NPB FINAL RESEARCH GRANT REPORT FORMAT

As a requirement of each research grant, a final report detailing the project results must be provided to the National Pork Board. Please write the industry summary with the producer in mind. The remaining content of the report can be written for a scientific audience. *ALL final reports must be submitted in the following format or the report will be returned for correction. PLEASE DO NOT INCLUDE ANY TYPE OF COVER PAGE.*

Project Title and NPB project identification number: Systematic discovery of ASFV determinants of virulence to improve the safety profile of first generation live-attenuated ASFV vaccines. NPB # 21-137

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Institution: Plum Island Animal Disease Center, ARS; USDA

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Industry Summary:

In this study we aimed to identify proteins in ASFV using serum containing antibodies from animals with live-attenuated vaccinated animals, that could be used as targets for a serological assay to differentiate between vaccinated and infected animals, these identified proteins were attempted to be deleted from a field isolate of highly pathogenic ASFV, to determine if they could be deleted. If they could be deleted, they were attempted to be deleted from the current live-attenuated vaccine, as a serological target, and resulted in a new next-generation live-attenuated vaccine containing a serological target. This same information is also used to identify proteins in ASFV that could be used for subunit vaccination. For example with coronaviruses it is known that the spike protein can be used for subunit vaccination, in ASFV there are over 160 proteins, the proteins for subunit vaccination are unknown, this study aimed at finding these proteins, and by using self-amplifying RNA system, were tested for subunit vaccination, however the subunit vaccination was not successful, as it was determined that the proteins expressed by self-amplifying RNA were not expressed in a similar manner as in the context of the virus, which is likely way the subunit vaccine was not effective. These findings are important to the swine industry as a serological marker in a vaccine is important to determine if an area is vaccinated but free of ASFV. In addition the identification of proteins in ASFV that could be used in future platforms for subunit vaccination, a gold standard for vaccination, and is preferred over live-attenuated vaccines, particularly for high-risk areas that are not currently in regions with ASF outbreaks.

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Key Findings:

- Development of a double deleted efficacious vaccine for ASFV, containing a serological marker
- Identification of 28 proteins in ASFV that are serological positive as targets for the serological marker and for subunit vaccines, information that was previously limited to only a few known proteins that were incapable of protection against ASFV

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.

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- Development of the first attempt at a mRNA vaccine using 28 protein targets, the most ASFV targets for any attempt at a subunit vaccine for ASFV.

Keywords: African swine fever; vaccines; DIVA; ASF; ASFV; African swine fever virus

Scientific Abstract: This should be a scientific description limited to one page in length to describe your project and its results.

Introduction: African swine fever virus (ASFV) is the causative agent of African swine fever (ASF), a lethal disease, which was first identified in 1921 in Kenya. ASFV is a large, structurally complex virus, harboring a large, double-stranded DNA genome of 180–190 kilobase (kb) pairs, which encodes more than 160 genes. We recently reported that there are six unique genotypes of ASFV, with the pandemic genotype 2 strain (previously classified as genotype II) gaining the most attention, as it is currently causing diseases in domestic and wild boar in Europe, Asia, Western Africa, and the island of Hispaniola. The genotype 2 strains are highly virulent and are severely affecting pork production across the globe. As commercial vaccines for genotype 2 have become available in Vietnam only recently, disease control is still mostly implemented by culling all of the infected animals and strictly limiting the movement of susceptible pigs.

Currently the immune response to specific genes during live-attenuated vaccination for ASF are unknown, understanding these is necessary in order to create a serological marker in live-attenuated vaccines, and to give genetic targets for subunit vaccination. In the case of several other viruses for example Coronaviruses, it has been well established that the spike protein is capable of inducing a protective immune response, and several subunit vaccines have been developed utilizing the spike protein. However, in ASF where there are over 160 genes in all isolates, there is no known protein or combination of proteins that are able to illicit a protective immune response, identifying these proteins is necessary for any successful subunit vaccine. Historically only a few genes have been shown to illicit an immune response, and those genes when tested in several subunit vaccine platforms have not offered any protection to ASF, suggesting that additional or different genes are required. In this study we set out to identify those proteins, and to use this information to introduce a serological marker into current ASF live-attenuated vaccine platforms.

The ASFV-G-ΔI177L vaccine platform is our most promising vaccine platform for ASF and was developed via the partial deletion of the I177L gene, is a safe and highly efficacious vaccine which has recently been approved for commercial use in Vietnam. It is a desirable characteristic in commercial vaccines that the immune response that they elicit may be differentiated from that induced by the infection with virus field isolates (DIVA capability) in order to facilitate the epidemiological management needed to control or eradicate the disease in either disease-free or endemic areas. A proof-of-concept genetic DIVA test based on qPCR to differentiate animals vaccinated with ASFV-G-ΔI177L from those infected with the parental virus ASFV Georgia 2010 isolate (ASFV-G) has been recently reported. However, no serological DIVA test has been developed to differentiate animals that have received ASFV-G-ΔI177L from those infected with ASFV field isolates. Here, we report the development of a novel serological test capable ASFV vaccines. And the addition of Ep402R deletion to the ASFV-G-ΔI177L vaccine platform. The resulting virus ASFV-G-ΔI177L/ΔEP402R, harboring the deletion of the EP402R gene that encodes for an antigenic envelope protein, the CD2 homolog, which mediates the adhesion to swine erythrocytes. ASFV-G-ΔI177L/ΔEP402R replicates well in swine macrophages and remains free of residual virulence when IM inoculated to domestic pigs, even to doses as high as 10^6 HAD_{50}. Furthermore, ASFV-G-ΔI177L/ΔEP402R induced protection against the appearance of a clinical disease in animals that were inoculated with a dose as low as 10^2 HAD_{50} and challenged with virulent parental ASFV-G. Importantly, a differential antibody response against the EP402R protein is absent in all ASFV-G-ΔI177L/ΔEP402R-inoculated pigs, which otherwise developed strong virus-specific antibody responses. Therefore, ASFV-G-ΔI177L/ΔEP402R constitutes the first potential DIVA-compatible, novel, safe and efficacious vaccine candidate.

Objectives:

Objective #1: Attempt to develop 8 single gene deletion recombinant viruses with 4 expected to be viable. Test these 4 viruses at a low dose, and any viruses where there is attenuation test at higher doses. Add one of these single deletions into ASFV-G-ΔI177L to serve as a additional Viral genetic determent of virulence (VGDV)

Objective #2: Attempt to develop 8 single gene deletion recombinant viruses with 4 expected to be viable. Test these 4 viruses at a low dose, and any viruses where there is attenuation test at higher doses. Add one of these single deletions into ASFV-G-ΔI177L to serve as a additional Viral genetic determent of virulence (VGDV) either to the virus produced in year one or to ASFV-G-ΔI177L

Materials & Methods:

Viruses and Cells

Primary cell cultures of swine macrophages were obtained from swine blood. Macrophages were seeded at a concentration of 5×10^6 cells/mL of culture media. Growth kinetic studies were comparatively performed between the original ASFV-G-ΔI177L, ASFV-G-ΔI177L/ΔVDG, and the parental virulent strain ASFV-G, using an MOI of 0.01 HAD_{50} or $TCID_{50}$, using standard procedures, with sample points obtained at 2, 24, 48, 72, and 96 h post infection (p.i.). Virus titrations were performed using swine macrophage cultures in 96-well plates, using standard procedures. Virus-infected cells were detected by either the presence of fluorescence (for ASFV-G-ΔI177L/ΔEP402R) or hemadsorption (HA) (for ASFV-G-ΔI177L, ASFV-G-ΔVFG and ASFV-G). The virus titers were calculated using the Reed and Muench method for virus quantification.

The ASFV-G-ΔI177L strain was previously developed in our laboratory. ASFV-G-ΔI177L/ΔVDG was developed by deleting the VDG gene from the genome of ASFV-G-ΔI177L by homologous recombination using a donor plasmid with positive selection fluorescent markers, following routinary procedures for developing new recombinant ASFV, deleting the VDG of interest.

Sequencing and Analysis of the ASFV recombinant genomes

Viral DNA from the infected macrophage cultures that showed 90–100% CPE was obtained using the Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA). After separation from the nucleus, the cytoplasmic fraction was used to obtain the viral DNA by following the manufacturer's protocol. Briefly, virus-infected cells were harvested and treated with hypotonic buffer (20 mM Tris-HCl, pH 7.4; 10 mM NaCl; 3 mM $MgCl_2$) on ice for 15 min (or until the cell membrane was dissolved). Then, the fraction containing the nucleus was separated by centrifugation, the cytoplasmic fraction was collected, and the DNA was extracted by adding 10% 3M NaOAc by volume to the sample (Sigma-Aldrich, St. Louis, MO, USA) and an equal volume of phenol–chloroform–isoamyl alcohol (25:24:1) with a pH of 6.5–6.9 (Sigma-Aldrich). These were then centrifuged at max speed in a tabletop centrifuge. Then, the aqueous phase was precipitated using 2 volumes of 100% ethanol, washed with the same volume of 70% ethanol, and dried. The obtained pellet of DNA was then resuspended in sterile water. The DNA library was then used for NGS sequencing using a Nextera XT kit in the NextSeq sequencer (Illumnia, San Diego, CA, USA), strictly following the manufacturer's protocol. Sequence analysis was performed using CLC Genomics Workbench software (CLCBio, Waltham, MA, USA).

Detection of ASFV

ASFV antibody detection was performed using an in-house ELISA previously described [34]. Briefly, the ELISA antigen was prepared from ASFV-infected Vero cells. Maxisorb ELISA plates (Nunc, St Louis, MO, USA) were coated with 1 μg per well of infected or uninfected cell extract. The plates were blocked with phosphate-buffered saline containing 10% skim milk (Merck, Kenilworth, NJ, USA) and 5% normal goat serum (Sigma, Saint Louis, MO, USA). Each swine serum was tested at multiple dilutions against both the infected and uninfected cell antigen. ASFV-specific antibodies in the swine sera were detected using an anti-swine IgG-horseradish peroxidase conjugate (KPL, Gaithersburg, MD, USA)

and SureBlue Reserve peroxidase substrate (KPL, Milford, MA, USA). Plates were read at OD630 nm in an ELx808 plate reader (BioTek, Shoreline, WA, USA). Sera titers were expressed as the log10 of the highest dilution where the OD630 reading of the tested sera at least duplicated the reading of the mock-infected sera.

Detection of Ep402R antibodies

The EP402R protein was expressed using a commercially available baculovirus system following the previously described routinary procedures [35]. Briefly, a truncated EP402R open-reading frame was amplified using the purified viral DNA from the ASFV Georgia 2007/1 isolate with primer set 5′-AGTCCACCACCTGAATCTAATGAA G-3′, and 5′-TGGCGGGATATTGGGTAGTAGC-3′. The amplicon was cloned into baculovirus using the Bac-to-Bac HBM TOPO Secreted Expression system (ThermoFisher Scientific, Waltham, MA, USA). The expression and purification of the recombinant antigen was performed as previously described [35]. Maxisorb ELISA plates (Nunc, St Louis, MO, USA) were coated with 0.1 μg per well of baculovirus-expressed EP402R protein. The plates were blocked with phosphate-buffered saline containing 10% skim milk (Merck, Kenilworth, NJ, USA). Swine sera were tested, their reactivity detected, and the titers calculated as described above for the anti-ASFV antibodies.

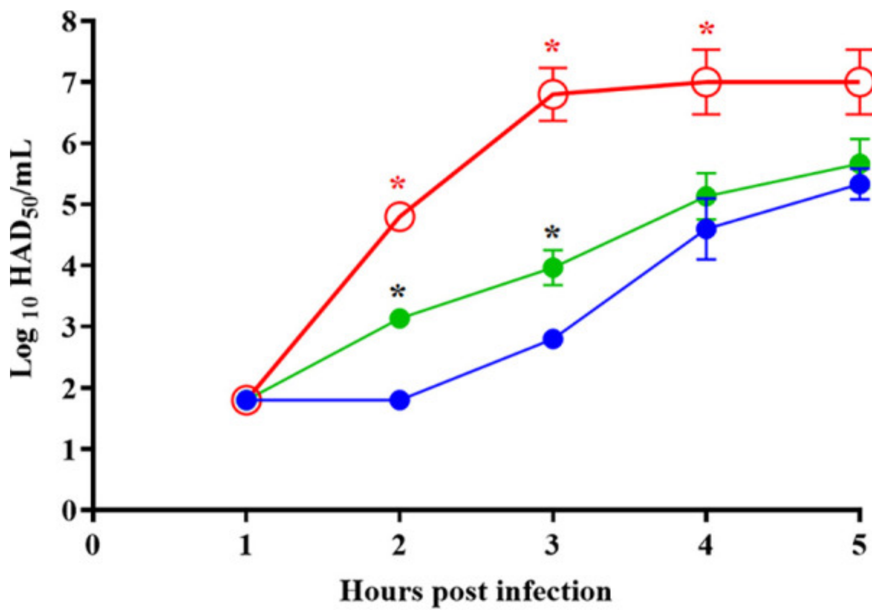
Evaluation of Virulence and Efficacy of ASFV-G-ΔI177L/ΔEP402R in Domestic Pigs

The virulence of the recombinant ASFV-G-ΔI177L/ΔEP402R was evaluated in 35–40 kg commercial-breed pigs. Groups of five pigs were experimentally infected via the intramuscular route (IM infected) with either 10^2 or 10^6 $TCID_{50}$ of ASFV-G-ΔI177L/ΔEP402R. The appearance of clinical signs (such as depression, anorexia, staggering gait, purple skin discoloration, diarrhea, and coughing), as well as changes in body temperature, were recorded daily throughout the experiment. Blood and serum samples were taken at days 0, 4, 7, 11, 14, 21, and 28 p.i.

Efficacy studies were performed by intramuscularly (IM) inoculating the animals 28 days post ASFV-G-ΔI177L/ΔEP402R inoculation with 10^2 HAD_{50} of virulent parental ASFV-G. An additional group of 5 naïve animals were incorporated as a control. All animals were observed daily for 21 days, sampled, and recorded as described above.All animal experiments were performed under biosafety level 3 conditions in the animal facilities at Plum Island Animal Disease Center, strictly following a protocol approved by the Institutional Animal Care and Use Committee (225.06-19-R_090716, approved on 9 June 2019).

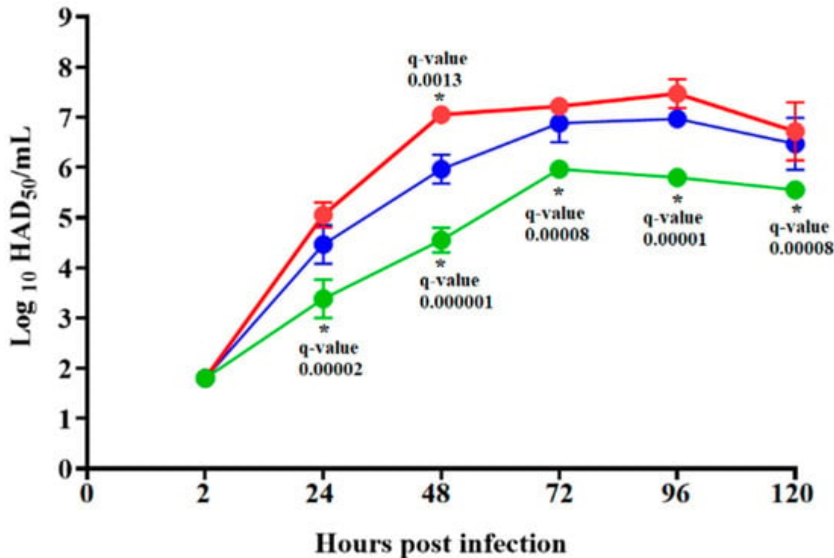
Results: We have successfully deleted the following genes from ASFV: A104R, MGF110 5L-6L, H108R, E165R, Ep296R, A151R, QP509L, E66L H240L, and O174L. We additionally tested 15other genes some of which couldn't delete, and some that we are continuing purification attempts. Deletion of E66L, 0174L, QP509L, E165R, MGF110 5L-6L and Ep269R did not have any effect on virus replication in primary cultures or virus virulence when tested in domestic swine. Deletion of A151R, H108R, A104R, and H240L resulted in partial attenuation, however in all cases there was a subset of animals that did not survive.

The addition of MGF-110-5L-6L to our vaccine candidate ASFV-G-ΔI177L had surprisingly reduced growth in primary swine macrophages as shown below in comparison to the parental vaccine.



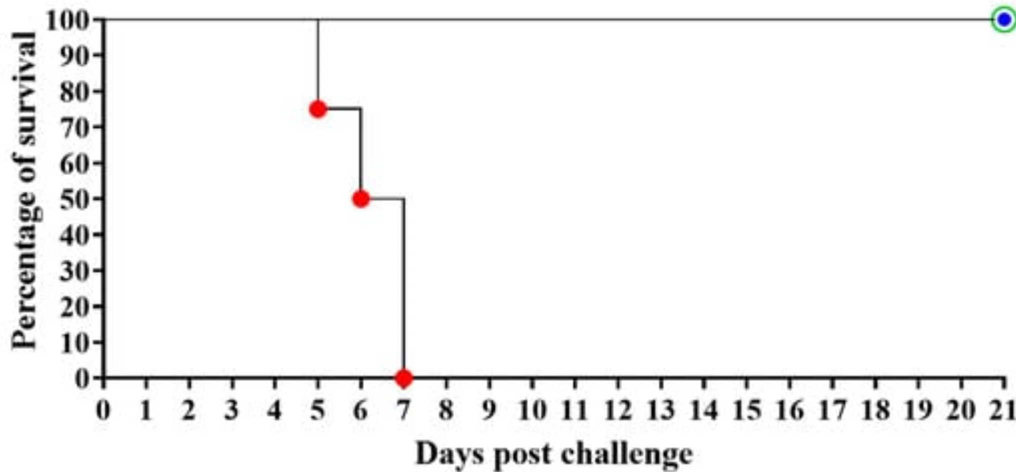
When tested in swine ASFV-G-ΔI177LΔMGF110-5L-6L reduced vaccine efficacy with 2/5 animals succumbing to ASFV, perhaps from the reduced growth in macrophages, however MGF110-5L-6L but was shown to be a potential serological candidate to differentiate vaccinated vs unvaccinated animals. We also attempted to delete E184L from ASFV-G-ΔI177L and were unsuccessful. E184L was successfully deleted in a different vaccine candidate ASFV-G-ΔMGF, and again reduced the efficacy of the parental ASFV-G-ΔMGF vaccine platform, however the combination of E184L and I177L is likely to be lethal for ASFV.

We then deleted another serological positive gene that was identified using sera from animals vaccinated with ASFV-G-ΔI177L, Ep402R, commonly known also as CD.. The addition of Ep402R, resulting in ASFV-G-ΔI177LΔEp402R did not negatively affect the growth of the ASFV-G-ΔI177L vaccine.

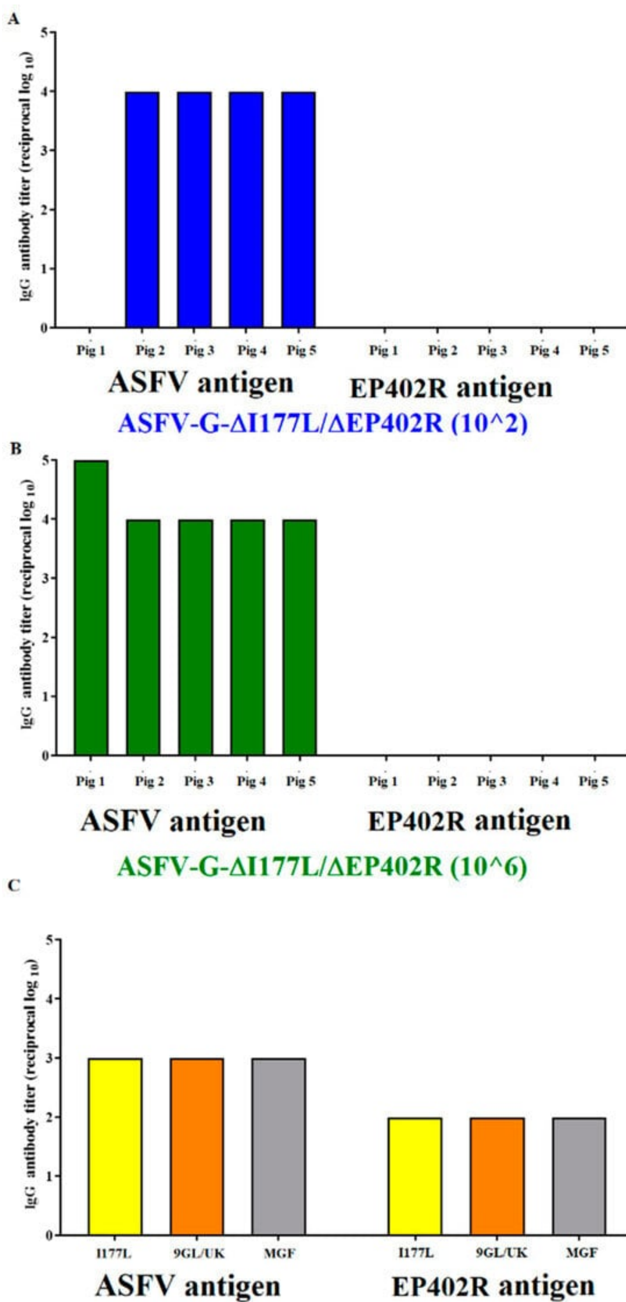


Furthermore, to evaluate the efficacy of ASFV-G-ΔI177L/ΔEP402R in mediating protection after the challenge with the highly virulent parental ASFV-G strain, the two groups of pigs inoculated 28 days earlier with either 10^2 or 10^6 $TCID_{50}$/mL of ASFV-G-ΔI177L/ΔEP402R were IM inoculated with 10^2 HAD_{50} of ASFV-G. The mock-vaccinated animals were also inoculated, under identical conditions, as a control group.

Animals in the mock-vaccinated group started presenting ASFV clinical signs by days 4–6 post challenge, quickly evolving to a severe form of the disease, with these pigs needing to be euthanized between days 5 and 7 post challenge. In opposition, the animals inoculated with ASFV-G-ΔI177L/ΔEP402R did not present clinical signs associated with ASF and continued to be clinically normal during the observation period of 21 days. Two animals in the group inoculated with 10^2 $TCID_{50}$/mL, at days 7 and 8 p.c., and one animal inoculated with 10^6 $TCID_{50}$/mL, at day 5 p.c., presented one-day rises in their body temperature, reaching or slightly exceeding 40 °C. These transitory rises in body temperature were not associated with any other clinical sign associated with ASF. Therefore, inoculation with ASFV-G-ΔI177L/ΔEP402R produced protection against the challenge of the virulent parental virus, solidly preventing the appearance of clinical ASF disease.



It was important, then, to evaluate if their reactivity to the EP402R protein could constitute the basis for the development of a DIVA test that would differentiate the systemic antibody response of animals inoculated with ASFV-G-ΔI177L/ΔEP402R from those infected with viruses harboring the EP402R gene. An in-house ELISA was used to detect the presence of anti-EP402R protein-specific antibodies (see the Materials and Methods section). The results demonstrated that all sera from the animals inoculated with ASFV-G-ΔI177L/ΔEP402R, obtained 28 days post infection, failed to recognize the EP402R protein (Figure 7). Conversely, sera obtained 28 days post infection from the animals with the recombinant ASFV vaccine candidates (ASFV-G-ΔI177L, ASFV-G-Δ9GL/ΔUK, or ASFV-G-ΔMG) reacted strongly against both the ASFV antigens and the EP402R protein. These results demonstrate that ASFV-G-ΔI177L/ΔEP402R functions as an antigenically marked vaccine, specifically not inducing antibodies to the EP402R protein, which is otherwise easily recognized by animals infected with viruses harboring the EP402R gene.



The NPB funding was used to leverage for Foundation for Food and Agriculture (FFAR) funding. I would like to provide NPB with a small update on this work as well. The FFAR project is also underway which involved the identification of serological targets by expressing all of the ASFV proteins individually and using immune sera. We have identified novel immunogenic targets and are continuing deletion studies for individual genes in ASFV. The goal of the combined projects is to find both a DIVA marker that is compatible for current live-attenuated vaccines (which resulted in testing MGF-110-5L-6L, E184L and Ep402R). We also used this information to identify targets for subunit vaccines. We have also used this information to leverage two collaborative projects with commercial partners to test novel saRNA vaccines, unfortunately in both cases the developed saRNA vaccines were not effective in protecting ASFV. After testing immune sera from both of these platforms, it was discovered that some of the individual ASFV protein targets were not producing an immunogenic response, even though in the context of the live attenuated vaccines these individual targets were immunogenic. Further investigation revealed that the likely cause of this, is that the saRNA constructs which express only one ASFV protein per construct, were not expressing protein at high levels, or the proteins were folding improperly. Additional investigation will have to be conducted in order to gain expression and proper folding so that the proteins expressed in saRNA vectors, act in a similar manner immunogenically as when expressed in ASFV proteins.

Discussion: Explain your research results and include a summary of the results that is of immediate or future benefit to pork producers.

In Conclusion, our research results have identified 28 novel VDG that are recognized by sera from vaccinated animals, the first study that has been used to determine antibody responses to individual proteins during ASFV vaccination using live-attenuated vaccines. Identifying these VDG, resulted in the creation of live-attenuated vaccines that contain a serological DIVA marker, in some cases there was a significant decrease in vaccine efficacy with the addition of the additional serological gene deletion. However, in the case of ASFV-GΔI177LΔEP402R the vaccine was highly effective, and protected all animals from ASF when challenged with the highly virulent strain ASFV-Gorgia. These results immediately benefit pork producers, as this new discovery can be used to develop a DIVA live-attenuated vaccine for ASF, a DIVA vaccine is necessary in areas that are not ASFV positive but are vaccinating animals for ASF, such as areas that have previously had ASFV outbreaks and are trying to establish ASF free status. This critical advancement for ASF live-attenuated vaccines can help to control ASF in outbreak areas.

Although the ASFV-GΔI177LΔEP402R identified in this report is still an experimental vaccine, and has not undergone the rigorous testing required for full commercialization, which requires a commercial partner. However, it is currently the most promising DIVA capable vaccine for ASF

This project resulted in several peer-reviewed publications:

1. Borca MV, Ramirez-Medina E, Espinoza N, Rai A, Spinard E, Velazquez-Salinas L, Valladares A, Silva E, Burton L, Meyers A, et al. Deletion of the EP402R Gene from the Genome of African Swine Fever Vaccine Strain ASFV-G-ΔI177L Provides the Potential Capability of Differentiating between Infected and Vaccinated Animals. *Viruses*. 2024; 16(3):376. https://doi.org/10.3390/v16030376
2. Ramirez-Medina, E., Velazquez-Salinas, L., Rai, A., Espinoza, N., Valladares, A., Silva, E., Burton, L., Spinard, E., Meyers, A., Risatti, G., Calvelage, S., Blome, S., Gladue, D. P., & Borca, M. V. (2023). Evaluation of the Deletion of the African Swine Fever Virus Gene O174L from the Genome of the Georgia Isolate. Viruses, 15(10), 2134. https://doi.org/10.3390/v15102134
3. Ramirez-Medina, E., Rai, A., Espinoza, N., Valladares, A., Silva, E., Velazquez-Salinas, L., Borca, M. V., & Gladue, D. P. (2023). Deletion of the H240R Gene in African Swine Fever Virus Partially Reduces Virus Virulence in Swine. Viruses, 15(7), 1477. https://doi.org/10.3390/v15071477
4. Gladue, D. P., Gomez-Lucas, L., Largo, E., Velazquez-Salinas, L., Ramirez-Medina, E., Torralba, J., Queralt, M., Alcaraz, A., Nieva, J. L., & Borca, M. V. (2023). African Swine Fever Virus Gene B117L Encodes a Small Protein Endowed with Low-pH-Dependent Membrane Permeabilizing Activity. Journal of virology, e0035023. https://doi.org/10.1128/jvi.00350-23
5. Ramirez-Medina, E., Vuono, E. A., Rai, A., Espinoza, N., Valladares, A., Spinard, E., Velazquez-Salinas, L., Gladue, D. P., & Borca, M. V. (2023). Evaluation of the Function of ASFV Gene E66L in the Process of Virus Replication and Virulence in Swine. Viruses, 15(2), 566. https://doi.org/10.3390/v15020566
6. Ramirez-Medina, E.; Vuono, E.A.; Pruitt, S.; Rai, A.; Espinoza, N.; Spinard, E.; Valladares, A.; Silva, E.; Velazquez-Salinas, L.; Borca, M.V.; Gladue, D.P. Deletion of an African Swine Fever Virus ATP-Dependent RNA Helicase QP509L from the Highly Virulent Georgia 2010 Strain Does Not Affect Replication or Virulence. Viruses 2022, 14, 2548. https://doi.org/10.3390/v14112548
7. Ramirez-Medina, E., Vuono, E.A., Pruitt, S., Rai, A., Espinoza, N., Valladares, A., Spinard, E., Silva, E., Velazquez-Salinas, L., Gladue, D. P., & Borca, M. V. (2022). ASFV Gene A151R Is Involved in the Process of Virulence in Domestic Swine. Viruses, 14(8), 1834. https://doi.org/10.3390/v14081834
8. Vuono, E. A., Ramirez-Medina, E., Pruitt, S., Rai, A., Espinoza, N., Spinard, E., Valladares, A., Silva, E., Velazquez-Salinas, L., Borca, M. V., & Gladue, D. P. (2022). Deletion of the EP296R Gene from the Genome of Highly Virulent African Swine Fever Virus Georgia 2010 Does Not Affect Virus Replication or Virulence in Domestic Pigs. Viruses, 14(8), 1682. https://doi.org/10.3390/v14081682
9. Vuono, E. A., Ramirez-Medina, E., Pruitt, S., Rai, A., Espinoza, N., Silva, E., Velazquez-Salinas, L., Gladue, D. P., & Borca, M. V. (2022). Deletion of the ASFV dUTPase Gene E165R from the Genome of Highly Virulent African Swine

Fever Virus Georgia 2010 Does Not Affect Virus Replication or Virulence in Domestic Pigs. Viruses, 14(7), 1409. https://doi.org/10.3390/v14071409
10. Vuono, E.A., Ramirez-Medina, E., Silva, E., Rai, A., Pruitt, S., Espinoza, N., Valladares, A., Velazquez-Salinas, L., Gladue, D. P., & Borca, M. V. (2022). Deletion of the H108R Gene Reduces Virulence of the Pandemic Eurasia Strain of African Swine Fever Virus with Surviving Animals Being Protected against Virulent Challenge. Journal of virology, 96(14), e0054522. https://doi.org/10.1128/jvi.00545-22
11. Ramirez-Medina, E., Vuono, E., Silva, E., Rai, A., Valladares, A., Pruitt, S., Espinoza, N., Velazquez-Salinas, L., Borca, M. V., & Gladue, D. P. (2022). Evaluation of the Deletion of MGF110-5L-6L on Swine Virulence from the Pandemic Strain of African Swine Fever Virus and Use as a DIVA Marker in Vaccine Candidate ASFV-G-ΔI177L. Journal of virology, 96(14), e0059722. https://doi.org/10.1128/jvi.00597-22
12. Ramirez-Medina, E., Vuono, E. A., Pruitt, S., Rai, A., Espinoza, N., Valladares, A., Silva, E., Velazquez-Salinas, L., Borca, M. V., & Gladue, D. P. (2022). Deletion of African Swine Fever Virus Histone-like Protein, A104R from the Georgia Isolate Drastically Reduces Virus Virulence in Domestic Pigs. *Viruses*, 14(5), 1112. https://doi.org/10.3390/v14051112

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