RESEARCH REPORT
SWINE HEALTH

Title: ***Exploiting the potential of leader proteinase coding sequence of foot-and-mouth disease virus to derive attenuated strains suitable for pathogenesis studies and development of improved countermeasures*** **– NPB** #12-023

Investigator: Dr Teresa de los Santos

Institution: Plum Island Animal Disease Center, North Atlantic Area, Agricultural Research Service, United States Department of Agriculture, Orient Point, NY, 11957

Date Submitted: June 2nd, 2013

Industry Summary: FMD is the one of the most feared viral diseases that can affect swine. Although this disease appeared to be eliminated from most developed nations by the end of last century, recent outbreaks in Europe, Japan, Taiwan, South Korea, Eastern Russia, etc, have demonstrated that infection can spread as wild fire affecting any nation and causing devastating economic and social consequences (Figure 1). Furthermore, post 9/11, a new era has emerged showing that any country is vulnerable to national and international terrorism. An outbreak of this disease would be devastating to the US economy which deals with the largest livestock market of the world. Therefore, it is essential to develop new control strategies that could confer very early protection and stop disease spread. FMD is a highly contagious disease that spreads very quickly among susceptible animals. The current FMD vaccine is a formulation of inactivated wild type (WT) virus antigen prepared in high containment bio-security level 3 facilities. This vaccine requires 7 days to induce protection, a time during which vaccinated animals are still susceptible to disease mostly when exposed to FMD virus (FMDV). In addition the vaccine is prepared from highly virulent circulating virus strains that despite extreme caution and care in the manufacturing process, could result in outbreaks by accidental virus release as it happened in the United Kingdom in 2007 (estimated cost $2B). It has been demonstrated for other viral diseases that live-attenuated vaccines are one of the best choices to obtain a strong early and long-lasting protection. Our goal is to develop alternative control strategies that could improve current FMD countermeasure programs based on the development of attenuated vaccines. An attenuated vaccine is expected to elicit more rapid innate immunity and a long lived adaptive immunity to effectively control disease. Moreover, induction of innate immunity could result in early protection against multiple FMDV serotypes. From the production perspective, use of an attenuated FMDV strain will reduce the consequences of accidental outbreaks caused by accidental release of virus from vaccine manufacturing facilities. Importantly, attenuated strains are excellent new tools to study the interactions between FMDV and the host immune system and ultimately could lead to the development of novel strategies to counteract FMD. In the last couple of years with the support of NPB we have successfully derived a mutant strain of FMDV that did not cause disease in swine (FMDV-SAP mutant). Interestingly inoculation of swine with this mutant strain induced a strong immune response that protected animals against infection with the parental WT virus, as early as two days post vaccination. However in rare occasions, revertants with increased virulence spontaneously arose, indicating that improvement is required for safety. Utilizing reverse genetic approaches we had explored the

These research results were submitted in fulfillment of checkoff-funded research projects. This report is published directly as submitted by the project's principal investigator. This report has not been peer-reviewed.

For more information contact:
National Pork Board • PO Box 9114 • Des Moines, IA 50306 USA • 800-456-7675 • Fax: 515-223-2646 • pork.org

possibility of adding more mutations in the SAP mutant to increase stability and attenuation. We had observed that some mutations in other Leader domains, like FHA domain, (e. g. H*) cause attenuation independently of SAP, however double mutants (SAP, H*) did not produce viable virus. In contrast, other SAP-derived strains with mutations in another domain of the L protein (CTE) are viable. We are currently characterizing the SAP-CTE mutants for further development into a live attenuated vaccine platform. Our results indicated that manipulation of the viral genome in the region that encodes for the leader protein is a viable alternative to derive less pathogenic FMDV strains that could be used as the basis for live attenuated vaccines against FMD or as seeds to grow the virus for manufacturing safer inactivated vaccines. Although this strategy is far from being used in countries that are FMD free without vaccination, it could be an affordable alternative to control FMD in regions of the world where the disease is enzootic thereby decreasing the risks of dissemination to disease-free nations. Ultimately a combination of strategies tailored to each region of the world will eventually succeed to eradicate this feared disease.

(Teresa de los Santos PhD, PIADC, ARS, USDA. TEL: (631) 323-3020. E-mail: teresa.delossantos@ars.usda.gov).

Keywords: Foot-and-Mouth Disease, FMDV, attenuated FMDV strains, leader protein, inflammatory response, SAP-mutant, H* mutant, CTE mutant.

Scientific Abstract: We have previously demonstrated that the foot-and-mouth disease virus (FMDV) leader proteinase (Lpro) is a virulence factor. Viruses with deletions of Lpro 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 Lpro SAF-A/B, Acinus and PIAS 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. Here we have evaluated the possibility of adding other mutations in the Lpro coding region to increase stability and safety. We observed that some mutations (H* mutants) were tolerated standing alone but not in combination with SAP, however other mutants (CTE) were viable and stable rendering viable viruses. We are currently characterizing these new viruses for further development into live attenuated vaccine candidates. On the other hand, we have evaluated the possible serotype cross protection rendered by SAP mutant attenuated virus in early challenge experiments.

Introduction: FMD is a highly contagious disease of domestic and wild cloven-hoofed animals including swine, cattle, sheep, goats, deer and buffalo that quickly replicates in the host spreading to susceptible animals by contact and aerosol. The disease is characterized by fever, lameness and vesicular lesions on the tongue, feet, snout and teats resulting in high morbidity but low mortality, except in young animals.

FMD is enzootic in all continents except for Australia and North America (Fig 1). Although the U.S. has been FMD free since 1929, recent natural outbreaks in previously disease-free countries have significantly increased the public awareness about this disease. In 2001, outbreaks in the United Kingdom (U.K.) that spread to several European countries resulted 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, world globalization and the events of September 11, 2001 have demonstrated that the

U.S. is vulnerable to the deliberate or accidental 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).

Currently, use of an inactivated vaccine is customary in non FMD-free countries but FMD-free countries are reluctant to use this vaccine for several reasons: vaccine manufacturing requires a biosafety level 3 (BSL3) containment facility, the vaccine does not allow for differentiation between vaccinated and infected animals, 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 FMDV-free status when vaccine is used. In addition, the current vaccine does not confer protection before 7 days post vaccination (dpv). 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. Although effective, protection induced by this new platform does not occur until 7 dpv, and duration of immunity is shorter than the one conferred by natural FMDV infection.

Scientists at Plum Island have also shown that FMD is highly susceptible to interferons (IFNs). Using the same Ad5 vector, Chinsangaram et al., (2003) demonstrated that swine inoculated with an Ad5 that delivers porcine type I IFN are completely protected when challenged one day after vaccination and protection lasted for 3-4 days. 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 5th and 7th 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 C 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 Lpro (leaderless virus). 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 Lpro 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. However in few cases we observed that the virus could revert to virulence causing disease. We hypothesized that if possible, addition of more mutations in the Lpro coding region could be tolerated and prevent reversion to virulence. We have identified other conserved regions within the Lpro coding sequence that are or may be involved in interactions with host factors (FHA and CTE domains). In preliminary studies we had found that mutation of a conserved residue in the FHA domain (H*) also yielded a viable virus with an attenuated phenotype in cell culture. Here, we report our results to develop new FMDV attenuated strains with mutations in the SAP, FHA and CTE domains. Although viruses containing FHA mutations alone could be derived, no double mutants SAP-FHA sustained viability. However double viable mutants SAP-CTE were obtained. These results suggest manipulation of the Lpro coding region to derive live attenuated

strains is a viable strategy however careful considerations and exhaustive experimental testing is required to develop effective vaccines candidates against FMD. On the other hand, since SAP mutant vaccine induced protection in 2 days, we tested whether this innate induced protection was strong enough to protect against other FMDV serotypes.

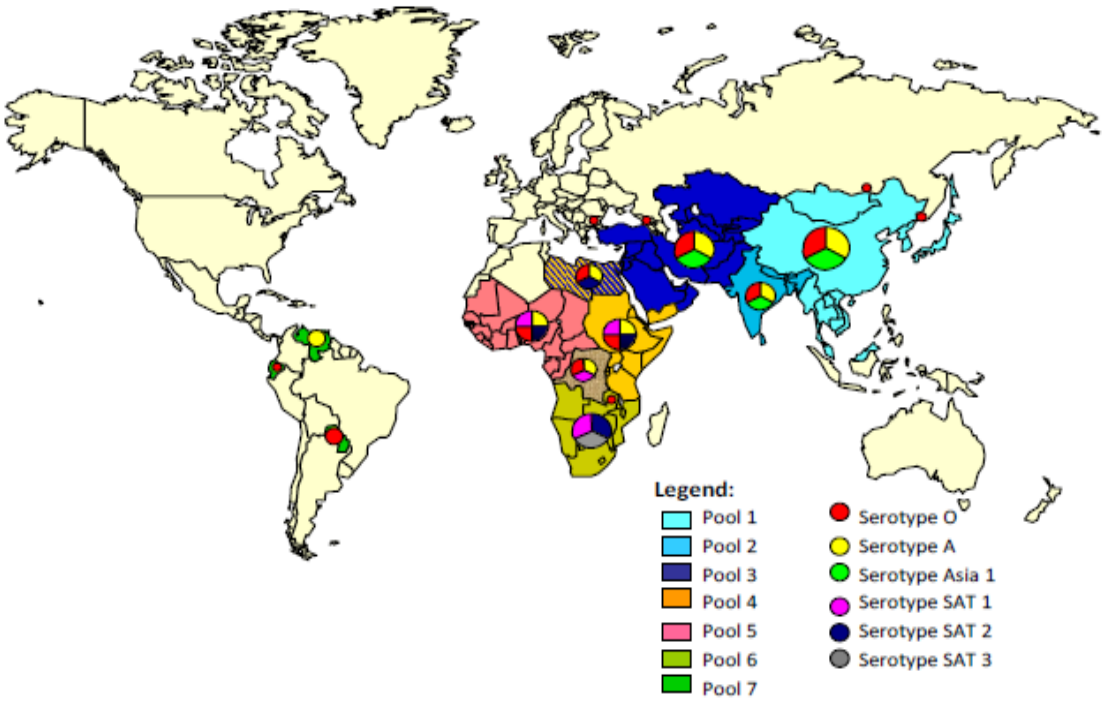


Fig. 1. FAO map showing worldwide FMD status in October 2012

Objectives:

1. Test the virulence in swine of FHA mutant FMDV.
2. Construct and characterize in vitro, FMDV strains with mutations in the L^{pro} CTE domain alone and in combination with mutations in the SAP and/or FHA domains.
3. Test the virulence in swine of FMDV strains with a combination of mutations in the L^{pro} coding region.
4. Test if the early protection detected after inoculation with SAP mutant virus is effective against challenge with different FMDV serotypes (cross-protection).

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 A12-WT and FMDV A24 WT were generated from the full-length serotype A12 an A24Cru infectious clones, pRMC35 and pA24IC as described below. A12-H* and A24-CTE mutant viruses,

were constructed by site directed mutagenesis using a QuikChange® (Qiagen, Valencia, CA) kit and following the manufacturer's direction.

Virus derivation using full length A24Cru FMDV clones containing combined mutations in the SAP and CTE of domains of the FMDV Lpro: Plasmids containing the full-length A24Cruzeiro genome with mutations in conserved residues of the SAP and CTE regions of Lpro 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 37C and at 24h were freeze-thaw and used for five further passages. A24-SAP/and two out of three CTE mutants transcript RNA yielded viable viruses and full sequences indicated that the mutations were maintained. Further passages of the viable and non-viable viruses in LFBK are currently being conducted.
All viable viruses were propagated in BHK-21 cells, concentrated by polyethylene glycol precipitation, titrated on the same cells and stored at -70^{o}C. Viruses of passage #6 for A12-WT and passage #5 for A12-H* were used for animal experiments and the full length sequences were confirmed by DNA sequencing of derived viral cDNA using an ABI prism 7000 (Applied Biosystems, Foster City, CA).

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 three 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 FMDV A12-WT (5x10^{5} pfu/animal) or two different doses of FMDV A12-H* mutant (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 we used 6 groups of 3 Yorkshire guilts each (five week old and weighing approximately 40 lbs). Three groups were subcutaneously (SC) vaccinated with attenuated FMDV A12-SAP (5x10^{5} pfu/animal) and 3 groups were inoculated with PBS to use as controls. All animals were challenged at 2 days post-vaccination (dpv) with 5x10^{5}pfu/animal of wild type FMDV serotypes A24, Asia 1 or O1 Manisa ID in the heel bulb. After the challenge, clinical disease was monitored daily during the first week and samples were collected as described for the first experiment.

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 (48). Neutralizing titers were reported as the serum dilution yielding a 70% reduction in the number of plaques (PRN70) induced by FMDV A12-WT in BHK-21 cells.

Results

1. Virulence of FHA mutant FMDV in swine.

To compare the virulence of FMDV WT, A12-WT, with an FMD mutant virus containing mutations in the FHA domain of Lpro (A12-H*), groups of three pigs were inoculated intradermally (ID) in the rear heel bulb with different doses of FMDV. Animals were inoculated with $5x10^5$ plaque forming units (pfu) per animal of A12-WT, a dose previously shown to cause clinical disease in swine, and with 10^6 and 10^7 pfu/animal of mutant A12-H* and followed as depicted in (Fig. 2)

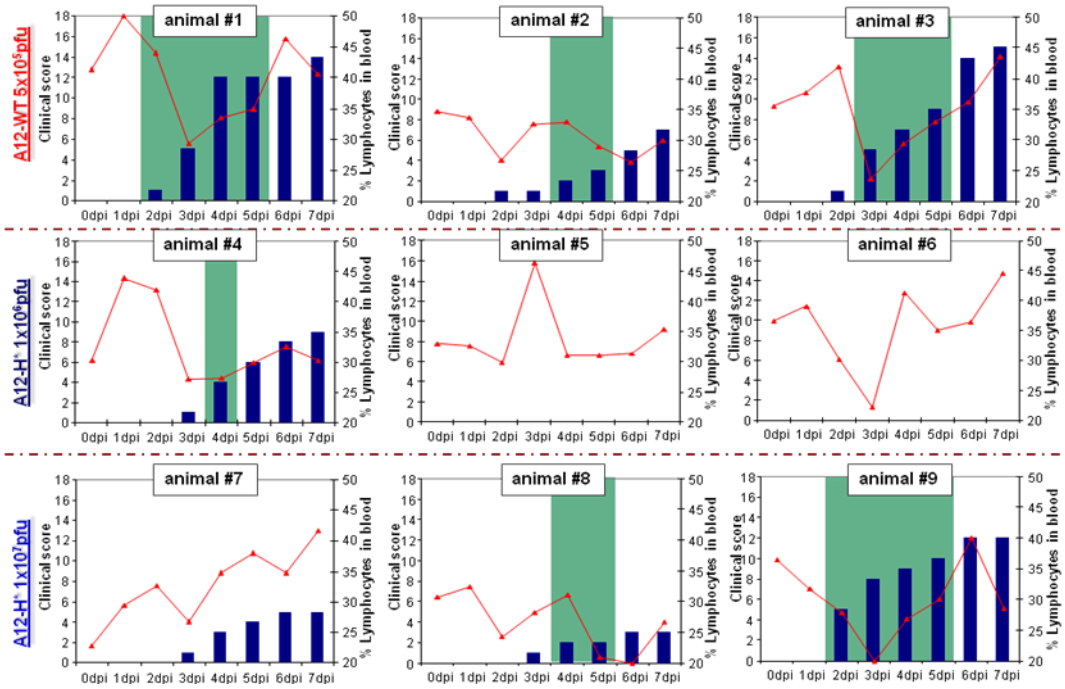


Figure 2: Clinical outcome after FMDV A12-WT or A12-H* inoculation. Clinical signs (bars), % of lymphocytes in blood (line) and body temperature (green area) were monitored daily during 7days post inoculation (dpi). Clinical score is expressed as number of toes with lesions plus one more point when lesions were present in the mouth/snout (maximum score is 17). % of lymphocytes in blood was detected using a Hemavet® equipment. Green area represents the period of time when animals had temperature over 40°C. Each graph represents data of an individual pig.

All three animals inoculated with $5x10^5$ plaques of A12-WT virus developed clinical signs of FMD by 2 days post inoculation (Fig. 2). In contrast, two animals inoculated with 2 times more (10^6 pfu) of FMDV A12-H* mutant did not develop clinical signs, and the third animal developed milder disease by 3 days post inoculation. All three animals inoculated with a 20 times higher dose of mutant virus (10^7 pfu) developed disease. However disease was of reduced severity and appeared a day later in 2 of the 3 animals of this group.

Consistently a decrease in the number of lymphocytes was detected prior to the peak of detectable levels of virus in blood (viremia) in both A12-WT and A12-H* inoculated animals (Fig. 2). In addition, two of the A12-H* inoculated animals did not develop fever (body temperature over 40°C) during the course of the experiment.

The presence of virus in blood and nasal secretions was determined by plaque assay. As shown in Fig. 3 all animals inoculated with A12-WT virus had peak levels of viremia prior to the onset of clinical disease. Interestingly 4 out the 6 animals inoculated with A12-H* did not develop viremia despite the presence of detectable virus in the nasal cavity.

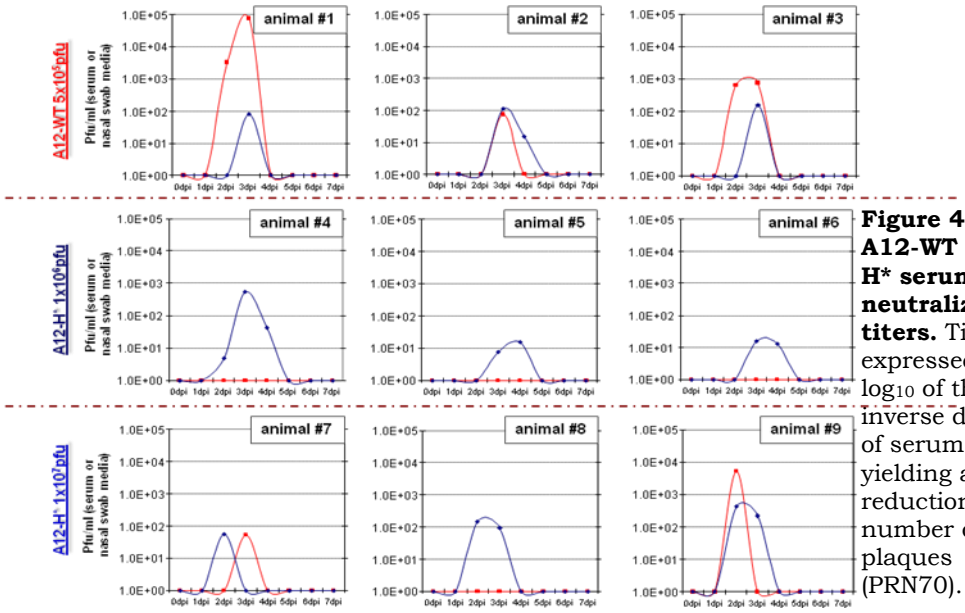
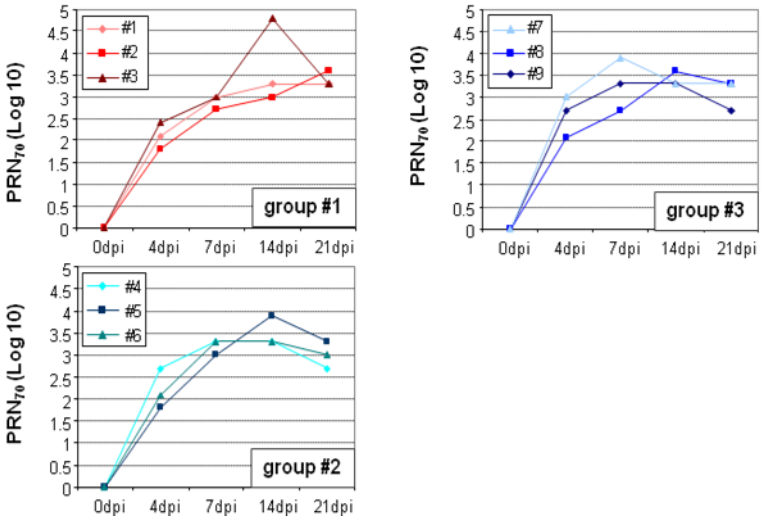


Figure 4. FMDV A12-WT or A12-H* serum neutralization titers. Titers are expressed as the $\log_{10}$ of the inverse dilution of serum yielding a 70% reduction in the number of plaques (PRN70).

Figure 3. Detection of viremia and virus shedding in swine after FMDV A12-WT or A12-H* ID inoculation. Virus titers were determined in blood and nasal swabs collected daily from animals inoculated with FMDV A12-WT or A12-H* from 0 to 7 days post infection. Each graph represents data of an individual pig.

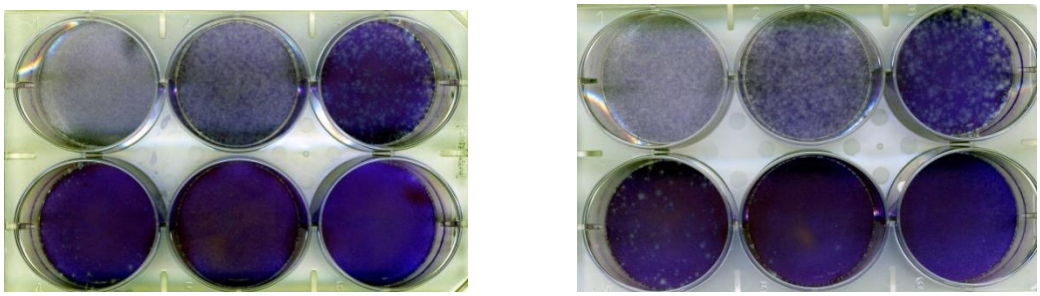
The levels of neutralizing antibodies were determined in all inoculated animals (Fig.4). As expected, all animals inoculated with A12-WT virus developed high antibody titers starting at 4 days and reaching a maximum titer by 14 days post inoculation. Interestingly, all the animals inoculated with A12-H* developed significant levels of FMDV specific neutralizing antibodies even those that did not develop clinical signs of disease and/or detectable levels of viremia (animals#5 and #6).



These data indicated that A12-H* FMDV displays reduced virulence in swine as compared to A12-Wild Type and elicit a strong antibody response.

2. Construction and characterization in vitro of FMDV strains with mutations in the L^{pro} CTE domain alone and in combination with mutations in the SAP and/or FHA domains.

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., 2012) we prepared new FMDV strains with mutations in specific conserved residues of the Lpro SAP domain and in a region of the protein known as C-terminal region (CTE) that is required for interaction with translation factor eIF-4G. SAP mutations included I55A and L58A, identical to the SAP mutant in serotype A12 since this region is conserved in both subtypes. Conserved mutations were introduced in the CTE region at three different locations.
Double mutants SAP-CTE were obtained in FMDV A24. Two mutations in the CTE region were tolerated in combination with I55A, L58A in the SAP domain. Virus stocks have been produced and are currently being thoroughly characterized *in vitro* for studies *in vivo* in the near future (Fig. 5).



A24-SAP (I55A,L58A); CTE #4 **A24-SAP (I55A,L58A); CTE #6**

Figure 5. Plaque phenotype of recovered viruses containing mutations in the SAP and CTE regions.

3. Test the virulence in swine of FMDV strains with a combination of mutations in the Lpro coding region.

Based on our previous results where we observed attenuation conferred by individual domain mutations in the Lpro SAP (I55A; L58A) or the FHA domains (H*) we attempted to construct FMDV strains containing SAP and FHA combined mutations. Unfortunately after several attempts no virus could be obtained. Back rub simulations were performed in order to determine the effect of mutations on the folding and spatial arrangement of amino acids in the structure of FMDV Lpro, concluding that the H* mutation was too disruptive. Possible substitutions predicted to be more tolerated were constructed. However no combinations of SAP mutations with FHA H* allowed for the generation of viable viruses. Given the results described in 2, we decided to proceed with SAP-CTE combination mutants which are currently being characterized in vitro for future analysis in vivo.

4. Test if the early protection detected after inoculation with SAP mutant virus is effective against challenge with different FMDV serotypes (cross-protection).

Vaccine early protection is often related with the induction of unspecific innate immune response. In our previous studies we demonstrated that FMDV SAP-mutant virus protected against homologous challenge in only two days in the absence of detectable neutralizing antibodies response (San Segundo et al., 2012). FMDV is a very variable virus and displays seven serotypes and multiple subtypes. In addition, the quasispecies nature of aphtoviruses enhances variability within each subtype (Domingo et al., 1990) and in general animals infected with a particular FMDV serotype/subtype are little or no protected against challenge with a different FMDV subtype.

Attenuated viruses are in general characterized by their ability of inducing early innate responses and therefore are expected to induce cross-protection between multiple serotypes and subtypes. In order to test this hypothesis, we designed an animal experiment vaccinating animals with attenuated FMDV SAP-mutant of serotype A12 and performing heterologous challenge two days later with FMDV serotypes A24, Asia 1 and O1Manisa (Table 1).
All animals in the controls group which were treated with PBS followed by challenge with each specific serotype, developed clinical disease starting at 2 dpc showing viremia and virus shedding between 2 and 3 dpc. Unexpectedly, all animals that had been vaccinated with FMDV SAP serotype A12 showed same clinical pattern than control animals, regardless the serotype of the virus used for the challenge. Furthermore, in the case of challenge with serotype A24, animals died during the experiment because of FMDV- induced myocarditis, indicating that in this particular experiment the challenge dose was very severe. In any case, we could not detect any heterologous protection induced by attenuated FMDV SAP-serotype A12 mutant virus, suggesting that the early protection seen in our previous experiments was probably a combination of innate and serotype specific adaptive immune responses.

Table 1: Clinical outcome in animals vaccinated with A12#SAP mutant FMDV or PBS (control) and challenged with different FMDV-WT serotypes.

Vaccine	*Animal*	*Challenge*	*Dose*[a]	*No. of lesions (day of onset)*[b]	*Viremia (dpc, day of onset, duration)*[c]	*PFU in nasal swabs (dpc, day of onset, duration)*[d]
A12SAP	25	A24	$1x10^5$	14 (2-D)	$1.8x10^5$ pfu/ml (2,2,2)	$3.6x10^2$ pfu/ml (3,1,3)
	26			14 (2-D)	$1.1x10^6$ pfu/ml (2,2,2)	$2.1x10^3$ pfu/ml (2,1,1)
	27			14 (3)	$7.8x10^2$ pfu/ml (3,3,1)	$1.8x10^2$ pfu/ml (2,2,4)
PBS	28	A24	$1x10^5$	9 (2-D)	$6.5x10^4$ pfu/ml (2,1,2)	$4.8x10^3$ pfu/ml (2,2,1)
	29			14 (2)	$1.3x10^5$ pfu/ml (3,1,4)	$1.6x10^2$ pfu/ml (2,2,3)
A12SAP	78	Asia-1	$1x10^5$	12 (2)	$1.3x10^5$ pfu/ml (2,2,2)	$6.3x10^3$ pfu/ml (2,2,2)
	79			15 (2)	$3.8x10^5$ pfu/ml (3,2,2)	$3.8x10^2$ pfu/ml (2,2,3)
	80			15 (2)	$2.2x10^5$ pfu/ml (2,2,2)	$6.3x10^3$ pfu/ml (2,2,2)
PBS	84	Asia-1	$1x10^5$	15 (2)	$1.5x10^6$ pfu/ml (2,1,2)	$5.5x10^1$ pfu/ml (2,1,2)
	85			15 (2)	$3.7x10^5$ pfu/ml (2,1,3)	$1.4x10^3$ pfu/ml (2,2,2)
	83			15 (2)	$5.3x10^3$ pfu/ml (2,2,2)	$7.4x10^1$ pfu/ml (2,2,2)
A12SAP	81	O1 Manisa	$1x10^5$	14 (2)	$8.3x10^5$ pfu/ml (2,2,2)	$1.1x10^4$ pfu/ml (2,2,3)
	82			15 (2)	$1.5x10^6$ pfu/ml (2,2,2)	$2.3x10^3$ pfu/ml (2,2,2)
	83			11 (2)	$4.0x10^5$ pfu/ml (2,2,2)	$1.1x10^3$ pfu/ml (2,2,3)
PBS	87	O1 Manisa	$1x10^5$	15 (2)	$1.8x10^6$ pfu/ml (2,1,2)	$5.5x10^2$ pfu/ml (2,2,2)
	88			15 (2)	$9.3x10^5$ pfu/ml (2,1,2)	$1.8x10^3$ pfu/ml (2,2,2)
	89			15 (2)	$1.1x10^6$ pfu/ml (2,1,3)	$1.1x10^3$ pfu/ml (2,2,2)

[a] Dose of challenge virus expressed as plaque forming units (PFU).
[c] Number of PFU per ml of serum. The dpc value is the day after challenge that the maximum level of viremia was detected; the onset value is the first day postchallenge that viremia was detected; and the duration value is the number of days of viremia.
[b] Number of toes with lesions plus the snout and tongue combined, if lesion present. The maximum score is 17. The day of onset is the first day after challenge that lesions were detected.
[d] Number of PFU per ml of nasal secretion. The dpc, onset and duration values are as defined in footnote b.
[f] Neg., less than 5 PFU/ml.

Discussion

The FMDV NS protein Lpro plays a key role in antagonizing the innate immune response. At the same time Lpro can be manipulated to render viable attenuated viruses. We have previously reported that FMDV lacking Lpro is attenuated in swine and cattle; however this virus was unable to completely protect animals against challenge with virulent FMDV (Chinsangaram et al., 1998, Mason et al., 1997). Recently we have constructed an FMDV variant containing mutations in a conserved domain of the

Lpro coding region, A12-SAP, which was attenuated *in vitro* and *in vivo* in swine. Remarkably, mutation of just two amino acid residues within the Lpro SAP domain prevented virus spread and disease, but was sufficient to induce complete protection against WT 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. Moreover we think that performing pathogenesis studies with this mutant will allow us to better comprehend the mechanism of attenuation and this information could be useful for the rational design of novel vaccine strategies. Furthermore, we think that inclusion of more mutations should increase the stability without severely affecting the degree of attenuation and indeed, increasing safety.

In previous studies we had demonstrated that other domains of Lpro, such as the FHA domain could be also mutated. Introduction of a mutation in the FHA domain also rendered a viable virus with a slight attenuated phenotype *in vitro*. Hence, we set up to study the virulence of this mutant in vivo. Interestingly, we found that animals inoculated with FMDV FHA H* mutant did not develop viremia or clinical signs but a strong neutralizing antibody response when administered at a dose that was twice the dose required to cause disease with WT virus. At a 20-fold higher dose, FMDV FHA mutant inoculated animals showed delayed and milder disease. These results suggested that perhaps a combination of Lpro SAP and FHA mutations could be tolerated and would improve safety of our live attenuated SAP vaccine platform. Unfortunately, our attempts to derive the SAP-FHA double mutant failed to derive viable viruses suggesting that these two domains of Lpro may somehow interact and potentiate in a still unknown function required for viability. Alternatively the double mutation could disrupt the overall architecture of the protein thus affecting all aspects of its biological activity. Further studies are underway to understand the molecular basis of this restriction and aiming to design novel mutants in these domains that presumably could be viable and stable.

In parallel studies we have now constructed other FMDV Lpro mutants that in addition to the SAP mutation contain changes in the CTE region of Lpro, a portion of protein that interacts with the host translation initiation factor eIF-4G. Interestingly, we have obtained viable viruses that are currently being characterized. Moreover, these viruses will be engineered to contain markers for differentiating vaccinated from infected animals (DIVA). We hope to be able to achieve such objectives with funds we have recently received from NPB.

Our last objective of the current proposal was to test the if the early protection conferred by inoculation with the FMDV SAP mutant (Diaz-San Segundo et al., 2012) would protect against challenge with other FMDV serotypes, such as A24, Asia 1 or O1 Manisa. We hypothesized that early protection could basically depend on induction of innate responses, i.e., production of IFN which we have previously proven effective against all 7 FMDV serotypes (Dias et al., 2011; Diaz-San Segundo et al., 2013). Our results showed that this was not the case. Vaccination with the SAP mutant serotype A12 did not protect when challenge with the other serotypes was performed 2 days post vaccination. It

is possible to assume that the challenge used in our experiments was too severe compared to our previous studies, since some animals died of FMDV caused myocarditis. Alternatively, these results may support that antibodies against FMDV appear early post infection and are the main cause of SAP vaccine induced protection. Recently Reid et al. (2011) have proposed that antibodies against FMDV may appear as early as 2 days post infection. These results were consistent with the appearance of significant levels of IFN in serum following the same pattern as viremia. Serum IFN could be produced upon internalization of immune-complexes FMDV-specific antibodies that bind the Fc receptors on plasmocytoid dendritic cells (Reid et al., 2011, Guzylac-Piriou et al., 2006; Lannes et al., 2012). In addition we and others have shown that neutralizing antibodies can be detected as early as 4 days post vaccination with inactive or Ad5 vectored vaccines (Moraes et al., 2003, Pacheco et al., 2010). Perhaps inoculation with attenuated strains of FMDV allow for the formation of antibodies as early as 2 days post infection.

Overall these results demonstrate that inoculation with live attenuated FMDVs induces early innate and adaptive immune responses and deserves further optimization to develop novel countermeasures against FMD.

References

Grubman, M. J., & Baxt, B. (2004). Foot-and-mouth disease. *Clinical Microbiology Reviews*, 17, 465-493

Moraes, M. P., Mayr, G. A., Mason, P. W. & Grubman, M. J. (2002). Early protection against homologous challenge after a single dose of replication-defective human adenovirus type 5 expressing capsid proteins of foot-and-mouth disease virus (FMDV) strain A24. *Vaccine,* 20, 1631-1639.

Chinsangaram, J., Moraes, M. P., Koster, M., & Grubman, M. J. (2003). Novel viral disease control strategy: adenovirus expressing alpha interferon rapidly protects swine from foot-and-mouth disease. *Journal of Virology,* 77, 1621-1625.

Terpstra, C. (1991) Hog cholera: an update of present knowledge. *Br Vet J,* 147, 397-406.

Chinsangaram, J., Beard, C., Mason, P. W., Zellner, M. K., Ward, G., & Grubman, M. J. (1998). Antibody response in mice inoculated with DNA expressing foot-and-mouth disease virus capsid proteins. *Journal of Virology*, 72, 4454-4457.

Mason, P. W., Piccone, M. E., McKenna, T. S., Chinsangaram, J., & Grubman, M. J. (1997). Evaluation of a live-attenuated foot-and-mouth disease virus as a vaccine candidate. *Virology*, 227, 96-102.

Diaz-San Segundo, F., Weiss, M., Pérez-Martín, E., Dias, C. C., Grubman, M. J., & de los Santos, T. (2012). Inoculation of swine with foot-and-mouth disease SAP-mutant virus induces early protection against disease. *Journal of Virology,* 86, 1316-27.

Dias, C. C., Moraes, M. P., Diaz-San Segundo, F., de los Santos, T., & Grubman, M. J. (2011). Porcine type I interferon rapidly protects swine against challenge with multiple serotypes of foot-and-mouth disease virus. *Journal Interferon Cytokine Research,* 31, 227-236.

Diaz-San Segundo, F., Montiel, N., de los Santos, T. & Grubman, M. J., (2013). Understanding the Mechanisms of Interferon-Induced Protection against Foot-and-Mouth Disease. *iConcept Press Ltd,* .ISBN 978-1-922227-20-1.

Reid, E., Juleff, N., Gubbins, .S, Prentice, H., Seago, J. & Charleston B. (2011). Bovine plasmacytoid dendritic cells are the major source of type I interferon in response to foot-and-mouth disease virus in vitro and in vivo. *Journal of Virology*, 85, 4297-4308.

Guzylack-Piriou L., Bergamin, F., Gerber, M., McCullough, K. C., & Summerfield, A. (2006). Plasmacytoid dendritic cell activation by foot-and-mouth disease virus requires immune complexes *European Journal of Immunology,* 36, 1674-1683.

Moraes, M. P., Chinsangaram, J., Brum, M. C. S., & Grubman, M. J. (2003). Immediate protection of swine from foot-and-mouth disease: a combination of adenoviruses expressing interferon alpha and a foot-and-mouth disease virus subunit vaccine. *Vaccine,* 22, 268-279.

Lannes, N., Python, S., & Summerfield A. (2012). Interplay of foot-and-mouth disease virus, antibodies and plasmacytoid dendritic cells: virus opsonization under non-neutralizing conditions results in enhanced interferon-alpha responses *Vet Res.*, 43, 64-81

Pacheco, J.M., Butler, J.E., Jew, J., Ferman, G.S., Zhu, J. & Golde, W.T. (2010). IgA antibody response of swine to foot-and-mouth disease virus infection and vaccination. *Clinical and Vaccine Immunology,* 17, 550–558.