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African swine fever vaccine: Turning a dream into reality

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Abstract

African swine fever (ASF) is currently threatening the swine industry at a global level. The disease originated in Africa has spread to Europe, Asia and Oceania, since 2007, reaching a pandemic dimension. Currently, the spread of ASF is unstoppable and that the development of a safe and effective vaccine is urgently required. The objective of this paper is to review the vaccine candidates tested during the 20th and 21st centuries, to identify the strengths and weaknesses of these studies and to highlight what we should learn. Several strategies have been explored to date, some of which have shown positive and negative results. Inactivated preparations and subunit vaccines are not a viable option. The most promising strategy would appear to be live attenuated vaccines, because these vaccine candidates are able to induce variable percentages of protection against certain homologous and heterologous virus isolates. The number of studies on live attenuated vaccine candidates has steadily increased in the 21st century thanks to advances in molecular biology and an in-depth knowledge of ASF virus, which have allowed the development of vaccines based on deletion mutants. The deletion of virulence-related genes has proved to be a useful tool for attenuation, although attenuation does not always mean protection and even less, cross protection. Therefore, ASF vaccine development has proved to be one of the top priorities in ASF research. Efforts are still being made to fill the gaps in the knowledge regarding immune response, safety and cross protection, and these efforts will hopefully help to find a safe and effective vaccine that could be commercialised soon, thus making it possible to turn a dream into reality.

KEYWORDS
ASF, control, immunisation, protection, review, vaccine

1 | INTRODUCTION

The World Organisation for Animal Health (OIE) lists African swine fever (ASF) as a notifiable disease (OIE, 2021a). This notification has given rise to restrictions in the pig and pork trade, with serious economic consequences (Brown et al., 2021; Niemi, 2020). ASF was first reported in Kenya in 1921, and was described as an acute haemorrhagic fever that caused high mortality in imported European pigs (Montgomery, 1921). The disease initially appeared to be restricted to the eastern part of the African continent, but was discovered to have spread to a location near Lisbon, in Portugal, in 1957, although it was rapidly controlled with an epidemiological silence of 3 years. However, in 1960, Portugal notified a second transboundary incursion of a more virulent ASF virus (ASFV) isolate. This time, it spread throughout the Iberian Peninsula and to several European countries (France, Sardinia, Malta, Belgium and the Netherlands) and Latin American countries

(Cuba, Brazil, Dominican Republic and Haiti) (Arias et al., 2002). All of these foci were eradicated, with the exception of the island of Sardinia, where the disease has remained endemic since 1978 (Jurado et al., 2018).

The disease has since been reported in most sub-Saharan countries (Penrith, 2013) and this expansion has facilitated a new spread to the European continent. In 2007, and specifically in the Georgian port of Poti, pigs were fed on contaminated meat residues transported in ships from Africa (Beltrán-Alcrudo et al., 2008). ASF subsequently began an unstoppable expansion from this Caucasus region across the European continent, with a slow but steady geographical advance (Sánchez-Vizcaíno et al., 2015; Sánchez-Vizcaíno et al., 2013). The disease affected up to 20 different countries on the European continent, namely Georgia, Armenia, the Russian Federation, Azerbaijan, Ukraine, Belarus, Lithuania, Poland, Latvia, Estonia, the Czech Republic, Moldova, Belgium, Bulgaria, Hungary, Romania, Slovakia, Serbia, Greece and Germany (OIE–WAHIS, 2021). The Czech Republic and Belgium have, however, recently been recognised by the OIE as ASF-free areas.

In 2018, the virus reached Asia for the first time (Zhou et al., 2018), and ASF has, to date, been reported in 12 Asian countries, including the People's Republic of China, Mongolia, Vietnam, Cambodia, the Democratic People's Republic of Korea, the Lao People's Democratic Republic, Myanmar, the Philippines, the Republic of Korea, Timor-Leste, Indonesia, India and Malaysia, affecting mostly domestic pigs. During 2020, the disease was also reported in Oceania, where it was notified in Papua New Guinea (OIE–WAHIS, 2021). Based on the current disease distribution, ASF has acquired a pandemic dimension affecting four different continents and is the greatest risk to the world's swine production.

ASF is caused by a complex and variable DNA virus, denominated as ASFV. ASFV is the only member of the *Asfarviridae* family (Dixon et al., 2019) and has an icosahedral multi-layered morphology and a diameter of about 200 nm (Alejo et al., 2018). The genome is a large double-stranded DNA molecule of 170–193 kbp that contains between 150 and 167 open reading frames depending on the ASFV isolates (Dixon et al., 2013). Due to the lack of neutralising antibodies, ASFV is classified on the basis of viral protein (vp) 72, which makes it possible to distinguish up to 24 different genotypes (Achenbach et al., 2017; Bastos et al., 2003; Quembo et al., 2018). This distinction is not related to the virulence of the virus isolates or cross protection between vaccine candidates. Moreover, virus isolates are frequently sorted as being homologous or heterologous. Although there is no consensual definition as to these concepts, 'homologous' appears to be related to genetically and antigenically similar ASFV isolates. According to Alejo et al. (2018), protein composition of ASFV is still largely unknown, although their results allowed them to identify 68 viral polypeptides by means of proteomic analysis, along with 21 cellular proteins. Interestingly, 44 new viral polypeptides were characterised, many of which still have unknown functions (Alejo et al., 2018). Other studies have focused on studying the structure of viral capsid with the objective of assisting in the development of vaccine and antiviral agents (Andrés et al., 2020; Liu et al., 2019; Wang et al., 2019). But despite all the studies conducted so far, only partial information regarding ASFV is known, and in addition, the effector mechanisms of protective immune response are not completely understood (Dixon et al., 2019). The relevance of the humoral and cellular immune response is widely accepted. Animals infected with ASFV develop specific antibodies against antigenic parts of the viral particle. As there is no vaccine against ASFV, the presence of antibodies is synonymous with ASF infection. Nevertheless, these antibodies are not able to fully neutralise the infection (Escribano et al., 2013), and the mechanism of virus neutralisation still, therefore, remains uncertain. High levels of antibodies have been related to a delay in the onset of clinical signs, a reduction in viremia titres and duration, thus increasing the survival rate (Gómez-Puertas et al., 1996; Neilan et al., 2004; Ruiz-Gonzalvo et al., 1986; Stone et al., 1968; Zsak et al., 1993).

TABLE 1 Characteristics of African swine fever virus (ASFV) that make vaccine development difficult

Complexity of ASFV particle only partially known.
Large ASFV genome, 170–193 kbp and large size of ASFV virion, 200 nm.
Scarce knowledge related to virulence genes and immune protection.
Gaps in knowledge on virus–host interactions.
High mortality in susceptible hosts such as domestic pigs and wild boar.
Not fully neutralising antibodies.
Gaps in knowledge regarding immune mechanisms of protection.
Genetic variation among ASFV isolates.
Gaps in knowledge regarding cross protection among ASFV isolates.
Lack of in vitro tests to assess protection.
Difficulty to obtain a stable cell line which supports virus growth.

Prevention, control and eradication measures have had limited success. If ASF enters a domestic pig farm, all the animals must be slaughtered, including those that are not affected (OIE, 2021a). This is owing to the lack of an effective vaccine and treatment against ASFV. That is the reason why the search for a vaccine against ASF has been always a priority. In fact, vaccine development has been identified as a major gap in ASF control and eradication (Arias et al., 2018). The genetic variation and complexity, the lack of fully neutralising antibodies, gaps in knowledge on the virus–host interactions and technical difficulties, such as the difficulty to obtain a stable cell lines with which to produce vaccines or the lack of in vitro tests to assess protection and cross protection, have been identified as some of the causes that have hindered vaccine development (Arias et al., 2017). Priorities in research, such as (i) the development of cell lines in order to replace primary cell cultures, (ii) the identification of new types and strategies for vaccine candidates, and (iii) conducting studies on existing live attenuated candidates so as to evaluate side effects, virus persistence, doses and safety parameters, have also been identified (Arias et al., 2018).

Therefore, the challenges that researchers must confront in order to develop a vaccine against ASF are summarised in Table 1.

Considering all of the above, the objective of this paper is to describe the main outcomes from vaccine research in the 20th and 21st centuries. Moreover, the strengths and weaknesses of these studies were

identified and what we have learnt from these experiments was highlighted.

2 | MATERIALS AND METHODS

The literature search was performed on the 24th January 2021 using PubMed database for scientific articles. Scientific papers written in English and Spanish (for reviewing convenience) from 1921 to 2021 were reviewed. A list of keywords was combined into a Boolean query to identify titles and/or abstracts of documents of interest. The key words used (and any word containing the stem presented) were 'African swine fever' and 'vaccine'. Based on this search and considering that not all articles had full text available, 77 scientific papers were used to conduct this review.

3 | ASF VACCINE HISTORY, 20TH CENTURY (1921–1999)

Since ASF was first described in 1921, researchers have reported various attempts to immunise pigs against this disease. During the 1930s, attenuated virus isolates and inactivated virus isolates were used to immunise domestic pigs with limited success (De Kock et al., 1940; Hess, 1971; Walker, 1933).

3.1 | Live attenuated vaccines

The first reports that pigs which had recovered from ASF could survive a second challenge with a related ASFV isolate appeared in 1957 (Detray, 1957). At that time, the use of attenuated viruses obtained by cell culture passage was shown to be the only possible way of providing pigs with solid protection against a homologous ASFV isolate challenge (Stone et al., 1968). The low number of survivors made studies on the characterisation and development of antibodies difficult (Stone et al., 1968).

During the 1960s, additional attempts based on live attenuated vaccine candidates were made (Coggins et al., 1968; Malmquist, 1963; Manso-Ribeiro et al., 1963; Petisca, 1965; Sánchez-Botija, 1963). Live attenuated vaccines were able to confer partial protection against homologous ASFV isolates, but not against heterologous virus isolates. In Portugal, Manso-Ribeiro et al. (1963) used a vaccine candidate attenuated after 60 cell passages in a porcine bone marrow culture. Vaccinated animals developed a protective response to the challenge with virulent virus isolates. However, side effects such as pneumonia, locomotor difficulties and skin ulcers were reported in over 7% of the animals vaccinated and caused death in around 4%. In Spain, Sánchez-Botija (1963) conducted similar experiments focused on attenuating virus isolates by means of passages in leukocytes and kidney cells. Their results showed that these attenuated ASFV isolates had lower virulence and infected pigs had few clinical signs or were able to survive. Some of these survivors were selected for clinical trials and challenged with virulent strains of ASFV. Variable percentages of these pigs developed a protective response (Sánchez-Botija, 1963). However, as stated by Manso-Ribeiro et al. (1963) mortality related to infection with attenuated ASFV isolate was reported in various experiments, along with side effects. Lesions had a certain affinity of the ASFV isolate to skin and nervous and/or lung tissues. Microscopically, skin lesions had a tumoral resemblance and were similar to the lesions observed in cases of myxomatosis (Sánchez-Botija, 1963). Moreover, some of these animals had persistent viremia and acted as carriers of the disease.

3.2 | Inactivated virus vaccines

Other approaches were used to design a vaccine against ASFV, such as inactivated viruses. In these studies, organ extracts treated with heat and chemicals, such as crystal violet, acetylethyleneimine and glyceraldehyde, among others, were used as vaccine candidates (Hess, 1971). According to the results from these studies, the vaccine candidate did not confer protection against the challenge (Bommeli et al., 1981; Stone & Hess, 1967). Forman et al. (1982) followed an immunisation strategy in which glutaraldehyde-fixed infected cells were employed. Vaccinated animals produced antibodies in sera with a slight reduction in viraemia and limited protection against splenic damage. However, according to the aforementioned authors, the animals were not protected against the challenge. Moreover, Bommeli et al. (1981) designed an inactivated vaccine based on an extract of infected pig spleen plus *n*-octylglucoside (non-ionic detergent). Most of the vaccinated animals were able to survive the challenge against homologous virus isolates but died when exposed to heterologous virus isolates.

3.3 | Subunit vaccines

Baculovirus-expressed haemagglutinin of ASFV, a virus protein, was used to immunise domestic pigs against ASF (Ruiz-Gonzalvo et al., 1996). The results from this study suggested a dose-dependent response. Vaccinated animals were able to develop a protective response similar to that obtained in passive antibody transfer experiments carried out on convalescent animals (Onisk et al., 1994; Wardley et al., 1985; Zsak et al., 1993). Moreover, Carrascosa et al. (1995) expressed vp12 in baculovirus, which was used to immunise domestic pigs. Likewise, Gómez-Puertas et al. (1998) produced vp54 and vp30, which are involved in virus attachment and antibody-mediated protective response. Unfortunately, vaccinated pigs did not develop protective immunity against lethal infection with virulent ASFV isolates.

3.4 | Summary on the main results of the 20th century

To summarise, the aforementioned experiments demonstrated that live attenuated vaccines were able to develop a certain amount of protection against homologous virus isolates (Manso-Ribeiro et al.,

1963; Sánchez-Botija, 1963). However, side effects and chronic forms were observed in vaccinated animals. Chronic forms are characterized by necrotic lesions of the skin and arthritis (Sánchez-Vizcaíno et al., 2015). Experiments with inactivated vaccines and subunit proteins were not able to induce a protective humoral response. In this respect, the importance of cellular response was highlighted by several authors (Martins et al., 1993; Revilla et al., 1992). Therefore, it is possible to state that vaccines should stimulate both humoral and cellular response.

4 | ASF VACCINE, 21ST CENTURY (2000–2021)

By the end of the last century, several authors had highlighted the importance of designing vaccines that would induce both antibodies and a cellular response. In 1994, a study showed that passively transferred antibodies conferred protection against challenge (Onisk et al., 1994). In order to confirm relevance of the cellular response, Oura et al. (2005) immunised domestic pigs with a non-virulent vaccine candidate which resisted a virulent challenge. The vaccinated animals were subsequently depleted of CD8+ T lymphocytes and then, they were exposed to the virulent isolate. Immunised domestic pigs were no longer fully protected against the challenge, which suggested that CD8+ T cells played a specific role in combating viraemia. Moreover, it has been proved that antibodies play a significant role in protection against the virus (Oura et al., 2005).

4.1 | Live attenuated vaccines

4.1.1 | Live attenuated vaccines based on naturally attenuated virus isolates

As had occurred in previous experiments, new vaccine candidates based on naturally attenuated virus isolates began to be tested. The experiments conducted with naturally attenuated virus isolates conferred up to 100% protection against homologous virus isolates in several experiments with domestic pigs (Gallardo et al., 2018, 2019; King et al., 2011; Leitão et al., 2001; Sánchez-Cordón et al., 2017; Sánchez-Cordón et al., 2020; Souto et al., 2016), wild boar (Barasona et al., 2019) and indigenous African pigs (Mulumba-Mfumu et al., 2016). Leitão et al. (2001) used a naturally occurring, non-haemadsorpting and non-fatal ASFV isolate to immunise domestic pigs. Vaccinated animals that developed significantly high levels of natural killer cell activity in the absence of clinical symptoms survived infection with the highly virulent isolate. However, previously observed side effects were also reported in some animals, such as a change in body temperature, skin necrotic lesions and the swelling of joints.

Vaccine studies on the same isolate were then continued by Gallardo et al. (2018). Vaccinated animals were fully protected against homologous and heterologous virus isolates and showed no clinical signs of the virus in either blood or tissues. Moreover, Boinas et al. (2004) isolated different ASFV isolates from *Ornithodoros* ticks (e.g. haemadsorpting and non-haemadsorpting), which were first described by Vigário et al. (1974). Domestic pigs experimentally infected with non-haemadsorpting virus isolates were subsequently shown to be resistant to the disease, or there was a delay in the onset of the disease when exposed to virulent isolate. Several authors discussed the potential relationship between these virus isolates and the vaccine candidate developed by Manso-Ribeiro et al. (1963). Moreover, King et al. (2011) developed an immunisation strategy from a naturally attenuated isolate that provided variable protection against homologous and heterologous virus isolates, protecting animals from both the disease and viraemia. Another naturally attenuated strain, Lv17/WB/Rie1, which was isolated from a wild boar in Latvia in 2017 (Gallardo et al., 2019), conferred solid protection against the challenge in domestic pigs. Further experiments proved that this isolate could be potentially used as a vaccine candidate for wild boar by oral administration (Barasona et al., 2019). Nevertheless, the authors acknowledged the need to assess the safety of repeated administration, overdoses, the genetic stability of the vaccine candidate and the characterising of long-term shedding animals. This last vaccine candidate is one of the vaccine prototypes that is being evaluated within the European Project named VACDIVA (H2020 Gran ID: 862874). The objective of this project is to obtain a safe and cross-protective DIVA vaccine (Differentiating Infected from Vaccinated Animals) for wild boar and domestic pigs. A total of three live attenuated candidates (two natural live attenuated viruses, Lv17/WB/Rie1 and NH/P68, and one attenuated by passage in tissue culture) and several deleted mutants integrate the candidates under evaluation. In the project, it will also produce and validate a DIVA tests and develop cost–benefit and effective surveillance and control-vaccination strategies in the different epidemiological scenarios. So far, the obtained results are promising and efforts continue to be done in order to achieve the final goal.

4.1.2 | Live attenuated vaccines based on deleting specific genes

Advances in genetic engineering allowed the development of live attenuated vaccine candidates based on deletion mutants. Genes related to virulence-associated genes were targeted in vaccine development (Abrams et al., 2013; Borca, O'Donnell, et al., 2020, Borca, Ramirez-Medina, et al., 2020; Carlson et al., 2016; Chen et al., 2020; Gallardo et al., 2018; Gladue et al., 2020; Lewis et al., 2000; López et al., 2020; Monteagudo et al., 2017; O'Donnell, Holinka, Gladue, et al., 2015; O'Donnell, Holinka, Krug, et al., 2015; O'Donnell et al., 2016, 2017; Ramirez-Medina et al., 2019; Reis et al., 2016, 2017, 2020; Sánchez-Cordón et al., 2018, 2020; Sanford et al., 2016; Zhang et al., 2021). Over 60% of the vaccine experiments conducted during this century have followed a vaccinating strategy focused on gene modification. The results achieved by different authors have, however, been variable. Around 80% of these experiments attained a wide variability of protection, and this range oscillated between 17% and 100% of protection in immunised animals. These results clearly show that deletion does not always mean protection. Interestingly, Carlson et al.

(2016) highlighted the possibility of vaccine candidate and challenge virus isolates persisting in surviving animals. The Chinese ASFV isolate (Pig/Heilongjiang/2018) was recently used to generate a gene-deleted vaccine candidate. A total of seven genes were deleted, resulting in a completely attenuated isolate that was able to confer dose-related protection against a homologous challenge (Chen et al., 2020). The authors stated that this vaccine candidate could be safely used with fattening pigs and pregnant sows, with no possibility of a reversion to virulence (Chen et al., 2020). Two different ASFV isolates have recently been identified in the People's Democratic Republic of China. These virus isolates had several deletions in the genome of the virus (e.g. MGF360 and CD2v genes). According to the official authorities, these virus isolates could be unlicensed vaccines that have been released in the currently affected areas. Veterinarians in the affected areas are reporting chronic forms (FAO, 2021; Reuters, 2021). Indeed, these mutants would also be able to develop acute forms of the disease.

4.1.3 | Live attenuated vaccines based on cell passages

In addition to the above, live attenuated vaccine candidates obtained from cell passages continued to be developed by Lacasta et al. (2015), Krug et al. (2015), Sereda et al. (2020) and Titov et al. (2017). These studies were able to demonstrate the protective potential of the attenuated isolate. However, Titov et al. (2017) observed that the challenge isolate could persisted for weeks in the blood and organs of pigs that had recovered. Tissue distribution and time of persistence were indicated as being critical in order to better understand the dynamics of this interaction. Moreover, virulent virus isolates could produce the disease after even 28 days in animals that had recovered from the challenge.

4.2 | Inactivated virus vaccines

Furthermore, Blome et al. (2014) attempted to provide protection by using an inactivated vaccine candidate with binary ethyleneimine (BEI) and different types of adjuvants (Poligen™ and Emulsigen), whereas Cadenas-Fernández et al. (2021) recently attempted to inactivate ASFV with BEI at a low temperature with new adjuvants (MF59®, Silica oil, mGNE, Montanide™ ISA201), high doses of the vaccine candidate and simultaneous double inoculation by two different routes (intradermal and intramuscular). As had occurred in previous experiments with inactivated virus isolates, the vaccinated animals were unable to survive the challenge carried out with the homologous virulent ASFV isolate.

4.3 | Subunit vaccines

Another strategy followed in order to discover a vaccine against ASFV was that of the subunit vaccine (Barderas et al., 2001; Neilan et al., 2004; Ivanov et al., 2011). Barderas et al. (2001) produced a quimeric vp54/vp30 expressed by a recombinant baculovirus which was used to immunise domestic pigs. Neilan et al. (2004) tested the ability of antibodies to neutralise vp30, vp54 and vp72, and discovered that they conferred insufficient protection. These experiments showed that vaccinated pigs were able to develop antibodies and survive the challenge, and that they even had lower viraemia with respect to the control pigs. Likewise, Burmakina et al. (2016) demonstrated that CD2v and C-type chimeric proteins were relevant for homologous protection. Moreover, Lopera-Madrid et al. (2017) developed a reverse vaccinology system with which to identify antigenic ASFV units produced in mammalian cells and in viral vectors. The limited success in this field attained in previous studies was favoured by the complexity of the virus and gaps in knowledge regarding the ASFV genome.

4.4 | Live vectored and DNA vaccines

Other authors have based their studies on DNA vaccines and/or vectors for gene transfer into mammalian cells, such as baculovirus and adenovirus, among others (Argilaguet et al., 2011, 2012, 2013; Cadenas-Fernández et al., 2020; Goatley et al., 2020; Jancovich et al., 2018; Lacasta et al., 2014; Lokhandwala et al., 2019; Netherton et al., 2019; Sunwoo et al., 2019). These were proved to induce antibody production, but only four experiments (Argilaguet et al., 2012, 2013; Goatley et al., 2020; Lacasta et al., 2014; Lokhandwala et al., 2019) achieved protection (20%–100%) against the challenge. Fortunately, Goatley et al. (2020) achieved 100% protection after challenge with a medium virulent isolate by using a pool of eight different vectored ASFV antigens.

4.5 | Summary on the main results of the 21st century

Live attenuated vaccines based on naturally attenuated virus isolates or deleting specific genes show the best results on protection. Most of them induced protection against homologous virus isolates and few against heterologous virus isolates. However, variable side effects were observed at some extent in the vaccinated animals. On the other hand, it has been demonstrated that, despite being theoretically the safest option, inactivated vaccines are currently not able to confer protection against ASFV. Results of subunit protein/peptide and DNA vaccines showed that animals were able to synthesise both specific antibodies and a T cell response. However, protection against challenge was scarce when compared with live attenuated vaccines.

Most of the experiments conducted in the 21st century (69%) have involved the use of attenuated vaccine candidates (e.g. naturally, by cell passages or by deleting specific genes). Almost 24% have consisted of naturally attenuated virus isolates, 12% attenuated by cell passages and 64% attenuated by deleting specific genes. Other strategies have included inactivated virus isolates (4%), subunit protein vaccines (6%), DNA vaccines (6%) and virus vectored vaccines (6%) (see Table 2).

TABLE 2 Percentage of studies based on strategies followed to develop an African swine fever (ASF) vaccine candidate during the 21st century

Type of vaccine candidate		Percentage of studies
Live attenuated vaccines (LAV)		69%
LAV based on naturally attenuated isolates	24%	
LAV based on cell passages	12%	
LAV based on deleting specific genes	64%	
Inactivated virus vaccines		4%
Subunit protein vaccines		6%
DNA vaccines		6%
Virus vectored vaccines		6%
Combined vaccination strategy		9%

Table S1 summarises the main characteristics of the vaccine experiments conducted during the 21st century.

According to the authors' experiences and based on the published studies, several strengths and weaknesses were identified (see Table 3).

5 | DISCUSSION

ASF is posing a major threat to both the global swine industry and wildlife (e.g. wild boar). ASF has reached pandemic proportions since it first began to spread throughout Europe in 2007. ASF is currently present on four different continents, namely Africa (1921), Europe (Sardinia-1978, 2007), Asia (2018) and Oceania (2020) and more than 50 countries (OIE–WAHIS, 2021). From 2007, only the Czech Republic and Belgium have been able to successfully eradicate ASF from wildlife, and their status as ASF-free countries is now recognised by the OIE (2021b). Nevertheless, most countries are still suffering from ASF outbreaks and/or cases, despite all the efforts made. The unstoppable spread of the disease is pushing researchers to search for a vaccine that could be used as an additional tool to control and eradicate ASF. Thus, ASF vaccine is one of the main priorities in world animal health.

The need for a vaccine has been recognised by researchers since the first report of ASF appeared, and studies began to be conducted at the beginning of the last century. Researchers have had to confront the difficulties previously mentioned in order to move forward. Some of the critical points identified when using live attenuated vaccines were carrier status, residual pathogenic action after attenuation of the vaccine candidate and vaccination failure. The following recommendations were also made: the sensitivity of domestic pigs to ASF should be tested, biosecurity measures should be ensured in order to avoid infection with circulating virus isolates and an adequate number of animals should be used in order to reach valid conclusions. These recommendations were partially followed in subsequent studies. Some of the reasons that could explain why these suggestions were followed only partially are as follows: (i) animal welfare limitations (e.g. justification of sample size, experiment duration), (ii) the huge economic costs owing to the biosecurity facilities required and (iii) an increased study complexity because of advances in science. Based on our experience, we recommend the inclusion of four additional critical points. First, establish a minimum number of animals when testing a vaccine in order to guarantee the validity of the results obtained according to what is established by the European Medicines Agency (EMA). Second, extend the time between vaccination and challenge in order to verify the duration of the protection and extend the time after challenge to evaluate potential side effects. Third, analyse the presence of vaccine and challenge viruses in target tissues. Fourth, evaluate cross protection against heterologous virus isolates as well as protection against homologous virus isolates.

Various additional strategies, technologies and methodologies have been implemented during the 21st century, such as the use of live

TABLE 3 Strengths and weaknesses of conducted studies based on the authors' experiences

Strengths
Complete attenuation of the virulence of certain vaccine candidates.
High level of protection achieved against homologous challenge.
Some vaccine candidates achieved protection against heterologous ASFV isolates (e.g. cross protection).
Sterile immunity was induced in a study.
Certain vaccine candidates have been adapted to stable cell lines.
DIVA approaches have been included.
Weaknesses
Reduced number of immunised animals in some studies.
Genes deletion might lead to over attenuation processes with no protection.
Most studies only challenged animals against homologous ASFV isolates (i.e. cross protection was not evaluated).
Lack of standardisation in number of days after challenge to evaluate the effectiveness and safety of the vaccine candidate.
Some studies did not analysed the persistence of vaccine and challenge virus in target tissues.
Scarce standardised methods to test efficacy and safety.
Lack of adaptation to stable tissue culture.

attenuated vaccines, development of attenuated virus isolates based on deletion mutants, subunit vaccines and DNA vaccine candidates. Results from these studies have shown variable protection (defined as surviving animals to challenge) against virulent virus isolates used as challenge viruses, as well as between homologous and heterologous virus isolates (see Table S1). In fact, the deletion of virulence related genes has proved to be a useful tool for attenuation. However, attenuation does not always correspond to protection. Results from studies on inactivated and subunit vaccines have shown that they do not generally confer protection or confer only partial protection against the challenge. However, it has been shown that natural or gene-deleted attenuated vaccine candidates have the capacity to induce a strong immune response and confer full protection against homologous challenge (see Table S1).

Strain diversity is one of the most serious difficulties involved in developing a vaccine against ASF (Arias et al., 2018). The vaccine candidates tested have conferred variable protection against heterologous virus isolates as well as against homologous virulent virus isolates (detailed information is shown in Table S1). The challenge of cross protection could be confronted by designing vaccine candidates based on ASFV isolates closely related to the circulating viruses. This approach could be supported by the fact that most of the current spread of ASF is local and, therefore, caused by the transmission of homologous virus isolates (Ge et al., 2018; Mazur-Panasiuk et al., 2019). Unfortunately, these attenuated vaccines were and are associated with safety issues, such as side effects, residual pathogenicity or viral persistence. The advances in molecular genetics have allowed us to identify virulence-associated genes. The deletion of these genes enhances the safety of the attenuated strains, but in some cases, over-attenuation has reduced their potential protection capabilities. Therefore, the balance between safety and protection needs to be achieved. Further research into ASF genomics and on the function of different genes including those related to the immune response is still necessary.

As evidenced above, a large number of experiments have been carried out in order to evaluate different vaccines candidates. Experimental designs have been diverse, varying in the type of host, number of animals, routes of inoculation of vaccine candidates and exposure to the challenge virus, doses or duration of experiments, among others. With regard to the immunised hosts, there are publications concerning animal trials with domestic pigs, wild boar and wild African suids of different ages, genders and breeds. Testing different types of hosts is current requirement when considering that wild boar is the most severely affected in Europe and also affecting populations from Asia.

Different routes of administration and doses, along with homologous and/or heterologous challenge viruses, have been used in vaccine trials. This large number of variables signifies that it is complicated to compare experiments and extrapolate any results if the experimental conditions were not the same. Therefore, it would be beneficial to harmonise certain experimental conditions in order to make the most of the results and conclusions obtained, especially regarding safety and protection (e.g. cross protection). This would allow researchers to compare their results with those of other experiments based on similar vaccine strategies. Some of these conditions are, for example, the number of animals immunised, time prior challenge, duration of the experiment, virus shedding routes (and, if applicable, vaccine and/or challenge viruses) or analysing the persistence of the vaccine and challenge virus in target tissues such as lymph nodes, lung or bone marrow.

Calculating the adequate sample size is a challenging task. In current experiments, there is no agreement on the appropriate number of animals that should be immunised in order to ensure that a vaccine is safe and effective. The sample size should be determined according to what is established by the EMA or any other competent institution. The present work has consequently found a great variability in the sample size employed to evaluate a vaccine candidate. When testing a vaccine with domestic pigs, the sample size has great variability. If animal trials are conducted with wild boar, it is necessary to take into account that these animals are more sensitive to stress and management. Moreover, hierarchical and aggressive behaviour has also been observed. All of this could lead to higher mortality rates than expected, signifying that larger sample sizes may be required.

Other critical factors are time to challenge and time to euthanasia. Most experiments use immunisation protocols in which pigs are challenged around 28 days after immunisation. Indeed, it has not been possible to evaluate the capability of vaccines as regards inducing long-term protection and side effects. The maximum number of days that an experiment has lasted during the 21st century was 130 days (Sánchez-Cordón et al., 2020). The study in question evaluated long-term immunity. However, the vaccinated animals did not develop a robust immunological memory that was able to induce protection and consequently manifested acute forms of the disease (Sánchez-Cordón et al., 2020). These results showed the importance of evaluating vaccine candidates over longer periods of time in order to test their effectiveness and safety. This information could additionally shed light on potential vaccination protocols.

Another point that should be borne in mind is that the duration of experiments is intrinsically related to animal welfare. Moreover, challenged animals have to be kept in the animal trials in order to test whether they develop the diseases. The time between challenge and euthanasia should be sufficiently long to test the efficacy of the vaccine candidate without compromising animal welfare. Finding the equilibrium between these two priorities might, to some extent, be controversial. Considering that the protocols related to animal welfare are currently much stricter, it is crucial to test the targeted organs and tissues of vaccinated and challenged animals. The potential persistence of the challenge virus and vaccine virus in live attenuated vaccines was previously proved by Manso-Ribeiro et al. (1963) and Sánchez-Botija (1963) but this has been debated by other authors who do not recognise the carrier status (Petrov et al., 2018). Bearing this precedent in mind, and according to the principle of maximum risk, the systematic analysis of specific tissues (e.g. tonsil, spleen, kidney, bone marrow, lymph nodes or lungs) should be included in order to fully validate the results obtained with regard to efficacy and safety.

Virus shedding routes in vaccinated animals are another parameter related to safety. Some experiments based on live attenuated vaccines have kept immunised animals and naïve pigs together in order to measure the transmissibility of the vaccine and/or challenge virus. It

is clear that the possibility of transmitting the challenge virus would be a tragedy in real scenarios. A recent study presented the first vaccine that was able to induce sterile immunity against the current ASFV strain responsible for recent outbreaks (ASFV Georgia 2010 isolate). It was shown that the replication of the challenge virus was avoided in animals immunised with moderate doses of the vaccine (Borca et al., 2020b). However, if this shedding is exclusively related to the vaccine virus, it could be used as an advantage. This has been highlighted by several authors owing to the potential reduction in costs of vaccination, as animals could be immunised by direct contact (Barasona et al., 2019). Moreover, the immunisation of wild boar populations could be easier if contacts between animals favour the transmission of the vaccine. Nevertheless, other difficulties might arise, such as vaccination routes, tracking vaccinated animals or overdoses, among other things.

Despite the efforts made to develop a safe and an effective vaccine against ASF, some new challenges are emerging. In the People's Republic of China, the disease was notified for the first time in August 2018. The isolate was denominated as ASFV-SY-18 and considered of high virulence and transmissibility and as belonging to genotype II. It was isolated from domestic pig samples obtained from an outbreak on a farm located near Shenyang city (Liaoning province) (Zhao et al., 2019; Zhou et al., 2018). Over 40% of the domestic pig population has since been lost as a result of mortality and mandatory sacrifices (FAO, 2021; OIE–WAHIS, 2021; Wu et al., 2020). This has led to several cases of the commercialisation of false ASF vaccines in an attempt to stop the disease from spreading (FAO, 2021; Reuters, 2021). In 2020, the presence of several ASFV strains with nucleotide insertions, deletions and mutations derived from Pig/Heilongjiang/2018-like viruses were reported. Some of these strains were shown to be non-haemadsorpting and to have a low virulence and high transmissibility. The presence of animals with subacute and chronic forms as well as persistent infection was also reported in animals infected with these ASFV isolates (Sun et al., 2021). These virus isolates are hypothesised to be non-authorised vaccines, as it has never been reported that ASFV could naturally mutate at such extreme levels (FAO, 2021; Reuters, 2021). In fact, the Chinese administration is attempting to educate the people involved in illegal vaccines through a prevention and control work plan for 2021 (FAO, 2021). This will place emphasis on the illegal production and use of ASF vaccines, with a particular focus on attempting to stop it. Far from helping, this situation is posing a great obstacle to the early detection of the disease and its subsequent control and eradication, owing to the increased number of subacute cases.

To conclude, ASF is the main threat to the world pig industry. In the past, it was possible to achieve eradication by implementing strict control and eradication measures. In our current context, connectivity works in favour of spreading any infectious diseases, especially in wildlife and also of spreading ASF. Despite the efforts made throughout history to find a vaccine against this complex virus, there are still huge knowledge gaps that have hindered the development of a vaccine. Developing a vaccine against ASFV has become a top priority in veterinary research and is correlated with the huge amount of scientific papers published recently. Researchers have, without any doubt, made great advances in this field. The road that must be travelled before a safe and effective vaccine can be commercialised is a little shorter every day. As Edison said, on his long road towards discovering the light bulb, 'I have not failed. I have just found 10,000 ways that don't work'.

6 | CONCLUSIONS

The development of a vaccine for ASF has been hampered by gaps in knowledge regarding host immunity and virus genomics, among others. Molecular biology has allowed the use of different vaccine strategies, focusing on subunit vaccines, gene deleted vaccines and genetically and naturally attenuated vaccines. The results obtained are shedding light on this field, with some promising results. Vaccines against ASF are no longer dreams, as we could be closer to a reality. However, the difficulties posed by this virus such as safety and the lack of cross protection may signify that a universal vaccine is not possible, although an effective and safe vaccine against the currently circulating virus isolates may be achieved. Safety and cross protection should be the key aspects in vaccine design. In order to overcome the remaining difficulties, a sufficient number of animals need to be vaccinated and challenged in animal trials, time prior challenge should be sufficiently long in order to test protection times and ASF targeted tissues should be analysed in order to prove sterile immunity.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required as this is a review article with no original research data.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analysed in this study.

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