Immunology

Currently, there is no vaccine available for ASF and the disease is strictly controlled by animal quarantine and slaughter. Attempts to vaccinate animals using infected cell extracts, supernatants of infected pig peripheral blood leukocytes, purified and inactivated virions, infected glutaraldehyde-fixed macrophages, or detergent-treated infected alveolar macrophages failed to induce protective immunity [1-4]. Pigs that survive infection can be protected against challenge with related virulent viruses. Pigs surviving acute infection with moderately virulent or attenuated variants of ASFV develop long-term resistance to homologous, but rarely to heterologous, virus challenge [5, 6]. However in these experiments the antigenic differences between isolates used for immunization and challenge was not well defined. Additional experiments have shown that immunization of pigs with an attenuated genotype I ASFV strain from Portugal, OURT88/3, could induce protection against other genotype I isolates (including from W. Africa) and against a genotype X isolate from Uganda but not against a genotype VIII isolate a[7, 8]. This demonstration suggests that vaccines with a broader cross-protection may be developed. However, more complete genome sequence information combined with knowledge of dominant protective antigens and mechanisms of immunity is required to understand the basis for cross-protection.

Pigs immunized with live attenuated ASF viruses containing engineered deletions of specific ASFV virulence/host range genes were protected when challenged with homologous parental virus [9-12]. Humoral and cellular immunity are significant components of the protective immune response to ASF. Passive transfer of antibodies from immune to naïve pigs has been demonstrated to protect pigs from lethal ASFV infection [5, 6, 13]. The mechanism by which antibodies can mediate protection is not clear. In some studies convalescent sera from pigs infected with low virulence isolates has been shown to neutralize virus by between 86 and 97% in Vero and pig macrophage cultures. ASFV neutralizing antibodies directed against virion proteins p30, p54, and p72 have been described [14-17]. Antibodies against p54 and p72 were shown to inhibit virus binding whereas antibodies against p30 inhibited virus internalization [15]. High passage in of ASFV isolates in tissue culture resulted in a loss of ability to neutralize virus and was correlated with alteration of the phospholipid composition of the virus particle [16-18]. This finding may help explain earlier reports that ASFV infection did not induce neutralizing antibodies. In addition the presence of antibodies
that inhibit complete virus neutralization has been reported [19]. The ability of antibodies to inhibit the haemadsorption of red blood cells to cells infected with different virus isolates, correlates with cross-protection. This implicates antibodies against the virus encoded CD2-like protein as having a role in cross-protection [20, 21]. In addition to virus neutralization, antibodies may mediate protection by other mechanisms. For example, antibodies that inhibit infection at a stage post-adsorption have been described: complement-dependent antibody mediated cytotoxicity and antibody dependent cell-mediated cytotoxicity may also play a role.

The importance of cellular immunity in protection has been shown in several studies. ASFV-specific cytotoxic T lymphocytes were demonstrated [22, 23]. In swine immunized with low virulence isolate NHP68. A role for CD8+ T cells in protection was clearly demonstrated since depletion of this cell subset abrogated the protection induced by the attenuated strain OURT88/3 (OURT88/3) [24]. Protection induced by this strain was correlated with induction of ASFV specific IFN gamma producing T cells [8]. DNA immunization has confirmed the relevance of specific CD8 T-cells in protection [25, 26]. Protection induced by the NHP68 strain was also shown to correlate with induction of higher numbers of NK cells [22].

ASFV, similar to other large DNA viruses, affects and modulates host immune responses and encodes many genes involved in this process. ASFV-infected macrophages mediate changes in cellular immune function, and they likely play a role in the severe apoptosis observed in lymphoid tissue [27-32]. ASFV inhibits phorbol myristic acid-induced expression of proinflammatory cytokines such as TNF-α, IFN-α, and IL-8 while inducing production of TGF-β from infected macrophages [33]. Conversely, increased TNF-α expression has been reported after ASFV infection in vitro and in vivo and TNF-α may play a key role in ASFV pathogenesis, including changes in vascular permeability, coagulation, and induction of apoptosis in uninfected lymphocytes [34, 35]. Notably, ASFV strains with different virulence phenotypes differ in their ability to induce expression of proinflammatory cytokine or IFN-related genes in macrophages early in infection [36-38]. The ASFV ankyrin repeat-containing protein pA238L (5EL) is, was initially described as an inhibitor of the NFκB/Rel family of cellular transcription factors, and was proposed to act as a viral homologue of the IκB inhibitor of NFκB. Subsequently A238L was shown to inhibit transcriptional activation mediated by additional transcription factors, including c-Jun, which interact with the p300 transcriptional co-activator [39, 40]. A238L was also shown to inhibit the cellular phosphatase calcineurin and thus activation of pathways dependent on calcineurin including activation of the NFAT transcription factor. Through these functions A238L is thought to be important in evading host immune responses [33, 41] through inhibition of transcriptional activation of a wide range of host immune response genes including proinflammatory and antiviral mediators and cytokines. Consistent with this role, pA238L is able to regulate expression of cyclooxygenase-2 (COX-2), TNF-α, and inducible nitric-oxide synthase (iNOS). COX-2 down-regulation occurs in an NFκB-independent, but NFAT-dependent, manner [42, 43]. Similarly, pA238L inhibits expression of iNOS, and ultimately production of nitric oxide. Interestingly, deletion of A238L from pathogenic ASFV does not affect viral growth in macrophages in vitro or viral pathogenesis and virulence in domestic swine [44] indicating that the virus may have other proteins which can compensate for loss of A238L. Additional ASFV-encoded proteins modulate or interfere with host immune responses. The ASFV 8DR (or pEP402R) is the only known viral homolog of cellular CD2, a T cell protein involved in co-regulation of cell activation. 8DR is necessary and sufficient for mediating hemoadsorption by ASFV-infected cells [45, 46]. Deletion of the 8DR gene from the ASFV genome led to decreased early virus replication and generalization of infection
in swine, and 8DR suppressed cellular immune responses in vitro [47]. The ASFV pEP153R (8CR) protein is similar to cellular and poxviral proteins resembling C-type lectin-like proteins, including membrane-bound immunoactivation and immunoregulatory proteins CD69 and NKG2 [48, 49]. The EP153R protein has also been demonstrated to modulate cells surface expression of MHC class I antigens [50][Hurtado et al., 2011]. A potential role for pEP153R in immunomodulation may be subtle, however, since pEP153R does not affect viral pathogenesis or virulence in domestic swine [48]. Evidence also suggests that ASFV affects Th2/B cell responses, including up-regulation of Th2 cytokines by a soluble virulence factor (p36) released from ASFV-infected monocytes and the nonspecific activation and apoptosis seen in B cell populations from ASFV-infected animals [30, 51]. ASFV multigene family 360 and 530 genes play a role in modulating host innate responses. Unlike wild type virus, infection of macrophages with Pr4Δ35, a mutant virus lacking MGF360/530 genes, resulted in increased mRNA levels for several type I interferon early-response genes [36]. Analysis of IFN-α mRNA and secreted IFN-α levels at 3, 8, and 24 hours post-infection (p.i.) revealed undetectable IFN-α mRNA and secreted IFN-α levels in mock and wild type-infected macrophages but significantly increased IFN-α levels at 24 hours p.i. in Pr4Δ35-infected macrophages, indicating that MGF360/530 genes either directly or indirectly suppress a type I IFN response. This effect may account for the growth defect of Pr4Δ35 in macrophages and its attenuation in swine [52]. ASFV encodes other genes with roles in evading the IFN response [53]. These include the I329L transmembrane protein which has some sequence similarities to TLR receptors including leucine rich repeats in the extracellular domain. I329L has been shown to act as an antagonist of TLR3 signalling and inhibits dsRNA stimulated activation of NFkB and IRF3 and transcription of IFNα and CCL5 [54, 55].

ASFV encodes inhibitors of other host intrinsic defence pathways including apoptosis, autophagy and stress-activated pathways. The ASFV apoptosis inhibitor A179L belongs to the Bcl 2 family and inhibits apoptosis through binding to Bid and Noxa [56]. A179L protein also targets the Beclin 1 autophagy related protein [57]. The DP71L protein targets the cellular phosphatase PP1 to dephosphorylate the eIF2α translation initiation factor and prevent the global shut-off of protein synthesis induced by cellular stresses including double-stranded RNA and ER stress [58, 59].

The development of rationally attenuated live vaccines for ASFV through targeted gene deletion(s) is possible. A number of genes have already been identified deletion of which reduces virus virulence. These include genes deletion of which reduces virus replication in macrophages and genes involved in suppressing the IFN response [60].

Vaccines

A commercial vaccine for ASF has never been available. Experimentally, protection can be achieved by inoculation of pigs with low-virulence isolates obtained by passage in tissue culture or by deletion of genes involved in virulence, as well as low-virulence isolates from the field [7, 9, 22, 61]. There is currently little information on the extent to which cross-protection can be achieved against heterologous isolates from the same or different genotypes. The importance of cellular immunity in protection has been shown in several studies. ASFV-specific cytotoxic T lymphocytes were demonstrated [22, 23] in swine immunized with low virulence isolate NHP68. A role for CD8+ T cells in protection was clearly demonstrated since depletion of this cell subset abrogated the protection induced by the attenuated strain OURT88/3 (OURT88/3)[24]. Protection induced by this strain was correlated with induction of ASFV specific IFN gamma producing T cells [8][King et al., 2011]. DNA
immunization has confirmed the relevance of specific CD8 T-cells in protection [25, 26]. Protection induced by the NHP68 strain was also shown to correlate with induction of higher numbers of NK cells [22].

A role for antibodies in protection had been shown since passive transfer of antibodies from immune pigs conferred partial protection to lethal challenge [13]. In experiments using recombinant proteins, partial protection was achieved using a combination of two proteins, p54 and p30, as well as with recombinant CD2-like protein [17, 62]. However, another study failed to achieve protection using a mixture of recombinant proteins p30, p54 and p72 [63]. The failure to achieve complete protection in these experiments may be because of the delivery method of the antigens and/or because more or different antigens are required to confer protection. Alternatively, it is possible that full protection can only be achieved by using live-attenuated replication competent ASF viruses as vaccines.

Pigs immunized with live attenuated ASF viruses containing engineered deletions of specific ASFV virulence/host range genes were protected when challenged with homologous parental virus [9-12]. Further research is required to develop effective vaccines. Identification of ASFV genes involved in virulence and in evasion of the host’s immune response (for review see [53, 60, 64, 65]) makes the development of rationally attenuated vaccines through sequential deletion of these genes realistic. However, extensive testing of the safety of such vaccines is required.

Alternative approaches for vaccine development that are based on expression of protective antigens requires the identification of antigens that can induce protection. DNA vaccination has been used as a tool to identify protective antigens. In one study immunisation of pigs with a plasmid expressing a fusion of the extracellular domains of the secretory hemagglutinin (or CD2-like protein coded for by EP402R), p30 (CP204L) and p54 (E183L) with a single chain variable fragment of an antibody specific for a swine leucocyte antigen II [66] induced a good antibody response but these were not protective. In another study DNA immunisation with these ASFV genes fused to ubiquitin protected a proportion of pigs from lethal challenge. Protection was correlated with induction of antigen specific CD8+ T cells in the absence of an antibody response [26]. In addition to DNA vaccination the development of high-throughput methods for constructing recombinant viral vectors opens a route for global analysis of the protective potential of all ASFV-expressed genes.

One concern about the use of ASFV vaccines is the genetic diversity of strains circulating in some countries. Recent experiments have demonstrated cross-protection between different genotypes ([8]Zsak personal communication), and therefore it may be possible to develop vaccines that can cross-protect against infection with several genotypes. The ability of diverse ASFV isolates to stimulate immune lymphocytes from ASFV immunised pigs was indicated to correlate with cross-protection [8]. In another study ability of sera from recovered pigs to inhibit haemadsorption was correlated with cross-protection [21, 67]. This suggests antibodies against the virus CD2-like protein (hemagglutinin) may be important for cross-protection. Further work is required to understand the immune mechanisms and antigens involved in determining cross-protection and establishing methods to predict and test this.

**DECISION MODEL**
The ASF Countermeasure working group (ASFCWG) used the quantitative Kemper-Trego (KT) decision model to assess ASF vaccine candidates for which published information was currently available. Consideration was only given to ASF experimental vaccine candidates for which publicly disclosed or published information was available as of April 2013. ASF vaccine candidates that are in the design phase or currently undergoing testing and evaluation, or ASF vaccine candidates that were tested in which the outcome was not publicly disclosed or published, were not ranked. Instructions for using the KT decision model were provided to the WG in advance of the discussion, however during this vaccine breakout session, criteria and weights in the model were modified for the purpose of assessing experimental ASF vaccines.

Criteria

The ASFCWG selected critical criteria to enable the comparison of experimental ASF vaccines (n=6) using a pertinent and valid analysis, as shown in the table below:

<table>
<thead>
<tr>
<th>Critical Criteria</th>
<th>Assigned Weight</th>
<th>Ideal Performance Metrics</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Efficacy</td>
<td>10</td>
<td>Prevents: mortality; morbidity; viremia; and transmission</td>
<td>Gap weight unchanged from 2009 review</td>
</tr>
<tr>
<td>Age Groups: all</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Safety</td>
<td>10</td>
<td>Age Groups: all no reversion to virulence (live vaccines) defined period of vaccine replication (live vaccines) acceptable injection site reactions short withdrawal period to slaughter</td>
<td>Gap weight increased from 2009 review (6)</td>
</tr>
<tr>
<td>Onset of Immunity</td>
<td>8</td>
<td>Rapid onset: 7-10 days</td>
<td>Gap weight decreased from 2009 review (10)</td>
</tr>
<tr>
<td>One Dose</td>
<td>8</td>
<td>Single inoculation site</td>
<td></td>
</tr>
<tr>
<td>DIVA Compatible</td>
<td>8</td>
<td>Will require companion assay</td>
<td></td>
</tr>
<tr>
<td>Distribution/Supply</td>
<td>8</td>
<td>Vaccine manufacturer has effective distribution capability</td>
<td>Gap weight increased from 2009 review (2)</td>
</tr>
<tr>
<td>Cross-Protection</td>
<td>6</td>
<td>Affords an acceptable level of efficacy against other genotypes/diverse geographic isolates</td>
<td>Criterion was not considered in 2009 review</td>
</tr>
<tr>
<td>Duration of Immunity</td>
<td>6</td>
<td>1 year minimum</td>
<td>Criterion was not considered in 2009 review</td>
</tr>
<tr>
<td>Cost to Implement</td>
<td>6</td>
<td>Comparable to other vaccine used in ASF endemic countries</td>
<td>Gap weight increased from 2009 review (2)</td>
</tr>
<tr>
<td>Mass Administration</td>
<td>4</td>
<td>Can be easily and rapidly administered to pigs of all ages; does not require special delivery device for administration</td>
<td>Criterion was not considered in 2009 review</td>
</tr>
<tr>
<td>Shelf-life</td>
<td>4</td>
<td>2 year minimum; no cold chain for deployment</td>
<td>Gap weight increased from 2009 review (2)</td>
</tr>
<tr>
<td>Withdrawal time</td>
<td>not considered</td>
<td>Short withdrawal period for food consumption</td>
<td>Gap weight in 2009 review gap weight = 2</td>
</tr>
</tbody>
</table>
Weight

As shown in the 2nd column in the table above, each criterion was weighted to allow a quantitative comparison of the impact of the selected interventions.

Product Profile

As shown in the 3rd column in the table above, each criterion had defined performance metrics to ensure a consistent and meaningful assessment.

Values

The values assigned by the ASFCWG for each of the interventions reflects the collective best judgment of ASFWG members (see Appendix II for results).

Immunology and Vaccines

There is no vaccine available for ASF. Attempts to vaccinate animals using inactivated virions have failed. Homologous protective immunity does develop in pigs surviving acute infection with moderately virulent or experimentally attenuated variants of ASFV. These animals develop long-term resistance to virus challenge with related isolates. Humoral and cellular immunity have been shown to be significant components of the protective immune response to ASF. However the actual immune mechanism(s) mediating that protection is still unclear. Additionally, the viral protein\proteins inducing the protective immune mechanism are still largely unknown. On the other hand, ASFV proteins have been shown to affect and modulate host immune responses in vitro. In some examples this can lead to virus attenuation.

Gaps

1) Identification of immune mechanism(s) mediating protection against the infection in swine.

2) Identification of the virus protein(s) responsible for the induction of protective immune mechanism.

3) Understanding the role of virus driven host immunomodulation in the process of virus infection in swine.

4) Permissive cell lines suitable for commercial development of live attenuated vaccines.

5) Appropriate licensed vectors for delivery of antigens to pigs.

Research needs

1) Discovery of the immune mechanism mediating effective homologous and heterologous protection against virus infection.
2) Functional genomics to identify viral determinants that correlate with presence/absence of homologous versus heterologous protection.


4) Better knowledge of the role of ASFV encoded proteins on virus pathogenesis and modulation of the host responses to infection.

5) Better knowledge of ASFV host cell interactions to underpin selection of cell lines suitable for producing live attenuated vaccines.

Vaccines

The President’s National Strategy for Homeland Security calls for the development of “high efficacy vaccines” to better protect our Nation from attack by the use of biological threat agents.

The ASFCWG determined that no ASF vaccines are commercially available. The current research into a suitable vaccine for ASFV is limited to only a few groups worldwide. The most promising, potential, candidates are rationally attenuated recombinant live viruses. The use of live attenuated viruses as vaccines is a well-established system with good protective attributes. None of the experimental candidates evaluated had been tested sufficiently to determine if they could be developed commercially. The potential for development of rationally attenuated live vaccines is good. Previous work has highlighted both virulence and immunomodulation genes, which if removed could provide a strong candidate vaccine strain. It would be important to carry out extensive testing of the candidate strains to confirm safety and efficacy. The development of DIVA vaccines would be particularly critical in any outbreak situation. This could readily be achieved by producing antibodies against proteins encoded by the genes deleted or use of recombination technology to insert suitable markers. Work is ongoing in several laboratories to develop candidate live attenuated vaccines with an acceptable safety and efficacious profile.

Another potential approach for producing a live vaccine with an improved safety profile is through development of single cycle infectious viruses. These could be produced using a complementing cell line expressing a gene essential for virus replication, by placing one or more essential virus genes under control of an inducible promoter or by manipulating the virus to enable entry and gene expression in pig macrophages but not production of infectious progeny. A number of viruses have been successfully engineered to place essential genes under control of an inducible promoter and some of these produce empty virus particles [68][]. However the potential of these viruses to induce protection in pigs has not been tested. The alternative to a live attenuated virus that would remove any risk of reversion to virulence is the use of a subunit vaccine. This would satisfy both safety issues and ensure good DIVA characteristics. Studies published to date have achieved only partial protection (up to 30%) using either recombinant proteins or by DNA vaccination. However these results are promising and are currently being pursued by testing of additional antigens and delivery
The current research is a long way from producing a working vaccine, although the information generated by the research will help generate effective vaccines in the future.

Summary

Vaccination against ASF is currently not an option. A focused drive on producing a rationally attenuated live virus vaccine would help to bring a viable vaccine online in the shortest timescale. Alternative approaches to produce vaccines, including identification of protective antigens and testing of different delivery systems should be pursued in parallel.

Assessment of Experimental Vaccines

The ASFCWG on Vaccines discussed the characteristics of the different available experimental vaccines for which published data was available at the date of assessment (April 2013). Following is a summary of the group’s opinion for each of them.

Introduction

As a general comment, four recurring themes provided the framework for the ranking process:

1. All current vaccine candidates are supported in most of the cases by only one or a very limited number of scientific reports, indicating that all candidate vaccines are at the discovery or exploratory stage.
2. The presence of potential residual virulence in the live, attenuated vaccines (LAV). Nevertheless, it was stressed that historical eradication of several swine diseases (e.g., CSF, pseudorabies) using LAVs has been achieved in which safety was a key attribute of the product profile.
3. The need of having a DIVA capability is relevant for the use in developed countries but less significant for developing countries.
4. Although the ideal profile for an ASF vaccine for use in an endemic versus disease free area will most likely differ, group consensus was that a single target profile would be used for both scenarios.

As a starting point, the ASFCWG performed a classification of the experimental vaccines tested so far. The working group acknowledged the practical limitations of the mandated score system due in large part to the relatively low number of published studies for each vaccine candidate. For example, some of the critical criteria evaluated and scored (onset of immunity, distribution, duration of immunity, and cost of implementation) were not assessed or discussed in these published studies.

A total of six vaccines candidates were evaluated – four live, attenuated viruses, one DNA-based, and one recombinant subunit.

In general, all four LAV strains present similar advantages and disadvantages. The main concern is the presence of possible residual virulence of the attenuated strains. None of the candidates have been designed harboring antigenic markers to support a DIVA strategy but knowledge of genes deleted provides a route to distinguishing infected from vaccinated animals. Also, none of the LAV strains are adapted to standardized cell lines for potential commercial vaccine production.

A general recommendation regarding the development of LAV candidates was the amplification of the functional genomics approach to further identify novel genes involved in virus attenuation and to evaluate the effects of deletions of more than one gene. Another recommendation
was to increase studies evaluating the level of virulence and transmission of the current LAVs. Another requirement is the development of well characterized cell lines that meet regulatory requirements for the replication and commercial production of the LAVs. It was also recommended to evaluate the development of LAVs based on single cycle infectious virus strains.

**Assessment of ASF vaccine candidates**

**A. Live attenuated ASF vaccine candidates**

*General Comments* - With the exception of the LAV homologous recombination at the Plum Island Research Centre (USA), the other three candidates are “classical” live attenuated viruses, two of them isolated *in vivo* (OURT88/3 and NHVP68) and the last one obtained after adaptation of a virulent, parental virus to an established cell line CV1 (E75CV1).

1. **Live attenuated ASFV strain OURT88/3**
   The OURT88/3 strain was isolated from *Ornithodoros erraticus* ticks collected on pigs in farms of the Ourique district located in the Alentejo province in Portugal in 1988 (Boinas *et al*., 2004). OURT88/3 is a natural non-haemadsorbing and non-pathogenic strain. Comparison of the genome of the OURT88/3 isolate with that of isolates Benin 97/1 demonstrates deletions of 8–10kbp (encoding five copies of the multigene family (MGF) 360 and two copies of the MGF 505/530. In addition, OURT88/3 isolate has interruptions in ORFs that encode CD2-like and C-type lectin protein [69](Chapman *et al*., 2008). OURT88/3 inoculation of pigs at a dose of $10^4$ TCID$_{50}$ by intramuscular (IM) route induced no detectable viremia and pigs were protected against challenge with virulent virus OURT88/1 after 21 days using $10^4$ HAD$_{50}$ intramuscularly with no disease signs or detectable viremia. Cross-protection experiments were also carried out and OURT88/3 induced partial protection against Lisbon ’57 isolate but no protection against Malawi LIL 20/1 isolate [7](Boinas *et al*., 2004). In another study pigs immunised with the OURT88/3 isolate followed 3 weeks later by the virulent OURT88/1 isolate were protected against the challenge 3 weeks later with both the West African genotype I isolate, Benin 97/1. In two experiments 100% protection ($\text{n}=9$) was observed and in another experiment 60% pigs ($\text{n}=6$) were protected. Pigs were also protected against challenge with the genotype X virulent Uganda 1965 (100% protection, $\text{n}=4$) isolate [8](King *et al*., 2011). Cross-protection induced by the OURT88/3 strain was correlated with the stimulation of lymphocytes from OURT88/3 immunised pigs by different ASFV isolates as measured by IFN gamma ELISPOT assay. In additional experiments [24](Oura *et al*., 2005) all 9 pigs immunized with OURT88/3 were protected against challenge with OURT88/1. Depletion of CD8+ T cells abrogated protection in another groups of pigs [24](Oura *et al*., 2005).

2. **Live attenuated strain MalΔ9GL**
   This virus (MalΔ9GL) is a recombinant virus obtained by genetic manipulation of the virulent strain Malawi [70]. MalΔ9GL is a recombinant virus obtained by genetic manipulation of the virulent strain Malawi [70]. MalΔ9GL is a recombinant virus obtained by genetic manipulation of the virulent strain Malawi [70]. The 9GL gene and the rat ALR. 9GL was removed from the Malawi genome by genetic recombination. 9GL is present in all ASFV isolates and is highly conserved at the protein level. Disruption of this gene was shown to lead to a growth defect in tissue swine primary cell cultures, in soft ticks and most importantly in swine. All pigs ($\text{n}=12$) inoculated with MalΔ9GL (doses $10^4$ to $10^6$ HAD50) survived the primary infection without showing clinical signs associated with ASFV and the subsequent challenge with homologous virulent Malawi isolate at 42 days post-primary inoculation. At this time circulating antibodies against ASFV were undetectable by binding assays. Subsequent experiments (Zsak *et al*; in preparation) in which two other virus isolates,
both geographically and temporally distinct from each other, were deleted at the 9GL gene, both yielded attenuated derivatives that elicited protection against the challenge with the homologous virulent virus. Additionally, when pigs (n=24) infected with a Pret4 were challenged with ASFV isolates obtained in Kruger National Park (where Pret 4 was isolated) all swine survived. The virulent challenge viruses have distinct restriction maps thus indicating the possibility of at least a limited heterologous protection as an attainable objective. Therefore, 9GL deletion strategy provides a promising basis for the generation of an efficacious rationally designed ASFV LAV.

3. Live attenuated strain E75CV
This virus isolate (E75CV1) was obtained after adaptation of ASF virulent virus isolate E75L in an established cell line CV1 (fibroblast derived from kidney of Cercopithecus aethiops). E75L virus was blindly passed on CV1 cells until cytopathic effect was apparent (normally three to four passages). The resulting virus grows well both in CV1 cells and in macrophages. E75CV has been used in CISA/INIA and PIADC for many years with up to 100% of the immunised pigs surviving the infection after challenge with the virulent parental strain E75L [13, 21, 71, 72](Ruiz Gonzalvo et al., 1986; Gómez-Puertas et al., 1996; Ramiro-Ibáñez et al., 1997; Onisk et al., 1994). Importantly, E75CV appears to have a very narrow safety margin with respect to immunizing dose. All animals which received an intramuscular dose at 10⁴ TCID50 survived, presenting only minor transient clinical signs. However, inoculation of pigs with a higher dose resulted in death of some pigs and lower doses reduced protection (Rodriguez et al., unpublished data). Although protection is consistently achieved against homologous E75 virus, no protection was detected against geographical related strains E70 or BA71 [21](Ruiz-Gonzalvo et al., 1986).

4. Live attenuated strain NH/P68
The NH/P68 isolate was obtained from a chronically infected pig in Portugal in 1968. Pigs immunised with NH/P68 can be protected against challenge with the virulent Lisbon 60 isolate [23](Martins et al., 1993) although some pigs develop a chronic form of disease characterised by late onset of fever, necrotic skin lesions and swelling of joints. These lesions were observed in 47% of pigs (n=19) immunised by the intramuscular route with 5X10⁶ CPE50 and in 25% of those (n=12) immunized by the oral nasal route. All of the healthy pigs survived challenge with Lisbon 60 virus [22](Leitao et al., 2001). A correlation was observed between increase in numbers of NK cells in healthy compared to diseased pigs. In addition the diseased pigs developed hypergammaglobulinaemia. The chronic form of disease observed in a high percentage of pigs raises concerns about the safety of this strain although it should be noted that a high dose of virus was administered and the minimum effective dose has not been determined.


B. ASF subunit vaccine candidates

1. Recombinant proteins p30, p54 and p72

Data in the mid-nineties described partial protection of pigs immunized with ASFV structural proteins. In one study administration of p54 and p30 proteins individually or expressed as a chimera, in a baculovirus system and administered without further purification together with Freund’s adjuvant, modified the course of disease and induced variable levels of protection from 50% in 6 pigs immunized with the individual proteins to 100% for 2 pigs immunized with the chimeric protein [17, 73]. In a second study administration of recombinant proteins p30 and p54 together with p72 protein produced in baculovirus induced neutralizing antibodies but no protection in 6 pigs [63].

2. DNA vaccination with chimeric p54, p30 and CD2-like gene

More recently the putative protective capabilities of p54, p30 and CD2-like proteins were extended into the field of DNA immunization. Immunization with a plasmid (pCMV-UbsHAPQ) encoding three ASFV antigens: p30, p54 and the extracellular domain of the CD2-like gene fused to ubiquitin (a genetic construct that allowed optimal intracellular presentation of the antigens in the SLAI context), induced partial protection against ASFV lethal challenge. Thirty-three percent of the immunized pigs (n=12) survived the lethal challenge in two independent experiments, while all controls died before day 8 post-infection [26]. It is important to note that the protection was evaluated using a heterologous ASFV-strain that shares the same HA amino acid sequence and the DNA vaccine sequence.

3. Baculovirus recombinant CD-2 like gene

A recombinant baculovirus encoding the ASF hemagglutinin (HA) gene, with homology to the thymocyte surface antigen CD2, was constructed. The baculovirus-expressed HA showed hemadsorption and erythrocyte-agglutinating activities characteristic of the CD2 homolog protein induced by the virus in infected macrophages. In a small pilot study, pigs immunized with the recombinant HA developed hemagglutination -inhibition and temporary infection-inhibition antibodies that recognize a 75-kDa structural protein and were protected against lethal infection in a dose dependent manner. Pigs were IM inoculated with three doses (the first using of Freund’s complete adjuvant and incomplete in the following doses) at monthly intervals using insect cells infected with the baculovirus expressing the HA protein obtained from the attenuated ASFV isolate E75CV. Results obtained with sera from immunized pigs suggest that epitopes in HA responsible for hemaglutination and infection-inhibition may be different. Immunized pigs were challenged intramuscularly with $4 \times 10^2$ TCID$_{50}$ of the virulent strain of ASFV E75. Control pigs died between days 7-8 post infection days 7–8 and the plasma virus titers on day 6 were from $10^6.69$ to $10^7.02$. In contrast, all pigs inoculated with the recombinant HA survived virulent challenge. In one vaccinate inoculated with the highest dose of recombinant HA, no viremia was detectable. In the remaining two vaccinates, pigs were viremic for at least 28 days with viral titers ranging between $10^5.35$ and $10^6.02$. No virus was isolated later than 42 days post infection [62].

Decision Model Summary.

Based on the available published data and the expert opinions of those attending the vaccine discussion group the vaccine candidates described above were ranked according to the scoring
matrix provided. As expected the profile of all the candidate live attenuated vaccines (LAVs) grouped quite closely and minor differences in scoring usually reflected the scope of the experiments reported. Generally LAVs scored highly for efficacy and the ability to induce protection with one dose. LAVs are expected to score highly for duration of immunity but this has only been tested for LAV-E75. The main concern with all of the LAVs is the safety profile and very little information is currently available on this. For the subunit vaccines recombinant CD2-like protein produced in baculovirus and the DNA vaccine based on the p54, p30 and CD2-like protein were considered. Each of these scored highly for safety and DIVA capability compared to LAVs. They scored much lower for efficacy, one dose delivery and onset of immunity. For the other criteria considered scoring of the LAVs and subunit vaccine were more comparable.

### Experimental Vaccines For ASF - April 3, 2013

<table>
<thead>
<tr>
<th>Weight</th>
<th>Critical Criteria</th>
<th>LAV - OURT88/3</th>
<th>LAV - Delta 9GL</th>
<th>LAV- NHVP68</th>
<th>LAV- E75</th>
<th>DNA Vx-CD2-p54-p30</th>
<th>Rec. subunit mix of cell extract -CD2</th>
</tr>
</thead>
<tbody>
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Rank each Criteria 2,4,6,8 or10 on each criterion -- no more than two "10" rankings allowed

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**Major Assumptions:**

Vaccine Profile

1. Highly efficacious: prevent mortality, prevent morbidity, prevent viremia, prevent transmission; efficacy in all age pigs, cross protection across all ASF viral strains; quick onset of immunity; one year duration for food consumption.

2. Safe in all age pigs; no reversion to virulence for live vaccines, defined period of replication for life vaccines.

3. 1 doses

4. Cross-protection

5. Rapid Onset of immunity 7 to 10 days.

6. Rapid speed of production and scale-up

7. Mass administration -

8. Duration of immunity - minimum of 1 year

9. DIVA -need

10. Shelf-life 2 years, no cold chain

11. Cost to implement