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New antigen identification in the African swine fever virus genome through a plasmid DNA library

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a) Background information

African Swine fever virus (ASFV) is the causative agent of African swine fever (ASF). ASFV is a devastating pathogen with a mortality rate of up to 100% in infected pig herds. It is prevalent and enzootic in 26 Sub-Saharan Africa (SSA) countries, reported in the years 2009-2011 (Penrith et al., 2013). Recently, outbreaks occurred in the Russian Federation resulting in economic losses estimated to be around 1 billion USD (FAO report, 2013) and the virus has now been reported in several other Eastern European countries, Asia and the American. This highlights the urgency of developing efficient countermeasures against ASF. There is no treatment nor commercial vaccine against ASF and the control of ASF is based on disease diagnostics, slaughter, and disposal of carcasses. Pigs are among the most profitable livestock for poor farmers. They are recognized as a species that can be used to improve livelihoods and contribute significantly to food security (Costard et al., 2009). With an estimated 34 million pigs in SSA, the development of an ASF vaccine could benefit about 6 to 17 million small-holder farmers, in this region alone. In addition, pig farming in SSA involves many women, since cultural norms regard this as a job for women and young people. Thus, tackling this disease through the development of a vaccine will have the added effect of improving the lives of SSA women.

Pigs that recover from infection are resistant to challenge against some ASFV isolates, indicating that these animals can develop a protective immune response (Lacasta et al., 2015). However, the complexity of ASFV, a virus encoding more than 150 different polypeptides (161 for “Kenya-1033” strain), many of them with unknown functions and others specialized in evading the immune system (Dixon et al., 2004), together with the variability of the virus isolates so far identified has complicated the identification of the mechanisms of protection against ASFV. However, seminal evidence has demonstrated the key role humoral responses (Onisk et al., 1994 and Schlafer et al., 1984) and CD8⁺ T-cells (Oura et al., 2005 and Lacasta et al., 2015) can play in protection. Thus, passive transfer of sera or colostrum from ASFV-infected and recovered pigs partially protected pigs against homologous ASFV challenge (Onisk et al., 1994 and Schlafer et al., 1984).

Three viral proteins were identified to be targets for neutralizing antibodies, p72/B646Lp, p54/E183Lp and p30/CP204Lp. Antibodies against p72 and p54 inhibit virus binding to cells, whereas those against p30 inhibit virus internalization. Other virion proteins present on the surface of intracellular mature or extracellular enveloped virus particles may be targets for neutralization by preventing virus entry or spread. These include the CD2v/EP402Rp, p12/O61Rp and D117Lp. However, there is a persistent ~10% of the virus that is not neutralized in the *in vitro* assays. Hence, it is not surprising that protection outcome using combinations of the mentioned antigens move from no protection to partial protection, either using Baculovirus expressed proteins or DNA vaccination (reviewed in Arias et al., 2017 and Escribano et al., 2012).

More recently, a virus expression library based on the avirulent ASFV strain Ba71V was used to screen the antibody specificity of polyclonal antisera from a surviving domestic pig infected with a virulent ASFV strain. Fourteen serological immunodeterminants were identified (Kollnberger et al., 2002). Unfortunately, no protection experiments followed these results.

Our approach in this project is the generation of a complete plasmid DNA library with all ORFs from “Kenya 1033” strain represented individually. The *de novo* synthesis of the genes and further cloning under the control of the CMV promoter will assure the accuracy of the gene sequences. GeneMill (University of Liverpool) is a leading expert in DNA design and cloning strategies. In the past, the construction of a library like the proposed was an important drawback because the difficulty of amplifying 161 ORF and cloning into individual plasmids. In most cases, the alternative was the construction of random libraries (Kollnberger et al., 2002 and Lacasta et al., 2014), with their intrinsic limitations. Using the automated DNA assembly technology in GeneMill allows the fast and efficient cloning of all 161 ASFV ORFs.

The ASFV DNA library will be a very valuable resource for antigen screening. After transfecting the plasmids into mammalian cells these ones will be screened against polyclonal sera from the natural reservoir of ASFV, warthogs (*Phacochoerus africanus*) and also from domestic pigs (African and European breeds) surviving the infection with either attenuated or virulent ASFV strains. The strategic position of ILRI allow us to have a vast

collection of serum samples available and the collaboration with IRTA-CReSA will increase even more this collection. Furthermore, the DNA library will be designed to facilitate its use as a DNA immunogen. Hence, in future the identified antigens will be used single or in plasmid pools in immunogenicity studies and eventually challenge experiments. The project presented is a comprehensive approach that considers the initial research steps identifying protective antigens and future steps of animal immunization with a very versatile tool such as a plasmid DNA library.

b) Objectives

The objective of this project is to identify new ASFV antigens that target the antibody neutralizing activity. More specifically, we will screen a DNA library encoding all ORFs from ASFV "Kenya 1033" to find serological immunodeterminants with potential protective capacity against ASF.

c) Experimental methodology

- 1) Generation of a complete ASFV ORF plasmid DNA library and validation of the sequences.

Proposed: All 161 ORFs from the virulent ASFV isolate Kenya 1033 (sequence available at ILRI) will be *de novo* synthesized by GeneMill (University of Liverpool). Hence, assuring the accuracy of the gene sequence. All ORFs will be cloned under the control of a cytomegalovirus (CMV) promoter. An extra plasmid will be generated with the sequence of the Green Fluorescent Protein (GFP) to be used as a general control for our assays. The generated plasmids will be expanded in bacteria, purified and sequenced. The final DNA library will be shipped to ILRI.

Current status: GeneMill provided the complete library in April 2021. The library was validated at the ILRI labs isolating the individual DNA plasmids from bacteria and digesting using *Bam*HI restriction enzyme. The quantity and the quality of the DNA plasmids was satisfactory, and the DNA plasmid library is ready to be used and stored at the ILRI Biorepository and inventoried in LabCollector LIMS software. A bacteria glycerol stock was also generated for every individual plasmid and it has been stored at the ILRI Biorepository facilities and inventoried in LabCollector LIMS software.

- 2) Transfection of HEK293 cells with the plasmid collection.

Proposed: The ASFV DNA plasmid library will be assessed for expression by transfecting the plasmids encoding GFP and the viral p72/B646Lp, p54/E183Lp and p30/CP204Lp proteins into HEK293 cells (transfection rates up to 90%). Expression of GFP protein and the efficiency of DNA transfection into HEK293 cells will be analysed by fluorescent microscopy and Flow Cytometry. Expression of p72, p54 and p30 will be assessed by Western Blot using the available anti-His and anti-Flag antibodies (every ORF is surrounded by a 6xHis and a Flag sequence at 5' and 3', respectively).

After the initial validation of expression and localization of the library-encoded proteins the rest of plasmids will be transfected into HEK293 cells, generating a collection of cells expressing all ASFV Kenya 1033 proteins in individual clones (HEK293_{ASFVLib}).

Current status: GFP-ASFV DNA plasmid was used to assess the efficiency of transfection. The transfection efficiency ranged 70-80% of the cells fluorescent after 48h (Figure1). The expression of 6xHis and Flag was also assessed by means of Western Blot using commercially available anti-Flag and anti-His antibodies. The expression of GFP and also the Tags (His and Flag) was satisfactory. A set of 4 plasmids encoding the ASFV proteins: p30, p54, p72 and CD2v were assessed after transfection to HEK293 with the optimized protocol. P30 and p54 were expressed demonstrating the suitability of the ASFV-library for antigen screening.

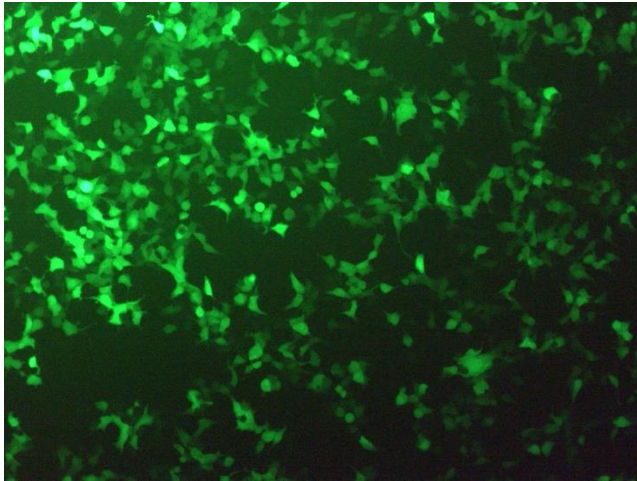


Figure 1. HEK293 cells transfected with a DNA plasmid encoding Green Fluorescent Protein (GFP). Cells in green are positive for transfection and protein expression.

3) Sera collection from warthogs (*Phacochoerus africanus*).

Proposed: Comparison of the pattern of antigen recognition between domestic ASFV susceptible pigs and resistant wild reservoirs (bushpigs and warthogs) will be of great interest. We will collect sera from wild animals in ASF positive areas in Kenya in collaboration with the Kenyan Wildlife Service (KWS). ILRI already has a big collection of sera samples from domestic animals positive for anti-ASFV antibodies from naturally infected animals and from experimental animals. Moreover, IRTA-CReSA will send to ILRI sera samples from recovered experimental domestic pigs infected with different ASFV isolates from different genotypes. We will also have access to the Friederich Loeffler institute (FLI) serum samples, they are current close collaborators with ILRI. Thus, there is no need to sample domestic animals for this project.

Current status: A set of around 200 samples from domestic animals have been identified. The animals were exposed to infection with ASFV attenuated virus, ASFV inactivated virus and ASFV subunit vaccination. A collection of 32 samples from warthogs were also identified from the ILRI Biorepository facilities and they are available for analysis. All samples were analysed to detect ASFV antibodies under both ELISA and Immunoperoxidase Monolayer Assay (IPMA). The positive samples were selected for further analysis. The relevant permits for wild animal sampling in collaboration with the KWS are in the process of obtention. However, the analysis of the already available samples will go ahead.

4) Antibody reactivity to HEK293_{ASFVLib} individual clones.

Proposed: In a first round of screening, sera from domestic African and European pigs recovered from either virulent or attenuated infection and sera from warthog will be screened against the HEK293_{ASFVLib} by means of IPMA. If necessary, a panning step to remove antibodies against p72, p30 and p54 proteins (immunodominant antigens already identified) and the concentration of the remaining antibodies will be performed. Sera detecting new antigens will be selected for further analysis.

Current status: The use of IPMA as read-out for the screening has been validated and the protocol is ready to be used routinely. The strategy for screening will comprise the generation of pools of DNA plasmids (including GFP in all pools to assess transfection efficiency). The efficiency of this strategy has already been validated.

5) ASFV neutralization assay and cross-reaction.

Proposed: The selected sera samples will be tested for their capacity to neutralize ASFV *in vitro*. In order to test cross-reactivity we will test the neutralizing capacity against the library parental strain Kenya 1033 but also

against other virulent and attenuated ASFV strains from different genotypes from our ILRI and IRTA-CReSA collection. A combination of sera recognizing different antigens will be also tested under the virus neutralization assay. Technology transfer will be provided in order to standardize the neutralization assay protocols between the two laboratories. We do not discard to expand the number of neutralization assays to assess additional antibody mechanisms of protection such as complement mediated cell lysis or antibody-dependant cell-mediated cytotoxicity (ADCC), potentially involved in protection against ASFV (reviewed in Escribano et al., 2012). Antigen-specific antibodies from the successful sera samples will be purified and concentrated and used in a neutralization assay single or in combination with other antigen-specific antibodies. The antigen-specific antibody purification is a standardized protocol at ILRI that has been used successfully for research on other diseases (e.g. East Coast fever). Successful antigens will be selected for future *in vivo* immunogenicity studies.

Current status: an ASFV seroneutralization assay has been developed and controlled rabbit antisera against inactivated ASFV, p30 and p54 are being used as positive and negative controls for neutralization (Figure 2). The rabbit sera were produced from a CGIAR Research Program project to be used across all ILRI ASFV projects. The ILRI seroneutralization assay is using an ASFV strain with GFP cloned in its genome. The ASFV-GFP virus is fluorescent allowing to use FACS analysis as a read-out for this assay, making it semi-quantitative and not only qualitative. All the ELISA and IPMA positive serum samples are in the process of being tested. IRTA-CReSA are also in the process of analysis of archived serum samples using the same assay.

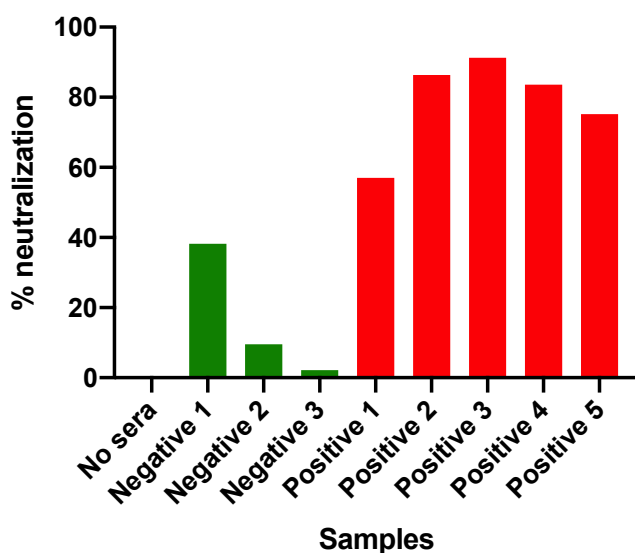


Figure 2. Neutralization capacity of sera from rabbits immunized with inactivated-ASFV, p30 or p54.

BUDGET STATUS REPORT (at 8 December 2021)

Donor:	International Veterinary Vaccinology Network (IVVN)
Project Period:	February 2019 to April 2022
Reporting Period:	September 2019-November 2021
Reporting Currency:	GBP
Project Budget:	GBP 94,692
Project OH Rate:	17%

General Comments

Funds Received to date from Donor	GBP 85,221
Expenditure to date	GBP 68,672
Commitments to date	GBP 12
Budget Balance to date exclusive of commitments	GBP 26,020
Budget Balance to date inclusive of commitments	GBP 26,008
Cofinancing total to date	N/A
Payment to Partner to date	GBP 35,077
Amount Available for Supplies	GBP 8,550
Amount Available for Travel	GBP 2,526
Linear Budget Projection Burn rate at close of period	92%
Linear Actual Project Burn rate at close of period	73%

References

- Arias, M, de la Torre, A, Dixon, L, Gallardo, C, Jori, F, Laddomana, A, Martins, C, Parkhouse, M, Revilla, Y, Rodriguez, F, Sanchez-Vizcaino, JM. 2017. *Approaches and Perspectives for Development of African swine fever virus*. Vaccines, 5 (4) 35.
- Argilaguuet, JM, Perez-Martin, E, Gallardo, C, Salguero, FJ, Borrego, B, Lacasta, A, Accensi, F, Diaz, I, Nofrarias, M, Pujos, J, Blanco, E, Perez-Filgueira, M, Escribano, JM, Rodriguez, F. *Enhancing DNA immunization by targeting ASFV antigens to SLA-II bearing cells*. Vaccine 29, 5379-5385.
- Costard, S, Wieland, B, Glanville, W, Jori, F, Rowlands, R, Vosloo, W, Roger, F, Pfeiffer, DU, Dixon, L. 2009. *African swine fever: how can global spread be prevented?* Philosophical transactions of the Royal Society of London. Series B, Biological sciences 364, 2683-2696.
- Dixon, L, Abrams, CC, Bowick, G, Goatley, LC, Kay-Jackson, PC, Chapman, D, Liverani, E, Nix, R, Silk, R, Zhang, F. 2004. *African swine fever proteins involved in evading host defence systems*. Vet. Immunol. Immunopathol. 100, 117-134.
- Escribano, JM, Galido, I, Alonso, C. 2012. *Antibody-mediated neutralization of African swine fever virus: myths and facts*. Virus research 173, 101-109.
- European Food Safety Authority report. doi:10.2903/j.efsa.2015.4163.
- FAO Report. <http://www.fao.org/docrep/018/aq240e/aq240e.pdf> (2013).
- Gil, F, Perez-Filgueira, M, Bardenas, MG, Pastor-Vargas, C, Alonso, C, Vivanco, F, Escribano, JM. 2011. *Targeting antigens to an invariant peptide of the MHC-II class II DR molecule potentiates the immune response to subunit vaccines*. Virus Res. 155, 55-60.
- Kollnberger, SD, Gutierrez-Castañeda, B, Foster-Cuevas, M, Corteyn, A, Parkhouse, RM. 2002. *Identification of the principal serological immunodeterminants of African swine fever virus by screening a virus cDNA library with antibody*. J. Gen. Virol., 82, 1331-1342.
- Lacasta, A, Ballester, M, Monteagudo, PL, Rodriguez, JM, Salas, ML, Accensi, F, Pina-Pedrero, S, Bensaïd, A, Argilaguuet, J, Lopez-Soria, S, Hutet, E, Le Potier, MF, Rodriguez, F. *Expression library immunization can confer protection against lethal challenge with African swine fever virus*. Jour. Virol. 88, 13322-13332.
- Lacasta, A, Monteagudo, PL, Jimenez-Marin, A, Accensi, F, Ballester, M, Argilaguuet, J, Galindo-Cardiel, I, Segales, J, Salas, ML, Dominguez, J, Moreno, A, Garrido, JJ, Rodriguez, F. 2015. *Live attenuated African swine fever viruses as ideal tools to dissect the mechanisms involved in viral pathogenesis and immune protection*. Vet Research 46, 135.
- Onisk, DV, Borca, MV, Kutish, G, Kramer, E, Irusta, O, Rock, DL., 1994. *Passively transferred African swine fever virus antibodies protects against lethal infection*. Virology, 198, 350-354.
- Oura CA, Denyer, MS, Takamatsu, H, Parkhouse RM. 2005. *In vivo depletion of CD8+ T lymphocytes abrogates protective immunity to African swine fever virus*. J. Gen. Virol, 86, 2445-2450.
- Penrith, ML, Vosloo, W, Jori, F, Bastos, AD. *African swine fever virus eradication in Africa*. Virus research 173, 228-246.
- Schlafer, DH, Mebus, CA, McVicar, JW. 1984. *African swine fever in neonatal pigs. Passively acquired protection from colostrum or serum of recovered pigs*. Am. J. Vet. 45, 1367-1372.