Project Title: Advancement in the development of ASFV vaccine technology for emergency vaccination
NPB project identification number: 21-131
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Date submitted: March 1st, 2023.

Industry Summary:

The evolving global spread of African Swine Fever Virus (ASFV) presents a high risk to the USA pork industry. Hence, it is important that innovative approaches are created to address existing challenges for ASFV vaccine development and production in the event of an emergency. An important area is the selection of a validated cell line that will serve as the substrate to produce the vaccine at a commercial scale. Aptimmune Biologics, Inc. is in a unique position to contribute to this area because it has a USDA CVB approved master cell stock (MCS) of the porcine macrophage cell line ZMAC-4 which is readily permissive to ASFV replication. The overall goal of this project was to establish the methods necessary to use ZMAC-4 cells to grow a leading ASFV modified live virus (MLV) vaccine candidate suitable for large scale emergency vaccine production. The goals of this project were: 1) create a founder virus stock of a ASFV vaccine candidate and optimization of virus culture; 2) ascertain the genome stability of the ASFV vaccine candidate after serial passage in ZMAC-4 cells; and 3) attain a prototype scaled-up method of virus culture that could enable the production of 5 to 10 million doses of vaccine per batch. To accomplish these goals, we use the ASF gene-deleted MLV vaccine candidate ASFV-G-Δ9GL/ΔUK and the ZMAC-4 cells to: i) determined kinetics of replication of the vaccine candidate; ii) optimize the method for virus culture, and iii) preform full genome sequence of serially passaged virus. The ASF MLV vaccine candidate was able to grow to high titers in the ZMAC-4 cell line without need of adaptation and without developing genomic changes over multiple passages. The results of this study indicate that the ZMAC-4 cell line is a sensible choice for ASFV commercial vaccine production. The main deliverable of this project was the establishment of methods and processes for the use of ZMAC-4 cells to produce a leading ASF MLV vaccine candidate suitable for production of a stockpile of emergency vaccine.

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Key Findings:

- ZMAC-4 cell line is unique in that it is the only porcine cell line supporting growth of ASFV that has been approved by USDA-CVB for production of biologics. Accordingly, it has been proven to be free of extraneous agents to 9 CFR/USDA standards.
- ZMAC-4 cell line supports the growth of both ASFV wild-type and gene-deleted virus vaccine candidates such as ASFV-G-Δ9GL/ΔUK and ASFV-G-ΔMGF over multiple passages without significant viral genomic changes.
- This ZMAC-4 technology can be used to produce 5 to 10 million emergency vaccine doses for stockpiling in the National Animal Vaccine Veterinary Countermeasures Bank

Keywords: ASFV, vaccine, ZMAC-4 cells

Scientific Abstract:
As a result of the alarming spread of the African swine fever virus (ASFV) in several parts of the world, research efforts on this virus have increased, including the development of several genetically engineered vaccine candidates. Although different continuous cell lines have been proposed for ASFV vaccine production, only one of them, the porcine macrophage ZMAC-4 cell line, has been approved by the USDA-CVB for commercial vaccine production. In this study, we examined ZMAC-4 cell line for its ability to support the replication of two ASF gene-deleted recombinant modified live virus (MLV) vaccine candidates, the ASFV-G-Δ9GL/ΔUK and ASFV-G-ΔMGF. As expected, because the natural host cell for ASFV consists of macrophages and monocytes, both viruses were able to readily replicate to high titers in ZMAC-4 cells without the need of adaptation. Further, sequence analysis of the virus genome after 5 and 10 serial passages of the ASFV-G-Δ9GL/ΔUK in the ZMAC-4 cell line showed no genomic changes, indicating that the ZMAC-4 cell line supports the replication of this vaccine candidate without the need for viral mutations to optimally replicate in this host cell. In addition, we optimized the scale-up virus culture conditions consisting of 1,500 ml volume capable of yielding a high titer of infectious virus in ZMAC-4 cells suitable for vaccine production. Accordingly, we obtained a titer close to 10^7 HAD_{50}/ml of the ASFV-G-Δ9GL/ΔUK, which by simply increasing the number of 2 L containers used for virus culture can produce several million doses of ASFV vaccine per 150 L batch. Our results confirm previous studies and demonstrate the value of the ZMAC-4 cell line for the stable propagation and production of ASFV MLV vaccine candidates. Based on these observations, the ZMAC-4 cell line provides a sensible choice for ASFV commercial vaccine production. A major deliverable of this project is the development of suitable methods to use the ZMAC-4 cell line as the substrate to grow a leading ASFV vaccine candidate to produce a stockpile of emergency vaccine doses.

Introduction:
In recent years, African swine fever virus (ASFV) has become a major crisis for the global pork industry causing massive losses in pig populations and major economic losses in Asia. ASFV has affected more than 50 countries in Africa, Europe, Asia, and the Pacific regions, with serious effects on food security (FAO, 2021). The recent confirmation of the presence of ASFV in the Hispaniola Island (Jean-Pierre et.al., 2022) has made the risk of ASFV entering the United States (U.S.) even more explicit than before. The potential spread of ASFV in the U.S. would cause tremendous economic damage to the American pork industry resulting in a loss of billions of dollars to pork producers (Carriquiry et.al ,2020). A stated goal of the Swine Health Team at the NPB is to help pork producers and the industry prepare to combat foreign animal diseases like ASFV by mitigating its transmission through biosecurity countermeasures along with the development of new vaccine/platform technology. In the event of an outbreak by a foreign animal disease, the primary method of control is culling infected herds, disinfecting the environment, and containment of healthy animals. This is complicated by the high level of resistance to environmental extremes seen with ASFV (Mazur-Panasiuk et.al., 2019). Euthanizing pigs and disposing of the carcasses is an economic disaster for farmers and deprives communities of an important source of animal protein. Vaccination, if available, is generally regarded as one of the most cost-effective measures to prevent or control livestock infectious diseases. (Roth, 2011). A recent review on the status of global ASFV vaccine development, covering all types of vaccine development efforts, identified the recombinant LAVs developed by gene knockout technology as the most optimistic prospects as a short/medium-term vaccine candidate in the future (Wu et al., 2020). At the present time, the most advanced ASFV LAV candidates are those developed by scientists at the United States Department of Agriculture (USDA) Plum Island Animal Disease Center (PIADC). Researchers at this center constructed several gene-deleted strains from the highly virulent ASFV Georgia 2007 (ASFV-G) by recombinant DNA technology. Among the viruses created were the ASFV-G-Δ9GL/ΔUK, which is a mutant with deletions in the 9GL (ORF B119l) and the UK (DP96R) genes (O'Donnell et.al. 2015); and the ASFV-G-ΔMGF, which was created by deleting six genes belonging to the multigene family 360 (MGF360) or MGF505 (O'Donnell et.al. 2016). These two engineered viruses were shown to replicate in pigs without causing disease (O'Donnell et.al. 2015, 2016), while eliciting protective immunity against challenge with the virulent ASFV-G. Importantly, these viruses only replicate

in porcine macrophages, and their adaptation to grow in heterologous cell lines would likely result in significant modification of the viral genome that could affect their vaccine efficacy (Krug et al., 2015). Then, a major limitation for commercial ASFV vaccine production has been the lack of a porcine cell line capable of supporting the replication of ASFV vaccine candidates. In vivo, ASFV primarily replicates in macrophages (Dixon, L. 2019). In vitro, efficient ASFV replication is observed in primary porcine macrophages and differentiated porcine monocytes. However, due to lot-to-lot variation, this type of cell cannot be used as a reliable platform for safe and efficient vaccine production. In addition, primary porcine macrophages are not suitable for the creation of a Master Cell Stock (MCS) that would be acceptable to the USDA Center for Veterinary Biologics (CVB). A USDA CVB-approved MCS must be used as the substrate to produce the vaccine virus to obtain a license for commercial vaccine production from the USDA. Alternatively, some African green monkey derived cell lines such as Vero and COS-7 cells have been used to adapt ASFV strains as experimental vaccines (Sánchez et.al., 2017; Krug et.al., 2015). However, adaptation of ASFV to replicate in non-porcine cell lines leads to viral genomic changes that are associated with lower viral yields, limited replication in vivo and/or failure to elicit a protective immune response (Sánchez et.al., 2017; Krug et.al., 2015; Hurtado et.al., 2010, Calzada-Nova et.al., 2012). Reportedly, another cell line capable of supporting the growth of an ASFV vaccine candidate is available (Borca et al., 2021). However, to our knowledge, it is not at the stage of commercial development since it is uncertain whether it will meet the strict USDA CVB requirements for the creation of a MCS. Our approach to produce a genome stable ASFV vaccine consisted of using its proprietary porcine alveolar macrophage cell line ZMAC-4 as the cell substrate to grow a suitable ASFV vaccine candidate. The ZMAC-4 cell line was approved by the USDA CVB for commercial vaccine production in 2008. A recent publication demonstrated that the ZMAC-4 cell line supports the growth of several ASFV field isolates, including the highly virulent ASFV-G without the need of adaptation (Portugal et al, 2020). Remarkably, this study also revealed that the replication of the wild-type ASFV-G in ZMAC-4 cells occurred with similar kinetics and yielded similar titers as in primary porcine bone marrow cells. Based on these observations, the ZMAC-4 cell line provides a sensible choice for ASFV commercial vaccine production. A major outcome of this project is the development the methods and process necessary to use the ZMAC-4 cell line as the substrate to grow a leading ASFV vaccine candidate suitable to produce a stockpile of emergency vaccine doses.

Objectives:

Objective 1. Creation of an ASFV-G-D9GL/DUK founder virus stock and optimization of virus culture.
Objective 2. Ascertain the genome stability of ASFV-G-D9GL/DUK after serial passage in ZMAC-4 cells.
Objective 3. Attain a scaled-up method of virus culture suitable to produce 5 to 10 million doses of vaccine per batch.

Materials & Methods:

Cells and viruses: ZMAC-4 cells were grown as previously been described (Portugal et al., 2020). The ASFV-Georgia 2007 (ASFV-G) gene-deleted mutant ASFV-G-Δ9GL/ΔUK (O'Donnell 2016) was licensed by Aptimmune from the USDA. Growth kinetics and titrations of ASFV-G-Δ9GL/ΔUK were assessed in ZMAC-4 cells. Virus titration was performed in 96-well plates. The presence of virus was determined by hemadsorption (HAD) performed as previously described (Portugal et al, 2020) and virus 50% HAD titers (HAD_{50}/mL) were calculated by using the Reed and Muench method (Reed and Muench, 1938).
Sequencing: The Next Generation sequencing was done by scientists at the U.S. Department of Homeland Security (DHS) Science and Technology Directorate (S&T). Briefly, DNA from ASFV-G-Δ9GL/ΔUK ZMAC-4 passages 5 and 10 was extracted using a modified HIRT method and quantified with a Qubit fluorometer using Thermo Fisher high sensitivity DNA assay. Libraries were prepared using Illumina's Nextera XT DNA library preparation kit and sequenced on either an Illumina Nextseq 500 or Nextseq 550. Sequencer 5.4.6 and GSNAP were used to generate p5 and p10 consensus sequences by aligning Illumina

data to the reference sequence ASFV isolate Georgia 2007/1 (GenBank: FR682468.2). Passage 5 and p10 alignment were analyzed in TABLET for discrepancies against the reference sequence.

Results:

The first step of Objective 1 was the creation of an ASFV vaccine founder virus stock. Initially, a limited dilution cloning process was completed using virus from a vial of the original ASFV-G-Δ9GL/ΔUK stock to assure the purity of the founder virus. The original ASFV-G-Δ9GL/ΔUK virus was first propagated by 2 blind passages on ZMAC-4 cells prior to cloning. As expected, the vaccine strain was able to grow in the cells without need of adaptation. After 6 days of starting the cloning procedure, seven potential clones were identified based on cytopathic effect (CPE) at the lowest dilutions. The presence of ASFV was confirmed by hemadsorption assay. The selected ASFV clones were sub-cultured in fresh ZMAC-4 cultures in T25 flasks. After 4 days, clones A and B showed faster virus growth, and a sample was taken for determining hemadsorption (HAD) titer. The results of HAD_{50} titration are shown below:

ASFV-G-Δ9GL/ΔUK CLONE	**HAD_{50}/ ML**
A	6.8E+06
B	2.2E+06

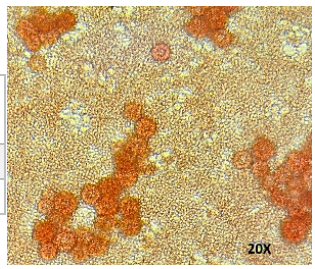

Fig 1. Hemadsorption in ZMAC-4 cells infected with ASFV-G-Δ9GL/ΔUK. Swine erythrocytes at 0.5% were added to a monolayer of ZMAC-4 cells together with the viral inoculum.

Based on the obtained titration results, clone A was selected and labeled as Pre-Founder seed virus. For the creation of the founder virus stock, ZMAC-4 cells were propagated in 75 cm^2 tissue culture flasks at 37ºC, 5% CO_2 humid environment. Then, cells were sub-cultured to two 300 cm^2 tissue culture flasks and maintained in a 37ºC, 5% CO_2 in air with a humid environment until reaching the cell concentration and volume for infection. Both 300 cm^2 cell culture flasks were infected with pre-founder seed ASF-G-Δ9GL/ΔUK at a MOI of 0.01. At 5 days after infection, viral fluids and cells were harvested when observed CPE was >80%. Cells were disrupted by two cycles of freezing and thawing. Virus fluids from both infected flasks were pooled, followed by filtration to clarify large cell debris. Bulk ASFV-G-Δ9GL/ΔUK was dispensed into pre-labeled cryotubes (1.2ml aliquots) keeping virus at ice-cold temperature by using a semi-automatic dispenser and immediately frozen in a -80ºC freezer. To verify that during the process of filling the founder virus stock was not contaminated with extraneous viable bacteria or fungi, 10 vials were randomly taken and pooled for sterility testing in soybean casein medium. At the same time, a Mycoplasma test was conducted by using a Mycoplasma Detection Kit (R&D Systems, MycoProbe) which is a colorimetric signal amplification system with sensitivity comparable to PCR. All tests were satisfactory, exhibiting no growth or a negative result. Afterwards, ten blind serial passages of the ASFV-G-Δ9GL/ΔUK founder stock were performed on ZMAC-4 cells in 25 cm^2 culture flask. Briefly, 2.5 x 10^6 ZMAC-4 cells were infected with 0.2 ml of the founder virus stock and maintained in a 37ºC, 5% CO_2 in air with a humid environment. After 4-5 days post infection, the viral fluid was harvested, and cells were disrupted by two cycles of freezing and thawing. This was labeled as ASFV-G-Δ9GL/ΔUK P1. The following virus passages were created in a similar manner and labeled with the subsequent number. Through all passages the ASFV-G-Δ9GL/ΔUK vaccine virus readily replicated in the ZMAC-4 cells with titers higher than 10^7 HAD_{50}/ml (Figure 2A), suggesting that the cell line is highly permissive and susceptible to ASFV infection. This result agrees with our previous observation with another ASFV-G gene-deleted vaccine candidate, ASFV-G-ΔMGF (Fig 2B), indicating that these vaccine viruses don't require adaptation to grow in the cells and it is likely that there will be no significant genetic changes in the virus genome when using ZMAC-4 cells as the substrate to grow a leading ASFV vaccine candidate.

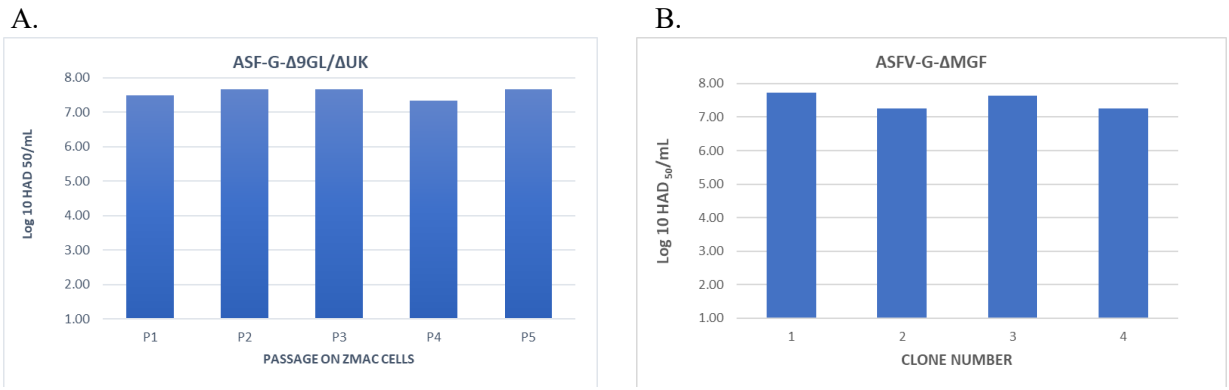

Figure 2. Titers of ASF-G-recombinant vaccine viruses on ZMAC-4 cells. (A) ASF-G-Δ9GL/ΔUK was passaged 5 times in ZMAC-4 cells. (B) ASFV-G-ΔMGF clones were obtained by limiting dilution and passed two times in ZMAC-4 cells. Titers were determined by HAD assay.

There are two critical parameters to consider for the optimal production of lytic viruses: multiplicity of infection (MOI) and time of harvest (Grein et al., 2017). To optimize the method of culture and determine the conditions that yield the highest possible titer of infectious virus, we performed multi-step growth curves of the ASF-G-Δ9GL/ΔUK mutant by infecting ZMAC-4 cells with a range of MOIs between 0.05 to 0.001. We identified that the MOIs 0.05 and 0.01 yielded the highest virus titers at 72h while the lowest MOIs took another 24h to reach similar titers (Fig.3A). To evaluate if we could reproduce similar results in a larger volume of cell culture, we infected 60 ml of ZMAC-4 cells at $3x10^5$ cells per ml in T75 flasks with either MOI of 0.05 or 0.01, and harvested the cultures at 0, 24, 48, 72 and 96h after infection. The results of this experiment showed that the kinetics of virus replication differed slightly in larger volumes, taking longer to reach the expected virus titers. This is an important consideration for setting up the conditions for scale-up for vaccine production.

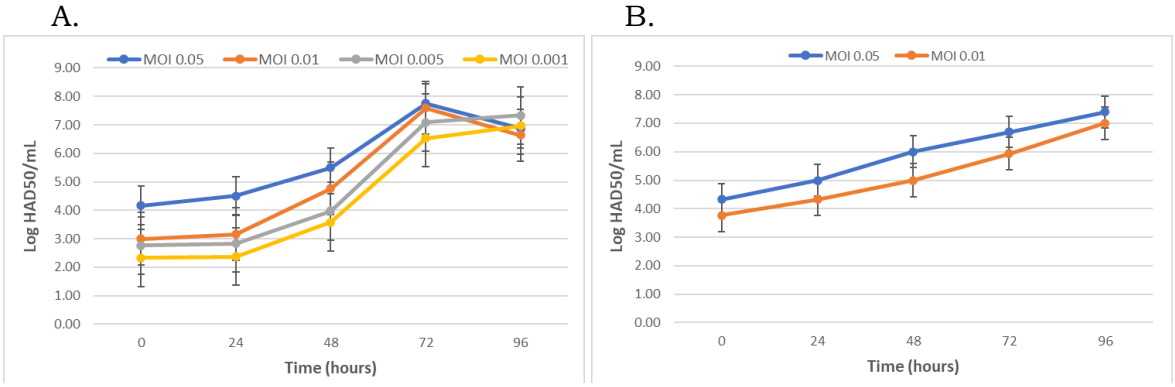

Fig 3. ASFV-G--Δ9GL/ΔUK growth curve kinetics in ZMAC-4 cells. (A) 24-well plates containing ZMAC-4 cells were infected at different multiplicities of infection. (B) T75 flasks containing ZMAC-4 cells were infected at either MOI=0.05 or 0.01. Aliquots were collected at the indicated time points and titrated on ZMAC-4 cells.

In Objective 2 we wanted to know if the plaque purification and continuous passaging of the ASFV-G-Δ9GL/ΔUK gene-deleted virus on ZMAC-4 cells caused significant mutations in the virus genome. For this purpose, whole genome sequencing was performed to establish the degree of genomic changes between samples. DNA was extracted from ASFV-Δ9GL/ΔUK ZMAC-4 passages 5 and 10 using a modified HIRT method, quantified with a Qubit fluorometer using a high sensitivity assay and sequenced using Illumina's Nextseq platform. Squencher 5.4.6 and GSNAP were used to generate consensus sequences by aligning Illumina data to the reference sequence ASFV isolate Georgia 2007/1. Each alignment was analyzed in

TABLET for discrepancies against the reference sequence and passages 5 and 10 consensus sequences were aligned using NCBI's Global Align program. The analysis showed that there were no sequence differences between the ASFV-Δ9GL/ΔUK ZMAC-4 passage 5 and 10 sequences, meaning the virus remained stable in between passages. Two large deletions corresponding to the ASFV 9GL and DP96R genes in both passages were identified. Table 1 lists the sequence difference in p5 and p10 with the ASFV Georgia 2007/1 reference sequence.

Variant Type	Location	Bases	Comment
Questionable	121, 129, 324, 351	G/C, A/T, G/C	Needs clarification
Questionable	14225 - 14237	CCC	Series of Cs needs clarfication
Questionable	15666 - 15682	CCC	Series of Cs needs clarfication
Questionable	17621 - 17632	GGG	Series of Gs needs clarfication
Questionable	19792 - 19799	GGG	Series of Gs needs clarfication
Questionable	19993 - 20008	GGG	Series of Gs needs clarfication
Substitution/Transversion	37432	G → C	
Deletion	96092 - 96264		173 base deletion - Corresponds with 9GL
Substitution/Transition	98378	A → G	
Undefined	106649	A or G	Needs clarification
Undefined	106680	T or C	Needs clarification
Undefined	106682	G or A	Needs clarification
Substitution/Transversion	167188	C → G	
Deletion	185341 - 185593		253 base deletion - corresponds to DP96R gene

Table 1. Summary of differences between the full genome sequence of ASFV-Δ9GL/ΔUK ZMAC-4 passages 5 and 10 with the ASFV Georgia 2007/1.

After establishing the processes for optimal growth of ASFV-G-Δ9GL/ΔUK in ZMAC-4 cells and confirming that multiple passaging the virus doesn't introduce significant genomics changes, we focused our effort to establish a scale-up process to produce a batch of vaccine enough to yield at least 5 million doses. Based on our experience, cell concentration at the time of infection (CCI) is critical for scaled-up production. For this experiment we selected two different concentrations, $1.3x10^5$ cells /mL and $2.0x10^5$ cells/mL. Two different containers with 1500 mL of cell at the indicated concentrations were infected at a MOI=0.03 and aliquots were collected at the indicated time points for titer determination by HAD assay. The results showed that both CCI tested were adequate for a scale-up process since the virus yield were very similar in both (Fig.4). Given that we expected to reach a titer close to 10^7 HAD_{50}/ml of ASFV-G-Δ9GL/ΔUK in ZMAC-4 cells, and that a dose of 10^2 HAD_{50} is sufficient to stimulate protective immunity (O'Donnell et.al., 2016), we are confident that using our current scale of cell culture, and by bottling 10^5 HAD_{50} per dose to allow for loss during production and shelf life, we should be able to produce between 6-10 million doses of ASFV vaccine per 150 L batch.

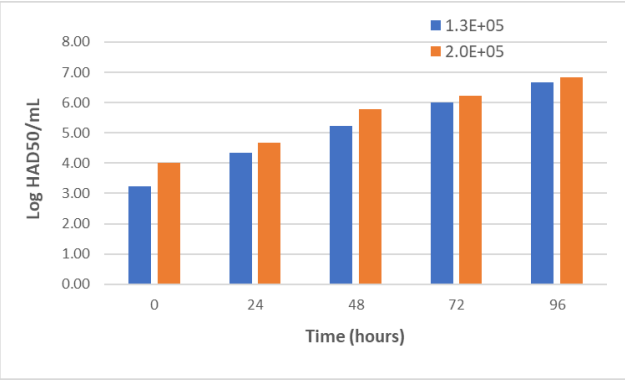

Fig. 4. ASFV-G-Δ9GL/ΔUK growth kinetics in scale-up volumes. ZMAC-4 cells at either 1.3 or 2.0x 10^5/mL in a 1.5L volume were infected at a MOI=0.03. Aliquots were collected at the indicated time points and titrated on ZMAC-4 cells.

Discussion:

The goal of this project was to support efforts to close gaps impeding the production of an ASFV vaccine listed in the DHS S&T Master Question List (MQL) under the medical veterinary countermeasures section (https://www.dhs.gov/publication/st-master-question-list-african-swine-fever). The MQL document mentions the need to identify a lead vaccine candidate that can be used to produce 5 to 10 million emergency vaccine doses for stockpiling in the National Animal Vaccine Veterinary Countermeasures Bank (NAVVCB). There are several promising modified live virus candidate vaccines which showed protection against homologous ASFV challenges with up to 100% protection (Brake D.A, 2022). Still, another challenge is the lack of a validated cell line that could allow propagation of the virus in a cost-effective manner. Several continuously cell lines have been reported as potential candidates for ASFV vaccine production. However, multiple passaging of attenuated ASFV strains on these cell lines led to genomic changes that potentially impact the biological properties of the virus (Krug P.W. et.al., 2015; Borca M. et.al., 2021, Sánchez et.al., 2017). A lead vaccine candidate needs a validated cell line to produce the ASFV vaccine doses for the NAVVCB. It has been reported that the ZMAC-4 cell line can propagate wild type ASFV and ASF vaccine candidate strains (Portugal et.al, 2020; Barrera, et.al. 2023) and until now, is the only USDA approved cell line for porcine vaccine production. The ZMAC-4 cell line offers a high-yield stable vaccine manufacturing process that in our estimation can easily provide up to 100 times more vaccine capacity than the methods used by others, making the vaccine extremely competitive from both supply chain and cost perspectives. The results presented demonstrate a process by which the porcine macrophage cell line ZMAC-4, can be used to produce millions of doses of a leading live attenuated ASFV vaccine candidate in a short period of time and without changes in the virus genome.

We fully acknowledge that the decision to use a live virus vaccine in the event of an ASFV outbreak in the U.S. is a debatable issue and will ultimately be dependent upon regulatory considerations. However, US pork producers must have all possible options available to combat such an unfortunate event. Of all technological alternatives being explored for an ASFV vaccine, the live attenuated virus vaccines are closest to the goal-line. Considering the rapid spread of ASFV since 2018, and the urgency of having a vaccine available considering the recent outbreak of ASFV in the Dominican Republic and Haiti, pursuing the development of modified live virus vaccine is warranted. Here, we have established procedures for preparation of media components, cell manipulations and ASFV virus infection using ZMAC-4 cells. Using these procedures, we create a prototype founder ASFV vaccine stock that can be used for rapid large-scale production of a commercial vaccine.

References

Barrera, J., Hurtle, W., Neilan, J., Brake, D. and Calzada, G. (2023). Permissiveness of the ZMAC-4 Cell Line for African Swine Fever Live Attenuated or Wild-type Viruses. Poster presented at the Conference of Research Workers in Animal Diseases; Jan 2023, Chicago, IL.

Borca MV, Rai A, Ramirez-Medina E, Silva E, Velazquez-Salinas L, Vuono E, Pruitt S, Espinoza N, Gladue DP. (2021) A Cell Culture-Adapted Vaccine Virus against the Current African Swine Fever Virus Pandemic Strain. J Virol., 95(14):e0012321.

Brake D. A. (2022). African Swine Fever Modified Live Vaccine Candidates: Transitioning from Discovery to Product Development through Harmonized Standards and Guidelines. Viruses, 14(12), 2619.

Calzada-Nova G, Husmann RJ, Schnitzlein WM, Zuckermann FA. (2012) Effect of the host cell line on the vaccine efficacy of an attenuated porcine reproductive and respiratory syndrome virus. Vet Immunol Immunopathol., 148(1-2):116-25.

Carriquiry, M., Elobeid, A., Swenson, D., Hayes, D. (2020) Impacts of African Swine Fever in Iowa and the United States. Working Paper 20-WP 600. Center for Agricultural and Rural Development, Iowa State University. https://www.card.iastate.edu/products/publications/pdf/20wp600.pdf .

Deutschmann, P.; Carrau, T.; Sehl-Ewert, J.; Forth, J.H.; Viaplana, E.; Mancera, J.C.; Urniza, A.; Beer, M.; Blome, S. (2022) Taking a Promising Vaccine Candidate Further: Efficacy of ASFV-G-ΔMGF after Intramuscular Vaccination of Domestic Pigs and Oral Vaccination of Wild Boar. Pathogens, 11, 996

Dixon, Linda K., Islam, Muneeb; Nash, Rachel H.; Reis, Ana L. (2019). African swine fever virus evasion of host defences. *Virus Research,* 266, 25-33

Grein TA, Weidner T, Czermak P. Concepts for the production of viruses and viral vectors in cell culture. (2017) In: Gowder,SJT, editor. New Insights into Cell Culture Technology. https://doi.org/10.5772/62590.

Hurtado, Carolina, Bustos María J.; Carrascosa, Angel L. (2010) The use of COS-1 cells for studies of field and laboratory African swine fever virus samples. J Virol Methods.;164(1-2):131-134.

Jean-Pierre RP, Hagerman AD and Rich KM (2022) An analysis of African Swine Fever consequences on rural economies and smallholder swine producers in Haiti. Front. Vet. Sci. 9:960344.

Krug, P. W., Holinka, L. G., O'Donnell, V., Reese, B., Sanford, B., Fernandez-Sainz, I., Gladue, D. P., Arzt, J., Rodriguez, L., Risatti, G. R., & Borca, M. V. (2015). The progressive adaptation of a georgian isolate of African swine fever virus to vero cells leads to a gradual attenuation of virulence in swine corresponding to major modifications of the viral genome. Journal of Virology, 89(4), 2324–2332.

O'Donnell, Vivian, Holinka, Lauren G.; Gladue, Douglas P.; Sanford, Brenton; Krug, Peter W.; Lu, Xiqiang; Arzt, Jonathan; Reese, Bo; Carrillo, Consuelo; Risatti, Guillermo R.; Borca, Manuel V. (2015) African Swine Fever Virus Georgia isolate harboring deletions of MGF360 and MGF505 genes is attenuated in swine and confers protection against challenge with virulent parental virus. J Virol.;89(11):6048-6056.

O'Donnell, Vivian, Risatti, Guillermo R.; Holinka, Lauren G.; Krug, Peter W.; Carlson, Jolene, Velazquez-Salinas, Lauro; Azzinaro, Paul A., Gladue, Douglas P., Borca, Manuel V. (2016) Simultaneous deletion of the 9GL and UK Genes from the African Swine Fever Virus Georgia 2007 isolate offers increased safety and protection against homologous challenge. J Virol.;91(1):1760-16.

Portugal, Raquel, Goatley, Lynnette C.; Husmann, Robert L.; Zuckermann, Federico A.; Dixon, Linda K. (2020) A porcine macrophage cell line that supports high levels of replication of OURT88/3, an attenuated strain of African swine fever virus. Emerging Microbes and Infections. 9(1):1245-1253.
Reed, L.J., Muench, H. A (1938) Simple Method of estimating Fifty percent endpoints, American Journal of Epidemiology; 27, 493–497.

Roth, James A. (2011) Veterinary vaccines and their importance to Animal Health and Public Health. Procedia Vaccinol.; 5:127-136.

Wu, Keke; Liu, Jiameng; Wang, Lianxiang; Fan, Shuangqi; Li, Zhaoyao; Li, Yuwan; Yi, Lin; Ding, Hongxing; Zhao, Mingqiu; Chen, Jinding (2020) Current State of Global African Swine Fever Vaccine Development under the Prevalence and Transmission of ASF in China. Vaccines, 8:531.