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6. African swine fever vaccines

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Abstract

African swine fever virus causes an acute haemorrhagic fever in domestic pigs and wild boar which is invariably fatal. Introduction of the disease into Georgia in 2007 has led to the deaths of tens of millions of animals across Eastern Europe, Asia and Oceania with serious effects on animal welfare and global food security. Control of the disease is impaired by the lack of an effective vaccine and is dependent on strict biosecurity at the farm gate, and rapid diagnosis, quarantine and slaughter of infected herds. The few pigs that do recover from disease are robustly protected from a subsequent encounter with the same virus isolate, showing that immunity is achievable. This review provides a historical perspective on the approaches that researchers have taken to develop African swine fever vaccines, as well as discussing promising modern techniques such as targeted gene deleted viruses and viral vectored vaccines.

Keywords: ASF vaccine, protective antigens, ASF, protective immunity, African swine fever

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6.1 Introduction and classic approaches to vaccination

African swine fever (ASF) was first described as an acute, invariably fatal disease in European breeds of domestic pigs. However, it was quickly realised that the few animals that did recover from acute fever were protected from a subsequent encounter with the virus, demonstrating the prospect of a protective immune response (Montgomery, 1921). These early studies also showed that wild suids, particularly warthogs and bushpigs, were associated with outbreaks of disease and that bushpigs were resistant to an inoculum that would normally kill a domestic pig. Since these early beginnings a number of different approaches have been taken to develop an effective African swine fever vaccine, but at the time of writing in June 2020 no licensed, commercial African swine fever vaccine is being used in the field.

6.1.1 Immune serum

Based on the observation that hyperimmune serum from animals recovered from European swine fever (now called classical swine fever) was able to protect against subsequent encounters with the virus, sera from the few animals that recovered from natural infection with African swine fever virus (ASFV) were tested for their immunological properties. These types of experiment were also used to show that African and classical swine fever were caused by two different aetiological agents. However, the results from these tests were not particularly promising as only a few animals survived (Montgomery, 1921), although both Walker and DeTray reported some positive results when serum and virus were inoculated at the same time (DeTray, 1957). Later experiments showed that transfer of serum from animals recovered from a moderately virulent isolate of ASFV (Malta 1978) reduced viraemia and clinical signs after homologous challenge and allowed survival of pigs after heterologous challenge (Uganda), although viraemia was not significantly reduced (Wardley *et al.*, 1985). Experiments also showed that feeding neonatal pigs with colostrum from sows recovered from ASF or injecting them with hyperimmune serum protected them from severe disease and death. Viraemia and clinical signs were much reduced compared to control colostrum deprived piglets or those fed normal colostrum, and piglets delivered by ASF recovered sows were also protected from challenge with homologous virus (Schlafer *et al.*, 1984). Immunoglobulin G (IgG) concentrated from animals that had been immunised with an attenuated strain (E75-CV1) and then challenged with a homologous virulent isolate (E75) was passively transferred into naïve pigs. Titres of ASFV-specific antibodies in the transferred pigs were similar to those from the pigs from which the serum was obtained, and in two separate experiments twelve out of fourteen pigs that had serum transferred survived challenge with E75. Although most of the animals were viraemic, this was 10,000 times less than controls and the duration and magnitude of fever were also significantly less (Onisk *et al.*, 1994). These experiments showed the importance of the antibody response to the protection mediated by the E75-CV1 strain, but did suggest that a vaccine that induced an antibody response alone might not be sufficient to generate a truly effective protective response. Later experiments would demonstrate the importance of the cellular response to protection mediated by live attenuated viruses (Oura *et al.*, 2005).

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6.1.2 Inactivated virus

African swine fever emerged just before development of the two principal techniques that would dominate vaccine development for around fifty years, inactivation and attenuation by repeated passage through either animals or tissue culture (Figure 6.1). In his initial description of the disease, Montgomery showed that heat treatment could inactivate the aetiological agent of ASF but with the exception of a single pig, did not protect animals from a subsequent inoculation with live virus (Montgomery, 1921). This approach was repeated using a number of different agents including toluene, crystal violet, formalin, β-propiolactone, glutaraldehyde and binary ethyleneimine, all without a clear indication of protection. Experiments with spleen extracts treated with the non-ionic detergent n-octylglucoside yielded better results, with 70% of animals surviving challenge with homologous virus, but these animals were persistently infected and transmitted disease to in contact animals. Similar experiments with n-octyl β-D-glucopyranoside showed reasonable levels of protection against challenge but, again, the recovered animals were

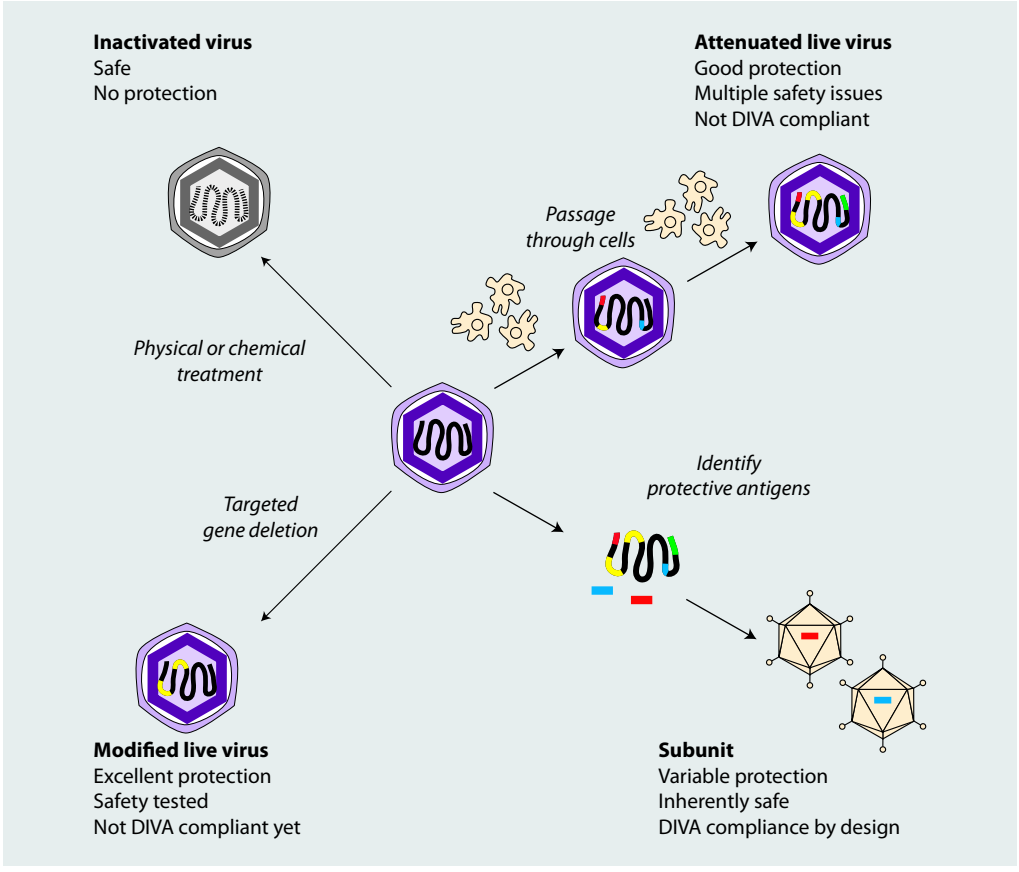

Figure 6.1. Approaches to African swine fever vaccine development. Schematic highlighting four approaches to generating an African swine fever virus vaccine. Inactivated virus, attenuated live virus (attenuation by passage through tissue culture), modified live virus (attenuation by targeted gene deletion) and subunit vaccines.

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persistently infected, although they did not transmit to in contact pigs (Kihm *et al.*, 1987). Both approaches failed to identify antibody responses to their respective immunogens and therefore the mechanism of protection in each case is unclear. Moreover, attempts by another group to repeat the experiments that showed a protective effect of n-octylglucoside treated ASFV were not successful (Forman *et al.*, 1982). More recent work used binary ethyleneimine treated virus in conjunction with the adjuvants Polygen™ and Emulsigen®-D and, although both inactivated preparations induced ASF-specific antibodies, they did not prevent disease by subsequent challenge with homologous virus (Armenia 2008 isolate) (Blome *et al.*, 2014). As discussed in later sections, experiments with individual proteins were able to offer some protection, but the prospects of an effective vaccine based on inactivated virus material or particles seem low.

6.1.3 Attenuation by passage

Initial attempts to passage the virus involved both embryonic and adult rodents, eggs, rabbits and goats, sometimes in conjunction with alternate passage through pigs. Using these techniques infectious virus could be maintained for up to 80 passages in the case of rabbits, without significant attenuation of the virus. It was not until the work of Malmquist in Kenya (Malmquist, 1962; 1963) and Manso Ribeiro and colleagues in Portugal (Manso Ribeiro *et al.*, 1963) during the mid-1950s with tissue culture that reproducible progress towards an attenuated virus was made (Figure 6.1). Malmquist and Hay's work (Malmquist and Hay, 1960) with porcine leukocytes and bone marrow led to the identification of the phenomenon of adsorption of red blood cells to cells infected with ASFV (called haemadsorption) and its subsequent adoption as a discriminating laboratory test between African and classical swine fever. They also passaged virus through these primary cell cultures as well as through pig kidney and testis cell lines. The progressive passage of the Kenyan Hinde strain of ASFV through pig kidney cells 75 times led to a virus that was attenuated in pigs, inducing only mild or inapparent signs, and these animals were protected from severe disease after subsequent challenge with the parental strain (Malmquist, 1962). Further passage through the porcine cell lines led to a virus that was essentially avirulent and was unable to protect pigs from the parental strain, an effect which Malmquist associated with the loss of pathogenicity (Malmquist, 1963). Much later experiments adapting the Georgia strain to African green monkey kidney Vero cells showed a similar loss of pathogenicity between the 80th and 110th passage (Krug *et al.*, 2015).

Manso Ribeiro's team passaged Portuguese isolate 1455 obtained in 1961 through bone marrow cells derived from piglets and showed that the virus was gradually attenuated by this process (Manso Ribeiro *et al.*, 1963). The Portuguese team noted that virus obtained between the 40th and 60th passage induced unspecified locomotive disorders as well as the typical signs of ASF in pigs; however, as the virus approached its 60th passage the clinical signs of ASF were less evident and by the 70th passage the locomotive disorders were also much reduced. Animals immunised with this attenuated strain that were subsequently challenged with virus circulating in both Portugal and Spain at the time were protected from acute ASF. Manso Ribeiro and colleagues did not observe significant clinical signs in the vaccinated animals nor an effect on in contact animals. Immunity was evident 14 days post immunisation with strain 1455, but not at 7 days. Both Malmquist and Manso Ribeiro's team explored the idea of cross-protection between different strains of virus. However, strain 1455 protected against selected isolates circulating in Spain and Portugal in the 1960s, but did not protect against isolates obtained from the original outbreak in Lisbon in 1957

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or from Katanga (now Democratic Republic of Congo). Similarly, the attenuated Hinde strain protected against homologous challenge, but not from viruses obtained from either Portugal or South Africa, although the numbers of animals used in these latter experiments were very low (Malmquist, 1963). Malmquist's work also provided the first hints of a potential mechanism to the observed protection, namely that serum from the animals that recovered from attenuated Hinde was capable of blocking the adsorption of red blood cells to cells infected with the parental Hinde isolate, but not to those infected with either of the Portuguese or South African isolates. While all of the viruses used in the Manso Ribeiro experiments were genetically related (p72 genotype I), they differed immunologically, as Katanga and Lisbon 1957 were haemadsorption inhibition assay serogroup 1 and Lisbon 60 was serogroup 4 (Malogolovkin *et al.*, 2015).

Although the performance of strain 1455 under experimental conditions appeared promising, unfortunately this was not replicated in field trials (Manso Ribeiro *et al.*, 1963). Between May and June 1962, 40,000 pigs were vaccinated, the number was increased to over half a million swine as the situation in Portugal worsened. During this time 128,684 animals across 1,247 herds were monitored closely and 3.4% of these suffered cases of acute ASF, out of which less than a third occurred more than three weeks after vaccination and could be assessed as vaccination failure (either through lack of protection or insufficient attenuation). However, there were significant complications in approximately 11% of vaccinated animals, principally concerning the respiratory, digestive and musculoskeletal systems as well as the skin. Between 7 and 10% of vaccinated animals died of these complications, depending on the age and breed, with Iberian pigs suffering the most. Pulmonary complications were particularly prevalent and were also seen in approximately 20% of the carcasses of pigs slaughtered in the area during early 1963. The vaccine also caused abortions, stillbirths and infertility in sows. The poorer performance of the vaccine in Iberian pigs was put down to the generally poorer health of these animals relative to other animals, rather than to the breed itself; however, Duroc-Jersey pigs were mentioned as being particularly sensitive to vaccine complications.

Around the same time researchers in Spain were also exploring the effect of passaging ASFV through pig kidney cells and leukocytes (Sánchez Botija, 1963). Isolate AL passaged through kidney cells 25 (AL-25), 30 or 60 times were compared to the same virus passaged 80 times (AL-80) through porcine leukocytes. AL-25 and AL-80 had some residual virulence, killing 3 to 6% of animals in the first 20 days after inoculation with the remainder recovering. However, between 10 and 50% of these recovered animals within a given experiment died either by a recurrence of acute disease or by chronic pulmonary disease. Sánchez Botija (1963) observed that the mortality from chronic pulmonary disease was much less in small scale laboratory animal experiments, an observation that he concluded was due to the better general health of the animals and the greater care that was taken with their husbandry. 'Hardy' pigs were also considered to be more susceptible to the vaccine strain. Recurrence of acute ASF and chronic forms of the disease was not seen in animals inoculated with the virus that had been passaged on kidney cells 60 times (AL-60), suggesting further passage through kidney cells had further attenuated the virus. All of the three attenuated viruses protected surviving animals from subsequent challenge with the parental strain. AL-25 and AL-60 were tested in field trials and natural cases of acute ASF were not seen in herds vaccinated with AL-25, whereas the protective efficacy of AL-60 showed more herd-to-herd variability. Sánchez Botija (1963) concluded that their attenuated strains required

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further analysis before wider usage and also highlighted that it was not possible to discriminate between vaccinated and naturally infected animals using the haemadsorption test.

The use of live attenuated ASF vaccines, particularly in Portugal where strain 1455 was used over much of the Southern part of the country, has often been linked with the endemicity of ASF in the Iberian Peninsula for 30 to 40 years (Costa, 1990). Although chronic forms of the disease were clearly associated with animals that were vaccinated during this time (Nunes Petisca, 1965), the presence of chronically affected animals had already been identified both in Spain (Sánchez Botija, 1962) and in Angola (Leite Velho, 1956), generally thought to be the origin of the European strains at that time. It also seems likely that at least two antigenically different strains of ASFV were circulating in Iberia at this point (Lacasta *et al.*, 2015; Malogolovkin *et al.*, 2015; Sereda *et al.*, 2020), which could explain some of the vaccine failures in the field. Additionally, the association of a suitable biological vector with the traditional farming practices in Iberia was something that no one could have predicted at the time. In short, ASF and Iberia was a case of the wrong virus in the wrong place and the unfortunate early use of vaccines is quite likely to have exacerbated the issue.

Researchers in the Soviet Union in the second half of the twentieth century used the haemadsorption inhibition assay as a tool to guide the generation of a panel of immunologically distinct attenuated strains of ASFV by passage through porcine bone marrow cultures (Sereda *et al.*, 2020). These viruses could then be used in the event of an outbreak of ASFV if the epidemiological situation permitted. Despite the outbreak of ASF in Odessa in 1977, this situation did not occur and the vaccines were never used outside of the laboratory. The process of building up the panel of attenuated strains also produced the most comprehensive map of ASFV immunotypes to date, with at least eleven different immunotypes identified based on the induction of antibodies capable of inhibiting haemadsorption to infected macrophages, in combination with immunisation and challenge studies in pigs (Malogolovkin *et al.*, 2015; Sereda *et al.*, 2020). An ASFV isolate from Rhodesia (now Zimbabwe) attenuated by serial passage was shown to have the same serogroup as the current Eurasian outbreak isolates and was capable of protecting pigs (Balyshev *et al.*, 2011) but it also induced chronic disease (Sereda *et al.*, 2020). Attenuated viruses were also used to test the effect of immune status of recipient animals and results supported earlier observations by Manso Ribeiro's team (1963) and Sánchez Botija (1962) that animals with meagre health had a poorer response to attenuated ASF vaccines (Budarkov *et al.*, 2017). Differences in the protection obtained with live attenuated viruses have been observed between farm animals and specific pathogen free animals (King *et al.*, 2011; Lacasta *et al.*, 2014). Host genetics are also likely to play a role, as differences in protection after OUR T88/3 immunisation were observed between different lines of minipigs inbred at the major histocompatibility locus (Oura *et al.*, 2005). Live attenuated viruses continued to be used extensively as tools to study the immune response to infection as discussed in Chapter 3.

Viruses with reduced virulence have been obtained from wild boar in the field during the present ASF panzootic. Notably one of them, a non-haemadsorbing strain from Latvia (Lv17/WB/Rie1), when delivered orally can protect wild boar from subsequent challenge with the virulent Armenia 2008 isolate (Barasona *et al.*, 2019). This strain is being considered as a potential vaccine; however, both intramuscular inoculation and natural transmission of this virus to domestic pigs causes non-specific clinical signs, including joint swelling, which are similar to those seen after infection

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with two Portuguese isolates NH/P68 and OUR T88/3, both of which cause chronic disease (Gallardo *et al.*, 2019). The differences between the Lv17/WB/Rie1 strain and the virulent parent are relatively minor compared to those seen in NH/P68 and OUR T88/3. Therefore, this naturally attenuated isolate from Latvia may represent an excellent candidate for further targeted genetic modification.

6.2 The recombinant revolution: targeted gene deletion

The emergence of the technology to genetically modify virus, along with the beginnings of the sequencing revolution of the late 1980s and early 1990s, greatly increased our ability to interrogate and understand a whole range of different aspects of ASFV biology, not least the identification of genetic factors related to virulence. The ability to excise genes from the ASFV genome meant that attenuated viruses could now be developed by targeted gene deletion rather than blind passage through tissue culture (Figure 6.1). The first targets for gene deletion were the very few ASFV genes which showed similarity to known cellular genes and/or genes from other viruses. The *DP71L* gene (also referred to as *NL*) is similar to the *ICP34.5* gene which is a known virulence factor from herpes simplex virus 1. Both *ICP34.5* and *DP71L* share similarity with a cellular protein called GADD34 that helps control a key step in the synthesis of proteins within the host. One of the host responses to cellular stress or viral infection is a decrease in protein synthesis within cells and GADD34 is part of the process that turns protein synthesis back on. By mimicking GADD34 activity *DP71L* can help maintain high levels of protein synthesis in the face of this host response and so ensure that ASFV can produce all of the proteins it requires to make new progeny (Barber *et al.*, 2017). Removal of the *DP71L* gene from the Spanish E70 ASFV isolate did not affect replication *in vitro*, but led to a significantly attenuated virus *in vivo*, with animals suffering a short fever and reduced viraemia. All of the animals survived the E70 deletion mutant and when they were subsequently challenged with the virulent parental virus all of them were protected without showing clinical signs or viraemia (Zsak *et al.*, 1996). This promising result led the authors to try and delete the *DP71L* gene from virulent isolates obtained from both Chalaswa in Malawi and Pretoriuskop in South Africa. Deletion of *DP71L* had no effect on the virulence of Malawi and only slightly altered disease progression of the Pretoriuskop strain (Afonso *et al.*, 1998). The observation that ASFV may encode multiple virulence factors and that different genes may be more or less important for virulence in a given isolate, led to a series of experiments where different genes were removed from these two African isolates. Individual ASFV genes or combinations of the genes *DP96R* (*UK*), *K196R* (*TK*), *B119L* (*9GL*) and a section of genome encoding six multigene family members were all shown to significantly attenuate different isolates in domestic swine (Lewis *et al.*, 2000; Moore *et al.*, 1998; Neilan *et al.*, 2002; Zsak *et al.*, 1998). A Malawi *B119L* deletion showed a significant growth defect in macrophages and in pigs viraemia was 100 to 10,000-fold lower than wild type virus, with a short fever duration and reduced clinical signs. All of the animals survived inoculation with the *B119L* deletion mutant and subsequent challenge with a lethal dose of the parental Malawi strain, although the dose of the deletion mutant affected the length of viraemia after challenge. The *B119L* gene encodes for a protein that is involved in formation of progeny viruses; the deletion of this gene did not block virus replication *per se*, but resulted in the assembly of aberrant capsids. This observation suggested that deletion of *B119L* might provide a route to attenuate any given strain of ASFV.

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The first complete genome sequence of an attenuated strain obtained from the field (Chapman *et al.*, 2008) allowed the identification of potential virulence factors by comparative genomic analysis. The Portuguese OUR T88/3 had a large deletion that meant the virus lacked members of MGF360 and MGF505 similar to those that had previously been shown to be important for the virulence of the Pretoriuskop isolate (Neilan *et al.*, 2002). These multigene family members were then deleted from the Benin 1997/1 isolate and this virus was attenuated in pigs and could protect those pigs from subsequent challenge (Reis *et al.*, 2016). In another experiment the *DP148R* (*MGF360-18R*) gene was deleted from the Benin 1997/1 isolate and this virus was also significantly attenuated in swine. After a transient fever, animals recovered and were protected after challenge with parental virulent virus (Reis *et al.*, 2017). The level of protection achieved with the *DP148R* deleted virus was practically 100%, although vaccinated animals did have persistent viraemia that lasted for several weeks. However, the pigs were clinically normal and this suggested that further modification of the *DP148R* deleted virus may yield a vaccine with an attractive safety profile.

6.2.1 Genotype II vaccines

Research into ASF vaccines intensified as the situation in Europe deteriorated and the disease subsequently spread into China. What had been learnt from previous experiments with the genotype I viruses obtained from Europe in the second half of the last century, as well as those from other geographical regions, was applied to the current panzootic with mixed results. The *EP402R* gene encodes for a protein responsible for the adsorption of red blood cells to the surface of infected cells and although deletion of this gene from the Georgia isolate did not attenuate its virulence in pigs (Borca *et al.*, 2020), it did attenuate the Spanish Badajoz 1971 strain (Monteagudo *et al.*, 2017). Interestingly, this deletion mutant was capable of protecting pigs from the genotype II Georgia strain. This protection was linked to the ability of CD8 T-cells to recognise heterologous virus, as the antibody response induced by the *EP402R* deletion mutant was highly variable. Nevertheless, some of the animals immunised with the Badajoz *EP402R* deletion mutant did become sick after challenge and ASFV was found in some of the animal's secretions, suggesting further optimisation was required. The Badajoz *EP402R* deletion mutant was also successfully maintained on a continuous African green monkey kidney cell line (COS-1) for twenty passages without significant genetic modification. Generation and culture of recombinant deletion mutants of ASFV field isolates are for the most part still carried out on primary macrophages derived from porcine bone marrow, leukocytes or lung washes. Current EU guidelines for veterinary medicinal products require freedom from 23 different extraneous agents and an ASF vaccine derived from primary cells may require a herd of specific pathogen free animals. A prospective ASF vaccine that could be propagated on a defined cell line would therefore have significant advantage over one that required primary cell culture.

In contrast to the experiments with the Malawi isolate, a Georgia *B119L* deletion was not completely attenuated in swine (Lewis *et al.*, 2000; O'Donnell *et al.*, 2015b) although animals survived a low dose and were protected from challenge with the virulent parental strain. A multigene family deletion did attenuate the Georgia strain (O'Donnell *et al.*, 2015a), but surviving animals challenged with parental virus still harboured the challenge virus despite appearing clinically normal. Different combinations of *B119L*, the multigene family region, *DP71L* and the *DP96R* genes were then deleted from the Georgia backbone (O'Donnell *et al.*, 2016, 2017;

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Ramirez-Medina *et al.*, 2019). A double deletion of *B119L* and *DP96R* was attenuated in swine, even at a dose 100 times greater than that which would have caused disease after infection with the single deletion of *B119L*. The double deletion also protected some pigs from challenge as early as 14 days post vaccination, but there was a marked difference in the response after challenge depending on the dose used for immunisation. The optimal dose protected all of the animals from severe disease with many of them showing minimal clinical signs, unfortunately a number of the animals still harboured virus three weeks later (O'Donnell *et al.*, 2017). However, a recent study has identified the *I177L* gene as a target for attenuating ASFV by gene deletion. This gene encodes for a highly conserved protein of unknown function that is a component of the virion. Interruption of this gene in the Georgia strain led to an interesting phenotype where viraemia was delayed by several days and the peak of circulating virus varied significantly from pig to pig. Crucially, despite some animals having as much virus circulating the blood as those infected with the wild-type virulent Georgia isolate, none of the pigs developed a temperature or exhibited any clinical signs (Borca *et al.*, 2020). All pigs were robustly protected when they were infected with the parental strain. Similarly, to what was seen in pigs infected with different Benin deletion mutants (Reis *et al.*, 2016, 2017), there was persistence of the virus in the blood stream; however, in all but one of the animals this was the vaccine and not the virulent parental virus. Preliminary data suggested that neither the vaccine nor the challenge virus was transmitted to sentinel pigs and even low doses of the *I177L* deletion mutant were capable of inducing robust protection, making this virus a potential vaccine candidate.

A number of deletion mutants were evaluated on a larger scale with a genotype II virus isolated from Heilongjiang in China in 2018 (Chen *et al.*, 2020) that is >99% identical to the Georgian strain used by other researchers. These experiments confirmed that deletion of *EP402R* did not attenuate Eurasian genotype II ASFV and also showed that the additional deletion of *DP96R* (*UK*) did not improve this attenuation. Furthermore, deletion of *DP148R* that attenuated the virulent Benin 1997/1 strain was not sufficient to attenuate the Chinese strain. However, deletions of the multigene family region and a double deletion of *B119L* and *DP96R* were attenuated, as was a combination of the multigene family region and *EP402R*. These three deletions were then tested for the ability to protect the immunised pigs against challenge with the virulent parental virus. Both of the multigene family-based deletions protected domestic pigs, but in contrast to the analogous virus based on the Georgia strain (O'Donnell *et al.*, 2017) the double deletion of *B119L* and *DP96R* did not induce a protective immune response. Pigs immunised with a high dose of the deletion mutant lacking the multigene family region and the *EP402R* gene only suffered a brief fever for one day after challenge, although ASFV was found in the tissues of animals three weeks later. Next, the multigene family deleted viruses were safety tested for reversion to virulence by passaging the viruses five times through pigs. Interestingly, the virus that did not have an *EP402R* deletion progressively replicated more efficiently as it was repeatedly passaged through pigs and by the fifth passage one animal died. This apparent reversion to virulence did not occur with the virus that had both the multigene family region and *EP402R* deleted and therefore the reason for the reversion is unknown. However, vaccinia virus can rapidly duplicate sections of its genome related to host immune evasion (Elde *et al.*, 2012). The *EP402R* deletion is not sufficient to attenuate genotype II ASFV on its own and therefore does not provide an additional safety measure if the attenuation conferred by the multigene family deletion can be reversed. A single dose of the multigene family and *EP402R* deletion mutant protected pigs from severe disease, but not infection, five months after immunisation, and this virus did not affect the health of a smaller

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number of pregnant sows or their piglets. The Chinese double deletion mutant is moving into field trials that may involve between 10,000 to 20,000 pigs and the results of these experiments will be of huge interest (Mallapaty, 2019). Previous experience in Spain and Portugal fifty years ago suggested that results in well husbanded pigs in clean animal isolation units did not translate into positive results in the field. However, none of the laboratories working with genotype II deletion mutants have reported any clinical signs that could be consistent with reactivation of the disease or chronic ASF, therefore it is to be hoped that second time round field trials of a live attenuated ASF vaccine will be successful.

6.3 Subunit vaccines: unfulfilled early promise

6.3.1 Protein vaccines

Antibody responses to specific ASFV proteins were first identified in the late 1970s and early 1980s (Black and Brown, 1976; Tabarés *et al.*, 1980). As the sequences of the genes encoding for these different antigens were deciphered, the possibility of generating immune responses in pigs without the use of ASFV became a reality (Figure 6.1). However, some of the first experiments of this type used protein purified directly from the supernatants of ASFV infected cells. Most ASFV isolates haemadsorb red blood cells to the surface of infected cells. However, Ruiz-Gonzalvo and Coll (Ruíz-Gonzalvo and Coll, 1993) identified an unusual strain which caused red blood cells to clump together (called haemagglutination) without needing to adhere to cells. In a series of experiments the haemagglutinating activity was separated, concentrated and used to immunise pigs. The immunisation induced antibodies in the serum of the animals that were capable of blocking the haemadsorption to infected cells *in vitro*. In a subsequent pilot study immunisation with the haemagglutinating activity protected one of two pigs from severe disease after contact challenge with a pig infected with the parental strain. Around the same time researchers in Russia had identified that the haemadsorption activity of virally infected cells was associated with glycoproteins (proteins modified by the addition of sugar residues). The haemadsorbing activity was then separated from other glycoproteins using anti-sera from recovered pigs. Similar to the results obtained by Ruiz-Gonzalvo and Coll (1993) this purified haemadsorbing activity could protect pigs from severe disease after infection with a related strain of ASFV. The ability of the protein to induce antibodies capable of blocking haemadsorption and protecting pigs from disease was also found to be dependent on the sugar residues attached to the protein (Sereda, 2013; Sereda *et al.*, 2018).

The identification of the gene encoding for the protein responsible for haemadsorption (Rodríguez *et al.*, 1993) enabled the generation of a recombinant baculovirus that could reproduce the haemadsorbing activity *in vitro*. The baculovirus was then used to immunise three pigs, which resulted in the animals having antibodies that could inhibit red blood cells sticking to infected macrophages and, in one of the pigs, antibodies were also capable of blocking infection of macrophages (Ruiz-Gonzalvo *et al.*, 1996). Strikingly, all three pigs survived challenge with the E75CV1 strain of ASF and the pig that had inhibiting antibodies had no circulating virus in the bloodstream throughout the course of the experiment. The cellular response to EP402R or ASFV was not tested in this study, but more recent work has identified T-cell epitopes in the EP402R protein (Argilaguet *et al.*, 2012; Burmakina *et al.*, 2019) and it is possible that immunisation

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with baculovirus infected cells also induced a cellular response against EP402R that could have contributed to protection.

As well as identifying the ASFV gene responsible for the haemadsorption phenomenon, significant progress has been made in identifying targets of neutralising antibodies against ASFV. In a series of experiments researchers showed that neutralising activity could be found in the sera of recovered pigs (Ruiz Gonzalvo *et al.*, 1986a,b) and that this activity targeted three different proteins that were part of the virus particle (Gómez-Puertas *et al.*, 1996). Antibodies against p72 and p54, encoded by the *B646L* and *E183L* genes respectively, inhibited the attachment of virus to the surface of susceptible cells, whereas antibodies against p30 (*CP204L* gene) were also capable of blocking the internalisation of virus that had already bound to cells (Gomez-Puertas *et al.*, 1996, 1998). Immunisation of pigs with cells infected with a baculovirus expressing p30 and p54, or a fusion of the two genes, induced antibodies against the two proteins that were capable of neutralising the virus (Barderas *et al.*, 2001; Gomez-Puertas *et al.*, 1998). Some of these pigs survived infection with virulent strain(s) and although the animals became sick and viraemic, both the clinical signs and levels of circulating virus in the bloodstream were lower than in control animals. All of the surviving vaccinated animals had cleared the virus by the end of the experiment. Unfortunately, these promising results did not translate to other strains of ASF. Baculoviruses expressing p30, p54, p72 and p22 (*KP177R* gene) derived from the South African Pretoriuskop isolate induced ASFV-specific antibodies capable of neutralising the virus. However, in contrast to the experiments with the E75 isolate the immunised pigs were not protected against challenge (Neilan *et al.*, 2004).

6.3.2 DNA vaccines

Immune responses can also be induced to individual proteins by immunising with the sequence of DNA that codes for that particular protein. Typically, genes in DNA vaccines are encoded by plasmids that can be directly injected into the target species or they can be introduced by specialist equipment, such as a gene gun. Manufacturing costs are typically a lot lower than producing the proteins themselves; lyophilised DNA is very stable and can be easily reconstituted prior to immunisation. DNA vaccination also lends itself to multi-valent vaccines or an approach called 'library' immunisation where many different genes or fragments of genes can be inoculated at the same time. This could be advantageous for complex pathogens like ASFV that have multiple genes and/or for which the antigenic determinants are unknown. DNA vaccines have been licensed for two different salmon viruses (APEX-IHN® and Clynav), avian influenza, West Nile virus in horses and canine melanoma (Oncept®).

Initial results with DNA vaccines against ASFV were not promising. A plasmid expressing the same p30 and p54 fusion protein described in the previous section was not immunogenic in pigs without the use of a molecular adjuvant (Argilaguet *et al.*, 2011). The p30 and p54 fusion protein were then fused to part of an antibody that specifically targeted ASFV proteins to pig macrophages. This led to the induction of immune responses to the two viral proteins and to the whole virus, but in contrast to the results with the baculovirus expressed proteins, the antibody response elicited did not inhibit the ability of the virus to infect macrophages, rather it enhanced it. Immunised pigs were not protected and had elevated viral titres three days after challenge, correlating with the infection enhancement seen *in vitro*. The difference in both the

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immune response and the clinical outcome with the same antigens after immunisation by DNA or baculovirus highlights the importance of the delivery system in the ultimate outcome of the vaccine. Disease and/or infection enhancement is an additional complication in designing ASFV subunit vaccines and it has been described by multiple groups using different antigens and vaccine platforms (Jancovich *et al.*, 2018; Netherton *et al.*, 2019; Sunwoo *et al.*, 2019).

The cellular immune response induced by the initial ASF DNA vaccine appeared to be biased toward a CD4 response (Argilaguet *et al.*, 2011); however, experiments with live attenuated ASFV had shown that a CD8 T-cell response was important for protection (Oura *et al.*, 2005). The protein responsible for ASFV haemadsorption encoded by the *EP402R* gene had already been shown as a protective antigen (Ruiz-Gonzalvo *et al.*, 1996) and so was also included in a follow up DNA vaccine study. A fusion between EP402R, p54 and p30 was created and combined with a different molecular adjuvant designed to direct the immune response toward a cellular response dominated by CD8 T-cells (Argilaguet *et al.*, 2012). Immunisation of pigs with the non-adjuvanted EP402R-p54-p30 fusion induced both antibody and cellular responses to the antigens, but again the antibody responses were not able to neutralise ASFV infectivity *in vitro* and were not able to protect pigs from challenge with virulent ASFV. Strikingly, the construct designed to target a cellular immune response did protect some of the pigs despite not inducing any detectable antibodies. This approach was expanded to include approximately three quarters of the entire ASFV genome by fusing a library of 4,000 separate fragments of ASFV genomic material to the molecular adjuvant (Lacasta *et al.*, 2014). Although there was no detectable ASF-specific immune response prior to infection 60% of the animals recovered from fever. An expansion of CD8 T-cells was observed after challenge suggesting that the cellular immune response played a role in the protection, although the exact nature of the protective response or the components of the ASFV genome that it targeted are unclear. This work suggested that with further improvement and optimisation a DNA vaccine approach might be feasible for ASF.

An alternative strategy was to combine DNA and protein immunisation. Plasmids and proteins for eight different ASFV proteins, including p30 and p54, were used to immunise pigs (Perez-Nunez *et al.*, 2019). Different combinations of antigens induced antibody responses that were capable of inhibiting or enhancing infection of macrophages in *in vitro* tests. Infection of pigs that had antibodies that enhanced infection also led to a more rapid appearance of viraemia and clinical signs compared to controls (Sunwoo *et al.*, 2019) and none of these immunised animals survived. Both studies where enhancement was observed included p54 and p30 in the pool, suggesting antibodies against these proteins might be responsible for infection enhancement (Argilaguet *et al.*, 2011; Perez-Nunez *et al.*, 2019). However, previous work with these two proteins showed that a protective response that included the induction of antibodies could be mounted when p54 and p30 were injected as a preparation of baculovirus infected cells (Barderas *et al.*, 2001; Gomez-Puertas *et al.*, 1998). This again highlights the complexities of developing subunit vaccines as individual ASFV proteins appear to induce immune responses that lead to diametrically opposed clinical outcomes depending on the delivery system.

6.3.3 Viral vectors

Many vaccination strategies require booster immunisations in order to generate effective immune responses. Normally a boost is identical to the original first immunisation, a procedure called

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homologous prime-boost immunisation. However, immunisation with antigens in one format followed by a boost with the same antigens in a different format, so called heterologous prime-boost, can prove advantageous. Heterologous prime-boost can avoid immune responses against the vaccine platform itself, preventing an effective recall response to the antigen of interest after boost and, in some cases, it can help shape the type of immune response. Viral vectors have been shown to be a potent way of generating both antibody and cellular responses in a variety of different species. Attenuated strains of vaccinia virus, the vaccine used to eradicate smallpox, and genetically engineered replication deficient adenoviruses have been used extensively over the last decade or so. A combination of DNA vaccination and vaccinia virus was used in a heterologous prime boost approach in the first instance to identify potentially immunogenic proteins and then to test their protective effects in pigs.

The work on ASF subunit vaccines described in Sections 6.3.1 and 6.3.2 focused on the few proteins that induced a clearly defined antibody response. However, as described in Chapter 2, ASFV encodes for more than 150 different proteins and the antigenicity and any potential role in protection had not been tested. Both the antibody and cellular responses to 41 different ASFV genes were determined in pigs after DNA plasmid prime followed by vaccinia virus boost. This approach identified thirteen different viral genes, eight of which were novel and capable of inducing consistent immune responses. However, immunised pigs were not protected from virulent ASFV in a subsequent challenge experiment and, as seen with other experiments, showed more rapid onset of clinical signs than the controls. In contrast to these other studies, however, viral load in the vaccinated animals was less than in the controls, suggesting there was also a protective effect. Increased clinical signs against the background of reduced viral replication has been observed in vaccination models with other viruses.

Live attenuated strains of ASFV were used in a series of experimental screens to understand the breadth and specificity of both the humoral and cellular immune response to natural infection (Jenson *et al.*, 2000; Kollnberger *et al.*, 2002; Netherton *et al.*, 2019; Reis *et al.*, 2007). Viral vectors were used to induce specific immune responses to these proteins in pigs and test for their protective efficacy. A range of immunogenic ASFV proteins were incorporated into adenovirus vectors and the majority of them proved immunogenic in swine (Goatley *et al.*, 2020; Lokhandwala *et al.*, 2016, 2017, 2019; Netherton *et al.*, 2019). However, the protective efficacy of these different combinations of viral vector antigens has been mixed. A pool of eleven different adenoviruses, containing 35 different ASFV proteins, did not protect wild boar from challenge with a virulent strain from Armenia (Cadenas-Fernandez *et al.*, 2020). Nine adenovirus vectored ASFV antigens induced enhanced disease in pigs after challenge with the virulent Georgia strain (very similar to the Armenia strain). However, immunisation with seven adenoviruses expressing eight different structural proteins showed more promise (Lokhandwala *et al.*, 2019). This second cocktail of adenoviruses induced antigen specific responses that were influenced by the choice of adjuvant, with the adjuvant BioMise inducing higher antibody responses than ZTS-01. However, the pigs that were immunised in combination with ZTS-01 had better clinical outcomes, with five of the nine pigs in the group surviving to the end of the study compared to two in the group immunised in combination with BioMise. Interpretation of this result is complicated, however, by the fact that not all of the control animals suffered acute disease after challenge despite some pigs having low level viraemia. Interestingly, all of the ASFV genes that were potentially protective were included in the 35 genes that were combined and trialled in wild boar. In the experiment

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that showed some promise the antigens were all delivered as individual genes, whereas in the wild boar study multiple fusions of the ASFV genes were used, but it is difficult to draw comparisons as these two studies used different challenge models as well as vector design (Cadenas-Fernandez *et al.*, 2020; Lokhandwala *et al.*, 2019).

A number of ASFV genes were identified as targets of the cellular immune response and these were also vectored using adenovirus in combination with a highly attenuated vaccinia virus in a heterologous prime-boost strategy (Netherton *et al.*, 2019). Two different pools were trialled and a combination of 12 ASFV proteins reduced viraemia and clinical signs in some pigs, but ultimately did not protect them from severe disease. Animals with reduced viraemia had higher ASF-specific cellular immune responses than those that did not, highlighting the potential role of the T-cell response in protection. Antibody responses, but not cellular responses, were observed in pigs immunised with a pool of nine ASFV antigens; however, these pigs suffered enhanced clinical signs compared to the controls. In a follow-up study eight antigens that were identified using the DNA-prime vaccinia virus-boost screen described above (Jancovich *et al.*, 2018) were also trialled using adenovirus vectors. The animals were then boosted with a combination of adenoviruses and highly attenuated vaccinia viruses and in one experiment all of the vaccinated animals survived challenge with a virulent strain of ASFV (Goatley *et al.*, 2020). The *caveat* to this positive result is the observation that all of the pigs got sick and although clinically normal at the end of the study were still viraemic. The strain of ASFV used as the challenge material in these studies was a virulent strain obtained from Portugal in 1988 and it will be interesting to see if these eight antigens are effective against the genotype II strains circulating in Europe and Asia.

6.4 Disabled infectious single cycle African swine fever vaccines

An alternative approach to vaccine development is to try and convert ASFV itself into a viral vector, also referred to as a disabled infectious single cycle (DISC) vaccine. The replication deficient adenoviruses described in the previous section typically lack the *E1* genes which are involved in early steps of adenovirus replication. Replication deficient adenoviruses are made with cell lines that contain these crucial E1 proteins, allowing the generation of progeny viruses that lack the *E1* gene. Therefore, an adenovirus vector can infect a cell in a pig and express the ASF gene of interest, but the adenovirus cannot make any more copies of itself. This type of strategy has been used with other large DNA viruses such as herpesvirus and therefore it may be possible to take a similar approach with ASFV. A replication deficient ASFV would have the potential to express all but one of the 150 or more genes that the virus encodes, allowing a full range of T-cell antigens to be displayed to the cellular immune system. Although the use of inactivated ASFV has not been successful as a vaccine, it is conceivable that inactivation methods may have induced structural changes to the virus particle that could have led to a loss of antigenicity (Schloer, 1980). Therefore, a replication deficient ASFV could conceivably induce both cellular and humoral immune responses that would be representative of infection with an attenuated strain of the virus without the attendant risk of causing disease. However, there are numerous technological roadblocks that need to be overcome before this approach can be brought to fruition (Figure 6.2). In the first instance one is the availability of suitable cell lines in which to grow the virus. As discussed above, field isolates of ASFV are typically grown in primary macrophages, which are not suitable for the generation of long-term cultures that express the ASFV genes required

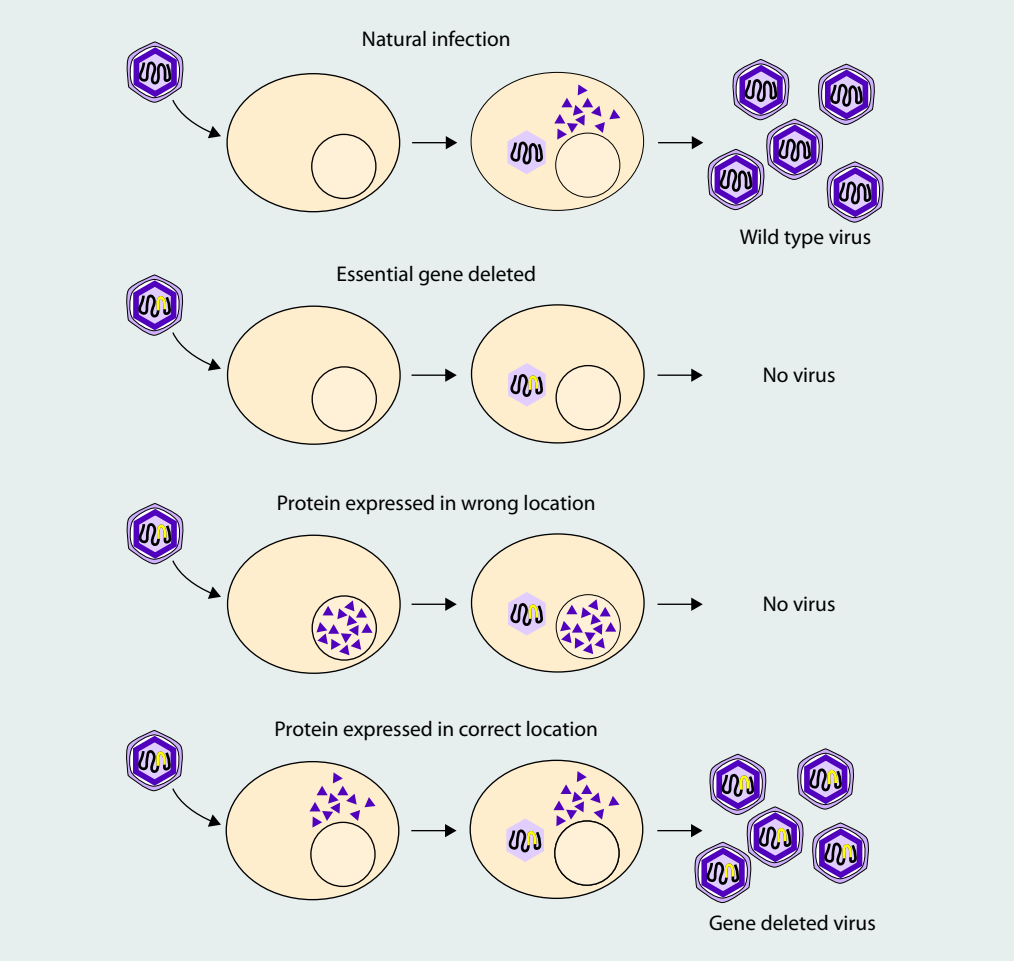

Figure 6.2. Challenges in developing defective infectious single cycle vaccines. Schematic highlighting of the principal challenges for developing a DISC vaccine for a complex pathogen like ASFV. Virus replication is compartmentalised within an infected cell with different viral proteins found in different locations. The essential ASFV gene selected as the target for the DISC vaccine therefore needs to be available in the correct location at the correct time in order to make gene deleted virus.

to support an ASFV DISC vaccine. Some field isolates can be grown in standard cell cultures or macrophage-like cell lines, but this could lead to adaptation of the virus to culture and loss of immunogenicity. The other problem is the choice of essential gene or genes to delete in order to block the ability of ASFV to replicate. Although a number of elegant studies have demonstrated the dependence of viral replication on particular genes, in order for a DISC vaccine to work the gene in question also has to be suitable for efficient expression in mammalian cells and crucially needs to be in the right place at the right time in the virus replication cycle. ASFV replication is compartmentalised both in space and time and viral structural proteins typically misfold or localise to cellular structures when expressed outside the context of an ASFV infected cell. However, recent work has suggested that the *A104R* gene that encodes for a protein involved in

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transcription of viral genes may be a suitable candidate for an ASF DISC vaccine (Frouco *et al.*, 2017). A proof of principle study showed that a genetically modified strain of ASFV lacking the *A104R* gene could be maintained on tissue culture cells that stably expressed the *A104R* gene for several passages (Freitas *et al.*, 2019). Further optimisation will be required to bring this technology to fruition and it is possible the other genes such as the *P1192R* gene may be more suitable targets than *A104R* (Coelho and Leitão, 2020).

6.5 Conclusions

ASF vaccine research did not get off to a particularly auspicious start and the experience in Spain and Portugal in the early 1960s cast a long shadow. Results of the field trials in China will be of great interest, with the hope that modern methods of targeted gene deletion are able to succeed where the classic approach of attenuation through passage in tissue culture did not. If the trials are successful, international acceptance of vaccination against ASF will most likely require development of a robust companion test to be able to differentiate vaccinated from naturally infected animals. Such a test will be crucial for allowing trade from countries that decide to include vaccination as part of their control strategies. A vaccine capable of eradicating virus from wildlife reservoirs is another complication, particularly relevant to the current European situation. Vaccination by injection is impractical when the virus is so widely dispersed in the wild, therefore an oral vaccine will be preferable and has shown to be particularly effective in the successful eradication of classical swine fever from wild boar. Of note, the attenuated Latvian strain has been shown to be effective when delivered orally to wild boar and therefore it may be possible to use live attenuated strains of ASFV in baited food for deployment in the field. An oral vaccine based on a poxvirus, a double stranded DNA virus related to ASFV, was used in the successful campaign to eradicate rabies from foxes in parts of Western Europe. As ASFV is also relatively stable in the environment it may be possible to carry out a similar vaccination campaign to eradicate ASF from European wild boar. Regardless of the success or failure of the scientific community to generate a safe and effective ASF vaccine, control of ASF will always require a multi-factorial approach based on the principles of biosecurity and rapid diagnosis in the case of breaches of that biosecurity. As Donald E. DeTray wrote of ASF nearly fifty years ago 'We should not be lulled into a false sense of security in the belief that a vaccine will solve the problem'.

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