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Review

Development of vaccines against African swine fever virus

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ABSTRACT

An outbreak in the Caucasus in 2007 initiated the spread of ASFV through Russia and Eastern Europe, subsequently affecting Ukraine, Belarus, Poland, the Baltic States, the Czech Republic, Moldova, Romania and Bulgaria. The declaration of outbreaks in China and Central Europe in August 2018, definitely confirms the serious threat that the extension of ASF represents for the global swine industry and the environment. Despite the efforts of several groups to generate a vaccine against ASFV, currently only control and eradication measures are available based mainly on the early detection and implementation of strict sanitary procedures, including the mass slaughter of animals, both domestic and wild boar. However, the rapid spread of the disease shows that these actions are clearly insufficient to control the current pandemic situation, and the development of a vaccine is urgently required.

1. Introduction

At present, the generation of vaccines can impede the spreading of ASF all over the world, as, despite other measures such as quick diagnostic, control and eradication have been implemented, the virus has expanded extremely fast from 2007 through Russia, EU and China, causing fatal ecosystem damage and grave economic consequences to stakeholders. African swine fever virus (ASFV), the only member of the Asfarviridae family, is a dsDNA virus of huge complexity and size that encodes more than 150 proteins, including both structural and host-induced immunoregulatory proteins. ASFV is the etiological agent of African swine fever (ASF), a devastating disease infecting monocytes and macrophages of both wild boar and domestic pigs. The outbreak in the Caucasus in 2007 (Rowlands et al., 2008) started the spread of ASFV across Russia and Eastern Europe. The declaration of outbreaks in China and central Europe during August 2018 enhances the serious problem that the extension of ASF poses for the global swine industry and for the environment. Currently, although there has been a successful eradication in some countries such as the Czech Republic, the OIE has notified during the period from February to March 2019, 13 countries with new or ongoing outbreaks: 9 in Europe (Belgium, Hungary, Bulgaria, Latvia, Moldova, Poland, Romania, Russia and Ukraine), 3 in Asia (China, Mongolia and Vietnam) and 1 in Africa (Zimbabwe) (OIE Report Nº11: February15-March 01, 2019).

Historically, failed attempts to develop vaccines against ASFV have included inactivated viruses, recombinant proteins, DNAs or recently combinations of DNAs plus viral proteins (Arias et al., 2017; Revilla et al., 2018; Rock, 2017). The approaches to protect pigs using inactivated vaccines were not successful, even if they were combined with specific adjuvants, revealing the poor role of antibodies in protection. In addition, other approaches such as the use of certain viral vectors for the expression (especially adenovirus-based vaccines) of ASFV antigens were likewise unsuccessful, whereas subunit vaccines showed only very little protection against exposure to virulent strains of ASFV. Faced with the general failure of the protocols mentioned above, and known the challenging vaccination scenario, a so-called "live attenuated vaccine" (LAV) emerges as a putative strategy in ASFV protection, on which there are already some patents, but no commercial product until now. The rapid dissemination of the disease shows these actions clearly insufficient to control the current pandemic situation, and the development of an efficient vaccine is urgently required. The development of a safe and effective vaccine will help hopefully in five years to the efficient immunization of domestic pigs and wild boar avoiding future outbreaks all over the world that put at risk the pig hut and entails great economic losses for the affected countries.

2. ASFV vaccines development: state of the art

The observation that pigs surviving ASFV infection are able to develop protective immunity against new infections indicates that an effective vaccine against ASFV might be possible. However, despite the fact that several research groups have implemented new and diverse vaccine technologies including inactivated viruses, recombinant proteins/peptides, DNA and DNA/protein combination vaccines, vectored

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vaccines and LAVs, at present there is not a completely safe and effective vaccine against ASFV on the market.

2.1. Inactivated and subunits ASFV vaccines

One of the major general disadvantages of these types of vaccines is the failure to generate a prolonged immune response. Inactivated vaccines do not confer protection even in the presence of adjuvants (Blome et al., 2014). Virion complexity and the intracellular and extracellular localization of infectious particles make viral neutralization a challenge, and the antibodies generated are not protective or may even enhance the disease (Blome et al., 2014; Detray, 1963; Mebus, 1988; Stone and Hess, 1967; Sunwoo et al., 2019)

Subunit vaccines which utilize the expression of specific ASFV genes and proteins to generate a specific immune response have also been reported. Identification of the ASFV antigens and epitopes responsible for induction of relevant immune responses is key to develop effective vaccines against ASFV. Although some research has been carried out on this topic, the 167 ORFs in the viral genome make it very difficult to select specific candidates based on "in silico" approaches or on IFNλ elispots.

It has been published that ASFV internalization is inhibited in vitro by antibodies against the p30 early viral protein, whereas antibodies against p12 (Angulo et al., 1993), p72 or p54 inhibit virus attachment, indicating the role these viral proteins play in the first steps of viral infection (Gomez-Puertas et al., 1996). Nevertheless, pigs immunized with either p30 or p54 were not protected against acute ASF despite the production of neutralizing antibodies (Gomez-Puertas et al., 1998). In this regard, the combination of p30 and p54 (or a chimeric construct) elicited partial protection (Barderas et al., 2001; Gomez-Puertas et al., 1998), though no protection was found when animals were immunized with baculovirus-expressed p30, p54, and p72 (Neilan et al., 2004). However, pigs vaccinated with baculovirus-expressed EP402R/CD2v elicited protection against homologous ASFV challenge (Ruiz-Gonzalvo et al., 1996). These results indicate that the antibody response to ASFV infection is not fully neutralizing, at least for the viral proteins tested so far (Escribano et al., 2013). Activation of T cell responses during ASFV infection has been also examined, with p32 described as an important target of cytotoxic T lymphocytes (Alonso et al., 1997). Oura and colleagues described that ASFV protection fails when cytotoxic T lymphocytes are depleted, suggesting an important role of T cells in ASFV protection (Oura et al., 2005).

DNA subunit vaccines have also been explored; one of the strategies used was to clone viral p30 and p54 genes in-frame with a segment encoding the immunoglobulin variable region recognizing SLA-II, in an attempt to direct the presentation of viral antigens. Although specific T cells against ASFV proteins were detected, neither neutralizing antibodies nor protection against virulent challenge was achieved (Argilaguet et al., 2011). In line with this, it was shown that vaccination of pigs with DNA encoding the extracellular domain of ASFV-hemagglutinin (HA) fused with viral p30 and p54 induced improvement of both cellular and humoral immune responses, though not conferring protection. However, when ubiquitin was expressed in-frame with these viral genes, partial protection was obtained, corresponding to a robust $CD8^+$ T cell response, in the absence of specific antibodies (Argilaguet et al., 2012; Lacasta et al., 2014).

A recent approach using a combination of selected virus-specific proteins and cDNAs was able to induce a robust immune response in terms of in vitro neutralizing antibodies and IFNγ production in vivo (Pérez-Núñez et al., 2018); however, vaccinated pigs were not protected against virulent challenge with the Armenia07 strain (Sunwoo et al., 2019).

In summary, DNA- and peptide-based vaccines induce specific antiviral immune responses including neutralizing antibodies or virus-specific T-cells, but show only low or partial protection after virulent ASFV challenge.

Very recently, viral vectors and expression platforms alone or in combination with other approaches have been implemented. The vectors used to date have been non-replicative vaccinia derivatives, such as modified vaccinia Ankara (MVA) or the highly attenuated vaccinia virus strain NYVAC, in addition to alpha- and adenoviruses. Recombinant MVAs expressing structural ASFV proteins have been found to generate a specific T-cell response in immunized animals (Lopera-Madrid et al., 2017). ASFV antigens produced by adenoviral vectors also produced specific antibody and cellular responses (Lokhandwala et al., 2017). Recently, a strategy using ASFV structural antigens expressed by alphavirus and a boost with the attenuated ASFV OURT88/3 suggested that the combination of vector-expressed antigens with attenuated ASFV can increase epitope recognition (Murgia et al., 2018). A combination of prime ASFV-DNA vectors together with a boost with ASFV-modified NYVAC has also been reported. These recombinants vectors contained multiple ASFV antigens, selected semi-randomly, including genes involved in replication, immune evasion, multigene family (MGF) members, or those with unknown function. However, animals vaccinated by this procedure were not fully protected against virulent challenge (Jancovich et al., 2018). Although subunit vaccines using vectors are promising and may be a safer option than LAVs for vaccinations, especially in non-endemic areas, there is still much effort remains to be done to generate prototypes that produce a truly effective protection.

2.2. Live-Attenuated ASFV vaccines (LAVs)

Due to the intricacy of infection and complexity of the porcine immune response against ASFV, development of a vaccine using live, replicating ASFV has been proposed. LAVs would presumably stimulate both innate and secondary cellular and humoral immunity, though they must be further attenuated by modification of viral genes responsible for host evasion, virulence, or immune inhibition, either in order to mitigate possible side effects or to establish DIVA markers.

2.2.1. LAVs obtained from virulent strains

One strategy to develop LAVs is to attenuate highly virulent strains by deleting specific genes related to virulence and/or blockage of the host immune response. Genes related to hemadsorption (EP402R) (Rodriguez et al., 1993), virulence such as 9GL (B119 L) (Lewis et al., 2000) and UK (DP96R) (Zsak et al., 1998), or regulation of the immune response [MGF 360/505; involved in inhibition of IFN production (Afonso et al., 2004)] have been deleted in order to attenuate virulent ASFV strains. Two recombinant viruses from the highly virulent ASFV Georgia07 strain were generated, one with six simultaneous deletions from MGF 360/505 and the other with the 9GL gene deleted. Both single-deletion mutants presented an attenuated phenotype and were able to protect against the parental virulent ASFV (O'Donnell et al., 2015a, b). However, when pigs were vaccinated with a Georgia07 isolate harboring both deletions, protection was lost although the safety level was improved (O'Donnell et al., 2016), suggesting that a certain level of viral replication is required to achieve protection. In comparison, simultaneous deletion of the 9GL and UK genes from the Georgia07 isolate offered 100% protection against the parental isolate, with safer levels compared to deletion of 9GL alone (O'Donnell et al., 2017), which may be due to the balanced function of the specific genes deleted. These results suggest that deletion of multiple functionally related genes may be key for protection and safety of the vaccine. Similar studies showed that animals vaccinated with Benin ΔDP148R and BeninΔMGF had a 100% survival rate against virulent challenge with Benin homolog (genotype I) (Reis et al., 2016, 2017). Another recent study showed that CD2v (EP402R) plays a key role in both viral attenuation and protection against infection. The Ba71 isolate carrying a deletion of CD2v/EP402R was able to induce a protective immune response in pigs challenged with homologous BA71 (genotype I) or heterologous Georgia07 (genotype II) and E75 (genotype I) virulent strains

Table 1
ASFV LAVs vaccines developed and their degree of protection conferred.

Type	Strain	ASFV/recombinant ASFV	Cell source of production	Challenge	Survival rate	Ref.
Naturally attenuated	NH/P68	NH/P68	PAM	Homologous L60	100%	(Gallardo et al., 2018)
				Heterologous Armenia07	100%	(Gallardo et al., 2018)
			COS-7 (Percoll Purified)	Heterologous Armenia07	33%	(Gallardo et al., 2018)
	OURT/88/3	OURT/88/3	BM	Homologous OURT/88/1	100%	(King et al., 2011)
				Heterologous UG65	100%	(King et al., 2011)
Genetically modified from naturally attenuated strains	NH/P68	NH/P68ΔA238L	COS-7	Homologous L60	100%	(Gallardo et al., 2018)
				Heterologous Armenia07	0%	(Gallardo et al., 2018)
		NH/P68ΔA224L	COS-7	Homologous L60	100%	(Gallardo et al., 2018)
				Heterologous Armenia07	50%	(Gallardo et al., 2018)
		NH/P68ΔEP153R	COS-7	Homologous L60	100%	(Gallardo et al., 2018)
				Heterologous Armenia07	0%	(Gallardo et al., 2018)
		NH/P68ΔA238L	COS-7 (Percoll Purified)	Heterologous Armenia07	40%	(Gallardo et al., 2018)
		NH/P68Δ276R	PAM	Heterologous Armenia07	0%	(Gallardo et al., 2018)
	OURT/88/3	OURT/88/3ΔDP71LΔ DP96R (ΔDP2)	BM	Homologous OURT/88/1	66%	(Abrams et al., 2013)
Genetically modified from virulent strains	Georgia07	Georgia07Δ9G L	PBM	Homologous Georgia07	100%	(O'Donnell et al., 2015b)
		Georgia07ΔMGF	PBM	Homologous Georgia07	100%	(O'Donnell et al., 2015a)
		Georgia07Δ9G LΔMGF	PBM	Homologous Georgia07	0%	(O'Donnell et al., 2016)
		Georgia07Δ9GLΔDP96R/UK	PBM	Homologous Georgia07	100%	(O'Donnell et al., 2017)
	Benin 97/1	BeninΔDP148R	BM	Homologous Benin 97/1	100%	(Reis et al., 2017)
		BeninΔMGF	BM	Homologous Benin 97/1	100%	(Reis et al., 2016)
	Ba71	BA71ΔEP402R	COS-1	Heterologous E75	100%	(Monteagudo et al., 2017)
				Heterologous Georgia07	100%	(Monteagudo et al., 2017)

PBM: Porcine blood macrophages, PAM: Porcine alveolar macrophages, BM: Bone marrow macrophages.

(Monteagudo et al., 2017). It is clear that some degree of infection is needed to confer protection, since deletion of essential genes such as thymidine kinase (TK) from the virulent Georgia07 strain induced complete viral attenuation, and was not able to further confer protection (Sanford et al., 2016). Interestingly, deletion of TK from the virulent Malawi strain attenuated while also inducing protection against homologous challenge (Moore et al., 1998).

Although the described modifications of some virulent strains result in viruses that can induce protection, the risk of vaccinating with such genetically modified viruses is high. In vivo homologous recombination between recombinant vaccines (lacking particular DNA sequences) and circulating wild type viruses cannot be discarded, thus potentially generating new disease outbreaks.

2.2.2. LAVS obtained from naturally attenuated strains

To avoid possibly catastrophic recombination, the modification of naturally attenuated ASFV strains would constitute a safer alternative to virulent ones. In this regard, the naturally attenuated OURT88/3 and NH/P68 strains have been shown to induce good levels of protection against virulent challenge (Leitao et al., 2001; Sanchez-Cordon et al., 2017), despite the presence of some undesirable side effects when inoculated into pigs. Nevertheless, even when naturally attenuated strains can confer protection without requiring further genetic manipulation, it is widely agreed that they cannot be used as vaccines without the addition of specific tags for tracking and without specific genomic modifications to ameliorate side effects.

A related study using ASFV-NH/P68 as a LAV against the virulent Armenia07 isolate currently circulating in Europe has been recently published (Gallardo et al., 2018). NH/P68 produced in porcine alveolar macrophages elicited 100% protection against homologous (Lisbon60) and heterologous (Armenia07) challenge, but with side effects in vaccinated animals. In an attempt to improve the side effects observed, viral genes were deleted such as A238L (Granja et al., 2006a, 2004; Granja et al., 2008, 2006b; Granja et al., 2009; Revilla et al., 1998), EP153R (Galindo et al., 2000; Hurtado et al., 2004), A224L (Nogal et al., 2001; Rodriguez et al., 2002), A276R (Correia et al., 2013), all involved either in the immune and inflammatory response, C-type lectin pathway, or apoptosis control, respectively. Single-deletion mutants NH/P68ΔA238L, NH/P68ΔEP153R and NH/P68ΔA224L produced in COS-7 cells were fully protective against homologous (Lisbon60) challenge. Surprisingly, this protection was almost completely lost when challenged with the heterologous Armenia07 strain. This loss of protection was more likely attributed to the cells than to the genetic deletions, as some degree of protection was recovered after immunization with purified NH/P68ΔA238L viruses (Gallardo et al., 2018). The A276R deletion mutant, which was produced in porcine alveolar macrophages, completely lost the protection observed in its wild type parent, suggesting a role for this specific gene in protection (Gallardo et al., 2018).

On the other hand, partial protection, based on the ability to stimulate IFNγ production, was observed in experiments in which vaccination with the attenuated OURT88/3 strain protected against heterologous strain (King et al., 2011). Strategies focused on reducing viremia and side-effects induced by OURT88/3 were reported unsuccessful, since deletion of genes such as DP71L and DP96R (involved in virulence and clinical signs), achieved in bone marrow macrophages, still reduced the ability of the attenuated recombinant viruses to protect against challenge (Abrams et al., 2013).

Recently new naturally attenuated isolates have been described. Deletion at the 5′-end of Estonian ASFV strains has been associated with an attenuated phenotype since 75% of minipigs and all of domestic pigs infected with Estonian strain were recovered after an acute course of disease (Zani et al., 2018). Moreover, pigs infected with a non-haemadsorbing ASFV genotype II isolated from wild boar in Latvia (Lv17/WB/Rie1), developed a nonspecific or subclinical form of the disease and were fully protected when pigs were exposed to other pigs previously infected with a related virulent HAD genotype II ASFV (Gallardo et al., 2019).

Table 1 shows the most relevant LAVs developed so far, indicating the vaccine type, cell type for production, type of protection and survival rate achieved in challenge experiments.

3. Culture cell lines for production of ASFV vaccines

An important issue for both the generation of experimental LAVs and their commercial production is the absence of a cell line that allows full replication of the recombinant prototypes while maintaining a good level of protection. Monocytes-derived macrophages and tissue macrophages, including alveolar macrophages are the main targets of ASFV infection in pigs, and they play a central role in the immune response through antigen presentation, cytokine secretion and phagocytosis (Gordon et al., 1995; van Furth et al., 1972). Although the use of primary monocytes-derived macrophages or alveolar macrophages for ASFV studies has several advantages for the study of host cell interactions, there are several disadvantages related to vaccine production. Batch-to-batch variations and laborious/costly cell extraction from animals would constrain future industrial virus production.

These problems have been partially overcome by using Vero or COS-7 cells as a model for productive ASFV infection (Enjuanes et al., 1976; Granja et al., 2006a; Hurtado et al., 2010); however, the use of these cells is not without its disadvantages. The adaptation of the virulent isolate Georgia07 to grow in Vero cells after several passages resulted in the loss of the replicative capacity of the ASFV in domestic pigs (Krug et al., 2015). In line with this, the ASFV strain Stavropol 01/08 9K lost their pathogenicity for pigs after 33 passages in the cell culture A4C2/9K (Balysheva et al., 2015). The naturally attenuated isolates OURT/88/3 -produced in bone marrow macrophages- and NH/P68 -produced in alveolar macrophages- confer 100% protection against heterologous UG65 (King et al., 2011) and Armenia07 (Gallardo et al., 2018) virulent isolates, respectively. Unfortunately, protection decreased to 33% when the NH/P68 isolate was produced in COS-7 cells, suggesting that the cellular platform selected to produce LAVs is not only important for production at industrial scales, but also to maintain vaccine immunogenicity (Gallardo et al., 2018). Recently COS-1 cells have been used to generate a deletion mutant lacking EP402R from virulent Ba71, without apparently affecting its protection (Monteagudo et al., 2017). Therefore, the use of cell systems that are more comparable to the monocyte/macrophage lineage is likely to be important for future generation of stable LAVs. In this regard, it has been described that IPAM 3D4/21 cells are more susceptible to infection by the ASFV-Lillie isolate than other clones such as 3D4/2 or 3D4/21. The authors suggest that the formulation of the culture medium is important for the optimal infection of the different clones (Weingartl et al., 2002). Moreover, it has been recently published that none of the porcine cell lines IPAM WT (CRL-2845) (Weingartl et al., 2002), IPAM-CD163 (Lee et al., 2010), WSL (wild boar lung cells) (Keil et al., 2014; Portugal et al., 2012) or CΔ2+ (Chitko-McKown et al., 2013) show a mature macrophage phenotype, and among them, only WSL are able to sustain productive ASFV infection (Sanchez et al., 2017), although in our hands, only NH/P68 was efficiently produced in these cells, whereas the growth of the virulent strains E70 and Armenia07 was much more limited (Fig. 1). Importantly, in vivo experiments showed that NH/P68 virus produced in WSL cells is able to infect and replicate in pigs (Sanchez et al., 2017) which is key to develop ASFV-LAV in the near future.

4. DIVA test

A fully efficient vaccine is dependent on the availability of a companion test that allows for differentiation between vaccinated and infected animals (DIVA). This capability is essential for eradication of infectious diseases in animal populations, as both specific protection and disease spread can be identified at the same time. As far as ASF is

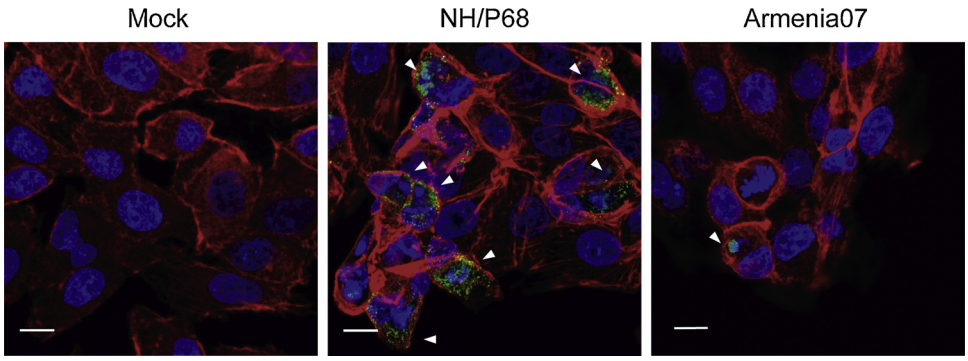

Fig. 1. ASFV infection in WSL cells. Cells were infected with NH/P68 and Armenia07 strains at an MOI of 1. At 16 hpi, cells were fixed and incubated with phalloidin-TRITC, anti-p72 antibody (17LD3) and DAPI to stain actin filaments, viral particles and cellular/viral DNA, respectively. Z-stack images were taken by Confocal Laser Scanning Microscopy (CLSM) and represented as a maximum of z-projection. Arrowheads show the infected cells; adapted from Sanchez et al., 2017.

concerned, it is broadly agreed that naturally attenuated strains cannot be used as vaccines without the ability to track them with specific tags.

To consistently differentiate between vaccinated animals and those that are naturally infected, and to ensure proper monitoring of the vaccination program and its impact on disease evolution, LAVs will need both positive and negative markers. Development of positive markers is straightforward, as elements present in the genome of engineered ASFV strains (GFP, β-Gal, β-Gus, others) facilitate discrimination by molecular or serological methods. On the other hand, negative markers must first be evaluated for induction of antibodies in the non-vaccinated, ASFV-infected animals. Faithful DIVA tests should therefore be considered in parallel to vaccine development and adapted to a context of vaccinating farmed (injected administration) and free-ranging populations of suids (oral administration).

5. Future perspectives

Further information is required in order to define the individual contributions of immune mechanisms and target the antigens involved in protection. Generation of a successful subunit vaccine will depend on the identification of viral targets which, together with an optimal delivery method, allow the correct presentation of viral antigens and activation of a protective immune response.

On the other hand, ASFV LAVs should improve the level of protection and safety while decreasing the side effects induced, while simultaneously initiating a companion DIVA test. As far as ASF is concerned it is broadly agreed that naturally attenuated strains cannot be used as vaccines without tracing them with specific tags. Additional studies of viral pathogenesis and immunogenicity are critically needed in order to develop an efficacious and safe ASF vaccine.

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