_{3B3D}$ conserved the characteristics of A24-SAP virus but it was more attenuated in vitro.

**Virulence of A24-SAP$_{3B3D}$ mutant in vivo.**

As mentioned above, A24-SAP$_{3B3D}$ mutant was more attenuated than A24-SAP mutant in vitro. Therefore we decided to test virulence in vivo in swine, a natural host. Groups of three pigs were inoculated ID in the rear heel bulb with $10^5$ pfu of A24-WT, A24-SAP or A24-SAP$_{3B3D}$ FMDV. All animals inoculated with A24-WT virus developed clinical signs of FMD by 2-3 days post inoculation reaching high scores, averaging from 14 to17 out of a possible maximum of 17 by 4dpi (Fig. 7). Animals inoculated with the A24-SAP showed a slight delay in the onset of disease but severity of disease was significantly lower than in the WT inoculated group; lesion scores reached average values of 4-7. In the case of pigs inoculated with A24-SAP$_{3B3D}$ mutant virus, disease appeared even later and was less severe than in the A24-SAP group with average scores of 2-6 appearing by 5-7 dpi. (Fig.7). These results confirmed that combination of mutations in SAP domain of L$^{pro}$ with mutations in other regions of the viral genome increase attenuation in vivo.

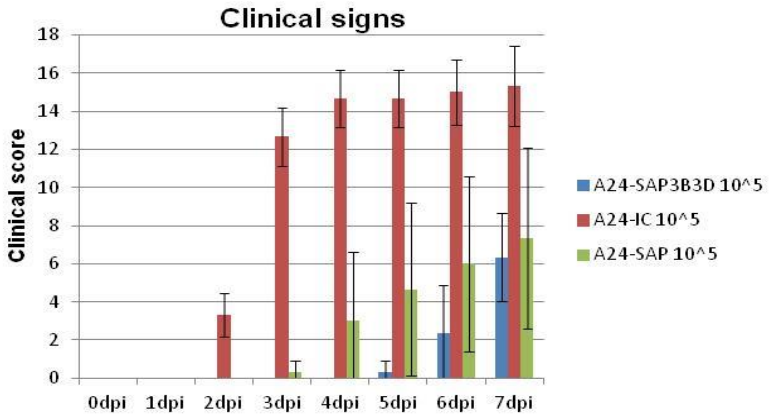


**Figure 7: Clinical outcome after FMDV A24-WT, A24-SAP or A24-SAP$_{3B3D}$ inoculation in swine.** Clinical signs were monitored daily during 7days post inoculation (dpi). Clinical score is expressed as number of toes with lesions plus one more point when lesions were present in the mouth/snout (maximum score is 17). Each data point represents the mean (± SD) of each group.

**Discussion:** As a virulence factor, FMDV L$^{pro}$ has a key role in antagonizing the innate immune response. In fact, virus genetically manipulated to lack L$^{pro}$ coding region, leaderless virus, is highly attenuated in cattle and swine due to the inability to block IFN production (Chisangaram et al., 2003; de los Santos et al., 2006). However, this attenuated virus could not be used as vaccine since it was not immunogenic enough to protect animals against homologous challenge (Chinsangaram et al., 1998, Mason et al., 1997). Recently we have constructed an FMDV variant containing mutations in a conserved domain of the L$^{pro}$ coding region, A12-SAP, which was attenuated *in vitro* and *in vivo* in swine. Remarkably, mutation of just two amino acid residues within the L$^{pro}$ SAP domain prevented virus spread and disease, but was sufficient to induce complete protection against WT FMDV homologous challenge. We showed that inoculation with FMDV A12-SAP mutant induces humoral and cellular immunity to equivalent levels found during infection with WT FMDV, and strikingly protection was observed as early as 2 days post vaccination (Diaz-San Segundo et al., 2012).
These observations suggested that the SAP mutant was able to induce early activation of cytokine networks to mount effective innate and adaptive immune responses against FMDV. In fact, no other vaccine platform evaluated up-to-date has been able to induce such an immune response. Live attenuated vaccines usually induce the same repertoire of immune responses as the wild type virus infection but without causing disease, in an equilibrium that might be hard to achieve experimentally. Nevertheless, such vaccines have been successfully developed and its use resulted in the eradication of two very important viral diseases, namely smallpox and rinderpest. One of the main challenges of making a live attenuated vaccine is to select and incorporate mutations that will be stable, thus having a low probability of reversion back to virulence. Deletion mutations are perhaps the most stable ones but usually they are not viable or they cause too much attenuation eliciting a poor immune response. Other mutations that include amino-acid substitutions and mutated viral proteins are usually less attenuated but can be subjected to reversion to wild type phenotype, since viral RNA polymerases are error prone and introduce a relatively high number of mutations to adjust for fitness in each particular environment. Tissue culture passage of SAP mutant virus displayed remarkable stability of the SAP mutation for at least 12 passages in tissue culture suggesting that this mutant could potentially be developed as a live attenuated vaccine candidate.
On the other hand, another big challenge with vaccination is to attain the capability of differentiating between vaccinated and infected animals. This is particularly important in the case of FMD, because in case of vaccination with the current commercial vaccine the country needs more time to gain FMD-free status. This would not be the case if vaccination were performed with a DIVA vaccine.

In this work we moved from working with a FMDV lab strain, A12, to using a field strain with potential of circulating in South America, namely A24Cruzeiro. This field strain is more virulent than the A12. Since the SAP domain is conserved in most serotypes and subtypes we introduced in A24 $L^{pro}$ the same mutations previously incorporated in A12 $L^{pro}$. Although A24-SAP virus was attenuated in vitro and in vivo, attenuation was milder than for A12-SAP. A12-SAP inoculated animals did not develop disease even at doses 100x higher than the A12-WT dose required to causing disease in pigs. Instead, A24-SAP viruses caused disease at a lower dose although disease developed later and with reduced severity. This was not surprising since there are many virulence factors involved, and although sequences of this two viral strains are similar, the sequence of the capsid, among other regions, differs.

Combination of mutation in $L^{pro}$ region with mutations in other location of the viral genome not only would result in further attenuation but also could be designed to include DIVA markers. Therefore, we attempted to introduce a deletion of two of the three 3B peptides encoded in the FMDV genome thus eliminating a dominant epitope encoded in the 3B1-3 non-structural proteins that could also result in further attenuation. Furthermore, this deletion has the potential for DIVA capabilities since it eliminates relevant antigenic sites contained in these peptides. Unfortunately, we were not able to derive virus from the infectious DNA clone. This was surprising considering that each of the two independent modifications built into the A24 backbone (A24-SAP, -and A24-3B3) had independently produced viable virus.

As a contingency plan, we constructed a mutant virus, A24-$SAP_{3B3D,}$ carrying only two of the three encoded 3Bs with mutations that eliminated a conserved antigenic site in the viral 3B2 and 3D polymerase that were previously described in Uddowla et al., 2012. The virus obtained was more attenuated than A24-SAP in vitro and in vivo. Interestingly animals showed a very mild disease that did not appear until 5 days post-inoculation.

Our results indicate that incorporation of DIVA markers in the FMDV SAP backbone is tolerated and attenuation could be augmented in vitro and in vivo. However, there is a need to achieve a higher level of attenuation. Structure-function data of FMDV $L^{pro}$ suggests that additional mutations in the A24 SAP $L^{pro}$ coding region should be tolerated to render viable viruses. We expect that these mutations will increase virus stability and attenuation thus improving safety. These live attenuated virus strains will ultimately be capable of rapidly controlling FMD, displaying DIVA properties and inducing long lasting protection, thus addressing the main shortfalls of current available FMD vaccines.

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