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African Swine Fever (ASF) vaccine development: progress and challenges

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Contents

Abstract

African Swine Fever (ASF) is a highly contagious and deadly disease that affects domestic and wild boar, representing a significant threat to the pork industry with economic consequences worldwide. As there are no available treatments, there is an urgent need for an ASF vaccine. To achieve that, several challenges must be overcome.

Even though gaps in the knowledge of ASF virology and immunology are hindering the development of vaccines, several candidates of different types have been proposed in recent years. Live-attenuated vaccines (LAVs) are the most advanced in the development pipeline and present promising results, but they still entail notable safety concerns. Subunit vaccines could circumvent most of LAVs' disadvantages; however, to achieve satisfactory protective immunity, more efforts need to be put on discovering protective antigens and appropriate adjuvants.

For a veterinary vaccine to be commercialized, a marketing authorisation application, comprising a comprehensive set of data demonstrating the quality, safety and efficacy of the candidate, has to be submitted to the competent authority, which emits its opinion regarding approval taking into consideration the scientific evaluation of the submitted dossier. The data on quality, safety and efficacy shall be obtained through the performance of assays, *in vitro* and *in vivo* studies that must follow the requirements established in the applicable regulatory documents and guidelines. Internationally harmonised guidelines specific for ASF vaccine testing would help streamlining this process.

This report addresses the global state of ASF vaccine progress, briefly overviewing the challenges that have been encountered, and the specific advantages and limitations of the different types of vaccines. As well, it summarises the regulatory framework around the development of veterinary medicinal products, with a focus on ASF vaccines, and **proposes a possible way forward to accelerate the development of ASF vaccines.**

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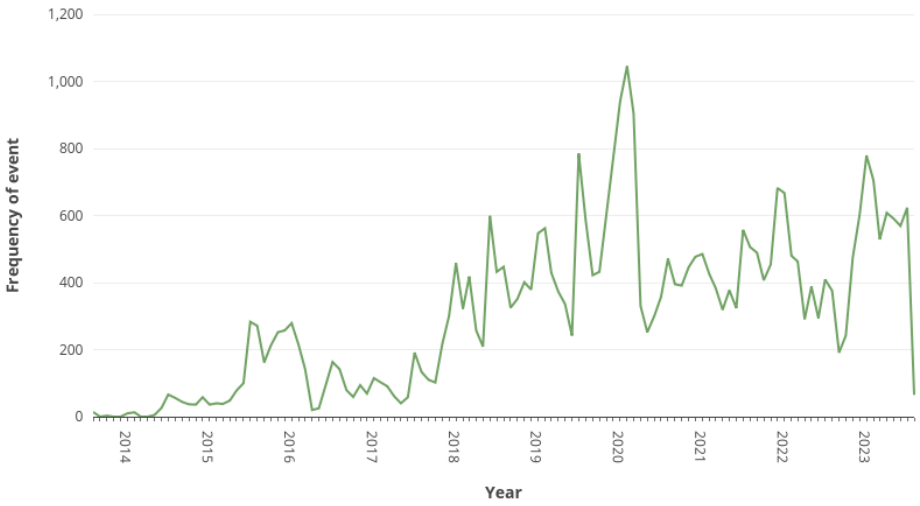
1 Introduction

African Swine Fever (ASF) is a devastating infectious disease that affects domestic swine and wild-boar of all breeds and ages.[1] ASF is highly contagious and the mortality rate of the most acute infections is almost 100%.[2,3] The causative agent of the disease is the African Swine Fever Virus (ASFV), a large linear double-stranded DNA arbovirus, exhibiting a complex multiple-layer structure, and the sole member of the *Asfarviridae* family.[2,4–6]

ASF was first reported in Kenya in 1921 and further spread across sub-Saharan African countries.[7] In 1957, ASFV shown to be able to cross continents, being detected in Portugal, and subsequently propagated to other European countries, to Brazil and to the Caribbean.[1,7] In the 1990's, ASFV genotype I, responsible for these first outbreaks, was eradicated in Europe (except in Sardinia) and America, after 30-40 years of efforts and containment measures.[1,8] However, a new round of transmission started in 2007, when ASFV genotype II was introduced to Georgia.[7] Afterwards, it spread though the Caucasus region into Russia and other Eastern European Countries.[1,7] By 2018, ASF reached Central Europe and appeared also in China, where it killed more than 4 million pigs.[1] ASF propagation continued, occurring in various regions of Asia, Oceania, and America. In fact, from 2020 to 2022, ASF outbreaks had been recorded in 35 different countries worldwide.[7] In Europe, ASF outbreaks have increased since 2020 (**Figure 1.A**), with the majority of the cases being reported in the eastern region (**Figure 1.B**).

A.



B.

Figure 1 – ASF outbreaks in Europe. **A.** 10-year-timeline of ASF in Europe. **B.** Geographic distribution of ASF cases in Europe showing 4451 events from 01.01.2023 to 01.08.2023. Source: FAO.2023.EMPRES-i PLUS [202308101331]. https://empres-i.apps.fao.org/diseases (Accessed 10/08/2023)

This rapid spread of ASF throughout the world has caused serious socio-economic impact in the farming sector and in trade.[3,6] Indeed, just in 2021, 142 million pigs were reared across the EU, representing the largest livestock category even before bovine, and the EU pig meat sector accounts for nearly half of the total EU meat production.[9] So far, no treatments or vaccines have been globally approved for ASF control. **Culling of infected animals and tight biosecurity rules are the only present countermeasures to prevent spreading, entailing important animal welfare concerns and economic consequences**.[10]

ASF is not a threat for human health, given the negligible risk for this stable virus to jump a species barrier. On the other hand, humans have a key role in the transmission of the disease. Spreading of ASF happens by direct contact between infected animals and by consumption of contaminated feed. Even though soft ticks are known among the African warthog populations for transmitting the disease and some speculation is made on the role of some flying arthropods as vectors, the major ASF "vector" in Europe and Asia is the human neglecting behaviour when it comes to biosecurity measures (transport vehicles, clothing) at farms and when feeding wild boar populations.[11,12] As the virus can survive in raw, dried and frozen pork products, the typical preventive measure (suggested recently also by FAO[13]) is the intensive border luggage control of passengers arriving from ASF-infected countries/regions, and instructing travellers to dispose waste containing pork products in a safe way. As humans are not susceptible to ASF, but its spread might have devastating socio-economic consequences, it was suggested to be an ideal candidate agent for biological warfare.[13] As such, ASF is also a popular element of fake news and subject of conspiracy theories generating anxiety regarding epidemic behaviour and undermining public trust in health policies.[14]

Considering all of these factors, a vaccine that could be deployed as needed and effectively used as preventive measure, without compromising safety, **seems to be the only option to completely eradicate the disease** in the long run and to put an end to the all the speculation around ASF.[15,16]

In spite of the host's ability to develop an immune response upon infection, ASFV encodes several proteins involved in mechanisms to escape both innate and acquired immunity, which contribute to virus survival and progeny production.[6,7] Further, there are some knowledge gaps related to these mechanisms, which challenge the development of an efficacious and safe vaccine. Namely, the specificities of different virus strains are not fully characterized, the processes involved in the development of immunity against ASFV are not well established, and no protective antigens have been identified so far. Besides, the efficacy of the ASF vaccine will depend on the induced immunity levels and duration of protection. High antibody levels (immune hyper-stimulation) appear detrimental with respect to clinical outcome and protection, therefore a shift towards fewer immunogenic ASF virus antigens might be useful. There is also a need to identify the antibodies that aggravate the disease. Moreover, the appearance of post-vaccination chronic symptoms must be monitored and the formulation and vaccination scheme to be used must be defined. Finally, the availability of stable cell lines for the ASF vaccine production, the selection of appropriate adjuvants and delivery vehicles, and the establishment of diagnostics to distinguish between infected and vaccinated animals (DIVA) are important factors to consider during vaccine development.[10]

Despite all these challenges, global research efforts are giving promising results and many vaccine candidates have been proposed over the last years.[10,17,18] These included inactivated, live attenuated and subunit vaccines, some of them resorting to biotechnological tools like recombinant DNA. However, any of these vaccines has been proved as unequivocally safe and efficacious in field trials.

For a candidate vaccine to be introduced in the market, quality, safety and efficacy need to be demonstrated. Marketing authorisations are granted by the competent authority responsible for the approval of veterinary medicinal products.[19-20] Annex II to the Regulation (EU) 2019/6 contains the requirements for immunological veterinary medicinal products, applicable to all vaccines. However, a **major drawback** to foster the development of vaccines against ASF **is that there are no internationally harmonised guidelines on how to assess their performance, in terms of purity, potency, safety and efficacy**.[21] Several ASF experts have highlighted the need of an in-depth safety characterization for life ASF vaccines to rule out reversion to virulence, [15,16] since prolonged forms of ASF with clinical characteristics below the detection limit have been observed to persist after vaccine testing, which could create infection cycles with increasing virulence.

In this context, the present report offers an overview of the main challenges encountered, as well as some insights into the current trends in ASF vaccine development. Moreover, it **proposes a way forward to speed up the development of promising vaccine candidates, for which specific funding should be made available**.

2 Vaccine candidates against ASF

2.1 ASF vaccine types. Advantages and limitations

Veterinary vaccines can be divided into 3 broad categories: inactivated, live attenuated and subunit vaccines. While live attenuated vaccines (LAVs) can be achieved through different procedures, subunit vaccines may resort to different technologies and delivery vectors and, therefore, both of these categories can be further subdivided into more specific types. **Table 1** presents an overview on the risks and benefits entailed by each of these vaccine types and subtypes.

Table 1 – Risks and benefits related to the use of various vaccine types

	Inactivated vaccines	**Live attenuated vaccines (LAVs)**				**Subunit vaccines**		
		Naturally attenuated	**Attenuated by passages**	**Modified virus**		**Antigen-based**	**DNA**	**mRNA**
				Deletion of genes	**Virus Vector**			
Genetic insertion, mutagenesis	No	Yes			Yes	No	Yes	No
Infectious	No	Yes			Yes	No	No	No
Mutation	No	No	Yes	Yes	Yes	No	No	No
Compatible with DIVA	No	No	No	Yes	Yes	Yes	Yes	Yes
Costs/production complexity	Low	Low	Moderate	High	High	High	Moderate	Moderate
Protective immunity	No	Yes	Yes	Yes	Partial	Partial	Partial	Not defined

2.1.1 Inactivated ASF vaccines

Traditional inactivated vaccines, containing toxins or cultures of microorganisms inactivated by chemical or physical means, are considered safe and usually involve low costs for production.[7] As such, several formulations have already been proposed as inactivated ASF vaccines, namely: ASFV-infected cell extracts; supernatant of ASFV-infected pig peripheral blood leucocytes; purified and inactivated ASF virions; glutaraldehyde-fixed ASFV-infected macrophages; and detergent-treated ASFV-infected alveolar macrophage cell cultures.[22,23] However, all of these attempts have failed to induce protection against ASFV infection.[1,8,22] In fact, they are not effective at inducing specific cytotoxic CD8+ T cell responses,[10,25] even when used together with adjuvants.[7,10,26]

2.1.2 Live attenuated ASF vaccines (LAVs)

LAVs involve the administration of a viable pathogen that presents reduced virulence. They can be divided into 4 different types: (1) naturally attenuated virus strains, (2) attenuated by serial cell line passaging, or (3) modified/recombinant agents obtained either through a) deletion of virulence-related genes or b) insertion of genes that code for specific immunising antigens into a nonvirulent vector microorganism.

Some positive results have been obtained with LAVs, with the use of both natural attenuated strains [27-29] and recombinant agents.[30–37] Indeed, **this type of vaccines confers a level of protection against experimental ASF infection far better than any other vaccine strategy tested so far**.[10]

While the use of **natural attenuated ASFV strains** complicates ASF eradication in endemic areas, given that chronically infected pigs might act as continuous reservoirs and are difficult to identify,[38] **ASFV strains attenuated by passages** may suffer genome modification, which could alter attenuation of virulence, safety, immunogenicity and protective efficacy.[1] Regarding **recombinant LAVs**, multiple **gene deletion** to attempt to completely remove any virulence from ASFV might not always be a good attenuation strategy, since it can lead to very weak viruses incapable of growing *in vivo* and of inducing the protective immunity.[1,10] In fact, deletion of a single gene can result in different phenotypes depending on the virus strand used and, thus, the effect of gene deletion on ASFV varies according to the strain.[1,31] As for LAVs corresponding to

modified **virus vectors** bearing genes that code for specific ASFV antigens, a major drawback is the lack of identified targets that would yield full protective immunity[4,10] Differentiating infected from vaccinated animals (DIVA) is a strategy in vaccine development that allows to distinguish between antigens and/or antibodies resulting from vaccination or from exposure to the real pathogen. This approach contributes to the proper monitoring – and finally eradication – of a disease, and can be implemented within the development of a recombinant LAV.

Nevertheless, LAVs also present some disadvantages which are hindering their progress in the development pipeline towards a marketing authorisation. Apart from the **concerns regarding their safety, due to association with some adverse side effects,[29,39] the potential of returning to virulence, of dispersing in the environment and of causing chronic or persistent infections,**[7,8] LAVs have shown limited effect when tested under field conditions.[1] Besides, despite being able to provide good homologous protection, **only partial/weak heterologous protection** seems to be achieved with this type of vaccines.[8,40] Moreover, **stable cell lines are still needed for LAVs large-scale production**, since standardisation of harvesting and culture protocols for primary porcine cells is difficult to attain and there is always the possibility of contamination by other animal pathogens.[1,7,8]

2.1.3 Subunit ASF vaccines

Subunit vaccines count on specific antigenic parts of the microorganism to yield immunity and are usually produced through recombinant DNA procedures. They rely on antigens or epitopes that have protective effects, such as the production of neutralizing antibodies and the induction of cellular immune responses.[18] This protective efficacy can be enhanced through the combination or fusion of protective antigens, together with appropriate adjuvants or delivery vehicles.[1] **These types of vaccines arise as ideal options for the future development of immunisation strategies against ASF, overcoming most of the risks inherent to LAVs in virtue of their better safety and stability**.[8,10] Besides, they are also compatible with DIVA strategy.[41]

Apart from the **antigen-based** vaccines (consisting in the direct delivery of a protein or epitope, with or without modifications), this category might also include other technologies for specific ASFV antigen production within the host cells, such as DNA plasmid vaccines, the novel family of mRNA vaccines, and the use of **viral vectors** for DNA/RNA delivery. All of these are promising platforms to be considered in future ASF vaccine design and development. Partial success in protecting pigs from infection was already shown by **DNA vaccines**,[42] but this type of technology also entails some risks such as insertional mutagenesis. On the other hand, the fast-expanding platform of **lipid nanoparticle-RNA vaccines** validated for human use during the COVID-19 pandemic might offer new solutions also in the fight against animal diseases, since they have no risks related to possible infection, mutation or mutagenesis. Because of the lipid nanoparticle formulation, no additional adjuvants are needed. Moreover, their development is supposed to be fast compared to other vaccine types, and production costs are considered to be moderate compared to LAVs, where production requires primary porcine alveolar macrophages or bone marrow cells.[43]

Yet, **most of subunit vaccine candidates are still in early phases of development**[7] and there is still a long way to go in this field.[8,10] The preliminary results on the efficacy of these vaccines are inconsistent,[42,44–48] most likely due to the great heterogeneity among the used antigens, virus strains, doses, vaccination strategies, delivery vectors, and challenge models.[8] A major drawback for the development of subunit vaccines for ASF in particular is that no protective antigens have been identified so far, and gaps in the knowledge of infection and immune response mechanisms remain.[8,10]

2.2 Important features for usable ASF vaccines

Overall, an ideal vaccine against ASF should have the following characteristics:

- It should be safe for the target animals, in-contact species and the surrounding environment.
- It should be able to yield long-term cross-protection against not only a single virus strain, but against different virus variants from different genotypes.
- If possible, the vaccine should enhance protective responses as well as avoid prejudicial immune responses (immune hyper-stimulation), through well established and understood mechanisms.

- In order to achieve better efficacy, it should incorporate adjuvants that can stimulate antigen-presenting cells and increase cellular immunity by secreting various Th1 cytokines (IFN-γ, IL-2, IL-12 and TNFα).[1]
- Importantly, the immune response produced by the vaccine shall be distinguishable from the one caused by natural infection (DIVA vaccine).
- Lastly, it would be desirable for the vaccine to be produced in a reproducible, high-quality and low-cost manner that would also allow for adaptation into bait formulations, compatible with wild-life immunization campaigns.[10]

2.3 Challenges to ASF vaccine development

ASFV is a very complex pathogen. The physical size of the virus is huge, implying that it has a lot of proteins that supply a high ability to avoid and escape the immune system. On the scientific-technical side, one of the main challenges is to **fully understand the mechanisms of ASF virus infection and immunity**.[10]

ASFV has around 190 genes coding a considerable amount of proteins, whose functions are not all known, and many immune escape genes. The precise nature of the host protective responses has not been fully determined. In most viral infections a humoral immune response, with neutralizing antibodies, is regarded as important and often sufficient to provide protection.[49] Conversely, in ASF, both humoral and especially cellular immunity seem to be important, since humoral immunity alone has not been effective in protecting animals from infection. In fact, various studies suggest that factors other than neutralizing antibodies play a role in protection against ASFV.[8] As highlighted before, **the identification of ASFV proteins inducing a fully protective immune responses in pigs, so called protective antigens, is still missing**. This hinders not only the development of effective vaccine candidates but also constitutes a major setback for designing robust and adequate studies to assess correlated of protection (immune response a vaccine needs to trigger so that the animal is protected), necessary to demonstrate the efficacy of a vaccine.

The extent of ASFV strain variation poses additional hurdles. ASFV establishes long-term persistent, latent infections in warthogs and in pigs surviving acute viral infections. The emergence of non-hemadsorbing naturally attenuated strains of ASFV that cause non-obvious symptoms hinders the diagnosis and identification of chronically infected pigs that might act as continuous reservoirs.[7] Furthermore, surviving pigs have been shown to develop long-term resistance to homologous virus infection, but not to heterologous.[1]

The European Food Safety Authority (EFSA) conducted a **gap analysis** on ASF (July 2019) [50], identifying further challenges in this context, such as: the lack of basic research in proteomics in this field; the high propensity for ASF transmission and spread due to the interaction of wild boar with domestic livestock; the fact that ASFV is capable of surviving in different matrices, which complicates its eradication and containment; the need for increasing awareness and compliance with existing control measures, to achieve suitable biosecurity levels.[51] Aiming to tackle the latter, EFSA launched the 'Stop African Swine Fever' campaign, in partnership with local authorities in 18 European countries, addressing farmers, veterinarians, and hunters.[52]

In view that ASF is a notifiable animal disease and wild boar can expand the infection front at rapid pace, another not negligible hurdle is the difficulty in finding locations inside Europe that would receive authorisation for performing field trials involving ASF. [73]

On the other hand, the lack of **internationally harmonised guidelines for assessing the quality, safety and efficacy of ASF vaccine candidates** has been identified as a major issue affecting current and future vaccine development.[21] Indeed, such guidelines would help focusing testing efforts, ensuring appropriate safety levels, and would allow for a comparability of results. ASF is a very particular viral disease, characterized by high lethality and fast transmissibility, therefore requiring stringent safety considerations during testing to avoid spreading. As recently suggested by Blome and co-workers[15], in-depth safety characterisation should be performed in the case of live ASF vaccines. Besides, the lack of internationally agreed parameters for vaccine testing in ASF may imply that criteria regarding quality, safety and efficacy differ worldwide, which could hinder an eventual acceptance of vaccines approved elsewhere. Recent efforts towards drafting harmonised international guidelines are being undertaken by the World Organisation for Animal Health (WOAH) [21], and an updated version of the ASF chapter within the Terrestrial Manual is expected to be released by the end of 2023.

3 Current trends in ASF vaccine development

A good indication of the efforts currently being made and the next steps to be taken for achieving an efficient tool in the fight against ASF can come from the evolution of submitted patent applications and the status of approval of vaccine candidates worldwide.

On the other hand, STAR-IDAZ International Research Consortium on Animal Health coordinates research at the international level in the context of new and improved animal health strategies.[53] Currently, it is working with the Global African Swine Fever Research Alliance (GARA) to coordinate research, establish and sustain global research partnerships for generation of scientific knowledge and roadmaps for prevention, control and ultimately eradication of ASF.[55] It is, thus, an excellent source for consultation of updates on worldwide ASF research advances.

3.1 Submitted patents of ASF vaccines

The landscape of patents for potential ASF vaccines directly reflects the current trends on ASF vaccine development. A search was performed on PatentSight® in January 2023, using 'ASFV' and 'vaccine' as keywords, in order to analyse the main features of the candidate vaccines that have been registered.

From the performed search, 481 patent registries were retrieved. Of these, only 87 (18%) accurately corresponded to ASF vaccines, while 38 (8%) corresponded to inventions that could potentially be used as an ASF vaccine. The first ASF vaccine patent was registered in 2013 and few others were published in the following years until 2019. After 2020 the number of published patents within this framework increased substantially (**Figure 2.A.**)

Most of the ASF vaccines with a published patent correspond to either LAVs obtained by deletion of virulence related genes from an ASFV strain (35%, n = 30) or subunit vaccines containing antigenic parts of the ASFV that were produced through biotechnology methods (44%, n = 38), as depicted in **Figure 2.B.** The published ASF vaccines' patents have been submitted by entities located in 12 different countries, and 3 patents were even registered by inventors from more than 1 country each. China stands out as the country with more registered patents (69%, n = 60), followed by the USA with 10 registered patents (12%) and South Korea (7%, n = 6). The majority of the patents were published between 2020 and 2022, especially by entities located in China and corresponding to both live attenuated and inactivated ASF vaccines (**Figure 2.C.**).

In 2021, five companies were reported to have ASF vaccine in their pipelines. MBF Therapeutics developed a DNA vaccine where the antigen was inserted into a plasmid using T-Max™ Platform and delivered using CaptaVax™ delivery system based on calcium phosphate nanoparticles; Inovio Pharmaceuticals together with Plumbline Life Sciences announced to be working on a DNA plasmid-based solution, Pacific Green Tech partnered with Epiwax targeting viral epitopes in combination with prime boost administration strategy to generate both cell-mediated response and humoral antibody response.[54]

3.2 Approved ASF vaccines

The National Veterinary Joint Stock Company (NAVETCO, Vietnam) with the USDA's Agricultural Research Service (ARS) has successfully developed a product which became the first ASF vaccine approved in Vietnam. It is based on genotype II pandemic strain, ASFV-G, which was modified to ASFV-G-ΔI177L by deletion of the I77L gene, a highly conserved genetic element with a predicted immune modulator function.[56] The prototype vaccine was safe under the conditions tested, however in 2022, about 600 pigs inoculated with this vaccine died in Vietnam. After temporary suspension of the use of the vaccine, it was concluded that the problem was caused by vaccination processes out of compliance with guidelines.[57] Investigations found some vaccinated animals that tested positive for a field strain of the ASF virus, or other pathogens. On one hand, the case underlines the **need of setting and following proper guidelines for vaccine administration**. On the other hand, it highlights the difference between fully controlled test and realistic field conditions that should be also considered at safety assessment. Although this ASFV-G-ΔI177L vaccine has a positive reporter gene (mCherry), no negative marker(s) were identified that could distinguish vaccinated from infected pigs in serological tests (DIVA strategy).[21]

A second vaccine approved in Vietnam is AVAC, a live attenuated multigene family strain virus vaccine[58], which has been developed entirely in Vietnam. In February 2023, promising news [59] appeared announcing that the efficacy rate of the AVAC vaccine, administered to pigs between the ages of 8 and 10 weeks, reached 95% and that Vietnam expected to begin nationwide distribution of the vaccine soon.

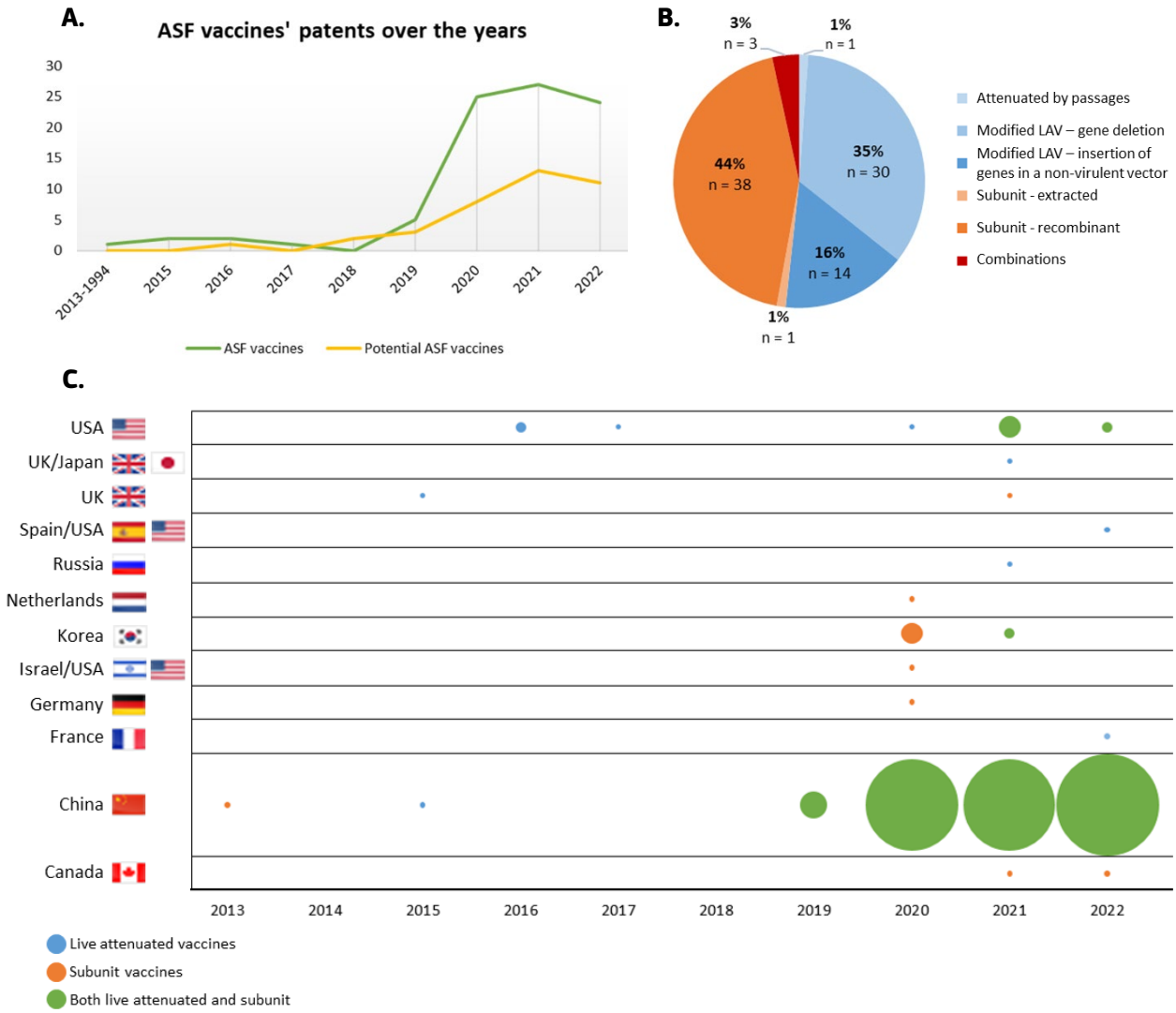


Figure 2 – Analysis of ASF vaccine patents registered on PatentSight®. **A.** Number of ASF vaccines and potential ASF vaccines' patents published over the years. **B.** Types of ASF vaccines with a published patent. **C.** ASF vaccines' patents submitted by entities in each country over the years. Circles represent the patents that have been published in each year. The circles' area is proportional to the number of patents. The circles are color-coded according to the type of vaccine.

3.3 EU-funded projects for developing an ASF vaccine

The European Union's Horizon 2020 research and innovation programme has funded two ongoing research projects focused on ASF vaccines, namely DEFEND[60] and VACDIVA[61].

The EU-funded DEFEND Horizon 2020 project gathered academic, industrial and governmental organisations in an international partnership to tackle ASF and lumpy skin disease in European livestock. The focus was on developing novel diagnostic tools and vaccines.

The EU-funded VACDIVA Horizon 2020 project was only focused in ASF and was planned to provide safe and effective vaccine prototypes against ASF, intended for both wild boar and domestic pigs, by the end of 2023. VACDIVA strategy consists in obtaining a LAV through gene deletion of virulence-associated genes from an already naturally attenuated strain, also compatible with DIVA testing. The project is planned to develop and validate cost-benefit and -effective surveillance and control-vaccination strategies in parallel. So far, several mutants have been produced from 2 naturally attenuated strains (genotype I and II) **and laboratory studies have been performed in domestic and wild swine in Spain, with successful results**. The next steps in the pipeline are the performance of field trials planned to be carried out in Kenya and Lithuania; of a possible pilot vaccination for domestic pigs in Asia; and of additional studies to further evaluate side effects. The final aim is to select a safe and efficacious prototype amenable to scaling up vaccine production.

4 Considerations to accelerate ASF vaccines development

4.1 Main requirements for the approval of ASF vaccines

Annex 1 provides a comprehensive overview of the regulatory framework around veterinary vaccines, including EU legislation and EU / International guidelines. According to Regulation (EU) 2019/6 an application for marketing authorisation shall contain technical documentation necessary for demonstrating the quality, safety and efficacy of the product. ASF vaccines fall into the category of immunological veterinary medicinal products, for which the requirements laid down in Section IIIb of Commission Delegated Regulation 2021/805, amending Annex II to Regulation (EU) 2019/6, apply.

In the case of LAVs, the most promising ASF vaccine candidates to date, strong evidence of its safety and efficacy has to be obtained in appropriate *in vivo* studies following all the requirements summarised in Annex 1. Briefly, in target animal safety studies, such as the ones to determine the safety of 1 dose, repeated single doses, 1 overdose and dissemination of the virus on the vaccinated animal, the parameters to be evaluated should include: presence of ASF clinical signs, preferably measured with a scoring system [e.g. the one proposed in King K. et al (2011)[39]]; daily body temperature post-vaccination; viremia measured in blood samples, and virus shedding, determined through virus detection assays performed on nasal and faecal swabs, all collected weekly for a considerable period after vaccination. For the spread of virus strain studies, especially important for LAVs, the assessments might include gross pathology and virus quantitation namely on tonsils, lungs, spleen, liver, kidneys and lymph nodes close to the inoculation site, and seroconversion, evaluated by antibody serology tests with ELISA.

Efficacy studies, namely the ones for determining the immunogenicity, protective dose and reduction of transmission, might mainly assess animals' clinical signs, fever, viremia, and seroconversion, as well as virus shedding (optional) after challenge with a lethal dose of a virulent virus.

Overall, a vaccine would be considered safe if it doesn't yield adverse effects (mortality, fever, clinical signs). Additionally, in the case of LAVs, it should be proved that it doesn't possess any residual virulence, and it is not able to spread within the organism and across animals. For it to be considered efficacious, it must prevent or reduce mortality, the clinical signs associated to the acute disease, fever, viremia and/or disease transmission.

4.2 Development of ASF-specific testing guidelines

Currently available guidance documents do not provide specific parameters or testing conditions that could be most relevant for ASF vaccine assessment. Guidelines on how best implement the above mentioned requirements would help to decide on the endpoints/scoring for checking clinical signs, the necessity of defining unreal worst-case scenarios, or what minimum criteria should be used to demonstrate acceptable safety and efficacy. A recent review by Brake D.A. (2022)[21] addressed some recommendations to support the development of harmonized international guidelines for ASF vaccines testing and provided some hints on the criteria to be used in *in vivo* studies' assessments. Moreover, the development of internationally harmonised guidelines would be very useful to foster ASF vaccine availability by facilitating interpretation and comparability of results, and ensuring long term safety.

4.3 Roadmap to accelerate ASF vaccine development

Several very promising vaccine candidates have been reported in recent years, but their levels of efficacy and safety need to be better demonstrated. **The development and deployment of ASF vaccines would benefit from an agreed protocol for vaccine evaluation and side-by-side comparison**, to be able to compare objectively the performance of different candidates. For that purpose, a roadmap to check vaccine performance and push candidate vaccines to enter into the market is proposed. The goal would be to validate the quality, safety and protective efficacy of the most promising vaccines against ASFV, based on the golden standard of double blind trials, and advance those that achieve established performance criteria. The roadmap consists of the following steps:

1. **Establish minimum acceptance criteria**, in compliance with EU legislation, for candidate vaccines in terms of quality, safety and efficacy. WOAH is currently developing guidelines specific for ASF vaccine testing, which could be the basis for such acceptance criteria.

2. **Select candidate vaccines**, e.g. through a dedicated call, **to be tested in a double blind set up** and shortlisted according the set of pre-agreed performance criteria. Beyond quality, safety and efficacy, important features to consider are the ability to protect from different strains of the virus, the availability of a stable cell line for vaccine production and the possibility of distinguishing vaccinated animals from infected ones. The double blind testing should be done in field trials under controlled biosafety conditions and in institutions from different countries, ideally from inside and outside Europe. For this, appropriate funding should be made available
3. Select the most promising candidates for submitting a marketing authorisation request and **establish a plan for scaling-up vaccine production** involving vaccine manufacturers.

5 Conclusions

This report overviews the main challenges of current technologies used for developing a vaccine against ASF, summarises the main requirements for applying for marketing authorisation and proposes a series of steps to support ASF vaccine development efforts. The roadmap involves establishing minimum acceptance criteria, shortlisting vaccine candidates based on results obtained in double blind field trials and advancing the most promising ones into marketing authorisation and deployment procedures.

ASF is an acute, highly contagious and deadly infectious disease and can be considered the threat to animal health with major potential economic and societal impact. ASF virus is a complex large double-stranded DNA virus that spreads quickly through contact with infected animals, ingestion of contaminated pig products and tick bites. Despite decades of ASF vaccine research, still some gaps in knowledge are hindering the development of a viable vaccine. Worth mentioning are gaps in the understanding of mechanisms of infection and immunity, as well as the fact that virus antigens responsible for inducing protective immune responses in pigs have not been identified yet. From the different approaches and categories of vaccines, the most promising, in the short term, seem to be deletion mutants of LAVs, which have been genetically modified to remove virulence genes.

The development and deployment of an ASF vaccine would profit from an agreed protocol for vaccine evaluation and side-by-side comparison, for which internationally harmonised test guidelines specific for ASF, as well as the agreement on minimum performance criteria, would be necessary. A major action that would accelerate vaccine development is the definition of harmonized international ASF vaccine testing guidelines concerning the assessment of quality, efficacy and safety. Ideally, test guidelines should include experimental procedures to proof the safety of vaccines for the target animals, for species in direct contact and for the environment. In addition, important aspects when considering promising vaccines would be the cross-protection ability against different virus strains, the stability and reproducibility of the vaccine manufacturing process and the availability of diagnostics tests to distinguish vaccinated animals from natural infections.

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List of abbreviations and definitions

ASF	African Swine Fever
ASFV	African Swine Fever Virus
DIVA	Differentiating Infected from Vaccinated Animals
EFSA	European Food Safety Authority
ELISA	Enzyme-linked Immunosorbent Assay
EMA	European Medicines Agency
LAV	Live-attenuated vaccine
OIE	Office International des Epizooties
SOP	Standard Operating Procedures
VICH	Veterinary International Conference on Harmonisation
VMP	Veterinary Medicinal Product
WOAH	World Organisation for Animal Health

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Annex 1 Regulatory framework for veterinary vaccines

In order to obtain a marketing authorisation, the company wishing to place the veterinary medicine product (VMP) on the market must submit an application to a competent authority accompanied by a comprehensive set of data demonstrating the quality, safety and efficacy of the candidate VMP.

The following entities provide guidance and support to the research and development of veterinary medicines and make available a set of resources and guidelines that must be considered when preparing a marketing authorisation for a VMP.

- **European Medicines Agency (EMA).** It is the EU body responsible for evaluating applications for centralised marketing authorisation.[62,63]
- **EFSA's Panel on Animal Health and Welfare**. Provides independent scientific advice to the European Commission (EC) and Member States (MS).[64]
- **World Organisation for Animal Health (WOAH).** It is the intergovernmental organisation that acts as global authority on animal health.[65]
- **Veterinary International Conference on Harmonization (VICH).** It is a trilateral programme, EU-USA-Japan, to harmonise technical requirements for VMP registration. It is done through the development of harmonised guidelines.[17]

Table A1 lists all the main legal, regulatory and guidance documents that shall be taken into consideration for the development of a veterinary vaccine against ASF. Noteworthy are:

- *Vaccine production and ASF*
  - **Regulation (EU) 2016/429**[66]**,** supplemented by **Commission Delegated Regulation (EU) 2023/361:** ASF is one of the diseases within the scope of the Regulation and for which rules for the use of VMPs for prevention and control apply (Article 5). It defines conditions for animal health in laboratories, the responsibilities of different stakeholders and how to manage transmissible diseases.
  - **Regulation (EU) 2019/6**[67] laying down rules for the placing on the market, manufacturing, import, export, supply, distribution pharmacovigilance, control and use of VMPs, supplemented by **Commission Delegated Regulation (EU) 2021/805** setting up the requirements for the technical documentation to be presented to the marketing authorisation (Annex II).
  - **Chapter 3.9.1. of the OIE/WOAH Terrestrial Manual – African Swine Fever**[68] – Overview on ASF, including epidemiology, aetiology, pathophysiology and clinical presentations, as well as the techniques and criteria available to diagnose it. There is a section referring to the requirements for testing vaccines, which despite not containing any information yet, is currently under development by WOAH.
  - **Chapter 1.1.8. of the OIE/WOAH Terrestrial Manual - Principles of Veterinary Vaccine Production**[69]**:** Overview of the requirements and procedures for guidance in the production of veterinary vaccines, including aspects on manufacture process, quality control and marketing monitoring after the product has been approved.
- *Guidelines on live vaccines*:
  - **VICH GL55**[70]: Criteria harmonisation to waive animal batch safety testing for live vaccines.
  - **VICH GL44**[71]: Target animal safety for veterinary live and inactivated vaccines.
  - **VICH GL41**[72]**:** Target animal safety: examination of live veterinary vaccines in target animals for absence of reversion to virulence.
- *Guidelines on clinical trials*:
  - **EMA/CVMP/IWP/260956/2021**[73]**:** Guideline on clinical trials with immunological veterinary medicinal products.

Table A1 – Main documents (including regulations, directives, guidelines, monographies and chapters) for guidance on developing Veterinary Medicinal Products,.

Document		Topic
A. General Regulations and Guidelines		
Regulation (EU) 2016/429 of the European Parliament and of the Council of 9 March 2016		Transmissible animal diseases and amending and repealing certain acts in the area of animal health ('Animal Health Law')
• Commission Delegated Regulation (EU) 2023/361 of 28.11.2022		Supplementing Regulation (EU) 2016/429 as regards rules for the use of certain veterinary medicinal products for the purpose of prevention and control of certain listed diseases
Regulation (EU) 2019/6 of the European Parliament and of the Council of 11 December 2018		Veterinary medicinal products and repealing Directive 2001/82/EC
• Commission Delegated Regulation (EU) 2021/805 of 8 March 2021		Amending Annex II to Regulation (EU) 2019/6
Chapter 3.9.1. of the OIE/WOAH Terrestrial Manual		African Swine Fever (Infection with African Swine Fever Virus)
B. Production and Control		
Volume 4 of EudraLex		EU Guidelines for Good Manufacturing Practice (GMP) for Medicinal Products for Human and Veterinary Use
Commission Directive 91/412/EEC of 23 July 1991		Laying down the principles and guidelines of good manufacturing practice for veterinary medicinal products
Chapter 1.1.8. of the OIE/WOAH Terrestrial Manual		Principles of veterinary vaccine production
EMA/CVMP/IWP/206555/2010-Rev.1 of 8 December 2016		Guideline on requirements for the production and control of immunological veterinary medicinal products
Analytical Validation	**VICH GL1** (Validation Definition)	Validation of Analytical Procedures: Definition and Terminology
	VICH GL2 (Validation Methodology)	Validation of Analytical Procedures: Methodology
Specifications *	**VICH GL39** (Quality)	Test procedures and acceptance criteria for new Veterinary Drug Substances and New Medicinal Products: Chemical Substances
Batch Sterility/Purity	**VICH GL11(R)** (Quality)	Impurities in new Veterinary Medicinal Products
	Monograph 2.6.1 of the European Pharmacopeia	Sterility
	VICH GL18(R) (Quality)	Impurities: residual solvents in new Veterinary Medicinal Products, active substances and excipients
	VICH GL25	Testing of residual formaldehyde
	VICH GL26 (Biologicals: Moisture)	Testing of residual moisture
	Monograph 2.6.2 of the European Pharmacopeia	Mycobacteria
	Monograph 2.6.3 of the European Pharmacopeia	Mycoplasmas
	VICH GL34 (Biologicals: Mycoplasma)	Testing for the detection of Mycoplasma contamination

Batch safety	**VICH GL50** (Biologicals: TABST)*	Harmonisation of criteria to waive target animal batch safety testing for inactivated vaccines for veterinary use
	VICH GL55 (Biologicals: TABST Live Vaccines)	Harmonisation of criteria to waive target animal batch safety testing for live vaccines for veterinary use
	VICH GL59 (Biologicals: LABST Veterinary Vaccines)	Harmonisation of criteria to waive laboratory animal batch safety testing for vaccines for veterinary use
Stability	**VICH GL3(3)** (Quality)	Stability: stability testing of new veterinary drug substances
	VICH GL5 (Stability)	Stability testing: photostability testing of new veterinary drug substances and medicinal products
	VICH GL45 (Quality)	Quality: bracketing and matrixing designs for stability testing of new veterinary drug substances and medicinal products
	VICH GL58 (Quality)*	Stability Testing of New Veterinary Drug Substances and Medicinal Products in Climatic Zones III and IV
C. Safety and Efficacy Assessment		
Target Animal Safety	**VICH GL41** (target animal safety) – reversion to virulence	Target animal safety: examination of live veterinary vaccines in target animals for absence of reversion to virulence
	VICH GL44 (target animal safety) - biologicals	Target animal safety for Veterinary live and inactivated Vaccines
Environmental Safety	**VICH GL6** (Ecotoxicity Phase I)	Environmental Impact Assessment (EIAS) for Veterinary Medicinal Products - Phase I Guidance
	VICH GL 38 (Ecotoxicity Phase II)*	Environmental Impact Assessment (EIAS) for Veterinary Medicinal Products - Phase II Guidance
	Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001	Directive on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC
Toxicology*	**VICH GL33** (safety: general approach)	studies to evaluate the safety of residues of veterinary drugs in human food: general approach to testing
	VICH GL54 (safety) – ARfD	studies to evaluate the safety of residues of veterinary drugs in human food: general approach to establish an acute reference dose (ARfD)
	VICH GL22 (Safety: Reproduction)	Studies to evaluate the safety of residues of veterinary drugs in human food: reproduction testing
	VICH GL23(R) (safety) - genotoxicity	Studies to evaluate the safety of residues of veterinary drugs in human food: genotoxicity testing
	VICH GL32 (safety: developmental toxicity) Revision 1	Studies to evaluate the safety of residues of veterinary drugs in human food: developmental toxicity testing
	VICH GL28 (safety: carcinogeniticy)	Studies to evaluate the safety of residues of veterinary drugs in human food: carcinogenicity testing
	VICH GL31 (safety: repeat-dose toxicity) Revision 1	Studies to evaluate the safety of residues of veterinary drugs in human food: repeat-dose (90 days) toxicity testing
	VICH GL37 (safety)	Studies to evaluate the safety of residues of veterinary drugs in human food: repeat-dose chronic toxicity testing

Efficacy	**EMEA/CVMP/682/99**	Duration of protection achieved by veterinary vaccines
Pre-clinical/ Laboratory studies	**DIRECTIVE 2004/10/EC** of the European Parliament and of the Council of 11 February 2004	Harmonisation of laws, regulations and administrative provisions relating to the application of the principles of good laboratory practice and the verification of their applications for tests on chemical substances
Field/Clinical Trials	**VICH GL9** (GCP)	Guideline on Good Clinical Practices
	EMA/CVMP/IWP/260956/2021	Guideline on clinical trials with immunological veterinary medicinal products
	EMA/CHMP/CVMP/3Rs/336802/2017	Recommendation to marketing authorisation holders, highlighting recent measures in the veterinary field to promote reduction, refinement and replacement (3Rs) measures described in the European Pharmacopoeia
	EMA/CHMP/CVMP/JEG-3Rs/746429/ 2012	Recommendation to marketing authorisation holders for veterinary vaccines, highlighting the need to update marketing authorisations to remove the target animal batch safety test (TABST) following removal of the requirement from the European Pharmacopoeia monographs
	EMA/CHMP/CVMP/3Rs/164002/2016	Reflection paper providing an overview of the current regulatory testing requirements for veterinary medicinal products and opportunities for implementation of the 3Rs

'*' – facultative, depending on: the type of vaccine; the intended market

A.1 Overview of the process for veterinary vaccine development and its regulatory and scientific requirements

Veterinary vaccines pipeline

The typical veterinary vaccine pipeline usually takes between 3 and 6 years[20] and can be divided into 4 main phases: (1) discovery, (2) development, (3) registration, and (4) commercialization (**Figure A.1**). During development (phase 2), the characterisation and quality assessment of the candidate is accomplished through the performance of a set of physical, chemical and biological analytical assays. On the other hand, in order to prove the safety and protective efficacy of the experimental vaccine, *in vivo* laboratory studies, and/or clinical/field trials must be conducted on the target animal species.[20]

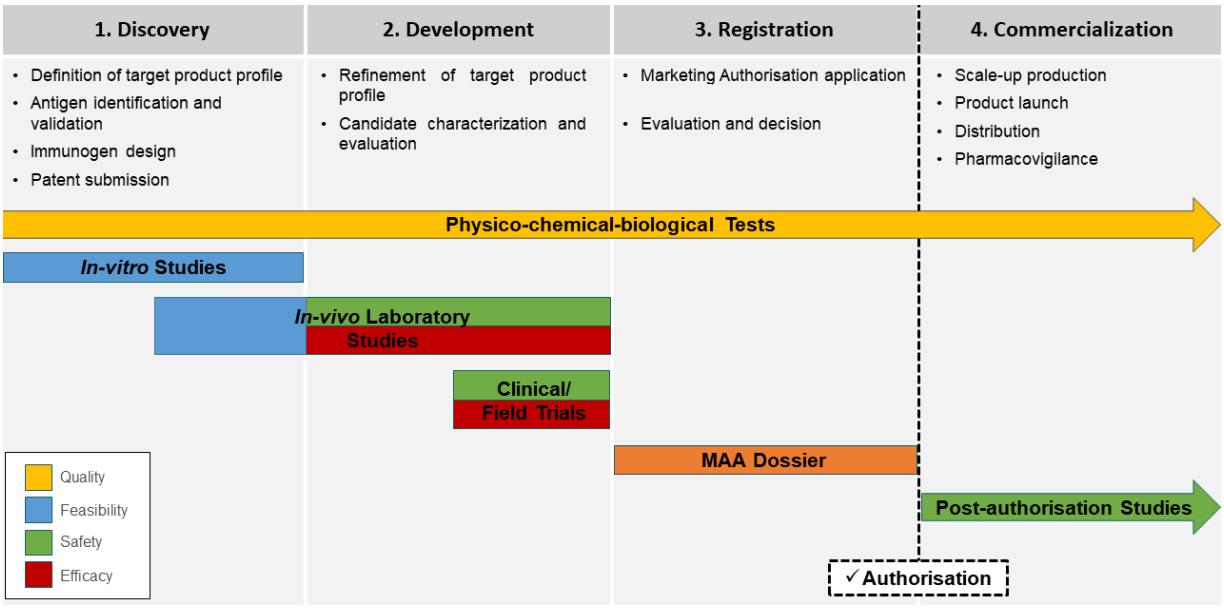

Figure A1 – Pipeline for veterinary vaccines' development for the European Union market. 'MAA' – Marketing Authorisation Application.

Vaccines classification

According to Chapter 1.1.8 of the OIE/WOAH Terrestrial Manual[69] vaccines may be prepared as live of inactivated products and can be classifies as presented in **Table A2**. Vaccines obtained using recombinant techniques, or biotechnological vaccines are divided into three broad categories: **Category I** – for products that pose negligible risks and no new or unusual safety concerns; **Category II** – for products in which the genetic modifications must not result in increased virulence, pathogenicity or survivability of the altered organism when compared with its wild-type form; and **Category III** – for products that contain recombinant-derived foreign genes that code for immunising antigens, which constitutes a new approach to vaccine development.[69] In this sense, it is possible to infer that ASF subunit, mRNA and DNA vaccines belong to category I, recombinant LAVs produced through ASFV gene deletion to category II, and live virus vectors containing genetic material coding for specific ASFV antigens to category III.

Table A2 - Types of vaccines according to Chapter 1.1.8. – Principles of Veterinary Vaccine Production - of the OIE/WOAH Terrestrial Manual.[69]

1. Live vaccines	**1.1.** Prepared from low virulence, mild, field isolates of a disease-causing agent. The exposure to the agent is safe and effective, causing immunisation instead of the disease		
	1.2. Prepared from isolates of disease-causing agents modified by passage through laboratory animals, culture media, cell cultures or avian embryos to select a variant of reduced virulence		
	1.3. Modified agents (rDNA)	**a)** Deletion of virulence-related genes from a microorganism	**Category II**
		b) Insertion of genes that code for specific immunising antigens from a disease-causing microorganism into a nonvirulent vector microorganism	**Category III**
2. Inactivated (killed) vaccines	**2.1.** Containing cultures of microorganisms inactivated by chemical or physical means		
	2.2. Containing inactivated toxins		
	2.3. Containing subunits (antigenic parts of microorganisms)	**a)** Extracted from cultures	
		b) Produced through rDNA procedures	**Category I**

As for the concrete case of ASF, the WOAH Terrestrial Manual (Chapter 3.9.1.)[68] classifies it as a non-zoonotic disease that does not affect public health. Also, according to the Commission Implementing Regulation (EU) 2018/1882[52], ASF is considered a **Category A** – 'listed disease that does not normally occur in the Union and for which immediate eradication measures must be taken as soon as it is detected' -, **D** – 'listed disease for which measures are needed to prevent it from spreading on account of its entry into the Union or movements between Member States' – and **E** – 'listed disease for which there is a need for surveillance within the Union – disease'. Being a notifiable disease, ASF is of mandatory declaration to the WOAH. Besides, and taking into account that the affected animals are pigs, the requirements concerning 'food-producing animals', meaning animals bred, raised, kept, slaughtered or harvested for the purposes of producing food, shall also be considered.

A.2 Quality, Safety and Efficacy Assessment

Quality assessment

The production of any VMP should be in accordance with the principles stipulated in the Good Manufacturing Practices (GMP), on Volume 4 of EudraLex[74]:

To ensure that the final product complies with the quality standards, several tests and assays must be conducted before, during and after the manufacturing process. **Figure A2** presents an overview of these required quality tests.

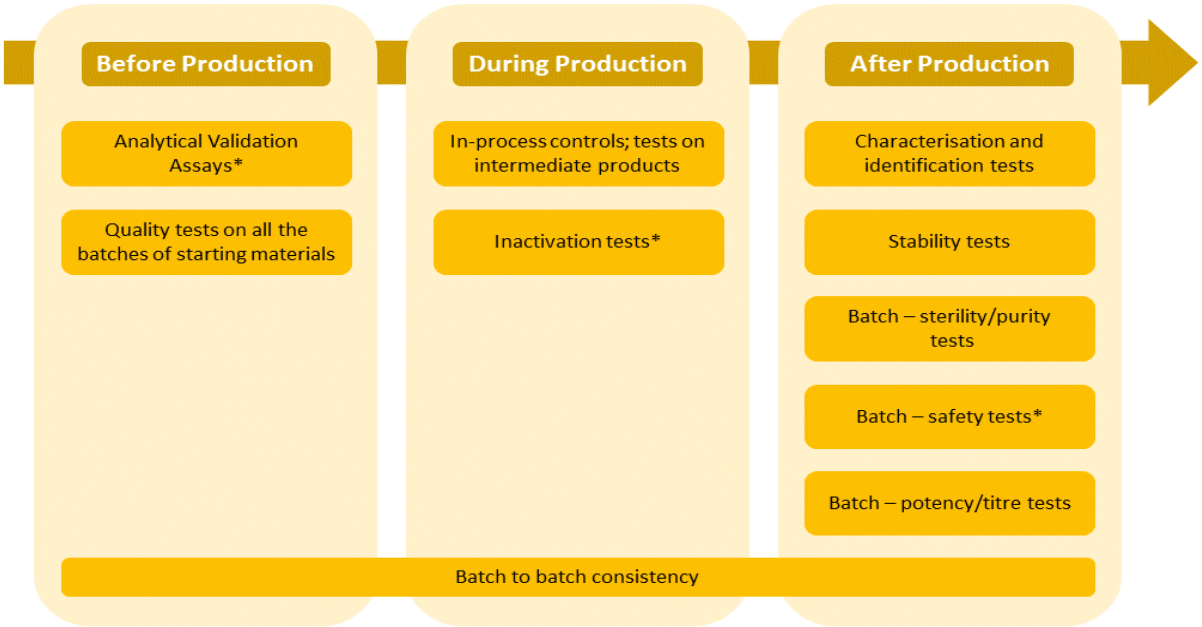

Figure A2 – Quality control tests required for marketing authorisation approval according to Commission Delegated Regulation (EU) 2021/805 of 8 March 2021[76]. (* - Facultative)

After production, it is necessary to perform tests that assess the **general characteristics** of the finished product, as well as **identification tests** for the active substances, the adjuvants and the excipient components, to show that these are in conformity with the limits set for the product.

Stability of the finished product shall also be assessed by performing biological and physico-chemical tests that support the shelf life proposed by the applicant. These studies should be carried out in a sufficient number of batches (not less than 3 representative consecutive batches) and also on products stored in the final container(s). The conditions of storage should be taken into account in the tests, including light, temperature, and the adhesive/absorptive properties of containers. Of note, the efficacy of any preservative system must be demonstrated as well.

Additionally, for each batch that is produced, a representative sample must be tested for purity, safety and potency/titre.

The **batch sterility/purity** of the final product is determined by testing for a variety of contaminants, namely extraneous agents such as viruses, bacteria, mycoplasma and fungi, and other substances.

Besides from the safety studies that need to be conducted to prove the overall safety of the VMP, it was also recommended to test and report on the safety of each batch. However, **batch safety tests** may be waived by the regulatory authority when a sufficient number of production batches have been produced under the control of a seed lot system and found to comply with the test. In fact, the European Pharmacopoeia no longer requires a batch safety test in target animal species for the release of vaccine batches/serials, and thus it is considered that the batch safety test is not mandatory as a control of the finished product.

Batch potency/titre tests allow for the quantification of the active substance and are designed to correlate with the host animal vaccination–challenge efficacy studies. For inactivated products, potency tests may be conducted in laboratory or host animals, or by means of reliable validated quantitative *in vitro* methods that correlate with *in vivo* efficacy. The potency of live vaccines is generally measured by means of virus titration. Importantly, the appropriate release titre is primarily dependent on the required potency and secondarily dependent on the rate of decay of the viruses in the vaccine, as indicated by the stability test.

Finally, to evaluate the consistency of the whole production process, all these tests must be performed in at least three consecutive batches and the results compared to assure satisfactory uniformity.[67,69,75,76]

Safety assessment

Figure A3 summarizes the safety studies that are required for the registration of a veterinary vaccine, taking into account the different types of vaccines and possible vaccination schemes.

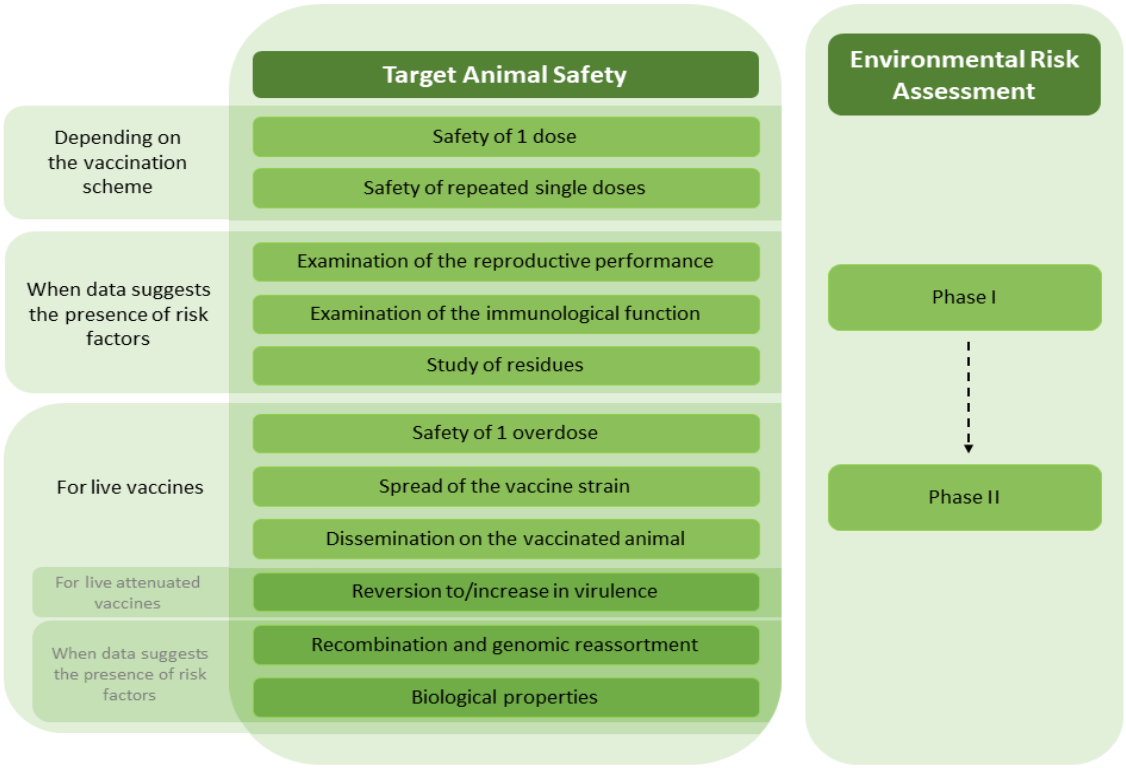

Figure A3 – Safety studies required for marketing authorisation approval according to Commission Delegated Regulation (EU) 2021/805[76] of 8 March 2021.

Target animal safety

Target animal safety studies are carried out on the species and categories in which the VMP is intended for use, and might vary according to the type of vaccine.

To assess the **safety of 1 dose**, the vaccine shall be administered at the recommended dose and by each recommended route, in each of the species present in the indications, including animals of the minimum age of administration. As for the **safety of the repeated administration of 1 dose**, in the cases in which the vaccine is intended to be administrated more than once, the studies shall be carried out in the most sensitive categories of the target species, using each recommended route of administration. The single dose study may be conducted as part of the repeated dose study. For this type of studies, the inoculated animals shall be observed and examined, for at least 14 days after administration, for signs of systemic and local reactions, and, when appropriate, for detailed *post-mortem* macroscopic and microscopic aspects of the injection sites.

When the data suggests that the starting materials may be potential risk factors for the reproduction of the animals, studies on the **examination of the reproductive performance** shall be carried out on males, non-pregnant and pregnant females, using the recommended dose and the most sensitive route of administration. In this framework, it is important to investigate potential harmful effects on the progeny, teratogenic and abortifacient effects. Likewise, when there is suspicion of possible adverse immunological effects and/or when adjuvants/preservatives are used, the **examination of the immunological functions** and/or the **study of the residues** shall be respectively performed.

When the final product corresponds to a live vaccine, such as the ASF LAVs mentioned in sections 2.1.2, 3.2 and 3.3, there are special safety requirements that must be addressed in the marketing authorisation application. For instance, it is required the performance of an **overdose safety study**, in which an overdose of the vaccine is administered by each recommended route of administration or, if suitable, by the most sensitive ones to animals of the most sensitive categories. Of note, if this study is performed with no signs of adverse reactions, the single dose safety study may be omitted. Another required variable to be tested is the **spread of the vaccine strain**, that is, the spread of the strain from vaccinated to unvaccinated target animals and, if necessary, to susceptible non-target animal species. For this, the recommended route of administration more likely to result in the spread must be used. Besides, it is necessary to study the **dissemination of the organism in the vaccinated animals**, by testing the animals' faces, urine, milk, oral, nasal and other secretions for its presence, as well as, if suitable, within the body of the animal with special focus on the predilection sites of replication of the organism. Also, it is required to study the **reversion to/increase in virulence** of attenuated vaccines. In these studies, the vaccine organism is propagated *in vivo* by inoculating a group of target animals of susceptible ages with the master seed or, if not available in sufficient quantity, the lowest passage seed used for production. Then, the organism shall be recovered from excretions or tissues and used for further inoculation of a new group of animals. This procedure is repeated for at least 5 passages and finally the isolated organism shall be fully characterised by the same procedures used to characterise the master seed, in order to assess if the level of attenuation is still acceptable. Further tests may be necessary, such as the precise determination of the **intrinsic biologic properties** of the strain and the probability of **recombination or genomic reassortment**, if applicable.[69,71,72,76]

Environmental risk assessment

The purpose of the environmental risk assessment is to evaluate the potential harmful effects which the use of the VMP may cause to the environment and to identify any necessary precautionary measures. This assessment is normally conducted in 2 phases.

The **Phase I** is mandatory and should always be performed. Its outcome is the level of risk associated with any potential exposure to the environment. This evaluation should take into account: (1) the target animal species and the pattern of use; (2) the method of administration, particularly the extent to which the VMP with enter directly into the environmental system; (3) the possible excretion of the product; and (4) the disposal of unused or waste product. In some cases, this study might be done in conjunction with the reversion to/increase in virulence tests.[77]

When the conclusions of the first phase indicate potential exposure to the environment, the **Phase II** assessment is required. It consists on the evaluation of the potential risk(s) that the VMP might pose to the environment, including investigations on the impact of the product on the soil, water, air, aquatic system, and non-target organisms.[78]

In the case of vaccines containing genetically modified organisms (GMOs), further environmental risk assessment procedure may be required in accordance with the principles of Directive 2001/18/EC. For category II and III vaccines, the competent authorities need to publish a notice to the public announcing that the vaccine does not pose significant impact on the environment and findings are available for public review.

If no substantive refuting comments are received, the competent authority may authorise the field testing or grant the regulatory approval.[69]

Efficacy assessment

The efficacy of a veterinary vaccine should be demonstrated by statistically valid vaccination–challenge studies in the host animal. For these studies, the most sensitive categories (usually the youngest) of seronegative (whenever possible) target animals have to be inoculated, using each recommended vaccination route and regimen, with the final product vaccine that has been produced at the highest passage level from the master seed, which should correspond to the minimum amount of antigen per dose throughout the entire shelf life of the vaccine. The challenge conditions shall mimic, as far as possible, the natural conditions of infection.[69,79]

These studies aim to determine the onset (if claimed) and duration of protection; to elucidate the immune mechanism which initiated after administration. The criteria for determining protection may vary with the immunising agent, but should have statistical significance. The primary efficacy criteria must derive from the main disease parameters, such as mortality, morbidity, clinical signs or lesions. As for the secondary efficacy criteria or efficacy indicators, there must be a correlation with the claimed protection in the target species. This means that the indicators must play a substantial role in the immune response that is relevant for the protection. These must be selected based on the literature and on results from early challenge trials in which protection was demonstrated.[69,73,79–81]

The aforementioned characteristics and requirements for the required safety and efficacy studies are summarized in **Table A3**.

Table A3 – Main requirements for each of the safety and efficacy studies, according to the VICH guidelines[79] and EMA/CVMP/IWP/260956/2021[73] and Ch. 1.1.8. of the OIE/WOAH Terrestrial Manual[69].

Vaccine Safety and Efficacy Assessment	
Study	**Requirements**
Safety of 1 dose	**Dose:** recommended **Route:** all the recommended **Potency/titre:** maximum **Animals:** all the species in the indications; including minimum age **Parameters:** local and systemic reactions **Follow-up:** at least 14 days
Safety of 1 overdose	**Dose:** overdose **Route:** all the recommended **Potency/titre:** maximum **Animals:** most sensitive categories **Parameters:** local and systemic effects **Follow-up:** at least 14 days
Safety of repeated single doses	**Doses:** recommended **Route:** all the recommended **Potency/titre:** maximum **Animals:** most sensitive categories **Parameters:** local and systemic reactions **Follow-up:** at least 14 days
Examination of the reproductive performance	**Dose:** recommended **Route:** most sensitive **Potency/titre:** maximum **Animals:** males, pregnant and non-pregnant females **Parameters:** reproductive performance; harmful effects on the progeny; teratogenic effects; abortifacient effects

Spread of the vaccine strain	**Dose:** recommended **Route:** most likely to result in the spread **Parameters:** presence/absence of the vaccine organism in secretions and within the body
Reversion to/increase in virulence	**Dose:** recommended **Route:** recommended **Product:** master seed or lowest passage seed **Animals:** target species of susceptible ages **Parameters:** characteristics of the organism isolated from excretions/ tissues **Follow up:** at least 5 passages
Vaccination-challenge	**Dose:** recommended **Route:** all recommended **Animals:** seronegative; most sensitive categories (youngest) **Potency/titre:** minimum **Parameters:** mortality; morbidity and clinical signs; serological antibodies • **Onset/Duration of protection** **Follow up:** as long as possible

A.3 Laboratory and field trials considerations

Scope for laboratory and field trials

The performance of laboratory and/or field trials might be mandatory or facultative, depending on the aim of the study and on the features of the candidate VMP (**Table A4**).

Table A4 – Scope for the laboratory studies and field trials to assess safety and efficacy of IVMPs, according to EMA/CVMP/IWP/260956/2021[73].

	Safety	Efficacy
Laboratory studies	**Mandatory**	**Facultative**; can be omitted if: - A valid laboratory challenge model is not available; - Data on the influence of passively acquired maternally derived antibodies is fully supported by clinical trials; - IVMPs with long-term duration of protection; - IVMPs only efficacious after re-vaccination and pre-clinical trials cannot be performed under these condition.
Field Trials	**Mandatory** (unless justified)	**Facultative**; can be omitted if: - A relevant laboratory challenge model (comparable disease presentation, distribution, severity, etc.) is available; - Method of administration can be mimic under laboratory conditions; - Laboratory results are sufficiently reliable to allow assessment of the benefit-risk. **Mandatory**, in cases of: - IVMPs with epidemiological effect; - IVMPs with effect on multifactorial disease outcomes; - IVMPs with effect on performance parameters (eg. weight gain).

Legal requirements for *in vitro* studies

All laboratory procedures and tests should be conducted in compliance with an international standard such as Good Laboratory Practice (GLP). Similarly, tests in animals should comply with Good Clinical Practice (GCP), on VICH GL 9[79], including compliance with international standards for animal care and use. **Clinical trials can**

be conducted outside the EU, but, in order for their results to be consider in support of marketing applications, they need to be conducted according to the GCP. For a field trial to be conducted, an application for its approval shall be submitted in accordance with the applicable national law to a competent authority of the country in which the trial is to take place. Approvals of clinical trials shall be granted on condition that food-producing animals used in the clinical trials or their produce do not enter the food chain unless an appropriate withdrawal period has been set by the competent authority.

Clinical field trials design

According to Regulation (EU) 2019/6[67], Art. 4, a clinical trial is a study which aims to examine under field conditions the safety or efficacy of a VMP under normal conditions of animal husbandry or as part of normal veterinary practice. In order for the results of a field trial to be reliable and scientifically relevant, it shall be planned and conducted according to the trial design and data analysis standards presented on **Table A5.**

Table A5 – General characteristics of field trials for assessing the safety and efficacy of veterinary medicinal products, according to guidance documents EMA/CVMP/IWP/260956/2021[73], VICH GL9 (GCP)[79] and Chapter 1.1.8. of the OIE/WOAH Terrestrial Manual[69].

Trial Design	
Location	• Multicentre; • Normal conditions, representing all animal husbandry practices for which the VMP is indicated to.
Study population	• Well defined and representative of the target population – all categories and ages for which the VMP is indicated to.
Treatment	• The treatment is the administration of the VMP by the recommended administration routes; • The product tested should come from 2 or more typical production batches.

Control	• The rationale for using controls must be defined;
	• A control is a group of animals unvaccinated or receiving a placebo;
	• Valid control consists on animals:
	  ○ investigated at the same time;
	  ○ in an equivalent environment – same farm, barn or similar setups;
	  ○ suffering a similar field challenge – if possible, negative controls and vaccinated animals should be mixed;
	• For modified live vaccines: if vaccine agents spread, separated housing between vaccinated and control groups is justified.

Randomisation	• A randomisation strategy must be used to assign animals to vaccinated and control groups.
Blindness	• **Double blindness is recommended;** • If not possible and if the parameter to be measured is subjective, any observer must be blinded to treatment.
Data analysis	
Sample size	• Calculated a priori; • Based on the effect size and variance.
Effect size	• Must be clinically relevant; • Described a priori.
Safety data	• Analysis should be related to the recommendations of use.
Efficacy data	• Analysis should be related to the intended indications and specific claims.

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