2022 African Swine Fever Virus Research Review

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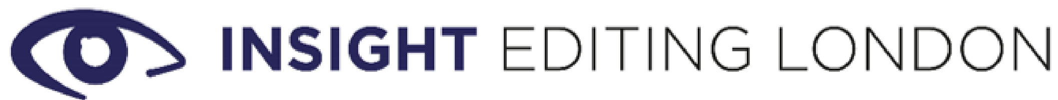

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Commissioning Body

This report was commissioned by the STAR-IDAZ International Research Consortium in collaboration with the Agricultural Research Service, United States Department of Agriculture, and the Global African Swine Fever Research Alliance.

The STAR-IDAZ International Research Consortium

The STAR-IDAZ International Research Consortium (IRC) is a global initiative aiming to coordinate research programmes at the international level and to contribute to the development of new and improved animal health strategies for priority diseases/infections/issues. The partners, research funders and programme owners, together form the Executive Committee which is supported by a Scientific Committee of 16 experts and an EU-funded Secretariat (SIRCAH – Horizon Europe Grant Agreement Number 727494).

The target deliverables of the STAR-IDAZ IRC include candidate vaccines, diagnostics, therapeutics, other animal health products and procedures, and key scientific information/tools to support risk analysis and disease control. To achieve these goals, the IRC partners agree to coordinate/align their research programmes to address identified research needs relating to the priority topics and to share results. Research gaps identified by expert Working Groups are organised into research roadmaps for the development of (i) candidate vaccines, (ii) diagnostics, (iii) therapeutics and (iv) disease control strategies, providing a structure to plot the identified research gaps and focus future investment (Entrican et al. 2021).

Agricultural Research Service, United States Department of Agriculture

The Agricultural Research Service (ARS) is the principal in-house research agency of the United States Department of Agriculture (USDA). ARS is one of four agencies in the Research, Education, and Economics (REE) mission and is charged with extending the nation's scientific knowledge with research projects in agriculture, human nutrition, food safety, natural resources, and the environment. ARS supports more than 2,000 scientists organized into approximately 660 permanent research projects at over 90 locations across the country and five laboratories overseas.

ARS conducts innovative research to find solutions to problems of high national priority that impact the American people daily. ARS often undertakes high-risk research endeavours to make significant

breakthroughs in important problem areas, including biodefence initiatives to detect, prevent, and mitigate the impact of especially dangerous infectious diseases that pose a threat to animals and public health.

Global African Swine Fever Research Alliance

The Global African Swine Fever Research Alliance (GARA) was founded in 2013 at the Plum Island Animal Disease Center in New York, US. Bringing together an international group of partners, collaborators, and stakeholders, the mission of GARA is to expand and maintain global research partnerships that will generate scientific data critical for the progressive prevention, control, and potential eradication of African swine fever. This mission is articulated through six strategic goals:

1. Identify research opportunities and facilitate collaborations within the Alliance
2. Conduct strategic and multi-disciplinary research to better understand ASF
3. Determine social and economic drivers and impact of ASF
4. Develop novel and improved tools to support the prevention and control of ASF
5. Determine the impact of ASF prevention and control tools
6. Serve as a communication and technology-sharing gateway for the global ASF research community and stakeholders

Purpose of the Report

African swine fever is currently the greatest single threat to global pork production, and our options for controlling and eradicating this disease remain highly limited. Stopping the current outbreak will require coordinated international research and biosecurity efforts. These efforts should be focused on the areas of greatest potential, and this requires regular updates and analyses to inform researchers, policymakers, and stakeholders of the current state of the field.

The primary background for this update is the African Swine Fever Gap Analysis Report published by the Global African Swine Fever Research Alliance (GARA) in November 2018 (GARA 2018), supplemented by the proceedings of the 3rd annual GARA Scientific Workshop in 2016 (GARA 2016). The purpose of this report is to revisit the research areas discussed in these resources, report relevant progress, and provide a general overview of the research that has been conducted across the major fields of African swine fever research since 2015. This report also incorporates research updates and input from leading scientists in the field, thereby providing an up-to-date picture of research around the world, enriched by the first-hand knowledge of researchers working at the cutting edge.

The findings of this report will be used to support future detailed gap analyses that will also incorporate expert opinion and review of current research and control measures, alongside knowledge of on-the-ground countermeasures (both in use and under development) and their efficacy. Importantly, this literature review does not attempt to rank the knowledge gaps identified, and this will therefore form a key part of future analyses.

About the Author

Dr Daniel Ackerman completed his Ph.D. in biological sciences and his subsequent post-doctoral studies at Carnegie Mellon University, Pittsburgh, USA. Since then, he has developed advanced communication and editing skills, and joined Insight Editing London in 2020. Daniel has assisted with the publication of diverse research articles during his time with IEL, rapidly building his reputation for writing and editing excellence across fields. He also recently co-authored the well-received and widely read Animal Influenza Research Review and Gap Analysis 2021.

Daniel Ackerman

About the Editor

Lucy Robinson

Dr Lucy Robinson gained her D.Phil. in veterinary immunology from the University of Oxford, UK, and continued her post-doctoral research into exotic infections at the Singapore Immunology Network. Following her editorial training and freelance experience, Lucy founded Insight in 2009 and has since successfully assisted with the publication of hundreds of articles and the construction of successful grant applications across diverse scientific fields. She has spoken at international conferences and delivered effective writing training to scientists from around the globe. Alongside Daniel Ackerman, she recently co-authored the Animal Influenza Research Review and Gap Analysis 2021.

Executive Summary

This report combines a comprehensive literature review with input from leading scientists across the field (for details of contributors, see here) to describe progress made in African swine fever virus research globally since 2015. By reference to previously identified knowledge gaps and expert consultation, we provide a literature-based update that identifies some of the areas in which future research and research funding should be targeted for maximum impact. The gap analysis presented here is intended to be used as a tool to supplement future in-depth gap analyses that include additional factors.

African swine fever has posed the greatest global threat to pig farming and pork production since its introduction to Georgia in 2007. The disease spread rapidly across Eastern and Central Europe, where it remained until 2015. Since then, it has spread into Western Europe, and in 2018, it was introduced into China. The pandemic has not slowed over the past 3 years, with further spread through Europe and East/Southeast Asia. In 2021, African swine fever reached the Dominican Republic and Haiti. The fight against this disease is in an urgent phase, and this report will summarize the substantial research progress that has been made in the face of this pandemic.

Research priorities by area:

Understanding African Swine Fever Virus

Epidemiology

African swine fever virus (ASFV) is a difficult pathogen to track and control due to its abilities to survive in the environment (e.g. within wild boar carcasses) and to be transmitted and maintained even within very low-density populations of swine. The transmission characteristics of ASFV also vary depending on geography – forest coverage, mountain ranges, number of water bodies, etc. – and local farm management systems, with smallholder/backyard farms more likely to facilitate untracked spread of the virus within domestic herds and to wild boar. One of the biggest challenges to ASFV epidemiology is the wide range of environments covered by the ongoing African swine fever (ASF) pandemic – many different ecological, geographical, and socioeconomic systems are currently dealing with ASF outbreaks, including Europe, Southeast Asia, Russia, Africa, and even island nations like Timor-Leste. This situation does not lend itself to one-size-fits-all solutions. Meanwhile, questions and controversies remain in many areas of ASFV epidemiology, and lack of standardization between study designs often makes direct comparisons difficult.

In Europe, the past 6 years have seen introductions of ASF to the Czech Republic, Romania, Belgium, Bulgaria, Hungary, Moldova, Serbia, Slovakia, Greece, Germany, North Macedonia, and Italy. Only two countries – the Czech Republic and Belgium, in which no domestic pigs were infected – now appear to have eradicated ASF via swift disease identification and biosecurity measures. Elsewhere (including Bulgaria, Hungary, Poland, Romania, and Slovakia in particular) the virus generally appears to be gaining ground, with numerous outbreaks especially on smallholder farms. Epidemiological investigations have revealed some details of the ASFV transmission patterns unique to these countries (e.g. Poland, where wild boar infections are dominant, vs. Romania, where domestic outbreaks are more common) and have also identified the apparent evolution of lower-virulence ASFV strains in Estonia and Latvia.

In Asia, the introduction of ASFV to China had dramatic socioeconomic consequences, and the country's pork production now appears to be stabilizing/increasing due to a shift in economic focus from smallholder farms to large, consolidated commercial producers. However, the virus has continued to cross international borders at a brisk pace, with subsequent introductions into Mongolia, Vietnam, Cambodia, Hong Kong, North Korea, Laos, the Philippines, Myanmar, Indonesia, Timor-Leste, South Korea, India, Papua New Guinea, Malaysia, Bhutan, and Thailand. Rural pig farms and smallholder operations dominate in many of these countries, and the resulting outbreaks have proven especially difficult to track. Epidemiological data on farm management and wild boar populations in Southeast Asia is lacking, and many lessons learned in Eurasia are unlikely to be applicable here.

Throughout the current pandemic, wild boar have played an important role in the transmission and maintenance of the disease, though the parameters involved (e.g. transmission speed, environmental contamination and spread to domestic pigs, the importance of boar population density, etc.) remain mostly unclear and may vary between geographical regions. Numerous risk factor assessments have been published for various European countries, with differing findings on the spatiotemporal correlation between wild boar infections and domestic outbreaks. Meanwhile, no conclusive role for carrier animals (wild boar that survive ASF and continue to shed the virus asymptomatically) has been demonstrated in the current pandemic, though this remains an active area of research. Environmental transmission (from infected wild boar carcasses to other boar or to domestic pigs) is also under intense study and may play a more significant role in colder climates.

Arthropods, the vectors responsible for half of ASFV's native sylvatic cycle in Africa, have also come under scrutiny in Europe. To date, no definitive link has been shown between Eurasian ticks and ASFV transmission in the current pandemic, but these studies continue to fill an important knowledge gap (particularly in Asia, where our understanding of tick populations is very limited).

The one factor that has proven relatively constant across the many environments of the current pandemic is human activity – anthropogenic factors have conclusively played a major role in the propagation and maintenance of ASF, with many recent reports describing routes of human-mediated transmission in Europe, Asia, Africa, and elsewhere. Within national borders, these routes include the transport and sale of infected domestic animals, feeding of untreated swill and food waste to pigs, inadequate biosecurity on farms, and uncontrolled hunting of infected boar leading to population dispersal and wider transmission patterns. Internationally, both legal and illegal transport of pigs can carry ASFV across great distances, with the recent introductions to Timor-Leste, the Dominican Republic, and Haiti proving that oceans are no barrier to anthropogenic transmission.

Virology/Molecular Biology

ASFV is a highly complicated virus, with a complex physical structure and large, G:C-rich genome that make virological studies inherently difficult. Sequencing of new ASFV isolates historically focused on specific regions of interest (e.g. the *p72/B646L* gene), but concentrating exclusively on such limited sequences may miss substantial genetic diversity. Since 2015, the continuing development and validation of next- and third-generation sequencing technologies has brought a dramatic uptick in the number of fully sequenced ASFV genomes. Many complete ASFV genome sequences have now been published, and the information in these papers is useful also for comparing the circumstance-specific efficacy and usability of the various new sequencing technologies now available on the market. The strengths and weaknesses of various instruments (e.g. for short-read vs. long-read sequencing) must be kept in mind when sequencing new isolates and resequencing old ones. Other recent studies have focused on the transcriptomics of ASFV infection (both from viral and host perspectives) and the potential roles of small non-coding RNAs in the infection process.

Understanding the functions of viral proteins is also critical for many areas of ASF research, including the study of viral evolution, the identification of the determinants of virulence and host immune response, and the development of new vaccine candidates. From a proteomic standpoint, approximately 50% of ASFV's genome remains functionally unresolved, with many protein products that are essentially uncharacterized. Though the picture is still not fully resolved, recent studies

focusing on individual viral proteins have begun to narrow this deficit, using structural biology and *in vitro* approaches to determine the functions of poorly understood proteins. Computational resources also continue to grow in both power and accessibility, allowing complicated protein modelling and interaction studies that explore the molecular-scale activities of virally encoded enzymes and transcription factors.

Pathogenesis

Different strains of ASFV can vary widely in virulence, but the determinants of these differences are not well-understood. High-resolution genomic assays are required to tease apart the often-subtle genetic differences between different ASFV isolates, and detailed *in vitro* studies can begin to identify the functions and interacting partners of viral proteins. Particularly critical functions for virulent strains are efficient entry and infection, manipulation of infected cells to avoid immune detection, and release of factors that lead to widespread lymphocyte death and general immunodeficiency. Our ability to generate new vaccine candidates is also dependent on understanding how individual proteins are used by ASFV to alter host cell immune responses.

Recent advances in sequencing and proteomic technologies, and in *in vitro* models, have allowed researchers to identify specific viral proteins that are required for infection, host immune evasion, and virulence - though many questions still remain. Over the past 6 years, studies have identified roles for viral proteins in regulating autophagy, host cell metabolism, and immune-related signalling pathways like the cGAS/STING and JAK/STAT1 pathways. Meanwhile, *in vitro* studies in primary porcine cells have begun to clarify the functions of particularly important proteins like CD2v and the MGF family proteins.

Another important area of ASF pathogenesis research is the African warthog, the second half of ASFV's native sylvatic cycle, which displays remarkable resistance to virulent ASFV infection. Several recent studies have addressed different potential sources (both environmental and genetic) of this resistance and the ways in which they may be applicable to domestic pigs.

Immunology

The most critical host-virus interactions occur at the interface between infected cells and the host immune system. ASFV preferentially infects porcine cells of the monocyte/macrophage lineage, and acute ASF is associated with massive apoptosis of lymphocytes leading to systemic immunodeficiency.

Knowledge of the immune response to ASFV infection, and the various proteins used by the virus to evade this response, is critical for developing an effective treatment and/or vaccine against ASFV. The nature of the anti-ASFV immune response remains unclear and occasionally controversial, with *in vivo* experimental infection studies giving different results regarding the importance of neutralizing antibodies, $CD8^+$ T cell responses, and other immunological factors in the effectiveness of the host immune response.

Since 2015, researchers have identified roles for viral proteins in a wide array of immunomodulatory activities including inhibition of type I interferons, and regulation of autophagy, apoptosis, and MHC protein expression. Inflammatory cytokine release is another important area of research, and several viral proteins are involved in the control of this process. Viral immune evasion is a complex process involving potential redundancy and/or combinatorial activity within the ASFV proteome – host- and strain-specific factors can combine to produce unpredictable outcomes, making it difficult to generalize specific experimental results. Increased standardization of ASFV gene characterization, and evaluation in multiple strains of varying virulence, will continue to be critical in the future to build our understanding of viral immunomodulation.

Meanwhile, transcriptomic studies have allowed high-resolution mapping of the response of porcine macrophages to ASFV infection, and *in vivo* analyses of infections with specific ASFV isolates have enabled us to begin to characterize strain-specific immune responses (including the importance of both humoral and cellular activity).

The historical system of genotyping new ASFV isolates based on *p72/B646L* gene sequence has also recently been called into question, and determinants of immunologically homologous vs. heterologous strains have been studied to guide vaccine development and boost our understanding of the requirements for immune protection against ASFV.

Controlling African Swine Fever

Biosecurity

With no commercially available vaccine or antiviral drug active against ASFV, biosecurity and depopulation remain our only lines of defence against the introduction of the virus and spread of the current pandemic. ASF has proven itself a very difficult disease to contain and eradicate, and strict control measures are necessary to provide the best possible chance of managing regional outbreaks.

It is critical that current biosecurity programmes are analysed and validated to determine their efficacy. However, it is also increasingly acknowledged that such measures should be tuned to the specific cultural and socioeconomic circumstances of individual nations and populations in order to be effective. Control measures that are successful in one region/country may not be successful in another.

During the last 6 years, many risk assessments, expert opinion studies, and reviews have been conducted to determine the effectiveness of the different ASF biosecurity measures applied across Eurasia. Studies on the practical effects of wild boar-focused biosecurity measures are critically important, potentially allowing location-specific planning by currently ASF-free countries and opening new avenues for disease control in epidemic regions. Epidemiological and surveillance data have been used to model the efficacy of wild boar containment measures including culling, feeding bans, hunting restrictions, and the construction of barriers to block wild herd movements. In domestic pigs, scientists have described the various risks present at the farm/environment boundary and the strategies that can be used to mitigate them. Meanwhile, key factors in human-mediated transmission have been identified, including inter-farm movements of people and animals and cross-contamination from wild boar habitats.

Participatory epidemiology has also begun to play an increasing role in ASF biosecurity research, with a growing understanding that smallholder pig farmers and other actors in the pork production chain are far more likely to comply with biosecurity regulations that do not place their cultural and economic livelihoods at risk. Studies of the knowledge, attitudes, and practices of farmers in resource-poor regions have expanded our understanding of the factors underlying biosecurity failure and the measures that can be taken to minimize these risks in different countries.

Surveillance

Unnoticed or unreported ASFV infections are an ever-present danger – once entrenched in regional wild and domestic pig populations, the disease is extremely difficult to eradicate, as demonstrated by the current epidemiological situation in Eurasia. Surveillance programmes allow us to monitor the spread of ASFV, facilitating the rapid identification of infected animals and efficient deployment of biosecurity resources in the event of an outbreak. Recent studies have described new approaches for ASF surveillance, including automated on-farm detection systems, novel sampling methods, and data collection techniques.

From an international standpoint, new computational models have been developed to simplify the collection and analysis of large surveillance datasets, thereby simplifying large-scale epidemiological research and potentially allowing faster governmental and regulatory responses to developing outbreaks. Meanwhile, web databases have been developed to make it easier for pork producers, academics, and regulatory officials to access publicly available surveillance data and design region-specific surveillance strategies.

Diagnostics

Rapid diagnosis of ASF in domestic pigs or wild boar is the first step in effective biosecurity, allowing farmers and regulators to react quickly to developing outbreaks and impose controls before the virus begins to spread unchecked. Today, several effective diagnostics are available to check for the presence of ASFV in various sample types. However, these tests are critically limited by their requirements for laboratory instrumentation and experienced users – this includes the current "gold standard", OIE-approved assays like qPCR, ELISA, and immunoperoxidase tests. Since 2015, substantial research effort has focused on the development of diagnostic tests with fewer laboratory requirements, variously detecting ASFV DNA, viral antigens, or anti-ASFV antibodies. New DNA tests include isothermal amplification methods, which gain field applicability by not requiring thermocyclers, and CRISPR/Cas-based assays that allow highly sensitive ASF diagnosis from even limited starting samples. Immunofluorescence and lateral flow assays for ASFV antigens or antibodies continue to be developed and refined to increase their sensitivity. Meanwhile, new sample collection techniques have also been described, aiming to reduce the difficulty of gathering samples (e.g. from wild boar carcasses) in the field and transporting them to diagnostic sites.

Isolation of infectious ASFV is also necessary for confirming a qPCR-positive sample. Currently, this process relies on the use of primary porcine cells, which necessarily reduces standardization due to the inherently donor-specific nature of primary cells. These cells are also generally difficult to culture, increasing labour requirements and introducing potential time delays into diagnostic processes. Stable cell lines have recently been validated for use in *in vitro* ASFV isolation techniques, avoiding these issues and increasing the reproducibility of standard diagnostic tests.

Vaccines

There is currently no ASF vaccine commercially available. This greatly limits our ability to control the ongoing pandemic, placing extreme pressure on biosecurity and control measures and necessitating

the costly depopulation of entire pig herds to prevent the spread of disease. Therefore, vaccine development has remained an active and dynamic field of research over the last 6 years, with studies on the design and testing of live attenuated vaccines (LAVs) somewhat dominating since 2015. However, there are many difficulties involved in developing a live attenuated vaccine (LAV) for ASFV, including the combinatorial nature of ASFV's complex gene program, the unpredictable effects of multiple gene deletions, and the differences between *in vitro* and *in vivo* viral characteristics. Nevertheless, substantial progress has been made since 2015, leading to the identification of several LAV candidates with great potential. ASFV strains with deletions to specific genes have been validated for attenuation *in vitro* and *in vivo*, expanding our knowledge of ASFV protein functions while also demonstrating homologous (and sometimes heterologous) protection against challenge with virulent viruses.

Limited advances have also been made toward different approaches to subunit vaccinations, which may avoid potential biosafety issues associated with LAVs. Elsewhere, researchers have begun to identify genes potentially useable as markers for DIVA (differentiating infected from vaccinated animals) tests, which will be critical in future vaccine deployment strategies. Several stable cell lines have also been proposed for the production of LAV candidates, removing the necessity of using primary porcine cells for this purpose. However, significant research challenges remain in all of these areas, and current ASFV vaccine candidates have yet to go through a full vaccine development plan subject to a robust regulatory process.

Drugs and Therapeutic Approaches

There are no commercially available antiviral drugs marketed for the treatment of ASF. This lack of antivirals for ASF control limits our options in outbreak response and control. As with vaccines, development of new anti-ASFV drugs (or validation of existing ones for anti-ASFV activity) is hampered by our incomplete understanding of the functional ASFV proteome. Since 2015, studies have begun to address this gap by characterizing the structural biology of important virally encoded enzymes and other factors that may be susceptible to small molecule treatment. Recent studies have tested antivirals both *in vitro* and *in vivo* for their ability to reduce ASFV replication, limit viral gene transcription, or otherwise interfere with the ASFV infection pathway.

Disinfectants

ASFV is a tenacious virus, capable of surviving on various surfaces (or within biological matrices like blood, urine, and faeces) for a considerable amount of time depending on environmental circumstances such as temperature and pH level. Disinfection is therefore a critical part of biosecurity, particularly on pig farms where thorough decontamination of affected premises is essential for halting an outbreak. There are many commercial products capable of inactivating ASFV, but their applicability to specific surfaces or contaminated environments is often untested, limiting the ability of farmers and pork producers to make informed decisions during disinfection. Numerous studies have been conducted over the past 6 years to close this gap by testing the efficacy of various disinfectants on relevant surfaces (e.g. steel and concrete) and in the presence of common biological contaminants.

Literature Review and Research Updates by Subject Area

Report approach

The primary literature review was conducted using the applied life sciences CAB Abstracts database (www.cabdirect.org) and the search terms ("African swine fever" OR "African swine fever virus"). When limited to studies published since 2015, the database returned a list of 1,522 papers. Papers were subsequently excluded from further consideration if they were not published in English or not relevant to African swine fever virus. The remaining papers were then manually screened for relevance to the scope of this report, finally leaving 1,145 papers for assessment for inclusion. These papers were allocated to the following topic areas as shown in Table 1.

Research Category	**Papers (n)**
Epidemiology	352
Virology/Molecular Biology	131
Pathogenesis	64
Immunology	58
Biosecurity	187
Surveillance	55
Diagnostics	130
Vaccines	107
Drugs and Therapeutic Approaches	35
Disinfectants	26
Total	1,145

Table 1: African swine fever virus peer-reviewed publications 2015-2021, sub-divided by topic area.

These studies formed the main structure of the report and were supplemented by 115 recently published studies retrieved from the PubMed.gov database, which has a shorter delay between publication and upload of article information than CAB Abstracts. Additional literature searches were performed during writing to provide appropriate citation for all material and, where needed, useful background. Studies were selected for inclusion based on the author's impressions of their potential impact within the field, their quality and novelty, and the relevance of their findings with respect to the knowledge gaps identified previously. More recent studies were given priority within the report. In total, 328 studies are referenced herein.

To generate an overview of ongoing research, African swine fever virus researchers and experts were contacted by email and invited to complete a survey that asked for brief summaries of their current and future projects in African swine fever virus research. These individuals were selected from attendance lists for the previous gap analysis workshops, personal connection with the commissioning team at STAR-IDAZ IRC, being on the GARA email distribution list, or through being among the list of most prolific authors within the "African swine fever" or "African swine fever virus" search of the CAB Abstracts database (defined as having contributed to >35 publications published between 2015 and 2021 inclusive): approximately one hundred individuals were contacted for input. Information provided by these researchers and experts is included within the appropriate report sections, but it should be noted that the representation of work is thus inherently biased towards those individuals and institutes that elected to respond to the request for information. Therefore, these sections should not be considered comprehensive in the same way as the rest of the report aims to be.

Two strategies were combined to identify current knowledge gaps: the knowledge gaps identified in previous gap analyses conducted by GARA were assessed to establish whether they have yet been filled or remain in need of further research; secondly, the African swine fever virus researchers contacted for ongoing research updates were also asked to submit their thoughts on current research knowledge gaps. Current knowledge gaps based on the literature review and expert opinion are summarised at the end of each literature review section.

Introduction

African swine fever (ASF) was first identified by Western veterinary science in Kenya in 1921 (Gaudreault et al. 2020). Its causative agent is African swine fever virus (ASFV), a unique and complex pathogen that is the only member of order Asfuvirales and the only known arbovirus (virus transmitted via arthropod vectors) with a genome composed of DNA (Galindo and Alonso 2017). In its ancestral habitat in Africa, where the virus has likely evolved for ~300 years (ASF-STOP 2021), ASFV exists in a sylvatic cycle between arthropod vectors – specifically soft ticks of the *Ornithodoros moubata* species – and wild Suidae such as *Phacochoerus* spp. warthogs. These ticks inhabit warthog burrows, where virus transmission occurs between juvenile ticks and warthogs (Gaudreault et al. 2020). Infection of these pigs results in viraemia without clinical disease, and horizontal transmission of ASFV between warthogs is not known to occur (ASF-STOP 2021). Therefore, ASF is considered a low pathogenic, persistent infection in its natural swine reservoir (McCleary et al. 2020).

The interjection of human activity into this sylvatic cycle resulted in the spread of ASFV to new environments and its introduction to immunologically naïve Suidae. The British colonization of Kenya in the late 1800s involved a massive influx of domestic pigs alongside a flourishing growth in the intercontinental swine trade (Alkhamis et al. 2018). When ASF was first reported by R. Eustace Montgomery in 1921, it was described as a highly virulent and contagious hemorrhagic disease that caused severe outbreaks and clinical symptoms in domestic pigs, with as much as 100% mortality. Since then, ASFV has rapidly expanded its geographic range on its home continent (Ståhl et al. 2019) and has caused major international outbreaks on two occasions. The first began in Portugal in 1957, likely via contaminated waste from airline flights that was fed to pigs near Lisbon airport (Brown and Bevins 2018). The virus then spread through Western Europe, Russia, Brazil, and the Caribbean over the next three decades until it was finally eliminated with great effort by the mid-1990s (with the exception of the island of Sardinia, where it remains endemic) (Gaudreault et al. 2020).

The second intercontinental outbreak of ASF began in 2007, when ASFV of genotype II (discussed in more detail below) was introduced to Georgia and infected nearly 20% of the country's domestic pig population within 2 months (Cwynar, Stojkov, and Wlazlak 2019). The virus quickly established a tenacious foothold in Europe, spreading through the Caucasus region, Russia, and the Eastern, Central, and Western regions of Europe. Introduction of the virus to China, the world's largest pork producer and consumer (Gaudreault et al. 2020), was a significant blow to the global pork industry and ASF biocontainment efforts. ASFV has since been reported in many other countries across Asia (Mighell and Ward 2021). In Europe, only Belgium and the Czech Republic have successfully eradicated the

disease (Penrith, Bastos, and Chenais 2021). In Asia, several countries appear to have controlled small initial outbreaks, but new cases have subsequently been reported (FAO 2022; Bacigalupo, Perrin, and Pacey 2022). Meanwhile, the introduction of ASFV to the USA, the world's third largest pork producer and consumer after China and the EU (USDA ERS 2019), remains a significant and growing threat. In July 2021, the USDA confirmed the introduction of ASF to the Dominican Republic (Cole and Stepien 2021), and by September, the virus had reached Haiti as well (Stepien and Cole 2021). These introductions mark a substantial geographical jump and highlight the risk of ASF introduction to mainland North America.

At each step, the current ASF pandemic has been marked by severe socioeconomic impacts encompassing animals lost to disease or culling, loss of trade revenue, and long-lasting impacts on the economic security of pig farmers and stakeholders in the pork production chain. The current global ASF situation is urgent and dynamic, requiring intense study of effective biosecurity and surveillance measures coupled with epidemiological studies that piece together the transmission patterns and risk factors associated with this pandemic. Much of ASFV's fundamental biology remains frustratingly elusive, with the functions of approximately 50% of its encoded proteins still mostly unknown. There are no commercially available vaccines or antivirals for the prevention/treatment of ASF, leaving farmers and regulators with few effective options in the face of this disease.

All areas of ASFV research can impact outcomes on the frontline – rational vaccine development requires functional knowledge of virology and immunology, and effective biosecurity depends on up-to-date epidemiological studies, risk assessments, and new surveillance technologies. The last 6 years have brought substantial advances in our understanding of ASFV's biology, its interactions with the host immune system, and the pathways by which it is transmitted and maintained in domestic and wild pigs. The ongoing pandemic requires an approach to future research that effectively integrates these new findings into existing scientific and regulatory frameworks, allowing coordinated ASF containment and control strategies that provide the best possible chance for disease eradication. With this perspective in mind, the following report provides updates on several critical areas of ASFV research, aiming to provide readers with an overview of recent scientific advances and to describe research avenues that continue to increase our understanding of and ability to control ASF.

Understanding African Swine Fever Virus

Epidemiology

Since the Eurasian ASF outbreaks in Georgia in 2007, the virus has continued to gain ground. Over the last 6 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 pandemic 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).

The "pig to pork" chain is by nature 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 pandemic 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).

Studies on the epidemiology of ASFV increase our capacity to understand viral evolution and transmission, the routes by which it spreads in new populations, and the various risk factors that are most highly associated with ASF outbreaks. Currently, many areas of knowledge lack clarity: in particular, the transmission patterns of ASF in different climates and environments are undefined, as are the potential roles of virus-shedding survivor pigs, the contribution of arthropods to Eurasian ASF transmission, and the ongoing effects of human activity on the intra- and international spread of ASFV.

Previously identified knowledge gaps

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

- *Complete viral genomes for origin tracking and identifying possible homologies*
- *Studies of wild Suidae as a reservoir for ASFV*
- *Molecular epidemiology studies in swine and soft ticks*
- *Study of soft tick distributions worldwide, including identification of ticks in new geographical areas as potential ASFV vectors*
- *Determination of tick infectivity by new ASFV isolates*
- *Increased study of wild hosts for genotyping*
- *Continuing characterization of circulating ASFV isolates in Africa and Europe*
- *Identification of phylogenetic markers associated with ASFV virulence*
- *Economic costs and socioeconomics of ASFV in pig/pork value chains, especially in low-biosecurity settings*
- *Role of environmental contamination and blood-sucking insects in ASFV cycle*
- *Role of survivor pigs as potential shedders*
- *Study of transmission rates between infected and contact animals for ASFV strains of varying virulence*

Literature review

Current Global Situation

The ASF pandemic that began in Georgia in 2007 continues to spread within and across national borders. Since the beginning of the period covered by this report (2015), genetically similar ASFV strains have been detected in the Czech Republic and Romania (2017); Belgium, Bulgaria, Hungary, and Moldova (2018); Serbia and Slovakia (2019); and Greece and Germany (2020) (Cwynar, Stojkov, and Wlazlak 2019; OIE 2022). Most recently, the virus has been detected on a farm in North Macedonia (2021) and in wild boar in the Piedmont and Liguria regions of northern Italy (2022) (OIE 2022).

Currently, ASF is present in domestic pig and wild boar populations across Eastern Europe. The most recent large-scale epidemiological analyses suggest that ASF is gaining ground in Bulgaria, Hungary, Poland, Romania, and Slovakia (Desmecht et al. 2021). Romania, in particular, reports particularly high numbers of outbreaks in smallholder farms (APHA Surveillance Intelligence Unit 2021). Meanwhile, the number of outbreaks and transmission levels appear to have stabilised in Latvia and Lithuania, and in Belgium and Greece, ASFV appears to be controlled (Desmecht et al. 2021). The Czech Republic and Belgium – both of which experienced ASF outbreaks only in wild boar, never in domestic pigs – issued self-declarations of ASFV-free status to the OIE in 2019 and 2020, respectively (Semerád 2019; Claeys 2020). In 2019, Estonia declared ASF-free status in domestic and captive wild pigs (Kalda 2019), though the virus persisted in wild boar. In July 2021, however, an Estonian domestic pig farm reported an ASF outbreak, ending nearly 4 years without ASF in the country's farms (Schulz et al. 2021). The recent discovery of ASF in wild boar (and later, domestic pigs) in Germany (Federal Ministry of Food and Agriculture 2021) also represents an intensified threat to the rest of Western Europe, and preventive surveillance and biosecurity measures are a high priority in disease-free nations (APHA Surveillance Intelligence Unit 2021).

In June 2018, the current ASFV pandemic spread to north-eastern China, emerging near Shenyang City at a farm where pigs had recently been fed table scraps (Zhou et al. 2018). The outbreak quickly spread to all mainland Chinese provinces and has had devastating socioeconomic consequences: the national pig herd was reduced by approximately 40% within 1 year of ASFV being introduced to the country (Gavier-Widén, Ståhl, and Dixon 2020). Further spread throughout Asia was seen as inevitable, and in 2019, the virus was reported in Mongolia, Vietnam, Cambodia, Hong Kong, North Korea, Laos, the Philippines, Myanmar, Indonesia, Timor-Leste, and South Korea (OIE 2022; Mighell and Ward 2021). This was followed by detection of introductions into India and Papua New Guinea in 2020, and into Malaysia, Bhutan, and Thailand in 2021.

Finally, ASF was reported in two Caribbean nations – the Dominican Republic and Haiti – in 2021, bringing the virus to the Western Hemisphere for the first time in nearly 40 years (Cole and Stepien 2021; Stepien and Cole 2021). These introductions mark a substantial geographical jump and highlight the risk of ASF introduction to mainland North America.

Epidemiology of the Current ASF Pandemic in Europe, Asia, and Africa

Though a relatively slowly mutating virus (Malogolovkin and Kolbasov 2019), ASFV has generated substantial genetic diversity within its natural African sylvatic cycle. Different strains of ASFV have historically been categorized into genotypes based on partial sequencing of the *p72/B646L* gene, which encodes the major viral capsid protein p72 (Netherton, Connell, et al. 2019). Based on this phylogeny, 22 separate genotypes of ASFV had been identified as of 2015: by early 2022, this number had risen to 26 as a result of the identification of two novel genotypes from Africa and two from Sardinia (Achenbach et al. 2017; Quembo et al. 2018; Ye et al. 2020). Only two of these genotypes have caused widespread outbreaks beyond Africa – genotype I, responsible for the 20th century outbreak, and genotype II, responsible for the current pandemic (ASF-STOP 2021). Though useful for epidemiology and evolutionary phylogenetics, these genotypes do not necessarily correlate with ASFV virulence or immune response (Gaudreault et al. 2020; Rock 2017). Different strains of ASFV cause substantially varying clinical presentations, ranging from peracute and acute infections with 90-100% mortality, to subacute and chronic forms that cause far lower rates of death (Sánchez-Cordón, Montoya, et al. 2018; Schulz, Conraths, et al. 2019; Gallardo et al. 2021; 2019; E. Sun, Zhang, et al. 2021).

Recent Findings – Europe

The ASFV strain introduced to Georgia in 2007 (Georgia 2007/1) is a highly virulent member of genotype II, and this strain and its derivatives are responsible for the current epidemics in Europe and Asia (Rock 2021). Since 2015, a substantial amount of research has focused on the epidemiological properties of the strains circulating in Eastern Europe, including their transmission properties, their maintenance within domestic pig and wild boar populations, and the routes by which they have spread.

Olesen *et al.* examined the transmission characteristics of POL/2015/Podlaskie/Lindholm, a Polish ASFV isolate obtained from an infected wild boar, finding that the virus was highly contagious and was efficiently transmitted to domestic pigs via aerosol or direct contact (Olesen et al. 2017). The course of disease was not identical in all infected pigs, with longer incubation and/or survival periods depending on the route of infection and, potentially, on inter-individual differences in virus susceptibility (Olesen et al. 2017). The researchers examined environmental infectivity of this isolate in a later study, finding that efficient transmission only occurred when pigs were exposed to contaminated surfaces $\leq$ 1 day post-contamination (Olesen, Lohse, Boklund, et al. 2018).

In neighbouring Russia, traditional backyard farms have been hit particularly hard by the ongoing ASF outbreak. The country's free-range farming systems have facilitated disease spread and transmission between domestic pigs and wild boar (Cwynar, Stojkov, and Wlazlak 2019), though a modelling and statistical analysis of ASF risk factors in the Samara Oblast (western Russia) did not find any significant spatiotemporal association between outbreaks in domestic pigs and those in wild boar (Glazunova et al. 2021). Human activity was instead the highest risk factor associated with domestic outbreaks, including transport of pigs/pig products from affected regions (Glazunova et al. 2021). Significant underreporting of wild and domestic ASF cases hampers epidemiological studies in Russia (Glazunova et al. 2021; Costard et al. 2015), where a shift in production systems from backyard farms to large commercial producers with improved biosecurity has allowed the pork industry to expand rapidly despite substantial losses to ASF (PW Reporters 2021)

In Estonia, two separate introductions brought ASFV to the southern and north-eastern regions of the country in 2014. Notably, these outbreaks exhibited different epidemiological properties: in the South, mortality in wild boar was high, and infected animals were typically positive for ASFV but negative for ASFV antibodies (seronegative); while in the Northeast, seropositivity was high in the wild boar population, and few deaths were reported (Nurmoja, Schulz, et al. 2017). Experimental infection with the north-eastern strain demonstrated 90% mortality in young wild boar, though high doses were required for oral infection and contagiousness was moderate (Nurmoja, Petrov, et al. 2017). These results led to the hypothesis that, rather than involving an attenuated ASFV strain, the north-eastern outbreak had simply begun earlier and gone undetected, allowing the local wild boar population to build up seropositivity over time. This was supported by analyses of the temporal trend of ASFV in Estonia, suggesting that the north-eastern introduction may have occurred several months before it was noticed and reported (Nurmoja, Schulz, et al. 2017; Schulz et al. 2021). Interestingly, later results from Zani *et al.* found that this Estonian strain, confirmed as highly virulent in wild boar, was survived by 75% of minipigs and 100% of domestic pigs under experimental conditions (Zani et al. 2018). Whole-genome alignment against Georgia 2007/1 revealed a deletion of ~14.6 kb from the 5' end of the genome, along with duplications of several genes including multigene family (MGF) 110 and 360 members. These MGF genes are responsible for relatively large inter-strain genomic variations (e.g. via duplication events) and have been implicated in critical functions in host immunomodulation that will be discussed in detail in later sections.

Since its introduction, ASFV in Estonia has evolved to moderate virulence strains, likely due to its maintenance within the wild boar population. Currently, infections range from acute to subclinical

forms of the disease, with recovered and potentially chronic animals reported (Gallardo, Nurmoja, et al. 2018; Gallardo et al. 2021). Nurmoja *et al.* later conducted a retrospective epidemiological analysis of 26 outbreaks in Estonian domestic pig herds between 2015 and 2017, finding that initial clinical signs were often mild and nonspecific despite the virulence of both Estonian isolates (Nurmoja et al. 2020). In contrast to results from Russia, proximity to ASF-infected wild boar populations was found to be the primary risk factor for farm infections in Estonia (Vilem et al. 2020). Further, there was not any difference in ASFV introduction risk observed between low-biosecurity backyard farms and moderate-biosecurity commercial farms, indicating that a higher biosecurity level does not always necessarily ensure a lower likelihood of ASFV introduction (Nurmoja et al. 2020).

A similar correlation between the occurrence of wild boar infections and domestic outbreaks was earlier observed in adjoining Latvia, where the southern Estonian ASFV strain likely originated (Nurmoja et al. 2020; Schulz et al. 2021). Here, ASFV continues to be maintained in wild boar, and recent domestic outbreaks have been linked to indirect contact with infected wild boar and/or a contaminated environment (Desmecht et al. 2021). Interestingly, research on Latvian ASFV isolates has suggested the emergence of mutated strains with substantially attenuated virulence. Gallardo *et al*. conducted experimental infections of domestic pigs with two Latvian strains, both isolated from wild boar: this revealed that the non-haemadsorbing (non-HAD) strain Lv17/WB/Rie1 caused only nonspecific symptoms or subclinical disease, thereby showing that circulating European ASFV strains are undergoing natural evolution which includes the emergence of attenuated forms over time. Interestingly, the Lv17/WB/Rie1 strain studied exhibited a point mutation in the *EP402R* gene that resulted in production of a truncated form of the viral CD2v protein (discussed in more detail in later sections).

In Poland, ASFV has been circulating since 2014, causing over 12,500 outbreaks in wild boar and 400 in domestic pigs (Woźniakowski, Pejsak, and Jabłoński 2021). These have occurred primarily in eastern Poland but have now spread to the west, likely as a result of human-mediated spread (Mazur-Panasiuk et al. 2020; Desmecht et al. 2021). Maintenance of the disease seems to depend on infected wild boar populations, as in other EU Member States (Bellini et al. 2021; Woźniakowski, Pejsak, and Jabłoński 2021), though definitive spatiotemporal correlations between wild boar density and domestic outbreaks have proven difficult to establish (discussed in more detail below) (Bellini et al. 2021; Podgórski and Śmietanka 2018; Jo and Gortázar 2021). Currently, ASF is continuing to gain ground in Poland, and only severe biosecurity measures have proven effective at limiting domestic outbreaks;

efforts to contain viral spread in wild boar have not thus far been successful (Woźniakowski, Pejsak, and Jabłoński 2021).

In neighbouring Germany, ASFV was first identified via passive surveillance of wild boar near the border with Poland in November 2020, though epidemiological study suggested that initial introduction may have occurred several months prior to detection (Sauter-Louis, Forth, et al. 2021). Since then, ASFV has continued to spread into the country, and outbreaks in domestic pig herds have been reported in Brandenburg (July 2021) and Mecklenburg-Western Pomerania (November 2021) (Federal Ministry of Food and Agriculture 2021).

Belgium, as mentioned above, appears to have eradicated ASFV after a total of 827 reported cases in wild boar (Dellicour et al. 2020). This required strict control measures, including a shutdown of activity (e.g. hunting) in the infected area, construction of a network of fences/barriers, and active surveillance for wild boar carcasses (Dellicour et al. 2020).

Romania, like Poland, appears to be experiencing an expanding ASF epidemic, with outbreaks linked particularly to human activities such as illegal movement of pigs, unsupervised slaughter of sick animals, and noncompliance with biosecurity measures (Desmecht et al. 2021). ASF in Romania appears to be dominated by domestic outbreaks at pig holdings (at various scales from rural smallholder farms to large commercial operations), suggesting that cases in wild boar may in fact be "spill-over" infections from domestic pigs (Sauter-Louis, Conraths, et al. 2021; Cwynar, Stojkov, and Wlazlak 2019). Comprehensive investigations of the risk factors driving Romanian ASF outbreaks have pointed to backyard holding populations, environmental characteristics (e.g. forests, rivers, and wetlands), and human activity, with the latter being particularly critical ("Land and water were identified as pivotal factors," noted Andraud *et al.*, "but they cannot hide the role of human activity") (Boklund et al. 2020; Andraud et al. 2021). The Danube River appears to drive ASF spread in Romania, consistent with its associated human population and transportation density (Andraud et al. 2021), though waterborne transmission cannot be excluded as a potential factor (Niederwerder et al. 2019). The epidemiological situation in neighbouring Bulgaria is similar to Romania, with an expanding ASF epidemic driven by human activity (Sauter-Louis, Conraths, et al. 2021; Desmecht et al. 2021).

Recent Findings – Asia and Africa

The first ASF outbreak in China was reported on August 3rd, 2018, when 400 pigs in a farm near Shenyang City (in the north-eastern Liaoning province) developed acute clinical disease after feeding on table scraps. Mortality was 100%, and so the farm was abandoned, but similar cases were soon observed in nearby farms (Zhou et al. 2018). By October 8th, ASF had spread to eight provinces and caused 33 outbreaks (T. Wang, Sun, and Qiu 2018). This isolate, dubbed ASFV-SY18, shares 100% nucleotide identity of the *p72/B646L* gene with Georgian, Russian, and Estonian isolates including Georgia 2007/1 (Zhou et al. 2018), while the complete genome shares the highest similarity with the Polish strain POL/2015/Podlaskie (Bao et al. 2019). The virus has since spread across Asia, largely in a north-east-to-south-east direction, via local movements combined with ineffective biosecurity (Mighell and Ward 2021). Early transmissions were attributable to human activity (including transport of infected pigs, products, and fomites), while later events have seen a higher-density of infections and have likely included direct contact spread (Mighell and Ward 2021).

Rapid economic growth in China has led to a high density of pigs being kept over large contiguous areas. A complex and densely interconnected pork industry has developed very quickly, with relatively little input from regulatory agencies and government authorities in rural areas (Dixon et al. 2020), creating a challenging epidemiological environment. Small farms (< 500 pigs in total) comprise more than 95% of the Chinese pig farming industry: these farms are often associated with poor sanitation, especially as untreated swill is often fed to pigs (You et al. 2021). From a biosecurity standpoint, small farms provide very little resistance to ASF introduction and spread; from a surveillance standpoint, their vulnerability and rural nature mean that virulent ASF outbreaks may completely destroy small farms, rendering accurate disease tracking and the generation of meaningful statistics difficult (You et al. 2021). The epidemiological situation in China thus remains complicated and uncertain, with new cases regularly reported (FAO 2022).

Various ASFV strains have spread to every mainland Chinese province over the past 3.5 years, massively impacting the national pork industry (Gavier-Widén, Ståhl, and Dixon 2020). As mentioned, molecular characterization of the initial SY18 strain revealed high homology with Georgia 2007/1, with an additional 10-bp insertion between the *I73R* and *I329L* genes that was identical to several strains circulating in Eastern Europe (Ge et al. 2018). Sun *et al.* reported their surveillance results on 22 ASFV field isolates from seven Chinese provinces between June and December of 2020 (E. Sun, Zhang, et al. 2021). Eleven of these isolates were non-HAD with *EP402R* mutations, and experimental infections with two of these strains (HLJ/HRB1/20 and HeB/Q3/20) caused non-lethal, persistent disease after low-dose infection. These naturally attenuated strains remained highly transmissible (E. Sun, Zhang,

et al. 2021). Further research from this group identified two field isolates (HeN/ZZ-P1/21 and SD/DY-I/21) from the Henan and Shandong provinces as belonging to ASFV p72 genotype I, with moderate virulence and efficient transmissibility (E. Sun, Huang, et al. 2021). Both viruses were similar to the Portuguese NH/P68 and OURT88/3 strains from the 20th century epidemic (E. Sun, Huang, et al. 2021). These findings complicate the Asian ASFV epidemiological landscape, highlighting the probability of harder-to-detect infections and raising questions about the evolution and introductions of ASFV in China.

Fast economic growth in Vietnam has also driven a sharp increase in the number of small farms without a concurrent improvement in farm biosecurity and hygiene, raising the risk of disease spread nationwide (Dixon et al. 2020). The first outbreak of ASFV in Vietnam occurred in 2019 at a family-owned backyard farm ~150 miles from the Chinese border, in a region where illegal movement of animals and meat products is common (Le et al. 2019). Partial genome sequences (including the *p72* gene) showed 100% homology with strains SY18 and Georgia 2007/1 (Le et al. 2019). Within a year after the initial outbreak, ASF had spread to 63 provinces and cities, resulting in the culling of ~6 million pigs (Pham et al. 2021). More recently, Nguyen *et al.* found that three different genotype II variants are currently circulating in Vietnam (Nguyen et al. 2022).

Elsewhere in Asia, ASFV continues to circulate in wild boar and on domestic farms. As discussed above, ASFV swept rapidly across Southeast Asia subsequent to its introduction into China, with the first outbreaks reported in Mongolia, Vietnam, Cambodia, Hong Kong, Laos, the Philippines, Myanmar, Indonesia, Timor-Leste, and the Korea Peninsula within a year (Mighell and Ward 2021). ASFV was likely introduced to South Korea via human activity, and large (>~25 miles) geographical jumps in the current transmission pattern indicate that human-mediated spread is continuing (Jo and Gortázar 2020). India, Papua New Guinea, Malaysia, Bhutan, and Thailand have also reported the introduction of ASFV within the past 2 years. Cambodia, Mongolia, Myanmar, and Hong Kong controlled small initial outbreaks (Mighell and Ward 2021), but new infections have been detected in Myanmar (OIE-WAHIS, evt_3751) and very recently in Hong Kong (FAO 2022). In addition, media reports suggest that the virus is still present in Cambodia (Bacigalupo, Perrin, and Pacey 2022). Information on Asian ASFV 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 urgently needed to track ongoing outbreaks and anticipate continuing spread.

In Africa, ASFV continues to spread to new environments and pig populations far beyond its region of origin in East Africa. Outbreaks have occurred from transmission between domestic pigs (Penrith et al. 2019) and from the sylvatic cycle, with warthogs being translocated into the south of the continent over the past 4 decades (Craig et al. 2021). As of 2017, 33 African countries had experienced ASF outbreaks (Penrith et al. 2019), and this number has continued to grow. Since 2015, outbreaks have been specifically reported to the OIE in Cabo Verde, Côte d'Ivoire, Zimbabwe, Kenya, the Central African Republic, Mali, Burundi, South Africa, Zambia, Chad, Sierra Leone, Namibia, Nigeria, and Tanzania (Penrith 2020; OIE 2022). Moreover, cross-border spread of ASF appears to be common: Hakizimana *et al.*, for instance, systematically reviewed ASFV transmission between Tanzania and nearby countries, finding that the virus had likely been introduced to Tanzania via multiple routes involving Kenya, Mozambique, Zambia, and Malawi (Hakizimana, Yona, et al. 2021). The researchers also found evidence for viral spread from Tanzania to Burundi (Hakizimana, Yona, et al. 2021).

The ongoing outbreak in Nigeria that began in 2020 has been particularly devastating, with nearly 1 million pigs culled within a month of the first reported cases (Sunday 2020). Adedeji *et al.* linked this outbreak to a genotype II strain, based on sequencing of the *p72/B646L* and *p54/E183L* genes, marking the first time that this genotype has been detected in Nigeria (Adedeji et al. 2021). Awosanya *et al.* also identified a new genotype I strain that is circulating in southwestern Nigeria; this strain seems to share ancestry with isolates from Spain and Brazil while showing mutations that may have decreased its morbidity and mortality (Awosanya et al. 2021). Meanwhile, in South Africa, the outbreak with the highest mortality occurred in 2016 in the Free State province (Mushagalusa, Etter, and Penrith 2021). Overall, the epidemiology of ASF in Africa remains unpredictable and difficult to control, and substantial gaps remain in our understanding of disease transmission patterns, risk factors, and viral evolution within this continent (Njau, Machuka, et al. 2021; Penrith et al. 2019; Penrith and Kivaria 2022).

Modes, Routes, and Drivers of ASFV Transmission

ASFV is thought to be maintained through four transmission cycles: (1) the sylvatic cycle between warthogs and soft ticks; (2) environmental transmission from wild boar carcasses to susceptible swine; (3) transmission between soft ticks and domestic pigs; and (4) transmission within domestic pig populations (Chenais et al. 2018; Dixon et al. 2020; Netherton, Connell, et al. 2019). These cycles may be interconnected to varying degrees depending on environmental and epidemiological factors (e.g. wild boar population movements, the degree of interaction between wild boar and domestic pigs, and

the presence of competent arthropod vectors), adding complexity to the daunting task of estimating likely ASFV transmission patterns and disease spread (Dixon et al. 2020; Brown and Bevins 2018). ASFV transmission dynamics are largely driven by domestic pigs in the pork production industry (Dixon et al. 2020). Human activity has a significant or even primary role in maintaining this cycle and long-distance transmission events (Busch et al. 2021; Chenais et al. 2019).

ASFV exhibits complex transmission dynamics in the field that make it difficult to generalize the epidemiological lessons learned from specific outbreaks or geographical locations. After the initial Georgian ASF outbreak in 2007, the virus's high virulence suggested that it might be self-limiting within pig populations (Schulz, Conraths, et al. 2019), but this has not been the case since. The environmental tenacity of ASFV (perhaps even in the carcasses of deceased animals) plays an important role in its persistence, particularly in wild boar populations, and increases the ease with which humans can unwittingly transfer the virus across distances (Chenais et al. 2019; Schulz, Conraths, et al. 2019).

ASFV has historically been viewed as a highly contagious disease, but recent studies have suggested that this is only true in certain situations – for instance, where substantial environmental contamination remains on domestic farms on which an ASF outbreak has not been adequately or efficiently controlled (Chenais et al. 2019). In the field, ASFV transmission between animals is often a slow process, with viral infectiousness depending on many factors including the virus strain, the infectious medium, and the route of transmission (Schulz, Conraths, et al. 2019; Pikalo et al. 2019). For instance, studies of ASFV's basic reproductive number (R_0) in field and experimental settings have returned values ranging from 0.5 to 18 depending on viral, host, and environmental parameters (Schulz, Conraths, et al. 2019). Below we discuss some of the most critical studies from the past 6 years on ASFV transmission drivers and pathways – these include wild boar, the potential role of survivors/carriers, environmental transmission, arthropods, and human activity.

Wild boar

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 circumstances is unclear as substantial knowledge gaps persist (Bellini et al. 2021). ASF epidemiology in wild boar is particularly important for biosecurity, as ASF control measures can have unintended impacts on the wild populations. Increasing hunting in response to an ASF outbreak, for instance, can lead to compensatory population growth and can disperse wild boar over a wider area (Gavier-Widén, Ståhl, and Dixon 2020; Desmecht et al. 2021). Over the past 6 years, many researchers have examined different aspects of ASF epidemiology in wild boar, including: viral maintenance in their populations, the potential correlation between wild boar density and ASF transmission risk, the amount of interaction between wild boars and domestic pigs, and the roles played by wild boar in environmental transmission. These studies have largely concentrated on Europe, as information about wild boar populations (and the epidemiology of ASFV in general) in Asia is limited (Gavier-Widén, Ståhl, and Dixon 2020).

Taylor *et al.* used European case data from 2018 to create a risk assessment framework, finding that the movement of wild boar was the highest-risk pathway for ASFV transmission to domestic pigs in Eastern Europe (Taylor et al. 2020). Similarly, in 2017, Bosch *et al.* estimated the risk of ASF introduction via wild boar into (at the time) disease-free European countries (Bosch et al. 2017). In their model, the highest risks were calculated for Slovakia, Romania, Finland, the Czech Republic, and Germany – a prediction now known to be 80% accurate. Interestingly, the most important risk factor identified in Bosch *et al.*'s model was the presence, not the density, of wild boar (i.e. large, dense populations of boar are not required for ASF risk) (Bosch et al. 2017).

The correlations between wild boar population density and routes of disease spread are inconsistent, although the finding and safe disposal of carcasses are considered to be critical biosecurity measures for ASF control (Bellini et al. 2021). Desmecht *et al.* modelled risk factors for ASF detection in wild boar within Romanian hunting grounds, finding that environmental factors, wild boar abundance, and the density of backyard pigs within the hunting ground were the primary risk factors (Desmecht et al. 2021). In Romania, Boklund *et al.* found that nearby wild boar abundance and proximity to wild boar cases were significant risk factors for backyard farms (Boklund et al. 2020).

In 2018, a study of ASF dynamics in north-eastern Poland showed that three independent domestic outbreaks had occurred in this region between 2014 and 2015, via transmission from wild boar. Wild

boar movements, however, were a poor spatiotemporal predictor of ASF dynamics, suggesting that wild boar social structure, a short duration of viral shedding, and ASF's high lethality may have instead contributed to this finding (Podgórski and Śmietanka 2018). In Russia, Glazunova *et al.* reported no significant colocation between domestic pig and wild boar outbreaks (though underreporting of wild boar cases is a potential confounding factor) (Glazunova et al. 2021). The role of wild boar in ASFV transmission is especially important in countries where free-ranging domestic pig populations are present. Cadenas-Fernández *et al.* used camera traps to estimate the level of interaction between wild boar and free-ranging pigs in Sardinia, and detected numerous daily interactions (particularly at water sources) (Cadenas-Fernández et al. 2019).

Estimates of the speed at which ASF naturally spreads within wild boar populations are generally comparable [e.g. ~2.9-11.7 km/year (Desmecht et al. 2021) or up to ~1-5 km/month (Gavier-Widén, Ståhl, and Dixon 2020; Dixon et al. 2020)]. Environmental factors can impact this rate, as seen in Belgium where the velocity of the ASFV wavefront is slower outside forested areas (Dellicour et al. 2020). Models of ASFV transmission in wild boar suggest faster spread in the winter months, when cold temperatures increase the environmental stability of the virus (Schulz, Conraths, et al. 2019). Epidemiological data from EU Member States support this pattern of seasonality, although differences are observed between countries (Desmecht et al. 2021). Proposed causes for such differences include variations in wild boar population distribution, human farming and recreational activities, and temperature/climatic factors (Chenais et al. 2019).

In 2020, O'Neill *et al.* derived a mathematical model of ASF in European wild boar to explain the observed epidemiological pattern of ASFV in wild boar (initial population crash followed by long-term, low-prevalence persistence in low-density hosts) that does not exhibit the expected self-limiting trajectory of a highly virulent disease (O'Neill et al. 2020). They found that direct, environmental, and survivor-based infections (transmission from wild boars who survive ASF and subsequently shed virus during a resurgence of viraemia) were all necessary to capture this phenomenon in their model (O'Neill et al. 2020).

Chronic disease and long-term carriers

The epidemiological significance of survivor/carrier animals is controversial and has received substantial attention over the past 6 years. Such animals would necessarily be difficult to identify and track. Moreover, the circumstances capable of producing survivors with chronic ASF infection may

vary depending on virus strain, individual animal parameters, and other epidemiological factors (Schulz, Staubach, et al. 2019). In the case of O'Neill *et al.*'s model of ASF persistence in wild boar (described above), the inclusion of a low rate of transmission from survivor animals was necessary to match observed outbreak data. As such, more detailed analyses of survivor animals in ASF epidemiology are needed (O'Neill et al. 2020).

In 2015, Gallardo *et al.* experimentally infected domestic pigs with the NH/P68 isolate and showed that in-contact pigs became seropositive with transient viraemia at 28 days post-exposure (Gallardo, Soler, et al. 2015). The clinical signs in these pigs were minimal, suggesting that such infections could escape notice under field conditions. These results were in line with previous studies from the 20th century epidemic in the Iberian Peninsula, demonstrating persistent infection in tissues by moderately virulent strains (Gallardo, Soler, et al. 2015). Later that year, Pietschmann *et al.* examined the risk of chronic disease and the establishment of carriers by experimentally infecting domestic pigs and European wild boar via the oronasal route with very low doses of the highly virulent strain Armenia08 (Pietschmann et al. 2015). The low dose regimen, however, led to detectable infection only in the weakest animals in each group, which then showed a typical onset, course, and disease outcome (Pietschmann et al. 2015). The question of whether there may be a prolonged or chronic disease course in domestic or wild animals under some circumstances thus remains open.

In 2019, Eblé *et al.* studied pigs experimentally infected with the moderately virulent ASFV strain Netherlands '86, looking for transmission from recovered pigs to naïve animals via direct contact (Eblé et al. 2019). The researchers observed direct contact transmission from clinically healthy survivor pigs to 2/12 naïve contact pigs, corresponding to a contribution of 0.3 to the virus's R_0 (Eblé et al. 2019).

In the same year, Ståhl *et al.* conducted a systematic review to resolve definitional uncertainties on the nature of carrier animals and to assess their potential role in ASFV epidemiology (Ståhl et al. 2019). They found that, 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. Overall, no link between ASF epidemiology and viral shedding by healthy 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).

ASFV tenacity and environmental transmission

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). The Georgia 2007/1 strain can persist in the faeces and urine of domestic pigs for ~8.5 and 13 days, respectively, at 4°C (Davies et al. 2017), although some have suggested lower stability in these matrices (Sauter-Louis, Conraths, et al. 2021). Notably, the persistence of ASFV in faeces is affected by bacterially produced enzymes present in this matrix, making it difficult to directly compare results obtained under field and laboratory conditions (EFSA et al. 2018). These features have consequences on ASFV's ability to remain within populations and geographical regions, potentially helping to maintain ongoing outbreaks and contributing to its long-term persistence in low-prevalence environments (Chenais et al. 2019; Busch et al. 2021).

Niederwerder *et al.* conducted a study of the infectious dose of the Georgia 2007/1 strain via oral exposure to pigs during natural drinking and feeding behaviours. The medium dose of infection via liquid intake was very low – 10 $TCID_{50}$ (50% Tissue Culture Infectious Doses) – likely due to viral contact with the tonsils. Meanwhile, the median dose of infection via plant-based feed was much higher, at $10^{6.8}$ $TCID_{50}$ (Niederwerder et al. 2019). These findings have relevance for the environmental epidemiology of ASFV, partly explaining the role of the Danube River in ASF outbreaks in Romania (Andraud et al. 2021). Despite the low infectious dose in liquid, Niederwerder *et al.* hypothesize that feed poses a higher risk of ASFV introduction compared to water sources due to the high-frequency, highly centralized nature of feed production and administration (Niederwerder et al. 2019).

In 2018, Chenais *et al.* defined an epidemiological pattern they called the "wild boar-habitat cycle," wherein wild boar transmit ASFV directly between animals and indirectly through carcasses (Chenais et al. 2018). Such indirect transmission could lead to low- or high-dose infections depending on the environmental parameters and the extent of carcass decomposition/degradation (Chenais et al. 2018). Colder environments translate to slower decomposition rates, with consequences for the persistence of ASFV in areas of Eastern Europe (O'Neill et al. 2020; Podgórski and Śmietanka 2018). Depending on farm management systems, environmental contamination by wild boar carcasses can also increase the risk of infection in domestic pigs (Dixon et al. 2020).

In 2017, Probst *et al.* conducted a behavioural study of wild boar near their dead fellows, and found that they displayed interest in and contact with the carcasses and the surrounding soil (Probst et al.

2017). Even if per-contact likelihood of infection in such cases is low, the prolonged survival of ASFV in the environment could raise the overall probability of transmission (Chenais et al. 2019). Findings from a recent study of ASF-infected carcasses in Lithuania, however, showed that no infectious virus could be isolated from 20 wild boar carcasses at various decomposition stages (Zani et al. 2020). The researchers hypothesized that differences in climate might impact on the persistence of infectious virus in wild boar carcasses (Zani et al. 2020). Further analyses are needed to separate the various environmental factors that impact ASFV survival in boar carcasses and allow a clearer understanding of the role of the wild boar-habitat cycle in long-term persistence of the current ASFV pandemic.

Arthropods

Ornithodoros soft tick species form half of ASFV's ancestral 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 Africa, as discussed above, 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; ASF-STOP 2021). 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).

The Georgia 2007/1 strain is capable of replicating in *O. erraticus* ticks, where it is maintained for at least 12 weeks, though transmission has not been observed (Guinat et al. 2016). In 2019, de Oliveira *et al.* compared the ability of three soft tick species – the Palearctic *O. erraticus* and *O. verrocosus* and the traditional Afrotropical *O. moubata* – to transmit virulent ASFV. They found that the Palearctic ticks were unable to transmit Eurasian ASFV strains (including Georgia 2007/1 and Ukraine2012 from the current outbreak), though successful infection of pigs via intramuscular (IM) inoculation with crushed tick homogenate indicated that the ticks maintained infectious virus for some time (de Oliveira et al. 2019). In Central Europe and the Baltic States, hard ticks are the major species, with soft ticks nearly absent (Bellini et al. 2021). In 2021, Herm *et al.* examined the presence of ASFV DNA in

hard ticks (Ixodidae family) from wild boar habitats on the Estonian island of Saaremaa. They found no viral DNA in any tick (despite swine DNA present in ~20% of samples), indicating that hard ticks are unlikely to serve as an epidemiologically relevant reservoir of ASFV (Herm et al. 2021). Recent results from China, however, have indicated the presence of ASFV DNA and transovarian transmission of ASFV within *Dermacentor silvarum* hard ticks (Z. Chen et al. 2019).

Herm *et al.* studied other arthropods in addition to hard ticks, including mosquitoes, biting midges, and tabanids), and found no ASFV DNA in any of these species (Herm et al. 2021). The possible role of non-tick arthropods in ASFV epidemiology has attracted substantial interest over the past 6 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, Lohse, Hansen, et al. 2018; Fila and Woźniakowski 2020). In the first study, the researchers found that ingestion of ASF-carrying flies (previously fed with blood containing 5.8 log_{10} $TCID_{50}$/mL of ASFV, a physiologically realistic titre) resulted in clinical disease in 25-50% of tested Danish domestic pigs (Olesen, Lohse, Hansen, et al. 2018). In the second, they showed that the flies are capable of carrying the infectious virus for at least 12 hours post-feeding (Olesen, Hansen, et al. 2018). 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 Woźniakowski 2020; Blome, Franzke, and Beer 2020). A 2021 study by Yoon *et al.*, for instance, examined nearly 29,000 arthropods (99.5% Diptera flies) from 14 South Korean pig farms experiencing ASF outbreaks – all were negative for ASFV DNA (Yoon et al. 2021).

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).

Anthropogenic factors

ASFV likely evolved in its ancestral habitat in Africa for ~300 years (ASF-STOP 2021), but the interjection of human activity into the sylvatic cycle resulted in the spread of ASFV to new

environments and naïve Suidae. The British colonization of Kenya in the late 1800s involved a massive influx of domestic pigs, and it was in these pigs that ASF was first described as a highly virulent and contagious haemorrhagic disease (Alkhamis et al. 2018). Today, human activity remains one of the most notable drivers of the continuing transmission of ASF (Bellini et al. 2021). These human activities include the legal and illegal transport of pigs and pig products, inadequate biosecurity on pig holdings, swill feeding of domestic pigs, and noncompliance with hunting restrictions and other control measures during outbreaks (Bellini et al. 2021; Sauter-Louis, Conraths, et al. 2021). In Europe, anthropogenic factors are considered to be the primary cause of long-distance transmission events and introductions into domestic pig farms (Schulz, Conraths, et al. 2019). A spatiotemporal modelling study of ASF outbreaks in Romania found that markers of human activity (population density, household density, and roads) were the main risk factor for the spread of ASF (Andraud et al. 2021). In Romania, meanwhile, visits from professionals working on farms were a significant risk factor for backyard farms during outbreaks (Boklund et al. 2020).

Anthropogenic factors can also interfere with studies of seemingly unrelated environmental factors. In Russia, for instance, the geographic density of bodies of water has been linked to increased ASF risk, hypothetically because of wild boar movements. Conversely, variables related to human activity are at least as likely to be the major contributing factor (Andraud et al. 2021). In neighbouring Estonia, 81% of domestic outbreaks between 2015 and 2017 were detected during July and August. One plausible explanation for this seasonality is the increased level of contact between farm workers and wild boar in the local environment (Nurmoja et al. 2020).

Feeding of pigs with contaminated table scraps was associated with the initial outbreak of ASF in China (Zhou et al. 2018), while contaminated pork products were likely responsible for the first outbreaks in Vietnam (Le et al. 2019). Anthropogenic factors have continued to play a major role in the transmission and maintenance of the disease in Asia (Jo and Gortázar 2020).

Transport of ASFV-contaminated feed is another possible route by which ASF can cross national barriers and oceans. A swine feed matrix stabilized the Georgia 2007/1 strain under simulated 30-day transoceanic shipping conditions (Stoian et al. 2019), and recent transboundary ASF outbreaks have been linked to ships' galley waste that was dumped into open landfills and left available to free-ranging pigs (Penrith, Bastos, and Chenais 2021). Similarly, the 2020 European risk assessment by Taylor *et al.* identified the legal trade of pigs as a major risk factor for introduction of ASF into Western Europe (Taylor et al. 2020). Indeed, Taylor *et al.* noted that "all risk assessments struggle to estimate the risk

by pathways which are very stochastic in nature, usually due to human behaviour" (Taylor et al. 2020). Over the past 6 years, the importance of human behaviour and decision-making to the epidemiology of ASFV has become increasing well-recognized (Penrith, Bastos, and Chenais 2021). Disease control measures are only as effective as the people implementing them, and the reasons underlying noncompliance with biosecurity regulations and farming best practices are often strongly rooted in long-standing socioeconomic and cultural realities. In Sardinia, for instance, where ASF has been endemic since the 20th century outbreak, farmers keep herds of unregistered free-ranging pigs despite the banning of free-range farming in 2012. This practice is entrenched in tradition and the economic situation of Sardinian farmers, and it unfortunately ensures regular contact between free-ranging pigs and potentially ASF-infected wild boars (Cadenas-Fernández et al. 2019).

Similar situations are common in Africa. Africa's pig population has doubled over the past 30 years, coinciding with an increase in the number of affected countries and outbreaks therein (Penrith et al. 2019). Tracking and surveying ASF in Africa is challenging, as underreporting ASF is very common due to multiple factors including poor communication channels and socioeconomic pressures on rural pig farmers. Regardless, it is clear that domestic pigs and human activity are the primary drivers in the current epidemiology of ASF in Africa – between 1989 and 2017, for instance, 88.5% of outbreaks in confirmed sources originated in domestic pigs (Penrith et al. 2019). Even in countries where the sylvatic cycle is well-established, domestic outbreaks have occurred, demonstrating that ASFV in Africa no longer requires warthogs and ticks for transmission (Penrith et al. 2019). Live pig markets have recently been identified as critical points of ASFV transmission in Nigeria (Adedeji et al. 2022). Emergency sales, where pigs suspected of having ASF are immediately sold to limit economic consequences, are also common in some regions (Bellini et al. 2021).

In 2015, Costard *et al.* examined and mathematically modelled the risk of ASFV release via such emergency sales. They found the risk to be high, and improving farmers' clinical diagnostic abilities did not effectively reduce it due to the relatively long incubation period of ASFV (infected animals are not necessarily symptomatic at the time of sale) (Costard et al. 2015). The researchers also emphasized the importance of ground-level socioeconomic factors on the spread of ASFV, particularly within resource-poor populations. In Russia, for instance, insufficient compensation to affected farmers leads to significant underreporting and illegal disposal or slaughtering of infected animals – the consequences of reporting ASF outbreaks are simply viewed as too costly (Costard et al. 2015). The developing field of participatory epidemiology, wherein disease control and outbreak response measures are designed and implemented in close coordination with local farmers, veterinarians, and

leaders, is a response to the growing understanding that "control measures, performing well in models or in theory, [are] useless without the support of relevant stakeholders" (Sauter-Louis, Conraths, et al. 2021). Such participatory approaches will be discussed in more detail in the Biosecurity section below.

Ongoing research

Below is a discussion of some of the many current and planned projects in the field of ASFV epidemiology. This section is not a comprehensive discussion of research in this field but provides a brief overview of selected research projects based on feedback from researchers surveyed during the writing of this report.

At the Food and Agriculture Organization (FAO) of the United Nations, researchers are examining the pig sector in North Macedonia, creating a computational model of ASF spread based on pig density and live pig movements to inform future risk assessments and modelling efforts (particularly critical after the December 2021 introduction of ASF to this country). Other studies at the FAO include an epidemiological investigation and risk assessment of ASF on hunting grounds in Kosovo, which incorporates a feasibility study to estimate the difficulty of implementing specific control measures in this environment.

In Madrid, at the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA, part of the Spanish National Research Council), the Centro de Investigación en Sanidad Animal (CISA) is involved in the One Health European Joint Programme's TELE-Vir project. Here, researchers are developing a point-of-incidence toolbox to rapidly identify and characterize emerging ASFV isolates via third-generation sequencing (ONT MinION) and existing bioinformatics platforms for data analysis and dissemination. Meanwhile, at the CIRAD in France, scientists are studying several aspects of ASF epidemiology within bushpig (*Potamochoerus larvatus*) populations in Southern Africa and Madagascar, including disease prevalence, interactions with domestic pigs, and genetics/hybridization between bushpigs and domestic pigs in different tropical settings.

At the University of Ibadan in Nigeria, researchers are investigating the source and subsequent spread of a major ASF outbreak that occurred in 2020 in the city of Lagos. Scientists at the Friedrich-Loeffler-Institut (FLI), meanwhile, are conducting epidemiological investigations of ASF outbreaks; these include several studies focused on wild boar, including: (a) determination of the post-mortem interval in wild boar; (b) spatiotemporal epidemiology, including reliable determination of wild boar

population densities; and (c) prediction of the epidemiological course of ASF in wild boar in Europe. FLI researchers are also studying the prevention and control of ASF in the backyard sector, considering the virus's unique biological characteristics (low contagiousness, high lethality, and high tenacity) and their implications in this environment. This research track also involves the evaluation of diagnostic and surveillance tools, with an emphasis on the socioeconomic circumstances of backyard holders.

At the Estonian University of Life Sciences (EMÜ), research is ongoing into the molecular characterization of Estonian ASFV strains and the potential roles of blood-sucking insects in the mechanical transmission of the virus. Finally, at the Federal Research Center for Virology and Microbiology (FRCVM) in Volginsky, an upcoming research project will model the spread of ASF in the Russian Federation via two primary routes: (1) predicting risk factors (e.g. for ASFV introduction, transmission, and endemicity) via spatial epidemiology approaches, with regard to relevant socioeconomic and ecological factors; and (2) clarifying the role of wild boar in this process and assessing wild boar population density thresholds for disease transmission (including analysis of existing methods for estimating wild boar density).

Future research priorities

Based on the above literature review and with reference to previously identified knowledge gaps and expert opinion, the following areas of ASF epidemiology should be considered priorities for future research:

- *Epidemiology of the global spread of genotype II ASFV, including ecology and evolution*
- *Routes and patterns of ASFV introduction into unaffected regions*
- *Dynamics of ASFV transmission in various population settings in wild and domestic Suidae*
- *Impact of environmental and climatic factors on wild boar populations and ASFV transmission*
- *Increasing knowledge of ASFV survival and transmission in different epidemiological settings including Eastern Europe, Western Europe, Southeast Asia, and Africa*
- *Role of the wild boar-habitat cycle in ASF-affected regions*
- *Propagation pathways of ASFV between wild and domestic herds (e.g. live pig transport, possible airborne or arthropod-mediated transmission, etc.)*
- *Potential role of symptomatic or asymptomatic carriers in ASF maintenance*
- *Sequencing and phylogenetic analysis of circulating Eurasian and African strains*
- *Harmonization of phylogenetic markers*

- *ASFV molecular epidemiology in domestic and wild Suidae*
- *Soft tick distribution in Europe and Asia*
- *Roles of arthropods (including flies) in ASFV transmission*
- *Risk factors for long-distance human-mediated ASFV spread (e.g. to the USA or Western Europe)*
- *Direct and indirect costs of ASF outbreaks and disease control*
- *Social and behavioural determinants of ASFV transmission in developing economies*
- *Human-animal interface studies at key sites, including social and behavioural sciences*
- *Deep studies of ASFV evolution related to pathogenicity*
- *Epidemiological gap-filling in under-surveyed regions (e.g. Russia, rural China, and Africa)*
- *Standardization and harmonization of ASFV epidemiology studies*
- *Rapid characterization of circulating ASFV in new outbreaks*

Virology/Molecular Biology

ASFV is a large icosahedral DNA virus with an average diameter of 200 nm. 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). The major ASFV capsid protein is p72 (encoded by the *B646L* gene), and genotyping of ASFV has historically been based on sequencing of a variable region within the C-terminal end region of this gene (Bastos et al. 2003; Gaudreault et al. 2020). 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).

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.

Previously identified knowledge 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*

Literature review

Recent Advances in ASFV Virology

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). Zhu *et al.* recently classified the MGF proteins into 31 groups based on protein sequence homology, followed by *in silico* investigation of their structure, function, and evolution. They found that MGF proteins within the same family tend to share similar structures and predicted functions, with ASFV more likely to lose MGF proteins than gain them during evolution. The researchers also established a web server for classifying MGF proteins (Z. Zhu et al. 2021).

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. 2019). 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. 2020). This unique, complicated structure reflects the complexity of the ASFV cell infection pathway (Andrés et al. 2020). 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. 2019).

Smaller-scale studies can also highlight the functions and biomechanics of specific ASFV proteins of interest. 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. 2020).

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. 2020). 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. 2020).

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. 2020).

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. 2020).

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. Gladue 2021). Below, 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 coding

sequence (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. 2020). 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. 2020). 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 ~3.20×10^{-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). 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).

Future research priorities

Based on the above literature review and with reference to previously identified knowledge gaps and expert opinion, the following areas of ASF virology should be considered priorities for future research:

- *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*

- *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*
- *Validation of historical ASFV genome sequences to remove possible artefacts.*

Pathogenesis

ASF is generally characterized by a severe clinical symptoms and high mortality, with the course of the disease depending on numerous factors including host immune responses and the virulence of the infecting viral strain. Highly virulent strains generally cause acute or peracute infections, which can be deadly as soon as 1-4 days post-infection. For instance, the virulent HLJ/18 strain, isolated from the spleen of an infected pig early in the Chinese ASF outbreak, exhibits an incubation period of 3-5 days in inoculated pigs, with a total time-to-death of $\leq$ 9 days (Zhao et al. 2019). By contrast, infection with low- and moderate-virulence strains is associated with subacute disease (similar to acute disease, but with less marked clinical signs and a longer time-to-death) and chronic disease (first observed in Iberia during the 20th century pandemic and of uncertain epidemiological relevance today) (Sánchez-Vizcaíno et al. 2015). Particularly important among the characteristic symptoms of ASF are (1) haemorrhages in multiple organs and (2) general immunodeficiency associated with a significant die-off in B and T lymphocytes and macrophages, thought to be caused by a proinflammatory cytokine storm associated with early-stage ASFV infection (Salguero 2020). Identifying the viral genes and proteins responsible for these differences in ASFV virulence, and the pathways by which the virus interacts with infected monocytes/macrophages and leads to lymphopenia, is critical for a deeper understanding of ASFV's biology and potential routes of control, treatment, and vaccination. Since 2015, substantial research efforts have focused on clarifying the pathogenesis of ASFV and the determinants of virulence and host immune responses.

Previously identified knowledge gaps

Previous 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*

Literature review

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 (Rock 2017). Studies 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. 2017). 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. 2020). 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. 2021). 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. 2021). 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. 2017). 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 below.

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.

Ongoing research

Below is a discussion of some of the many ongoing and planned projects in the field of ASFV pathogenesis. This section is not a comprehensive discussion of research in this field but is instead intended to provide a brief overview of selected research projects based on feedback from a survey of researchers conducted during the writing of this report.

At INIA, researchers are conducting several studies into host-virus interactions and the mechanisms of viral infection and replication. Among these is the African swine fever virus Interactome project (ASFVInt), which aims to identify the molecular mechanisms by which ASFV controls cellular signalling pathways during infection. Other research projects at INIA include an investigation of endosomal molecules involved in viral entry/fusion and a proteomic analysis of uncharacterized ASFV genes, which focuses on viral processes/strategies that ASFV may share with other enveloped viruses.

At the FRCVM, scientists are conducting a comprehensive study of the comparative and functional genomics of ASFV. Involved in this project is an analysis of genome sequences from different

spatiotemporal origins, with a primary goal of determining the role of individual genomic elements in the adaptive and evolutionary variability of this virus.

Future research priorities

Based on the above literature review and with reference to previously identified knowledge gaps and expert opinion, the following areas of ASF pathogenesis should be considered priorities for future research:

- *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*

Immunology

Antiviral immunity against ASFV involves both innate and adaptive responses and cell populations, including the monocyte/macrophages that the virus preferentially infects. Innate immunity to viral infection typically begins with ligation of intracellular pattern recognition receptors (PRRs) that bind to specific virus-associated molecular patterns (e.g. ASFV's cytoplasmic double-stranded DNA genome) and activate antiviral signalling cascades. The subsequent immune response can involve production of proinflammatory cytokines and chemokines, apoptosis, production of antibodies by B cells, and the recruitment of cytotoxic T cells to identify and destroy infected cells. Many viral pathogens evolve mechanisms by which to inhibit or avoid these immune responses, and ASFV is no exception.

However, as with many other aspects of ASFV's biology, we are still a long way from a complete picture of the interactions between the virus and the host immune response during infection. ASFV encodes a complex constellation of proteins that inhibit innate and adaptive immunity, including type I interferon inhibitors, regulators of MHC protein expression, and mediators of inflammatory cytokine production, autophagy, and apoptosis (Netherton, Connell, et al. 2019; L. Wu et al. 2021; Y. Wang, Kang, et al. 2021). Significant pieces of ASFV immunology remain unclear, including the roles of anti-AFSV antibodies, the interaction of ASFV with cell-mediated immunity (particularly $CD8^+$ T cells), and the roles of specific proteins in viral immune evasion. Many of ASFV's proteins are immunogenic, but their precise functions remain mostly unknown (Arias et al. 2018).

Since 2015, many studies have aimed to address some of the largest knowledge gaps in the field of ASFV immunology. Such studies are crucial not only for our understanding of the functional mechanisms of ASFV infection but also for the development of vaccines - one of the most urgent tasks facing the ASF research community today. Rational vaccine development requires the identification of ASFV's protective mechanisms, related proteins that may serve as vaccine targets, and delivery systems likely to induce high levels of protective innate and adaptive immunity (Gaudreault et al. 2020).

Previously identified knowledge gaps

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

- *Identification of immune mechanisms behind homologous/heterologous virus protection*

- *Host regulatory genes involved in antiviral (including innate) immune activity and their mechanisms*
- *Immunopathogenesis (e.g. T cell responses and MHC presentation)*
- *Viral proteins responsible for immune evasion and host immunomodulation*
- *The role of MGFs in antigenic variability and immune evasion*

Literature review

Host Immunogenetics and Virus-Host Interactions

Genome-wide transcriptomic studies are an increasingly popular method for obtaining a high-level picture of gene expression changes in ASFV-infected host cells, providing valuable information on altered signalling pathways, cytokine production levels, and other sets of immune-associated genes. Jaing *et al.* recently reported a complete transcriptomic RNA-seq analysis of whole blood RNA from domestic pigs infected with high-virulence (Georgia 2007/1) and low-virulence (OURT88/3) ASFV strains. They found substantial overlap between the two sets of upregulated host genes but noted a relative increase in expression of multiple genes associated with NK cell function during OURT88/3 infection, suggesting a link between viral virulence and NK cell inhibition (Jaing et al. 2017). In 2019, Zhu *et al.* conducted a transcriptomic analysis of primary swine macrophages after infection with the virulent Georgia 2007/1 strain (J. J. Zhu et al. 2019). Here, the researchers found that viral infection led to upregulation of proinflammatory and proapoptotic cytokines (including members of the TNF family, IFNs, and IL-17F) and downregulation of the anti-inflammatory mediator IL-10. These and other differentially expressed genes indicated numerous functional pathways involved in ASFV's immune avoidance, including (1) inhibition of MHC antigen processing and presentation, (2) decreased expression of neutrophil- and $CD8^{+}$ T cell-recruiting chemokines, (3) suppression of antiviral M1 activation in infected macrophages, (4) induction of the immune-suppressive cytokines IL-13 and IL-27, and (5) inhibition of macrophage autophagy and apoptosis (J. J. Zhu et al. 2019). Fan *et al.*, meanwhile, reported an infectomics study (combining comparative analysis of genome-wide expression profiles with proteomics) of tissues collected from ASFV-positive pigs in China, demonstrating cooperative functions in the host immune response (W. Fan et al. 2021). Lungs and spleen dominated the innate immune response (including host signal transduction and lymphocyte activation); liver and kidney primarily acted in metabolic regulation and inflammation; and the lymph nodes modulated energy metabolism in tandem with the liver (W. Fan et al. 2021).

Transcriptomics can also identify genetic patterns associated with viral gene products and potentially interacting host pathways. In the aforementioned transcriptomic study by Jaing *et al.*, the researchers detected viral gene expression in whole blood during infection with Georgia 2007/1, but not with OURT88/3 (Jaing et al. 2017). Notable among the expressed viral genes was *DP71L*, a phosphatase that dephosphorylates P-eIF2α and thereby prevents cells from blocking translation in response to infection. Also upregulated was expression of the host gene *PPP1R15A*, which encodes a homolog of this viral enzyme, suggesting a redundancy in ASFV's immune evasion system and potentially explaining the earlier discovery that *DP71L* is non-essential for ASFV infection (Jaing et al. 2017). Later, Ju *et al.* conducted a whole-genome transcriptome RNA-seq analysis of PAMs infected with the highly virulent Chinese isolate HLJ/18, finding that early-expressed ASFV genes were closely involved in suppressing host immunity (Ju et al. 2021). Viral infection also degraded host microRNAs (miRNAs) with putative antiviral functions, enhanced chemokine-mediated signalling pathways and neutrophil chemotaxis, and disordered host metabolic processes to promote viral transcription and replication (Ju et al. 2021). In a similar study, B. Yang *et al.* used RNA-seq and qPCR to analyse transcriptomic changes in PAMs during infection with the CN/GS/2018 strain, finding over 1,100 differentially expressed genes across a host of cellular processes including PRRs (e.g. RIG-I-like receptors and Toll-like receptors), chemokine expression, and host cell apoptosis (B. Yang et al. 2021).

As mentioned above, ASF is considered a low-virulence and persistent disease in native African Suidae including the common warthog (*P. africanus*) and the host factors underlying this resistance remain mostly unknown. In 2020, McCleary *et al.* studied the *RELA* gene, which encodes a subunit of NF-κB and differs by only 15 nucleotides (constituting 3 amino acid changes) between domestic pigs and wild boar (McCleary et al. 2020). The researchers produced gene-edited domestic pigs wherein the *RELA* gene contained these warthog amino acid substitutions. These pigs were then infected with the moderately virulent genotype X strain Ken05/Tk1, chosen to catch more subtle effects on virulence that might not be apparent in standard high-virulence genotype II viral infections. The gene-edited pigs were not resistant to infection, though the onset of clinical symptoms was delayed and levels of circulating viral DNA were lower, leading to the conclusion that the warthog *RELA* substitutions are involved in, but not sufficient for, their observed resilience against ASFV (McCleary et al. 2020).

Viral and Host Immune Determinants of ASFV Virulence

If differences can be identified in the host immune response against virulent vs. attenuated ASFV strains, then these mechanistic differences may be exploitable to enhance immune protection and

could aid in the development of ASFV vaccine candidates. In 2015, Lacasta *et al.* published a study comparing the *in vivo* pathogenesis of, and host immune responses against, virulent vs. live attenuated ASFV strains (E75 and E75CV1, respectively). They found substantially different courses of immunity – at 1 dpi, for instance, E75CV1 triggered significant up- or downregulation of the expression of ten immune-related genes, while the virulent E75 only upregulated expression of four (namely *IL-12p40*, *TGF-βR1*, *TNF-α*, and *IL-21*). By 7 dpi, however, E75 infection had resulted in a "dramatic imbalance of the immune system," with significant upregulation of numerous genes and secretion of soluble factors including TNF-α, IL-1β, and IL-12 (Lacasta et al. 2015). These proinflammatory cytokines are likely associated with the "cytokine storm" phenomenon that is frequently observed during infection with virulent ASFV strains. Meanwhile, Golding *et al.* analysed the interactions of virulent (including Georgia 2007/1 and BA71) and low-virulence (OURT88/3) strains with porcine IFN (Golding et al. 2016). *In vitro*, pre-treatment of DC-enriched porcine leukocytes with IFN-α reduced replication by OURT88/3 but not by virulent strains. Notably, OURT88/3 lacks the MGF genes *360-10L* thru *-14L* and *505-1R* and *-2R*. When the researchers tested a recombinant virus with comparable gene deletions (all of the above plus *MGF360-9L*), similar results were observed, suggesting that ASFV's sensitivity to type I IFNs is at least partially dependent on *MGF360* and *505* genes (Golding et al. 2016).

Such *in vitro* studies can allow in-depth analysis of ASFV infection dynamics in particular immune cell populations of interest. In 2018, Franzoni *et al.* examined the interaction between ASFV and immature or IFN-α/TNF-α-matured porcine monocyte-derived dendritic cells (moDCs), working specifically with the virulent Sardinian 22653/14, low-virulence NH/P68, and avirulent BA71v strains (Franzoni et al. 2018). The researchers found a complex pattern of interactions. At a high multiplicity of infection (MOI) of 1 virus/cell, all strains were capable of infecting immature moDCs, while maturation with IFN-α reduced susceptibility to the attenuated strains and maturation with TNF-α increased susceptibility to the virulent strain. Infection of moDCs with the attenuated strains (but not with 22653/14) led to downregulated MHC class I surface expression, indicating that certain attenuated ASFV strains may attract attention from NK cells via the "missing self" recognition pathway. Finally, infections by all strains appeared to downregulate expression of the low-affinity Fc receptor CD16, potentially impairing DC function (Franzoni et al. 2018). In a later study from the same group, Razzuoli *et al.* infected porcine moDCs with NH/P68 or 22653/14 and compared the expression of IFN-β and IFN-α genes (Razzuoli et al. 2020). They observed a significantly stronger type I IFN response to the attenuated NH/P68, suggesting that the virulent strain has developed mechanisms to inhibit expression of relevant genes including IFN-β and several IFN-α subtypes (Razzuoli et al. 2020). Meanwhile, García-Belmonte *et al.* infected PAMs with virulent Armenia/07 and attenuated NH/P68

strains and compared their molecular immune responses, finding that Armenia/07 specifically inhibited IFN-β production via control of the antiviral cGAS-STING signalling pathway (García-Belmonte et al. 2019). The cGAS-STING pathway is an important intracellular component of the innate immune system that begins with the sensing of cytosolic DNA (e.g. the ASFV genome), subsequently activating numerous downstream mediators and upregulating the transcription of proinflammatory genes. Several ASFV-encoded proteins are thought to interact with this significant signalling pathway, as discussed in the next subsection.

The roles of the adaptive immune system in host ASFV defence, including the functions of neutralizing antibodies, are also poorly understood. ASFV infection has long been known to induce ASFV-specific antibodies in pigs that survive the first few days of disease; these antibodies are not fully neutralizing but play a demonstrable role in protection, as serum transfer from pigs that have recovered from ASFV infection to naïve pigs confers partial protection against homologous viral challenge (Arias et al. 2018). However, neutralizing antibodies do not always confer such protection (and/or are not sufficient for protection), and their presence is not necessarily predictive of clinical outcomes (Hühr et al. 2020; Netherton, Goatley, et al. 2019). The cellular immune response to ASFV is also not well-defined, although the importance of T cells was previously demonstrated in a study showing that antibody-dependent depletion of $CD8^+$ T cells after priming with an attenuated ASFV strain abrogated protection against homologous challenge (Oura et al. 2005).

Recently, Hühr *et al.* investigated T cell responses in domestic pigs and wild boar after infection with the virulent Armenia08 strain, using a multicolour flow-based assay to identify various T cell subtypes and their functional impairment in infected pigs (Hühr et al. 2020). Domestic pigs showed lymphopenia and impaired proliferation of T cells; conversely, wild boar exhibited proliferation of $CD8^+$ and $CD4^+$ T cells at 5 dpi, though this response did not translate to positive clinical outcomes and all animals developed lethal disease (Hühr et al. 2020). Interestingly, expression of the cytolytic protein perforin was significantly decreased in $CD8^+$ T cells from both domestic and wild pigs (Hühr et al. 2020). In a later study from the same group, the researchers used a similar approach to examine the T cell response to the moderately-virulent ASFV strain “Estonia 2014,” which is lethal in wild boar but survivable in domestic pigs (Schäfer et al. 2021; Zani et al. 2018). Schäfer *et al.* found a wide array of differences in T cell responses, including (1) increased levels of $CD8^+$ and $CD4^+/CD8^+$ αβ T cells, substantial loss of perforin in $CD8^+$ T cells, and a regulatory T cell (Treg) response in both subspecies; (2) increased $ICOS^+/CD8^+$ invariant natural killer T cells (iNKTs) only in domestic pigs; and (3) differentiation of $CD8^+$ γδ T cells only in wild boar (Schäfer et al. 2021).

Sun *et al.*, meanwhile, used PBMCs from domestic pigs that survived infection with the virulent SY18 strain to screen for T cell-activating antigens (W. Sun et al. 2021). The viral capsid protein p72 caused one of the strongest responses, and results with a swine leukocyte antigen (SLA, also called MHC)-tetramer based on three positive p72 epitopes showed a gradual increase in both T cell and humoral immune responses during infection. The researchers also note that different breeds of domestic pig may recognize different viral peptides due to varying SLA genotypes, and breed should therefore be considered when conducting *in vivo* cellular immunity studies (W. Sun et al. 2021). Finally, Yue *et al.* recently examined the binding of ASFV antigens to the domestic-pig-specific SLA allele SLA-1*0101; finding that swine MHC class I presents viral peptides in an uncommon way that creates multiple bulged conformations of bound peptides, generating diversity for T cell receptor docking (Yue et al. 2021).

Viral Proteins Involved in Immune Evasion

Many viral proteins are known to be involved in immune evasion and host immunomodulation. ASFV encodes proteins that variously inhibit or activate NF-κB, for instance, suggesting that the virus precisely controls inflammation levels to support its replication pathway (e.g. inhibiting NF-κB during early infection to avoid an immune response, then upregulating it later to restrict stress-induced apoptosis) (Galindo and Alonso 2017). The viral MGF genes, as discussed above, also appear to play early roles in evasion of innate immunity (particularly type I IFNs) (Sánchez-Cordón, Montoya, et al. 2018; ASF-STOP 2021). However, much of ASFV's proteome (including immunomodulatory proteins) remains functionally uncharacterized, limiting our understanding of ASFV immunology and our ability to rationally develop potential live attenuated vaccines (LAVs). Therefore, the evaluation of ASFV protein functions is a vital component of ASFV control, and many important studies since 2015 have focused on characterizing single proteins within ASFV's repertoire.

In 2018, Borca *et al.* published a study on the L83L ORF, a protein that is highly conserved across all ASFV isolates and which, to date, had not been examined in detail (Borca, O'Donnell, et al. 2018). They found that L83L is transiently expressed during the early stage of viral infection and binds to host IL-1β, suggesting a role in modulating the innate immune response. However, the gene is non-essential, as its deletion from Georgia 2007/1 did not change replication or infection dynamics in primary cells *in vitro* or domestic pigs *in vivo*. The researchers note that further studies will be required using the more natural oronasal infection route, or a less virulent parental virus, to completely discount a role

for L83L in innate immune evasion (Borca, O'Donnell, et al. 2018), and the results of such a study are keenly anticipated.

One of the many potentially immunomodulatory proteins produced by ASFV is UBCv1 (also called pI215L), an E2 ubiquitin-conjugating enzyme that is the only enzyme of its kind known to be encoded by a virus (Barrado-Gil et al. 2020). In 2021, Barrado-Gil *et al.*, who had previously established that UBCv1 is an early-expressed protein that regulates host translation (Barrado-Gil et al. 2020), published an *in vitro* investigation of its possible roles in immune evasion (Barrado-Gil et al. 2021). Overexpression of UBCv1 in human cell lines revealed that the protein acts upstream of IκB kinase (IKK) to block nuclear translocation of the NF-κB subunit p65, thereby impairing cellular responses to proinflammatory cytokines. Interestingly, disabling UBCv1's catalytic activity via an alanine mutation of the active Cys85 residue did not impact this immunomodulatory activity, suggesting that these two functions are independent (Barrado-Gil et al. 2021). Later that year, Huang *et al.* showed that infecting PAMs with the virulent HLJ/18 strain inhibited cGAMP-induced type I IFN production (L. Huang et al. 2021) – a subsequent screen for involved genes identified UBCv1 as one of the strongest inhibitory effectors, acting via negative regulation of the cGAS-STING signalling pathway, and corroborated Barrado-Gil *et al*.'s observation of two independent functions for UBCv1 (L. Huang et al. 2021).

The type I interferon family of cytokines (including IFN-α and IFN-β) play crucial, wide-ranging roles in innate immunity and in the bridging of the innate and adaptive immune responses, and as the above results suggest, type I IFNs are likely some of the most important host factors governing the host response to ASFV infection (Dixon et al. 2020; Razzuoli et al. 2020; García-Belmonte et al. 2019; Golding et al. 2016). Modulating/restricting these factors is therefore important for productive ASFV infection, and research continues into the mechanisms by which viral-encoded proteins exert such control. The MGF proteins (particularly in the MGF360 and 505/530 families) suppress type I IFN responses and interfere with apoptosis (Dixon et al. 2019). In 2021, D. Li *et al.* studied infection with the CN/GS/2018 strain in PAMs and *in vivo*, finding that the MGF505-7R protein restricted type I IFN responses by inhibiting the cGAS-STING pathway. Deletion of the *MGF505-7R* gene via CRISPR-Cas9 caused higher IFN-β production and attenuated replication *in vitro*. This mutant strain was also completely attenuated in 7-week-old domestic pigs - an unusually strong result from deletion of a single gene (D. Li et al. 2021). In a similar study, J. Li *et al.* found that several members of MGF360 and 505 inhibited IFN-β production (even in the presence of strong broad-spectrum inducers like lipopolysaccharide) during infection of PAMs with the HLJ/18 strain, with MGF505-7R having the strongest effect (J. Li et al. 2021). The researchers observed that this protein also interacts with IKKα

to inhibit NF-κB activation and binds the intracellular sensor NLRP3 to block inflammasome formation. In this study, *MGF505-7R* deletion reduced, but did not eliminate, virulence, with 60% survival among 4-week-old piglets – possible explanations for this difference include the use of younger animals and viral strain-specific differences (J. Li et al. 2021).

In 2021, Shimmon *et al.* examined the function of viral protein A179L, previously shown to inhibit apoptosis and autophagosome formation by binding the key autophagy regulator Beclin-1 (Hernaez et al. 2013). Using the Vero cell-adapted BA71v strain, the researchers found that deleting the *A179L* gene did not stop the virus from disrupting autophagosomes, suggesting functional redundancy (Shimmon et al. 2021). Meanwhile, J. Yang *et al.* studied the previously uncharacterized protein F317L and demonstrated that it acts as an inhibitor of host innate immunity by suppressing the phosphorylation of IKKβ. This blocks the activation and nuclear translocation of NF-κB, decreasing the expression of proinflammatory cytokines (J. Yang et al. 2021). Finally, Gao *et al.* examined the effects of deleting a handful of immune-associated genes (*CD2v*, *MGF360-1R*, *MGF360-12L* thru *14L*, and *MGF505-2R* and *-3R*) on the immunopathology of the highly virulent GZ201801 strain in cultured PAMs (Gao et al. 2021). The mutant virus had impaired replication and decreased apoptosis-inducing abilities, associated with NF-κB inhibition and decreased IL-1β production. Interestingly, reporter assays suggested that MGF360-12L and -13L and MGF505-2R suppress NF-κB while CD2v activates it, illustrating the complicated and interconnected nature of ASFV's gene expression and host cell regulation (Gao et al. 2021). CD2v is a multifunctional adhesion protein that has also been associated with viral serospecificity and type-specific protective immunity (defining homologous vs. heterologous viral strains independent of p72 genotype) (Malogolovkin and Kolbasov 2019; Rock 2021). This will be discussed further in the Vaccines section below.

Ongoing research

Below is a discussion of some of the many ongoing and planned projects in the field of ASFV immunology. This section is not a comprehensive discussion of research in this field but is instead intended to provide a brief overview of selected research projects based on feedback from a survey of researchers conducted during the writing of this report.

In Madrid, researchers at the Centro de Biología Molecular Severo Ochoa (CBMSO) are conducting several investigations into the immunomodulatory activities of ASFV, combining cutting-edge *in vitro* and *in vivo* approaches to investigate the determinants of type I IFN modulation during viral infection.

The primary long-term goal of these studies is to facilitate rational live attenuated vaccine development by identifying genes critical for viral immune evasion. Elsewhere, CBMSO scientists are characterizing the viral factors responsible for modulating the cGAS-STING and JAK/STAT inflammatory pathways, aiming to describe the molecular mechanisms underlying their activity and the potential differences in these factors between virulent and attenuated strains. At The Vaccine Group in Plymouth, UK, researchers are investigating the role of T cells in ASFV immunity. Scientists at the FRCVM, meanwhile, plan to study the functional role of the *MGF110* genes in viral pathogenesis and host immune response evasion.

Future research priorities

Based on the above literature review and with reference to previously identified knowledge gaps and expert opinion, the following areas of ASF immunology should be considered priorities for future research:

- *Continuing immunological characterization of viral proteins with unknown functions*
- *Identifying correlates of immune protection*
- *Virus strain- and host subspecies-specific studies of host-virus interactions*
- *Identification of critical host genes for anti-ASFV innate and adaptive immunity*
- *Role of T cells in response to ASFV strains of varying virulence*
- *Determinants of warthog resistance to ASF*
- *Further characterization of MGFs and the functional consequences of inter-strain differences in these genes*
- *Translation of recently identified immunomodulatory genes into potential live attenuated vaccine candidates*

Controlling African Swine Fever

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).

Previously identified knowledge gaps

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

- *Multiscale epidemiological investigations of emergency control measures*
- *Risk assessments for ASFV control and spread*

- *Standardized protocols for cleaning/disinfecting locations and restricting movement of infected animals*
- *Improved tracking and surveillance networks*
- *Socioeconomic impacts of depopulation*
- *Sustainable and effective alternatives to stamping out/culling in developing countries with no compensation schemes*

Literature review

A wide array of biosecurity measures, with varying cost and invasiveness, have been implemented in the pork production sector and among wild boar populations in the many countries currently experiencing the ongoing ASF pandemic. For on-farm biosecurity, such measures include restrictions on contact with external pigs, disinfection of premises and farm vehicles, strict bans on swill feeding, and close veterinary supervision (ASF-STOP 2021). Culling of all infected herds and movement bans on neighbouring herds are commonly employed in response to outbreaks (Guinat et al. 2017). As discussed below, broad culling mandates can encounter resistance from local stakeholders in the pork production chain, particularly when compensation schemes are inadequate to ensure farmers' economic security (Ståhl et al. 2019). 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 the EU (Cwynar, Stojkov, and Wlazlak 2019).

Danzetta *et al.* published a systematic literature review of the strategies used by different countries to eradicate historical and current ASF outbreaks (Danzetta et al. 2020), emphasizing the need for rapid disease identification and response; swift implementation of control measures in direct collaboration with farmers, breeders, and international organizations; and financial compensation schemes and social programs for affected farmers. Biosecurity measures should also be tailored to region-specific epidemiological circumstances, as exemplified by Brazil's "garbage operation" in response to its 20th century epidemic – the elimination of pigs kept in public garbage plants was critical in controlling ASF transmission within small-scale, unregistered breeding programs (Danzetta et al. 2020). In the current pandemic, critical factors for eradication in the Czech Republic included (1) pre-outbreak application of passive surveillance on all dead pigs, (2) defining of risk-based geographical management zones for infected wild boar with strict controls on hunting in high-risk zones, and (3)

awareness campaigns and compensation schemes for affected hunters and farmers (Danzetta et al. 2020).

Wildlife Biosecurity

Wild boar are hardy and adaptable animals, and they are one of the most extensive and abundant human-spread mammals in the world (O'Bryan et al. 2022). They therefore pose a daunting challenge to ASF control. Populations of varying size and density are common throughout the various biomes of Eurasia, frequently serving as the means of ASF introduction into previously unaffected regions. Monitoring and surveillance of ASF in wild boar is difficult, and few conclusive connections have been drawn between specific boar control measures and epidemic outcomes. In Belgium, Dellicour *et al.* demonstrated that disease spread was faster inside forested areas, suggesting that tree coverage should be considered when installing fences and defining ASF containment areas (Dellicour et al. 2020; Jo and Gortázar 2021). This conclusion was supported by a spatial epidemiology study in Poland (Podgórski et al. 2020). Identification and removal of wild boar carcasses are also important for eliminating ASFV sources from infected areas (Desmecht et al. 2021; Bellini et al. 2021). Hunting and trapping are often employed to decrease wild boar populations, although several factors complicate control strategies that are based on boar culling. The persistence and environmental tenacity of ASFV allows it to be maintained and spread even in regions with very low wild boar density (< 1 individual per km^2) (Podgórski et al. 2020; Jo and Gortázar 2021). More *et al.* emphasized that a wild boar population density threshold sufficient for stopping the spread of ASF has not been established (due to unsolved epidemiological questions and the huge uncertainty introduced by varying boar population structures and anthropogenic transmission), and there is currently no indication that such a threshold even exists in the field (More et al. 2018). Meanwhile, as mentioned above, hunting programs can have the unintended effect of driving viral spread by dispersing wild boar populations over a wider area (Gavier-Widén, Ståhl, and Dixon 2020; Desmecht et al. 2021). More *et al.* recommended drastically reducing disease-free wild boar populations ahead of the ASF wavefront after introduction, then carefully managing infected populations to keep them undisturbed and avoid further spread (More et al. 2018).

Schulz *et al.* used surveillance data to statistically model and evaluate wildlife biosecurity measures in Latvia (Schulz, Oļševskis, et al. 2019). These measures included incentives for reporting dead boar and hunting female boar, collection and safe disposal of carcasses, feeding bans, and permission to use silencers and night vision devices for hunting. None of these measures had a significant short-term

effect on the number of wild boar hunted or found dead, and no change in the periodic prevalence of PCR-positive wild boar was observed, suggesting a critical need to consider additional wildlife biosecurity measures such as fencing and trapping (Schulz, Oļševskis, et al. 2019). As discussed in the Epidemiology section above, additional studies are also urgently required to build our understanding of ASFV survival in carcasses, the possible role of survivors/carriers, and the sometimes unusual transmission characteristics of ASF in boar (e.g. in the Czech Republic, where ASF spread at a slow rate of ~0.5 km/month despite a high regional wild boar density) (Danzetta et al. 2020).

Sauter-Louis *et al.* recently compared the epidemiological courses of ASF in Germany (during the first 6 months after introduction) and in two neighbouring countries – Belgium and the Czech Republic (Sauter-Louis, Schulz, et al. 2021). Since both of these countries had eradicated their own ASF outbreaks in wild boar, their biosecurity measures were used as a template for Germany. Here, however, the researchers found that Germany presented a very different epidemiological situation, with multiple introductions via wild boar crossing the Polish border in contrast to the single outbreak clusters observed in Belgium and the Czech Republic (Sauter-Louis, Schulz, et al. 2021). The transmission pattern of ASFV in Germany is closer to that experienced in Poland and the Baltic States, with continuous pressure and wavefront transmission across a lengthy shared border. For this reason, Sauter-Louis *et al.* recommended additional wildlife and country-level control measures, including fencing at the border area and joint disease control efforts with Poland (Sauter-Louis, Schulz, et al. 2021). When these researchers wrote their manuscript, ASF had only been detected in wild boar in Germany; the first case in domestic pigs was confirmed 5 days after its publication (Federal Ministry of Food and Agriculture 2021).

In South Korea, Jo & Gortázar evaluated wildlife biosecurity measures between October 2019 (shortly after the introduction of ASFV into the country) and October 2020. During this time, a total of 775 cases of ASF were found in wild boar across nine counties (Jo and Gortázar 2021). Implemented measures included trapping and hunting, local and large-scale fencing, and destruction of wild boar carcasses. Notably, carcass detection and removal are especially difficult in South Korea due to the presence of minefields in the Demilitarized Zone and Civilian Control Zone (Jo and Gortázar 2021). ASF was confined within a fenced area of three counties in 2019, but a change in management policy toward more disruptive culling led to the disease spreading east and south. This lead the researchers to conclude that wildlife biosecurity has unfortunately been unsuccessful on the national level, and they recommended immediate fencing and trapping as the most effective and cost-efficient control measures in South Korea (Jo and Gortázar 2021).

One general lesson from these studies is that, if an ASF outbreak in wild boar is not rapidly detected and controlled, it can quickly become a persistent epidemic that is extremely difficult to eradicate. Schulz *et al*. pose the question of whether affected countries may eventually have to accept/learn to live with the presence of ASF in wild boar, inevitably leading to a greater focus on the more easily controllable on-farm biosecurity (Schulz, Oļševskis, et al. 2019).

On-Farm Biosecurity

Without a commercially available vaccine or antiviral for ASF, farms must depend entirely on biosecurity measures to isolate domestic pigs from ASFV and prevent outbreaks (ASF-STOP 2021). The importance of specific measures differs between large commercial farms and the small backyard farms that are common across Eurasia and are often associated with higher-risk practices such as mixed pig sources, minimal segregation of animals, and lack of sufficient cleaning and disinfection (ASF-STOP 2021). Common on-farm biosecurity measures include physical isolation and movement restriction of ASF-affected herds, disinfection of vehicles and clothing immediately after transporting animals, and proper disposal of pig carcasses and associated fomites (Bellini, Rutili, and Guberti 2016). Security measures at farm entrances (e.g. disinfection of transport vehicles and personal protective equipment) are particularly important and can halt potential human-mediated spread of ASFV originating in the environment (including cross-contamination from wild boar hunting or from feed and bedding in areas inhabited by wild boar) (Bellini et al. 2021). Control measures on farms are typically regulated by national and international health and food safety agencies. Since 2015, substantial research has been conducted on the efficacy of these measures and the ways in which our current biosecurity systems may be inadequate to control ASF outbreaks.

In 2018, Jurado *et al.* conducted a literature review and expert assessment of control measures for preventing ASF spread in the EU domestic pig sector (Jurado et al. 2018). Among the most important perceived security measures were (1) identification of animals and farm records, (2) banning of swill feeding, (3) containment of pigs (disallowing undesired pig-pig or pig-wild boar contacts), (4) education of personnel, and (5) appropriate handling of food waste, carcasses, and associated residues. On non-commercial and outdoor farms, improved access to veterinarians and health services was also noted as crucial (Jurado et al. 2018). Meanwhile, Busch *et al.* reviewed some of the on-farm biosecurity paradigms in use around the world, making the argument that broadly applied control measures (ignoring the unique characteristics of ASFV and the specific circumstances and

environments in which domestic pigs are kept) are harmful and can be ineffective and/or disproportionate (Busch et al. 2021).

Depending on regional factors and farm management systems, substantial risk may also exist for domestic pigs at the interface between farm and environment/wild boar. Laddomada *et al.* examined the role of free-ranging pigs in maintaining ASF in Sardinia (Laddomada et al. 2019). This regional system of pig farming has long been associated with the persistence of the original 20th century ASF outbreak on the island, but stricter biosecurity/eradication measures were enacted in 2015-2018. A concerted push for increased education and eradication has had some promising results, with the recent culling of nearly 1,100 free-ranging pigs in the Orgosolo Municipality (considered the epicentre of ASF in Sardinia) (Laddomada et al. 2019). Interestingly, a 2018 depopulation initiative identified 53.4% ASFV seropositivity among culled free-ranging pigs, a very high proportion compared to both confined pigs and wild boar (Laddomada et al. 2019). These results confirm that free-ranging pigs play a critical role in maintaining the virus in Sardinia and may be useful for epidemiological studies in other countries with a relatively high potential for interaction between domestic pigs and wild boar. Conflict between the necessities of biosecurity and local socioeconomic, cultural, and traditional practices are an increasingly recognized source of ASF risk and are discussed in more detail below.

Proper disposal of dead domestic pigs is an important step in on-farm biosecurity. Pepin *et al.* recently studied the survival of common swine pathogens during pig carcass composting, a common practice in the pork industry (Pepin et al. 2021). Though unable to examine ASFV directly due to biosafety risks, the researchers collected time and temperature data relevant to the virus while using two other common porcine viruses for experiments. Composting of pre-processed (ground/mechanically crushed) carcasses achieved temperatures high enough to inactivate ASFV ($\geq$ 60°C) even in cold ambient weather down to -11°C. This temperature peak was reached regardless of biomass type or microbial digestion treatment, suggesting that this method of composting is relatively safe for mass disposal of pre-processed infected carcasses (Pepin et al. 2021).

In other areas, knowledge gaps may limit our ability to track potential pathways of ASFV introduction to domestic farms. Shurson *et al.* very recently reviewed the relative risks of ASFV contamination in swine feed ingredient supply chains, an area that is largely understudied due to the numerous factors that complicate our ability to detect the virus in feed (Shurson et al. 2022). Risks exist for many components of swine feed, including food waste and animal by-products, soybean meal, corn, and even vitamins and minerals. Feed fraud (including illegal component substitutions, mislabelling, and

grey market production) is another important potential risk factor, requiring additional guidance and education within the food safety industry (Shurson et al. 2022). Overall, the lack of national or international feed diagnostic and surveillance systems renders regulators more likely to adopt blanket, proactive requirements that may not accurately reflect the realistic risks of ASFV in feed components. More research will be required to evaluate component-specific risks and guide swine feed biosecurity measures (Shurson et al. 2022).

ASF Biosecurity in the USA

The current ASF outbreak has spread to the world's top consumer and producer of pork (China) and continues to transmit seemingly inexorably across the runner-up (the EU). The third-largest consumer and producer is the USA (USDA ERS 2019), which has never seen a confirmed case of ASF. ASFV's historical epidemics and current spread to Timor-Leste demonstrate that transmission is not constrained by oceans (Mighell and Ward 2021), and the recent cases identified in the Dominican Republic and Haiti have brought the virus to within 800 miles of US shores (Cole and Stepien 2021; Stepien and Cole 2021). Biosecurity in the USA – specifically focused on preventing introduction, but with contingencies and preparations made for potential outbreaks – is more critical than ever, as an ASF epidemic in the USA would have substantial economic impacts. In a 2020 report for the Center for Agricultural and Rural Development at Iowa State University, Carriquiry *et al.* modelled two potential 10-year scenarios for such an epidemic, both of which would follow an immediate 40-50% reduction in US live hog prices (Carriquiry et al. 2020). If the USA efficiently controlled the disease and re-entered the export market within 2 years, revenue lost would likely equal ~$15 billion USD, with almost no total jobs lost after 10 years. However, if ASF spread to wild boar and avoided eradication across the 10-year period, losses would likely be closer to $50 billion USD, with 140,000 jobs lost (Carriquiry et al. 2020).

A spate of recent studies have specifically addressed the risks, challenges, and control measures most applicable to the USA's situation. In 2017, Herrera-Ibatá published a quantitative risk assessment of ASF and CSF introduction into the USA via legal imports of live pigs and pig products. They found that the former posed a greater risk than the latter, and they identified the individual US states most vulnerable to ASFV introduction (Herrera-Ibatá et al. 2017). Importantly, this research was published prior to the 2018 introduction of ASF to China, which changed international risk factors significantly. Jurado *et al.* conducted a similar risk assessment in 2019, looking specifically at ASFV introduction via illegal entry in air passenger luggage (Jurado et al. 2019). Their quantitative stochastic model

suggested that the average risk of introduction via this route had increased by ~183% since the virus spread to Western Europe and Asia. This model associated the highest risk (~68%) with flights from China and Hong Kong, followed by Russia (~27%). Five US airports – Newark (EWR), Houston (IAH), LAX, JFK, and San Jose (SLC) – accounted for > 90% of the overall calculated risk (Jurado et al. 2019). Such studies may guide regulators and policymakers to apply stricter biosecurity measures at the points of greatest risk, targeting limited resources for the best possible efficiency.

Finally, Fanelli *et al.* published a short-term risk assessment of ASFV introduction to the USA, focusing on the significant changes imposed on human and pig movement patterns by the ongoing COVID-19 pandemic (Fanelli et al. 2021). Their findings point to an overall increase in connection between the USA and ASF-affected countries, including upward trends in incoming merchant ships and commercial flights from these regions. The introduction of ASFV to the Dominican Republic occurred while the risk assessment was in processing (and the virus was confirmed in Haiti after publication). This research review did not uncover any risk assessment studies published recently enough to include these outbreaks in the Caribbean region.

Preparation for a potential American ASF outbreak will involve studies of successful on-farm and wildlife biosecurity measures in other countries and of the particular geographical/environmental circumstances present across the USA. Arthropods, for instance, could potentially play a role in ASFV transmission in the USA. As discussed above, ticks have not been shown to play an important role in the current ASF pandemic, but their populations must nonetheless be carefully studied to ensure that we have a clear understanding of their habitats in case such a role is identified. In 2016, Donaldson *et al.* modelled the geographical distribution of *Ornithodoros turicata*, a soft tick found in several states across the southern USA. Their results suggest previously unrecognized potential habitats for *O. turicata* in states including Georgia, the Carolinas, and Nevada (Donaldson et al. 2016).

In 2018, Brown & Bevins comprehensively reviewed the risks of ASFV establishment and persistence in the USA, with specific attention paid to arthropods and wild boar populations (Brown and Bevins 2018). Their assessment predated ASF in China and may therefore be partially obsolete, but the identified epidemiological factors remain current. The vast majority of American pig production takes place indoors in high-security holdings, but backyard farms are still relatively common. Recent outbreaks in European high-security farms (Olesen, Hansen, et al. 2018; Nurmoja et al. 2020; Yoon et al. 2021) and the human-mediated spread of porcine epidemic diarrhoea virus in the USA in 2013 (Brown and Bevins 2018) are important reminders that anthropogenic factors can stymy even the

strictest on-farm biosecurity measures. The USA also hosts a population of about 6 million feral swine across at least 35 states. Findings from Europe suggest that fencing and careful control of hunting are especially important wildlife biosecurity measures in the event of an outbreak in the wild population, and country-level coordination with Mexico and Canada would be necessary to monitor the movement of herds across international borders (Brown and Bevins 2018).

Compliance, Feasibility, and Participatory Biosecurity

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 (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 (Penrith, Bastos, and Chenais 2021). Illegal activities such as swill feeding, nonreporting, and emergency sales are closely tied to tenuous economic situations and lack of trust in veterinarians and/or official authorities (Bellini et al. 2021). Currently, the COVID-19 pandemic is also having a significant impact on the pork production chain and associated stakeholder incomes, adding stress to an already difficult situation (Millet et al. 2021).

The "common denominator" of ASF spread throughout the current pandemic is the smallholder sector, which can account for > 80% of pig production in developing countries (Penrith, Bastos, and Chenais 2021). The factors responsible for poor on-farm biosecurity in resource-poor areas vary depending on local circumstances – in some cases, knowledge gaps may contribute to the problem, while in others, the knowledge of farmers and other stakeholders is simply superseded by socioeconomic factors (Penrith, Bastos, and Chenais 2021). Within the smallholder sector, even a future ASF vaccine with proven clinical efficacy might struggle to make a difference due to economic limitations, poor infrastructure, and frequent need for re-vaccinations (Penrith, Bastos, and Chenais 2021; Rock 2021). Chenais *et al.* note that poorly implemented farm biosecurity measures (e.g. enforcing culling without providing adequate compensation) may have the opposite of the intended effect, driving animal trade and sale into illegal, unregulated market systems (Chenais et al. 2019). These responses are to be expected, with farmers struggling to maintain their livelihoods under such circumstances (Ståhl et al. 2019). From a wildlife biosecurity perspective, national compensation

schemes may also backfire in unexpected ways. For example, Jo & Gortázar describe the problems associated with a South Korean program offering $1,000 USD bounties to civilians who identified ASF-positive wild boar carcasses – it is suspected that local hunters may have hidden carcasses in order to earn bounties, and the regular movement of such hunters between ASF-infected and uninfected areas helped spread the virus to distant boar populations (Jo and Gortázar 2021).

Tension is unavoidable between the necessity of effective on-farm biosecurity and the socioeconomic realities experienced by actors in the pork production chain. Guinat *et al.* found in their 2017 study that the use of heat-treated meat from culled pig herds in human or pet food chains is perceived as impractical by experts due to potential food safety risks (Guinat et al. 2017). Penrith *et al.*, however, note that disposing of meat may be culturally anathema or infeasible for food security in resource-poor communities (Penrith, Bastos, and Chenais 2021). Such tensions are unlikely to have easy solutions, but an increasing focus on the country- and region-specific feasibility of ASFV biosecurity strategies can improve our ability to respond to the current pandemic with effective control measures (Dixon et al. 2020).

In 2017, Chenais *et al.* described a Knowledge, Attitudes, and Practices (KAP) study among smallholder pig producers in Northern Uganda, conducting participatory rural appraisals to evaluate their understanding of and responses to ASF (Chenais et al. 2017). They found that knowledge of ASF clinical signs, transmission routes, and control measures was generally high, but that this did not guarantee implementation of proper on-farm biosecurity. 24.5% of the population of Uganda live below the national poverty line, particularly in rural areas, and the researchers therefore call for increased focus on management changes that can combine ASF biosecurity with efforts to reduce rural poverty. The importance of cultural habits and taboos was also stressed, with ASF outbreaks being associated with substantial negative impact on socioeconomic status; similar findings have been observed in other ASF-endemic regions including Russia, Georgia, and Sardinia (Chenais et al. 2017).

In Europe, Jori *et al.* studied the acceptance and perceived efficacy of wildlife biosecurity measures (e.g. zoning, surveillance, fencing, and intensive hunting) among selected European experts involved in the management of wildlife, animal health, and hunting, among other fields (Jori et al. 2020). In this study, the researchers focused primarily on evaluating the "World Café" method used for facilitating discussions between stakeholders with varying priorities and fields of expertise, and they identified several useful aspects of this paradigm including "promoting positive examples of solutions and producing constructive change" rather than focusing on simple solutions and consensus-finding (Jori

et al. 2020). The method was considered a success; recommendations from the event included an increased emphasis on fencing and trapping and the evaluation of wild boar poisoning and fertility control from legal, environmental, animal welfare, and feasibility perspectives (Jori et al. 2020).

Finally, Barnes *et al.* recently trialled participatory on-farm biosecurity measures against ASF in Timor-Leste as part of the ongoing development of a sustainable smallholder pig farming system in that country (Barnes et al. 2020). Tested control measures included fencing around pig pens, the use of dedicating outer clothing for farmers, and hand-washing before entering farm premises. These strategies were introduced as part of a collaborative process with local leaders, including public awareness meetings and Q&A sessions in local communities. Results were positive, with protective effects for fenced-in pigs relative to their unfenced counterparts and optimism about the biosecurity measures expressed by most trial farmers. These findings illustrate the importance of engagement and collective responses with local communities and may be applicable to countries with similar socioeconomic circumstances including Indonesia and Papua New Guinea (Barnes et al. 2020).

Ongoing research

Below is a discussion of some of the many ongoing and planned projects in the field of ASFV biosecurity and control measures. This section is not a comprehensive discussion of research in this field but is instead intended to provide a brief overview of selected research projects based on feedback from a survey of researchers conducted during the writing of this report.

At the FAO, scientists continue to explore region-specific ASFV on-farm and wildlife biosecurity, including (a) the composting of wild boar carcasses for ASFV elimination in Lithuania, and (b) a regional technical cooperation programme for ASF emergency preparedness in the Balkans. As part of their ongoing programmes in North Macedonia, FAO researchers are also quantifying biosecurity gaps and risk factors in the national pig sector and conducting a network analysis of live pig movements to inform disease mitigation and control strategies. From an economic perspective, FAO scientists are evaluating the impacts of ASF (particularly in the Philippines and Vietnam) using the newly developed Outbreak Costing Tool (OutCosT), which promotes a rapid outbreak response by evaluating stakeholder-specific financial costs alongside qualitative factors like animal welfare, environmental impact, and socioeconomic vulnerability.

In addition to their epidemiological studies, researchers at the FLI are evaluating the efficacy of wildlife biosecurity measures implemented to control ASF in wild boar. Meanwhile, at EMÜ, studies are ongoing into the awareness and acceptance of ASF control and biosecurity measures by pig farmers.

Future research priorities

Based on the above literature review and with reference to previously identified knowledge gaps and expert opinion, the following areas of ASF biosecurity should be considered priorities for future research:

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

Surveillance

Surveillance measures capable of early ASF detection are the "first line of defence against ASF" (GARA 2018) 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).

Arias *et al.* note that "there is no single recipe for preventing ASF" (Arias et al. 2018): the most appropriate surveillance strategies will vary depending on many factors including epidemiology (e.g. geographic dispersal of wild boar populations), socioeconomic resources, and the robustness of national communication/disease reporting channels (Dixon et al. 2020; Arias et al. 2018). Studies since 2015 have evaluated the efficacy of various surveillance measures across a range of epidemiological circumstances, providing new data that may help legislators and animal health organizations develop ASF prevention and control strategies.

Previously identified knowledge gaps

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

- *Development of global, coordinated surveillance systems*

- *Assessment and modelling of ASF epidemiology in emergency control programs within domestic pig and low- and high-density wild boar populations*
- *Evaluations of emergency control measure implementation*
- *Evaluation of diagnostic tests for detecting infected pigs in at-risk populations*
- *New assays for early detection of ASF*
- *Lower-cost sampling strategies to increase the efficiency of active surveillance*

Literature review

Integrated Data Collection Models for ASF Surveillance and Standardization

Tracking of ASF transmission, early detection of outbreaks, and disease control efforts all rely on the rapid and effective collection of national and international surveillance data. Ensuring the economically sustainable collection of high-quality surveillance data within and across national borders is a daunting task. In the last 6 years, researchers have begun to address this problem by developing new systems that leverage increasing computational resources to simplify, standardize, and increase the efficiency of ASF surveillance and data reporting. Peyre *et al.* published in 2019 on the development and testing of the RISKSUR EVA support tool, designed to guide members of swine disease surveillance efforts in designing evaluation and surveillance protocols (integrating technical, practical, and socioeconomic considerations), conducting evaluations, and communicating findings to decision-makers (Peyre et al. 2019). Freely available online, this tool comprises a web interface for evaluation plan development, a Wiki classroom for education, and a generic evaluation work plan. The researchers note that this tool is vulnerable to practical issues including limited data availability and resource scarcity, highlighting the continuing importance of harmonized data collection and socioeconomic epidemiological studies for the collection of ASF surveillance data (Peyre et al. 2019). In 2020, Clarke *et al.* developed a prevention-minded tool to guide the prioritization of animal health surveillance resources in Ireland, an ASF-free region (Clarke et al. 2020). The core of this tool is a user-friendly spreadsheet, flexible and adjustable to various epidemiological situations, that is used to collect expert opinions on high-risk diseases and the optimal allocation of surveillance resources. Interestingly, while validating the prioritization tool, Clarke *et al.* noted that expert respondents recommended a 50-50 allocation of resources to active and passive ASF surveillance activities (Clarke et al. 2020).

In 2018, the EFSA Panel on Animal Health and Welfare published a major report on ASF in European wild boar, including comparisons of EU wild boar culling strategies and population density estimates (More et al. 2018). Among other conclusions and recommendations, the researchers note that the only EU-wide wild boar information available at the time of writing were hunting data. Harmonization of data collection among EU Member States and validation of hunting data via more precise local surveillance techniques (such as camera trapping) are urgently needed to allow direct comparisons between datasets and improve predictive models of wild boar density (More et al. 2018). A subsequent report from EFSA's SIGMA project aimed to address such needs, presenting the SIGMA Animal Disease Data Model as a means to simplify the reporting and standardization of high-quality surveillance data on reportable diseases and reduce the effort required on the part of individual EU Member States (Zancanaro et al. 2019). The researchers note that much of the information required by EFSA for epidemiological studies/biosecurity recommendations are already collected by Member States – however, these data are decentralized and poorly harmonized, a key gap that the SIGMA model was developed to fill (Zancanaro et al. 2019).

In some cases, ASF data collection can be incorporated into existing national and international networks for other reportable swine diseases, allowing ASF surveillance to start from an already established data-gathering framework. In the EU, EFSA's SIGMA Consortium recently reported on the CSF & ASF wild boar surveillance database, into which ASF was integrated in 2014 (SIGMA Consortium 2019). This database combines rapid data recording and storage with multiple tools for analysing collected data, allowing EFSA and individual Member States to efficiently access and analyse country-specific and cross-border ASF data (SIGMA Consortium 2019). Brown & Bevins discuss a similar approach in their review of ASF risk factors facing the US, recommending that ASF surveillance be incorporated into ongoing active CSF data collection programs at the USDA. As swine samples are already collected and transported to a centralized diagnostic laboratory for analysis, such integration could be a relatively simple process (Brown and Bevins 2018).

New Methods for Sampling and Data Collection

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.* recently published a comparative study of biological sample matrices for ASF surveillance (Flannery et al. 2020). Domestic pigs were experimentally infected with virulent ASFV of genotype I (OURT88/1 strain) or II (Georgia 2007/1), and blood, bone marrow, ear biopsies, and oronasal/rectal swabs were collected from all pigs post-mortem. ASFV was detected in all ear samples (giving qPCR Ct values of 24.8-31.8 regardless of location in the ear), suggesting that ear biopsy punches may be a useful tool for rapid, high-throughput *en masse* surveillance during an outbreak. Bone marrow also contained the highest concentration of ASFV and gave the best probability of detection, making it a potentially useful matrix for surveillance of decomposed wild boar carcasses (Flannery et al. 2020). Meanwhile, Goonewardene *et al.* evaluated oral fluid collection as a non-invasive, less resource- and time-intensive method for active ASF surveillance (Goonewardene et al. 2021). The researchers collected aggregate oral fluid and individual oral swabs from groups of experimentally infected pigs housed under industry-standard conditions, finding that viral DNA could be detected in oral fluid samples by 3-5 dpi. Though slower than detection from blood samples (where viral DNA could be amplified as early as 1 dpi), the simple and non-invasive nature of oral fluid collection makes it potentially valuable as an industry-feasible supplement to blood testing, catching infections in aggregate samples that random individual testing might miss (Goonewardene et al. 2021).

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). In 2021, Kosowska *et al.* assessed the capacity of Dry-Sponges (manufactured by 3M), pre-hydrated with a new surfactant liquid, for sampling and inactivation of ASFV on various surfaces (Kosowska et al. 2021). The surfactant (a mix of alcohols, disodium phosphate, and 0.1% SDS in nuclease-free water) efficiently inactivated ASFV while preserving viral DNA, potentially eliminating the need for BSL-3 biosafety during validation and detection testing. The sponges could recover viral DNA from relevant surfaces including feeders, troughs, and the facility floor. Notably, DNA could also be recovered from animal skin, particularly important for surveillance of vehicles transporting live animals (Kosowska et al. 2021).

The increased focus on participatory epidemiology in low-income countries has also involved the development and testing of surveillance tools better suited to these environments. In 2015, Chenais *et al.* qualitatively evaluated three ASF surveillance methods in Uganda: (1) report-driven outbreak investigations, (2) participatory rural appraisals, and (3) household surveys via a smartphone app (Chenais et al. 2015). The researchers found that all three methods detected many times more

outbreaks than were reported to the OIE during the same time period, and the derived mortality estimates for each method matched reasonably well. Uganda is a resource-poor country with a large and rapidly growing pig population – the data obtained here may therefore be applicable to similar regions of East Africa, where standard surveillance strategies are ill-attuned to local socioeconomic and cultural realities (e.g. inadequate financial compensation schemes, distrust of local government, and stigmatization of ASF-affected farmers) (Chenais et al. 2015).

Periodic sampling and sentinel surveillance in at-risk pig populations are common strategies for early ASF detection, but the associated economic and logistical costs can be high. The development of automated surveillance systems, built with low-cost components and extensively validated, is a promising alternative. In 2015, Martínez-Avilés *et al.* described the Real-Time Monitoring System Online (RTMS-ON), which combines biosensors, eartag-embedded accelerometers, and 24-hour video monitoring to automatically detect and flag potentially diseased pigs in the field (Martínez-Avilés et al. 2015). This system was tested on pigs experimentally infected with the partially attenuated strain Ken05/Tk1 (chosen to induce milder, more easily missed clinical symptoms) and detected eight out of nine cases before or simultaneously with development of clinical signs, positive qPCR, or other common methods of ASF detection (Martínez-Avilés et al. 2015). A 2017 study from the same group focused specifically on automated, motion-based video detection of decreased movement in ASFV-infected pigs (Fernández-Carrión et al. 2017). Here, the researchers showed that pig mobility fell significantly below baseline levels by 4 dpi, with motion decreasing by ~10% prior to the detection of disease via clinical signs. Future studies will be required to further validate automated, data processing-based video surveillance systems under more complicated field conditions (Fernández-Carrión et al. 2017).

Automated surveillance can also be applied to the internet for rapid detection of ASF on national or regional scales. Arsevska *et al.* recently designed a platform (dubbed "PADI-web") that combines rule-based systems and data mining techniques to automatically collect, process, and extract epidemiological info from Google News (Arsevska et al. 2018). Dataset validation confirmed 80-95% accuracy for identifying information (e.g. location, case numbers, and hosts) on outbreaks of OIE-notifiable diseases including ASF. Experimentally, PADI-web was able to detect signals for 64% of all primary ASF outbreaks between January and June 2016, outpacing OIE notifications and identifying some potential outbreaks that lacked official confirmations. Overall, PADI-web could not match the specificity of human-verified biosurveillance systems such as HealthMap and ProMED, but the

potential efficiency gains promised by automation may drive increasing research attention in the future (Arsevska et al. 2018).

Finally, Leray & Ward recently published on the use of web-scraping techniques to automate the collection of publicly available, but hard-to-access, disease surveillance data (Leray and Ward 2021). Here, the researchers examined ASF outbreak data from the OIE's World Animal Health Information System (OIE-WAHIS), which currently suffers from slow request processing speeds and an unintuitive web interface and lacks a means to download bulk datasets. Leray & Ward developed a series of Python 3 scripts to automate collection of public OIE-WAHIS data through acquisition, cleaning, and selection and processing steps. This method allowed comparatively rapid collection of large, comprehensive datasets from OIE-WAHIS (including precise outbreak locations, numbers of deaths and culled animals, etc.), though the researchers note that analyses of such collected data may be complicated by the different biases and reporting methods of different countries (Leray and Ward 2021).

Wild Boar Surveillance Data in Epidemiological Studies

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 (discussed further in the Epidemiology and Diagnostics sections). 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.

In 2020, Schulz *et al.* used surveillance data submitted to the EU's CSF & ASF wild boar surveillance database to evaluate the status of the ASF outbreak in Estonia, which at the time had not yet experienced a re-emergence of the virus (Schulz et al. 2020). Among other conclusions, they note that age classifications within this database lack precision (only identifying wild boar as < 1 year, 1-2 years, or > 2 years old). This increases uncertainty when attempting to determine the temporal dynamics of ASF introduction or eradication in specific regions (Schulz et al. 2020).

In a similar recent study, Martínez-Avilés analysed wild boar surveillance (serological and PCR-based detections of ASF) in Estonia, Latvia, Lithuania, and Poland during 2014-2018 (Martínez-Avilés, Iglesias, and De La Torre 2020). The researchers collected data from the EU Animal Disease Notification System

to characterize the nature of wild boar infection patterns in areas with a persistent presence of ASF. They observed the expected positive correlation between "time with infection" and density of seropositive animals, with some regional variability, and they inferred that Lithuania and Poland likely experienced more acute forms of ASFV in 2017 and 2018 than did Latvia and Estonia. The researchers concluded that, despite the limitations imposed by the difficulty of wild boar surveillance, serological and virological data from these populations is critical for assessing regional patterns of ASF virulence and transmission (Martínez-Avilés, Iglesias, and De La Torre 2020).

Ongoing research

Below is a discussion of some of the many ongoing and planned projects in the field of ASFV surveillance. This section is not a comprehensive discussion of research in this field but is instead intended to provide a brief overview of selected research projects based on feedback from a survey of researchers conducted during the writing of this report.

At the FAO, researchers are developing a tool for early discovery of ASFV by creating a database of geospatial data on wild boar cases and ecological factors and modelling risk factors for the detection of infected wild boar carcasses. This model has applications for risk-based surveillance systems and can help guide the cost-effective deployment of biosecurity resources.

Researchers at the FLI are also involved in research efforts to improve the early detection of ASF in wild boar populations.

Future research priorities

Based on the above literature review and with reference to previously identified knowledge gaps and expert opinion, the following areas of ASF surveillance should be considered priorities for future research:

- *Standardization of national and international ASF surveillance programs, particularly in wild boar*
- *International guidelines for harmonizing surveillance data collection*
- *User-friendly online tools and databases for the collection, viewing, and analysing of publicly available surveillance data*

- *Validation of new methods for efficiently collecting samples from domestic pigs and wild boar*
- *Automated, low-cost surveillance systems for on-farm detection of ASFV-infected domestic pigs*
- *Epidemiological studies to identify likely points-of-entry into high-risk, currently ASF-free regions*
- *Continuing surveillance of ASF epidemiology and evolution in Africa*

Diagnostics

Reliable diagnostic tests for the presence of ASFV are essential for identifying infected animals as early as possible, allowing efficient biosecurity responses that increase the possibility of an outbreak being controlled. A large number of such tests are available for the detection of infectious ASFV, viral DNA, and anti-ASFV antibodies in various sample matrices. Real-time PCR (qPCR) is currently considered the gold standard for diagnosis, and OIE-recommended assays using primers against the viral *p72*/*B646L* gene form the backbone of international diagnostic capabilities (e.g. at national reference laboratories in the EU) (Gallardo, Fernández-Pinero, and Arias 2019; Gaudreault et al. 2020; Gallardo, Nieto, et al. 2015). For virus isolation, field isolates are used to infect cultures of primary porcine leukocytes, where the viruses replicate and produce the cytopathic effects and haemadsorption (HAD) reaction that are characteristic of most ASFV strains (Gallardo, Fernández-Pinero, and Arias 2019). Finally, detection of specific ASFV antigens (usually the p72 protein) is accomplished with commercially available enzyme-linked immunosorbent assays (ELISAs) (Arias et al. 2018; Sauter-Louis, Conraths, et al. 2021). Antibodies against ASFV are also detected with ELISAs, providing important information for epidemiological studies and low-virulence strain identification, though they typically require confirmation via immunofluorescence, immunoblotting, or indirect immunoperoxidase tests (IPT) (Gallardo, Fernández-Pinero, and Arias 2019).

ASF diagnosis is an established field with numerous comprehensively validated tests available. There are several commercially available qPCR tests that have been validated by national and international reference laboratories, and these tests are as yet unmatched in sensitivity (true positive rate), specificity (true negative rate), and robustness. However, our current array of diagnostic tests has substantial gaps that recent studies have begun to fill. qPCR, for instance, has technical requirements that limit its applicability in many field situations where the required laboratory equipment, trained personnel, and cold storage may not be available (Gaudreault et al. 2020). Antigen ELISAs are higher-throughput and more automatable than qPCR, but they lack sensitivity and may therefore fail to detect infections (Sauter-Louis, Conraths, et al. 2021; ASF-STOP 2021; Gallardo, Nieto, et al. 2015). ELISAs against anti-ASFV antibodies, though generally highly sensitive, are only suitable for serum samples, limiting their applicability in areas that lack standardized wild boar sample collection programmes (Gallardo, Fernández-Pinero, and Arias 2019). Finally, reliance on primary cells for viral replication reduces convenience and standardization (Arias et al. 2018). This section will discuss some of the progress made since 2015 in the development of rapid, reliable, sensitive, and convenient diagnostic tests with the potential to overcome the limitations of currently available assays.

Previously identified knowledge gaps

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

- *New assays based on cellular immunity for early disease detection*
- *Study and validation of current ELISAs and PCR tests under experimental and field conditions*
- *Commercial tests for large-scale and confirmatory diagnostics*
- *New pen-side tests; continuing evaluation and validation of commercially available pen-side tests*
- *New cell lines to replace primary cultures for ASFV replication*
- *Validation of ELISAs for antibody detection from alternative sample types*
- *Improved stability of reagents in commercial diagnostic tests*
- *Non-invasive sampling techniques for wild Suidae*
- *Validation (particularly field validation) of existing tests for various epidemiological situations*

Literature review

ASFV DNA Diagnostics

Isothermal DNA amplification is a promising avenue for new ASFV DNA diagnostics that is relatively simple and does not require an expensive laboratory-based thermocycler. However, isothermal amplification techniques can suffer from low sensitivity (Gallardo, Fernández-Pinero, and Arias 2019). Numerous recent studies have described new tests based on this technology, with varying diagnostic parameters and potential benefits (ease of use, pen-side compatibility, etc.). This subsection will discuss some of the most notable new ASFV DNA diagnostic tests that have been developed in the last 6 years, with an emphasis on demonstrated diagnostic parameters (e.g. sensitivity and detection limits).

In 2017, Woźniakowski *et al.* published a polymerase cross-linking spiral reaction for isothermal detection of ASFV DNA in blood and tissue samples (Woźniakowski et al. 2017). Testing in an array of sample matrices from infected boars and domestic pigs demonstrated a limit of detection (LOD) of 7.2×10^{2} copies/µL and complete specificity for ASFV DNA (Woźniakowski et al. 2017). Fan *et al.*

compared two similar approaches – recombinase polymerase amplification (RPA) and recombinase-aided amplification (RAA) – within a novel field-deployable assay format. Sensitivity in clinical blood and tissue samples was ~97% for both assays compared to qPCR, with LODs of 93.4 and 53.6 copies/reaction for RPA and RAA, respectively (X. Fan et al. 2020).

D. Wang *et al.* developed real-time and visual (colorimetric) loop-mediated isothermal amplification (LAMP) assays targeting the viral p10 (*A104R*) gene, reporting a LOD of 30 copies/µL and 100% agreement with qPCR on synthetic ASFV DNA samples from various genotypes (D. Wang et al. 2020). Meanwhile, Zhu *et al.* developed a multiplex visual detection platform based on Hive-Chip and direct LAMP (via primers targeting five viral genes including *p72/B646L*), aiming for onsite detection of ASFV (Y. S. Zhu et al. 2020). Notably, this assay does not require a DNA extraction step – NaOH-treated swine blood is mixed directly with LAMP reagents, and the reaction generates fluorescence that is detected with 365nm UV light after a 1-hour reaction at 63°C. The assay's LOD was 50 copies/µL in mock samples, with detection parameters comparable to common commercial tests (Y. S. Zhu et al. 2020). Finally, in 2021, Y. Wang and colleagues reported their development of a one-step visual LAMP assay. Testing against 126 clinical samples (covering 5 genotypes and various blood, serum, and tissue types) demonstrated a LOD of 10 copies/reaction and 100% sensitivity and specificity compared to OIE-recommended qPCR in clinical samples, though the researchers note that crude samples required heat treatment (95°C for 10 minutes) to improve these parameters (Y. Wang, Dai, et al. 2021).

DNA amplification without thermocycling gives isothermal amplification-based diagnostics a substantial head start on pen-side applications, and evaluating these new methods in the field is a critical step on the path to commercialization and practical implementation. Mee *et al.* reported the field validation of a previously published real-time LAMP assay (James et al. 2010) during an outbreak in Timor-Leste (Mee et al. 2020). Here, the researchers eliminated this protocol's DNA extraction step and added an internal amplification control to increase field feasibility. Results demonstrated a LOD of 400 copies/reaction, and of 37 total tested serum samples, two were false positives and two were false negatives compared to qPCR (Mee et al. 2020).

Tran *et al.* modified a colorimetric LAMP assay for ASFV detection in crude serum samples from Vietnamese domestic pigs. The assay's minimum time to detection was short (~20 minutes), with a LOD of 10 50% haemadsorption doses (HAD_{50})/mL in serum and 100% sensitivity compared to commercial qPCR (D. H. Tran et al. 2021). The same year, Z.-H. Wang *et al.* developed a portable instrument for isothermal, real-time recombinase-aided amplification, achieving ~97% sensitivity with

a LOD of 10 copies/reaction when detecting the *p72/B646L* gene. Importantly, detection based on the *EP402R* (CD2v) gene achieved the same LOD, making this a potentially useful test for identifying animals inoculated with live attenuated vaccines (LAVs) lacking the *CD2v* gene (Z.-H. Wang et al. 2021). Such tests capable of Differentiating Infected from Vaccinated Animals (DIVA; discussed in more detail in the Vaccine section below) will be critical for monitoring the eventual deployment of potential LAV candidates. Velazquez-Salinas *et al.* also recently published three qPCR DIVA assays that detect the genes *MGF360-12L*, *UK*, and *I177L* (deleted from the Georgia 2007/1 strain to produce LAV candidates as described in the previous section). Tested on a panel of blood samples, these assays all displayed sensitivity, specificity, and amplification efficiency comparable to the diagnostic reference qPCR against the *p72/B646L* gene (Velazquez-Salinas et al. 2021).

An important point in colorimetric isothermal amplification assays is the accurate distinction of positive samples from animals with low viraemia, where slight colour differences can cause subjective bias and possible false negatives. One solution is the use of more highly chromogenic dyes, with pH-sensitive dyes being a popular choice (Y. Wang, Dai, et al. 2021; D. H. Tran et al. 2021; Yu et al. 2021). Careful photo analysis offers another potential solution as demonstrated by Yu *et al.*, who validated a semi-quantitative ASFV diagnostic method that combines LAMP with specific image processing techniques (Yu et al. 2021). Smartphone photos of completed reaction tubes were processed using the hue-saturation-value colour model, which separates image luminance from colour information and is therefore well-suited for analysing images taken in the field. The researchers reported a LOD of 10 copies/reaction, providing an important proof-of-feasibility for sensitive determination of colorimetric LAMP assays in the field (Yu et al. 2021).

CRISPR/Cas effector technology has also been incorporated into ASFV DNA diagnostics, with the single-stranded DNA-degrading activity of activated Cas12a attracting particular interest. Bai *et al.* published an assay that combines RAA with CRISPR/Cas12a, using lateral flow immunochromatographic strip readout for on-site ASFV detection (Bai et al. 2019). Tested on synthetic DNA, this test demonstrated femtomolar diagnostic sensitivity after one hour at 37°C, with a LOD of 6×10^2 copies/µL and no cross-reaction with other common porcine viruses (Bai et al. 2019). The next year, X. Wang *et al.* published a portable, instrument- and extraction-free ASFV detection method that also combines CRISPR/Cas12a with immunochromatographic strips and lateral flow detection (X. Wang et al. 2020). Tested on 149 clinical samples, this assay showed a LOD of 20 copies/reaction and 100% agreement with qPCR results (X. Wang et al. 2020). Finally, Tian *et al.* reported a dual-gene ASFV diagnostic tool that uses Cas12a and Cas13a to simultaneously detect the amplified products of a

multiplex RPA reaction, specifically illuminating two DNA and RNA fluorescent probes (Tian et al. 2022). Built into a handheld device for point-of-care testing and combined with a smartphone-based fluorescence readout system, this test demonstrated 100% sensitivity and specificity in analysis of 35 blood samples from swine with suspected ASF infections (Tian et al. 2022).

CRISPR-based isothermal ASFV DNA detection assays do not necessarily require nucleic acid amplification. In 2020, He *et al.* described a high-throughput isothermal assay without an amplification step (He et al. 2020). This assay uses CRISPR-Cas12a programmed with a crRNA to detect target viral DNA. DNA binding activates Cas12's enzymatic activity, cleaving a fluorescent single-stranded DNA reporter present in the assay, and the process is measured within a novel point-of-care system based on a disposable cartridge and custom fluorometer. Testing this assay on DNA-spiked porcine plasma samples demonstrated high specificity and a LOD of 1 pM within two hours (and 100 fM – or $5.7x10^4$ copies/µL – within 24 hours), without requiring complex sample preparation (He et al. 2020).

Chen *et al.* recently demonstrated a portable magnetofluidic device for rapid (< 30 minutes) ASFV diagnosis in animal samples (L. Chen et al. 2021). This device incorporates external magnetic arms and a rapid thermocycler, allowing automated DNA extraction, purification, and amplification via magnetic beads within the assay cartridge assembly. Tested on 149 clinical samples from Chinese farms and slaughter facilities, the device reached 92.2% positive and 93.6% negative agreement with OIE-recommended qPCR (L. Chen et al. 2021). The next year, Li *et al.* published a duplex qPCR assay for differentiating genotype I and II ASFV isolates, demonstrating its efficacy on 84 tissue, blood, nasal swab, and environmental swab samples (X. Li et al. 2022).

As discussed above, the restricted range of biological matrices suitable for many ASFV diagnostic tests makes sample collection difficult, particularly from wild boar. Validation of additional useable, easy-to-collect sample types would increase the applicability of ASFV detection assays and simplify wild boar sample collection by hunters. Carlson *et al.* published a relevant study in 2018, evaluating the use of dried blood swabs (specifically GenoTubes from Thermo Fisher) for the detection of viral DNA. qPCR tests on Estonian field samples gave 98.9% sensitivity and 98.1% specificity compared to anticoagulated (EDTA) blood, indicating that such fast-drying swabs may be an excellent alternative for wild boar sample collection (Carlson et al. 2018). Filter paper is another easy method for storing and transporting sample materials. This matrix suffers from numerous limitations, including limited nucleic acid recovery and inability to isolate infectious virus or antibodies (Sauter-Louis, Conraths, et al. 2021), but its simple and low-cost nature makes it useful in remote/rural field situations. Elnagar

et al. compared seven FTA Whatman filter paper cards, seven nucleic acid extraction methods, and 11 experimental eluates for optimal DNA extraction properties (Elnagar et al. 2021). Best results were obtained with GenSaver 2.0 FTA cards, and the optimal eluate was Chelex Resin 100 buffer (from Bio-Rad) mixed with either 1x Tris-EDTA (TE) buffer or TE+DMSO (Elnagar et al. 2021). In all such studies, it is important to remember that there may be strain-specific differences that impact the suitability of various matrices (e.g. swabs) for specific diagnostic techniques. Comprehensive validation is necessary to ensure that new assays and sample matrices can meet appropriate sensitivity thresholds in various epidemiological contexts.

ASFV Antigen Diagnostics

Commercially available ASFV antigen ELISAs currently lack sensitivity – Gallardo *et al.*, for instance, tested the INgezim PPA DAS K2 ELISA on a total of 171 experimental and field serum samples, finding only 77.2% sensitivity compared to the Universal ProbeLibrary (UPL)-PCR (Gallardo, Nieto, et al. 2015). New ASFV antigen detection tests have been developed to address this gap while maintaining simplicity, efficiency, and ease of use.

Lateral flow assays (LFAs) are one such category of tests with the potential to facilitate rapid, pen-side diagnosis from whole blood samples without requiring laboratory instrumentation. In 2016, Sastre *et al.* described an LFA for the detection of circulating ASFV p72 antigen in serum and anticoagulated blood samples (Sastre et al. 2016). This test demonstrated comparable or slightly higher sensitivity compared to the PPA DAS antigen-ELISA in experimental and field samples, respectively, though it remained inaccurate compared to UPL-PCR (detecting 35 out of 52 PCR-positive field samples) (Sastre et al. 2016). Practicality (e.g. speed, ease of use, and field usability) and accuracy are often at cross-purposes in pen-side tests, where rapid applicability is especially important at the start of suspected outbreaks (Gallardo, Fernández-Pinero, and Arias 2019; Blome, Franzke, and Beer 2020). As with molecular diagnostics, validation is critical to ensure that newly developed assays are suitable under different environmental conditions. This LFA, for instance (now commercialized as the INgezim ASFV CROM Ag) was recently tested by Deutschmann *et al.* for field diagnosis of wild boar carcasses (Deutschmann et al. 2021). Though reaching 100% diagnostic specificity and 77% sensitivity in high-quality experimental freeze-thawed matrices, the assay achieved only 12.5% sensitivity in field or “field-like” samples. This rose to 44% in freeze-thawed samples, suggesting that accessibility of viral antigens is a potential problem to be solved in future LFA development (Deutschmann et al. 2021).

Knowledge of immunodominant epitopes is important for increasing the sensitivity of serological assays. In 2019, Petrovan *et al.* characterized a panel of antibodies against p30, an immunogenic viral phosphoprotein that stimulates higher antibody responses than p72 during infection (Giménez-Lirola et al. 2016). These antibodies recognized the genotype I BA71v and genotype II Georgia 2007/1 strains *in vitro*, and the latter in tissue samples from infected pigs, via binding to p30 epitopes within amino acids 61-93 or in the protein's hydrophilic C-terminal region (Petrovan et al. 2019). Expanding on this study, Wu *et al.* comprehensively mapped the antigenic regions of the p30 protein, finding two highly conserved and immunodominant epitopes on the p30 protein (amino acids 116-125 and 146-160, both within the C-terminal region) (P. Wu et al. 2020). Epitope studies like these can increase our understanding of the immune response to various ASFV antigens and may provide useful tools for the development of new diagnostic antigen-ELISAs.

Meanwhile, Zhang *et al.* described the development of a signal-amplified sandwich colloidal gold test strip for detecting ASFV based on antibodies against the p30 protein (including one against the C-terminal region) (X. Zhang et al. 2021). Testing of 153 field samples (including serum, blood, and tissue) showed 96.8% specificity and 90% sensitivity compared with qPCR, though detection was not efficient in pigs with low levels of viraemia. Importantly, these test strips were stable for at least 1 year at room temperature or 4°C, increasing potential applicability pen-side and/or in remote regions (X. Zhang et al. 2021).

Detecting Antibodies against ASFV

ASF-infected Suidae produce antibodies against ASFV in all but peracute cases, and these antibodies can persist in the bloodstream for months or years (ASF-STOP 2021). Antibody-based diagnostics are therefore critical for tracking viral transmission, identifying previously infected animals, and detecting low-virulence ASFV isolates (Gallardo, Fernández-Pinero, and Arias 2019; Schulz, Staubach, et al. 2019). Currently, the gold standards for ASFV antibody detection are ELISAs (with validated commercial tests available) in conjunction with the IPT. These methods are widely used and extensively validated, but current methods suffer from some limitations including lack of ability to detect antibodies in non-serum samples and high resource/time requirements (Giménez-Lirola et al. 2016; P. Wu et al. 2020).

In 2015, Pourquier *et al.* presented the ID SCREEN, an ELISA based on three recombinant viral antigens (p32, p62, and p72) (Pourquier et al. 2015). This method was nearly 100% specific in various samples

from disease-free animals and correctly identified a small set of positive samples as well (including sera on filter paper) (Pourquier et al. 2015). Meanwhile, Giménez-Lirola *et al.* published a new recombinant p30 dual matrix ELISA for detection of ASFV antibodies in serum and oral fluid (Giménez-Lirola et al. 2016). This test was highly diagnostically specific and was able to identify oral fluid antibodies as early as 8 dpi in domestic pigs inoculated with replicon particles expressing the *p30* gene (Giménez-Lirola et al. 2016).

Developments in immunoassay design have also brought new paradigms to ASFV antibody detection. Liu *et al.* very recently described a rapid, semiautomated luciferase immunoprecipitation assay for ASFV antibody detection via magnetic beads modified with protein A/G and a recombinant ASFV p30/luciferase fusion protein (H. Liu et al. 2021). Testing in swine sera revealed 96.3% agreement and four-fold higher sensitivity compared to a commercial ELISA, though testing was not performed in non-optimal/field-like samples (H. Liu et al. 2021). Later that year, Yang *et al.* reported a chemiluminescence immunoassay for detecting antibodies against the viral p54 protein. Tests of 49 clinical serum samples determined a 100% consistency rate between this assay and the OIE-recommended commercial kit (Y. Yang et al. 2021). Future studies will be required to validate these new tests on various samples and conditions.

Finally, sample collection and the range of appropriate biological matrices are priorities for antibody detection assays as for DNA. In their previously discussed study, Carlson *et al.* also evaluated fast-drying swabs for ASFV antibody sample collection and ELISA analysis, finding 94.7% sensitivity and 96.1% specificity compared to anticoagulated blood (Carlson et al. 2018). Giménez-Lirola *et al.*'s results are also notable, since oral fluid collection is non-invasive and comparatively easy to collect from wild boar (Giménez-Lirola et al. 2016).

In Vitro Viral Isolation Techniques

Before a qPCR-positive sample is considered virologically positive, qPCR results must be confirmed by isolation of infectious virus. *In vitro* viral replication is therefore required both to diagnostically confirm the presence of live infectious virus and to produce sufficient amounts of a particular isolate for downstream analyses such as whole-genome sequencing (Rai et al. 2020). Historically, several cell lines have been used for the production and titration of individual ASFV strains – these include IPAM, COS-1, WSL, and ZMAC-4 cells (T. Wang et al. 2021). However, changes to the viral genome and immunogenicity during passaging are a significant concern, as are cell line-specific differences in ability

to propagate specific ASFV strains (Sánchez et al. 2017). Currently, *in vitro* viral isolation relies on cultured susceptible primary cells (e.g. porcine macrophages) to avoid these issues. Primary cell culture is an enduringly challenging process, and the unpredictable nature of primary cells introduces a variability that hinders the standardization of ASFV replication and isolation techniques (Arias et al. 2018). Replacing these primary cells with a stable cell line capable of supporting productive ASFV infection and replication would reduce these issues and allow new ASFV isolates to be characterized with greater ease and reliability.

One such cell line was identified in 2020, when Rai *et al.* reported the successful use of MA-104 cells (a commercially available African green monkey kidney epithelial cell line) for isolation of several infectious ASFV strains (Rai et al. 2020). Using these MA-104 cells to test for the presence of infectious virus, the researchers achieved a sensitivity ~10-fold lower than with primary swine macrophages but ~10-fold higher than a qPCR assay. Importantly, MA-104 cells infected with HAD isolates also exhibited haemadsorption in the presence of swine red blood cells, and cells infected with non-HAD isolates were identifiable via immunostaining (Rai et al. 2020). Rai *et al.* later published a detailed protocol describing infection of MA-104 cells for detection and quantification of infectious isolates via HAD assays or immunostaining (Rai et al. 2021). The same year, Wang *et al.* published their adaptation of the HLJ/18 strain to HEK293T cells, though the adapted strain displayed reduced infectivity in PAMs that correlated with stepwise losses to *MGF300* and *360* genes during passaging (T. Wang et al. 2021).

Masujin *et al.* described another stable cell line for ASFV infection and replication, using IPKMs (immortalized porcine kidney macrophages) that they had previously developed from primary macrophages by transducing cells with the SV40 large T antigen and porcine telomerase reverse transcriptase genes (Masujin et al. 2021). IPKMs supported high levels of viral replication (> 10^7 $TCID_{50}$/mL) and displayed the expected haemadsorption reactions and cytopathic effects during infection with the Armenia/07, Ken05/Tk1, and Espana75 isolates (Masujin et al. 2021)

Ongoing research

Below is a discussion of some of the many ongoing and planned projects in the field of ASFV diagnostics. This section is not a comprehensive discussion of research in this field but is instead intended to provide a brief overview of selected research projects based on feedback from a survey of researchers conducted during the writing of this report.

Within INIA, the CISA is a European Reference Laboratory for ASF and is therefore involved in ongoing development and harmonization of ASF diagnostic tests, training of experts for laboratory diagnosis, and deployment of experienced personnel in emergency situations. In 2022, researchers at CISA will begin developing a rapid, portable metal nanoparticle-based assay for the simultaneous detection of viral antigens and anti-ASFV antibodies as part of a partnership funded by the NIFA/USDA Agriculture & Food Research Initiative Competitive Grants Program.

At Kansas State University, researchers are developing molecular-, antigen-, and antibody-based point-of-care diagnostics. Meanwhile, at the University of Ibadan, scientists are collaborating with the Nigeria Veterinary Research Institute, conducting serological and molecular ASFV detection on Ibadan slaughterhouse/slab samples to investigate the potential role of asymptomatic carrier pigs in ASFV maintenance and spread. Future research directions include the development of an affordable, rapid pen-side diagnostic kit for ASFV.

Future research priorities

Based on the above literature review and with reference to previously identified knowledge gaps and expert opinion, the following areas of ASF diagnostics should be considered priorities for future research:

- *Field validation of portable, low-cost diagnostic assays for pen-side ASF testing under various environmental conditions*
- *Using clinical samples to validate cell lines to replace primary cultures for virus isolation and diagnosis*
- *Development of ASFV antigen diagnostic tests with increased sensitivity*
- *Standardization and commercialization of validated isothermal amplification- and/or CRISPR-based diagnostic tests*
- *Continued development and validation of DIVA tests that correspond to promising LAV candidates*
- *Comprehensive validation of novel or modified ELISA tests for antibody detection from non-serum samples (e.g. blood, oral fluids, meat juice, filter papers, etc.)*
- *Improved stability of reagents in commercial diagnostic kits*
- *Expanded field validation of novel assays (portable and non-portable) under various environmental conditions*
- *Validation of non-invasive sampling methods in wild Suidae*

- *Standardization of low-cost, easy-to-use sample collection and transport methods*
- *Ongoing training and outreach for international harmonization of diagnostic tests*
- *Rapid characterization of circulating ASFV strains in new outbreaks*

Vaccines

No effective commercial vaccine is or has ever been available for ASFV. Without a vaccine, costly and imperfect surveillance, biosecurity, and depopulation measures are our only means of controlling the disease. Detection of ASF is a death sentence for domestic herds, requiring the culling of both affected and unaffected pigs to limit viral spread as much as possible (Muñoz-Pérez, Jurado, and Sánchez-Vizcaíno 2021). Large-scale slaughter of pigs is a disaster from economic, sustainability, and animal welfare standpoints (Millet et al. 2021), and, as discussed above, it can drive human behaviours (e.g. emergency sales) that further the spread of disease. The development, validation, and deployment of an effective ASF vaccine is therefore of paramount importance and is one of the most active and fast-moving areas of ASFV research. We are currently closer than ever before to achieving this goal, but significant and unavoidable problems remain to be solved.

Many roadblocks hinder the development of a vaccine. One issue already mentioned is the lack of a stable cell line that can effectively propagate ASFV without forcing adaptive changes in the viral genome (Blome, Franzke, and Beer 2020). Meanwhile, critical questions remain unsolved within ASFV immunology and the correlates of protection against homologous viral strains. Not the least of these questions is: “what defines homologous and heterologous strains?” As discussed above, the genotyping of ASFV isolates based on the *p72/B646L* gene does not correlate with immunological response (i.e. there is no guarantee that infection with a specific genotype II strain will protect against infection with any other genotype II strain, even a very closely related one) (Malogolovkin et al. 2015). 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).

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).

Past attempts to produce an ASF vaccine have involved three primary strategies: (1) inactivated vaccines (virus particles "killed" via various treatments); (2) subunit vaccines (using immunoprotective antigens to induce an immune response capable of neutralizing the complete live virus); and (3) live attenuated vaccines (viable viruses genetically altered to prevent clinical symptoms while still inducing a protective immune response). Inactivated vaccines were quickly found to be ineffective, and more recent studies revisiting them in the context of the ongoing pandemic have not improved their prospects (Sánchez-Cordón, Montoya, et al. 2018; Cadenas-Fernández et al. 2021). Subunit vaccines have been hindered by our lack of knowledge on ASFV PAs, and studies involving single or multiple pooled antigens have almost universally had disappointing results (Rock 2021; Muñoz-Pérez, Jurado, and Sánchez-Vizcaíno 2021; Pérez-Núñez et al. 2019). It is possible that the complex nature of the ASFV particle may preclude the development of sufficient protective responses against any subunit vaccine (Rock 2021), but research is ongoing into potential subunit candidates as our knowledge of ASFV PAs continues to expand. Live attenuated vaccines (LAVs) form the vast majority of currently promising vaccine candidates, and recent advances in viral genome editing technology have greatly

expanded our ability to rapidly generate new LAV candidates. However, critical safety concerns (e.g. possible recombination with field strains or reversion to virulence) continue to persist for this class of vaccine.

Developments since 2015 in the field of ASF vaccine candidates are too broad and dynamic to be comprehensively summarized in any single literature review. Instead, the below section will discuss a selection of the most pertinent positive and negative results from LAV research, potentially promising subunit vaccine studies, and other vaccine-associated research developments.

Previously identified knowledge gaps

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*
- *Different routes of vaccination (e.g. intramuscular vs. oronasal)*

Literature review

Live Attenuated Vaccines

Cell 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 (10^3 HAD_{50} 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 (10^6 HAD_{50}). 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 10^2-10^4 $TCID_{50}$ 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 (10^2-10^6 HAD_{50}), 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).

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 $2x10^6$ HAD_{50}, 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 (10^2 HAD_{50}) 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). Finally, as mentioned above, Borca *et al.* demonstrated the utility of the PIPEC 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.

Ongoing research

Below is a discussion of some of the many ongoing and planned projects in the field of ASFV vaccine development. This section is not a comprehensive discussion of research in this field but is instead intended to provide a brief overview of selected research projects based on feedback from a survey of researchers conducted during the writing of this report.

At the CBMSO in Madrid, scientists are investigating the molecular mechanisms of ASFV's immunomodulation (of type I IFN pathways, in particular) and are developing cell lines to sustain production of new LAV candidates. Researchers at this institute are also developing new LAV

candidates, using CRIPSR/Cas9 to generate recombinant gene-deleted viruses for subsequent characterization by next-generation sequencing and *in vivo* testing. Upcoming projects at CBMSO will continue this line of research through (a) development of new LAVs with improved biosafety characteristics (e.g. limited replication *in vivo*), and (b) investigation of ASFV's monocyte-macrophage tropism and its implications for vaccine design.

At Kansas State University, researchers are tackling the challenges of ASFV modified live vaccine and subunit vaccine development from several directions, including (a) the identification of viral antigens capable of eliciting granzyme B^+ T cell responses, (b) the evaluation of live-vectored (e.g. modified leukaemia virus) subunit vaccines that express rationally selected ASFV antigens, and (c) the optimization of ASFV antigen delivery *in vivo* for induction of cytotoxic T lymphocyte responses. Meanwhile, scientists at the FRCVM are identifying and characterizing ASFV PAs and their variability among heterologous strains. Upcoming research projects at this institute include a study focusing on the theoretical and experimental justification of methods for comprehensive evaluation of candidate LAV strains.

Researchers at INIA are involved in the EU-funded VACDIVA project, which aims to develop three safe pilot vaccines for domestic pigs and wild boar alongside companion DIVA tests and cost-effective vaccination plans.

Future research priorities

Based on the above literature review and with reference to previously identified knowledge gaps and expert opinion, the following areas of ASF vaccine development should be considered priorities for future research:

- *Continuing study of ASFV protein functions/antigenicity and the mechanisms of homologous and heterologous protective immunity*
- *Cross-protective studies of LAV candidates*
- *Standardization of LAV testing protocols to facilitate comparisons and ensure reproducibility*
- *Studies of biosafety and long-term protection in previously validated LAV candidates*
- *Safety testing of new and validated LAV candidates (e.g. testing for presence of virus in tissues, evaluating vaccine strain shedding, etc.)*

- *Identification of DIVA markers*
- *Testing of dose- and route-specific protective efficacy*
- *Identification of specific ASFV subunit combinations with protective effects*
- *Development of an effective antigen delivery platform*
- *Working with stakeholders and government officials to encourage sufficient economic investment in eventual ASF vaccine deployment*

Drugs and Therapeutic Approaches

There are currently no effective antiviral drugs marketed for the treatment of ASF (Y. Wang, Kang, et al. 2021). The development of new anti-ASFV drugs depends on a thorough understanding of the functions of viral proteins, including structural studies to identify important active domains that may be susceptible to small molecule inhibition. As discussed above, many ASFV proteins remain functionally uncharacterized. As with vaccines, the lack of effective antiviral agents places more pressure on biosecurity and depopulation measures, driving up economic costs in the event of an infection and limiting our capacity for outbreak control. Fortunately, recent studies have begun to make inroads toward effective ASFV antivirals, clarifying the functions and potential vulnerabilities of proteins critical for viral replication and testing various small molecules for their therapeutic efficacy.

Previously identified knowledge gaps

Previous reports identified the following priority research knowledge gaps in ASF drug/therapeutic development over the past 6 years:

- *Physiologically relevant analyses of experimentally identified anti-ASFV compounds*
- *Potential role of anti-ASFV drugs in reducing viral transmission*
- *Continuing movement toward licensed antiviral drugs for ASF treatment*

Literature review

The replication cycle is a common target of antiviral drugs when this process involves viral enzymes that are structurally/functionally distant enough from host cell machinery to allow targeted, selective inhibition. In 2018, Arabyan *et al.* published a study on the flavonoid molecule genistein, which they had previously found to inhibit ASFV replication *in vitro* via an unknown mechanism (Arabyan et al. 2018). Here, the researchers tested the effect of genistein on infection of PAMs with the Armenia/07 strain and found a 50% inhibitory concentration (IC_{50}) of 17 µM, below cytotoxic levels. Best results were achieved with treatment at 8 hours post-infection, associated with a significant decrease in viral DNA levels in the perinuclear virus factories. Subsequent molecular docking studies indicating that genistein binds strongly to four specific residues at the ATP-binding site of the viral type II topoisomerase (ASFV-topo II), leading to DNA fragmentation and blocking late viral gene transcription. ASFV-topo II, encoded by the *P1192R* gene, is highly conserved among ASFV strains, giving genistein potential as a cross-strain antiviral drug (Arabyan et al. 2018). In 2020, Fan *et al.* described an

alternative, immunological approach, testing combined recombinant porcine IFN-α and IFN-γ for emergency preventive treatment of pigs infected with the virulent SY18 strain (W. Fan et al. 2020). *In vitro*, treatment of PAMS with 100 or 1,000 U/mL doses significantly inhibited viral replication and triggered the production of IFN-induced genes and MHC-I transcription. *In vivo*, pigs were inoculated orally with 10^2 $TCID_{50}$ of SY18, after which IFNs were injected intramuscularly once/day for 3 days. Low-dose treatment (10^5 U/kg) gave better results than high doses (10^6 U/kg), significantly reducing viral load in treated pigs (W. Fan et al. 2020).

In the same year, Liu *et al.* conducted a structural analysis of pA104R (ASFV's histone-like protein), solving crystal structures for its apo (unbound) and DNA-bound states (R. Liu et al. 2020). The researchers found that pA104R has a DNA-binding pattern distinct from its bacterial homologs (Frouco et al. 2017), with β-ribbon arms facilitating binding to the major groove instead of the minor groove. Two stilbene derivatives disrupted this DNA binding, with one in particular (called "SD4") exhibiting an IC_{50} of 6.1 µM for this molecular inhibition. *In vitro*, this translated to an IC_{50} of 0.48 µM for virus production (corresponding to a 95% reduction in viral DNA levels with 50 µM treatment), with very low associated cytotoxicity (50% cytotoxic concentration [CC_{50}] of 263 µM) (R. Liu et al. 2020). Another potential drug target is pE165R, the viral-encoded dUTPase for which unique structural features were previously reported (G. Li et al. 2020). Liang *et al.* conducted structural comparisons between this protein and porcine dUTPase, confirming that pE165R employs a unique two-subunit active site in contrast to the host enzyme's classical three-subunit structure (Liang et al. 2021). These researchers subsequently tested a compound (here called simply "compound 1") that was originally developed as an inhibitor of the *Plasmodium falciparum* dUTPase. Enzymes kinetics and surface plasmon resonance analysis indicated that compound 1 competitively inhibited ASFV dUTPase (equilibrium dissociation constant = 15.6 µM), with two-fold selectivity for the viral over the host protein (Liang et al. 2021).

Meanwhile, Sirakanyan *et al.* tested drugs that target microtubules, which are used during infection to move virions to the perinuclear space (viral factories), recruit synthesized viral proteins for virion assembly, and transport newly-made viruses to the cell surface (Sirakanyan et al. 2021). *In silico* screening against tubulin's colchicine binding site identified a promising compound, called "6b", that reduced *in vitro* viral replication in a dose-dependent manner (IC_{50} = 17.1 µM for infection of PAMs with Armenia/07). Unusually for microtubule-targeting agents, 6b also showed minimal cytotoxicity (CC_{50} > 500 µM) and *in vivo* toxicity (up to 100 mg/kg in mice). Mechanistically, the compound appeared to interfere with ASFV attachment, internalization, and egress, showing greater efficacy when added within 2-8 hours post-infection (Sirakanyan et al. 2021). *In silico* models are growing

increasingly popular in drug discovery research as our computational resources continue to expand. Choi *et al.* recently applied machine learning models to the prediction of ASFV inhibitors (Choi et al. 2021), focusing specifically on the viral reparative DNA polymerase X (*Asfv*PolX). The researchers conducted molecular docking studies of FDA-approved drugs with crystal structures of *Asfv*PolX in its apo and DNA-bound forms, identifying ten top-ranked compounds via principal component analysis and *k*-means clustering. One of these compounds, pentagastrin, inhibited the activity of purified *Asfv*PolX by 60% at a concentration of 100 µM (Choi et al. 2021). These results serve as an interesting proof-of-concept for molecular docking and machine learning models, which may play an increasing role in identifying antiviral candidates.

Galindo *et al.*, meanwhile, studied a panel of experimental and FDA-approved compounds known to target the endosomal membrane (Galindo et al. 2021). Blocking endosomal membrane proteins can cause "entry reduction" in viruses like ASFV that use the endosomal pathway to enter cells, reducing their infectivity. Here, the researchers observed >80% entry reduction in ASFV infectivity (BA71v strain) of Vero cells treated with four drugs individually: Tetrandrine, an inhibitor of the TPC-1 and -2 endosomal calcium channels; Verapamil, which non-specifically modifies intercellular calcium levels; Apilimod, an inhibitor of the endosomal kinase PIKfyve; and Raloxifen, a selective oestrogen receptor modulator. Raloxifen treatment had a particularly low IC_{50} (0.91 µM) and high sensitivity index (CC_{50}/IC_{50} = 26.42) and correlated with reduced expression of the p30 protein. Interestingly, this effect was independent of the oestrogen receptor pathway and endosomal acidification, with proposed explanations instead including altered endosomal cholesterol accumulation and calcium flux (Galindo et al. 2021).

Later that year, Huang *et al.* tested the antiviral activity of GS-441524, a 1-cyano-substituted adenine C-nucleoside ribose analogue that inhibits viral RNA transcription by competing with natural nucleosides (Z. Huang et al. 2021). This compound was previously shown to be effective against coronaviruses, Ebola, and other RNA viruses, but its efficacy against DNA viruses like ASFV had not yet been tested. In this study, the researchers measured *p72/B646L* mRNA levels and p30 protein levels in treated PAMs, finding that GS-441524 inhibited transcription with a 50% effective concentration (EC_{50}) of 73.2 µM (and a CC_{50} of 287.5 µM). This mechanism was unrelated to the levels of IFN-α, IFN-β, TNF-α, and IL-6 (Z. Huang et al. 2021).

Future research priorities

Based on the above literature review and with reference to previously identified knowledge gaps and expert opinion, the following areas of ASF drug/therapeutic development should be considered priorities for future research:

- *Further testing of FDA-approved drugs and natural products for ASFV inhibition*
- In vivo *validation of putative antivirals tested* in vitro
- In silico *screening for new ASFV replication inhibitors*
- *Scalable methods for the economical deployment of antivirals to infected animals in the field*

Disinfectants

The environmental tenacity of ASFV allows transmission via contact between animals and contaminated surfaces. Disinfection therefore plays an important role in ASF biosecurity, particularly in preventing viral transmission to uninfected animals after post-outbreak restocking and in keeping environmental contaminants from entering pig holdings (Guinat et al. 2016; Dixon et al. 2020). Commercial disinfectants are available for the elimination of ASFV, but important research continues into new methods for rapid and effective disinfection of a variety of sample matrices. Especially important is the evaluation of organic matter (e.g. faeces, blood, etc.) that are often present within pig holdings and can interfere with the activity of many chemical disinfectants (Krug et al. 2018).

Previously identified knowledge gaps

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

- *Low-cost commercial disinfectants that inactivate ASFV on contaminated surfaces and carcasses*
- *Environmental impact studies on existing disinfectants*

Literature review

In 2018, Krug *et al.* evaluated several disinfectants on surfaces in pork-packing plants, finding that their efficacy was substantially reduced in the presence of blood and meat juices. Acidic substances (e.g. citric acid) did retain efficacy in the presence of faeces, but sodium hypochlorite did not (Krug et al. 2018). Importantly, the researchers also reported difficulties recovering control ASFV from porous concrete, a commonly encountered surface that lacks validated disinfection protocols (Krug et al. 2018). Gabbert *et al.* later addressed this gap, publishing a laboratory method for recovering pH-sensitive viruses (including ASFV, here using strain BA71v) from unsealed concrete (Gabbert, Neilan, and Rasmussen 2020). Using this protocol, the researchers demonstrated that inactivating ASFV on carbonated concrete required ten minutes of contact with 5% Virkon S disinfectant solution (compared with 2% for five minutes on stainless steel) (Gabbert, Neilan, and Rasmussen 2020).

Similarly, Juszkiewicz *et al.* tested the effects of four commercial disinfectants – sodium hypochlorite, potassium peroxymonosulfate, glutaraldehyde, and quaternary ammonium compounds – against

ASFV (BA71v strain). The latter two were too cytotoxic to allow proper interpretation of results (involving *in vitro* infection of Vero cells with inactivated virus solutions), but the former were both effective at inactivating ASFV – sodium hypochlorite at 0.5% and 1% in low-level soiling, and potassium peroxymonosulfate at 1% in high-level soiling (Juszkiewicz et al. 2019). The same group later expanded on this study, evaluating eight disinfectants and using microfiltration to reduce the cytotoxicity of inactivated virus solutions. Here, sodium hypochlorite, glutaraldehyde, caustic soda, and potassium peroxymonosulfate gave the best results (> 5-log reduction in viral titre), though removal of soiling remained a critical step (Juszkiewicz et al. 2020).

Pan *et al.* evaluated highly complexed iodine (HPCI) as a disinfectant, comparing it to the commercially available povidone-iodine (PVP-I) (Pan et al. 2021). They reported that that HPCI and PVP-I were both minimally cytotoxic, and 0.25% HPCI was able to completely inactivate 10^5 $TCID_{50}$/mL of ASFV via spray disinfection within five minutes – 0.25% PVP-I, conversely, failed to completely inactivate the virus under the same conditions (Pan et al. 2021). Finally, McCleary *et al.* recently characterized the ability of 1% Virkon S, BD FACS Lysing buffer, and Qiagen AVL buffer to reduce the infectivity of an 8.05 log_{10} HAD_{50}/mL solution of the Ken05/Tk1 ASFV strain (McCleary et al. 2021). A 30-second incubation with a 20:1 proportion of 1% Virkon S completely inactivated the virus, and similar results were achievable by both commercial buffers after a ten-minute incubation, validating their use for the safe transport of treated infected samples from BSL-3 facilities (McCleary et al. 2021).

As discussed above, environmental transmission of ASFV via contact with boar carcasses is a significant concern in regions with endemic wild boar outbreaks. Carlson *et al.* conducted a relevant study of the stability of ASFV in soil and various chemical options for disinfecting this matrix (Carlson et al. 2020). The researchers spiked soil samples with blood from wild boar experimentally infected with the Armenia08 strain and were able to isolate infectious virus from sterile sand for at least 3 weeks; from beach sand for up to 2 weeks; from yard soil for 1 week; from swamp soil for 3 days; and not at all from two acidic forest soils. They note, however, that forest soils are heterogeneous both within and between regions, so these results on forest soil from Northern Germany are not necessarily applicable in different environments. One-hour treatments with citric acid or calcium hydroxide completely inactivated virus within all matrices (Carlson et al. 2020).

ASFV can also survive in components of pig feed, and several recent studies have been published on methods of inactivating virus these matrices. Kalmar *et al.* tested the sensitivity of ASFV (specifically the Vero cell-adapted Lisbon/60 strain) to various physical and chemical conditions that can be applied

to blood used in the production of spray dried porcine plasma, a pig feed ingredient; they reported that combined treatment with 48°C heat, pH of 10.2, and 20.6 mM hydrogen peroxide for ten minutes inactivated 4.17 log_{10} $TCID_{50}$ of ASFV/mL plasma (Kalmar, Cay, and Tignon 2018). Later, Jackman and colleagues evaluated MCFAs (specifically caprylic, capric, and lauric acids, in a dry mix of 65% MCFA to 35% silica) and glycerol monolaurate (GML) as ASFV-inhibiting feed additives (Jackman et al. 2020). GML had higher efficacy than MCFAs when included in feed – treatment of feed samples with 2% GML resulted in 88% reduction in the infectivity of the BA71v strain after 30 minutes. Notably, GML also appeared to decrease the amount of structurally intact p72 protein present, potentially altering viral antigenicity (Jackman et al. 2020). Tran *et al.* also studied the effect of MCFAs in an *in vitro* feed model, reporting no cytotoxicity in PAMs at 100 µg/mL and strong antiviral activity at 0.375% and 0.5% concentrations (H. T. T. Tran et al. 2021). Niederwerder and colleagues applied a similar strategy to test the ability of two chemical additives – (1) 37% aqueous formaldehyde and propanoic acid, and (2) an equal-volume blend of the hexanoic, octanoic, and decanoic MCFAs – to inactivate Georgia 2007/1 ASFV in an experimental model of 30-day transoceanic shipment (Niederwerder et al. 2021). Inclusion of 1% MCFA mix or 0.33% formaldehyde-based additive into feed samples inoculated with 10^5 $TCID_{50}$ of ASFV lowered viral titres to undetectable levels after "shipment". Out of 24 pigs tested in a subsequent bioassay, only one showed evidence of ASFV infection, providing evidences that chemical feed additives can at least mitigate the risk of viral introduction via feed transport (Niederwerder et al. 2021).

Future research priorities

Based on the above literature review and with reference to previously identified knowledge gaps and expert opinion, the following areas of ASF disinfection should be considered priorities for future research:

- *Evaluation of disinfectants under field conditions*
- *Increasing understanding of ASFV survival in the environment*

Conclusions

The studies described in this report were selected based on practical parameters (excluding papers not published or available in English) and the degree to which they directly addressed previously identified research gaps in ASFV research (GARA 2018; 2016). The ongoing threat posed by African swine fever has galvanized the ASFV research field, leading to a massive upswing in associated publications. A search of the PubMed database for “African swine fever”, for instance, shows that 45 relevant papers were published in 2014 compared to 398 in 2021. At the time of writing this report (early February 2022), 59 relevant papers had already been published this year. ASFV research is very active and fast-moving. Even this breakneck pace, however, has had trouble matching the speed at which the ongoing pandemic has moved through Eurasia and beyond. Many valuable research papers and reviews were published between 2015 and 2018, but this timeframe accounts for only ~25% of the total papers cited in this report. Far from reflecting a lack of quality or importance in these papers, this is simply attributable to many of them being superseded by recent events. Genomic studies, for instance, have been transformed by new techniques like third-generation sequencing and CRISPR/Cas; novel vaccine candidates have been validated and refined; and previously cutting-edge reviews and risk assessments have been rendered outdated by the introduction of ASF to China and its subsequent spread through Asia. At the time of writing, it is still too early for published risk assessments to have incorporated the recent outbreaks in the Dominican Republic and Haiti, and if ASF reaches the American mainland, it will bring another significant shift in the epidemiological outlook of the current pandemic. The next ASFV research review/gap analysis may look very different from this one.

Meanwhile, the enduring COVID-19 pandemic continues to strain national resources and place additional burdens on international collaboration and research efforts. In this situation, increased research harmonization and international collaboration are a necessity to allow the fastest possible responses to new ASF developments. This includes standardization of viral genome sequencing techniques and diagnostic tests, streamlining of pipelines for reporting ASF detections and accessing these data, and increased integration of research, policy, and resource implementation. For the time being, biosecurity and surveillance remain our only means of preventing and controlling the spread of ASFV. At the local level, particularly in the many resource-poor regions currently suffering with endemic ASF, biosecurity solutions cannot be disentangled from the socioeconomic needs of pig farmers and smallholders – any control measures or strategies that do not take this reality into account are likely doomed to failure. An effective response to this disease will therefore require sustained coordination between researchers, regulators/policymakers, and stakeholders at all levels of the pig production chain.

With this situation in mind, the substantial progress reported here encourages optimism about the future of the ASFV research field. In particular, we are nearer than ever before to the deployment of an effective live attenuated vaccine, and essential studies of biosafety and long-term efficacy are proceeding apace. Rapid advances in sequencing technology, genomics, transcriptomics, and proteomics are rapidly closing knowledge gaps in the ASFV genome and viral protein functions. Computational modelling will continue to open new doors in epidemiology, structural biology, surveillance and risk assessment, and other critical fields of ASFV research. Meanwhile, increasing integration of social sciences into the fields of participatory biosecurity and epidemiology are beginning to address the foundational socioeconomic factors underpinning poor biosecurity.

Altogether, we hope that this report will provide a useful resource for increasing understanding of the advances made between 2015-2021 and for focusing ASF research on areas of critical need. ASFV is a complex and mysterious virus at every level, but the progress made since 2015 has answered many questions relating to the virus itself, the host response to infection, and the interactions between the two. The pace of ASFV research holds great promise for the future, and effective measures for the control and eradication of this disease appear closer now than ever before.

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Appendices

Abbreviations

ANSES	The French Agency for Food, Environmental and Occupational Health & Safety
ASF	African Swine Fever
ASFV	African Swine Fever Virus
bp	base pair
CBMSO	Centro de Biología Molecular Severo Ochoa (Centre for Molecular Biology “Severo Ochoa”)
CC_{50}	50% Cytotoxic Concentration
cGAS-STING	cyclic GMP-AMP Synthase-Stimulator of Interferon Genes
CIRAD	Centre de cooperation Internationale en Recherche Agronomique pour le Développement (French Agricultural Research Centre for International Development)
CISA	Centro de Investigación en Sanidad Animal (Centre for Animal Health Research)
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
crRNA	CRISPR RNA
CSF	Classical Swine Fever
Ct	Cycle threshold (in qPCR)
DIVA	Differentiating Infected from Vaccinated Animals
DNA	Deoxyribonucleic Acid
dpi	days post-infection/immunization
dsDNA	double-stranded DNA
EC_{50}	50% Effective Concentration
EDTA	Ethylenediaminetetraacetic Acid
EFSA	European Food Safety Authority
ELISA	Enzyme-Linked Immunosorbent Assay
ELIspot	Enzyme-Linked Immunosorbent spot
EMÜ	Eesti Maaülikool (The Estonian University of Life Sciences)
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FLI	Friedrich-Loeffler-Institut (the Friedrich Loeffler Institute)
FMD	Foot and Mouth Disease
FRCVM	Federal Research Center for Virology and Microbiology
GARA	Global African Swine Fever Research Alliance

GO	Gene Ontology
HAD	Haemadsorbing/Haemadsorption
HAD_{50}	50% Hemadsorption Dose
HAI	Hemadsorption Inhibition
hpi	hours post-infection
IC_{50}	50% Inhibitory Concentration
IFN	Interferon
Ig	Immunoglobulin
IKK	IκB Kinase
IL	Interleukin
IM	Intramuscular
INIA	Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (National Centre for Agricultural and Food Research and Technology)
iNKT	invariant Natural Killer T cell
IPT	Immunoperoxidase Test
ITR	Inverted Terminal Repeat
KAP	Knowledge, Attitudes, and Practice
kb	kilobases/kilobase pairs
KEGG	Kyoto Encyclopedia of Genes and Genomes
LAMP	Loop-Mediated Isothermal Amplification
LAV	Live Attenuated Vaccine
LFA	Lateral Flow Assay
LOD	Limit Of Detection
LVR	Left Variable Region
MCFA	Medium-Chain Fatty Acid
MGF	Multigene Family
MHC	Major Histocompatibility Complex (also called SLA in pigs)
miRNA	microRNA
moDC	monocyte-derived Dendritic Cell
MOI	Multiplicity Of Infection (viruses per cell)
MVA	Modified Vaccinia Ankara
NF-κB	Nuclear Factor-κB
NGS	Next-Generation Sequencing
NK	Natural Killer

OIE	World Organization for Animal Health
OIE-WAHIS	OIE World Animal Health Information System
ONT	Oxford Nanopore Technologies
ORF	Open Reading Frame
PA	Protective Antigen
PAM	Porcine Alveolar Macrophage
PBMC	Peripheral Blood Mononuclear Cell
PRR	Pattern Recognition Receptor
qPCR	quantitative real-time Polymerase Chain Reaction
RAA	Recombinase-Aided Amplification
RNA	Ribonucleic Acid
RPA	Recombinase Polymerase Amplification
RVR	Right Variable Region
scnRNA	small noncoding RNA
SDS	Sodium Dodecyl Sulphate
SLA	Swine Leukocyte Antigen (also called MHC)
spp.	Species
$TCID_{50}$	50% Tissue-Culture Infectious Dose
TNF	Tumour Necrosis Factor
Treg	Regulatory T cell
UPL	Universal ProbeLibrary
US/USA	United States/United States of America
USD	US Dollars
USDA	US Department of Agriculture

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Conflict of Interest Statement

The authors declare no conflict of interest.

Additional Resources

Please see the following websites for additional information and/or resources around the content and aims of this report:

OIE-WAHIS

GARA

STAR-IDAZ

ASFVdb (from Zhu & Meng 2020)