DYNAMICS OF PROTECTION AGAINST VIRULENT CHALLENGE IN SWINE VACCINATED WITH ATTENUATED AFRICAN SWINE FEVER VIRUSES

by

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B.S., University of Connecticut, 2005
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Abstract

African swine fever (ASF) is a lethal hemorrhagic disease of swine caused by a double-stranded DNA virus. ASFV is endemic in Sardinia and Saharan Africa and has been recently expanded from the Caucasus to Eastern Europe. There is no vaccine to prevent the disease and current control measures are limited to culling and restricted animal movement. Swine infected with attenuated strains are protected against challenge with a homologous virulent virus, but there is limited knowledge of the host immune mechanisms generating that protection. Swine infected with Pret4 virus develop a fatal severe disease, while a derivative strain lacking virulence-associated gene 9GL (Pret4Δ9GL virus) is completely attenuated. Swine infected with Pret4 Δ9GL virus and challenged with the virulent parental virus at 7, 10, 14, 21, and 28 dpi showed a progressive acquisition of protection (from 40% at 7 dpi to 80% at 21 and 28 dpi). This animal model was used to associate the presence of host immune response and protection against the challenge. The presence of anti-ASFV antibodies, as well as ASFV-specific IFN-γ production in PBMCs, and cytokines in serum were assessed in each group. Interestingly, with the exception of ASFV-specific antibodies in the surviving swine challenged at 21 and 28 dpi, no solid association between any of the parameters assessed and the extent of protection could be established. These results were corroborated using a similar model based on the use of a rationally attenuated derivative of the highly virulent strain Georgia 2007. These results, encompassing data from over 114 immunized swine, underscore the complexity of the system under study where it is very plausible that protection against disease or infection relies heavily on the concurrence and or interaction of different host immune mechanisms.

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Dedication

To my mother Christine, no matter where I am going or what I am doing I will always have my memories of you.

"Those we love don't go away, they walk beside us everyday. Unseen, unheard, but always near, still loved, still missed and forever dear." –Unknown

Chapter 1 - Introduction to African swine fever virus

1.1 African swine fever

1.1.1 African swine fever virus

African swine fever (ASF) is a highly contagious hemorrhagic disease of domestic swine, feral swine, and wild boar (*Sus scrofa*) first described in 1921 in Kenya by Montgomery (Eustace Montgomery, 1921). This disease is transmitted directly between infected swine by the oro-nasal route, indirectly by ingestion of contaminated food or through feeding by infected tick vectors. Aerosol transmission occurs only over very short distances. Unlike domestic swine, infections of other susceptible members of the *Suidae* family, including the warthog (*Phacochoerus aethiopicus*), giant forest hog (*Hylochoerus meinertzhageni*), Red River hogs (*Potamochoerus porcus*), and Bushpigs (*Potamochoerus larvatus*) are generally asymptomatic with low viremia (Anderson *et al.*, 1998; Luther *et al.*, 2007; Oura *et al.*, 1998; Sanchez-Vizcaino *et al.*, 2012). It is thought that these asymptomatic carriers are reservoir hosts for the virus in Africa, but further investigation is necessary since several species of suids have different roles in the epidemiology of ASF depending on taxonomy, geography, and the extent of contact between domestic swine and wildlife (Jori and Bastos, 2009). Although the literature describes a specific relationship between soft ticks and wild suids including warthogs and bushpigs in East and Southern Africa (Plowright, 1981; Thomson, 1985), this does not apply to all of Africa. For instance, in West African nations a feral swine epidemiology of ASF has not been established and the existence of a sylvatic cycle involving the connection between soft ticks and warthogs is still in question (Jori *et al.*, 2013). ASFV can be maintained in a sylvatic cycle between *Suidae* species and argasid

ticks including *Ornithodoros moubata* and *erraticus*, but two additional virus cycles are also recognized in Africa's endemic areas including a pig-tick cycle where warthogs are not a part of the cycle and another where ASFV persists in domestic swine without any sylvatic host, invertebrate or vertebrate (Jori and Bastos, 2009; Penrith and Vosloo, 2009). Young warthogs become infected when bitten by infected ticks and develop a transient viremia for a few weeks that is sufficient to infect more ticks (Thomson, 1985; Thomson *et al.*, 1980). Both wild boar and feral swine are highly susceptible to ASFV and develop clinical disease with mortality rates similar to domestic swine (Denis, 2014). The exact role of bushpigs still eludes us, although there are reports demonstrating them as free-living hosts infected with ASF experimentally and under natural conditions (Denis, 2014; Luther *et al.*, 2007; Oura *et al.*, 1998). The giant forest hog in Africa has also demonstrated active infection but its role in ASF spread still remains to be understood (Penrith and Vosloo, 2009).

Following the spread of ASF into Portugal in 1957 as a result of waste from airline flights being fed to pigs near Lisbon, the disease was not eradicated from the Iberian Peninsula until the mid 1990's (Costard *et al.*, 2009). The tick vector was implicated as a long-term reservoir host, as the disease reappeared on a Portuguese farm where infected ticks were discovered in 1999 (Costard *et al.*, 2009). Adult ticks and large nymphs can survive for up to 5 years in the presence of swine to feed on (Oleaga-Perez *et al.*, 1990). These factors alone make the control and eradication of ASF extremely difficult.

ASF is a severe disease with drastic socio-economic consequences, and it is notifiable to the World Organization for Animal Health (OIE). There are no vaccines

available and the only methods of control are surveillance, epidemiological investigations, strict quarantine and biosecurity measures, control of animal movement, and finally slaughter of all infected animals (Beltrán-Alcrudo *et al.*, 2009). These control measures significantly impact the national and international trade of pigs and pig products.

1.1.2 Classification

The etiologic agent of ASF is African swine fever virus, an enveloped double-stranded DNA virus of the genus *Asfivirus* within the *Asfarviridae* family. This virus shares features of *Poxviridae*, *Iridovividae*, *Phycodnaviridae*, and *Mimiviridae* (Tulman *et al.*, 2009). It is the only arthropod-borne (arbo) virus with a double-stranded DNA genome.

1.1.3 Morphology and structure

ASFV particles have a complex multi-layered structure (Carrascosa *et al.*, 1984). The virion is approximately 200 nm in diameter (Breese and DeBoer, 1966) and contains more than 50 polypeptides. The virion core is about 80 nm in diameter and it is comprised of an electron-dense nucleoprotein enclosed by a matrix or core shell (Andres *et al.*, 2002; Andres *et al.*, 1997). The core contains the virus genome, enzymes, and other proteins necessary for the early stages of infection after viral entry (Dixon *et al.*, 2008). The core or thick protein layer is surrounded by two lipid bilayers, called the inner membrane. The icosahedral capsid is just external to this inner membrane and is composed of the structural protein p72 encoded by B646L (Salas and Andres, 2013). The capsid is formed by 1892 to 2172 hexagonal capsomeres (Andres *et al.*, 2001; Breese and DeBoer, 1966; Carrascosa *et al.*, 1984). This makes up about

one third of the protein content of the virion (Tulman *et al.*, 2009). ASFV capsomeres are in a hexagonal lattice arrangement individually consisting of 13 nm hexagonal arrangements with a central hole (Carrascosa *et al.*, 1984). The outside of the capsid is a loose external membrane obtained by virion budding through the plasma membrane, which is not necessary for virus infection. Two-dimensional analysis of Percoll gradient purification of extracellular ASFV revealed 54 structural proteins with molecular weights from 10,000 to 150,000 Daltons (Esteves *et al.*, 1986).

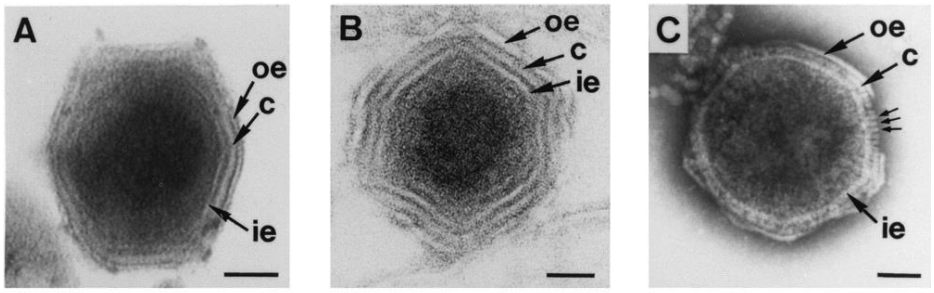

Figure 1.1 Structure and assembly of extracellular ASFV particles

Electron microscopy with epon sectioning (A), cryosectioning (B), and negative staining (C) (Andrés *et al.*, 1998). Electron microscope methods show a central core surrounded by three layers: the inner envelope (ie), the capsid (c), and the outer envelope (oe). Small arrows in C are individual capsomeres. Bars: 50 nm. (Andrés *et al.*, 1998).

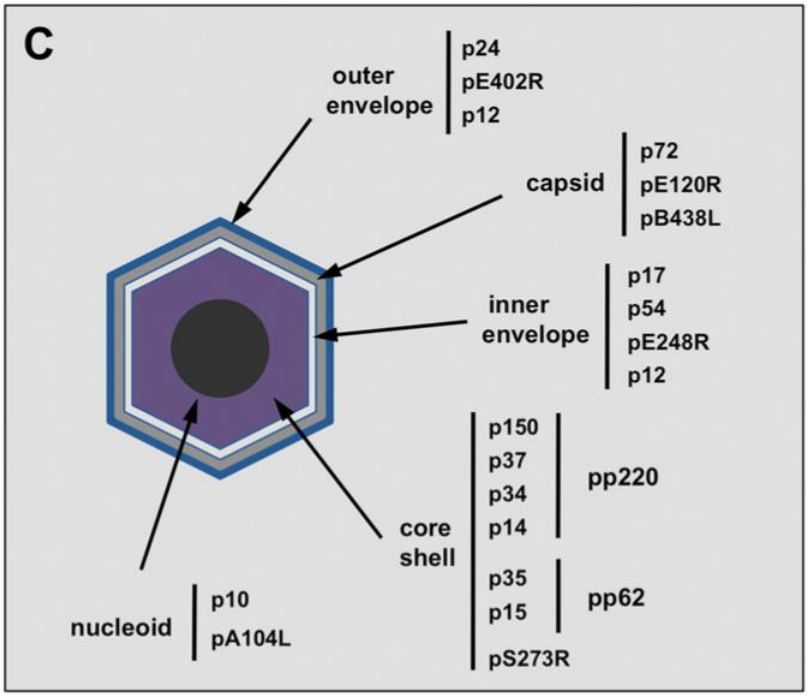

Figure 1.2 Structure and protein composition of ASFV (Salas and Andres, 2013).

1.1.4 Genome

The linear double-stranded DNA genome has 170-193 kbp in 151-167 open-reading frames (ORFs) located on both strands (Blasco *et al.*, 1989b; Dixon *et al.*, 2013b; Tabares *et al.*, 1980; Tulman *et al.*, 2009). The length of the genome varies due to the gain or loss of sequences from the 5' and 3' ends of the genome, which encode copies of six multigene families (MGF100, MGF110, MGF300, MGF360, MGF530, and P22) (Dixon *et al.*, 2008). The organizational makeup of these gene families may shed light on viral evolution by gene duplication and antigenic variation. Multiple copies of multigene families may give the virus a selective advantage allowing immune evasion (Dixon *et al.*, 2008). The large ASFV genome comprises several genes involved in nucleotide metabolism, transcription, DNA replication and repair, immune evasion, and the regulation of apoptosis (Dixon *et al.*, 2013b).

The genomic locations of some of the structural proteins have been identified. P220 (CP2475L) and pp60 (CP530R) form part of the core shell. P54 (E183L) is an important membrane protein in the internal envelope. The external envelope of the virus particle contains CD2v, the hemadsorption protein encoded by ORF EP402R (Rodriguez *et al.*, 1993). P72 (B646L) is a major virus capsid component that is used for genotyping.

Previously, twenty-two genotypes had been described based on the C-terminal end of the P72 gene (Bastos *et al.*, 2003; Lubisi *et al.*, 2005; Michaud *et al.*, 2007). ASFV genotyping mainly involves genes that code for the viral proteins p54 (E183L), p72 (B646L) and the central variable region (CVR) within B602L. Isolates from eastern and southern Africa are more diverse and separate into 21 genotypes; Western Africa, Western Europe including Sardinia, and South America cluster in genotype I (Bastos *et al.*, 2003; Boshoff *et al.*, 2007; Lubisi *et al.*, 2005). Genotype II mostly contains eastern European isolates (Gallardo *et al.*, 2014). More recently, Achenbach (Achenbach *et al.*, 2016) analyzed ASFV from domestic swine from 2011 to 2014 identifying a new genotype 23, sharing ancestry with genotypes 9 and 10 commonly found in eastern Africa and the Republic of the Congo. Phylogenetic studies reveal relative strain homogeneity among isolates from all sources in Western Africa, Europe, and America, and strain heterogeneity with isolates from Southern and Eastern Africa.

Malogolovkin (Malogolovkin *et al.*, 2015) has proposed a serogrouping classification of worldwide strains based on the extracellular portion of the CD2v protein using hemadsorption inhibition (HAI). HAI typing places ASFV into discrete serogroups that are not related to P72 capsid protein genotypes. For example, ASFV of serogroups

1, 2 and 4 fall into P72 genotype 1 (Malogolovkin *et al.*, 2015). The use of HAI is impractical for routine ASFV serogrouping since it requires live virus and convalescent serum from rarely surviving animals, because HAI antibodies appear late and at low titers during infection (Malmquist, 1963; Malogolovkin *et al.*, 2015; Ruiz Gonzalvo *et al.*, 1986b; Ruiz-Gonzalvo and Coll, 1993; Vigario *et al.*, 1974).

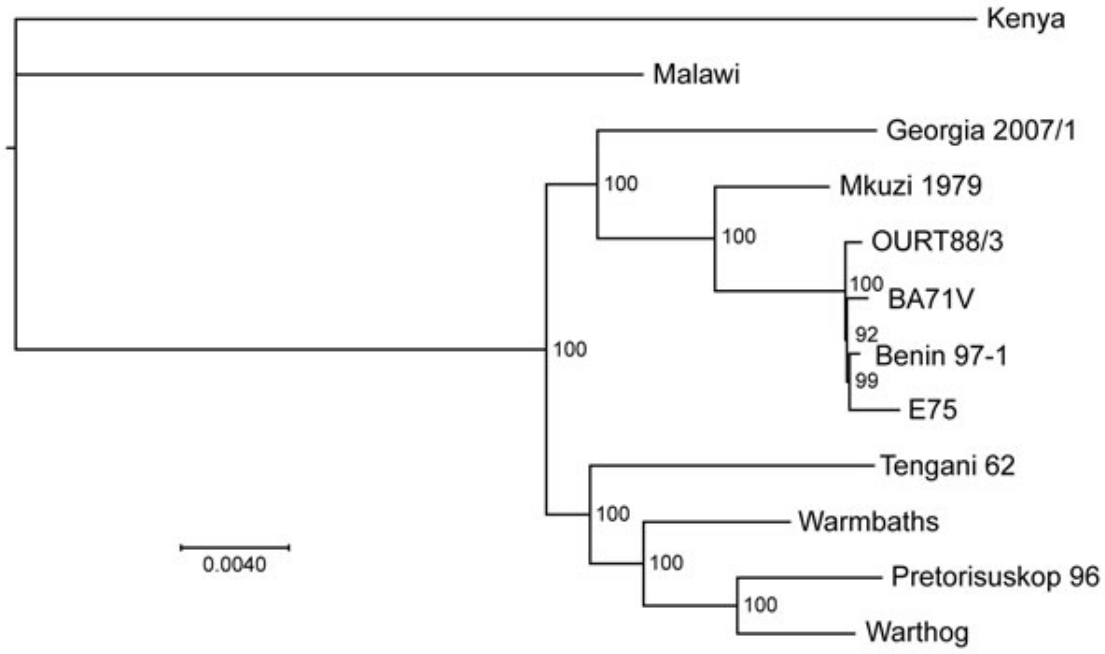

Figure 1.3 Comparison of the Georgia 2007/1 ASFV genome with those of other ASFV isolates (Chapman *et al.*, 2011).

The ASFV phylogeny midpoint was rooted in a neighbor-joining tree on the basis of 125 conserved open reading frame regions (40,810 aa) from 12 viruses. Node values show percentage bootstrap support (n = 1,000). The isolates shown and accession numbers are Kenya AY261360, Malawi Lil20/1 AY261361, Tengani AY261364, Warmbaths AY261365, Pretoriuskop AY261363, Warthog AY261366, Warmbaths AY261365, Mkuzi AY261362, OurT88/3 a.m.712240, BA71V NC_001659, Benin97/1 a.m.712239, and E75 FN557520. Scale bar indicates nucleotide substitutions per site (Chapman *et al.*, 2011).

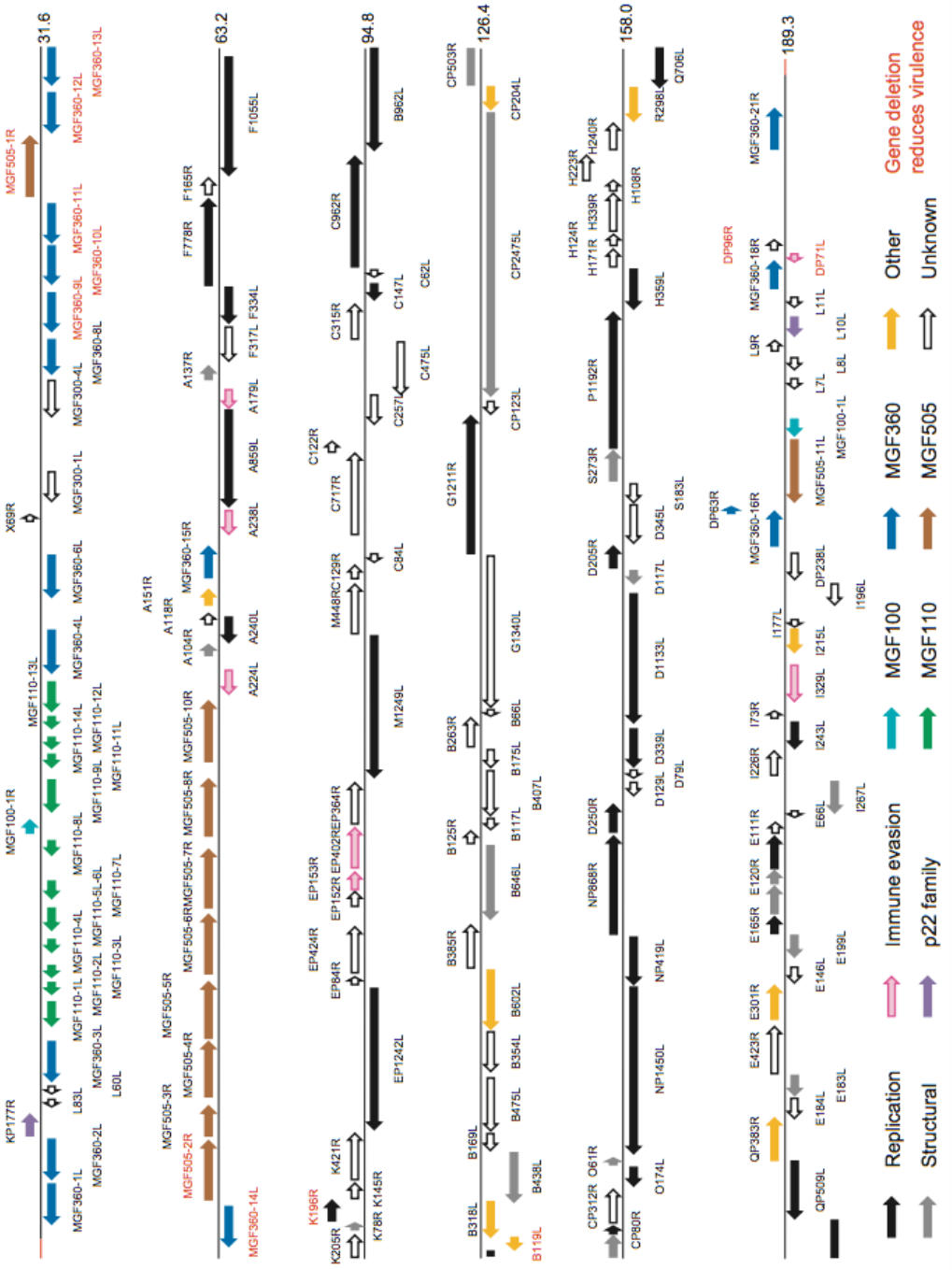

Figure 1.4 Organization of ASFV genomes (Dixon et al., 2013b).

The genomic organization of the Georgia 2007/1 isolate is shown in Fig 1.4 (Dixon *et al.*, 2013b). Black arrows indicate ORFs encoding enzymes involved in DNA replication, repair, or transcription. Grey represents ORFs for structural proteins. Pink indicates ORFs for evading host immune defenses. Multigene families are indicated by turquoise, blue, green, brown, and mauve. Yellow represents proteins with various other predicted functions, and white represents proteins of unknown function. Red represents ORFs whose deletion reduces virulence (Dixon *et al.*, 2013b).

1.1.5 Virus replication

ASFV infects monocytes in peripheral blood and macrophages found throughout the host. Infection has also been demonstrated in hepatocytes, endothelial cells, and renal tubular cells. The virus can also replicate in soft ticks of the genus *Ornithodoros* (Burrage, 2013; Casal *et al.*, 1984; Coggins, 1974; Fernandez *et al.*, 1992). Peripheral-blood mononuclear cell (PBMC) cultures infected with ASF virus show cytopathic changes (cell rounding and margination of nuclear chromatin) leading to degeneration of cells at 2-3 days and finally cell detachment at 4-8 days (Gomez-Villamandos *et al.*, 2013; Moulton and Coggins, 1968b). ASFV DNA replication occurs at 5-10 hours post-infection in cell culture (Tulman *et al.*, 2009), and from 8-10 hours post-infection onwards these cultures generally show hemadsorption or forming of rosettes after the addition of swine erythrocytes. ASFV morphogenesis occurs at the perinuclear viral factories near the Golgi complex and microtubule organizer center, surrounded by endoplasmic reticulum cisternae and mitochondria (Salas and Andres, 2013). A number of studies investigating viral entry used tissue culture cells. DNA replication and virus morphogenesis occurs within the cytoplasm close to the cell nucleus. Earlier studies

demonstrated that extracellular ASFV enters macrophages by receptor-mediated endocytosis (Alcami *et al.*, 1989, 1990). Receptor-mediated endocytosis, dynamin-dependent and clathrin-mediated endocytic pathways were also identified as the principal ways for ASFV entry in Vero cells, wild boar lung cells (WSL), and macrophages (Galindo *et al.*, 2015; Hernaez and Alonso, 2010). The presence of cholesterol in cellular membranes was found to be key in a successful ASFV infection. Phosphoinositide-3-kinase (PI3K) activity and actin-dependent endocytosis are also required (Galindo *et al.*, 2015). Other studies stated that ASFV triggers its own uptake by macropinocytosis (Hernaez *et al.*, 2016; Sanchez *et al.*, 2012).

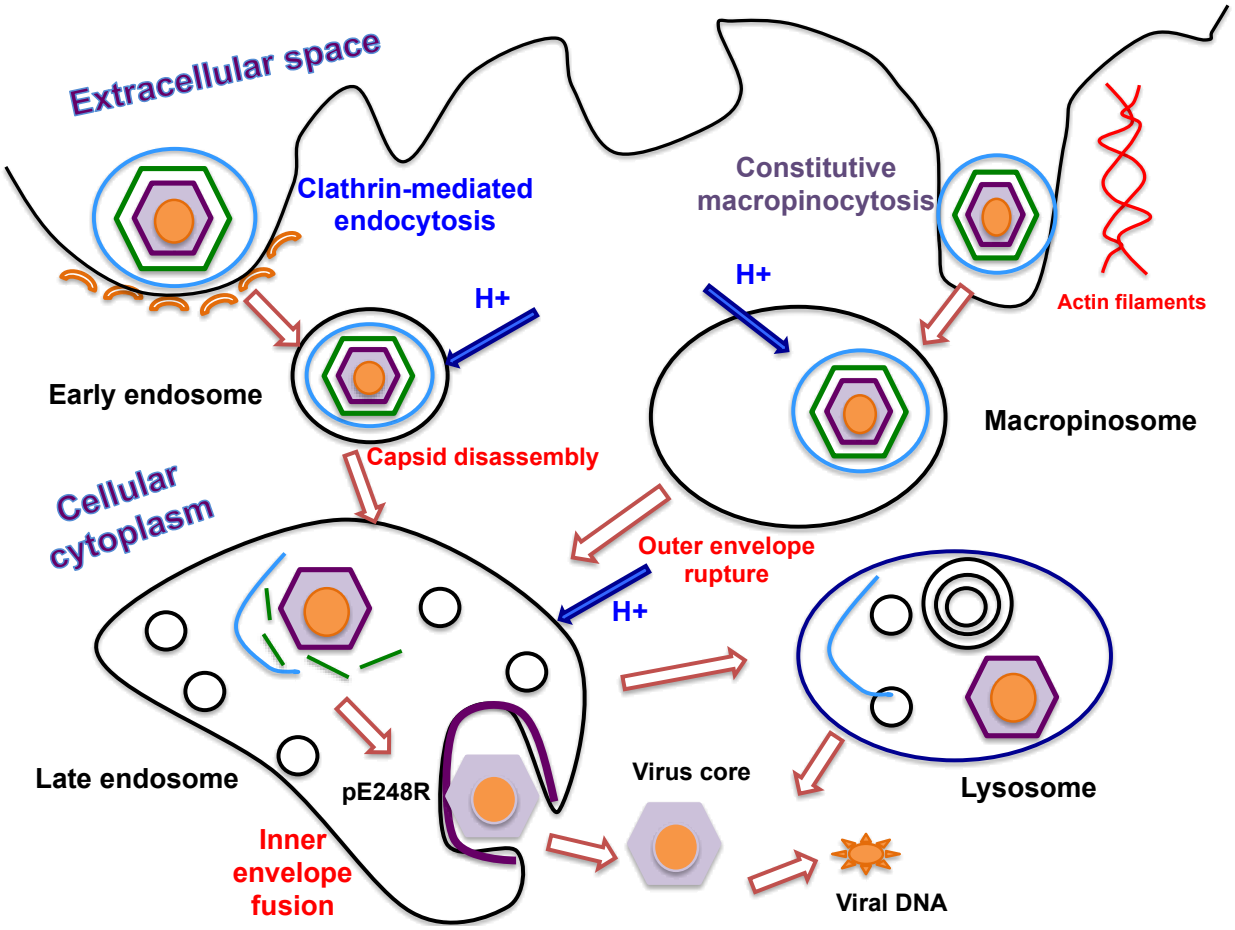

Figure 1.5 Diagram of ASFV internalization and uncoating, adapted from (Hernaez *et al.*, 2016)

In figure 1.5, ASFV is shown entering a macrophage by clathrin-mediated endocytosis and constitutive macropinocytosis. Once the virus is taken up by the cell, the particles are transported from early endosomes or macropinosomes to late endosomes. Next, these transported viruses undertake the un-coating process in low pH conditions necessary for capsid disassembly and disruption of the outer viral membrane. Afterwards, the inner viral envelope will fuse with the endosomal membrane where the genome within the naked core enters the cellular cytosol. Some of the disrupted particles may reach lysosomes, they may fuse or be degraded, as lysosomal hydrolases may contribute to virus disruption or un-coating (Hernaez *et al.*, 2016).

Once the virus enters the cells, the outer membrane is lost. ASFV is internalized in endosomes and then a low pH induces fusion between the viral membrane and vesicle membrane, causing the release of virus cores into the cytoplasm. Early mRNA synthesis begins in the cytoplasm using enzymes and factors packaged in the virus core. ASFV transcription occurs independently of host RNA polymerase, as productive infection requires the cell nucleus. The mechanisms of ASFV replication and transcription are similar to poxviruses, with the exception of viral DNA in the nucleus. Early virus proteins encode enzymes necessary for DNA replication, and subgenomic fragments at early stages of DNA replication of are found in the cell nucleus. Microtubules are necessary in the formation of perinuclear factories. DNA replication in the factories is followed by a shift to gene transcription to late virus genes. These genes encode structural proteins, enzymes, and factors required for virus particles in the next round of infection. The virus assembly takes place in factories, then the mature virions move on microtubules to the plasma membrane. ASFV particles exit from infected cells once transported from the virus factories to the plasma membrane. Other theories suggest the virus release and spread with the observation of ASFV inducing apoptosis at late stages of infection. Viral particles in apoptotic bodies may provide a means to evade the immune system by avoiding exposure of extracellular virions to the host immune system. Interestingly it has been speculated that macrophages also recognize scavenger receptors and may take up apoptotic bodies with virus particles (Dixon *et al.*, 2008).

In other studies, ASFV infection has been related to the maturation stage of the macrophage which is linked to the expression of the CD163 scavenger receptor

(Sanchez-Torres *et al.*, 2003), however it has also been found that ASFV can replicate in cells that do not express CD163 (Dixon *et al.*, 2013a; Lithgow *et al.*, 2014). This highlights the importance of further investigation in understanding ASFV replication and cell receptor usage.

1.2 African Swine Fever History, Global Distribution

1.2.1 Epidemiology and global impact

ASFV was first described in Kenya in 1910 (Eustace Montgomery, 1921) with the earliest outbreaks outside of Africa in Lisbon, Portugal, in 1957 (Blasco *et al.*, 1989a; Manso Ribeiro *et al.*, 1958; Vinuela, 1985). In 1959, a second outbreak occurred resulting in ASF spreading from the Iberian Peninsula to France, Italy, Malta, Belgium, and the Netherlands (Penrith *et al.*, 2009). In the late 1970s, Brazil (1978), Cuba (1977), the Dominican Republic (1978), Haiti (1979), and other Caribbean islands also reported ASF outbreaks. ASF first spread to West Africa in Senegal in 1978 and Cameroon in 1982, and recently information has emerged about the presence of ASF in Nigeria and Cape Verde in the 1960-70s (Penrith *et al.*, 2009). ASF historically has been introduced to disease-free areas by feeding of domestic animals with contaminated pork products imported via airports or seaports. ASF then establishes itself in the domestic pig population from where it disseminates further. The distribution of ASF changed in the 1990s and 2000s, as many countries including Côte d'Ivoire (1996), Nigeria (1997), Togo (1997), Ghana (1999), Burkina Faso (2003), Chad (2010), Madagascar (1998), Portugal (1999) and Mauritius (2007) reported outbreaks.

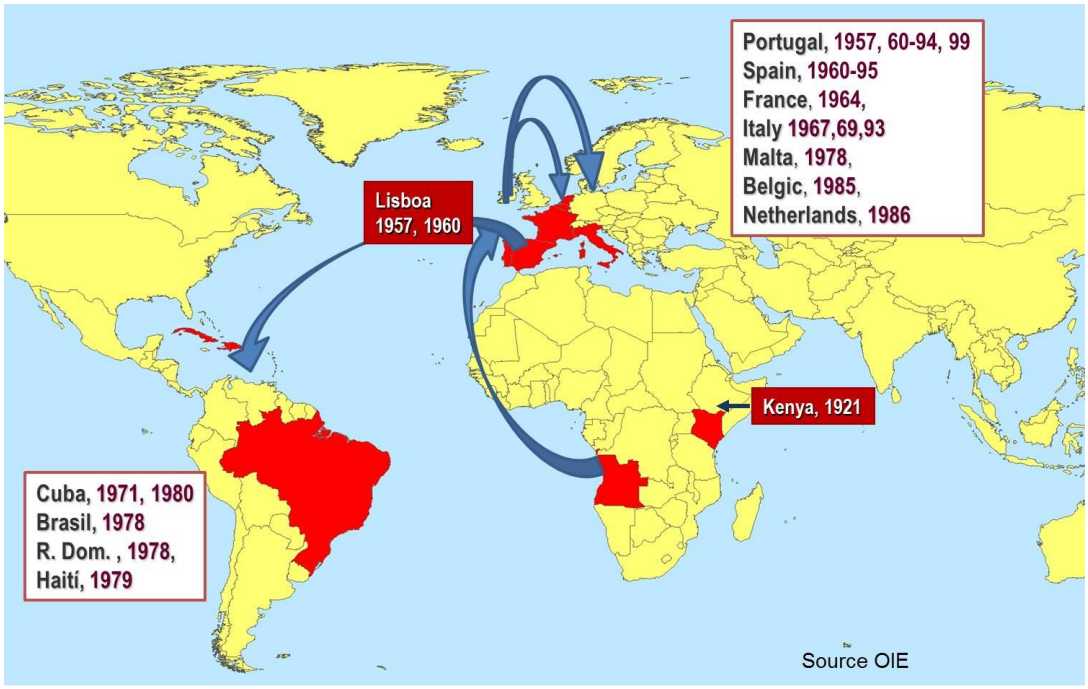

Figure 1.6 Map illustrating different ASFV outbreaks prior to the 2000's, from ASForce.org.

Several factors contributed to the spread of ASF to new territories, including globalization, the financial crisis forcing small farmers to feed swill or garbage to their animals, the high tenacity of ASFV in the environment and in meat products, as well as the occurrence of asymptomatic carrier animals. By the late 1990s many European countries had managed to eradicate ASF, with the exception of Italy (Sardinia). ASF is still endemic in Sardinia and affects domestic pigs, wild boar, and feral pigs. Carrier ticks are not found in Sardinia and are not involved in the transmission or persistence of ASF in this case. It is likely the free-range or backyard-farming systems that increase the risk of outbreaks (Mannelli *et al.*, 1997; Sanchez-Vizcaino *et al.*, 2015).

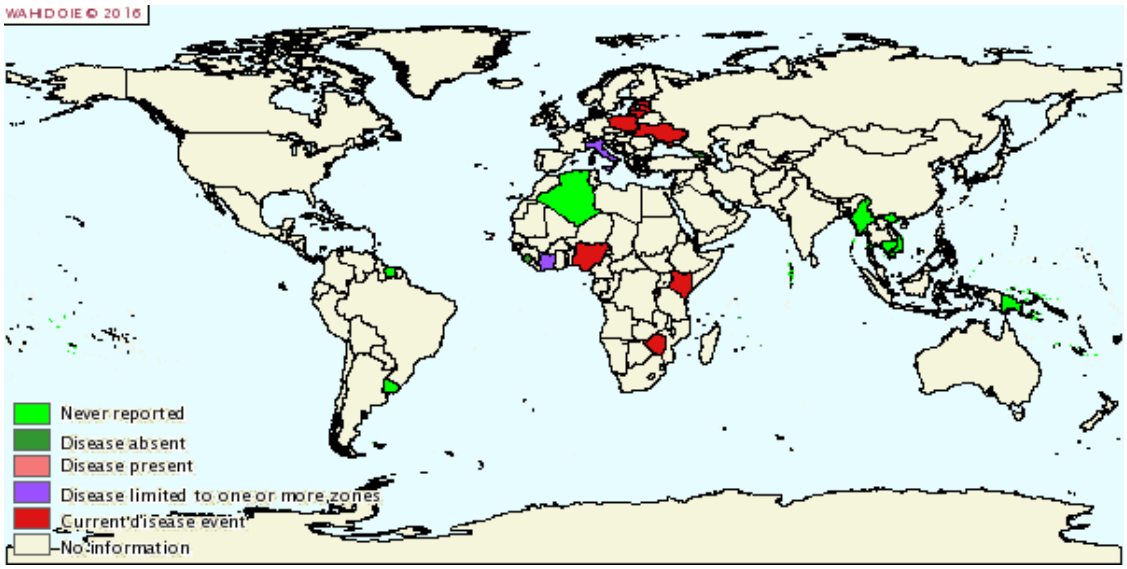

Figure 1.7 World Animal Health Information System (WAHIS) African swine fever distribution map January-June 2015 (OIE Accessed Feb 2016)

In April 2007, the Caucasus region (Georgia) reported several ASF outbreaks and since then the virus has spread to Armenia, Azerbaijan, Russia, Ukraine, Belarus, Estonia, Latvia, Lithuania, and Poland. Genetic studies of ASFV isolates from Russia and the Caucasus region suggest that one strain arrived in 2007 and subsequently spread. These isolates closely resemble p72 genotype II found in Mozambique, Madagascar, and Zambia (Rowlands *et al.*, 2008). It is thought that infected swill from international ships was fed to swine near the port of Poti on the eastern shore of the Black Sea, the site of first entry of ASF in the Caucasus region (Beltrán-Alcrudo *et al.*, 2009).

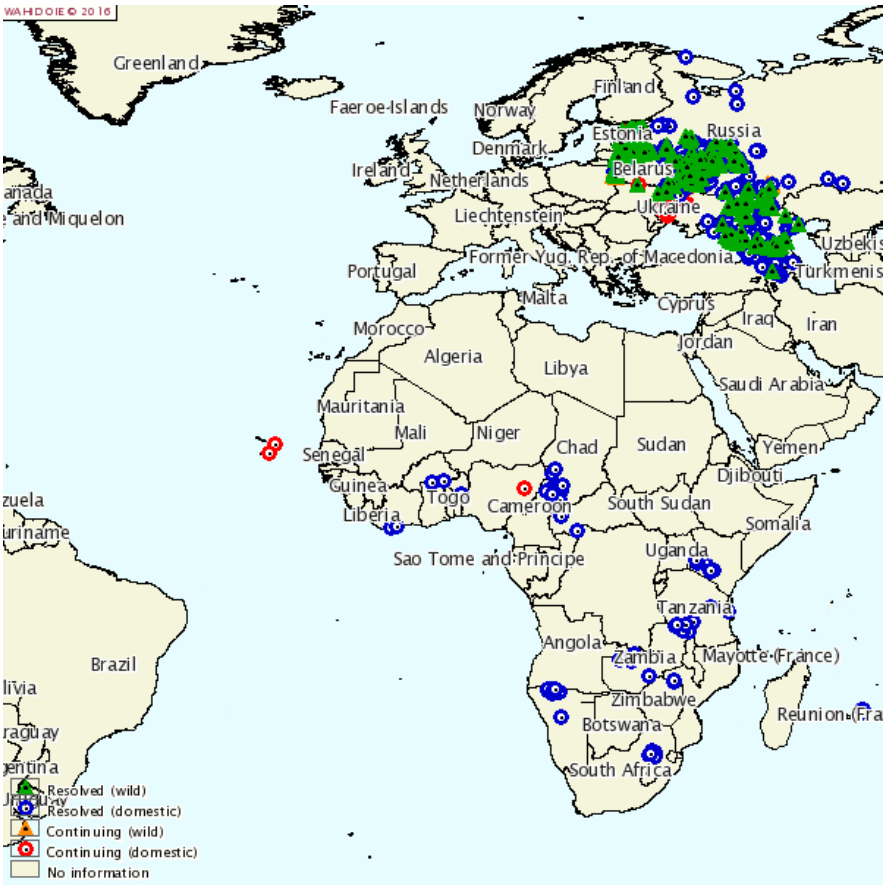

Figure 1.8 WAHIS African Swine Fever outbreak map April 2007-January 2016 (OIE, accessed Feb 2016)

1.3 ASFV pathogenesis

1.3.1 Clinical signs and lesions

The incubation period of ASF varies from 5 to 15 days with morbidity and mortality ultimately reaching 100%. Clinical disease is usually peracute or acute, but depending on the virulence of the virus strain, the clinical signs, duration, and course of an ASFV infection can vary from a highly acute clinical presentation with death in under 10 days to a long-term persistent infection. Many animals suffer from severe thrombocytopenia and lymphopenia during this clinical course, but ASF may manifest as subacute or chronic when less virulent strains are involved (Penrith *et al.*, 2009).

1.3.1.1 Peracute presentation of ASF

If swine are peracutely infected with ASFV, they are often found dead without prior clinical signs, but they can also be pyrexic, have rapid shallow breathing, can be recumbent or huddling together with other pigs, and fair-skinned pigs in particular may appear flushed.

1.3.1.2 Acute presentation of ASF

Swine suffering from acute ASF develop a persistent fever up to 42°C (107.6°F). Clinical signs may last two to seven days. Animals appear flushed to cyanotic with erythema on the tips of the ears, and pinpoint to ecchymotic hemorrhages may be visible on the skin. They may be recumbent or listless, and anorexic. Mucopurulent ocular and nasal discharges may be apparent. They appear to be uncomfortable, arch their backs, or show ataxia or lameness in their limbs. Infected swine may present with vomiting and bloody diarrhea. Swine may also suffer from dyspnea and bloody froth from the mouth and nostrils indicative of lung edema. Some swine may also develop central nervous signs including convulsions. Abortions can occur at any stage and usually result from the high fever; vertical transmission does not occur (Penrith *et al.*, 2009).

Figure 1.9 Swine inoculated with Pretoria parental strain 5 days post-challenge (own photos)

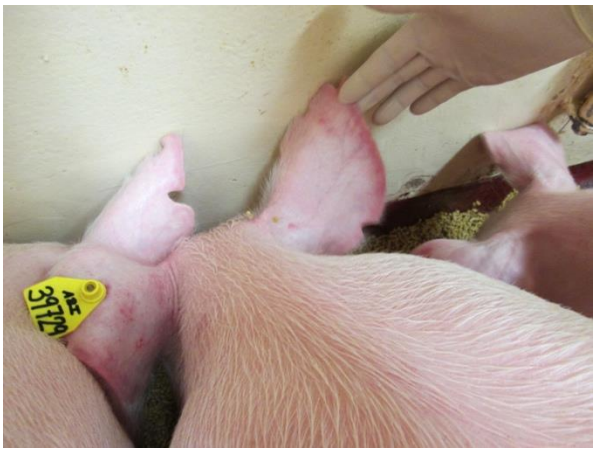

Figure 1.10 Swine inoculated with Pretoria parental strain, red tipped (erythema) ears 8 days post-challenge (own photo)

1.3.1.3 Subacute presentation of ASF

Subacute presentation of ASF with longer survival is seen in pigs when they are infected with a less virulent strain. These animals will have undulating fever and lose their body condition. Often interstitial pneumonia is observed and clinically presents as respiratory distress and moist coughing. Frequently secondary bacterial infections will ensue. Pigs can have swollen and painful joints. Pigs may die in weeks or months or they may recover and progress to a chronic form of the disease. Cardiac lesions may be present and result in acute or congestive heart failure (Penrith *et al.*, 2009).

1.3.1.4 Chronic presentation of ASF

Chronically infected swine lose body condition, may appear emaciated and their growth is stunted. Animals commonly have lameness and ulcers or pressure wounds over bony points. Often these pigs are prone to secondary bacterial infections and signs of pneumonia may also occur. These animals can survive for many months but a full recovery is unlikely (Penrith *et al.*, 2009).

1.3.1.5 Necropsy and lesions of ASF

The most common post-mortem lesion is an enlarged, friable, dark red to black spleen. Lymph nodes are enlarged and hemorrhagic. Most commonly lesions are found in the tonsils, and the gastrohepatic, trachealbronchial, mediastinal, renal, and mesenteric lymph nodes. Kidneys often have petechial hemorrhages. The stomach may be deeply congested or hemorrhagic on the mucosal surface. Hemorrhages may also be observed on the gall bladder and urinary bladder. Focal skin necrosis, fibrinous pericarditis, pericardial effusion, multifocal epicardial hemorrhages, ascites, swollen joints, and lungs with consolidation and edema often occur.

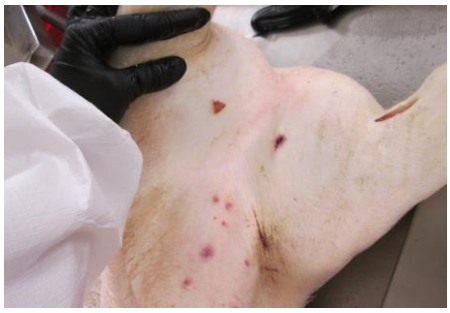

Figure 1.11 Swine inoculated with Pretoria parental strain: multifocal hemorrhages on skin (own photo)

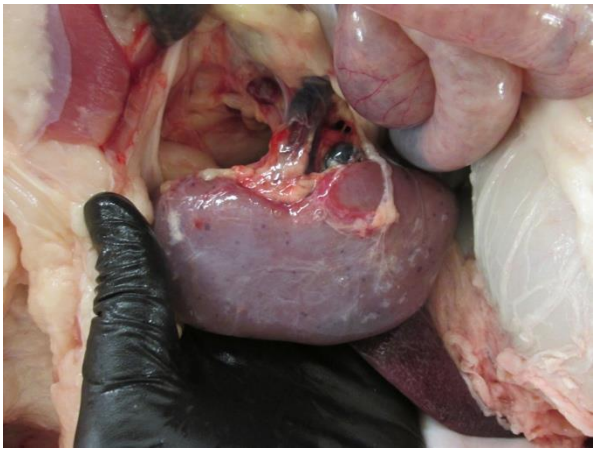

Figure 1.9 Swine inoculated with Pretoria parental strain: pinpoint focal hemorrhages on kidney, and hemorrhagic renal lymph nodes (own photo)

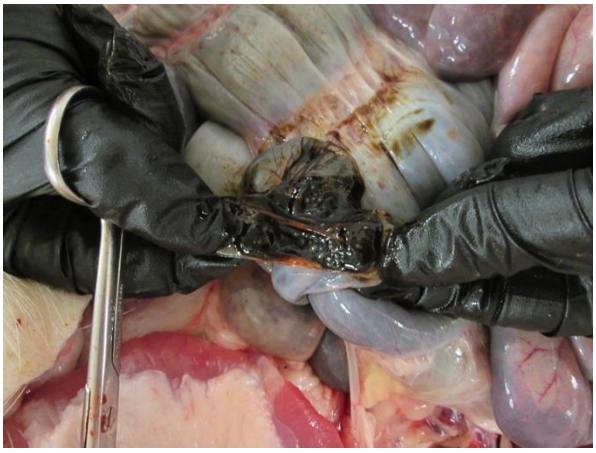

Figure 1.10 Swine inoculated with Pretoria parental strain, melena in intestines (own photo)

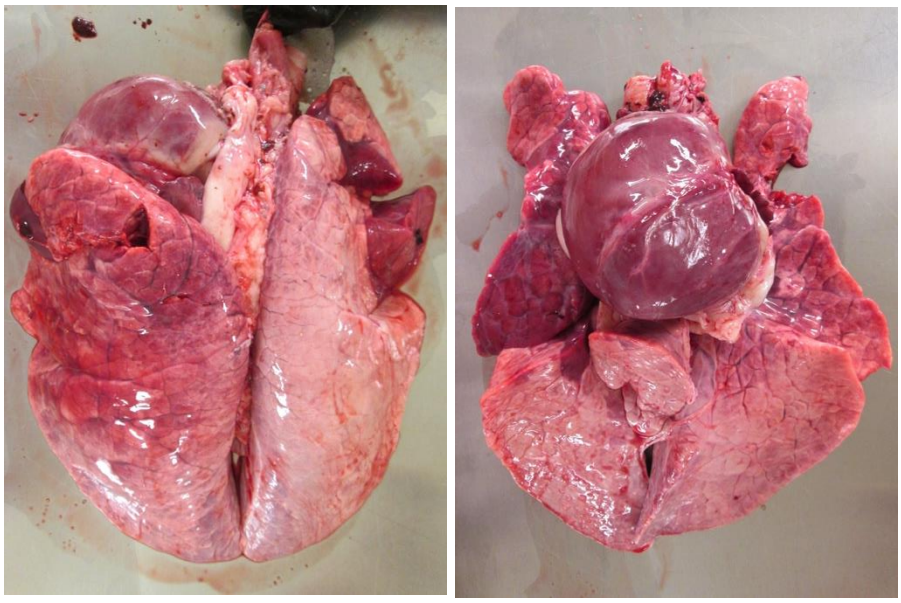

Figure 1.11 Swine inoculated with Pretoria parental strain: interlobular pulmonary edema and consolidation (own photos)

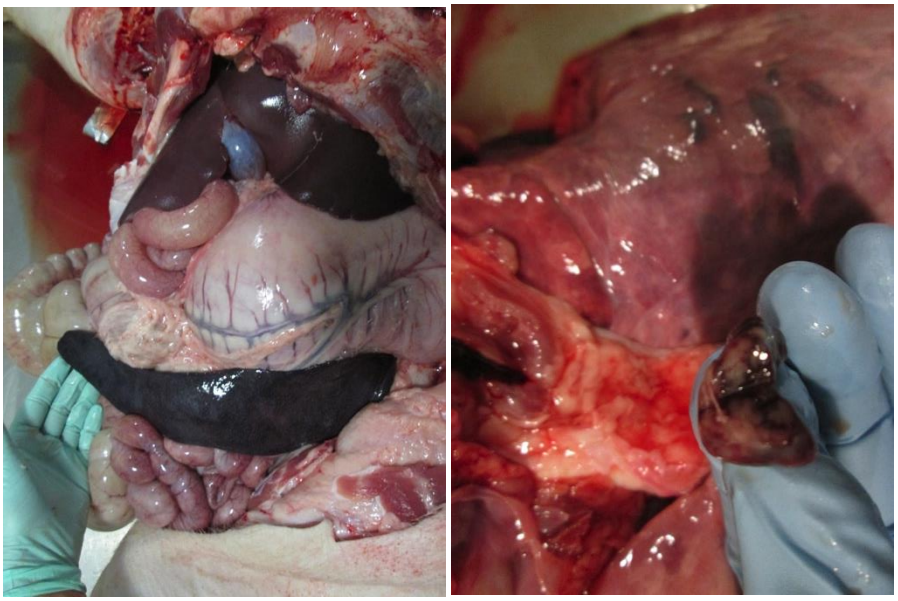

Figure 1.12 Swine inoculated with Pretoria parental strain swine, enlarged spleen (left), hemorrhagic tracheobronchial lymph node (right) (own photos)

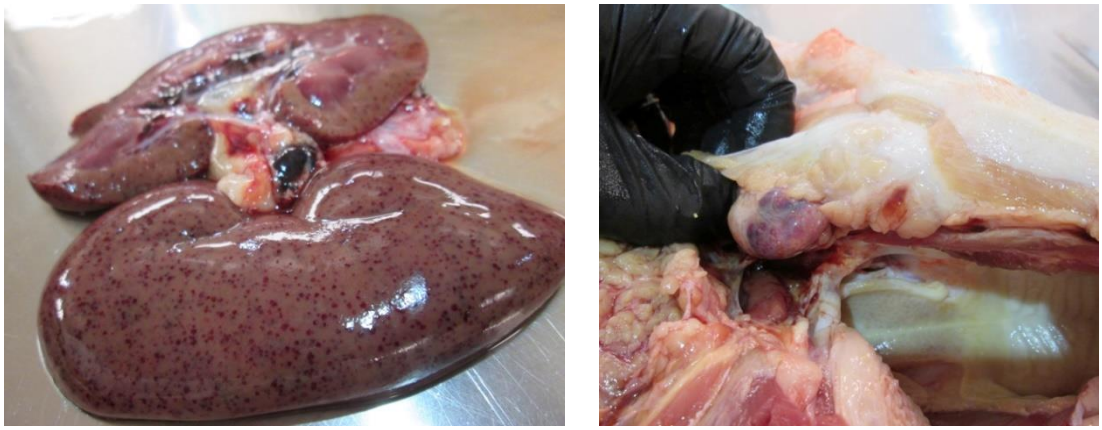

Figure 1.13 Swine inoculated with Pretoria parental strain: pinpoint focal hemorrhages on kidney (left) and enlarged hemorrhagic retropharyngeal lymph node (right) (own photos)

1.3.1.6 Histopathological lesions of ASF

ASF viral infection in tissues often results in massive destruction of macrophages and extensive apoptosis of lymphocytes and lymphopenia (Gomez-Villamandos *et al.*, 1995; Gonzalez-Juarrero *et al.*, 1992; Sanchez-Cordon *et al.*, 2008; Sanchez-Vizcaino *et al.*, 1981). Karyorrhexis in the lymphoid tissues is often a remarkable feature. Fibrinoid changes are often found in blood vessel walls as a result of necrosis of the endothelium and leakage of inflammatory mediators. Interstitial pneumonia with fibrin and macrophage infiltrate is commonly found in the lungs. Within the kidneys, renal tubular degeneration with hyaline droplet absorption is observed. The portal tracts of the liver will be infiltrated with macrophages. In the brain, lymphocytic meningoencephalitis can be visible.

1.4 Strategies for control

1.4.1 Disinfection of ASF

Preventing the spread of ASFV is a critical aspect and most importantly should focus on regulating the import of pork products and ensuring proper disposal or decontamination of fomites on planes and ships.

ASFV is stable in the environment over a wide range of temperatures and pH (2-11). ASFV can be inactivated by sunlight and desiccation, but is relatively stable in excretions of infected swine, carcasses, meat products; putrefaction, freezing, or thawing does not inactivate the virus. ASFV can remain infective in feces for at least 11 days and for months in bone marrow (Beltrán-Alcrudo *et al.*, 2009). ASFV can survive in chilled meat for 15 weeks and 3-6 months in cured hams and sausages that have not been cooked or smoked at high temperatures (2010; Mebus *et al.*, 1993; William A. Geering, 2001).

Sodium hypochlorite bleach (10%), citric acid (2%) and some iodine and quaternary ammonium compounds have been reported as effective against ASFV on nonporous surfaces (Krug *et al.*, 2012). Higher concentrations can be used to disinfect the virus on wood surfaces.

There are recommendations that otherwise unprocessed meat must be heated to 70°C for 30 minutes to inactivate ASFV. To inactivate virus in serum and body fluids heating to 60°C for 30 minutes is sufficient. Altering the pH to less than 3.9 or greater than 11.5 in serum-free medium also can inactivate the virus (Spickler, 2015). Since it is difficult to determine if all aspects of swill are cooked properly, most countries forbid its use.

1.4.2 Laboratory diagnostics

Rapid laboratory diagnosis is key to distinguishing ASF from other diseases such as classical swine fever, bacterial septicemia, porcine dermatitis and nephropathy syndrome, acute porcine reproductive and respiratory syndrome, erysipelas, Glaesser's disease, pseudorabies, thrombocytopenic purpura, and other general septicemic or hemorrhagic conditions (Jean Gladon, 2011).

ASF can be diagnosed via virus isolation on primary cultures of porcine macrophages (cultured from peripheral blood, bone marrow, or alveolar lavage) using samples from blood or tissues such as spleen, kidney, liver, tonsil, and lymph nodes. Virus isolation of ASFV is typically performed on primary culture of porcine macrophages isolated from the peripheral blood or lungs of swine. Primary leukocyte cultures may also be derived from bone marrow or lung lavages. ASFV-infected cells are detected by adding homologous swine erythrocytes. Once macrophages are actively infected, the erythrocytes form rosettes around infected cells, this is known as hemadsorption. Hemadsorption is a sensitive and specific method, but it is important to note that some isolates are non-hemadsorbing (Gonzague *et al.*, 2001; Pini and Wagenaar, 1974). PCR or immunofluorescence are often used to detect the virus. The fluorescent antibody test (FAT) allows for fast detection of antigen on cryopreserved sections or impression smears obtained at necropsy.

The viral genome may also be detected by polymerase chain reaction (PCR). PCR is particularly useful when samples are unsuitable for virus isolation. It should be noted that ASFV is not found in aborted fetuses, but blood from the sow should be sampled.

Serology may be useful where the disease is endemic, but often pigs die before developing antibodies against ASFV. However, animals that survive will have antibodies that will persist for long periods. Current serological assays include ELISA, immunoblotting, and indirect fluorescent antibody (IFA) test.

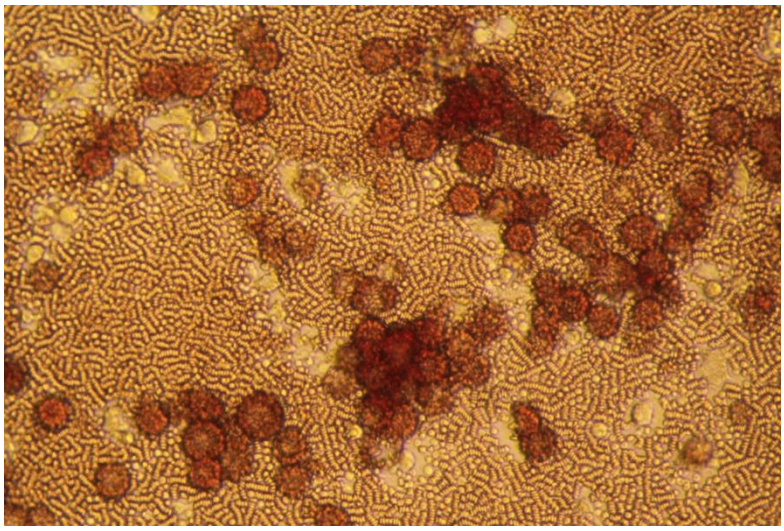

Figure 1.14 Hemadsorption detection of ASFV-infected macrophages cultured from porcine peripheral blood (own photo)

1.4.3 Vaccine strategies

Currently there are no vaccines or effective treatments available against ASF. The lack of heterologous protection among strains of ASFV represents an obstacle in our research. Vaccine development began in the 1960s when scientists first attenuated ASF virus by cell passage. These attenuated viruses (e.g. strain $Lisbon_{60}$) were able to stimulate an antibody response and prevent acute death after challenge at 117 dpi. (Manso Ribeiro *et al.*, 1963; Stone *et al.*, 1968). There have been several failed attempts to vaccinate animals using infected cell extracts, supernatants of infected PBMCs, inactivated virions, glutaraldehyde-fixed infected macrophages, or detergent-treated infected alveolar macrophages (Coggins, 1974; Forman *et al.*, 1982; Kihm *et al.*, 1987; Mebus, 1988; Neilan *et al.*, 2004).

It has been said that ASF was able to establish itself enzootically in Portugal and Spain after the use of an unsafe ASF vaccine in the 1960s and 1970s (Coggins, 1974; Dixon *et al.*, 2013a; Hess, 1971; Manso Ribeiro *et al.*, 1963). Europe observed more chronic and subclinical infections in domestic pigs in the 1960-1970s than East Africa (Coggins, 1974; Hess, 1971; Scott, 1965). A large number of carrier animals were generated, hindering subsequent attempts to eradicate ASF (Hess, 1971). Since the use of this vaccine caused adverse post-vaccination reactions including pneumonia, locomotor changes, skin ulcers, and even death, no further field trials of attenuated vaccines were carried out (Dixon *et al.*, 2013a; Manso Ribeiro *et al.*, 1963).

Swine that survive primary ASFV infection or have been inoculated with attenuated ASFV often resist homologous challenge with the parental strain (Coggins, 1974). Coggins also reported that vaccinated pigs frequently had transitory protection against the challenge virus, and often develop a chronic form of the disease with lesions in the lungs, pericardium, and skin over the bony prominences. Even pigs that did not respond clinically to ASFV may still have histopathologic lesions such as hyperplasia of reticular cells and lymph nodes, where virus was often isolated (Coggins *et al.*, 1968; Moulton and Coggins, 1968a).

Swine that survive ASFV infection against one genotype generally have solid immunity to challenge from a homologous strain but not against heterologous strains even if they are considered the same genotype (King *et al.*, 2011; Leitao *et al.*, 2001; Manso Ribeiro *et al.*, 1963; Mulumba-Mfumu *et al.*, 2015). Effective control of ASF will hinge on the availability of several vaccines based on specific isolates in found in each region.

1.4.3.1 Inactive and subunit vaccines

Experimental inactivated and subunit vaccines have been consistently unsuccessful in protecting swine from ASFV infection (Coggins, 1974; Forman *et al.*, 1982; Mebus, 1988). Kihm *et al.* demonstrated some level of protection (50%) of pigs against ASFV using an inactivated vaccine from spleen mixed with Freund's adjuvant, however all pigs were found to be viremic between 4 and 18 days post-challenge. The virus persisted in spite of high antibody titers until at least 91 days post-inoculation and early antibody detection had no correlation with protection (Kihm *et al.*, 1987).

In 2004, Neilan *et al.* immunized swine with baculovirus-expressed p30, p54, p72, and p22 from Pretoria (Pret4) (Neilan *et al.*, 2004). Although ASFV specific neutralizing antibodies were detected in vaccinates, swine exhibited only a two-day delay to the onset of clinical disease with reduced viremia at 2 DPI. However, naïve animals and vaccinates both died between 7 and 10 days post-infection.

Lacasta *et al.* demonstrated that a DNA vaccine encoding p54, p30, and the hemagglutinin extracellular domain fused with ubiquitin provided partial protection in the absence of detectable antibodies prior to virulent challenge (Lacasta *et al.*, 2014). In this case, only 50% of the vaccinated pigs survived the challenge, and 2 out of 8 swine showed no viremia or viral excretion post-infection. The subunit vaccines induced partial protection with 33-50% of immunized animals surviving challenge (Argilaguet *et al.*, 2012; Lacasta *et al.*, 2014).

More recently, Blome et al. conducted an experiment to re-assess binary ethyleneimine (BEI) inactivated ASFV preparations (strain Armenia 08) formulated with current adjuvants Polygen or Emulsigen-D. The modern adjuvants did not enhance the

efficacy of BEI-inactivated ASFV vaccines. Antibodies were detected in vaccinates, but no neutralization or protection was observed (Blome *et al.*, 2014).

Unfortunately, there is no vaccine available for ASFV despite many attempts with inactivated viruses or subunit vaccines. Previous attempts using attenuated vaccines demonstrated good potential in protecting swine against virulent homologous challenge.

1.4.3.2 Live attenuated viruses as vaccines

Protective vaccination is feasible because complete protection can be achieved by infection with low-virulence isolates of ASFV. Convalescent swine develop strain-specific immunity that can withstand challenge with antigenically related virulent strains. ASFV strains attenuated naturally, by cell culture passage, and targeted gene deletions have been used to create rationally attenuated strains that elicit protective immune responses (Abrams *et al.*, 2013; Hamdy and Dardiri, 1984; Leitao *et al.*, 2001; Lewis *et al.*, 2000; Mebus and Dardiri, 1980; Neilan *et al.*, 2004; O'Donnell *et al.*, 2015a; O'Donnell *et al.*, 2015b)

In the past, the main issue with attenuated viral strains was biosafety. Some attenuated strains retained virulence and produced sub-clinical and possibly chronic infections in swine (Coggins *et al.*, 1968; Sanchez Botija, 1963). Now with advances in molecular virology we can genetically attenuate ASFV in a more controlled manner.

Pigs immunized with live attenuated ASFVs containing genetically engineered deletions of specific virulence-associated genes are protected when challenged with homologous parental viruses. Specifically, individual deletions of 23-NL (DP71L), UK (DP69R), TK (A240L, 9GL (B119L), or multiple genes from MGF 530/505 and MGF 360 from the genomes of virulent ASFV viruses including Malawi Lil-20/1 (Mal),

Pretoriuskop/96/4 (Pret4), E70 and Georgia 2007 resulted in significant attenuation of these isolates in swine (Lewis *et al.*, 2000; Moore *et al.*, 1998; O'Donnell *et al.*, 2015a; O'Donnell *et al.*, 2015b; Zsak *et al.*, 1998; Zsak *et al.*, 1996).

Previously it was described that NL-S with similarity to the neurovirulence-associated gene (ICP34.5) in herpes simplex virus is not essential for viral replication in swine macrophages in culture (Zsak *et al.*, 1996). Deletion of NL-S resulted in attenuation of the virus in domestic swine, signifying its relevance as a viral virulence factor (Zsak *et al.*, 1996). Swine infected with ΔNL-S E70 remained clinically normal for 30 days and with 1,000 fold reduction in mean and maximum viremia titers compared to virulent E70 (Zsak *et al.*, 1996).

Interestingly, it should be noted that the same deletion of NL-S in Pret4 and Malawi Lil 20/1 did not cause attenuation and mortality was 100% in domestic swine (Afonso *et al.*, 1998). This data suggests that the E70 strain isolated in Spain in 1970, years after its introduction to the Iberian Peninsula, may already be more attenuated or adapted to domestic swine than African strains (Afonso *et al.*, 1998). Viruses isolated from different regions will have different selection pressures e.g. when cycling of the virus occurs in different hosts found in Europe (domestic swine, wild boar, possibly ticks) versus Africa (domestic swine, warthogs, bushpigs, ticks) (O'Donnell *et al.*, 2015a).

A second E-70 mutant generated by the deletion of the UK gene, a gene encoding for a 15-kDa protein that is highly conserved between African and European ASFV isolates, was significantly attenuated in domestic pigs when administered at 10^2 HAD_{50} IM (Zsak *et al.*, 1998). Although replication kinetics and viral yields in vitro were

indistinguishable from the parental strain, E-70ΔUK-infected swine had viremia titers 300- to 100,000-fold lower at 4 dpi than swine infected with the parental strain (Zsak *et al.*, 1998). Swine inoculated with E-70ΔUK and challenged 42 days later remained clinically normal with no detectable viremia (Zsak *et al.*, 1998).

The first description of a 9GL deletion was in ASFV Malawi Lil 20/1 using homologous recombination (Lewis *et al.*, 2000). The 9GL gene encodes p14, a late non-structural viral protein of 119 amino acids with high conservation at both the nucleotide and the amino acid level among several ASFV field isolates, including 22 viruses representative of African, European, and Caribbean strains (Lewis *et al.*, 2000). The 9GL gene has similarity to the Essential for Respiration and Vegetative Growth (ERV1) gene in yeast, which is essential for oxidative phosphorylation, the cell cycle, and the maintenance of mitochondrial genomes. ERV1 homologues have been found in a variety of organisms ranging from protozoa to plants, and in addition to ASFV, they are also present in a number of other cytoplasmic DNA viruses. GFER, the human homolog of ERV1, functions as a growth factor in the regeneration of the liver and in spermatogenesis (Lewis *et al.*, 2000). Deleting the 9GL gene from ASFV negatively affects viral growth and virulence, but the mechanisms of this attenuation are unknown.

The 9GL deletion reduced virus yield in swine macrophage culture by 90 to 99% compared to the parental strain, and a majority of the viral particles observed by electron microscopy resembled intermediate stages in normal virion morphogenesis. Based on these observations, it was speculated that p14 performs energy- and redox-related functions in infected cells that are critical for efficient virion assembly and maturation (Lewis *et al.*, 2000).

Swine experiments demonstrated that the deletion of 9GL in Malawi-Lil 20/1 and Pretoria attenuated the virus in vivo (Lewis *et al.*, 2000; Neilan *et al.*, 2004). Swine receiving 10^2, 10^4, or 10^6 HAD_{50} of MalawiΔ9GL had solid protection when challenged with its parental isolate 42 days later. Only two swine, one each from the MalawiΔ9GL dose groups 10^4 and 10^6 HAD_{50} had detectable viremia and transient fever, where as all 4 receiving 10^2 had transient fever and viremia (Lewis *et al.*, 2000). Swine inoculated with 10^4 HAD_{50} Pret4Δ9GL and challenged 42 days post-inoculation all survived, with half of the swine presenting with elevated temperatures following challenge (Neilan *et al.*, 2004).

When 9GL was deleted from ASFV Georgia 2007, however, it was found that the mutant virus was still virulent at a dose of 10^4 $HAD_{50,}$ unlike MalawiΔ9GL, which was attenuated at 10^2, 10^4, and 10^6 HAD_{50}. GeoΔ9GL at 10^4 HAD_{50} induced a lethal disease in swine similar to its parental isolate. Swine receiving 10^2 or 10^3 HAD_{50} GeoΔ9GL IM were partially protected when challenged with Georgia 2007 at 21 and completely protected at 28 days post-inoculation.

The 9GL gene deletion reduces virulence of Pretoriuskop/96/4 and Malawi Lil-20/1 while to a lesser degree with Georgia 2007, indicating an isolate-specific effect of gene deletions on attenuation (O'Donnell *et al.*, 2015b) that is of great importance for the design and validation of attenuated ASFV vaccines (Sanford *et al.*, 2015).

Similar observations were made with another set of deletion mutants. Like other large DNA viruses, ASFV encodes enzymes involved in the synthesis of deoxynucleoside triphosphates, including thymidine kinase (TK). The inactivation of the TK gene in poxviruses and herpesviruses showed the gene to be nonessential for

growth in cultured cells, but ΔTK viruses exhibited a reduction in virulence and pathogenicity (Moore *et al.*, 1998). For ASFV Haiti, Malawi and Georgia, the loss of the TK gene severely impaired the growth on swine macrophages *in vitro* and reduced virulence in vivo (Moore *et al.*, 1998; Sanford *et al.*, 2015). Pigs inoculated with 10^4 $TCID_{50}$ of MalawiΔTK became clinically ill but 75% (3 of 4) survived, while the same dose of the parental virus was invariably fatal (4 of 4). For Georgia, on the other hand, doses of Vero-adapted GeoΔTK as high as 10^6 TCID50 did not lead to any clinical signs or viremia in pigs. At 56 dpi, the 3 surviving MalawiΔTK-inoculated swine were challenged intramuscularly with 10^4 $TCID_{50}$ of the parental Malawi virus and were protected (Moore *et al.*, 1998), while all Vero-adapted GeoΔTK-inoculated pigs died upon challenge with virulent Georgia 2007 (Sanford *et al.*, 2015).

ASFV contains at least five multigene families (MGF), all of which lack similarity to other genes and lack genetic variability among different ASFV isolates. Experiments have demonstrated that MGF530/360 is associated with macrophage host range specificity, and these genes work to promote infected cell survival (Zsak *et al.*, 2001). Most recently it has been reported that deleting six genes belonging to MGF360 or MGF505 completely attenuated Georgia 2007, a highly virulent strain spreading through the Caucasus and Eastern Europe (O'Donnell *et al.*, 2015a). One hundred percent of swine subsequently challenged with 10^3 HAD_{50} virulent Georgia survived (O'Donnell *et al.*, 2015a). However, it was recently found that a double deletion of Δ9GL and ΔMGF (O'Donnell *et al.*, 2016) was highly attenuated but did not elicit a detectable antibody response nor protection against challenge with the virulent parental Georgia 2007 virus.

Understanding the role of ASFV genes using single deletion virus mutants is very complex when comparing isolates. Although the NL gene deletion attenuated E75, it did not attenuate Malawi Lil 20/1 (Afonso *et al.*, 1998). The 9GL deletion attenuated isolates Malawi and Pretoria in swine, but in Georgia at doses of 10^4 HAD_{50} the deletion did not attenuate the virus to prevent clinical disease and death.

The deletion of these genes often reduces viral replication in primary macrophage culture and swine studies with these deletion mutants have shown that limiting the viral replication in macrophages dramatically reduces the virulence in domestic pigs. Reduced viral replication may avoid the induction of immunopathological processes and allow host defense mechanisms to clear the virus, but our understanding of how virulent or attenuated viruses interact with the host is limited. The reduced viral replication may allow host defense mechanisms to clear virus or it may be that some pathological processes are not induced. It is thought that viral replication in macrophages is reduced by deleting genes encoding enzymes involved in nucleotide metabolism such as the thymidine kinase gene and dUTPase (Dixon *et al.*, 2008). At this point, the host mechanisms mediating the immune response to ASFV are not well understood. Further research evaluating host parameters are necessary to understand the early events in the replication of attenuated strains.

1.5 Host immune response to ASFV infections

1.5.1 The role of antibodies in protection

Humoral and cellular immunity are significant components in mounting an immune response to ASF. It has been shown that antibodies are sufficient to protect swine from a lethal challenge in some early studies (Hamdy and Dardiri, 1984; Onisk *et*

al., 1994; Ruiz Gonzalvo *et al.*, 1986b). In a study by Zsak *et al*., convalescent serum from pigs infected with a low virulent isolate from Spain E75, neutralized culture adapted variants E75, E70, Lisbon60, Malawi Lil20/1 and a low passage variant of E75 by 86-97% in Vero and macrophage cell cultures (Zsak *et al.*, 1993). It was also found that a monoclonal antibody, mAb-135D4, recognizing ASFV protein p72, also exhibited strong neutralizing activity with these viruses (Zsak *et al.*, 1993). Ruiz et al. demonstrated that sera from pigs inoculated with attenuated isolates and later challenged with a heterologous isolate inhibited infection of PBMCs by different heterologous viruses (Ruiz Gonzalvo *et al.*, 1986b).

Based on results from early experiments, it was hypothesized that neutralizing antibodies were not completely effective or even induced by ASFV (Hess, 1981; Vinuela, 1985). However, it was demonstrated that different isolates of ASFV could be neutralized by convalescent swine sera and monoclonal antibodies (Ruiz Gonzalvo *et al.*, 1986a; Ruiz Gonzalvo *et al.*, 1986b; Zsak *et al.*, 1993). These authors described a persistent fraction of non-neutralized virus of 10% in their assays.

Other roles for antibodies should be considered. Both Onisk and Wardley et al. examined the role of antiviral antibodies by passive antibody transfer experiments within a homologous system (Onisk *et al.*, 1994; Schlafer *et al.*, 1984b; Wardley *et al.*, 1985). These studies observed a reduction in virulence, mortality, and a delayed onset to infection in pigs treated with anti-ASF immunoglobulin. Passive transfer of antibodies to naïve pigs could provide protection against a virulent challenge. Protective antibodies are known to function in virus neutralization, antibody-dependent cell-mediated cytotoxicity (ADCC), and complement-dependent cell lysis (CDCL) (Wardley *et al.*,

1985; Wardley and Wilkinson, 1985). A reduction in viremia is only secondary to the clinically protective effects of antibody as viremia levels and survival appear to show little correlation (Wardley *et al.*, 1985).

Our understanding of neutralizing antibodies is limited. Previously it was shown that antibodies transferred by colostrum provided a degree of protection to suckling piglets against virus challenge (Schlafer *et al.*, 1984a; Schlafer *et al.*, 1984b). Piglets given colostrum collected from a sow that had recovered from ASFV infection or Ig precipitated from ASFV antiserum had a delayed onset of clinical disease and viremia. Piglets fed passively transferred antibodies from the colostrum of recovered pigs or by parental administration of antiserum had 10,000-fold lower viremia titers than the naïve control piglets (Schlafer *et al.*, 1984b). In this study 9/10 swine given colostrum from ASFV recovered swine survived virulent challenge (Schlafer *et al.*, 1984b).

Onisk *et al.* reported that 85% of swine receiving anti-ASFV immunoglobulin (Ig) survived challenge, had a 3-day delay in viremia and a 10,000-fold reduction in mean and maximum virus titers. This is one of few studies reporting direct *in vivo* evidence where the acquisition of humoral protective immunity by naïve pigs against ASFV was achieved after passive transfer of ASFV-specific antibodies obtained from pigs previously infected with attenuated ASFV (Onisk *et al.*, 1994).

Blocking antibodies can inhibit neutralization and allow for the persistence of a small fraction of non-neutralized virus. This phenomenon was found by other groups (Ruiz-Gonzalvo *et al.*, 1996; Zsak *et al.*, 1993). It is thought that incomplete neutralization of ASFV could lead to chronic ASFV infection in pigs and possible persistence of the virus in swine in the situation of an excess of antibodies. One group

demonstrated a long-term persistent ASF infection of 100% of domestic swine infected for more than 500 days post-inoculation (Carrillo *et al.*, 1994). This subject of persistent infections and the role of antibody neutralization in ASF infections need further investigation.

Gomez-Puertas et al. showed that ASFV induces antibodies that neutralize the virus before and after binding to susceptible cells, inhibiting both virus attachment and internalization. He showed that antibodies to p72 and p54 are involved in the inhibition of virus attachment, while antibodies to p30 inhibited virus internalization in Vero cells and macrophages (Gomez-Puertas *et al.*, 1996). Later it was shown that neutralization by specific antibodies depends on the phospholipid composition of the viral particle (Gomez-Puertas *et al.*, 1997).

However, the role of these proteins in a protective immune response in swine could not be demonstrated when pigs were immunized with baculovirus-expressed p30, p54, p72, and p22 from Pret4 (Neilan *et al.*, 2004). Specific neutralizing antibodies could be detected in immunized animals but clinical disease ensued with swine dying between 7 and 10 dpc. Pigs immunized with baculovirus-expressed recombinant proteins yielded contradictory results in regards to protection against virulent challenge (Gomez-Puertas *et al.*, 1998; Neilan *et al.*, 2004). It is possible that these results are due to differences in viral stains used. Antibodies certainly play a significant role in protection against ASF, but other active components of the immune response including the cellular immunity should be considered.

1.5.2 Role of cellular immunity in protection

African swine fever virus productively infects macrophages, causes apoptosis in lymphocytes and encodes a variety of immune invasion genes. Cellular immune responses to ASFV infection have been detected and quantified, but whether they are critical in protection against clinical disease still remains to be elucidated.

Proliferation of ASFV-specific lymphocytes was initially reported by (Wardley and Wilkinson, 1980) 10 days after infection with a non-virulent Uganda isolate, but not with a virulent Kiurawira isolate because swine died 5 to 10 after inoculation. Scholl *et al*. similarly showed a strong antigen-specific blastogenic response in PBMCs of swine infected with an attenuated strain of ASFV (NH/P68) (Scholl *et al.*, 1989).

Later experiments examined the function of ASFV-specific cytotoxic CD8+ T lymphocytes (CTL). Martins et al. showed MHC class I restricted CTL activity against ASFV-infected cells in swine infected with the non-fatal, non-hemadsorbing ASFV isolate NH/P68 (Martins *et al.*, 1993).

The cellular immune recognition of peptides expressed by a random genomic library of ASFV Malawi (LIL20/1) was studied to better understand the targets of that cellular immune response. The viral DNA was randomly sheared, cloned into plasmids and expressed in skin fibroblasts to serve as antigen presenting cells. Each clone was screened for recognition by matched CD8+ lymphocytes from ASFV-sensitized swine, and T cell proliferation was detectable for 14/72 clones (Jenson *et al.*, 2000).

The importance of CTLs for protection against virulent ASFV was demonstrated by the depletion of CD8+ cells in swine vaccinated with the non-pathogenic OUR/T88/3 variant (Oura *et al.*, 2005). This depletion abrogated protection against challenge with

the related virulent isolate OUR/T88/1, indicating that CD8+ lymphocytes play an important role in the protective immune response to ASFV infection and that antibody alone is not protective.

This was confirmed by the observation that pigs that remained asymptomatic after infection with NH/P68 developed high levels of natural killer (NK) cells and resisted subsequent challenge with virulent ASFV Lisbon 60. Pigs that developed lesions after NH/P68 infection, on the other hand, had normal NK cell counts but developed high titers of ASFV-specific antibody (Leitao *et al.*, 2001)

IFN-γ, predominantly produced by NK cells and effector T cells, is an activator of the effector functions of monocytes and macrophages. Activation of macrophages by IFN-γ enhances direct antimicrobial activity and proinflammatory responses, including cytokine and chemokine production, and promotes further IFN-γ production at the site of inflammation. Considering that ASFV mainly targets professional APCs, IFN-γ might have a direct impact on virus-host interactions, especially during the early stages of infection (Takamatsu *et al.*, 2013).

Esparza et al. were among the first to demonstrate the reduction of ASFV replication in monocytes and alveolar macrophages by bovine IFN-α and porcine IFN-γ (Esparza *et al.*, 1988). Paez et al. demonstrated that human IFN-α and IFN-γ inhibited ASFV replication in Vero cells (Paez *et al.*, 1990). They also found that continuous treatments with IFN-α “cured” Vero cells from persistent infections with ASFV.

A few studies investigating IFN-γ production in swine PBMCs after exposure to vaccine and/or mutant virus studies exist. In one of the first studies, PBMCs from swine immunized with attenuated, Vero-cell adapted ASFV BA71 proliferated and produced

interleukin-1 (IL-1) and IFN-γ after homologous restimulation in vitro (Revilla *et al.*, 1992).

Two reports (Argilaguet et al., 2012; King et al., 2011) state that there is a direct correlation between circulating IFN-γ-producing PBMCs and protection against virulent challenge in animals. However, our studies outlined in this dissertation with attenuated viruses including ∆9GL Pretoria and ∆9GL-UK Georgia have demonstrated no correlation between IFN-γ-producing PBMCs in vaccinated animals and protection against virulent challenge.

For these reasons, we looked into measuring other cytokines, chemokines, or immune mediators to find host response parameters that could be correlated with swine surviving virulent challenge.

1.5.3 Cytokines in ASFV infection

ASFV infects macrophages, which are antigen-presenting cells (APCs) that play an important role in the immune system. APCs detect pathogen-associated molecular patterns (PAMPs) through their array of pattern recognition receptors (PRRs), including toll-like receptors (TLRs), and produce cytokines and chemokines, which aid in signaling to clear pathogens by phagocytosis. Macrophages are essential components of the host defense system, but their activation must be controlled since the cytokines and mediators they produce can lead to host tissue damage.

ASFV encodes over 150 proteins, many of which have been shown to modulate host immune responses (Dixon *et al.*, 2004; Tulman *et al.*, 2009). ASFV has proteins that inhibit interferon induction, host transcription factor activation, stress responses and apoptosis. During ASFV infection, changes occur in coagulability and vascular

permeability due to alterations in chemokine secretion that play a role in disease pathogenesis (Gomez del Moral *et al.*, 1999; Salguero *et al.*, 2002; Salguero *et al.*, 2005).

Not all immune responses contribute to protective immunity, and some of these responses contribute to pathological changes in ASFV-infected swine. In our research we attempted to decipher the role of different cytokines in mediating protection against ASF.

To date there are only a limited number of *in vivo* studies reporting on the cytokine response in swine infected with attenuated strains of ASFV, and they report conflicting results. In vitro data has shown that non-virulent strains (NH/P68, ΔMGF360/530 Pretoria) induce higher levels of monocyte chemoattractant protein (MCP-1), CXCL10, IFNα, IL-6, IL-12p40, IL-15 and TNFα when compared to their virulent (Lisbon 60, Pret4, E75) counterparts by PCR or microarray analysis (Afonso *et al.*, 2004; Gil *et al.*, 2003; Gomez del Moral *et al.*, 1999).

TNFα has been considered as a major player in the clinical manifestation of ASF. Gomez del Moral found that tissue macrophages appeared to be the main source of TNFα in spleen and lymph nodes of ASFV-infected animals. This increase in TNFα was correlated with the expression of viral genes encoding p30 or p54 (Gomez del Moral *et al.*, 1999). Interestingly, Esparza reported that bovine tumor necrosis factor α (TNF-α) increased ASFV production in monocytes (Esparza *et al.*, 1988).

In swine infected with virulent ASFV, concentrations of TNFα increased at 3 to 4 dpi and remained elevated until death. It has been suggested that the coagulation and vascular disorders seen in ASFV infection are not due to a direct effect of the virus, but

due to factors released by infected macrophages. TNFα affects endothelial cells, ultimately decreasing anticoagulation, increasing permeability and expression of adhesion molecules for leukocytes and platelets.

TNFα has also been attributed to inducing apoptosis of surrounding cells, and a component of shock as a stimulator of IL-1 and IL-6. IL-12p40 and IFNα/β are secreted after in vitro infection with non-virulent isolates, while TNFα is secreted after infection by both non-virulent and virulent isolates (Afonso *et al.*, 2004; Gil *et al.*, 2008; Gil *et al.*, 2003; Gomez del Moral *et al.*, 1999; Zhang *et al.*, 2006).

MCP-1 attracts monocytes and promotes mast cell activation, and CXCL10 also attracts monocytes, T lymphocytes, and NK cells while having the capacity to control viral replication. In Afonso et al., it was also found that the attenuated ΔMGF360/530 Pretoria virus induced the expression of the interferon-stimulated genes ISG15 and ISG43 (Afonso *et al.*, 2004). ISG15 stimulates the production of IFN-γ by CD3+T cells to enhance the proliferation and cytotoxicity of NK cells (Zhang and Zhang, 2011), while ISG43 is a protease whose function is to specifically remove ISG15.

Infection of blood-derived macrophages with low (OURT88/3) and high virulence (Benin 97/1) ASFV isolates results in the down-regulation of mRNA levels of chemokines CCL2, CCL2L, CXCL2, and chemokine receptors CCR1, CCR5, CXCR3, CXCR, and the up-regulation of CCL4, CXCL10, and chemokine receptor CCR7. Macrophages infected with low-virulence isolated had higher mRNA expression of CCL4, CXCL8, and CXCL10 (Fishbourne *et al.*, 2013a).

These parameters were further investigated in blood from pigs infected with low (OURT88/3) and high virulence (Benin 97/1) ASFV isolates (Fishbourne *et al.*, 2013b).

CCL2 mRNA was increased in swine infected with Benin 97/1 by 30-fold compared to those infected with OURT88/3, the opposite of what was found *in vitro.* CCL2, which is a chemoattractant for macrophages, may increase monocyte recruitment from the bone marrow. An increase in CCL2 could represent a mechanism by the virus to attract susceptible cells to an area of infection in order to increase viral dissemination.

In the same study, CXCL10 mRNA was increased by up 15-fold compared to naïve swine. CXCL10 is an interferon-stimulated gene. It has an important role during viral infections, and may contribute to lymphocyte priming toward Th1, promote the survival and expansion of T lymphocytes and induce apoptosis in lymphocytes lacking specific co-stimulating signals (Fishbourne *et al.*, 2013b).

Overall, the diversity of findings emphasizes that the virulence of ASFV isolates may be dependent on their capacity to regulate the expression of macrophage-derived cytokines that influence the development of protective host responses. The differences in cytokine expression between virulent and non-virulent isolates suggest the action of viral factors capable of regulating the cytokine expression at the macrophage level. Such interactions are highly relevant to understanding ASF pathogenesis and protective responses against infection.

1.6 Summary and rationale for the studies

1.6.1 Significance of our work

ASF is a severe, multi-systemic, hemorrhagic viral disease that threatens the swine economy globally. ASF is enzootic to most countries of Sub-Saharan Africa. Since 2007, ASF has been reported in Russia, Ukraine, and Belarus. In 2014 it entered the European Union and still currently affects the Baltic region and Poland. Since the

entry of ASF into the European Union, there is a constant risk for ASF to continue its spread throughout Europe and globally. Currently no vaccine is available to prevent ASF, and control measures are limited to culling of infected swine and restricted animal movement.

With no vaccines available, it is critical that we improve our understanding of the protective immune response against ASFV. No subunit vaccine has induced 100% protection, and over the years several attenuated strains have been developed. Specifically, individual deletions of UK (DP69R), 23-NL (DP71L), TK (A240L), 9GL (B119L), or multiple genes from MGF 530/505 and MGF 360 from the genomes of virulent ASFV viruses including Malawi Lil-20/1 (Mal), Pretoriuskop/96/4 (Pret4), E70 and Georgia 2007 resulted in significant attenuation of these isolates in swine (Lewis *et al.*, 2000; Moore *et al.*, 1998; O'Donnell *et al.*, 2015a; O'Donnell *et al.*, 2015b; Zsak *et al.*, 1998; Zsak *et al.*, 1996).

Pigs immunized with live attenuated ASFVs containing genetically engineered deletions of specific virulence-associated genes are generally protected when challenged with homologous parental viruses. Nevertheless, there are gaps of knowledge in understanding the complex virus-host interaction, the role of humoral immunity, and the significance for T-cell immunity in protective vaccination against ASFV.

1.6.2 Why we chose Pretoria ∆9GL for this model

The animal model described in this thesis uses swine inoculated with an attenuated strain, Pret4∆9GL, challenged with the parental Pret4 at different times post-

vaccination. Swine were infected with Pret4Δ9GL virus and challenged with the virulent parental Pret4 virus at different times post-inoculation (7, 10, 14, 21, and 28 dpi).

We chose Δ9GL Pretoria for multiple reasons. Pret4Δ9GL is a well-established ASFV deletion mutant that has been used in earlier studies (Lewis *et al.*, 2000; Neilan *et al.*, 2004). Those experiments demonstrated that the deletion of 9GL from ASFV Malawi and Pretoria attenuated the virus in swine. One of the first experiments demonstrated that swine inoculated with 10^4 HAD_{50} Pret4Δ9GL and challenged at 42 days post-inoculation were protected with half of swine showing no fever following challenge (Neilan *et al.*, 2004).

Previous research has shown much success with attenuated strains obtained naturally, by adaptation to cell cultures, or by genetic modifications as effective experimental vaccines that protect against homologous strains. Currently there is no strong understanding of the host immune mechanisms mediating protection in swine immunized with attenuated strains.

The Pret4Δ9GL virus was previously generated by homologous recombination (Lewis *et al.*, 2000; Neilan *et al.*, 2004). The 9GL gene deletion reduces virulence and replication in cell culture (Lewis *et al.*, 2000). The 9GL gene has similarity to Essential for Respiration and Vegetative Growth (ERV1) in yeast. Although the specific mechanism in how the 9GL deletion attenuates ASFV in swine is unknown, the deletion causes defects in virion assembly and maturation as well as causing viral interference *in vivo* (Lewis *et al.*, 2000).

We selected the Pret4 isolate since it was less virulent than Georgia 2007 or Malawi Lil 20/1 and we hypothesized that Pret4Δ9GL would not be a strong

immunogen. This increased our chances of obtaining protected and unprotected individuals by the same treatment, cumulating the chances of identifying host mechanisms and protection with groups of animals receiving the same treatment at different time points. Within this model, we sought to identify host immune mechanisms (virus specific antibodies, IFN-γ responses in ASF-stimulated PBMCs, and patterns of systemic cytokines) that could be associated with presence of protection. With this challenge model, we were able to observe and evaluate early protection of swine against ASF, while also providing an opportunity to understand different host immune mechanisms that may take place as early as 7-14 days post-inoculation and later at 21-28 days post-inoculation.

1.6.3 Cross validation of Pretoria ∆9GL with Georgia ∆9GL/∆UK swine studies

To further extend our knowledge and cross-validate our results, this vaccination/challenge model was also utilized with a double-deletion mutant of Georgia 2007, Geo∆9GL∆UK, and its parental strain.

Deletion of the *9GL (B119L)* gene from ASFV Malawi Lil-20/1 and Pretoriuskop/96/4 attenuated both viruses in swine and provided a protective immune response against lethal challenge with parental isolates. However, the Δ9GL version of the Georgia isolate (ASFV-G), although still attenuated, had to be administered at low doses (10^2 to 10^3 HAD_{50}) to induce a protective response and not cause clinical signs in inoculated swine while still inducing a protective response. In order to attenuate GeoΔ9GL further, our lab deleted a second gene, UK *(DP96R)*, which had previously been shown to attenuate ASFV E70. Swine inoculated with Geo∆9GL∆UK and

challenged at 14 and 28 days post-inoculation had 100% survival (5/5 and 10/10). With this model we also investigated immune factors associated with early protection at 7 to 14 days post-inoculation and later onset at 21 and 28 days post-inoculation and compared the results to those of the Pret4Δ9GL model.

1.6.4 Goals of this research

In this thesis we focused on studies to find correlates of protection against ASFV. The animal model described in this thesis used swine infected with attenuated, deleted mutant and virulent wildtype strains.

Our first aims were to compare growth curves *in vitro* while also investigating the replication and pathogenesis of Pret4 versus Pret4∆9GL virus *in vivo*. We compared viral replication with a growth curve over a four-day period in primary swine macrophage culture. Next we investigated differences in the replication of these two viruses in swine. We compared the clinical outcome of infection (fever, mortality) between Pret4 and Pret4∆9GL. Following clinical examination, we compared the level of viremia and nasal shedding in swine infected with either virus and analyzed differences in early viral replication in tissues collected from swine at 2,4, 6, 8, and 10 days post-inoculation.

Our next aim was to generate information to explain dynamics of protection, optimize our detection of immune responses, while increasing our knowledge in the design of a rational vaccine using a rationally attenuated mutant. In order to detect different elements of immunes response associated with swine protected from virulent challenge, samples were taken at the time of challenge of 114 vaccinated swine with 65 from the Pret4∆9GL group and 54 from the Geo∆9GL∆UK group. In this model, antibody was measured via immunoperoxidase and ELISA, and IFN-γ was quantified by

ELISPOT with stimulated PBMCs. Circulating cytokines were measured in swine sera at the time of challenge.

Time-course antibody data were also collected from the start of the study until 21 days after challenge to characterize seroconversion and our ability to measure it with our in-house anti-ASFV IgG ELISA and immunoperoxidase assays. Within the groups of animals inoculated with Pret4Δ9GL, IFN-γ responses in ASFV-stimulated PBMCs were characterized one week before challenge, at challenge, and one week after challenge in all 65 inoculated animals. To further supplement this information in determining host factors critical for the induction of a protective immune response against lethal challenge, 18 cytokines were tested in swine sera at the time of challenge. These cytokines included MCP2, TGF-β1, IFN-α, IFN-β, IFN-γ, IL1-α, IL1-β, IL-2, IL-5, IL-8, IL-10, IL-12 p35, IL-12 p40, OAS, PKR, TNF, MX-1, and VCAM using commercial ELISAs.

The existence of correlates of immunity and protection against ASF still remains controversial. The determination of these correlates is a critical step in the development of a vaccine strategy against ASFV as it provides different criteria to understand the host's immune response. The use of attenuated ASFV strains generated by genetic manipulation is a reasonable approach as model to investigate protective immune responses and to develop an effective ASFV vaccine.

Chapter 2 - Materials and Methods

2.1 Introduction

Since common methods were used in many of the experiments described in this dissertation, this chapter provides the collected technical approaches and research material used to avoid unnecessary duplication.

2.2 Viruses used in these studies

As shown in Table 2.1, multiple viruses were used from the experiments described in this dissertation. The preparation of these viruses, including the generation of deletion mutants is described in below. Further details of the viruses and rationale and their selection are provided in chapter 1.

Table 2.1 Viruses and their characteristics

Isolate	Gen Bank no.	Genotype (P72 basis)	Source	Phenotype
Pretoriuskop/96/4 (Pret4)	AY261364	1	Tick South Africa 1996	Highly virulent (lesser compared to Geo)
Pret4Δ9GL		1	Macrophage culture 2004	Attenuated by deletion of B119L
Georgia 2007/1 (Geo)	FR682468	2	Domestic pig Georgia 2007	Highly virulent
GeoΔ9GL/ΔUK		2	Macrophage culture 2016	Attenuated by deletion of B119L and DP96R

2.2.1 Primary culture of macrophages

Healthy donor swine older than eight months were bled one to two times per month to obtain PBMCs for primary culture. The blood was collected using 4-6 inch 12-14 gauge stainless steel needles via the right jugular vein with a vacuum pump and

tubing primed with heparin. At each collection, 1-2 liters of blood containing 50 ml/l of heparin were harvested.

Primary swine macrophage cultures were prepared from defibrinated blood as described by Zsak et al (1996). The blood was incubated at 37°C for 1-2 hours until the erythrocytes sedimented to the bottom and almost half of the total volume was retained as plasma resting at the top. In up to 25 parallel preparations, 35 mL of plasma were underlaid with Ficoll-Paque Plus at a specific density of 1.077 g/ml. Plasma and Ficoll were spun at 400 × g for 30 minutes at room temperature with brake and acceleration set to zero. After centrifugation, the cell-free plasma was collected and spun again to remove remaining cells and debris, filtered through 0.45µm and 0.22µm filters and stored at 4°C. Mononuclear leukocytes from the plasma-Ficoll interface were pipetted into new 50-mL conical tubes and washed twice with RPMI-1640 media containing 1% antibiotics and antifungal agents.

The washed cells were then seeded into 20-25 75 cm^2 filter-cap flasks (Primaria) containing macrophage media composed of RPMI, 30% (v/v) L929 cell supernatant, 20% (v/v) fetal bovine serum, 1% antibiotics and 0.1% gentamicin. The flasks were incubated for 24 hours at 37°C with 5% CO_2. On the following day, adherent cells (i.e., macrophages) were detached from the flasks using 10mM EDTA in sterile phosphate-buffered saline (PBS) and reseeded into 6- and 96-well plates (Primaria) at a density of $1X10^5$ cells per well in 96-well plates and $2X10^7$ per well in 6 well plates for use in assays 24 hours later.

2.2.2 Homologous recombination

2.2.2.1 Pretoria

Pretoriuskop/96/4 (Pret4) and its attenuated isolate containing a deletion of the 9GL gene (B119L) Pret4∆9GL were kindly provided by Dr. John Neilan (Neilan *et al.*, 2004) of the Plum Island Animal Disease Center. Recombinant ASF viruses were generated by homologous recombination between the parental ASFV genome and a recombination transfer vector after infection and transfection of primary swine macrophage cultures (Lewis *et al.*, 2000; Neilan *et al.*, 1997; Zsak *et al.*, 1996). The Pretoria ∆9GL (Pret4∆9GL) virus was derived from the Pretoriuskop/96/4 isolate of ASFV by deleting 173 bp within the 9GL (B119L) gene encompassing amino acid residues 11-68 (Lewis *et al.*, 2000; Neilan *et al.*, 2004). The deleted region was replaced with a gene cassette containing the β-glucuronidase (β-GUS) gene and an ASFV p72 promoter sequence via homologous recombination when macrophage cultures were infected with ASFV Pret4 and subsequently transfected with p72GUSΔ9GL. Recombinant Pret4Δ9GL viruses were purified by plaque purification.

2.2.2.2 Georgia

Geo∆9GL/UK was derived from the highly virulent Georgia 2007 (Geo 2007) isolate of ASFV by two successive homologous recombination procedures. First, a 173 bp region within the 9GL gene was deleted from the Geo 2007 virus as described above and replaced with the p72GUS∆9GL reporter cassette. The single-deletion mutant was obtained after a series of plaque purifications on primary swine macrophage cultures (O'Donnell *et al.*, 2015b). The second recombination step replaced the UK gene (Zsak et al., 1998) with a cassette containing a fluorescent gene (mCherry) under the control

of the p72 promoter. This recombinant virus was also plaque purified and amplified in primary swine macrophage cultures.

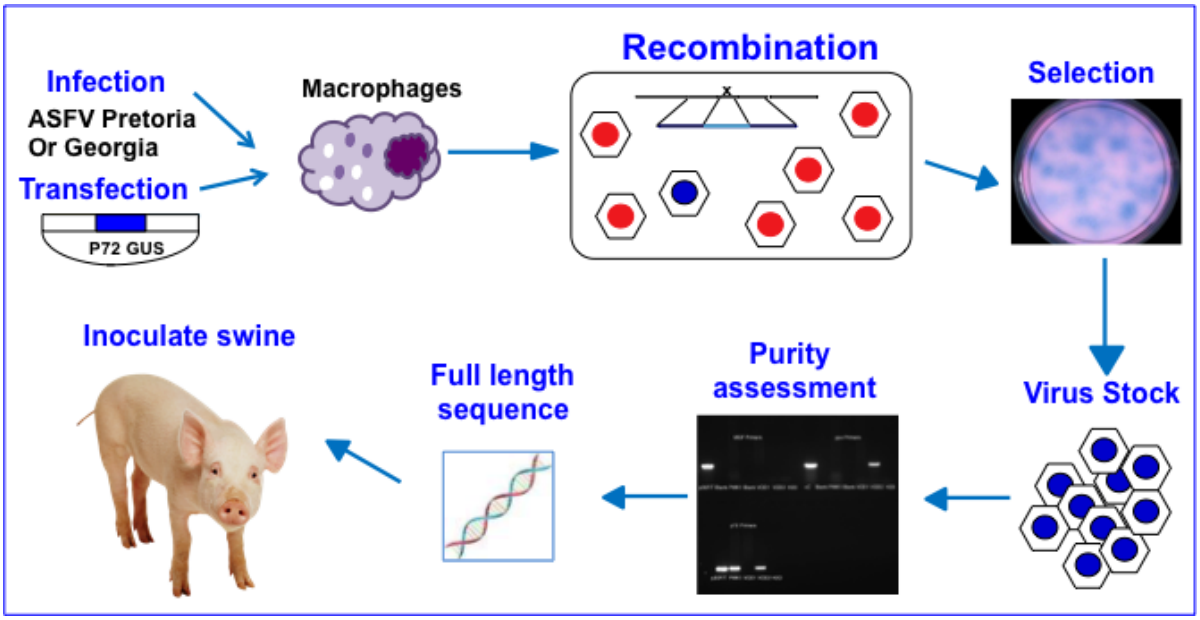

Figure 2.1 Homologous Recombination, targeted gene deletion for ASFV attenuation

2.2.3 Sequencing

ASFV DNA was extracted from infected macrophage cultures by Trizol, quantified with the Qubit® dsDNA HS assay kit and enzymatically fragmented using the Ion Shear™ Plus kit. Fragmented DNA was barcoded using the Ion Plus Fragment Library kit, size selected, and the DNA library was clonally amplified onto Ion Sphere™ Particles, generating template-positive ISPs using the Ion PGM™ Template OneTouch™ 2 200 Kit with the Ion OneTouch™ 2 Instrument. The template-positive ISPs were prepared for sequencing, loaded onto an Ion 314™ Chip v2 and run on the Ion PGM™ Sequencer. Obtained sequences were trimmed using the Galaxy (https://usegalaxy.org/) NGS QC workflow, then aligned and analyzed using Sequencher 5.2.2 (Genecodes) and CLC Genomics Workbench (Qiagen).

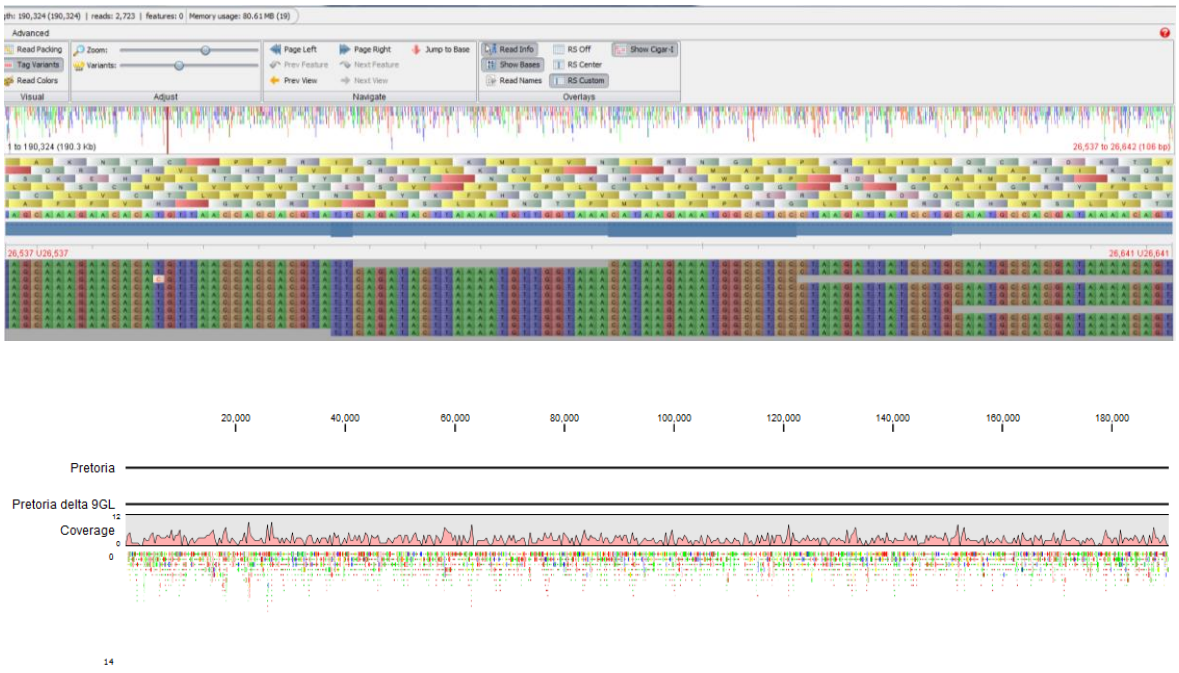

Figure 2.2 Next-Generation Sequencing (NGS) of Pretoria Genome

2.2.4 Growth curves *in vitro*

Pret4 and Pret4Δ9GL were titrated on primary swine macrophages. The presence of virus was assessed by hemadsorption. Virus titers were calculated by the method of Reed and Muench (Reed and Muench, 1938) and expressed as 50% hemadsorption doses (HAD_{50}). For the growth curves, preformed monolayers of primary swine macrophages in 6-well plates were infected at a multiplicity of infection (MOI) of 0.01 HAD_{50} per cell. Virus was adsorbed for 1 hour at 37°C with 5% CO_2. The inoculum was removed and cells were rinsed two times with PBS before 2 mL of macrophage media were added to each well. The supernatant from one of the 6 wells on the plate was collected after 0, 2, 24, 48, 72, and 96 hours of incubation at 37°C with 5% CO_2. Supernatants were frozen at -70°C, and all samples were titrated at the same time to avoid inter-assay variability. Viral titration was performed on primary swine macrophage

cell culture in 96-well plates. Virus dilutions and cultures were performed using macrophage media.

2.3 Viral detection assays from swine studies

2.3.1 Virus isolation and titration

Primary swine macrophages were seeded on 96-well plates at a density of $1X10^5$ cells per well in a volume of 100 µl. For the titrations, 96-well deep-well plates were filled with 630 µl of macrophage media per well. In the top row of the deep-well plate, 70 µl of thawed whole blood were added to the media, creating a 1/10 dilution. The blood and media were mixed by pipetting up and down 10 times, and 70 µl were transferred to the next row. This was repeated five more times to create a dilution series with seven steps, from 10^{-1} to 10^{-8}. Tips were changed before transferring each new dilution to the next row. All samples and plates were kept on ice to prevent blood from clumping. After the dilutions had been prepared, 100 µl of each dilution were transferred to the 96-well plates containing the macrophages in replicates of four, with each plate of macrophages receiving a total of 3 titrated samples.

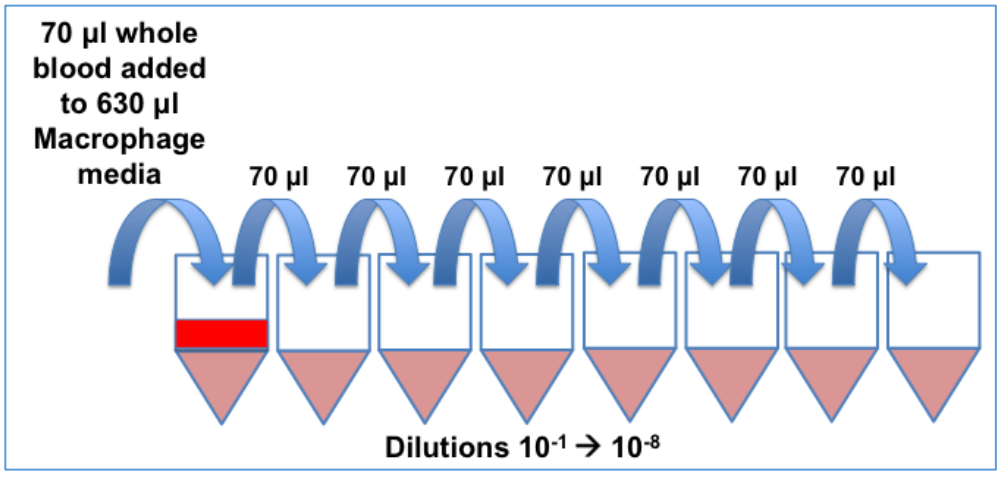

Figure 2.3 Virus titration and dilution scheme

2.4 Animal experiments

For all ASFV experiments castrated male Yorkshire pigs were purchased from a herd free of porcine reproductive and respiratory virus. Swine were dewormed and vaccinated against common porcine pathogens, including porcine circovirus 2, prior to arrival on Plum Island. Pigs were approximately 3-4 months old and weighed 33-36kg on arrival. All animal procedures were performed following a protocol approved by the Plum Island Animal Disease Center Institutional Animal Care and Use Committee (IACUC), ensuring ethical and humane treatment of experimental animals. The animal experiments were carried out in BSL-3 Ag isolation rooms at the Plum Island Animal Disease Center. Animals were kept within the facilities for at least 5 days prior to the start of the experiments to allow for acclimation to the new environment. All animals were fed a grower diet daily and had unlimited access to water.

Swine were clinically assessed, with rectal temperatures recorded at least once per day. Moribund animals were sedated with an intramuscular injection of tiletamine hydrochloride and zolazepam hydrochloride (Telazol, Zoetis; 3 mg/kg), ketamine (8 mg/kg), and xylazine (4 mg/kg), placed in dorsal recumbency and humanely euthanized intravenously via the right jugular vein with 85.8 mg/kg of sodium pentobarbital. Animals without clinical disease were humanely euthanized at the end of the study at 21 days post-challenge.

2.4.1 Clinical evaluation of swine

A clinical score was calculated for each pig in the comparative pathogenesis study to compare differences in swine responses to the Pret4Δ9GL and Pret4 viruses. Clinical signs and scoring are found in Table 2 below. The clinical signs were assigned

numerical values based on severity and significance as described by (Howey *et al.*, 2013). Clinical scores were recorded daily for each pig.

Table 2.2 Clinical Scoring of swine inoculated with ASFV adapted from (Howey *et al.*, 2013)

Characteristic	Score	
Behavior and Mentation:	0	Normal, alert, responsive
	1	Mildly obtunded. Slightly reduced liveliness, stands up unassisted, resists restraint or rectal thermometer
	2	Obtunded. Reluctant to stand but will do so when assisted, decreased resistance to restraint or rectal thermometer
	3	Intermittent ataxia, disorientation, can still stand/walk or Will not stand/walk even when assisted, still conscious
	4	Moribund. Nonambulatory, unconscious/nonresponsive
Neurologic signs		
	0	Normal
	2	Unambiguous neurologic signs, convulsions, seizures
Defecation		
	0	Normal to mildly soft stools
	1	Profuse watery diarrhea +/- mild hematochezia or melena
	2	Severe to marked hematochezia or melena
Body Temperature		
	0	38-40°C
	1	Temperature greater than or equal to 40°C
	2	Temperature greater than or equal to 40°C for at least 2 subsequent days
	3	Temperature greater than or equal to 41°C
	4	Temperature than or equal to 38°C

2.4.2 Sample collection

Swine were restrained by a rope. Sample collection included whole blood with EDTA for titrations and PCR analysis, whole blood with heparin for ELISpot, and clotted blood for serum for ELISA (Enzyme Linked Immunoassay), IPA (Immunoperoxidase Assay), and cytokine ELISAs. All blood samples were collected from the jugular vein. Nasal swabs were collected from both nostrils using sterile cotton swabs that extended

just caudal of the alar fold. All samples were stored in cryovials and kept at -70°C until processing.

2.4.3 Comparative pathogenesis study with Pret4 and Pret4Δ9GL

Experiments were performed to compare pathogenesis between swine inoculated with virulent Pret4 and Pret4Δ9GL via intramuscular inoculation. Twenty pigs were inoculated into the right semimembranosus muscle with 10^4 50% hemadsorption doses (HAD_{50}) of virulent Pret4 or Pret4Δ9GL in 1 mL (two groups, n=10 per group). Swine were evaluated daily for fever and clinical signs. Blood samples and nasal swabs were collected every other day for 10 days post-infection (DPI). Two animals from each group were euthanized and necropsied 2, 4, 6, 8, and 10 days post-infection. Tissues collected during postmortem examination included tonsil of the soft palate, lingual tonsil, nasal tonsil, dorsal nasopharynx, lung, liver, spleen, as well as retropharyngeal, gastrohepatic, and superficial cervical lymph nodes.

Tissue samples collected were stored in cryomolds, embedded in Optimal Cutting Temperature (OCT) medium (Tissue-Tek O.C.T. compound, Sakura Finetek, CA) and then frozen in liquid nitrogen vapor.

2.4.4 Protective immunity after vaccination with Pret4Δ9GL: onset of protection

Eighty-five pigs were used in a second set of experiments to assess the onset of protective immunity against virulent homologous challenge after vaccination with PretΔ9GL. Sixty-five swine were inoculated with 1 mL 10^4 HAD_{50} of Pret4Δ9GL and subsequently challenged with 1 mL 10^4 HAD_{50} of virulent Pret4 at 7, 10, 14, 21, or 28 days post-inoculation. The experiments were carried out in 4 batches, and each

challenge time point was repeated at least once. In each experiment, the Pret4∆9GL inoculations were staggered so that all groups were challenged with Pret4 at the same time. A control group of 5 naïve swine was included in each batch; overall, 20 swine were used as unvaccinated challenge controls. Each vaccination group contained 5 swine, with the exception of the 28-day challenge where the first experiment contained four swine and the third contained six swine.

2.4.5 Protective immunity after vaccination with Geo∆9GL/UK: dose effect and onset of protection

At total of sixty-four swine were used in a third set of experiments with the Georgia strain of ASFV. This included 15 naïve controls for three separate challenge experiments and 49 vaccinates receiving the double deletion mutant Geo∆9GL/UK before challenge with virulent homologous Georgia 2007. For the dose study, groups of 4 or 5 swine were vaccinated with either a low dose of 10^2 HAD_{50} (two experiments, n = 9 overall), a medium dose of 10^4 HAD_{50} (two experiments, n = 10), or a high dose of 10^6 HAD_{50} of Geo∆9GL/UK (three experiments, n = 15). All vaccinated swine were challenged with 10^4 HAD_{50} of virulent Georgia intramuscularly at 28 days post-inoculation with Geo∆9GL/UK. For the onset of protection study – similar to what was described for Pret4∆9GL above – 15 pigs (in 3 groups of 5) were inoculated with the medium dose of Geo∆9GL/UK (10^4 HAD_{50}) and challenged with virulent Georgia 2007 at 7, 14 or 21 days post-inoculation.

2.5 Tissue maceration technique for virus isolation

Ten tissues were collected from 20 swine that were infected with either Pret4∆9GL or Pret4 during necropsy and frozen at -70°C. Tissues weighing 1 gram or

less were used for titration. Each tissue was place in an individual mortar with a small pinch of aluminum oxide (Ward's Science, Rochester NY) and ground to a paste with a pestle. For every 0.5 g of tissue 1 mL of DMEM with 10% FBS was added to the tissue paste. The tissue was ground further with media and pipetted into 2 mL microcentrifuge tubes and centrifuged for 10 minutes at 4°C at 8,000 × g. Supernatant from the tissue macerate was used for titrations as described previously.

2.6 PCR for detection and typing of Pret4 and PretΔ9GL

Total DNA was extracted from 200 µl of whole blood from infected swine or virus stocks with the Qiagen DNeasy blood and tissue kit, using 20 µl proteinase K and 200 µl buffer AL. For improved lysis, blood samples were incubated at 56°C for 10 min, but this was not done for the virus stocks.

After lysis, 200 µl of 100% ethanol were added and the samples were mixed thoroughly by vortexing. The mixture was transferred into a DNeasy Mini spin column in a 2-mL collection tube, centrifuged at 6000 × g for 1 min and the flow through was discarded. Washes with AW1 and AW2 were completed in a similar manner following the manufacturer's protocol. DNA was eluted with 50 µl of nuclease-free water at 6000 × g for 1 min.

Identification of virus in the blood of infected/challenged animals was performed by a differential PCR based on the recognition of the 9GL and the β-GUS gene in Pret4 and PretΔ9GL, respectively.

Thermocycler settings were 95°C for 1 minute, followed by 32 cycles of 95°C for 30 seconds and 68°C for 1 minute.

Detection of a 160-bp fragment of the 9GL gene was performed using the following pair of primers:

Forward: 5’ GTAAGATACGAAAAGGCGTG 3’

Reverse: 5’ CATTGGGGACCTAAATAC 3’

Detection of a 485-bp fragment of the β-GUS gene was performed using the following pair of primers:

Forward: 5’ GCAATTGCTGTGCCAGGCAGTT 3’

Reverse: 5’ TGCCAGTCAACAGACGCGTG 3’

Table 2.3 PCR mixture was set up as follows:

Reagent	Volume
10X Buffer	5 µl
Water	40 µl
dNTPs	1 µl
Taq Polymerase	1 µl
Forward Primer 100 picomoles/µl	1 µl
Reverse Primer 100 picomoles/µl	1 µl
Sample DNA	1 µl

2.7 Serological assays

2.7.1 In-house indirect ELISA

Antigen preparation and ELISA procedures were based on the methods of (Katz *et al.*, 2012) with minor adjustments. Briefly, Vero cells were infected with Vero-adapted ASFV Georgia (Krug *et al.*, 2015) and incubated until cytopathic effect reached 100%. The infected cells were resuspended in water containing protease inhibitor (Roche), and then Tween 80 (G-Biosciences) and sodium deoxycholate (Sigma) were added to a final concentration of 1% (v/v) each. Uninfected Vero cells were treated in the same manner

and all antigen preparations were stored at -70°C. Maxisorb ELISA plates (Nunc) were coated overnight at 4°C with 1 µg per well of either infected cell or uninfected cell antigen. Antigen concentration was determined by the Bradford assay. The plates were blocked with PBS containing 10% (w/v) skim milk powder (Merck) and 5% (v/v) normal goat serum (Sigma) for 2 hours at 37°C. Then plates were washed with PBS 3 times. Each serum sample was tested at multiple dilutions (1/10, 1/100, 1/1000, and 1/10,000) against both infected and uninfected cell antigen and incubated at 37°C for 1 hour. Plates were washed 3 times with PBS. ASFV-specific antibodies in the swine sera were detected by an anti-swine IgG-horseradish peroxidase conjugate (KPL) using a 1:1500 dilution and incubated at 37°C for 1 hour, detected by peroxidase substrate (SureBlue Reserve, KPL). Plates were read at a wavelength of 630 nm in an ELx808 plate reader (BioTek). Swine sera were considered positive for ASFV-specific antibodies if the OD_{630} ratio of the reaction against infected cell antigen to uninfected cell antigen was higher than 2.2. The serum dilution was considered negative if the ratio was less than 1.5.

2.7.2 Svanovir commercial ELISA

The Svanovir ASFV-Ab Indirect ELISA kit was used to detect swine antibodies against p30 ASFV antigen. Serum samples were diluted 1/100 in sample dilution buffer by adding10 µl of serum to 990 µl buffer. Serum was tested against control antigen and ASF viral antigen by adding 100 µl of diluted serum to the corresponding wells in duplicate. Plates were sealed, gently mixed in a rocker and incubated at 37°C for 1 hour. Subsequently, plates were rinsed four times with PBS 0.05% Tween buffer. One hundred µl of HRP conjugate was added to each well, and again incubated at 37°C for 1 hour. Plates were rinsed three times with PBS/Tween and 100 µl of substrate solution

was added to each well and incubated for 10 minutes at room temperature. The color reaction was terminated by adding 50 µl of Stop Solution to each well and mixing thoroughly.

The optical density of each well at 450 nm was measured in a microplate photometer (using air as blank). Plates were read at OD_{450} in an ELx808 plate reader (BioTek).

Calculations:

OD ASFV – OD control = OD corrected

Percent Positivity

PP = (OD corr. Sample or Negative Control / OD corr. Positive Control) * 100

Control values should fall within these limits:

OD Positive Control > or = 0.5

OD Negative Control < or = 10

2.7.3 Immunoperoxidase assay for detecting anti-ASFV antibodies

Anti-ASFV antibodies in sera of infected animals were quantified with an in-house immunoperoxidase assay, where Vero cells in 96-well plates were infected (at MOI 0.1) with Vero-adapted ASFV Pret4. Infected cell monolayers were fixed in 50% acetone and 50% methanol for 10 minutes at room temperature. Plates were blocked with 5% (w/v) skim milk powder (Millipore, Billerica, MA) and 0.05% (v/v) Tween 20 (Sigma, St Louis, MO) for a 1 h at 37°C. Two-fold dilutions (1/50→1/6400) of the sera were diluted in the milk + tween + PBS1X blocking buffer and subsequently incubated on the infected cell monolayers in duplicates for 1 h at 37°C. After washing with PBS, the presence of anti-ASFV antibodies was detected by using Vector biotinylated goat anti-swine IgG (H+L) and the Vectastain ABC HRP kit (Vector Laboratories, CA). Titers

were expressed as the inverse $\log_{10}$ of the highest serum dilution with positive cytoplasmic staining. A positive control (a serum previously showing strong staining in 1/6400) and a naïve serum for a negative control were diluted in two fold dilutions and added to every plate for quality control. See setup below.

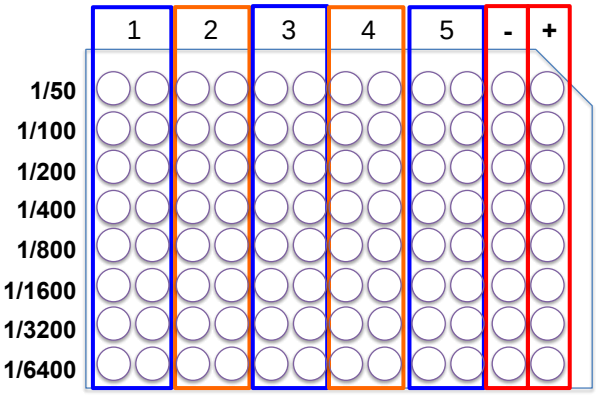

Figure 2.4 Immunoperoxidase dilution scheme

2.8 Peripheral blood mononuclear cell isolation technique and IFN-γ ELISpot

Detection of ASFV-specific IFN-γ producing cells was performed using a modification of the ELISpot porcine IFN-γ method by R&D Systems. A monoclonal antibody specific for porcine IFN-γ is pre-coated onto polyvinylidene difluoride-backed microplates. When the plates are incubated with stimulated PBMCs, this antibody binds and immobilizes IFN-γ produced by the cells. Bound IFN-γ is then visualized and quantified.

Fifteen mL of porcine blood were mixed with 20 mL of PBS at room temperature in 50-mL conical tubes. Twelve mL of Ficoll Paque Plus at a density of 1.077 g/mL was carefully pipetted under the blood/PBS mixture and spun at 420 × g for 32 minutes at room temperature with the acceleration and brake set to zero.

The cells were diluted in trypan blue 1/20 and counted manually on a hemocytometer, and concentration was adjusted to 5×10^6/ml. Cells in a volume of 100 µl were placed in a round-bottom 96-well plate and then spun at 640 × g for 4 minutes. The supernatant was removed carefully without disturbing the cell pellet. One hundred µl of Vero-adapted Pret4 (2.5×10^6 per ml; MOI of 0.5), a non-specific cell stimulant (25ng/mL phorbol 12-myristate 13-acetate [PMA] and 25ng/mL calcium ionomycin; positive control) or sterile media (negative) were added to the cells in triplicates. The mixtures were immediately transferred to the ELISpot plate and incubated for 18 hours at 37°C with 5% CO_2. Cell lysis, washing and detection by anti-IFN-γ antibody, streptavidin-AP and BDIP/NBT chromogen were sequentially performed as recommended by the manufacturer. Spot counts were performed with an ELISpot plate reader (Immunospot, Cellular Technology Limited) with the following settings: counting mask size 100%, normalize counts of mask: Off, sensitivity: 130, min. spot size: 0.086 sq.mm, max. spot size: 0.2596 sq. mm, oversized spots were estimated at spot separation: 1, diffuseness: large, background balance: 67. Cell counts were expressed as a number of spots per 5 × 10^5 PBMC.

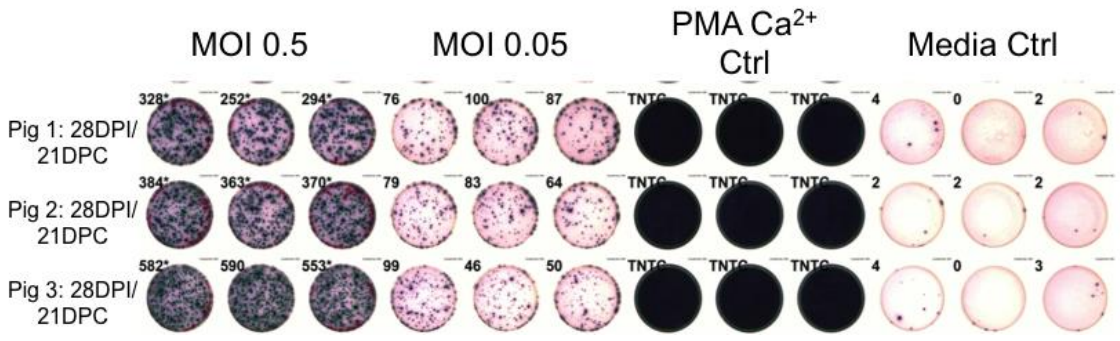

Figure 2.5 ELISpot cell count from CTL plate reader

2.7 Cytokine ELISA

Levels of serum MCP2, TGF-β1, IFN-α, IFN-β, IFN-γ, IL1-α, IL1-β, IL-2, IL-5, IL-8, IL-10, IL-12 p35, IL-12 p40, OAS, PKR, TNF, MX-1, and VCAM were assessed using commercial ELISAs following manufacturer protocols (MyBioSource, San Diego, CA).

Table 2.4 Cytokines, chemokines, ELISA kits

	Cytokine Kit	Manufacturer #
1	Porcine VCAM-1/CD106 (soluble Vascular Cell Adhesion Molecule 1	MBS2515499
2	Porcine Total Tumor Necrosis Factor	MBS019118
3	Porcine 2',5'-Oligoadenylate Synthetase	MBS060350
4	Porcine Interferon-induced GTP-binding protein Mx1	MBS2601462
5	Porcine Interleukin 12 p35	MBS754052
6	Interleukin 6 (IL6)	MBS2021271
7	Porcine Interferon b, IFN-b	MBS9300061
8	Porcine Monocyte Chemotactic Protein 2 (MCP2)	MBS9308754
9	Porcine Protein Kinase R	MBS058542
10	TGF-beta1	MBS9302475
11	Interferon alpha	MBS738080
12	Interleukin 10	MBS761474
13	Interleukin 12 p 40	MBS268094
14	Interleukin 5	MBS265447
15	Interleukin 8	MBS2506194
16	Interleukin 2	MBS9304562
17	Interleukin 1 alpha	MBS9301200
18	Interleukin 1 beta	MBS01994

2.8 Immunocytochemistry for ASFV p30

ASFV antigen was detected in cyrosectioned tissues by using a primary mouse monoclonal antibody targeting ASFV p30 (1D9) (Cuesta-Geijo *et al.*, 2012; Galindo *et al.*, 2012) (kindly provided by F. Javier Dominguez Juncal, Instituto Nacional de

Investigación y Tecnología Agraria y Alimentaria [INIA], Spain) and a commercial biotinylated anti-mouse IgG with the VECTASTAIN Avidin Biotinylated Enzyme Complex and Vector VIP horseradish peroxidase kits (Vector Laboratories, CA).

Table 2.5 Reagents for ASFV p30 Detection

Blocking Buffer (BB) with Normal Horse Serum	ASFV p30	Conjugate Anti-mouse IgG
92 mL MEM 2X	Dilute p30 antibody 1:300	Dilute anti-mouse IgG 1:200 or 1 drop per 5 ml
92 mL Sterile Water	Blocking buffer with NHS	Blocking buffer with NHS
10 mL HEPES 1M		
5.3 mL BSA 7.5%		
For every 5mL BB add 1 drop Normal Horse Serum (NHS)		

Table 2.6 Reagents for ASFV p30 Detection

A+B Reagent	VIP Substrate
133 µl Bovine Serum Albumin (7.5%)	3 drops Reagent 1
9.8 mL PBS1X	3 drops Reagent 2
4 Drops ABC Reagent A	3 drops Reagent 3
4 Drops ABC Reagent B	3 drops H_2O_2
For every 10 mL total	For every 5 mL PBS1X

Infected cells on 96-well plates were fixed with 50% acetone and 50% methanol for 10 minutes at room temperature. Plates were blocked with 100 µl blocking buffer for 20 minutes at room temperature. Next, 65 µl of diluted anti-ASFV p30 antibody 1D9 were added and incubated at room temperature for 30 minutes. A+B Reagent was prepared in advance and incubated at room temperature for 30 minutes prior to use. The plates were washed 3 times with PBS, 65 µl/well of AB substrate solution was added and incubated for approximately 30 minutes at room temperature. AB Reagent was

discarded and the plates were washed 3 times with PBS. VIP substrate was made fresh in PBS, and 65 µl were pipetted in each well. Plates were incubated for 3-10 min while repeatedly checking for developing background straining. To stop the color reaction, the VIP substrate was discarded and distilled water was used to wash the plate 3 times.

2.9 Immunohistochemistry staining of tissue sections with 1D9 (p30)

For IHC, OCT-embedded tissue samples were cryosectioned and mounted on electrostatically charged glass slides. The slides were fixed in acetone for 10 min at -20°C. IHC staining was performed as previously described by (Howey *et al.*, 2013). Slides were blocked for 2 h at room temperature with PBS 0.05% Tween (PBST) containing 6% (v/v) mixed serum and 2% (w/v) powdered milk. Primary mouse monoclonal antibody 1D9, targeting ASFV p30, was diluted 1:300 in PBST and 60 ul volume was applied to tissue sections on each slide that were then incubated for 20 h at 4°C. For IHC, specific anti-ASFV immunoreactivity was detected using a commercial micropolymer alkaline phosphatase detection system (Mach 3 AP; Biocare, CA) as per the manufacturer's recommendation with an alkaline phosphatase substrate (Vector Red; Vector Laboratories, CA). Slides were counterstained with Gill's hematoxylin and cover-slipped using routine methods. An adjacent section of each screened tissue was treated with a mouse monoclonal antibody against foot-and-mouth-disease virus (10GA4) as a negative control.

Chapter 3 - Pretoria Swine Experiments

3.1 Introduction

There is no vaccine available against ASF, and the only means of control of ASF are strict quarantine and biosecurity measures, control of animal movement, and slaughter of exposed and affected herds. Experimental vaccines based on the use of inactivated virus or virus subunits have failed to induce solid protective immunity (Forman, A. J., 1982; Kihm et al., 1987; Mebus, C., 1988, Chapman et al., 2011, Lacasta, A. 2014), but homologous protective immunity does develop in swine surviving infections with moderately virulent or attenuated ASFV isolates (Mebus, C., 1988; Hamdy and Dardiri, 1984; Ruiz-Gonzalvo et al., 1981). Swine immunized with live attenuated ASFVs with genetically engineered deletions of specific virulence-associated genes are similarly protected when challenged with homologous parental viruses. Previous reports of individual deletions of the *UK* (*DP69R*), *23-NL* (*DP71L*), *TK* (*A240L*) or *9GL* (*B119L*) genes from the genome of virulent ASFV resulted in significant attenuation in swine, where swine immunized with these modified viruses demonstrated protection when challenged with homologous ASFV (Zsak et al., 1998; Moore et al., 1998; Lewis et al., 2000; Zsak et al., 1996). These observations are the only experimental evidence supporting a rational development of effective live attenuated virus against ASFV.

The deletion of 9GL (*B119L*) in the highly virulent ASFV isolates Malawi Lil-20/1, Pretoriuskop/96/4 (Pret4), and more recently Georgia 2007, resulted in complete attenuation of these viruses in swine (Lewis et al., 2000; Neilan et al., 2004; O'Donnell et al., 2015a). Therefore, targeting the highly conserved *9GL* (*B119L*) gene for genetic

modifications was seen as a reasonable approach for developing attenuated viruses as vaccine candidates. Animals infected with Pret4 lacking the 9GL gene (PretΔ9GL) are all protected when challenged with Pret4 at 42 days post-infection (dpi) (Neilan et al., 2004). Challenging Pret4∆9GL-infected animals with the parental virulent Pret4 virus earlier than 42 dpi showed a progressive acquisition of protection starting with 40% of animals surviving a challenge at 7 dpi and reaching around 80% of protection against a challenge between 21 and 28 dpi. Based on these results, we developed an animal model to investigate correlates of protection against virulent challenge with Pret4.

3.2 Analysis of the Pret4∆9GL genome and the parental Pret4 genome

To evaluate the status of genetic modification and the integrity of the genome of the Pret4∆9GL recombinant virus, full genome sequences of Pret4∆9GL and the parental Pret4 viruses were generated using NGS on the Ion Torrent PGM™ and compared. From the analysis we found that the Pret4∆9GL virus did not accumulate any significant mutations during the process of homologous recombination and sequential plaque purification steps.

3.2 Analysis of the replication of Pret4 and Pret4∆9GL *in vitro*

In vitro growth characteristics of Pret4∆9GL and the parental Pret4 were evaluated in primary swine macrophage cell culture and compared in a multistep growth curve (Figure 3.1). Pret4∆9GL's growth was significantly delayed compared to the parental Pret4 virus in early time points prior to 96 hours. The 9GL-gene-deleted virus exhibited titers 10 to 1000-fold lower relative to the parental virus. The deletion of the 9GL gene significantly affected the ability of the Pret4∆9GL virus to replicate in vitro in primary swine macrophage culture.

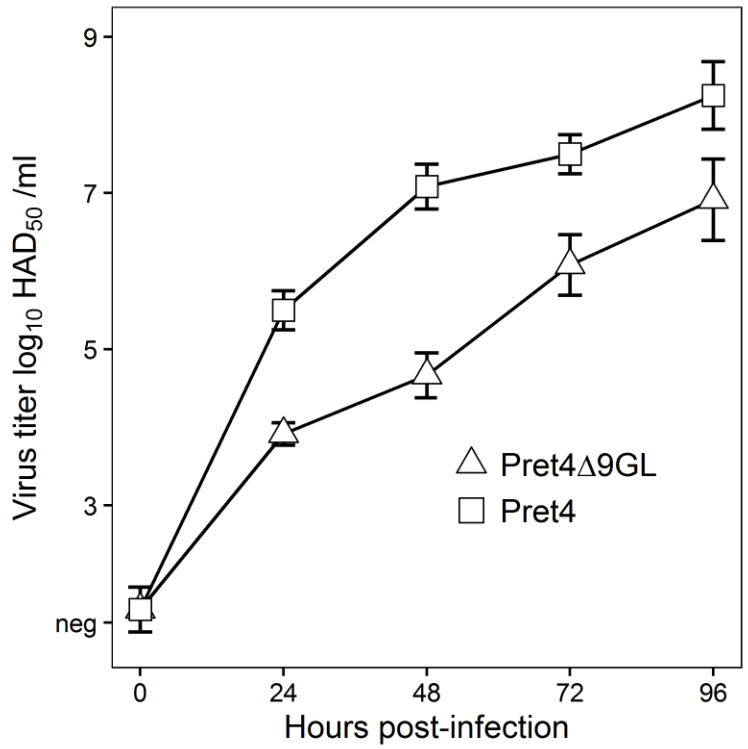

Figure 3.1 In vitro growth characteristics of Pret4Δ9GL (triangles) and parental Pret4 (squares). Primary swine macrophage cell cultures were infected with either Pret4Δ9GL or Pret4 at MOI 0.01 and the virus yield was titrated at different times post-infection. Data represents mean and standard deviation (SD) from three independent experiments as described in Chapter 2.4. The limit of detection was Log_{10} 1.5 HAD_{50}/mL.

3.3 Experimental design and results of comparative pathogenesis experiment

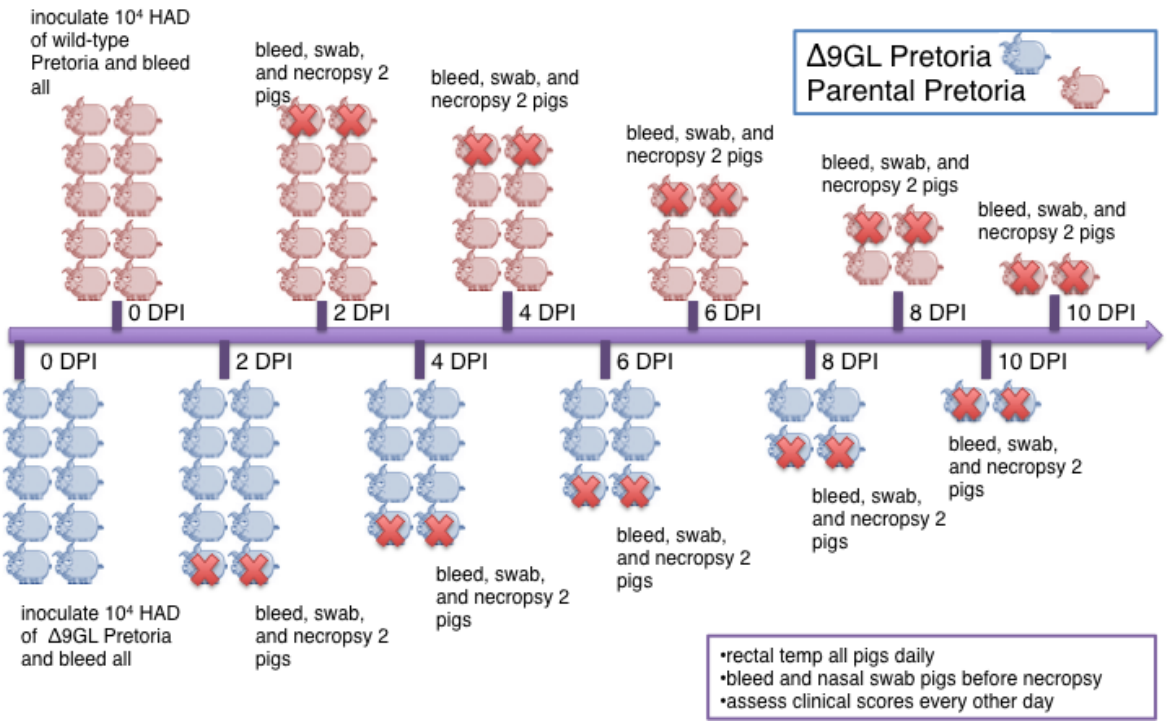

Figure 3.2 Experimental Design of comparative pathogenesis study between the virulent Pret4 and its attenuated derivative Pret4Δ9GL[1]

To investigate growth characteristics *in vivo*, twenty 33-36 kg pigs were inoculated intramuscularly with 10^4 50% hemadsorption doses (HAD_{50}) of virulent Pret4 or Pret4Δ9GL. Animals were observed daily for fever and clinical signs (see Table 6 below). Blood samples and nasal swabs were collected every other day for 10 days post-infection (DPI). Two animals from each group (n=10) were euthanized and necropsied on 2, 4, 6, 8, and 10 days post-infection. Whole blood, nasal swabs, and tissue homogenates were titrated on primary peripheral-blood macrophages as

[1] Pig clip art is in the public domain. Downloaded from http://cliparts.co/clipart/2303191.

previously described (Zsak *et al.*, 2005). Presence of virus was detected by hemadsorption and virus titers were calculated by the method of Reed and Muench (Reed and Muench, 1938)

3.3.1 Comparative pathogenesis between the virulent Pret4 and its attenuated derivative Pret4Δ9GL

3.3.1.1 Clinical signs

To assess virus replication *in vivo,* swine were inoculated with either Pret4∆9GL or parental Pret4. Swine were observed daily for fever and ASFV-related clinical signs (see Table 2 in Chapter 2.4.1). Swine infected with Pret4 presented with increased body temperature of greater than 40°C by 3 to 4 days post-infection. Clinical signs associated with ASF became apparent as early as four days post-infection. As ASF progressed, swine either died or were humanely euthanized in 6 to 10 days post-infection. Swine inoculated with Pret4∆9GL remained clinically normal during the 10-day observational period (Fig 3.4). Swine inoculated with Pret4Δ9GL did not have detectable clinical signs such as skin hemorrhages, neurologic signs, melena, or pyrexia.

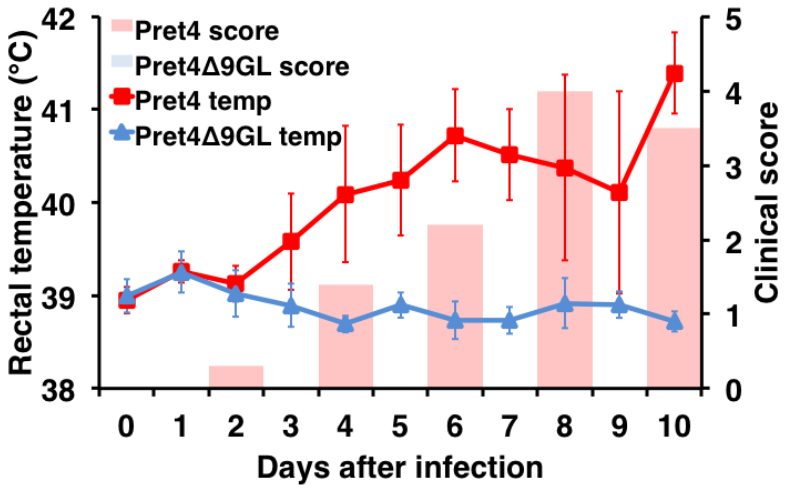

Figure 3.3 Comparative pathogenesis in twenty swine: Rectal temperatures (lines) and clinical scores (bars) of Pret4 (red squares and pink) bars and Pret4Δ9GL (blue triangles) infected swine. See clinical scoring in table 2.2

3.3.1.2 Viremia and nasal shedding

Blood samples and nasal swabs were collected every other day for 10 days from each group (n=10). Whole blood and nasal swabs were titrated on primary swine macrophage cell cultures. Viremia in animals inoculated with Pret4Δ9GL peaked transiently by day 4 post-infection and remained at significantly lower levels compared to swine inoculated with Pret4. The limit of detection was Log_{10} 1.5 HAD_{50}/mL. Swine inoculated with Pret4Δ9GL had significantly lower viremia than those inoculated with Pret4. No Pret4Δ9GL was detected in nasal swabs. Clinical signs of ASF progressed in Pret4-infected swine, nasal shedding of virus was detected as early as 4 dpi. No nasal viral shedding was detected by virus isolation in Pret4Δ9GL-infected animals.

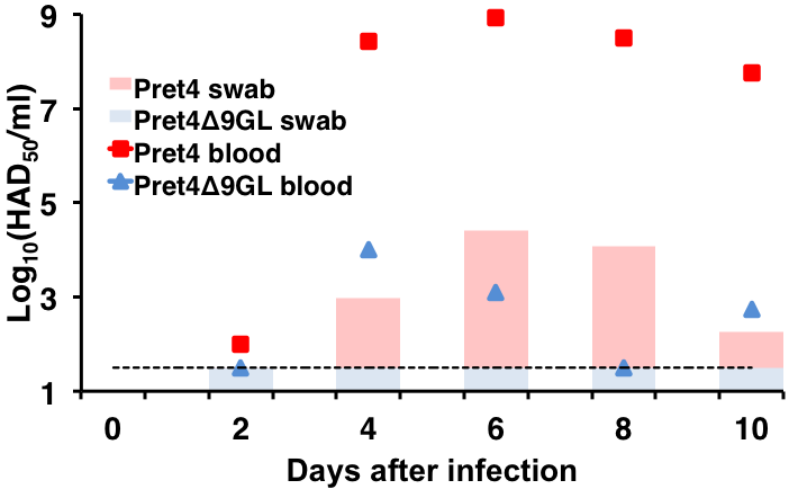

Figure 3.4 Comparative pathogenesis in twenty swine: Virus titers in nasal swabs (blue triangles Pret4Δ9GL and red boxes Pret4) and blood samples (light blue bars Pret4Δ9GL and pink bars Pret4). Limit of detection was Log_{10} 1.5 HAD_{50}/mL.

3.3.1.3 Virus detection in tissues by virus isolation and immunohistochemistry

The same group of swine that were inoculated with 10^4 HAD_{50} of either Pret4 or Pret4Δ9GL were humanely euthanized (2 swine per time point) from each group at 2, 4, 6, 8, and 10 days post-inoculation. Ten tissues were collected for virus isolation (Fig 3.5) and in OCT medium for IHC (3.6) for further characterization of viral antigen present in tissues. The 10 tissues collected from each pig included the lingual tonsil, palatine tonsil, nasopharyngeal tonsil, epithelium of the dorsal nasal pharynx, lung, a submandibular lymph node, liver, spleen, gastrohepatic lymph node, and superficial cervical lymph node. The highest Pret4 titers were observed in the spleen, liver, and gastrohepatic lymph node. Pret4Δ9GL was found only in spleen at days 2, 4, 6, and 10 dpi, where as virus was isolated from liver at 4, 6, and 10 dpi. Lastly, Pret4Δ9GL virus was detected the lung of one pig at 6 dpi. The limit of detection was Log_{10} 1.5 HAD_{50}/mL.

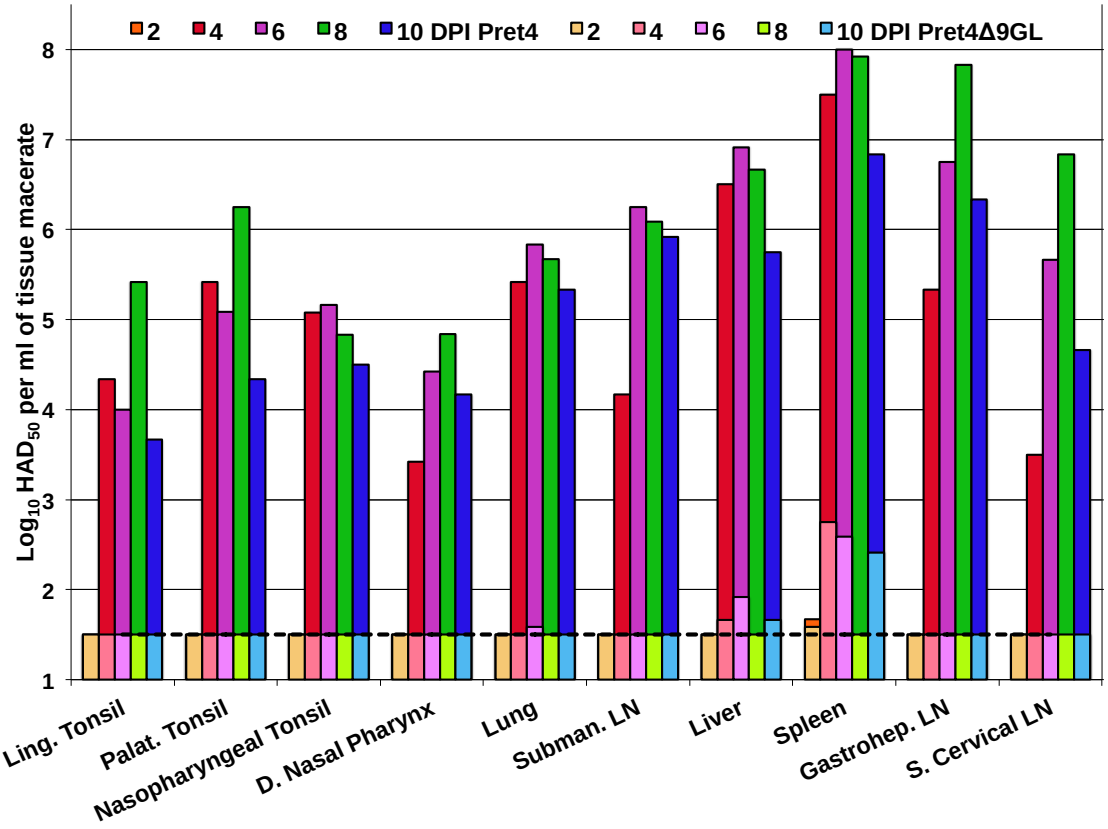

Figure 3.5 Comparative Pathogenesis in 20 swine: Virus titers of tissue macerates collected from 2 swine per time point at 2, 4, 6, 8, and 10 days post-inoculation. Bars with dark colors set to the back represent an average titer from each tissue macerate from two individual animals inoculated with Pret4 at each time point. Light pastel colored bars set in the front represent an average titer from each tissue macerate from two individual animals inoculated with Pret4Δ9GL. Virus titer limit of detection: ≤ Log_{10} 1.5 HAD_{50}/mL of tissue macerate.

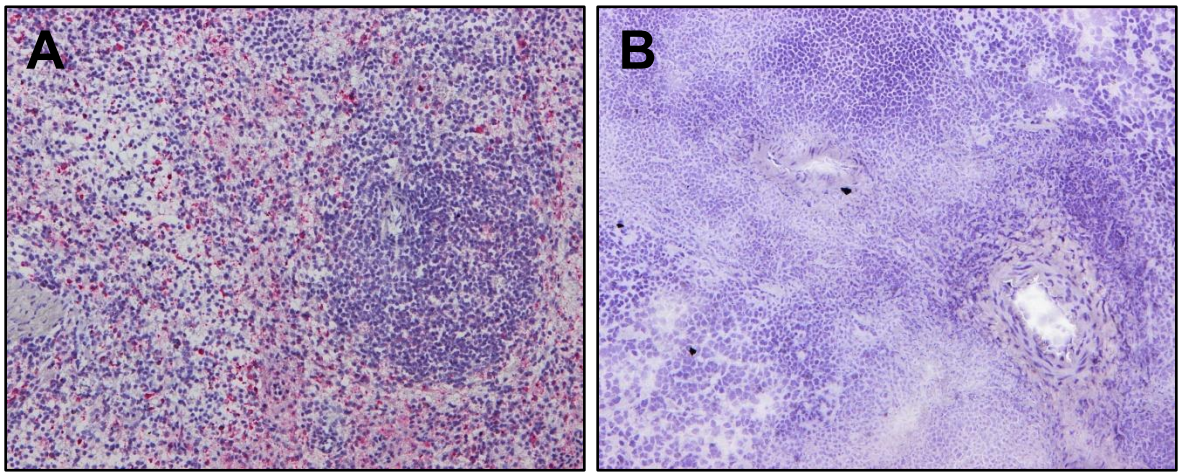

Figure 3.6 Immunohistochemistry staining of frozen spleen section with anti-ASFV-p30 and hematoxylin at 4 days post-infection with Pret4 (A) and Pret4Δ9GL (B) 10X magnification.

3.3.1.4 Descriptions of IHC from frozen spleen sections

Spleen from swine infected with Pret4 stained positive extensively for p30 at time points between 4 and 10 dpi. The p30 antigen was not detected in any swine infected with Pret4Δ9GL despite the successful isolation of virus via macrophage culture. IHC was only run on spleen samples since the highest titers of Pret4Δ9GL were found in the spleen. Further IHC of other Pret4Δ9GL tissues was not pursued after multiple sections had been cut and multiple staining attempts to detect PretΔ9GL in the spleen had been made with no result.

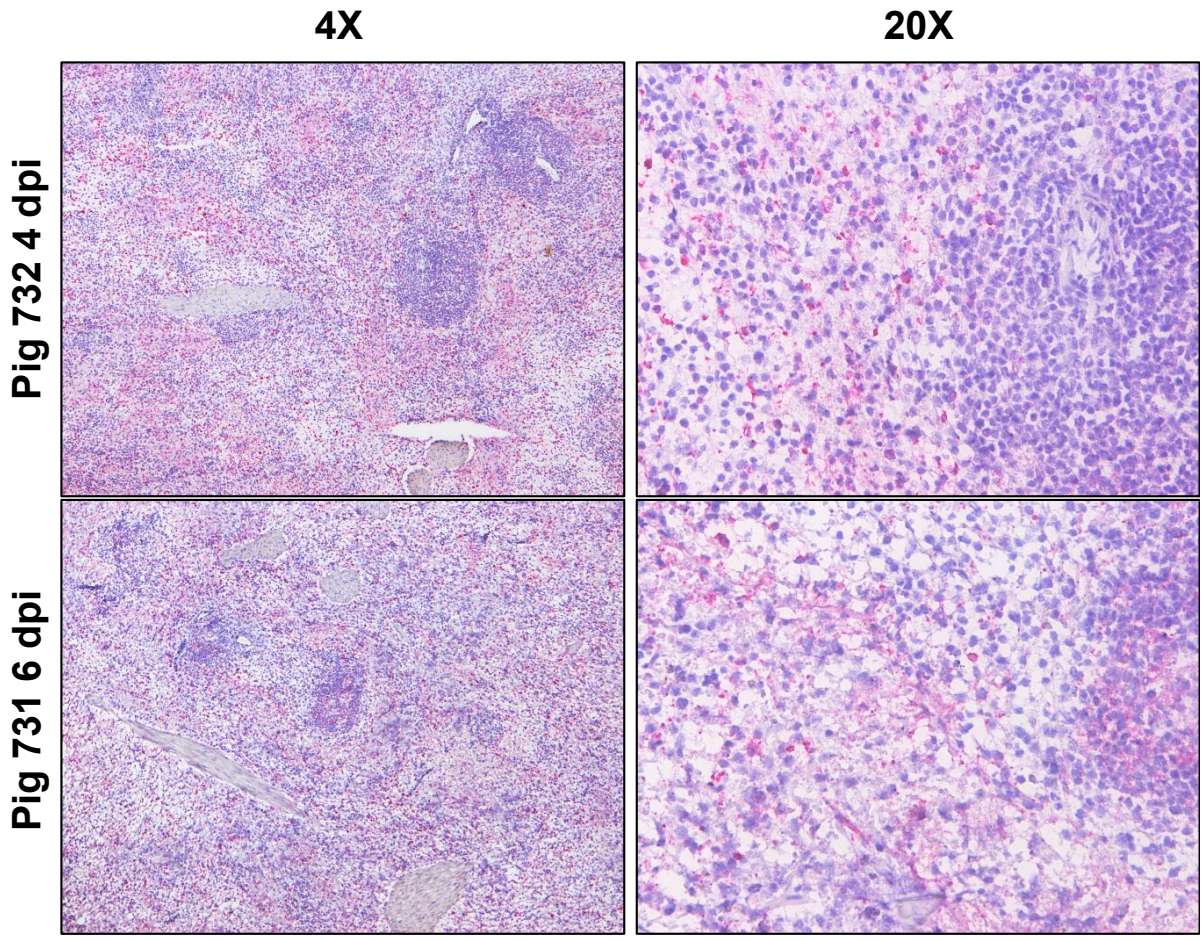

Figure 3.7 Immunohistochemistry staining of frozen spleen section with anti-ASFV-p30 and hematoxylin at 4 (top) and 6 days post-infection (bottom) with Pret4. Taken at 4X (left) and 20X (right).

These images above in Fig 3.7 show the extensive staining of anti-ASFV p30 throughout the spleen in swine infected at four and six days post-infection with Pret4. ASFV antigen is found within and surrounding the white pulp and red pulp.

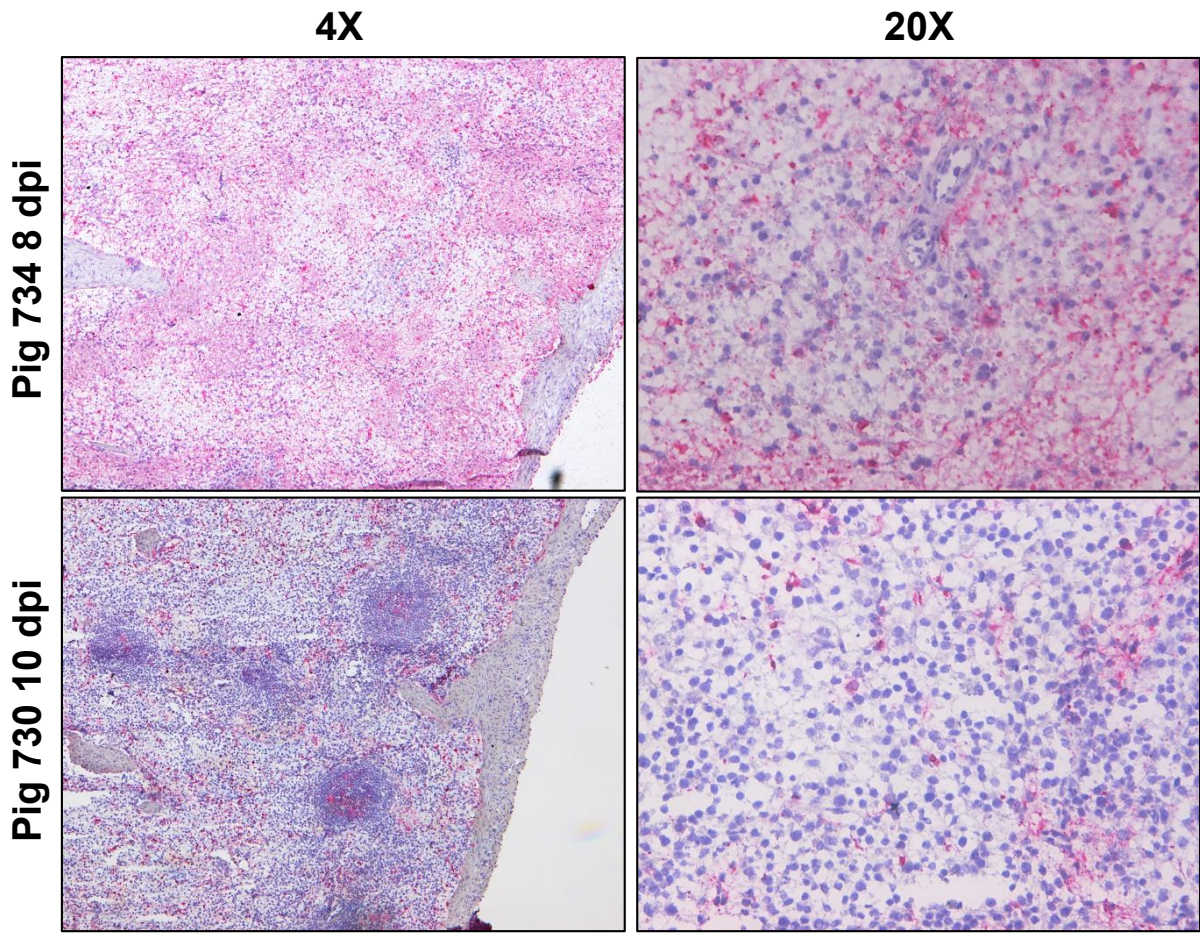

Figure 3.8 Immunohistochemistry staining of frozen spleen section with anti-ASFV-p30 and hematoxylin. Spleen from swine infected with Pret4 at 8 (top) and 10 dpi (bottom) at 4X (left) and 20X (right).

The images in Fig 3.8 above show the extensive staining of anti-ASFV p30 throughout the spleen of swine infected with Pret4 at 4 and 6 days post-infection. ASFV is found within and surrounding the white pulp and red pulp. In pig 734, the lack of purple staining indicates severe depletion of lymphocytes throughout the spleen. Lymphoid depletion was also visible in pig 730 from 10 dpi but not to the same degree. Which organs were most severely affected varied among infected swine.

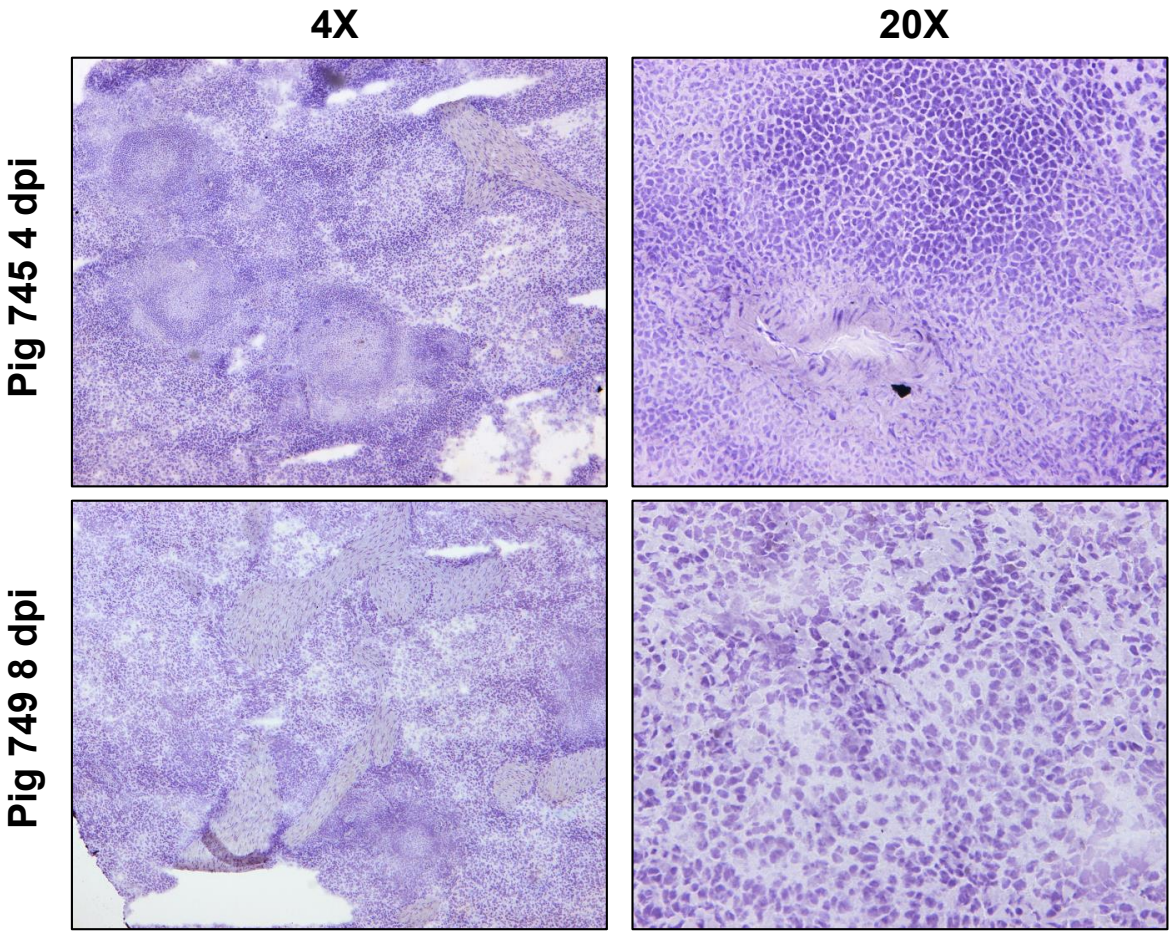

Figure 3.9 Immunohistochemistry staining of a frozen spleen section with anti-ASFV-p30 and hematoxylin. Spleen from swine infected with Pret4Δ9GL at 4 (top) and 8 dpi (bottom) at 4X (left) and 20X (right).

These images in Fig 3.9 above representing swine infected with Pret4Δ9GL at 4 and 8 days post-infection are negative for anti-ASFV p30. White spaces in the section are an artifact of the sectioning technique.

3.4 Experimental design of onset of protective immunity in Pr4Δ9GLv-inoculated animals

Swine infected with Pret4Δ9GL virus were all protected against challenge with the parental virulent Pret4 virus at 42 days post-inoculation in an experiment done previously by (Neilan *et al.*, 2004). We sought to understand the mechanisms mediating protection against ASF at different time points. Using swine weighing 36-55 kg, protection was assessed in Pret4Δ9GL-inoculated animals challenged 7, 10, 14, 21, or 28 days later with 10^4 HAD_{50} of Pret4. A group of 15 naïve swine were only challenged with Pret4. In each Pret4Δ9GL-inoculated group, animals were challenged at 7, 10, 14, 21, and 28 DPI with Pret4. Sample size was n=15 for the 7-day challenge group, n=10 for the 10-day challenge group, n=15 for the 14-day challenge group, n=10 for the 21-day challenge group, and n=15 for those challenged at day 28. All swine were bled at 4, 7, 11, 14, 21, 28 days post-inoculation (DPI), and 4, 7, 11, 14, and 21 days post-challenge (DPC) unless otherwise noted. Whole blood samples were titrated on primary swine peripheral blood mononuclear cells.

3.4.1 Naïve swine controls challenged with Pret4

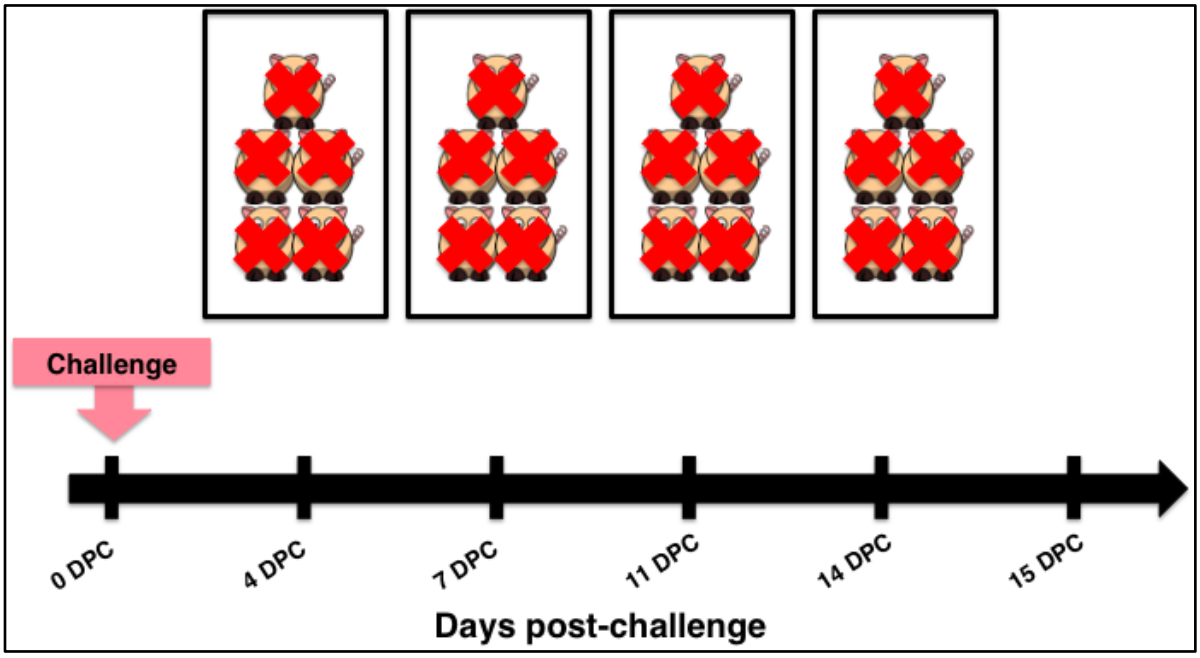

Figure 3.10 Twenty naive swine served as controls, groups of 5 swine were challenged with Pret4 in four separate experiments. None of the animals survived. Non-survivors are marked with a red 'X'.[2]

Twenty swine were used in four separate experiments, with five animals in each group. All swine were challenged with 1 mL of 10^4 Pret4 HAD_{50} intramuscularly. All swine in the control group died or were humanely euthanized between 5 and 15 days post-challenge.

[2] Pig clip art is from http://www.clker.com/clipart-29067.html

3.4.2 Swine inoculated with Pret4∆9GL and subsequently challenged 7 days post-inoculation with Pret4

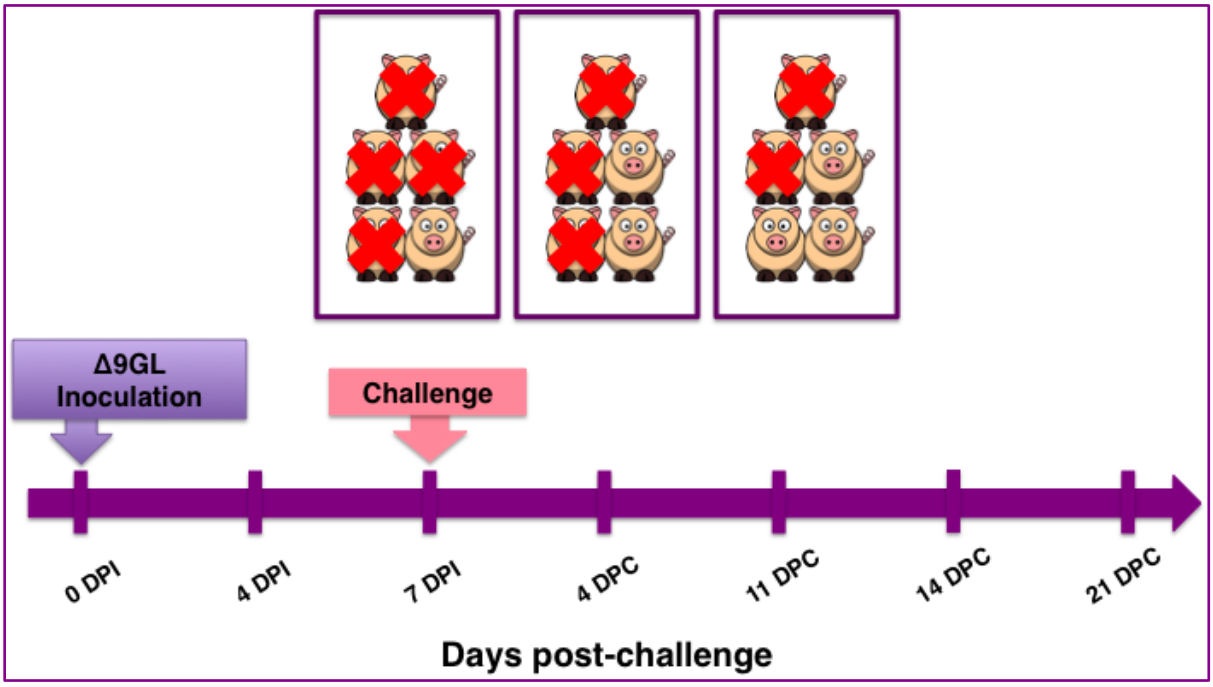

Figure 3.11 Fifteen swine in 3 groups of 5 were inoculated with Pret4Δ9GL and challenged with Pret4 7 days later. Swine were observed for 21 days following challenge. Non-survivors are marked with a red 'X'.

Fifteen swine were used in three separate experiments, with five animals in each group. All swine were inoculated with 10^4 HAD_{50} Pret4∆9GL and challenged seven days later with 10^4 HAD_{50} Pret4. Nine swine or 60% in the 10-day group died or were humanely euthanized between 9 and 12 days post-challenge.

3.4.3 Swine inoculated with Pret4∆9GL and subsequently challenged 10 days post-inoculation with Pret4

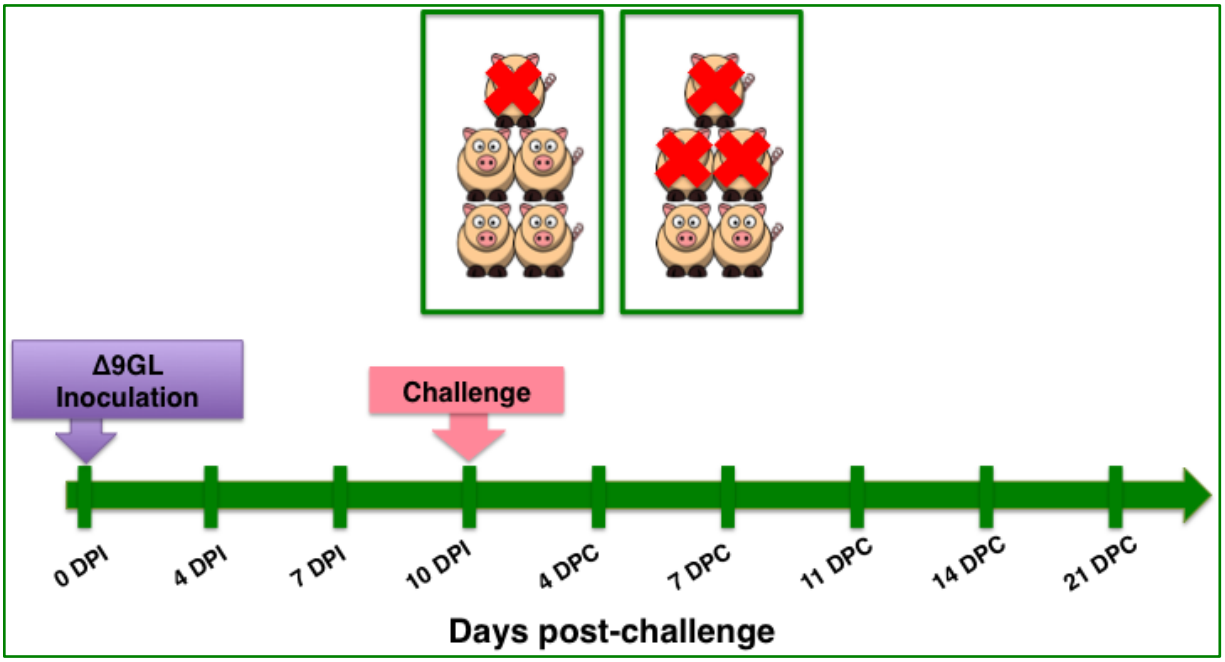

Figure 3.12 Ten swine in 2 groups of 5 were inoculated with Pret4Δ9GL and challenged with Pret4 10 days later. Swine were observed for 21 days following challenge. Non-survivors are marked with a red 'X'.

Ten swine were used in two separate experiments, with five animals in each group. All swine were inoculated with 10^4 HAD_{50} Pret4∆9GL and challenged ten days later with 10^4 HAD_{50} Pret4. Four swine or 40% in the 10-day group died or were humanely euthanized between 6 and 9 days post-challenge.

3.4.4 Swine inoculated with Pret4∆9GL and subsequently challenged 14 days post-inoculation with Pret4

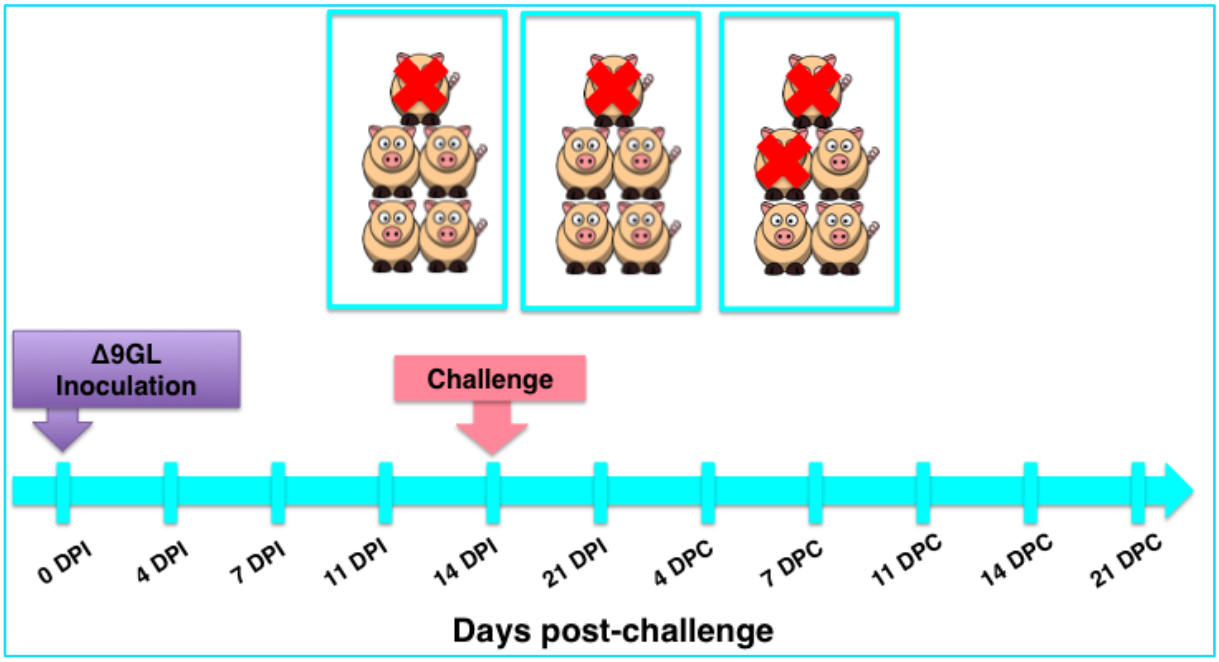

Figure 3.13 Fifteen swine in 3 groups of 5 were inoculated with Pret4Δ9GL and challenged with Pret4 14 days later. Swine were observed for 21 days following challenge. Non-survivors are marked with a red 'X'.

Fifteen swine were used in three separate experiments, with five animals in each group. All swine were inoculated with 10^4 HAD_{50} Pret4∆9GL and challenged ten days later with 10^4 HAD_{50} Pret4. Four swine or 27% in the 14-day group died or were humanely euthanized between 8 and 18 days post-challenge.

3.4.5 Swine inoculated with Pret4∆9GL and subsequently challenged 21 days post-inoculation with Pret4

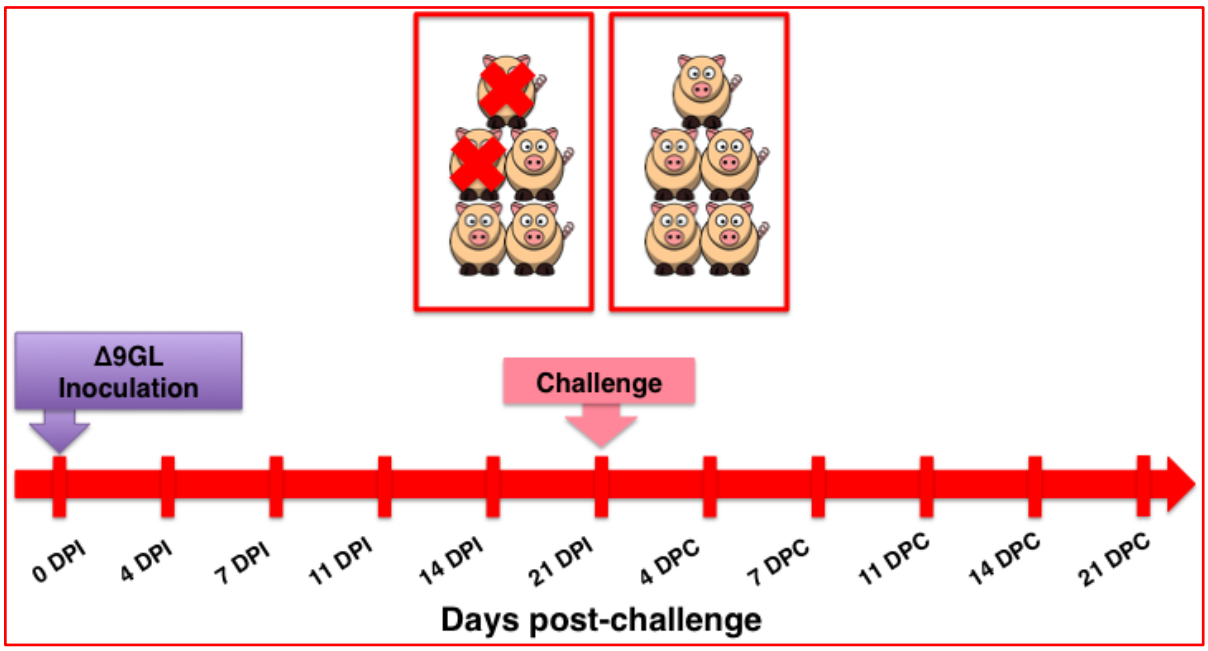

Figure 3.14 Ten swine in 2 groups of 5 were inoculated with Pret4Δ9GL and challenged with Pret4 21 days later. Swine were observed for 21 days following challenge. Non-survivors are marked with a red 'X'.

Ten swine were used in two separate experiments, with five animals in each group. All swine were inoculated with 10^4 HAD_{50} Pret4∆9GL and challenged ten days later with 10^4 HAD_{50} Pret4. Two swine or 20% in the 21-day group died or were humanely euthanized between 11 and 14 days post-challenge.

3.4.6 Swine inoculated with Pret4Δ9GL and subsequently challenged 28 days post-inoculation with Pret4

Figure 3.15 Fifteen swine in 3 groups of 5 were inoculated with Pret4Δ9GL and challenged with Pret4 28 days later. Swine were observed for 21 days following challenge. Non-survivors are marked with a red 'X'.

Fifteen swine were used in three separate experiments, with 4, 5, and 6 animals in each group. All swine were inoculated with 10^4 HAD_{50} Pret4Δ9GL and challenged 10 days later with 10^4 HAD_{50} Pret4. Three swine or 20% in the 28-day group died or were humanely euthanized between 6 and 11 days post-challenge.

3.5 Results of onset of protective immunity in Pr4Δ9GLv-inoculated animals

3.5.1 Assessment of survival status of swine in individual challenge groups

Swine infected with Pret4Δ9GL are all protected when challenged with Pret4 at 42 days post-infection (Neilan *et al.*, 2004). Challenging Pret4Δ9GL infected animals with the parental virus Pret4 showed a progressive acquisition of protection starting with 40% of the challenged animals at 7 dpi and reaching 80% surviving between 21 and 28 dpi.

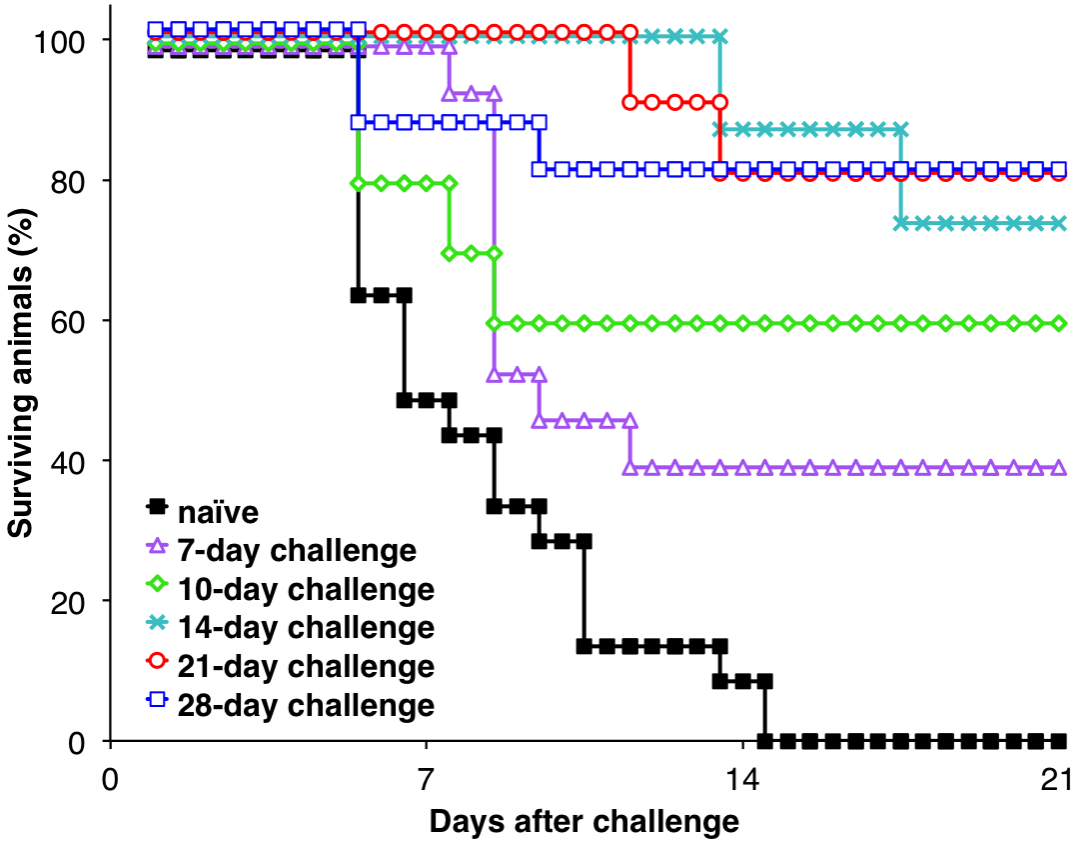

Figure 3.16 Percentage of surviving animals after challenge with Pret4 at different times post-inoculation with Pret4Δ9GL.

Swine were inoculated with 10^4 HAD_{50}/mL Pret4ΔGL virus and challenged with virulent Pret4 via the same dose and route at either 7 (n=15), 10 (n=10), 14 (n=15), 21 (n=10), or 28 (n=15) days later and clinically observed for 21 days post-challenge (see Fig 3.16). In our study, 6 out of 15 (40%) animals challenged as early as 7 days post-inoculation with Pret4ΔGL survived the challenge. When swine inoculated with Pret4ΔGL virus were challenged at later time points, an increase in the portion of animals surviving the virulent challenge occurred. Six out of 10 (60%), 11 out of 15 (73%), 8 out of 10 (80%), and 12 out of 15 (80%) swine survived when challenged at 10, 14, 21, and 28 days post-inoculation with Pret4ΔGL.

3.5.2 Clinical signs and rectal temperatures

Swine surviving challenge presented with transient pyrexia at times (see Appendix A Fig 5.1-5.7), typically around 1-6 days post-challenge. On average all surviving swine in the different challenge groups remained at temperatures below 40°C, with the exception of swine in the 14-day challenge group, where many surviving swine had elevated temperatures following challenge in the first week (see Fig 3.17- 3.22). These swine appeared to be generally healthy as they were active and eating.

Non-survivors had a mostly consistent presentation of clinical signs associated with ASF. Fever, lack of appetite, inability to stand, and staggered gait were observed in all non-protected swine. It appears that the clinical presentation of the disease did not differ between animals challenged at early or later times.

3.5.3 Viremia of swine in the different challenge groups

The surviving swine presented with a broad range of viremia titers from 10^3 HAD_{50}/mL to 10^8 HAD_{50}/mL. Many of the swine that succumbed to the virulent challenge

had temperatures, viremia kinetics, and clinical signs similar to naïve challenged swine by 3-5 days post-challenge, with euthanasia or death mainly occurring at 8-11 days post-challenge.

3.5.3.1 Viremia and rectal temperatures of naïve control swine challenged with Pret4

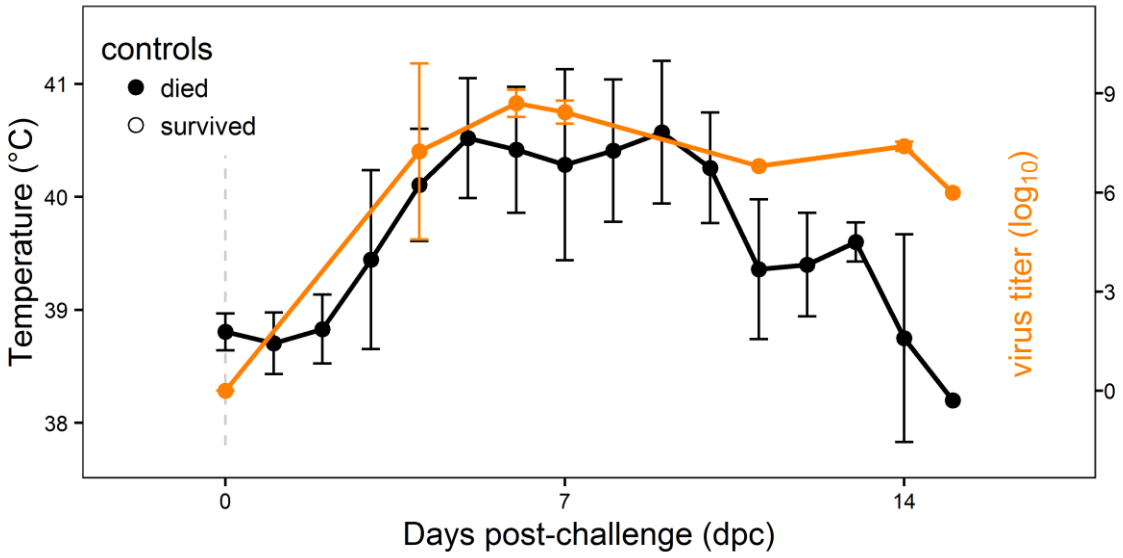

Figure 3.17 Average rectal temperature (black) and viremia titer (orange) of naïve control swine infected with Pret4. Filled circles represent mean values for non-survivors. Data represents mean values, error bars are standard deviation (SD) from 20 individual animals. Limit of detection: Log_{10} 1.5 HAD_{50}/ml.

Naïve control swine all developed clinical signs of ASF, including fever, loss of appetite, staggering gait, petechial hemorrhage, melena, and cyanosis of ears. Viremia was detected as early as 4 days post-inoculation in most swine. Fever was observed as early at 3-4 days post-challenge.

3.5.3.2 Viremia and rectal temperatures of survivors and non-survivors inoculated with Pret4Δ9GL and challenged 7 days post-inoculation

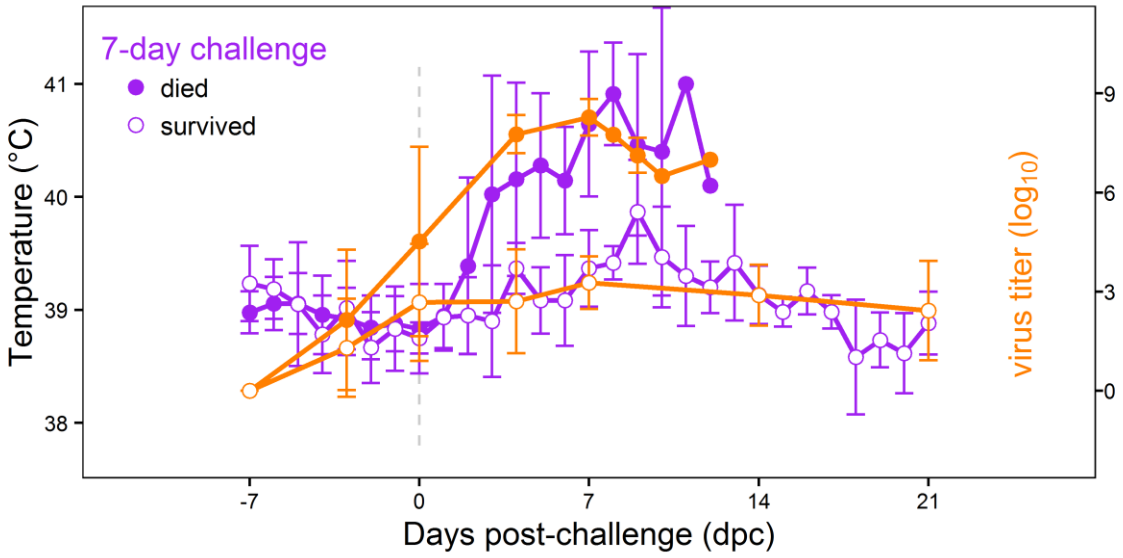

Figure 3.18 Average rectal temperature (purple) and viremia titers (orange) of 7-day challenge swine. Filled circles represent mean values for non-survivors, while open circles are mean values for survivors. The chart shows means and SD from 15 individual animals, with 6 survivors and 9 non-survivors. Limit of detection: Log_{10} 1.5 HAD_{50}/mL.

All non-survivors in the 7-day challenge group had clinical signs of ASF and viremia that were indistinguishable from naïve control animals. Surviving swine had viremia and temperatures that, on average, were much lower than non-survivors. This divergence was visible within a few days following challenge. Interestingly, the onset of fever was on average at 11 days post-challenge. Observing temperatures of animals individually, it was apparent that 2 out of 6 surviving swine had transient temperature peaks above 40°C with an average duration of almost 2 days (see Fig A.5.3 and Table 3.1). These two animals also had transient peaks of viremia greater than 10^3 HAD_{50}/mL.

3.5.3.3 Viremia and rectal temperatures of survivors and non-survivors inoculated with Pret4Δ9GL and challenged 10 days post-inoculation

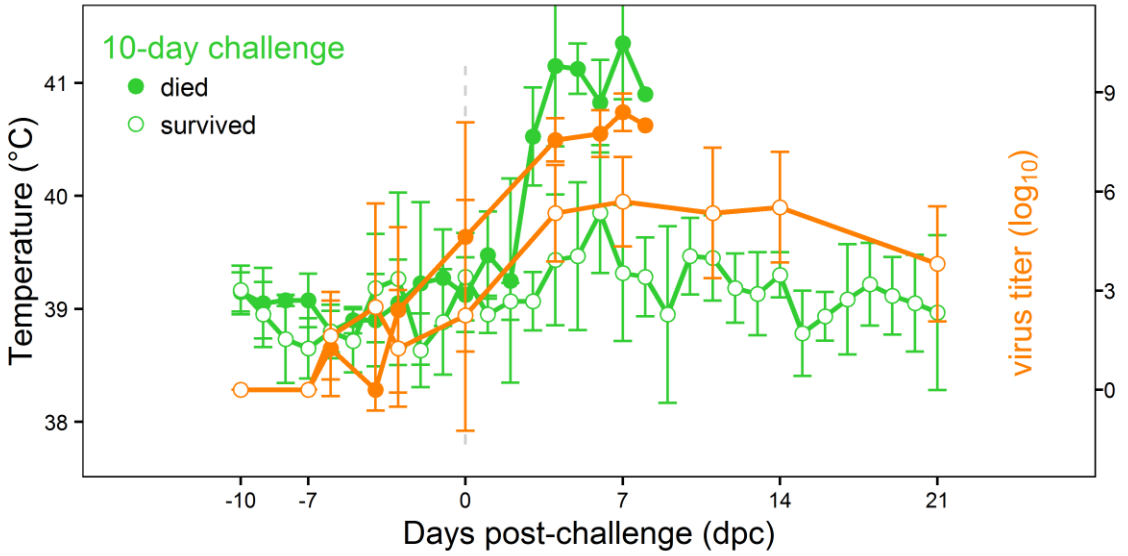

Figure 3.19 Average rectal temperature (green) and viremia titers (orange) of swine inoculated with Pret4Δ9GL and subsequently challenged with Pret4 10 days later. Filled circles represent and mean values for non-survivors, while open circles are mean values for survivors. The chart shows means and SD with 5 survivors and 4 non-survivors Limit of detection: Log_{10} 1.5 HAD_{50}/mL.

All non-survivors in the 10-day challenge group had clinical signs of ASF indistinguishable from naïve control animals. In the swine surviving the challenge, half did not have viremia peaking higher than 10^6 HAD_{50}/ml, or temperatures peaking above 40°C (Fig A.5.4). By 21 days, viremia decreased to 10^3 HAD_{50}/mL in four out of the six survivors. Non-surviving swine had higher viremia titers reaching above 10^6 HAD_{50}/mL within 7 days post-challenge and rectal temperatures were also elevated to above 41°C following challenge.

3.5.3.4 Viremia and rectal temperatures of survivors and non-survivors inoculated with Pret4Δ9GL and challenged 14 days post-inoculation

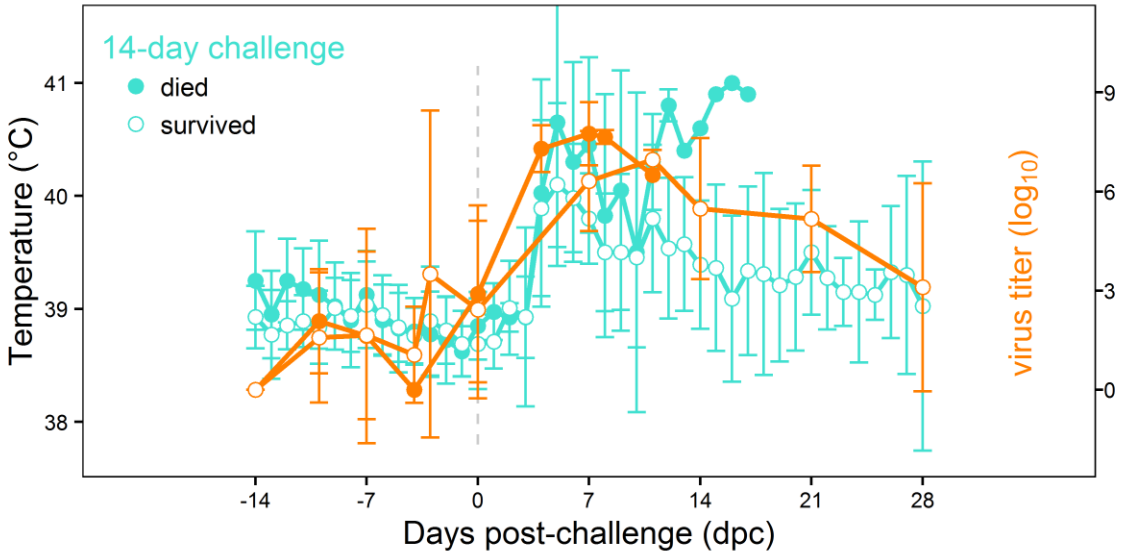

Figure 3.20 Average rectal temperatures (turquoise) and viremia titers (orange) of swine inoculated with Pret4Δ9GL and challenged at 14 days post-inoculation. Filled circles represent and mean values for non-survivors, while open circles are mean values for survivors. The chart shows means and standard deviation (SD) from 15 individual animals, with 11 survivors and 4 non-survivors. The limit of detection was Log_{10} 1.5 HAD_{50}/mL.

Differences between non-surviving and surviving swine were less apparent, with the exception of the rising temperatures of non-survivors around 10 days post-challenge. At least eight out of eleven survivors had viremia peaking above 10^6 HAD_{50}/mL post-challenge, with all but 2 swine presenting with transient temperatures above 40°C (Fig A.5.5). These transient peaks in temperatures lasted longer in the 14-day challenge group, on average 6.2 days (see Table 3.1). All non-survivors had clinical signs of ASF, temperatures, and viremia levels indistinguishable from the naïve control animals.

3.5.3.5 Viremia and rectal temperatures of survivors and non-survivors inoculated with Pret4Δ9GL and challenged 21 days post-inoculation

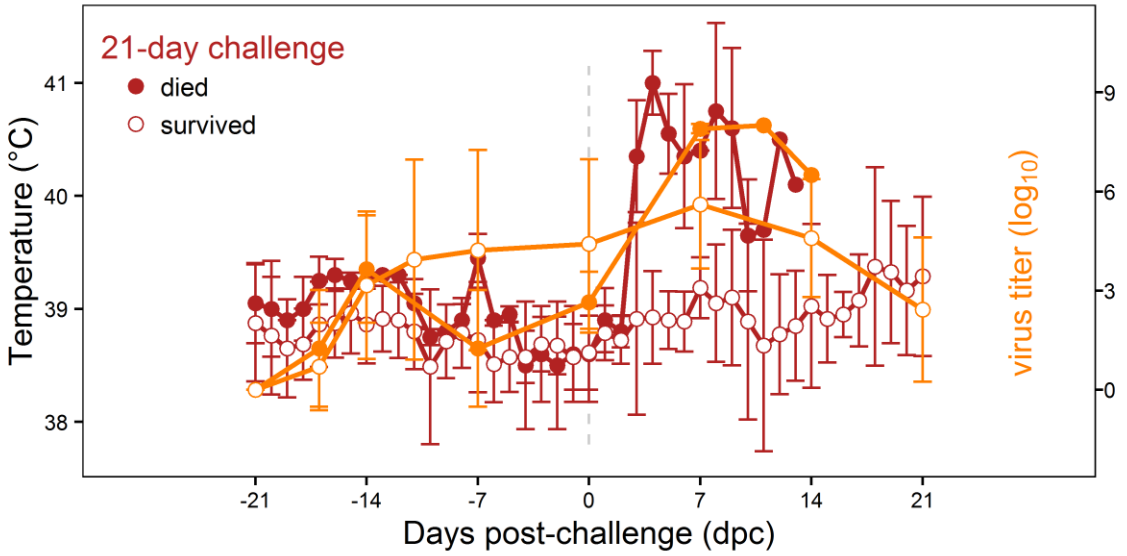

Figure 3.21 Average rectal temperature (red) and viremia titers (orange) of swine inoculated with Pret4Δ9GL and subsequently challenged with Pret4 21 days later. Filled circles represent and mean values for non-survivors, while open circles are mean values for survivors. The chart shows means and SD from 10 individual animals, with 8 survivors and 2 non-survivors. The limit of detection was Log_{10} 1.5 HAD_{50}/mL.

There is a clear divergence between the groups of survivors and non-survivors. Of the survivors, half had viremia titers peak above 10^6 HAD_{50}/ml, and a majority of these animals also had a short peak in temperatures above 40°C, on average lasting about 2 days (see Fig A.5.6 and Table 3.1). The 2 non-survivors had sharp increases in viremia, followed by clinical signs of ASF indistinguishable from naïve control animals.

3.5.3.6 Viremia and rectal temperatures of survivors and non-survivors inoculated with Pret4Δ9GL and challenged 28 days post-inoculation

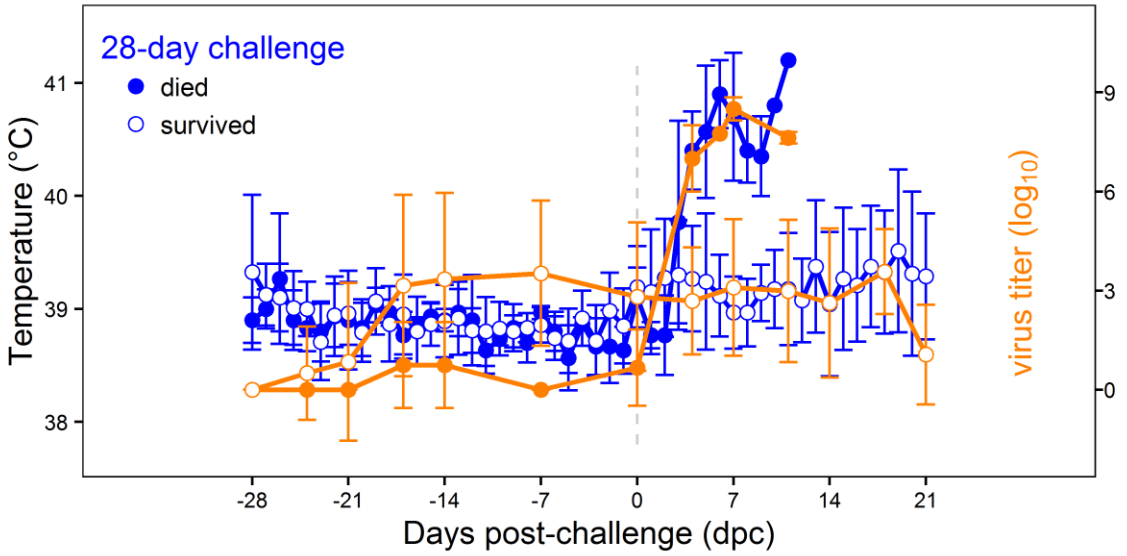

Figure 3.22 Average rectal temperature (blue) and viremia titers (orange) of Pret4Δ9GL swine challenged 28-days post-inoculations with Pret4. Filled circles represent and mean values for non-survivors, while open circles are mean values for survivors. This chart shows the means and SD from 15 individual animals, with 12 survivors and 3 non-survivors. The limit of detection was Log_{10} 1.5 HAD_{50}/mL.

All non-survivors had clinical signs of ASF indistinguishable from naïve control animals. Only 2 out of 10 swine surviving the challenge had viremia greater than 10^6 HAD_{50}/mL prior to challenge, while most viremia titers were at 10^3 HAD_{50}/mL or undetectable by 21 days post-challenge (See Fig A.5.7). On average fever began 11 days post-challenge and lasted for almost 4 days in the 12 survivors.

3.5.3.7 Summarizing viremia post-challenge of survivors and non-survivors previously inoculated with Pret4∆9GL

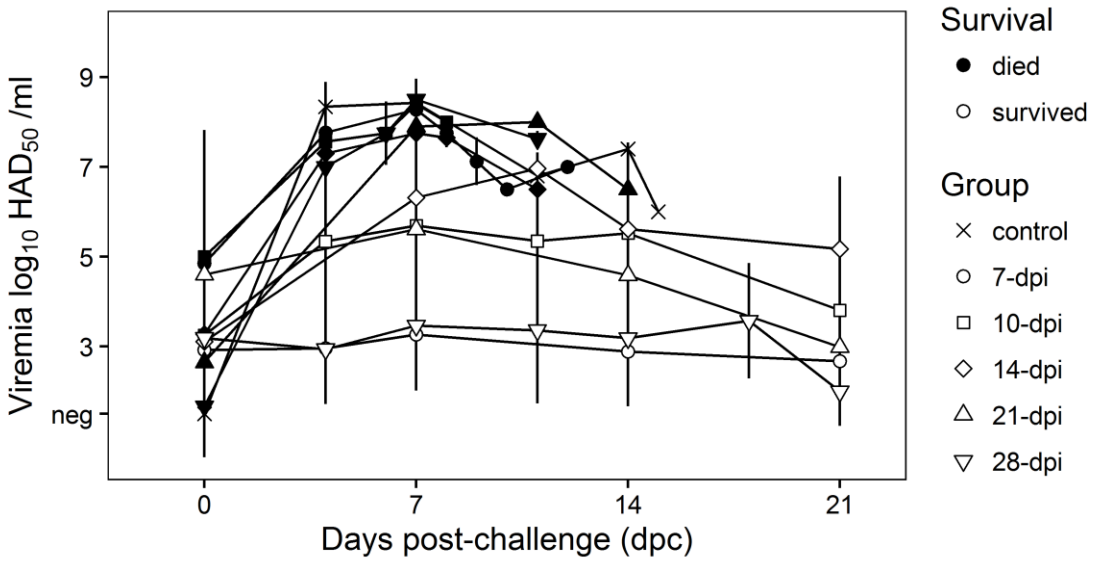

Figure 3.23 Summary of viremia in animals challenged with Pret4 virus at different times after inoculation with Pret4∆9GL. Black filled shapes represent animals that did not survive; white open shapes represent animals that survived challenge.

In Fig 3.23 the contrast between the viremia in surviving and non-surviving animals is apparent. Among the groups we also see that the 28-, 7-, and 21-day challenge groups have the lowest viremia among surviving swine. The 14-day challenge group had the highest viremia among surviving animals. Non-surviving swine are indistinguishable between groups.

3.5.3.7 Summarizing rectal temperatures post-challenge of survivors and non-survivors previously inoculated with Pret4Δ9GL

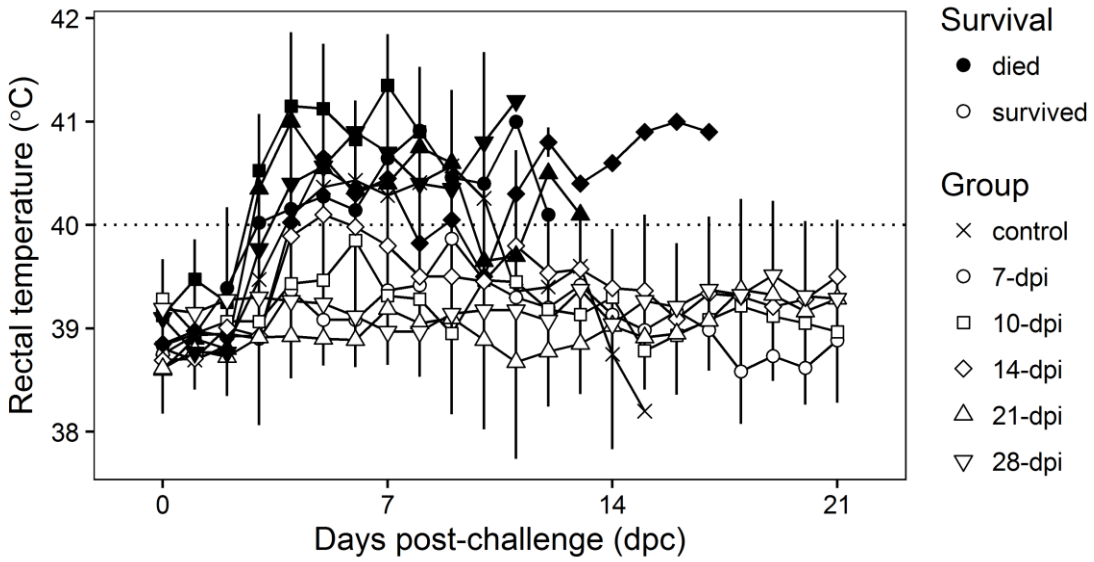

Figure 3.24 Summary of temperatures in animals challenged with Pret4 virus at different times after inoculation with Pret4Δ9GL. Black filled shapes represent animals that did not survive; white open shapes represent animals that survived challenge. The mean and SD of each group were shown in the previous figures.

Among the average temperatures, stark differences are clear between swine that survived challenge and swine that did not. In general, we observe that surviving swine in the 21- and 7-day challenge groups had the lowest temperatures on average.

3.5.4 Virus typing by PCR post-challenge for swine previously inoculated with Pret4Δ9GL

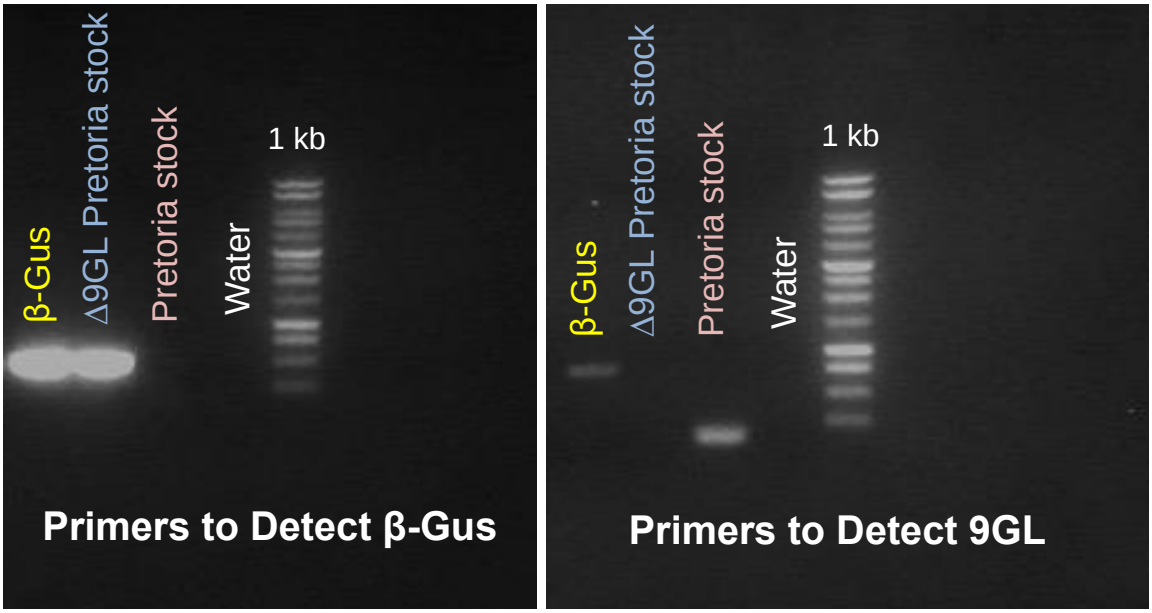

Figure 3.25 PCR detection of Pret4Δ9GL and Pret4 virus DNA using specific primers. (A) Assessment of the presence of β-GUS genes to detect Pret4Δ9GL. The first band is a plasmid containing β-GUS, the second band is the PretΔ9GL Virus, the third band is parental Pretoria stock virus, and fourth is the water control. (B) Assessment of the presence of 9GL gene to detect parental Pret4. The first band is a plasmid containing β-GUS, the second band is the PretΔ9GL Virus, the third band is parental Pretoria stock virus, and fourth is the water control.

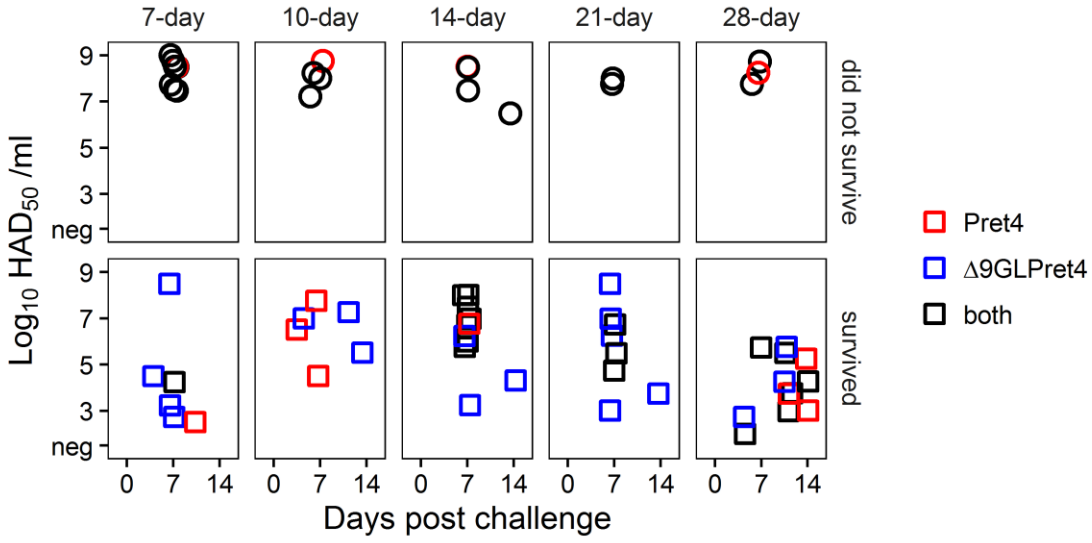

Figure 3.26 Virus detection by PCR, determining the presence of the 9GL (B119L) or β-GUS genes. Blue boxes represent positive result for Pret4Δ9GL virus, red boxes represent positive results for the parental Pret4 virus, and black boxes indicate that the PCR was positive for both viruses.

Figure 3.27 illustrates the characterization of circulating virus after the challenge of swine inoculated with Pret4Δ9GL. Only the blood sample with the highest viremia titer was tested from each animal. Sample time points ranged from 4 to 14 days following challenge. Specific detection of Pret4 and/or Pret4Δ9GL was performed by differential PCR based on the detection of the 9GL (B119L) or β-GUS genes.

In general, there was a heterogeneous distribution of finding both or either one of the viruses in all groups, with the exception of the 21-day challenge group where Pret4 was never found by itself.

3.5.5 Analysis of immune responses and viremia in swine infected with Pret4Δ9GL at the time of challenge

3.5.5.1 Viremia at the time of challenge

An infection with Pret4Δ9GL may be a competitive factor in the process of protection against the challenge with virulent Pret4 virus. We looked for a correlation between survival and the presence of Pret4Δ9GL viremia at the time of challenge at 7,10, 14, 21, and 28 days post-inoculation with Pret4Δ9GL. In general, it was found that the viremia levels varied from pig to pig independent of the time post-challenge and independent of their survival following challenge. Swine surviving challenge had viremia titers ranging from as low as undetectable (<1.5 $\log_{10}$ HAD_{50}/ml) to values peaking at 4-7 $\log_{10}$ HAD_{50}/mL. Consequently, detection of viremia at a particular level was not associated with protection against a virulent challenge with Pret4.

3.5.5.2 Detection of anti-ASFV antibody at the time of challenge

We focused on antibody levels at the time of challenge in Pret4Δ9GL-inoculated swine using two different in-house developed assays, indirect ELISA and an immunoperoxidase assay (IPA). In all 15 swine challenged at 7 dpi, antibodies were undetectable by ELISA independent of survival status (Fig. 3.27) We detected antibody in only 2 of the 6 surviving swine at 10 days post-inoculation by ELISA, while 4 of the 6 survivors were detected by IPA (Fig 3.28). Comparably, in 11 swine surviving challenge at 14 days post-inoculation, 4 were positive by ELISA, and 8 were positive by IPA (Fig 3.29). Consequently the detection of antibody by ELISA or IPA did not correlate with swine surviving challenge.

Analyzing the detection of antibody titers in swine challenged at later times seemed to indicate an association with survival. The antibody detected by either IPA or ELISA were lower or absent in swine challenged at 21 days post-inoculation that did not survive (Fig 3.30). Evidently, in the group of swine challenged at 28 days post-inoculation with Pret4Δ9GL, all surviving swine had detectable antibody by both ELISA and IPA (Fig 3.31). The 3 swine that did not survive the challenge in this group did not have detectable antibody.

3.5.5.2 Detection of IFN-γ in ASFV-stimulated PBMCs at the time of challenge

PBMCs from swine were stimulated with Vero-adapted Pret4 to measure IFN-γ responses by ELISPOT. We focused on the IFN-γ at the time of challenge for each group to investigate any T-cell sensitization to ASFV. We found no significant differences in the number of IFN-γ-producing PBMCs on the day of challenge between swine surviving challenge and those that did not, independent of the time of challenge (See Fig 3.21 to 3.31 for IFN-γ measurements on the day of challenge). Results of the IFN-γ ELISA for more time points can be found in Appendix A.

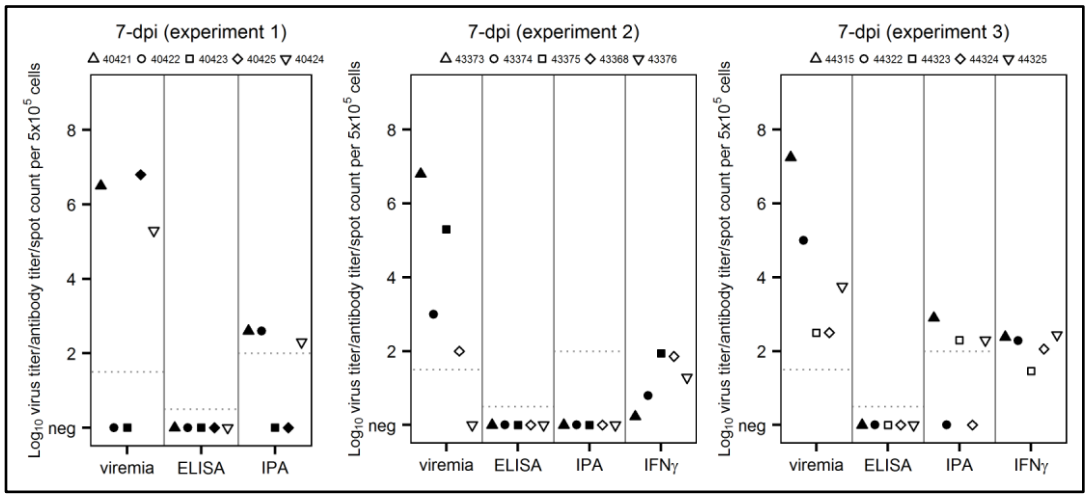

Figure 3.27 Viremia and immune parameters 7 days after infection with Pret4Δ9GL (day of challenge with parental Pret4). Log_{10} values of viremia, anti-ASFV antibody titer detected by ELISA and immunoperoxidase (IPA), and the number of circulating ASFV-specific IFN-γ-producing PBMCs are represented for each individual pig. Survival status of swine is indicated as "survived" (white open shapes), or "did not survive" (black filled shapes). Virus titer limit of detection: ≤ Log_{10} 1.5 HAD_{50}/mL. ELISA limit of detection:≤ Log_{10}(1/10). IPA limit of detection:≤ Log_{10}(1/200).

No antibody was detected by ELISA at 7 days post-inoculation with Pret4Δ9GL (see Fig 3.27). The IPA assay detected antibody in 6 of 15 swine 7 days after inoculation. Of the animals with detectable antibody, 3 out of 6 survived the challenge. The presence of IFN-γ production in PBMCs did not differ between survivors and non-survivors. Viremia on the day of challenge (7 dpi) varied in all swine and had no bearing on survival status.

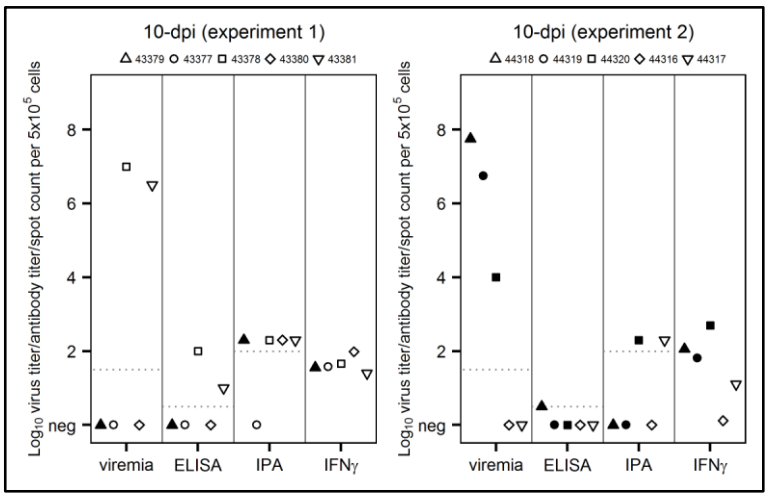

Figure 3.28 Viremia and immune parameters 10 days after infection with Pret4Δ9GL (day of challenge with parental Pret4). Log_{10} values of viremia, anti-ASFV antibody titer detected by ELISA and IPA, and the number of circulating ASFV-specific IFN-γ-producing PBMCs are represented for each individual pig. Survival status of swine is indicated as "survived" (white open shapes), or "did not survive" (black filled shapes). Virus titer limit of detection: ≤ Log_{10} 1.5 HAD_{50}/mL. ELISA limit of detection:≤ Log_{10}(1/10). IPA limit of detection:≤ Log_{10}(1/200).

The ELISA assay detected antibodies in 3 of 10 pigs, while IPA detected antibodies in 6 of these animals (see Fig 3.28). Of the swine that survived, 4 out of 6 had antibody detectable by IPA and 2 were also positive by ELISA. The presence of IFN-γ production in PBMCs did not differ between survivors and non-survivors.

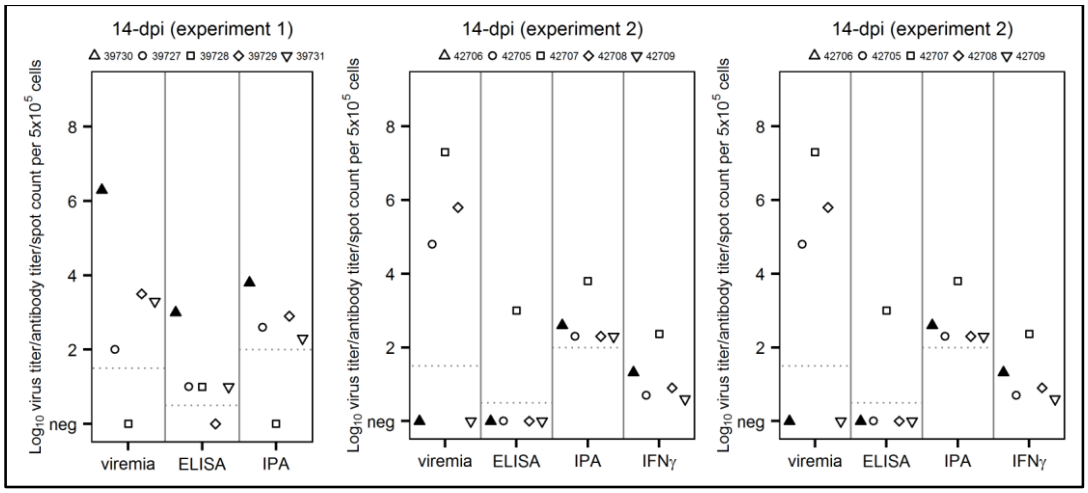

Figure 3.29 Viremia and immune parameters 14 days after infection with Pret4Δ9GL (day of challenge with parental Pret4). Log_{10} values of viremia, anti-ASFV antibody titer detected by ELISA and IPA, and the number of circulating ASFV-specific IFN-γ-producing PBMCs are represented for each individual pig. Survival status of swine is indicated as "survived" (white open shapes), or "did not survive" (black filled shapes). Virus titer limit of detection: ≤ Log_{10} 1.5 HAD_{50}/mL. ELISA limit of detection:≤ Log_{10}(1/10). IPA limit of detection:≤ Log_{10}(1/200).

Antibodies were detected in 6 out of 15 animals by ELISA and in 11 out of 15 by IPA. Of the 11 swine that survived the challenge, 4 were positive by ELISA, and 8 were positive by IPA. The presence of IFN-γ production in PBMCs did not differ between survivors and non-survivors, nor did the detection of viremia.

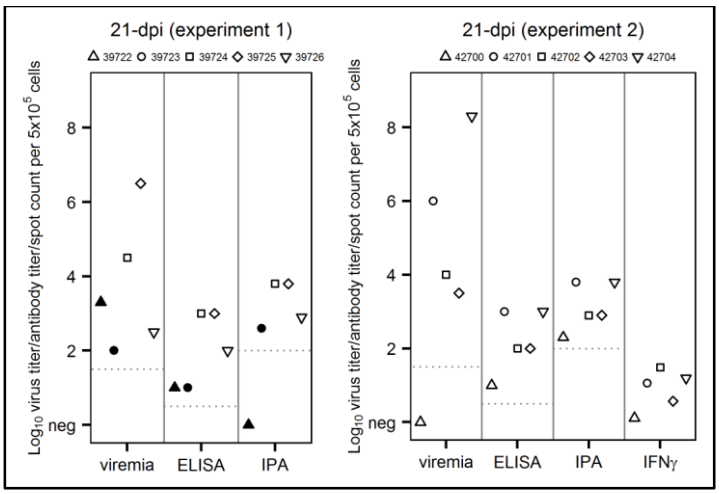

Figure 3.30 Viremia and immune parameters 21 days after infection with Pret4Δ9GL (day of challenge with parental Pret4). Log_{10} values of viremia, anti-ASFV antibody titer detected by ELISA and IPA, and the number of circulating ASFV-specific IFN-γ-producing PBMCs are represented for each individual pig. Survival status of swine is indicated as "survived" (white open shapes), or "did not survive" (black filled shapes). Virus titer limit of detection: ≤ Log_{10} 1.5 HAD_{50}/mL. ELISA limit of detection:≤ Log_{10}(1/10). IPA limit of detection:≤ Log_{10}(1/200).

Antibody was detected in all 8 survivors by ELISA and IPA. The two non-survivors had low or undetectable antibody by IPA and ELISA. IFN-γ was only tested in the second experiment in which all swine survived.

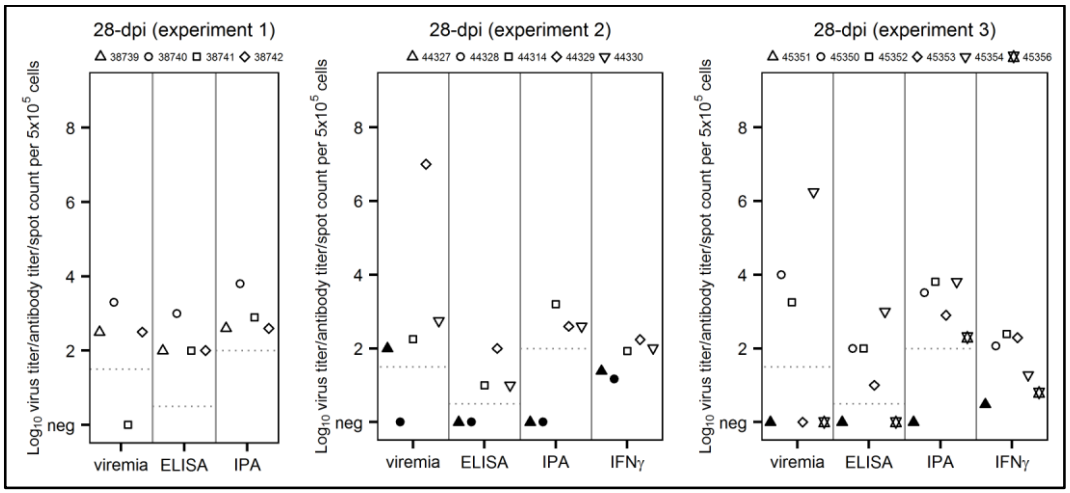

Figure 3.31 Viremia and immune parameters 28 days after infection with Pret4Δ9GL (day of challenge with parental Pret4). Log_{10} values of viremia, anti-ASFV antibody titer detected by ELISA and IPA, and the number of circulating ASFV-specific IFN-γ-producing PBMCs are represented for each individual pig. Survival status of swine is indicated as "survived" (white open shapes), or "did not survive" (black filled shapes). Virus titer limit of detection: ≤ Log_{10} 1.5 HAD_{50}/mL. ELISA limit of detection:≤ Log_{10}(1/10). IPA limit of detection:≤ Log_{10}(1/200).

Antibody was detected in all 12 survivors by ELISA and IPA. The 3 non-survivors had low or undetectable antibody by ELISA and IPA. IFN-γ detected in stimulated PBMCs was higher in all 8 surviving swine that were tested. The IFN-γ ELISPOT assay was not used in the first experiment.

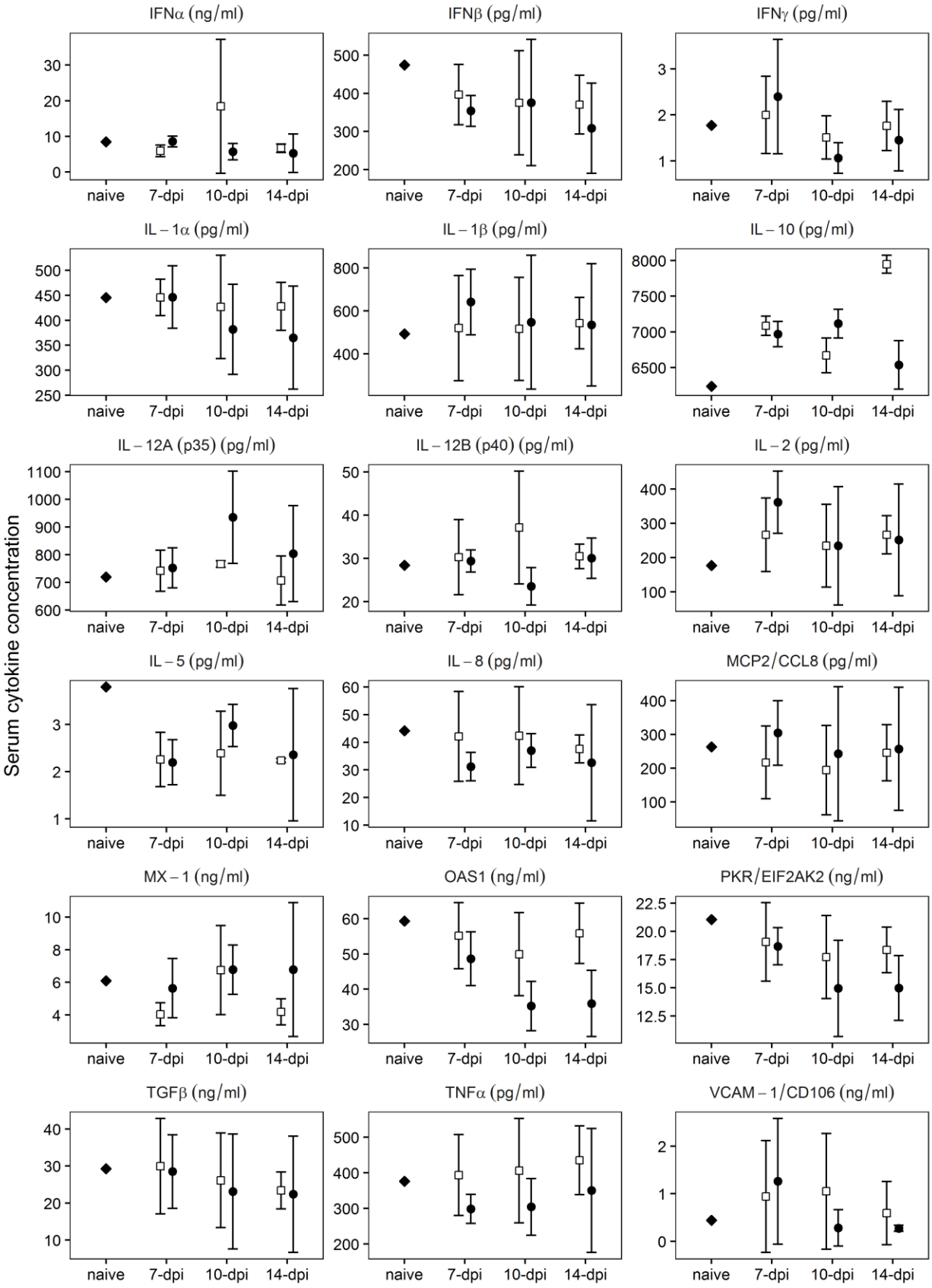
Serum cytokine concentration
IFNα (ng/ml)
IFNβ (pg/ml)
IFNγ (pg/ml)
IL – 1α (pg/ml)
IL – 1β (pg/ml)
IL – 10 (pg/ml)
IL – 12A (p35) (pg/ml)
IL – 12B (p40) (pg/ml)
IL – 2 (pg/ml)
IL – 5 (pg/ml)
IL – 8 (pg/ml)
MCP2/CCL8 (pg/ml)
MX – 1 (ng/ml)
OAS1 (ng/ml)
PKR/EIF2AK2 (ng/ml)
TGFβ (ng/ml)
TNFα (pg/ml)
VCAM – 1/CD106 (ng/ml)
naive
7-dpi
10-dpi
14-dpi

Figure 3.32 Evaluation of systemic levels of different host cytokines in swine inoculated with Pret4∆9GL virus. Average values and 95% confidence intervals of 15, 10, and 15 animals are shown for the groups challenged at 7, 10, 14 days post-inoculation with Pret4∆9GL. All values are expressed as a concentration per mL of serum. Survival status of swine is indicated as "survived" (white open shapes), or "did not survive" (black filled shapes).

No specific or unique patterns of cytokine levels in serum were observed in swine that did or did not survive challenge.

Table 3.1 Summary of Pretoria swine studies

Group	No. of Surviving	% Fever	Mean days until fever (±SD)	Mean duration of fever in days (±SD)	Max Temp °C	Mean days until death (±SD)
Control	0/20	100	4.1±1.0	4.4±1.7	41.6	8.3±3.2
7-day challenge	6/15	S: 33	11.0±2.8	1.5±0.7	40.7	
		N:100	3.7±1.4	6.0±2.1	41.8	9.3±1.1
10-day challenge	6/10	S: 50	6.0±2.7	2.7±1.2	40.7	
		N:100	3.0±0.8	4.8±1.5	41.7	7.3±1.6
14-day challenge	11/15	S: 64	5.4±2.9	6.2±4.3	41.3	
		N:100	6.0±3.4	6.0±2.6	41.4	13.0±5.0
21-day challenge	8/10	S:25	9.2±6.7	2.0±1.4	40.7	
		N:100	3.0±0.0	8.5±2.1	41.3	12.5±1.5
28-day challenge	12/15	S: 50	11.0±7.7	3.8±1.2	41.1	
		N:100	7.0±6.1	6.0±2.6	41.2	9.0±2.6

S: Swine surviving challenge with Pret4

N*: Swine that did not survive challenge with Pret4*

This table summarizes all Pretoria swine experiments conducted to determine the time of onset of protective immunity. In general, fever in non-survivors began around 3 days post-challenge, whereas surviving swine had fever from around 5 to 11 days post-challenge. Between 25% and 64% of surviving challenge presented with fever.

Chapter 4 - Georgia Swine Experiments

4.1 Introduction

The epidemic ASFV strain Georgia 2007/1 is a highly virulent virus that entered the Caucasus region in early 2007 and since then has spread to Armenia, Azerbaijan, and Russia. Further outbreaks have also been reported in Ukraine, Belarus, Lithuania, Latvia, and Poland, ultimately posing a risk to the whole of Europe. Developing a vaccine against the Georgia isolate is critical. To further extend our knowledge and cross-validate our results, the vaccination/challenge model used in the Pretoria experiments was also utilized with a double-deletion mutant of Georgia 2007, GeoΔ9GL/ΔUK, and its parental strain.

In order to attenuate Georgia 2007, our lab deleted two genes: 9GL *(B119L)* and UK *(DP96R)*. The double mutant containing these two deletions was completely attenuated in swine. It was found that swine inoculated with GeoΔ9GL/ΔUK and challenged with parental Georgia 2007 at 28 days post-inoculation had 100% survival.

To determine the appropriate vaccination regimen, we tested doses of 10^2, 10^4, and 10^6 HAD_{50} and assessed antibody levels as well as IFN-γ production in stimulated PBMCs for all animals at the time of challenge. We found that 10^4 HAD_{50} was a sufficient vaccine dose. All swine inoculated with that dose were clinically normal for the 28-day observational period and all survived challenge with virulent Georgia at 28 dpi.

With this model, we also investigated immune factors (antibody, IFN-γ in PBMCs, and 18 serum cytokines) associated with protection against virulent challenge as early as 7 to 14 days post-inoculation and later at 21 and 28 days post-inoculation and compared the results to those observed with the Pret4Δ9GL model.

4.2 Experimental design of GeoΔ9GL/ΔUK dose-response study in swine

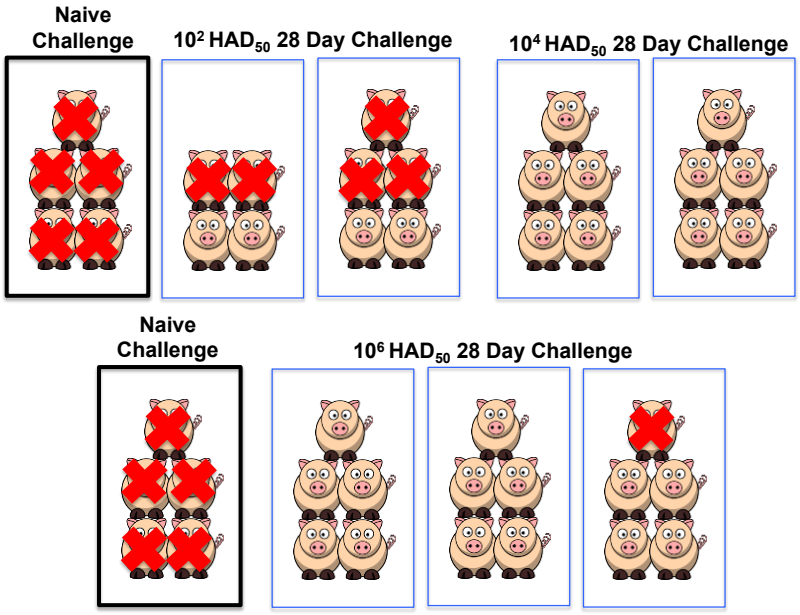

Figure 4.1 Dose-response study of GeoΔ9GL/ΔUK and subsequent challenge at 28 days with 10^3 HAD_{50} Georgia 2007. Swine marked with a red X did not survive challenge.

Swine were inoculated with 10^2, 10^4, or 10^6 HAD_{50} of GeoΔ9GL/ΔUK and subsequently challenged with virulent Georgia 2007 28 days later to find an appropriate vaccine dose. Experiments were conducted in groups of 4 or 5 pigs. Swine receiving 10^2 HAD_{50} had 44% survival (4/9), compared to 100% (10/10) for 10^4 HAD_{50} and 93% (14/15) for 10^6 HAD_{50}. Control animals showed ASF-related signs as early as 3 days post-infection with a majority of them being euthanized at 5 or 6 days post-challenge.

4.2 Experimental design of GeoΔ9GL/ΔUK onset of protective immunity in swine

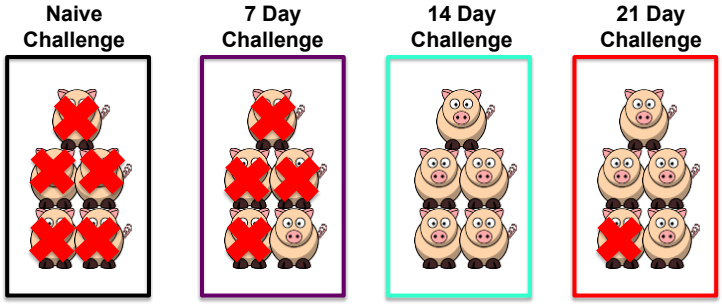

Figure 4.2 Analysis of the onset of protection for inoculation with 10^4 HAD_{50} of GeoΔ9GL/ΔUK and subsequent challenge at 7, 14, 21 days with 10^3 HAD_{50} of Georgia 2007. Swine marked with a red X did not survive challenge.

Of the 5 swine challenged 7 days post-inoculation with GeoΔ9GL/ΔUK, one survived the challenge. When challenged at 14 days post-inoculation, all 5 of 5 swine survived, while 1 out of 5 swine challenged at 21 days did not survive.

4.3 Analysis of immune responses of swine infected with Geo∆9GL/∆UK

Here we investigated the immune mechanisms present in swine surviving ASF challenge with a focus on circulating antibody, IFN-γ production in ASFV-stimulated PBMCs, as well as cytokines and chemokines in serum.

The 5 swine that had been inoculated with 10^2 HAD_{50} of Geo∆9GL/∆UK and did not survive when challenged 28 days later did not have measurable antibody by ELISA and were either low or undetectable by IPA. When swine were inoculated with a 10^4 HAD_{50} dose and challenged 28 days later, all swine survived the challenge with virulent Georgia 2007. Antibody titers were generally higher in these animals compared to those inoculated with a lower dose. Finally, when swine were inoculated with 10^6 HAD_{50}, 14 out of 15 swine survived the challenge and had the highest antibody levels at the time of challenge (28 days post-inoculation). The single non-survivor in the 10^6-vaccine group had the lowest antibody titers, around 10- to 100-fold less compared to the survivors receiving the same dose.

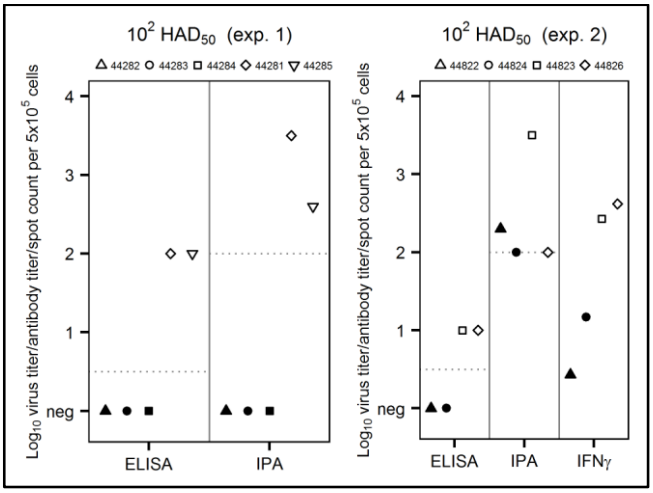

Figure 4.3 Anti-ASFV antibodies detected by ELISA and IPA, and IFN-γ-producing PBMCs 28 days post-inoculation with 10^2 HAD_{50} of Geo∆9GL/∆UK. Survival status of swine after challenge is indicated as "survived" (white open shapes), or "did not survive" (black filled shapes). Log_{10} values of anti-ASFV antibody titer detected by ELISA and IPA, and the number of circulating ASFV-specific IFN-γ-producing PBMCs are represented for each individual pig. ELISA limit of detection:≤ Log_{10}(1/10). IPA limit of detection:≤ Log_{10}(1/200).

Swine inoculated with 10^2 HAD_{50} had antibodies detectable in 4 out of 9 animals by ELISA and 6 out of 9 by IPA. Of the 6 swine presenting with antibody, 4 survived and 2 did not. The IFN-γ production in PBMCs was lower in non-survivors when compared to survivors.

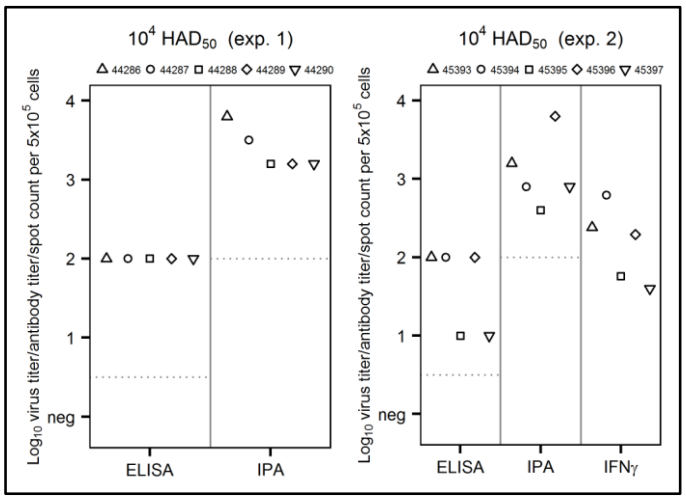

Figure 4.4 Anti-ASFV antibodies detected by ELISA and IPA, and IFN-γ-producing PBMCs 28 days post-inoculation with 10^4 HAD_{50} of Geo∆9GL/∆UK. Survival status of swine after challenge is indicated as "survived" (white open shapes), or "did not survive" (black filled shapes). Log_{10} values of anti-ASFV antibody titer detected by ELISA and IPA, and the number of circulating ASFV-specific IFN-γ-producing PBMCs are represented for each individual pig. ELISA limit of detection:≤ Log_{10}(1/10). IPA limit of detection:≤ Log_{10}(1/200).

Swine inoculated with 10^4 HAD_{50} had antibodies detectable in all 10 animals by ELISA and IPA. The IFN-γ production in PBMCs was elevated in all surviving swine.

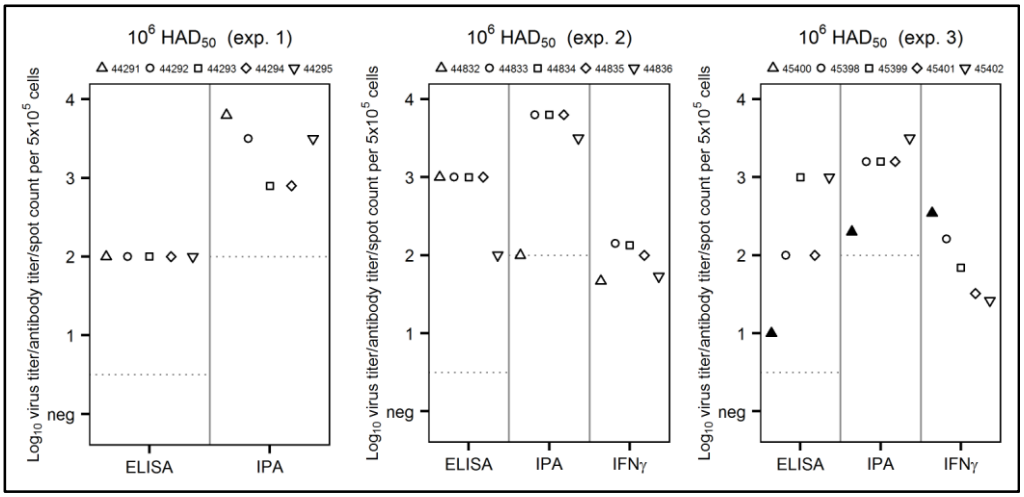

Figure 4.5 Anti-ASFV antibodies detected by ELISA and IPA, and IFN-γ-producing PBMCs 28 days post-inoculation with 10^6 HAD_{50} of Geo∆9GL/∆UK. Survival status of swine after challenge is indicated as "survived" (white open shapes), or "did not survive" (black filled shapes). Log_{10} values of anti-ASFV antibody titer detected by ELISA and IPA, and the number of circulating ASFV-specific IFN-γ-producing PBMCs are represented for each individual pig. ELISA limit of detection:≤ Log_{10}(1/10). IPA limit of detection:≤ Log_{10}(1/200).

In swine inoculated with 10^6 $HAD_{50,}$ antibodies were detected in all 15 animals by ELISA and IPA, with the one non-survivor having the lowest antibody titer (Fig 4.5). The IFN-γ production in PBMCs was elevated in all surviving swine but highest in the single non-survivor.

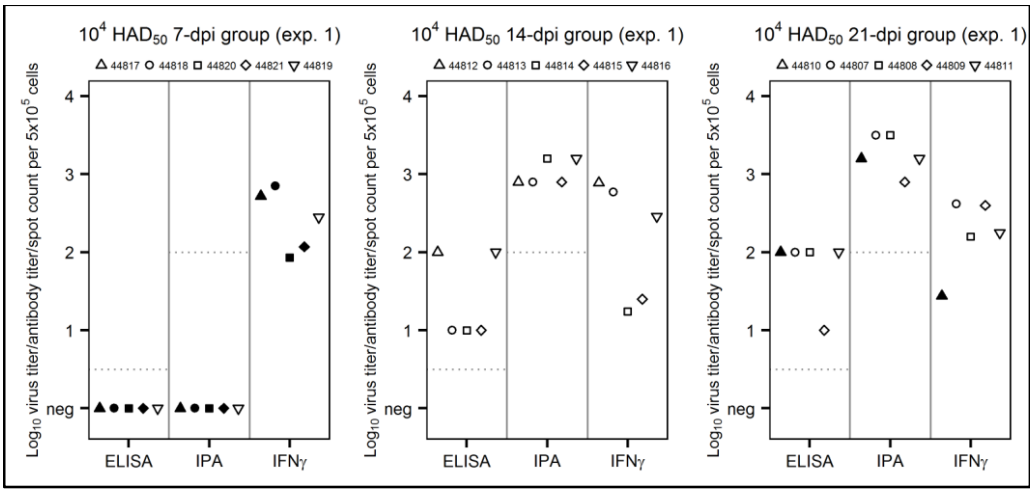

Figure 4.6 Anti-ASFV antibodies detected by ELISA and IPA, and IFN-γ-producing PBMCs 7, 14 or 21 days post-inoculation with 10^4 HAD_{50} of Geo∆9GL/∆UK. Survival status of swine after challenge is indicated as "survived" (white open shapes), or "did not survive" (black filled shapes). Log_{10} values of anti-ASFV antibody titer detected by ELISA and IPA, and the number of circulating ASFV-specific IFN-γ-producing PBMCs are represented for each individual pig. ELISA limit of detection:≤ Log_{10}(1/10). IPA limit of detection:≤ Log_{10}(1/200).

ASFV antibodies were undetectable in all 5 animals challenged at 7 days post-inoculation. Only one animal survived challenge, but antibody and IFN-γ did not differ between survivors and non-survivors in this challenge group.

In the 14-day challenge group, all swine survived and had measurable levels of antibody by IPA and ELISA, along with the presence of IFN-γ production by the PBMCs.

Swine in the 21-day group all had antibody detected by IPA and ELISA in addition to the IFN-γ production in stimulated PBMCs. One pig out of 5 did not survive the challenge in the 21-day challenge group and presented with the lowest count of PBMCs producing IFN-γ for that group.

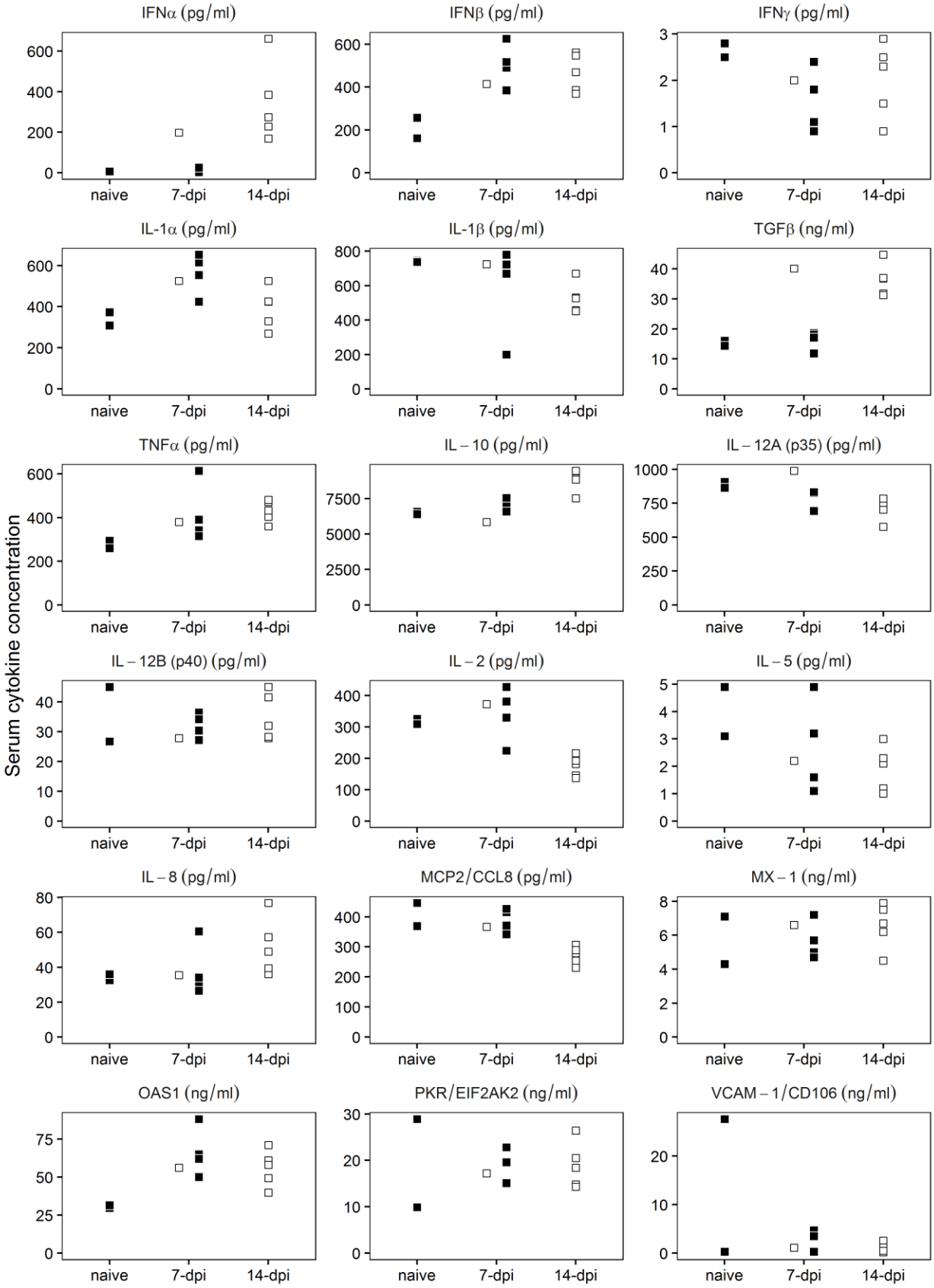
Serum cytokine concentration
IFNα (pg/ml)
IFNβ (pg/ml)
IFNγ (pg/ml)
IL-1α (pg/ml)
IL-1β (pg/ml)
TGFβ (ng/ml)
TNFα (pg/ml)
IL – 10 (pg/ml)
IL – 12A (p35) (pg/ml)
IL – 12B (p40) (pg/ml)
IL – 2 (pg/ml)
IL – 5 (pg/ml)
IL – 8 (pg/ml)
MCP2/CCL8 (pg/ml)
MX – 1 (ng/ml)
OAS1 (ng/ml)
PKR/EIF2AK2 (ng/ml)
VCAM – 1/CD106 (ng/ml)
naive
7-dpi
14-dpi

Figure 4.7 Evaluation of systemic levels of different host cytokines at the time of challenge in swine inoculated with Geo∆9GL∆UK virus. Individual points represent data from each pig in the groups challenged at 7, 14, and 21 days post-inoculation with Georgia 2007. All values are expressed as a concentration per mL of serum. Survival status of swine is indicated as "survived" (white open shapes), or "did not survive" (black filled shapes).

Interferon-α (IFN-α) was 200-fold higher in surviving animals compared to non-surviving animals, and transforming growth factor β (TGF-β) in surviving swine ranged from 1.5- to 2.5-fold higher compared to non-surviving swine in the 7- and 14-dpi groups. In general, the mean values did not vary greatly between protected and non-protected swine. Other than IFN-α and TGF-β, no specific or unique patterns of cytokine levels in serum were observed in swine that did or did not survive.

Table 4.1 Survival status and fever responses in swine inoculated with GeoΔ9GL/ΔUK and subsequently challenged with Georgia 2007

Group	No. of Surviving	Mean days until fever (±SD)	Mean duration of fever in days (±SD)	Maximum daily temp °C (±SD)	Mean days until death (±SD)
Control	0/5	3.6±0.6	5±0.5	40.8±0.7	7.6±0.1
Geo Δ9GL/ΔUK 10^2	2/5	3.3±0.7[a]	3.7±0.6[a]	41.2±0.9[a]	7.0±0.0[a]
Geo Δ9GL/ΔUK 10^4	5/5	-	-	39.4±1.8	-
Geo Δ9GL/ΔUK 10^6	5/5	10.4±4.3[b]	4.0±2.8[b]	40.5±0.8[b]	-
Control	0/5	4.8±0.5	0.2±0.5	41.2±0.9	5.0±0.0
Geo Δ9GL/ΔUK 10^2	2/4	5.0±0.0[c]	1.5±0.7[c]	41.0±0.7[c]	5.5±0.7[c]
Geo Δ9GL/ΔUK 10^4	5/5	-	-	39.7±0.9	-
Geo Δ9GL/ΔUK 10^6	5/5	10[d]	3[d]	40.4±0.0[d]	-
Control	0/5	3.8±0.5	1.2±0.5	40.9±0.3	5.0±0.0
Geo Δ9GL/ΔUK 10^6	4/5	11.0±2.6[e]	3.0±1.0[e]	40.2±0.6[e]	11.0[e]

(a) Data is based on 3 out of 5 swine or (c) 2 out of 5 swine presenting severe clinical ASF in the first two experiments. The remaining 2 and 3 swine in the first and second experiments did not present ASF-related clinical signs. Their mean maximum daily rectal temperature was 39.5°C with an SD of 0.9.

(b) Data are based on 2 out of 5 swine that had transiently elevated rectal temperatures and mild clinical presentation. The remaining 3 swine did not present with any ASF-related clinical signs and their mean maximum daily rectal temperature was 39.2 °C with an SD of 0.2.

(d) The data represents 1 out of 5 pigs that had a transient fever. The remaining four animals did not have any clinical signs of ASF, their mean rectal temperature was 39.5°C with an SD of 0.5.

(e) These data representing 2 out of 5 swine with transient rectal temperature elevations. One pig presented with severe clinical ASF and was euthanized 11 days post-challenge. The remaining 2 pigs did not show any ASFV-related clinical signs or fever.

All swine were challenged at 28 days post-inoculation with 10^3 HAD_{50} Georgia 2007

Chapter 5 - Discussion

5.1 Importance of controlling foreign animal diseases

Viral diseases of livestock are an important cause of food insecurity, economic losses and animal suffering across the globe. Producers in North America and Europe benefit from the fact that many of these diseases are absent from the hemisphere. Nevertheless, these ‘foreign’ animal diseases cause severe losses in other parts of the world, often depressing agricultural and human development in the poorest countries. Globalization and climate change increase the risk of re-introducing these diseases into free areas, especially diseases that can be spread by fomites or arthropod vectors. Their long-term control requires the application of effective measures in the most affected countries, which are the reservoirs of infection. In the meantime, countries with highly industrialized systems of densely stocked and fully susceptible livestock need contingency plans to deal with incursions. Reliance on slaughtering animals to halt epizootics is increasingly unacceptable on both economic and ethical grounds, making vaccination a critical element of both scenarios (Paton and Taylor, 2011).

5.2 Why vaccinating against ASFV is so important

African swine fever (ASF) is a lethal hemorrhagic disease of swine caused by a double-stranded DNA virus. ASF virus (ASFV) is among the most important pathogens of domestic swine globally. There are 23 recognized genotypes of ASFV. This devastating disease can cause tremendous economic hardship due to the high mortality rate and the severe trade implications once ASFV is detected in a country.

Since there is no vaccine available, our options for the containment and eradication of ASF are limited. In order to successfully eradicate ASF, an early detection

and notification system must be in place, teams of trained veterinarians must be able to recognize the disease in the field and send samples for rapid diagnosis at capable laboratories. This is critical in countries with high swine density populations, such as the U.S., in order to reduce economic losses. If the diagnosis of ASF is confirmed, the affected farms must be depopulated immediately with proper disposal of carcasses and a complete disinfection of the farm, vehicles, and other fomites.

There are no effective vaccines against ASF, and their development is a high priority. Protective vaccination is feasible because complete protection can be achieved by infection with low-virulence isolates of ASFV. Convalescent swine develop strain-specific immunity that can withstand challenge with antigenically related virulent strains. However, because of this strain specificity and a lack of heterologous protection, the goal must be to develop a vaccine capable of protection against multiple isolates. ASFV strains attenuated by cell culture passages, or targeted gene deletions have been used as experimental vaccines that elicit protective immune responses (Boinas *et al.*, 2004; Hamdy and Dardiri, 1984; Leitao *et al.*, 2001; Lewis *et al.*, 2000; Mebus and Dardiri, 1980; O'Donnell *et al.*, 2015a; O'Donnell *et al.*, 2015b). The use of naturally occurring attenuated ASF viruses has been explored as well, but unfortunately protection was variable with different challenge viruses (Souto *et al.*, 2016).

Pigs immunized with attenuated ASFVs containing genetically engineered deletions of specific virulence-associated genes are protected when challenged with homologous parental viruses. Specifically, individual deletions of UK (DP69R), 23-NL (DP71L), TK (A240L), or 9GL (B119L) genes from the genomes of virulent ASFVs resulted in significant attenuation of these isolates in swine (Lewis *et al.*, 2000; Moore *et*

al., 1998; Zsak *et al.*, 1998; Zsak *et al.*, 1996). The deletion of these genes often reduces viral replication in primary macrophage culture and swine studies with these deletion mutants have shown that limiting the viral replication in macrophages dramatically reduces the virulence in domestic pigs. Reduced viral replication may avoid the induction of immunopathological processes and allow host defense mechanisms to clear the virus, but our understanding of how virulent and attenuated viruses interact with the host is limited.

5.3 Investigating the use of two attenuated mutants and determining an appropriate dose

In this thesis we investigated those host defense mechanisms in swine protected against homologous challenge using four different ASFV strains including Pretoriuskop/96/4 (genotype I), its derivative Pret4Δ9GL, Georgia 2007/1 (genotype II) and its derivative GeoΔ9GL/ΔUK (See Table 1 in Chapter 2).

We first characterized the differences between Pret4 and Pret4Δ9GL replication *in vitro*. A comparative growth curve at an MOI of 0.01 demonstrated Pret4Δ9GL was delayed in replication compared to the parental Pret4 (Fig 3.1). These results are similar to those reported previously (Lewis *et al.*, 2000; Neilan *et al.*, 2004; O'Donnell *et al.*, 2015b; O'Donnell *et al.*, 2016) where 9GL was deleted from Malawi Lil 20/1, Pret4, and Georgia 2007/1. In all these cases, growth curves of 9GL-deleted mutants and double mutants (Δ9GL/ΔMGF or Δ9GL/ΔUK (see Appendix Fig A.8) demonstrated a delay in viral replication in swine macrophages when compared to the parental strains. ASFV infection results in the induction of mitochondrial stress-response proteins (Rojo *et al.*, 1998). With 9GL's homology to ERV1, which is important for oxidative phosphorylation

and the maintenance of mitochondrial genomes in yeast, it is possible that p14, encoded by 9GL, may play a role in important mitochondrial functions in infected cells mediating that stress response (Lewis *et al.*, 2000; Rojo *et al.*, 1998).

Interestingly, deleting the 9GL gene does not attenuate all isolates equally. We found that when 9GL was deleted from Georgia 2007, it was still highly virulent at high doses. Infection with 10^6 or 10^4 HAD_{50} was 100% fatal, but all swine survived when given 10^3 or 10^2 HAD_{50} (O'Donnell *et al.*, 2015b). In contrast, when Malawi∆9GL was given at 10^6 HAD_{50} or 10^4 HAD_{50} and Pret4∆9GL at 10^4 HAD_{50}, all swine survived and did not present with any clinical signs.

Understanding the role of deleted ASFV genes is extremely complex because different ASF isolates behave differently with each deletion. Since ASFV Georgia 2007 was not completely attenuated by deleting just 9GL, the double mutants Geo∆9GL/∆MGF (O'Donnell *et al.*, 2016) and Geo∆9GL/∆UK (unpublished data) were generated. By itself, the deletion of 9GL or six genes of the MGF360/505 group created two attenuated ASFV strains that were able to induce a protective immune response against homologous challenge with virulent Georgia 2007. Unfortunately, the Geo∆9GL/∆MGF double mutant was over-attenuated. It did not cause any detectable viremia or antibody response in swine after administration of doses as high as 10^6 HAD_{50} nor was there any protection against a subsequent virulent challenge (O'Donnell *et al.*, 2016).

Swine can be given Geo∆9GL/∆UK doses as high as 10^6 HAD_{50} to induce an antibody response by 28 days without any clinical disease. We found that the 10^4 HAD_{50} dose was sufficient to stimulate a protective immune response when challenged at 28

days (100% survival), while a lower dose of 10^2 HAD_{50} only protected 40% of swine (4/10). This is significantly different compared to swine receiving 10^2 HAD_{50} of MalawiΔ9GL, where 100% survived virulent challenge at 42 days, or GeoΔ9GL, where 100% of swine survived virulent challenge at 28 dpi after inoculation with 10^2 or 10^3 HAD_{50} (Lewis *et al.*, 2000; O'Donnell *et al.*, 2015b). Swine administered 10^4 HAD_{50} Pret4Δ9GL and challenged 28 days later had an 80% survival rate (12/15) after repeating the experiment three times. Higher doses (10^6 HAD_{50}) of GeoΔ9GL/ΔUK appeared to be less effective, as one animal did not survive a 28-day challenge, and 4/15 of these animals had transient fever.

The variation in survival could be influenced by several factors. The initial virulence of the parental strain probably influences viral replication and the ability to stimulate a protective immune response. Also the genetics of swine can vary and influence how they respond to the attenuated virus. The Pret4Δ9GL and GeoΔ9GL/ΔUK were repeated multiple times to gain a more representative sample of how domestic swine will respond to different doses of these attenuated viruses and at various times post-inoculation.

5.4 Comparative pathogenesis of Pret4 and Pret4Δ9GL

Our comparative pathogenesis experiment (see Fig. 3.2) demonstrated that during our 10-day observational period, Pret4Δ9GL was attenuated in swine. Just four days after infection with either Pret4 we saw viremia peaks above 10^7/ml, whereas Pret4Δ9GL was detected at a 1000-fold lower level (see Fig. 3.3). No nasal shedding of the Pret4Δ9GL virus was detected, while from 4 to 10 days post-infection Pret4 was detected in all nasal swab samples (see Fig. 3.3). These results are similar to our recent

publication with GeoΔ9GL, where swine inoculated with 10^2 or 10^3 did not present with any nasal shedding of the attenuated virus (O'Donnell *et al.*, 2015b), although shedding was apparent with the parental Georgia 2007 strain (Guinat *et al.*, 2014).

None of our swine inoculated with Pret4Δ9GL presented with any clinical signs of disease (changes in mentation, neurologic signs, or melena) (see Fig. 3.4). This was shown previously by (Neilan *et al.*, 2004) for a 42-day period. Swine infected with Pret4, on the other hand, developed fever and clinical signs including diarrhea, lack of appetite, and a listless attitude as early as 4 dpi.

As a part of our study to investigate differences in virus distribution at early time points of infection with Pret4 or Pret4Δ9GL, we performed necropsies on swine every other day for 10 days. What we found was that at 2, 4, 6, and 10 days post-infection we were able to detect low level of virus in spleen macerate in swine infected with either virus (Fig 3.5). Other than spleen, lung or liver we did not detect the PretΔ9GL virus in the other 7 organs collected. The PretΔ9GL virus did not replicate well in swine during the 10-day observational period, unlike its virulent parental strain Pret4, which was isolated from all organs beginning at 4 dpi. Although we detected virus in spleen macerate, we were unable to detect ASFV antigen in the spleen by IHC. The spleen is a very blood-rich organ and it is possible that our virus isolation from the spleen could be a direct result of viremia, and not necessarily local replication of virus. Individual animal data (Appendix A Fig 6.2-5) illustrates the heterogeneity in Pret4Δ9GL replication between animals, which makes it difficult to predict a certain level of replication necessary for a protective response. As seen with OUR T88/4, sporadic viremia and

less efficient transmission was also characteristic of non-pathogenic non-hemadsorbing isolates (Boinas *et al.*, 2004).

Having a virus that is attenuated adequately to not cause clinical disease, but still able to replicate sufficiently to induce protection against challenge with a homologous virulent strain, is an important step toward understanding how the host responds to ASFV.

5.5 Onset of protective immunity of PretΔ9GL and GeoΔ9GL/ΔUK

Protective immune responses induced by attenuated viruses have been investigated previously. It has been suggested that passive transfer of antibodies from immunized swine to naïve swine could confer protection against virulent challenge (Onisk *et al.*, 1994). Other reports suggest that antibodies cannot completely neutralize ASFV, and the presence of neutralizing antibodies has been controversial (Gomez-Puertas and Escribano, 1997; Neilan *et al.*, 2004; Zsak *et al.*, 1993).

To understand the basis of protection when swine are challenged at different time points (7, 10, 14, 21, 28 days post-inoculation), we sought to find correlates of different virological and immunological parameters and the survival status after a 21-day observational period following homologous challenge. Our focus was to quantify PretΔ9GL viremia, circulating antibodies, ASFV-stimulated PBMCs producing IFN-γ, and several cytokines and chemokines present in the serum at the time of challenge.

This model allowed us to analyze the immunological status of swine that did or did not survive the challenge at different times post-vaccination. By challenging swine early (7, 10, 14 dpi) and late (21, 28 dpi) we could monitor the presence of different immune parameters that may change during the maturation of the host response

between 0 and 28 days after inoculation with Pret4Δ9GL. We were astounded to find 40-73% of Pret4Δ9GL-inoculated swine survived challenge at 7, 10, 14 dpi. **This is the first report of swine surviving when challenged earlier than 3 weeks after inoculation.** The existence of protection so early indicates that partially effective early host immune mechanisms are at play. It is not clear what host immune mechanisms are providing such early protection. However, none of the host parameters analyzed in the Pret4Δ9GL animals correlated with an animal surviving challenge. When we looked at the early challenges of swine inoculated with GeoΔ9GL/ΔUK, we found 20-100% of swine surviving challenge with virulent Georgia at 7 and 14 dpi.

Viremia in Pret4Δ9GL swine surviving the challenge was present with a broad range of titers (10^3 to 10^8 $\log_{10}$ HAD_{50}/mL peaking at day 7-10 post-challenge) in all groups at almost all the time points tested during the observational period of 21 days post-challenge. It appears that the clinical presentation of ASF, as well as kinetics of virus replication systemically in swine, whether surviving virulent challenge or not, are very similar regardless the time of challenge. These results were unanticipated as we might expect that immune mechanisms develop at different times post-infection with Pret4Δ9GL would be quantitatively or qualitatively different. Consequently, we expected that the level or type of immune response protecting swine from a virulent challenge would be different. With this expectation, we though this difference may be shown in the outcome following challenge with swine protected or not protected from the Pret4 challenge. Conversely, the analysis of viremia in GeoΔ9GL/ΔUK-infected animals in the 28-day challenge groups revealed that, regardless of the dose received, swine had different viremia kinetics after challenge (O'Donnell *et al* 2016, submitted). These swine

had a small transient peak of viremia, then decreased until viremia titers were low (10^3 HAD_{50}/ml) or undetectable in 17 of the 25 survivors by 21 days post-challenge (O'Donnell *et al* 2016, submitted).

The presence of anti-ASFV antibodies appears to be associated with protection. Although absent at 7 and 10 dpi, they were detected in swine inoculated with Geo∆9GL/∆UK at 14, 21, and 28 dpi and in swine inoculated with Pret4∆9GL at 21 and 28 dpi. Among swine inoculated with 10^2 HAD_{50} Geo∆9GL/∆UK and challenged at 28 dpi, non-survivors generally did not have detectable anti-ASFV antibodies, whereas the four survivors did. With swine inoculated with Pret4∆9GL and challenged at 21 or 28 days, all surviving animals (80%) had detectable antibodies. In the Pret4∆9GL 14-day challenge where 73% survived, one of the 3 non-survivors had antibody levels higher than any survivor. These variations between individuals are difficult to explain. We suspect that as more time passes between inoculation with an attenuated strain and the virulent challenge, the immune response matures to respond more specifically and possibly quicker. Looking at the big picture, swine challenged later with Pret4∆9GL (21 and 28 days) and Geo∆9GL/UK (14,21, and 28) demonstrated an association between the presence of anti-ASFV antibodies and protection from virulent challenge.

Previously it was reported that $CD8^+$ T cells were essential for a protective immune response when induced by OUR T88/3 and challenged with OUR T88/1 (Oura *et al.*, 2005). Further evidence suggesting the importance of lymphocytes in a protective immune response was reported in experiments demonstrating cross protection induced by the OUR T88/3 isolate against challenge with virulent isolates from different

genotypes, where protection was correlated with the capacity to stimulate IFN-γ in PBMCs from immunized swine (King *et al.*, 2011).

Based on previous reports correlating the presence of IFN-γ in PBMCs of vaccinated animals with protection (Argilaguet *et al.*, 2012; King *et al.*, 2011), we expected to find some correlation between survival status after challenge and the number of IFN-γ-producing PBMCs. However, in both models (Pret4Δ9GL and GeoΔ9GL/ΔUK), IFN-γ production in PBMCs alone was not associated with protection. In many of the Pret4Δ9GL swine the IFN-γ ELISPOT was performed weekly to further investigate any patterns associated with protection. In many non-survivors, the number of IFN-γ-producing PBMCs declined as the health of the animal deteriorated (see appendix A, swine numbered in red). In many of the surviving swine, the challenge virus acted as a booster in the IFN-γ response in PBMCs, as the number of IFN-γ-producing cells increased in the weeks after challenge (see appendix A, swine numbered in green).

5.6 Open questions about the role of cytokines

Reports suggest elevations of TNF-α, and IL-1β play a major role in ASF pathogenesis due to their proinflammatory, proapoptotoic, and procoagulant action (Gomez-Villamandos *et al.*, 2013; Zakaryan *et al.*, 2015). Cytokines and chemokines measured at 7, 10, and 14 days post-inoculation in swine sera with Pret4Δ9GL did not yield any particle pattern related to survival. Among the groups tested there were variations in cytokine and chemokine levels in all groups regardless of each group's clinical outcome. More recently, Reis *et al.* (2016) also looked at IL-1β, IL-4, TNFα, IFN-γ, and IL-10 in serum, and did not find significant differences in cytokine levels in swine

infected with ASFV BeninΔMGF or OUR T88/3, with the exception of elevated IFN-γ at 5 and 7 dpi in swine immunized with BeninΔMGF (Reis *et al.*, 2016).

In swine inoculated with GeoΔ9GL/ΔUK, IFN-α and TGF-β levels were elevated in the only animal surviving challenge at 7 days post-inoculation when compared to other animals that did not survive. Two groups have demonstrated a reduction of ASFV replication in monocytes/macrophages by IFN-α and porcine IFN-γ (Esparza *et al.*, 1988) as well as by human IFN-α and IFN-γ in Vero cells (Paez *et al.*, 1990). However, Golding *et al*. (2016) found that virulent ASFV continues to replicate in the presence of circulating IFN-α. Afonso and Golding reported that MFG360/530 genes affect the IFN-α production in macrophage cultures when comparing Pret4Δ35 and Pret4 (Afonso *et al.*, 2004; Golding *et al.*, 2016). Attenuated viruses lacking MGF genes (Pret4Δ35 and OURT88/1) are unable to block the host IFN-α response (Golding *et al.*, 2016).

Attenuated strains of ASFV are currently the most reasonable approach to develop an ASF vaccine. Several attenuated strains obtained by genetic manipulation have demonstrated the ability to induce protective immune responses against virulent strains of ASF. In this thesis we explore a number of immunologic and viral parameters in association with protection against challenge. We have developed an in-house immunoperoxidase assay and an indirect ELISA assay to detect ASFV-specific swine IgG in sera. Both assays were able to detect an IgG response against ASFV as early as 7 or 11 dpi. Results from the ELISA and IPA demonstrate that the presence of antibodies at later challenge time points (21 and 28 dpi, as well as 14 dpi for GeoΔ9GL/ΔUK) is associated with survival in a majority of animals.

Although IFN-γ did not correlate with a protective immune status, we found that IFN-γ increased continuously for the 3 weeks following challenge. These results, encompassing data from over 114 immunized swine, underscore the complexity of the system under study where it is very plausible that protection against disease or infection relies heavily on the concurrence and or interaction of different host immune mechanisms.

5.7 Potential problems

Effective vaccines against ASF must prevent viral replication to minimize the effects of the virulent virus causing damage to the host (Wardley and Wilkinson, 1985).

It is well known that the vaccine strain used in the 1960's in Portugal killed up to 7% of vaccinated swine and at times chronic carriers of ASFV ensued (Boinas *et al.*, 2004; Nunes Petisca, 1965). A substantial portion of these animals had reactions ranging from skin ulcers, pneumonia and abortion to death (Manso Ribeiro *et al.*, 1963). The vaccine was the Lisbon 60 isolate passaged up to 150 times in swine PBMCs (Manso Ribeiro *et al.*, 1963).

The naturally attenuated OURT T88/3 isolate has been shown to induce a protective immune response against virulent homologous challenge (Boinas *et al.*, 2004; King *et al.*, 2011; Oura *et al.*, 2005). Adverse reactions including fever and joint swelling have been observed in some pigs post-inoculation. A transient fever was described in 5 out of 12 swine immunized with OUR T88/3 in one study (King *et al.*, 2011).

Research investigating the potential existence of an ASFV carrier state, where swine are shedding ASFV without presenting clinical signs is necessary. Chronic forms

of ASF have been described in swine experimentally infected with attenuated strains from previous outbreaks in Europe (de Carvalho Ferreira *et al.*, 2012; Gallardo *et al.*, 2015; Wilkinson *et al.*, 1981). If any attenuated virus is to be licensed as a vaccine, extensive studies will be needed to look at the safety of the vaccine and the absence of the development of chronic ASF. Swine inoculated with the vaccine virus should be necropsied at early and late times to observe the tropism of the virus and potential debilitating lesions associated with that virus.

5.8 Future experiments and outlook

Immunological and pathological aspects of ASFV remain to be elucidated. This thesis sought to further our knowledge of host factors that influence protection against death against ASFV. Based on the results presented here, similar experiments in the future should put an emphasis on the collection of more clinical pathology data.

Flow cytometry to characterize changes in defined immune cell types including cytotoxic T cells (CD3+CD8+), γδ T cells (CD3+γδTCR+), helper T cells (CD3+CD4+), or NK cells (CD3-CD4-CD6-CD8α+) (Gerner *et al.*, 2009) may help to delineate changes in the immune response to the attenuated vaccine virus and the challenge virus. The IFN-γ responses measured by ELISPOT could be further characterized by a flow cytometry assay with gating on different T cell subsets, macrophages (CD163+/CD172+) and NK cells in combination with intracellular staining for IFN-γ.

Incorporating complete blood counts (CBCs) and blood chemistry could help to predict the survival outcome of the animals. With a CBC we would have general counts of monocytes, lymphocytes, neutrophils, eosinophils, basophils and erythrocytes. As reported previously, (Zakaryan *et al.*, 2015), swine succumbing to an ASFV challenge

will have lymphopenia and varying numbers of monoblasts (immature monocytes) and lymphoblasts. With this information we may be able to better gauge an animal's health status and aid our veterinarians and technicians in deciding to euthanize experimental animals to avoid unnecessary suffering. As we get closer to a more promising vaccine such parameters will be critical to monitoring swine health after vaccination and challenge.

Improving our methods for virus isolation and growth is another critical need related to the development of potential vaccines. Currently we rely on primary macrophage cultures to grow our attenuated viruses, because Vero-adapted ASFV virus did not stimulate protective immune responses in swine. We need a cell line that allows us to scale up the quantities of virus we grow, while giving us excellent quality control for a clean and reliable cell line.

It would be important to test our mutant viruses after a booster injection with the same attenuated virus around 2-3 weeks to see how that changes the effectiveness of the immune response. If better success is found with booster vaccination schedules, an attempt to booster animals with two different attenuated strains and challenging animals with either parental virus could provide interesting results.

Better descriptions of pathogenesis and possible mechanisms for persistent or chronic ASF infections should be investigated. Understanding this development is relevant for disease control as well as for vaccine design and efficacy. Further studies are necessary to investigate chronic and persistent infections, because it is little understood how they impact their host and the epidemiology of ASF in various regions.

The studies here investigate protection against a virulent challenge with a 21-day observational period. In future experiments, swine should be monitored longer for any significant health changes as swine suffering from chronic forms of ASF do not have specific lesions and may linger for months. In our experiments, we have live attenuated and virulent strains replicating in our swine after challenge, therefore further investigation of how this relates to an animal's long-term health status should be pursued.

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ASForce Accessed July 2016

www.ASForce.org

Appendix A - Supplemental Figures

The figures below show individual animal data from the Pretoria onset of protection experiments. All swine that did not survive challenge are numbered in red. Swine that did survive challenge are numbered in green.

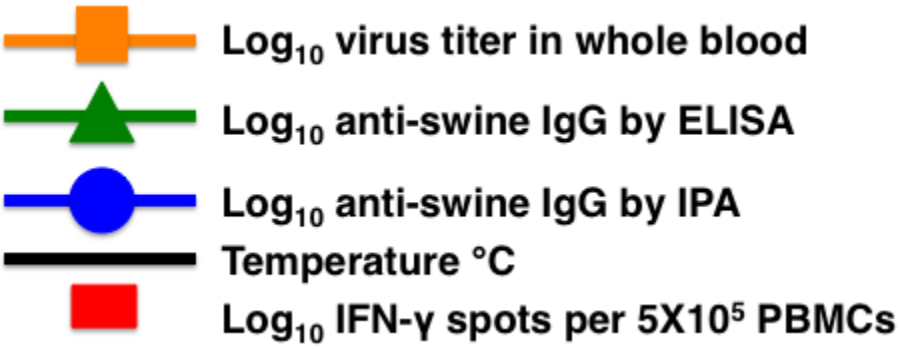

Figure A.5.1 Legend for Individual graphs

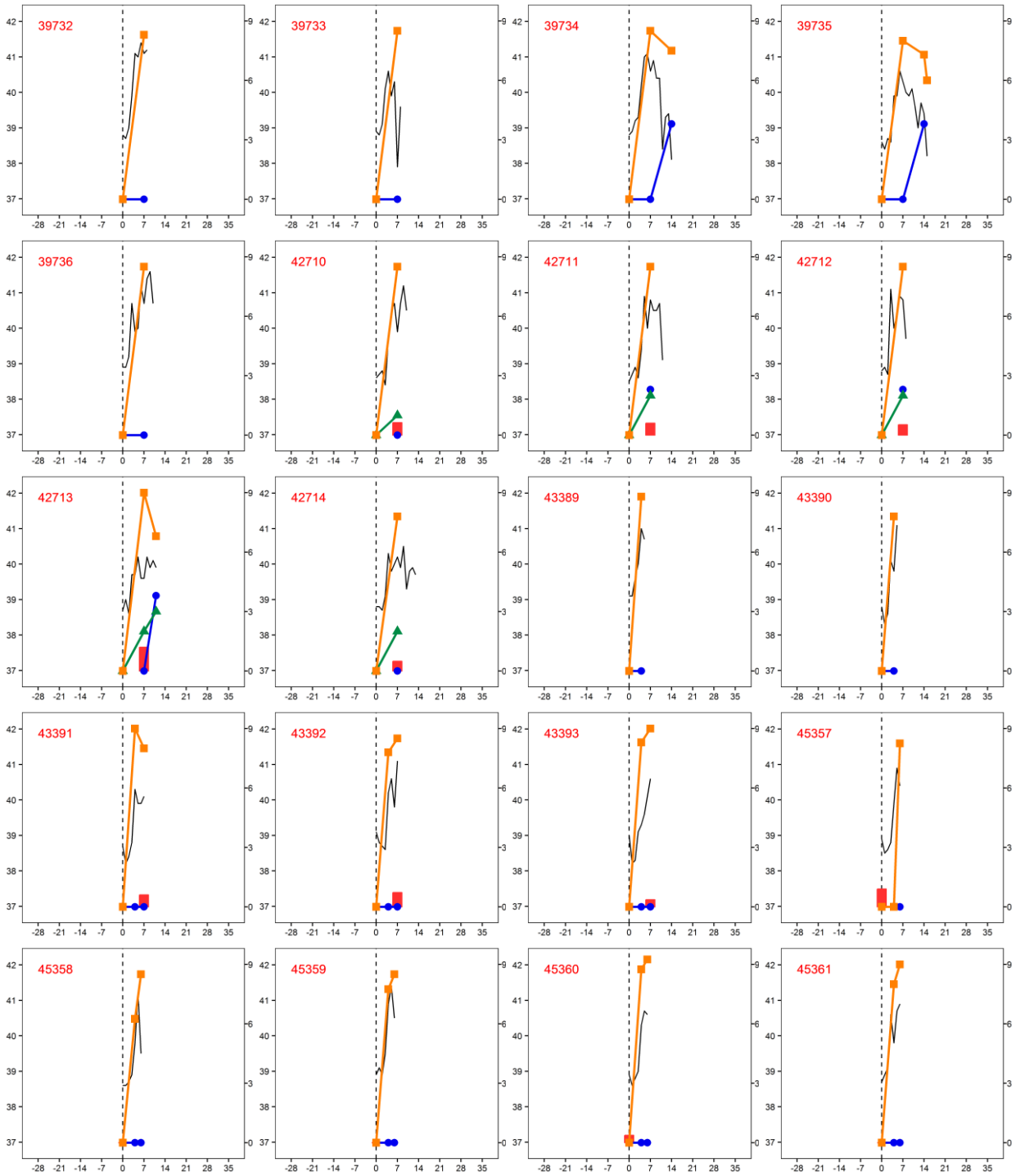

Figure A.5.2 Individual animals in control group. Left axis: rectal temperature (black) (°C). Right axis: Log_{10} of viremia titer (orange), anti-ASFV antibody by IPA (blue), anti-ASFV antibody by ELISA (green), and IFN-γ spots per $5X10^5$ PMBCs (red bars) of naïve swine challenged on day 0.

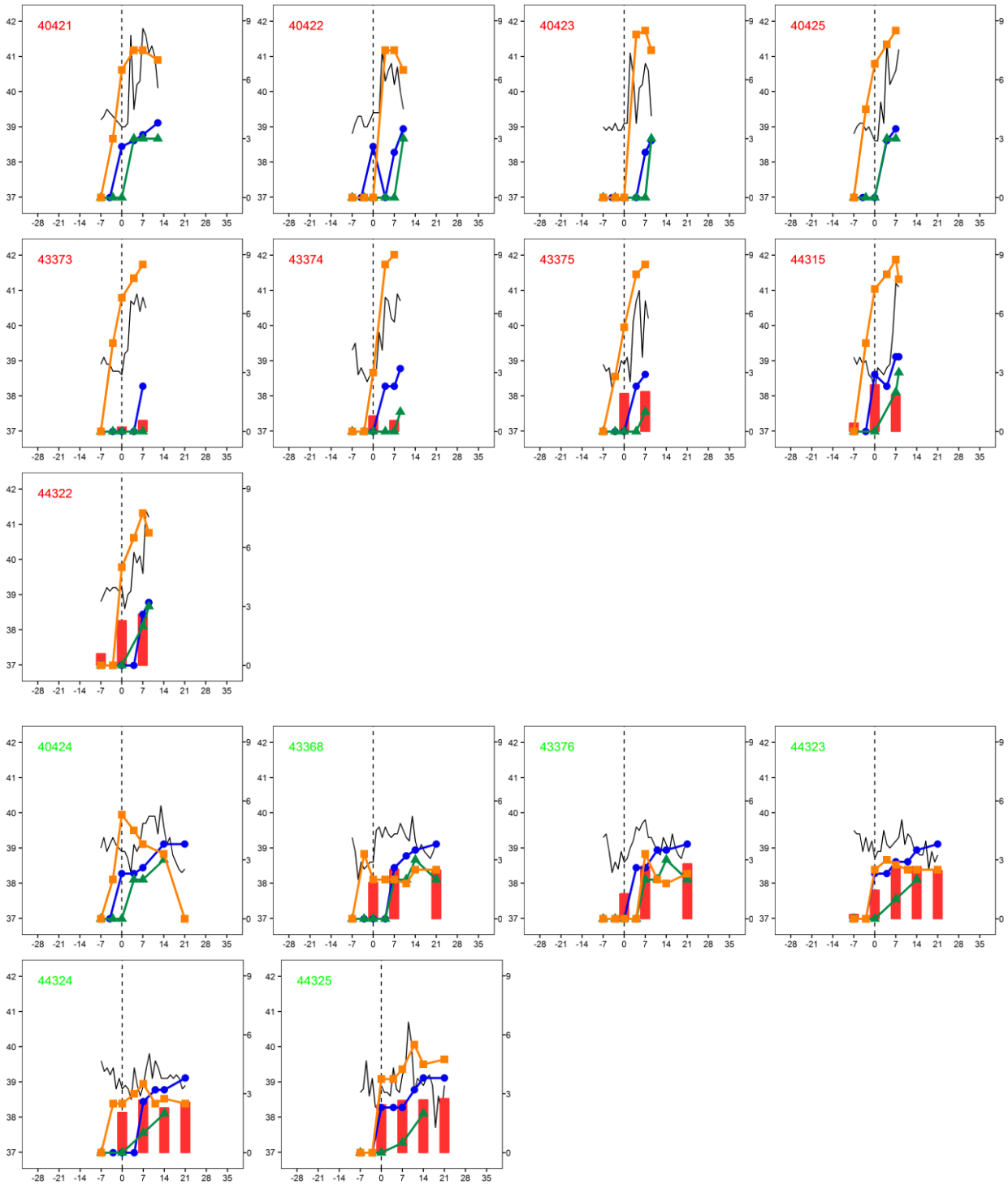

Figure A.5.3 Individual animals in 7-day group. Left axis: rectal temperature (black) (°C). Right axis: Log_{10} of viremia titer (orange), anti-ASFV antibody by IPA (blue), anti-ASFV antibody by ELISA (green), and IFN-γ spots per $5X10^5$ PMBCs (red bars) of naïve swine challenged 7 days post-inoculation (=day 0).

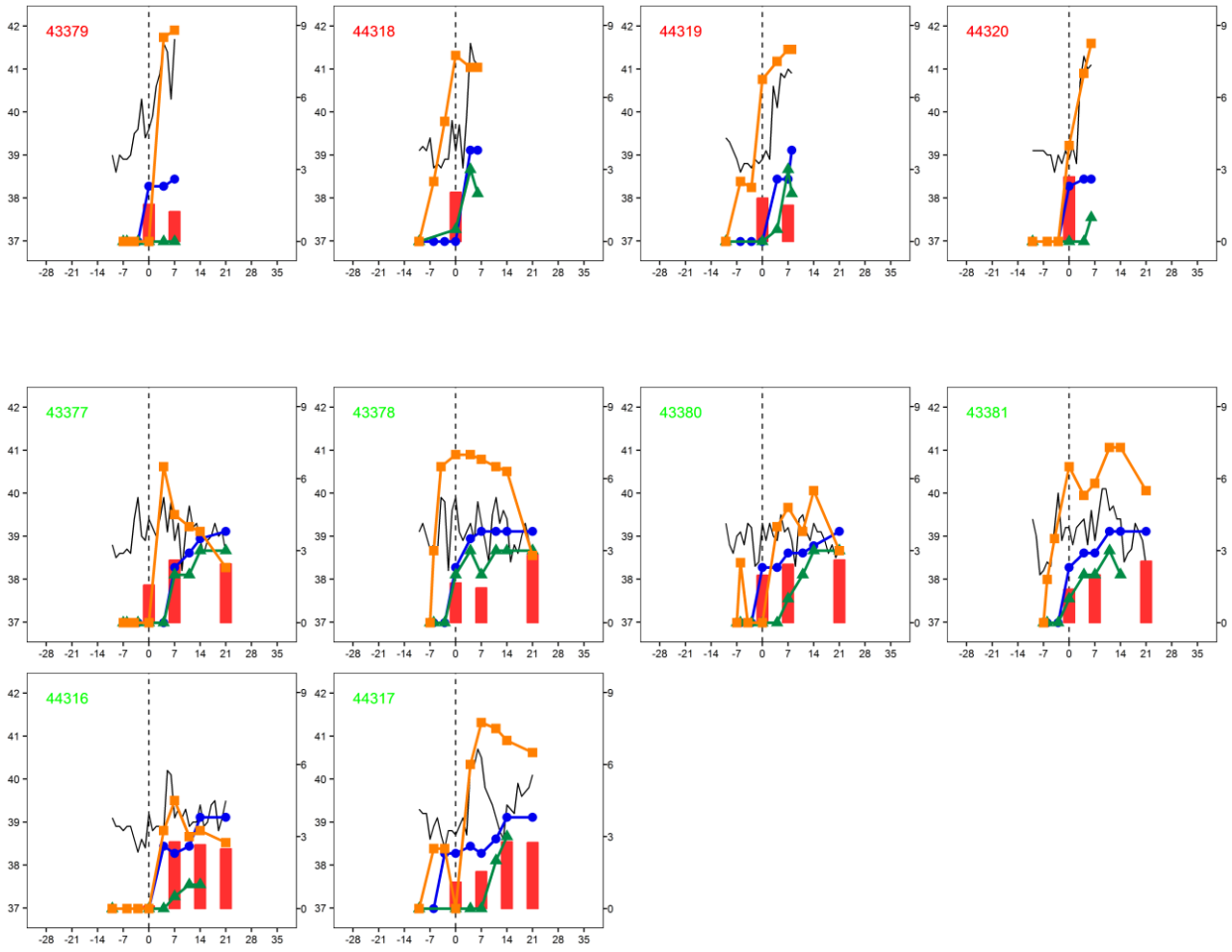

Figure A.5.4 Individual animals in 10-day group. Left axis: rectal temperature (black) (°C). Right axis: Log_{10} of viremia titer (orange), anti-ASFV antibody by IPA (blue), anti-ASFV antibody by ELISA (green), and IFN-γ spots per $5X10^5$ PMBCs (red bars) of naïve swine challenged 10 days post-inoculation (=day 0).

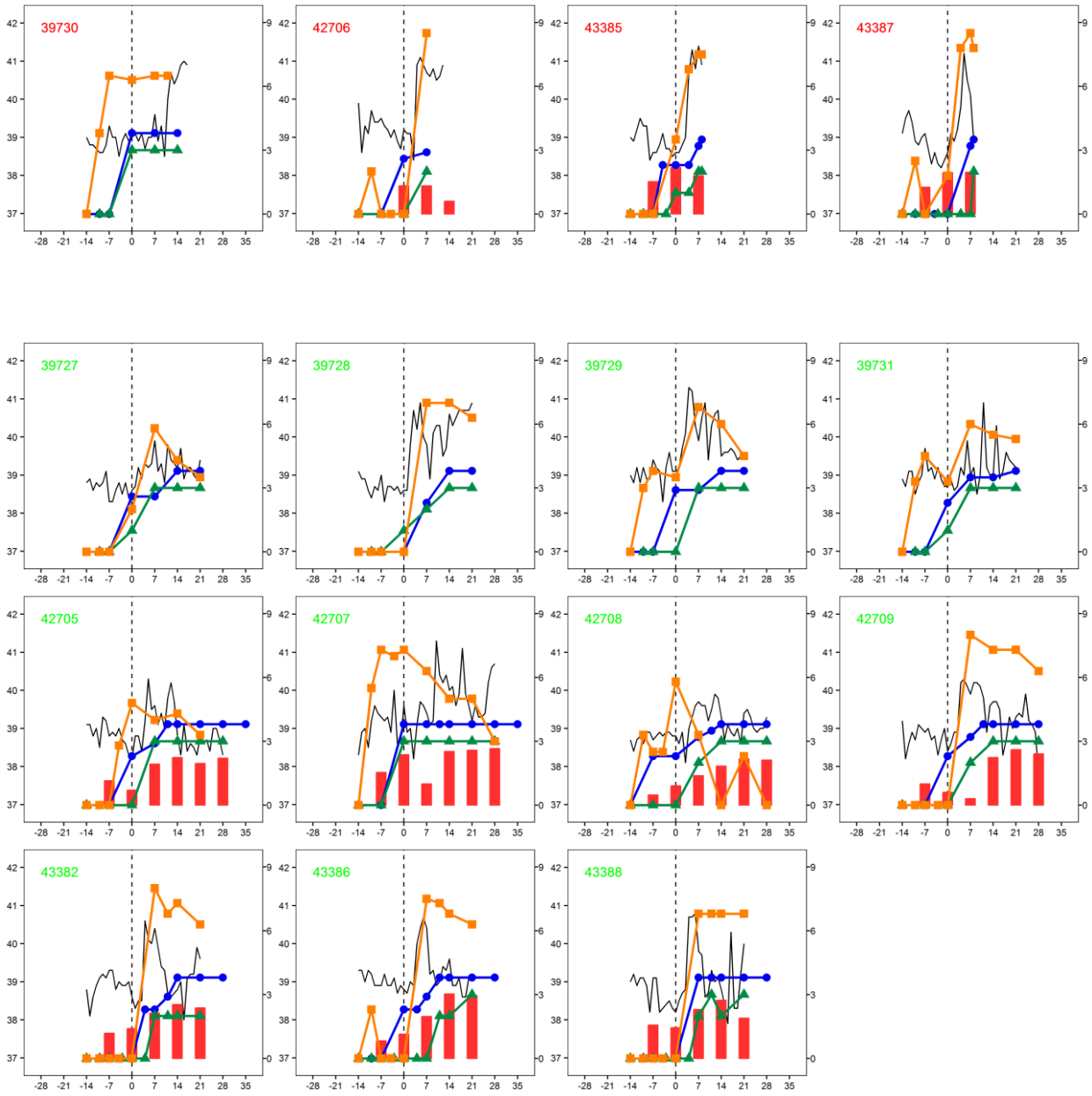

Figure A.5.5 Individual animals in 14-day group. Left axis: rectal temperature (black) (°C). Right axis: Log_{10} of viremia titer (orange), anti-ASFV antibody by IPA (blue), anti-ASFV antibody by ELISA (green), and IFN-γ spots per $5X10^5$ PMBCs (red bars) of naïve swine challenged 14 days post-inoculation (=day 0).

*Note that IFN-γ was not tested in swine 3927 through 39731.

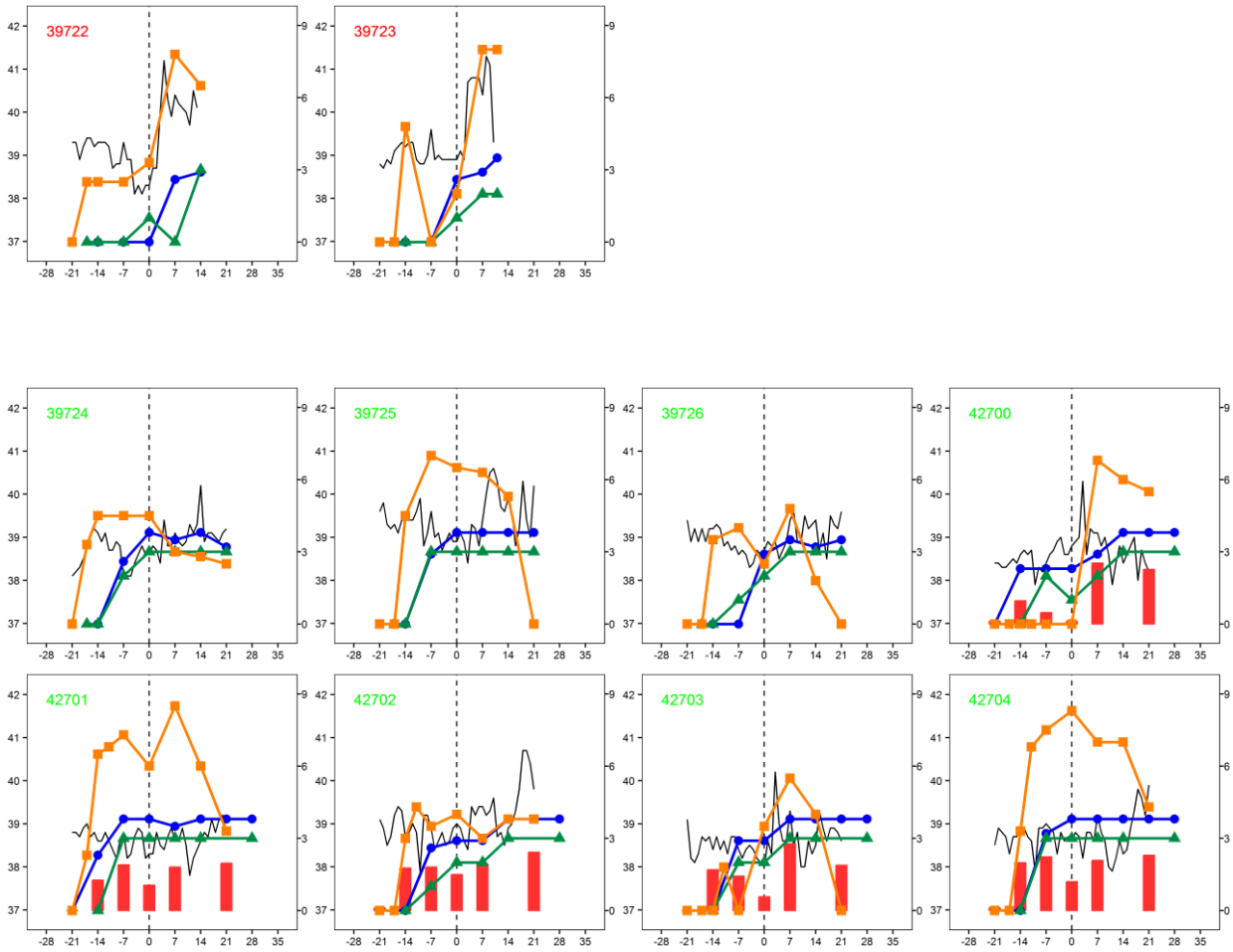

Figure A.5.6 Individual animals in 21-day group. Left axis: rectal temperature (black) (°C). Right axis: Log$_{10}$ of viremia titer (orange), anti-ASFV antibody by IPA (blue), anti-ASFV antibody by ELISA (green), and IFN-γ spots per 5X10^5 PMBCs (red bars) of naïve swine challenged 21 days post-inoculation (=day 0).

*Note that the two non-survivors and three survivors were not tested for IFN-γ by

ELISPOT. The 14-dpc samples were not tested in this group.

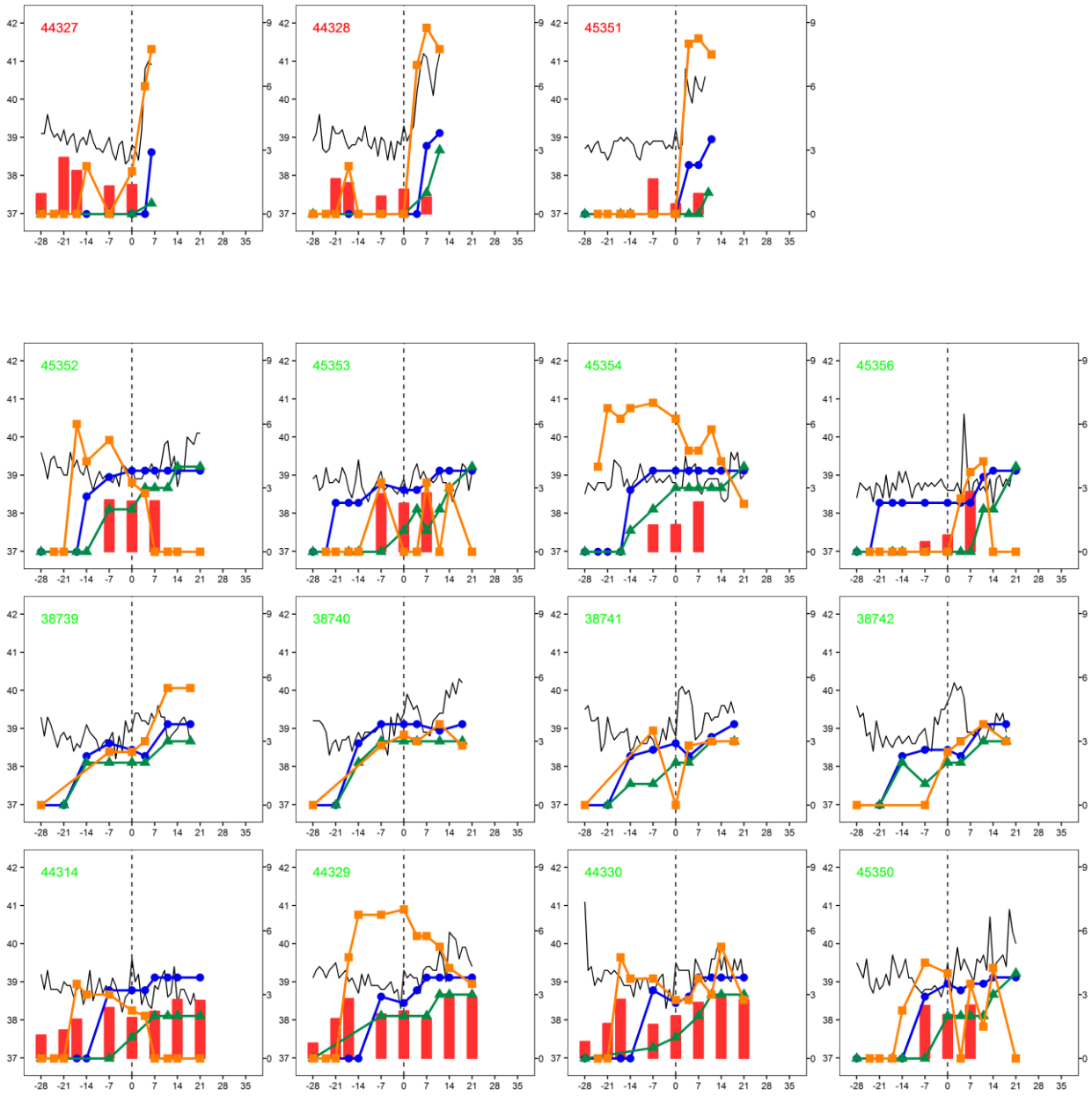

Figure A.5.7 Individual animals in 28-day group. Left axis: rectal temperature (black) (°C). Right axis: Log$_{10}$ of viremia titer (orange), anti-ASFV antibody by IPA (blue), anti-ASFV antibody by ELISA (green), and IFN-γ spots per 5X10^5 PMBCs (red bars) of naïve swine challenged 28 days post-inoculation (=day 0).

*Note, IFN-γ was only tested at -7, 0, 7 dpc in swine 45350 through 45356.

IFN-γ was tested once a week throughout the entire study for swine 44313, 44327,

44328, 44329, 44330.

IFN-γ was not tested in the first experiment in swine 38739-38742.

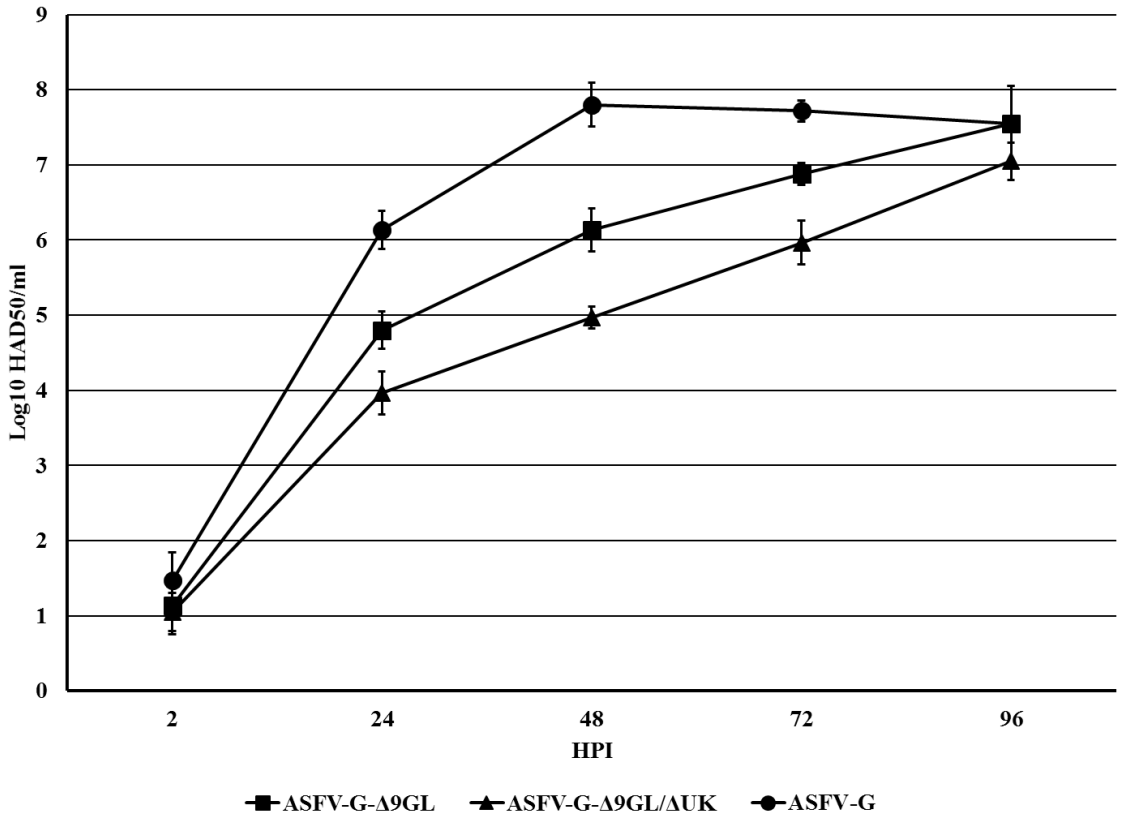

Figure A.5.5.8 In vitro growth characteristics of GeoΔ9GL, GeoΔ9GL/ΔUK, and parental strain ASFV-Georgia 2007. Swine macrophage cultures were infected (MOI 0.01), with each virus and titrated at the 2, 24,48,72, and 96 hours post-infection. Data represent means and SD from three independent experiments. Limit of virus detection ≥ 1.8 Log_{10} HAD_{50}/mL.

Appendix B - Reagents

Table 5.1 List of supplies used throughout this thesis

Reagents/Supplies	Manufacturer	Catalogue Number
DPBS 1X	Gibco/Invitrogen	14190-144
Antibiotic/Antimycotic	Gibco/Invitrogen	15240-062
EDTA 0.5 M	Corning Cellgro	46-034-CI
Fetal Bovine Serum (Gamma Irradiated)	HyClone	SH30071-03
Ficoll-Plaque Plus	GE Healthcare	17-1440-02
Filter 0.45 um for 500 ml	Corning	430770
Filter 0.22 um for 500 ml	Corning	430769
Gentamicin	Gibco/Invitrogen	15750-060
HEPES	Gibco/Invitrogen	15630-0808
Primaria 6-Well	Falcon Becton Dickinson	353846
Primaria 96-Well	Falcon Becton Dickinson	353872
Primaria T75	Falcon Becton Dickinson	353810
Roller Bottle 2 L	Corning	430849
RPMI Media 1640	Gibco/Invitrogen	21870-076
Trypsin EDTA 0.05%	Gibco/Invitrogen	25300-054
Vectastain (ABC kit) Peroxidase Mouse IgG	Vector	PK-4002
Vectastain Peroxidase Standard	Vector	PK-4000
Vector VIP Peroxidase Substrate Kit	Vector	SK-4600
1kb DNA Ladder	Promega	G571A
Biotyinylated Anti-Swine IgG (H+L)	Vector	BA-9020
Blue/Orange Loading Dye	Promega	G190A

Appendix C - Co-Authored Publications

Below is a list of publications I was a part of as a Ph.D. student at Kansas State University. Shown on the following pages is a selection of these publications I chose to include in this thesis.

1. Gladue, D.P., O'Donnell, V., Fernandez-Sainz, I.J., Fletcher, P., Baker-Branstetter, R., Holinka, L.G., Sanford, B., **Carlson, J.,** Lu, Z., Borca, M.V., 2014. Interaction of structural core protein of classical swine fever virus with endoplasmic reticulum-associated degradation pathway protein OS9. Virology 460-461, 173-179.

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African Swine Fever Virus Georgia 2007 with a Deletion of Virulence-Associated Gene *9GL* (B119L), when Administered at Low Doses, Leads to Virus Attenuation in Swine and Induces an Effective Protection against Homologous Challenge

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ABSTRACT

African swine fever virus (ASFV) is the etiological agent of an often lethal disease of domestic pigs. Disease control strategies have been hampered by the unavailability of vaccines against ASFV. Since its introduction in the Republic of Georgia, a highly virulent virus, ASFV Georgia 2007 (ASFV-G), has caused an epizootic that spread rapidly into Eastern European countries. Currently no vaccines are available or under development to control ASFV-G. In the past, genetically modified ASFVs harboring deletions of virulence-associated genes have proven attenuated in swine, inducing protective immunity against challenge with homologous parental viruses. Deletion of the gene *9GL* (open reading frame [ORF] B119L) in highly virulent ASFV Malawi-Lil-20/1 produced an attenuated phenotype even when administered to pigs at 10^6 50% hemadsorption doses (HAD_{50}). Here we report the construction of a genetically modified ASFV-G strain (ASFV-G-Δ9GLv) harboring a deletion of the *9GL* (B119L) gene. Like Malawi-Lil-20/1-Δ9GL, ASFV-G-Δ9GL showed limited replication in primary swine macrophages. However, intramuscular inoculation of swine with 10^4 HAD_{50} of ASFV-G-Δ9GL produced a virulent phenotype that, unlike Malawi-Lil-20/1-Δ9GL, induced a lethal disease in swine like parental ASFV-G. Interestingly, lower doses (10^2 to 10^3 HAD_{50}) of ASFV-G-Δ9GL did not induce a virulent phenotype in swine and when challenged protected pigs against disease. A dose of 10^2 HAD_{50} of ASFV-G-Δ9GLv conferred partial protection when pigs were challenged at either 21 or 28 days postinfection (dpi). An ASFV-G-Δ9GL HAD_{50} of 10^3 conferred partial and complete protection at 21 and 28 dpi, respectively. The information provided here adds to our recent report on the first attempts toward experimental vaccines against ASFV-G.

IMPORTANCE

The main problem for controlling ASF is the lack of vaccines. Studies on ASFV virulence lead to the production of genetically modified attenuated viruses that induce protection in pigs but only against homologous virus challenges. Here we produced a recombinant ASFV lacking virulence-associated gene *9GL* in an attempt to produce a vaccine against virulent ASFV-G, a highly virulent virus isolate detected in the Caucasus region in 2007 and now spreading though the Caucasus region and Eastern Europe. Deletion of *9GL*, unlike with other ASFV isolates, did not attenuate completely ASFV-G. However, when delivered once at low dosages, recombinant ASFV-G-Δ9GL induces protection in swine against parental ASFV-G. The protection against ASFV-G is highly effective after 28 days postvaccination, whereas at 21 days postvaccination, animals survived the lethal challenge but showed signs of ASF. Here we report the design and development of an experimental vaccine that induces protection against virulent ASFV-G.

African swine fever (ASF) is a contagious viral disease of swine. The causative agent, ASF virus (ASFV), is a large enveloped virus containing a double-stranded DNA (dsDNA) genome of approximately 190 kbp. ASFV shares aspects of genome structure and replication strategy with other large dsDNA viruses, including the *Poxviridae*, *Iridoviridae*, and *Phycodnaviridae* (1). ASF causes a spectrum of disease that ranges from highly lethal to subclinical, depending on host characteristics and the virulence of circulating virus strains (2). ASFV infections in domestic pigs are often fatal and are characterized by high fever, hemorrhages, ataxia, and severe depression.

Currently, the disease is endemic in more than 20 sub-Saharan African countries. In Europe, ASF is endemic on the island of Sardinia (Italy), and outbreaks of ASF have been recorded in the Caucasus region since 2007, affecting Georgia, Armenia, Azerbaijan, and Russia. Isolated outbreaks have been recently reported in Ukraine, Belarus, Lithuania, Latvia, and Poland, posing the risk of further dissemination into neighboring countries. The epidemic

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virus, ASFV Georgia 2007/1, is a highly virulent isolate that belongs to genotype II (3).

Currently, there is no vaccine available against ASF, and disease outbreaks are usually controlled by quarantine and slaughter of affected and exposed herds. Past attempts to vaccinate animals against ASF 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 have failed to induce protective immunity (4–6). However, homologous protective immunity does develop in pigs surviving ASFV infections. Pigs surviving acute infections with moderately virulent or attenuated ASFV isolates develop long-term resistance to homologous viruses but rarely to heterologous viruses (7, 8). Pigs immunized with live attenuated ASFVs containing genetically engineered deletions of specific virulence-associated genes were protected when challenged with homologous parental viruses. Specifically, individual deletions of the *UK* (open reading frame [ORF] DP69R), *23-NL* (ORF DP71L), *TK* (ORF A240L), or *9GL* (ORF B119L) genes from the genomes of virulent ASFVs resulted in significant attenuation of these isolates in swine. Animals immunized with these modified viruses showed protection when challenged with homologous ASFVs (9–11). So far, these observations are the only experimental evidence supporting a rational development of effective live attenuated virus against ASFV.

In particular, deletion of the *9GL* (B119L) gene in highly virulent ASFV isolates Malawi Lil-20/1 and Pretoriuskop/96/4 resulted in complete attenuation of these viruses in swine (10, 12). Intramuscular (i.m.) administration of Malawi Lil-20/1-Δ9GL mutants to pigs at a relatively high virus dose (10^6 50% hemadsorption doses [HAD_{50}]) did not induce clinical disease, with all animals surviving the inoculation. Furthermore, i.m. inoculation of pigs with these viruses even at a relatively low dose (10^2 HAD_{50}) induced protection against challenge with virulent Malawi Lil-20/1 virus (10). Therefore, targeting of the highly conserved *9GL* (B119L) gene for genetic modifications appeared to be a reasonable approach for developing attenuated viruses that can be used as vaccine candidates. Here we report the construction of a recombinant Δ9GL virus of the highly virulent and epidemiologically relevant ASFV Georgia 2007 (ASFV-G) isolate. *In vitro*, as observed with Malawi Lil-20/1-Δ9GL mutants (10), ASFV-G-Δ9GL has a decreased ability relative to the parental virus to replicate in swine macrophage primary cultures. However, unlike Malawi Lil-20/1-Δ9GL virus, with i.m. administration of ASFV-G-Δ9GL to swine at relatively high doses (10^4 HAD_{50}), the virus retained a virulent phenotype similar to the parental virus. Inoculation of pigs with lower doses (10^2 to 10^3 HAD_{50}) of ASFV-G-Δ9GL did not induce clinical disease. Thus, deletion of the highly conserved *9GL* (B119L) gene from the ASFV-G isolate resulted in a lesser degree of reduction of the virulent phenotype relative to the degree of attenuation observed with Malawi Lil-20/1-Δ9GL and Pretoriuskop/96/4-Δ9GL, indicating an isolate-specific effect on ASFV attenuation. Interestingly, animals inoculated with these sublethal doses of ASFV-G-Δ9GL were partially protected against challenge at 21 days postinfection (dpi) but completely protected at 28 dpi. To our knowledge, along with our recent report regarding the development of an attenuated ASFV-G deletion mutant lacking genes from multigene families (MGF) 360 and 505, these are the first reports of experimental vaccines that induce protection against highly virulent ASFV-G.

MATERIALS AND METHODS

Cell cultures and viruses. Primary swine macrophage cell cultures were prepared from defibrinated swine blood as previously described by Zsak et al. (11). Briefly, heparin-treated swine blood was incubated at 37°C for 1 h to allow sedimentation of the erythrocyte fraction. Mononuclear leukocytes were separated by flotation over a Ficoll-Paque (Pharmacia, Piscataway, NJ) density gradient (specific gravity, 1.079). The monocyte/macrophage cell fraction was cultured in plastic Primaria (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ) tissue culture flasks containing macrophage media, composed of RPMI 1640 medium (Life Technologies, Grand Island, NY) with 30% L929 supernatant and 20% fetal bovine serum (HI-FBS; Thermo Scientific, Waltham, MA) for 48 h at 37°C under 5% CO_2. Adherent cells were detached from the plastic by using 10 mM EDTA in phosphate-buffered saline (PBS) and were then reseeded into Primaria T25 6- or 96-well dishes at a density of 5×10^6 cells per ml for use in assays 24 h later.

ASFV Georgia (ASFV-G) was a field isolate kindly provided by Nino Vepkhvadze, from the Laboratory of the Ministry of Agriculture (LMA) in Tbilisi, Republic of Georgia.

Comparative growth curves between ASFV-G and ASFV-G-Δ9GL viruses were performed in primary swine macrophage cell cultures. Preformed monolayers were prepared in 24-well plates and infected at a multiplicity of infection (MOI) of either 0.1 or 0.01 (based on the HAD_{50} previously determined in primary swine macrophage cell cultures). After 1 h of adsorption at 37°C under 5% CO_2, the inoculum was removed, and the cells were rinsed two times with PBS. The monolayers were then rinsed with macrophage medium and incubated for 2, 24, 48, 72, and 96 h at 37°C under 5% CO_2. At appropriate times postinfection, the cells were frozen at −70°C, and the thawed lysates were used to determine titers by HAD_{50} per milliliter in primary swine macrophage cell cultures. All samples were run simultaneously to avoid interassay variability.

Virus titration was performed on primary swine macrophage cell cultures in 96-well plates. Virus dilutions and cultures were performed using macrophage medium. Presence of virus was assessed by hemadsorption (HA), and virus titers were calculated by the Reed and Muench method (13).

Construction of the recombinant ASFV-G-Δ9GL. Recombinant ASFVs were generated by homologous recombination between the parental ASFV genome and a recombination transfer vector following infection and transfection of swine macrophage cell cultures (11, 14). Recombinant transfer vector (p72GUSΔ9GL) contained flanking genomic regions, which included portions of *9GL* mapping to the left (1.2 kbp) and right (1.15 kbp) of the gene and a reporter gene cassette containing the β-glucuronidase (GUS) gene with the ASFV p72 late gene promoter, p72GUS (11). This construction created a 173-nucleotide deletion in the *9GL* open reading frame (ORF), B119L (amino acid residues 11 to 68). Recombinant transfer vector p72GUSΔ9GL was obtained by DNA synthesis (Epoch Life Sciences, Sugar Land, TX). Macrophage cell cultures were infected with ASFV-G and transfected with p72GUSΔ9GL. Recombinant viruses representing independent primary plaques were purified to homogeneity by successive rounds of plaque assay purification.

PCR. The purity of ASFV-G-Δ9GL in the virus stock as well as in virus isolated from infected animals was assessed by PCR. All PCRs were designed to amplify internal regions of each of the tested genes. Detection of a *9GL* (B119L) 357-bp gene fragment was performed using the forward primer 5′-TAGAGATGACCAGGCTCCAA-3′ and reverse primer 5′-GTTGCATTGGGGACCTAAATACT-3′. Detection of a β-GUS gene 471-bp gene fragment was performed using the forward primer 5′-GACGGCCTGTGGGCATT-3′ and reverse primer 5′-GCGATGGATTCCG GCAT-3′. Detection of a p72 (B646L) 256-bp gene was performed using the forward primer 5′-GTCTTATTGCTAACGATGGGAAG-3′ and reverse primer 5′-CCAAAGGTAAGCTTGTTTCCCAA-3′.

Sequencing of PCR products. PCR products were sequenced using the dideoxynucleotide chain-termination method (15). Sequencing reactions were prepared with the Dye Terminator cycle sequencing kit (Ap-

plied Biosystems, Foster City, CA). Reaction products were sequenced on a PRISM 3730xl automated DNA sequencer (Applied Biosystems). Sequence data were assembled with the Phrap software program (http://www.phrap.org), with confirmatory assemblies performed using CAP3 (16). The final DNA consensus sequence represented an average 5-fold redundancy at each base position. Sequence comparisons were conducted using BioEdit software.

Next-generation sequencing of ASFV genomes. ASFV DNA was extracted from infected cells and quantified as described earlier (17). Full-length sequencing of the virus genome was performed as described elsewhere (17). Briefly, 1 μg of virus DNA was enzymatically sheared, and the resulting fragmented DNA size distribution was assessed. Adapters and library bar codes were ligated to the fragmented DNA. The appropriate size range of adapter-ligated library was collected using the Pippin Prep system (Sage Science) followed by normalization of library concentration. The DNA library was then clonally amplified onto intracellular serine proteases (ISPs) and enriched. Enriched template ISPs were prepared for sequencing and loaded onto Ion chips and sequenced with an Ion PGM sequencer (Life Technologies, Grand Island, NY). Sequence analysis was performed using Galaxy (https://usegalaxy.org/) and CLC Genomics Workbench (CLCBio).

Detection of anti-ASFV antibodies. Anti-ASFV antibodies in sera of infected animals were quantified using an in-house-developed assay. Vero cells were infected (MOI of 0.1) with an Vero-adapted ASFV Georgia strain (ASFV Vero) (17) in 96-well plates and fixed. Two-fold dilutions of the sera were incubated for 1 h at 37°C in the 96-well ASFV Vero-infected cell monolayer. After washing, the presence of anti-ASFV antibodies was detected by using a commercial anti-swine peroxidase-labeled mouse immunoglobulin and a peroxidase substrate (Vector; Vector Laboratories, Burlingame, CA). Titers were expressed as the $\log_{10}$ value of the inverse of the highest serum dilution showing a reaction with the infected cells.

Animal experiments. Animal experiments were performed under biosafety level 3 conditions in the animal facilities at Plum Island Animal Disease Center (PIADC) following a protocol approved by the Institutional Animal Care and Use Committee.

ASFV-G-Δ9GL was assessed for its virulence relative to the parental ASFV-G virus using 80- to 90-pound commercial breed swine. Five pigs were inoculated intramuscularly (i.m.) with either 10^2, 10^3, or 10^4 HAD_{50} of ASFV-G-Δ9GL or ASFV-G. Clinical signs (anorexia, depression, fever, purple skin discoloration, staggering gait, diarrhea, and cough) and changes in body temperature were recorded daily throughout the experiment.

ASFV-G-Δ9GL was assessed for its protective effect using 80- to 90-pound commercial breed swine. Groups of five pigs were inoculated intramuscularly (i.m.) with either 10^2 or 10^3 HAD_{50} of ASFV-G-Δ9GL. At either 21 or 28 days postinfection, animals were i.m. challenged with 10^3 HAD_{50} of highly virulent parental ASFV-G. Clinical signs (as described above) and changes in body temperature were recorded daily throughout the experiment.

Detection of ASFV-specific IFN-γ-producing cells. Detection of ASFV-specific interferon gamma (IFN-γ)-producing cells was performed using a modification of the enzyme-linked immunosorbent spot (ELISpot) porcine IFN-γ method (R&D, Minneapolis, MN). Peripheral blood mononuclear cells (PBMCs) were isolated from 15 ml of porcine blood by Ficoll-Paque Plus gradient (density, 1.077) and washed twice with 1× PBS at room temperature. Cell counts were adjusted to 5×10^6 cells/ml, and 96-well plates were seeded with the cells. After seeding, cells were stimulated with a buffer containing 25 ng/ml of phorbol myristate acetate (PMA) and 25 ng/ml of calcium ionomycin or were exposed to ASFV-G virus at an MOI of 0.5. The cells and stimulators were then immediately transferred to ELISpot plates (as provided in the kit) and incubated for 18 h at 37°C. The steps for washing as well as for using the detection antibody, streptavidin-alkaline phosphatase (AP), and 5-bromo-4-chloro-3-indolyl-phosphate–nitroblue tetrazolium (BCIP/NBT) chromogen were sequentially performed as recommended by the kit's manufacturer. Reading was performed with an Immunospot ELISpot plate reader (Cellular Technology Limited) with the following settings: counting mask size of 100%, normalize counts of mask off, sensitivity of 130, minimum spot size of 0.086 mm^2, and maximum spot size of 0.2596 mm^2. Oversized spots were estimated at a spot separation of 1, diffuseness setting of "large," and background balance of 67. Cell counts were expressed as number of spots per 5×10^6 PBMCs/ml.

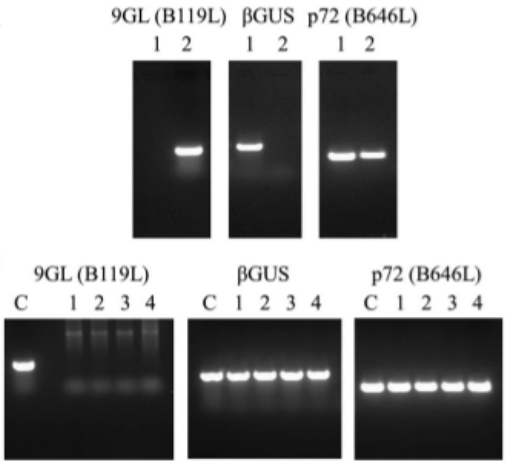

FIG 1 PCR analysis of ASFV-G-Δ9GL virus DNA using specific primers targeting the *9GL* (B119L), p72 (B646L), or β-GUS genes. (A) Assessment of purity of the ASFV-G-Δ9GL virus stock by PCR. Lane 1, ASFV-G-Δ9GL; lane 2, ASFV-G. (B) Identification of the presence of parental ASFV-G (lanes 1 to 4) in viruses isolated from animals infected with 10^4 HAD_{50} ASFV-G-Δ9GL virus. The control (lane C) consists of a plasmid containing the respective target genes.

RESULTS

Development of the ASFV-G-Δ9GL deletion mutant. ASFV-G-Δ9GL was constructed by genetic modification of the highly virulent ASFV Georgia 2007 isolate (ASFV-G). A 173-bp region, encompassing amino acid residues 11 to 68, within the *9GL* (B119L) gene was deleted from ASFV-G virus and replaced with a gene cassette containing the β-glucuronidase (β-GUS) gene under the control of the ASFV p72 late gene promoter (p72GUS) by homologous recombination (see Materials and Methods). The recombinant virus was obtained after 11 successive plaque purification events on monolayers of primary swine macrophage cell cultures. The virus population obtained from the last round of plaque purification was amplified in primary swine macrophage cell cultures to obtain a virus stock. To ensure the absence of parental ASFV-G, virus DNA was extracted from the virus stock and analyzed by PCR using primers targeting the p72 (B646L), *9GL* (B119L), and β-GUS genes. Only amplicons for the p72 (B646L) and β-GUS genes were detected in DNA extracted from the virus stock; no amplicons were generated with primers targeting the *9GL* (B119L) gene (Fig. 1A), indicating the lack of contamination of the ASFV-G-Δ9GL stock with ASFV-G.

Analysis of the ASFV-G-Δ9GL genome sequence relative to parental ASFV-G genome sequence. To evaluate the accuracy of the genetic modification and the integrity of the genome of the recombinant virus, full genome sequences of ASFV-G-Δ9GL and

TABLE 1 Summary of differences between the full-length genome sequence of ASFV-G-Δ9GL and the parental ASFV-G compared with ASFV Georgia 07/1

NPN[a]	Type of modification[b]	Result for virus	
		ASFV-G	ASFV-G-Δ9GL
433	T insertion	+	+
411	A insertion	+	+
1602	MGF *360-1L* TT deletion FS	+	+
1620	MGF *360-1L* T insertion FS	+	+
36465	MGF *505-4R* G to C Glu224Gln	−	+
97391	B438L A to G SM	+	+
166192	E199L C to G Ala85Pro	+	+
183303	T insertion in NCR	+	+

[a] NPN, nucleotide position number based on the sequence of the ASFV Georgia 2007/1 isolate published by Chapman et al. in 2011 (3).
[b] FS, nucleotide modification causes frameshift in the corresponding ORF; SM, nucleotide modification causes silent mutation; NCR, noncoding region.

parental ASFV-G were obtained using NGS on the Ion Torrent PGM and compared. First, a full-length genome comparison between parental ASFV-G and ASFV Georgia 2007/1 (3) was performed. The following differences were observed between these two viruses (nucleotide positions are provided based on ASFV Georgia 2007/1 GenBank accession no. FR682468): (i) two nucleotide insertions, T at position 433 and A at position 441, in a noncoding segment of the genome; (ii) two nucleotide deletions, T at position 1602 and T at position 1603, in the MGF *360-1L* gene ORF resulting in a frameshift; (iii) a nucleotide insertion, T at position 1620, in the MGF *360-1L* gene ORF resulting in a frameshift; (iv) a nucleotide mutation of A to G at position 97391 resulting in a silent mutation in ORF B438L; (v) a nucleotide mutation of C to G at position 166192 resulting in a residue substitution (Ala to Pro) at residue position 85 in ORF E199L; and (vi) a nucleotide insertion of T at position 183303, a noncoding segment of the genome (Table 1). Second, a full-length genome comparison between ASFV-G-Δ9GL and parental ASFV-G was performed. The DNA sequence of ASFV-G-Δ9GL revealed a deletion of 173 nucleotides in ORF B119L (*9GL*) relative to parental ASFV-G that corresponds with the introduced modification. The consensus sequence of the ASFV-G-Δ9GL genome showed an insertion of 2,324 nucleotides in ORF B119L corresponding to the p72GUS cassette sequence introduced to generate a 173-nucleotide deletion in the targeted gene. Besides the insertion of the cassette, only one additional difference was observed between ASFV-G-Δ9GL and ASFV-G genomes: a G-to-C point mutation at position 36465 resulting in amino acid substitution E224Q in ORF MGF *505-4R*. In summary, ASFV-G-Δ9GL virus did not accumulate any significant mutations during the process of homologous recombination and consequent plaque purification steps.

Replication of ASFV-G-Δ9GL in primary swine macrophages. *In vitro* growth characteristics of ASFV-G-Δ9GL were evaluated in cultures of primary swine macrophages, the primary cell targeted by ASFV during infection in swine, and compared relative to parental ASFV-G in multistep growth curves (Fig. 2). Cell cultures were infected at an MOI of either 0.1 or 0.01, and samples were collected at 2, 24, 48, 72, and 96 h postinfection (hpi). ASFV-G-Δ9GL virus displayed a growth kinetic significantly slower than that of the parental ASFV-G virus. Depending on the time point and MOI utilized to infect macrophages, the recombinant virus exhibited titers 10- to 10,000-fold lower than those of the parental virus. Therefore, and as observed with ASFV Malawi Lil-20/1-Δ9GL, deletion of the *9GL* (B119L) gene significantly affects the ability of the virus to replicate *in vitro* in primary swine macrophage cell cultures.

Assessment of ASFV-G-Δ9GL virulence in swine. Deletion of the *9GL* (B119L) gene from the genomes of ASFV isolates Malawi Lil-20/1 and Pretoriuskop/96/4 has been shown to drastically reduce virulence in swine (10, 12). In those reports, it was observed that i.m. inoculation of pigs with the recombinant deletion mutant at doses as high as 10^4 (10, 12) or even 10^6 HAD_{50} (10) induced only a transient rise in body temperature.

Here, 80- to 90-pound pigs inoculated i.m. with 10^4 HAD_{50} of ASFV-G exhibited increased body temperature (>104°F) by 3 to 4 days postinfection. Pigs presented clinical signs associated with the disease, including anorexia, depression, purple skin discoloration, staggering gait, and diarrhea. Signs of the disease aggravated progressively over time, and animals either died or were euthanized *in extremis* by day 7 or 8 postinfection (Table 2). Animals inoculated i.m. with 10^2 or 10^3 HAD_{50} of virulent ASFV-G developed a clinical disease comparable in severity to that observed in animals infected with 10^4 HAD_{50} of the same virus, with the exception that both clinical signs and the onset of death were slightly delayed by 1 to 3 days. Pigs presented a short period of fever starting by day 6 to 7 postinfection, with animals dying or euthanized *in extremis* around 8 to 9 days postinfection. The severity of the clinical signs observed in these animals was similar to those inoculated at higher dose (10^4 HAD_{50}) (Table 2).

Interestingly, animals inoculated i.m. with 10^4 HAD_{50} of ASFV-G-Δ9GL developed clinical disease similar to that observed in animals inoculated i.m. with 10^4 HAD_{50} of parental ASFV-G, the only difference being a slight delay in the onset of fever. Conversely, pigs inoculated i.m. with 10^2 or 10^3 HAD_{50} of mutant ASFV-G-Δ9GL did not present any signs of clinical disease during the entire observation period (21 days). Therefore, the degree of

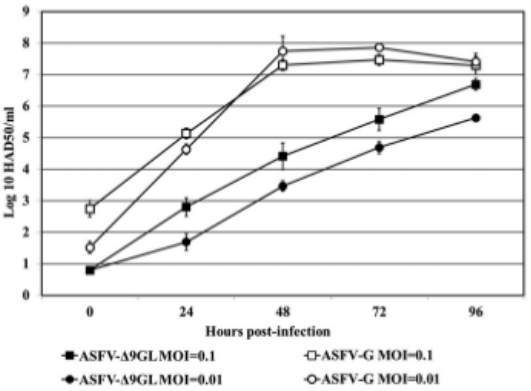

FIG 2 *In vitro* growth kinetics of the ASFV-G-Δ9GL and parental ASFV-G viruses. Primary swine macrophage cell cultures were infected (MOI of 0.1 or 0.01) with either ASFV-G-Δ9GL or parental ASFV-G virus. Virus yields were estimated at the indicated times postinfection by titration in primary swine macrophage cell cultures. Data represent means and standard deviations from two independent experiments. The sensitivity of virus detection was ≥1.8 HAD_{50}/ml.

TABLE 2 Swine survival and fever response following infection with different doses of ASFV-G-Δ9GL and parental ASFV-G

HAD_{50} of virus	No. of survivors/total	Mean (SD) time to death, days	Mean (SD) characteristic of fever		
			No. of days to onset	Duration, days	Maximum daily temp, °F
10^2					
ASFV-G	0/5	9.4 (1.22)	7 (0.0)	1.2 (0. 82)	106.7 (0.58)
ASFV-G-Δ9GL	10/10[a]				103.0 (0.17)
10^3					
ASFV-G	0/5	8.8 (1.1)	5.6 (0.89)	3.2 (0.43)	106.2 (0.27)
ASFV-G-Δ9GL	10/10[a]				103.4 (0.32)
10^4					
ASFV-G	0/10[a]	7.25 (0.7)	3.5 (0.76)	3.75 (0.71)	107 (0.47)
ASFV-G-Δ9GL	0/5	8.25 (1.6)	5.25 (1.91)	3.25 (0.46)	106.5 (0.46)
ASFV Mal Δ9GL	4/4		9 (0.0)	1 (0.82)	104.2 (0.59)

[a] Data from two similar experiments are presented together.

virulence of ASFV-G-Δ9GL virus administered i.m. into swine appears to depend on the amount of infectious virus used in the experimental inoculation.

A control group of animals ($n = 4$) were inoculated with 10^4 HAD_{50} of Malawi Lil-20/1-Δ9GL (10). As previously described, these animals remained clinically normal throughout the experimental period, showing a transient and rather mild rise in body temperature (Table 2).

Viremia in experimentally inoculated animals was quantified at different days postinfection by hemadsorption. As expected, animals inoculated with 10^2, 10^3, or 10^4 HAD_{50} of virulent parental ASFV-G had very high virus titers in blood until the day of their death (Fig. 3C, F, and H). Conversely, animals inoculated with ASFV-G-Δ9GL at any of the utilized doses had relatively low virus titers in blood compared with those of the ASFV-G-inoculated animals (Fig. 3A, B, D, E, and G). Animals inoculated with 10^4 HAD_{50} of mutant ASFV-G-Δ9GL presented virus titers in blood 1,000- to 10,000-fold lower than those at the corresponding time point in animals inoculated with similar dose of the ASFV-G virus (Fig. 3G). Therefore, although the severities and kinetics of the presentation of clinical disease were similar, blood titers in both groups were significantly different. Thus, despite a low titer in blood that might indicate limited replication *in vivo*, ASFV-G-Δ9GL induces a lethal disease in pigs without reaching the viremia levels observed in animals inoculated with parental ASFV-G.

Generally, animals inoculated with either 10^2 HAD_{50} or 10^3 HAD_{50} of mutant ASFV-G-Δ9GL had relatively low virus titers in blood compared with those of the ASFV-G-inoculated animals (Fig. 3A, B, D, and E). Animals inoculated with 10^2 HAD_{50} of ASFV-G-Δ9GL showed very heterogeneous viremia titers. While 5 of the 10 inoculated animals showed undetectable virus titers in blood until 14 to 21 dpi, 2 of the 10 pigs showed intermediate virus titers (ranging from 10^3 to 10^4 HAD_{50}/ml), while 3 of 10 animals exhibited high virus titers in blood (ranging from 10^6 to 10^7 HAD_{50}/ml). In general, regardless of the observed virus titers, viremia tended to peak around 21 dpi (Fig. 3A and B). Similarly, 6 of 10 animals inoculated with 10^3 HAD_{50} of ASFV-G-Δ9GL presented high titers (ranging from 10^6 to10^7 HAD_{50}/ml), 2 of 10 presented intermediate titers (approximately 10^4 HAD_{50}/ml), and 3 of 10 presented low titers (ranging from 10^2 to 10^3 HAD_{50}/ml), while the remaining pig presented undetectable titers in blood until the time of challenge (Fig. 3D and E). Despite the observed heterogeneity of viremia titers among animals inoculated with ASFV-G-Δ9GL, it is interesting to notice the absence of clinical signs in infected animals, demonstrating a lack of correlation between the presence and severity of disease with virus titers in blood. It is clear that this lack of correlation between viremia titers and severity of disease is present in animals dying after being inoculated with 10^4 HAD_{50} of ASFV-G-Δ9GL as well as in animals inoculated with sublethal doses of ASFV-G-Δ9GL, which although presenting variable virus titers in blood are clinically normal.

Since viremia titers in animals infected with sublethal doses of ASFV-G-Δ9GL are rather heterogeneous, with most of the animals presenting medium to low virus titers in blood compared to animals inoculated with the wild-type virus, it is unlikely that these animals will shed virus. Virus shedding was assessed by virus isolation, using primary cell cultures of swine macrophages, from nasal swab samples obtained from pigs ($n = 4$) inoculated i.m. with either 10^2 or 10^3 HAD_{50}/ml of ASFV-G-Δ9GL (Fig. 4). Virus was not detected in the nasal cavities of inoculated pigs during the monitoring period, although viremia titers in these animals (Fig. 4) resemble those observed in other infected pigs (Fig. 3). Also, virus was not detected in blood or nasal swabs obtained from unninoculated sentinel pigs brought in contact with each group of inoculated animals. Interestingly, these contact pigs did not seroconvert as they were ASFV-specific antibody negative by 28 dpi (data not shown). Therefore, contact pigs show neither viremia nor ASFV-specific antibodies, suggesting that ASFV-G-Δ9GL is not being eliminated by exposed animals.

To rule out that the disease observed in the animals inoculated i.m. with 10^4 HAD_{50} of ASFV-G-Δ9GL was caused due to contamination of the inoculum with parental ASFV-G (previously shown to be undetectable by PCR in Fig. 1A), viruses isolated from blood at 7 dpi were tested by PCR using primers that target the p72 (B646L), *9GL* (B119L), and β-GUS genes. All four ASFV-G-Δ9GL viruses isolated from blood of inoculated animals tested negative for parental ASFV-G. The *9GL* (B119L) gene was not detected in these viruses, whereas amplification of the p72 (B646L) and β-GUS genes was recorded in all instances (Fig. 1B). Furthermore, sequencing was conducted on blood-isolated viruses to assess the integrity of the p72GUS cassette inserted by homologous recom-

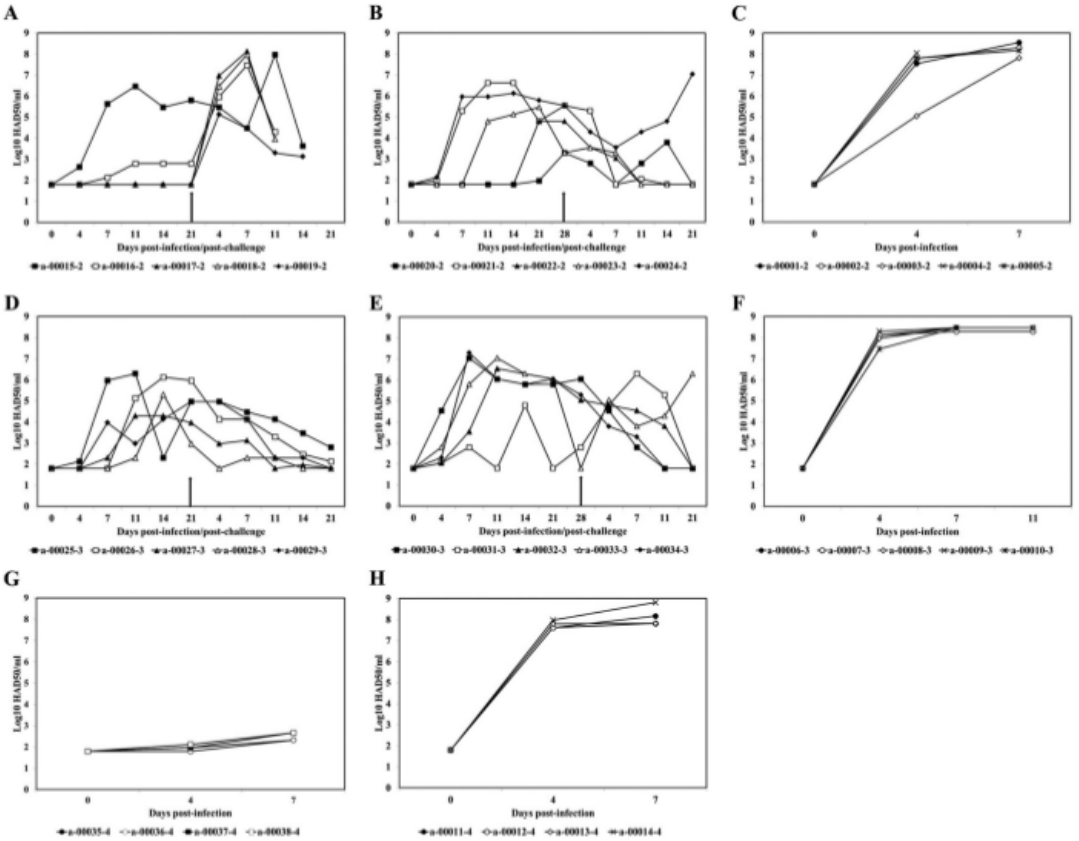

FIG 3 Virus titers in blood samples (e.g., a-00015-2), obtained from pigs that were infected with either 10^2 (A and B), 10^3 (D and E), or 10^4 (G) HAD_{50} ASFV-G-Δ9GL and challenged (time of challenge indicated by arrows) at either 21 dpi (A and D) or 28 dpi (B and E). Also shown are viremia titers in pigs infected with either 10^2 (C), 10^3 (F), or 10^4 (H) HAD_{50} ASFV-G. Values are expressed as $\log_{10}$ HAD_{50} per milliliter. The sensitivity of virus detection was $\geq\log_{10}$ 1.8 HAD_{50}/ml. Numbers with the "a-" prefix are animal identification numbers.

bination into ASFV-G. Obtained sequences revealed that p72GUS and both flanking regions were not modified in these viruses (data not shown). Since these data indicated the absence of contamination of the inoculum with parental ASFV-G, it is concluded that ASFV-G-Δ9GL virus inoculated at high doses (10^4 HAD_{50}) is able to induce a clinical disease indistinguishable from that induced by the parental virus.

The ASFV 9GL (B119L) gene is highly conserved. Sequence analysis of the *9GL* (B119L) genes from several ASFV isolates obtained from various temporal and geographic origins, including those from tick and pig sources, reveals a high degree of conservancy. Isolates compared include Malawi Lil-20/1 (1983), Crocodile/96/1 (1996), Crocodile/96/3 (1996), Pretoriuskop/96/5 (1996), Pretoriuskop/96/4 (1996), Fairfield/96/1, and Wildebeeslaagte/96/1 from ticks, domestic Georgia 2007/1 (2007), Killean 3, European-70 (1970), European-75 (1975), Kimakia (1964), Victoria Falls, La Granja (1963), Lisbon60 (1960), Spencer (1951), Tengani (1962), Zaire (1967), and Haiti 811 (1980) from domestic pigs, Uganda (1961) from a warthog, and Lee (1955) from a bush pig. Among these isolates, the amino acid identity for *9GL* (B119L) ranges between 93% and 100%. In the particular case of the Malawi Lil-20/1 or Pretoriuskop/96/4 isolates, identity with the Georgia 2007 isolate is 93%. Clearly, the *9GL* (B119L) gene is highly conserved, suggesting a common and conserved function for the gene across ASFV isolates. Therefore, evidence suggests that differences in virulence between Δ9GL Malawi Lil-20/1 and ASFV-G mutant viruses may be a multigenic effect involving viral genes other than the *9GL* (B119L) gene. Amino acid identities of translational products of predicted open reading frames (ORFs) of ASFV-G and Malawi Lil-20/1 genomes were compared using CLC Genomics Workbench (CLC Bio) and the Basic Local Alignment Search Tool (BLAST) (18). A total of 189 predicted ORFs were used for this analysis. It was observed that 102/189 of the predicted proteins encoded by these ORFs retain a high percentage of identity that is over 90%; 29/189 have identities ranging between 80% and 90%, 16/189 are 70% to 80% identical, 7/189 predicted proteins have identities that range between 60% and 70%, and 35/189 seem to be dis-

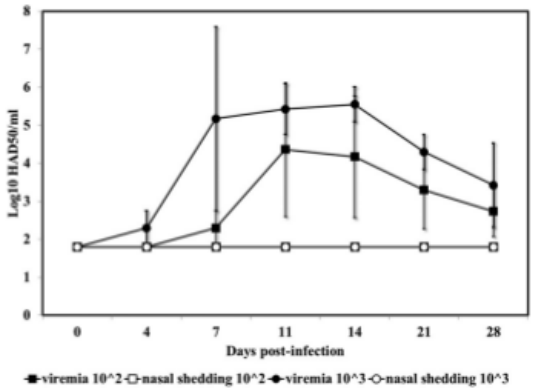

FIG 4 Viremia and virus shedding (nasal swabs) observed in animals ($n = 4$ per group) infected with either 10^2 or 10^3 HAD_{50} ASFV-G-Δ9GL. The average and standard deviation of values from each group along a 28-day observation period are presented. Values are expressed as $\log_{10}$ HAD_{50} per milliliter. The sensitivity of virus detection was $\geq \log_{10}$ 1.8 HAD_{50}/ml.

similar, with percentages of identity of less than 60%. Altogether the observed genotypic difference between Malawi Lil-20/1 and ASFV-G should account for the phenotypic differences observed between the derived Δ9GL mutants. In fact, this analysis supports a previous phylogenic analysis of known ASFV isolates in which Georgia 2007/1 and Malawi Lil-20/1 were found to be distantly related (3).

Animals inoculated with sublethal doses of ASFV-G-Δ9GL virus are protected against challenge with virulent parental virus. In order to assess induction of protection of the mutant virus against challenge with the virulent parental virus, animals inoculated i.m. with ASFV-G-Δ9GL (Table 3) were challenged with the parental virus ASFV-G. Groups 1 and 2, which received 10^2 HAD_{50} and 10^3 HAD_{50} of ASFV-G-Δ9GL, respectively, were challenged i.m. with 10^3 HAD_{50} of ASFV-G at 21 dpi. Groups 3 and 4, which received 10^2 HAD_{50} and 10^3 HAD_{50} of ASFV-G-Δ9GL, respectively, were challenged i.m. with 10^3 HAD_{50} of ASFV-G at 28 dpi. After challenge, animals were monitored daily for clinical signs and changes in body temperature. Five additional naive animals in a control group were challenged i.m. with 10^3 HAD_{50} of ASFV-G. In this control group, the onset of ASF-related signs was observed by 5 days postchallenge (dpc), evolving to a more severe disease in the following days, with all animals dying or being euthanized by 8 dpc.

Animals in group 1 (10^2 HAD_{50} ASFV-G-Δ9GL/challenge at 21 days) started showing clinical signs of the disease by 6 dpc. Progress toward a more severe clinical stage of the disease was observed in 3 pigs that died by 8 dpc, whereas the remaining two animals of the group displayed a milder form of the disease without a rise in body temperature. These animals survived the challenge with virulent ASFV-G during the entire observation period (21 days) (Table 3 and Fig. 5). All animals in group 3 (10^2 HAD_{50} ASFV-G-Δ9GL/challenge at 28 days) survived the infection with the parental virulent virus. In this group, four pigs remained clinically normal during the observational period, while the remaining pig presented a late onset of body temperature increase (14 dpc) that lasted until the end of the observational period (Table 3 and Fig. 5).

In group 2 (10^3 HAD_{50} ASFV-G-Δ9GL/challenge at 21 days), four pigs remained clinically normal during the observational period, while the remaining pig presented a transient increase in body temperature for 3 days starting by day 9 dpc without additional signs of ASF (Table 3 and Fig. 5). In a similar experiment performed previously under the same conditions described here, complete protection against ASF was observed in 4 of 5 animals (data not shown). Pigs in group 4 (10^3 HAD_{50} ASFV-G-Δ9GL/challenge at 28 days) survived challenge, remaining clinically normal throughout the observational period (Table 3 and Fig. 5). This experiment was repeated under exactly the same conditions in which 5 of 5 animals were completely protected against ASFV-G (data not shown). In summary, inoculation of pigs with sublethal doses of ASFV-G-Δ9GL effectively induced protection against clinical disease and death induced by challenging pigs with parental virulent ASFV-G. This phenomenon was even more pronounced when higher doses of ASFV-G-Δ9GL were used in the inoculation of pigs, and it was not fully observed before 28 dpi.

Viremias observed after challenge could be caused by the primary infection with ASFV-G-Δ9GL or represent the replicative activity of ASFV-G in challenged pigs. The presence of ASFV-G or ASFV-G-Δ9GL in blood samples taken after challenge was determined by conventional PCR (Table 4). In group 1 (10^2 HAD_{50} ASFV-G-Δ9GL/challenge at 21 days), a drastically increased

TABLE 3 Swine survival and fever response in animals infected with ASFV-G-Δ9GL after challenge with parental ASFV-G

Treatment by day challenged	No. of survivors/total	Mean (SD) time to death, days	Mean (SD) characteristic of fever		
			No. of days to onset	Duration, days	Maximum daily temp, °F
21 dpi					
10^2 HAD_{50} (group 1)	2/5	8.33 (0.58)[a]	6.4 (0.55)[a]	2.6 (0.55)[a]	106.2 (0.87)[a]
10^3 HAD_{50} (group 2)	5/5		9.0 (0)[b]	3 (0)[b]	105.8 (0)[b]
28 dpi					
10^2 HAD_{50} (group 3)	5/5		14 (0)[b]	7 (0)[b]	106.2 (0)[b]
10^3 HAD_{50} (group 4)	5/5				103.0 (0.17)
Control	0/5	8.2 (1.1)	5.20 (1.31)	3.0 (0.70)	106.5 (0.46)

[a] Values calculated considering only animals dying from the disease. The two surviving animals, although presenting ASF-related clinical signs, did not present body temperatures that were ≥ 104°F.
[b] Values calculated considering only the sick animals.

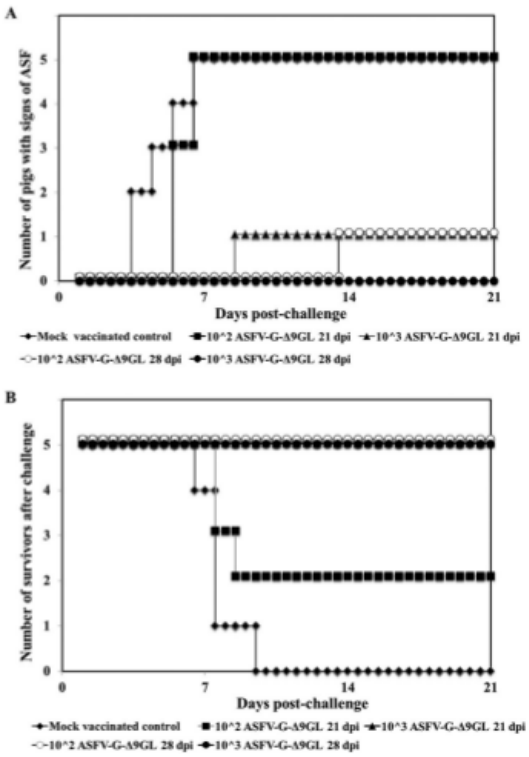

FIG 5 Morbidity (A) and mortality (B) observed in animals infected with either 10^2 or 10^3 HAD_{50} of ASFV-G-Δ9GL and challenged at either 21 or 28 dpi with the parental virulent ASFV-G virus.

viremia was observed in four of five pigs by 4 dpc (Fig. 3A). Three of these four animals developed severe clinical disease and died or were euthanized *in extremis*. One of these four animals became sick transitorily and survived challenge. The virus detected in the blood of these 4 animals at 4 dpc was parental ASFV-G, but it was not detected in the remaining surviving animal of the group. Animals in group 3 (10^2 HAD_{50} ASFV-G-Δ9GL/challenge at 28 days) presented postchallenge viremia titers similar to the virus titers observed at 0 dpc (Fig. 3B). ASFV-G was not detected in the blood of these animals at 4 dpc. A similar situation was observed with animals in group 2 (10^3 HAD_{50} ASFV-G-Δ9GL/challenge at 21 days) (Fig. 3D). Also animals in group 4 (10^3 HAD_{50} ASFV-G-Δ9GL/challenge at 28 days) showed the same general patterns of viremia after challenge observed in groups 2 and 3. In group 4, only one animal showed high virus titers in blood after challenge, and ASFV-G was the detected virus, while only ASFV-G-Δ9GL was detected in blood from the four remaining pigs in the group (Fig. 3D).

The immune response to ASFV-G-Δ9GL infection was evaluated in pigs at different time points until day 28 postinfection. Three groups of pigs were i.m. inoculated ($n = 4$) with either 10^2 or 10^3 HAD_{50} of ASFV-G-Δ9GL or mock infected, and their immune responses were evaluated by assessing the presence of ASFV-specific antibodies and the presence of ASFV-specific IFN-γ-producing cells in blood. Pigs in the groups inoculated with ASFV-G-Δ9GL developed a robust antibody response against ASFV (detected at 21 and 28 dpi) with no significant differences in the antibody titers between the two sampling time points (Fig. 6). Similarly, ASFV-specific IFN-γ-producing cells were detected in ASFV-G-Δ9GL-infected animals (with the sole exception of animal 296, who remained nonresponsive). IFN-γ-producing cells were detectable by day 14 postinfection, generally peaking by day 21 postinfection and decreasing toward day 28 postinfection. Control animals did not show ASFV-specific antibody or IFN-γ responses (Fig. 6). At 28 dpi, animals in all groups were i.m. challenged with 10^3 HAD_{50} of ASFV-G. The ASFV-G-Δ9GL-exposed groups each included an uninoculated contact pig. As expected, all animals in the control group as well as the contact pigs in the ASFV-G-Δ9GL-exposed groups became sick and died or were euthanized *in extremis* by days 8 to 9 postchallenge. Pigs in the 10^3-HAD_{50} ASFV-G-Δ9GL group survived challenge, although showing transient periods of fever (1 to 2 days). Similarly, 3/4 pigs in the 10^2 HAD_{50} ASFV-G-Δ9GL group survived challenge, showing transient periods of fever, with the exception of animal 296, who developed severe ASF (euthanized on day 10 postchallenge). Noticeable is the fact that this animal was the only one among all ASFV-G-Δ9GL-infected swine that did not develop ASFV-specific antibody or IFN-γ responses.

TABLE 4 Virus detected in blood, clinical signs, and outcome of disease in pigs exposed to ASFV-G-Δ9GL and challenged with parental ASFV-G

ASFV-G-Δ9GL dose/day of challenge for blood sample shown	Virus detected at 7 dpc[a]		Disease presentation and outcome	
	ASFV-G-Δ9GL	ASFV-G	Clinical signs	Survival
10^2 HAD_{50}/21 dpi				
a-00015-2[b]	+	−	Yes	Yes
a-00016-2	+	+	Yes	No
a-00017-2	+	+	Yes	No
a-00018-2	+	+	Yes	No
a-00019-2	+	+	Yes	Yes
10^2 HAD_{50}/28 dpi				
a-00020-2	+	−	No	Yes
a-00021-2	+	−	No	Yes
a-00022-2	+	−	No	Yes
a-00023-2	+	−	No	Yes
a-00024-2	+	−	Yes	Yes
10^3 HAD_{50}/21 dpi				
a-00025-3	+	−	No	Yes
a-00026-3	+	−	No	Yes
a-00027-3	+	−	No	Yes
a-00028-3	+	−	No	Yes
a-00029-3	+	−	No	Yes
10^3 HAD_{50}/28 dpi				
a-00030-3	+	−	No	Yes
a-00031-3	+	+	No	Yes
a-00032-3	+	−	No	Yes
a-00033-3	+	−	No	Yes
a-00034-3	+	−	No	Yes

[a] Virus detection by PCR: +, positive; −, negative.
[b] Numbers with the "a-" prefix are animal identification numbers.

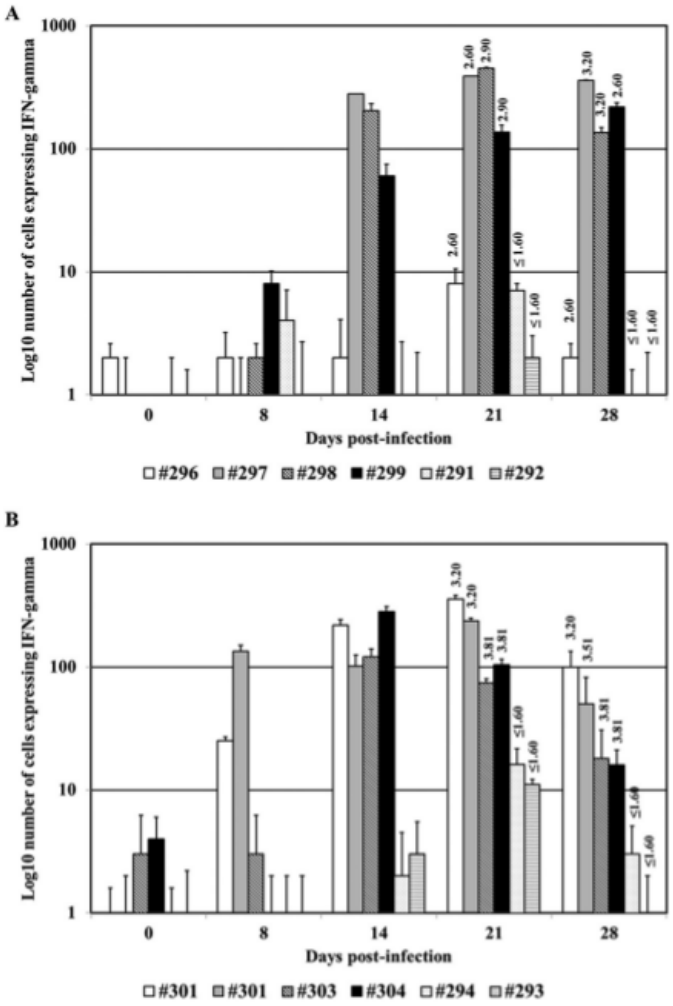

FIG 6 Assessment of ASFV-specific antibodies and IFN-γ-producing cell responses in pigs infected with either 10^2 (animals 296 to 299) or 10^3 (animals 301 to 304) HAD_{50} ASFV-G-Δ9GL or mock infected (animals 291 to 294). Bars represent the IFN-γ-producing cells in each of the animals at different times postinfection (expressed as number of cells producing IFN-γ per 5×10^5 cells). Anti-ASFV antibody titers are shown on the top of the bars (titers measured at 21 or 28 dpi) and are expressed as the $\log_{10}$ value of the inverse of the highest serum dilution still recognizing ASFV-infected cells (as described in Materials and Methods).

DISCUSSION

No vaccines are available to prevent ASFV infection. Only live attenuated virus strains have been useful in protecting pigs against challenge with homologous virulent isolates. These attenuated viruses have been regularly produced by sequential passages in cell cultures and, more recently, by genetic manipulation. Attenuated viruses obtained by genetic manipulation involve the deletion of specific genes by a process of homologous recombination. Independent deletion of four different genes from ASFV has been shown to attenuate virulent viruses. Independent deletions of the *NL* (DP71L) (11) or the *UK* (DP69R) (19) genes from ASFV E75, deletion of the *TK* (A240L) gene (9) from ASFV adapted to Vero cells, Malawi Lil-20/1, and Haiti, and deletion of the *9GL* (B119L) gene also from Malawi Lil-20/1 (10) and Pretoriuskop/96/4 (12) isolates rendered recombinant deletion mutant viruses with significantly reduced virulence in swine. In all of these cases, animals inoculated with each of these genetically modified viruses survived the infection and became protected against ASFV when challenged with the corresponding virulent parental virus (homologous challenge) (9–12, 19). Those findings suggest that develop-

ment of attenuated ASFV recombinant viruses by genetic manipulations of target genes is an effective approach for vaccine development.

The *NL* (DP71L) gene product exists in two different forms: a long form (184 amino acids) and a short form (70 to 72 amino acids), depending on the ASFV isolate (11). Although deletion of this gene in the ASFV E70 isolate (short form) rendered an attenuated virus, the deletion of the *NL* (DP71L) gene from ASFV Malawi Lil-20/1 (long form) or Pretoriuskop/96/4 (short form) did not result in attenuation of the virus (20). A deletion of the *TK* (A240L) gene, a highly conserved gene among all ASFV isolates that is involved in DNA synthesis, has been introduced into the genome of the pathogenic Vero cell-adapted Malawi Lil-20/1 and Haiti H811 viruses. The Malawi Lil-20/1 mutant virus was less virulent *in vivo* than a revertant virus (wild-type-like virus), but it was not completely attenuated in swine (9). The *UK* (DP69R) gene is located in the right variable region of certain ASFV isolates. Deletion of this gene from the ASFV E70 isolate rendered a virus exhibiting reduced virulence (19). Although the *UK* (DP69R) gene is conserved, it is not present in every ASFV isolate (e.g., Malawi Lil-20/1), limiting its use as a candidate target gene for producing attenuated viruses.

The *9GL* (B119L) gene is highly conserved among the ASFV isolates sequenced thus far, including those from both tick and pig sources. The fact that deletion of the gene from virulent Malawi Lil-20/1 (10) or Pretoriuskop/96/4 (12) effectively reduced virulence in swine and induced protection makes *9GL* (B119L) a candidate target gene for modification to produce an attenuated virus that can confer effective protection against ASFV. Interestingly, here we observed that deletion of *9GL* (B119L) from the ASFV-G isolate does not have the same effect in terms of attenuation reported for Malawi Lil-20/1 or Pretoriuskop/96/4. Only when ASFV-G-Δ9GL was administered at a low dose to swine was it possible to observe a significant reduction in virus virulence. Data presented here indicate that the *9GL* (B119L) gene is not absolutely required for ASFV-G virulence, suggesting that other virulence factors may be involved in the process. As observed with deletions of *NL* (DP71L) in the E70, Malawi Lil-20/1, and Pretoriuskop/96/4 isolates that lead to different phenotypes (11, 20), deletions of *9GL* (B119L) have produced similar outcomes, suggesting that virulence of ASFV is the result of a multigene effect.

The NL proteins encoded by E70 (short form) and Malawi Lil-20/1 (long form) differ significantly, and that may explain the phenotypic differences observed in swine inoculated with the respective deletion mutant viruses. However, protein identity matrices indicate that the 9GL protein is highly similar among ASFV isolates, where ASFV-G, Malawi Lil-20/1, and Pretoriuskop/96/4 share over 93% amino acid identity, making it unlikely that ASFV attenuation relies solely on protein divergence. Since the observed phenotypes are most likely mediated by the effect of multiple genes (9–12, 19), the evidence accumulated so far makes it difficult to speculate what is indeed the spectrum of genes mediating virulence in the ASFV Georgia 2007 isolate. It is possible that the number or function of additional virulence-associated genes among different ASFV strains may alter the intrinsic effect of the *9GL* (B119L) gene on the general balance of the virulence in a particular virus strain. Therefore, it remains to be determined why the deletion of *9GL* (B119L), a gene that has been associated with virus virulence in Malawi Lil-20/1 and Pretoriuskop/96/4 isolates, does not drastically alter virulence of ASFV-G. Sublethal doses of ASFV-G-Δ9GL were effective at inducing protection against challenge with the virulent parental isolate.

The protection induced by ASFV-G-Δ9GL delivered either at 10^2 or 10^3 HAD_{50}/ml was more effective when animals were challenged at 28 dpi, suggesting the need for some degree of maturation of the host immune mechanism(s) mediating protection against ASFV. The observed ASFV-specific antibody levels in ASFV-G-Δ9GL-exposed pigs were not significantly different at 21 or 28 dpi, whereas circulating ASFV-dependent IFN-γ-producing cells appear to peak at 21 dpi and decrease toward 28 dpi. Thus, there is no evident association between the measured immune parameters at 28 dpi and the observed protection against ASFV-G challenge. Challenging at the same time naive sentinel pigs commingling with ASFV-G-Δ9GL-exposed pigs resulted in less effective protection against ASFV-G. Clearly under these conditions, cohabitation of ASFV-G-Δ9GL-inoculated pigs with contact naive animals that developed ASF while shedding large amounts of ASFV-G resulted in highly stringent challenge. This was evidenced by the fact that some of the ASFV-G-Δ9GL-inoculated pigs developed transient fever or even ASF (i.e., pig 296). Interestingly, this particular pig failed to develop an IFN-γ response against ASFV. This observation is in agreement with previous reports that indirectly support the role of the T-cell response in the protection against ASFV (21–23). The immune mechanisms mediating protection against ASF are still not well understood. The results presented here show no substantial differences in the antibody or T-cell responses observed in pigs at 21 or 28 dpi (with the exception of the T-cell response in pig 296). It is possible that under the experimental conditions tested here, still unidentified immune mechanisms mediating protection against ASFV in ASFV-G-Δ9GL-infected animals evolve or mature after a certain period of time. In fact, we have observed a similar progressive acquisition of immunity to challenge with parental homologous virus until 28 dpi in animals infected with ASFV-Pret4-Δ9GL (unpublished data).

Our findings hinder the possibility of using the deletion of the *9GL* (B119L) gene as the sole target for developing a live attenuated vaccine candidate against the ASFV-G isolate. The results shown here, demonstrating that sublethal doses of ASFV-G-Δ9GL did induce protection against ASF, open the possibility for using the ASFV-G-Δ9GL genome as platform for incorporating additional genetic modifications that could lead to a safer and useful vaccine candidate against ASF.

In summary, we present evidence of the differential attenuation effect of the deletion of the ASFV *9GL* gene in the ASFV Georgia isolate. We have shown complete protection of domestic pigs against challenge with the highly virulent, epidemiologically relevant, ASFV Georgia isolate.

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Deletion of the thymidine kinase gene induces complete attenuation of the Georgia isolate of African swine fever virus

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ABSTRACT

African swine fever virus (ASFV) is the etiological agent of a contagious and often lethal viral disease of domestic pigs. There are no vaccines to control Africa swine fever (ASF). Experimental vaccines have been developed using genetically modified live attenuated ASFVs obtained by specifically deleting virus genes involved in virulence, including the thymidine kinase (TK) gene. TK has been shown to be involved in the virulence of several viruses, including ASFV. Here we report the construction of a recombinant virus (ASFV-G/V-ΔTK) obtained by deleting the TK gene in a virulent strain of ASFV Georgia adapted to replicate in Vero cells (ASFV-G/VP30). ASFV-G/P-ΔTK demonstrated decreased replication both in primary swine macrophage cell cultures and in Vero cells compared with ASFV-G/VP30. *In vivo*, intramuscular administration of up to 10^6 $TCID_{50}$ of ASFV-G/V-ΔTK does not result in ASF disease. However, these animals are not protected when challenged with the virulent parental Georgia strain.

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1. Introduction

African Swine Fever Virus (ASFV), a large enveloped virus with a double-stranded (ds) DNA genome of approximately 180–190 kilobase pairs (Costard et al., 2008), causes a spectrum of disease (from highly lethal to sub-clinical) depending on the virus strain (Tulman et al., 2009). Virulent ASFV infections in domestic pigs are fatal and characterized by fever, hemorrhages, ataxia and severe depression.

The current epidemiological situation of ASF (endemic in several sub-Saharan African countries and in Sardinia) is particularly important for outbreaks recorded in the Caucasus region since 2007 (affecting Georgia, Armenia, Azerbaijan and Russia and more recently in Ukraine, Belarus, Lithuania, Latvia and Poland), threatening to disseminate into neighboring Western European countries (Chapman et al., 2011).

There is no vaccine available for ASF and the disease is controlled by quarantine and elimination of affected animals. Protective immunity does develop in pigs surviving viral infection against reinfection with homologous viruses (Hamdy and Dardiri, 1984; Ruiz-Gonzalvo et al., 1981). Pigs immunized with live attenuated ASF viruses developed by deleting specific ASFV virulence-associated genes (*UK*, *23-NL*, *TK*, *9GL* or *MGFs*) were protected when challenged with homologous virulent parental virus (Lewis et al., 2000; Moore et al., 1998; Zsak et al., 1996,1998; O'Donnell et al., 2015a,b). These reports are the only experimental evidence describing the rational development of an effective live attenuated virus against ASFV.

ASFV thymidine kinase (TK), a viral enzyme involved in synthesis of deoxynucleoside triphosphates (Martin Hernandez and Tabares, 1991) has been shown to not be essential for virus replication in cell cultures (Martin Hernandez et al., 1995; Rodriguez et al., 1992) but recombinant ASFV having the TK gene removed could not replicate in swine macrophages (Moore et al., 1998). Deletion of the TK gene both in herpesviruses and poxviruses showed the gene is not essential for replication in cultured cells (Dubbs and Kit, 1964; Panicali and Paoletti, 1982), but was associated with a reduction in virus virulence (Buller et al., 1985; Field and Wildy, 1978; Kochneva et al., 1994). Similarly, deletion of the TK gene from the ASFV Malawi isolate results in significant virus attenuation (Moore et al., 1998).

Here we report the development of a recombinant virus (ASFV-G/V-ΔTK) engineered by deleting the TK gene from the genome of a virulent ASFV Georgia isolate that has been adapted to replicate in Vero cells (ASFV-G/VP30) while still causing disease in domestic pigs inoculated with the virus. Compared with the parental virus,

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ASFV-G/P-ΔTK demonstrated decreased replication efficiency both in primary swine macrophage cell cultures and in Vero cells. ASFV-G/V-ΔTK is completely attenuated when administered IM to swine at a dose as high as 10^6 $TCID_{50}$ although, interestingly, these, animals are not protected when challenged with the virulent parental Georgia strain.

2. Materials and methods

2.1. Cell cultures and viruses

ASFV Georgia (ASFV-G) was a field isolate kindly provided by Dr. Nino Vepkhvadze, from the Laboratory of the Ministry of Agriculture (LMA) in Tbilisi, Republic of Georgia. ASFV-G/VP30 was developed by serial passages in Vero cells as described by Krug et al. (2015).

Growth kinetics was assessed either in Vero cells or in primary swine macrophage cell cultures. Vero cells were obtained from ATCC and sub-cultured in DMEM with 10% FC while primary swine macrophage cell cultures were, prepared as described by Zsak et al. (1996). In either case, preformed monolayers were prepared in 24-well plates and infected at a MOI of 0.1. After 1 hour of adsorption at 37 °C under 5% CO_2 the inoculum was removed and the cells were rinsed two times with PBS. The monolayers were then rinsed with media and incubated for 2, 24, 48, 72 and 96 h at 37 °C under 5% CO_2. At appropriate times post-infection, the cells were frozen at ≤−70 °C and the thawed lysates were used to determine titers by $HAID_{50}$/ml in primary swine macrophage cell cultures or $TCID_{50}$/ml in Vero cell cultures. All samples were run simultaneously to avoid inter-assay variability. Virus titration was performed on primary swine macrophage or Vero cell cultures in 96-well plates. Presence of virus was assessed by hemadsorption (HA) or immunocytochemistry (using an anti-ASFV p30 monoclonal antibody produced in APHIS, PIADC); virus titers were calculated by the Reed and Muench method (1938).

2.2. Construction of recombinant ASFV-G/VP-ΔTK

Recombinant ASFVs were generated by homologous recombination between the parental ASFV genome and a recombination transfer vector following infection and transfection of swine macrophage cell cultures (Zsak et al., 1996). The recombinant transfer vector (p72GUSΔTK) contained flanking genomic regions, which included the left arm, located between genomic positions 63,236 to 64,282, and the right arm, located between genomic positions 64,602 to 65,674 and a reporter gene cassette containing the β-glucuronidase (GUS) gene with the ASFV p72 late gene promoter, p72GUS (Zsak et al., 1998). This construction created a 312 nucleotide deletion within the TK gene (K196R) between nucleotide positions 64,289 to 64,601 (Fig. 1). Recombinant transfer vector p72GUSΔTK was obtained by DNA synthesis (Epoch Life Sciences, Sugar Land, TX, USA). Vero cell cultures were infected with ASFV-G/VP30 and transfected with p72GUSΔTK. Recombinant viruses representing independent primary plaques were purified to homogeneity by successive rounds of plaque assay purification.

2.3. Polymerase chain reaction (PCR)

Purity of ASFV-G/VP-ΔTK in the virus stock was assessed by PCR. Detection of the TK genes was performed using the following pair of primers: forward 5′ CACTCCGGTATACAGCTACG 3′; reverse 5′ GGCCAATATACTTAACCC AGC 3′. Detection of the β-Gus gene was performed using the following pair of primers: forward 5′ GCCGATTATCATCACCGAATAC3′; reverse 5′TGCGCCA GGAGAGTTGTTGATTC3′. Presence of ASFV DNA was detected using the following pair of primers: forward 5′CGTAGAGCTGTTGTTGTCAT 3′; reverse 5′CAGAGAGAATACAGCTG TAGGTCT3′, which detect the presence of the MGF505 3R gene.

2.4. Sequencing of PCR products

PCR products were sequenced using the dideoxynucleotide chain-termination method (Sanger et al., 1977). Sequencing reactions were prepared with the Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Reaction products were sequenced on a PRISM 3730xl automated DNA sequencer (Applied Biosystems). Sequence data were assembled with the Phrap software program (http://www.phrap.org), with confirmatory assemblies performed using CAP3 (Huan and Madan, 1999). The final DNA consensus sequence represented an average five-fold redundancy at each base position. Sequence comparisons were conducted using BioEdit software (Tom Hall, Ibis Biosciences, Carlsbad, CA, Copyright 1997–2013).

2.5. Next generation sequencing (NGS) of ASFV genomes

ASFV DNA was extracted from infected cells and quantified as described earlier (Krug et al., 2015). Full-length sequencing of the virus genome was performed as described elsewhere (Krug et al., 2015). Briefly, one microgram of virus DNA was enzymatically sheared and the resulting fragmented DNA size distribution was assessed. Adapters and library barcodes were ligated to the fragmented DNA. The appropriate size range of the adapter-ligated library was collected using the Pippin Prep™ system (Sage Science) followed by normalization of library concentration. The DNA library was then clonally amplified onto ISPs and enriched. Enriched template ISPs were prepared and loaded onto Ion chips for sequencing. Sequence analysis was performed using Galaxy (https://usegalaxy.org/) and CLC Genomics Workbench (CLCBio).

2.6. Animal experiments

Animal experiments were performed under biosafety level 3 conditions in the animal facilities at PIADC following a protocol approved by the Institutional Animal Care and Use Committee.

ASFV-G/VP-ΔTK was assessed for its virulence phenotype relative to the parental ASFV-G/VP30 virus using 80–90 pound commercial breed swine. Five pigs were inoculated intramuscularly (IM) either with 10^4 $TCID_{50}$ of ASFV-G/VP-ΔTK or ASFV-G/VP30 (additional experiments were performed using 10^6 $TCID_{50}$ of ASFV-G/P-ΔTK). Clinical signs (anorexia, depression, fever, purple skin discoloration, staggering gait, diarrhea and cough) and changes in body temperature were recorded daily throughout the experiment.

To assess the protective effect of ASFV-G/VP-ΔTK, ASFV-G/P-ΔTK-infected animals were IM challenged with 10^3 HAD_{50} of highly virulent parental ASFV-G at 28 days post-infection. Clinical signs (as described above) and changes in body temperature were recorded daily throughout the experiment.

2.7. Detection of anti-ASFV antibodies

Anti-ASFV antibodies in sera of infected animals were quantified using an in-house immunohistochemistry assay. Vero cells were infected (MOI = 0.1) with ASFV-G/VP30 (Krug et al., 2015) in 96 well plates. Two-fold dilutions of the sera were incubated for 1 hour at 37 °C in the 96-well ASFV-infected cell monolayer. After washing, the presence of anti-ASFV antibodies was detected by using a commercial anti-swine peroxidase labeled mouse immunoglobulin and a peroxidase substrate (Vector Laboratories, CA). Titers were

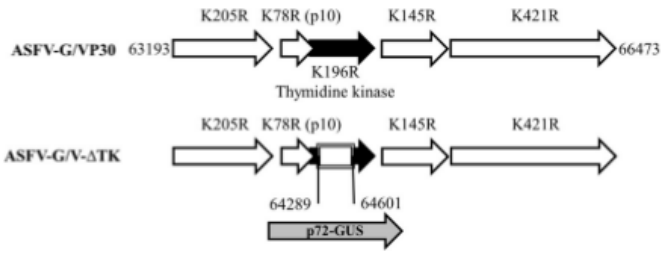

Fig. 1. Schematic representation of the thymidine kinase (TK) gene (*K196R*) region in ASFV-G/VP30 showing adjacent open reading frames. A 312 bp nucleotide deletion was introduced between positions 64289 to 64601 within the ORF of the *K196R* gene by inserting a p72-GUS reporter cassette using homologous recombination. This insertion resulted in the production of a TK-recombinant virus named ASFV-G/V-ΔTK.

expressed as the $\log_2$ of the inverse of the highest serum dilution showing reaction with the infected cells.

3. Results

3.1. Development of the ASFV-G/P-ΔTK deletion mutant

ASFV-G/P-ΔTK was constructed using ASFV-G/VP30 as the parental strain, a derivative of ASFV Georgia 2010 (Krug et al., 2015). ASFV-G/VP30, although adapted to replicate in Vero cells after 30 successive passages, is still virulent when intramuscularly (IM) inoculated into swine at a dose of 10^4 $TCID_{50}$ (Krug et al., 2015). From the 591-bp TK gene, a 312-bp region was deleted (between nucleotide positions 64,289 and 64,601) from ASFV-G/VP30 and replaced with a cassette containing the p72GUS reporter gene (p72GUSΔTK) by homologous recombination (see Section 2 and Fig. 1). The recombinant virus was obtained after 12 successive plaque purification events on monolayers of Vero cell cultures. The virus population obtained from the last round of plaque purification was amplified in Vero cell cultures to obtain a virus stock. To ensure the absence of parental ASFV-G, virus DNA was extracted from the virus stock and analyzed by PCR using primers targeting the MGF505 3R, β-Gus and TK genes. Only amplicons for the MGF- and β-Gus genes were detected in DNA extracted from the virus stock, whereas no amplicons were generated with primers targeting the TK gene (Fig. 2), indicating the lack of contamination of the ASFV-G/VP-ΔTK stock with ASFV-G/VP30.

3.2. Analysis of the ASFV-G/P-ΔTK genome sequence relative to parental ASFV-G/VP30 genome sequence

To evaluate the accuracy of the genetic modification and the integrity of the genome of the recombinant virus, the full genome sequence of ASFV-G/VP-ΔTK was obtained using NGS on the Ion Torrent PGM™ and compared to that of the parental ASFV-G/VP30 (Krug et al., 2015). A full-length genome comparison between parental ASFV-G/VP-ΔTK and ASFV-G/VP30 demonstrated no significant differences with the exception of the 312-bp deletion (between nucleotide positions 64,289 and 64,601) of the central area of the TK gene (K196R), which has been replaced by the p72GUSΔTK cassette. In addition, the location (nucleotide positions 178,631 to 183,475) and length (4,845 bp) of the gap present in the genome of the ASFV-G/VP30 (when compared with its parental virus ASFV-G) was also present in the genome of ASFV-G/VP30 (Krug et al., 2015). Besides the insertion of the cassette, no additional differences were observed between ASFV-G/VP-ΔTK and ASFV-G/VP30 genomes. Therefore, ASFV-G/VP-ΔTK virus did not accumulate any significant mutations during the process of homologous recombination and plaque purification.

3.3. Comparative replication of ASFV-G/VP-ΔTK and ASFV-G/VP30

In vitro growth characteristics of ASFV-G/VP-ΔTK was first evaluated in Vero cell cultures and compared to parental ASFV-G/VP30 in a multistep growth curve. Cell cultures were infected with either virus at a MOI of 0.01 and samples were collected at 2, 24, 48, 72 and 96 h post-infection (hpi). ASFV-G/VP-ΔTK displayed a decreased growth kinetic when compared with that of the parental ASFV-G/VP30 (Fig. 3A). ASFV-G/VP-ΔTK yields were 10–100 times lower than those of ASFV-G/VP30 regarding the time point considered.

In addition, the ability of ASFV-G/VP-ΔTK to replicate in primary swine macrophage cell cultures, the primary cell targeted by ASFV during infection in swine, was compared relative to the parental ASFV-G/VP30. Growth analysis was performed under similar conditions as described when Vero were used (MOI = 0.01, with sampling times at 2, 24, 48, 72 and 96 hpi). Results demonstrated (Fig. 3B) that ASFV-G/VP-ΔTK displayed a decreased growth kinetic when compared with that of the parental ASFV-G/VP30 (Fig. 3B), showing yields 100–1000 times lower than those of ASFV-G/VP30 regarding the time point considered. Therefore, deletion of the TK gene causes a clear disadvantage in virus replication regardless of the cell type considered.

3.4. Assessment of ASFV-G/VP-ΔTK virulence in swine

In order to evaluate the effect of the deletion of the TK gene in ASFV-G/VP-ΔTK virulence, 80–90 pound pigs (n = 5) were IM inoculated with 10^4 $TCID_{50}$ of either ASFV-G/VP-ΔTK or the parental ASFV-G/VP30. As previously described (Krug et al., 2015), animals infected with 10^4 $TCID_{50}$ of ASFV-G/VP30 exhibited increased body temperature (>104 °F) by 6–8 days post-infection followed by the appearance of clinical signs associated with the disease including anorexia, depression, purple skin discoloration, staggering gait and diarrhea. Signs of the disease aggravated progressively over time and animals either died or were euthanized *in extremis* by days 8–9 post-infection (Table 1). Conversely, animals infected with ASFV-G/VP-ΔTK did not present any signs of clinical disease during the entire observation period (21 days). An additional experiment was then performed where animals with the same characteristics ($n = 5$) were IM inoculated with a higher dose (10^6 $TCID_{50}$) of ASFV-G/VP-ΔTK. This group of animals also remained clinically normal throughout the 21-day observation period (Table 1). Therefore, partial deletion of the TK gene completely attenuated the virulent ASFV-G/VP30.

Viremia in experimentally inoculated animals was quantified at different days post-infection in swine macrophage cell cultures. As expected, animals inoculated with 10^4 $TCID_{50}$ of virulent parental ASFV-G/VP30 had very high virus titers in blood with viremia titers reaching values as high as 10^7 to 10^8 $TCID_{50}$/ml by the time of death

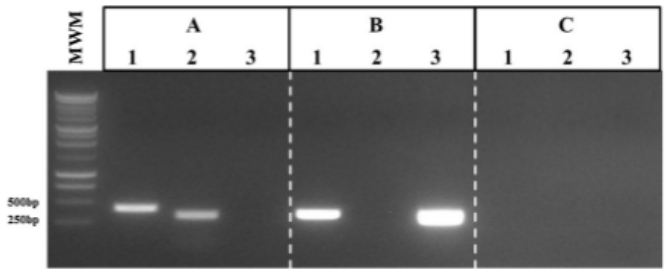

Fig. 2. PCR analysis of ASFV-G/VP-ΔTK virus stock DNA using specific primers targeting MGF505 3R (lane 1), βGus (lane 2), or TK (lane 3) genes. DNA from ASFV-G/VP-ΔTK (A) or parental ASFV-G/VP30 (B) was assessed along with non-DNA template control (C) samples. MWM: molecular weight marker.

Table 1
Swine survival and fever response following infection with ASFV-G/VP-ΔTK or parental ASFV-G/VP30.

Virus	No. of survivors/total	Mean time to death (days ± SD)	Fever		
			No. of days to onset (days ± SD)	Duration No. of days (days ± SD)	Maximum daily temp (°F ± SD)
ASFV-G/VP30 10^4 TCID	0/4	8.75 (0.96)	7.2 (0.84)	2 (0.71)	106.4 (0.67)
ASFV-G/VP-ΔTK 10^4 TCID	4/4	–	–	–	102.7 (0.19)
ASFV-G/VP-ΔTK 10^6 TCID	4/4	–	–	–	103.7 (0.4)

(–) Animals did not die or they did not present fever.

(Fig. 4). Conversely, animals inoculated with 10^4 or 10^6 $TCID_{50}$ of mutant ASFV-G/VP-ΔTK had almost undetectable virus titers in blood throughout the experimental period. Therefore, although ASFV-G/VP-ΔTK is able to replicate in swine macrophage cell cultures, it appears that it is not efficient in replicating in animals since no viremia titers could be detected.

3.5. Animals inoculated with ASFV-G/VP-ΔTK are not protected against challenge with virulent parental virus

In order to assess the effect of inoculation with ASFV-G/VP-ΔTK on the induction of protection against challenge with the virulent parental virus, those animals surviving ASFV-G/VP-ΔTK infection were IM challenged with 10^3 HAD_{50} of ASFV-G. Both groups, animals inoculated with either 10^4 or 10^6 $TCID_{50}$ of ASFV-G/VP-ΔTK, were challenged at 28 dpi with 10^3 HAD_{50} of virulent parental ASFV-G. Animals were monitored daily for clinical signs and body temperature.

Two naive animals that were challenged using the same route and dose served as the control group. These animals started displaying ASF-related signs by 5 days post-challenge (dpc), evolving to a more severe disease in the following days and all animals dying or being euthanized around 8 dpc. Similarly, all animals receiving either 10^4 or 10^6 $TCID_{50}$ of ASFV-G/VP-ΔTK developed a disease indistinguishable in severity and kinetics to that observed in the mock vaccinated control animals (data not shown). Therefore, infection with ASFV-G/VP-ΔTK does not induce protective immunity against challenge with the virulent parental ASFV-G. In accordance with the lack of protection all animals infected with either 10^4 or 10^6 $TCID_{50}$ of ASFV-G/VP-ΔTK lack any detectable ASFV-specific antibodies at the time of challenge (28 dpi) detected using an in-house immunohistochemistry assay or ELISA (data not shown).

4. Discussion

There are no commercial vaccines to prevent ASF. Experimental protection against challenge with virulent ASFV isolates has been partially achieved by immunizing swine with experimental subunit vaccines, as expressed proteins or DNA vaccines (Lacasta et al., 2014; Argilaguet et al., 2012,2013; Barderas et al., 2001), and in a more effective way by pre-infecting pigs with homologous live attenuated virus strains. Attenuated ASFVs have been developed by either adaptation to grow in different cell substrates (Enjuanes et al., 1976; Pires et al., 1997; Ruiz Gonzalvo et al., 1986; Tabares et al., 1987; Krug et al., 2015) or by genetic manipulation, deleting specific genes by homologous recombination (Zsak et al., 1996; Zsak et al., 1998; Moore et al., 1998; Lewis et al., 2000; Neilan et al., 2004; O'Donnell et al., 2015a,2015b). These recombinant deletion mutant viruses have significantly reduced virulence in swine and animals inoculated with each of these genetically modified viruses survived the infection when challenged with the virulent parental virus (Lewis et al., 2000; Moore et al., 1998; Neilan et al., 2004; Zsak et al., 1996,1998; O'Donnell et al., 2015a,2015b). Therefore, development of attenuated ASFV recombinant viruses by genetic manipulation of specific genes appears to be an effective approach for vaccine development.

Among these viral genes is the one encoding for the enzyme thymidine kinase (TK), involved in synthesis of deoxynucleoside triphosphates (Martin Hernandez and Tabares, 1991). The ASFV TK gene has been shown to not be essential for virus growth in cell cultures (Martin Hernandez et al., 1995; Rodriguez et al., 1992) but recombinant ASFV having the TK gene removed could not replicate in swine macrophages (Moore et al., 1998).

As described, ASFV-G/VP-ΔTK possesses a reduced ability to replicate when compared with its parental virus. This disadvantage was evidenced when evaluated either in Vero cells or primary cultures of swine macrophages. Similarly, recombinant ASFV-ΔTK produced in strains Malawi and Haiti (which were also partially adapted to replicate in Vero cells) also exhibited yields 100 to 1000 time lower than the parental viruses in swine primary macrophages (Moore et al., 1998).

In swine, these ΔTK strains, as it is reported here for ASFV-G/VP-ΔTK, presented an attenuated phenotype producing a very mild form of the disease (Moore et al., 1998). In fact, based on the reported data, ASFV-G/VP-ΔTK appears to present a more attenu-

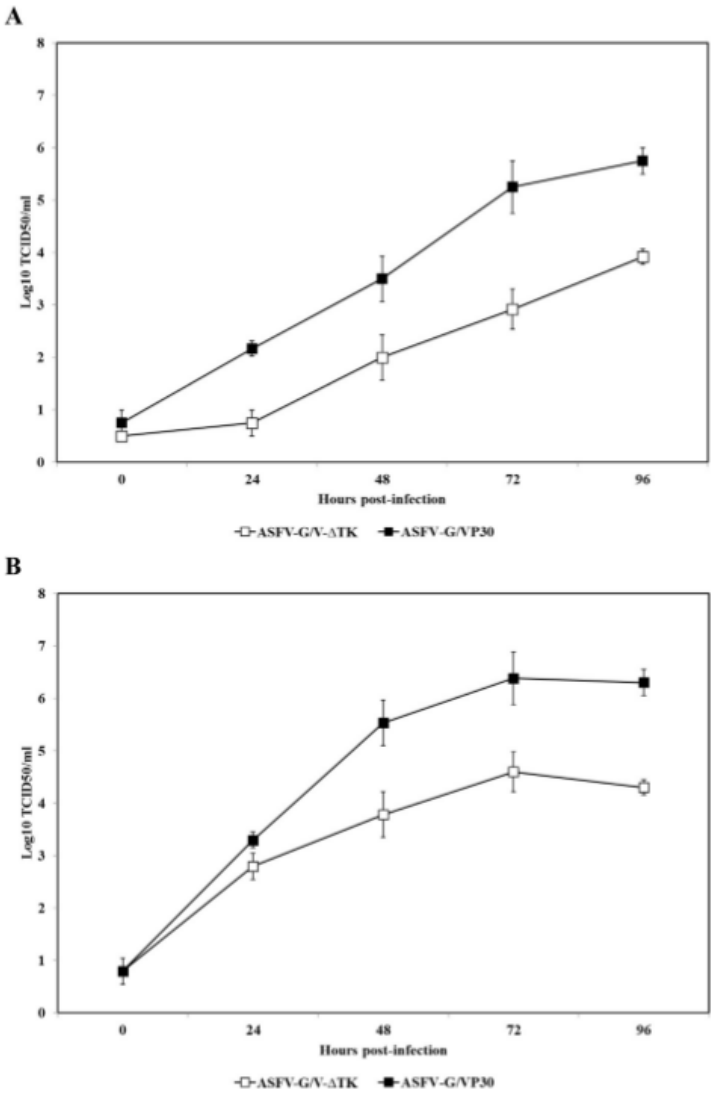

Fig. 3. *In vitro* growth kinetics of ASFV-G/VP-ΔTK or parental ASFV-G/VP30. Vero cell line (A) or primary swine macrophage cell (B) cultures were infected (MOI = 0.01) with either ASFV-G/VP-ΔTK or ASFV-G/VP30 viruses. Virus yields obtained at the indicated times post-infection were titrated in Vero cell cultures. Data represent means and standard deviations from two independent experiments. Sensitivity of virus detection: ≥1.8 $TCID_{50}$/ml.

ated phenotype than Malawi or Haiti ΔTK strains since no presence of clinical signs were detected in ASFV-G/VP-ΔTK-infected animals even when they were inoculated with a dose of 10^6 $TCID_{50}$, while some of the animals infected with the Malawi-ΔTK virus did not survive the infection or presented a transient fever (Moore et al., 1998). Accordingly, levels of viremia in animals infected with ASFV-G/VP-ΔTK were almost undetectable while levels above 10^4 $TCID_{50}$ were achieved in animals infected with Malawi-ΔTK virus (Moore et al., 1998). Importantly, while animals surviving the Malawi-ΔTK infection were protected against challenge with parental virulent virus (Moore et al., 1998), none of those infected with ASFV-G/VP-ΔTK survived challenge with ASFV-G.

Possible differences between the pathogenesis and protective effect of infection with ASFV-G/VP-ΔTK and Malawi-ΔTK viruses may be due to the different genetic background of the strains used as parental virus in each of the cases. Comparing the full length genomic sequences of the Vero-adapted parental viruses and corresponding non-Vero-adapted field isolates showed interesting differences. While no notable differences were found between the field isolate Malawi LiL-20/1 and its Vero-adapted version, Malawi LiL-20/1V (data not shown), significant differences

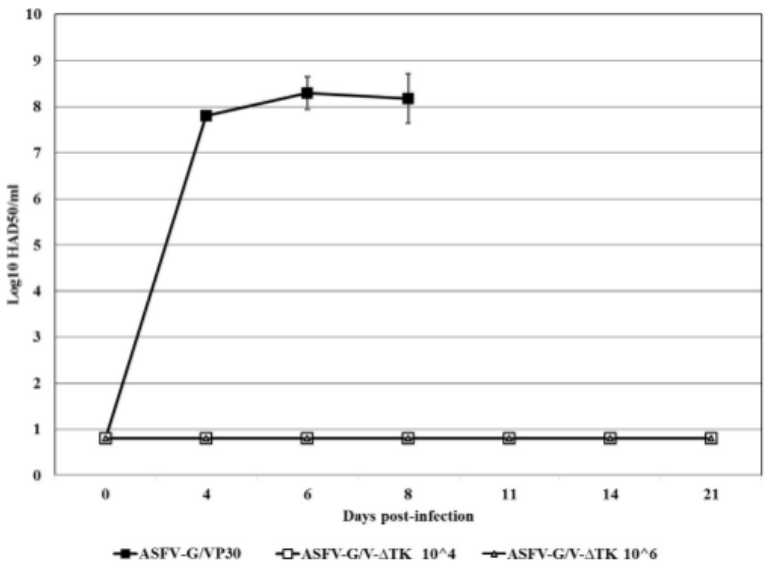

Fig. 4. Virus titers in clinical blood samples from pigs infected with either 10^4 (A) or 10^6 (B) $TCID_{50}$ of ASFV-G/VP-ΔTK compared with those from pigs infected with parental ASFV-G/VP30. Values are expressed as log_{10} HAD_{50}/ml. Sensitivity of virus detection: ≥log_{10} 0.8 HAD_{50}/ml.

were observed between ASFV-G and its Vero adapted derivative ASFV-G/VP30 (Krug et al., 2015). These observations may indicate that Malawi LiL-20/1V is less divergent from its parental virus than ASFV-G/VP30 is from ASFV-G, supporting the concept that Malawi LiL-20/1 V is less attenuated than ASFV-G/VP30. Therefore, it could be expected that their corresponding ΔTK derivatives will behave similarly to the corresponding parental viruses, with ASFV-G/VP-ΔTK being completely attenuated but also replicating less aggressively *in vivo*, failing to mount an effective immune response in the infected animal as the Malawi-ΔTK does (Moore et al., 1998).

The study of the role of ASFV genes using single gene deletion virus mutants has demonstrated certain heterogeneity in regards to the function of a similar gene in the context of different virus isolates. Thus, deletion of the NL gene has been shown to attenuate virulent isolate E75 (Zsak et al., 1996) but does not alter virulence of isolate Malawi (Afonso et al., 1998). In addition, deletion of 9GL is very effective in attenuating virulence of Malawi isolate, even at doses of 10^6 HAD_{50} (Lewis et al., 2000), while does not abolish virulence in isolate Gerogia2007 when administered at just 10^4 HA_{50} (O'Donnell et al., 2015b). In fact, most of the studies involving deletion of a particular gene have been carried out using only one virus isolate opening the question if a gene would play exactly the same role in different virus isolates. This is very relevant issue considering that different ASFV isolates may present a wide phenotypic variability particularly in terms of their virulence in swine. This issue is also important since it may affect the development of live attenuated vaccines, particularly when you consider the existing limitation in the inability to induce effective cross-protection between different ASFV isolates. This causes the need to develop a homologous attenuated strain to each of the virulent field isolates.

In summary, we report here the first evidence that deletion of the thymidine kinase gene is able to completely attenuate a virulent ASFV isolate although animals infected with the attenuated strain do not develop a protective immune response when challenged with the virulent parental ASFV.

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African swine fever virus Georgia isolate harboring deletions of 9GL and MGF360/505 genes is highly attenuated in swine but does not confer protection against parental virus challenge

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ABSTRACT

African swine fever virus (ASFV) produces a contagious disease of domestic pigs that results in severe economic consequences to the swine industry. Control of the disease has been hampered by the unavailability of vaccines. We recently reported the development of two experimental vaccine strains (ASFV-G-Δ9GL and ASFV-G-ΔMGF) based on the attenuation of the highly virulent and epidemiologically relevant Georgia2007 isolate. Deletion of the 9GL gene or six genes of the MGF360/505 group produced two attenuated ASFV strains which were able to confer protection to animals when challenged with the virulent parental virus. Both viruses, although efficient in inducing protection, present concerns regarding their safety. In an attempt to solve this problem we developed a novel virus strain, ASFV-G-Δ9GL/ΔMGF, based on the deletion of all genes deleted in ASFV-G-Δ9GL and ASFV-G-ΔMGF. ASFV-G-Δ9GL/ΔMGF is the first derivative of a highly virulent ASFV field strain subjected to a double round of recombination events seeking to sequentially delete specific genes. ASFV-G-Δ9GL/ΔMGF showed a decreased ability to replicate in primary swine macrophage cultures relative to that of ASFV-G and ASFV-G-ΔMGF but similar to that of ASFV-G-Δ9GL. ASFV-G-Δ9GL/ΔMGF was attenuated when intramuscularly inoculated into swine, even at doses as high as 10^6 HAD_{50}. Animals infected with doses ranging from 10^2 to 10^6 HAD_{50} did not present detectable levels of virus in blood at any time post-infection and they did not develop detectable levels of anti-ASFV antibodies. Importantly, ASFV-G-Δ9GL/ΔMGF does not induce protection against challenge with the virulent parental ASFV-G isolate. Results presented here suggest caution towards approaches involving genomic manipulations when developing rationally designed ASFV vaccine strains.

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1. Introduction

African Swine Fever (ASF) is a highly lethal contagious viral disease of swine caused by ASF virus (ASFV), a large enveloped virus containing a double-stranded DNA genome of approximately 180–190 kilobase pairs (Costard et al., 2008). ASF causes a spectrum of disease, from highly lethal to sub-clinical, depending on host characteristics and the virus strain (Tulman et al., 2009). Virulent ASFV infections in domestic pigs are fatal and characterized by fever, hemorrhages, ataxia and severe depression.

Currently, ASF is endemic in several sub-Saharan African countries. In Europe, the disease is endemic in Sardinia (Italy) and outbreaks have been recorded in the Caucasus region since 2007, affecting Georgia, Armenia, Azerbaijan and Russia and more recently in Ukraine, Belarus, Lithuania, Latvia, Estonia and Poland, threatening to disseminate into neighbouring West European countries (Chapman et al., 2011).

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There is no vaccine available for ASF and outbreaks are usually controlled by animal quarantine and elimination of affected animals. Experimental vaccines based on the use of different inactivated virus preparations have failed to induce protective immunity (Coggins, 1974; Kihm et al., 1987; Mebus, 1988). Protective immunity does develop in pigs surviving viral infection against reinfection with homologous, although rarely to heterologous, viruses (Hamdy and Dardiri, 1984; Ruiz Gonzalvo et al., 1986). Pigs immunized with live attenuated ASF viruses containing engineered individual deletions of ASFV virulence-associated genes (*UK*, *DP69R*, *23-NL*, *TK* or *9GL* genes) were protected when challenged with homologous virulent parental virus (Moore et al., 1998; Lewis et al., 2000; Zsak et al., 1996, 1998). Thus far, these studies constitute the only experimental evidence describing the rational development of an effective live attenuated virus against ASFV.

Following this rationale, we recently reported the development of two experimental vaccine strains (ASFV-G-Δ9GL and ASFV-G-ΔMGF) based on the deletion of either the 9GL gene or six genes of MGF360/505 (O'Donnell et al., 2015a,b). Both vaccine strains protected animals when challenged with the parental virus, the highly virulent Georgia2007 isolate. However, both viruses may present deficiencies regarding their safety. The ASFV-G-Δ9GL protective dose is dangerously close to its lethal dose (O'Donnell et al., 2015b) while the genomic area modified in ASFV-G-ΔMGF is the target of active recombination events in ASFV (Krug et al., 2015; de la Vega et al., 1990; Pieres et al., 1997; Tabares et al., 1987; Yanez et al., 1995), creating potential genetic instability. In order to develop a safer vaccine strain we decided to develop a novel virus where all genetic modifications present in ASFV-G-Δ9GL and ASFV-G-ΔMGF were included in one strain, ASFV-G-Δ9GL/ΔMGF. ASFV-G-Δ9GL/ΔMGF showed a decreased ability to replicate in primary swine macrophage cultures relative to that of ASFV-G and ASFV-G-ΔMGF and was drastically attenuated when intramuscularly inoculated in swine, even at doses as high as 10^6 HAD_{50}. Animals infected with doses ranging from 10^2 to 10^6 HAD_{50} presented no detectable viremias or anti-ASFV antibodies. Accordingly, ASFV-G-Δ9GL/ΔMGF does not induce protection against challenge with virulent parental ASFV-G. Results presented here suggest caution towards approaches involving multiple genomic manipulations when developing rationally designed ASFV vaccine strains.

2. Materials and methods

2.1. Cell cultures and viruses

Primary swine macrophage cell cultures were prepared from defibrinated swine blood as previously described by Zsak et al. (1996). Briefly, heparin-treated swine blood was incubated at 37 °C for 1 h to allow sedimentation of the erythrocyte fraction. Mononuclear leukocytes were separated by flotation over a Ficoll-Paque (Pharmacia, Piscataway, N.J.) density gradient (specific gravity, 1.079). The monocyte/macrophage cell fraction was cultured in plastic Primaria (Falcon; Becton Dickinson Labware, Franklin Lakes, N.J.) tissue culture flasks containing macrophage media, composed of RPMI 1640 Medium (Life Technologies, Grand Island, NY) with 30% L929 supernatant and 20% fetal bovine serum (HI-FBS, Thermo Scientific, Waltham, MA) for 48 h at 37 °C under 5% CO_2. Adherent cells were detached from the plastic by using 10 mM EDTA in phosphate buffered saline (PBS) and were then reseeded into Primaria T25, 6- or 96-well dishes at a density of 5×10^6 cells per ml for use in assays 24 h later.

ASFV Georgia (ASFV-G) was a field isolate kindly provided by Dr. Nino Vepkhvadze, from the Laboratory of the Ministry of Agriculture (LMA) in Tbilisi, Republic of Georgia (Krug et al., 2015). ASFV-G-Δ9GL has been previously developed and reported (O'Donnell et al., 2015b). Basically, ASFV-G-Δ9GL was developed by deleting the 9GL gene from ASFV-G by homologous recombination, replacing it with the β-glucuronidase (β-GUS) gene with the ASFV p72 late gene promoter (O'Donnell et al., 2015b).

Comparative growth curves between ASFV-G, ASFV-G-Δ9GL, ASFV-G-ΔMGF and ASFV-G-Δ9GL/ΔMGF viruses were performed in primary swine macrophage cell cultures. Preformed monolayers were prepared in 24-well plates and infected at a MOI of 0.01 (based on HAD_{50} previously determined in primary swine macrophage cell cultures). After 1 h of adsorption at 37 °C under 5% CO_2 the inoculum was removed and the cells were rinsed two times with PBS. The monolayers were then rinsed with macrophage media and incubated for 2, 24, 48, 72 and 96 h at 37 °C under 5% CO_2. At appropriate times post-infection, the cells were frozen at ≤−70 °C and the thawed lysates were used to determine titers by HAD_{50}/ml in primary swine macrophage cell cultures. All samples were run simultaneously to avoid inter-assay variability.

Virus titration was performed on primary swine macrophage cell cultures in 96-well plates. Virus dilutions and cultures were performed using macrophage medium. Presence of virus was assessed by hemadsorption (HA) and virus titers were calculated by the Reed and Muench method (1938).

Construction of the recombinant ASFV-G-Δ9GL/ΔMGF

Recombinant ASFV-G-Δ9GL/ΔMGF was generated by homologous recombination between the parental ASFV-G-Δ9GL genome and a recombination transfer vector following infection and transfection of swine macrophage cell cultures (Zsak et al., 1996). The recombinant transfer vector (p72GFPΔMGF) contained flanking genomic regions, which included the amino terminus of the MGF505 1R gene mapping to the left (left arm is located between genomic positions 24566–27928) and the carboxyl end of the MGF505 3R gene to the right (right arm is located between genomic positions 35487–36515) and a reporter gene cassette containing the green fluorescent protein (GFP) gene with the ASFV p72 late gene promoter (Zsak et al., 1996). This construction created a 7558-nucleotide deletion in the left variable region of ASFV-G (between nucleotide positions 27928–35481) (Fig. 1). Recombinant transfer vector p72GFPΔMGF was obtained by DNA synthesis (Epoch Life Sciences, Sugar Land, TX, USA). Macrophage cell cultures were infected with ASFV-G and transfected with p72mGFPΔMGF. Recombinant viruses representing independent primary foci were purified to homogeneity by successive rounds of limiting dilution purification.

2.2. Polymerase chain reaction (PCR)

Purity of ASFV-G-Δ9GL/ΔMGF in the virus stock was assessed by PCR. Detection of the MGF350/MGF505 genes (as presence of parental genome DNA) was performed using the following pair of primers: forward 5′GAGGATGATTTGCCCTTCACTCA3′; reverse 5′CGCCACTAGTAAA CATTGTTCTATCT3′. These primers amplified a 422 bp fragment of the ORF MGF505-1R gene. Detection of the β-Gus gene (as control of DNA availability), amplifying a 471 bp fragment, was performed using the following pair of primers: forward 5′ GCCGATTATCATCACCGAATAC3′; reverse 5′TGCGCCAGGAGAGTTG TTGATTC3′.

2.3. Next generation sequencing (NGS) of ASFV genomes

ASFV DNA was extracted from infected cells and quantified as described earlier (Krug et al., 2015). Full-length sequencing of the virus genome was performed as described elsewhere (Krug et al., 2015). Briefly, one microgram of virus DNA was enzymatically sheared and the resulting fragmented DNA size distribution was assessed. Adapters and library barcodes were ligated to the

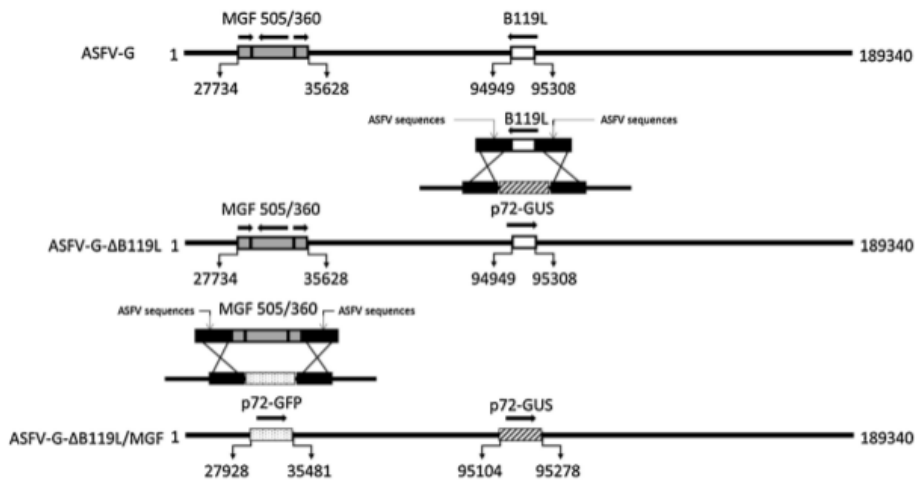

Fig. 1. Schematic representation of the 9GL (*B119L*) and MGF360/MGF505 gene regions deleted in ASFV-G-Δ9GL/ΔMGF. Nucleotide positions indicating the boundaries of the deletion relative to the ASFV-G genome are indicated.

fragmented DNA. The appropriate size range of the adapter-ligated library was collected using the Pippin Prep™ system (Sage Science) followed by normalization of library concentration. The DNA library was then clonally amplified onto ISPs and enriched. Enriched template ISPs were prepared and loaded onto Ion chips for sequencing using an Ion Torrent PGM™ instrument. Sequence analysis was performed using Galaxy (https://usegalaxy.org/) and CLC Genomics Workbench (CLCBio).

2.4. Animal experiments

Animal experiments were performed under biosafety level 3 conditions in the animal facilities at PIADC following a protocol approved by the Institutional Animal Care and Use Committee.

ASFV-G-Δ9GL/ΔMGF was assessed for its virulence phenotype relative to the parental ASFV-G virus using 80–90 pound commercial breed swine. Five pigs were inoculated intramuscularly (IM) either with 10^2, 10^4 or 10^6 HAD_{50} of ASFV-G-Δ9GL/ΔMGF or 10^4 HAD_{50} of ASFV-G. Clinical signs (anorexia, depression, fever, purple skin discoloration, staggering gait, diarrhea and cough) and changes in body temperature were recorded daily throughout the experiment.

To assess the protective effect of the different doses of ASFV-G-Δ9GL/ΔMGF at 28 days post-infection, ASFV-G-Δ9GL/ΔMGF-infected animals were IM challenged with 10^3 HAD_{50} of highly virulent parental ASFV-G. Clinical signs (as described above) and changes in body temperature were recorded daily throughout the experiment.

2.5. Detection of anti-ASFV antibodies

Anti-ASFV antibodies in sera of infected animals were quantified using an in-house developed ELISA. Antigen preparation and ELISA procedure was based on the method of Katz et al. (2012) with minor adjustments. Briefly, Vero cells were infected with an ASFV-G strain adapted to replicate in Vero cells (Krug et al., 2015) until cytopathic effect reached 100%. The infected cells were resuspended in water containing protease inhibitor (Roche, New York, NY), followed by the addition of Tween 80 (G-Biosciences, Saint Louis, MO) and sodium deoxycholate (Sigma, Saint Louis, MO) to a final concentration of 1% (v/v). Uninfected Vero cells were treated in the same manner and these antigens were stored at −70 °C. Maxisorb ELISA plates (Nunc, Saint Louis, MO) were coated with 1 μg per well of either infected cell or uninfected cell antigen. The plates were blocked with phosphate buffered saline containing 10% skim milk (Merck, Kenilworth, NJ) and 5% normal goat serum (Sigma). Each swine serum was tested at multiple dilutions against both infected and uninfected cell antigen. ASFV-specific antibodies in the swine sera were detected by an anti-swine IgG-horseradish peroxidase conjugate (KPL, Gaithersburg, MD) and SureBlue Reserve peroxidase substrate (KPL). Plates were read at OD630 in an ELx808 plate reader (BioTek, Shoreline, WA). Swine sera were considered positive for ASFV-specific antibodies if the OD630 ratio of the reaction against infected cell antigen to uninfected cell antigen was higher than 2.2.

3. Results and discussion

3.1. Development of the ASFV-G-Δ9GL/ΔMGF deletion mutant

We hypothesized that a recombinant virus, ASFV-G-Δ9GL/ΔMGF, harboring all gene deletions individually present in ASFV-G-Δ9GL and ASFV-G-ΔMGF would potentially present a more attenuated phenotype. ASFV-G-Δ9GL/ΔMGF was generated by homologous recombination between the recombinant ASFV-G-Δ9GL, previously developed by O'Donnell et al. (2015b), and recombination transfer vector p72mGFPΔMGF by infection and transfection procedures using swine macrophage cell cultures (Zsak et al., 1996; O'Donnell et al., 2015a,b). ASFV-G-Δ9GL has replaced its native 9GL gene with the ASFV p72 late gene promoter/β-glucuronidase (GUS) gene cassette (O'Donnell et al.,2015b). Therefore, ASFV-G-Δ9GL was used as parental virus in infection/transfection procedures performed on macrophage cell cultures using recombinant transfer vector p72mGFPΔMGF as previously described (see Material and Methods). The resulting recombinant virus, besides the deletion of the 9GL gene, harbors a deletion encompassing the carboxyl terminal half of the MGF505-1R gene, the complete deletion of MGF360-12L, 13L, 14L, and MGF505-2R genes along with the deletion of the amino terminal half of the MGF505-3R gene (O'Donnell et al.,2015a)

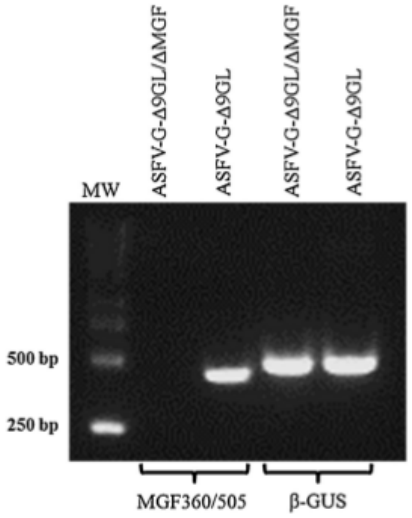

Fig. 2. PCR analysis of ASFV-G-Δ9GL/ΔMGF stock using specific primers targeting MGF360/MGF505 or β-GUS genes. Viral DNA from ASFV-G-Δ9GL/ΔMGF or ASFV-G-Δ9GL were tested for the presence of MGF360/MGF505 and β-GUS gene.

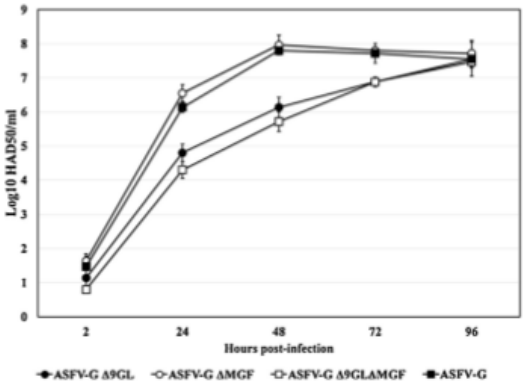

Fig. 3. *in vitro* growth kinetics of ASFV-G-Δ9GL/ΔMGF, ASFV-G-Δ9GL, ASFV-G-ΔMGF, and parental ASFV-G. Primary swine macrophage cell cultures were infected (MOI 0.01) with virus and virus yield, obtained at the indicated times pi, were titrated in primary swine macrophage cell cultures. Data represent means and standard deviations from two independent experiments. Sensitivity of virus detection: ≥ $\log_{10}$ 1.8 HAD_{50}/ml.

(Fig. 1). The recombinant virus was obtained after eight successive limiting dilution purification events on primary swine macrophage cell cultures using GFP as the fluorescent selectable marker. The virus population obtained from the last round of purification was amplified in primary swine macrophage cell cultures to obtain a virus stock.

To ensure the absence of parental ASFV-G-Δ9 G in the ASFV-G-Δ9GL/ΔMGF stock preparation, virus DNA was extracted from the virus stock and analyzed by PCR using primers targeting genes MGF and β-Gus. While amplicons for β-Gus genes were detected in DNA extracted from the virus stock, no amplicons were generated with primers targeting the MGF505-1R gene (Fig. 2), indicating the lack of contamination of the ASFV-G-Δ9GL/ΔMGF stock with parental ASFV-G-Δ9GL.

Analysis of the ASFV-G-Δ9GL/ΔMGF genome sequence relative to parental ASFV-G-Δ9GL genome sequence

To evaluate the accuracy of the genetic modification and the integrity of the genome of the newly developed recombinant virus, full genome sequences of ASFV-G-Δ9GL/ΔMGF and parental ASFV-G-Δ9GL were obtained using NGS on the Ion Torrent PGM™ and compared (Krug et al., 2015). The full-length genome comparison between ASFV-G-Δ9GL/ΔMGF and parental ASFV-G-Δ9GL revealed a deletion of 7558 nucleotides corresponding with the introduced modification in the MGF360/505 area (Fig. 1). Additionally, the consensus sequence of the ASFV-G-Δ9GL/ΔMGF genome showed an insertion of 1256 nucleotides corresponding to the p72mGFP cassette sequence (Fig. 1). Besides the insertion of the p72mGFP ΔMGF cassette, no additional differences were observed between the ASFV-G-Δ9GL/ΔMGF and parental ASFV-G-Δ9GL genomes, confirming ASFV-G-Δ9GL/ΔMGF did not accumulate undesired mutations during the process of homologous recombination and plaque purification. It is interesting to note that successive passages of ASFV in swine macrophages cell cultures is often accompanied by genome modifications as we previously observed with several recombinant viruses produced under a process that included in some cases more than 15 passages.

3.2. Replication of ASFV-G-Δ9GL/ΔMGF in primary swine macrophages

While viruses lacking the 9GL gene have been described as having a decreased ability to replicate in swine macrophages, viruses lacking the MGF genes located in the left variable region of the ASFV genome are still able to replicate in swine macrophages (Zsak et al., 2001; Krug et al., 2015; O'Donnell et al., 2015b). Therefore, we analyzed the *in vitro* growth characteristics of ASFV-G-Δ9GL/ΔMGF in swine macrophages, the primary cell targeted by ASFV during infection in swine, in comparison to those of ASFV-G-Δ9GL, ASFV-G-ΔMGF and field isolate ASFV-G (Krug et al., 2015; O'Donnell et al., 2015a,b). Macrophage cell cultures were infected with one of these viruses at a MOI of 0.01 and samples were collected at 2, 24, 48, 72 and 96 h post-infection (hpi). As expected, ASFV-G-ΔMGF presented growth kinetics indistinguishable from ASFV-G (O'Donnell et al., 2015a); however, both ASFV-G-Δ9GL/ΔMGF and ASFV-G-Δ9GL displayed slower kinetics. Although all four viruses reached similar titers by the end of the experiment, ASFV-G-Δ9GL/ΔMGF and ASFV-G-Δ9GL yields were 10–100 times lower than those of ASFV-G-ΔMGF and ASFV-G virus (Fig. 3) at some of the intermediate sampling time points. Therefore, as it happens with ASFV-G-ΔMGF (O'Donnell et al., 2015a), deletion of these particular six MGF360/MGF505 genes does not significantly affect the ability of the mutated virus to replicate in primary swine macrophage cultures when compared with its corresponding parental virus (ASFV-G for ASFV-G-ΔMGF and ASFV-G-Δ9GL for ASFV-G-Δ9GL/ΔMGF).

3.3. Assessment of ASFV-G-Δ9GL/ΔMGF virulence in swine

ASFV-G-Δ9GL virus has been shown to be attenuated when intramuscularly (IM) inoculated in swine at doses of 10^2 or 10^3 HAD_{50} while animals inoculated with 10^4 HAD_{50} developed clinical disease similar to that observed in animals IM inoculated with 10^4 HAD_{50} of parental ASFV-G, the only difference being a slight delay in the onset of fever (O'Donnell et al., 2015b). In order to evaluate the additive effect of the deletion of MGF360/MGF505 genes to the ASFV-G-Δ9GL genome, ASFV-G-Δ9GL/ΔMGF was IM inoculated into three different groups (n = 5) of 80–90 pound pigs at doses of 10^2, 10^4 or 10^6 HAD_{50} and compared with a group of

Table 1
Swine survival and fever response following infection with different doses of ASFV-G-Δ9GL/ΔMGF.

Virus and Dose(ASFV-G-)	No. of survivors/total	Mean time to death days (±SD)	Fever		
			No. of days to onset (±SD)	Duration, No. of days (±SD)	Maximum daily temp, °F (±SD)
Δ9GL/ΔMGF 10^2 HAD	5/5	–	–	–	102.9 (0.79)
Δ9GL/ΔMGF 10^4 HAD	5/5	–	–	–	103.1 (0.68)
Δ9GL/ΔMGF 10^6 HAD	5/5	–	–	–	102.7 (0.47)
Parental 10^4 HAD	0/5	7 (0.71)	3.6 (0.55)	3.4 (1.1)	105.9 (0.62)

(–) Animals did not die or they did not present with fever.

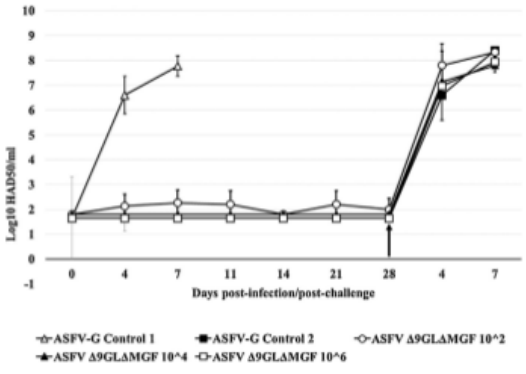

Fig. 4. Virus titers in blood samples obtained from pigs infected IM with either 10^2, 10^4 or 10^6 HAD_{50} of ASFV-G-Δ9GL/ΔMGF and challenged at 28 days pi (arrow) with 10^3 HAD_{50} of ASFV-G. Control 1 and Control 2 represent animals inoculated with 10^3 HAD_{50} of ASFV-G at day 0 post-infection or challenge, respectively. Values are expressed as $\log_{10}$ HAD_{50}/ml. Sensitivity of virus detection: $\geq \log_{10}$ 1.8 HAD_{50}/ml.

animals inoculated with 10^3 HAD_{50} of ASFV-G. As expected, animals infected with 10^3 HAD_{50} of ASFV-G exhibited increased body temperature (>104 °F) by 3–4 days post-infection followed by the appearance of clinical signs associated with the disease including anorexia, depression, purple skin discoloration, staggering gait and diarrhea (Table 1). Signs of the disease aggravated progressively over time and animals either died or were euthanized *in extremis* by around 7 days post-infection (pi). Animals infected with 10^2, 10^4 or 10^6 HAD_{50} of ASFV-G-Δ9GL/ΔMGF did not present any signs of clinical disease associated with ASF during the entire observation period (21 days) (Table 1). Therefore, deletion of MGF360-12L, 13L, 14L and MGF505-1R, 2R, 3R genes (ΔMGF construct) significantly increased attenuation of ASFV-G-Δ9GL since at doses that ASFV-G-Δ9GL became virulent (10^4 or 10^6 HAD_{50}) (O'Donnell et al., 2015b), ASFV-G-Δ9GL/ΔMGF is completely attenuated.

Viremia in experimentally inoculated animals was quantified at different days pi in swine macrophage cell cultures. As expected, animals inoculated with 10^3 HAD_{50} of virulent parental ASFV-G had very high virus titers in blood (as high as 10^7–10^8 HAD_{50}/ml) until the day of their death (Fig. 4). Conversely, animals inoculated with 10^2, 10^4 or 10^6 HAD_{50} of mutant ASFV-G-Δ9GL/ΔMGF had either very low or undetectable (test sensitivity is $\geq 10^{1.8}$ HAD_{50}/ml) virus titers in blood (Fig. 4). Therefore, although deletion of the MGF360/505 genes in the ASFV-G-Δ9GL genome does not affect the ability of ASFV-G-Δ9GL/ΔMGF to replicate in swine macrophage cultures, it certainly provokes a decreased replication during the infection in swine.

Presence and magnitude of viremias in animals infected with either ASFV-G-Δ9GL or ASFV-G-ΔMGF is quite heterogeneous, ranging from undetectable, particularly when low doses are used, to high titers (10^5–10^7 HAD_{50}/ml) (O'Donnell et al., 2015a,b). In addition, no correlation was established between level of viremia and presence of any ASFV-related clinical signs, since all inoculated animals remained clinically normal regardless of their viremia values. Interestingly, all ASFV-G-Δ9GL/ΔMGF-infected animals presented almost undetectable viremias regardless if their virus yields in swine macrophage cultures were not drastically different from those of ASFV-G-Δ9GL or ASFV-G-ΔMGF, suggesting that either our swine macrophage cultures are not an accurate representation of the macrophage population in vivo or, there are other unknown host immune factors besides the availability of cell types susceptible to infection that govern the kinetics of virus replication during the infection in the animal.

Infection with ASFV-G-Δ9GL/ΔMGF does not provide protection against challenge with virulent ASFV-G

In order to assess the effect of inoculation with ASFV-G-Δ9GL/ΔMGF on the induction of protection against disease, ASFV-G-Δ9GL/ΔMGF exposed animals were challenged with parental virulent ASFV-G. Animals previously infected IM with 10^2, 10^4 or 10^6 HAD_{50} of ASFV-G-Δ9GL/ΔMGF were IM challenged at 28 dpi with 10^3 HAD_{50} of virulent parental ASFV-G. Animals were monitored daily for clinical signs and changes in body temperature. Five naïve animals that were challenged with parental ASFV-G using the same route and dose served as a control group. These animals inoculated with ASFV-G displayed ASF-related signs by 3–4 days post-challenge (dpc), evolving into a more severe disease in the following days and all animals dying or being euthanized around 6–7 dpc (Table 2). All animals receiving 10^2, 10^4 or 10^6 HAD_{50} of ASFV-G-Δ9GL/ΔMGF succumbed to the challenge with the parental virulent virus. Kinetics of appearance of clinical signs, including rise of the body temperature, were very similar to those of the control animals challenged with ASFV-G and no significant differences were found in the length of the disease or time in which animals were euthanized between animals previously infected with ASFV-G-Δ9GL/ΔMGF and those just challenged with virulent parental ASFV-G (Table 2).

The presentation of ASF clinical signs, replication kinetics and magnitude of the viremia titers in the ASFV-G-Δ9GL/ΔMGF-infected animals after the challenge with virulent ASFV-G did not differ from that of the challenged control animals (Fig. 4). In general, absence of virus titers in blood upon challenge is a good indicator of the protective potency of the immunity elicited by a vaccine virus. In our experience, this is not always the case with ASFV and, particularly, with animals infected with ASFV-G-Δ9GL or ASFV-G-ΔMGF which were protected against the disease/lethality produced by challenge with virulent ASFV-G (O'Donnell et al., 2015a,b). In the particular case of ASFV-G-ΔMGF-infected animals protected against the challenge, 30–40% of those showed presence of challenge virus in blood (O'Donnell et al., 2015b).

Presence of anti-ASFV antibodies in serum of animals inoculated with ASFV-G-Δ9GL/ΔMGF was detected at the time of challenge using an in-house developed direct ELISA (see Materials and Methods). While animals inoculated with ASFV-G-Δ9GL or ASFV-G-ΔMGF presented at the time of challenge an average (n = 8)

Table 2
Swine survival and fever response of ASFV-G-Δ9GL/ΔMGF-infected animals challenged with parental ASFV-G.

Virus and Dose(ASFV-G-)	No. of survivors/total	Mean time to death, days (±SD)	Fever		
			No. of days to onset (±SD)	Duration, No. of days (±SD)	Maximum daily temp, °F (±SD)
Δ9GL/ΔMGF 10^2 HAD	0/5	7 (0)	4.4 (1.14)	2.6 (1. 14)	105.5 (1.69)
Δ9GL/ΔMGF 10^4 HAD	0/5	6.8 (0.45)	3.2 (0.45)	3.6 (0.9)	106.4 (0.58)
Δ9GL/ΔMGF 10^6 HAD	0/5	7 (0)	3.2 (0.45)	3.8 (0.54)	106.1 (0.45)
Mock infected	0/5	6.6 (0.55)	3.8 (0.45)	2.8 (0.84)	105.7 (0.55)

All animals were IM challenged with 10^3 HAD of parental ASFV-G.

antibody titer of $\log_{10}$ 2.1 (SD ± 0.32) and $\log_{10}$ 2.1 (SD ± 0.57), respectively, none of the ASFV-G-Δ9GL/ΔMGF-infected animals had detectable levels of antibodies at 28 days pi. As expected, induction of virus-specific antibodies is directly related to the presence of virus replication in the ASFV-G-Δ9GL/ΔMGF-infected animals. In this regard, it should be mentioned that all ASFV-G-Δ9GL- or ASFV-G-ΔMGF-infected animals protected against disease after challenge with parental ASFV-G presented, at some point before challenge, significant viremia values, which was completely absent in all ASFV-G-Δ9GL/ΔMGF-infected animals. Host mechanisms mediating protection against ASFV infection or disease still remain unknown and the possible relationship between replication of attenuated strains and the protection achieved by them cannot be ruled out at this time.

Therefore, infection with any of the doses of ASFV-G-Δ9GL/ΔMGF tested equally failed in inducing protection against clinical disease, resulting in virus replication and death upon challenge with parental virulent ASFV-G.

Genomic manipulation of ASFV as presented here has not been reported before; therefore, predictability of the obtained phenotype has some degree of uncertainty. Perhaps the only report providing comparable data was presented by Abrams et al. (2013) where it was shown that deletion of the NL (DP71L) and UK (DP96R) genes from the genome of the naturally attenuated OUR T88/3 strain reduced its ability to protect pigs against challenge with virulent virus. As reported here, deletion of virus genes previously associated with virus virulence (Zsak et al., 1996, 1998) in the genome of an attenuated virus strain produced a deleterious effect in its ability to confer protection against challenge.

4. Conclusions

The additive genetic effect on virus attenuation sought by deleting 9GL and MGF505/360 genes in ASFV-G led to a significant decrease of virus virulence in swine. ASFV-G-Δ9GL/MGF double-deletion mutant virus administered at doses 100-fold over the dose that causes disease in swine exposed to ASFV-G-Δ9GL resulted in attenuation. However this particular combination of deletions in the ASFV-G genome led to a virus with limited immunogenic capability precluding the use of it as an effective modified live vaccine. Although pursuing additive variations for specific traits by genetically modifying ASFV is feasible as shown here, the outcome of those modifications is still unpredictable and at each instance it will require a thorough experimental corroboration.

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Recoding structural glycoprotein E2 in classical swine fever virus (CSFV) produces complete virus attenuation in swine and protects infected animals against disease

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ABSTRACT

Controlling classical swine fever (CSF) mainly involves vaccination with live attenuated vaccines (LAV). Experimental CSFV LAVs has been lately developed through reverse genetics using several different approaches. Here we present that codon de-optimization in the major CSFV structural glycoprotein E2 coding region, causes virus attenuation in swine. Four different mutated constructs (pCSFm1-pCSFm4) were designed using various mutational approaches based on the genetic background of the highly virulent strain Brescia (BICv). Three of these constructs produced infectious viruses (CSFm2v, CSFm3v, and CSFm4v). Animals infected with CSFm2v presented a reduced and extended viremia but did not display any CSF-related clinical signs. Animals that were infected with CSFm2v were protected against challenge with virulent parental BICv. This is the first report describing the development of an attenuated CSFV experimental vaccine by codon usage de-optimization, and one of the few examples of virus attenuation using this methodology that is assessed in a natural host.

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1. Introduction

Classical swine fever (CSF) is a highly contagious disease of swine. The etiological agent, CSF virus (CSFV), is an enveloped virus with a positive-sense, single-stranded RNA genome, classified as a member of the genus *Pestivirus* within the family *Flaviviridae* (Becher et al., 2003). The 12.5 kb CSFV genome contains a single open reading frame that encodes a 3898-amino-acid polyprotein and ultimately yields 11–12 final cleavage products (NH_2-Npro-C-E^{rns}-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH) through co- and post-translational processing of the polyprotein by cellular and viral proteases (Leifer et al., 2013). Structural components of the CSFV virion include the Core (C) protein and glycoproteins E^{rns}, E1 and E2. E1 and E2 are type I transmembrane proteins with an N-terminal ectodomain and a C-terminal hydrophobic anchor; E^{rns} loosely associates with the viral envelope (Thiel et al., 1991; Weiland et al., 1990; Weiland et al., 1999). E2 is the most immunogenic of the CSFV glycoproteins (Weiland et al., 1999; Konig et al., 1995; van Gennip et al., 2000), inducing neutralizing antibodies that provide protection against lethal CSFV challenge. Several studies have demonstrated the presence of genetic determinants of virulence within the E2 gene. Using reverse genetics, it has been demonstrated that amino acid substitutions introduced at specific positions within the E2 gene lead to partial or complete attenuation of virulent viruses in swine (Risatti et al., 2005a, 2005b, 2006, 2007a, 2007b; Van Gennip et al., 2004). Most of these attenuated viruses have been shown to

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induce protection against the virulent parental strains, opening the possibility to use this approach to develop experimental vaccine strains.

An alternative approach to produce live attenuated viruses consists of the systematic introduction of synonymous mutations by manipulating the codon usage bias or the pair codon usage. Codon usage bias refers to the phenomenon where specific codons are used more often than other synonymous codons during translation of genes, the extent of which varies within and among species. This variation is typically manifested by a balance between mutational and transitional pressure (Behura and Severson, 2013). In general the overall extent of codon usage bias in RNA viruses is low; the strong correlation between base and dinucleotide composition and codon usage bias suggests that mutational pressure rather than transitional pressure might be the most important factor shaping codon usage bias in viral RNA genomes (Jenkins and Holmes, 2003). Despite low codon usage in RNA viruses, the experimental de-optimization of codon usage bias or pair codon usage carried out in different viral genomes such as influenza A virus (Baker et al., 2015; Nogales et al., 2014; Luan et al., 2009), arenavirus (Cheng et al., 2015), respiratory syncytial virus (Meng et al., 2014; Le Nouen et al., 2014), porcine reproductive and respiratory syndrome virus (Ni et al., 2014), poliovirus (Mueller et al., 2006; Burns et al., 2006) and vesicular stomatitis virus (Wang et al., 2015), has produced not only viral attenuation in cell culture and animals, but also protection when animals have been challenged with the parental strain. Although the exact mechanism of action remains unknown, the use of this approach might represent a good platform for the development of live attenuated vaccines.

Here we present the development of several different constructs using mutational approaches based on the genetic background of the highly virulent CSFV strain Brescia (BICv). Most of the mutant constructs led to the production of infectious viruses and, interestingly, one of the viruses was completely attenuated in swine. Virus attenuation correlated with a decreased ability to replicate in primary swine macrophage cell cultures, the main target cell type during the infection *in vivo*. Animals infected with the attenuated strain were protected against the presentation of clinical CSF and infection after the challenge with the virulent parental BICv.

2. Materials and methods

2.1. Viruses, antibodies and cells

Swine kidney cells (SK6) (Terpstra et al., 1990), free of BVDV, were cultured in Dulbecco's minimal essential media (DMEM) (Gibco, Grand Island, NY) with 10% fetal calf serum (FCS) (Atlas Biologicals, Fort Collins, CO). CSFV strain Brescia was propagated in SK6 cells and was used for the construction of an infectious cDNA clone (Risatti et al., 2005). Growth kinetics was assessed using either SK6 cells (Risatti et al., 2005) or primary swine macrophage cell cultures prepared as described by Zsak et al. Zsak et al., (1996). Titration of CSFV from clinical samples was performed using SK6 cells in 96-well plates (Costar, Cambridge, MA). After 4 days in culture, viral infectivity was assessed using an immunoperoxidase assay utilizing the CSFmonoclonal antibody WH303 (mAb WH303) (Edwards et al., 1991) and the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) (Risatti et al., 2005). Titers were calculated according to the method of Reed and Muench (Reed and Muench, 1938) and expressed as $TCID_{50}$/ml. As performed, test sensitivity was $\geq \log_{10}$ 1.8 $TCID_{50}$/ml.

2.2. Codon usage de-optimization strategy

Codon usage de-optimization was carried out *in silico* using the web server Optimizer (http://genomes.urv.es/OPTIMIZER/) (Puigbo et al., 2007). The procedure was conducted along the 373 codons of the glycoprotein E2 coding region of CSFV (nucleotides 2435-3553 from CSFV isolate Brescia; GenBank Accession number: AY578687.1). The reference host codon usage dataset was obtained from the Codon Usage Database (Nakamura et al., 2000). It included 1,168,059 codons contained in 2953 coding regions, which represent the most abundant codons for each amino acid in these regions in the species *Sus scrofa*. Two different algorithms were used to design the mutant viruses. First, the mutant CSFm1 was designed using the one amino acid-one codon algorithm, which is based on the codon adaptation index (CAI = 1) theory (Pesole et al., 1988). Basically, all the E2 codons that encode the same amino acid were substituted by the less commonly used synonymous codon in the reference host dataset (Puigbo et al., 2007). Second, the mutant CSFm2 was designed using the guided random method that is based on the Monte Carlo algorithm. Essentially based on the reference dataset, a new pool of codons was created for each amino acid of E2 that was equal to the number found in the target sequence, and that was in accord with their low occurrence in the reference dataset, so that the de-optimized sequence contained codons in the opposite proportions in which they are found in the adjusted reference dataset (Jayaraj et al., 2005). Mutant CSFm3 was de-optimized just in the first 123 codons of the E2 coding region (nucleotides 2435-2803), using (as with CSFm1) the one amino acid-one codon algorithm (Pesole et al., 1988).

With mutant CSFm4, we wanted to determine the potential effect of modifying synonymous sites which have remained conserved during the evolution of natural populations of CSFV in the E2 coding region. For this propose, we conducted a sequence alignment considering full-length sequences available in the GenBank database corresponding to all CSFV genotypes. Twelve codons were found highly conserved among all different genotypes, and based on low frequencies of codon usage found in the reference dataset for the respective amino acids, synonymous mutations were manually introduced at the corresponding nucleotide positions. Nucleotide sequences for each of the four CSFm constructs, compared with the parental virus, is provided in Fig. 1.

2.3. Development of mutant viruses

A full-length infectious cDNA clone (IC) of the BICv virus (pBIC) (Risatti et al., 2005) was used as a DNA template in which mutations were introduced to alter codon usage as described earlier. Areas of CSFV glycoprotein E2 containing the desired nucleotide changes were commercially synthesized (Epoch, Missouri City, TX) and further directionally cloned into pBIC using the Infusion (In-Fusion cloning, Takara-Clontech, Mountain View, CA) methodology as described by the manufacturer using the appropriate primers in the 5′ and 3′ ends of the cloned construct (CSFm1: 5′ CTGGGGCACAAGGCCGGCTAGCGTGATAAGAAGATCATCG 3′ and 5′ GCCTAACTGTAGACCCGCGGCTAATTGTTCCGTT AAT 3′; CSFm2: 5′ CTGGGGCACAAGGCCGGCTAGCTTGCAAAGAAGATCATC G 3′ and 5′ GCCTAACTGTAGACCCGCGGCCAACTGCTCCGTTAAGACAA 3′; CSFm3: 5′ CTGGGGCACAAGGCCGGCTAGCGTGTAAAGAAGATCATCGTTA 3′ and 5′ GCCTAACTGTAGACCCGCGGCGAGTTGTTCTGTTAGAACT 3′; CSFm4: 5′ CTGGGGCACAAGGCCGGCTAGCCTGCAAGGAAGATCACA 3′ and 5′ GCCTAA CTGTAGACCCGCGGCGAGTTGTTCTGTTA 3′) respectively. Fidelity of the produced constructs was assessed by full-genome sequencing. The resulting vector was linerized using BssHII and *In vitro* RNA synthesis, SK6 cells transfections and rescue of the mutant viruses were performed exactly as previously described (Risatti et al., 2005).

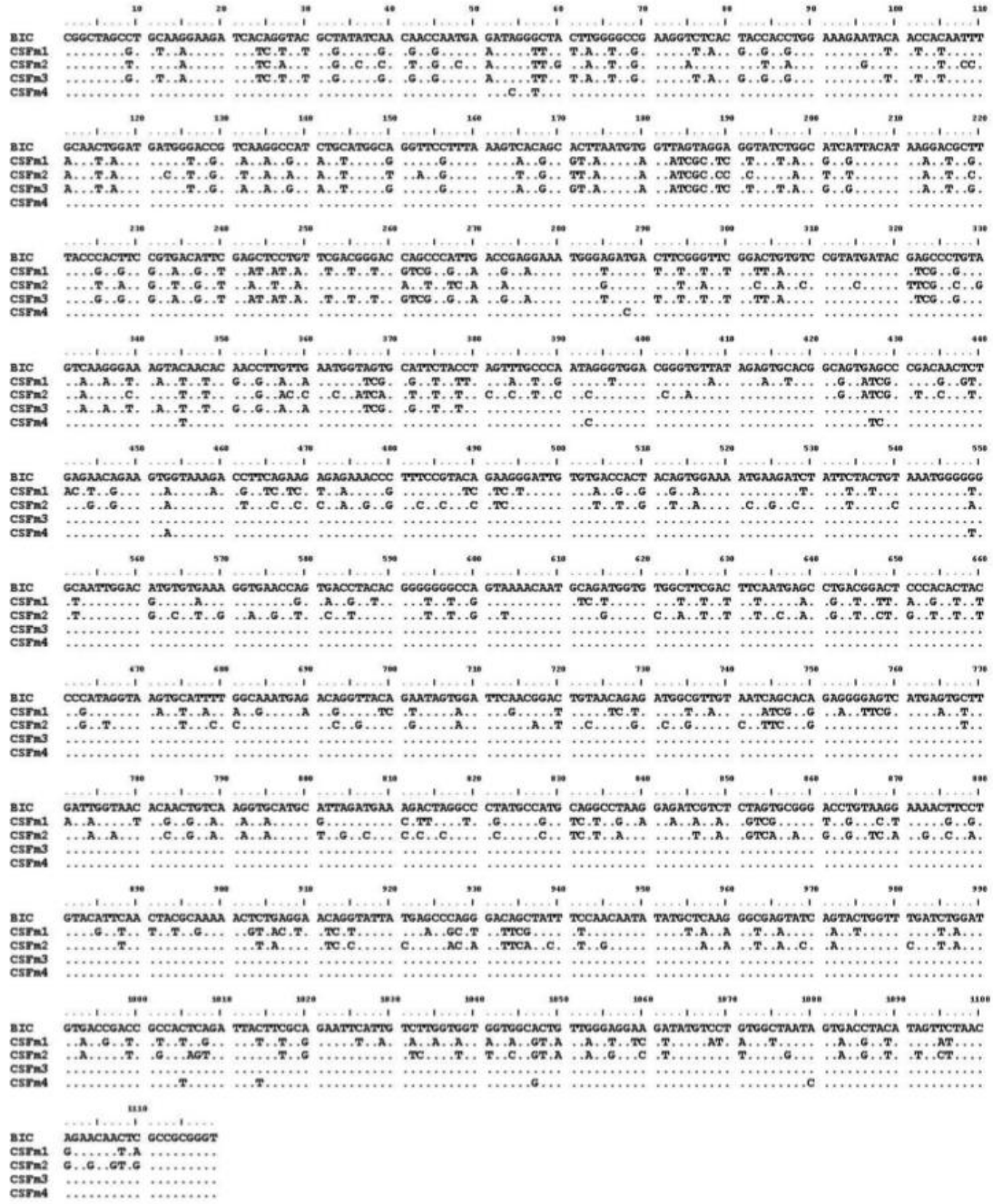

Fig. 1. Complete nucleotide sequence of CSFV E2 glycoprotein in CSFm1–CSFm4 constructs. All substituted nucleotides in the development of all mutated constructs are presented compared with the native sequence of E2 BICv.

2.4. Bioinformatic evaluation of mutant viruses

Different statistical methods were used to analyze *in silico* de-optimization of codon usage in the mutant viruses.

Relative synonymous codon usage (RSCU) and Codon adaptation index (CAI) calculations: RSCU is an index to assign a numerical value to individual codons that indicates how biased each of these codons is with reference to a random expectation codon usage, so that RSCU values larger than 1.0 indicate that one synonymous codon is favored over the rest; RSCU values less than 1.0 indicate an unfavored codon; and RSCU values of 1.0 indicate no preference (Sharp et al., 1986). CAI estimates the deviation of RSCU of a given protein coding sequence with respect to a reference dataset or relative adaptiveness. Values range from 0 to 1, with higher values indicating a higher proportion of the most abundant codons (Sharp and Li, 1987). Both analyses were conducted using the web server CAIcal (http://genomes.urv.es/CAIcal/) (Puigbo et al., 2007).

Codon pair bias (CPB): CPB for an entire reading frame has been defined as the arithmetic mean of the individual codon pair scores (CPS). The CPS of each codon pair is the natural log of the ratio of the observed over the expected number of occurrences over all coding regions in the genome. A positive CPB score indicates a prevalent use of overrepresented codon pairs, while a negative CPB score represents a predominant use of underrepresented codon pairs (Coleman et al., 2008). Following a methodology previously described (Tulloch et al., 2014), a reference codon pair usage table was developed based on the analysis of 25,883 *Sus scrofa* mRNA sequences (obtained from ftp://ftp.ensembl.org/pub/release-82/fasta/sus_scrofa/cds/). Based on the use of that table, we obtained CPB scores of all mRNA sequences used in this study from *Sus scrofa* as well as CPB scores from the E2 coding region of BICv and CSFm1-4v. All calculations were conducted using the program Composition Scan in the SSE software version 1.2 (Simmonds, 2012).

Additionally nucleotide and dinucleotide frequencies were obtained using the program Composition Scan in the SSE package (Simmonds, 2012).

2.5. DNA sequencing and analysis

Full-length clones of BICv and mutant constructs and derived *in vitro*-rescued viruses were completely sequenced. CSFV-specific primers were used to partially PCR amplify cDNA obtained from virus RNA by reverse transcription. PCR products were sequenced using the dideoxynucleotide chain-termination method (Sanger et al., 1977). Sequencing reactions were prepared with the Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Reaction products were sequenced on a PRISM 3730xl automated DNA sequencer (Applied Biosystems). Sequence data was assembled with the Phrap software program (http://www.phrap.org), with confirmatory assemblies performed using CAP3 (Huang and Madan, 1999). The final DNA consensus sequence represented an average five-fold redundancy at each base position. Sequence comparisons were conducted using BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

Full-length virus genome sequencing for all recovered viruses was obtained by Next Generation Sequencing. Full-length sequencing of the virus genome was basically performed as described elsewhere (Krug et al., 2015). Viral RNA was obtained from the supernatant of infected swine kidney tissue culture cells (SK6 cells) using the TRIzol method (Life Technologies, Grand Island, NY, USA). RNA concentration was determined using the Qubit® RNA high-sensitivity (HS) assay kit (Life Technologies) and read on a Qubit 2 fluorometer (Life Technologies). One microgram of viral RNA was enzymatically fragmented to obtain blunt-end fragments in a length range of 100–300 bp using the enzyme Rnase III (Life Technologies) and incubated at 37 °C in a Peltier DNA Engine Tetrad 2 thermal cycler. Ion adapters were hybridized to fragmented RNA, hybridized fragmented RNA was reverse transcribed to cDNA and cDNA was ligated to ion library barcodes and amplified using the Ion total RNA-seq v2 kit (Life Technologies). 1 ul of amplified cDNA was loaded onto Agilent DNA 1000 chip (Agilent, Santa Clara, CA, USA) and quantified using 2100 bioanalyzer (Agilent). Using the 2100 expert software, a smear analysis was performed to quantify the molar concentration (nM) of the cDNA libraries in order to determine the library dilution required for template preparation. After dilution of cDNA library to a concentration of 20 pM was performed, the cDNA library was clonally amplified onto Ion Sphere particles (ISPs), generating template-positive ISPs using the Ion PGM template OneTouch 2 200 kit (Life Technologies) with the Ion OneTouch 2 instrument (Life Technologies). Before proceeding to enrichment, quality assessment of nonenriched template-positive ISPs was performed using the Ion Sphere quality control (QC) assay kit (Life Technologies) and a Qubit 2 fluorometer. The template-positive ISPs were then enriched using the Ion PGM template OneTouch 2 200 kit (Life Technologies) and Ion OneTouch enrichment system (ES) instrument (Life Technologies) to eliminate template-negative ISPs and to denature cDNA on template-positive ISPs. Using the Ion PGM 200 sequencing v2 kit (Life Technologies), enriched template ISPs were prepared for sequencing, loaded onto either an Ion 314 or Ion 316 chip, v2 (Life Technologies), and run on the Ion PGM sequencer (Life Technologies). The sequences obtained were then trimmed using Galaxy (https://usegalaxy.org/) NGS QC and manipulation tools. Sequences were aligned and analyzed using Sequencher 5.2.2 (Genecodes) and CLC Genomics Workbench (CLCBio) software.

2.6. Animal studies

Animals were randomly allocated into the corresponding groups following each of the protocols. All animals were acclimated to their environment for seven days prior to beginning each experiment. Animal experiments were performed in accordance with protocols approved by Plum Island Animal Disease Center Institutional Animal Care and Use Committee.

Virulence of CSFm mutant viruses relative to BICv was initially assessed in 10–12 week old, forty-pound commercial breed pigs inoculated intranasally (IN) with 10^5 $TCID_{50}$ of each virus. Animals were randomly allocated into 4 groups ($n=5$) and were inoculated with a mutant virus or BICv. Clinical signs (anorexia, depression, purple skin discoloration, staggering gait, diarrhea and cough) and changes in body temperature were recorded daily throughout the experiment and scored as previously described (Risatti et al., 2007). Blood was collected at times post-infection from the anterior vena cava in EDTA-containing tubes (Vacutainer) and quantification of viremia by virus titration was as previously described.

For infection-challenge studies, 15 pigs were randomly allocated into three groups ($n=5$). Pigs in groups 1 and 2 were IN inoculated with CSFm2v, and pigs in group 3 were mock-infected. At 3 days post-inoculation (DPI) (group 1) or 28 DPI (group 2), animals were challenged with BICv along with animals in group 3. Clinical signs and body temperature were recorded daily throughout the experiment as described above. Blood samples were collected at times post-challenge for quantification of viremia as described earlier.

2.7. Quantitative real-time PCR (qPCR)

qPCR was performed as previously described (Borca et al., 2008). Briefly, swine macrophage primary cell cultures were infected at MOI = 1, and total cellular RNA was extracted at 36 h post-infection. Contaminant genomic DNA was removed by DNase

Table 1
RSCU values of native CSFvE2 as well as the CSFm1-4v related to Sus scrofa codon usage.

Amino acid	Codon	*Sus scrofa*	BICv	CSFm1v	CSFm2v	CSFm3v	CSFm4v
Phe	UUU	0.79	0.38	**2.00**	**1.25**	1.00	0.38
Phe	UUC	**1.21**	**1.63**	0.00	0.75	1.00	**1.63**
Leu	UUA	0.37	0.53	**6.00**	**1.76**	**2.82**	0.53
Leu	UUG	0.77	1.41	0.00	1.06	0.71	1.41
Leu	CUU	0.75	0.35	0.00	0.35	0.00	0.35
Leu	CUC	1.55	0.88	0.00	0.88	0.53	0.88
Leu	CUA	0.38	1.24	0.00	1.59	1.06	1.24
Leu	CUG	**3.07**	**1.59**	0.00	0.35	0.88	**1.59**
Ile	AUU	0.91	0.64	0.00	**1.29**	0.64	0.64
Ile	AUC	**1.67**	0.64	0.00	0.43	0.43	**1.29**
Ile	AUA	0.42	**1.71**	**3.00**	**1.29**	**1.93**	1.07
Val	GUU	0.57	0.63	0.00	1.50	0.50	0.63
Val	GUC	1.06	0.88	0.00	0.75	0.50	0.88
Val	GUA	0.34	0.63	**4.00**	**1.63**	1.38	0.75
Val	GUG	**2.03**	**1.88**	0.00	0.13	**1.63**	**1.75**
Ser	UCU	0.99	0.35	0.00	0.35	0.35	0.38
Ser	UCC	1.50	1.06	0.00	0.71	0.35	1.13
Ser	UCA	0.72	1.41	0.00	1.76	0.71	**1.50**
Ser	UCG	0.39	0.00	**6.00**	**2.12**	**2.82**	0.00
Ser	AGU	0.77	1.41	0.00	1.06	0.71	**1.50**
Ser	AGC	**1.62**	**1.76**	0.00	0.00	1.06	**1.50**
Pro	CCU	1.05	1.11	0.00	1.11	0.89	1.11
Pro	CCC	**2.11**	0.89	0.00	1.11	0.67	0.89
Pro	CCA	1.37	**1.33**	0.00	0.44	1.11	**1.33**
Pro	CCG	0.80	0.67	**4.00**	**1.33**	**1.33**	0.67
Thr	ACU	0.83	0.76	0.00	1.08	0.54	0.76
Thr	ACC	**1.63**	1.30	0.00	0.65	0.54	1.30
Thr	ACA	0.89	**1.41**	0.00	0.76	0.97	**1.41**
Thr	ACG	0.56	0.54	**4.00**	**1.51**	**1.95**	0.54
Ala	GCU	0.95	0.44	0.00	**1.33**	0.00	0.44
Ala	GCC	**2.17**	0.89	0.22	0.44	0.22	0.89
Ala	GCA	0.89	**2.22**	0.00	0.89	1.33	**2.00**
Ala	GCG	0.61	0.44	**3.78**	**1.33**	**2.44**	0.67
Tyr	UAU	0.73	0.76	**2.00**	0.76	**1.14**	0.95
Tyr	UAC	0.93	**1.24**	0.00	**1.24**	0.86	**1.05**
His	CAU	0.70	0.86	**2.00**	**1.71**	**1.43**	**1.14**
His	CAC	**1.57**	**1.14**	0.00	0.29	0.57	0.86
Gln	CAA	0.44	**1.43**	**2.00**	**1.43**	**1.43**	**1.43**
Gln	CAG	**1.92**	0.57	0.00	0.57	0.57	0.57
Asn	AAU	0.79	**1.14**	**2.00**	0.71	**1.43**	**1.14**
Asn	AAC	0.80	0.86	0.00	**1.29**	0.57	0.86
Lys	AAA	0.76	0.89	**2.00**	**1.11**	**1.44**	0.89
Lys	AAG	0.83	**1.11**	0.00	0.89	0.56	**1.11**
Asp	GAU	0.80	**1.24**	**2.00**	**1.43**	**1.52**	**1.14**
Asp	GAC	0.95	0.76	0.00	0.57	0.48	0.86
Glu	GAA	0.73	0.96	**2.00**	**1.04**	**1.22**	0.96
Glu	GAG	**1.22**	**1.04**	0.00	0.96	0.78	**1.04**
Cys	UGU	0.78	0.93	**2.00**	0.93	**1.20**	0.88
Cys	UGC	**1.40**	**1.07**	0.00	**1.07**	0.80	**1.13**
Arg	CGU	0.44	0.00	**6.00**	0.95	0.95	0.00
Arg	CGC	**1.82**	0.32	0.00	**1.58**	0.32	0.32
Arg	CGA	0.84	0.00	0.00	1.26	0.00	0.00
Arg	CGG	1.79	0.00	0.00	0.63	0.00	0.00
Arg	AGA	1.55	**2.84**	0.00	0.00	**2.84**	**2.84**
Arg	AGG	1.71	**2.84**	0.00	**1.58**	1.89	**2.84**
Gly	GGU	0.57	1.16	**4.00**	1.16	**2.19**	**1.42**
Gly	GGC	**1.60**	0.65	0.00	0.52	0.65	0.65
Gly	GGA	1.00	0.90	0.00	**1.29**	0.52	0.90
Gly	GGG	1.15	**1.29**	0.00	1.03	0.65	1.03

RSCU values are presented for different codons encoding for the 18 amino acid residues used in the different versions of E2. In Bold are represented the highest RSCU value for each amino acid.

treatment and genomic DNA contamination of RNA stocks assessed by qPCR. cDNA was synthesized in 100 µl reactions containing 2500 ng of total RNA. For gene expression qPCR was performed using *Power* SYBR® Green with primer pairs described in (Borca et al., 2008). Melting curve analysis was performed to verify specificity of the amplification. Normalization of gene expression was performed with β-actin mRNA. Relative quantities (RQ) of mRNAs were estimated using the $2^{-\Delta\Delta Ct}$ method (described in ABI PRISM® 7700 Sequence Detection System User Bulletin #2 (PN 4303859)). The normalized mRNA expression level of a cellular gene in the infected cells was considered significant when it departed from its level in uninfected cells threefold, in either direction. Swine genes targeted included: AMCF-2, CD46, DAP12, ICAM, ICE, IL-1α, IL-1β, IL-1R, IL-2, IL-2α, IL-4, IL-5, IL-6, IL-6R, IL-7, IL-8, IL-8R, IFNα, IFNβ, IFNγ, IL-10, IL-12p35, IL-12p40, IL-12R, IL-13, IL-15, IL-16, IRF-1, IRF-3, IRF-6, IRF-7, IRF-9, MCP-1, MCP-2, OAS, NKG2D, NCP-1, NCP-2, NOS, PKR, RANTES, TGF-β1, TGF-β2, TGF-B3R, TLR-1, TLR-2, TLR-3, TLR-5 and TLR-10.

3. Results

3.1. Bioinformatic evaluation

To evaluate the effect produced by codon usage de-optimization along the glycoprotein E2 coding region of CSFV we employed two different algorithms (see Section 2) to produce mutant viruses in which codons that encode the same amino acid were substituted by the less commonly used synonymous codon in the reference host dataset (*Sus scrofa*). After the de-optimization process the total number of synonymous mutations varied among the mutants, with mutant CSFm1v having the highest number of synonymous mutations with 312, followed by CSFm2v with 298, and CSFm3v with 109. Finally, CSFm4 has just 12 synonymous mutations manually introduced in highly E2 conserved codons corresponding to nucleotide positions: $2482^{(I)ATA\text{-}ATC}$, $2485^{(G)GGG\text{-}GGT}$, $2716^{(D)GAT\text{-}GAC}$, $2773^{(Y)TAC\text{-}TAT}$, $2821^{(I)ATA\text{-}ATC}$, $2855^{(S)AGC\text{-}TGC}$, $2881^{(V)GTG\text{-}GTA}$, $2977^{(G)GGG\text{-}GGT}$, $3433^{(H)CAC\text{-}CAT}$, $3442^{(Y)TAC\text{-}TAT}$, $3475^{(A)GCA\text{-}GCG}$, and $3508^{(I)ATA\text{-}ATC}$. The exact positions of all synonymous mutations introduced in the CSFm mutant constructs are shown in Fig. 1.

3.2. Effects on relative synonymous codon usage in CSFm1v to CSFm4v mutants

To evaluate the impact on synonymous codon usage after the de-optimization process we evaluated BICv and CSFm1v-CSFm4 E2 by

using RSCU and CAI indexes (see Section 2). RSCU index indicates how biased each of these codons is with reference to a random expectation codon usage. In general, the average RSCU of the coding regions analyzed from *Sus scrofa* was characterized for the high preference of usage of C/G ending codons (20 out of 23 with RSCU values > 1), with the CUG codon being one of the six codons to encode the amino acid leucine which has the highest RSCU (3.07), while there is a low codon usage pattern especially of codons containing the dinucleotides CpG (UCG, CCG, ACG, GCG, CGU, CGA) and UpA (UUA, AUA, GUA, UAU, UAC) in their composition. On the other hand, codon adaptation index (CAI), which measures the relative adaptiveness of a given protein coding gene sequence with respect to a reference set of genes, was calculated between the reference data set of genes from *Sus scrofa* and BICv (E2 coding region).

Results indicated that there is a 66% similarity in the RSCU pattern between *Sus scrofa* and native CSFV E2 of BICv (Table 1). BICv showed the same low preference for the usage of codons that contain CpG and UpA in their composition. However, after substituting the mutant viruses native codons by the less synonymous codons used by *Sus scrofa* CAI scores changed to different levels according to the de-optimization strategy employed to obtain each mutant, with CSFm1v being the one with the lowest CAI value (0.38), followed by CSFm3v (0.55), CSFm2v (0.56) and finally CSFm4v that remained at the same value as BICv (0.66). As expected, due to the used algorithm, the highest decrease of relative adaptiveness regarding *Sus scrofa* was found in the CSFm1 E2 sequence.

It has been previously shown that by altering codon pair bias (CPB) throughout the viral genome can cause differences in viral virulence in the natural host (Coleman et al., 2008). Although our de-optimization strategies were not based on altering CPB in these mutant viruses, we wanted to evaluate any potential effect that could occur on CPB by using the two algorithms employed in this report. Our analysis showed that there was a normal distribution of the *Sus scrofa* genes, with an average CPB score of 0.067 ± 0.035 (Fig. 2). The CPB score of BICv was calculated to be -0.023, which remained very similar to that for CSFm1v (-0.025) and CSFm4v (-0.028). Nonetheless, while the one amino acid-one codon algorithm does not have significant effects on the CPB value of CSFm1v, in the case of CSFm3v (developed using the same algorithm but just in 1/3 of E2 coding sequence) produced a positive CPB value of 0.007, which made this mutant closer to the average CPB scores from *Sus scrofa* genes. Conversely, the algorithm used to design CSFm2v had the highest negative impact in CPB (-0.086) among all CSFm mutants, by increasing the usage of unrepresented pair codons in *Sus scrofa* (Fig. 2).

Increasing CpG and UpA dinucleotide frequencies in the genome of some viruses has been linked with viral attenuation

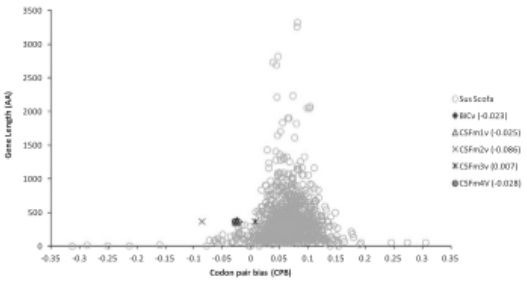

Fig. 2. Codon pair bias (CPB) scores of swine genes and CSFV E2 from BICv and CSFm1-4 mutants. Comparison was performed based on the CPB score of 25,883 *Sus scrofa* genes. Each mark represents the calculated CPB score of a gene plotted against its amino acid length. CPB scores of the CSFm1-4 mutants are indicated. Calculation of CPB values were performed as described in Section 2.

(Tulloch et al., 2014). Since our de-optimization strategy increases the usage in the mutant viruses of less commonly used synonymous codons in *Sus scrofa* associated with codons containing CpG and UpA dinucleotides in their composition, we expected an increase of the frequencies of these two dinucleotides in the genomic composition of the mutant viruses. Comparing odds ratios of observed/expected frequencies of these two dinucleotides between BICv E2 and those in the CSFm mutant viruses, we found a disruption at different levels in the frequency patterns associated with these two dinucleotides along the E2 coding region of the mutant viruses. The highest changes in the observed/expected odds ratios in these two dinucleotides were found in CSFm1v, followed by CSFm2v and CSFm3v, while CSFm4v remained very similar to BICv (Fig. 3).

It has been demonstrated that low levels of G/C content are associated with decreased gene expression in mammalian cells (Kudla et al., 2006). Comparison of G/C nucleotide compositions between BICv and mutant viruses indicated that the most important changes occurred in mutant CSFm1v E2, where total G/C composition decreased 9% in contrast with BICv E2, while no significant changes were detected in the other three CSFm E2 mutants (CSFm2v and CSFm3v have a decrease of 2 and 3%, respectively, while no change was detected for CSFm4v).

3.3. Production of CSFm viruses

To assess the importance of the introduced mutations in the process of virus replication and virulence a CSFm construct series of recombinant CSFVs containing mutations in E2 was designed using the cDNA infectious clone (IC) of the Brescia strain (BICv) as a template. A total of 4 cDNA IC constructs containing the distinctive areas of E2 mutated as described above were developed, pCSFm1-pCSFm4. Infectious RNA was *in vitro* transcribed from each mutated full-length cDNA and used to transfect SK6 cells. Infectious virus was rescued by day 4 post-transfection from cells transfected with viral RNA derived from constructs pCSFm2, pCSFm3, and pCSFm4. In contrast, after three independent transfection procedures, pCSFm1 viral RNA did not produce infectious virus. Whole genome sequencing was performed by next generation sequencing (described in the Section 2) for each of the mutant viruses, and no changes were detected at the nucleotide level in the viral stock of viruses pCSFm2v-pCSFVm4v when compared to the initial designed construct.

3.4. Replication of the CSFm mutant viruses in vitro

The importance of the introduced mutations in the process of virus replication was primarily assessed in SK6 cells. *In vitro* growth characteristics of the CSFm2v, CSFm3v, and CSFm4v viruses relative to parental BICv were evaluated in a multistep growth curve. SK6 cell cultures were infected at an MOI of 0.01 $TCID_{50}$ per cell. Viruses were adsorbed for 1 h (time zero), and samples were collected at 72 h post-infection and titrated in SK6 cell cultures using immunoperoxidase staining, calculation of titers was previously described by Risatti et al. (2005). Results indicate that all three mutant CSFm viruses exhibited an almost indistinguishable replication kinetics compared to that of the parental BICv (Fig. 4).

3.5. Virulence of CSFm mutant viruses in vivo

To examine whether the genomic changes introduced into E2 of CSFm2v, CSFm3v, and CSFm4v viruses may affect virulence, four different groups of pigs (n = 5) were intranasally (IN) inoculated with 10^5 $TCID_{50}$ of each of the CSFm mutant viruses (CSFm2v, CSFm3v, and CSFm4v) and monitored for clinical disease, evaluated relative to parental BICv (Table 2, Fig. 5A and B). All animals

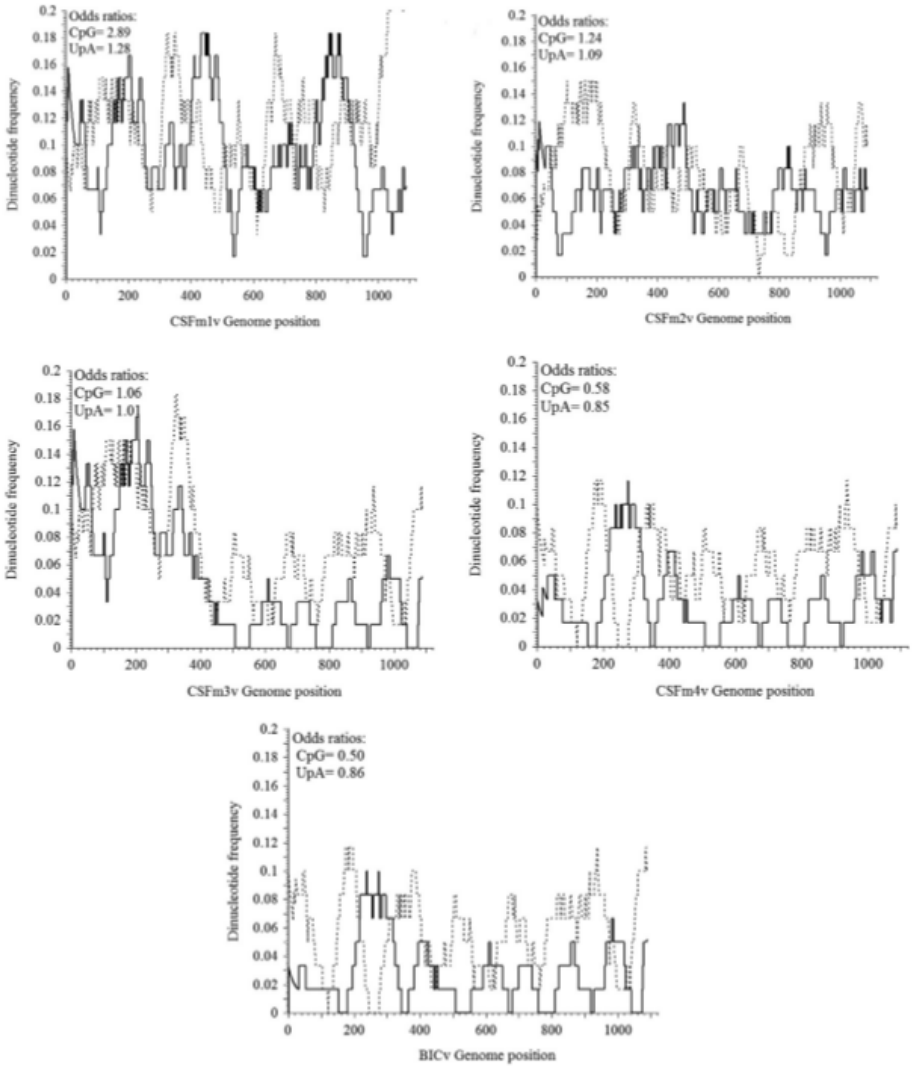

Fig. 3. Comparison of CpG and UpA dinucleotide frequencies along the E2 genomic region between BICv and CSFm1-4 mutants. Solid and dotted lines represents CpG and UpA dinucleotides respectively. Frequency values and odds ratio calculations were obtained using the program Composition Scan in the SSE package version 1.2 (Simmonds, 2012) as described in Section 2.

infected with BICv presented a rise in body temperature and clinical signs of CSF starting around 3 DPI. Animals quickly developed classical symptoms of severe disease, being euthanized around 5 to 6 DPI. CSFm3v and CSFm4v viruses presented a virulence phenotype almost indistinguishable from that of the parental BICv (Table 2, Figs. 5A and B and 6). All animals infected with these two viruses presented clinical signs of CSF starting at 3–4 DPI, with clinical presentation and severity similar to those observed in animals inoculated with BICv. Conversely, animals inoculated with CSFm2v survived the infection and remained clinically normal throughout the observation period (21 days). No rise in body temperature was observed, and only a transient and very mild diarrhea during the first week after infection was observed in these animals (Table 2, Figs. 5A and B and 6).

As expected, in general viremias accompanied the evolution of the clinical disease. Therefore, viremias in animals inoculated with BICv are clearly detected by 3 DPI reaching the highest titers by the time of death at 5–7 DPI (Fig. 6). Animals infected with mutant viruses CSFm3v or CSFm4v exhibited viremia kinetics almost indistinguishable from that induced by parental BICv, presenting high titers that remained until death of the animal. In contrast, mutant virus CSFm2v induced a mild and transient viremia lasting during the first week post-inoculation, with virus titers being significantly lower (1000 to 10,000 folds depending of the sample time considered) than those found in animals inoculated with parental BIC virus (Fig. 6).

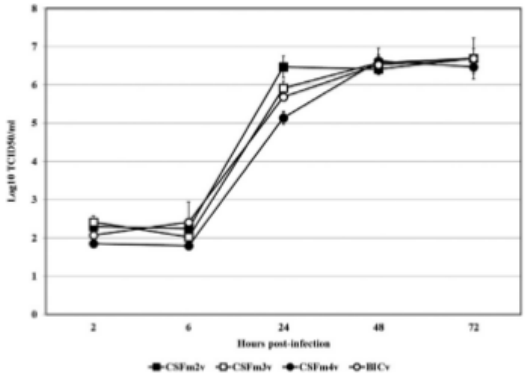

Fig. 4. *In vitro* growth characteristics of CSFm2-4v mutant viruses and parental BICv. SK6 cell cultures were infected (MOI=0.01) with CSFm2-4v with CSFm2v or BICv. Virus yield obtained at the indicated times post-infection were titrated in SK6 cell cultures. Data represent means and standard deviations from three independent experiments. Sensitivity of virus detection: ≥ 1.8 $TCID_{50}$/ml.

Table 2
Swine survival and fever response following infection with CSFm mutant viruses or virulent parental BICv.

Virus	No. of survivors/total No.	Mean time to death (days ± SD)	Fever	
			No. of days to onset (days ± SD)	Duration (days ± SD)
BICv	0/5	5. 6 (1.34)	3.4 (0.89)	2.2 (1.79)
CSFm2v	5/5	–	–	–
CSFm3v	0/5	6.4 (0.89)	3.6 (0.89)	2.82 (0.84)
CSFm4v	0/5	5.8 (1.1)	3.4 (0.55)	2.4 (1.14)

3.6. Comparative growth of CSFm2 mutant virus in swine macrophage cell cultures

Cells derived from the macrophages lineage are the main target for CSFV replication during the infection in swine. Since the CSFm2 mutant virus clearly replicates with lower efficiency than BICv during swine infection it was important to assess the comparative ability of CSFm2v to replicate in swine macrophages. *In vitro* growth characteristics of the CSFm2v mutant virus relative to parental BICv were evaluated in a multistep growth curve. Primary swine macrophage cell cultures were infected at an MOI of 0.01 $TCID_{50}$ per cell. Viruses were adsorbed for 1 h (time zero), and samples were collected during 72 h post-infection. Samples were titrated in SK6 cells and the presence of virus detected by immunoperoxidase staining. The growth of CSFm2 virus was significantly lower than BICv, showing titers approximately 100-fold lower than the parental virus, depending on the sampling time considered (Fig. 7). Similar results were obtained in a single-step growth curve experiment comparatively performed in primary swine macrophage cell cultures using CSFm2v and BICv at a MOI of 1 and harvested at 36 h post-inoculation. CSFm2v reached titers approximately 100 times lower (4.25_{log10} $TCID_{50}$ ± SD = 0.16) than the parental BICv (6.42_{log10} $TCID_{50}$ SDL: 0.16) (data not shown). Therefore, attenuated CSFm2 virus has an evident disadvantage replicating in swine macrophages compared with the parental virulent BICv.

3.7. Transcriptional activation profile of immunologically relevant genes in swine macrophages infected with CSFm2 virus

To further understand possible mechanisms responsible for CSFm2v attenuation, the pattern of activation of immunologically relevant genes in swine macrophages infected with mutant and parental viruses was analyzed using quantitative real-time PCR followed by melting curve analysis. To assess changes in cellular

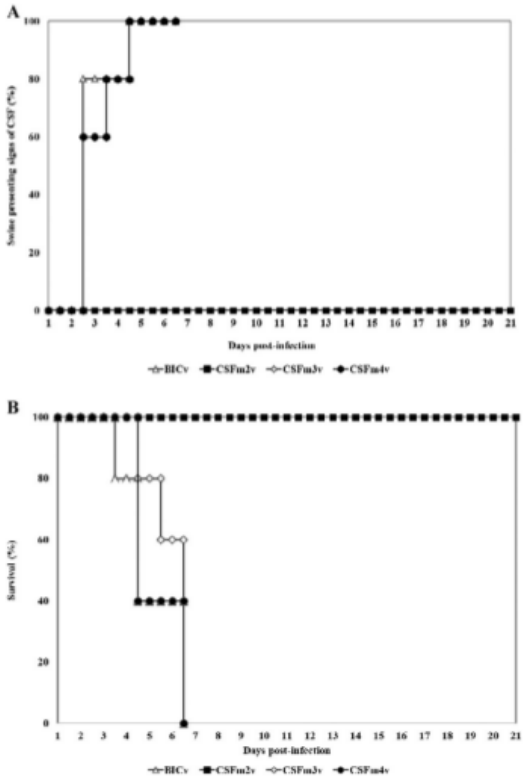

Fig. 5. Morbidity (A) and mortality (B) in swine infected with mutant viruses CSFm2-4v or parental BICv. Groups of animals ($n=5$) were infected intranasally (IN) with 10^5 $TCID_{50}$. Presence of clinical signs was recorded for an observational period of 21 DPI.

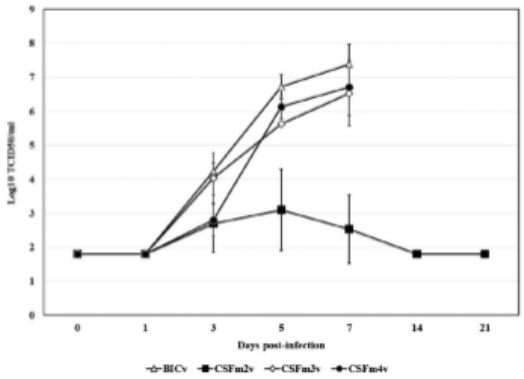

Fig. 6. Viremia titers in swine infected with mutant viruses CSFm2-4v or parental BICv. Groups of animals ($n=5$) were infected IN with 10^5 TCID $TCID_{50}$. Viremia data represent the mean log_{10} TCID $TCID_{50}$/ml and standard deviations of titers from all animals in the group. Sensitivity of virus detection: ≥ log_{10} 1.8 TCID $TCID_{50}$/ml.

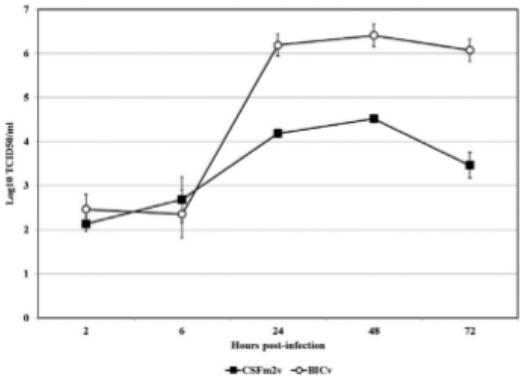

Fig. 7. *In vitro* growth characteristics of CSFm2 mutant virus and parental BICv in swine macrophages. Primary swine macrophages cell cultures were infected (MOI= 0.01) with CSFm2v or BICv. Virus yield obtained at the indicated times post-infection were titrated in SK6 cell cultures. Data represent means and standard deviations from three independent experiments. Sensitivity of virus detection: ≥ 1.8 $TCID_{50}$/mL

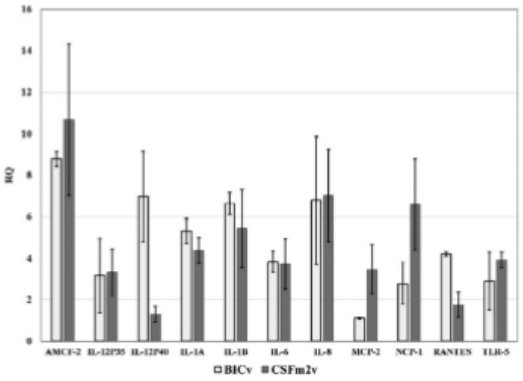

Fig. 8. Gene expression changes in peripheral blood-derived macrophages after infection with either CSFm2 or BIC viruses. Gene expression quantification was assessed by quantitative reverse transcription real-time polymerase chain reaction (qrt-PCR). Values are represented as relative quantities (RQ) of mRNA accumulation (estimated by $2^{-\Delta\Delta Ct}$) with their corresponding SD. RQ values over 3 are considered as positive.

gene expression upon infection, primary porcine macrophage cell cultures were infected at MOI=1 $TCID_{50}$ per cell with either CSFm2 virus or BICv. Total cellular RNA was extracted from infected and mock-infected cells at 36 hpi. Steady state levels of mRNA accumulation were determined for 49 swine immunomodulatory genes as described elsewhere (Borca et al., 2008). This approach identified only eleven genes (AMCF-2, IL-12p35, IL-12p40, IL-1α, IL-1β, IL-6, IL-8, MCP-2, NCP-1, RANTES and TLR-5) actually showing a significant expression increase in macrophages infected with either CSFm2v or BICv when compared with the pattern of gene expression of uninfected macrophages (Fig. 8). Among these genes only four of them presented a differential expression between CSFm2v and BICv viruses. MCP-2, and NCP-1 has increased expression in macrophages infected with CSFm2 virus while IL-12p40 and RANTES were more actively transcribed in BICv-infected macrophages (Fig. 8).

3.8. CSFm2 mutant virus protects pigs against lethal CSFV challenge

The ability of attenuated CSFm2v virus to induce protection against virulent BICv was assessed in early and late vaccination-exposure experiments. Groups of pigs ($n=5$) were IN inoculated with 10^5 $TCID_{50}$ of CSFm2v and IN challenged at 3 or 21 DPI with 10^5 $TCID_{50}$ virulent BICv. Mock-vaccinated control pigs receiving BICv ($n=5$) developed anorexia, depression and fever by 5 days post-challenge (DPC) (Table 3), and died or were euthanized *in extremis* by 7 DPC. CSFm2 virus induced complete clinical protection in animals challenged at 21 days post-immunization. All pigs survived infection with the virulent parental BICv and remained without fever or demonstrating any other CSF-associated clinical signs during the 21 day observational period (Table 3). Animals infected with CSFm2v and challenged with BICv 3 days later presented a heterogeneous behavior. Three of the animals remained clinically normal with the exception of a very weak and transient rise in body temperature in one of them during the observational period. The other two animals became sick at the same time that the animals in the mock-vaccinated group did although they presented a clinical disease more extended, being euthanized around 10 DPC.

Viremia in vaccinated and challenged animals was examined at different times post-challenge. As expected, in mock-vaccinated control animals, viremia was observed within 4 dpc, with virus titers remaining typically high (6.84_{log10} $TCID_{50}$ ± SD=0.42) at the last time point tested before animals died or were euthanized (Fig. 9). Conversely, animals inoculated with CSFm2v and challenged with BICv at 21 DPI did not produce detectable viremia. BICv was not detected in blood samples during the 21-day observation period (Figs. 7 and 8). Alternatively, in the group of CSFm2v-infected animals challenged with BICv at 3 DPI, viremia's kinetics follows the presence of clinical signs. Animals showing severe disease and death presented viremias similar to those in animals mock-vaccinated (7.12_{log10} $TCID_{50}$ ± SD=0.71 at day 7 post challenge) while in those remaining clinically normal, transient viremias were detected at low levels (3.29 $TCID_{50}$ ± SD=0.29) by 4 dpc (Fig. 8). These results indicate that protection induced by CSFm2v was complete, preventing both the presentation of CSF-related clinical signs and the replication of the challenge virus when challenge was conducted at 21 days after initial infection.

Table 3
Swine survival and fever response in CSFm-vaccinated animals after challenge with parental virulent BICv.

Challenge group (at dpi)	No. of survivors/ total	Mean time to death (± SD)	Fever		
			No. of days to onset (± SD)	Duration no. of days (± SD)	Maximum daily temp, °F (± SD)
Mock	0/5	6.8 (0.45)	5 (0.0)	1.8 (0.45)	105.8 (0.22)
CSFm2v 3 dpi	3/5	10 (1.41)	5 (0.0)	5 (1.41)	105.6 (0.81)
CSFm2v 21 dpi	5/5	–	–	0	102.2 (0.27)

4. Discussion

The introduction of synonymous mutations in virus genomes has been used to experimentally develop attenuated virus strains by de-optimizing the codon usage bias or the codon pair bias. This

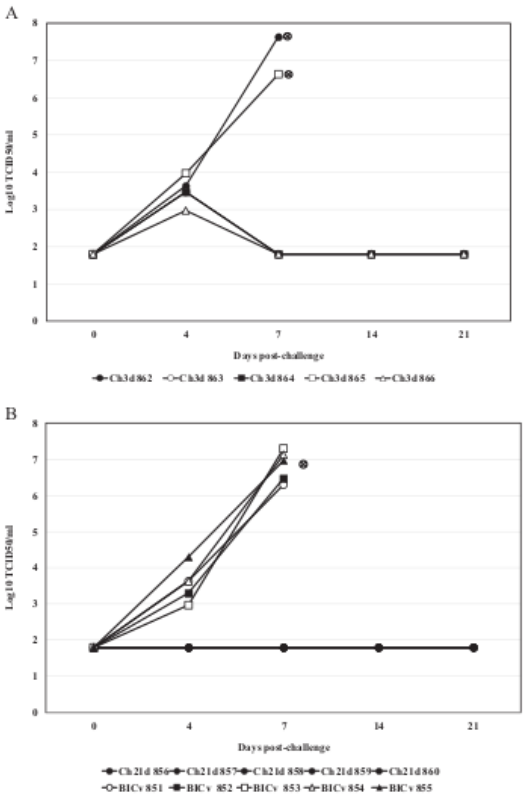

Fig. 9. Viremia titers in swine infected with CSFm2v virus and challenged with BICv. Three groups of animals ($n=5$) were either infected IN with 10^5 $TCID_{50}$ CSFm2v virus (animal tags # 856–860 and 862–866) or mock infected (animal tags # 851–855) and challenge IN with 10^5 $TCID_{50}$ of BICv at either (A) 3 (Ch3d 862–866) or (B) 21 (Ch21d 856–860) days post-CSFm2v infection. Viremia data represent individual animal data for those mock vaccinated (851–855) or challenged at 3 DPI (Ch3d 862–866) and the mean log10 TCID50/ml and standard deviations of animals challenged at 21 DPI (Ch21d 856–860). Sensitivity of virus detection: $\geq \log_{10}$ 1.8 TCID50/ml.

methodology has been used to develop several attenuated RNA virus strains utilizing poliovirus (Nogales et al., 2014; Mueller et al., 2006; Burns et al., 2006), PRRSV (Gao et al., 2015), dengue virus (Shen et al., 2015), influenza Mueller et al. (2010), respiratory syncytial virus (Le Nouen et al., 2014; Meng et al., 2014) and vesicular stomatitis virus (Wang et al., 2015).

The mechanisms causing virus attenuation remain poorly understood and may depend on many factors. Initial reports indicated that virus attenuation can be due to translation impairment although more recently it has been suggested that changes in the RNA sequence may increase innate immune responses in the host. Different parameters raised from the analysis of the nucleotide sequence of the mutated genes have been used to predict virus attenuation.

Although RSCU and CPB represent two different concepts, our methodology based on de-optimizing the RSCU indirectly altered the CPB. CPB de-optimization has been correlated with alteration in the ability of the mutant viruses to replicate and produce disease in diverse viruses such as PRRS (Gao et al., 2015), influenza (Mueller et al., 2010), dengue (Shen et al., 2015), human respiratory syncytial virus (Le Nouen et al., 2014) and VSV (Wang et al., 2015). Importantly, the increase in the usage of the unrepresented codon pairs of the attenuated CSFm2 virus, which showed the lowest CBP score among all CSFm mutants, is consistent with those findings. Interestingly, it has been reported that clustering of CSFV isolates based on their CPBs clearly discriminates between highly virulent strains, vaccine strains and moderately virulent strains (Leifer et al., 2013).

It has been reported that genomes of RNA viruses have low CpG and UpA dinucleotide frequencies (Rima and McFerran, 1997; Rothberg and Wimmer, 1981) compared with mammalian genomes (Alff-Steinberger, 1987). Previous reports showed that poliovirus (Mueller et al., 2006; Burns et al., 2006) and echovirus mutants (Atkinson et al., 2014) that have increased CpG and UpA frequencies display decreased replication kinetics compared to wild-type virus. Conversely, virus mutants where CpGs and UpAs were removed displayed increased replication compared to parental wild-type virus (Atkinson et al., 2014). A comparative analysis of CpG and UpA dinucleotide frequencies and attenuation in different virus models indicates correlation between increased frequencies and attenuation (Tulloch et al., 2014). As has been shown using a PRRSV system (Gao et al., 2015), the CSFm mutant construct that displayed the highest CpG and UpA dinucleotide frequency, CSFm1, was not able to produce viable virus progeny. Importantly, of the CSFm mutant viruses that were able to replicate, the mutant presenting the highest frequency of CPG and UPA dinucleotides, CSFm2v, is the only one with an attenuated phenotype in swine.

It is not clear what the molecular mechanisms are underlying decreased virus replication and, more importantly, attenuation of viruses suffering from these synonymous de-optimizations. It has been proposed that de-optimization of codon pair bias produces an alteration in the translation efficiency, affecting the rate of protein production which may result in slower virus replication rates (Coleman et al., 2008; Mueller et al., 2010; Yang et al., 2013). Alternatively, it has been suggested that virus attenuation induced by increased CpG and UpA frequencies can be mediated by an enhanced host innate immune response (Burns et al., 2006; Zsak et al., 1996; Atkinson et al., 2014). Both hypotheses may be combined and virus attenuation may result because viruses replicating at much slower rate, not causing significant damage to a host cell, will subsequently allow the induction of an efficient innate immune response.

We do not know the host/virus mechanisms mediating attenuation during the infection of CSFm2v virus in swine. Attenuated CSFm2v yield in swine macrophage cultures was clearly reduced when compared to parental virulent BICv. Accordingly, CSFm2v virus replication during infection in swine is severely decreased when compared with BICv. Similar results were reported in de-optimized attenuated viruses tested in experimental (Nogales et al., 2014; Meng et al., 2014; Mueller et al., 2006; Wang et al., 2015) as well as natural hosts (Le Nouen et al., 2014; Gao et al., 2015). In regards to a possible stimulation of the innate immune response by the attenuated de-optimized virus as described by others (Burns et al., 2006; Tulloch et al., 2014; Atkinson et al., 2014; Blaszczyk et al., 2000), we found a few changes in the pattern of immune-relevant genes activated in swine macrophages infected by CSFm2v. Increased expression of chemokines MCP-2 and NCP-1 may account for increased recruitment of cells mediating the host innate immune response which in turn may result in decreased virus replication. For example the monocyte chemotactic protein 2 (MCP-2), has been described as a potent inhibitor of HIV-1 strains by interacting with the cell surface receptor CCR5 that is used by this virus as a co-receptor (Blaszczyk et al., 2000).

In summary, it is shown here that introduction of an specific

type of silent mutations in massive amounts through determined area of CSFV genome encoding for structural glycoprotein E2 produced a severe attenuation of the parental virulent phenotype during swine infection. Importantly, infection with the attenuated virus induced protection of the infected animals against challenge with the virulent parental virus. This is the first report showing the development of an attenuated CSFV strain by codon usage de-optimization, and one of the few examples of virus attenuated using this methodology that is assessed in its natural host.

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