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Review article

Current strategies, advances, and challenges in multi-epitope subunit vaccine development for African swine fever virus

Ella Mae Joy S. Sira[1], Edward C. Banico[1], Lauren Emily Fajardo[1], Nyzar Mabeth O. Odchimar[1], Alea Maurice Simbulan[1] and Fredmoore L. Orosco[1,2,*]

[1]*Virology and Vaccine Research and Development Program, Industrial Technology Development Institute, Department of Science and Technology, Bicutan, Taguig City 1634, Philippines.*

[2] Department of Biology, College of Arts and Sciences, University of the Philippines Manila, Manila 1000, Philippines.

Abstract

African Swine Fever (ASF), a highly contagious and lethal viral disease affecting swine populations, presents a critical global threat with no approved vaccine. Traditional approaches such as whole virus-based vaccines have several limitations, prompting interest in peptide-based subunit vaccines. However, the inefficacy of existing peptides and the complexity of the ASFV genome further complicate antigen screening. Immunoinformatics has addressed this challenge by utilizing bioinformatics tools for the design and evaluation of multi-epitope subunit vaccines. Although multi-epitope subunit vaccines offer safety advantages, their potential to induce both humoral and cellular immune responses is crucial for protective immunity against ASFV infection. Despite the growing interest in computational vaccine design, a notable gap exists in *in vivo* confirmation studies. This review addresses the challenges and advances in ASFV multi-epitope subunit vaccine development, underlining the urgency of a safe and effective vaccine given ASF's global impact on swine populations and associated economic losses.

Keywords: African swine fever virus, Multi-epitope vaccine, Reverse vaccinology, Subunit vaccines,

*Corresponding author: Fredmoore L. Orosco, Virology and Vaccine Research and Development Program, Industrial Technology Development Institute, Department of Science and Technology, Bicutan, Taguig City, Philippines. Department of Biology, College of Arts and Sciences, University of the Philippines Manila, Manila 1000, Philippines. E-mail: orosco.fredmoore@gmail.com.

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INTRODUCTION

African Swine Fever (ASF) is a highly contagious and deadly viral disease that affects domestic and feral swine, warthogs, bushpigs, and wild boars (Dixon et al., 2019; Njau et al., 2021). Although non-zoonotic, ASF poses a serious threat, as its mortality rate can reach 100%, with cases in 52 countries from Asia, Europe, Africa, and the Americas (World Organisation for Animal Health, 2023). From 2016 to 2022, ASF has caused more than 19,000 outbreaks, losing almost 10 million domestic pigs (Orosco, 2023a; Orosco, 2023b). This exerted a significant global impact, particularly in China, the world's largest pork producer. In September 2019, China suffered direct economic losses estimated at $141 billion, affecting both the meat and animal feed markets worldwide (Berthe, 2020) Due to its international spread and significant impact on the health of domestic swine and wild boars, the World Organisation for Animal Health included ASF in “listed diseases”, along with classical swine fever, Nipah virus encephalitis, porcine respiratory and reproductive syndrome, porcine cysticercosis, and transmissible gastroenteritis. This classification includes diseases that are acknowledged as issues of global significance (World Organisation for Animal Health, 2023).

The causative agent of the ASF is African Swine Fever Virus (ASFV). It is the only member of the family Asfarviridae and the sole DNA arbovirus, that infects soft tick species from the genus *Ornithodoros* (Dixon et al., 2019; Gaudreault et al., 2020; Zhu, 2022). The virion has a complex structure, including an internal nucleoprotein core, an internal lipid membrane, an icosahedral capsid, and an outer lipid envelope (Alejo et al., 2018; Blome et al., 2020).

ASFV has developed various mechanisms to antagonize host immune responses, enabling persistent infections (Niu et al., 2023; Orosco, 2023a). This rapid evolution of pathogens hinders the development of an effective viral vaccine. Currently, vaccine development strategies for ASFV are broadly classified into whole virus-based (whole-inactivated and live-attenuated) and subunit vaccines (Sang et al., 2020).

Whole-inactivated vaccines for ASFV were proven ineffective in stimulating cellular responses, and thus, were not considered a viable strategy for a vaccine (Cadenas-Fernández et al., 2021). In contrast, live-attenuated vaccines for ASFV have induced adequate levels of protection but retained residual virulence in the proportion of the immunized population (Gallardo et al., 2015). Subunit vaccines circumvent the possible persistence of residual virulence (Lopera-Madrid et al., 2021) and have been found to confer more extended protection than live-attenuated vaccines (Bosch-Camós et al., 2020; Centers for Disease Control and Prevention, 2023). The traditional method of subunit vaccine development involves the use of large proteins; however, this approach results in unnecessary loads in the vaccine that increase its overall allergenicity (Chauhan et al., 2019). Therefore, there has been growing interest in the development of peptide-based subunit vaccines. However, in terms of effectiveness, the current set of tested peptides has shown inadequate protective immunity against ASFV (Cadenas-Fernández et al., 2020), implying the need to discover novel antigens.

The screening of potential antigenic determinants within the ASFV remains a challenge owing to its complex nature, which encodes more than 150 ORFs from which no expression or functional data are available (Bosch-Camós et al., 2020).

Advancements in vaccinology have led to a new era in vaccine research and development. New approaches, such as immunoinformatics, have gained popularity because of their ability to analyze genomic and proteomic information from target pathogens using bioinformatics tools and screen potential epitopes that

can induce both humoral and cellular immune responses (Kanampalliwar, 2020). One central approach in immunoinformatics is epitope mapping, which localizes potential epitopes on the surface of antigens recognized by immune receptors (Nilvebrant and Rockberg, 2018) and integrates them to design a subunit vaccine. This methodology offers a rapid and inexpensive approach to vaccine design.

More than a hundred years have passed since ASFV was first identified; however, there is still no commercially available vaccine to date. The need to develop an effective vaccine for ASFV is underlined by the absence of existing vaccines that are fully approved and licensed for use in commercial pig populations (Rock, 2017). Therefore, further research and development of ASFV vaccines remains essential in the fight against this disease. Previous review papers (Chathuranga and Lee, 2023; Orosco, 2023b) have discussed progress on ASFV vaccine research; however, none have delved into the details of the current progress of a multi-epitope subunit vaccine against ASFV. This review provides a summary of the current state of research on multi-epitope subunit vaccine development for (ASFV including the challenges in the area and recent advances in the broader field of vaccinology that can be harnessed to create safe and effective vaccines against ASFV. This review aims to provide insights into innovative approaches and potential solutions, bridging the gap between the current challenges and promising advancements in ASFV multi-epitope subunit vaccine development.

VACCINE STATUS

Current vaccines for ASFV can be broadly classified into two types: whole virus-based vaccines and subunit vaccines. The differences between these vaccine types, including their composition, safety profiles, effectiveness, and development processes, will be explored in subsequent sections.

Whole virus-based Vaccines

Vaccines based on a whole pathogen offer a significant advantage over other forms of vaccines in terms of stability and preservation of a substantial portion of the antigenic determinants of the pathogen (Chambers et al., 2016). Whole virus vaccines use either an inactivated or an attenuated (weakened) form of the virus.

Inactivated vaccines

Inactivated vaccines are produced by rendering virulent viruses non-infectious while preserving their ability to trigger an immune response (Lopez et al., 2023). Since the pathogen is completely inactivated, this type of vaccine guarantees the prevention of any reversion to a virulent phenotype of the virus in the recipient. Past efforts to develop an inactivated vaccine for ASFV that is capable of conferring protection have been unsuccessful (Forman et al., 1982; Kihm et al., 1987; Mebus, 1988; Stone and Hess, 1967). Inactivated forms of the pathogen were observed as antigenic, but could not stimulate the body to produce complete immunity. Hence, exploring inactivation methods that retain the essential antigens required to trigger a sufficient immune response has been a subject of interest over the years. Various modern inactivation methods have been explored in pursuit of a comprehensive solution (Table 1).

Table 1 Inactivated strains of ASFV for vaccine development and their methods of inactivation.

Strain	Method of inactivation	Challenge	Protection	References
Armenia08	binary ethyleneimine (BEI)	Homologous (Armenia08)	0%	Blome et al., 2014
Pol16/DP-OUT21	binary ethyleneimine (BEI)	Homologous (Pol16/DP-OUT21)	0%	Cadenas-Fernández et al., 2021
Estonia2014	gamma-irradiation	Heterologous (Armenia08)	0%	Pikalo et al., 2022

Cutting-edge adjuvants such as MF59®, silica oil, mGNE, MontanideTM ISA201 VG (Cadenas-Fernández et al., 2021), and Polygen™ or Emulsigen®.D (Blome et al., 2014) were used in combination with the inactivated strains to induce complete protection against challenges of homologous strains of ASFV. However, these attempts were unsuccessful, indicating that the failures of inactivated ASFV vaccines extend beyond the choice of adjuvants.

The negative results of these studies suggest that an inactivated virus as a strategy for the ASFV vaccine may not be a viable option. The insufficiency of inactivated vaccines in generating protection against ASFV has been attributed to the absence of a cellular immune response (Cadenas-Fernández et al., 2021; Orosco, 2023b). An *in vitro* study further confirmed that anti-ASFV antibodies were insufficient to effectively inhibit viral replication, and the interaction between humoral and cellular immune responses is indeed needed to combat the disease (Walczak et al., 2022). However, viral replication within the host is essential to elicit a potent and lasting cellular immune response (Cadenas-Fernández et al., 2021).

Protection from ASF heavily relies on the host's cellular immune response, which is not induced by the inactivated form of the pathogen. The viral antigens required to activate this response need an active but less efficient viral replication within the host. This may explain the superior efficacy of the attenuated virus over its inactivated counterpart in *in vitro* studies (Liu et al., 2021).

Live attenuated vaccines

Attenuated vaccines are weakened versions of the target pathogen, engineered to maintain the capability for limited replication within the host. Since they present a broader array of relevant antigenic determinants, including those reliant on active metabolism, this type of vaccine can induce more robust T-cell responses, high titers of virus-neutralizing antibodies, and provide a longer duration of protection from clinical disease compared to inactivated vaccines (Chambers et al., 2016).

In a recent study conducted by Bourry et al. (2022), unintended attenuation of the Georgia 2007/1 strain (ASFV-989) was generated because of the supposed thermal inactivation of the strain. Inoculation of pigs with this attenuated form led to the rapid development of anti-ASFV antibodies and specific cellular immune responses. Notably, this attenuated strain exhibited the same replication kinetics within the host as the parental Georgia 2007/1 strain did. However, it is important to note that this study did not establish a clear correlation between viral replication and the presence of cellular immune responses. Nevertheless, during the challenge with the homologous strain (Georgia 2007/1), viremia remained low to negligible, and pigs survived. In addition, virus attenuation for vaccine studies has traditionally

been achieved through natural, subculture, or recombinant methods (Liu et al., 2021).

Naturally attenuated (NA) strains occur naturally during epidemics, and are primarily driven by genetic mutations and adaptive changes. These strains are less harmful or less capable of causing severe disease than the typical virulent strains of ASFV (Wang et al., 2021). While at least six well-known naturally attenuated strains of ASFV have been identified (Azarpajouh, 2023), only three strains (NH/P68, OUR T88/3, and Lv17/WB/Rie1) have been properly explored for vaccine design studies (See Table 2). The other three naturally attenuated strains of ASFV are Estonia 2014, HuB20, and Pig/Heilongjiang/HRB1/2020.

Table 2 Naturally attenuated strains of ASFV.

Strain	Challenge	Protection	References
NH/P68	Heterologous (L60)	100%	Leitão et al., 2001
OUR T88/3	Homologous (OURT88/1)	100%	Oura et al., 2005
	Homologous (OURT88/1)	100%	Boinas et al., 2004
	Homologous (OURT88/1)	50-100%	Sánchez-Cordón et al., 2017
	Homologous (OURT88/1)	50%	Mulumba-Mfumu et al., 2016
	Heterologous (DRC 085/10)	100%	Mulumba-Mfumu et al., 2016
	Heterologous (Uganda 1965)	100%	King et al., 2011
	Heterologous (Benin 97/1)	86%	King et al., 2011
	Heterologous (Lisbon-57)	25%	Boinas et al., 2004
	Heterologous (Malawi)	0%	Boinas et al., 2004
Lv17/WB/Rie1	Homologous (Latvian)	100%	Gallardo et al., 2019

The results of these experiments provide valuable insights into the host response to a range of parameters involved in conferring immune protection. Aside from the challenge virus, other parameters such as administration dose and route of administration, were found to affect the protection conferred by naturally attenuated strains of ASFV (Sánchez-Cordón et al., 2017). Moreover, observations from the study by Oura et al. (2005) revealed the importance of the cytotoxic T-cell population in eliminating ASFV-infected cells. Pigs with high levels of $CD8^+$ cells showed reduced levels of viremia or any clinical symptoms of ASF compared with pigs with depleted levels of $CD8^+$ cells after challenge with a homologous strain.

The utilization of naturally attenuated strains as a vaccine strategy for ASFV requires further investigation because of the documented toxic side effects that could potentially extend to the quality of pigs for pork production. These side effects are caused by high residual virulence and are expected in pigs vaccinated with naturally attenuated strains (Gladue and Borca, 2022). Efforts to reduce residual virulence are required to facilitate the adoption of this vaccine in clinical settings.

The second attenuation strategy involves successive subculturing of virulent strains into sensitive cells for multiple generations. This process leads to spontaneous deletion of specific portions of the viral genome, significantly reducing viral virulence (Zhang et al., 2023). The use of an attenuated form of the virus as a vaccine strategy was initiated following the successful attenuation of ASFV by Manso-Ribeiro et al. (1963) through successive subculturing on porcine bone marrow cells. Subsequently, pigs vaccinated with these attenuated strains showed resistance to virulent strain attacks. Multiple replicate studies using the updated strains and cell lines are presented in Table 3.

Table 3 Subculture-attenuated strains of ASFV.

Strain	Cell lines	Challenge	Protection	References
Georgia 2007/1	Vero cells	Homologous (Georgia 2007/1)	0%	Krug et al., 2015
Stavropol 01/08	A4C2, CV-1	Homologous (Stavropol 01/08)	0%	Balysheva et al., 2015
Georgia 2007/1-ΔI177L	PIPEC	Homologous (Georgia 2007/1)	100%	Borca et al., 2021

Among the three well-known cell-passage attenuated strains of ASFV, all displayed considerable potential for attenuation. However, two of them yielded disappointing results in terms of protection from homologous strains, except for a study involving the Georgia 2007/1-ΔI177L strain adapted to the porcine fetal kidney cell line (PIPEC). The strain displayed attenuation but efficient protection efficacy, similar to that of the virulent parental strain. While this discovery offers promise for the large-scale production of the ASFV vaccine in cell lines, it is important to note that the safety and genetic stability of the Georgia 2007/1-ΔI177L/ΔLVR strain still requires comprehensive evaluation (Wang et al., 2021)

Further reduction of the residual virulence of attenuated vaccines can be achieved by targeted knockout of virulence-associated genes (VAGs) or immunosuppressive immune escape-related genes (Liu et al., 2021). Notably, the reported virulence-associated genes of ASFV mainly include TK (K196R), UK (DP96R), 23-NL (DP71L), 9GL (B119L), and CD2v (EP402R) (Tulman et al., 2009; Liu et al., 2021), and the immune escape-related genes mainly include MGF 360/530, A238L (5EL), 8DR (pEP402R), 8CR (pEP153R), and caspase 3 (pA224L) (Tulman et al., 2009). Additionally, the deletion of genes inhibiting interferon (γ) activity presents another avenue for investigation, focusing on A276R from MGF360, A528R from MGF530, and I329L (Correia et al., 2023, 2013). Although new VAGs, such as I177L, I226R, and A137R, have been identified, the precise mechanisms through which these genes influence virulence are still not fully understood (Wang et al., 2021). Strains with deletions of these genes, either individually or in combination, have been considered potential candidates for vaccine evaluation (Table 4).

Ongoing clinical evaluations of Georgia 2007/1-ΔI177L and HLJ/18-7GD as potential vaccines for ASFV have been documented (Han et al., 2023). Notably, Georgia 2007/1-ΔI177L was able to induce protection against virulent Vietnamese ASFV field strains (Tran et al., 2022). This VAG has been used to create a commercially available vaccine within the ASF-endemic regions of Vietnam (Lai et al., 2022).

While certain knockout VAGs, including Georgia 2007/1-ΔI177L, have shown promise in protecting both homologous and heterologous viruses, safety concerns remain essential. These concerns include post-viral shedding, post-vaccination complications, recombination with field viruses, and insufficient protection of immunocompromised pigs, requiring a cautious approach to the widespread promotion of an attenuated vaccine (Chambers et al., 2016; Sereda et al., 2020; Han et al., 2023). Achieving a fully attenuated vaccine requires the deletion of multiple VAGs. Nonetheless, the simultaneous deletion of VAGs may risk over-attenuating ASFV, potentially leading to a reduced or complete loss of protection (Wang et al., 2021). Furthermore, the molecular basis of ASFV virulence can differ among strains, and it is essential to note that biological alterations resulting from gene deletions in one ASFV strain may not necessarily mirror those in other strains (Chen et al., 2020).

In addition, according to Zhang et al. (2023), the development of live attenuated vaccines is costly, with prolonged development cycles and inherent instability, imposing constraints on their rapid progress.

Table 4 Recombinant attenuated strains of ASFV through deletion of virulence-associated genes (VAGs).

Strain	Deleted Gene	Challenge	Protection	Reference
Malawi Lil-20/1	B119L	Homologous (Malawi Lil-20/1)	100%	Lewis et al., 2000
Georgia 2007/1	B119L	Homologous (Georgia 2007/1)	100%	O'Donnell et al., 2015
Pretoriuskop/96/4	B119L	Homologous (Pretoriuskop/96/4)	100%	Carlson et al., 2016
Benin 97/1	DP148R	Homologous (Benin 97/1)	94%	Reis et al., 2017
Georgia 2007/1	E184L	Homologous (Georgia 2007/1)	60%	Ramirez-Medina et al., 2022
BA71	EP402R	Homologous (BA71)	100%	Lopez et al., 2020
BA71	EP402R	Heterologous (E75)	100%	Lopez et al., 2020
BA71	EP402R	Heterologous (RSA/11/2017)	83%	Lopez et al., 2020
BA71	EP402R	Heterologous (Ken06.Bus)	33%	Lopez et al., 2020
BA71	EP402R	Homologous (BA71)	100%	Monteagudo et al., 2017
BA71	EP402R	Heterologous (E75)	100%	Monteagudo et al., 2017
BA71	EP402R	Heterologous (Georgia 2007/1)	100%	Monteagudo et al., 2017
BA71	EP402R	Heterologous (Georgia 2007/1)	50-100%	Bosch-Camós et al., 2022
OURT88/3	I329L	Homologous (OURT88/1)	33%	Reis et al., 2020
Georgia 2007/1	I177L	Homologous (Georgia 2007/1)	100%	Borca et al., 2020
Georgia 2007/1	K196R	Homologous (Georgia 2007/1)	0%	Sanford et al., 2016
Kenya-IX-1033	A238L	Homologous (Kenya-IX-1033)	67%	Abkallo et al., 2022
Kenya-IX-1033	A238L, EP402R	Homologous (Kenya-IX-1033)	50%	Abkallo et al., 2022
NH/P68	A238L, A224L, EP153R	Homologous (NH/P68)	100%	Gallardo et al., 2018
NH/P68	A238L, A224L, EP153R	Heterologous (Arm07)	17%	Gallardo et al., 2018
OUR T88/3	DP71L, DP96R	Homologous (OURT88/1)	66%	Abrams et al., 2013
HLJ/18	MGF360 (12L, 13L, 14L), MGF505 (1R, 2R, 3R), EP402R	Homologous (HLJ/18)	100%	Chen et al., 2020
Benin 97/1	MGF360 (9L, 10L, 11L, 12L, 13L, 14L), MGF505 (1R, 2R, 3R, 4R)	Homologous (Benin 97/1)	100%	Reis et al., 2016
SY18	MGF100 (7L-11L)	Homologous (SY18)	100%	Zhang et al., 2021

Subunit Vaccines

While live attenuated vaccines have demonstrated promising results in challenge studies, recent research has shifted its focus to the development of safer vaccine alternatives. Subunit vaccines offer a targeted approach with fewer side effects and increased safety compared with live attenuated virus vaccines (Orosco, 2024). Numerous immunogenic ASFV proteins have been identified and examined for their potential role in protecting against ASF, which are summarized and discussed in greater detail in this review.

ASFV structural proteins, namely, p30 (*CP204L*), p54 (*E183L*), p72 (*B646L*), pp62 (*CP530R*), and CD2v (*EP402R*), have been the targets of subunit vaccine approaches, either as individual components or in combination in a multi-target formulation (Gaudreault and Richt, 2019). These proteins exist in vaccine formulations as whole proteins or only their antigenic peptides and are combined with bacterial, viral, or functional proteins to increase immunogenicity (Han et al., 2023). These proteins or peptides can be expressed in bacterial or yeast cells or can be synthesized within a virus-like particle (VLP). Li et al. (2023) reported high titer antigen-specific antibody response upon inoculation of VLPs displaying the antigenic proteins p30, p54, p72, CD2v, and K145R on the surface of T7 phages.

Stimulation of immune responses to specific proteins can be accomplished through immunization with DNA sequences. DNA vaccines commonly utilize plasmids that contain the target genes and can be introduced into the recipient through injection or using specialized equipment like gene guns (Netherton, 2021). Initial experiments with DNA vaccines targeting ASFV using a plasmid expressing the p30 and p54 fusion protein yielded unsatisfactory outcomes in pigs (Argilaguet et al., 2012; Argilaguet et al., 2011). Similar proteins were expressed using baculovirus, a viral vector, and an increase in protection was observed (Barderas et al., 2001; Gómez-Puertas et al., 1998). This highlights the importance of the delivery system for the outcome of the vaccine (Netherton, 2021).

Several viral vector vaccines employ a diverse range of viruses, including retroviruses, lentiviruses, adenoviruses, poxviruses, alphaviruses, arenaviruses, herpesviruses, flaviviruses, paramyxoviruses, and rhabdoviruses (Ravilov et al., 2022). These viral vectors have been optimized to improve their capacity to package genetic material, target specific cells, and enhance viral replication. Viral vectors offer a highly versatile platform for vaccine development owing to their capacity to elicit consistent antigen-specific humoral and cellular immune responses, mitigating the risk of reverting to a virulent state, and ensuring clear differentiation between vaccinated and infected animals (Gaudreault and Richt, 2019). In ASFV, a limited range of viral vectors have been studied for vaccine design. These include baculoviruses, alphaviruses, adenoviruses, lentiviruses, pox vaccinia virus, and Newcastle Disease Virus (Ravilov et al., 2022). Thus, only a few have been tested against severe ASFV infection (Table 5).

Table 5 Viral vectors used for protein expression of ASFV genes for vaccine development.

Vector	(Strain) Gene	Challenge	Protection	Reference
Baculovirus	(E70) O61R	Homologous (E70)	0%	Carrascosa et al., 1995
	(E75) EP402R	Homologous (E75)	100%	Ruiz-Gonzalvo et al., 1996
	(E75) E183L, CP204	Homologous (E75)	50%	Gómez-Puertas et al., 1998
	(E75) CP204L, E183L	Homologous (E75)	100%	Barderas et al., 2001
	(Pr4) CP204L, E183L, B646L, KP177R	Homologous (Pr4)	0%	Neilan et al., 2004
	(E75) E183L, CP204L, EP402R	Homologous (E75)	67%	Argilaguet et al., 2013
Adenovirus	(Georgia 2007/1) A151R, B119L, B602L, EP402RΔPRR, B438L, K205R, A104R, CP530R, B646L	Homologous (Georgia 2007/1)	0%	Lokhandwala et al., 2019
	(strain not identified) I215R, I73R, CP530R, CP204L, MGF110-5L, B646L, MGF110-4L, M448R, L8L, E146L, C129R, A151R, MGF 110-1L, L10L, K78R, E184L, E165R, CP312R	(strain not identified) OUR T88/1	0%	Netherton et al., 2019
	(Georgia 2007/1) CP204L, E183L, CP530R, B646L, CP2475L	Homologous (Georgia 2007/1)	56%	Lokhandwala et al., 2019
	(OUR T88/3) B602L, B646L, CP204L, E183L, E199L, EP153R, F317L, MGF505-5R	Homologous (OUR T88/1)	100%	Goatley et al., 2020

The development of a successful ASFV vaccine requires more comprehensive research to understand the functions of ASFV genes, identify protective proteins, and determine the most suitable combinations for a vaccine. Although no one-size-fits-all vector exists, baculoviruses and adenoviruses have shown promise as potential vaccine delivery platforms for ASFV. To date, no study has directly compared the protection conferred by different viral vectors using the same set of ASFV genes, indicating the need for further research in this area.

CURRENT CHALLENGES IN ASFV VACCINE DEVELOPMENT

The challenges encountered in the development of ASFV vaccines primarily stem from the complex nature of the virus (Gaudreault and Richt, 2019; Zhu, 2022). With a genome ranging from 170 to 193kb encoding 150 to 167 genes (Blome et al., 2020; Zhu, 2022), the task of identifying essential elements to induce a robust and protective immune response is challenging. This difficulty is compounded by the limited known functions of many of the identified viral genes (Bosch-Camós et al., 2022).

The high genetic variability of ASFV strains poses a challenge in developing a universal vaccine that provides broad protection against diverse viral variants. The immune response generated by a vaccine is often strain-specific (Urbano and Ferreira, 2022). This is a common issue when using live attenuated vaccines via deletion of VAGs, where rational deletion does not consistently yield the desired outcome. Lopez et al. (2020) reported that the removal of the *EP402R* gene from the

genotype I BA71 isolate resulted in the attenuation of the virus, providing protection against challenges from both homologous and heterologous (E75 and RSA/11/2017) virulent viruses. However, the same deletion did not protect against the heterologous strain (Ken06.Bus). A similar result was observed in a study by Gallardo et al. (2018), where deletion of *A238L*, *A224L*, and *EP153R* genes from the NH/P68 strain protected pigs from the challenge of the virulent homologous strain but not from a challenge of a heterologous (Arm07) strain.

Aside from the significance of strain-specific immunity conferred by previous vaccine designs for ASFV, the understanding of interactions among multiple genes remains incomplete (Gaudreault and Richt, 2019). Various gene combinations resulted in different protection rates, either by inhibiting or enhancing immune responses, as demonstrated by *in vivo* tests. For example, a single deletion of *EP402R* confers complete protection against the challenges of homologous strains (Monteagudo et al., 2017; Lopez et al., 2020). Additionally, a single deletion of *A238L* led to partial protection against homologous strain challenge (Abkallo et al., 2022). However, double deletion of these two genes resulted to a lesser protection than *A238L* and *EP402R* single deletion mutants (Abkallo et al., 2022). Given these findings, further investigation of the complex interactions among the viral genes is recommended to refine the understanding of immunity against ASFV. There is also a need to understand the host response, especially viral receptors, innate immune responses, and the interaction of the virus with the host at the cellular level (Blome et al., 2020).

ASFV PROTEOMICS

Proteins capable of inducing antibody or cell-mediated immunty

The ASFV genome has a 170–194 kb linear dsDNA genome encoding 68 structural proteins and 150-200 non-structural proteins (Dixon et al., 2019; Tesfagaber et al., 2021; Miao et al., 2023). In particular, p30 (*CP204L*), p54 (*E183L*), p72 (*B646L*), and p22 are highly immunogenic, which is attributed to their antibody-inducing capabilities. These proteins have been widely used in immunogenicity studies, vaccines, and diagnostics (Gaudreault and Richt, 2019; Imatdinov et al., 2020). Moreover, the fusion of the recombinant forms of the four proteins stimulated the production of neutralizing antibodies. However, neutralizing antibodies are only able to delay the onset of the disease and are insufficient in protecting animals against the infection, underscoring the involvement of cellular immune responses in the protection against ASFV, such as the $CD8^+$ lymphocyte subset (Schäfer et al., 2022; Zhang et al., 2023).

The immune systems of pigs have a large number of $CD4^+CD8^+$ double-positive (DP) T cells. The role of DP T-cells in ASFV infection remains unclear; however, Oura et al. (2005) reported that anti-CD8 antibodies can nullify the protective immunity of swine against highly virulent ASFV (Sun et al., 2021), implying the important role of $CD8^+$ T-cells in protection. Jancovich et al. (2018) screened 47 ASFV antigens to identify those capable of eliciting cellular responses and to gauge their potential to induce protective immunity. Antibody responses to antigen 127 (*CP204L*/p30) were consistently high across all groups, 3- to 4-fold above background levels. Antigens 194 (*L10L*), 145 (*D117L*), and 205 (*EP153R*) also showed a more than 2-fold increase in response. Although *CP204L*/p30 is expected to induce mainly $CD4^+$ T cells, which have shown to induce the greatest IFN-γ-

producing cells among the antigens, P30 is also recognized by CTL in ASFV-immune pigs (Netherton et al., 2019).

Another noteworthy protein is P22, a structural protein encoded by the *KP177R* gene that resides in the inner envelope of the ASFV virion. Recent genomic research has shed light on the multifaceted role of the P22 protein, revealing its interactions with host proteins involved in various cellular functions, such as cell signaling transduction, cell structure maintenance, and virus binding processes. Although the absence of p22 in a recombinant ASFV does not impact the virus's pathogenicity or virulence, immunization with p22 can elicit a heightened antibody response (Li et al., 2023).

p72 (*B646L*) is a highly conserved structural protein of ASFV and is involved in viral capsid assembly, virus adsorption, and susceptible cell invasion. It is one of the major antigens detected in pigs, constituting approximately 31 - 33% of the total mass of the virion (Liu et al., 2019). Twelve linear B-cell epitopes and one conformational neutralizing epitope have been identified in p72 (Yin et al., 2022; Miao et al., 2023). Along with pp62, p72 has also been shown to stimulate the strongest IFN-γ production in live pigs, which is attributed to its large size. Macrophages or dendritic cells (DC) degrade p72 into polypeptides, which are then presented by SLA-I indicating its role in cellular immune response. This is further confirmed by the $CD8^+$ lymphocyte depletion assays conducted which showed an increase in $CD8\alpha^+$ $tetramer^+$ double-positive T cells after infection, signifying the activation of specific $CD8^+$ T cell immune responses (Sun et al., 2021).

p54 is a key protein in ASFV which plays a vital role in viral morphogenesis and infection. Notably, research has shown that anti-p54 sera can hinder ASFV attachment to susceptible cells, indicating its involvement in viral entry. Likewise, the p54/E183L gene is indispensable for virus viability and recruitment of envelope precursors to assembly sites (Tesfagaber et al., 2021). Thirteen nanobodies against p54 (Nbs) were identified with Nb8 (GenBank accession no. OM459819), which shows high affinity and specificity. One linear B-cell, 76QQWVEV81 epitope, was identified using Nb8-horseradish peroxidase (Nb8-HRP). The identified epitope is conserved across different ASFV strains (Zhao et al., 2022; Zhao et al., 2023). Other notable proteins with reported immunogenic responses to ASFV are listed in Table 6.

Table 6 Proteins identified to be immunogens/antigens that are able to or have potential to induce humoral, cellular, or both immune responses.

Gene	Protein	Function	Ab (+/-)	T Cell (+/-)	Disease Enhancement (+/-)	References
Structural						
B438L	p49	Necessary for vertices formation of the icosahedral capsid	+	+	+	Lokhandwala et al., 2017; Alejo et al., 2018; Gaudreault and Richt, 2019; Bosch-Camos et al., 2020
B646L	p72	Major component of viral icosahedrons and play a crucial role in the formation of viral capsid during the later stages of viral expression. Also involved in viral entry.	+	+	+	Jia et al., 2017; Gaudreault and Richt, 2019; Bosch-Camos et al., 2020
CP204L	p30	Antigenic structural proteins.	+	+	+	Jia et al., 2017; Gaudreault and Richt, 2019; Bosch-Camos et al., 2020; Xu et al., 2023
CP530R	pp62	Precursor for polyproteins that can be proteolytically yielding mature virions.	+	+	+	Jia et al., 2017; Gaudreault and Richt, 2019; Bosch-Camos et al., 2020
CP530R/partial	p15	Involved in the assembly of viral capsid	+	-	+	Jia et al., 2017; Gaudreault and Richt, 2019; Bosch-Camos et al., 2020
	p35	Involved in the assembly of viral capsid	+	-	+	Jia et al., 2017; Gaudreault and Richt, 2019
CP2475L	pp220	P150, p37, p14, and p34 precursor. Important for packaging of nucleoprotein core,	NA	+	NA	Gaudreault and Richt, 2019; Bosch-Camos et al., 2020
CP2475L/partial	p37	Involved in the assembly of viral capsid	+	+	+	Bosch-Camos et al., 2020
D117L	p17	Transmembrane protein found within the internal envelope of the virus that is vital for facilitating the transition of viral membrane precursors into icosahedral intermediates and ensuring the virus's viability.	+	low	+	Jia et al., 2017; Gaudreault and Richt, 2019; Bosch-Camos et al., 2020
E120R	p14.5	DNA-binding protein that is necessary for the transport of virions to plasma membrane.	+	NA	+	Jia et al., 2017; Gaudreault and Richt, 2019; Bosch-Camos et al., 2020
E183L	p54	Antigenic structural proteins.	+	+	+	Gaudreault and Richt, 2019; Bosch-Camos et al., 2020; Xu et al., 2023
E199L	j18L	Necessary for viral core entry. Downregulates PYCR2 expression resulting in the activation of autophagy.	NA	+	+	Gaudreault and Richt, 2019; Bosch-Camos et al., 2020; Matamoros et al., 2020; Chen et al., 2021; Miguel Ángel Cuesta-Geijo et al.; 2022
EP402R	CD2v	ASFV hemagglutinin. Protective T-cell epitopes housed in the extracellular domain. Plays a role in viral persistence in blood.	+	+	+	Lokhandwala et al. 2017; Gaudreault and Richt, 2019; Bosch-Camos et al. 2020; Vlad Petrovan et al., 2022; Xu et al., 2023
H108R	Inner envelope	Involved in the virulence of ASFV.	+	low	+	Gaudreault and Richt, 2019; Bosch-Camos et al., 2020; Vuono et al., 2022

Gene	Protein	Function	Ab (+/-)	T Cell (+/-)	Disease Enhancement (+/-)	References
KP177R	p22, outer envelope		+	+	+	Gaudreault and Richt, 2019; Bosch-Camos et., 2020; Xu et al., 2023
O61R	p12,	P12 plays a role in the attachment of the virus to host cells, and the membrane proteins present on the surface of permissive cells serve as receptors for ASFV.	+	+	+	Jia et al., 2017; Gaudreault and Richt, 2019; Bosch-Camos et al., 2020
A104R	Viral histone-like	Primes strong antibody responses	+	+		Lokhandwala et al., 2017; Gaudreault and Richt, 2019; Bosch-Camos et al., 2020
K78R	p10	Involved in virus attachment.	low			Jia et al.. 2017; Gaudreault and Richt, 2019; Bosch-Camos et al., 2020; Xu et al., 2023
A137R	P11.5	Involved in virus attachment	+			Jia et al. 2017; Bosch-Camos et al., 2020; Urbano and Ferreira, 2022
B602L	P72 chaperone	Major capsid protein that plays a role in virus entry.	+	+		Jia et al. 2017; Bosch-Camos et al. 2020; Urbano and Ferreira, 2022; Xu et al., 2023
CP530R	pp62	p35 and p15 polyprotein precursor.	+	+		Jia et al., 2017; Bosch-Camos et al., 2020, Sun et al., 2021
CP530R/partial	p35	Important in the assembly of the viral capsid.	+	-	+	Jia et al., 2017; Bosch-Camos et al. 2020
CP530R/partial	p15	Important in the assembly of the viral capsid.	+	-	+	Jia et al., 2017; Bosch-Camos et al., 2020
EP153R	C-type lectin	Increase red blood cells and ASFV-infected cells binding, inhibits cell surface expression of SLAI and inhibits apoptosis.	+	+	+	Bosch-Camos et al., 2020;Vlad Petrovan et al., 2022
E248R	Transmembrane myristoylated protein	Involved in the fusion step that releases the naked viral core from the late endosome (LE) to the cytoplasm.		+		Bosch-Camos et al., 2020; Cuesta-Geijo et a., 2022
Nonstructural						
A151R	viral replication	Necessary for virus replication and morphogenesis. Involved in viral transcription.	+	+		Lokhandwala et al. 2017; Gaudreault and Richt, 2019
B119L	9GL	Requirement in virus assembly.	+	+		Lokhandwala et al., 2017; Gaudreault and Richt, 2019; Bosch-Camos et al., 2020
B602L	P72 chaperone	Deletion leads to severe alteration of viral assembly.	+	+	+	Lokhandwala et al., 2017; Gaudreault and Richt, 2019; Bosch-Camos et al., 2020
F1055L	Helicase	Involved in DNA replication specifically in the initiation point replication	NA	+	+	Gaudreault and Richt, 2019; Bosch-Camos et al., 2020; Wang et al., 2021
G1211R	DNA polymerase	Involved in DNA replication specifically in the initiation point replication	NA	+	+	Gaudreault and Richt, 2019; Wang et al., 2021
L10L	KP117R-related	Function remains unknown but may have a role in signaling pathways. Simultaneous deletion of I7L, I8L, I9L, I10L, and I11L led to a decrease in virulence.	+	NA	+	Gaudreault and Richt, 2019; Bosch-Camos et al., 2020; Cackett et al., 2022

Gene	Protein	Function	Ab (+/-)	T Cell (+/-)	Disease Enhancement (+/-)	References
MGF360-11L	KP362L	Hypothesized to have a role in immune evasion and infection tropism.	NA	+	+	Gaudreault and Richt, 2019; Bao et al., 2021
MGF505-4R		Hypothesized to have a role in immune evasion and infection tropism.	NA	+	+	Gaudreault and Richt, 2019; Bao et al., 2021
NP419L	DNA ligase	Involved in base excision repair.	NA	+	+	Gaudreault and Richt, 2019; Bosch-Camos et al., 2020; Wang et al., 2021
NP1450L	RNA polymerase subunit 1	Contains genes coding for the attachment protein of ASFV to vero cells.	NA	+	+	Yáñez et al., 1993; Gaudreault and Richt, 2019
F334L	RNA reductase	Facilitates the formation of deoxyribonucleotides.	+	+		Gaudreault and Richt, 2019; Bosch-Camos et al., 2020; Avagyan et al., 2022
K196R	Thymidine kinase	Plays a roles in nucleotide and DNA synthesis. Deletion of this gene significantly reduces replication in macrophages.	NA	+		Cubillos et al., 2013; Gaudreault and Richt, 2019; Bosch-Camos et al., 2020; Peng-hao, 2020; Avagyan et al., 2022
I215L	Ubiquitin conjugating enzyme	May play a role in the suppression of cyclin synthesis in PAM,		+		Gaudreault and Richt, 2019; Bosch-Camos et al., 2020; Avagyan et al., 2022
C475L	Poly(A) polymerase	Involved in mRNA modification specifically in 3' end modification.		+		Bosch-Camos et al., 2020; Wang et al., 2021
A179L	N/A	Promotes virus proliferation by Inhibiting ASFV infection-induced apoptosis.		low		Bosch-Camos et al., 2020; Jun Yi Shi et al., 2021; Yang et al., 2023
C962R	DNA primase	Involved in DNA replication specifically in the initiation point replication and modifies the DNA relocation mode		+		Bosch-Camos et al., 2020; Wang et al. 2021
DP71L	Protein phosphatase 1 regulator	Inhibits apoptosis by suppressing both the induction and activation of CHOP.		low		Bosch-Camos et al., 2020; Yang et al., 2023
E165R	dUTPase	Plays a role in DNA repair specifically in the prevention of the incorporation of uracil into DNA.		+		Bosch-Camos et al., 2020; Vuono et al., 2022
E296R	AP endonuclease	It has endonucleolytic activity specific for abasic sites.		+		Redrejo-Rodríguez et al., 2006; Bosch-Camos et al., 2020
G1340L	VACV A7 early transcription factor large subunit-like	Involved in early viral transcription.		+		Alejo et al., 2018; Bosch-Camos et al.; 2020; Urbano and Ferreira, 2022
H339R	pH339R	Alpha-NAC binding protein		+		Bosch-Camos et al., 2020
H359L	RNA polymerase subunit 3-11	Involved in virus transcription		+		Bosch-Camos et al., 2020; Duan et al., 2022; Zhang et al., 2023
I329L	Type I transmembrane protein	General TLR responses inhibitor		low		Bosch-Camos et al., 2020; Correia et al., 2023
NP868R	mRNA-capping enzyme	Involved in mRNA capping thus provides mRNA stability and translation efficiency		+		Bosch-Camos et al., 2020; Wang et al., 2021
O174L	DNA polymerase X (polX)	Involved in base-excision repair.		+		Bosch-Camos et al., 2020; Forth et al., 2022

Gene	Protein	Function	Ab (+/-)	T Cell (+/-)	Disease Enhancement (+/-)	References
D205R	RNA polymerase subunit	Involved in RNA transcription	+	N/A	N/A	Katarzyna et al., 2023; Luong et al., 2023
Unassigned						
K205R		Induces strong antibody response.	+	+	+	Lokhandwala et al., 2017; Gaudreault and Richt, 2019; Bosch-Camos et al., 2020
EP364R		Target Cyclic GMP-AMP To Inhibit the cGAS-STING Signaling Pathway	NA	+	+	Gaudreault and Richt, 2019; Dodantenna et al., 2022
F317L		Inhibitor of the NF-κB pathway	NA	+	+	Gaudreault and Richt, 2019; Xu et al., 2023
E184L		Virulence factor	NA			Gaudreault and Richt, 2019; Bosch-Camos et al., 2020; Dolata et al., 2023
CP312R			+		+	Gaudreault and Richt, 2019; Bosch-Camos et al., 2020; Hagoss et al., 2023
C44L			NA	low		Gaudreault and Richt, 2019; Bosch-Camos et al. 2020
C129R		Enhance DNA virus replication by negatively regulating the production of type 1 IFNs and proinflammatory cytokines.	+			Netherton et al., 2019; Bosch-Camos et al., 2020; Dodantenna et al., 2022
K145R		Escodes an abundant late cytoplasmic protein.	+			Kollnberger et al., 2002; Bosch-Camos et al., 2020; Rathakrishnan et al., 2021
M448R		Involved in tRNA repair using their RNA ligase activity.	-	+		Bosch-Camos et al., 2020; Laia Bosch-Camós et al., 2021
Multigene families						
MGF110-4L		XP124L/ Hypothesized to be involved in the redistribution of resident ER proteins aiding in immune evasion.	+	+		Netherton et al., 2004; Netherton et al., 2019; Bosch-Camos et al., 2020
MGF110-5L		VB2L/Hypothesized to be involved in the redistribution of resident ER proteins aiding in immune evasion.	+			Netherton et al., 2004; Netherton et al., 2019; Bosch-Camos et al., 2020
MGF110-1L		Hypothesized to be involved in the redistribution of resident ER proteins aiding in immune evasion.	-	+		Netherton et al., 2004; Netherton et al., 2019; Bosch-Camos et al., 2020
MGF110-2L		Hypothesized to be involved in the redistribution of resident ER proteins aiding in immune evasion.		low		Netherton et al., 2004; Bosch-Camos et al., 2020

Note. Table is adapted and modified from Gaudreault and Richt (2019)

The extensive characterization of key proteins such as p30, p72, p54, and p22 in ASFV underscores their roles in inducing humoral and cellular immune responses. While the highly immunogenic nature of these proteins, particularly their ability to elicit antibody responses, has been instrumental in vaccine development and diagnostics, the reliance on neutralizing antibodies alone has proven insufficient for complete protection against ASFV. The involvement of cellular immune responses, notably $CD8^+$ T cells, is crucial in conferring full protection. In the pursuit of effective ASFV control strategies, a comprehensive approach that

integrates both humoral and cellular immune responses is important for the development of vaccines.

RECENT DEVELOPMENTS IN IMMUNOINFORMATICS AND REVERSE VACCINOLOGY FOR ASFV VACCINE DEVELOPMENT

Advances in vaccine development have been attributed to bioinformatics and immunoinformatics. Recent strategies for antigen discovery and design have used computational techniques to speed up vaccine evaluation processes, including reverse vaccinology, systems biology, and structural vaccinology (Ahmad et al., 2019; Muhammad et al., 2020; García-Machorro et al., 2022). Reverse vaccinology refers to the use of genetic information as the starting point of the design process rather than examining the activity of the pathogen itself. It has consistently surpassed other technologies in antigen of interest detection since its first application in the identification of vaccine candidates against serogroup B meningococcus (Martinelli, 2022). With the curation of comprehensive databases and continuous benchmarking studies, reverse vaccinology is continuously improving. Reverse vaccinology works by identifying specific protein components, called epitopes, within the pathogen's genome that can induce strong immunological responses. The predicted and selected epitopes were then incorporated into the design for the development of a multi-epitope vaccine (Khalid and Poh, 2023). A multi-epitope vaccine allows for simultaneous delivery of specific epitopes from different antigens. It also allows the selection of desirable antigens using accurate algorithms to be included in the vaccine formulation (Bahrami et al., 2019). To induce successful protective immunity against ASFV, it is important to attain a balance in CTL, HTL, and antibody immune responses (Kollnberger et al., 2002; Imatdinov et al., 2020; Zhang et al., 2023a).

Designing of multi-epitope ASFV vaccines using computational methods

Several studies have designed epitope-based vaccines or have predicted epitopes using immunoinformatics and bioinformatics. approaches to be included in vaccine formulations against ASFV (Ros-Lucas et al., 2020; Herrera and Bisa, 2021; Fagbohun, 2021; Buan et al., 2022; Nguyen et al., 2022; Rowaiye et al., 2023). The *in silico* design of a multi-epitope vaccine can be generally divided into ten key steps: (1) protein retrieval in FASTA format, (2) sequence analysis, (3) epitope prediction, (4) vaccine construction, (5) structure prediction and validation, (6) molecular docking, (7) molecular dynamics, (8) immune simulation, (9) codon optimization, and (10) *in silico* cloning (Figure 1).

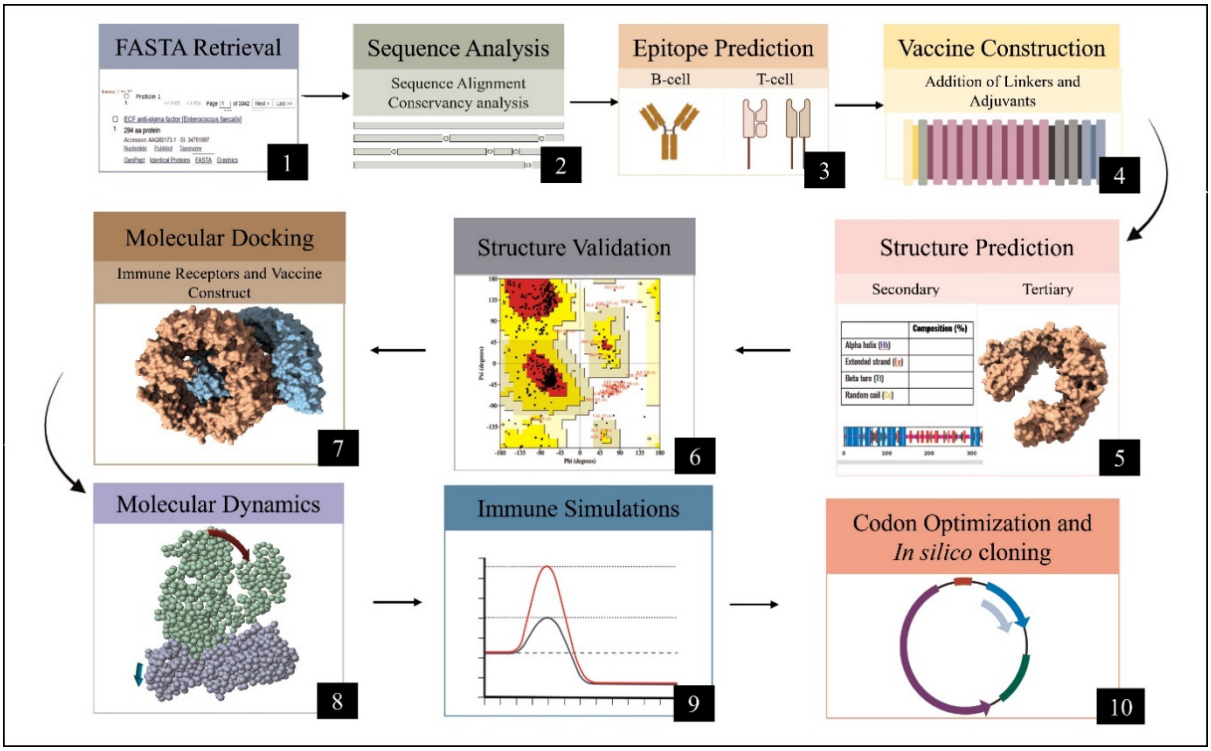

Figure 1 General methodology employed for designing a multi-epitope vaccine against ASFV.

The design of a multi-epitope vaccine usually starts with the identification of conserved and significant proteins as sources of epitopes to be incorporated into the candidate vaccine construct. UniProt and NCBI are the most common databases for the retrieval of these proteins. Proteins are then clustered using CD-HIT to identify representative sequences. After retrieval, protein sequences are aligned using ClustalW or MUSCLE and analyzed for conservation using the Protein Variability Server (PVS), which computes the absolute variability at each site (Díez-Rivero and Reche, 2009). The protein sequence with masked variable residues is then fragmented into ≥15-mer and ≥9-mer to predict HTL and CTL epitopes, respectively.

Both HTL and CTL epitope predictions start by assessing the binding affinity of potential epitopes with major histocompatibility complex class II (HTL) using MHC II binding prediction or with major histocompatibility complex class I CTL) using NetMHCcons 1.1 or NetMHCpan 4.1. The main difference between HTL and CTL epitope prediction is the processing step. This step considers the processing of the antigens into peptides through the endogenous pathway. The endogenous pathway includes the processing of antigens into small peptides that occur in the cytosol, attach to MHC class I molecules in the endoplasmic reticulum, and then transported to the surface of the cell (Seder and Mascola, 2003). Since the antigen-presenting capabilities of MHC-I for $CD8^+$ T cells are limited to those processed via the endogenous pathway, CTL epitope prediction requires consideration of the proteasomal cleavage sites of the human proteasome and transporter associated with antigen processing (TAP) transport efficiency. NetChop 3.1 and Proteasomal cleavage/ TAP transport/MHC class I combined predictor can be used for this analysis.

Unlike HTL and CTL epitopes, linear B-cell epitopes do not have an identified optimal length to induce an immune response; hence, multiple B-cell epitope servers are used for prediction such as ABCpred and BepiPred 2.0. Another approach to epitope collection is through the direct retrieval of experimentally validated epitopes as performed by Ros-Lucas et al. (2020) for B-cell epitopes.

The predicted HTL, CTL, and B-cells epitopes will be linked using "GPGPG", "AAY", and "KK", respectively with the addition of an adjuvant linked using "EAAK" (Rowaiye et al., 2023). The addition of adjuvants into subunit vaccines is recommended for the enhancement of immune responses and improvement of vaccine efficacy (Orosco and Espiritu, 2024). Other combinations may also be used such as in the case of Buan et al. (2022) where he used only two linkers "AAY" for CTL and adjuvant, and "GPGPG" for HTL and B-cell epitopes. Previous adjuvants used in a multi-epitope vaccine design, specifically against ASFV, include a synthetic Toll Like Receptor-4 (TLR-4) agonist peptide "HELSVLL" (Rowaiye et al., 2023), A6 ("TNGDILNYY") and F3 ("SVDSPTITY") peptides (Buan et al., 2022).

The vaccine construct should be checked for antigenicity, non-allergenicity, solubility, and stability using Vaxijen 2.0, AllerTop v2.0, SOLpro, and ProtParam respectively. The secondary structure of the construct can be predicted using GOR IV, GlobPlot2, SOPMA, and PSIPRED. For tertiary structure prediction and/or refinement, Galaxy TBM and RaptorX can be used. The quality of the predicted model will then be assessed using MolProbity, ERRAT, and Verify 3D.

Cluspro 2.0 is used for the docking of the vaccine construct to TLR(s). The binding affinity, which is related to the binding free energy, of the model produced can then be determined using PRODIGY or HawkDock. Molecular dynamics using iMODS or MDWeb can be used to assess the stability of the dock. The final steps in vaccine design include immune simulation using C-ImmSim, codon optimization, and *in silico* cloning. A complete list of the servers and their corresponding purposes used in the ASFV computational vaccine design studies is listed in Table 7.

Table 7 Servers and softwares used in the vaccine design and/or epitope prediction studies against ASFV.

Tool/Server	Purpose	Link	Reference
iVax toolkit	T-cell prediction	https://epivax.com/	Gutiérrez et al., 2015
MHCII Binding Prediction	CD4+ T-cell epitopes prediction	https://tools.iedb.org/mhcii/	Herrera and Bisa, 2021; Buan et al., 2022
NetMHCcons 1.1	CTL epitope mapping	https://services.healthtech.dtu.dk/services/NetMHCcons-1.1/	Karosiene et al., 2011; Herrera and Bisa, 2021
Proteasomal cleavage/ TAP transport/MHC class I combined predictor	CTL prediction	http://tools.iedb.org/processing/	Tenzer et al., 2005; Buan et al., 2022
NetChop 3.1	Cleavage sites prediction of the human proteasome	https://services.healthtech.dtu.dk/services/NetChop-3.1/	Nielsen et al., 2005; Ros-Lucas et al., 2020

Tool/Server	Purpose	Link	Reference
NetMHCpan 4.1	MHCI binding prediction	https://services.healthtech.dtu.dk/services/NetMHCpan-4.1/	Reynisson et al., 2020; Rowaiye et al., 2023
ABCpred	B-cell epitope prediction	https://webs.iiitd.edu.in/raghava/abcpred/	Saha and Raghava, 2006; Buan et al., 2022
BepiPred 2.0	B-cell epitope prediction	https://services.healthtech.dtu.dk/services/BepiPred-3.0/	Ros-Lucas et al., 2020; Clifford et al., 2022
GalaxyPepDock	SLA-epitope docking	https://seoklab.org/GalaxyPepDock/	Lee et al., 2015; Herrera and Bisa, 2021
PRODIGY	Binding affinity prediction	https://wenmr.science.uu.nl/prodigy/	Herrera and Bisa, 2021; Honorato et al., 2021
VaxinPAD	Screening of peptide based adjuvants	http://crdd.osdd.net/raghava/vaxinpad/	Nagpal et al., 2018; Rowaiye et al., 2023
Design 2.0	Disulphide engineering	http://cptweb.cpt.wayne.edu/DbD2/index.php	Craig and Dombkowski, 2013; Rowaiye et al., 2023
C-ImmSim	Immune simulation	https://kraken.iac.rm.cnr.it/C-IMMSIM/	Rapin et al., 2010; Buan et al., 2022
JCat	Codon optimization	https://www.jcat.de/	Grote et al., 2005; Buan et al., 2022
VaxiJen v2.0	Antigenicity prediction	https://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html	(Doytchinova and Flower, 2008; Rowaiye et al., 2023
AllerTop v.2.0	Allergenicity prediction	https://www.ddg-pharmfac.net/AllerTOP/feedback.py	Dimitrov et al., 2014; Rowaiye et al., 2023
ToxinPred	Toxicity prediction	https://webs.iiitd.edu.in/raghava/toxinpred/index.html	Gupta et al., 2013; Herrera and Bisa, 2021
SCRATCH suite SOLpro	Protein solubility prediction	https://scratch.proteomics.ics.uci.edu/	Magnan et al., 2009; Rowaiye et al., 2023

Among the servers mentioned, it is important to note that computational tools used in vaccine design are limited to non-human species. This is attributed to the lack of available experimental data required for the development of prediction models. To date, only NetMHCpan has been trained and evaluated for SLA class I alleles prediction while none is available for SLA class II alleles. To address this,

prediction tools for swine utilize similarities between human and swine immune system orthologs. One notable example is PigMatrix, a subscription-based tool developed by EpiVax. PigMatrix is integrated into the workflow of iVAX toolkit used in computational vaccine design for humans. PigMatrix uses the pocket profile method, the similarities of the secondary structures of HLA and SLAs, and the HLA binding preference to generate SLA epitope predictors. Another tool integrated into the iVAX toolkit to ensure the specificity of the T-cells predicted is the T cell Epitope Content Comparison tool (EpiCC). It compares the epitope content predicted in a strain to other strains to assess whether the epitope predicted is strain-specific or shared among many strains which allows to estimate whether the vaccine can be used for circulating or newly emerging strains of a pathogen (Gutiérrez et al., 2015; Moise et al., 2020). While there are a lot of open-access computational tools available, most of it are trained on human datasets highlighting the need for the development of swine-specific predictors.

Computational-based vaccine design studies against ASFV

Studies have focused on a specific protein while others used a proteome-wide approach for epitope prediction. Rowaiye et al. (2023) focused on the major capsid protein (p72) of 42 ASFV strains originating from 28 different countries to select immunodominant epitopes for vaccine design. These sequences were then subjected to predictions for their binding to MHC proteins. Subsequently, epitopes recognized by T and linear B lymphocytes were carefully chosen and assessed for antigenicity, immunogenicity, allergenicity, and toxicity. The selected epitopes were subjected to modeling and molecular docking with the corresponding MHC proteins. Addition of adjuvant and multiple linkers was employed to construct a multi-epitope vaccine candidate (VC). After evaluating the physicochemical and immunological properties of the VC, the predicted tertiary model was further refined, validated, and subjected to mutation analysis. Molecular dynamics simulations were then conducted, particularly focusing on the interaction of VC with TLR4. Additionally, the VC was virtually cloned into a plasmid vector. The resulting 130 amino acid multi-epitope vaccine candidate had a weight of 14.60 KDa and an isoelectric point of 10.63. Notably, the VC is characterized by its hydrophilic nature and lack of allergenic properties. It exhibits MHC I immunogenicity and antigenicity values of 1.43 and 0.72, respectively. Furthermore, it has an instability index value of 33.11 and a half-life of 1 hour, 0.5 hours, and over 10 hours in the reticulocytes of mammals, yeast, and *E. coli*, respectively. Overall, the vaccine construct displays favorable characteristics, but further testing of its safety and effectiveness is needed, especially since immune simulation was not implemented.

Buan et al. (2022) conducted the same analysis using p12, p17, p22, p54, CD2v, and p72 from fifty-six ASFV isolates from different countries resulting in a 398 amino acid multi-epitope vaccine with a molecular weight of 42, 485.82g/mol. Docking analysis was performed to predict the interaction of the construct with the swine leukocyte antigen (SLA)-1. Immune simulation of the designed ASFV vaccine showed an immune response compared to that of the control group which received the adjuvant alone. The ASF vaccine demonstrated significantly elevated levels of both primary and secondary immune responses compared to the control. Similarly, B-cell and T-cell populations were higher in the ASFV vaccine group. Interestingly, the adjuvant alone exhibited relatively higher levels of helper T-cells and most cytokines compared to the vaccine.

Herrera and Bisa (2021) focused on the prediction of cytotoxic T-cell epitopes (CTL) in the structural proteins of ASFV: pp220, pp62, p72, p30, and CD2v. Due to the high mutation rate of viral genomes, the identification of highly conserved sequences is important. This study used CD-HIT, Clustal Omega, and protein variability server (PVS) to identify highly conserved > 9-mer fragments. These fragments are then used for CTL epitope mapping which were then validated through molecular docking and analysis. The binding affinity, binding energy, and the root-mean-square deviation (RMSD) were generated through docking analysis of CTL epitopes with SLA-1*0401 and then used for further validation.

Ros-Lucas et al. (2020) also focused on the epitope prediction for future components of an epitope-based vaccine formulation against ASFV but included the prediction of HTL and B-cell epitopes. Ros-Lucas and colleagues identified putative-exposed proteins from 7,088 ASFV proteins coming from 46 ASFV proteomes. They identified a total of 49 unique and conserved epitopes, with 6 $CD8_+$ T cell epitopes, 14 $CD4_+$ T cell epitopes, and 29 B-cell epitopes. Sequence-based and structure-based approaches were used to predict new B cell epitopes. The sequence-based approach started with the retrieval of ASFV conserved B-cell epitopes and mapping to assess their localization. Those "outside" the protein were kept and those in the inner side or within transmembrane locations were discarded. Structure-based approach in predicting epitopes relies on the flexibility and relative solvent accessibility (RSA) of residues in the structure. The two B-cell epitopes predicted using the structure-based approach were also predicted in the sequence-based approach. No further simulations were conducted.

Another study that extends the epitope mapping to nonstructural proteins, Simbulan et al. (2024) designed a multi-epitope subunit vaccine against ASFV using the immunogenic peptides screened from 100 well-annotated ASFV proteomes. Their study focused on proteins from genotypes I and II, given the involvement of these genotypes in recent global ASFV outbreaks. A total of 6 CTL, 4 HTL, and 4 LBL epitopes were predicted and used as components for their vaccine design. This vaccine exhibited notable immunogenicity in *in silico* evaluations such as immune simulation and molecular docking analyses. Additionally, the vaccine demonstrated stable profiles in secondary and tertiary structure assessments.

Although these studies provide valuable insights into epitope prediction for vaccine development, there is a consistent need for *in vivo* validation to ensure the safety and efficacy of the proposed vaccine candidates. Ros-Lucas et al. (2020) and Simbulan et al. (2024) also underscore the importance of a pan-proteomic approach in epitope screening to consider a wide array of ASFV proteins increasing the potential success of vaccine candidates by capturing a more complete range of potential immunodominant epitopes.

OTHER FACTORS INFLUENCING THE ADVANCEMENT OF PROTEIN SUBUNIT VACCINES

Aside from *in silico* methods, several advancements have been made in the methods of expression, types of adjuvants, and routes of immunization of protein subunit vaccines over the last half decade alone. This section will discuss these recent advancements in detail and how they factor into the creation of effective protein subunit vaccines against ASFV.

Novel Vectors/Delivery Systems

Prokaryotic Vectors

Escherichia coli is the most commonly used vector for the production of heterologous proteins, owing to its rapid growth, quick and easy expression, low production cost, and high product yield (Ma et al., 2020). *E. coli* BL21 (DE3) pLysS cells have previously been used to express ASFV fusion proteins in immunization studies in mice (Zhang et al., 2022) and pigs (Zhang et al., 2023).

Various lactic acid bacteria (LAB) have also been used as expression systems. *Lactococcus lactis*, the model organism of the LAB group, has been widely used to express and distribute antigens and bioactive polypeptides to mucosal systems and has also served as a distribution system for DNA vaccines (Ma et al., 2020). Its ability to regulate intestinal flora to maintain intestinal stability and boost immunity, as well as specific biological activities that promote mucosal immunity, make the use of this carrier advantageous. The optimized structural domains of p30, p54, and p72 were successfully expressed in *L. lactis* using the expression vector pMG36e (Zhang et al., 2023b). Another LAB group member, *Lactobacillus plantarum*, has been used to express p54 (Chen et al., 2021) and p14.5 (Huang et al., 2022).

The Salmonella Typhimurium (ST) JOL912 (*ΔlonΔcpxRΔasd*) strain has good *in vivo* plasmid stability and can specifically invade antigen-presenting cells, making its use advantageous over viral vectors. As ST does not require additional adjuvants or formulation additives, vaccine preparation is simple and inexpensive compared to nucleic acid vaccines. Recombinant ST vectors were used to express antigen cocktails CD2v/CTL/9GL, p54/p12/p72, or a combination of both (Bhilare et al., 2023).

Eukaryotic Vectors

Yeast

The advantages of yeast expression systems include high yield, fast growth rate, low production cost, and assistance in protein folding (Ma et al., 2020; Opriessnig et al., 2021). These advantages are evident in Meng et al. (2022), where the correctly folded trimeric p72 was expressed in *Saccharomyces cerevisiae* with high similarity to the native p72 in the ASFV virion. In an earlier study, *S. cerevisiae* was used to express p30-Fcc and p54-Fca fusion proteins (Chen et al., 2021).

Mammalian Cells

As mammalian cell expression systems are capable of proper protein folding and post-translational modifications, as well as the emission of signals that can be easily recognized by other mammalian cells (Khan, 2013), the use of this kind of expression system may be more appropriate for ASFV immunization studies. Mammalian expression systems that have recently been used to express ASFV proteins include rabbit epithelial kidney RK13 cells for a cocktail of 15 ASFV proteins (Bosch-Camós et al., 2021), baby hamster kidney cells (BHK-21) expressing CD2v, p30, or K205R (Hua et al., 2023), and Chinese hamster ovary (CHO) cells expressing p17 (Li et al., 2023).

Baculoviruses

Baculoviruses are arthropod-specific viruses that naturally regulate insect populations. Their high level of gene expression directed by the very late polyhedrin gene (*polh*) promoter makes them highly suitable expression systems for recombinant protein production. The insect cells used in the baculovirus expression system can recognize and process signal peptides, assist in oligomerization, and allow for post-translational modifications, permitting the correct formation of

eukaryotic proteins. Several baculovirus vectors with various modifications are available for protein expression.

The BaculoDirect™ expression system bypasses the cloning and recombination steps and directly inserts the gene of interest into the baculovirus genome *in vitro*, after which it can be expressed in insect cells (Invitrogen, 2012). BaculoDirect™ was previously used to express a cocktail of ASFV proteins (Sunwoo et al., 2019). In the BacMam vector, the *polh* promoter is replaced with a mammalian cell or virus promoter for more efficient delivery and expression in mammalian cells (Chambers et al., 2018). The ASFV CD2v-p30-p54 fusion antigen was previously expressed using BacMam (Zhou et al., 2022). The pFastBac™ vector contains two promoters, *polh* and p10, which allow for the simultaneous expression of two proteins in insect cells. Using this vector, B602L and the B602L-Fc fusion protein could be simultaneously expressed (Yang et al., 2023).

Nanocarriers

Poly (lactic-co-glycolic acid) (PLGA) nanoparticles are synthetic biodegradable polymers that are effective drug delivery systems for various therapeutic agents, including proteins (Rezvantalab et al., 2018). CpG and mannose-modified PLGA (CMR-PLGA) nanoparticles have been previously used to deliver p54 to mice (Huo et al., 2022).

Virus-like particles (VLPs) are formed by the spontaneous self-assembly of viral structural capsid proteins. Since they do not contain genetic material, they are non-replicating and non-pathogenic. However, they can elicit an immune response similar to that elicited by natural viruses (Opriessnig et al., 2021). An engineered T7 phage VLP displaying the SpyTag peptide-p10 fusion protein (T7-ST) was also used to create five VLPs displaying p30, p54, p72, CD2v, and K145R (Li et al., 2023).

Replicating Mammalian Viruses

A number of studies have employed the pseudorabies virus (PRV), a mammalian herpesvirus, for the expression of antigenic ASFV proteins: the PRV-ΔgE/ΔgI/ΔTK strain expressing CD2v (Feng et al., 2020); the PRV strain Bartha-K61 expressing p30, pp62, p54, p72 and CD2v (Chen et al., 2022); and the PRV-XJ-ΔTK strain also expressing p72 and pB602L in BHK-21 cells (Deng et al., 2023).

Replication-Defective Viruses

Replication-defective viruses lack the functions needed for viral genome replication and progeny virus particle assembly. They are propagated in cell lines containing viral gene components that allow replication. This tightly controlled replication confers a higher level of safety, an advantage over other replicative expression systems. In an amplicon or replicon vaccine, an amplicon or replicon (RP) genome contains an origin of replication and packaging signals, the minimal cis-acting signals for viral genomic replication. A helper virus or cell line supplies the viral proteins that enable genomic replication and virion formation (Dudek and Knipe, 2006). In recent years, two kinds of replication-deficient viruses have been employed in ASFV protein expression studies: alphaviruses (Murgia et al., 2018; Fang et al., 2022; Huang et al., 2023) and adenoviruses (Lokhandwala et al., 2019; Cadenas-Fernández et al., 2020; Goatley et al., 2020; Lu et al., 2021; Zajac et al., 2022; Zhou et al., 2022; Liu et al., 2023; Zajac et al., 2023).

Novel Adjuvants

Oil emulsions

Oil emulsions act as adjuvants by forming a depot at the injection site, which helps enhance the immune response to peptide antigens by protecting them from degradation and prolonging their exposure to the immune system by facilitating their slow release and enhancing antigen uptake by immune cells (Brewer et al., 2018). Various types of oil emulsions have been used as adjuvants in ASFV vaccine studies: oil-in-water (O/W) emulsions such as ISA 25 (Sunwoo et al., 2019), water-in-oil (W/O) emulsions such as Freund's adjuvant (Huo et al., 2022; Li et al., 2023; Yang et al., 2023) and ISA 61 VG (Hua et al., 2023), and water-in-oil-in-water (W/O/W) emulsions such as ISA 201 VG (Zajac et al., 2023) and ISA 206 VG (Zhang et al., 2023).

Fusion proteins are a common method of increasing adjuvanticity in subunit vaccines, with an antigen and an adjuvant fused into a single molecule. The fragment crystallizable region (Fc region) of an antibody can be fused to an antigen to facilitate Fc receptor-mediated antigen transport across the membrane barrier of antigen-presenting cells (Yang et al., 2023). ASFV antigen-Fc fusion proteins that have previously been synthesized are p30-Fcγ and p54-Fcα (Chen et al., 2021) and B602L-Fc (Yang et al., 2023), which have been shown to have an enhanced immune response compared to their antigen-only counterparts.

Peptide Adjuvants

In addition to mediating antigen transport, other adjuvants can directly enhance certain aspects of the immune response in combination with an antigen. The heat-labile enterotoxin B (LTB) subunit from *E. coli* is a known mucosal adjuvant that enhances local mucosal immunity, humoral immunity, and Th2 cellular immunity in rabbits when fused to p30, p54, or p72 (Zhang et al., 2023b). The mucosal adjuvanticities of two toll-like receptor (TLR) ligands, FlaB flagellin from *Vibrio vulnificus* and heat shock protein 70 from *Mycobacterium tuberculosis* (Hsp70), were also evaluated in a fusion expression with the ASFV CD2v-p30-p54 fusion antigen. Immunization in pigs demonstrated that Hsp70 induced a significantly more robust immune response than FlaB (Lu et al., 2021).

The major outer membrane lipoprotein I (OprI) of *Pseudomonas aeruginosa* is a TLR-2 ligand capable of prompting dendritic cells to secrete proinflammatory ecytokines cytokines *in vivo* and eliciting strong humoral and cellular immune responses against the peptides or proteins it fuses with. The fusion of OprI to various ASFV fusion proteins has been shown to activate dendritic cells and elevate proinflammatory cytokine secretion (Zhang et al., 2022; Zhang et al., 2023).

CTA1-DD is an artificial adjuvant composed of the CTA1 subunit of cholera toxin and the D-domain dimer of protein A of *Staphylococcus aureus*. It induces robust $CD4^+$ T cell responses, specific antibody production, germinal center B cells, and Tfh responses. A CTA1-p14.5-D-D fusion protein previously induced T lymphocyte, B lymphocyte, and dendritic cell differentiation and maturity, as well as specific antibody production in mice (Huang et al., 2022).

Z12, a novel cell-penetrating peptide from the ZEBRA protein, enhances $CD4^+$ and $CD8^+$ T cell-mediated immunity when fused to an antigen. Z12 fused to p30 and p54 has been shown to have higher transduction efficiency in RAW264.7 cells and significantly stronger humoral and cellular responses upon immunization in mice (Zhang et al., 2021).

Universal T cell epitopes can also enhance immune responses (Diethelm-Okita et al., 2000). When fused to ASFV antigens along with Z12 (Zhang et al., 2021) or OprI (Zhang et al., 2022), constructs containing universal T cell epitopes had

significantly more robust immune responses compared to constructs without universal T cell epitopes.

In an immunoinformatics approach to develop a vaccine candidate against ASFV, an adjuvant was selected by screening synthetic TLR-4 agonists using the VaxinPAD web server with the SVM threshold set at 0.0 and the dipeptide composition method was selected. The peptide "HELSVLL" had the highest score and was selected as the adjuvant (Rowaiye et al., 2023).

Cytokines

Interleukin 21 (IL-21) is a cytokine produced by natural killer cells and differentiated mature CD4+ T cells. It plays a role in B cell activation and proliferation, and in germinal center and humoral immunity. IL-21 fused with p54 induces specific humoral and cellular immune responses in mice (Chen et al., 2021). IL-33 is secreted during cell damage and can directly interact with dendritic cells to stimulate the differentiation of naive T cells. It can also promote the expansion of NK and NKT cells and enhance Th1 and CD8+ T cell responses during viral infections. IL-33-Mus fused with p14.5 induced a more robust immune effect than fusion with CTA1-DD (Huang et al., 2022).

As different combinations of vectors/delivery systems and adjuvants have been used to create several protein subunit vaccines against ASFV (See Table 8), each containing different combinations of epitopes, it is difficult to assess how much of the effectiveness of the immune responses of the various vaccines can be attributed to the protein expression, the adjuvanticity, or the effectiveness of the epitopes themselves. However, the vectors/delivery systems and adjuvants mentioned above have all been shown to be effective to various extents. Further studies may be conducted to directly compare the efficacy of different vectors/delivery systems and adjuvants using the same vaccine.

Table 8 Novel vaccines used in immunization studies against ASFV.

Vector/ Expression System	Type (Protein, Vector prime-boost)	Adjuvant	Dosage and Interval	Immune Response(+/-)			Reference
				Innate	Antibody	T Cell	
Alphavirus	Alphavirus RP prime + live attenuated ASFV boost	N/A	Prime: 2×, 2-4.5 × 107 RPs, 3-week intervals; Boost: 104 TCID50 OURT88/3, 1-week after second immunization	N/A	+	N/A	Murgia et al., 2018
Adenovirus	Protein	BioMize 0226	Prime: 8 × 1010 IFU, Boost: 8 × 1011 IFU, 4-week interval	N/A	+	N/A	Lokhandwala et al., 2019
Adenovirus	Protein	BioMize 0226	Prime: 7 × 1010 IFU, Boost: 2×, 7 × 1011 IFU, 3-week intervals	N/A	+	N/A	Lokhandwala et al., 2019
Adenovirus	Protein	ZTS-01	Prime: 7 × 1010 IFU, Boost: 2×, 7 × 1011 IFU, 3-week intervals	N/A	+	N/A	Lokhandwala et al., 2019
Baculovirus	DNA-Protein	ISA25	3×, 100 μg DNA, 100 μg protein, 0/21/35 dpv	N/A	+	+	Sunwoo et al., 2019

Vector/ Expression System	Type (Protein, Vector prime-boost)	Adjuvant	Dosage and Interval	Immune Response(+/-)			Reference
				Innate	Antibody	T Cell	
Adenovirus	Protein	BioMize	2×, 5-week intervals	N/A	-	N/A	Cadenas-Fernández et al., 2020
Adenovirus	Protein	BioMize	single dose	N/A	-	N/A	Cadenas-Fernández et al., 2020
Adenovirus	Protein	N/A	2×, 5-week intervals	N/A	-	N/A	Cadenas-Fernández et al., 2020
Adenovirus (prime) and Poxvirus (boost)	Protein prime-boost	N/A	Prime: 5 × 109 IU, Boost: 7.5 × 107 PFU, 4-week interval	N/A	+	+	Goatley et al., 2020
Adenovirus (prime) and Poxvirus (boost)	Protein prime-boost	N/A	Prime: 5 × 109 IU, Boost: 7.5 × 107 PFU, 4-week interval	N/A	+	+	Goatley et al., 2020
Adenovirus (prime) and Poxvirus (boost)	Protein prime-boost	N/A	Prime: 1.5 × 1010 IU, Boost: 2 × 108 PFU, 4-week interval	N/A	+	+	Goatley et al., 2020
Adenovirus (prime) and Poxvirus (boost)	Protein prime-boost	N/A	Prime: 1.5 × 1010 IU, Boost: 2 × 108 PFU, 4-week interval	N/A	+	+	Goatley et al., 2020
Herpesvirus	Protein	N/A	2×, 1 × 10^5 TCID50, 1-week interval	N/A	+	+	Feng et al., 2020
Yeast	Protein	Fcγ	4×, 1 × 109 CFU, 2-week intervals	N/A	+	N/A	Chen et al., 2021
Yeast	Protein	Fcα	4×, 1 × 109 CFU, 2-week intervals	N/A	+	N/A	Chen et al., 2021
Adenovirus	Protein	FlaB	2× 1 × 108 TCID50, 3-week interval	+	+	+	Lu et al., 2021
Adenovirus	Protein	Hsp70	2× 1 × 108 TCID50, 3-week interval	+	+	+	Lu et al., 2021
Prokaryotic vector	Protein	IL-21	3×, 1 × 109 CFU, 3 days each dose, 2-week intervals	N/A	+	+	X. Chen et al., 2021
Prokaryotic vector	Protein	Z12		N/A	+	+	Zhang et al., 2021
Prokaryotic vector	Protein	Z12 and universal T-cell epitope		N/A	+	+	Zhang et al., 2021
Mammalian cell line	DNA prime + recombinant live attenuated ASFV boost	N/A	Prime: 2×, 2-week intervals; Boost: 1 × 103 PFU BA71ΔCD2	N/A	-	+	Bosch-Camós et al., 2021
Adenovirus	Protein	ENABL®	2×, 3 × 1011 IFU, 14-week interval	N/A	+	+	Zajac et al., 2022
Adenovirus	Protein	ZTS-01	2×, 3 × 1011 IFU, 14-week interval	N/A	+	+	Zajac et al., 2022
Nanoparticles	Protein	Freund's adjuvant (with free p54 only)	single dose	+	+	+	Huo et al., 2022

Vector/ Expression System	Type (Protein, Vector prime-boost)	Adjuvant	Dosage and Interval	Immune Response(+/-)			Reference
				Innate	Antibody	T Cell	
Prokaryotic vector	Protein	IL-33-Mus	3×, 1 x 109 CFU, 2-week intervals, 3 days each	+	+	+	Huang et al., 2022
Prokaryotic vector	Protein	CTA1-DD	3×, 1 x 109 CFU, 2-week intervals, 3 days each	+	+	+	Huang et al., 2022
Alphavirus	Protein	N/A	3×, 1 × 103.0 TCID50/0.1 mL, 2-week intervals	N/A	+	+	Fang et al., 2022
Alphavirus	Protein	N/A	3×, 1 × 103.5 TCID50/0.1 mL, 2-week intervals	N/A	+	+	Fang et al., 2022
Baculovirus /Adenovirus	Protein	N/A	2×, 2 × 108 PFU, 3-week interval	+	+	+	Zhou et al., 2022
Herpesvirus	Protein	N/A	Mice: single dose, 1 × 105.0 TCID50; Pigs: 2×, 1 × 105.0 TCID50, 2-week interval	N/A	+	N/A	Chen et al., 2022
Prokaryotic vector	Protein	OprI	2×, 30 ug, 2-week interval	+	+	+	Zhang et al., 2022
Prokaryotic vector	Protein	OprI and universal tetanus toxoid CD4+ T cell epitope P2 (TT-P2)	2×, 30 ug, 2-week interval	+	+	+	Zhang et al., 2022
Baculovirus	Protein	Fcγ, Freund's adjuvant	2×, 200 pM, 3-week interval	N/A	+	+	Yang et al., 2023
Vaccine-like particles (VLPs)	Protein	Freund's adjuvant	4×, 1 × 109 PFU, 1-week intervals	N/A	+	N/A	Y. Li et al., 2023
Prokaryotic vector	Protein	heat-labile enterotoxin B (LTB)	2×, 1 × 108 CFU, 2-week intervals, 3 days each	+	+	+	Zhang et al., 2023b
Mammalian cell line	Protein	ISA61 VG	2×, 50 μg (per protein), 4-week interval	N/A	+	N/A	Hua et al., 2023
Mammalian cell line	Protein	ISA61 VG	2×, 50 μg (per protein), 4-week interval	N/A	+	N/A	Hua et al., 2023
Adenovirus	Protein	ISA201 or BioMize	3×, 4.2 × 1011 IFU, at 0/3/7 weeks	N/A	+	-	Zajac et al., 2023
Adenovirus	Protein	N/A	2×, 2 × 1010, 8-week interval	+	+	+	Liu et al., 2023
Alphavirus	Protein	N/A	3×, 2-week intervals	+	+	+	Huang et al., 2023
Prokaryotic vector	Protein	N/A	2×, 1 × 108 CFU, 2-week interval	N/A	-	+	Bhilare et al., 2023
Herpesvirus	Protein	N/A	2×, 200 uL, 2-week interval	N/A	+	+	Deng et al., 2023
Prokaryotic vector	Protein	OprI and ISA206 (O-Ags-T formulation)	2×, 2 mL, 3-week interval	+	+	+	Zhang et al., 2023

ASFV VACCINATION ROUTES

ASF remains a critical concern for the swine industry globally, necessitating the development of effective vaccines to combat its devastating impact. Importantly, identifying the most appropriate vaccination route is crucial for vaccine development and implementation, with implications for the effectiveness, safety, and practicality of immunization programs. Important factors that should be considered include the identification of the pathogen, desired immune response, practical constraints, and characteristics of the target population of pigs to enhance the overall success of immunization programs and contribute to the prevention and control of infectious diseases. In this section, the vaccination route/s administered for subunit ASFV vaccines, including the vaccine parent strain and protein incorporated, dosage, challenge dose and strain, and major findings were outlined (Table 9). This knowledge serves as a foundational step in advancing our quest for robust prevention and control strategies against ASF and related diseases.

Table 9 Vaccine routes of ASFV subunit vaccines administered in pre-clinical studies on ASFV.

Vaccination Route	Vaccine Strain (protein used)	Experimental Animal	Vaccine Dosage+	Challenge Strain and Dose	Findings	References
Intramuscular	Pig/HLJ/2018 (vector expressed P72, CD2v, pB602L, or p30 epitopes)	BALB/c female mice	30 μg	-	Protective	Song et al., 2023
Intramuscular	Pig/HLJ/2018 (vector expressed CD2v or CD2v-IR)	Mice	Prime: 1 mg Booster: 1 mg 21 dpi	-	Protective	Lu et al., 2023
Intramuscular	Georgia 2007/1 (vector expressed p32, p54, pp62, and p72)	Pigs	Prime: 4×10^{10} IFU* Booster: 4×10^{10} IFU 14 weeks pi *10^{10} IFU each protein	-	Protective	Lokhandwala et al., 2016
Intramuscular	Georgia 2007/1 (vector expressed A151R, B602L, EP402RΔPRR, B438L, or K205R-A104R)	Pigs	Prime: 10^{11} IFU Booster: 10^{11} IFU 8 weeks pi	-	Protective	Lokhandwala et al., 2017
Intramuscular	Georgia/2007; Ba71V; E70 recombinant proteins (p15 + p35 + p54, +/− p17) with pcDNAs (CD2v + p72 + p32, +/− p17)	Pigs	100 μg 3x IM (0, 21, and 35 dpi)	360 HAU Arm07 IM 3 weeks after 3rd vaccination	Protective	Sunwoo et al., 2019

Vaccination Route	Vaccine Strain (protein used)	Experimental Animal	Vaccine Dosage+	Challenge Strain and Dose	Findings	References
Intramuscular	E75CV1 (vector expressed Hemagglutinin (HA))	Pigs	2 dose groups: 10^7 HAU 5 x 10^6 HAU	4 x 10^2 $TCID_{50}$ E75	Not protective	Ruiz-Gonzalvo et al., 1996
Intramuscular	1207VR15 (vector expressed p54 and/or p30)	Pigs	5 x 10^7 to 5 x 10^8 baculovirus-infected sf cells	5 x 10^2 $TCID_{50}$ E75 IM	Protective	Gomez-Puertas et al., 1998
Intramuscular	Pr4Δ9GL and Pr4 (vector expressed p30, p54, and p72)	Pigs	2 groups 10^4 $TCID_{50}$ Pr4Δ9GL Prime: 1-2 x 10^8 Sf21 cells Booster: 1-2 x 10^8 4-week pi	10^4 $TCID_{50}$ Pr4Δ9GL IM 42 dpi	Nonprotective	Neilan et al., 2004
Intramuscular	Subunit ASFV vaccine E183L-HEK B646L-HEK O61R-HEK MVA-B646L MVA-EP153R MVA-EP402R	Pigs	Prime: 10^7 $TCID_{50}$ MVA OR 200 g/antigen HEK Booster: 10^7 $TCID_{50}$ MVA OR 200 g/antigen HEK 28 dpi	N/A	Protective	Lopera-Madrid et al., 2017
Intramuscular	E75 (vector expressed p54, p30, and HA (pCMV-UbsHAPQ))	Pigs	Expt 1: 10^4 HAU_{50} IM 1x pCMV-UbsHAPQ Expt 2: 10^4 HAU_{50} IM 2x and 4x pCMV-UbsHAPQ	10^4 HAU_{50} E75 IM	Nonprotective Less protective	Argilaguet et al., 2012
Intramuscular (and intravenous)	CongoFrance CD2v/Lectin (KK-262)	Pigs	Expt 1: Prime: 10^6 HAU50 IM Booster: 10^6 HAU_{50} IM 7 dpi Expt 2: Prime: 2 x 10^6 HAU_{50} IM Booster: 2 x 10^6 HAU_{50} IM 21 dpi	10^3 HAU_{50} Congo K49 IM 42 dpi	Protective	Burmakina et al., 2016
	FranceCongo CD2v/Lectin (FK-32/135)		Expt 3: Prime: 2 x 10^6 HAU_{50} IM Booster: 2 x 10^6 HAU_{50} IM 21 dpi Expt 4: Prime: 3 x 10^6 HAU_{50} IM and IV Booster: 3 x 10^6 HAU_{50} IM 21, 42, 63, & 77 dpi	10^3 HAU_{50} Congo K49 IM 90 dpi	Nonprotective	
Intramuscular	Malawi Lil-20/1Δ8CR or	Pigs	10^2 $TCID_{50}$	10^2 $TCID_{50}$ Malawi Lil-20/1 IM		Neilan et al., 1999

Vaccination Route	Vaccine Strain (protein used)	Experimental Animal	Vaccine Dosage+	Challenge Strain and Dose	Findings	References
	Malawi Lil-20/1					
Intramuscular	ASFV strain not stated (vector expressed CD2v, CTL, 9GL, p54, p12, and p72)	Pigs	Prime: 10^8 CFU Booster: 10^8 CFU 14 dpi	-	Protective	Bhilare et al., 2023
Intramuscular	Ba71v and OUR T88/1 (vector* expressed E199L, EP153R, EP364R, F317L, I329L, MGF360-11L, MGF505-4R and MGF505-5R) *rAd and MVA vectors		Expt 1: Prime A: 5×10^9 IU rAd Booster A: 5×10^9 IU rAd 28 dpi Prime B: 7.5×10^7 PFU MVA Booster B: 7.5×10^7 PFU MVA 28 dpi Expt 2: Prime A: 1.5×10^{10} IU Booster A: 1.5 × 1010 IU 28 dpi Prime B: 2 × 108 pfu MVA Booster B: 2×10^8 PFU MVA 28 dpi	10,000 HAD OUR T88/1 IM 4 weeks pi	Protective	Goatley et al., 2020
Intramuscular	OUR T88/3	Pig	Expt 1: 10,000 IU OUR T88/3 Expt 2: 10,000 IU OUR T88/3 Expt 3: 3×10^{10} IU of rAd-GFP 5×10^9 IU of rAd expressing ASFV gene	1st: 10,000 HAD OUR T88/1 IM 3 weeks after vaccination. 2nd: 10,000 HAD Georgia 2007/1 IM 3 weeks after 1st challenge 10,000 HAD OUR T88/1 IM 3 weeks after vaccination 10,000 HAD OUR T88/1 IM 5 weeks after vaccination	Less protective	Netherton et al., 2019
intraperitoneal	Vector expressed p30	Mice	Prime: 80 ug 2X Booster: 60 ug	-	Protective	Liberti et al., 2023
intramuscular	A151R, B119L, B602L, 110 EP402RΔPRR, B438L, K205R-A104R, p32,	Pigs	Expt 1: Prime: 8 X 10^{10} IFU Booster: 8 X 10^{11}	10^3 or 10^4 $TCID_{50}$ IN Georgia 2007/1	Nonprotective	Lokhandwala et al., 2019

Vaccination Route	Vaccine Strain (protein used)	Experimental Animal	Vaccine Dosage+	Challenge Strain and Dose	Findings	References
	p54, pp62, and p72		IFU 4 weeks pi; 2X 3-week interval Expt 2 and 3: Prime: 7 X 10^{10} IFU Booster: 7 X 10^{11} IFU 4 weeks pi; 2X 3-week interval			
intramuscular	BA71 (vector expressed M448R and MGF505-7R)	Pigs	Expt 1: (15 individual plasmids) Prime: 0.6 mg 2X 2-week interval Booster: 10^3 PFU BA71ΔCD2 14 dpi Expt 2: (M448R + MGF505-7R) Prime: 0.6 mg 2X 2-week interval Booster: 10^3 PFU 14 dpi BA71ΔCD2	Lethal dose of Georgia2007/1 3 weeks after boost	Protective	Bosch-Camós et al., 2022

*protective – presence of anti-ASFV antibody and/or absence of ASFV related clinical signs
+Safe – no clinical symptoms upon vaccine overdosing and no reversion to virulence and ASFV vaccine strain genome stability is established
**No access – no publicly accessible article or data
– – not stated
dpi or pi, days post inoculation (vaccination); $TCID_{50}$,50% Tissue Culture Infectious Dose; HAD_{50}, 50% hemadsorption doses; PFU, Plaque-Forming Unit; IFU, Infectious focus units; IU, International Unit.

Traditionally, intramuscular injection (IM) has been a common route for animal vaccine delivery (Ols et al., 2020). Table 9 shows that the IM vaccination route is the dominant mode of vaccine administration of subunit ASFV vaccines, with intranasal vaccination as an alternative route. The recommended injection site (in pigs) for IM vaccination is the neck because it has a well-developed muscle and is an accessible area that minimizes the risk of accidental injury. The injection of the vaccine into the muscle tissue serves as a robust means of delivering antigens, initiating a cascade of immune reactions throughout the circulatory system. Thus, IM vaccination aims to induce a systemic immune response, including the production of neutralizing antibodies. These antibodies play a pivotal role in recognizing and neutralizing the ASFV particles, thereby preventing the establishment of viremia. By impeding viremia, IM vaccination disrupts the virus's ability to travel through the bloodstream, limiting its ability to reach different organs and tissues. In summary, the emphasis on inducing a systemic immune response through IM vaccination is grounded in its effectiveness in generating neutralizing antibodies. This not only protects individual pigs from systemic ASFV infection but also holds the potential to mitigate the broader impact of the virus by reducing

viremia and limiting its spread within the swine population. However, few pre-clinical investigations on ASFV subunit vaccines using intramuscular vaccination have shown less to no protection (Ruiz-gonzalvo et al., 1996; Neilan et al., 2004; Argilaguet et al., 2012; Burmakina et al., 2016; Lokhandwala et al., 2019; Netherton et al., 2019). In contrast, ASFV vaccines exhibiting protective immunity via IM administration have varying responses relative to ASFV strains against various challenge ASFV strains (Gómez-Puertas et al., 1998; Sunwoo et al., 2019; Goatley et al., 2020; Bosch-Camós et al., 2022). Notably, among the compiled studies, no study has utilized a vaccine containing multiple ASFV strains, even within the context of subunit vaccines. This necessitates the development of subunit vaccines with multiple antigens from various strains.

In addition to intramuscular vaccination, other widely used vaccination routes have been applied in *in vivo* studies such as intradermal, intranasal, oral, and/or a combination (Figure 2). Intradermal vaccination involves delivering the vaccine directly into the dermal (dermis) layer of the skin (Figure 2). The use of intradermal vaccination has several benefits including removal of needle disposal, fewer accidents, reduced vaccine volume, greater antigen dispersion, faster administration, and reduced pain and distress (Tizard et al., 2020); thus, it has gained popularity among swine producers. Intradermal vaccination is administered using an intradermal application liquids (IDAL) needle-free injector. A study conducted by Salman et al. (2023), revealed that intradermally administered Diluvac (inactivated *E. coli* suspension) to pigs was significantly protective than the intramuscularly administered Diluvac. Though unrelated to ASFV subunit vaccine, this study can confer the effectiveness of intradermal vaccination using needle-free injector and the necessity to explore the intradermal vaccination route for swine.

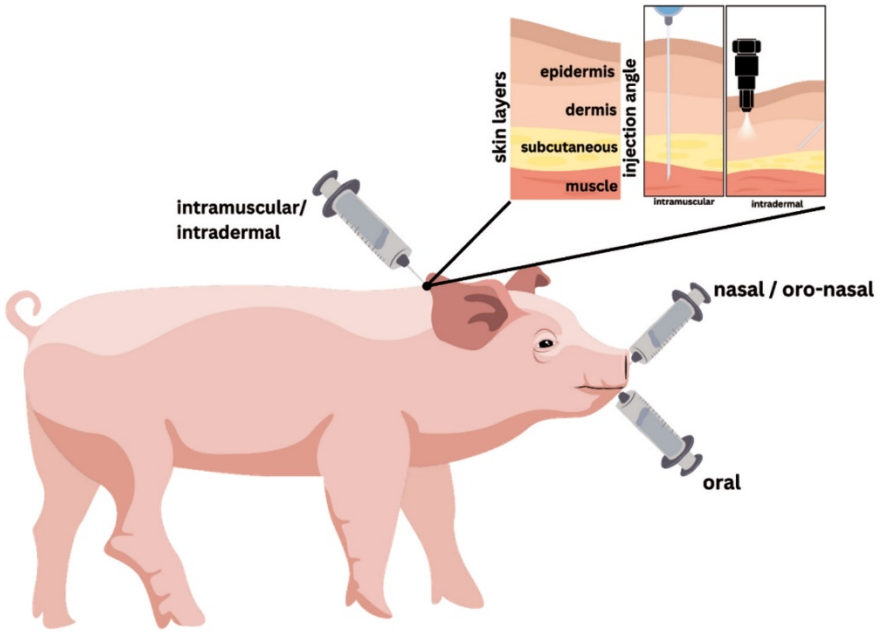

Figure 2 Common vaccination routes of ASFV vaccine employed in pre-clinical studies. This also shows the recommended injection site (neck) and the injection angle for intramuscular and intradermal vaccination (with needle or needle-less injection)

ASFV primarily enters the host through the respiratory tract. A strong mucosal immune response at the site of entry can be highly effective in preventing initial viral entry or limiting viral infection. Thus, intranasal vaccination (IN) route emerged as a preferable method for stimulating a potent mucosal immune response. This was observed in the ASFV study wherein the IN administration of attenuated ASFV on pigs showed higher protection compared to intramuscular vaccination with a live attenuated Genotype I OURT88/3 strain (Sánchez-Cordón et al., 2017).

Liu et al. (2023) have demonstrated the effectiveness of combined administration: intramuscular and intranasal, of a subunit vaccine utilizing HLJ/18 strain. The subunit vaccine contains CP204L (p30), E183L (p54), EP402R (CD2v), B646L (p72), and B602L (p72 chaperone) expressed by a type 2 adenovirus. This *in vivo* study showed high levels of IgA, IgG, and IFN-gamma, suggesting a potentially protective ASFV vaccine in both in pigs and mice. To note, this study did not employ challenge lethal dose, thus, this only imply safety and toleration of the vaccine administered.

Lastly, oral vaccination routes have gained popularity because of their convenience especially when dealing with wildlife (Cross et al., 2007) and their immune-protection potential. Barasona et al. (2019) conducted the first oral vaccination in wild boars. The study used a live attenuated ASFV (Genotype II Lv17/WB/Rie1 strain) and demonstrated protection against the challenge strain Arm07. A study conducted by Deutschmann et al. (2022) compared the efficacy of the orally administered attenuated ASFV (ASFV-G-ΔMGF) vaccine in domestic pigs and wild boars. Their findings revealed no significant difference in the protective efficacy of the oral vaccine against the challenge of ASFV Arm08 strain between the two tested groups. In pig farms, oral vaccines can be conveniently administered by incorporating them into the feed or drinking water however, feeds and water systems must be free of water treatments (e.g., chlorinated), medications, and/or disinfectants (Tizard, 2021). Additionally, when using the water system, a proportioner must be employed to ensure the correct dosage.

The considerable appeal of oral vaccines stems from the heightened recognition of ASFV's oral transmission potential. Oral vaccination targets both the (gut) mucosal immune system, providing a means to combat ASFV at its entry points and systemic immune system. This route holds promise for preventing infection via the digestive and respiratory tracts especially for subunit vaccines. In addition, other advantage of orally administered subunit vaccines is the storage requirement which usually do not require low temperature storage (Van der Weken et al., 2020). Though an oral ASFV subunit vaccine has not been explored, a research on PEDV oral subunit vaccine, containing S protein, has shown to be protective in pigs (Choe et al., 2020). This recent study necessitates the exploration of oral ASFV subunit vaccines.

In essence, in the development of effective vaccines against ASFV, choosing the correct vaccination route is crucial. This section has provided crucial insights into various vaccination routes for subunit ASFV vaccines, serving as a fundamental stepping stone in our pursuit of robust prevention and control strategies against ASFV. While intramuscular vaccination has been a conventional approach, recent pre-clinical investigations have revealed variable protective effects, highlighting the need for advancements such as subunit vaccines encompassing multiple ASFV strains. Moreover, alternative routes, such as intradermal, intranasal, and oral vaccination, have gained attention for their unique advantages in immune response initiation and convenience of administration. In summary, the exploration of diverse vaccination routes not only enhances our

understanding of ASFV immunization but also paves the way for innovative strategies in mitigating its impact, thereby safeguarding swine populations worldwide. Continued research and development in these varied approaches promises to strengthen our arsenal against ASFV.

FUTURE PERSPECTIVES

There is a growing interest in the development of subunit vaccines because of their safety. Multi-epitope subunit vaccines, in particular, are gaining attention because they are a cost-effective and time-efficient approach for vaccine design. These characteristics have allowed for the discovery of novel immunogenic peptides in the ASFV's complex genome as current peptides are ineffective in inducing an immune response against ASFV. Aside from its advantage over safety, multi-epitope subunit vaccine allows for simultaneous delivery of humoral and/or cellular-inducing epitopes to attain a balance of cellular and humoral immune responses that is needed to reach protective immunity against ASFV.

A pan-proteomic approach in epitope screening coupled with the innovative application of reverse vaccinology, demonstrates promising strides in epitope screening despite the challenges posed by the virus's complex genome. One major challenge in computational swine vaccine design is the absence of specialized prediction tools tailored for swine stemming from the lack of experimental data on SLAs. The curation of information on SLAs may be hastened through the collaboration of experts from various fields, specifically environmental, animal science, and human health. By making use of the similarities between the human and swine immune systems, swine-specific predictors can be developed. Currently, there is still a lack of open-access servers for non-human vaccine designs. Additionally, it is crucial to bridge the gap between computational design and *in vivo* confirmation studies, including delivery systems and viral vectors, to ensure the efficacy and practical application of these novel vaccines. The promising advantage of oral and mucosal vaccine administration over intramuscular administration as well as the use of adjuvants or nanoparticles for targeted delivery is worth noting considering that ASFV enters through the respiratory tract. A mucosal-specific immune response can be effective in inhibiting viral entry and limiting viral infection. As research continues to unfold, the integration of comprehensive safety assessments and experimental validations will be pivotal in advancing our understanding and ultimately achieving protective immunity against ASFV.

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AUTHOR CONTRIBUTIONS

FLO, AMS, and EMJSS conceptualized and designed the study. EMJSS, ECB, LEF and NMOO drafted the paper. EMJSS, ECB, LEF, NMOO, and FLO revised the paper. All authors approved the final version of the manuscript for submission. EMJSS revised the paper based on the reviewers' comments and suggestions.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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