African Swine Fever

Gap Analysis Report

2022

Global African Swine Fever

Research Alliance

The Global African Swine Fever Research Alliance (GARA) aims to expand ASF research collaborations worldwide and maximize the use of resources and expertise to achieve its five strategic goals:
1. To facilitate research collaborations and serve as a communication gateway for the global ASF research community.
2. To conduct strategic research to increase our understanding of ASF.
3. To develop the next generation of control measures and strategies for their application.
4. To determine social and economic impacts of the new generation of improved ASF control
5. To provide evidence to inform development of policies for safe trade of animals and animal products in ASF-endemic areas.

Additional information on the GARA and the work of the alliance can be found on the following website: http://www.ars.usda.gov/GARA

The purpose of this ASF Gap Analysis Report is to assess current scientific knowledge and the available countermeasures to effectively control and mitigate the impact of an ASF outbreak in countries experiencing outbreaks, and also support global control and eradication initiatives in ASF-endemic countries.

This ASF Gap Analysis Report is the compilation of four workshops organized by the GARA with the support of its partners.

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EXECUTIVE SUMMARY

The Global African Swine Fever Research Alliance (GARA) organized five scientific conferences from 2013 to 2022 and 3 virtual webinars that are available on the GARA you tube channel. These activities to conduct gap analyses of our current knowledge and the available veterinary medical countermeasures to effectively control and mitigate the impact of a disease outbreak cause by African swine fever (ASF).

The key and consistent conclusion from these workshops is that although ASF was historically confined to the continent of Africa, the risk of an introduction of ASF into free areas of Europe, North America, South America, Eurasia, or Asia is significant and would be economically devastating. African swine fever is one of the most complex viral diseases affecting domestic pigs, wild boars and other wild suids. Soft ticks are considered a biological reservoir and transmission vector. In African wild suids such as African warthog and bush pig, African swine fever virus (ASFV) usually induces an unapparent infection.

Currently ASF is present in more than 20 sub-Saharan African countries. Moreover, since the report of the virus into Georgia in the Caucasus region in 2007 ASF has extended its geographical distribution and is currently present in large parts of Eastern, Central and Southern Europe. Furthermore, in 2018 ASF was introduced into China, and has since spread widely in the region. In 2021, a new long distance jump occurred, and ASF was reported first from the Dominican Republic and later from Haiti. With this development during the past 15 years, we now have a global ASF epidemic and more ASFV in the world than ever before. The risk for further dissemination of the disease to free areas is considered high.

The initial expression of ASF in swine can be variable due to various host factors and the diversity of virulence among ASFV strains, it typically presents as an acute hemorrhagic fever, with high case fatality rate. Viral mechanisms involved in induction of disease, tissue tropism, host range, and induction of immune responses are still not well understood. The disease occurs in several forms, ranging from acute lethal to chronic clinical disease. Antibody response elicited by infection with highly virulent strains of the virus begins to appear to detectable levels until at least 7-14 days post infection. Serology aiming at antibody detection, is thus not appropriate for early detection of ASF, which rather should be achieved with molecular tests targeting the viral genome. Serological diagnosis, however, has an important role in the diagnostic toolbox for ASF as it will provide data for a better understanding of the epidemiological context, in particular in regions where the disease is endemic.

The GARA determined that the following countermeasures were important but several weaknesses were identified.

Surveillance

Routine surveillance for early detection, based on passive (clinical) surveillance using virus detection techniques, is crucial in both domestic pigs and wild boar. Rapid and accurate detection affects the time when control measures can be implemented and affects the extent of the disease outbreak. Strains of ASFV can vary from low to highly virulent; and clinical signs

may range from very acute and serious disease with high case fatality rates to more prolonged ("*persistent*") infections with less severe signs. During the ongoing global epidemic caused by ASFV genotype II, the initial expression of infection in pigs in previously ASF-free countries has typically been acute disease with fatal outcome. However, with recent experiences it has become more and more clear that ASFV is not a highly contagious virus (as it historically has been described), and thus the progression of the disease within an affected herd is not necessarily rapid, which may cause a delay in detection, in particular in large herds. With this in mind, a system of enhanced passive surveillance based on systematic weekly testing of pigs found dead has been suggested, and this has also been implemented in affected countries in the EU (EFSA, 2018).

Recent experience with detection of ASFV in illegally imported food and feed warrants the onset of a new branch of testing and validating methods to detect ASFV in foods, food scraps, and agricultural processed products as part of the laboratory-based surveillance programs. However, caution with this kind of testing needs to be considered due to vulnerabilities associated with sampling size and the fact that a negative result does not tell you anything, while conversely a viral genome positive result does not provide sufficient information to judge the true risk of a possible introduction.

Depopulation

Depopulation is the primary countermeasure to reduce virus shedding and stop the spread of ASF virus. In most parts of the world, minimum control measures will include depopulation of infected units, surveillance and movement restriction within established control zones together with surveillance in herds that have been in contact with infected herds. Preemptive depopulation of contact herds and neighboring herds might be established, based on a risk-assessment. However, this method of control results in significant financial implications and the culling of thousands of animals has also become ethically debatable. The feasibility and effectiveness of stamping out in the absence of a fair and timely compensation scheme is highly dubious; i.e., in the absence of compensation there is no incentive for pig owners to report, who will instead sell or slaughter their pigs further spreading the disease. There is therefore a need to come up with sustainable and effective alternatives to stamping out for countries that cannot afford compensation (see eg Dixon et al 2020).

Biosecurity

On-farm biosecurity is a critical countermeasure for preventing the introduction and spread of ASF. Optimal biosecurity is effective by controlling the movement of pigs, people, equipment and supplies, and the potential biological or mechanical carriers of ASF. Priority biosecurity measures include the banning of swill feeding and the containment of scavenging pigs, which may be a challenge in developing countries. The identification of the source of transmission and entry into a target herd is a critical step in the implementation of an effective biosecurity program. However, after measures to curtail the spread of the disease are implemented, the most likely routes of transmission of ASFV may change. Since ASFV is an Arbovirus, a biosecurity plan should address procedures for cleaning and disinfecting facilities when *Ornithodoros* ticks are present; although a comprehensive biosecurity plan should include the control of insects and pests. Animal contacts as source of the virus may decrease, and transport trucks, people contacts, and pick-up for rendering services may contribute equally in the spread of the disease

between premises. In endemic areas in South Africa, the ideal solution for commercial pig farms has been the use of compartmentalization which has been used successfully in ASF control areas.

Vaccines

Currently, only one commercially produced ASF vaccine has been approved in the field. Except for this vaccine, approved by the Vietnamese government, there is currently no commercial vaccine available for ASFV. Although not systematically evaluated and classified, ASF scientists know there is a lack of cross protection among animals becoming immune to a certain virus isolate and subsequently exposed to another heterologous strain. This constitutes an important issue that will need to be addressed by both the ASF research community and veterinary authorities when considering vaccination strategies for the control and eradication of ASF. If vaccination of wild boar populations is considered, only safe, live-attenuated vaccines may be feasible.

Diagnostics

African swine fever is usually suspected based on clinical signs, but clinical evidence is nonspecific and difficult to differentiate from other infectious diseases of swine; e.g., Classical Swine Fever. Diagnosis is based on a pathogen detection test such as PCR, which may be combined with and a serological test for specific antibodies, usually performed by ELISA, for a better understanding of the epidemiological context. In industrialized countries, both detection tools are commercially available and allow disease detection from three to four days post infection by PCR, and roughly day 7 to 14 by ELISA. Confirmatory tests are so far not commercially available.

GROUP PICTURES

1st GARA Gap Analysis Workshop
Plum Island Animal Disease Center
Orient Point, New York
April 6-8, 2013

2nd GARA Gap Analysis Workshop
ARC - Onderstepoort Veterinary Institute
Pretoria, South Africa
November 11-14, 2014

3nd GARA Gap Analysis Workshop
ANSES - Laboratoire de Ploufragan-Plouzané
Ploufragan, France
September 6-8, 2016

4th GARA Gap Analysis Workshop
Istituto Zooprofilattico Sperimentale
Cagliari, Sardinia, Italy
April 11-13, 2018

5th GARA Gap Analysis Workshop
Melia Cribe
Punta Cana, Dominican Republic
May 24-27, 2022

GLOSSARY

AHT: Animal Health Technician

APHIS: Animal and Plant Health Inspection Service

ARS: Agricultural Research Service

BSL: BioSafety Level

ELISA: Enzyme-linked immunosorbent assay

ASF: African swine fever

ASFV: African swine fever virus

DIVA: Differentiating between infected and vaccinated animals

FADDL: Foreign Animal Disease Diagnostic Laboratory

GMP: good manufacturing practice

HSPD-9: Homeland Security Presidential Directive Nine

Ig: Immunoglobulin

MLV: Modified live virus vaccine

NAHLN: National Animal Health Laboratory Network

NVS: National Veterinary Stockpile

OIE: World Organization for Animal Health

PCR: Polymerase Chain Reaction.

qPCR: Real-time PCR

cPCR: Conventional PCR

PPE: Personal Protective Equipment

INTRODUCTION

African swine fever (ASF) is a contagious viral disease of domestic pigs with significant economic consequence. In Africa, ASF virus (ASFV) produces unapparent infections in wild suids: wart hog (*Phacochoerus africanus*), bush pigs (*Potamochoerus larvatus, P. porcus*) and the giant forest hog (*Hylochoerus meinertzhageni*) (a single report). The natural reservoir of ASFV is considered the soft tick *Ornithodoros moubata* (Dixon *et al*., 2005).

African swine fever virus is a large enveloped virus containing a double stranded (ds) DNA of approximately 190 kilobase pairs. African swine fever virus shares aspects of genome structure and replication strategy with other large dsDNA viruses, including the *Poxviridae, Iridoviridae,* and *Phycodnaviridae*. Although initially classified as an iridovirus, based largely on virion morphology, increasing knowledge of ASFV molecular biology led to its reclassification as the sole member of a new DNA virus family, *Asfarviridae* (*Asfar*, African swine fever and related viruses) (Costard *et al*. 2009).

African swine fever virus infections in domestic pigs are often fatal and are characterized by fever, hemorrhages, ataxia and severe depression. However, the course of infection varies depending on host characteristics and the particular virus strain. African swine fever occurs in several forms, ranging from highly lethal to sub-clinical. Acute forms of ASF, associated with highly virulent ASFV strains, are characterized by high fever, purple discoloration of the skin, multiple hemorrhages, respiratory distress, incoordination and death 3 to 7 days post-infection. Only a low percentage of animals will survive. Sub-acute and chronic forms of the disease are characterized by high fever, staggering gait, cough, diarrhea, purple discoloration of the skin, and death in 20 to 45 days post infection. These forms are accompanied by a higher proportion of surviving animals, and can be associated with ASFV strains of moderate and low virulence, respectively [1].

African swine fever was considered a disease of sub-Saharan Africa. However, in 1957, ASF was introduced into Portugal and later on to other European countries and some States of Central and South America. From Europe, ASF was eradicated at the end of the 1990s with the exception of the Italian island of Sardinia. Eradication was also achieved in the other affected countries outside Europe. However, in 2007, a very virulent viral strain of ASF was introduced into the Republic of Georgia, probably through untreated food waste from international ships in the harbor of Poti. Subsequently, the virus (Georgia 2007) started to spread in the Trans-Caucasian region and reached the Russian Federation. From the beginning, this new introduction affected both domestic pigs and European wild boar. The latter proved to be as susceptible as domestic pigs and the disease established self-sustaining cycles within the wild boar population. This was unprecedented as so far, any previous introduction into the European wild boar population had been self-limiting, unless sustained by co-infection and spillover from domestic pigs.
Since the introduction of the virus into Georgia in the Caucasus region in 2007 ASF has extended its geographical distribution and is currently present in large parts of Eastern, Central and Southern Europe. Furthermore, in 2018 ASF was introduced into China, and has since spread widely in the region. In 2021, a new long distance jump occurred, and ASF was reported first from the Dominican Republic and later from Haiti. With this development during the past

15 years, we now have a global ASF epidemic and more ASFV in the world than ever before. The risk for further spread towards additional countries must be considered very high, and the multi-sectoral nature of the situation necessitates inclusion of all stakeholders into the design of control measures (OIE, ASF Handbook).

In most parts of the world, minimum control measures will include depopulation of infected units, surveillance and movement restriction within established control zones together with surveillance in herds that have been in contact with infected herds. Preemptive depopulation of contact herds and neighboring herds might be established, based on a risk-assessment. However, this method of control results in significant financial implications and the culling of thousands of animals has also become ethically debatable. The feasibility and effectiveness of stamping out in the absence of a fair and timely compensation scheme is highly dubious; i.e., in the absence of compensation there is no incentive for pig owners to report, who will instead sell or slaughter their pigs further spreading the disease. There is therefore a need to come up with sustainable and effective alternatives to stamping out for countries that cannot afford compensation (see eg Dixon et al 2020). Currently, there are two approved vaccines for ASFV in Vietnam, however these vaccines are currently not available outside of Vietnam. Consequently, detection and elimination of infected animals is so far the only methodology to control and eradicate ASF (Costard *et al*. 2009).

BACKGROUND

GARA GAP ANALYSIS WORKSHOPS

This gap analysis report is the compilation of gap analyses conducted during scientific conference organized by the GARA 2013-2022:

1st GARA Scientific Conference, Plum Island Animal Disease Center, Orient Point, New York, United States of America, April 6-8, 2013

2nd GARA Scientific Conference, Agricultural Research Council, Pretoria, South Africa, November 10-14, 2014

3rd GARA Scientific Conference, ANSES, Ploufragan, France, September 6-8, 2016

4th GARA Scientific Conference, Istituto Zooprofilattico Sperimentale, Cagliari, Sardinia, Italy, April 11-13, 2018

5th GARA Scientific Confrence, Melia Caribe Conference , Punta Cana, Dominican Republic, May 24th- 27th, 2022

The gap analyses conducted by ASF experts were determined both by presented research updates reported from 44 research institutes from 34 different countries from across the world, coupled with scientific literature reviews. Using this information, priority areas for ASFV research were determined.

Report Updates

This report will be updated periodically with new scientific information, research breakthroughs, and/or the successful development of veterinary medical countermeasures. This report was last updated September 2022.

REFERENCE MATERIAL

The GARA recommends the following websites and reports as background information on the biology, epidemiology, and control of ASF:

1. https://www.ars.usda.gov/GARA/ (GARA official website)
2. http://www.fao.org/docrep/004/y0510e/y0510e00.HTM (FAO: ASF Contingency Plans)
3. http://www.oie.int/wahis/public.php?page=disease (WAHIS Interface)
4. http://athena.bioc.uvic.ca/organisms/Asfarviridae (Viral Bioinformatics: Asfarviridae)
5. http://www.fao.org/3/a-i7228e.pdf (FAO Manual: Detection and Diagnosis)
6. http://www.fao.org/ag/againfo/programmes/en/empres/ASF/index.html (ASF Resources)
7. http://web.oie.int/RR-Europe/eng/eng/Regprog/docs/docs/GF-TADs%20Handbook_ASF_WILDBOAR%20version%202018-09-25.pdf ((Handbook on ASF in wild boar and biosecurity during hunting)

8. http://asf-referencelab.info/asf/en/procedures-diagnosis/sops (European Union Reference Laboratory for ASF)
9. https://www.ASFVgenomics.com (individual ASFV protein information, including structure predictions

DEFINITION OF THE THREAT

The current threat for an introduction of African swine fever (ASF) into new geographical locations has never been higher. Since the introduction of ASF into the Republic of Georgia in 2007, ASF has been reported from large parts of Europe and Asia as well as from the island Hispaniola in the Caribbean. With the continued spread of ASF in Africa, Russia, Europe, Asia and the Caribbean, there is an unbroken perpetual threat of introducing ASF in previously ASF-free countries. Potential routes of infection include 1) the importation of infected pork or wild boar products fed as contaminated swill to domestic pigs, 2) the spread of the virus to new geographical areas from infected pigs and wild boars, and 3) accidental or intentional nefarious events.

ECONOMIC IMPACT

The introduction of ASF into previously free countries or regions has had important economic consequences for swine industries. A significant consequence of the introduction of ASF is the loss of status for international trade and the implementation of drastic and costly control strategies to eradicate the disease (Costard *et al*., 2009). In Cuba, the introduction of the disease in 1980s led to a total cost of U.S $9.4 million (Simeon-Negrin and Frias-Lepoureau 2002). In Spain, the final 5 years of the eradication program alone were estimated to have cost $92 million (Arias and Sanchez-Vizcaino 2002). Given the effect on pork production and trade as well as the costs of eradication, Rendleman and Spinelli estimated in 1994 that the net benefit of preventing ASF introduction in the United States would amount to almost $450 million, nearly 5 per cent of the value of total sales of pork products. In endemic countries, ASF has huge socioeconomic implications both for the individual farmers, especially the smallholder producers and at national scales. (Fasina *et al*., 2012; Chenais et al 2017).

EPIDEMIOLOGY

African swine fever may show regional patterns of presentation, associated with regional risk factors that should be assessed to establish proper surveillance and control strategies. However, more importantly, the presentation of the disease in pigs is strongly linked to the properties of local/regional pig value chain, which may vary within a region as well as between regions. Although to date four separate epidemiological cycles of ASF have been described, the so called domestic pig cycle, in which transmission between domestic pigs occur independently from contact with wild pigs or the argasid tick vector, dominates globally. In addition, an ancient sylvatic cycle, involving wild African suids and argasid ticks, and a domestic pig-tick cycle occurs in parts of the world. To this, an additional cycle has recently been added: the wild boar-habitat cycle, which includes the issue of wild boar and persistence in the environment as observed in large parts of Eastern Europe. Albeit the initial expression of ASF in a previously ASF-free country potentially could vary, the disease typically presents as an acute to peracute disease with high case fatality affecting pigs or wild boar. The presence of competent arthropod vectors (argasid ticks) may impact the maintenance of the virus in the environment.

SURVEILLANCE

Routine surveillance for early detection, based on passive (clinical) surveillance using virus detection techniques, is crucial in both domestic pigs and wild boar. Rapid and accurate

detection affects the time when control measures can be implemented and affects the extent of the disease outbreak. Viral strains of ASF can vary from low to highly virulent; and clinical signs may range from very acute and serious disease with high case fatality rates to more prolonged ("*persistent*") infections with less severe signs. During the ongoing global epidemic caused by ASFV genotype II, the initial expression of infection in pigs in previously ASF-free countries has typically been acute disease with fatal outcome. However, with recent experiences it has become more and more clear that ASFV is not a highly contagious virus (as it historically has been described), and thus the progression of the disease within an affected herd is not necessarily rapid, which may cause a delay in detection, in particular in large herds. With this in mind, a system of enhanced passive surveillance based on systematic weekly testing of pigs found dead has been suggested, and this has also been implemented in affected countries in the EU (EFSA, 2021).

Recent experience with detection of ASFV in illegally imported food and feed warrants the onset of a new branch of testing and validating methods to detect ASFV in foods, food scraps, and agricultural processed products as part of the laboratory-based surveillance programs. However, caution with this kind of testing needs to be considered due to vulnerabilities associated with sampling size and the fact that a negative result does not tell you anything, while conversely a viral genome positive result does not provide sufficient information to judge the true risk of a possible introduction.

BIOSECURITY

Implementing biosecurity measures on the farm is one of the most important countermeasures to prevent and protect commercial swine operations, but specific measures need also to be included and integrated in an eradication campaign to prevent further transmission and geographical spread through transport and person-to-person contacts. The main goal of a biosecurity plan is to decrease the probability of infection and significantly reduce the cost associated with losses. A set of zoo-sanitary measures should be put in place to accomplish the goals set by the biosecurity plan. The more measures are implemented, the higher the cost, but warranted in a disease outbreak situation. The OIE *Terrestrial Animal Health Code* (Chapter 15.1 and Chapters 4.3 and 4.4) provides guidelines for the establishment of compartments free of ASF.

DEPOPULATION

Depopulation is the primary countermeasure to reduce virus shedding and stop the spread of ASF virus. Minimum control measures will include depopulation of infected herds, surveillance and movement restriction within established control zones together with surveillance in herds that have been in contact with infected herds. Depopulation of contact herds and neighboring herds might be established. Thus, this method of control has resulted in significant financial implications and the culling of thousands of animals has also become ethically debatable. The effectiveness of stamping out in the absence of a fair and timely compensation scheme is highly dubious; i.e., in the absence of compensation there is no incentive for pig owners to report, who will instead sell or slaughter their pigs further spreading the disease. Lack of funds to compensate owners, particularly for culling of healthy pigs on neighboring premises, also constitutes a challenge in less wealthy countries. There is a need to come up with sustainable and effective alternatives to stamping out for countries that cannot afford compensation. When implemented, the speed of depopulation of infected herds including disposal of carcasses, and

disinfection of premises may have an effect on disease spreading, duration of the outbreak, and overall effectiveness of the control measure (Boklund *et al*., 2009). This control measure is effective in countries or geographic areas where pigs are housed in well-defined premises or pig farms. In areas where domestic pigs are kept on free-ranging scavenging systems, depopulation might be difficult.

VACCINES

There is no worldwide commercially available vaccine for ASF and the control of the disease is strictly dependent on animal quarantine, biosecurity measures, and slaughter. Recently, a live-attenuated ASF vaccine has passed regulatory approval in Vietnam and is being used on commercial farms. However, it is not yet available in other countries. This vaccine has a genetic backbone of Genotype II and has not yet been tested if it offers cross protection to other genotypes currently circulating in Africa. This presents a major gap for most of the world in the availability of veterinary medical countermeasures to effectively prevent, control, or eradicate an ASF outbreak. Some of the challenges in developing a vaccine include the following technical hurdles: 1) ASFV is one of the largest DNA virus known with the large majority of the viral genes uncharacterized or known functions; 2) there are no production cell lines that have been incorporated into a commercial vaccine that is readily available for vaccine production; 3) there are several ASFV genotypes with different phenotypic characteristics, cross protection with experimental vaccines has not been tested to date; 4) vaccine are needed for parenteral administration for domestic pigs, and administration to vaccinate feral swine and wild boars, and potentially other wild suids where a sylvatic cycle exists, although wild suids in Africa are known to be fairly resistant to ASF viruses.

DIAGNOSIS

ASF is usually suspected based on clinical signs or increased mortality, but clinical evidence may be nonspecific and difficult to differentiate from other endemic infectious diseases and transboundary diseases such as classical swine fever. For this reason, reliable laboratory diagnosis is crucial. It is usually based on real-time or conventional PCR in combination with antibody detection, where needed. Serological and virological differentiation of other etiological agents producing ASF-like diseases is critical. Pen-side tests can be useful to take decisions and actions under resource limited or remote conditions.

GAP ANALYSIS

The following section summarizes in brief what we know about African swine fever virus, gaps in our knowledge, and research needs.

VIROLOGY

African swine fever virus (ASFV) is a large, enveloped virus containing a double stranded (ds) DNA The length of the ASFV genome varies from ~170-190 kb depending on the viral strain, and encodes 150-200 viral proteins (Karger et al. 2019; Y. Wang, Kang, et al. 2021). ASFV has a low natural mutation rate (Malogolovkin and Kolbasov 2019) due to its DNA genome and employment of relatively accurate DNA polymerase proofreading and base-excision repair systems to facilitate viral replication in the highly oxidizing environment of the cytoplasm (Blome, Franzke, and Beer 2020; Netherton, Connell, et al. 2019)... The virions comprise an outer envelope, capsid, inner membrane, core shell, and inner core/nucleoid containing a single molecule of linear, covalently close-ended dsDNA (Gaudreault et al. 2020; Galindo and Alonso 2017). ASFV encodes novel genes involved in host immune response modulation, viral virulence for domestic swine, and in the ability of ASFV to replicate and spread in its tick vector. ASFV shares aspects of genome structure and replication strategy with other large dsDNA viruses, including the *Poxviridae*, *Iridoviridae*, and *Phycodnaviridae* (Dixon *et al*., 2000 and 2008). ASFV and poxviruses replicate in the cytoplasm of the infected cell, primarily in discrete perinuclear assembly sites referred to as virus factories. They also exhibit temporal regulation of gene expression and have similar genome structures, including terminal inverted repeats, terminal crosslinks, a central conserved region and variable regions at each end of the genome [1]. Although initially classified as an iridovirus based largely on virion morphology, increasing knowledge of ASFV molecular biology led to its reclassification as the sole member of a new DNA virus family, *Asfarviridae* (*Asfar*, African swine fever and related viruses) (Dixon *et al*. 2000).

The ASFV virion is comprised of more than 50 polypeptides and has a complex but regular structure by electron microscopy, icosahedral in symmetry and containing several concentric layers for an overall diameter of approximately 200 nm (Breese and DeBoer 1966; Carrascosa *et al*., 1984, 1985; Estevez *et al*., 1986 and 1987; Schloer, GM, 1985). The 80-nm virion core is composed of a nucleoid, (Andres *et al*., 1997 and 2002). Surrounding the nucleoid are two lipid bilayers, (Andres *et al*., 1997 and 1998; Rouiller *et al*., 1998). External to the inner membrane is the capsid, composed of the structural protein p72 (also referred to as p73), which comprises approximately one-third the protein content of the virion, and providing the icosahedral structure to the virion (Andres *et al*., 1997; Carrascosa *et al*., 1986; Garcia-Escudero *et al*., 1998; Tabares *et al*., 1980a). Covering the capsid is a loose external membrane obtained by virion budding through the plasma membrane, which is not required for virus infection (Andres *et al*., 2001; Breese and DeBoer 1966; Carrascosa *et al*., 1984; Moura Nunes *et al*., 1975).

In swine, ASFV primarily infects monocyte/macrophage-lineage cells (Sánchez-Vizcaíno et al. 2015), with secondary targets including vascular endothelial cells, hepatocytes, and epithelial cells (Y. Wang, Kang, et al. 2021). Viral entry is poorly understood, though it is thought to involve both the clathrin-mediated endocytosis and macropinocytosis pathways (Sánchez, Pérez-

Núñez, and Revilla 2017; Galindo et al. 2015; Hernaez and Alonso 2010). The viral infection process then proceeds through the endosomal pathway, where essential functions (including viral uncoating, endosomal fusion, and escape to the cytoplasm) depend on numerous factors including acidic pH, cholesterol, Rab7 GTPase activity, and the endolysosomal protein Niemann-Pick C type 1 (Cuesta-Geijo et al. 2012; 2022). Once in the cytoplasm, ASFV begins replicating in perinuclear "virus factories" (Simões et al. 2019; Gaudreault et al. 2020; Cuesta-Geijo et al. 2017).

At least 50% of ASFV's genes have unknown functions (Dixon et al. 2020), and large gaps remain in our understanding of its cell entry pathways (including required cell-surface receptors), transcriptional dynamics during infection, and functional genomics. As the current ASF pandemic continues to rage, virological studies are critical for expanding our knowledge of ASFV gene functions so that we can predict the effects of gene mutations or deletions on viral activity and infection dynamics. Recent advances in sequencing technology allow us to generate complete ASFV genome sequences much faster than previously possible, facilitating study of antigenic diversity and viral genome plasticity and evolution.

Structural analyses of ASFV and its components are another means by which we might better understand the mechanisms of viral infection. We know, for example, that both extracellular (enveloped) and intracellular (unenveloped) forms of ASFV are infectious, suggesting that both the envelope and protein capsid haveroles in viral infection and potential host immune responses (Andrés et al. 2020; N. Wang et al.In 2020, two separate studies published high-resolution cryo-EM structures of the ASFV particle. In their paper, Andrés et al. noted that the ASFV virion (specifically, strain BA71v) combines architectural elements of the Faustovirus (its closest evolutionary relative) and those of other membrane-containing viruses, like Pacmanvirus (Andrés et al.This unique, complicated structure reflects the complexity of the ASFV cell infection pathway (Andrés et al.Meanwhile, Wang et al. published the cryo-EM structure of the HLJ/18 strain virion using an optimized block-based reconstruction strategy to resolve the capsid structure up to 4.1 Å (N. Wang et al. 2019). Among other findings, they identified four exposed regions on the p72 major capsid protein that likely define neutralizing epitopes within the ASFV capsomers (N. Wang et al.
The lack of characterization of many ASFV proteins remains a significant hindrance to our understanding of the virus-host interface and the mechanisms underlying infection and virulence. The ASFV genome contains five groups of genes termed multigene family (MGF) genes – MGF100, 110, 300, 360, and 505 – and although their protein products have important roles in viral infection and host interactions, most have not been functionally characterized (Z. Zhu et al. 2021). Nevertheless, the majority of ASFV proteins are quite conserved across the different viruses, in particular the central genomic core which is highly conserved among different virus isolates. These include membrane and other structural proteins known to be present in the virus particle, and those that have been shown to affect different stages of virion morphogenesis in the infected cell (Afonso *et al*., 1992; Alcami *et al*., 1992 and 1993; Brookes *et al*., 1998b; Camacho and Viñuela 1991; Lopez-Otin *et al*., 1988 and1990; Munoz *et al*., 1993; Rodriguez *et al*., 1994; Simon-Mateo *et al*., 1995; Sun *et al*., 1995 and 1996). Other ASFV proteins share sequence similarity to cellular proteins or enzymes, including those involved in aspects of nucleotide metabolism, DNA replication and repair, transcription, and protein modification, and those that likely account for enzymatic activities present in ASFV virions or induced in infected cells

(Baylis *et al*., 1992, 1993a; Blasco *et al*., 1990; Boursnell *et al*., 1991; Freije *et al*., 1993; Hammond *et al*., 1992; Lu *et al*., 1993; Martin Hernandez and Tabares 1991; Martins *et al*., 1994; Rodriguez *et al*., 1993b; Yanez 1993; Yanez *et al*., 1993a, 1993b and1993c). Several of these proteins appear to be distantly related to homologs identified in poxviruses (Baylis *et al*., 1993b; Blasco *et al*., 1990; Boursnell *et al*., 1991; Freije *et al*., 1993; Martin Hernandez and Tabares 1991; Roberts *et al*., 1993; Yanez *et al*., 1993b). Additional enzymatic components encoded in the ASFV genome include homologs of cellular ubiquitin conjugating enzyme, trans-prenyltransferase, NifS-like protein, and components of a base-excision repair pathway (Hingamp *et al*., 1992; Rodriguez *et al*., 1992). ASFV also encodes proteins predicted to mediate virus–host interaction, virulence, and mechanisms that enhance the ability of the virus to successfully replicate within the host, including homologs of cellular inhibitor of apoptosis (IAP), Bcl-2, I Kappa B (IKB) myeloid differentiation primary response antigen MyD116, lectin-like, and CD2 proteins (Borca *et al*., 1994b; Neilan *et al*., et al. 1993a; Rodriguez *et al*., 1993a; Sussman *et al*., 1992). Notably, several of these putative virulence/host range proteins, along with certain multigene family (MGF) proteins, the central variable region protein 9-RL (pB602L as annotated in BA71V), and the variable tandem repeat-containing structural protein p54 (pE183L) (Irusta *et al*., 1996; Rodriguez *et al*., 1994; Sun *et al*., 1995), are among the most variable among multiple field isolates.

In 2019, Chen et al. published a structural and functional analysis of four crystal structures of AsfvLIG, the error-prone viral DNA ligase, in complex with DNA (Y. Chen et al. 2019). They identified a unique N-terminal domain and four critical active site residues important for enzymatic activity, opening new avenues for potential small molecule viral inhibitor design (Y. Chen et al. 2019). Li et al. conducted a similar study of the ASFV dUTPase, encoded by the E165R ORF. The researchers found that this viral enzyme contains a novel, two-subunit active site and has low primary sequence similarity (~23%) with porcine dUTPase, providing another possible route of ASFV-specific inhibition (G. Li et al. 2020). Banjara et al. investigated the complexed crystal structure of the viral A179L protein that binds to the mammalian proapoptotic Bcl-2 proteins; they identified A179L as the first known "panprodeath" Bcl-2 binder, binding to all major porcine proapoptotic Bcl-2 proteins (e.g. BH3-only proteins, Bak, and Bax) to block programmed cell death in response to viral infection (Banjara et al. 2017). Finally, Frouco et al. reported the DNA-binding properties of the ASFV protein pA104R, which is the only known histone-like protein encoded by a mammalian virus (Frouco et al. 2017). Interestingly, the researchers found 25-50% sequence identity with two families (HU and HF) of bacterial histone-like proteins, as well as a marked stability across temperature and pH ranges that likely supports ASFV's environmental tenacity. Immunostaining revealed that pA104R localizes in both cytoplasmic viral factories and the nucleus, suggesting a possible role in host genome heterochromatization (silencing pro-immune genes) and/or viral nuclear replication (Frouco et al. 2017). Recent data have suggested a possible nuclear replication stage, complementing the canonical perinuclear cytoplasmic process, as part of the ASFV infection pathway, but this remains debated (Frouco et al. 2017; Dunn et al. 2020; Cackett et al. 2020). For example, small ASFV DNA fragments have been detected in the nucleus, but their purpose is unclear (Rojo et al. 1999; Simões, Martins, and Ferreira 2015).

Other efforts over the past 6 years have aimed to shine a light on the broad transcriptomic and proteomic dynamics of ASFV infection. For example, Alejo *et al.* constructed a "proteomic

atlas" of the ASFV particle via mass spectrometry of purified extracellular virions, followed by immunoelectron microscopy to localize detected proteins (Alejo et al. 2018). They identified 68 viral proteins (39% of the putative genome coding capacity), including almost all previously described proteins and 44 newly identified polypeptides (half with unknown functions). Twenty-one host proteins were also reliably detected in the virion, most likely recruited during virus budding (Alejo et al. 2018). At the transcriptional level, Cackett and colleagues used a combination of RNA-seq, 3'RNA-seq, and RNA 5'-end cap analysis gene expression sequencing (CAGE-seq) to determine total ASFV RNA abundance and transcription start and termination sites at the single-nucleotide resolution (Cackett et al. 2020). Among many promising results, the researchers: (1) characterized DNA consensus motifs of early and late ASFV core promoters and a polythymidylate sequence determinant for transcription termination; (2) identified an apparent downregulation of MGF genes during the course of infection, with a corresponding upswing in the expression of genes containing putative transmembrane domains or signal peptide genes; and (3) described the use of alternative transcription start sites between early and late viral infection stages, potentially increasing viral protein diversity (Cackett et al. 2020; Cackett, Sýkora, and Werner 2020). This multistage temporal regulation of gene expression (divided into immediate-early, early, intermediate, and late gene classes) is a hallmark of ASFV and is similar to the infection dynamics of poxviruses. In general, early-expressed genes (~4-6 hours post-infection [hpi]) tend to be involved in viral genome replication, immune evasion, and requirements for late gene expression; these late-expressed genes (~8-16 hpi) include structural proteins for new virions and early transcription factors to be packaged into new virus particles (Y. Wang, Kang, et al. 2021; Sánchez et al. 2013). Olasz *et al.* used next-generation short-read (Illumina MiSeq) and third-generation long-read sequencing (Oxford Nanopore MinION) to produce a detailed map capturing the transcription dynamics of ASFV (specifically the highly virulent Hungarian isolate ASFV-HU_2018) within these classes, profiling total RNA from infected porcine macrophages at 4, 8, 12, and 20 hours hpi (Olasz et al.

Dunn *et al.* conducted an *in vitro* study to identify the potential functions of host and viral small noncoding RNAs (sncRNAs) in the viral infection process (Dunn et al.While only a small effect on host sncRNAs was observed, the researchers discovered three potential novel small RNAs encoded by the virus itself. One of these (dubbed ASFVsRNA2) was detected in the lymphoid tissue of ASFV-infected pigs. Overexpression of this small RNA *in vitro* led to ≤ 1-log reduction in viral growth, suggesting that ASFV might use virus-encoded sncRNA to disrupt its own replication via an unknown mechanism (Dunn et al. 2020). Meanwhile, Zhu & Meng developed the African Swine Fever Virus database (ASFVdb), a platform for online data visualization and analysis including comparative genomics and proteomics (Z. Zhu and Meng 2020). This database integrates data from NCBI, UniProt, ViralZone, and published literature, and performs various annotation and functional predictions based on these data. The ASFVdb has already been leveraged in numerous studies (Z. Zhu et al. 2021; Cackett et al. 2020; Chastagner et al. 2020) and may serve as a useful collaborative resource in ongoing and future projects.

The viral entry pathway of ASFV is another active area of research with seemingly more questions than answers. In line with previous studies (Hernaez and Alonso 2010; Sánchez et al. 2012), Galindo *et al.* reported in 2015 that ASFV enters host cells via dynamin-dependent, clathrin-mediated endocytosis; related factors necessary for entry included the presence of cholesterol in cell membranes and the activity of phosphoinositide-3-kinase (Galindo et al.

2015). The researchers observed that specific inhibitors of macropinocytosis did not inhibit viral entry into swine macrophages (Galindo et al. 2015). The following year, Hernáez *et al.* used flow cytometry and electron microscopy to conduct a high-resolution study of the viral entry pathway and subsequent movement through the endocytic network. Differing from the findings of Galindo *et al.*, they found that ASFV enters host cells via both constitutive macropinocytosis and clathrin-mediated endocytosis (Hernáez et al. 2016). Other findings included the requirement of pE248R, a type II transmembrane polypeptide in the viral inner envelope, for the final steps (viral fusion and core delivery to the cytoplasm) in the pH-dependent pathway of ASFV in endosomes (Hernáez et al. 2016; Andrés 2017). A later study from this group showed that pE199L, a viral cysteine-rich structural polypeptide, is also required for the viral entry process (Matamoros et al. 2020). Specifically, this protein mediates membrane fusion and core penetration steps. pE199L and pE248R both display weak sequence similarity to members of the poxvirus membrane fusion complex, pointing to a potential similarity in the viral entry mechanisms of these two types of virus as well (Matamoros et al.

These and similar studies of the viral entry pathway have led to the general consensus that ASFV entry can involve both endocytosis and macropinocytosis (Galindo and Alonso 2017; Gaudreault et al. 2020; Y. Wang, Kang, et al. 2021), though many questions remain. No specific cell-surface receptor for ASFV has been identified – blocking of CD163, for instance, inhibited viral infection *in vitro* but not *in vivo* in genetically-modified pigs (Popescu et al. 2017). Thus, the determination of ASFV-specific receptors (and potential redundancy and interactions between multiple receptors) remains another open question. Data from other recent studies have suggested a possible Fc-receptor-mediated endocytosis pathway for ASFV, though further research is needed to conclusively evaluate this (Y. Wang, Kang, et al. 2021; Gaudreault et al.

The generation of complete ASFV sequences is critical for phylogenetics, evolution and transmission tracking, and functional analyses. Indeed, many recent efforts have focused on developing new, efficient protocols for this difficult process. Forth and colleagues developed a deep-sequencing workflow for the rapid generation of high-quality whole genome sequences, combining a target enrichment step with Illumina and long-read Nanopore sequencing, and used this workflow to generate an improved Georgia 2007/1 sequence with 71 corrected homopolymer errors and additions to the inverted terminal repeats (ITRs) (Forth, Forth, et al. 2019). They noted the importance of using sequencing tools appropriate for the task at hand – in this case, using shorter Illumina reads (~99.9% accuracy) for better precision while using longer but less accurate (~90%) Nanopore reads for correct assembly (Forth, Forth, et al. 2019). Ji et al. recently published a protocol for sequencing from PCR-positive clinical tissues, covering all steps from virus extraction, through host sequence removal and data assembly, to gene prediction and functional analysis (Ji et al. 2021). Meanwhile, Olasz et al. published an efficient whole ASFV genome sequencing workflow including a DNase treatment step, monitoring of sample preparation via qPCR, and whole genome amplification, with a focus on avoiding time-consuming specific PCR-Sanger sequencing steps (Olasz et al. 2019). The researchers also compared Illumina and Ion Torrent next-generation sequencing systems and found that an Illumina NextSeq 500 provided fewer ambiguous reads (Olasz et al. 2019). Relatedly, Masembe et al. described an alignment-free tool for documenting viral diversity via genome-scale hidden Markov model domains, and made it openly available as a platform-independent Docker image (Masembe et al.

Recent efforts have shown that many individual genes of ASFV can be deleted without changing Recent efforts have shown that many individual genes of ASFV can be deleted from highly virulent field isolates without changing the virulence of the virus. Therefore, genes as Ep152R (***Virus Research*** (2016) 223:181-189), L83L (***Virus Research*** (2018) 249:116-123), MGF360-16R ORF (doi.org/10.3390/v12010060), C962R (doi.org/10.3390/v12060676), X69R (doi.org/10.3390/v12090918), MGF110-1L (doi: 10.3390/v13020286), KP177R (doi:10.3390/v13060986), A859L (doi.org/10.3390/v14010010), E165R (doi.org/10.3390/v14071409), MGF110-5L-6L (doi.org/10.1128/jvi.00597-22), EP296R (doi.org/10.3390/v14081682), QP509L (doi.org/10.3390/v14112548) have been deleted from the highly virulent Georgia 2010 isolate without altering its virulence in domestic pigs.

However, several reports have contributed to understand the role of several viral proteins that were previously characterizedthat as critical for virus replication. Some examples of these newly characterized proteins are the E2 ubiquitin conjugation enzyme I215L (Freitas *et al*., 2018), the viral decapping enzyme D250R or g5R (Quintas *et al*., 2017), a virus histone like protein pA104R (Frouco *et al*., 2017), the apoptosis inducing protein A179L (Banjara *et al*., 2017), the virus topoisomerase II protein (Freitas *et al*., 2016), and the viral protein Ep152R of unknown function, except for its specific binding to host protein BAG6 (Borca *et al*., 2017) Interestingly, some of those genes, as the structural protein KP177R (doi:10.3390/v13060986), RNA Helicase Gene A859L (doi.org/10.3390/v14010010), the dUTPase gene E165R (doi.org/10.3390/v14071409), or the ATP-dependent RNA helicase QP509L (doi.org/10.3390/v14112548) have been shown to not be essential for the process of ASFV replication in macrophages. In addition, the role of cellular vesicular system has been shown to be critical during virus replication (Cuesta-Geijo *et al*., 2012, 2016, 2017), as well as the ubiquitine proteasome system (Barrado Gil *et al*., 2017).

Gaps

Previous reports (GARA 2018; 2016) identified the following priority research knowledge gaps in ASF virology over the past 6 years:

- Complete ASFV genome sequences
- Automation/standardization of ASFV genome sequencing workflows and enrichment techniques
- Generation of corroborated reference sequences
- Establishment of large-scale bioinformatics resources/databases
- ASFV and host transcriptomics during infection
- Functional genomics of ASFV proteins

Complete ASFV sequences since 2015

The ASFV genome is difficult to sequence due to its high G-C content, complex ITRs (Olasz et al. 2019), and length up to ~190 kb that all render traditional Sanger sequencing slow and laborious (Forth, Forth, Blome, et al. 2020). Recent advances in next- and third-generation sequencing technologies have spurred a dramatic increase in the number of fully sequenced ASFV genomes available in the literature. O'Donnell et al., for instance, recently combined the

Oxford Nanopore (ONT) MinION sequencer with a new companion software script (dubbed "ASF-FAST") for real-time output data analysis (O'Donnell et al. 2020). Regardless of starting sample type (e.g. cell culture isolates or swine blood samples), >90% genome resolution was achieved within 10 minutes after enrichment (removal of host-methylated DNA) (O'Donnell et al. 2020). Only 19 full-length ASFV sequences were available in 2018, most of which were generated using Sanger sequencing techniques; by October 2021, this number had increased to 114 and is expected to continue to rise (D. GladueBelow, a selection of the important sequences gathered over the past 6 years is presented, with an emphasis on the various sequencing technologies used.

In 2015, Rodríguez et al. published the complete sequence of BA71 (the virulent parental strain of the attenuated Vero cell-adapted strain BA71v), obtained via an API PRISM 3700 automated DNA sequencer (Rodríguez et al. 2015). They identified a relatively small number of changes between the parental and attenuated strains, including an ~8 kb deletion affecting six members of the MGF360 family. In 2016, Granberg et al. used a combination of Illumina MiSeq and PacBio RSII (for long-read sequence data) to sequence the Sardinian 47/Ss/08 strain, which belongs to the same virulent subgroup as Benin 97/1 and E75 (Granberg et al. 2016).

Olesen et al. described the complete sequence of POL/2015/Podlaskie in 2018, using an Illumina MiSeq (with confirmatory PCR and Sanger sequencing) to sequence the virus directly from blood-derived nucleic acid samples from an experimentally infected pig (Olesen, Lohse, Dalgaard, et al. 2018). Meanwhile, Masembe et al. sought to rectify a gap in East African ASFV sequencing data (comprising only 3/20 complete sequences at the time of this study) using an Illumina NextSeq 500 to sequence five genotype IX isolates from domestic pigs in Uganda (Masembe et al. 2018).

2019 brought a spate of complete sequences from Europe as the virus continued to spread across the continent and into Western Europe. Gilliaux et al. used an Illumina MiSeq to sequence the newly emerged Belgian strain Belgium/Etalle/wb/2018, providing valuable information for phylogenetic analyses and viral tracking after its geographical jump over >600 miles from the nearest outbreak in the Czech Republic (Gilliaux et al. 2019). Forth et al. analysed the complete genome of Belgium 2018/1, finding 15 differences compared to Georgia 2007/1 (Forth, Tignon, et al. 2019). Mazur-Panasiuk et al. also used an Illumina MiSeq to completely sequence seven Polish isolates collected 2016-2017 (Mazur-Panasiuk, Woźniakowski, and Niemczuk 2019). They found "minor, but remarkable" variability in the published sequences, demonstrating a slow and steady evolution of ASFV in Poland, though the observed sequence diversity was not sufficient to track the origins of the seven isolates (Mazur-Panasiuk, Woźniakowski, and Niemczuk 2019). Meanwhile, Kovalenko et al. used an ONT MinION Mk1b third-generation sequencing platform to completely sequence the Ukrainian isolate Kyiv/2016/131 from the spleen of an infected domestic pig. Among other findings, they observed a 10 bp insertion between the isolate's I73R and I329L genes present in the Chinese 2018/AnhuiXCGQ genome but not in POL/2015/Podlaskie (Kovalenko et al. 2019). Bao et al. analysed the codingsequence (via the BGISEQ-500 protocol) of this China/2018/AnhuiXCGQ strain and found potentially significant mutations in DNA repair genes compared to POL/2015/Podlaskie (Bao et al. 2019).

In 2020, Ndlovu et al. published the results of two studies with a total of six ASFV genome sequences from Africa. The first reported the LIV 5/40 strain (genotype I) from Zambia and the South African RSA/2/2008 (genotype XXII) and SPEC 57 (genotype III) strains, all isolated from Ornithodoros soft ticks, generated using an Illumina HiSeq (Ndlovu, Williamson, Malesa, et al. 2020). In the second, the researchers sequenced (via Illumina MiSeq) the strains Zaire (genotype IV), RSA/W1/1999 (genotype XX), and RSA/2/2004 (also genotype XX), which was isolated from a European wild boar in South Africa (Ndlovu, Williamson, Heath, et al. 2020). In both of these studies, the viral genomic termini were not sequenced. Chastagner et al. used Proton Ion Torrent technology to describe the coding-complete sequence of Liv13/33, a genotype I strain originally isolated in 1983 from Ornithodoros moubata in Zambia (Chastagner et al.Elsewhere, Forth et al. reported the complete genome sequence of Czech Republic 2017/1, the causative strain of the 2017-2018 outbreak in that country, via Illumina MiSeq (Forth, Forth, Václavek, et al.As with other reports of European complete genomes, the researchers noted very high sequence identity with other Eastern European strains. Now, in-depth virological and pathogenicity studies are required to identify the potential functional effects of observed mutations (for instance, a nonsynonymous mutation in the D1133L-ORF, a member of helicase superfamily II and putative transcription factor) (Forth, Forth, Václavek, et al. 2020).

By 2021, ASF had transmitted extensively across Asia, causing widespread outbreaks and heavy economic losses. Truong et al. delineated the sequence of the Vietnamese isolate VNUA-ASFV-05L1/HaNam, isolated from the spleen of an infected pig during a 2020 outbreak, via Illumina NovaSeq6000 (Truong et al. 2021). Mileto et al., meanwhile, completely sequenced ASFV/Timor-Leste-2019-1, using a combination of Illumina MiSeq 150PE and ONT MinION long-read sequencing to resolve the terminal repeats (Mileto et al. 2021). In Africa, Bisimwa et al. reported the sequence (missing only the termini) of Uvira B53, a genotype X strain from the Democratic Republic of the Congo, using an Illumina HiSeq X (Bisimwa et al. 2021). Njau et al. published the first complete genome sequence of a genotype II ASFV from Africa – specifically Tanzania/Rukwa/2017/1 – via an Illumina MiSeq. They found that this isolate was closely related to Georgia 2007/1-derived viruses, which differences including the length/copy number changes in the MGF360 and 110 families (Njau, Domelevo Entfellner, et al. 2021). Later, Hakizimana et al. used an Illumina NovaSeq6000 to completely sequence the genotype X BUR/18/Rutana and genotype II MAL/19/Karonga (responsible for outbreaks in domestic pigs in Burundi and Malawi, respectively) (Hakizimana, Ntirandekura, et al. 2021). Finally, Fiori et al. released the largest single batch of complete ASFV sequences to date, describing 58 genomes from laboratory virus archives in Sardinia (Fiori et al. 2021) via Illumina HiSeq 2500. The researchers used this data to estimate the evolutionary rate of ASFV in Sardinia at ~3.20x10-6 substitutions/site/year, approximately two orders of magnitude below previously reported values for Eurasian and African ASFV outbreaks between 1960 and 2015 (Alkhamis et al. 2018). Though unable to be directly compared due to differences in sequence datasets, these results suggest that the insularity of Sardinia and its unique farm management styles (including the aforementioned free-ranging pig populations) may place constraints on the virus's evolution (Fiori et al. 2021). These findings were corroborated by Torresi et al., who published the complete sequences (obtained via Illumina MiSeq and HiSeq 3000 instruments) of 12 Sardinian isolates collected between 1978 and 2012. They found a remarkable genomic stability among these isolates, with no indication of attenuation or changes in virulence (Torresi et al. 2020).

As more complete ASFV genome sequences have been published, the research focus has shifted from quantity to quality. Of the 114 complete sequences available in October 2021, for instance, most were Georgia 2007/1 derivatives with unknown depth and quality of reads (D. Gladue 2021). Unfortunately, most of these strains are only derivatives of the current outbreak strain, originated in the Republic of Georgia, therefore the full picture of current strains circulating in Africa is not available, and that presents a current GAP. The table below summaries the original samples from 2018. including their collection date, country of origin and the host they were isolated from. However, it remains a gap that there is the very limited sequence data from isolates obtained from species other than domestic swine, particularly in Africa, where there are only six isolates that originated from ticks, one from warthogs, and one from wild boar. The lack of diverse sequence information allows for only very limited interpretation of the differences between different isolates and does not allow for tracking genetic evolution of new strains, as most of the sequenced pandemic strains are very closely genetically related to the original outbreak strain in Georgia, with very few mutational changes present. Sequencing studies often suffer from a lack of standardization in sample selection, sequencing method and validation, and bioinformatics (Forth, Forth, Blome, et al. 2020). The strengths and weaknesses of the various next-generation and third-generation sequencing platforms must be kept in mind, with an emphasis on reporting methodological details and gathering high-quality, comparable genome sequences to ensure harmonization within the literature (Forth 2021; D. Gladue 2021).

The genomes of susceptible host species are an additional a major gap in ASFV research. While there are fourteen individual genomes from breeds of *Sus scrofa* on NCBI, the genome sequences of the wild and domestic pigs, boar and warthogs in outbreak areas of Africa and Europe remains largely unknown. In order to conduct genomic studies as to what factors contribute to resistance of some of these breeds, a large-scale sequencing effort would have to be conducted. The major GAPs would include the species and subspecies of both domestic and wild pigs in endemic or outbreak areas, and the genomic sequences of animals that are able to survive an outbreak.

Although recent advances in next-generation sequencing have proven to be of value for both the sequencing of ASFV and host genomes, the problem still exists, largely due to the extensive cost and amount of work involved not only in sampling but also in sequencing and building these large genomes. In the case of ASFV genome sequencing, better protocols to separate the viral DNA away from the host DNA could make the sequencing effort easier and more cost effective. Without this information functional genomic studies are limited only to a particular strain being used by an individual laboratory. As this information becomes more readily available it will allow for a better prediction of the potential cross protection between isolates and virus evolution, both over time and during individual outbreaks.

Transcriptomics of ASFV and of the host during various stages of infection

ASFV has 150-170 ORF (open reading frames), however the majority of these ORFs are only predictions and very few have any experimental evidence on either the RNA or protein level. While it is likely that the majority of these ORFs do produce a protein product, it is possible that the expression profiles of these viral genes could vary between isolate and could differ depending on which host is infected, which could explain the varying outcome of ASFV varying

from 100% mortality to sub-clinical infections. However, to date there is no published information on the RNA or protein profiles of ASFV expressed genes, even at the experimental or in vitro level.

In Ploufragan (Jaing *et al*., 2016) presented the gene expression profile analysis of whole blood RNA from pigs infected with low and high pathogenic ASFV. RNAseq analysis identified 395 genes most differently expressed at euthanasia day in the highly pathogenic Georgia 2007 strain and 181 genes modified at 7 days post infection in the attenuated OURT88/3 group. The top 20 common genes that had the highest differential expression between both groups were genes related with macrophage markers, natural killer cell markers, chemokines and other important immune response markers.

There is a gap in knowledge for which are the receptor(s) that the virus uses to infect swine macrophages. It is possible that a number of molecules act as receptors and co-receptors at infection. One of the candidate genes CD163, a scavenger receptor which is expressed by mature macrophages and correlates with permissiveness to ASFV infection. However, using CRISPR/Cas9 gene-edited pigs with a mutated CD163 demonstrated that the recombinant pigs lacking CD163 were still susceptible to infection with no changes observed in viral virulence, suggesting that CD163 is not the receptor for ASFV [2]. The use of a wild boar isolated stable cell line (WSL) to grow adapted ASFV from several different isolates was reported as a useful tool for virus growth in cell culture as no observed large deletions in the genome were detected with full length sequencing of these viral genomes after successive growth passages of several ASFV virus isolates. (Keil *et al*., 2016).

Functional Genomics of ASFV proteins

The majority of ASFV proteins, have limited experimentally proven functions; functional genomics has been limited to mostly prediction, either by conserved protein sequences or domains in other virus families or host proteins. The lack of experimentally proven functions is a major gap in ASF research. Understanding the role for ASFV proteins during infection is critical to understanding both the pathogenesis of ASF, but also understanding how ASFV is able to avoid detection by the host immune system and cause disease. Understanding the functionality of any particular ASFV protein shouldn't stop at functional prediction, and identification of the protein partners both viral and host for a particular protein is a major gap in ASFV research. To date there has been very little information reported for host-viral protein interactions.

Some relevant recent information was released at the GARA Gap Analysis Workshop in Ploufragan, France, referring to the previously uncharacterized ASFV Ep152R gene functionality and its interaction with cellular protein Bag6 [3]. It was also reported the identification the mechanism of virus uncoating at viral entry by endocytosis and some cellular molecules that are relevant to this process (Cuesta-Geijo *et al*., 2016). Also, they reported the previously unidentified role of natural innate immunity mechanisms related to interferon induced proteins that are able to inhibit virus entry to the cytoplasm from the endosome called IFITM proteins (Muñoz-Moreno *et al.*, 2016). In addition the purification of ASFV particles and characterization of the proteome of mature extracellular ASF virions using a mass spectrometry

approach was reported, and the identification of new viral and host-derived ASFV structural proteins was also presented (Kessler *et al*, 2016).

A clear understanding of the functionality of ASFV proteins and the role they play during infection, in particular how ASFV evades the immune response, is critical for the development of rationally designed live-attenuated vaccines. Large scale functional genomic studies represent a significant part of this gap that could be accomplished either by direct protein-protein identification methods such as yeast two-hybrid or Co-immunoprecipitation (Co-IP) followed by mass spectroscopy. Purification of viral proteins and in vitro assays to confirm their functional prediction functions would be of additional value.

Host Genomic Screens to determine virulence factors for ASFV

In light of today's technology, in which large scale genomic screens have been accomplished for many other viruses, to date no such screens have been reported for ASFV, representing a large Gap in ASFV research. These genomic screens need swine specific reagents, for example loss of gene function screens both either by siRNA screening or CRISPR/Cas9 screening would require a library that is targeted against the swine genome. Development of these swine specific libraries would be necessary for reliable large scale *in vitro* genomic screens for ASFV. These screens would help gain an insight into the host pathways that are critical for ASFV replication and could lead to the discovery of the virus cellular receptors, immune markers of infection, and the pathways involved in virus replication and virus virulence. This information would contribute to understanding virus cell tropism and thus to development of cell lines that support virus replication as well as improving understanding of virus pathogenesis.

The major gaps identified in 2022 are as follows:

- Computational characterization and experimental validation of ASFV proteins with unknown functions
- Host and viral transcriptomics and proteomics throughout infection
- Host-virus interactions throughout the infection process
- Viral entry pathways and potential cell-surface receptors for ASFV
- Sequence-to-phenotype prediction models
- Collection of complete ASFV genome sequences, particularly those causing outbreaks in Africa
- Standardization of ASFV genome sequencing workflows to ensure comparable data, including the use of online bioinformatics databases
- Increased integration of next- and third-generation sequencing techniques to produce sequence data of the highest possible quality

- Sequencing of historical ASFV genome sequences using next-generation sequencing (NGS). To validate previously sequenced isolates using NGS technology, and to sequence genomes where full-length sequencing was not performed.

Sequencing of wild pig genomesWith current DNA sequencing technologies it would be relatively easy and cheap to sequence the complete genomes from 1) 1-3 isolates from each genotype, 2) a series of viruses (>10) with different virulence and 3) a series of viruses (>5) that have replicated exclusively in domestic pigs, wild pigs and ticks.

2) ASFV bioinformatics resource:
There is a need to continue the annotation and analysis of ASFV genomes. The size range of ASFV is difficult and requires specialized tools. The acquisition of more genome sequences will make the management and comparison of the gene complement even more complicated. Although there is a good amount of sequencing data available for ASFV, using current, very robust technologies, it has been possible to develop a comprehensive database, which includes full length genome sequence of large number of isolates to replace the current less meaningful genotype based classification: https://virology.uvic.ca/organisms/dsdna-viruses/asfarviridae/

3) CRISPR/Cas9 or siRNA libraries targeting swine genomes.
Development of a swine specific knockout libraries are critical for *in vitro* genomic screens for ASFV. Libraries targeting the swine genome would be highly valuable to perform host genomic screens for ASFV for a wide range of experimental avenues that could lead to the discovery of potential receptors, pathways modulated to avoid immune detection or for increased virus virulence.

4) Viral Transcriptomic Studies

- Genomic wide either on the RNA or Protein level data for ASFV gene expression would be relatively easy with current technologies and could provide data to determine differences in ASFV gene expression *in vitro* and *in vivo* in different hosts.

PATHOGENESIS

African swine fever virus infection of domestic swine results in several forms of the disease, ranging from highly lethal acute manifestations to subclinical depending on contributing viral and host factors (Tulman *et al*., 2009). In Africa, highly virulent viruses produce a broad range of responses in populations of pigs in endemic areas. At the herd or population level, infections may result in 50-100 percent of the pigs seroconverting, but showing no signs of disease, with variable proportion of the pigs dying of acute ASF. Unlike domestic swine, wild African suids infected with ASFV are generally asymptomatic with low viremia titers (Heuschele and Coggins 1969; Montgomery 1921; Plowright 1981; Thomson 1985). These features of ASF presentation and the resemblance of the clinical manifestation to other diseases in swine such as Erysipelas and Classical Swine Fever hamper syndromic surveillance in domestic swine based exclusively on clinical signs.

Infection usually occurs through the oronasal route with primary virus replication in tonsils followed by a viremia with further secondary replication of all organs of the hemolymphatic

system. In the acute form of the disease, the incubation period ranges from 5 to 15 days. Affected animals exhibit fever and anorexia followed by congestion and cyanosis of the skin, increased respiratory and heart rates, nasal discharge, incoordination, vomiting and, finally, coma and death. Hemorrhage may be observed clinically in multiple forms and secretions including epistaxis, melena, hematochezia, and hematemesis. Survival times for animals infected with African ASFV strains range from 2 to 9 days (Conceicao 1949; Creig and Plowright 1970; Haresnape *et al*., 1988; Mendes 1961; Thomson *et al*., 1979; Howey et al, 2013). Typical clinical pathological findings in acute ASF include leukopenia (Detray and Scott 1957; Edwards *et al*., 1985; Wardley and Wilkinson 1977), B and T cell lymphopenia (Sánchez Vizcaino *et al*., 1981; Wardley and Wilkinson 1980), thrombocytopenia (Anderson *et al*., 1987; Edwards 1983; Edwards *et al*., 1985), lymphocyte and mononuclear cell apoptosis (Carrasco *et al*., 1996; Gomez-Villamandos *et al*., 1995; Oura *et al*., 1998c; Ramiro-Ibañez *et al*., 1996; Salguero *et al*., 2004). Morphologic lesions may include hemorrhage in lymph nodes, spleen, kidneys, and respiratory and gastrointestinal tracts, congestion of skin and serosae, severe interlobular lung edema, and cavitary effusions that may range from serofibrinous to hemorrhagic. (DeKock *et al*., 1994; Detray 1963; Konno *et al*., 1972; Manso Ribeiro and Rosa Azevedo 1961; Maurer *et al*., 1958; Montgomery 1921; Nunes Petisca 1965; Steyn 1928 and 1932; Howey et al, 2013). The extensive necrosis in affected tissues and severe hemostatic and hemodynamic changes are likely important factors leading to death. Acute ASF also induces significant changes in acute-phase proteins (Carpintero *et al*., 2007; Sanchez-Cordon *et al*., 2007). Subacute cases last 3–4 weeks and the most prominent signs include remittent fever, loss of condition, pneumonia, dyspnea, cardiac insufficiency and swelling of the joints. While hemorrhage of lymph nodes and other tissues may be found, it is not as prominent as in acute ASF (Moulton and Coggins 1968a). The primary cell types infected by ASFV are those belonging to the mononuclear- phagocytic system, including fixed tissue macrophages and specific lineages of reticular cells (Colgrove *et al*., 1969; Konno *et al*., 1971a and 1971b; Mebus 1988; Moulton and Coggins 1968a). Affected tissues show extensive damage after infection with highly virulent viral strains. Moderately virulent ASFV strains also appear to infect these cell types, but the degree of tissue involvement and the resulting tissue damage are much less severe. The ability of ASFV to replicate and efficiently induce marked cytopathology in macrophages *in vivo* in numerous porcine tissues (Howey *et al*, 2013) appears to be a critical factor in ASFV virulence.

Long term persistence following infection of pigs with genotype I isolates of reduced virulence has been demonstrated (Wilkinson 1984; Carrillo *et al*., 1994). These persistent infections have been demonstrated to be transmissible from pigs persistently infected with the low virulence genotype I NH/P68 isolate to contact pigs (Gallardo *et al*. 2015). Low virulence isolates can cause chronic forms of the disease, which are characterized by the absence of typical acute-phase lesions and low mortality rates, but distinct clinical signs including delayed growth, emaciation, joint swelling, skin ulcers and secondary bacterial infections are common (Sanchez-Vizcano 2015). Pigs that survive infection have been shown to carry virus in tissues or blood for long periods of time, which may contribute to virus transmission, disease persistence, sporadic outbreaks and sudden reactivation of the disease (Costard *et al*. 2013; Gallardo *et al*., 2015). Some studies in Africa have identified ASFV nucleic acid in apparently healthy pigs (Kalenzi Atuhaire *et al*., 2013; Thomas *et al.*, 2016) that were positive for ASFV by PCR in tissues but negative in blood by PCR and serology (Okoth *et al*. 2013; Abworo *et al*., 2017). There is limited experimental evidence for transmission from persistently infected to naïve animals, and

the relevance of persistently infected animals as carriers of ASF in the field is not clear, but data on healthy infected animals keeps accumulating (Titov *et al*. 2017, Abworo *et al*., 2017, Thomas *et al*., 2016, Muhangi *et al*., 2015, Braae *et al*., 2015, Athuaire *et al.*, 2013), suggesting that the virulent virus could survive for long periods of time in the recovered pigs and a recrudescence of virulence may occur at later times (Titov *et al*., 2017).

Persistent infection with ASFV has been reported to occur in warthogs and in domestic pigs surviving acute viral infection [DeKock *et al*., 1940; Detray 1957; Plowright et al., 1969). Under experimental conditions, long-term persistent infection is the sequel to infection with ASFV (E75-L7 administered at low dose intramuscularly (im), E75-CV1 on and challenged twice with E75-L7, and E75-CV1 challenge once with E75-L7] in domestic pigs (Carrillo *et al*., 1994). In these animals, viral DNA was detected in the peripheral blood monocyte fraction more than 500 days post inoculation (p.i.) by PCR in intermittent periods; however, infectious virus could not be isolated from these samples and transmission was never demonstrated. Recent data using a moderately virulent isolate of different genotype during a shorter period of time (Petrov *et al*., 2018), do not support the establishment of a carrier status in animals surviving infection, though long term detection of viral genome in blood (for at least 90 pi) is consistent with many other reports (McVicar 1984; Mebus and Dardiri, 1980; Carrillo *et al*., 1994; Gallardo *et al.*, 2015; Carvalho Ferreira *et al*., 2012).

In sub-Saharan Africa, ASFV is maintained in a sylvatic cycle between wild suids (warthogs) and argasid ticks of the genus Ornithodoros (Plowright *et al*., 1969a and 1969b; Thomson *et al*., 1983; Wilkinson 1989). However, other wild pigs such as bush pigs do not inhabit burrows, and therefore would most likely spread ASFV via direct transmission, although evidence for such occurrences is limited (Jori and Bastos 2009; Jori *et al.* 2013). Unlike domestic swine, wild suids infected with ASFV are generally asymptomatic with low viremia titers (Heuschele and Coggins 1969; Montgomery 1921; Plowright 1981; Thomson 1985). Most adult warthogs in ASFV enzootic areas are seropositive and are likely to be persistently infected. Like warthogs, bush pigs develop subclinical infection and are more resistant to direct-contact transmission than are domestic species; however, the duration of ASFV viremia may be extended (Anderson *et al*., 1998). Although ASFV replication in blood leukocytes of domestic swine, warthogs, and bush pigs *in vitro* is similar, ASFV replication, spread, and induction of lymphocyte apoptosis *in vivo* is reduced in bush pigs when compared to domestic swine (Anderson *et al*., 1998; Oura *et al*., 1998a and 1998b).

Deletion of this gene, which is clearly involved, as MGF360/530 genes, in interferon response modulation, reduces virulence of the Benin isolate in pigs and induces protection against challenge with the homologous virus.

These studies have made significant contributions in increasing our understanding of the molecular basis of ASFV pathogenesis and the particular role of viral proteins in the outcome of the disease.

GapsPrevious reports (GARA 2018; 2016) identified the following priority research knowledge gaps in ASF pathogenesis over the past 6 years:

- Mechanisms of host-to-host infection in swine and ticks

- Determinants of virulence for different genotypes/strains in various hosts
- Identification of phylogenetic markers associated with evolving ASFV virulence, host range, and pathogenicity in endemic areas
- Activation patterns of host immune genes, especially early in infection
- Host genomic screens to identify ASFV virulence factors

ASFV virulence is a relative phenomenon, with observed differences depending on multiple variables including the viral strain, the route and dose of infection, and the host animal (RockStudies of the genomes and *in vivo* infection dynamics of individual virus strains can provide valuable information on potential virulence determinants and inter-strain differences in pathology. Portugal *et al.* compared the genomes of two Portuguese ASFV strains from the 20th century epidemic – the high-virulence Lisboa60 and the naturally attenuated NH/P68 – identifying several genes with significant differences between the strains (Portugal et al. 2015). Notable findings included left variable region genes present (e.g. MGF110-2L and -9L, MGF505-5R and -8R, and 86R) or deleted (MGF360-6L) in NH/P68. This strain also displayed mutations in the *B119L*, *I215L*, and *CP312R* genes (Portugal et al. 2015). In 2017, Gallardo *et al.* published an examination of the infection kinetics caused by the Lithuania 2014 (LT14/1490) field isolate, finding 94.5% mortality (with one in-contact pig remaining asymptomatic and surviving infection) (Gallardo et al. Later, this group studied the evolution of ASFV virulence by comparing the moderately virulent southern Estonian strains Es15/WB-Tartu-14 and Es15/WB-Valga-6 (Gallardo, Nurmoja, et al. 2018). The Tartu strain exhibited a much shorter incubation period and severe clinical pathology – interestingly, however, pigs that were "in-contact" (not experimentally inoculated, but exposed to pigs that were) with either strain developed varying disease courses covering acute, subacute, and chronic presentations with 50% mortality overall. Survivor pigs experienced recurring disease/viraemia, though none were able to transmit ASFV to sentinels introduced 137 days post-exposure (Gallardo, Nurmoja, et al. 2018).

Meanwhile, Sehl *et al.* studied experimental infections of domestic pigs and wild boar with the moderately virulent "Estonia 2014" strain, previously associated with the high number of clinically healthy but seropositive wild boar discovered in northeast Estonia, as discussed above (Nurmoja, Schulz, et al. 2017). The virus was highly virulent in wild boar and only moderately virulent in domestic pigs (Sehl et al. 2020). The determinants of this difference are unknown, though a high viral antigen load in wild boar at 7 days post-infection (dpi) (at which point domestic pigs had already cleared the infection) suggested that early viral clearance was more effective in domestic pigs (Sehl et al.In 2021, Gallardo *et al.* conducted a comparative study of pathology in three Eurasian virus isolates: the Polish Pol16/DP/OUT21, Estonian Est16/WB/Viru8, and non-haemadsorbing Latvian Lv17/WB/Rie1. The viruses demonstrated an increasing curve of virulence and clinical pathology – the traditional acute, lethal presentation in domestic pigs infected with the Polish strain, a delayed and slightly more survivable presentation with the Estonian strain, and a minimally symptomatic, non-lethal disease with the Latvian strain (Gallardo et al.Interestingly, infection with the Latvian and Estonian strains also led to persistence of virus for over 2 months in primary (e.g. tonsils and lymph nodes) and some secondary replication sites (Gallardo et al. 2021).

As mentioned above, virulent ASFV infection is associated with lymphocyte depletion and massive cell death (apoptosis and necrosis) in lymphoid tissues (Salguero 2020), but the

molecular determinants of this pathology remain unknown. Li *et al.* conducted an *in vitro* study of 94 viral proteins to identify contributing functional factors, showing that the protein pE199L (a late-stage protein involved in viral entry and cell autophagy) interacts with the pro-apoptotic host effector Bak to promote cell death via permeabilization of the mitochondrial outer membrane (T. Li et al. 2021). pE199L also promotes autophagy by interacting with the autophagy-associated host protein PYCR2 and downregulating its expression (S. Chen et al. 2021). The role of autophagy in ASFV infection is unclear, but these results suggest that the virus manipulates this process to promote survival (S. Chen et al. 2021). Meanwhile, Wang *et al.* characterized the *in vivo* kinetics of cytokine release in domestic pigs infected with the SY18 ASFV strain, identifying three stages in acute infection: (1) a primary phase (0-2 dpi): no symptoms and no change in cytokine levels; (2) a clinical phase (3-7 dpi): "cytokine storm" with extensive upregulation of expression of proinflammatory cytokines and chemokines including TNF-α, IFN-α, and several interleukins (ILs); and (3) a terminal phase (7-8 dpi): additional upregulation of expression of multiple cytokines (e.g. TNF-α, IL-1β, and IL-10) (S. Wang et al. 2021). IFN-γ expression was absent throughout the study, possibly reflecting an impaired activation of natural killer (NK) cells (S. Wang et al. 2021), though this has yet to be formally demonstrated.

In 2018, Keßler *et al.* used mass spectrometry and a recombinant mutant of the naturally attenuated OURT88/3 ASFV strain to produce an *in vitro* catalogue of expressed viral proteins, identify core proteins required to support infection, and clarify host-specific differences in expression profiles (Keßler et al. 2018). Among other findings, the researchers identified the expression of 23 uncharacterized ASFV ORFs, including three functionally unknown proteins (pK145R, pC129R, and pI73R) that were highly expressed in a wild boar cell line (Keßler et al. 2018). Later, Yang *et al.* constructed an interaction network between the viral protein MGF360-9L (a highly conserved protein previously shown to impact virulence in domestic pigs) and host factors in transfected PK-15 porcine kidney cells (B. Yang et al.Immunoprecipitation and liquid chromatography-mass spectrometry identified 268 host proteins that interact with MGF360-9L; subsequent GO and KEGG analyses showed that these proteins were enriched in the proteasome, ribosome, spliceosome, carbon metabolism, and host metabolic response pathways (B. Yang et al. 2021).

Many recent studies have focused on the important task of testing the functional effects of individual gene deletions on the virulence and infectivity of ASFV, with positive and negative results alike critical for expanding our knowledge of the poorly understood ASFV proteome. In 2020, Ramirez-Medina et al. published a string of reports on the in vitro and in vivo virulence of Georgian ASFV strains with deletions in previously uncharacterized genes. The C962R, X69R, and MGF360-1L genes were all dispensable for ASFV infectivity, with no impact on viral replication kinetics in primary swine macrophage cultures or on clinical disease in vivo (Ramirez-Medina, Vuono, Rai, Pruitt, Ediane, et al. 2020; Ramirez-Medina, Vuono, Pruitt, et al. 2020; Ramirez-Medina, Vuono, Rai, Pruitt, Silva, et al. 2020). Hübner et al., continuing the proteomic work by Keßler et al. described above, examined the uncharacterized proteins p285L and pK145R in the virulent Armenia08 strain. They found that the former localized in purified ASFV virions, while the latter was present diffusely in the cytoplasm of infected cells, and neither protein was essential for in vitro viral propagation (Hübner et al. 2021). Meanwhile, Li et al. evaluated the in vitro and in vivo functions of MGF505-7R, previously found to degrade the

innate immunity-related STING protein (D. Li et al. 2021), and found that this protein inhibited the IFN-γ-mediated JAK-STAT1 proinflammatory signalling pathway (J. Li et al. 2021). Deletion of this gene from the virulent CN/GS/2018 strain reduced viral replication in primary porcine alveolar macrophages (PAMs) and attenuated its pathology in vivo (100% survival with moderate clinical symptoms in infected pigs) (J. Li et al. 2021).

In 2017, Reis et al. found that deleting the early gene DP148R from the virulent genotype I Benin 97/1 strain substantially reduced virulence in vivo without impacting replication in vitro (Reis et al.Following up on this study, Rathakrishnan et al. tested the impact of deleting this gene, alone or in combination with K145R deletion, on the virulence of Georgia 2007/1 (Rathakrishnan et al. 2021). In contrast to Benin 97/1, DP148R deletion did not impact the Georgia strain's virulence in vitro or in vivo: co-deletion of K145R delayed the onset of disease and viraemia in experimentally infected pigs by 3 days, but clinical symptoms and mortality remained unchanged (Rathakrishnan et al. 2021). In another related study from this group, Petrovan et al. tested deletions of the EP153R and EP402R genes in DP148R-deleted Benin 97/1 (BeninΔDP148R). They found that deleting EP153R had no additional effect, while deleting EP402R substantially reduced virus and viral genome persistence in vitro. Deleting both (in addition to DP148R) reduced viraemia and clinical signs to nil, but protection against virulent challenge was also reduced (Petrovan et al. 2022). Immunological protection will be discussed in more detail in the Vaccines section.

In 2021, Vuono et al. investigated the KP177R gene (encoding the viral inner membrane structural protein p22) in the Georgia2010 strain, finding that deletion did not impact replication and infection dynamics in vitro and in vivo (Vuono et al. 2021). Studies of two MGF gene deletions – MGF110-1L and MGF100-1R in Georgia 2007/1 and the virulent Chinese strain GZ201801, respectively – showed that both genes were non-essential, with no impact on in vitro replication kinetics or in vivo disease course (Ramirez-Medina et al. 2021; Y. Liu et al. 2021).

Finally, Chaulagain et al. conducted an in vitro study of the viral CD2v adhesion protein (encoded by the EP402R gene), previously implicated in virulence, cell entry, and immunomodulation (Dixon et al. 2019; Netherton, Connell, et al. 2019; Rock 2021; Pérez-Núñez et al. 2015) and essential for viral replication in ticks (Chaulagain et al. 2021). CD2v deletion has been observed in several naturally attenuated non-HAD strains (including OURT88/3, Lv17/WB/Rie1, and NH/P68), but its effect appears to be strain-dependent and can result in attenuation or have no effect (Borca, O'Donnell, et al. 2020; Chaulagain et al. 2021). Here, the researchers transfected a porcine cell line and swine peripheral blood mononuclear cells (PBMCs) and macrophages with the EP402R gene from the virulent genotype I Congo K-49 strain. They found that CD2v expression activates the key inflammatory transcription factor NF-κB, induces the transcription of IFN-β and interferon (IFN)-stimulated genes, and promotes an antiviral state and apoptosis in primary host cells (potentially involved in the extensive lymphoid cell death associated with ASF) (Chaulagain et al. 2021).

As previously mentioned, in vitro studies suggested that CD163, a cell-surface marker expressed on mature tissue macrophages, acts as a viral receptor during ASFV infection (Dixon et al. 2019). In 2017, however, Popescu et al. used CRIPSR-Cas9 to generate CD163 knockout pigs, finding that this deletion was not protective against infection with the Georgia 2007/1 strain.

Possible compensatory upregulation of expression of other macrophage surface markers was not observed, though it cannot be comprehensively ruled out (Popescu et al. 2017).

In Africa, warthogs (Phacochoerus africanus) are the natural mammalian host of ASFV; as discussed above, they display remarkable resilience to ASFV infection, showing no clinical signs of disease when infected with strains that induce acute haemorrhage and death in domestic pigs (Arias et al. 2018). The provenance of this resistance is unclear, with genetic and environmental characteristics proposed as possible explanations. In 2019, Correa-Fiz et al. addressed the latter category, comparing the faecal microbiota of various domestic pigs and warthogs from Africa and a Spanish zoo (Correa-Fiz et al. 2019). Among other results, the researchers found six operational taxonomic units present only in resistant animals, including members of the *Anaeroplasma*, *Petrimonas*, and *Moraxella* genera (Correa-Fiz et al. 2019). In a follow-up study, this research group transplanted faecal microbiota from warthogs to domestic piglets and monitored for any associated changes in response to infection with virulent (E75 strain) or attenuated (E75CV1) ASFV (J. Zhang et al. 2020). Surprisingly, transplantation did not substantially alter the microbiota of the transplanted animals, and no difference was observed in response to virulent viral infection. However, increased total IgA levels were observed in transplanted animals, and the transplant conferred partial protection against infection with the attenuated strain (J. Zhang et al. 2020).

Research is ongoing into the determinants of warthog resistance to ASFV, as the relevant biological mechanisms may have promise for disease control in Eurasian swine populations. Individual gene deletions and larger-scale proteomics studies also continue to define ASFV's highly complex pathogenetic landscape. The virulence determinants and host-pathogen interactions of ASFV (including immunological factors such as the roles of IFN-γ and CD8+ T cells) are far from being fully understood (Pikalo et al. 2019), and more research will be required to definitively identify crucial viral proteins and inter-strain differences in proteins necessary for infection.

Information presented in the 2022 meeting has demonstrated that a field isolate (DR21) obtained from domestic pigs presenting ASF clinical disease in Dominican Republic showed a decreased ability to produces lethal disease and transmit the virus to cohabitating naïve pigs. DR21 virus only produced an acute lethal disease if parenterally inoculated while induce a mild and protracted form of the disease if it is administrated oronosally or by contact (.doi.org/10.3390/v14051090). The characterization of the virulence and transmissibility of the ASFV isolate (DR2021) causing disease outbreaks in Dominican Republic (DR) was presented. Results demonstrated that, unless parenterally inoculated, the DR2021 produced a protracted form of clinic disease being lethal in approximately in only 50% of the animals inoculated oronasally or by contact. In addition, most of the animals develop a strong virus specific antibody response. Therefore although, full length sequence of the DR2021 isolate showed subtle differences with the potentially parental Georgia derivatives, it appears to present clear differences in terms of virulence and transmissibility.

The following areas of ASF pathogenesis were identified as GAPS in 2022:

- Proteins required for virulence in various strains of ASFV
- Transcriptional dynamics of the ASFV genome across the four temporal stages of viral infection
- Genetic mutations and functional mechanisms underlying the decreased virulence observed in some naturally attenuated circulating strains
- Functions of MGFs in virus-host interactions and virulence
- Determinants of warthog and bushpig resistance to virulent ASFV
- Standardization of pathogenesis models and experimental ASFV delivery routes to ensure comparability of data

Research needs

IMMUNOLOGY

A key hurdle in developing a safe and effective ASF vaccine has been the lack immunological information. Attempts to vaccinate animals using infected cell extracts, supernatants of infected pig peripheral blood leukocytes, purified and inactivated virions, infected glutaraldehyde-fixed macrophages, or detergent-treated infected alveolar macrophages failed to induce protective immunity (Coggins 1974; Forman *et al*., 1982; Kihm *et al*., 1987; Mebus 1988). Homologous protective immunity does develop in pigs surviving viral infection. Pigs surviving acute infection with moderately virulent or attenuated variants of ASFV develop long-term resistance to homologous, but rarely to heterologous, virus challenge (Hamdy and Dardiri 1984; Ruiz-Gonzalvo *et al*., 1981). Pigs immunized with live attenuated ASF viruses containing engineered deletions of specific ASFV virulence/host range genes were protected when challenged with homologous parental virus (Lewis *et al*., 2000; Moore *et al*., 1998; Zsak *et al*., 1996 and 1998). Humoral and cellular immunity are significant components of the protective immune response to ASF. Antibodies to ASFV are sufficient to protect pigs from lethal ASFV infection (Hamdy and Dardiri 1984; Onisk *et al*., 1994; Ruiz-Gonzalvo *et al*., 1981). Although ASFV neutralizing antibodies directed against virion proteins p30, p54, and p72 have been described (Borca *et al*., 1994a; Gomez-Puertas *et al*., 1996; Zsak *et al*., 1993), they are not sufficient for antibody-mediated protection (Neilan *et al*., 2004). CD8 + lymphocytes also appear to have a role in the protective immune response to ASFV infection (Oura *et al*., 2005).

ASFV, similar to other large DNA viruses, affects and modulates host immune responses. ASFV-infected macrophages mediate changes in cellular immune function, and they likely play a role in the severe apoptosis observed in lymphoid tissue (Childerstone *et al*., 1998; Oura *et al*., 1998c; Ramiro-Ibañez *et al*., 1996; Takamatsu *et al*., 1999). ASFV inhibits phorbol myristic acid-induced expression of proinflammatory cytokines such as TNF-α, IFN-α, and IL-8 while inducing production of TGF-β from infected macrophages (Powell *et al*., 1996). Conversely, increased TNF-α expression has been reported after ASFV infection *in vitro* and *in vivo* and TNF-α may play a key role in ASFV pathogenesis, including changes in vascular permeability, coagulation, and induction of apoptosis in uninfected lymphocytes (Gomez del Moral *et al*., 1999; Salguero *et al*., 2002 and 2005). Notably, ASFV strains with different virulence phenotypes differ in their ability to induce expression of proinflammatory cytokine or IFN-

related genes in macrophages early in infection (Afonso *et al*., 2004; Gil *et al*., 2003; Zhang *et al*., 2006). The ASFV ankyrin repeat-containing protein pA238L (5EL) is the only known viral homolog of cellular IκB proteins, the cytoplasmic inhibitors of the NFκB/Rel family of cellular transcription factors, and it is thought to be important in evading host immune responses (Miskin *et al*., 1998; Powell *et al*., 1996). The activity of pA238L provides a novel mechanism for ASFV to modulate the response of host cells to infection, especially considering the role of NFκB transcriptional pathways in inducing expression of a wide range of proinflammatory and antiviral mediators and cytokines. Consistent with this role, pA238L is able to regulate expression of cyclooxygenase-2 (COX-2), TNF-α, and inducible nitric-oxide synthase (iNOS). COX-2 downregulation occurs in an NFκB-independent, but NFAT-dependent, manner (Granja *et al*., 2004b). Similarly, pA238L inhibits expression of iNOS, and ultimately production of nitric oxide, by a mechanism likely involving p300 transactivation. Interestingly, deletion of A238L from pathogenic ASFV does not affect viral growth in macrophages *in vitro* or viral pathogenesis and virulence in domestic swine (Neilan *et al*., 1997b). Additional ASFV-encoded proteins modulate or interfere with host immune responses. The ASFV 8DR protein (pEP402R) is the only known viral homolog of cellular CD2, a T cell protein involved in co-regulation of cell activation (Borca *et al*., 1994b; Rodriguez *et al*., 1993a). 8DR is necessary and sufficient for mediating hemoadsorption by ASFV-infected cells (Borca *et al*., 1994b; Rodriguez *et al*., 1993a). Deletion of the 8DR gene from the ASFV genome led to decreased early virus replication and generalization of infection in swine, and 8DR suppressed cellular immune responses *in vitro* (Borca *et al*., 1998). Side by side comparative *in situ* hybridization tests demonstrated that deletion of CD2 homologue absolutely abolished ASF viral replication in Thymus (Borca et al. 1998). The ASFV pEP153R (8CR) protein is similar to cellular and poxviral proteins resembling C-type lectin-like proteins, including membrane-bound immunoactivation and immunoregulatory proteins CD69 and NKG2 (Neilan *et al*., 1999; Yanez *et al*., 1995). A potential role for pEP153R in immunomodulation may be subtle, however, since pEP153R does not affect viral pathogenesis or virulence in domestic swine (Neilan *et al*., 1999). Evidence also suggests that ASFV dramatically affects Th2/B cell responses, including upregulation of Th2 cytokines by a soluble virulence factor (p36) released from ASFV-infected monocytes and the nonspecific activation and apoptosis seen in B cell populations from ASFV-infected animals (Takamatsu *et al.,* 1999; Vilanova *et al*., 1999). ASFV multigene family 360 and 530 genes play a role in modulating host innate responses. Unlike wild type virus, infection of macrophages with Pr4Δ35, a mutant virus lacking MGF360/530 genes, resulted in increased mRNA levels for several type I interferon early-response genes (Afonso *et al*., 2004). Analysis of IFN-α mRNA and secreted IFN-α levels at 3, 8, and 24 hours post infection (p.i.) revealed undetectable IFN-α in mock and wild type-infected macrophages but significantly increased IFN-α levels at 24 hours p.i. in Pr4Δ35-infected macrophages, indicating that MGF360/530 genes either directly or indirectly suppress a type I IFN response. This effect may account for the growth defect of Pr4Δ35 in macrophages and its attenuation in swine (Zsak *et al*., 2001).

In this regard, several reports support the importance of the function of MGF genes in the modulation of IFN responses, and their importance in virus virulence. The original experiments from Neilan *et al*., 2002, showing that deletion of MGF360/MGF530 genes reduce viral replication in macrophages, have recently been extended by others to show that these deletions also closely associate with decrease virus virulence in experimental infection studies in pigs (Krug *et al*., 2015; O'Donnell *et al*., 2015b; Reis *et al*., 2016; Sanchez-Cordon *et al*., 2016).

Similarly, deletion of a recently characterized gene, DP148R, also involved in interferon response modulation, was also shown to reduce virulence of the Benin isolate in pigs (Reis *et al*., 2017).

Important progress has also been made in the characterization of immunogenicity of ASFV proteins in pigs. For example, several reports are now available describing cellular and antibody responses elicited by ASFV antigens individually expressed using a diversity of expressing vectors: vaccinia virus (Lopera-Madrid *et al*., 2017); DNA immunization (Argilaguet *et al*., 2012; Argilaguet *et al*., 2013; Lacasta *el al*., 2014); adenovirus (Lokhandwala *et a*l., 2016; Lokhandwala *et al*., 2017); or the combination of these expression vectors (Jancovich *et al*., 2018). Some of these reports have gone further and also evaluated the ability of these viral proteins to induce a protective immune response in pigs (Argilaguet *et al*., 2012; Argilaguet *et al*., 2013; Lacasta *el al*., 2014; Jancovich *et al*., 2018). However, to date, most of these studies have only shown protection that does not reach values higher than 50% of the individuals under study, indicating the need of further work in order to expand the identification of virus antigens involved in the induction of protective immune responses.

In conclusion, a significant amount of data has now been accumulated toward the immunogenic characterization of several ASFV proteins. Importantly, in some cases, these virus proteins have also been evaluated for their ability to induce a protective immune response in pigs, which is the first step on the road towards the potential development of subunit vaccines.

Gaps

Attempts to induce protective immunity using different vaccine platforms have to date failed. Homologous protective immunity does develop in pigs surviving acute infection with moderately virulent or experimentally attenuated variants of ASFV. These animals develop long-term resistance to homologous, but rarely to heterologous, virus challenge. Humoral and cellular immunity have been shown to be significant components of the protective immune response to ASF. Although ASFV neutralizing antibodies have been described to be directed against particular virus proteins, they are not sufficient for antibody-mediated protection. Additionally, CD8+ lymphocytes also appear to have a role in the protective immune response to ASFV infection. Thus, although humoral and cellular immune response are involved in contributing to the protection against the infection, the actual immune mechanism(s) mediating that protection is still unclear. Additionally, the viral protein\proteins inducing the protective immune mechanism are still largely unknown. On the other hand, ASFV proteins have been shown to affect and modulate host immune responses *in vitro*.

As described above, advances have been achieved in identifying and understanding the function of virus genes modulating the host response and its direct effect during the process of infection in the natural host. Additionally, important progress has been achieved in the study of the immunogenicity of many previously uncharacterized viral proteins when administered to the natural host. Some of the key gaps that remain include:

1) The identification of immune mechanism(s) mediating protection against infection in swine remains one of the major questions to be answered.

2) Advances in the identification of the virus protein(s) responsible for the induction of protective immune mechanism.
2) Understanding the actual role of virus driven host immunomodulation in the process of virus infection in swine.
3) Correlation of protection between heterologous viral strains remains unclear.

Mechanisms of protection

Currently there is a large gap in understanding the mechanisms of protection induced by experimental vaccines for ASFV. Currently several reported experimental single or double gene deletions in the ASFV genome have resulted in live attenuated vaccine candidates, however how these vaccines protect against ASF is largely unknown. There is a lack of knowledge of the specific role for individual ASFV proteins in induction of protection, beyond a basic protein functional prediction. The cellular and antibody mediated mechanisms involved in protection are also largely uncharacterized. In addition the mechanisms of virus (or experimental vaccine) persistence is unknown. This is important to understand in order to avoid the persistence of live attenuated vaccines in the field and to understand and predict the persistence of filed isolates of reduce virulence.

The cellular immune response to ASFV or to any experimental vaccine is largely unknown, in part due to the lack of available knowledge on swine immunology. Currently it is largely unknown what specific cell types are involved in inducing an immune response or what cell types are involved in inducing long term protection to ASFV. To date there has been very little discovery of neutralizing or T-cell epitopes for ASFV. Recently p30, p54, p72 when expressed could produce neutralizing antibodies, but these antibodies did not confer protection to ASFV [4]. This suggests that we need to understand more broadly the role of antibodies in protection or disease enhancement, as there is potential for antibodies produced that are not neutralizing to be involved in disease, perhaps inhibiting the spread of the virus. It is also possible that antibody mediated enhancement of disease could occur as virus uptake into macrophages could be mediated by Fc receptors. Understanding the role for both antibodies to ASFV and the immune response to ASFV could allow us to understand the differences in both virulence between different ASFV isolates, and potentially an understanding why some wild African pigs become infected, but clinically do not exhibit any clinical signs of ASF. Understanding these mechanisms of protection will allow the generation of safer vaccines, including the possibility of creating subunit vaccines.

Correlation of protection between heterologous viral strains

The understanding of cross protection between heterologous viral strains is largely unknown, in part due to the lack of available genomic sequences of the different ASFV strains; thus, the diversity of ASFV is not clear. Largely, there has been very little work involving field strains in Africa, especially in current endemic areas. In Mozambique, serologically positive pigs were resistant to at least two highly virulent viruses, a genotype II and a genotype VIII virus, which are not closely related to one another, suggesting cross protection events under field conditions (Penrith *et al*., 2004). Possible countries identified for conducting cross protection studies include Uganda, Tanzania, Mozambique, and Kenya. In these countries, using endemic strains

for experimental vaccines could allow the possibility of conducting long term vaccine studies involving a large number of animals.

Understanding the mechanisms of cross protection will allow better cross protective next generation vaccines, and the ability to predict which vaccine to use if a new emerging ASFV strain were to cause an outbreak. Understanding the specific viral proteins involved in cross protection would involve a large effort in sequencing current circulating ASFV strains, and large cross protection studies with divergent strains.

Research needs

1) Discovery of the immune mechanism mediating effective homologous and heterologous protection against virus infection.
2) Identification of viral genetic patterns that correlate with presence/absence of homologous versus heterologous protection.
3) Identification of virus protein\s involved in the induction of protective immune response.
4) Identify regulatory genes involved in pro-inflammatory cytokines and antibodies production and the assessment of their actual role in the process of virus infection\virulence in swine.
5) Explore the development of new assays based on cellular immunity for the early detection of the disease.
6) Improve our understanding of the role of multigene families in antigenic variability and evasion of immune response.

7) Identify and characterize genes related to host protection.

VACCINES

There is currently no commercial vaccine available for ASFV available worldwide with the only vaccine approved for use being ASFV-G-ΔI177L in Vietnam. This vaccine is based on the genotype II Georgia/ 2010 strain that is currently circulating in Europe, Asia and the Dominican Republic. Cross protection with other genotypes such as those circulating in Africa have not been tested. Although this vaccine is available for use in Vietnam, a vaccine is not available in other countries at the time of this report.. ASFV-G-ΔI177L is a recombinant vaccine with a specific deletion in ASFV protein I177L, similar approaches have been performed with other genetic deletions. A review of live attenuated vaccines for ASF is presented in Gladue *et. al. 2022*. Historical gene deletions causing virus attenuation are provided in Table III and deletions specifically attenuating the ASFV Georgia/2007 isolate are presented in Table IV .

An alternative method to produce attenuation of virulent viruses has been to pass a virulent virus in tissue culture, however, for ASFV, most of these studies have resulted in the loss of replication in swine, not having good efficacy to protect animals from virulent challenge (reviewed in Sereda *et al 2020*). Another approach to obtain attenuated virus strains has been the use of low-virulence field isolates. However, to date, all low virulence field isolates evaluated have shown to have residual virulence and attempts to decrease this virulence introducing additional genomic modification, have not eliminated residual virulence or have affected their protective efficacy.

Cross-protection of live-attenuated vaccines is largely unknown, however studies that were performed using surviving animals of low-virulence strains has shown that although ,isolates from the field (Lewis *et al*., 2000; Leitao *et al*., 2001; Boinas *et al*., 2004). Usually these animals develop long-term resistance to homologous but rarely to heterologous virus challenge (Hamdy and Dardiri 1984; Ruiz-Gonzalvo *et al*., 1981). This lack of cross protection among different isolates constitutes an important issue to be considered in the development of ASF vaccine candidates as vaccines may have to be targeted to specific areas where homologous ASFV strains are causing outbreaks in the field.

The mechanism of protection involves cell-mediated immunity, since depletion of CD8+ T cells abrogates protection (Oura *et al*., 2005; Denyer *et al*., 2006). A role for antibodies in protection had been shown since passive transfer of antibodies from immune pigs conferred partial protection to lethal challenge (Onisk*et al*., 1994). In experiments using recombinant proteins, partial protection was achieved using a combination of two proteins, p54 and p30, as well as with recombinant CD2-like protein (Ruiz-Gonzalvo *et al*., 1996; Gomez-Puertas *et al*., 1998). However, some of these results could not be repeated by others using highly virulent ASFV isolates (Neilan *et al*., 2004). The failure to achieve complete protection in these experiments may be because of the delivery method of the antigens and/or because more or different antigens are required to confer protection. Alternatively, it is possible that full protection can only be achieved by using live-attenuated replication competent ASF viruses as vaccines.

Pigs immunized with live attenuated ASF viruses containing engineered deletions of specific ASFV virulence/host range genes (for review see Dixon *et al*., 2008 and Tulman *et al*., 2009) were protected when challenged with homologous parental virus (Lewis *et al*., 2000; Moore *et al*., 1998; Zsak *et al*., 1996 and 1998). This preliminary work, mostly produced using historical virus strains, has been recently extended to virus isolates with current epidemiological significance. In addition, novel genes have now been identified as additional targets for deletions leading to virus attenuation. For example, deletion of 9GL, previously described with the Malawi isolate (Lewis *et al*., 2000), was deleted in the Georgia 2007 isolate (O'Donnell *et al*., 2015a), resulting in virus attenuation and demonstrated use as an experimental vaccine to protect against homologous challenge. Other genetic manipulations including deletion of a group of MGF genes either in the Georgia 2007 isolate (O'Donnell *et al*., 2015b), or Benin (Sanchez-Cordon *et al*., 2018; Reis *et al*., 2016), or the previously uncharacterized DP148R gene (Reis *et al*., 2017) attenuated the parental virus and protected against challenge with the virulent homologous virus.

Interestingly, for the first time, complex genetic manipulations with multiple gene deletions in the same virus were introduced in the Georgia 2007 isolate. Some of these attempts were initially unsuccessful, resulting in profound attenuation but failing in inducing a protective immune response (O'Donnell *et al*., 2016a; Abrams *et al*.,Another important problem for ASFV vaccine development is the safety profile of vaccine candidates, with historical experience demanding great caution in their validation and field application. In the 1960s, during the 20th century ASF pandemic, early live vaccine candidates were extensively field-tested in Spain and Portugal – unfortunately, these viral strains induced chronic symptoms in many affected animals and led to an increase in the number of infections (Blome, Franzke, and Beer 2020). Recently, in China, ASF outbreaks on large pork producer farms led to the identification of two new attenuated

strains lacking MGF360 and CD2v genes (Patton 2021b). These and other circulating attenuated strains – including those identified by Sun et al. (E. Sun, Zhang, et al. 2021), discussed above – have raised the possibility of unauthorized ASF vaccines circulating in China (Rock 2021; Muñoz-Pérez, Jurado, and Sánchez-Vizcaíno 2021; FAO 2021), where the government is currently cracking down on the suspected practice (Patton 2021a). These and other historical examples stress the importance of comprehensively testing the safety of live vaccine candidates. Context-specific variables related to the viral strain, the route and dose of immunization, individual host factors, and other sources can impact host immunity and ASFV virulence, necessitating extremely thorough safety testing under as wide an array of field or field-like conditions as possible (Rock 2021). In the urgent and competitive rush to develop an ASF vaccine, there are concerns that a vaccine may be released to the market before it has been adequately tested (Ståhl et al. 2019), and it is critical that efficacy and safety testing not be deprioritized (Gavier-Widén, Ståhl, and Dixon 2020; Muñoz-Pérez, Jurado, and Sánchez-Vizcaíno 2021).

The identification of protective antigens (PAs) has been described as "perhaps the single greatest ASFV research challenge" (Rock 2021), and experimental investigations must currently contend with two unknowns: the PAs themselves and the optimal way to present these antigens to the immune system for a protective response (Rock 2021). Antibodies produced by infected animals do not fully neutralize the virus, complicating the study of PAs (Blome, Franzke, and Beer 2020). Currently, eight serogroups have been distinguished based on haemadsorption inhibition (HAI) serologic typing (Rock 2021), and recent studies of viral strains with experimental or naturally occurring mutations/deletions to CD2v (discussed below) indicate that this protein is an important marker for HAI serologic specificity. CD2v, the multifunctional adhesion protein encoded by the EP402R gene, is the only known viral homolog of CD2, a host protein expressed by T and NK cells with roles in immunomodulation and protective immune responses (Chaulagain et al. 2021). However, other viral antigens may be necessary for complete protective immunity, and the importance of various proteins on immunogenicity may also be strain-dependent (Rock 2021).

Previous reports (GARA 2018; 2016) identified the following priority research knowledge gaps in ASF vaccine development over the past 6 years:

- Virology/genomics studies for vaccine discovery research
- Viral genetic patterns associated with the presence/absence of homologous vs. heterologous protection
- Impact of antigenic diversity on variable vaccine cross-protection against heterologous strains
- Determination of live attenuated vaccine safety characteristics
- Engineering of gene-deleted ASFV
- Standardization and inter-laboratory testing of vaccine candidates
- Full sequencing of new vaccine candidates
- Potential markers for DIVA vaccines
- New effective subunit vaccines

- Development of immortalized cell lines for ASFV vaccine production

One concern about the use of ASFV vaccines is the potential genetic diversity of strains circulating within the same geographical area. Although anecdotal information exists suggesting the existence of cross-protection between viruses belonging to different genotypes, it was not until recently that this has been experimentally demonstrated. Deletion of CD2-like gene from the virulent Badajoz71 isolate produced an attenuated virus that induced protection to the homologous parental virus but also to the heterologous Spain75 and Armenia2010 isolates (Monteagudo *et al*., 2017). Interestingly, this particular virus possesses the unique characteristic of growing in an established cell line, which is paramount for a potential vaccine strain that will need to grow at high titers for vaccine production. Therefore, it may be possible to develop vaccines that can cross-protect against infection with several genotypes.

Identification and characterization of novel ASFV genes involved in virulence and in evasion of the host's immune response is still needed to facilitate and improve the development of rationally attenuated vaccines through sequential deletion/modifications of these genes. Although further research is required, it appears that to development of effective vaccines is now more realistic than just a few years ago.

Alternative approaches investigating subunit vaccines based on the expression of protective antigens have not significantly progressed due to the lack of investigative work to identify viral antigens inducing protection. The recent development of high-throughput methods for constructing recombinant viral vectors opens a route for global analysis of the protective potential of all ASFV-expressed genes. In this regard, several reports studied the cellular and antibody response elicited by ASFV antigens individually expressed using a diversity of expressing vectors. Thus, vaccinia (Lopera-Madrid *et al*., 2017), DNA immunization (Argilaguet *et al*., 2012; Argilaguet *et al*., 2013; Lacasta *el al*., 2014), adenovirus (Lokhandwala *et a*l., 2016; Lokhandwala *et al*., 2017) or their combination (Jancovich *et al*., 2018) have been used to immunize pigs with several different viral proteins that were selected using different criteria. Some of the reports are restricted to the characterization of the immune response elicited by each of the virus antigens expressed in the different vectors (Lokhandwala *et a*l., 2016; Lopera-Madrid *et a*l., 2017; Lokhandwala *et al*., 2017), without assessing the protective effect of immunization against challenge with virulent virus in pigs. In those reports where challenge studies were included (Argilaguet *et al*., 2012; Argilaguet *et al*., 2013; Lacasta *el al*., 2014; Jancovich *et al*., 2018), protection values have not reach higher than 50% of the individual animals under study. These results indicate that further work is needed in order to expand the identification of virus antigens involved in the induction of protective immune response. Future success using subunit vaccine platforms may require optimization of the immunization protocols, including the selection of an effective vaccine vector. Importantly, it should be mentioned that it has been reported that successive immunization using vaccinia and adenovirus as vectors expressing eight undisclosed specific virus proteins protected 100% of immunized pigs from the challenge with the virulent Benin isolate (Netherton *et al*., 2018). This constitutes so far the only report presenting an ASFV subunit vaccine with total protective efficacy.

Live Attenuated Vaccines

culture passaging is a historical method for generating LAVs and is still in modern use. Over time, passaging places adaptive pressure on the viral genome, increasing its replication *in vitro* while reducing its fitness *in vivo*. Such mutations can potentially result in a LAV candidate and can also provide valuable information on the effects of adaptive pressure on the viral genome. In 2015, Krug *et al.* described the impact on the Georgia 2007/1 strain of long-term culturing (up to 110 passages) in Vero cells (Krug et al. 2015). This resulted in complete attenuation of the virus, associated with major deletions in both genomic variable regions (including many members of the *MGF100*, *360*, and *505* families) and point mutations. This viral strain did not protect against the virulent parental virus *in vivo* (Krug et al. 2015). *MGF* gene mutations are common in naturally attenuated (e.g. OURT88/3 and NHV) and cell-culture adapted (e.g. BA71v) ASFV strains, and substantial effort has gone into determining the vaccine potential of ASFV with specific *MGF* mutations. Such mutations are often accomplished via homologous recombination methods, though new genome-editing techniques have recently enabled more specific and convenient strategies that will be discussed below. O'Donnell *et al.* examined the activity of Georgia 2007/1 with *MGF* mutations (specifically *MGF360-12L* thru *-14L* and *505-1R* thru *-3R*) drawn from the Vero culture-adapted strain described above (Krug et al. 2015), creating the "ASFV-G-ΔMGF" candidate strain (O'Donnell, Holinka, Gladue, et al. 2015). This virus replicated efficiently in primary swine macrophages, was completely attenuated *in vivo*, and provided protection against parental Georgia 2007/1, though 30-40% of the tested pigs harboured the parental virulent virus after challenge (O'Donnell, Holinka, Gladue, et al. 2015). Another study from this research group evaluated deletion of 9GL, a protein involved in virion assembly, to produce ASFV-G-Δ9GL (O'Donnell, Holinka, Krug, et al. 2015). This strain conferred complete protection only over a small range of doses (103 HAD50 intramuscularly [IM], with lower doses insufficient and higher doses causing lethal disease). No correlation was observed between protection and anti-ASFV antibody levels or IFN-γ-producing immune cells (O'Donnell, Holinka, Krug, et al. 2015).

Next, O'Donnell and colleagues combined the mutations from the two strains above, aiming to increase the virus's protective dose range while limiting potential genetic instability. The resulting ASFV-G-Δ9GL/ΔMGF was over-attenuated *in vivo* and did not produce detectable circulating virus or protect against parental challenge (O'Donnell et al. 2016). The next year, this group described simultaneous deletion of 9GL and *UK*, a poorly characterized virulence-related gene. The resulting strain, ASFV-G-Δ9GL/ΔUK, was the first attenuated virus to confer protection against virulent challenge at 14 dpi, and it did not induce clinical disease even at high doses (106 HAD50). This protection correlated with serum anti-ASFV antibodies but not with circulating ASFV-specific IFN-γ-producing cells (O'Donnell et al. 2017).
Meanwhile, Reis *et al.* evaluated a mutant of the virulent Benin 97/1 strain with several deleted or inactivated *MGF* genes (*360-9L* thru *-14L* and *530/505-1R* thru *-4R*), including deletions present in the naturally attenuated OURT88/3 strain. This BeninΔMGF strain induced higher levels of IFN-β transcription in cultured macrophages compared to the parental strain and conferred 100% protection *in vivo* (though transient fever was observed at 5-6 dpi) (Reis et al. 2016). A later study from the same group described the deletion of *DP148R*, an early gene with potential roles in immune evasion, from Benin 97/1. This attenuated the virus *in vivo* without affecting replication *in vitro*, and vaccination trials demonstrated 100% and 83% protection against parental challenge after IM or oronasal inoculation, respectively (Reis et al. 2017).

Several years later, *DP148R* deletion was shown to have no effect on the virulence of Georgia 2007/1, emphasizing that the effects of specific gene deletions can be strain-specific (Rathakrishnan et al. 2021). Similarly important are the dose and route of vaccination, and different inoculation strategies can produce different results. Sánchez-Cordón tested routes of immunization with attenuated OURT88/3, finding 100% protection against virulent OURT88/1 from oronasal administration and only 50-66% protection (dose-dependent) from the IM route. However, oronasal administration was associated with persistent viraemia and mild clinical signs, leading the researchers to recommend the IM route for feasibility and safety reasons (Sánchez-Cordón et al. 2017). Next, the researchers dose-dependence with their BeninΔMGF strain, finding that IM administration of 102-104 TCID50 conferred increasing (50-83%) protection against challenge. Notably, no correlation was observed between protection levels and serum IgM or IgG levels (Sánchez-Cordón, Jabbar, et al. 2018).

Also in 2018, Gallardo *et al.* published the construction and evaluation of LAVs based on the NH/P68 strain, finding that individual deletions of the *A238L*, *A224L*, and *EP153R* genes (all involved in virus-host interaction and immunomodulation) produced strains capable of protecting against challenge with the homologous L60 strain (Gallardo, Sánchez, et al. 2018). Meanwhile, Borca *et al.* presented the application of CRISPR-Cas9 to the development of recombinant ASFV, resulting in a significantly higher recombination frequency (and therefore easier production of recombinant viruses) compared to traditional homologous recombination methods (Borca, Holinka, et al. 2018).

In 2020, Sánchez-Cordón *et al.* published a study of BeninΔMGF and OURT88/3, both of which had previously been shown to induce high levels of homologous protection over short timeframes (Sánchez-Cordón et al. 2020). Here, the researchers focused specifically on long-term protection, a rarely examined attribute that is critical for the field applicability of LAV candidates (Muñoz-Pérez, Jurado, and Sánchez-Vizcaíno 2021). Neither strain conferred protection against Benin 97/1 challenge at 130 dpi. Initial immunization led to a transient increase in circulating NK cells, CD8+ T cells, and IFN-γ-secreting memory cells that peaked at 24 dpi and decreased to preimmunization levels by the time of challenge. Levels of Tregs and the anti-inflammatory cytokine IL-10 were also elevated at the end of the experiment, suggesting that immunoregulatory processes may inhibit effective protection (Sánchez-Cordón et al. 2020). Meanwhile, Borca *et al.* reported that deleting *CD2v* (here called 8DR) from the Georgian ASFV strain did not reduce its virulence – the ASFV-G-Δ8DR strain induced clinical disease indistinguishable from the parental strain (Borca, O'Donnell, et al. 2020). Meanwhile, Chen *et al.* published their development of HLJ/18-7GD, a LAV candidate with deletions of seven genes that play important roles in virulence (specifically *MGF505-1R* thru *-3R*, *MGF360-12L* thru *-14L*, and *CD2v*) (W. Chen et al. 2020). This strain conferred complete protection against parental challenge at 28 dpi. Importantly, deletion of *CD2v* was crucial for safety, limiting the potential for reversion to virulence that was observed in a strain lacking this deletion (HLJ/18-6GD) (W. Chen et al. 2020). Gladue *et al.* subsequently tested the effects of incorporating this deletion into their ASFV-G-Δ9GL candidate (D. P. Gladue et al. 2020). Two viruses were tested – ASFV-G-Δ9GL/ΔCD2v and -Δ9GL/ΔCD2v/EP153R, from which the viral C-type lectin-like viral gene *EP153R* was also deleted. Only the latter displayed decreased replication *in vitro*, and neither induced viraemia or provided protection against challenge with Georgia 2007/1 (D. P. Gladue et al. 2020). Finally, this research group tested the deletion of *I177L*, an uncharacterized but highly

conserved late gene. The resulting candidate (ASFV-G-ΔI177L) was completely attenuated across a range of IM doses (102-106 HAD50), and immunization conferred effective protection against virulent challenge. Notably, this was the first published LAV candidate capable of inducing sterile immunity against the ASFV Georgia strain, restricting replication of the parental virus after challenge *in vivo* (Borca, Ramirez-Medina, et al. 2020). This vaccine strain was recently approved for use in Vietnam where large batches of the vaccine are currently being used.

A number of studies published in 2021 followed up on these promising results. First, Borca *et al.* tested the ASFV-G-ΔI177L strain as an oronasal vaccine (total dose of 2x106 HAD50, split between oral and nasal delivery routes), finding it equally effective to IM administration. Interestingly, oronasal administration caused comparatively lower viraemia in immunized animals, though circulating antibody responses were unchanged from IM administration (Borca, Ramirez-Medina, et al. 2021). Next, this group adapted ASFV-G-ΔI177L to cell culture via 11 passages in the PIPEC stable swine epithelial cell line (Borca, Rai, et al. 2021). The resulting strain, dubbed ASFV-G-ΔI177L/ΔLVR, had an additional deletion of ~11 kb in the left variable genomic region (LVR), including several *MGF300* and *360* genes – similar deletions were previously observed in other cell culture-adapted strains such as L60V and BA71v (Ye et al. 2020). ASFV-G-ΔI177L/ΔLVR maintained the *in vivo* attenuation, immunogenicity, and protective efficacy of its parental strain, and its ability to replicate effectively in PIPECs makes it a practical candidate for future large-scale manufacture (Borca, Rai, et al. 2021). Finally, Tran *et al.* tested ASFV-G-ΔI177L against the virulent strain currently circulating in Vietnam, finding that low-dose (102 HAD50) inoculation was protective in both European and native Vietnamese domestic pig breeds (X. H. Tran et al. 2021).

Research into other LAV candidates has continued apace. Koltsova *et al.* produced a *CD2v*/*EP402R*-deleted version of the Congo-a virus (a cell culture-adapted version of the virulent genotype I Congo strain) (Koltsova et al. 2021). This LAV candidate (ΔCongoCD2v) had unchanged growth kinetics in primary swine macrophages and COS-1 cells *in vitro* – however, replication *in vivo* was significantly reduced. ΔCongoCD2v did not protect against challenge with the original Congo strain, highlighting the unpredictable nature of ASFV's growth characteristics (Koltsova et al. 2021). Gladue *et al.* tested deletion of the *A137R* gene (which encodes the late protein p11.5, localized to the perinuclear virus factories during infection) from ASFV-G. The resulting ASFV-G-ΔA137R conferred complete and sterile protection against parental virus challenge after low-dose IM inoculation (D. P. Gladue et al. 2021). Finally, Zhang *et al.* deleted *L7L* thru *L11L*, uncharacterized genes in the viral genome's right variable region (RVR), from the virulent SY18 strain to produce the LAV candidate SY18ΔL7-11 (J. Zhang et al. 2021). This candidate replicated normally in primary bone marrow-derived macrophages *in vitro*, while its presentation *in vivo* was promising but unpredictable: several animals exhibited low viraemia post-immunization, and one had high viraemia and died at 14 dpi (with elevated levels of IL-1Ra from 3-7 dpi, as observed during infection with virulent SY18). All surviving animals were protected and asymptomatic when challenged with parental virus (J. Zhang et al. 2021).

Cross-protection – the ability of a LAV to protect against viruses from heterologous serogroups, not just the homologous parental strain – is an important open question in ASF vaccine development, with ASFV antigenic diversity remaining a confounding factor (Malogolovkin and

Kolbasov 2019). As discussed above, the determinants of serogroup specificity are not well understood, and reliably predicting the ability of a given vaccine candidate to protect against heterologous strains will require substantial research into ASFV protein functions, protective antigens, and correlates of immune response (Rock 2021). In 2017, Monteagudo *et al.* published on BA71ΔCD2, a *CD2v/EP402R* gene-deleted strain, demonstrating dose-dependent protection against parental BA71 and the heterologous genotype I E75 and genotype II Georgia 2007/1 strains (Monteagudo et al. 2017). Cross-protection against E75 correlated with the induction of CD8+ T cells responsive to both BA71 and E75. The researchers also noted residual virulence of this LAV candidate, with small amounts of virus detectable in the blood and nasal secretions of some immunized pigs (Monteagudo et al. 2017). This group later used the BA71ΔCD2 strain to investigate the mechanisms behind cross-protection (Lopez et al. 2020). BA71ΔCD2 immunization protected 5/6 pigs against tick-borne challenge with the genotype XIX strain RSA/11/2017, but only 2/6 were protected against the more phylogenetically distant genotype IX Ken06.Bus. In subsequent prime-boosting experiments, a homologous strategy (BA71ΔCD2□BA71ΔCD2) improved this survival rate to 50%, while heterologous prime-boosting (BA71ΔCD2□parental BA71) conferred 100% protection. These findings highlight the complexity of the biological processes that underlie heterologous protection, with outcomes depending on factors beyond sequence similarity (Lopez et al. 2020).

Our ability to monitor the efficacy of a commercialized vaccine (and, eventually, to confirm ASF eradication) will depend on reliably identifying vaccinated animals (Velazquez-Salinas et al. 2021). For this to be possible, LAV candidates must be compatible with tests that can differentiate infected from vaccinated animals (DIVA). Ramirez-Medina *et al.* very recently evaluated the *E184L* gene, which encodes an uncharacterized but immunogenic structural protein (Mazur-Panasiuk, Woźniakowski, and Niemczuk 2019; Jaing et al. 2017), as an antigenic DIVA marker (Ramirez-Medina et al. 2022). Deletion of this gene (producing ASFV-G-ΔE184L) moderately attenuated viral virulence, and sera from inoculated animals had no detectable antibody response against E184L peptides, making this deletion a promising functional marker for use in LAV candidates (Ramirez-Medina et al. 2022).

Finally, another important factor in the eventual deployment of an ASF vaccine is the means of vaccination. This may be a relatively simple task in domestic pigs, but vaccination of wild boar is more difficult (Gavier-Widén, Ståhl, and Dixon 2020; Rock 2021). In 2019, Barasona *et al.* reported the first successful oral vaccination of wild boar against ASFV, using the attenuated Lv17/WB/Rie1 strain (Barasona et al. 2019). Eleven of 12 wild boar housed in a BSL3 facility were protected against challenge with the virulent Armenia/07 strain – importantly, three of the wild boar were immunized through contact with the other nine, indicating that these orally vaccinated animals were able to shed the vaccine virus. Preliminary results suggested low risk of infectivity after viraemic periods, but further study is needed to ensure the safety profile of such approaches (Barasona et al. 2019).

Subunit Vaccines

The foremost advantage of a subunit vaccine over a LAV is its safety profile: using viral proteins rather than attenuated replication-competent viruses removes the risks of reversion to virulence, delayed viraemia, and potential shedding of vaccine strains that plagued 20th century vaccination

strategies (Blome, Franzke, and Beer 2020). Unfortunately, most historical and modern attempts to develop subunit vaccine candidates have generated at best only partial protection when compared with LAVs (Muñoz-Pérez, Jurado, and Sánchez-Vizcaíno 2021; Dixon et al. 2020). The main factor confounding subunit vaccine development is our lack of knowledge on ASFV protective antigens (PAs) (Rock 2021; Gavier-Widén, Ståhl, and Dixon 2020). Subunit vaccine candidates have been shown to induce specific antibodies and T cell responses – however, these have not been found capable of conferring strong protection, likely due to the complex and combinatorial nature of the host anti-ASFV immune response (Muñoz-Pérez, Jurado, and Sánchez-Vizcaíno 2021; Arias et al. 2018). In spite of these setbacks, numerous studies over the past 6 years have continued to explore new pathways toward PA identification and subunit vaccines.

Jancovich *et al.* screened 47 viral proteins from Georgia 2007/1 for immunogenicity and protective activity, selecting proteins that covered various known functions and temporal expression patterns (Jancovich et al. 2018). Pooled antigens were delivered to pigs using a DNA prime/recombinant vaccinia virus boost strategy, and cell-mediated responses were measured via IFN-γ ELIspot to identify the most immunogenic peptides. Fourteen proteins, including p30, E183L, E199L, and F317L, induced consistently high immune responses. Prime-boost vaccination with all 47 antigens, however, did not protect pigs against Georgia 2007/1 challenge, although viral genome levels were significantly reduced in blood and some target tissues (Jancovich et al. 2018). The next year, Netherton *et al.* constructed an IFN-γ ELIspot with 133 predicted proteins from OURT88/3, using it to screen for antigens recognized by lymphocytes from pigs immunized with this strain (Netherton, Goatley, et al. 2019). Based on the results from this assay, 18 particularly immunogenic ORFs were cloned into adenovirus and Modified Vaccinia Ankara (MVA) vectors and used in immunization-challenge experiments. Again, viraemia was reduced in a proportion of the challenged pigs, but the antigen pool did not protect against severe disease (Netherton, Goatley, et al. 2019).

Sunwoo *et al.*, meanwhile, reported on a combined DNA-protein subunit vaccination strategy, inoculating pigs three times with ASFV plasmid DNA (genes encoding CD2v, p72, p32, + p17) and recombinant proteins (p15, p35, p54, + p17) (Sunwoo et al. 2019). Subsequent challenge with Armenia/07 showed that this treatment did not confer protection – disease kinetics and time-to-death were in fact faster. Although circulating antigen-specific antibodies were present, sera from these animals also enhanced ASFV infection *in vitro*, suggesting an antibody-dependent enhancement of viral infection that has previously been observed in similar contexts (Sunwoo et al. 2019; Brown and Bevins 2018; Gaudreault et al. 2020).

These results indicate that, while subunit vaccine candidates can induce humoral and cellular immune responses, the choice of antigens is critical for inducing a protective response and avoiding antibody-dependent enhancement effects. In 2020, Goatley *et al.* described an adenovirus-prime/MVA-boost strategy to inoculate pigs with pooled antigens derived from the OURT88/3 and Benin 97/1 strains (Goatley et al. 2020). One pool – comprising *B602L*, *B646L* (p72), *CP204L* (p30), *E183L*, *E199L*, *EP153R* (C-type lectin), *F317L*, and *MGF505-5R* – protected 100% of pigs from fatal disease after challenge with virulent OURT88/1 at 28 dpi. Clinical signs in immunized/challenged pigs were enhanced over controls, with the researchers suspecting an immune overreaction, and animals required NSAID/antipyretic treatment to

manage symptoms (Goatley et al. 2020). The immunological correlates of protection remain unclear – ASFV-specific IFN-γ-secreting memory cells were observed in all protected and some unprotected animals, for instance, suggesting that their activation may be necessary but not sufficient for protection. Further studies will be necessary to tease apart the complex responses to this antigen pool and the mechanisms of the protection it affords (Goatley et al. 2020).

In 2021, Bosch-Camós *et al.* reported *in vivo* experiments using a heterologous prime-boost system, testing the effect of plasmid DNA priming on the protection conferred by a suboptimal dose of the BA71ΔCD2 strain (Bosch-Camós et al. 2021). In the first study, pigs were immunized with 15 plasmids encoding ubiquitin-tagged ASFV proteins chosen based on *in vitro* MHC I-binding studies. Prime-boost afforded 60% protection (3/5 animals) against Georgia 2007/1 challenge, compared to 20% from BA71ΔCD2 alone. Subsequently, priming with only two plasmids (encoding *M448R* and *MGF505-7R*) gave similar results (increase in survival from 20% to 60%), indicating that these proteins are CD8+ T cell antigens with protective potential. Meanwhile, Lopera-Madrid *et al.* tested the importance of promoter selection in subunit vaccine efficacy (specifically the p30 protein), using recombinant MVA vectors with a set of promoters that drive different expression levels and timings. Of the five vectors tested, the natural poxvirus promoter PrMVA13.5L produced the highest levels of p30 mRNA and anti-p30 antibodies in mice (Lopera-Madrid et al. 2021), indicating that selection of an appropriate promoter is another critical aspect of subunit vaccine design. Finally, Zhang *et al.* tested the immunogenicity of recombinant ASFV proteins p30 and p54 fused to the cell-penetrating peptide Z12, finding that sera from mice immunized with this construct neutralized >85% of ASFV *in vitro*.

Cell Lines for Production of LAV Candidates

As discussed above, cell lines suitable for productive ASFV infection are critical for solving issues of standardization, variability, and high required labour inherent in the use of primary macrophages. Cell lines (e.g. COS-1 cells) are available for the propagation of certain ASFV strains, but viral genome instability and changes to virulence and/or immunogenicity during passaging are a significant issue. In 2017, Sánchez *et al.* published their comparison of four porcine cell lines – IPAM-WT, IPAM-CD163, CΔ2+, and WSL – against primary PAMs for virus production, using virulent Armenia/07 and E70 and attenuated NHV/P68 strains as examples. The cultured cells expressed low levels of monocyte/macrophage-specific surface receptors and were minimally susceptible to infection with the exception of WSL, which efficiently produced NHV/P68 but not virulent strains (Sánchez et al. 2017).

Portugal *et al.* evaluated the growth factor-dependent ZMAC-4 porcine macrophage cell line for ASFV replication (Portugal et al. 2020). These cells were susceptible to infection with eight isolates (including OURT88/3, NH/P68, and Georgia 2007/1), which subsequently replicated to high titres comparable to primary porcine bone marrow cells. Additionally, 12 passages of OURT88/3 in ZMAC-4 cells did not reduce the virus's ability to induce protection against challenge with virulent OURT88/1, indicating that these cells can produce high levels of LAV strains without impacting protective mutations (Portugal et al. 2020).

As mentioned above, Borca *et al.* demonstrated the utility of the PIPEC (Plum Island Porcine Epithelial cells) as stable cell line for producing the ASFV-G-ΔI177L/ΔLVR strain (Borca, Rai,

et al. 2021). Notably, the genomic changes (ΔLVR) induced by adaptation to PIPEC cells were stable after 30 passages, demonstrating that PIPECs can maintain the proliferative ability of ASFV strains in primary cells.

IPKM is an immortalized porcine kidney macrophages that has been shown to support high levels of viral replication of ASFV producing hemadsorption reactions and cytopathic effects when inoculated with virulent field isolates as Armenia07, Kenya05/Tk-1, and Espana75 (doi: 10.1038/s41598-021-84237-2). Serial passages of some field isolates does not alter the virulence of the virus (doi: 10.3390/v14081794).

At the time of writing this report, live attenuated viral vaccines appear to be the most promising option to be used as a control tool for disease dissemination. One major concern is the presence of residual virulence with live-attenuated viral vaccines.

There is a lack of established guidelines for testing the safety and efficacy of live-attenuated vaccines so the approval will be dependent on the standardization of ASF vaccine clinical study design, which is still lacking. Although it appears that all ages of susceptible species have similar susceptibility to with ASF infection, however it is unknown if attenuated strains have a different degree of attenuation depending on age. The weight/age of other swine vaccines is 8-10 weeks (25kg), the weight/age tested will determine the label claim of the vaccine. The most common route of testing ASFV vaccines remains with parenteral inoculation, either intramuscular (IM) or subcutaneous (SC), although some studies have used oral/bait inoculation. There is also no standard for minimum protective/immunizing dose vs. maximum release dose, or what would be considered a safety overdose range. Many of the vaccine trials published lack a dose response study to determine the minimum dose required for the tested vaccine platform.

Challenge studies also vary between research group from IM challenge to contact challenge studies where one unvaccinated pig receives an IM dose of virulent virus and is allowed to remain in contact with vaccinated animals for a set period of time. Some of the studies conducted do not provide 100% mortality in the control group, causing the results to be difficult to interpret. Due to these inconsistencies a centralized biorepository for a subset of challenge viruses was recommended, however, currently, this seems unlikely due to the complexity of sharing specific isolates that may be subject to restrictive material transfer agreements.

It is also necessary the standardization of scoring system (including fever, viremia, and clinical signs) to evaluate presence/absence of disease.

What is considered a safe vaccine could depend on the area it is going to be deployed; for example, deploying a vaccine with an identical backbone to ASFV that is circulating in an endemic area, or during an outbreak situation, is very different than deploying a vaccine in an uninfected area. Currently there has been no vaccine with a marker for differentiation of infected from vaccinated animals (DIVA).

Identification of antigenic markers is a critical preliminary step to engineer a vaccine with negative markers to differentiate infected from vaccinated animals (DIVA). To date the introduction of DIVA markers to ASFV vaccine candidates has caused the loss of efficacy

(MGF5/6, E184L) making the only DIVA capable assays the qPCR assays, which have the limitation that they are unable to detect animals that do not have the active presence of either vaccine virus or field virus strains.

Some of the key research gaps that impede the discovery of safe and effective ASF vaccines, and in particular, vaccines that have been engineered for the purpose of control and eradication, include:

- Lack of understating of the molecular/antigenic bases governing cross-protection between different strains of ASFV
- Lack of a standardized protocols for the evaluation of vaccine safety and efficacy.
- An immortalized cell line for ASFV vaccine production without requiring virus adaptation
- ASFV gene products that can induce a protective immune response to engineer subunit vaccines
- Mechanisms of homologous and heterologous protective immunity.
- Identification of antigenic markers to engineer a vaccine with a negative marker that is capable of differentiating infected from vaccinated animals (DIVA).

- **Research needs**Lack of Cross protection studies to understand how many different vaccines may be required for the broad coverage of acting field isolates.
- ASFV virology and functional genomics studies to identify targets that will produce important information for vaccine research, including novel determinants of virulence in the ASFV genome, antigenic targets required for immunity, and understand mechanisms of immune evasion
- Studies of biosafety and long-term protection in previously validated LAV candidates
- Working with stakeholders and government officials to encourage sufficient economic investment in eventual ASF vaccine deployment

DIAGNOSIS

A wide variety of laboratory techniques are available either for ASF virus and antibody detection, and a combination of both is the recommended approach for detecting ASF. It is important to point out that ASF presents three significant advantages for detection: i) viremia begins usually at 2-3 dpi, and it is maintained for several weeks; ii) specific antibodies appear detectable in blood from the 8-15th day post infection at high levels and persist for long periods of time, even years; iii) specific antibodies (if they appear before the animal dies) are a very good marker of infection if vaccines and vaccine candidates are not used in the respective region. Regarding antibody detection, one must keep in mind, however, that experimental infections of domestic pigs and wild boar with virulent isolates frequently resulted in acute forms of ASF with 100% lethality within less than 12 days, with no detectable antibody response in serum (Gabriel *et al.,* 2011; Blome *et al.,* 2012, Pikalo *et al*., 2021).

The persistence of specific ASF-IgG antibodies for long periods of time in infected pigs provide the primary strategy to detect the sub-acute and chronic forms of ASF, which is essential for ASF eradication programs in endemically infected regions. Several techniques have been adapted to ASF antibody detection, but the most common, practical and inexpensive tests are enzyme-linked-imunosorbent assays (ELISA). For confirmatory diagnosis, immunoblotting (IB);

indirect immunofluorescence antibody test (IFA), and the immunoperoxidase test (IPT) are available.
From suspect live animals, blood samples are the matrix of choice. However, alternative sample matrices such as tonsil scrapings or tonsil swabs may be suitable under certain conditions. From pig carcasses, blood, spleen, lymph nodes, lung, kidney or tonsils should be sampled. Bone marrow may be used when the carcass is found in advanced stages of decomposition (e.g., wild boar carcasses), and bloody swabs or tissue exudates can be suitable for the detection of viral genome and antibodies. Tissues are used for virus isolation (HA test), viral antigen detection (DIF test), and detection of viral genome (PCR test), while blood, tonsil scrapings and tonsil swabs are only used for virus isolation and viral genome detection. Serum (or plasma) is used for antibody detection byELISA, IB or other confirmatory assays.

Nowadays, PCR is the most widely used technique for the detection of ASFV. Virus isolation on primary macrophages (usually done as haemadsorption test, HA) is subsequently carried out for comprehensive strain characterization and bio-banking. In addition, direct immunofluorescence (DIF) is used. With the panzootic spread of ASF, numerous commercial real-time PCR kits have been developed and several kits are validated and/or licensed, especially on the European market. Recently, comparative studies have been conducted and published (Schoder *et al*,, 2020; Pikalo *et al.*, 2022). It has been shown that the tests are very robust, specific and sensitive. Additionally, tests have been developed that contain all reagents in a dried-down format (e.g., the PCR initially described by Zsak *et al.* (2005). An internal control (endogenous and/or heterologous) is an important prerequisite for reliable testing.

Virus Detection Techniques

Virus detection and isolation.

The hemadsorption test (HA) is used for confirmatory purposes and to produce virus isolates for downstream characterization. The test is both sensitive and specific. HA is based on the hemadsorption characteristics that most of the ASF virus isolates induce when pig macrophages are infected in the presence of the porcine erythrocytes. A characteristic rosette around the infected macrophages develops before the cytopathic effect appears. It is important to point out that it has been observed that a small number of field strains show only cytopathic effect without producing the hemadsorption phenomenon. These strains are identified using PCR and/or DIF test on the sediments of these cell cultures.

Detection of ASF viral genome.

Since 2000, laboratory PCR tests, based on conventional and real-time procedures, have been developed and some of them have already been validated (OIE, 2000; Agüero *et al.*, 2003; King *et al*., 2003; Zsak *et al*., 2005). These techniques use primer pairs selected from a highly conserved region of the viral DNA, usually within the VP72 genome region, detecting a wide range of ASF isolates belonging to all the known virus genotypes. It is a fast and reliable technique to be included in epidemiology surveillance and diagnosis of ASF. As mentioned above, there is a growing number of fully validated commercial kits and in-house protocols targeting alternative genome regions (useful to exclude amplicon contaminations). Summaries

can be found in recent publications such as Netherton *et al.*, 2022. Among the commercially available kits licensed in at least one country are: INgene q PPA (Ingenasa), virotype ASFV and virotype ASFV 2.0 (Indical Bioscience), ID Gene ASF Duplex and Triplex (IDvet), Kylt ASF (Anicon), ADIAVET ASFV FAST TIME (Bio-X), virellaASFV seqc (gerbion), VetMAX ASFV (Thermofisher), VetAlert ASFV (Tetracore), and RealPCR ASFV (IDEXX). Some kits allow simultaneous detection of ASF and other pathogens such as classical swine fever (e.g., Kylt ASF/CSF RTU (Anicon) or SwineFever combi (gerbion)). To ensure reliability of negative results, internal controls should be mandatory.

Direct immunofluorescence (DIF).

The DIF assay is based on the demonstration of viral antigen on impression smears or frozen tissues section with an immunoglobulin conjugated against ASF virus. It is a very fast (one hour) and economic test with high sensitivity to the acute ASF form. For subacute or chronic forms, DIF test presents a sensitivity of only 40 %. This decrease in sensitivity seems to be related to the formation of antigen-antibody complexes, which do not allow the reaction with the ASF conjugate.

Additionally, there is a unique commercial ELISA viral antigen detection test, the Ag-ELISA. Both antigen detection techniques DIF and Ag-ELISA exhibit a very low sensitivity in case of chronic forms of the disease, while antigen-antibody complex are present. These techniques are only recommended for the diagnosis of acute forms of the disease. The antigen detection techniques are not recommended in case of chronic forms of the disease, in endemic areas, or for an individual diagnosis of the disease.

Additional Tests.

In recent years a number of diagnostic platforms have been adapted to ASF diagnosis, most of them based on DNA detection, either as part of multiplexed techniques (Lung *et al*., 2018, Xiao *et al.*, 2018, Erickson *et al.*, 2018, Hu *et al.*, 2015, Shi *et al.*, 2016, Sastre *et al*., 2016) or single detection, which includes portable PCR systems (Liu *et al*., 2017), lateral flow devices for antigen detection (Sastre *et al*., 2016), and new platforms such as use of biosensors (Mujibi *et al.*, 2018), the droplet digital PCR ddPCR (Wu *et al*., 2018), the recombinase polymerase amplification, RPA (Wang *et al*., 2017), the Polymerase cross-linking spiral reaction, PCLSR (Wozniakowski *et al*., 2017), or the isothermal cross priming amplification CPA (Fraczyk *et al.*, 2016), some of them with times as short as 10 minutes for the result, and sensitivity and specificity as high as the OIE recommended ULP-PCR. In addition, CRISPR-Cas12a and fluorescent-based point-of-case systems and nanofluidic chip digital PCRs have been described recently.

Antibody Detection Techniques

Antibody ELISA.

This is the most useful method for large-scale serological studies and a growing number of suitable tests is available. At present, at least four commercial ELISAs are in routine use in Europe and beyond (two from Ingenasa, two from IDVet) and more are being validated. Other

ELISAs are in house or in development and validation process, and all have their strengths and weaknesses, which should be considered when testing samples of doubtful or bad quality. The procedure of an "in house" OIE ELISA as well as a standardized/validated soluble antigen for the OIE ELISA test can also be provided by CRL upon request.

Immunoblotting assay (IB).

This is a highly specific, sensitive and easy to interpret technique which is successfully used as an alternative method to IFA recommended as a confirmatory test of the positive or doubtful results by ELISA. There is no commercial IB Kit available, and standardized/validated IB antigen strips should be prepared by the own laboratory. It could be provided by CRL previous a request. However, due to the complexity of the IB antigen-strip production, the annual amount of it is limited.

Indirect immunofluorescence antibody test (IFA).

The IFA test is a fast technique with high sensitivity and specificity for the detection of ASF antibodies from either sera or tissue exudates. It is based on the detection of ASF antibodies that bind to a monolayer of cell lines (e.g. Vero cells MS) infected with an adapted ASF virus. The antibody-antigen reaction is detected by a labelled fluorescein A-protein.

Immunoperoxidase test (IPT). The IPT is an immune-cytochemistry technique on fixed infected cells to determine the antibody-antigen complex formation through the action of the peroxidase enzyme. In this procedure, permanent cells such as Vero, WSL or MS are infected with ASFV isolates that are adapted to these cell cultures. The infected cells are fixed and are used as antigens to determine the presence of the specific antibodies against ASF in serum, blood swab or tissue exudate samples (Gallardo et al., 2015).

Pen-side Tests. Recently, Cappai *et al.*, (2017) reported on the use and validation of a commercial serological pen-side test in Sardinia, Italy. The implemented test is a lateral flow device (LFD) that is produced by Ingenasa (INgezim PPA CROM). The study on hunted wild boar showed a sensitivity of 82 % and a specificity of 96 % under field conditions (better performance under laboratory conditions). It was demonstrated that the use of pen-side tests was less expensive and laborious, while still providing expedited results. Thus, these tests could be considered under certain conditions.

For the general diagnostic workflow, the use of a combination of virological detection techniques (PCR test is recommended since Ag detection techniques such as DIF and antigen ELISA show very limited sensitivity in chronic cases) simultaneously with the use of serological test (ELISA, and confirmation of positives and doubtful results by IPT/IFA or IB), makes it possible to detect all ASF epidemiology situations (acute, subacute and chronic) in very short time with accuracy and confidence.

The characterisation of ASFV isolates is performed by standardised protocol established at the international level and by the EU Regional Laboratory by genotyping. The genotyping strategy involve sequencing of three independent regions on ASFV genome; i) the C-terminal end of the

gene encoding the VP72; ii) the full-gene sequencing of the VP54; and iii) the variable region within ASFV genome named CVR (central variable region) marked by the presence of tandem repeat sequences (TRS). The partial VP72 and full-length sequencing of VP54 places ASFV isolates into major subgroups prior to CVR analysis to resolve the intra-genotypic relationships of viruses causing ASF outbreaks. This method has provided additional information about strains of viruses circulating in Europe, America and Africa over a 45-year period. Furthermore, these methods have allowed determination of the genetic relationships and origin of viruses responsible for disease outbreaks occurred in the last years in Europe (Italy and Caucasus countries) and Africa.
Over the last years, generation of whole genome sequences has become an affordable and beneficial option for genomic epidemiology and full strain characterisation. Use of this technique has led to the discovery of viral variants, e.g., in Poland and Germany and is the basis for tailored sequencing approaches (see e.g., Forth *et al*., 2022).

Recent Advances in the Development of ASF Diagnosis

Advances in pathogen detection.

Recently, a study was undertaken to compare the available diagnostic test systems in the framework of the current outbreak in Eastern Europe [5]. In this study, the Universal Probe Library (UPL) PCR [5,6] was the most sensitive method followed by the OIE prescribed real-time PCR [7] and the conventional PCR. In general, agreement among the methods was fair to good (94% between UPL and real-time PCR; 88% between UPL and conventional PCR). The commercial antigen ELISA (INgezim PPA DAS K2; Ingenasa) showed a sensitivity of roughly 77%.

The above-mentioned comparisons (Schoder *et al.,* 2021; Pikalo *et al.,* 2022) have shown that several real-time PCR kits are suitable for ASFV detection with minor variations. Kits can be chosen to fit the general workflow of the respective laboratory.

Auer et al., 2022: Comparison of the sensitivity, specificity, correlation and inter-assay agreement of eight diagnostic in vitro assays for the detection of African swine fever virus

Overall, it is advisable to choose test kits that have been validated by a reference laboratory on international or national scale.
It must be noted that special attention should be placed on extraction methods, both manual and automated. An unpublished study has very recently shown high variability with some matrices. Furthermore, direct PCR assays have been revisited and described for the simultaneous detection of ASF and CSF (Nishi *et al.,* 2022). Additional approaches were developed using a fluorescent biosensor and lateral flow assay based on direct PCR combined with a CRISPR/Cas12a system (Cao *et al.,* 2022). Further validation is needed to explore the potential of these new approaches.

Different approaches have been reported to differentiate field and vaccine strains (Zhu et al., 2022; Yang et al., 2022; Huang et al., 2022).
Isothermal amplification methods can aid diagnosis under basic laboratory or even field settings and have been investigated over the last years. Among the isothermal methods that have been

tried for ASFV is cross-priming amplification (targeting the p72 gene). Fraczyk *et al*., (2016) could demonstrate that the method was as sensitive as the UPL PCR, at least under the chosen conditions. Quite similar results were obtained for a loop-mediated isothermal amplification (LAMP) assay targeting the topoisomerase II gene. Apart from standard settings, visualization through the use of dual-labelled biotin and fluorescein amplicons on lateral flow devices was demonstrated (James *et al*., 2010). Recently, promising LAMP assays were also described targeting the p10 gene of ASFV (Wang *et al.*, 2020) and a portable LAMP assay coupled with a CRISPR/Cas12a system (Yang *et al.,* 2022). Other LAMP applications were combined with Lateral Flow Dipsticks (Zuo *et al.*, 2021). LAMP has been used as diagnostic technique in Timor-Leste (Mee et al., 2020).

Apart from other isothermal methods such as RPA as implemented e.g., by TwistDx or RAA as developed by Qitian (Fan *et al*., 2020) or Wu *et al.* (2022), different field-applicable PCR machines (e.g., Daigle *et al.,* 2021; Elnagar *et al.,* 2022) and point-of-care isothermal methods have been tested over the last years. Among them are a combination of RPA with a gold nanoparticle test strip (Wang et al., 2021), RPA-CRISPR-based assays (Ren *et al*., 2021) and a field-deployable C-SAND assay kit (MatMaCorp, Zurita *et al.*, 2022). In addition, techniques of digital PCR and CRISPR-Cas related assays have been explored. An example of the latter is a point-of-care system that employs a CRISPR-Cas12a and fluorescence system (He *et al.*, 2020). Another recent approach was the design of ladder-shape melting temperature isothermal amplification (LMTIA) assays (Wang et al., 2022) or graphene oxide-based accelerated strand exchange amplification (GO-ASEA) (Zhuang et al., 2022). AlphaLISA technology has recently been explored as well (Chen et al., 2021). However, all these methods need further assessment when it comes to field application.

Despite the availability of fully validated WOAH-recommended PCRs and commercial kits, in-house PCR protocols are still developed with the availability of additional sequences and are being published (e.g., Wang *et al.*, 2020). Their performance has to be further explored in the field.

Advances in antibody detection.

For ASF antibody detection, five serological methods were recently tested, including three commercial ELISAs (from Ingenasa, IDVet, and Boehringer Ingelheim Svanova), the OIE-ELISA, and the confirmatory immunoperoxidase test (IPT). The IPT was shown to be the most sensitive assay that also allowed testing of tissue exudates [5]. The IPT was able to detect ASF antibodies at an earlier point in the serological response, when few antibodies were present. This is in line with field experience from affected countries in Europe.

Generally, knowledge was gained and collated on suitable antigens and their expression for serodiagnosis of ASF. Evaluation of the available data also revealed strengths and weaknesses in terms of broad range of detection (Perez-Filgueira *et al.*, 2006; Cubillos *et al.*, 2013). For the African setting, it was demonstrated that recombinant p30 of Morara/Georgia was able to mirror the overall situation. These data fed (see Svanovir ELISA and IDScreen) and will feed into new test developments towards early and reliable detection of ASF antibodies. At present at least four commercial ELISAs are in routine use (two from Ingenasa, two fromIDVet), and others are

under development and validation. All these tests have different strengths and weaknesses, especially when it comes to testing of bad quality wild boar sera. The results were shown to vary among laboratories. A large-scale comparison is currently under way in Germany with six/seven kits under evaluation. Preliminary results indicate good performance with domestic pig samples and wild boar samples of adequate quality. More problems are seen with samples of bad quality. Very recently, a flow cytometry-based multiplex system (suspension microarray technology) has been described to detect antibodies against ASFV CD2v, p30, p54, and p22 (Li *et al*., 2022). Moreover, additional blocking ELISAs have been published (Yu *et al.*, 2021). Along similar lines, a bead-based multiplex assays for the simultaneous detection of antibodies against ASF (p72 and p30) and CSF (E2) has been established (Aira et al., 2019).

Additional methods include luciferase immunoprecipitation (Ding et al., 2022), adapted indirect ELISAs (Nah et al., 2022), QDM based immunosensors (Li et al., 2022), and chemiluminescence immunoassays (Yang et al., 2021).

Recent developments in pen-side tests and alternative sampling and testing schemes.

Following the above-mentioned studies using antibody LFDs, promising results were also obtained under experimental conditions and in combination with alternative sample matrices such as dry blood swabs (Pikalo *et al.*, 2021; Carlson *et al.*, 2020).
Apart from the single lateral flow device (LFD) for antibody detection to ASF, multiplexing has been reported with CSF, which would facilitate surveillance for both diseases (INgezim ASFV-CSFV-CROMAb) (Sastre *et al*., 2016a). Furthermore, related techniques have been used such as colloidal-gold dual immunochromatography for the detection of p30 and p72 specific antibodies (Wang *et al.*, 2021), and fluorescent immunochromatography test strips (Li et al., 2020).

While antibody detection by LFD is quite sensitive, much lower sensitivity was observed when detecting viral antigen by LFD. However, field experience showed that the combination of an antigen LFD (INgezim PPA CROM Ag LFD, Ingenasa) used on sick animals and an antibody LFD (INgezim PPA CROM Ab LFD, Ingenasa) can help to prioritize sampling and control efforts in pig farms affected by ASF (Lamberga et al., 2022). Yet, field experience from Germany showed that negative results must be regarded with great care (Deutschmann *et al*, 2020). Even samples with very high viral loads detected in real-time PCR did not result in positive reactions.
New pen-side tests have been reported and commercialized in recent years. Among them are the PenCheck (Silver Lake Research) and the Eradikit African Swine Fever Lateral Flow Assay (In3diagnostic). The former is suitable for both blood and tissues, the latter mainly for tissues. Both tests have shown potential under experimental conditions and with defined field materials (Onyilagha *et al.*, 2022; Friedrichs *et al.*, manuscript in preparation). Field use must show their performance in more detail.

Non-invasive sampling strategies could mean an optimization for wildlife surveillance by circumventing the necessity of fitness-biased hunting/capture sampling schemes that can in the worst case even further disperse the virus. Recently, different approaches for the in-life sampling have been evaluated both under experimental and field conditions.

One option for a non-invasive approach could be the collection of faeces from the wild boar habitat. Along these lines, de Carvalho Ferreira *et al*., (2014) tested the suitability of fecal samples. They demonstrated that, in comparison with virus detection in blood, virus can be detected in faeces 50-80% of the time. For the subacute/chronic phase, this percentage decreases below 10%. Despite this rather variable detection in the course of the infection, ASFV DNA was proven to be rather stable in faeces (half-life of more than two years at 12°C or ~15 days at 30°C, respectively) and thus, testing of faeces could supplement the toolbox of monitoring methods. Apart from genome detection, it was recently shown that faeces could also be suitable for ASFV specific antibody detection (Nieto-Pelegrín *et al*., 2015).

Another option is the use of (bait) ropes for the collection of oral fluid. Oral fluids were shown to be suitable for antibody detection (Mur *et al*., 2013; Giménez-Lirola *et al.*, 2016) and genome detection (Grau *et al*., 2015).

With regard to bait ropes, published studies cover mainly CSF (Mouchantat *et al*., 2014; Dietze *et al.*, 2017) and FMD (Mouchantat *et al*., 2014), but similar approaches have been followed for ASF under both field and laboratory conditions. In Russia, ropes were left at feeding places and wild boar were shown to chew on the ropes.

Oro-pharyngeal fluids collected on ropes have recently shown high potential for herd diagnosis of ASF (Goonewardene *et al.*, 2021).

Braae *et al.*, (2013) investigated the use of FTA cards for blood collection and subsequent testing by qPCR under field conditions in Tanzania. Detection of viral DNA was demonstrated in a subset of clinically healthy animals and the principle was confirmed under laboratory settings.

These results are in line with the work published by Randriamparany *et al.*, (2016) and Michaud *et al.*, (2007). Here, ASFV diagnosis (and characterization) was successfully performed from dried-blood filter papers (experimental and field samples) over extended periods of time. Especially under tropical conditions, these approaches ensure suitability and stability for downstream applications without a cold chain and sophisticated transport. Randriamparany *et al*., (2016) could additionally demonstrate the suitability for antibody detection. In detail, the study showed that real-time UPL PCR from filter papers is as sensitive as conventional testing by virus isolation and conventional PCR, and ELISA from filter papers was comparable with the same assay from serum. No problems with specificity were encountered.

Along the same lines as the above-mentioned FTA cards and filter papers, Petrov *et al*. [8] could show that dry blood swabs (in general using different cotton, foam or tissue swabs) can be a valuable, stable and easy-to-handle method to test carcasses for ASFV (and CSFV) genomes. The advantage is that the swab is already combined with a shipment-suitable receptacle, and no direct contact or further equipment is needed. In the reported studies, so-called Genotubes (Thermofisher) were the optimum in terms of handling and stability. In subsequent studies, the suitability of these swabs for ASFV antibody detection by ELISA (in the protocol meant for filter paper punches) was demonstrated in a proof-of-concept study (Blome *et al.*, 2014).

Recently, these initial data were supplemented by a broader validation study that also included the combination with antibody lateral flow devices (Carlson *et al*., 2020). The mentioned validation study showed the following performance characteristics for the Genotube based samples (when compared to routine diagnostic sample matrices and tests): qPCR 98.8% sensitivity [CI 93.4, 100.0] and 98.1% specificity [CI 90.1, 100.0] under laboratory conditions (85.7% [CI 71.5, 99.6] with stored field samples), and for serology by ELISA 93.1% sensitivity [CI 83.3, 98.1] and 100% specificity [CI 95.9, 100.0]. Good agreement was found when using the above-mentioned antibody LFDs from Ingenasa. This concept is particularly interesting as it was shown that it had almost no problems with bad sample quality.

For pathogenesis and immune response studies (correlates of protection and virus distribution in vaccination/challenge trial) but also diagnosis from necropsies, an optimized in situ hybridization protocol for the detection of African swine fever virus (ASFV) DNA in formalin-fixed, paraffin-embedded tissues using digoxigenin-labeled probes has been described by Ballester *et al*., [9].

Superficial inguinal lymph nodes (SILNs) have shown potential for the screening of dead pigs (Goonewardene et al., 2022). These samples can be collected in minutes with no to minimum environmental contamination. It was demonstrated that viral genome copy numbers in SILNs highly correlate with those in the spleen and, by sampling SILN, all pigs were detected that succumb to highly virulent and moderately virulent ASFV strains (100% sensitivity). ASFV was isolated from all positive SILN samples. Along similar lines, minimally invasive samplers have been used to detect ASFV in inguinal lymph node samples (Li et al., 2022).

Advances in cell cultures: It was recently shown that an immortalized porcine macrophage cell line competent for the isolation of ASFV (Masujin et al., 2021).

Genotyping

To better understand the molecular epidemiology of the recent outbreaks, additional genome markers are under investigation. Among them are different intergenetic regions.
Next-generation sequencing could be aided by enrichment through targeted sequence capture technology (Fernández Pinero, unpublished).
Variants have arisen in some countries (e.g., Germany) that can be traced by tailored PCR and sequencing approaches (Forth *et al.*, 2022).
Rapid Sequence-Based Characterization of African Swine Fever Virus by Use of the Oxford Nanopore MinION Sequence Sensing Device and a Companion Analysis Software Tool
Vivian K O'Donnell

Ressources:
Center of Excellence for African Swine Fever Genomics
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Conclusion

In conclusion, our toolbox of diagnostic tests has grown considerably over the past years but there is still a need for harmonization, situation-adapted diagnostic workflows, and general knowledge of disease biology that helps us in further adjusting our methodologies. Optimized and harmonized workflows are needed for next-generation sequencing. However, it is important to note that in the last years the field of diagnostics has expanded into the search for new models of detection. Not only for the use of current techniques in new types of samples like feces (de Carvalho Ferreira *et al*., 2014; Nieto-Pelegrin *et al.*, 2015), FTA cards (Braae *et al*., 2013), dry blood swabs (Petrov *et al*., 2014: Blome *et al.*, 2014), and oral fluids (Mur *et al*., 2013; Grau *et al.*, 2015; Gimenez-Lirola *et al*., 2016), but also searching for new models as air samples (de Carvalho Ferreira *et al*., 2013) and feed (Dee *et al*., 2018). This line of expansion will help to reach a more environmental and less invasive diagnostic as the industry desires.

Gaps

ASF is usually suspected based on clinical signs, but clinical evidence is usually nonspecific and would be difficult to differentiate from other diseases of swine, including Classical Swine Fever, Erysipelas, Salmonellosis, Eperythrozoonosis, Pasteurellosis, Pseudorabies, thrombocytopenic purpura, warfarin poisoning, and heavy metal toxicity. Regional labs in endemic countries lack the infrastructure and/or expertise for reliable diagnostic services. Some of the existing regional laboratories in Africa have limited capacity and most of them use the fluorescent tests and not real time RT-PCR.

The overarching gaps for diagnostics are:

1) There is a lack of commercial tests for large scale and confirmatory diagnostics
2) Validation of serological and virological tests for different epidemiological situations (e.g., low versus virulent ASFV strains).
3) Need to perform biological characterization and determine serotypes and pathotypes of current ASF strains that will provide the knowledge to extrapolate such characteristics using in vitro tests in the laboratory
4) In order to replace laborious and homogeneous primary cultures for virus isolation, cell cultures need to be found that support ASFV replication and the cell lines recently described have to be validated
5) The global ASF situation has to be considered when developing diagnostic assays. Tailored approaches could be an option for some scenarios.
6) For an early detection of the disease by serological methods, ELISA systems should be improved including the possible use of alternative sample matrices.
7) To understand the genetic diversity, studies targeting the sylvatic cycle hosts in Africa should be undertaken.
8) Pen-side tests and other field-deployable diagnostic tests need further investigation
9) There is an urgent need to increase the knowledge of the survivor pigs from the clinical and ASF diagnosis point of view.
10) New phylogenetic markers associated with pathogenicity should be looked at.
11) Field validation of new assays is needed considering fitness-for-purpose and the overall situation.

12) There is a need to intensify training and follow-up activities for international harmonization of diagnostic tests.

Research needs

1) Identify/develop cell lines that replace primary cultures for improved virus isolation techniques.
2) Full validation of novel or modified ELISA tests for detection of antibodies in alternative sample types (e.g., blood, exudate´s tissues, oral fluids, meat juice, filter papers, etc.).
3) Improved stability of reagents in commercial diagnostic kits (molecular virological and serological assays) regarding shipment and expiration issues. This could be overcome by exploring different strategies such as gelification lyophilization and others.
4) Automation and standardization of viral genome sequencing for subtyping ASFV strains.
5) Expanded field validation of novel assays, taking into consideration the worldwide scenarios.
6) Development and evaluation of non-invasive sampling methodologies in wild suids.
7) Validate available penside diagnostic tools to enhance detection and improve surveillance in wild life in Africa.
8) Development, evaluation and field validation of commercial confirmatory serological tests.
9) Standardization and validation of ELISA tests to detect antibodies against *Ornithodoros* tick saliva antigens in bitten animals.
10) Improved knowledge of the role of the survivor pigs as potential shedders by the use of appropriated diagnostic serological and virological tests for identification/detection of these animals.
11) Study the effects and detection of low virulent isolates and persistent infections

EPIDEMIOLOGY

African swine fever was first reported in Africa in 1909 following the introduction of European domestic pigs in Kenya. It was characterized at the time as an acute hemorrhagic disease with mortality rates of 100 percent in domestic pigs (Montgomery *et al.* 1921). It was subsequently recognized that ASF had been present in southern and eastern Africa all along in wild suids (Penrith *et al.*, 2013). Many countries in sub-Saharan Africa have remained endemically infected and continue to experience a significant number of ASF outbreaks annually (recently reviewed in Mulumba et al 2019).

The first spread of ASF outside Africa was in Portugal in 1957 as a result of waste from airline flights being fed to pigs near Lisbon airport (Costard *et al*., 2009; Gallardo *et al.,* 2015). Similar means of ASFV introduction were reported for the outbreak in Brazil in 1978 (Lyra 2006). All ASF introductions outside Africa were successfully eradicated, with the exception of the infection on the Island of Sardinia, Italy. However, in June 2007, an ASF outbreak was notified to the OIE in the Caucasus region by the Republic of Georgia, presumably caused by feeding pigs with ASFV contaminated pork brought in on ships from Africa (Rowlands et al., 2008).

Since the introduction of the virus into Georgia in the Caucasus region in 2007 ASF has extended its geographical distribution and is currently present in large parts or Eastern, Central and Southern Europe. Furthermore, in 2018 ASF was introduced into China, and has since spread widely in the region. In 2021, a new long-distance jump occurred, and ASF was reported first from the Dominican Republic and later from Haiti. Among the countries affected during the current epidemic, only three – Czechia and Belgium, in which no domestic pigs were infected, and Greece, in which only one outbreak, in domestic pigs, occurred– have managed to control and eradicate ASFV via swift disease identification and biosecurity measures.

With this development during the past 15 years, we now have a global ASF epidemic and more ASFV in the world than ever before. The risk for further spread towards additional countries must be considered very high and the disease currently represents the greatest threat facing the world's swine production industry .

The epidemiology of ASF may vary substantially between affected countries, regions and continents. However, more importantly, the epidemiology of the disease is strongly linked to the properties of local/regional pig value chain and the host population, which may vary within a region as well as between regions. Although to date four separate epidemiological cycles of ASF have been described, the so called domestic pig cycle, in which transmission between domestic pigs occur independently from contact with wild pigs or the argasid tick vector, dominates globally. In addition, in Eastern and Southern Africa an ancient sylvatic cycle, involving wild African suids (warthogs) and argasid ticks of the genus *Ornithodoros*, prevails, which can serve as source of virus responsible for outbreaks in domestic pigs (for review: Tulman *et al*., 2009). Also a domestic pig-tick cycle, in which the argasid tick serves as a reservoir of the virus in the local environment of the domestic pig has been described in parts of the world (Costard *et al*., 2009). To this, an additional cycle has recently been added: the wild boar-habitat cycle, which includes wild boar and persistence in the environment as observed in large parts of Eastern Europe (Chenais et al 2018). The presence/absence of the arthropod vector (ticks of the genus *Ornithodoros*) in the affected area will impact maintenance of the virus in the environment but not the spread (Plowright *et al*., 1994). Thus, depending on the properties of the regional/local pig value chain and the host population ASF may show regional patterns of presentation, associated with risk factors that should be assessed to establish proper surveillance and control strategies.

At least twenty-four different *p72 genotypes* have been identified among virus isolates from sub-Saharan African countries. However, the use of *p72* for genotyping only provides an initial characterisation and does not directly provide data on cross immunity between the genotypes or their virulence. Previous to the appearance of the *p72 genotype* II in Georgia in 2007 only isolates belonging to the *p72 genotype I,* had been detected outside the African continent.

Although the importance of ASFV genotypes in the biology of the virus is not well understood, it has contributed to our knowledge of the distribution and evolution of ASFV. It will be important to continue to assess ASF strains in endemic region, as demonstrated in recent reports that have identified new genotypes in Mozambique (Quembo *et al.*, 2017), and Ethiopia (Achenbach *et al.*, 2017).

Gaps

1) Since the start of the current ASF epidemic in Georgia in 2007, the virus has continued to gain ground. Over the last few years, ASF has moved across Eastern Europe and into Western Europe, inflicting substantial socioeconomic losses on pig farming and pork production industries. The introduction of ASF to China in 2018 was a "worst case scenario" (Gallardo et al. 2021), as China is the world's largest producer and consumer of pork, responsible for approximately 50% of the global pork supply (Gaudreault et al. 2020). This event was quickly followed by a string of new outbreaks across Southeast Asia and India. ASF is now a global epidemic affecting five continents and shows no signs of slowing; it currently represents the greatest threat facing the world's swine production industry (Muñoz-Pérez, Jurado, and Sánchez- Vizcaíno 2021). A better understanding of this threat and the risk it constitutes for free regions, together with improved capacity to predict, is needed.
2) The "pig to pork" value chain is a slow and deliberate process, with an average time of ~9-10 months from insemination-to-slaughter, making it difficult for the pork industry to quickly adapt to changing epidemiological circumstances (Millet et al. 2021). Consequently, the current ASF epidemic has brought severe disruption and socioeconomic loss to the pig production industries of affected nations. These losses may take the form of direct death of animals (from the disease or from culling of infected/at-risk pigs), market interruptions, and strict international trade restrictions. Economic modelling of the outbreak in China, for instance, has estimated total losses (including direct, indirect, and government losses) of ~111 billion USD, evidenced by a 3.67 million metric ton reduction in the national supply of pork between August 2018 and July 2019 (You et al. 2021). These losses are often felt most sharply by low-income farmers and rural pig producers, with long-term impacts on the livelihood and economic security of the community (Penrith, Bastos, and Chenais 2021; Dixon et al. 2020). However, a better understanding of the impact and costs of the disease as well as its control in different contexts, is needed
3) Early detection is a critical factor for controlling outbreaks, and there is an urgent need to understand the local incentives and impediments to early detection and reporting. This warrants better understanding of outbreaks' social, economic (value of the incentive, need for compensation, insurance systems for farmers/indemnity systems), and public impacts. Some aspects to take into consideration are that farmers face multiple disease panoramas, and ASF might not be their top priority. Some farmers (e.g. organic productions in developed countries) might have decreased their contact with veterinarians leading to lack of knowledge about diagnosis and clinical signs. In new epidemiological scenarios, new strategies to reduce the timeliness of detection are needed. Improved awareness of ASF along the value chain in both ASF-endemic and free areas will be needed.
4) The epidemiological significance of survivor/"carrier" animals is controversial and has received substantial attention over the past few years. While shedding of infectious virus by survivor animals is theoretically possible (though unlikely), there is currently no evidence for any significant role played by clinically healthy survivor animals (Ståhl et al. 2019). Overall, no link between ASF epidemiology and viral shedding by healthy survivors ("carriers") has been established (Blome, Franzke, and Beer 2020), though the topic remains active in the literature and questions related to low-dose infections,

chronic/persistent disease courses, and wild boar epidemiology remain (Pietschmann et al. 2015; Ståhl et al. 2019). As such, more detailed analyses of survivor animals in ASF epidemiology are needed (O'Neill et al. 2020).

5) The spread of ASFV between domestic pigs (facilitated by human activity) is the primary driver of the current ASF pandemic (Dixon et al. 2020). However, wild boar are a natural reservoir of ASFV in the current Eurasian pandemic, and their importance to regional transmission patterns varies depending on their geographic distribution, movement patterns, and other regional variables (Sauter-Louis, Conraths, et al. 2021). The wild boar population maintains ASF within it via direct (animal-to-animal) and indirect (environment-to-animal) transmission, with the latter also facilitating human-mediated transmission (e.g. via contaminated vehicles, clothing, and feed) (Gavier-Widén, Ståhl, and Dixon 2020). Wild boar are highly implicated in the epidemiology of the current Eurasian pandemic (Penrith, Bastos, and Chenais 2021; Desmecht et al. 2021), but the degree of risk they present in various scneraios and settings that currently prevail is unclear (Bellini et al. 2021).
6) In most parts of Europe it is now clear that wild boar is not (usually) affected by ASF as spillover from domestic pigs, but rather that they are driving the disease dynamics. The most important knowledge gap concerns which control strategy to apply for ASF in wild boar in the absence of an effective and safe vaccine. There are currently few successful experiences of elimination. Intensively applied hunting in large areas has not produced useful results, but on the contrary may contribute to spreading the infection.
7) While the cost for the different management components of ASF in domestic pigs is more or less understood, the same does not apply to the management of wild boar populations, which is less standardized and involves other stakeholders. The successful experiences of ASF elimination from the Czech Republic and Belgium were very costly. If applied in larger areas the costs would be higher and elimination times longer. Questions therefore arise about the sustainability of control of ASF in wild boar.
8) Information on Asian ASF epidemiology and wild boar populations is scarce (Gavier-Widén, Ståhl, and Dixon 2020), with less data (e.g. on animal movements and farm management) and fewer studies available relative to Europe – more research and surveillance are needed to track ongoing outbreaks and anticipate continuing spread.
9) ASFV is a highly tenacious virus, capable of surviving for extended periods of time in the environment and on various biological matrices (Dixon et al. 2020). The virus can persist for more than 1 year in blood at 4°C, for several months in boned meat, and for several years in frozen carcasses (Chenais et al. 2019; Probst et al. 2017). However, further analyses are needed to separate the various environmental factors that impact ASFV survival in wild boar carcasses and its environment and allow a clearer understanding of the role of the wild boar-habitat cycle in long-term persistence of the current ASFV epidemic.
10) *Ornithodoros* soft tick species form half of ASFV's ancestral sylvatic cycle and can maintain ASFV at high titres for long periods – notably, experimental transmission to pigs has been observed >19 months post-infection of a tick vector (Gaudreault et al. 2020). In parts of Africa, ASFV is maintained and transmitted between neonatal warthogs and *Ornithodoros moubata* ticks that colonize warthog burrows (Gaudreault et al. 2020). In the 20th century ASF epidemic, *Carios erraticus* ticks (formerly known as *Ornithodoros erraticus*, which is used below to match the literature) were important in

the disease's transmission and maintenance within the Iberian Peninsula (Gaudreault et al. 2020). The identification of any potential role of arthropod vectors in the current ASF pandemic has, therefore, been an important goal of recent epidemiological research. Soft tick species (including *Ornithodoros* spp.) are widespread in ASF-affected regions of Europe and China (T. Wang, Sun, and Qiu 2018), but no conclusive link to the current ASF pandemic has yet been demonstrated (Gaudreault et al. 2020), and the determinants of tick competence for various ASFV strains remain unknown (de Oliveira et al. 2019).

11) The possible role of non-tick arthropods in ASFV epidemiology has attracted substantial interest over the past years as researchers attempt to identify potentially overlooked transmission pathways. In 2018, Olesen *et al.* published two studies on *Stomoxys calcitrans* (stable flies), a blood-feeding fly that can mechanically transmit ASFV (Blome, Franzke, and Beer 2020) and thus might drive the introduction of ASFV into high-biosecurity pig holdings (Olesen et al, 2018; Fila and Wozniakowski, 2020). Though experimentally capable of transmitting infection, however, *S. calcitrans* (and other livestock-associated flies such as tabanids and other *Stomoxys* spp.) have not been linked to any specific outbreaks in the current Eurasian pandemic (Fila and Wozniakowski, 2020; Blome, Franzke, and Beer 2020). Overall, arthropod vectors do not seem to play a significant role in the current epidemiology of ASFV outside Africa (Blome, Franzke, and Beer 2020), but the possibility cannot be completely excluded. Our understanding of soft tick distribution in Eurasian ASF-endemic regions is limited (Bellini et al. 2021), and climate change may also provide unexpected opportunities for interactions between arthropods and pigs (Arias et al. 2018).

Research needs

1) Epidemiology of the global spread of genotype II ASFV, including ecology and evolution
2) Risks, including routes and patterns, of further spread of ASFV into unaffected regions
3) Dynamics of ASFV transmission in various population settings in wild and domestic Suidae
4) Impact of environmental and climatic factors on wild boar populations and ASFV transmission
5) Increasing understanding of ASFV survival and transmission in different epidemiological settings including Eastern Europe, Western Europe, Southeast Asia, and Africa
6) Role of the wild boar-habitat cycle in ASF-affected regions, in particular in areas with susceptible wild suids outside Europe for which the importance of this cycle to date is unknown
7) Better understanding of the possible routes of transmission of ASFV between wild boar and domestic pigs (e.g. the possible role of arthropod-mediated transmission)
8) Potential role of symptomatic or asymptomatic survivors (so-called "carriers") in ASF maintenance
9) Sequencing and phylogenetic analysis of circulating Eurasian and African strains - ASFV molecular epidemiology in domestic and wild Suidae
10) Possible roles of arthropods (e.g. biting flies) in ASFV transmission
11) Risk factors for long-distance human-mediated ASFV spread to free areas

12) Direct and indirect socioeconomic consequences of ASF outbreaks and disease control – with particular focus on the situation in developing economies and low biosecurity settings
13) Societal determinants of ASFV transmission, with particular focus on the situation in developing economies and low biosecurity settings
14) Human-animal interface studies at key sites, including social and behavioural sciences
15) Deep studies of ASFV evolution related to pathogenicity
16) Epidemiological gap-filling in under-surveyed regions (e.g. Russia, rural China, and Africa)
17) Standardization and harmonization of ASFV epidemiology studies
18) Rapid characterization of circulating ASFV in new outbreaks

SURVEILLANCE

The clinical presentation of ASF in domestic pigs, ranging from highly lethal acute manifestations to subclinical, depends on the virulence of circulating virus, administration route, dose and host factors (Tulman *et al*., 2009).

Unlike domestic pigs, wild African suids infected with ASFV are generally asymptomatic with low viremia titers (Heuschele and Coggins 1969; Montgomery 1921; Plowright 1981; Thomson 1985). These features of ASF presentation and the resemblance of the clinical manifestation to other diseases of swine hamper surveillance based exclusively on clinical signs. Based on the complexity of the epidemiology of ASF and multiple clinical manifestation of the disease it is necessary to develop surveillance activities based on diagnostic testing.

Surveillance measures capable of early ASF detection are the "first line of defence against ASF" and are critical for responding to new outbreaks and controlling existing ones. These measures are typically divided into passive (observer-initiated) and active (investigator-initiated) systems (Dixon et al. 2020). In the former, encounters with potentially diseased pigs are reported from within the pork production system (by farmers, hunters, veterinarians, etc.). The efficacy of passive strategies depends on the knowledge levels of actors in the pig production chain, their willingness to report disease, and regional farm management practices (on smallholder farms, for instance, higher in-herd mortality levels may be considered normal, giving an ASF outbreak more time to spread before it is noticed). Active surveillance involves more resource-intensive efforts to conduct diagnostic tests in at-risk domestic and wild pig populations for markers of ASFV infection (Dixon et al. 2020). Passive surveillance is generally considered superior to active measures for the detection of ASF in wild boar (Cwynar, Stojkov, and Wlazlak 2019; Sauter-Louis, Conraths, et al. 2021; More et al. 2018), but both strategies are important for ASF control and were important components of 20th century ASF eradication efforts (Danzetta et al. 2020). In 2017, Guinat *et al.* conducted an expert opinion study on the ASF surveillance strategies that best combine efficacy and practicality. The most optimal was considered to be enhanced passive surveillance of domestic pigs and of wild boar (hunted or found dead). Active surveillance and carcass removal in wild boar were considered highly effective but less practical strategies (Guinat et al. 2017).

Gaps

1) As efforts continue to streamline and harmonize ASF surveillance programs, new sampling and data collection methods are critical to ensure that actors in the pork production chain can gather accurate, reliable farm-level information on ASF outbreaks and disease prevalence with minimal effort and maximum sensitivity. Research into the practical aspects of pig sample collection has the potential to increase the quality of field surveillance data and the likelihood of detecting ASF outbreaks before substantial within-herd transmission has occurred (Flannery et al. 2020; Goonewardene et al. 2021).
2) Rapid and accurate sampling methods are also important in the monitoring and surveillance of potential environmental transmission (e.g. from contaminated pig holdings, vehicles, and associated fomites) (Dixon et al. 2020; Kosowska et al. 2021). The increased focus on participatory epidemiology in low-income countries may also involve the development and testing of surveillance tools better suited to these environments (Chenais et al. 2015).

3) Monitoring and detecting ASF cases within wild boar populations is challenging due to the dynamic nature of wild animal movement and the many unknowns involved in ASFV transmission between animals and via environmental contamination. Large-scale studies focusing on the use of wild boar surveillance data to track outbreaks and analyse disease control measures can provide useful information on the state of available surveillance data and potential methods for improving it (Schulz et al. 2020; Martínez-Avilés et al 2020).).

Research needs

1) Standardization of national and international ASF surveillance programs, particularly in wild boar
2) International guidelines for harmonizing surveillance data collection
3) User-friendly online tools and databases for the collection, viewing, and analysing of publicly available surveillance data
4) Validation of new methods for efficiently collecting samples from domestic pigs and wild boar
5) Automated, low-cost surveillance systems for on-farm detection of ASFV-infected domestic pigs
6) Continuing surveillance of ASF epidemiology and evolution in Africa
7) Develop tests for detecting ASFV in ticks.
8) To validate outbreak surveillance measures, epidemiological investigations need to be performed on the implementation of emergency control measures and the use of ‘diagnostic tests to detect infected pigs in exposed populations.

BIOSECURITY

ASFV is a tenacious virus, stable in a wide range of environments, fomites, and pig products. It is also capable of long-term, low-prevalence maintenance in wild boar and is therefore very difficult to eradicate once it establishes a foothold. Meanwhile, there is currently no vaccine available to protect domestic pigs or wild boar against infection. ASFV biosecurity and disease control (including depopulation) are therefore of the utmost importance. Disease control resources are limited, particularly in low- and middle-income countries, and the economics of biosecurity measures must be studied as well. These measures consist of three primary categories: (1) on-farm biosecurity, (2) regional biosecurity for wildlife/wild boar, and (3) country-level biosecurity (e.g. trade and international movement restrictions). The importance of each category depends on the specific epidemiological circumstances faced in a given region or nation.

Disease control studies commonly reveal a conflict between efficacy and practicality – in wildlife biosecurity, for instance, active surveillance and carcass removal are considered some of the most effective strategies for ASF control, but they are also among the least practical (Danzetta et al. 2020). Alongside such studies, there is a growing understanding that technical knowledge is not itself sufficient to achieve disease control (Penrith, Bastos, and Chenais 2021). On-farm biosecurity measures in particular require the cooperation and assistance of actors within the pork food system (e.g. farmers, breeders, veterinarians, etc.) who are unlikely to act against their own economic security and livelihood. Many recent studies have therefore focused on the “participatory” aspect of on-farm biosecurity, wherein local actors are specifically

engaged in the development and implementation of economically and regionally feasible biosecurity measures (Penrith, Bastos, and Chenais 2021; Dixon et al. 2020; Chenais et al. 2019).

Gaps

1) Biosecurity regulations from animal health authorities are critical standards against which international control programs can be compared, allowing individual countries to develop policies appropriate for their national circumstances. Currently, these regulations rarely take regional socioeconomic and cultural factors into account (Chenais et al, 2017; Dixon et al. 2020). Such factors are increasingly recognized as critical components of ASF control, linked unavoidably to the behaviour of local actors in the pork production chain and to the success of on-farm biosecurity strategies that are often implemented without taking the culture and livelihood requirements of local stakeholders into account (Guinat et al, 2017; Penrith, Bastos, and Chenais 2021).
2) For wildlife biosecurity significant challenges are posed by the inherently uncontrollable nature of wild animal populations (Guinat et al. 2017). Strategies like fence construction, bans on feeding, and carefully controlled hunting programs have seen success in parts of the EU, whereas control of the disease has been hard to achieve in other parts (Cwynar, Stojkov, and Wlazlak 2019; Jori et al, 2020).

Research needs

1) Local/regional/national analyses on the efficacy of specific biosecurity measures adapted to the local context
2) Impact of environmental factors (e.g. forests, rivers, and mountain barriers) on ASF spread within wild boar populations
3) Continuing study of ASFV epidemiology in wild boar, including environmental transmission and ecological factors associated with enhanced viral survival in carcasses
4) Risk factors for domestic farms with varying biosecurity levels
5) Pathways of greatest risk for introduction of ASFV into disease-free regions, including the USA
6) Participatory studies of local socioeconomic and cultural factors impacting ASF prevention and control in low-income endemic regions
7) Increasing coordination between government actors, animal health agencies, and pig production communities to develop locally appropriate control measures

COUNTERMEASURES ASSESSMENT

ASSUMPTIONS

The following captures assumptions made by GARA working groups to assess potential countermeasures to enhance our ability to contain and eradicate an outbreak of ASF.

Situation
Countermeasures assessed for worst case scenario: A coordinated intentional distribution of ASFV-contaminated material in a high density highly populated pig region of an ASF-free country.

Target Population
Countermeasures assessed for target pig production segments in priority order:

1. Backyard pigs
2. Comprehensive commercial swine operations (farrowing, nursery, and finishing)
3. Commercial indoor farrowing operations
4. Large intensive indoor pig farms
5. Valuable commercial genetic swine stock

Scope of Outbreak
Countermeasures assessed for multiple outbreaks occurring simultaneously in backyard pigs, three farrowing commercial operations, a finishing pig commercial operation, a sow replacement operation, and evidence of infection in feral swine.

Vaccine Administration
No vaccine available, therefore the only control strategy would be based in the early detection of infected animals and their elimination, and strict control of the movement of pigs.

DECISION MODEL

The gap analysis working groups used the quantitative Kemper-Trego (KT) decision model to assess available vaccines and diagnostics, including experimental products. Instructions for using the model were provided prior to GARA scientific workshops (see Appendix I). Criteria and weights in the model were modified by the working groups for the purpose of assessing available countermeasures as well as experimental ASF vaccines and diagnostics (See Appendices II, III, IV, V and VI).

Criteria
The working groups selected critical criteria to enable the comparison of countermeasures using a pertinent and valid analysis, as follows:

Vaccines
- Efficacy
- Safety
- One dose
- Speed to scale-up
- Storage
- Distribution/Supply
- Mass administration
- DIVA compatible
- Withdrawal period
- Cost to implement (cost of goods, cost of replacement, inventory costs, cost to administer)

Diagnostics
- Sensitivity
- Specificity
- Direct (antigen/DNA) detection, DIVA during outbreak
- Indirect (antibody) detection DIVA general and post-outbreak surveillance
- Validation to purpose
- Speed of scale-up
- Throughput
- Pen-side test
- Rapid result
- Need for a confirmatory test
- Easy to perform
- Storage/Distribution/Supply
- Cost to implement

Weight
Each criterion was weighted to allow a quantitative comparison of the impact of the selected interventions.

Product profile
To ensure a consistent and meaningful assessment, the desired product profile (i.e., the benchmark) was identified for each countermeasure:

Desired Vaccine Profile
1. Highly efficacious: prevents transmission; efficacy in all age pigs, including maternal antibody override; one-year duration of immunity
2. Safe in all age pigs; no reversion to virulence for live vaccines
3. Only one dose is required
4. Rapid speed of production and scale-up, can deliver finished product quickly, and manufacturing method yields high number of doses
5. Expiration date of 24 months or greater
6. Manufacturer has effective storage and distribution capability
7. Quick onset of protection, 7-days or less
8. DIVA compatible: Can effectively and reliably differentiate infected from vaccinated animals
9. Short withdrawal period for food consumption
10. Cost of goods, cost of administration, cost of storage

Desired Diagnostic Test Profile
1. Detect all ASF genotypes
2. Direct tests for control and eradication
3. Indirect tests for post-control monitoring/detection subclinical cases
4. Rapid test- early detection
5. >95% specificity
6. >95% sensitivity
7. Pen-side test
8. DIVA Compatible
9. Field validated
10. Easy to perform/easily train personnel
11. Scalable
12. Reasonable cost

Values
The values assigned for each of the interventions reflect the collective best judgment of ASF working group members.

VACCINES

The GARA gap analysis working groups noted that current research into a suitable vaccine for ASFV is limited to only a few research groups worldwide. A summary of experimental ASF vaccines reported in peer-reviewed scientific publications 2012-2018 is provided in Table II. To date, the most promising candidate vaccines are rationally attenuated gene-deleted recombinant live viruses. Previous work has highlighted both virulence and immunomodulation genes, which if removed would provide a strong candidate vaccine strain. The use of live attenuated viruses as vaccines is a well-established system with good protective attributes but evidence of reversion-to-virulence in some of the experimental vaccines tested to date is a concern. The use of recombination technology also allows for the insertion of suitable markers for the development of DIVA vaccines that would be particularly critical in any outbreak situation. There is currently no candidate isolate appropriately attenuated to ensure both safety and efficacy, but in the last few years several laboratories have progressed and are potentially getting closer to developing safe and effective experimental vaccine candidates. The alternative to a live attenuated virus that would remove any risk of reversion to virulence is the use of a subunit vaccine. This would satisfy both safety issues and ensure good DIVA characteristics. Although previous data indicated that such a strategy did not provide efficient protection against ASFV infection, there is currently some promising ongoing research into the feasibility of using such a strategy for producing an ASF vaccine. Collectively, progress in the development of a working vaccine and the preliminary results obtained to date indicate that a first generation vaccine may be feasible in the near future.

Summary

Vaccination against ASF is still not an option, but progress has been made towards the production of a rationally attenuated live virus vaccine.

Assessment of Experimental Vaccines (see Appendix II)

The GARA gap analysis working groups discussed the characteristics of the different available experimental vaccines and assessed their performance against a list of criteria linked to the ideal product profile for an ASF vaccine. The following is a summary of the group's opinion for each of them.

1) *ASFV recombinant live attenuated gene-deleted vaccine:* Attenuated by deletion of specific genes that have been identified as virulence determinants. As result, attenuated virus strains are produced which has been shown to effectively prevent disease in animals challenged with the parental virulent virus around 28 days post vaccination. The WG recognizes the effectiveness of this experimental vaccine in terms of inducing efficient protection with only one dose, the rapid onset/duration of the induced immunity and the safety of the product along with the molecular basis for the development of DIVA test. Lack of heterologous protection is recognized as its main deficiency, although recent results obtained with some of these vaccines showed the presence of cross protection among genotypically different isolates.

2) *Subunit recombinant ASFV proteins expressed in different vaccine vectors:* Different recombinant vectors containing individual ASFV gene/s have been used; e.g., vaccinia, raccoon pox, Ankara, swine pox, and human adenovirus. Safety, rapid onset of immunity,

possibility of developing a DIVA test and the cost of implementation are recognized as the strength of theses vaccine platforms. It is important to remark that so far, with the exception of a very recent report showing preliminary data, there is no experimental evidence that an individual or a group of AFSV genes vectorized in any way can significantly protect domestic swine against the challenge with the homologous virus. Therefore, development of an ASFV subunit vaccine will depend on further research to identify protective antigens and the virus structures able to induce protection against the infection.

3) *ASFV DNA vaccines:* This is technically also a subunit vaccine where ASFV gene/s is cloned into DNA constructs that are used as immunogens. Its safety and the possibility to develop DIVA compatible diagnostic tests are the only strengths remarked by experts during the GARA Gap Analysis Workshops. As with vaccine candidates analyzed in *(2)* above, no candidate ASFV genes have to date been successfully identified so far to be used in a subunit vaccine.

Based in this assessment the GARA gap analysis working groups have decided that the most promising experimental vaccines are based on the use of rationally attenuated strains of ASFV. Nevertheless, the working groups recognized that this candidate vaccine platform needs a great deal of experimental assessment in several aspects of its basic development, including induction of early immunity, development of a compatible DIVA test, and no reversion to virulence.

DIAGNOSTICS

The GARA gap analysis working groups determined the effectiveness of this countermeasure is high. Early detection of ASF is important to minimize spread of disease and reduce the economic impact. ASF surveillance in the U.S. is accomplished through a combination of passive and active surveillance programs. Diagnostic designed during the recovery phase post-outbreak are also essential.

Summary

- In case of any suspicious of the disease, virus and antibody detection techniques should be performed simultaneously.
- Antibody response to ASFV takes from 7-10 days. Animal surviving
- ASF virus can be detected from 2-3 dpi. The disease antibodies persist for long periods of time.
- Incubation periods, is around 3-15 days. The incubation period is usually 3–15 days. The more virulent strains produce peracute or acute haemorrhagic disease characterized by high fever, loss of appetite, haemorrhages in the skin and internal organs, and death in 3–10 days, sometimes even before the first clinical signs are observed.

Assessment of Laboratory and Commercial Diagnostic Tests (see Appendices III and IV). The GARA working groups identified and assessed six diagnostic tests to be used for surveillance, confirmation, and recovery. These tests are available for use in laboratories worldwide and one test is commercially available. The value of these tests was assessed against the desired diagnostic test profile for ASF control and eradication (See Decision Model, Appendix I).

1) *Virus isolation (VI)*
   Virus isolation in swine macrophages primary cell cultures is a classic technique for the detection of infectious virus. Virus infection is detected by hemadsorption or presence of cytopathogenic assay. The ASFCWG stressed the attributes of VI, including the specificity and sensitivity of the technique as well as the fact that results do not need further confirmation. However, the technique presents disadvantages as it takes several days to run the test, is difficult to scale up, the impossibility to adapt the technique in a throughput system, and the need for technical expertise to perform the test.

2) *Conventional RT-PCR.* This technique is based in the use of specific primers for conserved areas of p72. The technique present good specificity and sensitivity, has been validated, is easily scaled up and results are quickly obtained. Unfortunately, results need to be corroborated by a confirmatory technique and it is necessary to have technical expertise to perform the test.

3) *Real time RT-PCR.* The test present good specificity, results are quickly available, is easy to be adapted in a throughput system and easily scaled up. As with the conventional PCR, results need to be corroborated by a confirmatory technique and it is necessary to have technical expertise to perform the technique.

4) *Fluorescent antibody tests (FAT).* The assay consists of detecting virus in tissues of infected animals using fluorescent anti-ASFV specific antibodies. This test has high specificity, results quickly available, has been validated, is inexpensive, and provides definitive results. The disadvantages of this test are the difficulties for scaling up or set up in a throughput system, and it needs to be performed by a highly trained operator.

5) *Antigen ELISA.* This assay allows the detection of virus using a capture ELISA based on the use of anti-ASFV antibodies on the plate. The specificity is good, although the sensitivity is poor. This technique is easily scaled up as well as adapted to a throughput system. Additionally, it is easy to perform and results are obtained quickly. Beside its poor sensitivity, another disadvantage of the technique is lack of validation, it is expensive and results need to be confirmed by a second technique.

6) *Multiplex PCR assays:* A multiplex conventional RT-PCR is available for simultaneous and differential detection of ASFV and Classical Swine Fever Virus (CSFV) (Agüero *et al.*, 2004). The method is highly sensitive and specific and has been validated using field and experimental porcine clinical material. This test can be useful in case of clinical suspicion of swine hemorrhagic disease, as well as in those countries/areas where both viruses can be co-circulating at any time.

Assessment of Experimental Diagnostic Tests (see Appendices V and VI)
The GARA gap analysis working groups identified and discussed several new technologies that are being considered for the detection of ASF in the laboratory or as pen-side tests for field use.

1) *Loop mediated isothermal amplification (LAMP)*: LAMP is based on amplification of nucleic acids without the need of PCR equipment. It requires only the combined use of a DNA polymerase with strand-displacement activity and four-six specially designed primers towards six regions of the DNA target (Notomi *et al.,* 2000). LAMP is described as a highly specific and sensitive tool, which allows the detection of amplified products even by the naked eye. The comparative simplicity of the technology makes LAMP adaptable to front-line testing in regional laboratories, simple diagnostic situations and even to pen-side testing as a rapid first-line tool. Several LAMP assays have been developed recently for ASFV detection, and standardisation and validation are currently ongoing (Hertjner and Allan, QUB, Belfast, UK).

2) *Real-time PCR assays using commercial universal probe libraries (UPL)***:** UPL was recently commercialized by *Roche Applied Science*, and is a collection of short hydrolysis DNA probes, originally designed for gene expression analysis and offered as a universal detection system. Currently, UPL probes are being applied also for pathogen detection, main advantages being reasonably low cost, short time of delivery, and ready-to-use format. The combination of a specific primer set and an appropriate UPL probe will allow specific and sensitive detection of ASFV by real-time PCR at a comparably lower cost. Two UPL real-time PCR assays, designed in different viral genome regions, have been developed and standardised recently for ASFV detection (Fernández-Pinero, Gallardo, and Arias, CISA-INIA, Valdeolmos, Spain). Validation for their suitability in diagnosis is in progress.

3) *Linear-After-The-Exponential (LATE)-PCR:* LATE-PCR is an advanced asymmetric PCR producing huge amount of ssDNA molecules, which are detected by the incorporation of a specific low-Tm probe. This tool provides several advantages, such as increased multiplexing capacity and faster thermocycling, compared to currently used PCR chemistries (Sánchez *et al*., 2004). A LATE-PCR method has just been developed for ASFV detection (Hakhverdyan, Stahl, and Belák, SVA, Uppsala, Sweden; in cooperation with Ronish and Wangh, Brandeis University, USA). The LATE technology is exclusively licensed by *Smiths Detection*, and the developed ASF assay will be adapted to their portable PCR platform *BioSeeq* to provide a robust, powerful and simple-to-use diagnostic system for onsite detection of ASFV in a wide range of environmental conditions.

4) *Lateral flow device (LFD)*: A one-step immunochromatographic strip (pen-side test) capable of specifically detecting anti-ASF antibodies in serum specimens is under development. The qualitative assay is based on a direct immunoassay in which the detector reagent is latex micro particles covalently coated with a purified ASFV protein. The capture reagent is a viral protein adsorbed on the nitrocellulose membrane strip to form a test line. A second line created above the test line, by the immobilization of anti-control protein antibodies, is used as a control of the test. A serum specimen is applied to the sample pad. The anti-specific antibodies present in the sample specifically bind to the labelled micro

particles. The antibody-protein binding complex formed migrates until the nitrocellulose membrane by the flow caused by capillary action and reacts with the immobilized viral protein, which generate a visible test line.

DISINFECTANTS/INACTIVATION

The survivability of ASFV in feces and urine of experimentally infected animals was recently investigated by Davies *et al.*, 2017. Based on the calculated half-lives it can be assumed that ASPV remains infectious at 37°C for almost four (urine) or three (feces) days. In a study by Turner and William in 1999, it was shown that at 40°C, the inactivation of ASFV in pig manure is realized after 4 hours, and within 5 minutes at 65°C (See Table IV).

Disinfectants

The use of effective disinfectants for cleaning infected premises, trucks, and fomites is an important measure for preventing new introductions of ASF. However, many of the common disinfectants are ineffective. Care should be taken to use a disinfectant specifically approved for ASFV. A number of inactivation methods and disinfectants have been tested and reported for various materials, including animal waste. See Table IV for a complete list of disinfectants, field application, effective concentrations, exposure time, and references.

Rendering

Rendering of animal byproducts is heavily regulated in Europe and involves sterilization at 3 bar and 133°C for at least 20 min. Any process that exceeds 70°C for 20 min (or 60°C for 30 min) will inactivate ASFV; therefore, the rendering process will inactivate ASFV.

Biogas plant

African swine fever viruses can also be inactivated in a correctly operated biogas plant within hours (thermophilic) or days (mesophilic). The process does not only have temperature effects that inactivate the virus (pH value, metabolites, etc.). Nevertheless, it must be kept in mind that biogas plants are generally not designed in such a way that a strict black/white separation can take place (in contrast to high-security laboratories or modern rendering plants). For this reason, additional measures (preheating, adaptation of the process organization) would be necessary to achieve safe inactivation and protection against recontamination. In the doctoral thesis of Andres Moss (Moss A. 2001), a preheating of materials of animal origin to at least 70°C was proposed. However, this would be difficult to implement under field conditions.

Carcass Burial

Studies to assess ASFV inactivation in buried carcasses and soil are on-going but not yet completed at the time of publishing this report. For soil, the pH found in forest seems to be a limiting factor for virus survival. This report will be updated when the results of these experiments are completed.

ACARICIDES

Acaricides for controlling the soft tick may not be useful as the tick lives off the host and burrows underground as well as crevices in buildings. The best ASF method is to remove the pigs from infected premises.

DRUGS

There are no licensed anti-viral drugs available to treat pigs against ASF. Several publications reported compounds with potent anti-ASFV activity (Quetglas *et al*., 2012; Hakobyan *et al*., 2017; Barrado-Gil *et al*., 2017, etc.). Some of them are FDA-approved drugs but also active natural products (Fabregas *et al*., 1999; Galindo *et al*., 2011). However, the analysis of their potential use or applications in ASF remain unexplored. Antiviral drugs could be useful to complement vaccination or stamping out strategies from an epidemiological and economic point of view. One example was demonstrated for classical swine fever (CSF), another epizootic disease of pigs where outbreaks in areas of high pig density were controlled effectively with antiviral drugs combined with other common countermeasures such as massive culling and emergency vaccination (Backer *et al*., 2013). Possible benefits in ASFV would be the potential reduction of virus titers in blood and virus excretion from infected animals, and the reduction of the risk of dissemination until culling is possible. Thus, antivirals could potentially reduce transmission and prevent dissemination in susceptible animals around affected farms (belt-containment strategy). These compounds might be used as supplements in combined antiviral plus vaccination strategies to reduce virus persistence of live attenuated vaccines; however, an assessment of their potential use remains a gap. One antiviral tested, lipophilic statins, showed some activity against ASFV (Quetglas *et al*., 2012), demonstrating its potential use as a vaccine adjuvant (Xia *et al.*, 2018). Further studies are needed to understand the mechanisms of action of antivirals, which may in turn inform the development of new vaccine candidates.

PERSONAL PROTECTIVE EQUIPMENT (PPE)

ASF is not a human pathogen. PPE should be suitable to prevent farm-to-farm virus spread by animal health officials involved in eradication.

RECOMMENDATIONS

RESEARCH

The GARA recommends the implementation of the following research priorities to advance our ability to rapidly detect, control and respond to an ASF outbreak, including the progressive control and eradication of ASF in endemic settings.

Virology

- African swine fever virus complete genomic sequences from each genotype, viruses with different virulence, and viruses that have replicated exclusively in domestic pigs, wild pigs and ticks
- Generation of reference sequences that have been confirmed by different techniques in different laboratories to account of sequencing errors in repeat regions and other difficult stretches.
- Harmonization of sequencing workflows and validation of different enrichment techniques and host exclusion.
- ASFV bioinformatics resource to establish a comprehensive database that will include full length genome sequence of large number of isolates to replace the current less meaningful genotype based classification.

Viral Pathogenesis

- Basic parameters governing host to host infection, including domestic and wild swine as well as the arthropod host.
- Study the pathogenesis of ASFV isolates with different virulence in diverse susceptible host.
- Determine patterns of activation of immunologically relevant host genes particularly at early stages after infection.
- Identify ASFV genes and genetic determinants (group of genes like multigene families) involved in host range, virulence and pathogenicity. Correlate transcriptomics and proteomics.
- Continued investigation of determinants of virulence for different genotype and ASFV strains.

Immunology

- Discovery of the immune mechanism mediating effective homologous and heterologous protection against virus infection.
- Identification of viral genetic patterns that correlate with presence/absence of homologous versus heterologous protection.
- Identification of virus protein\s involved in the induction of protective immune response.
- Identify regulatory genes involved in pro-inflammatory cytokines and antibodies production and the assessment of their actual role in the process of virus infection\virulence in swine.
- Explore the development of new assays based on cellular immunity for the early detection of the disease.
- Explore immune-pathogenesis including T-cell responses and MHC presentation.

- Improve our understanding of the role of multigene families in antigenic variability and evasion of immune response.
- Identify and characterize genes related to host protection.

Epidemiology

- A global ASF surveillance system that provides high quality, accurate, and real-time information on ASF risk is needed to cover critical gaps of information of the ASF situation worldwide and to support ASF control and eradication on a global scale.
- Continuing molecular epidemiology studies to monitor both captive and wild suid populations as well as soft tick distribution is essential to effectively address the ASFV problem in endemic areas. These studies are also of great importance for preventive and surveillance programs.
- Conduct field validation of existing ELISA for the detection of tick presence.
- Need to intensify virus detection, isolation and characterization from sylvatic cycle hosts in Africa for genotyping purposes.
- Ongoing biological and molecular characterization of currently circulating isolates in Africa and Europe.
- To identify and apply new phylogenetic markers associated with virus virulence to understand virus evolution in endemic areas.
- To better understand the socioeconomics of the disease and pig and pork value chains, particularly those related to low biosecurity settings.
- To better understand the costs (direct and indirect) of ASF, both in epidemic and endemic situations.
- Identify management tools to control the disease in wild boar
- To better understand the role played by the environmental contamination and blood sucking insects in the disease cycle.
- Improved knowledge of the role of the survivor pigs as potential shedders by the use of appropriated diagnostic serological and virological tests for identification/detection of these animals.

Surveillance

- Further evaluate performance and overall accuracy of currently available ELISAs and PCR tests under experimental and field conditions. Evaluate under experimental conditions the performance and overall accuracy of currently available ELISAs and PCR tests.
- Automation and standardization of viral genome sequencing for subtyping ASFV strains
- Assess the rate of transmission of strains of ASFV of different virulence in infected-contact animal experiments.
- The epidemiology of ASF in emergency control programs needs to be assessed and modelled on the level of the individual pig, the herd, and the demographics of the region (low versus high density pig populations).
- Risk assessments need to be performed with regard to control or spread of ASFV

Diagnostics

- Support the development of new technologies for pen-side testing
- Evaluate and validate commercially available pen-side tests to "fit for purpose" for surveillance, response, and recovery
- Identify/develop cell lines that replace primary cultures for improved virus isolation techniques.
- Full validation of novel or modified ELISA tests for detection of antibodies in alternative sample types (e.g., blood, exudate´s tissues, oral fluids, meat juice, filter papers, etc.).
- Improved stability of reagents in commercial diagnostic kits (molecular virological and serological assays) regarding shipment and expiration issues. This could be overcome by exploring different strategies such as gelification lyophilization and others.
- Expanded field validation of novel assays, taking into consideration the worldwide scenarios.
- Development and evaluation of non-invasive sampling methodologies in wild suids.
- Validate available penside diagnostic tools to enhance detection and improve surveillance in wild life in Africa.
- Development, evaluation and field validation of commercial confirmatory serological tests.
- Standardization and validation of ELISA tests to detect antibodies against *Ornithodoros* tick saliva antigens in bitten animals.
- Study the effects and detection of low virulent isolates and persistent infections

Vaccines

- ASFV virology and functional genomics studies to inform vaccine discovery research.
- Determine safety characteristics associated with experimental live attenuated vaccines.
- Identify alveolar macrophage genes that enable ASF viral growth to inform the development of a cell line for vaccine production.
- Further explore the engineering of gene-deleted ASFV as potential vaccine candidates.
- There is a need for inter-laboratory testing of vaccine candidates.
- Harmonize challenge tests and read-outs.
- Continue to explore the potential for effective subunit vaccines.
- Research potential antigenic vaccine markers to differentiate infected from vaccinated animals (DIVA).
- Develop baits to enable the effective oral vaccination of wild boars.
- Develop and validate effective parenteral routes for live vaccine administration
- Proof-of-concept testing of needle-free systems for the delivery of new ASF molecular vaccines.

Biotherapeutics

- Testing Ad5-IFN distribution and expression in swine for rapid onset of protection against ASFV infection.

Disinfectants

- Development of low cost commercially available disinfectants for use in the inactivation of ASFV on contaminated surfaces found in farm settings and other susceptible environments.
- Explore the use of disinfectants to reduce the risk of ASFV infections from ASFV-infected carcasses.

Feral Swine and Wild Suidae

- Conduct research projects to further our understanding of the potential role of feral swine and wild suids as a reservoir for ASF.

Tick Vector

- Identify if the ticks in new geographical areas where ASF outbreak occur could become biological vectors or not.
- Determine whether new ASFV isolates can productively infect local ticks and whether they become persistently infected.
- Research is needed to further understand the distribution of soft ticks.

PREPAREDNESS

Many of the countermeasures discussed in this report will require planning, preparation, and integration into a coordinated disease control program. Critical will be funding for veterinary medical countermeasures to be stockpiled for use in an emergency response plan for an outbreak of ASF. The GARA recommends investing in the implementation of research priorities to support preparedness plans and ensure the effective use of countermeasures to prevent, control, and eradicate ASF.

CONCLUSION

African swine fever is a transboundary animal disease that currently threatens swine production worldwide. Even though ASF is an African disease, it is now well entrenched in the Caucasus, Russia, Europe, Asia and currently threatening the Americas. The most significant cause of this recent geographical spread is most likely due to the illegal movement of animals, trade, and contaminated products. This places other countries that trade in pig and pig products in danger, including Europe, South America, and North America. Furthermore, the epidemiological implications of ASF outbreaks in new geographical and ecologically unique environments are unknown, complicating control measures. Surveillance programs will be the first line of defense against ASF. Diagnostic tests are available and need to be incorporated in diagnostic laboratories. A key control measure will be vaccines but they are, with a unique exception, currently unavailable, a major gap in the availability of countermeasures to control ASF outbreaks.

TABLE I: SUMMARY OF COMPLETE GENOMES AVAILABLE ON NCBI BEFORE 2014

Name	Assession	Collection date	Country	Host
Odintsovo_02/14	KP843857.1	2014	Russia	Wild Boar
26544/OG10	KM102979.1	2010	Italy: Sardinia	Domestic pig
47/Ss/2008	KX354450.1	2008	Italy: Province of Sassari, Sardinia)	Domestic pig
Georgia 2007/1	FR682468.1	2007	Georgia	Domestic pig
Ken06.Bus	KM111295.1	2006	Kenya	Domestic pig
Ken05/Tk1	KM111294.1	2005	Kenya	Tick
OURT 88/3	AM712240.1	1988	Portugal: Alentejo	Tick
Pretorisuskop/96/4	AY261363	1996	South Africa: Kruger National Park	Tick
Malawi Lil-20/1	AY261361	1983	Malawi:Calaswa	Tick
Mkuzi 1979	AY261362.1	1979	South Africa: Mkuzi Game Resercve	Tick
BA71V	NC_001659.2 U18466.2	1971	Spain	Vero adapted
NHV	KM262846	1968	Portugal	Domestic pig
Tengani 62	AY261364	1962	Malawi: Tengani, Nsanje District	Domestic pig
L60	KM262844	1960	Portugal	Domestic pig
Kenya 1950	AY261360	1950	Kenya	Domestic pig
E75	FN557520.1	1975	Spain	Domestic pig
warthog	AY261366.1	Pre-2003	Namibia	Warthog
Warmbaths	AY261365	Pre-2003	South Africa: warmbaths	Tick
Benin 97/1	AM712239.1	1997	Benin	Domestic pig

TABLE II: BS FOR INACTIVATING AFRICAN SWINE FEVER VIRUS

Disinfectant	Field of application	Concentration	Exposure time	Reference
Sulfuric acid	surface disinfectant	1%	15 min	Fauser-Leiensetter, 2000
	liquid manure	1%	recommendation: 1 week	Fauser-Leiensetter, 2000
Formic acid	surface disinfectant	1%	15 min	Fauser-Leiensetter, 2000
	liquid manure	4%	recommendation: 1 week	Fauser-Leiensetter, 2000
Peracetic acid	surface disinfectant	2%	15 min	Fauser-Leiensetter, 2000
Formaldehyde	surface disinfectant	0%	15 min	Fauser-Leiensetter, 2000
	liquid manure	1%	recommendation: 1 week	Fauser-Leiensetter, 2000
	liquid manure	0.50%	> 4 days	Shirai et al., 1999, zitiert im EFSA Scientific Review
Sodium dodecyl sulfate	surface disinfectant	3%	15 min	Fauser-Leiensetter, 2000
	liquid manure	3%	recommendation: 1 week	Fauser-Leiensetter, 2000
Glutaraldehyde solution	surface disinfectant	1%	30 min	Fauser-Leiensetter, 2000
	liquid manure	1%	recommendation: 1 week	Fauser-Leiensetter, 2000
	tissue	0.2 %	11 days	Cunliffe et al., 1979, zitiert im EFSA Scientific Review
Sodium hydroxide solution	surface disinfectant	0.50%	30 min	Fauser-Leiensetter, 2000
	liquid manure	4%	recommendation: 1 week	Fauser-Leiensetter, 2000
	liquid manure	1%	150 s (4°C)	Turner und Williams, 1999
	liquid manure	1%	30 min (4 °C)	Turner und Williams, 1999
Citric acid	surface disinfectant	2%	30 min (22°C)	Krug et al., 2012
Caustic lime	dung pack			Bergerdorf et al., 1989, cited by Haas et al., 1995
Iodine		0,015 bis 0,0075 % (potassium iodide)		Shirai et al., 1999
Ortho-phenylphenol		1%	1 h	EFSA Scientific Review; OIE; Stone und Hess, 1973
Chloride, Hypochlorite	surface disinfectant	0,03 bis 0,0075 % sodium hypochloride		Shirai et al., 1999, zitiert im EFSA Scientific Review
	surface disinfectant	2,3 % Chlor	30 min	OIE

	surface disinfectant	0,15 % / 0,2 % als Natrium hypochloride		Krug et al., 2012
Quarterary ammonium compounds	surface disinfectant	0.003%		Shirai et al., 1999, zitiert im EFSA Scientific Review
Lime $Ca(OH)_2$	liquid manure	1%	150 s (4°C)	Turner und Williams, 1999
	liquid manure	1%	30 min (4 °C)	Turner und Williams, 1999
Heat	pig slurry	65 °C	5 min	Turner und Williams, 1999

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