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Blueprint and Roadmap on the possible development of a vaccine for African Swine Fever prepared by the African Swine Fever EU reference laboratory[1] on Commission request

This document does not necessarily represent the views of the Commission Services

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Blueprint and Roadmap (BRMP) on the possible development of an African Swine Fever (ASF) vaccine

SCOPE

African Swine Fever (ASF) is a devastating viral disease of swine which is currently spreading in Africa and Europe. Many factors, including the complex epidemiology, with the presence of natural ASF reservoirs, the carrier animals, its potential for endemicity and the resistance of the virus in the environment represent significant challenges for ASF control. No vaccine is currently available. The availability of effective and safe ASF vaccines is an urgent requirement to re-inforce control and eradication strategies. Work leading to the rational development of a protective ASF vaccine and vaccination programmes in the different scenarios, therefore, should be a priority.

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TABLE of CONTENT

Annexes.

ACRONYMS AND ABBREVIATIONS

ADCC	Antibody-dependent cell-mediated cytotoxicity
ASF	African Swine Fever
ASFV	African Swine Fever virus
BSL	Biosafety level
CTL	Cytotoxic T cell
CVMP	Committee for Medicinal Products for Veterinary Use
DISC	Disabled infectious single cycle
DNA	Deoxyribonucleic acid
DIVA	Differentiating Infected from Vaccinated Animals
EMA/EMEA	European Medicine Agency's
EP	European Pharmacopoeia
EU	European Union
GMP	Good Manufacturing Practices
HA	Hemagglutinin
HAD	Hemagglutination/haemadsorption
IFN	Interferon
LAV	Live attenuated vaccine
Kbp	Kilo-base pair
MA	Marketing Authorization
MAH	Marketing Authorisation Holders
MFG	Multigene families
MS	Member states
MUMS	Minor Use Minor Species
NF	Nuclear factor
NFAT	Nuclear factor of activated T cells
NK	Natural killer
ORF	Open reading frame
VIMP	Veterinary Immunological Medicinal Products

DEFINITIONS

HOMOLOGOUS PROTECTION	Viral isolate induces protection to the parental virus isolate.
HETEROLOGOUS PROTECTION	Viral isolate also induces protection against other virus isolates.
VIRULENCE	The ability of the virus to produce disease.
RESIDUAL VIRULENCE	Capacity of a modified virus in causing or inducing disease as identified by the development of clinical and/or pathological signs.
SIDE EFFECT	Adverse clinical effects induced by vaccine administration (virus, adjuvant, site inoculation, etc).

1. INTRODUCTION

African Swine Fever (ASF) is an important haemorrhagic viral disease affecting swine whose notification is mandatory due to its high mortality, efficient transmission rates and the great sanitary and socioeconomic impact has on international trade in animal and swine products.

The **causative agent** of the disease is the ASF virus (ASFV), the only member of the *Asfaviridae* family [1]. The ASFV double-stranded DNA genome varies in length from about 170 to 190 kbp depending on the isolate and contains between 150 and 167 open reading frames (ORFs) [2-6]. Like other complex DNA viruses, ASFV has a number of strategies to evade the host's defence systems, including innate and intrinsic immune mechanisms such as type I IFN responses, apoptosis, inflammation and activation of host immunomodulatory gene expression [7, 8].

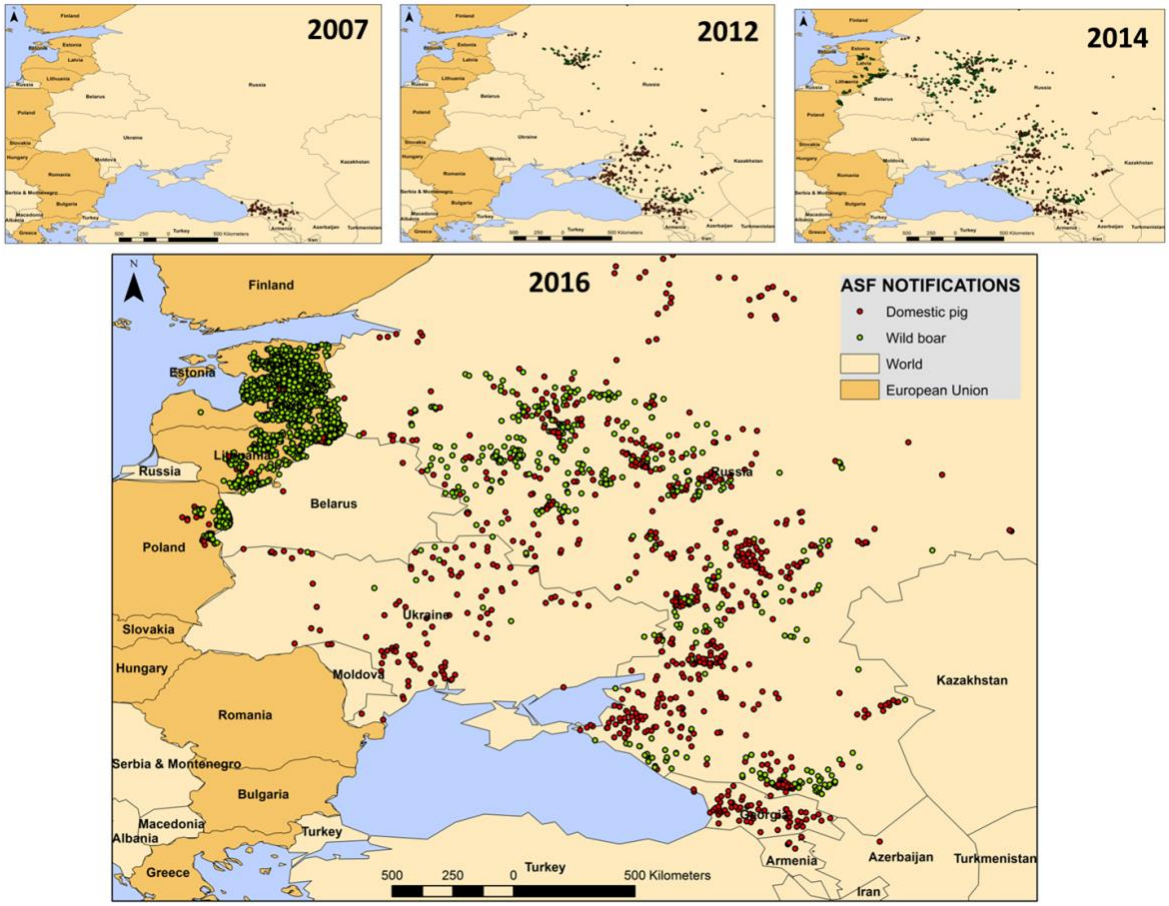
ASF affects only species of the suidae family (both wild and domestic) of all breeds and ages, giving rise to a **variety of clinical signs and lesions** that vary in terms of the virus virulence, host species affected and their immunological-status. European wild boar (*Sus scrofa*) and feral pigs are very susceptible to the disease and exhibit similar clinical signs and lethality to domestic pigs. By contrast, infected wild African Suidae develops subclinical and asymptomatic long-term persistent infections, acting as virus reservoirs. ASFV may cause a persistent infection in surviving animals.

When introduced into disease-free regions or domestic pig population, peracute and acute forms of the disease are predominant. These forms of ASF result in high mortality rates of up to 95-100% within 4–9 days post-infection. However, after several years of ASFV presence, subacute or chronic forms may be present, and mortality rates decline over time. In infections with low virulent ASFV isolates, the clinical manifestations of the disease are more variable and difficult to recognize in the field. The infection can persist for several months without obvious clinical signs in the infected animals [9-23]. Sub-clinically infected, chronically infected, or surviving pigs are likely to play an important role in the epidemiology of the disease, for example resulting in disease persistence in endemic areas or in sporadic outbreaks of ASF into previously ASFV free zones. The relevance of this is stressed by the observation that under experimental conditions, viremic pigs that survived from sub-acute infections were able to shed virus from their oropharynx for at least 70 days [24-26]. Other experimental studies have identified porcine tissues as a source of infectious virus at up to 180 days post infection during persistent infections with moderately virulent isolates [27-30].

Until recently, ASF was mainly endemic in sub-Saharan Africa, with foci in Sardinia, Italy (since 1978). Importantly, and starting from a single introduction of ASFV in Georgia from East Africa in 2007 [31, 32], the disease spread rapidly throughout the Caucasus and thousands of kilometres north-westward into the Russia Federation reaching Ukraine in 2012 and Belarus in 2013. Continued spread towards the West, resulted in notification of ASF in Lithuania and Poland in early 2014, the first notification of ASF cases in wild boar within the EU. This was probably due to the multiple introductions of virus by infected wild boar from neighbouring countries to the east along ecological corridors [33]. Since then, ASFV has spread to Estonia, Latvia, Lithuania and Poland, affecting both domestic and wild animals [34]. The seriousness of this threat is exemplified by recent outbreak of ASFV in Moldova in September 2016 [35], (**figure 1**). Similarly in

Sub-Saharan African, ASFV has re-emerged after over 15 years of silence in countries such as Ivory Coast and Cape Verde [36, 37] and the number of countries reporting ASF outbreaks has significantly grown in the last decade with currently more than 25 African countries infected with ASFV [20]. Thus, ASFV currently poses a threat to the EU trading area not only from within its borders but also from the risk of its introduction from the growing list of endemic African countries.

Figure 1: Spatio temporal evolution of the ASF notifications in continental Europe from 2007 to 2016.



Virus entrance into free regions usually occurs as a result of ingestion of meat or meat products from infected animals as uncooked pork waste (especially from ships and aircraft) being fed to pigs or by wild boar. Once the disease is established in an area, it mainly **spreads** via oral or nasal routes of dissemination and exposure by direct contact between sick and healthy animals (domestic pigs and wild suids), recovered carrier animals and soft ticks or, for example, through indirect transmission by lorries, at drinking and eating troughs, via surgical and personal equipment, rodents, or other farm animals, that act as mechanical ASFV carriers (only porcine species and *Ornithodoros spp.* can productively be infected).

In Eastern Europe the disease has become endemic in two regions of southwest and central Russia [38] and both domestic pigs (mainly free-range domestic pigs) and wild boar populations are widely affected recording 40% and 60% of ASF notifications respectively (2007-2016). Domestic pigs appear to play the primary role in the subsequent disease transmission [39] that

seems to have been influenced by factors such as contaminated swill, garbage and vehicles, and/or free-range farming [38]. These factors contributed to ASFV transmission within domestic backyard farms and wild boar populations. Wild boar appears to play a secondary role in disease transmission. Nevertheless, the fact that wild boar can transmit the virus even in the absence of domestic pig [40] implies that they can maintain infectivity and provide positive feedback that sustains virus circulation between wild boar and free-range pigs [41].

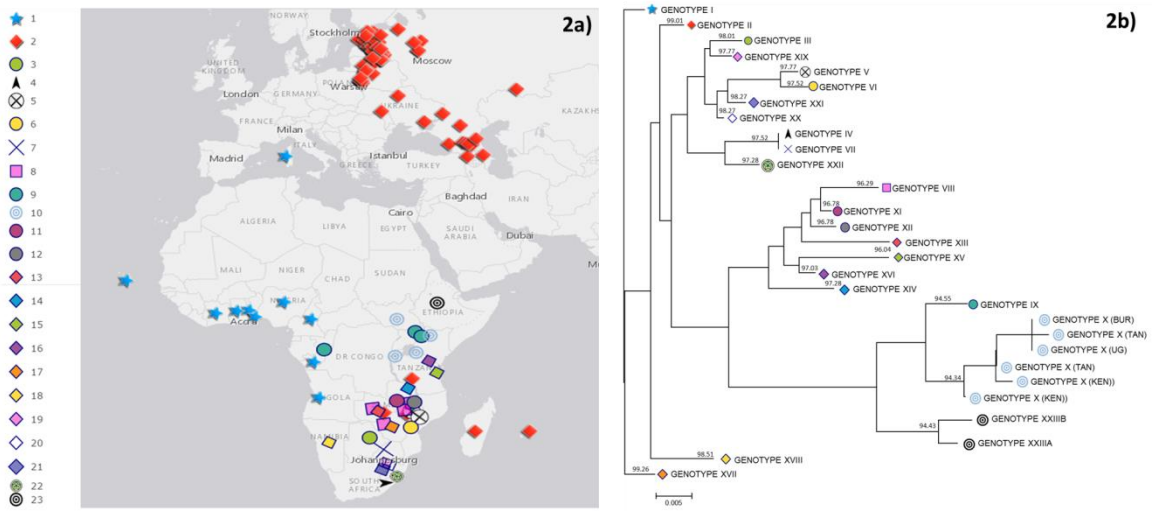
In the Baltic countries and Poland, wild boar appear to play a relevant role in the entrance and subsequent local spreading whereas long distance spreading has been associated to human factors [33, 42]. Practices such as illegal swill [43] and the use of freshly harvested grass from infected areas as feed [33], are thought to be the most likely entry route into low-biosecurity farms. Special attention should be paid to natural landscapes where wild boar populations play an important role in maintaining disease infectivity and in buffer monoculture areas where the opportunities to transmit the virus to domestic pigs through direct or indirect contacts are higher [44].

The clinical picture of ASF reported in Eastern Europe shows acute forms of the disease associated with virulent virus isolates [45-47]. The viraemia starts a few days after infection and antibody response can be usually detected from the second week post-infection onwards. More recently, the presence of seropositive wild boar has been described [48] suggesting that, despite the virulent nature of current ASFV circulating strains affecting Eastern Europe, some animals can survive for weeks and even may be recovering from the infection as it has been demonstrated in recent in vivo experiments with circulating strains [49].

The **epidemiology of ASF is very complex** and varies significantly between countries, regions and continents showing different epidemiological scenarios. This depends on the characteristics of virus circulating, the presence and role of wild and domestic hosts and reservoirs, and environmental, social and cultural factors. Furthermore, a specific host will not necessarily always play the same active role in the spread and maintenance of ASF in a given area.

As a further complication, in Eastern and Southern African countries, the ASFV has been maintained, for centuries, in a sylvatic cycle involving soft ticks (*Ornithodorus* genus) and asymptomatically infected wild African pigs, mainly warthogs (*Phacochoerus* spp). These can act as potential long-term carriers allowing the virus to spill over into domestic species when the two interact. Two additional cycles have been described in endemic areas, namely a domestic pig/tick cycle, without warthog involvement, and a domestic pig/pig cycle in which the virus persists in domestic pigs in the absence of any other vertebrate or invertebrate hosts [19, 20, 23, 50-58]. The epidemiological complexity of ASF has been clearly demonstrated in Eastern and Southern Africa, where genetic characterization of the ASFV based on the sequencing of the C-terminal end of the major protein p72, has identified 23 genotypes (**figure 2)** [59-61]. This rich genetic diversity is promoted through the sylvatic cycle and extended by the domestic cycle with open borders and unrestricted movement of swine in conflict areas. In contrast, Western and Central Africa, which lack the ancient sylvatic cycle, have traditionally been shown to have genotype I isolates circulating with low genetic variability although transfer and dissemination of ASFV genotypes from eastern to western Africa has recently been demonstrated [62].

Figure 2: (a) *Geographical distribution of the 23 ASFV p72 genotypes****; (b)*** *Phylogenetic relationship of the 23 ASFV genotypes based on the analysis on the C-terminal end of the p72 protein****.***



Outside Africa, the disease circulates among domestic pigs (*Sus scrofa domesticus*) and European wild boar (*Sus scrofa*), causing similar clinical signs and mortality in both populations, and it is highly probable that ASFV has spread through movements of infected wild boar and domestic pigs, and contaminated pig products. In the past, ASFV genotype I was the only one found in Europe, America, and the Caribbean, still circulating in Sardinia since its introduction in 1978 [63, 64]. In 2007, a new genotype, the genotype II, was introduced into Georgia from East Africa, spreading across the Trans Caucasus, Russian Federation and Eastern European countries as mentioned above [31, 33, 65]. Two central variable region (CVR) genetic variants within the p72 genotype II have been recently identified in the wild boar population that are co-circulating since 2015 in Estonia [66].

The protective immunity to ASF is still poorly characterised although it has been of major interest for researchers. The observations that pigs are resistant to challenge against some ASFV isolates indicate that animals can develop a protective immune response [67]. However, the complexity of ASFV, a virus encoding more than 160 different polypeptides, many of them specialized in evading different aspects of the immune system [68], together with the variability of the virus isolates so far identified has complicated this task.

Results have shown that animals that survive infection with less virulent isolates can be protected against challenge with related virulent viruses [18, 69, 70]. The extent of cross-protection against different genotypes has been little studied although there are reports of cross-protection between certain genotypes [71-74]. Currently the virus antigens important for cross-protection have not been fully characterised although antibodies against the virus CD2-like protein have been shown to be involved with cross-protection [72, 75, 76].

There is evidence that the protective immune response includes both cellular and serological immunity [67]. Some findings, such as the lack of fully neutralising antibodies, remain controversial [77]. Nevertheless, some protection related to antibody-mediated immunity is

observed. Thus, passive transfer of sera from ASFV-infected and recovered pigs, partially protected pigs against homologous AFSV challenge infection and the potential fatal consequences of infection by delaying the onset of the ASF clinical signs and reducing the levels of viremia [78-82].

A variety of *in vivo* and *in vitro* studies performed to date indicate a potential protective role of antibodies by using additional mechanisms including complement mediated cell lysis or antibody-dependent cell-mediated cytotoxicity (ADCC) [67, 83]. An interesting correlation has been established between the presence of haemadsorption (HAD) inhibitory antibodies in a serum with its capacity to inhibit the infection of ASFV *in vitro* and to partial protect against ASFV challenge *in vivo* [72, 84] .

Together with the protective role of the antibodies, evidences also exist about the key role that both the innate immunity, including the induction of NK responses [85] and the specific T-cell responses, can also play in protection [67]. Using pigs recovered from experimental infection with the naturally attenuated ASFV-isolate NHP68 as experimental model, it was possible to demonstrate the key protective role of the specific CD8-T cell subset capable to eliminate the virus intracellularly as a result of the cytotoxic activity [86]. Antibody depletion of the CD8+ cell population abrogated the protection induced by the natural attenuated strain OURT88/3 demonstrating an essential role for this cell subset in protection [87]. Thus it is clear that immune protection related to antibody mediated and cell mediated immunity can be obtained.

Since no vaccine is currently available, prevention, control and eradication measures are based on early detection, and on the implementation of strict sanitary measures, including surveillance, epidemiological investigation, tracing of pigs, and stamping out in infected holdings. These measures must be combined with strict quarantine and biosecurity measures in domestic pig holdings and animal movement control [88]. However, in some affected areas, governments and farmers are not being able to afford or implement such intense controls. This fact, combined with the re-emergence of ASF in the European Continent has increased the **interest in the development and release of a vaccine against ASF as additional tool to support current control efforts**.

Vaccination to contain viral infections in livestock has been tested over time as being the most cost efficient measure applicable. The availability of effective and safe ASF vaccines would allow improved ASF disease control and eradication programmes as well as reduced economic losses in the endemic regions affected, including the African continent. Controlling the disease where endemic, reduces further the threat and the possibility of ASF introduction into disease-free regions. Work leading to the rational development of a protective ASF vaccine, therefore, should be a priority.

2. OBJECTIVE

The aim of this ASF blueprint and roadmap is to give an overview of the research efforts needed and help to direct investments towards specific areas. The general approach is to attain an agreement among leading experts on a description of the baseline, identify the gaps and draft a

strategic plan to address the deficiencies and barriers identified, particularly in vaccine development.

The **STRATEGIC GOALS** to be identified might include:

- Identification of **potential strategies for vaccine development** based on existing data/knowledge in a view of an effective and safe vaccine.
- Identification of **gaps and needs for vaccine development and manufacturing** following the Good Manufacturing Practice Standards.
- Identification of a **network of dedicated laboratories** directed towards vaccine development

3. BACKGROUND ON VACCINE DEVELOPMENT. CURRENT KNOWLEDGE STATUS

During the past few years, several research groups have been working in vaccine development using many technologies ranging from inactivated viruses and recombinant proteins/peptides and viral vectors to live-attenuated vaccines. As yet all these experimental vaccines have been tested in domestic pigs and none of them have been taken forward for evaluation of their potential for commercial production. In principle, the same vaccine strain could be used for domestic pig and wild boar. Natural populations of wild boar have been successfully vaccinated against other infectious diseases through the use of LAVs administered orally by the distribution of palatable baits.

The main findings from the experimental vaccine approaches are described below.

3.1. APPROACHES BASED ON INACTIVATED VIRUS.

To date inactivated preparations of ASFV have not conferred protection even in the presence of adjuvants. An indication of antibody-mediated enhancement of the infection seemed to be observed [89-93]. The complexity of the virus particle which contains more than 50 proteins in several layers and the fact that there are two infectious forms, an intracellular mature and extracellular form, is most likely the reason for this failure.

Conversely, passive transfer of ASFV antibodies from pigs recovered from the infection with an attenuated ASFV strain protected pigs against a lethal ASFV challenge [80]. These results clearly demonstrate quantitative and/or qualitative differences in the induction of antibodies after immunization with either live attenuated viruses or inactivated ASFV preparations, an issue worthy to better explore in the future.

3.2. SUBUNIT VACCINE APPROACHES.

Several ASFV proteins have been reported to induce a kind of neutralizing antibodies in immunized pigs, including the p72, p54 and p30 proteins [94-96]. The protective potential of the induced antibodies resulted controversial. While co-immunization with p54 and p30 expressed in baculovirus conferred significant protection against the lethal challenge with E75 [94], combination of p54+p30+p72 baculovirus expressed proteins did not protect against lethal challenge with Malawi [96]. These contradictory results migh be partially explained by the virus strain used, albeit more recent work with DNA vaccines encoding p54 and p30 did not show any

protection against lethal infection with E75, neither the induction of specific neutralizing antibodies [97]. These results are difficult to compare due to the very different nature of protein versus DNA immunization protocols. On the other hand, the ASFV hemagglutinin (HA) protein encoded by the *EP402R* gene (also named CD2v), when functionally expressed in a baculovirus system, showed some degree of protection against a virulent challenge, in this occasion correlating with the induction of antibodies that inhibited HAD and temporarily inhibited infection [84]. Recently results have provided evidence for CD2v and/or C-type lectin proteins as being important for protection against homologous ASFV infection [72].

Strategies based on DNA vaccines have been also performed: ASFV genes p30 and p54 were cloned in-frame with a single chain variable fragment of a specific antibody against swine leukocyte antigen II. While specific T-cells against ASFV proteins were detected, neither neutralizing antibodies nor protection against a virulent challenge was reported [97]. Vectors displaying the extracellular domain of HA fused to viral p30 and p54 exponentially enhanced both humoral and cellular responses in pigs, without conferring protection. However, fusion of these three ASFV-determinants (HA, p54 and p30) to ubiquitin, induced strong CTL response and conferred partial protection in the absence of specific antibodies, correlated with the proliferation of HA (CD2v)-specific CD8+ T-cells [98]. Further immunization with a DNA expression library containing several other viral ORFs fused to ubiquitin also conferred partial protection against a virulent challenge [99]. Once again, this protection correlated with the presence of ASFV specific T-cells and the absence of detectable antibodies, highlighting the role of T-cell responses in protection and revealing the existence of multiple ASFV antigens with potential protective capacity. Despite the utility that these strategies might have in the future for dissecting both the immune mechanisms and the ASFV-antigens involved in protection, they are today far from providing the level of protection required to be useful in the field.

Very recent approaches, dealing with combinations of specific ASFV proteins and DNA, (heterologous prime-boost vaccine) as a chance in protection, have been developed. Although robust immune response in terms of neutralizing antibodies and IFN production were found, pigs were not protected against virulent challenges with Armenia strain [100].

Other prime and boost vaccination strategies have involved immunization of pigs with pools of recombinant adenoviruses expressing individual ASFV proteins and boost with either the same vectors or with recombinant modified vaccinia Ankara strain (MVA) expressing the same antigens. In these experiments, robust cellular and antibody responses have been obtained albeit pigs were not challenged with ASFV [101]. In recent studies, evidence of partial protection was provided [Netherton and Dixon unpublished data].

In conclusion, DNA and peptide-based and viral vectored vaccines have been shown to induce a specific ASFV immune response involving antibodies and/or T-cells, but to date resulted only in a partial protection against challenge [99]. Further work will be needed in order to identify both the antigens to be included in a potential subunit vaccine and the optimal immune mechanisms to be triggered after vaccination in order to confer solid protection against ASFV.

3.3. LIVE-ATTENUATED VACCINES (LAVs).

3.3.1. LAVs obtained from virulent and naturally occurring low virulent ASFV isolates

The use in the field of LAVs produced by the attenuation of naturally occurring virulent strains has been limited to the extensive experience in Portugal and Spain during the early sixties. At that time, a large number of animals were vaccinated in field conditions with LAVs, with presence of wild boar and ticks. Field conditions included the animals were exposed to multiple infection and re-infections with heterologous viruses. From the field experiment in Spain some animals showed chronic clinical signs. These vaccines were not used anymore mainly due to safety problems derived from their inherent infectious nature [103].

Other strategy has involved the immunization of pigs with the naturally attenuated ASFV strains OURT88/3 or NH/P68. Immunized pigs were protected against challenge with homologous virulent strains [18, 70, 85, 87], albeit partial cross-protection has been shown against heterologous viruses [72-75]. The protection levels varied from 66% to 100% dependent on the pigs and the challenge virus, as well as the delivery route and administration dose [18, 71, 73, 74, 102]. As described for the subunit vaccines, both specific antibodies [80] and specific CD8+ T-cells [87], seem to play a crucial role in the protection afforded by LAVs. Cross-protection induced by the OURT88/3 isolate, against challenge with virulent isolates from different genotypes, was correlated with the ability of those isolates to specifically stimulate IFNγ producing lymphocytes from the immunised pigs [71]. **Despite the correlation between the induction of specific T-cell responses and protection [67, 71, 99], this is far from being a confirmed prediction and other mechanisms are been studied to identify key players in protection**. However, the attempts using either naturally attenuated ASFV strains as vaccines have demonstrated so far several side-effects, at least at certain doses, since a substantial proportion of the vaccinated pigs developed unacceptable post-vaccination reactions including pneumonia, locomotor disturbances, necrotic foci, abortion and death. In the best scenario, pigs do not show significant clinical signs with exception of transient fever and low viremia that coincides with low albeit detectable nasal shedding in some vaccinated pigs [73, 74, 85].

The ASFV genome encodes a large number of genes including non-homologous genes which are not essential for virus replication but identified as playing a role in host immune evasion, such as the NFκβ and NFAT inhibitor *A238L*, the apoptosis inhibitors *A179L* and A224L, the protein phosphatase 1 activator *DP71L* and those genes involved in inhibiting the induction of IFNαβ including the multigene family (MGF) 360 and 505 genes or the non-homologous *I329L*, *K205R, DP148R* and *A276R* genes [8, 104-121]. The D96R (also referred to as UK) gene is also a potential immune evasion gene, although its mechanism of action is unclear [122]. Such genes are good candidates for the development of an attenuated gene deletion mutant virus for vaccine development, either based on virulent or naturally attenuated ASF viruses. This strategy could enhance safety and efficacy profiles over those observed for traditionally generated LAVs.

3.3.2. Recombinant LAVs obtained from virulent viruses

Recombinant ASFVs containing specific single deletions of genes involved in the evasion of the immune response, such as the thymidine kinase (TK), 9GL (*B119L*) gene, NL (*DP71L*) gene, and multiple members of multigene families 360 and 505 (MGF 360/505), induces complete

attenuation of virulent ASFV isolates and protective immune responses against homologous parental virus challenge, albeit some residual virulence remained [123-127]. However, the effects of gene deletion on ASFV attenuation and protection may be strain dependent and, in some cases, the deleted viruses exhibit virulence phenotype indistinguishable from the parental virus, or are not able to protect against homologous challenge [123, 127].

Recent studies have demonstrated that multiple-gene mutants in ASFVs can variable affect viral immunogenicity. The multiple deletion of 6 members of MGF360 and 505 combined with 9GL gene produced an attenuated Georgia ASFV strain with improved safety, but unable to confer protection to animals when challenged with the virulent parental virus [128]. In contrast, the virulent Georgia isolate modified by deletion of the 9GL and UK virulence factors showed improved safety and protection compared to the deletion of 9GL alone [129]. These results clearly demonstrated that the serial deletion of a second virulence factor might render much safer recombinant live attenuated ASFV-vaccines, thus opening hopes for the near future.

Recent studies using a live attenuated vaccine (BA71ΔCD2), obtained by deleting the CD2v (HA) gene from the virulent BA71 ASFV isolate have demonstrated the feasibility of obtaining a very solid protection against homologous and heterologous virus [130]. Pigs immunized with the BA71ΔCD2 vaccine, originated from a genotype I were protected against the challenge with the homologous virulent ASFV Ba71 strain, against the heterologous virulent genotype I E75, and against Georgia07 ASFV strain, an heterologous strain belonging to genotype II ASFV [131]. Combining some of the different mutations so far described might yield a vaccine prototype with potential field applications. A balance is required such that some virus replication occurs in the host to induce an efficient immune response while avoiding pathological effects associated with too much virus replication.

3.3.3. Recombinant LAVs obtained from attenuated viruses.

The strategies developed under EU projects, especially ASFRISK and ASFORCE, to improve safety of attenuated strains (OURT88/3 or NH/P68) by deletion of several genes have provided variable results. The deletion of genes such as *DP71L* and *DP96R* (involved in virulence and clinical signs), *DP148R* or the *A276R* (an inhibitor of IFNβ), reduced the ability of the attenuated viruses to protect against challenge [132, Reis et al., in preparation, Revilla et al., in preparation]. In contrast, some of these mutants showed a good degree of protection (60-100%) against challenge with the virulent strain Armenia 2007. But, in agreement with previous studies, the vaccine candidates induced (low) viremia and side-effects such as arthritis and necrotic foci in most of the vaccinated pigs [73, 74] which would prevent their commercial use and which confirm the fact that efficient and safe vaccine against ASFV are not available. The main antiviral response, type I IFN is critical for the virus attenuation and induction of protection. However it is critical to achieve a balance such that efficient viral replication occurs to induce an effective immune response but avoid clinical signs [74, 111, 127]. Protection induced by immunizations of pigs with low virulence ASFV isolates, especially protection mechanisms induced by the naturally low-virulent NH/P68 and OURT88/3 isolates, have been also described in vivo by using inbred and outbred pigs [18, 71, 73, 74, 102, 132].

3.4. VACCINE DEVELOPMENT: SUMMARY OF CURRENT STATE OF ART.

From the currently available data on vaccine development, **the LAVs seem the most promising candidates in the short-term**. The solid protection so far demonstrated by a number of LAVs (up to 100%), the increase safety achieved by the multiple gene deletion approach together with their potential to confer solid cross-protection, allow being very optimistic about their field implementation in the near (medium term) future. **Table 1 summarizes the most promising LAV candidates for vaccine development** based on existing data/knowledge. In spite of their experimental success, further research is needed to confirm their safety, DIVA-capabilities and efficacy in long term controlled experiments; essential requisite to offer optimal LAVs.

Parallel efforts performed with ASFV subunit vaccines recommend being more cautious regarding their prompt commercial implementation. Conversely to their intrinsic safer nature and DIVA-potential, the protection levels afforded against ASFV experimental challenge resulted very poor when compared with LAVs thus allowing their recommendation as the long-term choice for ASFV vaccine development. A continuous research effort focused both on antigen discovery and on better understanding the mechanisms involved in ASFV protection will guarantee succeeding in this longer-term objective.

Table 1: Promising progress towards the development of a ASFV LAV.

Parental ASFV	Vaccine type	ASFV vaccine	Cell production system	PROTECTION	References
NH/P68 (att)	Naturally attenuated	NHV/P68	PBM	HETEROLOGOUS STRAIN (L60, Arm07)	Leitao *et* al., 2001; Gallardo *et* al., 2012
OURT88/3 (att)	Naturally attenuated	OURT88/3	BM	HOMOLOGOUS/ HETEROLOGOUS STRAIN (OURT88/1, Ug65)	Boinas *et* al., 2004, King *et* al., 2011, Sanchez-Cordon et al 2016)
Georgia07 (vir)	Genetically modified	Georgia07Δ9GL &UK	PAM	HOMOLOGOUS STRAIN	O´Donnel et al 2016
Ba71 (vir)	Genetically modified	Ba71ΔCD2	COS	HOMOLOGOUS AND HETEROLOGOUS STRAIN (E75, Georgia07)	Patent. Fernando Rodriguez and Maria Luisa Salas WO 2015091322 A1
Benin (vir)	Genetically modified	BeninΔMGF	BM	HOMOLOGOUS STRAIN	Reis et al., 2016
Benin (vir)	Genetically modified	BeninΔDP148R	BM	HOMOLOGOUS STRAIN	Reis et al 2016
NH/P68 (att)	Genetically modified	NHV/P68 TETβGUS,	COS + 4 passages in PAM	HOMOLOGOUS AND HETEROLOGOUS STRAIN (Arm07)	Revilla Y. unpublished data
NH/P68 (att)	Genetically modified	NH/P68ΔA238L	COS + 4 passages in PAM	HOMOLOGOUS AND HETEROLOGOUS STRAIN (Arm07)	Gallardo et al 2015

Att = attenuated, Vir = virulent
Cell systems: Porcine blood monocyte/ macrophages (PBM), pig bone marrow cells (BM), monkey kidney tissue derived cells (COS) or porcine alveolar macrophages (PAM)

4. IDENTIFICATION OF GAPS AND NEEDS FOR VACCINE DEVELOPMENT AND MANUFACTURING.

LAV have demonstrated to be able confer solid protection (up to 100% surviving) against ASFV experimental challenge. Despite the fact that some LAVs have been identified as promising

vaccines (table 1), the experts have identified **some gaps that might constrain the development of an effective and safe DIVA-LAV vaccine against ASF in the mid-term.**

On the other hand, developing an efficient vaccine based on individual determinants from the ASFV (subunit vaccine) would require also parallel research efforts. Conversely to LAVs, subunit vaccines present the advantage of their innocuous nature but however would require a long-term effort in terms of research on antigen discovery and immune protective mechanism identification.

4.1. GAPS AND NEEDS IDENTIFIED USING LAVs.

Despite recent successes in the use of LAVs, there are still some important gaps/uncertain that should be considered.

→ **Safety of ASF LAVs:**

- *LAVs based on naturally attenuated strains of ASFV:* even though these vaccines induce very solid protection against the closely related virus challenges, the use of naturally attenuated ASFV strains as vaccines have some **side effects**. This was clearly demonstrated in the past with the only vaccine tested (1962-64) in Portugal and Spain so far, which showed chronic clinical signs and lesions, and intermittent low viremia. Depending on the dose, a substantial proportion of the vaccinated pigs could develop **post-vaccination reactions** as it has been shown by experimental conditions, mainly due to secondary infections that can lead to the development of the chronic ASF form. From the experimental studies the difference between a safe and virulent dose appears to be small and ASFV strain dependent, **thus a safe immunizing dose may be a concern and point out this as an important point to be established for the vaccine candidates.**

- *LAVs based on virulent ASF viruses containing engineered deletions:* Since ASFV is a stable DNA virus and the attenuation usually results from deletion of several genes, the reversion to virulence is very unlikely. However, the vaccinated animals may undergo an un-detected, subclinical ASFV infection and the recombination among DNAs from the LAV and the natural strain may occur. Multiple deletions in one single LAV candidate should reduce this possibility to the minimum, albeit long-term controlled experiments of vaccination and challenge should be performed in large groups of animals to ensure their safety.

An optimal equilibrium between attenuation and infection capability should be reached in order to obtain a **SAFE and EFFICACIOUS** vaccine. Too much attenuation could lead to non-pathogenic viruses that are non-efficient for vaccine purposes but too low attenuation would result in avoiding its use in the field for safety reasons.

→ **Selection of targeted virulence genes to be deleted→** Current vaccine strategies followed to develop LAVs for ASF rely on the deletion of one or more virulence genes. However, it is not always clear which virulence genes to target, since the effects of gene deletion on ASFV attenuation and protection seem to be strain dependent. For example, from virulent strains the deletion of the NL (*DP71L*) gene completely attenuates the European E70 strain in animals but has no effect in two African ASFV strains [126]. Additionally, ASFV strains Malawi and

Georgia are both attenuated by TK gene-deletion but only the TK deleted Malawi virus was capable of inducing a protective immune response in inoculated animals [123]. In attenuated ASF viruses, the deletion of virus genes previously associated with virus virulence can produced a deleterious effect in its ability to confer protection against challenge. **Thus testing gene deleted viruses from different genotypes may be needed.**

Further work is required to optimise the combinations of genes that can be deleted to produce a LAV that can meet safety standards required for registration and induce a good level of protection. For this, a better understanding of the mechanisms of evasion genes, such as *I329L, K205R, A276R*, MGF360 and 505 genes and others, is needed to identify candidate virus genes for construction of a gene deletion LAVs, that may, 1) define novel mechanisms for virus manipulation of IFN induction, 2) suggest novel strategies to control de the virus, 3) improve our knowledge of the IFN-induction pathway, and 4) improve our understanding of the mechanisms of ASFV pathogenesis.
(The safety issues of all kind of veterinary vaccines to be authorised in Europe will be further evaluated under the conditions foreseen on current European legislation, guidelines and Ph. Eur. monographs on Veterinary Medicinal Products.)

→ **Cell line development for vaccine commercialization →** An important issue for the commercial production of ASF vaccine, mainly for the LAVs, is to have a suitable cell line available for *in vitro* virus replication of vaccine candidates. In ASFV infected pigs, monocytes and alveolar macrophages are the main targets of ASFV, a fact that further hampers vaccine development since macrophages play a central role in the immune response, regulating phagocytosis, antigen presentation and cytokine secretion [133, 134]. Although primary culture systems such as monocytes or alveolar macrophages are frequently used in many laboratories for biological and immunological studies of ASFV, experimentation with primary cells has several drawbacks, such as the difficulty of reproducible results due to batch to batch variations and the laborious and costly methods to obtain cells from animal donors that also would in the future constrain the virus production at industrial scale. These issues were partially overcome several years ago by the adaptation of some ASFV isolates to grow in different monkey-derived stable cell lines, such as Vero or MS cells, which have been routinely used for biological studies, production and purification of the adapted virus [135-137]. However, the adaptation of ASF viruses has always resulted in genomic changes to the point of inactivating the virus [138]. **The use of a cell system is required for the analysis of the immune response and generation of future vaccines**. In relation to this, five different porcine cell lines of monocyte origin have been developed so far: ZMAC, IPAM WT, IPAM-CD163, WSL and CΔ2+ [139-144], although the COS-cell line system have shown to be highly efficient to sustain the *"in vitro"* replication of ASF viruses without the adaptation procedure [137, 143, 145]. The genetically modified LAV, the BA71ΔCD2 produced in COS cell, resulted in an effective vaccine able to confer homologous and heterologous protection. The COS cell line was successfully used for the generation of the LAV without significant genome changes [131]. However, some *"in vivo"* experimental studies based on the attenuated NH/P68 strain have shown that the LAV produce in COS cells were not able to maintain the capacity to confer protection [146]. Therefore, **further evaluation studies are required for the potential**

use of COS cells in vaccine production. Furthermore, no studies have been published so far determining the behaviour of ASFV strains generated from cell lines such as WSL and COS-1.

4.2 GAPS AND NEEDS IDENTIFIED IN SUBUNIT VACCINES.

A subunit vaccine against ASFV is possible but much further work is needed due to several factors:

→ Several ASFV proteins have been associated with protection, but **no specific viral protein(s) has been shown sufficient to confer full robust protective immunity in pigs**. This failure likely indicates that additional viral antigens should be identified and their antigenic diversity known to achieve a solid protection. Previous studies [99] clearly demonstrated that inactivated and purified virions, did not protect against the infection.

→ **Optimized delivery/vector systems are required to induce good levels of immune responses.** Several immunisation strategies and delivery/vector systems have been used to immunise pigs with a variety of different ASFV antigens. Either results from these experiments are difficult to compare and interpret since the antigens or the delivery/vector system may not be optimal to induce a protective immune response. **Further work is required to define protective antigens and to optimise delivery systems including viral particles, attenuated viral vectors, etc, and strategies for vaccination to induce protective responses.** Eventually systems that can be applied in the field commercially will also need to be evaluated. Viral vectors that can deliver several antigens/genes should be investigated.

4.3 GAPS AND NEEDS IDENTIFIED IN THE IMMUNE CORRELATES OF PROTECTION

The mechanisms involved in immune protection against ASFV are still poorly characterised. There are two models for protective immunity to ASFV: the resistant, natural wildlife hosts, the bush pig or warthog, and the recovered, susceptible domestic pig. It is probable that effective elimination of ASFV requires both serological and cellular immunity. Host immune and/or concomitant co-pathogen infection status appear to impact ASFV virulence. Examples of ASFV attenuated in conventional pigs, but retaining virulence in specific-pathogen-free pigs, have been described [71, 99]. Therefore, **deeper research in the immunity developed by the natural hosts**, including bushpig and warthog, by ASFV infection **should be addressed**. However, this approach is very difficult since few reagents are available for wild African pigs and some experts consider the efforts should be focused on pigs as the target species of interest and put effort into the development of reagents for pigs. Unfortunately, many gaps exist in this knowledge and in most occasions, vaccines are launched to the market without a real knowledge of the real mechanisms involved in immune protection. Besides this, identification of the correlates of immune protection would allow applying the three Rs (3Rs) guiding principles of animal science and reducing unnecessary painful challenges with ASFV (see 4.4)

Additional research on virus biology and virus host interactions at cellular level is required to underpin vaccine research. Gaps in knowledge include transcriptome analysis to identify those virus genes that are transcribed at different stages of the replication cycle, better knowledge of

the virus structural proteins including those on the surface. In addition, the characterization of cell receptor(s) on pig macrophages can be an interesting issue to be addressed, in order to focus molecules involved and thus neutralizing virus entry.

4.4 GAPS AND NEED IDENTIFIED IN *"IN-VIVO"* EXPERIMENTAL VACCINE TRIALS.

The development of new vaccines is dependent upon robust preclinical animal models in order to select those vaccine candidates, which should progress to clinical development. The **programme designs to evaluate vaccine candidate in experimental conditions** should be prepared according to the requirements of the European veterinary immunological legislation guidelines and Ph. Eur texts and monographs, taking into account the critical requirements for vaccine registration in the EU such as:

- Target species (swine, wild boar), categories (young/ older animals, pregnant…)
- Routes of the vaccine administration*
- Well-fare for the in vivo experiments
- Vaccine dose (depending on vaccination schedule proposed)
    - One, two or more doses (onset of immunity)
    - Revaccination (duration of immunity) and overdose (safety tests) (if needed)
- Standardize clinical data collection and analysis (techniques, target samples, etc).
- Routes of infection, Challenge doses, challenge virus strain/s to be used
- Vaccination period
- Safety studies (including absence of reversion to virulence)

**Natural populations of wild boar have been vaccinated against several infectious diseases through the use of oral vaccines administered by the distribution of palatable baits. For attempting vaccination of wild boar, ideally an oral vaccine should be developed. Domestic pig can be vaccinated by using parenteral route*

The gaps identified through the consultation mainly revolve around two aspects: i) to harmonise and standardise clinical data collection and analysis and, ii) to reduce the size, lengths and costs of clinical trials. A weak point found at the time to ASF vaccine development comes from the **absence of a real correlation between *in vitro* detected immune responses and *in vivo* protection**. Thus, the only unarguable proof for an antigen to become a real vaccine candidate comes from its potential to clinically protect target species (pigs and wild boar). This is considered one of the major challenges to progress in the ASF vaccine development since **there is no animal model that could mimic swine**. Vaccines are tested and selected using vaccination-challenge experiments in pigs which require strict biosafety level 3 (BSL3) animal facilities. Such procedures are not only extremely expensive, but are also environmentally and ethically problematic, considering the severe animal suffering associated with disease development, and the requirement that all animals be slaughtered at the end of the experimentation. The development of techniques that could correlate *"in vitro"* with protection *"in vivo"* might be also considered in order to reduce in a rational manner the *"in vivo"* experimental infections. Preliminary in vitro tests are absolutely necessary (e.g. continued ability to replicate in pig macrophages for LAV). This will reduce the suffering of animals, reduce the biosafety risks associated with challenge

experiments, and reduce the costs of vaccine testing both for research and routine purposes in the field of ASF.

4.5 GAPS AND NEEDS IDENTIFIED IN "FIELD" EXPERIMENTAL VACCINE TRIALS.

According the European legislation including in the EP general tests and guidelines for immunological veterinary medicines, following the*" in vivo"* laboratory tests, the efficacy and safety of the future vaccines should be evaluated by field trials. Following the guidelines of the Good clinical practise (GCP) specified in the VICH GL-9, VICH GL-44 and in the EMEA/CVMP/852/99 document field trials should be well planned, controlled and monitored as well as carried out in representative animal houses/husbandry practices and geographical regions using a using a vaccine to be authorised.

It will be required to identify confined livestock facilities, experimental farms under strict biosafety measures for vaccine candidate testing with a higher number of animals, and for a long period of time in confined conditions.

Field trials are a key point to evaluate the risk-benefit of vaccine candidates. Their design will depend on the characteristics of the vaccine (DIVA vaccine, formulation…) and the infection under different virus–host scenarios (different virulence, different densities of hosts, and different durations of viremia and probabilities of developing chronic infections).

4.6 DEVELOPMENT OF DIVA TEST.

The application of a vaccine is dependent on the availability of an accompanying discriminatory test (DIVA test) allowing differentiation between vaccinated and infected animals. **Reliable DIVA tests should be therefore considered in parallel to vaccine development**. It might be relatively easy to be designed using subunit vaccines and LAVs based on the deletions of targeted virulence factors which provide a **suitable DIVA test based on a negative marker**. In addition, the absence in some LAVs (by specific deletion or due to natural deletion) of the CD2v gene responsible of the HAD phenomenon would facilitate the use of a simple method such as the HAD assay to differentiate the non-vaccinated animals, although some non-HAD field strains have been described in endemic areas. This fact would hamper the differentiation using the virus isolation test. Finally, a positive selection could be used since markers present in the genome of manipulated strains (BGal, BGus, others), easy the discrimination between vaccine and natural strains with different strategies ranging from molecular to serological methods.

In order to ensure a proper monitoring of the vaccination campaign and of its impact on disease evolution in a vaccinated wild boar population, the vaccine will need both a positive and a negative marker to make possible a very reliable differentiation between vaccinated animals and those infected by natural infection (DIVA).

4.7 GAPS AND NEED IDENTIFIED IN VACCINE WILD BOAR DEVELOPMENT.

The unprecedented geographical spread of ASF and its tendency to persist in many wild boar populations of Eastern Europe justifies **the need to invest in the development of a specific vaccine for the wild boar**.

In principle, the same vaccine strain can be used for domestic pig and wild boar. However, to be successfully used in the wild boar further specific investigations related to the oral administration route, efficacy, safety and its use in the environment are required. For instance, the candidate wild boar vaccine needs to be immunogenic after oral administration and this will probably require a higher virus titre in the vaccine. In addition, the oral vaccine needs to be **stable in the external environment** to avoid losing potency when it is exposed to low and hot temperatures, sunshine, etc. For a feasible oral immunization scheme, a **suitable delivery vehicle in the form of bait is needed**.

Candidate wild boar vaccines fulfilling the above requirements should be initially tested in controlled laboratory conditions, followed by experiments in "controlled field conditions" - e.g. large enclosures emulating free ranging populations - before vaccination of natural populations.

All these specific adaptations will need a considerable amount of time and resources before they are achieved.

5 CONCLUSION

The recent alarming spread of ASF in Eastern Europe demands immediate countermeasures, with development of a vaccine as a high priority, as an additional good tool accompanying sanitary control measures.

Considerable progress has been made in the last decade leading to the development of ASF attenuated strains that have the potential to be used as candidate vaccines in a short/medium term. However there are a number of important issues to clarify before a LAV is available for commercial development. In particular the following gaps should be addressed in parallel:

- Additional genome modifications of the vaccine candidates may be required.
- Further *in vivo* testing to confirm acceptable levels of safety and efficacy against relevant field strains is a mandatory step that will require adequate financing.
- More studies are needed to develop in vitro correlates of protection in order to select the most promising vaccine candidates and reduce the number of animal challenge experiments.
- A suitable cell line for vaccine production needs to be identified.

The development in the longer term of subunit vaccines that in principle are safer than LAVs will require the identification of dominant antigens and to optimise delivery systems that can induce good levels of protection. Further work will be needed in order to identify both the antigens to be

included in a potential subunit vaccine and the optimal immune mechanisms to be triggered after vaccination in order to confer solid protection against ASFV.

For use of any vaccine in the field a DIVA test is needed, but this is not expected to be a major constraint.

In principle the same vaccine strain can be used for domestic pig and wild boar. However, the development of a **specific vaccine to be successfully used in the wild boar** will probably pose additional challenges, related to vaccine administration route, efficacy, safety and vaccination strategy.

6 IDENTIFICATION OF A NETWORK OF DEDICATED LABORATORIES DIRECTED TOWARDS VACCINE DEVELOPMENT.

Scientists have been working for decades to develop an effective vaccine for ASF. While research studies have successfully demonstrated that such vaccine is possible in a short term, many challenges continue to impede progress on the road to an effective ASF vaccine. The lack of a process to foster collaboration among researchers has slowed – and sometimes blocked – the development of an effective ASF vaccine. Gaps remain between scientists, the research groups who develop tests to characterise new vaccines, and the groups who manufacture vaccines. Moreover, there is very limited collaboration between groups working on vaccines against ASF, with this lack of sharing resulting in the loss of information, knowledge, and expertise. To connect these different groups and accelerate the development and testing of promising new ASF vaccines, it is essential to connect these groups and further strengthen the vaccine research infrastructure and ensure the highest quality research possible in this field. The aim is to address building capacity and capability by identifying networks of dedicated laboratories that cover a range of themes, and build on and develop existing strengths and collaborations. Encourage interdisciplinary working between research institutions worldwide is critical to maintain and further develop expertise and competencies as well as to ensure the future of ASF vaccine development and manufacture. **Table 2** shows research institutions with BSL-3 laboratories that are currently dedicated to ASF vaccine development and/or experimental vaccine trials for ASF vaccine.

Table 2: Research institutions with BSL-3 laboratories dedicated to ASF vaccine development and experimental vaccine trials .

Institution		Address	Country	Contact person	Email
ANSES	Ploufragan-Plouzané Laboratory/swine immunology and virology unit ZOOPOLE	LES CROIX - BP 53 Ploufragan 22440	France	Marie Frederique Le Poitier	marie-frederique.lepotier@anses.fr
CBMSO- CSIC	Consejo Superior de Investigaciones Científicas - Centro de Biología Molecular Severo Ochoa.	C/Nicolas Cabrera, 1 Cantoblanco, Madrid 28049	Spain	Yolanda Revilla	yrevilla@cbm.uam.es
CIRAD	Centre de coopération internationale en recherche agronomique pour le développement	Avenue Agropolis, 34398 Montpellier Cedex 5	France	Ferran Jori	ferran.jori@cirad.fr
CReSA-IRTA	Centre de Recerca en Sanitat Animal	Universidad Autonoma de Barcelona, 08193 Cerdanyola del Vallès, Barcelona,	Spain	Fernando Rodríguez	fernando.rodriguez@irta.cat
FLI	Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health,	Institute of Molecular Biology Sudufer 10 Greifswald-Insel Riems 17493	Germany	Günther Keil/ Sandra Blome	guenther.keil@fli.bund.de./ Sandra.Blome@fli.bund.de
FMV-ULisboa	Faculdade de Medicina Veterinaria.	Avenida da Universidade Técnica, 1300-477 Lisboa	Portugal	Carlos Martins	cmartins@fmv.ulisboa.pt
IGC	Instituto Gulbenkian de Ciência	Rua da Quinta Grande, 6, Apartado 14 Oeiras 2780-156	Portugal	Michael Parkhouse	parkhous@igc.gulbenkian.pt
INIA-CISA	Centro de Investigación en Sanidad Animal	Crta de Algete el Casar s/n CP 28130, Madrid	Spain	Marisa Arias/Carmina Gallardo	arias@inia.es, gallardo@inia.es
IZS-Peruggia	Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche	Via G. Salvemini, 1 Perugia 06126	Italy	Gian Mario de Mia	gm.demia@izsum.it
IZS-Sardegna	Istituto Zooprofilattico Sperimentale della Sardegna	Via Duca degli Abruzzi 8, Sassari	Italy	Alberto Laddomada	albertolad@LIVE.COM
Vet.Kansas state	College of Veterinary Medicine Kansas State University	30 West Pershing Road, Kansas City, MO 64108	USA	Juergen A. Richt	jricht@ksu.edu
PIR	The Pirbright Institute	Ash Road Pirbright Woking GU24 0NF	United Kingdom	Linda Dixon/Chris Netherton	Linda.dixon@pirbright.ac.uk
USDA	United States Department of Agriculture Agricultural Research Service (USDA), Foreign Animal Disease Research	40550 ROUTE 25 USDA ORIENT POINT WAREHOUSE NY 11957	USA	Manuel Borca	manuel.borca@ars.usda.gov
VISAVET-UCM	Centro de Vigilancia Sanitaria Veterinaria (VISAVET). Universidad Complutense de	Av. Puerta del Hierro Madrid 28040	Spain	Jose Manuel Sánchez-Vizcaíno	jmvizcaino@ucm.es

	Madrid.				
VNIIVViM*	The National Research Institute for Veterinary Virology and Microbiology	PETUSHKI DISTRICT Pokrov 601120	Russian Federation	Dennis Kolvasov	kolbasovdenis@gmail.com

*BSL-2 plus restricted area

7 KEY VETERINARY VACCINOLOGY INTERNATIONAL INDUSTRIES/COMPANIES with a potential interest on ASF vaccine development and release.

Table 3 shows the International Companies that have manifested a potential interest on ASF vaccine development, and initial contacts have been established with their local focal points through a meeting to know more specifications about , i.e the need for BSL3, will be required. Companies have been contacted through the Spanish Animal Health Industry Association, VETERINDUSTRIA (Manager, Santiago de Andrés [mailto: sdandres@veterindustria.com]), as IFAH EUROPE MEMBER.

Table 3: Key veterinary vaccinology industries/companies with a potential interest on ASF vaccine development and release

Company	Local Contact focal Point	Corporative Contact	Email
Zoetis	Félix Hernaez	Alicia Urniza, Director EU R&D Biologicals at ZOETIS. Mercuriusstraat 20, Zaventem, 1930 Belgium. Office: +32 27468224| Mobile: +32 497052281 ; +34 649815040	felix.hernaez@zoetis.com alicia.urniza@zoetis.com
Ceva	Alex Martino		alex.martino@ceva.com
Laboratorios Calier	Francisco Díaz-Varela		fdv@calier.es
CZ Veterinaria	Andrés Fernández		a.fernandez@czveterinaria.com
MSD Animal Health	Juan Carlos Castillejo		juan.carlos.castillejo@merck.com
Laboratorios Hipra	Joan Tarradas		joan.tarradas@hipra.com
Boehringer Ingelheim España, S.A	José Maria Salleras		jose-maria.salleras@boehringer-ingelheim.com;
IDT Biologika	Sergio Barrabés		sergio.barrabes@idt-biologika.com
IMICROQ, S.L	Katia Uliaque Cugat		
Rekom Biotech	Ana Camacho Páez	Ana Camacho Páez CSO and CEO of Rekom Biotech	info@rekombiotech.com agcamacho@rekombiotech.com
BIOORGANIC RESEARCH AND SERVICES S.L (BIONATURIS)	Victor Infante		Victor.infante@bionaturis.com
Vacunek, S.L.	Isbene Sánchez		comercial@vacunek.com
Thermofisher	Gabriel Rodríguez Alarcón	Nardy Robben Global Product Manager Phone: +31 6 54602148	gabriel.rodriguez-alarcon@thermofisher.com
Aquilón CyL, S.L.	Claudia de León Salazar		media@aquiloncyl.com
IDEXX LABORATORIOS	Christian Shelp		ChristianSchelp@idexx.com
Laboratorios Larrasa	José Larrasa	José Larrasa Rodríguez General Manager Polígono Industrial El Chaparral 06170 La Albuera, Badajoz-Spain Phone : +34 629 37 35 27	jlarrasa@laboratorioslarrasa.com
CALIER	Joaquim Tarés Ubiergo	Joaquim Tarés Ubiergo Business Development c/ Barcelonès, 26, PI El Ramassar. Les Franqueses del Vallès, 08520 Barcelona phone:+34 93 849 51 33 F.	qtares@calier.es
HIPRA	Elia Torroella	Elia Torroella R&D and Regulatory Affairs phone:+34666400302	Elia.torroella@hipra.com

A meeting with the Companies took place at INIA headquarters on 18th January. A questionnaire was prepared and agreed to be sent to the local contacts in order to identify the international company contact points and a brief feedback on vaccine development. Questionnaires received are gathered in ***Annex I*.**

8 ROADMAP ON VACCINE DEVELOPMENT AND MANUFACTURING

Regulatory approval for new vaccines is often complex, time consuming and costly. Although general EU legislation, monographs and guidelines exist, there are no specific guidelines or E.P. monographs applicable to ASF vaccines.

The ASF vaccines at present under research should be manufactured by Marketing Authorisation Holders (MAH) that comply with Good Manufacturing Practices (GMP) requirements, and presented for Marketing Authorisation to the competent authorities (Commission-European Medicine Agency –EMA- and/or National Medicines Competent Authorities) by following, the centralized, decentralized or national registration procedures.

Once the vaccine candidate has been preauthorised selected, its quality, safety and efficacy should be demonstrated by the MAH and will be evaluated by the Competent Authorities according to the harmonized criteria at the EU level (authorisation phase).

Wherever possible, a centralised procedure is preferable to allow a better exchange of the data available between Member States (MS).

The **table 4** summarizes the steps to be fulfilled for vaccine development and manufacturing according to general guidelines.

Table 4: STEPS FOR VACCINE DEVELOPMENT AND MANUFACTURING.

Studies performed during research for VACCINE DEVELOPMENT	"*In vitro*" and "*In vivo*" laboratory studies are required to identify the viability of the developed vaccine for commercial purposes. Pharmaceutical companies will identify viable candidates based on these laboratory research results (type and quantity of active substance, type and quantity of adjuvants and other excipients, dose, etc). STUDIES REQUIRED: • *"In vitro"* trials • *"In vivo"* laboratory trials (non- target and target species) **At these previous stages, these studies are not mandatory in general to fully comply with European legislation and guidelines for Veterinary Immunological Medicinal Products (VIMP) but it is recommended to perform them according to these criteria**	
VACCINE MANUFACTURING	LABORATORY AND FIELD TRIALS	Previous to the presentation of the dossier for authorization of the final products, the vaccine candidate is needed to be proved in general in field large scale studies. These field studies with the product under research should be authorized previously by the Competent Authorities,

		following in general GCP VICH Gl-9 indications.
	AUTHORISATION	STUDIES REQUIRED to be included in the dossier for authorization (that should have been performed with the final product to be registered): • *"In vitro"* laboratory trials • *"In vivo"* laboratory trials • Field trials All these studies should be conducted with the final vaccine to be authorized, fulfilling with the European requirements for VIMP. To be discussed with EMA and other medicine regulatory bodies the possibilities to facilitate the authorization of ASF vaccines ("under exceptional circumstances", authorization as Minor Use Minor species (MUMS products)-for example for wild boar.
	POST AUTHORISATION	STUDIES REQUIRED: As indicated in European legislation and guidelines, Post-authorization safety and or efficacy studies could/ should be done

In accordance with the European legislation and guidelines applicable to VIMP steps summarized in table 4 for VACCINE DEVELOPMENT AND MANUFACTURING, it has been drafted a **roadmap for ASF vaccine development that is shown in table 5 with the aim to define priorities and to make recommendations to support and accelerate the development of an effective and safe ASF vaccine**. This roadmap is expected to guide and inform the future European research commitments and investment priorities to create an appropriate environment to advance ASF vaccine research, know-how, and final development.

In principle, the same vaccine strain can be used for domestic pig and wild boar. The development of a specific **vaccine to be successfully used in the wild boar will probably pose additional challenges** as it is specified in point 5 and 6 related to vaccine administration route, efficacy, safety and vaccination strategy.

The table shows a number of activities and sub-activities that should be addressed in parallel. The activities have been classified according to the following priority criteria:

- (1) High: vaccine development with promising results.
- (2) Medium: vaccine development which require higher effort of research.

Table 5: ROADMAP FOR VACCINE DEVELOPMENT.

PRIORITY (1) High (2) Medium	ACTIVITY /SUB-ACTIVITY	DESCRIPTION	TIME SCHEDULE (YEARS)								PROBABILITY OF SUCCESS
			1	2	3	4	5	6	7	8	
(1)	**1. Evaluation of existing cell lines.**	**Large scale production of current available cell lines successfully used for ASF vaccine development**									**>80%**
	1.1. Large scale production of cell lines	Large scale production of current available cell lines such as COS cells successfully used for ASF vaccine development									
	1.2. Modification of existing macrophage-like cell lines (IPAM,WSL)	Phenotypic characterization of IPAM, WSL, (others). Analysis of cellular factors involved in ASFV susceptibility in IPAM, WSL, others.									
	1.3. Stability testing of LAVs genomes in cell line										
(1)	**2. Cell line development.**	**Establishment of cell culture systems for vaccine development and production maintaining the stability of the virus genome**									**>80%**
	2.1. Development and screening of new cell lines for ASFV susceptibility including pig monocytes/macrophages.	Identification of potential new cell lines sustaining productive ASFV infection including strategies to generate porcine macrophages lines susceptible to productive ASFV infection.									
(1)	**3. LAV *in vitro* development based on genetically modified ASFV strains.**	***In vitro* production and characterization of LAVs based on genetically modified ASFV strains, including attenuated and virulent viruses.**									**>80%**
	3.1. Further *In vitro* evaluation of **genetically modified ASF viruses already published.**	*In vitro* evaluation of already published LAVs based on genetically modified ASFV strains with single or multiple gene deletions known to attenuate virus and induce good levels of protection against challenge.									
	3.2. Further *In vitro* production of **genetically modified ASF viruses under development.**	*In vitro* production and characterization of LAVs based on genetically modified ASFV strains with single or multiple gene deletions known to attenuate virus and induce good levels of protection against challenge.									
(1)	**4. Deeper exploring of naturally attenuated ASFV strains as LAVs.**	**Further evaluation of naturally ASFV strains, such as the NH/P68 (NHV) known to induce good levels of protection against challenge, as potential LAVs candidates.**									**>80%**

(1)	**5. LAV *in vivo* testing in domestic pigs and in wild boar.**	***In vivo* testing of LAVs in order to select the vaccine candidate to be used in domestic pigs and wild boar based on protection level and safety studies.**									**>80%**
	5.1. Combinations of doses of immunization of pigs and wild boar.	Evaluation of clinical signs, virus shedding and protection against challenge at a range of doses including one dose, overdose and repeated administration of one dose.									
	5.2. To evaluate the cross-protective efficacy.	Evaluation of clinical signs, virus shedding and protection against challenge using the homologous and heterologous viruses.									
	5.3. Evaluation of reversion to virulence.	Virus stability by genome analysis after pig passages and virus recombination.									
	5.4. Long-term experiments.	Long-term in vivo experimental studies on safety, efficacy and duration of protection.									
(1)	**6. Identification of effective delivery systems, routes and vaccination formulas to induce protective responses in wild boar and in domestic pigs.**	**Evaluation of vaccination formulas for domestic pig and wild boar vaccination including administration routes (intramuscular, oral), adjuvants, etc.**									**>80%**
	6.1. Assessment of the immunization of domestic pigs by different administration routes.	Evaluation of the immunity induced after different immunization routes.									
	6.2. Assessment of the immunization of wild boar by oral administration of the vaccine	Evaluation of the immunity induced after oral immunization using different formulations.									
	6.3. Oral bait testing for wild boar vaccination	Bait formulation and design to allow intake of ideal vaccine doses to achieve sufficient protection in wild boar populations.									
(1)	**7. Research on protective immune responses to identify correlates for protection in vaccine testing.**	• Defining innate and cellular and serological adaptive immune responses correlating with protection *vs* virulence • Measure ASFV specific cellular responses • Measure antibody responses to ASFV									**>80%**
(1)	**8. Development of DIVA test**	**Development of DIVA test in parallel to the LAV development based on both negative and positive markers.** (Time is scheduled for a pre-prototype vaccine candidate).									**>80%**

	8.1. DIVA -serological tests. Negative markers	Testing antibody responses to proteins from genes deleted from LAVs as targets for negative markers.									
	8.2. DIVA - serological tests. Positive markers.	Testing antibodies to markers inserted into genetically modified LAVs as targets for positive selection.									
	8.3. Development of genetic DIVA test	Development of DIVA test based on PCR molecular techniques targeted to negative or positive markers									
(1*)	**9. Development of SUBUNIT vaccines**										**>50%**
	9.1. Selection of viral antigenic proteins and methods of delivering to the immune system	Comparison of effective delivery system able to efficiently present selected viral antigens for immune system activation and protection.									
	9.2. Selection of specific adjuvant and vehicles to induce a protective response	In vitro and in vivo test of viral antigens and adjuvant for protection									
(2)	**10. Development of disabled infectious single cycle (DISC) vaccines**	**Generation of replication-defective virus based-vaccines.**									**>50%**
	10.1. Identification of ASFV gene targets for DISC virus construction.	Characterization of ASFV essential late genes and construction of helper cell lines expressing these									
	10.2. Generation and propagation of ASFV DISC mutants	Construction of ASFV deletions of essential genes complimented by helper cell lines. Confirm abortive infection in porcine macrophages.									
	10.3. *In vivo* testing of ASFV DISC mutants	Testing DISC deletion mutants by immunisation of pigs.									

FOR RESEARCH IT IS AS IMPORTANT AS LAV but with less success probability

To produce and market a new *vaccine*, it is necessary to prove to the authorities that the product fulfills with the **European legislation and guidelines for VIMP**. This passes by the submission of *Marketing Authorization* (*MA*) dossier. This consists of a dossier with data proving that the vaccine has passed the stages of research, development, production and quality control, as well as clinical testing, and that the quality, safety and efficacy required of the vaccine to be used has been established. The structure of the MA dossier for marketing authorization of an immunological product for veterinary use comprises four sections including the Administrative (1), Quality (2), Safety (3) and Efficacy (4). In the **Annex II** is shown a summary of requirements for marketing authorization of a VIMP (veterinary immunological medicinal product). **This document has been prepared in collaboration with the Spanish Veterinary Medicine Agency.**

The **vaccine manufacturing** laying down the principles and guidelines of GMP in respect of medicinal products for human and Veterinary Use which consider several aspects such as:

- Validated Cell line and cell line scale up capacity
- Validated Master and Working Seed Virus and virus scale up capacity
- Excipients and other starting materials in compliance with Ph. Eur or other Pharmacopoeia.
- Freeze-drying capacity
- Consistency of production batches
- Validated In process-controls
- Validated finished product controls: e.g. final product batch release potency tests (in vitro/ in vivo)
- Adequate stability of the final product to cover the proposed validity period of the vaccine

The expected time to achieve it is between 8-10 years in total. If the vaccines are classified as MUMS or "under exceptional circumstances" timelines for the first authorisation can be reduced to less than 8 years since field trials could not be required (or required post-authorisation). Once the dossier is submitted to the Authorities, it has to be taken into account that standard 210 days for assessment (plus 3-6 months of clock stop for MAH to answer) will be required to approval of the vaccine.

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ANNEX I

QUESTIONNAIRES BPRM

Valdeolmos, Madrid, 31 January 2017

M.Arias

Technical Director EURL for ASF

The following questions try to get a feedback on your potential interest in the case of a possible development of an ASF vaccine:

1. **Has your company previous experience in development and/or manufacturing of**

Cell lines	YES
Modified Live and Live Attenuated Vaccines, subunits vaccines….	YES
Differentiating infected from vaccinated animals (DIVA) or serological test	YES
Oral bait vaccine/delivery systems	NO

for other swine diseases?

2. **Has been your company involved in ASF vaccine or ASF diagnosis research and development during the last years?** YES

3. **Would your company interested in the development of an ASF vaccine or ASF diagnosis test?**

a. Modified Live and Live Attenuated Vaccines, subunits vaccines….	YES
b. Differentiating infected from vaccinated animals (DIVA) or serological test	YES

COMMENTS:

Under which conditions would your company be interested in the development and manufacturing of an ASF vaccine and/or ASF diagnosis test?

Defined scenario where the vaccine can be applied:

- Emergency vaccination, i.e. targeted use to directly combat an outbreak of ASF in an EU member state or in case of a perceived immediate threat of infection occurring.
- Preventive vaccination, i.e. longer term vaccination to try to reduce the risk of the disease spreading into populations considered to be at risk.

Flexible approach to facilitate rapid authorisation of vaccines and provide information to manufacturers on the minimum data requirements for authorization of potential ASF vaccines for emergency use. For example considering the minor use minor species (MUMS)/ limited market guideline.

Future vaccine banks.

Funded research by the EC to identify a candidate with a demonstrated "POC" (Prove Of Concept).

Further spread of the disease in the EU countries.

Which capabilities and infrastructure are needed for the development and manufacturing of an ASF vaccine?

Access to challenge material and models

Established permissive cell line

BSL-3 R&D labs and animal facilities

BSL-3 pilot plant and manufacturing facilities

Please, indicate the company contact point details (name, position, address, e-mail, phone…) related to this issue at corporate level:

Alicia Urniza, Director EU R&D Biologicals at ZOETIS.
Mercuriusstraat 20, Zaventem, 1930 Belgium. Office: +32 27468224| Mobile: +32 497052281 ; +34 649815040 ; alicia.urniza@zoetis.com

The following questions try to get a feedback on your potential interest in the case of a possible development of an ASF vaccine:

1. **Has your company previous experience in development and/or manufacturing of**

Cell lines	
Modified Live and Live Attenuated Vaccines, subunits vaccines….	YES
Differentiating infected from vaccinated animals (DIVA) or serological test	
Oral bait vaccine/delivery systems	

   for other swine diseases?

2. **Has been your company involved in ASF vaccine or ASF diagnosis research and development during the last years?** NO

3. **Would your company be interested in the development of an ASF vaccine or ASF diagnosis test?**

a. Modified Live and Live Attenuated Vaccines, subunits vaccines….	YES
b. Differentiating infected from vaccinated animals (DIVA) or serological test	

COMMENTS:

Under which conditions would your company be interested in the development and manufacturing of an ASF vaccine and/or ASF diagnosis test?

Under financial instrument for research and development

When economic perspective will provide benefits for the company

When the European Commission allow vaccination or when a vaccine bank to be used will be created

Which capabilities and infrastructure are needed for the development and manufacturing of an ASF vaccine?

Development needs partnerships with research centres that can perform basic research with vaccine candidates and provide vaccine prototypes

Efficacy testing needs biosafety facilities for *in vivo* testing

Manufacturing will need level 3 of contention (in case of live attenuated vaccine)

Manufacturing will need a well stablished system for virus replication (i.e cell line adapted to PPA)

Please, indicate the company contact point details (name, position, address, e-mail, phone…) related to this issue at corporate level:

LABORATORIOS CALIER

Name: JOAQUIM TARÉS UBIERGO

Position: Business Development

Address: c/ Barcelonès, 26, PI El Ramassar. Les Franqueses del Vallès, 08520 Barcelona

e-mail: qtares@calier.es

phone:+34 93 849 51 33 F. +34 93 840 13 98

The following questions try to get a feedback on your potential interest in the case of a possible development of an ASF vaccine:

1. **Has your company previous experience in development and/or manufacturing of**

Cell lines	
Modified Live and Live Attenuated Vaccines, subunits vaccines….	
Differentiating infected from vaccinated animals (DIVA) or serological test	x
Oral bait vaccine/delivery systems	

   for other swine diseases?

2. **Has been your company involved in ASF vaccine or ASF diagnosis research and development during the last years?** x

3. **Would your company interested in the development of an ASF vaccine or ASF diagnosis test?**

a. Modified Live and Live Attenuated Vaccines, subunits vaccines….	
b. Differentiating infected from vaccinated animals (DIVA) or serological test	x

COMMENTS:
Under which conditions would your company be interested in the development and manufacturing of an ASF vaccine and/or ASF diagnosis test?

We would be interested in participation in development of ASF diagnostic test methods, potentially DIVA testing, based on molecular technologies or other technologies, except serology.

Which capabilities and infrastructure are needed for the development and manufacturing of an ASF vaccine?

Please, indicate the company contact point details (name, position, address, e-mail, phone…) related to this issue at corporate level:
Contactperson information:
Nardy Robben
Global Product Manager
Email: nardy.robben@thermofisher.com
Phone: +31 6 54602148

The following questions try to get a feedback on your potential interest in the case of a possible development of an ASF vaccine:

1. **Has your company previous experience in development and/or manufacturing of**

Cell lines	
Modified Live and Live Attenuated Vaccines, subunits vaccines….	YES
Differentiating infected from vaccinated animals (DIVA) or serological test	YES
Oral bait vaccine/delivery systems	

for other swine diseases?

2. **Has been your company involved in ASF vaccine or ASF diagnosis research and development during the last years?** NO

3. **Would your company interested in the development of an ASF vaccine or ASF diagnosis test?**

a. Modified Live and Live Attenuated Vaccines, subunits vaccines….	YES
b. Differentiating infected from vaccinated animals (DIVA) or serological test	YES

COMMENTS:

Under which conditions would your company be interested in the development and manufacturing of an ASF vaccine and/or ASF diagnosis test?

We are interested in participating in a collaborative project. We would like to be a partner to execute part of the work needed and assuming direct costs (labor, researchers, materials, equipments,..), between €5-10 millions.

Which capabilities and infrastructure are needed for the development and manufacturing of an ASF vaccine?

We have a Level-3 laboratory, a complete facility to produce viral/bacterial proteins using diferent expressions systems in an industrial manner, a sequence platform based on NGS, a high-throughput MALDI-TOF platform for discovery of genomic sequence polymorphisms, high-throughput ELISA platform for immune response evaluation.

Please, indicate the company contact point details (name, position, address, e-mail, phone…) related to this issue at corporate level:

JOSE LARRASA RODRIGUEZ
GENERAL MANAGER
POLIGONO INDUSTRIAL EL CHAPARRAL
06170 LA ALBUERA
BADAJOZ-SPAIN
PHONE: +34 629 37 35 27

The following questions try to get a feedback on your potential interest in the case of a possible development of an ASF vaccine:

1. **Has your company previous experience in development and/or manufacturing of**

Cell lines	
Modified Live and Live Attenuated Vaccines, subunits vaccines….	
Differentiating infected from vaccinated animals (DIVA) or serological test	YES
Oral bait vaccine/delivery systems	

   for other swine diseases?

2. **Has been your company involved in ASF vaccine or ASF diagnosis research and development during the last years?** NO

3. **Would your company interested in the development of an ASF vaccine or ASF diagnosis test?**

a. Modified Live and Live Attenuated Vaccines, subunits vaccines….	
b. Differentiating infected from vaccinated animals (DIVA) or serological test	YES

COMMENTS:

Under which conditions would your company be interested in the development and manufacturing of an ASF vaccine and/or ASF diagnosis test?

We are interested in collaborating in the development of an ASF diagnosis test providing the recombinant antigens used as raw material for the manufacturing of the tests. We have previous experience designing and producing rec. antigens intended as biomarkers for the diagnosis of Leptospirosis in swine. We also have previous experience in designing and producing rec. antigens that have been used in the production of vaccines.

Which capabilities and infrastructure are needed for the development and manufacturing of an ASF vaccine?

Please, indicate the company contact point details (name, position, address, e-mail, phone…) related to this issue at corporate level:

Dr. Ana Camacho Páez
CSO and CEO of Rekom Biotech
agcamacho@rekombiotech.com
+34 607 861 573

The following questions try to get a feedback on your potential interest in the case of a possible development of an ASF vaccine:

1. **Has your company previous experience in development and/or manufacturing of**

Cell lines	Yes
Modified Live and Live Attenuated Vaccines, subunits vaccines….	Yes
Differentiating infected from vaccinated animals (DIVA) or serological test	Yes
Oral bait vaccine/delivery systems	Yes

for other swine diseases?

2. **Has been your company involved in ASF vaccine or ASF diagnosis research and development during the last years?** No

3. **Would your company interested in the development of an ASF vaccine or ASF diagnosis test?**

a. Modified Live and Live Attenuated Vaccines, subunits vaccines….	Yes
b. Differentiating infected from vaccinated animals (DIVA) or serological test	Yes

COMMENTS:

Under which conditions would your company be interested in the development and manufacturing of an ASF vaccine and/or ASF diagnosis test? We are open at this step of the process as it is too early to put too much conditions. We wold need to be involved in the development and produce the product in our production facilities.

Which capabilities and infrastructure are needed for the development and manufacturing of an ASF vaccine? P3 level Facilities to conduct studies, and depending on the kind of vaccine to be developed, new P3 level production facilities.

Please, indicate the company contact point details (name, position, address, e-mail, phone…) related to this issue at corporate level:

Elia Torroella
R&D and Regulatory Affairs Director
HIPRA
Elia.torroella@hipra.com
+34666400302

ANNEX II

SUMMARY OF REQUIREMENTS FOR MARKETING AUTHORIZATION BPRM

Valdeolmos, Madrid, 31 January 2017

M.Arias

Director EURL for ASF

Summary of requirements for marketing authorization of a VIMP (veterinary Immunological medicinal product) as indicated DIRECTIVE 2001/82/EC OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 6 November 2001 on the Community code relating to veterinary medicinal product.

These requirements are specified in the Annex I "Requirements and analytical protocol, safety test, pre-clinical and clinical of VIMP" TITLE II "Requirements for immunological veterinary medicinal products" ***of the 2001/82/EC DIRECTIVE, pg 47.***

PART 5. Summary

A. ADMINISTRATIVE DATA of the dossier
B. SUMMARY OF PRODUCT CHARACTERISTICS
C. EXPERT REPORTS

PART 6. Analytical (physico-chemical, biological or microbiological) tests of immunological veterinary medicinal products

ACTIVITY	DESCRIPTION
A. QUALITATIVE AND QUANTITATIVE PARTICULARS OF THE CONSTITUENTS	
1. Qualitative particulars	Qualitative particulars. The designation or description of: • the active substance(s), • the constituents of the adjuvants, • the constituent(s) of the excipients, whatever their nature or the quantity used, including preservatives, stabilisers, emulsifiers, colouring matter, flavouring, aromatic substances, markers, etc., • the constituents of the pharmaceutical form administered to animals.

2. Usual terminology, to be used in describing the constituents of immunological veterinary medicinal products	
3. Quantitative particulars	It is necessary to specify whenever possible the number of organisms, the specific protein content, the mass, the number of International Units (IU) or units of biological activity, either per dosage-unit or volume, and with regard to the adjuvant and to the constituents of the excipients and, the mass or the volume of each of them.,.
4. Development pharmaceutics	An explanation shall be provided with regard to the composition, components and containers, supported by scientific data on development pharmaceutics.

B. DESCRIPTION OF MANUFACTURING METHOD OF THE FINISHED PRODUCT

The description of the manufacturing method shall include at least:

- the various stages of manufacture (including purification procedures) so that an assessment can be made of the
- reproducibility of the manufacturing procedure and of the risks of adverse effects on the finished products, such as
- microbiological contamination,
- in the case of continuous manufacture, full details concerning precautions taken to ensure the homogeneity and
- consistency of each batch of the finished product,
- mention of substances which cannot be recovered in the course of manufacture,
- the details of the blending, with the quantitative particulars of all the substances used,
- a statement of the stage of manufacture at which sampling is carried out for in-process control tests.

C. PRODUCTION AND CONTROL OF STARTING MATERIALS

Starting materials. means all components used in the production of the immunological veterinary medicinal product. Culture media used for the production of the active substance are considered as one single starting material. In the case of:

- an active substance not described in the European Pharmacopoeia or in the pharmacopoeia of a Member State,
  or
- an active substance described in the European Pharmacopoeia or in the pharmacopoeia of a Member State when prepared by a method liable to leave impurities not mentioned in the pharmacopoeial monograph and for which the monograph is inappropriate to adequately control its quality, which is manufactured by a person different from the applicant, the latter may arrange for the detailed description of the manufacturing method, quality control during manufacture and process validation to be supplied directly to the competent authorities by the manufacturer of the active substance.

D. SPECIFIC MEASURES CONCERNING THE PREVENTION OF THE TRANSMISSION OF ANIMAL SPONGIFORM ENCEPHALOPATHIES

E. CONTROL TESTS DURING PRODUCTION

1. The particulars and documents accompanying an application for marketing authorization, pursuant to Article 12(3)(i) and (j) and Article 13(1), shall include particulars relating to the control tests which are carried out on intermediate products with a view to verifying the consistency of the production process and the final product.
2. For inactivated or detoxified vaccines, inactivation or detoxification shall be tested during each production run immediately after the inactivation or detoxification process.

F. CONTROL TESTS ON THE FINISHED PRODUCT

1. General characteristics of the finished product

2. Identification and assay of active substance(s)

3. Identification and assay of adjuvants

4. Identification and assay of excipient components

5. Safety tests

6. Sterility and purity test

7. Inactivation

8. Residual humidity

9. Batch-to-batch consistency

G. STABILITY TESTS

A description shall be given of the tests undertaken to support the shelf life proposed by the applicant.

PART 7. Safety testing

A. INTRODUCTION	
1. The safety tests shall show the potential risks from the immunological veterinary medicinal product which may occur under the proposed conditions of use in animals: these shall be evaluated in relation to the potential benefits of the product. Where immunological veterinary medicinal products consist of live organisms, especially those which could be shed by vaccinated animals, the potential risk to unvaccinated animals of the same or of any other potentially exposed species shall be evaluated. 2. The particulars and documents which shall accompany the application for marketing authorization pursuant to Article 12(3)(j) and 13(1) shall be submitted in accordance with the requirements of section B. 3. Member States shall ensure that the laboratory tests are carried out in conformity with the principles of good laboratory practice laid down in Council Directives 87/18/EEC and 88/320/EEC.	
B. GENERAL REQUIREMENTS	
1. The safety tests shall be carried out in the target species. 2. The dose to be used shall be that quantity of the product to be recommended for use and containing the maximum titre or potency for which the application is submitted. 3. The sample used for safety testing shall be taken from a batch or batches produced according to the manufacturing process described in the application for marketing authorization.	
C. LABORATORY TESTS	
1. Safety of the administration of one dose	The immunological veterinary medicinal product shall be administered at the recommended dose and by each recommended route of administration to animals of each species and category in which it is intended for use, including animals of the minimum age of administration
2. Safety of one administration of an overdose	An overdose of the immunological veterinary medicinal product shall be administered by each recommended route of administration to animals of the most sensitive categories of the target species
3. Safety of the repeated administration of one dose	Repeated administration of one dose may be required to reveal any adverse effects induced by such administration. These tests shall be carried out on the most sensitive categories of the target species, using the recommended route of administration.
4. Examination of reproductive performance	Examination of reproductive performance shall be considered when data suggest that the starting material from which the product is derived may be a potential risk factor. Reproductive performance of males and non-pregnant and pregnant females shall be investigated with the recommended dose and by each of the recommended routes of administration. In addition, harmful effects on the progeny, as well as teratogenic and abortifacient effects, shall be investigated.
5. Examination of immunological functions	Where the immunological veterinary medicinal product might adversely affect the immune response of the vaccinated animal or of its progeny, suitable tests on the immunological functions shall be carried out.
6. Special requirements for live vaccines:	***6.1. Spread of the vaccine strain*** Spread of the vaccine strain from vaccinated to unvaccinated target animals shall be investigated, using the recommended route of administration most likely to result in the spread. Moreover, it may be necessary to investigate the spread to non target species which could be highly susceptible to a live vaccine strain. ***6.2. Dissemination in the vaccinated animal*** Faeces, urine, milk, eggs, oral, nasal and other secretions shall be tested for the presence of the organism. ***6.3. Reversion to virulence of attenuated vaccines*** Reversion to virulence shall be investigated with material from the passage level which is least attenuated between the master seed and the final product. The initial vaccination shall be carried out using the recommended route of administration most likely to lead to reversion to virulence. At least five serial passages through animals of the target species shall be undertaken. Where this is not technically possible due to failure of the organism to replicate adequately, as many passages as possible shall be carried out in the target species. If necessary, in vitro propagation of the organism may be

	carried out between passages in vivo. The passages shall be undertaken by the route of administration most likely to lead to reversion to virulence. ***6.4. Biological properties of the vaccine strain*** Other tests may be necessary to determine as precisely as possible the intrinsic biological properties of the vaccine strain (e.g. neurotropism). ***6.5. Recombination or genomic reassortment of strains*** The probability of recombination or genomic reassortment with field or other strains shall be discussed.
7. Study of residues	For immunological veterinary medicinal products, it will normally not be necessary to undertake a study of residues. However, where adjuvants and/or preservatives are used in the manufacture of immunological veterinary medicinal products, consideration shall be given to the possibility of any residue remaining in the foodstuffs.
8. Interactions	Any known interactions with other products shall be indicated.
D. FIELD STUDIES	
Unless justified, results from laboratory studies shall be supplemented with supportive data from field studies.	
E. ECOTOXICITY	
The purpose of the study of the ecotoxicity of an immunological veterinary medicinal product is to assess the potential harmful effects which the use of the product may cause to the environment and to identify any precautionary measures which may be necessary to reduce such risks. An assessment of ecotoxicity shall be compulsory. This assessment shall normally be conducted in two phases. The first phase of the assessment shall always be carried out: the investigator shall assess the potential extent of exposure of the environment to the product, its active substances, or relevant metabolites, taking into account: • the target species and the proposed pattern of use (e.g. mass medication or individual animal medication), • the method of administration, in particular the likely extent to which the product will enter directly into environmental system, • the possible excretion of the product, its active substances or relevant metabolites into the environment by treated animals, persistence in such excretia, • the disposal of unused or waste product. Where the conclusions of the first phase indicate potential exposure of the environment to the product, the applicant shall proceed to the second phase and evaluate the potential ecotoxicity of the product. For this purpose, he shall consider the extent and duration of exposure of the environment to the product, and the information about the physical/chemical, pharmacological and/or toxicological properties of the compound obtained during the conduct of the other tests and trials required by this Directive. Where necessary, further investigations on the impact of the product (soil, water, air, aquatic systems, non-target organisms) shall be carried out. These further investigations shall be carried out in accordance with the test protocols laid down in Annex V to Council Directive 67/548/EEC or where an end point is not adequately covered by these protocols, in accordance with other internationally recognized protocols on the immunological veterinary medicinal product and/or the active substances and/or the excreted metabolites as appropriate. The number and types of tests and the criteria for their evaluation shall depend upon the state of scientific knowledge at the time the application is submitted.	

PART 8. Efficacy trials

A. INTRODUCTION
1. The purpose of the trials described in this Part is to demonstrate or to confirm the efficacy of the immunological veterinary medicinal product. All claims made by the applicant with regard to the properties, effects and use of the product, shall be fully supported by results of specific trials contained in the application for marketing authorization.

2. The particulars and documents which shall accompany applications for marketing authorizations pursuant to Article 12(3)(j) and 13(1) shall be submitted in accordance with the provisions below.
3. All veterinary clinical trials shall be conducted in accordance with a fully considered detailed trial protocol which shall be recorded in writing prior to commencement of the trial. The welfare of the trial animals shall be subject to veterinary supervision and shall be taken fully into consideration during the elaboration of any trial protocol and throughout the conduct of the trial. Pre-established systematic written procedures for the organization, conduct, data collection, documentation and verification of clinical trials shall be required.
4. Before the commencement of any trial, the informed consent of the owner of the animals to be used in the trial shall be obtained and documented. In particular, the animal owner shall be informed in writing of the consequences of participation in the trial for the subsequent disposal of treated animals or for the taking of foodstuffs from treated animals. A copy of this notification, countersigned and dated by the animal owner, shall be included in the trial documentation.
5. Unless the trial is conducted with a blind design, the provisions of Articles 58, 59 and 60 shall apply by analogy to the labelling of formulations intended for use in veterinary clinical trials. In all cases, the words for veterinary clinical trial use only shall appear prominently and indelibly upon the labelling.

B. GENERAL REQUIREMENTS

1. The choice of vaccine strains shall be justified on the basis of epizoological data.
2. Efficacy trials carried out in the laboratory shall be controlled trials, including untreated control animals. In general, these trails shall be supported by trials carried out in field conditions, including untreated control animals. All trials shall be described in sufficiently precise details so as to be reproducible in control trials, carried out at the request of the competent authorities. The investigator shall demonstrate the validity of all the techniques involved. All results shall be presented as precisely as possible. All results obtained, whether favourable or unfavourable, shall be reported.
3. The efficacy of an immunological veterinary medicinal product shall be demonstrated for each category of each species recommended for vaccination, by each recommended route of administration and using the proposed schedule of administration. The influence of passively acquired and maternally derived antibodies on the efficacy of a vaccine shall be adequately evaluated. Any claims regarding the onset and duration of protection shall be supported by data from trials.
4. The efficacy of each of the components of multivalent and combined immunological veterinary medicinal products shall be demonstrated. If the product is recommended for administration in combination with or at the same time as another veterinary medicinal product, they shall be shown to be compatible.
5. Whenever a product forms part of a vaccination scheme recommended by the applicant, the priming or booster effect or the contribution of the product to the efficacy of the scheme as a whole shall be demonstrated.
6. The dose to be used shall be that quantity of the product to be recommended for use and containing the minimum titre or potency for which the application is submitted.
7. The samples used for efficacy trials shall be taken from a batch or batches produced according to the manufacturing process described in the application for marketing authorization.
8. For diagnostic immunological veterinary medicinal products administered to animals, the applicant shall indicate how reactions to the product are to be interpreted.

C. LABORATORY TRIALS

1. In principle, demonstration of efficacy shall be undertaken under well controlled laboratory conditions by challenge after administration of the immunological veterinary medicinal product to the target animal under the recommended conditions of use. In so far as possible, the conditions under which the challenge is carried out shall mimic the natural conditions for infection, for example with regard to the amount of challenge organism and the route of administration of the challenge.
2. If possible, the immune mechanism (cell-mediated/humoral, local/general classes of immunoglobulin) which is initiated after the administration of the immunological veterinary medicinal product to target animals by the recommended route of administration shall be specified and documented.

D. FIELD TRIALS

1. Unless justified, results from laboratory trials shall be supplemented with data from field trials.
2. Where laboratory trials cannot be supportive of efficacy, the performance of field trials alone may be acceptable.

PART 9. Particulars and documents concerning safety testing and efficacy trials of immunological veterinary medicinal products

A. INTRODUCTION

As in any scientific work, the dossier of safety and efficacy studies shall include an introduction defining the subject and indicating the tests which have been carried out in compliance with Parts 7 and 8, as well as a summary, with references to the published literature. Omission of any tests or trials listed in Parts 7 and 8 shall be indicated and discussed.

B. LABORATORY STUDIES

The following shall be provided for all studies:

1. a summary;
2. the name of the body having carried out the studies;
3. a detailed experimental protocol giving a description of the methods, apparatus and materials used, details such as species, breed or strain of animals, categories of animals, where they were obtained, their identification and number, the conditions under which they were housed and fed (stating inter alia whether they were free from any specified pathogens and/or specified antibodies, the nature and quantity of any additives contained in the feed), dose, route, schedule and dates of administration, a description of the statistical methods used;
4. in the case of control animals, whether they received a placebo or no treatment;
5. all general and individual observations and results obtained (with averages and standard deviations), whether favourable or unfavourable. The data shall be described in sufficient detail to allow the results to be critically evaluated independently of their interpretation by the author. The raw data shall be presented in tabular form. Byway of explanation and illustration, the results may be accompanied by reproductions of recordings, photomicrographs, etc.;
6. the nature, frequency and duration of observed side-effects;
7. the number of animals withdrawn prematurely from the studies and reasons for such withdrawal;
8. a statistical analysis of the results, where such is called for by the test programme, and variance within the data;
9. occurrence and course of any intercurrent disease;
10. all details concerning medicinal products (other than the product under study), the administration of which was necessary during the course of the study;
11. an objective discussion of the results obtained, leading to conclusions on the safety and efficacy of the product.

C. FIELD STUDIES

Particulars concerning field studies shall be sufficiently detailed to enable an objective judgement to be made. They shall include the following:

1. a summary;
2. name, address, function and qualifications of the investigator in charge;
3. place and date of administration, name and address of the owner of the animal(s);
4. details of the trial protocol, giving a description of the methods, apparatus and materials used, details such as the route of administration, the schedule of administration, the dose, the categories of animals, the duration of observation, the serological response and other investigations carried out on the animals after administration;
5. in the case of control animals, whether they received a placebo or no treatment;
6. identification of the treated and control animals (collective or individual, as appropriate), such as species, breeds or strains, age, weight, sex, physiological status;
7. a brief description of the method of rearing and feeding, stating the nature and quantity of any additives contained in the feed;
8. all the particulars on observations, performances and results (with averages and standard deviation); individual data shall be indicated when tests and measurements on individuals have been carried out;
9. all observations and results of the studies, whether favourable or unfavourable, with a full statement of the observations and the results of the objective tests of activity required to evaluate the product; the techniques used must be specified and the significance of any variations in the results explained;
10. effect on the animals' performances (e.g. egg laying, milk production, reproductive performance);
11. the number of animals withdrawn prematurely from the studies and reasons for such withdrawal;

12. the nature, frequency and duration of observed adverse reactions;
13. occurrence and course of any intercurrent disease 14. all details concerning medicinal products (other than the product under study) which have been administered either prior to or concurrently with the test product or during the observation period; details of any interactions observed;
15. an objective discussion of the results obtained, leading to conclusions on the safety and efficacy of the product.

D. GENERAL CONCLUSIONS

General conclusions on all results of tests and trials carried out in compliance with Parts 7 and 8 shall be given. They shall contain an objective discussion of all the results obtained and lead to a conclusion on the safety and efficacy of the immunological veterinary medicinal product.

E. BIBLIOGRAPHICAL REFERENCES

The bibliographical references cited in the summary mentioned under Section A shall be listed in detail.