Antigenic characterization of African swine fever virus (ASFV) p30 and p54 proteins

by

Vlad Petrovan

B.S., University of Bucharest, 2012
M.S., University of Bucharest, 2014

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine and Pathobiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2019

Abstract

African swine fever (ASF) is a highly contagious and lethal viral disease of swine with significant socio-economic impact in the developed and developing world. In the absence of a vaccine, recent outbreaks in Europe and Asia have drawn interest in developing diagnostics tools that are critical for early detection and implementation of strict biosafety measures. African swine fever virus (ASFV) has a complex organization, containing more than 100 proteins, including those with a structural role and enzymes that are packed in the virus core for use in early infection. Among the structural proteins, p30 and p54 are also highly immunogenic, representing serological candidates for conducting ASF detection and surveillance. Production of monoclonal antibodies (mAbs) and recombinant proteins will help characterize the antigenic regions which, in turn, will lead to the development of novel diagnostic tests against this disease.

In this study, a panel of mAbs was generated against recombinant p54 and p30. First, we developed a screening methodology for the resulting hybridomas using enzyme linked immunosorbent assay (ELISA) and confirmatory immunofluorescence assay (IFA) on ASFV infected cells. Our results identified five mAbs against p54 and three mAbs against p30 which were positive on both assays. Based on the screening methodology and criteria, we further characterized our mAbs by immunoprecipitation, Western blot analysis, ELISA, and immunohistochemistry (IHC) in a wide variety of tissues collected from ASFV-infected pigs. Second, the epitopes recognized by those mAbs were identified using recombinant polypeptide fragments expressed in bacteria or mammalian cells and oligopeptides. These assays identified several linear epitopes which were also recognized by sera from ASFV-infected pigs. Interestingly, the anti-p30 mAbs also recognized a region that has similar characteristics similar

to an intrinsically disordered protein (IDP). Third, we evaluated the efficacy of our generated mAbs in a highly sensitive blocking ELISA, using known positive and negative serum samples.

The results presented in this thesis provide valuable tools for improving ASFV diagnostics, surveillance, and vaccine development.

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Acknowledgements

I would like to express my sincere gratitude to Dr. Bob Rowland for his wisdom in mentoring me during my PhD program. I would like to thank my committee members, Dr. Ying Fang, Dr. Jishu Shi, Dr. Jodi McGill and Dr. Frank Blecha for their wisdom, valuable advice, and for reviewing my work. Also, I would like to thank my committee chair, Dr. Paul Smith for his support. I must thank the people that supported my research: Ana Stoian, Luca Popescu, Maria Murgia, Maureen Sheahan, Becky Eaves, Francine Rowland, Pengcheng Shang, Fangfeng Yuan, and Sailesh Menon. I would like to thank my friends that helped me throughout this journey: Philip Freda, Renan Donadelli, John Coffin, Matt Galliart, Jake Alsdurf, and Em Collin. Lastly, I would like to acknowledge the support of my friends from Romania, especially Dr. Mihaela Zaulet who helped me through the first steps in my career.

Dedication

I would like to dedicate this work to my parents, Emilia and Vania, who supported me unconditionally through my entire life and through my Ph.D. journey. I can’t thank you enough!

Chapter 1 - Introduction

1.1 History and distribution

East African swine fever was first described in the early 1900s, as a disease that caused high morbidity and mortality among domestic pigs (*Sus scrofa domesticus*). The first infection experiments revealed that warthogs (*Phacochoerus africanus*), bush pigs (*Potamochoerus porcus*) and giant forest hogs *(Hyclochoreus meinerizahageni*) are resistant to the disease; however, they could play a role in disease transmission to the domestic pig (Montgomery, 1921). Montgomery also provided pioneering studies regarding the nature of the causative agent, survival in the environment, transmission and host range. Subsequently, the disease was reported in Northern parts of the African continent, between Algeria and Morocco (Donatien and Lestoquard, 1940). Since the initial discovery, the disease name has changed to African swine fever (ASF).

The first known transcontinental spread of ASF occurred when it was found in Portugal in 1957, but the outbreak was confined by slaughtering all the infected pigs (Ribeiro et al., 1958). However, a second outbreak occurred again in Portugal in late 1960, caused by contaminated pig products from Angola. This allowed the introduction into neighboring countries (Spain, France, Italy, Belgium and the Netherlands) and from there into South America and the Caribbean. As of today, ASF still remains endemic in Africa and on the island of Sardinia, in Italy.

A second transcontinental spread occurred more recently, in 2007, from Senegal to Georgia, with subsequent spread to neighboring Caucasus countries (Rowlands et al, 2008). Currently, the disease is present in the Trans-Caucasus region, parts of the Russian Federation and Ukraine, Poland, Latvia, Lithuania, Estonia, Moldova, the Czech Republic, Romania and Belgium.

Following an outbreak in Russia in 2018, a third transcontinental spread occurred into China, the biggest swine producer worldwide and from there into Mongolia, Vietnam and Cambodia (Figure 1.1; OIE WAHIS, 2019; Zhou et al, 2018).

Overall, ASF spread into Europe and China now endangers the pig industry worldwide. Outbreaks in swine-producing countries result in severe economic losses due to pig mortality, costs associated with disease control and eradication, and trade banning. It is estimated that an ASF outbreak in the US would be catastrophic with costs of over 15 billion dollars in the first year of outbreak (OIE WAHIS 2019).

1.2 Etiologic agent

ASF is caused by the African swine fever virus (ASFV), a large, icosahedral, double stranded DNA (ds DNA) virus, which is the only known member of the *Asfarviridae* family and the only known vector-borne DNA virus. ASFV belongs to a virus super-family, along with *Poxviridae, Iridoviridae, Phycodnaviridae, Mimiviridae* and other giant virus families (Dixon et al., 2005; Iyer et al., 2006; Colson et al., 2013). Formerly classified with the *Iridoviridae,* based on its capsid morphology, ASFV was reclassified in 2001 and is now the only known member of the family *Asfarviridae* in the genus Asfivirus, which occupies a separate clade between the *Poxviridae* and *Iridoviridae* (Fauquet & Mayo, 2001). Moreover, the capsid resemblance between ASFV and the iridoviruses has led to the idea of an evolutionary relationship between the viruses (Solas et al., 1999). Recently, an icosahedral ds DNA virus was isolated from an environmental sample and shares structural similarities with ASFV, Faustovirus and Kaumoebavirus (Andreani et al., 2017). Due to this recent expansion of newly described giant amoeba viruses, the *Asfaviridae* family may soon contain other members.

ASFV has a wide range of genetic variation (24 different genotypes) as shown by the sequencing of the C-terminal end of the major capsid protein (B646L/p72) and full sequencing of p54. Variation between the same genotypes is shown by sequencing the central variable region (CVR) (Bastos et al., 2003; Achenbach et al., 2017; Quembo et al., 2018). Of the contemporary strains, Genotype I is currently circulating through West and Central Africa, was introduced to Europe in 1957 and 1960, and is currently present in Sardinia. More recently, Genotype II was introduced in Georgia in 2007, spreading throughout most of Europe and into Russia by 2017, and is now found in South East Asia. (Rowlands et al., 2008; Zhou et al, 2018).

1.3 Pathogenesis and clinical signs

ASFV can cause a broad spectrum of clinical outcomes, ranging from hyperacute or acute infection (with mortality up to 100%) to subclinical or chronic infections, depending on the virulence of the strain, exposure or doses (Kleiboeker, 2002). The incubation period can vary from two to five days in experimental infections and five to seven days in natural-infected cases and is usually correlated with acute or subacute disease presentations (Gallardo et al., 2018; Sánchez-Cordón et al., 2018). Interestingly, ASFV causes persistent chronic infection, in African wild pigs and in soft ticks of the genus *Ornithodoros* (Heuschele and Coggings, 1969; Parker et al., 1969). However, this is not the case regarding infection of domestic pigs. ASFV enters the body via the tonsils or pharyngeal mucosa and after 24 to 30 hours post infection (hpi) the virus can be found in all the lymphoid tissues (Greig, 1972; Blome et al., 2013).

Highly virulent strains, such as the currently circulating strain, Georgia/07, may cause hyperacute disease, in which pigs die between three and five days after infection (Guinat et al., 2016). Under those circumstances, the disease is associated with anorexia, high fever, nasal

hemorrhages, cutaneous erythemas and skin cyanosis, melena and, in some cases, diarrhea (Kleiboeker, 2002). Pathological observations, presented in Figure 1.2 can include pulmonary oedema (Figure 2-A), hemorrhagic heart (Figure 2-B), multifocal cortical hemorrhages (petechiae) on kidney (Figure 2-C), hemorrhagic lymph nodes with marked size increase (Figure 2-D), enlarged tonsils (Figure 2-E), and splenomegaly (Figure 2-F). Tissues presented in Figure 1.2 were collected from a pig, infected with the strain Georgia/07, Genotype II (3 x $\log_{10}$ $TCID_{50}$/ml at 5 days post infection), that exhibited the acute form of the disease.

Infections with moderate/low virulence strains (e.g. attenuated strains, like OURT 88/3) can cause a wide range of mortalities from 0 to 60%. Clinically recovered animals, depending on the strain used for infection, may be persistently infected for a long time (Villeda et al., 1993; Leitao et al., 2001 Figure 1.3). Even after apparently recovery, death can occur between days 15 and 20 after the infection, with presentation of milder clinical signs and lesions than the acute form. However, this outcome is characterized by extensive hemorrhages in lymph nodes, kidneys, and spleen, with diffuse organ enlargement and swollen joints due to antibody-antigen depositions. In the chronic form of ASF, which can also be caused by attenuated strain, OURT 88/3, few if any respiratory clinical signs are seen (Gómez-Villamandos et al., 2003). Nevertheless, lesions such as pleuritis, pleural adhesions, pneumonia and reticuloendothelial hyperplasia of lymph nodes are often found in the chronic form, however, a number of these lesions may be attributable to bacterial secondary infections.

1.4 Direct and indirect transmission

ASFV can be transmitted *via* direct contact with infected animals or indirect contact *via* contaminated fomites, uncooked meat from infected animals, or through arthropod vectors

(Penrith & Vosloo, 2009 and Figure 1.4). The natural arthropod host is the soft tick from the genus Ornithodoros. In sub-Saharan Africa (Tanzania), *Ornithodoros moubata* (Walton) serves as the ASFV vector between ticks living in underground burrows and different species of wild suids, thereby creating the sylvatic cycle of the disease (Plowright et al., 1969). As mentioned before, warthogs (*Phacochoerus africanus*), bush pigs (*Potamochoerus porcus*) and giant forest hogs (*Hyclochoreus meinerizahageni*) are resistant to disease. Identifying possible resistance markers between the above-mentioned species and the domestic swine (*Sus scrofa*) still merits further investigation.

The sylvatic cycle is maintained through trans-stadial, venereal and trans-ovarian transmission of the virus in the tick population (Plowright et al., 1974). In Europe, ASFV was isolated from *O. erraticus* after the Spanish outbreak, in 1960 (Sanchez-Botija, 1963). Other studies demonstrated transmission between pigs and ticks from different sub-species belonging to different geographical regions: *O. coriaceus*, *O. turicata* (in North America), *O. savigny* (in Africa), and *O. puertoicensis* (in the Carribean) (Groocock et al 1980, Mellor and Wilkinson 1985, Hess et al, 1989). During the feeding process, ticks salivary glands secrete more than 100 secretory proteins that facilitate the feeding process through a rich repertoire of hemostatic and inflammatory compounds, and various blood modulators (Mans et al. 2008). Overall, *Ornithodoros* ticks have a very broad geographical distribution, can live for extended periods of time without feeding and can feed without causing an inflammatory response; therefore, posing a risk of (re)introducing ASF long after the disease was believed to have been eradicated from an area.

ASFV is currently the only known DNA arbovirus, and replication and transmission are adapted to the tick's anatomy. Kleiboeker et al., 1998 showed that the first indication of viral

replication in ticks occurs at 3 days post infection (dpi) in the midgut compartment where cells also lack ribosomes, vesicles, microtubules and filaments. At 21 days post feeding, many of these digestive cells are ASFV positive by immunohistochemistry (Kleiboeker et al., 1999). While at 42 dpi, evidence of ASFV replication could be found in the midgut, hemocyte, coxal gland, connective tissue and salivary gland; with salivary gland secretions containing 2–3 $\log_{10}$ of ASFV (Kleiboeker et al., 1998). However, other studies showed primary viral localization to the midgut (Greig, 1972) and hemocytes (Endris et al., 1987).

Experimentally infection of tick with an ASFV isolate from the same geographic region results in a high-titer, persistent infection with no gross differences from uninfected ticks (Kleiboeker et al., 1998). However, exposure of European and North American ticks to a variety of ASFV isolates results in high mortality but also long-term persistence (Endris et al., 1991; Hess et al., 1987). The mechanism of tick death, other than the metabolic pressure placed upon adult females laying eggs while carrying a large viral load (Kleiboeker et al., 1998) and the rupture of the gut while taking in an ASFV infected blood meal (Rennie et al., 2000) is unknown. The lack of ASFV generalization in ticks after oral infection with non-adapted isolates (e.g. Malawi strain grown in South African ticks) may be attributed to the premature death of infected cells in the gut (Kleiboeker et al., 1999). The reason for ASFV clearance from ticks in lab colonies has not been determined (Hess et al., 1989). Recently it was shown that ASFV present in blood-fed *Stomoxys* flies or *Tabanidae* flies could potentially result in ASF infection (Olesen et al., 2018).

Indirect transmission can occur if healthy pigs ingest infected meat products or have contact with contaminated fomites (Mur et al., 2012). The virus persists for more than 1000 days in frozen meat, and is it highly resistant to inactivation in the environment in the presence of

organic material. As an example, the 2007 outbreak in Georgia has been attributed to the improper disposal of infected pork from a ship at the port of Poti (Beltrán-Alcrudo et al., 2008). A recent study described that ASFV can survive in different feed ingredients or feed products, when exposed to a simulated transboundary transportation model shipment from Europe to the United States (Dee et al., 2018). Moreover, ASFV has a higher risk of introduction in five major US airports, based on the origin of the flight and passengers' habits of bringing potentially contaminated pork products (Washington-Dulles, George Bush-Houston, John F. Kennedy-Queens, Warwick and San Juan) (Jurado et al., 2018).

Transmission can also occur through direct contact between sick and healthy animals or by contact with infectious excretions and contaminated equipment. Feces, urine, blood and oral fluids can easily contaminate water sources, soil and animal pens. Recent transmission studies revealed that infected pigs had high titers of virus in blood, nasal, and rectal fluid, independent of inoculation route, dose or contact between pigs (Gallardo et al., 2015). Similar infection kinetics were observed in transmission studies using wild boars, revealing that even a low dose was enough to establish a persistent infection (Pietschmann et al., 2015).

1.5 Virus structure, virulence and antigenic variation

The ASFV particle consists of multilayered domains: the internal core formed by the central genome contains the nucleoid, which is coated by a thick protein layer named the core shell; an inner lipid envelope surrounding the core; and finally, the capsid protein, which is the outermost layer of the intracellular virion, ranging in size from 170-190 nanometers (nm) in diameter. Electron micrographs of negatively stained and shadowed capsids revealed capsomers with an icosahedral structure (Carrascosa et al., 1984). The extracellular virus acquires an

external membrane after budding out through the cisterna derived from the ER, increasing the size of the virion to 175 - 215 nm (Carrascosa et al., 1984; Germán-Andrés et al., 1998; Breese Jr. & DeBoer, 1966). These ER-derived viral membranes represent the first morphological evidence of virus assembly being the precursors of the inner viral envelope (Germán-Andrés et al., 1998). The envelope precursors will assemble into icosahedral viral particles through a process involving mitochondria recruitment supplying the ATP- and calcium-dependent processes that the virus morphogenetic processes may require (Cobbold et al., 1996).

The virus encodes between 150-165 proteins, depending on the strain, which play a role in viral architecture, viral replication and evasion of host defenses (Figure 1.5; Dixon et al., 2013). The genome is a dsDNA molecule, varying in length between different isolates from 170 to 190 kbp (Chapman et al., 2008). The first study that investigated the complexity of ASFV genome was conducted in 1995, when the Vero adapted strain, Badajoz 1971 Vero (BA71V), was sequenced (Yanez et al., 1995). The sequencing of the additional non-pathogenic and highly pathogenic isolates allowed comparative genomic analyses for understanding molecular mechanisms associated with cell tropism and virulence (Dixon et al., 1994; Yozawa et al., 1994).

Variation between the genomes is observed in the number of copies of different multigene families (MGF) that are present in the left or right variable regions (LVR/RVR) of the genome and in 14 viral proteins (including CD2-like, p54, B602L and α-like DNA polymerase) (Portugal et al., 2015). Moreover, sequence analysis revealed some similar characteristics between poxviruses and iridoviruses (Yanez et al., 1995; Rodriguez et al., 1994). To date, studies show that ASFV contains five MGF's: 100, 110, 300, 360 and 505/530, named according to the average number of amino acids in the encoded proteins (Chapman et al., 2011). Several studies showed that genes that are present in MGF 360 and 505/530 have evolved by gene duplication

and determine host range and virulence for field isolates of ASFV. Adaptation of those wild type strains or highly virulent ones (e.g. Georgia/07) to cell cultures led to loss of members of the MGF 110, 360 and 505 families (De la Vega et al., 1990; Krug et al., 2015). Therefore, deletion of MGFs allowed the generation of recombinant viruses with attenuated virulence and induction of protection against challenge with different strains.

Other genes have been attributed as playing a role in the natural hosts' (ticks and/or African wild pigs): immune evasion, including A238L as an inhibitor for NF-kB and NF-AT (Neilan et al., 1997; Granja et al., 2006); and A179L, A224L EP153R as apoptosis inhibitors (Hurtado et al., 2004). Moreover, MGF's 360 and 505 are believed to play a role in inhibiting the induction of IFN β/α and affecting the viral replication in ticks (Neilan et al., 2002; Afonso et al., 2004; Burrage et al., 2004). These are some of the ASFV genes that are important in virulence; however, they are insufficient to fully explain it, indicating that other viral determinants or a combination of these should play a key role. Detailed information regarding host evasion is discussed in section 1.9 and 1.10. An important aspect between virulent and non-virulent strains lies in the differences of C-type lectin-like protein, and pEP402R, a homologue of CD2-type receptors of T lymphocytes, which are found in truncated forms in non-virulent isolates (Portugal et al., 2015).

These interesting aspects regarding genomic variation suggest that if pigs survive challenge, the challenge strain should be sequenced for genomic comparison and characterization of viral phenotype.

1.6 Structural and non-structural ASFV proteins

The complexity of ASFV is given by the architecture of the virus. The genes encoded by the ASFV genome are closely spaced and encoded on both DNA strands with no clear bias for coding of genes on either strand. As mentioned previously, sequencing of different ASFV isolates including tissue culture adapted strain BA71V, 10 field isolates from Europe (OURT 88/3, OURT 88/1, isolated between 1980-1990 from ticks in Portugal) and Africa (Kenya 1950, Malawi, etc.) and recently the contemporary isolate Georgia/07, allowed the identification of more than 110 conserved open reading frames (ORF's) (de Villiers et al., 2010; Chapman et al., 2011). The nomenclature relies on the *EcoRI* fragment at the 5' end of each ORF, followed by the number of amino acids encoded and a letter, indicating the direction of transcription (leftwards or rightwards). Around 25 proteins that are encoded play a role in either the structure or the morphogenesis of the viral particle (summarized in Table 1.1).

Construction of ASFV recombinants expressing copies of some of those genes under inducible isopropyl-β-D-thiogalactopyranoside (IPTG) combined with the use of specific antisera raised against the protein(s), allowed the identification of their roles in virion morphogenesis. More exactly, the system consists in the *lacI* gene, encoding the repressor protein, introduced in the thymidine kinase (TK) locus, under the control of the ASFV early/late promoter pU104L, and an IPTG-inducible promoter, formed usually by the p72 promoter, followed the gene of interest (Almazan et al., 1992; Garcia-Escudero and Vinuela, 2000). The most relevant structural proteins are: p72, p54 (will be discussed in Chapter 2), p30 (will be discussed in Chapter 3), pp220, pp62, CD2v, p10, p12, p14.5, p29, p27, p34, p17. Generation of recombinant viruses which express the protein p72 revealed its role as the major capsid protein on the external surfaces of membranous structures, it interacts with ER membranes and the

requirement for a viral chaperone for correct assembly, encoded by B602L (Cobbold et al., 1996; Garcia Escudero et al., 1994). Moreover, p72 is one of the most immunogenic ASFV proteins and a target for neutralizing antibodies (Borca et al., 1994; Kollnberger et al., 2002). As mentioned previously, p72 is also used to genotype different ASFV isolates (Bastos et al., 2003).

Two of the biggest proteins based on their molecular weight are pp220 and pp62, ASFV polyprotein precursors, that are cleaved into the mature virion proteins. The polyprotein pp220, encoded by CP2475L, is cleaved to yield the mature virion proteins p150, p37, p14, p34, p5 and the polyprotein pp60, encoded by CP530R, is cleaved to p35 and p15, p8 by a viral SUMO-like protease recognizing Gly-Gly-X motifs (Dixon et al., 2013; Alejo et al., 2018; Figure 1.6). Experiments also revealed that pp220 and pp62 interact with each other to form the core shell below the inner lipid envelope (Salas et al., 2013).

The p10 structural protein is encoded by the K78R gene and codes for a DNA binding protein for both double- and single-stranded DNA. The protein is extremely hydrophilic and enriched in lysine residues (23%) (Muñoz et al., 1993). Using a yeast-based nuclear import assay it was shown that p10 is actively imported into the nucleus of yeast cells, therefore implying that it could play a role during the viral infection cycle (Nunes-Correia et al., 2008).

The viral attachment protein p12 was initially localized on the external part of the envelope, above the capsid, based on the treatment with β-D-octylglucopyranoside (Carrscosa et al., 1993; 1991). However, recent studies revealed that p12 localizes also in the virus factories as well as into virus particles spread throughout the cytoplasm then through the cell surface (Salas et al., 2013). Interestingly, a p12 peptide was able to inhibit virus attachment, but anti p12 antibodies did not neutralize the virus (Angulo et al., 1993).

Another protein that is localized on the outer envelope is the CD2v (EP402R) homologue with the T and NK cells proteins, which plays a role in hemadsorption to erythrocytes and is involved in establishing a partial protective immune response (Rodriguez et al., 1993; Burmakina et al., 2016). CD2v is a glycosylated, type II membrane protein, which contains three functional domains: a signal peptide, a trans-membrane region, and two immunoglobulin-like domains. Previously, it has been shown that CD2v binds mABp1, an actin binding adaptor protein implicated in vesicular transport, implying a role in ASFV transport inside the cell. The C-terminus part of CD2v contains a hypervariable region that does not share any amino acid similarity with the cellular CD2v cytoplasmic domain. Recently is was shown that together with C-type lectin, CD2v is mediating haemadsorption inhibition (HAI) serological specificity, therefore representing a good candidate to serotype ASFV isolates (Malogolovkin et al., 2015). However, not all ASFV strains have the CD2v protein, therefore classical genotyping should be used to characterize ASFV strains. On the other hand, C-type lectin (EP153R) induced increased apoptosis after infection of macrophages, therefore playing a role in regulating cell death. The role of those two proteins in vaccine development will be discussed later.

Another relevant protein involved in virus morphogenesis is p14.5, encoded by the gene pE120R, which is localized in the capsid layer of the virions. The protein interacts with its binding partner, the major capsid protein p72 and with ss/ds viral DNA in an independent manner (Martinez-Pomares et al., 1997). Therefore, this protein could play a role in encapsidation of ASFV during late stages of the replication cycle.

Protein p17, encoded by the late gene D117L, is an abundant structural protein that is expressed late in the viral infection cycle (Simón-Mateo et al., 1995). The protein contains a type

I transmembrane region, is localized on the viral internal envelope and plays a role in interactions of capsid proteins with viral membrane precursors (Suárez et al., 2010).

However, the above-mentioned proteins were described in early reports using classical approaches to study structural ASFV proteins which were laborious and sometimes inconsistent. As mentioned previously, p12 protein was initially described as being incorporated into the viral membranes, but now it is generally accepted that it plays a role in attachment to host cells membranes. Due to the lack of knowledge of other ASFV structural proteins it is not known if they have any role in morphogenesis or immunity. The latter will represent the "golden ticket" towards developing better vaccines. In the light of recent advancements in proteomic analysis using nano-liquid chromatography (nLC), matrix-assisted laser desorption/ionisation tandem time-of-flight (MALDI-TOF/TOF) and reversed-phase high performance liquid chromatography (HPLC) coupled via a nano-spray source to a quadrupole time of flight (QTOF) spectrometer for tandem mass spectrometry (MS/MS), we now have a better understanding of biological and structural analysis and host response during ASFV infection both *in vitro* (using BA71V strain) and *in vivo* (using E75 strain) (Alejo et al., 2018; Herrera-Uribe et al., 2018; Keßler et al., 2018). As a result, the study conducted by Alejo et al. identified 44 new viral proteins (from which the representative ones are p5 and p8, part of pp220 and pp60, respectively). Another study also identified three viral proteins K145R, pC129R, pI73R which are abundantly expressed in three different cell lines and they do not have any homology with known viral proteins (Herrera-Uribe et al., 2018). This brings the percentage of proteins with unknown function to 34% of the viral mass. Moreover, those proteins do not have any homology with other viral families. The results also revealed that the virus is dedicating around 24% of its proteins towards structure and morphogenesis, 19% of its proteins are involved in viral transcription, 6% are maintaining the

genome integrity, 4% are involved in virus attachment and entry and 3% are directed towards evading host immunity and the rest are represented by proteins with unknown function. A summary of the enzymes and non-structural proteins with different functions regarding DNA replication, nucleotide metabolism, host evasion, DNA repair and transcription are presented in Table 1.2

ASFV infects cells belonging to the monocytes/macrophage lineage, which are rich in reactive oxygen species (ROS) inducing breaks and nicks in the viral DNA. Mammalian pathways can repair the damages induced by ROS by a base excision repair pathway (BER). ASFV does not possess a BER pathway, therefore has evolved its own repair mechanism. The major players involved in base excision repair are DNA polymerase (ASFV *pol X*) and ligase (ASFV *LIG*) (Oliveros et al., 1997). However, the fidelity is low, and they tolerate base mismatches; therefore, this mechanism is advantageous for the virus providing genotype evolution or viral recombinants. Using X-ray crystallography and nuclear magnetic resonance, now we can visualize the structure of those 2 enzymes involved in DNA repair and replication. A recent study revealed that 4 amino acid residues (two residues in the adenylation domain and two in the oligonucleotide/oligosaccharide-binding fold domain) are important in catalytic activity of ASFV *LIG*, therefore representing a new strategy of developing live mutant viruses that could be used in vaccine development (Chen et al., 2019).

In adition, *in vivo* proteomic analysis of lymph nodes from pigs infected with high virulence or low virulence homologous isolate revealed different signaling pathways correlated with tissue destruction, downregulation of immune responses (including Rho GTPases, a key player in regulating migration of Toll Like Receptors). Interestingly, expression of the host nuclear ribonucleoprotein complex (hnRPC) was upregulated by day 1 on both strains but

remained, surprisingly, in the attenuated strain at 3- and 7-days post infection (Herrera-Uribe et al., 2018). This is important because in Chapter 3 the properties of ASFV structural protein p30, which interacts with hnRPC and possibly plays a role in downregulation of host mRNA translation, will be discussed.

A possible hypothesis between the differences in upregulation of hnRPC can be related to the fact that ASFV p30 can interact differently with its binding partner, which can be strain dependent.

1.7 Mechanism(s) of attachment and entry

The ASFV infectious cycle starts with viral attachment and entry into the host cell. ASFV interaction with cellular receptor/s or ligands promotes subsequent entry steps involving the activation of signaling pathways and endocytosis. Early studies compared wild type and tissue culture adapted strains and showed that the entry mechanism is a low pH- and temperature-dependent process, which is consistent with saturable and specific receptor-mediated endocytosis (Alcami et al. 1989; 1990). However, the receptors are necessary but not sufficient for efficient viral production, as they are not the only factors affecting productive infection (Carrascosa et al., 1999).

One proposed receptor is cluster of differentiation (CD) 163, a member of the scavenger receptor cysteine-rich (SRCR) family, whose expression is restricted to a subpopulation of macrophages described as anti-inflammatory (having an M2 phenotype which will be discussed later). In addition, CD163 it is used as a marker for monocyte differentiation.

Macrophages incubated with an anti-CD163 antibody, blocked infection in a dose-dependent manner (Sánchez-Torres et al., 2003). However, the role of CD163 in ASFV was

resolved since several studies showed that in non-permissive cells, CD163 expression is not required for infection; secondly, there is no correlation between monocyte/macrophage subsets and CD163 expression and ultimately, the gene-edited pigs lacking CD163 were not resistant to infection with ASFV Georgia 2007/1 (Lithgow et al., 2014; Franzoni et al., 2017; Popescu et al., 2017). Moreover, the permissiveness of the cell lines^{CD163+} could be strain dependent (Sanchez et al., 2017). These observations indicate that CD163 is not essential for ASFV infection both *in vitro* and *in vivo*. More, ASFV can replicate in cells that do not express CD163, such as Human embryonic kidney (HEK) cells, suggesting that the virus can bind to other receptors or it can use alternative pathways to enter cells (Herrera-Uribe et al., 2018).

One alternative pathway used by different viruses to enter cells is related to the presence of antibodies that could facilitate cell entry using Fc receptors (or a both viral and Fc receptors), complement receptors or C1qR receptor, a process called antibody-dependent enhancement (ADE). The effect of ADE will lead to an increase in target cell infection, which could be associated with an exacerbation of the disease (Sauter & Hober, 2009). Antibodies generated during vaccination generated a response similar to ADE after challenge and the vaccinees showed exacerbated clinical signs compared to the controls (Argilaguet et al., 2011). A more recent study showed that there is a correlation between enhanced clinical signs and *in vitro* enhancement following vaccination using a combination of DNA vaccination and recombinant proteins (Sunwoo et al., 2019). Those results are building upon older *in vitro* studies where it was shown that ASFV entry is not mediated by Fc receptors (Alcami & Vinuela, 1991). A tempting hypothesis is that the viral replication would use an alternative ADE mechanism.

ASFV has been shown to internalize into the cells by using two distinct endocytic pathways: macropinocytosis and clathrin-mediated endocytosis (CME). CME is regulated by

several proteins and lipids which take part in the membrane bending and elongation. The viral protein p30 was shown to be inhibited when cells were treated with either chlorpromazine (CPZ-2), a CME inhibitor, or with Dynasore, a dynamin inhibitor; which has an important role in cellular membrane fission, required only for CME (Hernaez & Alonso, 2010).

Cells treated with different micropinocytosis inhibitors such as EIPA (Na+/H+ channel inhibitor), IPA-3 (Pak-1 inhibitor) or Cytochalasin D (inhibitor of actin filament polymerization), affected viral internalization and production of the early protein p30 (reviewed in Sanchez et al., 2017). Based on recent results, a model for ASFV entry is presented in Figure 1.7. Other components have been used to inhibit virus attachment, such as thermally reduced graphene oxide (TRGO) and linear polyglycerol sulfate (IPGS). However, the exact mechanisms of inhibition and the potential biological application are still unknown (Ziem et al., 2017).

Knowledge of the viral and binding host partners involved in entry/internalization and the role of antibodies in enhancing the infection could offer new potential therapies against the virus.

1.8 Viral replication and transcription

As already mentioned, ASFV replicates predominantly in cells belonging to monocyte/macrophage lineage and some specific lineages of reticular, polymorphs, and megakaryocytic cells (Casal et al., 1984; Wardley and Wilkinson, 1978); replication has been observed to a lesser extent in endothelial cells (Wilkinson and Wardley, 1978), hepatocytes (Sierra et al., 1987), renal cells (Gomez-Villamandos et al., 1995) and neutrophils (Carrasco et al., 1996). ASFV provides a perfect study model to better understand its viral replication and how it modulates the host immune system.

The replication cycle is split into two distinct phases: an early phase, at 4-6 hours post infection (hpi) that occurs inside the nucleus, and it declines after 8-12 hpi. The nucleus involvement has been detected by bromodeoxyuridine pulse experiments, *in situ* hybridization, and autoradiography on infected Vero cells and monocyte derived macrophages (Rojo et al., 1999; Simoes et al., 2015). As seen in other large DNA viruses (like Poxvirus or Adenovirus), the short viral fragments (~2000 nucleotides) found in the nucleus might be used as replication templates after diffusion into cytoplasmic factories or for generation of mRNA transcripts. The need for the nucleus was initially described using enucleated Vero cells that upon infection with ASFV did not maintain viral replication (Ortin & Vinuela, 1977).

The second phase of replication starts between 12-19 hpi and takes place in the perinuclear area close to the microtubule organizing center (MTOC), for both virulent and tissue culture adapted strains (Rojo et al., 1999; Simoes et al., 2015). ASFV has its own DNA replication/repair system consisting of an AP endonuclease (APE), an extremely poor prone repair polymerase (*Pol X*) (Figure 1.9 lower graph), and an extremely error tolerant ATP-dependent DNA ligase. After the DNA replication starts apoptotic markers are detected both *in vitro* and *in vivo*. ASFV encodes three proteins that inhibit apoptosis (A224L, A179L, and EP153R), and one protein (E183L) that can induce it (Reis et al., 2017a). From a virus perspective this strategy is advantageous because it creates a temporal coordination between viral replication and induction of cell death. Also, both virulent and non-virulent isolates inhibit apoptosis in the same manner, therefore not representing a virulence marker.

The similarities between ASFV and Vaccinia virus structure promoted researchers to establish a model for replication (Figure 1.8). Poxvirus genes that are in variable regions are often nonessential for viral replication *in vitro*, but they present functions related to viral host

range (Massung et al., 1993). Initiation of replication starts with the introduction of a single-strand nick in the genome near the 5' or 3' termini. The exposed 3' OH group acts as a primer for DNA polymerase and DNA synthesis proceeds towards the genome termini. This generates an intermediate in which termini of nascent and template strands are self-complementary and fold-back to form a self-priming hairpin structure. The replication is initiated by a DNA primase encoded by C962R leading to suggestions of a modified model for ASFV replication (Dixon et al., 2013).

Around 19% of the genome capacity codes for genes that are currently considered to be involved in transcription and modification of viral mRNA, although the function of most of these has been postulated from comparative studies with different viruses (Rodríguez & Salas, 2013; Alejo et al., 2018) (Table 2.2). Upstream of each gene is a short promoter sequence rich in adenine and thymine (A/T), which is recognized by the viral RNA polymerase complex (Figure 1.9) during various stages of viral gene expression: early, intermediate and late. ASFV early gene expression in infected cells is detectable as early as 1 hpi, reaching a plateau at 2–6 hpi. However, detectable level of pre-replicative transcripts has been observed through the late times and has been attributed to a basal activity of their promoters (Almazán et al., 1992). Finally, the reactivation of the expression in late infection stages (20 hpi) is a common characteristic shared by most of the early and immediately early genes that have been characterized (Almazán et al., 1992; Rodríguez et al., 1993). After the DNA replication starts, the pattern of transcription changes at about 6 hpi (Salas et al., 1986). In contrast, other regulatory elements involved in viral replication are still unknown (Ex. involvement of different transcription factors). The whole replication repertoire is similar to poxviruses, where gene expression is controlled at the level of transcription initiation through a variety of mechanisms (Broyles, 2003). ASFV transcripts are

modified by addition of a 5' cap and 3' poly-A tail. Interestingly, the gene encoding the mRNA capping enzyme (NP868R) encodes a polypeptide containing all three catalytic domains, (triphosphatase, guanyl transferase and methyltransferase) required for this function (Iyer et al., 2006). As expected, ASFV takes advantage and recruits all the components of the translation machinery to the "viral factories".

Taken together, ASFV possesses a remarkable independence from its host for the temporal control gene expression.

Although basic information is available regarding the mechanisms of ASFV replication, many details of the process are lacking. The role of the nucleus, during first steps in viral replication, especially the role of p30 and the interaction with the hRNP, merits more in-depth exploration, since early experiments using enucleated cells are four decades old.

1.9 Innate immunity

ASFV modulates the host's innate and adaptive responses, thus providing a strategic advantage to overcome these responses. However, this strategy is costly for the host, since ASFV infects cells belonging to monocyte/macrophages lineage. In turn, those immune cells maintain tissue homeostasis; playing a key role in the clearance of senescent cells, destruction of pathogens and activation of different defense mechanisms. Once activated, macrophages will differentiate into functionally specialized subsets defined as 'classically' or 'alternatively' activated macrophages, also known as M1 and M2. Also, different ASFV strains varying in virulence influence the cytokine responses. ASFV can infect dendritic cells (DCs), which are the most important antigen presenting cells (APC), capable of activating a naïve T-cell response (Gregg et al., 1995).

The first line of defense against viral pathogens is represented by the innate immune responses. The components of the innate immunity also orchestrate the adaptive immune response. The antiviral response triggered is part of the innate immune response to viral pathogens. Viruses have evolved counteracting measures against the host's innate immunity, where IFN I (IFN-α and IFN-β) produced by the infected cells plays a crucial role (reviewed in Hoffmann et al., 2015).

The IFN I signaling pathway is triggered upon binding and entry of viruses or by IFN secretion by other cells, which cause modifications of cellular membranes, cytoskeleton and endocytic pathways. Initially, the cell response starts with the recognition of specific pathogen associated molecular patterns (PAMPs), which are a distinct set of molecular features of invading pathogens. The PAMP signatures are detected by a set of specialized proteins called pattern recognition receptors (PRRs), which are expressed by among others, macrophages and DCs (Diebold, 2010; Christensen and Paludan, 2017). Among the PRRs that are stimulated by dsDNA viruses like ASFV include the TLR's 3 and 9. As expected, ASFV genome encodes a TLR-3 antagonist that can keep the TLR-3 signaling pathway under control in the first stages of infection, especially in the highly virulent forms of the virus (de Oliveira et al., 2011).

Once activated, TLR's initiate a cascade of pathways through a variety of unique adaptor proteins, like MyD88, which converge in the activation by phosphorylation of the transcription factor IFN regulatory factor 3 (IRF3) and activating the nuclear factor-kappa B (NF-κB). These translocate to the nucleus and activate the expression of IFN-β at a first stage, which then via an autocrine loop can induce IRF7 expression. IRF7 in its turn activates the expression of both IFN-α and β subtype genes (Hoffmann et al., 2015). The secreted IFN I (α/β) bind to its receptor on the infected and neighboring cells and activates the Janus kinase/signal transducer and activator

of transcription (JAK-STAT) pathway, leading to activation of central mediators STAT1 and STAT2 by phosphorylation. These form heterodimers with IRF9, assembling in the complex interferon stimulated gene factor 3 (ISGF3), which translocate to the nucleus and activates the transcription of interferon stimulated genes (ISGs) encoding hundreds of molecules involved in inhibition of viral gene expression, degradation of nucleic acid, regulation of transcriptional events, thus creating the anti-viral state (Sadler and Williams, 2008). Figure 1.10 from Bowie & Unterholzner illustrates the general mechanism of TLR pathway activation.

Using different evasion strategies, ASFV has evolved a complex set of mechanisms that inhibit transcription of type I interferon (IFN), different cytokines and chemokines, adhesion molecules and other immunomodulatory genes. For example, A238L has a homology to IκBα, and it interacts with p65 of the NF-κB family of transcription factors, thereby acting as an inhibitor of its activity (Almeida et al., 2012). This was shown using two strains of different virulence infecting porcine macrophages. Protein A238L was detected in both cases at high levels, however higher mRNA expression and production of IFNα, TNFα and IL12 was seen in the highly virulent strain only (Gil et al., 2008). This implies that highly virulent strains might have additional components that help downregulating the immune responses.

Upregulation of several ISG's has been observed during infection with wild type ASFV, compared with infection with a deletion mutant lacking six genes from MGF360 and two genes from MGF530. Moreover, the deletion of those genes severely reduces viral replication in ticks (Burrage et al., 2004). One of the genes, A528R, was shown to play a dual role in the inhibition of both IFN-β and NF-κB. It might also play a role of virulence marker between different virulent strains since IFN-β is easily detected in macrophages following infection with low

virulence (OURT 88/3), but barely detected when macrophages are infected with a virulent strain (Reis et al., 2016).

However, less is known about host's innate immune response that occurs after challenge with strains of different virulence. Lacasta et al. (2015) identified immune modulators triggered at day 1-day post infection with a low virulent E75 strain. Several cytokine genes are upregulated after infection including IFN-γ, IL-12, IL-10, IL-21, IL-6, TGF-β, IL-1β and TNF-α. However, the absence of IFN-γ and IL-10 in vaccinated pigs with OUR/T883 and challenged with homologous virulent 88/1, may favor survival (Sanchez-Cordon et al., 2017). These results underline the failure of the innate immune system to detect the initial steps of virus replication, thus allowing the rapid spread of the virus to different lymphoid tissues. IFN-γ was originally called macrophage-activating factor because it up-regulates macrophage functions including antigen processing and presentation. Although many cell types secrete IFN-γ, the main producers are activated T cells, natural killer (NK) and NK-T cells.

An important cellular component is represented by NK cells since they are a bridge between innate and adaptive immunity and play a key role in combating viral infections. Leitão et al. (2001) demonstrated a significant increase in NK cell cytotoxicity at 7 days post infection in pigs that remained asymptomatic during ASFV infection; however, a decrease is observed in non-protected pigs. The difference between low virulent OURT/883 and high virulent Georgia/07 challenged pigs revealed twelve upregulated genes that are associated with monocytes and NK cells phenotype (Jaing et al., 2017).

In conclusion innate immunity during ASFV infection is dependent on different lymphocyte subsets and signaling molecules. Moreover, high virulent strains of ASFV have

evolved strategies to bypass this immune barrier, thus in this case protection is correlated with presence of antibodies and T-cells.

1.10 Adaptive immunity

As discussed, the mechanisms of innate immunity are easily bypassed by ASFV, an important player in keeping the infection under control is the adaptive immunity, especially the presence of antibodies and cytotoxic T-cells (CTL's).

Antibody response against ASFV can be detected as early as 7-8 DPI, but there are controversies about the ability of these antibodies to provide protection (reviewed in Escribano et al., 2013). Specific antibodies have different functional roles in protection; therefore transfer of anti-ASFV IgG from ASFV-surviving pigs allowed naive pigs to survive homologous challenge (Onisk et al., 1994). One experiment showed that when those antibodies are transferred through colostrum, they also confer partial protection to piglets challenged with ASFV (Schlafer et al., 1984). However, when evaluated *in vitro*, virus neutralization activity is relatively weak, difficult to measure and dependent on the strain used. In order to overcome this problem, mutant viruses containing different inducible marker genes were generated for conventional plaque reduction assays (Gomez-Puertas et al., 1995). Serum from a convalescent swine infected with E75 neutralizes the infectivity of multiple isolates (E75, E70, Lisbon60, Malawi 20/1) and low-passage cell culture adapted viruses by more than 80% in cell cultures. However, the same immune sera failed to neutralize high passaged cell adapted viruses (Zsak et al., 1993). This implies that during passaging, some isolates are associated with phenotypic changes that can allow them to escape neutralization. A later study revealed that the differences between low and

high passaged tissue culture adapted viruses are also correlated with changes in the phospholipid composition of the virus (Gómez-Puertas et al., 1997).

Convalescent swine serum revealed some proteins with roles in neutralization: p72/B646L, p54/E183L, and p30/CP204L. Antibodies against p72 and p54 inhibited a first step of the virus binding to cells, while anti-p30 antibodies inhibit a second step related to virus internalization (Gómez-Puertas et al., 1996). Surprisingly, antiserum raised against the dynein binding domain on the p54 was able to neutralize more than 60% in an *in vitro* assay (Escribano et al., 2013). Alternatively, antibodies that inhibit virus spread could create the offset of different mechanisms of entry as macropinocytosis and clathrin mediated endocytosis (Sanchez et al., 2012; Hernaez et al., 2010). When tested as vaccine candidates, both p30 and p54 reduced the course of the disease after challenge with E75 isolate (Gómez-Puertas et al., 1998). However, when p30, p54 and p72 were tested again as vaccine candidates, there were not sufficient to confer protective immunity to challenge against a different virulent isolate (Neilan et al., 2004). The discrepancies between studies can be explained by the ASFV strain used. Therefore, other viral components present on the surface of intracellular mature or extracellular enveloped virus particles may be targets for neutralization by preventing virus entry. These include the immunodominant proteins comprising structural proteins, non-structural proteins and proteins with unknown function (Kollenberger et al., 2002). It is tempting to hypothesize that cooperation of multiple antibodies recognizing multiple epitopes could correlate with protection; therefore, identification of virus proteins which may be targets of antibody mediating neutralization still merits further investigation. The role of antibodies against p54 will be discussed in Chapter 3.

Other mechanisms were also described during ASFV infection, such as complement-dependent antibody-mediated cytotoxicity (CDAC) and antibody-dependent cell-mediated cytotoxicity (ADCC) (Wardley et al., 1985).

Antibodies alone are not sufficient for protection against ASFV infection suggesting that there are other cells involved in protection, such as CD8+ lymphocyte subset. The first *in vitro* indication of CD8 involvement was during a proliferative assay of PBMC from challenged pigs stimulated with p72 capsid truncations, were inhibited using at a higher percentage using anti-SLA class I antibody rather than anti-SLA class II antibody, suggesting the importance of SLA I presentation pathway (Leitão et al., 2000). ASFV specific CTL's were first described *in vivo* in pigs infected with highly virulent ASFV Uganda isolate. In another study, pigs exposed to OUR/T88/3 and then depleted of CD8+ lymphocytes were no longer fully protected from virus-related OUR/T88/1. In this experiment, pigs immunized with 88/3 were administered an anti-CD8 mAb. After challenge, the CD8-depleted pigs showed clinical signs typical of ASFV infection; whereas, the untreated pigs were protected (Oura et al., 2005). Using a targeted vaccination approach, Argilaguet et al. (2012) revealed that immunized pigs are partially protected in the absence of antibodies and identified some CTL epitopes. However, a definitive conclusion cannot be drawn in regards with protective CTL epitopes, since that is dependent on how the antigen is presented due to the variability of MHC alleles within a pig population.

Another interesting aspect regarding cellular immunity is that pigs that survive challenge with low virulent isolates can be protected against homologous strains. Therefore, the memory immune response is developed prior to challenge. Using a T-cell proliferation assay, Wardley and Wilkinson (1980) were able to demonstrate the existence of memory T-cells in infection with a low virulent isolate. Moreover, when lymphocytes were collected from pigs challenged

with a virulent strain, they did not detect any proliferative cells, suggesting that memory can be strain specific. Using a similar experiment, Canals et al. (1992) showed that proliferation of an inactivated virus could be blocked by anti-CD4 mAb and inhibited by 60% using an anti-CD8 mAb. Since porcine memory helper T-cells have a phenotype of CD4+ $CD8_{lo}$ the results are concluding the involvement of memory during ASFV infection. The fact that only a few ASFV antigens can confer protection, in the absence of antibodies, seems to underline the importance of T-cells in ASFV infection. Whether cross protection can be achieved by stimulating T-helper cells still requires attention in future studies.

In conclusion, the failure of innate and adaptive immune responses to protect against ASFV replication leads to hemorrhagic fever pathology in infected pigs. A better understanding of the host's immune response and different cellular subsets is needed to fully understand the immune mechanisms involved in ASFV infection.

1.11 Vaccines

The search for an efficient and safe vaccine against ASFV has proven to be difficult, mainly from poor understanding of the immune system during infection. Moreover, the complexity of ASFV, a virus encoding more than 160 polypeptides, most of them directed towards evading immune system, together with the variability of the virus isolates so far identified has complicated this task (Dixon et al., 2004). Attempts to create ASFV vaccines have included inactivated viruses, recombinant proteins/peptides, library immunizations, DNA vaccines (or a combination of DNA and recombinant antigens to stimulate both humoral and cell mediated immunity), viral vectors for antigen delivery, live-attenuated vaccines (LAVs), gene

deletion mutants or even low virulent isolates (reviewed in Arias et al., 2017 and summarized in Table 1.3).

The initial approach based on inactivated viruses gave non-satisfactory results. Moreover, tissue culture adapted strains provided partial protection and did not confer protection against heterologous viruses (Forman et al., 1982). The use of modern adjuvants, such as Polygen^{TM}, which helped in achievement of protection against other viral diseases, did not increase the efficacy of ASFV inactivated vaccines (Blome et al., 2014).

The use in the field of LAVs produced by the attenuation of naturally occurring virulent strains has been limited to the extensive experience in Portugal and Spain during the early 1960's. Field isolate viruses were serially passed through primary bone marrow or blood macrophage cell cultures and then used to vaccinate pigs. A big part of the vaccinated herds developed unacceptable post-vaccination reactions, including high morbidity or mortality and development of antibody-antigen depositions that led to inflammation, causing a slow commercial production. In addition, many carrier animals were generated, without the possibility of differentiation from actually infected individuals, therefore hindering subsequent attempts to eradicate the disease.

Immunization of pigs with a plasmid coding for the extracellular domain of HA (CD2v/EP402R) fused upstream to the p30/CP204L and p54/E183L genes enhanced both humoral and cellular responses, without conferring protection. However, when those three genes were fused to ubiquitin, induced strong CTL responses, and conferred partial protection in the absence of specific antibodies. Moreover, the protection was correlated with the presence of protective CTL epitopes of HA (CD2v/EP402R), p54 and p30 (Argilaguet et al., 2012). However, in a follow up study baculovirus expressed p54, p30 fused with the extracellular

domain of the viral hemagglutinin, under the control of the human cytomegalovirus immediate early promoter (CMV), enhanced the T cell response, but did not confer protection. As mentioned previously immunization with p30 and p54 changed the disease outcome in pigs challenged with E75 strain (Gomez Puertas et al., 1998). The next experimental set up revealed that immunization with baculovirus-expressed p30, p54, p72 and p22, although capable of inducing neutralizing antibodies *in vitro*, failed to be protective (Neilan et al., 2004). Since the absence of protective B cell epitopes is still under debate, a library containing several viral ORF's fused to ubiquitin (excluding p54, p30 and CD2v) was used to target the proteasome pathway and stimulate T cell responses, protected 50% of the challenged animals (Lacasta et al., 2014). Combinations of recombinant proteins and DNA were used as prime boost strategy, to stimulate both branches of the immune system, but no protection against challenge was observed despite induction of robust immune responses (Sunwoo et al., 2019). Taken together, those results highlight the importance of antigens recognized by both T and B cells, revealing the existence of multiple ASFV antigens correlated with protection. However, attention should be directed towards rational selection of antigens used in vaccines to avoid ADE (discussed above) (Sunwoo et al., 2019; Lokhandwala et al., 2019- manuscript in preparation).

Recombinant viruses expressing different ASFV antigens are a suitable alternative in terms of safety and efficacy. Recently, a recombinant Newcastle disease virus expressing the p72 protein was constructed. Although it elicited strong immune responses in mice, the experiment should be carried in pigs to evaluate the immunogenicity (Chen et al., 2016). Another approach was the use of adenovirus-vectored ASFV multi-antigen cocktail that elicit strong B and T cell responses in pigs (Lokhandwala et al., 2016; 2017). However, when challenged, the vaccinated pigs showed exacerbated clinical signs, leading towards a hypothesis that vaccination generated

antibodies level that enhanced the immune response (Lokhandwala et al. 2019-manuscript in preparation). A prime boost approach using modified vaccinia virus Ankara expressing ASFV antigens (B646L, EP153R, and CD2v), and HEK 293-produced antigens (p72, p54, p12) generated a good T-cell response in pigs (Lopera-Madrid et al., 2017). However, further challenge experiments are required to assess the immunogenicity. A different alternative using this approach is the incorporation of prime boost strategy using prototype vaccines, followed by challenge with low or moderately virulent strains of ASFV (NH/P68 or OURT 88/3). This creates a window to evaluate the possible synergistic effects of the vaccine and wildtype strains used for challenge (Murgia et al., 2018). A similar approach used a library immunization of 47 gene candidates either as DNA prime prime or vaccinia virus boost to identify different protective antigens, based on timing of expression and functions. This approach has the advantage to characterize a large pool of ASFV antigens (around 30% of ASFV genome) Moreover, this experiment confirmed that p30 contains several T cell epitopes (Jancovich et al., 2018).

Another approach was the generation of ASFV deletion mutants lacking genes involved in virulence to reduce the infectivity. This approach will likely be used as a backup strategy during an outbreak. One example is the deletion of the thymidine kinase (TK) gene of highly virulent Georgia/07, which affects replication in macrophages and generates non-virulent virus that has no protective potential (Monteagudo et al., 2017; Sanford et al., 2016). However deletion of TK seems to be strain dependent also, since Malawi strain lacking this region conferred transient fevers, lower viremia titers, and reduced mortality to inoculated pigs (Moore et al., 1998).

In another experiment, depletion of genes involved in the evasion of the immune response (NL gene), and MGF's 360 and 505, or genes involved in virus replication or morphogenesis and 9GL (B119L) gene, have resulted in attenuation of virulent ASFV isolates and induction of protective immune responses against virulent parental virus challenge, but the protection is shown to be strain dependent. Another mutant (BeninΔMGF) was attenuated in pigs and immunization with this virus protected against challenge with a lethal dose of Benin 97/1, suggesting that deletion of IFN modulators is a promising route for rational attenuation of virulent ASFV isolates to construct candidate vaccine strains (Reis et al., 2016). A similar outcome was also observed when a gene with unknown function (DP148R) was deleted from the genome without affecting viral replication and inducing high protective immune responses (Reis et al., 2017b). Cross protection was achieved using a deletion mutant lacking CD2v that attenuated the virulent BA71 strain *in vivo,* conferring full protection against parental strain BA71, homologous virulent strain E75 and surprisingly against heterologous strain, Georgia/07 (Monteagudo et al., 2017). An interesting correlation exists between *in vitro* haemadsorption (HAD) inhibitory antibodies against CD2v, protection against challenge *in vivo* correlated with different T cell epitopes (Burmakina et al., 2016; 2019). Another experiment revealed that the deletion of DP71L and DP96R from the OURT88/3 genome did not protect pigs after challenge with OURT88/1 (Abrams et al., 2013). ASFV gene 8-DR has previously been deleted using traditional homologous recombination and has been determined to be non-essential.

Taken together, the MLV approach needs some "fine tuning" of the genes that can be deleted, since there is a thin balance between rendering a truly attenuated isolate that could elicit good immune responses and a partial attenuation that can't protect the animals after challenge. The use of deletion mutants, in particular the ones lacking CD2v, could represent an advantage

in order to establish a DIVA (differentiation of vaccinated versus infected animals) vaccine prototypes and are candidates that can be used where an endemic status is implemented. An interesting idea to pursue will be the integration of inducible “genetic switches” across vaccine prototypes, therefore limiting the virus replication in the host for a desired period of time. The latter could be easily accomplished since gene editing techniques such as CRISPR/Cas9 are becoming more popular as means to fight viral diseases. So far, the use of CRISPR for the deletion of 8-DR or p30 led to a reduction inhemadsorbtion of swine red cells or to a reduction of almost 4 logs in viral titers (Borca et al, 2018; Hübner et al., 2018). CRISPR/Cas9 system may represent a significant increase in the efficiency for developing a new generation of recombinant ASFVs or to identify different cellular ligands during early steps of infection.

1.12 Diagnostic techniques

Due to the explosive nature of ASFV due to different disease outcomes, rapid and highly specific diagnostic tests are important for implementation strict biosafety measures. In the US, in case of an ASF outbreak, laboratory diagnostic testing will be performed at the National Veterinary Services Laboratories, Foreign Animal Disease Diagnostic Laboratory (NVSL FADDL) at Plum Island. ASF can’t be diagnosed based solely on clinical evaluation, since other viral or bacterial diseases (like Classical swine fever, Salmonellosis, PDNS) present similar symptomatology. Therefore, ASFV diagnostics are based on virus detection and antibody presence (summarized in Tables 1.4A and 1.4B). The transport of suspected materials for initial or confirmatory diagnosis of ASF infection requires the use of a cold chain or the addition of preservative agents that do not interfere with the diagnostic procedures. However, in remote

regions where cold storage is limited, shipping methods are relying on dried blood on different types of filter papers (Randriamparany et al., 2016).

When talking about the development of ASFV diagnostics, there are two advantages that have to be taken under consideration: a) appearance of viremia begins after 24-48 hours post infection (hpi); and b) serum specific antibodies can be detected starting 8-14 DPI. Current methods for virus detection approved by the Office International des Epizooties (OIE; 2012; GARA report 2013) include: virus isolation, hemadsorption test (HA), polymerase chain reaction (PCR) in various formats, fluorescent antibody test (FAT), antigen enzyme-linked immunosorbent assays (Ag-ELISA). Current methods used to detect antibodies against ASFV include: ELISA, immunoblot (IB), immunofluorescence antibody test (IFA), immunoperoxidase test (IPT). According to the OIE, ELISA test based on semi-purified antigen followed by an IB is the preferred standard for international trade.

Virus isolation is currently the "gold standard" for initial confirmation of outbreak and it relies on the inoculation of the suspected sample material into primary macrophages or monocyte derived macrophages of porcine origin (Malmquist & Hay, 1960). Another approach to this "gold standard" relies on the propriety of some ASFV isolates to generate "rosettes" of pig erythrocytes around infected cells. This phenomenon is referred as hemadsorption, based on the presence of viral CD2v; however, some isolates do not have this capacity, therefore requiring a confirmatory test. Overall, those two approaches will provide the highest sensitivity and specificity for an initial outbreak screening. There are several drawbacks when using those tests. The most important one is the fact that high containment facilities are required to work with suspected samples and reference viruses. Another drawback is represented by the laborious work time (several days from receiving the sample until the test is completed) combined with the use

of primary cell lines (monocytes or macrophages) which present batch to batch variation. Finally, as mentioned previously some strains might not cause hemadsorption, therefore a sequential test is recommended (either PCR or FAT that will be discussed below).

Fluorescence antibody test (FAT) can be used as confirmatory test for the strains that do not cause hemadsorption, albeit having low sensitivity (Bool et al., 1969). It can be used when no clinical signs are present (during sub-acute forms of the disease), but there should be a good understanding of the current circulation of ASFV strains. The test relies on the inoculation of porcine macrophages or monocytes with serum, blood, tissue homogenate, meat juice or any other suitable sample collected. After 4-6 days, the cells are fixed and permeabilized to expose the viral antigens that are detected with specific monoclonal antibodies. However, the availability of monoclonal antibodies is limited, with data generated a few decades ago and no knowledge on how many strains they can possibly recognize. Therefore, one of the goals of this thesis was to develop and characterize monoclonal antibodies that can be used as diagnostic reagents for ASFV detection (for detection of antigen and antibodies).

In recent years, different polymerase chain reaction (PCR) assays have evolved to answer the need of high sensitivity, specificity and time. The technique is fast, and it relies on the amplification of a specific viral fragment that is conserved across different ASFV genotypes (based on the major capsid protein p72, which is the most conserved) from tissues, blood, serum, oral fluids (OF) and even tick homogenates. Several PCR test have been already fully validated, taking into account different epidemiological situations and that have no cross reactivity with other swine diseases. Moreover, portable PCR machines are becoming available (from Tetracore), making it possible to use them in field conditions. However, the major drawbacks are represented by the potential of cross contamination of the samples, reagents resulting in false

positive results or the limit of detection of the assay. A novel PCR technique is the droplet digital PCR (ddPCR) that enables the quantification of nucleic acids without using the standard samples, therefore having a high sensitivity.

Antigen-ELISA tests are based on capture of viral antigen, with either a pair of monoclonal or polyclonal antibodies. The advantage of this technique is that it can detect circulating virus, before the appearance of an antibody response. It is also advantageous to use this test due to the high number of samples that could be run on a single plate and the fact that multiple sample types are compatible (serum, oral fluids, blood, and tissue homogenates). Therefore, one commercial kit is in the validation process (Ingezim K2), however the sensitivity and specificity of the assay in limited (only 16 field samples and from inoculated animals at 7 DPI), therefore it should only be used in combination with other virological/serological diagnostic assays. Other antigen capture ELISAs were previously developed using a set of monoclonal antibodies, but the results were inconsistent (Wardley et al., 1979; Vidal et al., 1997).

Since molecular tests are not yet fully validated for field conditions other approaches for detection of ASFV genome have been developed. Isothermal amplification tests are a cheaper and faster alternative than PCR, relying on the use of one temperature step that could be achieved using a water bath, without the need for additional equipment. Two such tests were evaluated for ASF: loop-mediated isothermal amplification (LAMP), Invader® and recombinase polymerase amplification (RPA) (James et al., 2010; Hjertner et al., 2005; Wang et al., 2017). However, currently the sensitivity is lower than PCR, but seems enough for detection of acute cases. Nonetheless, validation in endemic areas is still required for those tests.

Another approach to detect viral antigen is an immunochromatographic test using specific monoclonal antibodies (against the ASFV capsid protein). This could be incorporated into a lateral flow device that could be used as a pen side diagnostic test, relying on the capture of antigen using anti p72 mAb and detection with a different HRP labeled antibody which recognizes a different epitope. Validation for this test is ongoing.

Since no vaccine against ASFV is currently available, the detection of specific ASFV-antibodies is a good indicator of previous infection. The anti-ASFV specific antibodies are detected as early as 8 days post infection and they persist for long periods of time (Reis et al., 2007; Giménez-Lirola et al., 2016). However, in the acute phase of disease the pig dies before antibodies are detectable. It is therefore recommended that in the early stages of an outbreak, samples are taken for detection of viral DNA as well. A comparative study between OIE ELISA and different ELISA's using different soluble antigens from different genotypes revealed that the antibody response against different isolates might be directed against multiple shared epitopes, which are recognized regardless of the virus isolate and antigenic polymorphism (Gallardo et al., 2013).

Many potentially useful viral protein candidates (recombinant proteins produced either in *E.coli* or insect cells) in ASFV serology (either ELISA or Western blot) have been described; among them, p30, p54 and p72, were shown to also be very immunogenic since antibodies are detected at early times post infection and they can neutralize the attachment and entry and reduce the viremia (Kollnberger et al., 2002; Gomez-Puertas et al., 1996, 1998). Other recombinant proteins comprising both structural and non-structural ASFV proteins included pp62, precursor protein of the core shell, that showed high reactivity against poorly preserved sera, pA104R, pB602L, and pK205R, p48, C-type lectin, p10 and others (Gallardo et al., 2006 and Tables 1 and

2). In addition, CD2v protein mediates HAI serological specificity, providing a simple method for serotyping.

The most commonly used serological tests for detection of anti-ASFV antibodies, that are validated by the OIE are the indirect ELISA, based on semi-purified viral antigen, followed up by immunoblotting (IB) assay to confirm the samples with doubtful results. The main disadvantages using the OIE-approved ELISA are based on the use of live virus as antigen, which involves biosafety level 3 facilities, standardization of reagents, and the use of an alternative serological test (IFA, IB) or an antigen-detection test for diagnostic confirmation, resulting in a time consuming process until a validated result is obtained (OIE manual, 2012). Although not fully validated, three commercial ELISA kits are available for the detection of ASFV antibodies (blocking Ingezim PPA from Ingenasa, ID Screen ASFV indirect ELISA from IDvet, and ASFV-Ab assay from Svanovir).

Western blotting (WB or IB) is a rapid and sensitive assay for the detection of specific antibodies and provides a better recognition of weak positive samples visualization of antibody binding to different ASFV antigens. However, the use of live virus for preparation of IB implies the same issues as described for OIE ELISA, namely standardization (virus strain, cell line used etc.) and use of high containment facilities. Therefore, numerous recombinant proteins are currently used as antigens in WB, especially as confirmatory test. The best example is the use of recombinant p54 produced in *E. coli*. In this assay p54 requires solubilization in 8M Urea to reach optimal signals, suggesting that anti-p54 antibodies recognize mainly linear epitopes (Alcaraz et al., 1995). In a recent study by Kazakova et al. (2016), recombinant p30 was used to prepare immuno-strips. The results revealed 100% specificity against different strains or tissues

and early detection of antibody response (8 DPI). However, one of the major drawbacks of the WB techniques relies on the fact that only individual testing can be performed.

A novel technique which has become more popular in recent years for measuring antibody response or nucleic acids is the fluorescent microsphere immunoassay (FMIA), commonly known as Luminex. This technique incorporates antigen-coated microbeads, which contain differing ratios of two fluorescent dyes and was initially used for genotyping the single nucleotide polymorphisms from the major capsid ASFV protein (LeBlanc et al., 2012).

As a serological test, Luminex offers several advantages, including: 1) the detection of antibodies to multiple antigen targets in a single sample; therefore assessing the quantity and quality of immunity; 3) incorporation of mAbs for antigen detection 4) rapid delivery of a semi-quantitative result; 5) use of different samples such as oral fluids, swabs exudates or meat juice; 6) possibility to create a porcine panel for porcine emerging diseases (such as ASFV, CSFV, SVA, FMD); and 7) good sensitivity and specificity for the detection of early or mucosal immune responses. Based on those characteristics Giménez-Lirola et al. (2016) included the most immunogenic proteins (p30, p54 and p72) into the Luminex assay. Results not only showed that p30 provided the best diagnostic performance, but antibodies against this protein were detected in oral fluids as early as 8 DPI, which is equivalent to the performance reported for the OIE ELISA. This shows that this sample type would be a better alternative based on the simplicity of collection and on the fact that represents a population sample, rather than individual one.

As described for detection of viral antigen, pen side diagnostics representing a faster and cheaper alternative of detecting anti ASFV antibodies. The first dot immunobinding assay (DIA) for detection of ASFV antibodies used strips dotted with a cytoplasmic soluble antigen. The

assay showed high sensitivity be used under field conditions, but was not further validated (Pastor et al., 1992). Therefore, a one-step multiplex ASFV/CSFV lateral flow assay was developed that specifically differentiates between anti-ASF and anti-CSF antibodies in serum. The assay is not currently validated; however, the preliminary results show 100% sensitivity (Sastre et al., 2016). Recently a commercial kit from Ingenasa (PPA CROM) was validated in Sardinia using wild boar samples resulting in 100% specificity (Cappai et al., 2017).

Considering that ASF is a very complex disease with different clinical outcomes, genetic complexity, and no vaccine available, prevention and control are based on rapid diagnosis and implementation of bio-sanitary policies. Therefore, the use of the most novel and robust diagnostic tools that are updated to be applicable to all scenarios is critical for the implementation of effective control programs. Under those circumstances the simultaneous detection of antigen and antibodies should always be performed.

1.13 Importance of developing novel diagnostic tests using monoclonal antibodies

Although ASFV is not present in the US, the current outbreaks in Europe and Asia have drawn increasing interest in developing rapid and specific diagnostic assays for surveillance programs. Moreover, a better understanding of the immune system after infection is required for generating novel vaccine approaches. The overall goal of this research was to develop and characterize monoclonal antibodies against two of the most immunogenic ASFV proteins, p30 and p54.

The results presented in this thesis were used to both establish novel sensitive diagnostic assays and identify immunodominant epitopes. Previous research revealed the importance of our selected antigens which contain both T- and B- cell epitopes. Independently of B- and T- cell stimulation, the studies revealed that p30 and p54 can cause a delay in appearance of clinical signs. However, there is little information about which regions are preferentially recognized by infected pigs. Epitope mapping is an important technique used to determine regions within a protein that can be recognized by the immune system. Therefore, identifying the exact epitopes will create the opportunity to design epitope-based vaccines and genotype specific diagnostic tests.

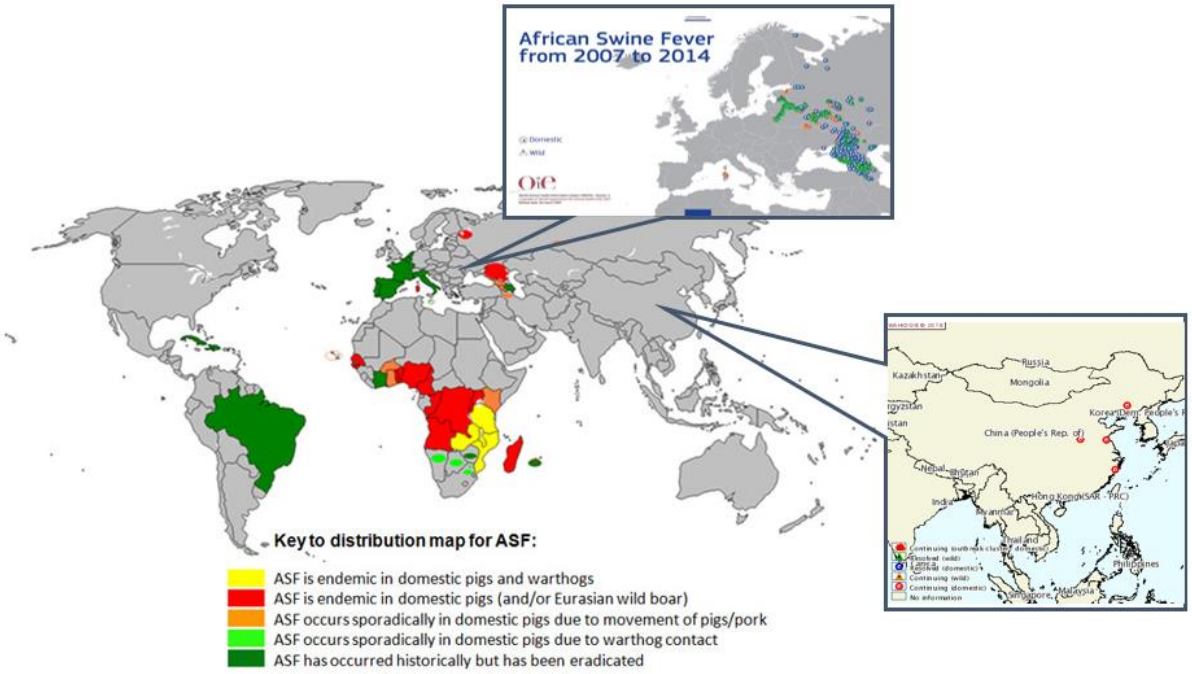

Figure 1-1 ASF historical and current distribution.

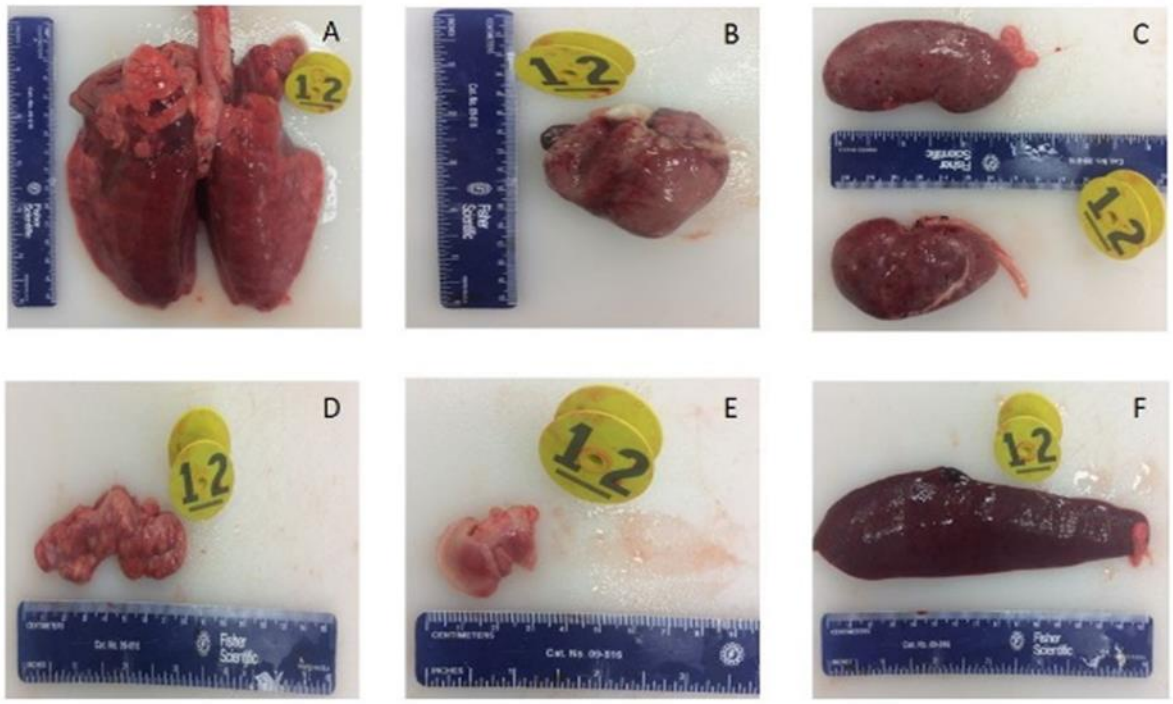

Figure 1-2 Gross pathological observations from a pig infected with highly virulent Georgia/07 strain. Pulmonary oedema (2-A), hemorrhagic heart (2-B), multifocal cortical hemorrhages (petechiae) on kidney (2-C), hemorrhagic lymph nodes with marked size increase (2-D), enlarged tonsil (2-E) and splenomegaly (2-F)

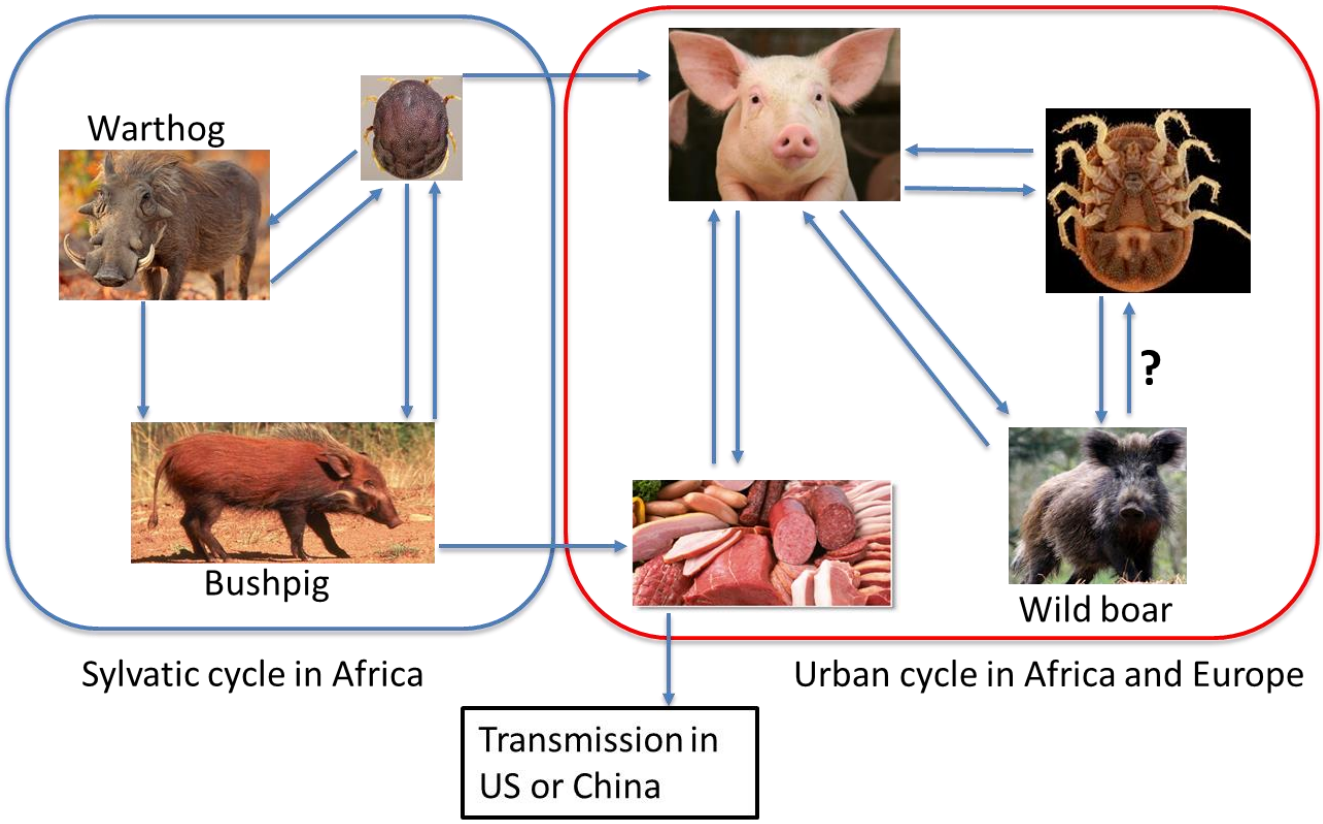

Figure 1-3 ASFV transmission routes in Africa. Sources of infection in Africa include warthogs (*Phacochoerus africanus*) and bushpigs (*Potamochoerus larvatus*) which become persistently infected, acting as a source of infection. *Ornithodoros* spp. ticks inhabiting warthog burrows or pig housing can also be involved in transmission in East Africa. Urban cycle that is currently present in Europe and Asia include infectious domestic pigs (*Sus scrofa domesticus*) and wild boar (*Sus scrofa*), contaminated carcasses, food waste, contaminated vehicles, equipment or possible through a sub-species of *Ornithodoros* spp. (Sanchez-Cordon et al., 2018).

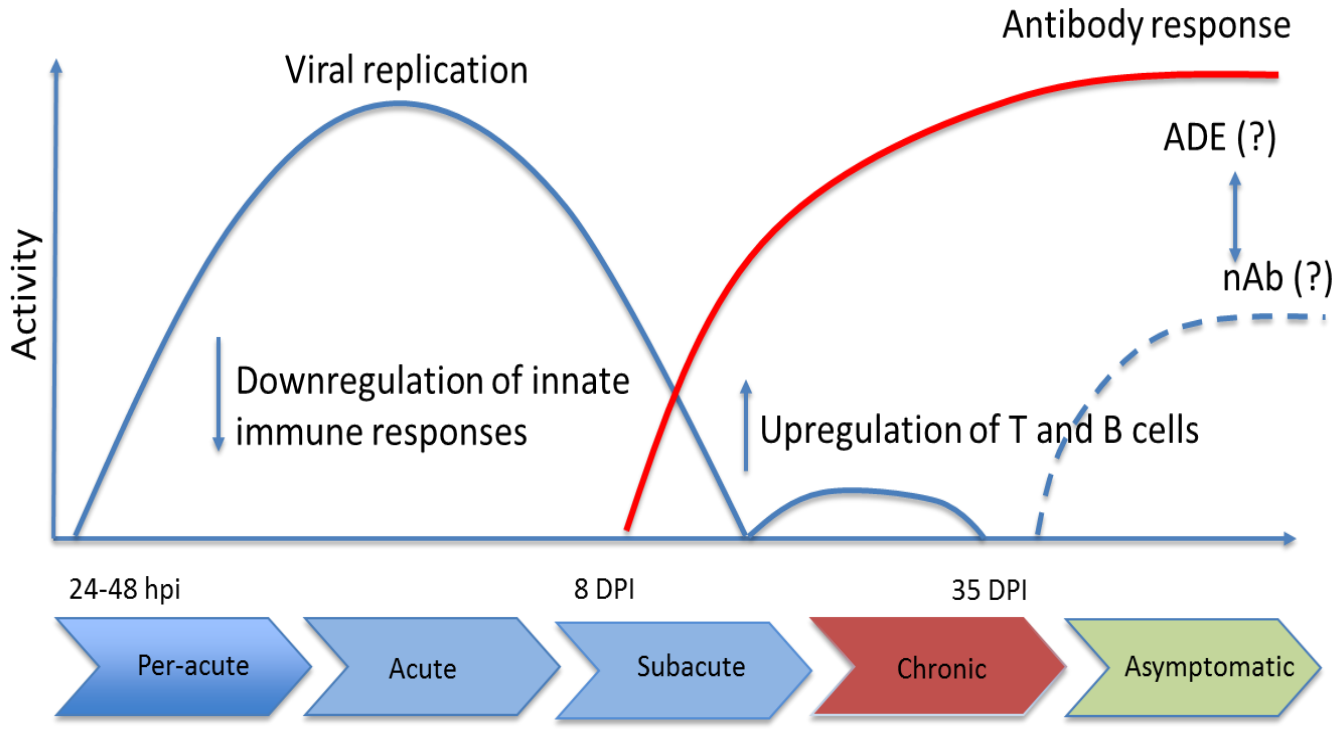

Figure 1-4 Clinical ASF presentation correlated with disease outcomes. Viremia can be detected between 24-48 hpi where it peaks at 4-5 DPI, correlated with the per-acute and acute forms of the disease. After 8 DPI an upregulation of adaptive immunity (plasma B cells and cytotoxic T cells) is seen, followed by a short phase of intermittent viremia up to 35 DPI. Levels of polyclonal antibodies generated after vaccination and infection will induce antibody dependent enhancement mechanism (ADE). After the chronic phase of the disease the pigs will become asymptomatic and they will generate neutralizing antibodies. A tempting hypothesis will be to follow the levels of neutralizing antibodies (nAb) induced after vaccination and disease enhancement.

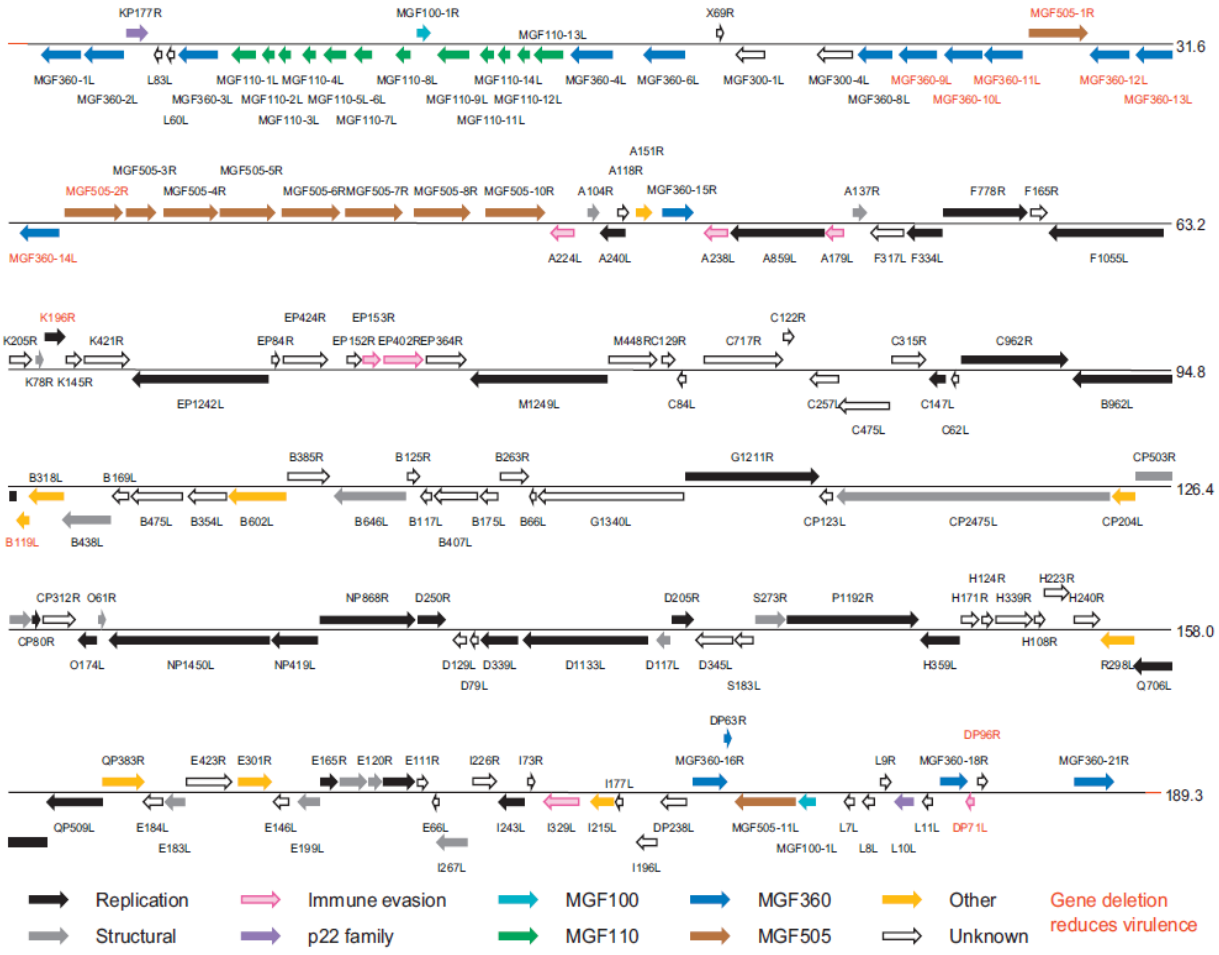

Figure 1-5 ASFV genome organization. The organization of open reading frames (ORFs) on the genome of the virulent ASFV isolate Georgia/07 is shown. ORFs are shown as arrows to indicate their size and direction they are read. The colours indicate ORFs with known functions. Black indicates ORFs encoding enzymes and factors involved in genome replication, repair or transcription. Grey indicates ORFs encoding structural proteins. Pink indicates ORFs encoding proteins involved in evading the host defences. Turquoise, blue, green, brown and mauve indicate members of multigene families. ORFs encoding proteins with other predicted functions are shown in yellow. ORFs encoding proteins of unknown function are shown in white. Red text indicates ORFs whose deletion reduces virus virulence. (Taken from Dixon et al., 2013)

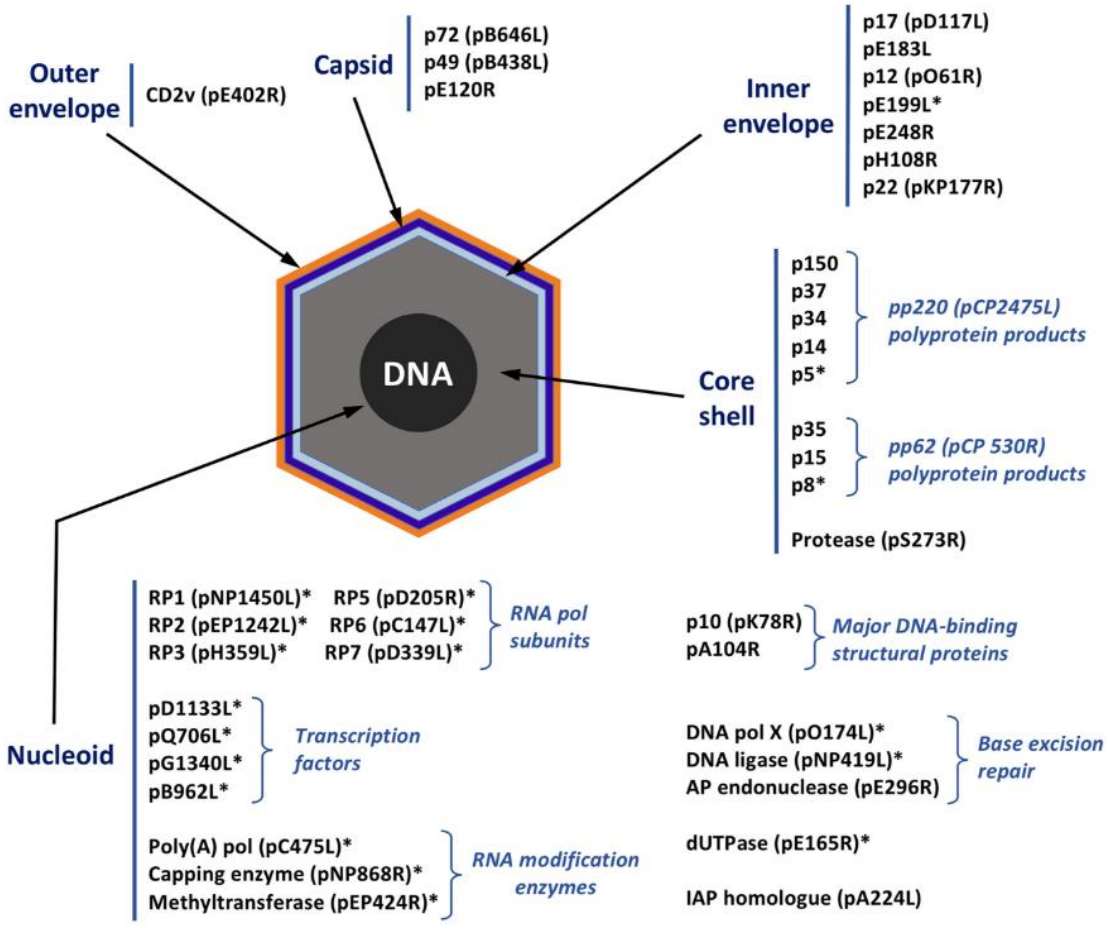

Figure 1-6. ASFV structure based on tissue culture adapted BA71V strain. The subviral localization of 40 viral proteins among the five structural domains of the ASFV particle is shown. The distribution of proteins marked with an asterisk was inferred from the predicted or known role, while that of the remaining proteins was determined by immunoelectron microscopy. pol, polymerase. (Taken from Alejo et al., 2018)

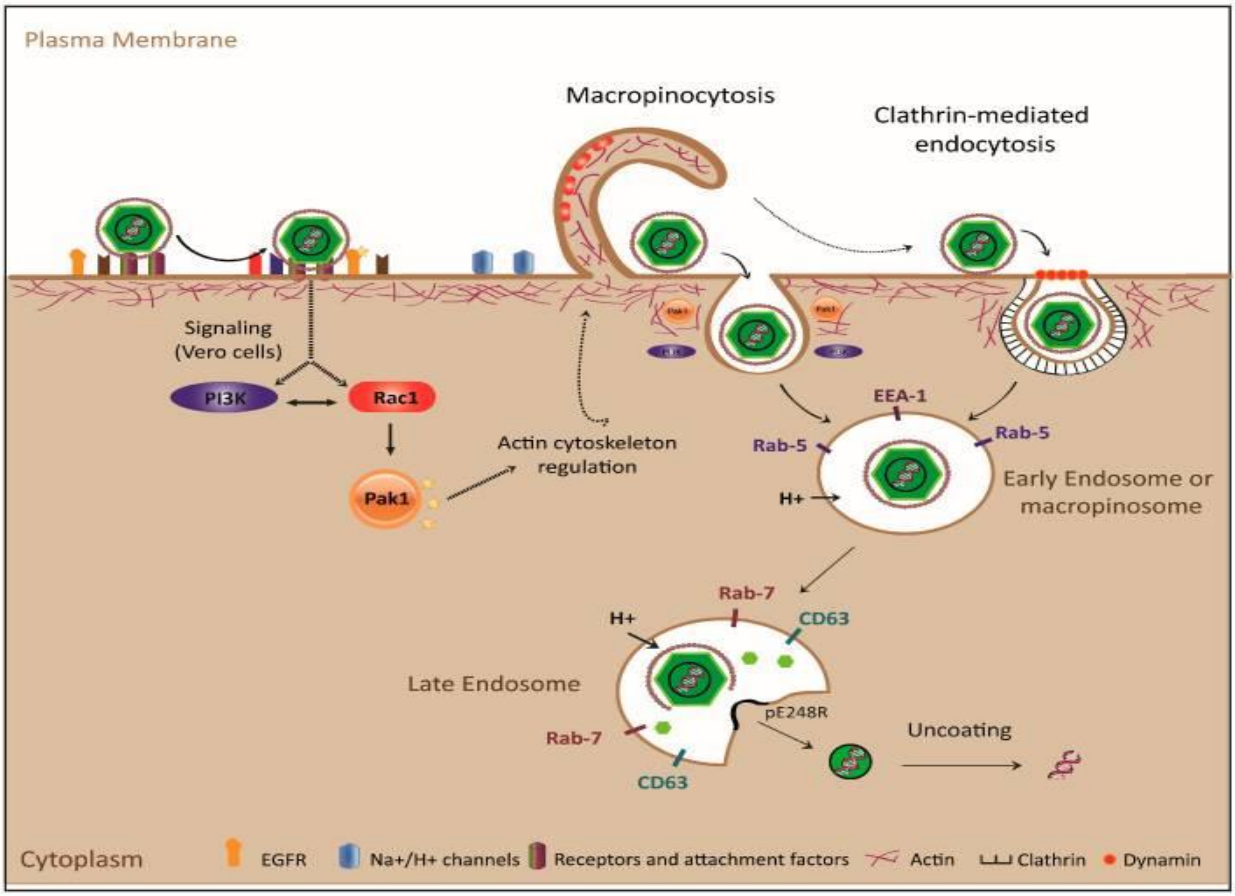

Figure 1-7 Model for ASFV entry and uncoating. Interaction of the viral particle with membrane receptors and attachment factors activates PI3K, EGFR, Rac1 and Pak-1 signaling pathways, which regulate actin dynamics, forming ruffles to internalize by macropinocytosis in Vero cells. In the case of swine macrophages, although the virus uses macropinocytosis, it does not actively induce the pathway; ASFV is also able to enter cells by CME. After viral uptake, particles are endocytosed in early endosomes or macropinosomes and transported to late endosomes where the pH-dependent uncoating process takes place. The viral outer envelope is disassembled and the inner envelope fuses with the endosomal membrane, delivering viral cores to cytosol, where viral protein pE248R plays an important role (Taken from Sanchez et al., 2017).

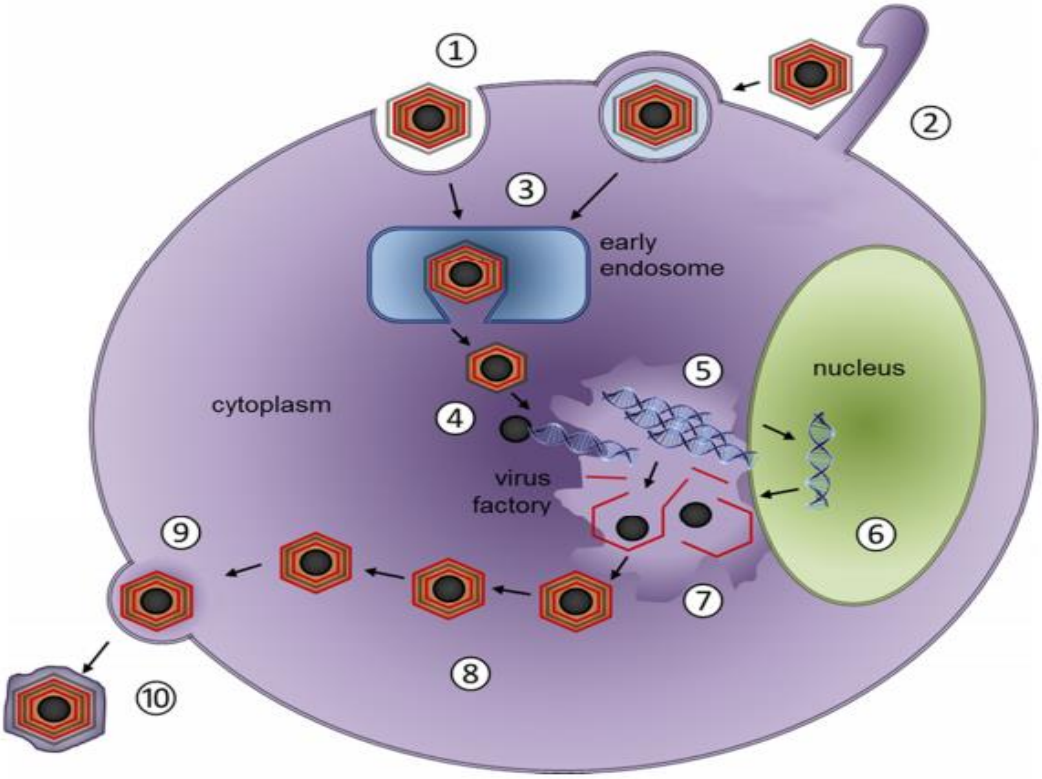

Figure 1-8 ASFV replication cycle. The virus particle enters the cell by (1) clathrin-mediated endocytosis or (2) macropinocytosis and (3) enters the endosomal-lysosomal system. (4) It then exits through fusion of the viral envelope with the vesicle membrane and virus particles are directed to perinuclear regions through interaction between structural protein p54 and the dynein motors of the microtubule network. Early viral gene transcription begins in partially uncoated cores following entry of the core into the cytoplasm, using enzymes and factors packaged into the virus core, independently of host RNA polymerase. (5) Replication of viral DNA occurs in the cytoplasm, in perinuclear virus factories, although (6) early sub-genomic length fragments are produced in the nucleus. (7) New virions are assembled through wrapping of the nucleoproteincore by a single lipid bilayer on which the virus capsid is formed. (8) Fully assembled virus particles are transported to the cell surface by kinesin motor proteins of microtubules where (9) they exit by budding through the plasma membrane (10) and acquire an outer envelope (Taken from Arav, 2014)

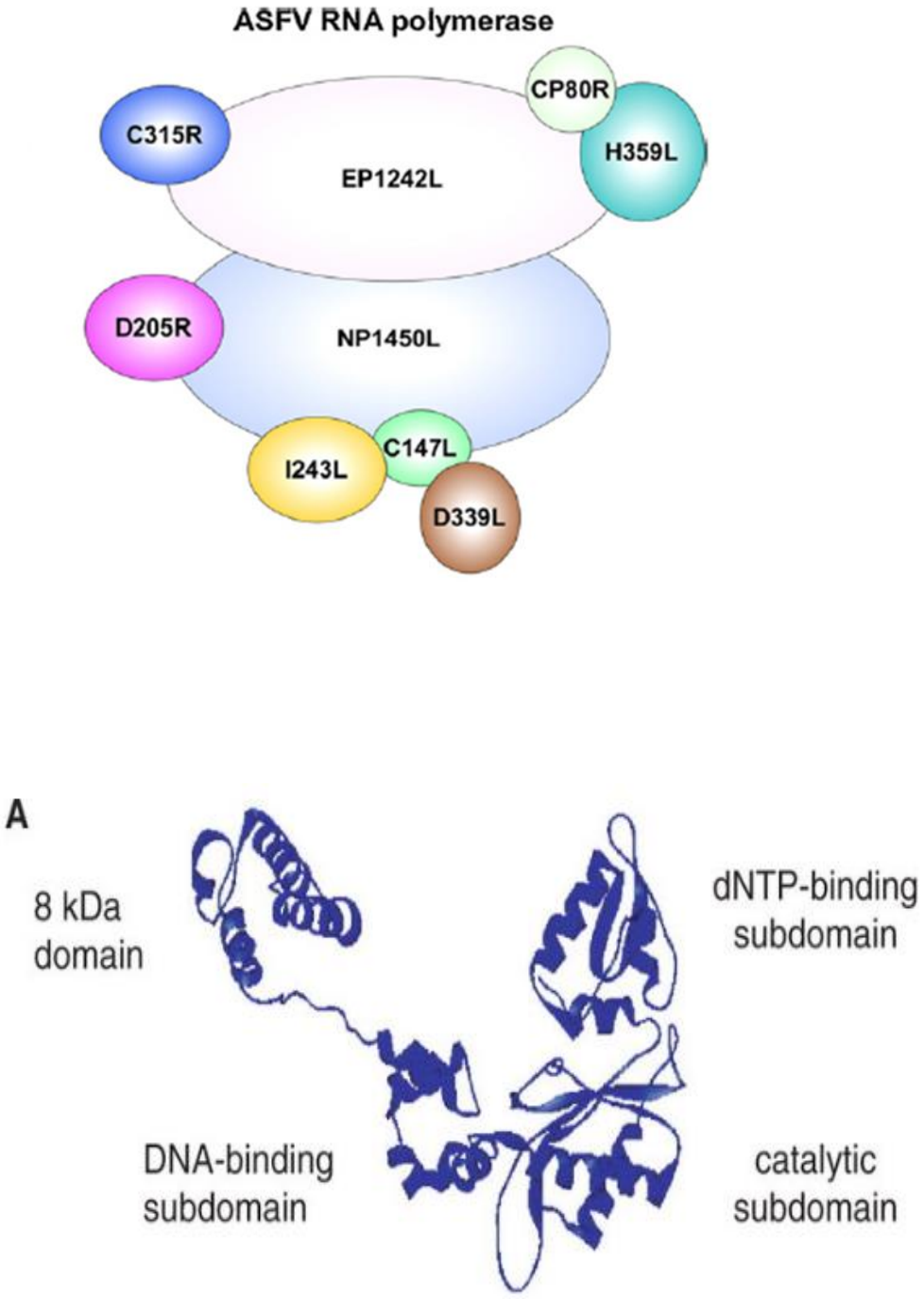

Figure 1-10 ASFV RNA polymerase complex (upper image) and ASFV DNA polymerase (lower image). (Taken from Martinez & Salas, 2012; Tang et al., 2008)

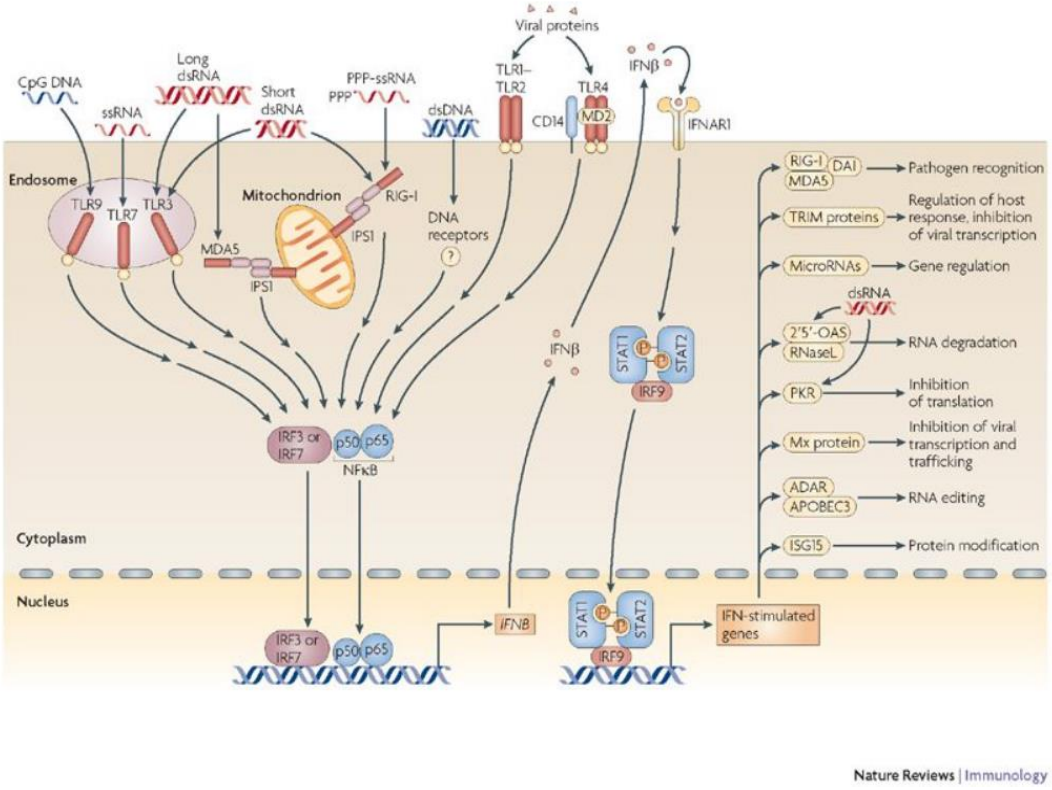

Figure 1-11 PRRs pathway. All of the pattern-recognition receptors (PRRs) initiate signalling pathways that converge at the activation of the transcription factors interferon (IFN)-regulatory factor 3 (IRF3), IRF7 and/or nuclear factor-κB (NFκB); this leads to the expression of IFNβ. IFNβ then initiates an antiviral effector programme in the infected cell and neighbouring cells, which involves the expression of numerous IFN-stimulated genes (ISGs). Some of the ISGs shown here, such as RIG-I (retinoic-acid-inducible gene I), MDA5 (melanoma differentiation-associated gene 5), DAI (DNA-dependent activator of IRFs), some microRNAs and the TRIM (tripartite motif-containing) family of proteins, are involved in the amplification and regulation of the IFN response. Other ISGs shown here, such as 2′5′-oligoadenylate synthetase (OAS) and ribonuclease L (RNaseL), IFN-inducible dsRNA-dependent protein kinase (PKR), myxovirus resistance (Mx) protein, adenosine deaminase RNA-specific (ADAR) and apolipoprotein B mRNA-editing enzyme, catalytic polypeptide 3 (APOBEC3), are involved in antiviral mechanisms that interfere with the life cycle of individual viruses . OAS and PKR are further activated by double-stranded RNA (dsRNA). IFNAR1, interferon-α receptor; IPS1, IFNB-promoter stimulator 1; ISG15, IFN-stimulated protein of 15 kDa; MD2, myeloid differentiation protein 2; PPP, 5′ triphosphate; ssRNA, single-stranded RNA; STAT, signal transducer and activator of transcription; TLR, Toll-like receptor (taken from Bowie & Unterholzner, 2008).

Table 1-1 Genes encoding proteins with roles in viral architecture and morphogenesis. (Adapted from Dixon et al., 2013; Alejo et al., 2018)

ORF	***Function***	***Description***
CP2475L	Core shell/pp220	p150
CP2475L	Core shell/pp220	p34
CP2475L	Core shell/pp220	p37
CP2475L	Core shell/pp220	p14
CP2475L	Core shell/pp220	p5
B646L	Capsid/VN	p72
CP204L*	Attch./VN/Binds to host ribonucleoprotein-K	p30 (p32)
E183L*	Internalization/VN/Virus transport	p54 (j13L)
EP402R	Hemadsorption to infected cells/Serotype specific neutralization	CD2 like
EP153R	Apoptosis inhibitor	C-type lectin
B602L	Correct folding of capsid	Chaperone
O61R	Attch.	p12
KP177R	Membrane protein	p22
K78R	DNA binding protein	p10
A137R	Unknown	p11.5
A104R	DNA binding protein	Histone-like
A151R	Interaction with the viral structural protein pE248R	Component of redox pathway
B438L	Formation of capsid morphogenesis	p49
D117L	Membrane protein	p17
CP530R	Core shell/pp62	p15
CP530R	Core shell/pp62	p35
CP530R	Core shell/pp62	p8
H108R	Membrane protein (strain specific)	j5R
E199L	Membrane protein (strain specific)	J18L
E120R	Formation of capsid morphogenesis	p14.5
E248R	Membrane protein	Component of redox pathway
XP124L	Possible interaction with ER	MGF 110
Y118L	Possible interaction with ER	MGF 110
EP152R	Transmembrane domain	Host protein interaction
CP123L	Membrane protein; Unknown function	Unknown
I177L	Membrane protein; Unknown function	Unknown
E146L	Membrane protein; Unknown function	Unknown
C257L	Membrane protein; Unknown function	Unknown
B117L	Membrane protein; Unknown function	Unknown
K145R	Possible membrane prot.; Unknown fct	Unknown
B169L	Two transmembrane domains; Unkn fct	Unknown
EP84R	Two transmembrane domains; Unkn fct	Unknown

**Note: ***discussed in Chapters 2 and 3; ORF in italics are also used in serology diagnostics

Table 1-2 Genes involved in DNA replication, transcription, host evasion and other functions (Adapted from Dixon et al., 2013; Alejo et al., 2018).

ORF	***Function***	***Description***
A240L	Nucleotide metabolism	Thymidylate kinase
K196R	Nucleotide metabolism	Thymidine kinase
F334L	Nucleotide metabolism	Ribonucleotide reductase (small)
F778R	Nucleotide metabolism	Ribonucleotide reductase (large)
B962L	Transcription	VACV I8 like RNA helicase
D1133L	Transcription	VACV D6 like RNA helicase
F1055L	Transcription	Helicase superfamily II
A859L	Transcription	Helicase superfamily II
Q706L	Transcription	VACV D11-like helicase
QP509L	Transcription	Helicase superfamily II
NP1450L	Transcription	RNA polymerase subunit 1
EP1242L	Transcription	RNA polymerase subunit 2
H359L	Transcription	RNA polymerase subunit 3
G1340L	Transcription	VACV A7 early transcription factor
NP868R	Transcription	mRNA-capping enzyme
D339L	Transcription	RNA polymerase subunit 7
D205R	Transcription	RNA polymerase subunit 5
C147L	Transcription	RNA polymerase subunit 6
R298L	Protein phosphorylation	Serine/threonine protein kinase 1
O174L	DNA replication	DNA polymerase X
NP419L	DNA repair	DNA ligase
G1211R	DNA replication	DNA polymerase family B
P1192R	DNA replication	Topoisomerase type II
EP424R	Transcription	Putative RNA methyltransferase
H339R	Binding protein	Alpha-NAC
E296R	DNA repair	AP endonuclease
E165R	DNA repair	dUTPase
B119L	Protein folding	FAD-linked sulfydryl oxidase
A224L	Host cell interaction	IAP apoptosis inhibitor
A179L	Host cell interaction	Bcl2 apoptosis inhibitor
A238L	Host cell interaction	Shut down of gene transcription
DP71L	Host cell interaction	Neurovirulence factor
QP383R	Host cell interaction	Nif S-like
B318L	Enzyme	Prenyltrasnferase
I215L	Enzyme	Ubiquitin conjugating enzyme
D250R	Enzyme	Nudix hydrolase
C962R	DNA replication	DNA primase
B175L	Transcription factor	VV VLTF2
G1340L	Transcription factor	VV A8L
B385R	Transcription factor	VV A2L
Note: ORF in italics are also used in serology diagnostics		

Table 1-3 Approaches for the development of ASFV vaccines.

Type of vaccine	Antigen(s)	Protection
Live attenuated on porcine bone marrow	Different strains	Partial; Strain dependent
Inactivated	Different strains	No
Recombinant proteins	p54, p72, p30, p22, CD2v	Partial (delay in the appearance of disease)
DNA	CD2v, p54, p72	Partial (delay in the appearance of disease)
DNA+protein	CD2v, p72, p30, p54 (plasmid DNA) or p15, p35, p54, p17 (recombinant protein)	No; ADE observed
Viral vectors (Adenovirus, Poxvirus, Alphavirus)	Antigen cocktail	No
Attenuated isolates	Naturally low virulent, collected during outbreaks	Partial; Homologous protection
Gene deletion mutants	CD2v, TK, MGF, 9GL, DP148R	Full homologous/heterologous

Table 1-4A Virus detection tests currently available for ASFV (Adapted from GARA 2013).

Detection	Available tests		Type (in house/ commercial)	Recommendation	Status
Virus detection	Virus isolation		VI and haemadsorption (in house)	Gold standard for initial outbreak	Labor intensive; use of primary cells
	Antigen detection		Immunofluorescence assay (FAT) (in house)	Individual testing	Labor intensive; Low SN for non HA strains
			Capture ELISA Ingezim K-2	Surveillance	Low SN; only serum
			In house capture ELISAs	Individual testing	Not validated
	PCR	Conv.	OIE PCR	Surveillance	In use
			In house ASF-CSF	AFS/CSF outbreaks	Validated
		Real Time	OIE TaqMan probe(s)	Surveillance	In use
			UPL* probe	Surveillance	Validation ongoing
			MGB* probe	Not in use	Not validated
			Tetracore (lyophilized kit); QIAGEN Virotype	Field	Not validated
			Multiplex ASF-CSF	Surveillance	In use
			LATE*-PCR	Not in use	Not validated
			Field deployable PCR (T-Core)	Not in use	Recently validated
		Digital droplet (ddPCR)		Not in use	Not validated
	Isothermal		LAMP*, Invader® assay	Not in use	Validation ongoing
			recombinase polymerase amplification (RPA)	Not in use	Not validated
	Pen-side		lateral flow devices	Not in use	Low SN

*UPL=Universal probe library; MGB= minor groove binder; LATE=Linear After The Exponential; LAMP=loop mediated isothermal amplification

Table 1-5 B Antibody detection tests currently available for ASFV (Adapted from GARA 2013).

Detection	Available tests	Type	Recommendation	Status
Antibody detection	ELISA	OIE indirect	Surveillance	High contain. facilities
		Recombinant proteins as ELISA antigens	Surveillance	In use
		ID Screen ASFV indirect ELISA	Surveillance	Not validated
		Blocking ELISA Ingezim K3 PPA	Surveillance	Not validated
		Ab ELISA from Svanovir	Surveillance	Not validated
		In house ELISAs	Surveillance	Not validated
	Confirmatory	Immunoblot (IB)	Confirmatory	High contain. facilities
		Immunofluorescence test (IFA)	Confirmatory	High contain. facilities
		Indirect immunoperoxidase test (IPT)	Confirmatory	High contain. facilities
		Recombinant proteins as western blot antigens (strips)	Individual testing/ Confirmatory	Validated
	Luminex	Porcine panel for emerging swine diseases using multiple antigens	Multiplex panel for swine diseases	Not validated; Elevated costs
	Penside	Lateral flow devices	Individual testing	Validated
		Dot blot	Individual testing	Not validated

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Chapter 2 - Antigenic properties of African swine fever virus (ASFV) structural protein, p54

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Abstract

In the absence of a vaccine for the control of infections caused by African swine fever virus (ASFV), diagnostic tools are critical for early detection and implementation of control measures. Along with other immunogenic proteins, such as p30 and p72, p54 is a potential serological target for conducting ASF detection and surveillance. In this study, a panel of mouse monoclonal antibodies (mAbs) were prepared against a baculovirus-expressed p54(60-178) polypeptide, which lacked the predicted hydrophobic domains. Recombinant polypeptide fragments expressed in bacteria and synthesized oligopeptides were used to map linear epitopes. The results identified three different domains, which were also recognized by sera from ASFV-infected pigs. However, none of the antibodies showed virus neutralizing activity. The results from this study provide some new tools for improving ASFV diagnostics and for vaccine development.

Key words: African swine fever virus, p54, monoclonal antibodies, epitope mapping

1. Introduction

African swine fever virus (ASFV) is an important threat to a global agricultural economy. The recent outbreak of ASF in China, along with the diagnosis of ASFV infection in wild boar in Belgium, have created a renewed sense of urgency for effective measures that can prevent the entry of ASFV into negative regions, such as the U.S. and provide effective diagnosis following an outbreak. Acute ASF disease is characterized by lethargy, anorexia, high fever, and death within in a few days after infection (Gallardo et al., 2015a). ASFV infection can also result in a chronic or subclinical form of disease, which can make early detection difficult. Since there are no effective vaccines, disease control is heavily dependent on surveillance and early diagnostic detection. Serological approaches incorporating virus-derived or recombinant ASFV antigens are important alternatives to the classical approaches, such as immunoperoxidase assay (IPA) of infected cells, which is the confirmatory test, validated by the EU reference laboratory (Gallardo et al., 2015b). Although the serological tests currently validated and approved for ASF diagnosis provide a good diagnosis, the complexity of the disease and the antigenic variation created by 24 different genotypes can influence the sensitivity and specificity of the assays.

Current serological tests, when combined with clinical disease, can provide a confident diagnosis. However, the use of serology for surveillance creates the possibility of false positive results, which can trigger a foreign animal disease investigation. This can be avoided by the incorporation of multiple antigen targets as a means to derive a consensus. ELISA-based serological tests have been developed that incorporate immunodominant ASFV proteins, such as p72 and p30. Another potential target is the 183 amino acid structural protein, p54, a product of the viral gene, *E183L*. The protein is located in the inner envelope and is essential for virus replication and viability (Rodriguez et al., 1996). As illustrated in Figure 2.1, p54 is a type I membrane protein with the inner N-terminal 60 amino acids followed by a transmembrane

domain and a 131 amino acid C-terminal ectodomain which contains several predicted glycosylation and phosphorylation sites (Rodriguez et al., 2004; Mima et al., 2015; Sun et al., 1995). The protein possesses both conserved and variable regions therefore representing an evolution marker to study ASFV diversity. Heterogeneity among different ASFV isolates is related to repetitions of a Pro-Ala-Ala-Ala motif located near the C-terminal end. However, the repetitive motif is not involved in virulence or infectivity (Alcaraz et al., 1992; Rodriguez et al., 1994). Together with p30, p54 participates in the binding of the virion to target cells (Gomez-Puertas et al., 1998). After virus internalization, p54 interacts with the host protein, dynein, resulting in the transport of the virion to the perinuclear region of the cell (Alonso et al., 2001).

The dynein binding domain (DBD) is also involved in the activation of caspase-3 and the induction of apoptosis (Hernáez et al., 2004). The DBD, located between amino acids 149-161 (see Figure 2-1), is a target for neutralization by convalescent serum from ASFV-infected pigs (Escribano et al., 2013). Anti-p54 antibodies appear as early as eight days after infection and persist for several weeks (Pastor et al., 1990; Alcaraz et al., 1995; Perez-Filgueira et al. 2006). A p54 ELISA-based serological test, described by Gallardo et al., (2009), showed a 98% sensitivity and 97% specificity compared to the OIE ELISA. In addition, the ASFV antibody-positive samples remained positive for p54 reactivity after storage for one month at 37˚C. Therefore, p54 antibody is among the good targets for detection of ASFV antibodies (Oviedo et al., 1997).

In this study, we generated a panel of mAbs against a baculovirus-expressed p54 recombinant protein. The results showed that the mAbs and sera from ASFV-infected pigs recognized conserved and variable regions on p54, which are consistent with linear epitopes. In turn, the results can be incorporated into the development of ASF serological assays and vaccines.

2. Materials and methods

2.1 Ethics statement on the use of animals. Experiments involving animals and virus were performed in accordance with the Federation of Animal Science Societies Guide for the Care and Use of Agricultural Animals in Research and Teaching, the USDA Animal Welfare Act and Animal Welfare Regulations, or according to the National Institutes of Health's Guide for the Care and Use of Laboratory Animals, and were approved by the Kansas State University and Plum Island Animal Disease Center institutional animal care and use and institutional biosafety committees. Animals were humanely euthanized by pentobarbital overdose following the American Veterinary Medical Association (AVMA) guidelines for the euthanasia of animals, and all efforts were made to minimize suffering.

2.2 Serum samples. Experimental infection of pigs with ASFV was performed in the BSL3-Ag facilities at the Biosecurity Research Institute (BRI). Six five-week-old Large White X Landrace pigs were inoculated *via* intramuscular injection (IM) with 10^4 $TCID_{50}$ of the low virulent isolate, ASFV OURT88/3. Blood was collected in serum separator tubes before infection (0 DPI) and at 17 days post infection (17 DPI). For normalization of results, the internal standards included on each plate were serum samples obtained before (0 DPV) and after immunization (55 DPV) with a replication deficient alphavirus expression p54 antigen (based on BA71V strain). Serum samples were stored at -80^o C prior to use.

2.3 Production of anti-p54 monoclonal antibodies. The nucleotide sequence of the p54 gene encompassing amino acids 60 to 178 (without the predicted transmembrane region) was amplified from ASFV Georgia 2007/1 strain (GenBank Accession # FR682468.1) using the

following primers: Forward 5´-TTG GCC CAA GAC TTG CTG AAT AGC-3´ and Reverse 5´-ATA CGT TGC GTC CGT GAT AGG AGT -3´. The amplicon was cloned into the expression cassette of pFastBac/HBM TOPO vector (Invitrogen Bac-to-Bac HBM TOPO), and then shuttled into the baculovirus shuttle vector (bacmid). The recombinant bacmid DNA was then used to transfect SF9 and Hi-Five insect cells to express p54 fusion protein with 6×His-tag attached to the C-terminus, according to the manufacturer's instructions. The protein was expressed in baculovirus-infected cells and purified using HisPurTM Cobalt Chromatography Cartridge (Thermo Fisher). The immunization of mice and preparation of mAbs is the same as previously described in Heimerman et al. (2017). Briefly, the p54 immunogen was emulsified with Montanide ISA 206 VG adjuvant (Seppic) at 1:1 ratio. BALB/c mice were immunized with 0.2 mL (10 µg) of immunogen and boosted at two-week intervals. The antibody titer was assessed by indirect ELISA on plates coated with baculovirus-expressed p54(60-178). Splenocytes were isolated and fused to Sp2/O myeloma cells (kindly provided by Dr. Lucy, F. Lee, at Avian Disease Oncology Laboratory, Agriculture Research Service, USDA). Hybridomas were screened by indirect ELISA against the same baculovirus-expressed p54(60-178) antigen used for immunization. For the IFA, Lisbon/60 infected Vero cells were fixed with cold acetone:methanol (50:50%, v/v) for 5 min at room temperature. The monolayers were rinsed once with PBS, then incubated with hybridoma supernatant at 37°C for 30 min. After washes, the Alexa Fluor 488–conjugated goat anti-mouse IgG (Life Technologies, Thermo Fisher), diluted 1:500 in PBS, was added to the plate and incubated at 37°C for 30 min. Cells were washed again and viewed under a fluorescence microscope. Positive clones were amplified and sub-cloned to produce monoclonal hybridoma cell lines and mAbs.

2.4 Expression of p54 polypeptide fragments. The ASFV p54 gene sequence from ASFV BA71V strain (GenBank Accession # U18466.1) (see Figure 1B) was used for the preparation of codon optimized p54 polypeptide fragments (Integrated Technologies). Synthesized DNA fragments were prepared for five polypeptide fragments; p54(54-113), p54(83-143) and p4(113-183), p54(1-183) and p54(54-183). *SacII* and *EcoRI* restriction sites were incorporated into the 5′ and 3′-ends of each DNA fragment and cloned into a pHUE bacterial expression vector, which expresses a 5xHis ubiquitin fusion protein (Catanzariti et al., 2004). Plasmids were transformed into *E. coli* BL21 (DE3) competent cells (Cat #C2527H, New England Biolabs). Expression and purification of polypeptides was the same as described in Heimerman et al. (2017). Briefly, the soluble recombinant proteins were purified from the bacterial pellet using PrepEase His-Tagged Protein Purification Kit (USB), under native conditions according to the manufacturer's instructions. Polypeptides that remained insoluble were purified under denaturing conditions using 8 M urea. Partially soluble proteins were purified using a modified protocol incorporating 0.3% Sarkosyl and 0.5 M CAPS buffer. Affinity purification was performed on a nickel column and the purity assessed by SDS-PAGE. Protein concentration was determined by the Bradford assay. Oligopeptides, 15-16 amino acids in length were commercially synthesized by 21st Century Biochemicals and coupled to ovalbumin (OVA) for a better exposure of antigen during ELISA. The oligomer sequences were based on the ASFV Georgia 2007/1 isolate (GenBank Accession # FR682468.1).

2.5 ELISA. Indirect ELISA was performed as previously described by Heimerman et al. (2017). Briefly, recombinant polypeptides or OVA-coupled oligopeptides were adjusted to a concentration of 4 µg/ml in carbonated coating buffer (pH 9.6), and 100 µl added to each well of

a flat-bottom polystyrene plate (Costar). Wells were blocked overnight with 10% goat serum in PBS (PBS-GS). Dilutions of serum (1:400) or mAb (1:4) were prepared in PBS-GS and 100 µl added to each well. After incubation for 1 hr at 37°C, the plates were washed 3x with PBS with 0.01% Tween 20 (PBST), and secondary antibody added, and incubated for 1 hr at 37°C. Secondary antibodies, purchased from ICN Biomedicals, included HRP-conjugated goat anti-swine IgG and HRP-conjugated goat anti-mouse IgG , diluted 1:2000 and 1:500 in PBS-GS, respectively. After washing, 100 µl of ABTS substrate (KPL) was added to each well and incubation continued at room temperature in the dark for 20 min. The reaction was stopped by adding 1% SDS and absorbance measured using a microplate reader (FLUOstar Omega BMG Labtech). The results were reported as A_{405}–A_{650} nm. ELISA plates included a negative control (a p72 mAb). A sample was considered positive if the OD value was greater than 3 standard deviations above the anti-p72 irrelevant mAb.

2.6 Western blots. Polypeptides were separated by SDS-PAGE and then transferred to 0.2 µm PVDF membranes (Amersham) using a Mini Trans-Blot Electrophoretic Transfer Cell (BIO-RAD). The western blot procedure was performed as described in Heimerman et al. (2017). After transfer, membranes were incubated overnight at 4°C in blocking buffer (PBST with 5% nonfat dry milk) followed by three washes with PBST. Anti-p54 mAbs diluted 1:100 or a Penta-His (QIAGEN) mAb diluted 1:1000 in blocking buffer was added to the membrane and incubated for 1 hr at room temperature. After washing with PBST, HRP-conjugated goat anti-mouse IgG (ICN Biomedicals), diluted 1:3000 in blocking buffer, was added and incubation continued for 1 hr at room temperature. Peroxidase activity was detected with 4-chloro-1-naphtol/3,3'-diaminobenzidine, tetrahydrochloride substrate (CN-DAB; Thermo Fisher) for 2–10

min at room temperature. The reaction was stopped by rinsing the membrane with double-distilled water.

2.7 Virus neutralization assay. Monoclonal antibodies were serially diluted 1:4 in MEM supplemented with 7% FBS and antibiotics. Each mAb was assayed in triplicate. Each assay included a virus only control (no mAb) and an unrelated mAb of the same isotype (anti-E2 CSFV). One hundred microliters of mAb were mixed with 100 µl of ASFV BA71V at a concentration of 500 $TCID_{50}$/ml on a 96 well plate and incubated for 2 hrs at 37°C. Samples were transferred to a 96-well plate of confluent Vero cells and incubated for 3-4 days at 37°C, 5% CO_2. Infected cells were detected by IFA with an anti-p30 mAb prepared by us. Using an EVOS FL Auto microscope (Thermo Fisher), the number of fluorescent cells were counted in 10 fields per well. The percent neutralization was calculated using the following formula: ((1-(Mean number of positive cells / Mean number of positive cells in isotype control)) *100). Positive/negative cutoff was set to 90% inhibition.

2.8 Data analysis. For screening the mAb's data was normalized based on optical density (OD) value of the test sample minus background of the negative control. All clones with optical OD two times or higher than the negative control were considered positive. Normalized absorbances for ELISA using polyclonal serum were calculated based on the following formula: (A_{405}-A_{650}) of sample / positive control mean. A sample was considered positive if its normalized absorbance was above the mean of the negative control +3 SD. Serum from a pig immunized with an alphavirus construct expressing p54 was used as positive control for data normalization.

3. Results

3.1 Expression and purification of p54 recombinant proteins. All recombinant proteins were expressed in *E. coli* as 5xHis-ubiquitin fusion proteins. The inclusion of 5xHis-ubiquitin was for the purpose of affinity purification and to improve expression in *E. coli* (Catanzariti et al., 2004). The full-length protein, p54(1-183), was cloned into a vector that lacked the Ubiquitin tag and was insoluble under native buffer conditions. Therefore, purification was performed in the presence of 8 M Urea. The p54(54-183) polypeptide was also insoluble but could be purified under milder conditions using CAPS/Sarkosyl buffer. The remaining p54 fragments (54-113, 83-143, 113-183) were affinity purified under native conditions with the buffers supplied in the kit. As shown in Figure 2.2 and Table 2.2, SDS PAGE followed by immunoblotting with a 5xHis mAb showed that all p54 constructs migrated as expected and according to their predicted molecular weights. The rationale behind using p54 (1-183) in western blots (WB) relies on the fact that 8M Urea will disrupt the potential conformational epitopes, therefore will represent a better alternative to detect linear epitopes (Oviedo et al., 1997).

3.2 Characterization of anti-p54 mAbs. Putative mAbs were initially screened against the baculovirus expressed p54(60-178) Georgia 2007/1 antigen used for the immunization of mice. The 12 positive mAbs were then screened by IFA on Vero cells infected with Lisbon/60. The results produced seven IFA-positive clones. The five IFA-negative clones were not subjected to any further study. A third round of screening was conducted using ELISA, and WB against BA71V p54 recombinant polypeptides expressed in *E. coli*. Of the seven IFA-positive mAbs, five clones, #101, #117, #7, #143-1 and #154-1, reacted with the *E. coli*-expressed antigen (see Table 2.2). The two negative mAbs, #61 and #8, where not subjected to further analysis, but

likely recognize conformational epitopes in p54 present in infected cells but absent in the bacterial expressed products.

3.3 Epitope mapping against p54 polypeptides and oligopeptides. The remaining five mAbs were screened by ELISA against the polypeptide fragments listed in Table 2.2. As expected, all mAbs recognized the largest polypeptide fragment, p54(54-183). Of the smaller fragments, MAb #154-1 recognized only p54(54-113) and was placed in Group 1. The mAbs #7 and #143-1 reacted with p54(54-113) and p54(83-143). The polypeptides have an overlap of 30 amino acids, located between amino acids 83 and 113. These two mAbs were placed in epitope recognition Group 2. Finally, mAbs #117 and #101 recognized p54(83-143) and p54(113-183) and were placed in Group 3. The overlap between the two polypeptides covers the 31-amino acid sequence between amino acids 113 and 143. For the purpose of completeness, the seven remaining mAbs, #25, #42, #61, #75, #8, #12-2, #2-18, listed in Table 2.1, were negative for reactivity against the p54 polypeptides (data not shown).

Finer epitope mapping studies consisted of reacting mAbs with three sets of overlapping oligopeptides diagrammed in Figure 2.3. The three oligopeptide groups covered peptide sequences p54(60-84), p54(83-113), and p54(113-150). The Group 1 antibody, #154-1, recognized two oligopeptides, p54(60-75) and p54(65-79), which contained the sequence overlap, 65-EDIQFINPYQD-75. The two mAbs in Group 2 each recognized a different oligopeptide: mAb #143-1 reacted with 93-ATTASVGKPVTGRPA-107 and mAb #7 reacted with the partially overlapping oligopeptide, 98-VGKPVTGRPATNRPAT-113. Based on this difference, mAb #143-1 was placed in Group 2a and mAb #7 in Group 2b. The Group 3 mAbs,

#117 and #101, reacted with oligopeptides p54(113-127) and p54(118-132). The overlapping peptide sequence consisted of 118-TDNPVTDRLV-127.

3.4 Comparison of p54 antigenic regions among representative ASFV isolates. The p54 peptide sequences, listed in Table 3, show sequences from 26 viruses that represent 20 of the 24 known ASFV genotypes. The results showed that the peptide sequence in the proposed Group 1 antigenic region, recognized by mAb #154-1, is highly conserved among the different ASFV isolates. The identity predicts that mAb #154-1 should recognize all ASFV genotypes. The peptide sequence recognized by the Group 2a antibody, #143-1, was conserved for 5 isolates representing viruses in genotypes II, IV, XVII, XXa, XXI. In addition, peptide sequences for genotypes I, V and VI were also identical to the Georgia strain except for a single conserved alanine to valine substitution. Recognition of infected cells with BA71V and Lisbon/60 peptide sequences by mAb #143-1, indicates that the presence of an alanine found in the genotype 1 viruses does not affect antibody recognition. The Group 2b epitope was conserved in only two isolates belonging to genotypes II and XVII. A single conserved valine to alanine substitution at position 98 was present for genotype I viruses. Since mAb #7 recognized BA71V and Lisbon/60 peptide sequences, this amino acid substitution does not affect mAb recognition. Genotypes IV, Va-b, VI, XXa and XXI possessed a single threonine to valine substitution at position 113. The peptide sequences recognized by Group 3 mAbs were identical only for genotypes I, II and Xa.

3.5 Virus neutralization activity of mAbs. The mAbs were tested for neutralizing activity using the Vero-adapted virus BA71V. mAbs belonging to Grps I, IIb and III were negative for the presence of neutralizing activity at a dilution of 1:4 (neutralization activity <90%). Interestingly,

mAb belonging to Grp IIa (143-1) showed the highest neutralization activity, 94%. Moreover, when all 5 antibodies were combined, neutralizing activity was present above background at a dilution of 1:16 (Table 2.4).

3.6 Recognition of polypeptides and oligopeptides by sera from ASFV-infected pigs. Sera from 6 pigs, experimentally infected with ASFV isolate OURT88/3, were tested against the p54 polypeptides described in Table 2.3. OURT88/3 is a low virulent isolate that induces a robust antibody response (Mulumba-Mfumu et al., 2016). For this analysis, we selected sera collected at 17 days after infection when pigs tested negative for virus in serum. The results in Figure 2.4A showed the highest mean absorbance for the largest polypeptide, p54(54-183). Among the smaller polypeptide fragments, the greatest reactivity was against the C-terminal polypeptide, p54(113-183), followed by p54(83-143) and p54(54-113). Sera were also reacted with the panel of oligopeptides described in Figure 3. The results showed that the p54(113-127) and p54(118-132) oligopeptides reacted with the most sera, with 62.5% and 37.5% of sera being positive, respectively (Figure 2.4B). These two oligopeptides were also recognized by the Group 3 mAbs (see Figure 2.3). Only 12.5% of sera reacted with p54(98-113), which was recognized by the Group 2b mAb, #7. Only one pig (6%) reacted with p54(65-75) and p54(65-79), which were recognized by the Group 1 mAb, #154-1. Only 20% of the pigs reacted with p54(83-97) and 10% with p54(88-102), regions that were not recognized by none of the mAb's. Together, these data show that antibody response during ASFV infection is directed towards the N-terminal region of p54, between amino acids 113 and 132, which is an immunodominant region.

4. Discussion

In the absence of an effective vaccine, the control of ASF relies on accurate, efficient, and low-cost detection strategies for active surveillance of negative populations, detection of infected herds following an outbreak, and for demonstrating freedom from disease. Along with p72, p30 and pp62, p54 is a major structural protein which is immunogenic and often incorporated in the formulation of vaccines and for serology-based detection methods (Neilan et al. 2004; Gallardo et al., 2006; and 2009). The characterization of antigenic domains in p54 provides valuable information which can be incorporated into improved diagnostic assays and for the design of vaccines. Previous studies identified a linear epitope in the p54 DBD region which covered amino acids 149-161. Sera from mice immunized with a DBD oligopeptide showed a small but detectable amount of virus neutralizing activity (Escribano et al., 2013). In this study, mAbs were prepared following immunization of mice with a baculovirus-expressed polypeptide that covered the p54(60-178) region. However, none of the mAbs produced in this study recognized the DBD region of p54. Even though the DBD peptide sequence is antigenic, it may not be immunodominant.

We used two expression systems to produce recombinant p54: baculovirus and *E. coli*. The baculovirus system has the advantage of being able to produce proteins with post translational modifications, and since the p54 is predicted to contain 23 glycosylation sites, it was a preferred choice for the immunization of mice. For the initial epitope mapping studies, we used an *E. coli* expression system since it had been previously used in the production of p54 for diagnostic assays development (Alcaraz et al., 1995; Reis et al., 2007).

Based on the reactivity of overlapping polypeptides, three antigenic regions were identified in this study, p54(54-113), p54(83-143), and p54(113-143) (see Table 2.3). The single

Group 1 mAb, #154-1, is predicted to react with a peptide encompassed by p54(65-75), which is located in a highly conserved region of p54 (Table 2.3). The conserved nature of the antigen recognized by mAb #154-1 creates the opportunity to incorporate this antibody into blocking and antigen-capture or blocking ELISA-based tests. One drawback in the use of mAb#154-1 for the detection of anti-ASFV antibodies in pigs is that the p54(65-75) region does not appear to be very immunogenic, since only a small percentage of infected pigs recognized this region (Figure 2.4B). The Group 2b and Group 3 mAbs, along with sera from OURT 88/3 infected pigs, preferentially recognized a region covering amino acids 97 to 132 (Figure 2.4B). Also, one mAb 143-1, showed a high neutralization activity *in vitro*. However, the region recognized by this mAb, 93-107 is not very antigenic. This could be explained in two ways: 1) *in vitro*, phospholipid composition of the virion changes after several passages, potentially masking neutralizing epitopes and 2) other neutralizing regions on p54, such as DBD are not very antigenic, requiring multiple challenges to generate an antibody response. Therefore, we can conclude that, as the virus is replicating, the outside architecture changes after each cycle and that could be the reason why the region 93-107 is not recognized by ASFV serum. Moreover, as in the case with the DBD, an antibody response might be directed towards this region after several consecutive challenges.

As described in Table 3, this region has considerable peptide sequence variability. In fact, genotypes V and VI, lack the p54(118-127) peptide sequence altogether, which means that isolates within these genotypes would not be recognized by the Group 3 mAbs. Therefore, Group 3 antibodies might be useful to differentiate between different circulating strains. The high specificity of the mAb's that reacted with variable epitopes could be exploited to capture ASFV

envelope protein in an antigen-capture ELISA. The development of this assay is utmost important when antibodies against virulent ASFV strains are not present in the serum

5. Conclusion

The anti-p54 mAbs developed in this study recognized several conserved and variable regions consistent with linear epitopes. The epitopes recognized by mAbs were also recognized by sera from ASFV-infected pigs. These reagents will be useful to develop different diagnostic assays for detection of anti-ASFV antibodies and viral antigen in infected pigs.

Acknowledgements

Funded was provided by the Kansas National Bio and Agro-Defense Facility Transition Fund and the Kansas Bioscience Authority through a matching grant to Kansas State University's Center of Excellence for Emerging and Zoonotic Animal Diseases, Award # 15-130. Monoclonal antibody preparation was funded by the U.S. Department of Homeland Security under IAA Award # HSHQDC-12-X-00122. We would like to thank Dr. Baker for the contribution of the pHUE expression vector.

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Table 2-1. Summary of results for mAbs prepared against baculovirus-expressed p54 (60-178)

mAb	p54(60-178)	IFA[*1]	Western/ ELISA[*2]
101	+	+	+
117	+	+	+
25	+	-	-
42	+	-	-
61	+	+	-
7	+	+	+
75	+	-	-
8	+	+	-
12-2	+	-	-
143-1	+	+	+
154-1	+	+	+
2-18	+	-	-

[*1] Immunofluorescent antibody (IFA) staining performed against Vero cells infected with Lisbon/60.
[*2] ELISA performed on plates coated with *E. coli*-expressed p54(54-183). Western blots performed with *E. coli*-expressed p54(1-183). Recombinant proteins were based on ASFV BA71V (See Figure 1B for peptide sequence). For the ELISA, a positive result was based on an absorbance value greater than 3 standard deviations above the negative control.

Table 2-2. Reactivity of mAbs against p54 polypeptide fragments*

Location	Purif. method	~size (kDa)	mAb (Epitope Group) 154-1 (1)	143-1 (2)	7 (2)	117 (3)	101 (3)	p54 protein/polypeptide fragment
1-183**	8M urea	20	+	+	+	+	+	1 — 183
54-183	CAPS/ Sarko	35	+	+	+	+	+	54 — 183
54-113	Native	17	+	+	+	-	-	54 — 113
83-143	Native	16	-	+	+	+	+	83 — 143
113-183	Native	18	-	-	-	+	+	113 — 183

*mAb's were screened by ELISA against fragments corresponding to the BA71V (GenBank Accession # U18466.1) peptide sequence (see Figure 1 for sequence). Fragments were identified as positive based on a greater than 3 standard deviation increase in OD_{450} above the background. The epitope group assigned to each mAb is shown in parentheses.
** expression vector without ubiquitin

Table 2-3 Comparison of p54 antigenic regions with peptide sequences from representative ASFV isolates.

ASFV Strain (GenBank Accession #)	p54 Genotype	Grp1 (65-75)	Grp2a(93-107) Grp2b(98-113)	Grp3 (118-127)
Georgia 2007 (AM999765)	II	EDIQFINPYQD	ATTASVGKPVTGRPA VGKPVTGRPATNRPAT	TDNPVTDRLV
BA71V (FJ174390)	Ia	. . . . .	. . . . . A	. . . . .
Lisbon/57 (FJ174420)	Ib	. . . . .	. . . . . A	. . . . .
Lisbon/60 (EU874321)	Ic	. . . . .	. . . . . A	. . . . .
Mkuzi 1979 (AY261362)	Id	. . . . .	. . . . . A	. . . . .
Warmbaths (AY261365)	III	. . . . .	. . . . G - - . . . . . D . . . . . L V	A . N R . . M . N P
Wharthog (AY261366)	IV	. . . . .	 V	A . N . . . . .
Tengani 62 (AY261364)	Va	. . . . .	V	- - - - - - - - - .
Moz64 (FJ174422)	Vb	. . . . .	V	- - - - - - - - - .
SPEC/265 (EU874344)	VI	. . . . .	V	- - - - - - - - - .
SPEC/154 (EU874359)	VII	. . . . .	. . . . G - - . . . . . D . . . . . L V	A . N R . . M . N P
Malawi Lil-20/1 (AY261361)	VIII	. . . . .	. . S . G . A . . . . M . . . V . . K . -	- - - - - . . . . .
Ug03H.1 (FJ174431)	IX	. . . . .	. . . . G N . . . . I . D . . . . D . . V	. . . - - - - - . .
Ug64 (FJ174430)	Xa	. . . . .	. . . . G N . . . . I . D . . . . D . . V	. . . . .
Kenya 1950 (AY261360)	Xb	. . . . .	. . . . G N . . . . I . D . . . . D . . V	- . N . . . . .
M KAB/62 (EU874331)	XI	. . . . .	. . . . G . . . . . M . . . V . . K . I	. . N . . . . .
SUM/1411 (EU874357)	XIII	. . . . .	. . . . G G . . . . . M . . . V . . K . V	V . N . . . I N N S
NYA/1/2 (EU874330)	XIV	. . . . .	. . . . G . . . . . M . . . V . . K . -	. . N . . I . . . .
TAN/08/MAZIMBU (GQ410767)	XV	. . . . .	. . . . G . . S . . . M D . . I . . N . V	H P V . . R L . . .
TAN/03/1 (EU874354)	XVI	. . . . .	. . . . G . . S . . . - - - - - . H . . V	V . N . . . T N N P
ZIM/92/1 (EU874345)	XVII	. . . . .	. . . . .	. . - - . . . . .
RSA/96/3 (EU874375)	XIX	. . . . .	. . . . G - - . . . . . D . . . . . L V	A . N R . . M . N P
Pretorisuskop/96/4 (AY261363)	XXa	. . . . .	 V	A . N . . . . .
RSA/96/1 (EU874339)	XXI	. . . . .	 V	A . N . . . . .
SPEC/245 (EU874381)	XXII	. . . . .	. . . . G - - . . . . . D . . . . . L V	A . N R . . M . N P
ETH/1a (KT795363)	XXII	. . . . .	. . A . G G . . R . I . D . . . . D . . -	V . N R L . T N S P

Table 2-4 Neutralization activity of p54 mAbs against BA71V strain.

mAb	mAbs*						
Dilution	154-1	143-1	7	117	101	all	CSF
1/4	100+/-18 (78)	28+/-13 (94)	59+/-7 (87)	79+/-1 (82)	81+/-3 (82)	30+/-4 (93)	>450 (0)
1/8	106+/-38 (76)	49+/-9 (89)	59+/-1 (87)	99+/-7 (78)	207+/-60 (54)	38+/-4 (92)	>450 (0)
1/16	>450 (0)	365+/-120 (19)	277+/-28 (38)	>450 (0)	>450 (0)	38+/-8 (92)	>450 (0)

*The upper number shows the number of infected cells +/- one standard deviation for three replicates. The percent inhibition is in parenthesis. For wells without antibody, the mean number of cells was 450+/-49. Shaded boxes indicate positive neutralization results; percent inhibition of BA71V greater than 90%. A an anti-CSF E2 mAb was used as an isotype control.

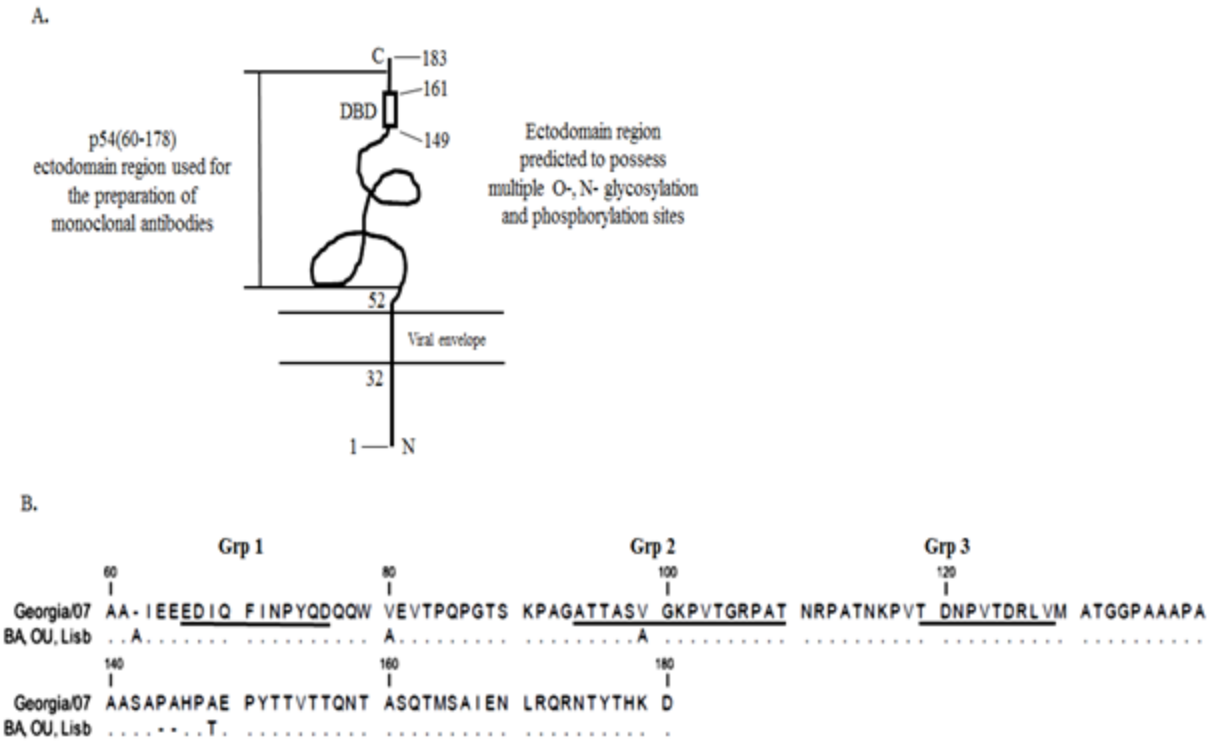

Figure 2-1 ASFV p54 structure and peptide sequence. (A) p54 is a type I transmembrane protein located in the inner envelope of the virion. The C-terminal ectodomain region is predicted to possess several glycosylation and phosphorylation sites (Mima et al., 2015; Sun et al., 1995). Near the outer C-terminal end is the dynein binding domain (DBD). **(B)** Antigenic domains of the p54 recognized by the mAbs used in this study. Sequences from other isolates used in this study (OURT 88/3, Lisbon/60) are identical with BA71V.

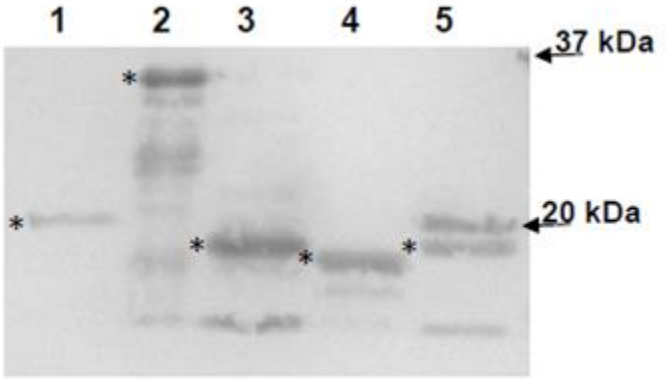

Figure 2-2 Expression of p54 polypeptide fragments. (A) Diagram of polypeptide fragments, purification conditions and predicted molecular weight. **(B)** Anti-His tag mAb staining of ASFVp54-ubiquitin fusion polypeptides. The predicted position of each protein is identified by an asterisks Key: Lane 1, p54(54-183); Lane 2, p54(1-183); Lane 3, p54(54-113); Lane 4, p54(83-143); Lane 5, p54(113-183).

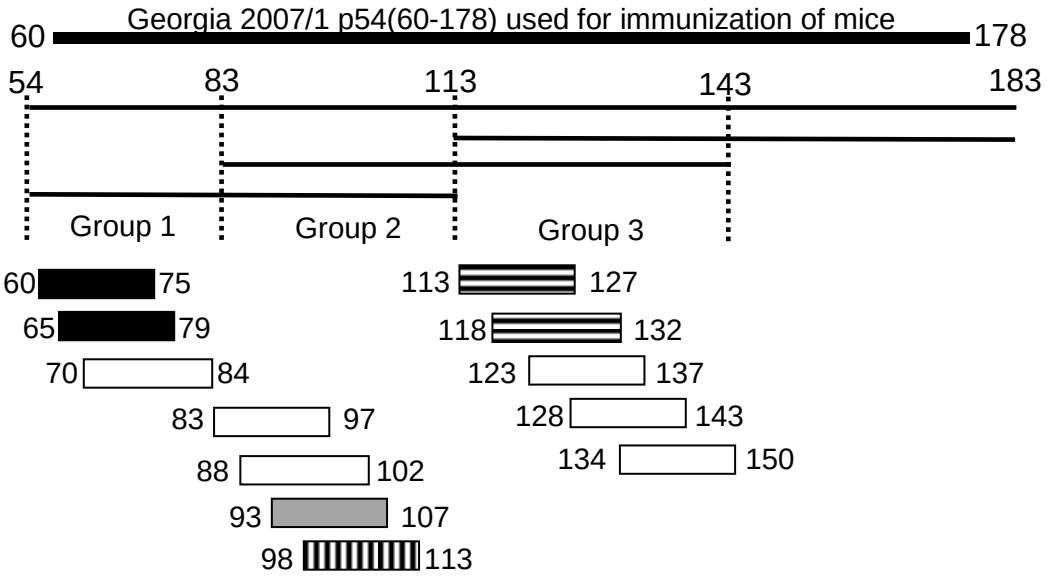

Figure 2-3 Reactivity of mAbs against p54 oligopeptides. Horizontal lines show the same polypeptides diagrammed in Figure 2.2. Rectangles show synthesized oligopeptides. Oligopetide sequences were prepared according to BAV71V (see Figure 2.1B for comparison with Georgia). Key: reactivity with mAb #154-1, black; mAb #143-1, gray; mAb #7, vertical lines; mAbs #117 and #101, horizontal lines.

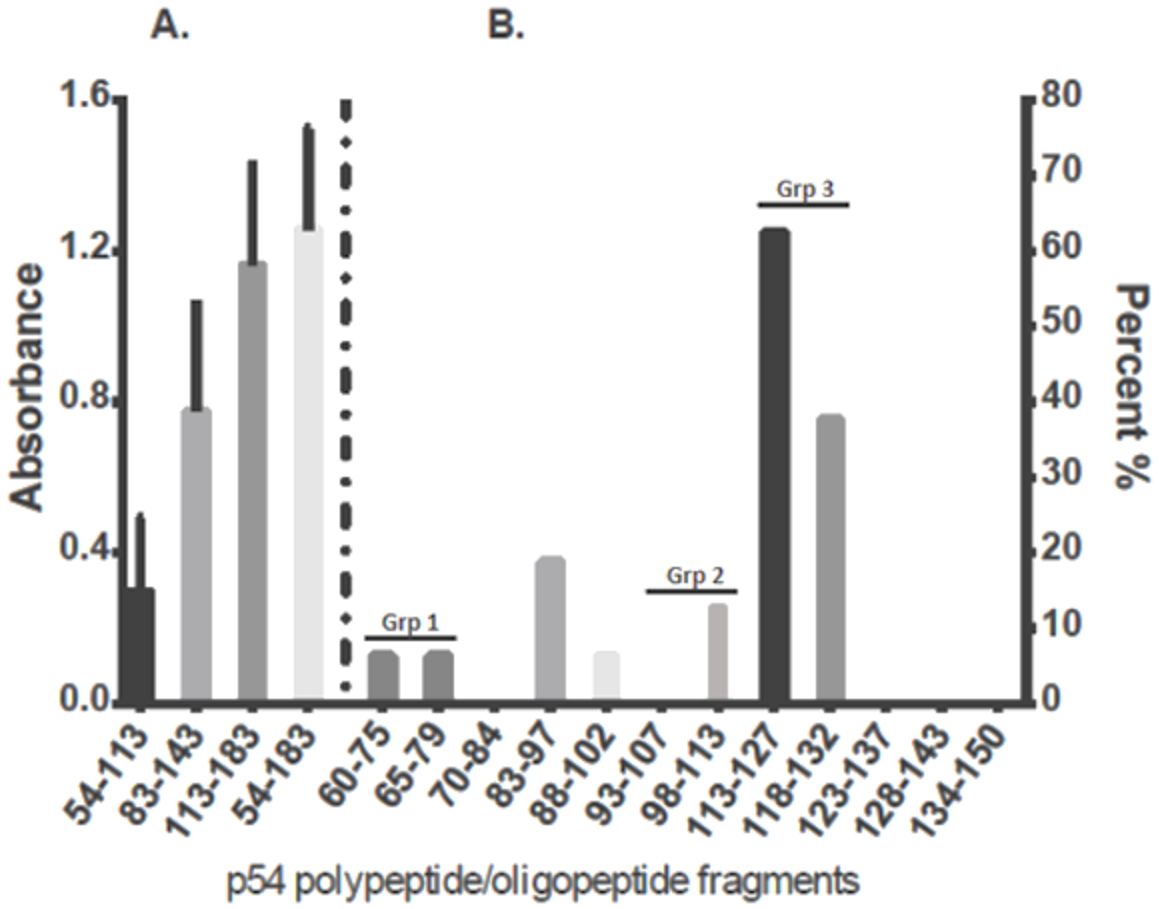

Figure 2-4. Reactivity of sera from ASFV-infected pigs with p54 peptide fragments. Serum samples were obtained from 6 pigs at 17 days after infection with OURT 88/3. (A) The mean and standard deviation for absorbance against the polypeptide fragments described in Figure 2. (B) Reactivity of sera against oligopeptide fragments described in Figure 3. Results are shown as percent positive, which was determined by an absorbance value greater than 3 standard deviations above the absorbance for negative sera. The horizontal lines above the bars show oligopeptides recognized by the different groups of mAbs.

Chapter 3 - Development and characterization of monoclonal antibodies against p30 protein of African swine fever virus

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Abstract

Among the structural proteins composing the virion of African swine fever virus (ASFV), p30 is one of the most immunogenic proteins and is produced early during ASFV infection. These two characteristics made it a good target for the development of diagnostic assays. In this study, a panel of p30-specific monoclonal antibodies (mAbs) was generated. The reactivity of these mAbs was confirmed by immunoprecipitation and Western blot analysis in Vero cells infected with alphavirus replicon particles expressing p30 (RP-p30). Furthermore, this panel of mAbs recognized ASFV strains BA71V (Genotype I) and Georgia/2007 (Genotype II) by immunofluorescence assay with virus-infected Vero cells and swine macrophages. They also detected p30 expression by immunohistochemistry in tissue samples from ASFV-infected pigs, including lung, tonsil, spleen and lymph nodes. Epitope mapping revealed a linear epitope within the 32-amino acid region, 61-93, which is also recognized by ASFV infected pigs. In contrast, two of the mAbs recognized the C-terminal region of the protein, which is highly hydrophilic, enriched in glutamic acid residues, highly immunogenic and possessing properties consistent with an intrinsically disordered protein region (IDPR). The availability of this panel of mAbs and mAb-based diagnostic assays provide valuable tools for ASFV surveillance and disease control.

Keywords: monoclonal antibodies, African swine fever virus, p30 protein, diagnostic assays

1. Introduction

African swine fever virus (ASFV) is a causal agent of lethal hemorrhagic fever in domestic pigs, characterized by high mortality and morbidity. The virus is directly transmitted by contacting with other infected animals and contaminated feed or fomites and is also transmitted indirectly through the soft ticks of genus *Ornithodoros* (Sánchez-Vizcaíno and Neira, 2012). ASFV originated in Africa, where warthogs and bushpigs are reservoirs (Montgomery, 1921; Oura et al., 1998). The virus initially spread from West Africa to Europe in the middle of the last century. It was finally eradicated from most areas of Europe during the mid-1990s, but ASFV remains endemic in the island of Sardinia, Italy (Martinez-Lopez et al., 2015). Since 2007, ASFV again spread out of Africa to Caucasus and then Eastern Europe, causing outbreaks in the Russian Federation and several neighboring countries, including Belarus, Ukraine, Lithuania, Estonia, Poland, Latvia, Czech Republic, Romania and Hungary. Recently, ASFV has outbreaks in major swine producing countries in Europe, China and Mongolia. In Europe, the most affected country is Romania with 1062 ASFV outbreaks that have been reported by the Romania National Sanitary-Veterinary and Food Safety Authority (ANSVSA) to OIE, while the number of confirmed cases of ASFV-infected wild boars has been increasing in Belgium (http://business-review.eu/news/african-swin-fever-expanded-to-276-localities-with-1062-active-outbreaks-189738; https://www.fwi.co.uk/livestock/health-welfare/livestock-diseases/28-wild-boar-infected-with-african-swine-fever-in-belgium). In China, following the confirmation of the first case on August 3, 2018, almost 1 million pigs were culled (Ge et al., 2018). These new outbreaks pose the potential pandemic threat to global swine industry.

ASFV is a large double-stranded DNA virus that belongs to the family *Asfarviridae*, genus Asfivirus (Dixon et al., 2013). The virus is enveloped with two membranes at its inner and

outer sides, wrapped around an icosahedral capsid. The virion is composed of several concentric domains that are made of more than 50 polypeptides (Alejo et al., 2018; Esteves et al., 1986). The p30 protein is assembled in the inner membrane of the viral envelope. It is a phosphoprotein, encoded by the gene CP204L. The protein is abundantly expressed before DNA synthesis takes place and continues until the end of the virus life cycle (Afonso et al., 1992; Prados et al., 1993).

Among the ASFV proteins that have been analyzed, p30 protein was determined to be a highly immunogenic protein and stimulates the highest level of antibody response during ASFV infection (Giménez-Lirola et al., 2016). Antibodies against p30 can be detected as early as 8 days post infection in pigs (Giménez-Lirola et al., 2016; Gomez-Puertas et al., 1998; Gomez-Puertas et al., 1996). Moreover, recombinant p30 fused with p54 induced neutralizing antibodies correlated with a reduction in viremia levels in vaccinated pigs (Barderas et al., 2001). These proprieties allow the use of p30 as an antigen in a variety of diagnostic tests (Barderas et al., 2000; Oviedo et al., 1997).

Since no vaccine and treatment are currently available, highly sensitive and specific diagnostic reagents and assays are urgently needed for rapid detection and implementation in animal quarantine and elimination. Monoclonal antibody (mAb) is a key reagent for the diagnosis of viral infection. Based on the immunogenic nature of ASFV p30 protein, in this study, we developed and characterized a panel of monoclonal antibodies (mAbs) against p30 protein. Moreover, we have compared our results with the ones obtained by our group from a prime boost vaccine study, incorporating an alphavirus vaccine platform expressing ASFV p30 as prime and low virulent strain OURT 88/3 as boost.

These mAbs were further applied in the development of various diagnostic assays for detection of ASFV infection.

2. Material and Methods

2.1 Source of serum samples. Experimental vaccination and/or infection of pigs with ASFV was performed in the BSL3-Ag facilities at the Biosecurity Research Institute (BRI). Sixteen five-week-old Large White X Landrace pigs were inoculated or vaccinated as described in section 2.2. Blood was collected in serum separator tubes before infection (0 DPI) and at 17 days post infection (17 DPI). For normalization of results, the internal standards included on each plate were serum samples obtained before (0 DPV) and after immunization (55 DPV) with a replication deficient alphavirus expression p30 antigen (based on BA71V strain). Serum samples were stored at -80˚ C prior to use

2.2 Prime boost approach using Alphavirus vaccine platform and ASFV OURT 88/3. Sixteen Large White x Landrace pigs were immunized intramuscularly (IM) with 2 ml containing 10^7 replicon particles (RP) of RP-30. Six pigs were mock inoculated as negative controls. One week following the second prime with RP-30 all sixteen pigs were inoculated 10^4 $TCID_{50}$ of low virulent OURT 88/3 strain. Serum was collected as described in section 2.1.

2.3 Production of recombinant p30 in *E. coli.* The ASFV p30 (631 bp) gene sequence from BA71V strain (GenBank Accession # U18466.1) was used for the preparation of p30 recombinant protein fragments. The corresponding nucleotide sequences were codon optimized for expression in *E. coli*, and synthesized by Integrated DNA Technologies (IDT, Coralville, IA). The His-tagged full-length or truncated p30 was cloned into pHUE vector and recombinant proteins were expressed in *E. coli* as described previously (Catanzariti et al., 2004; Heimerman et al., 2018). Nine recombinant overlapping proteins were purified with PrepEase His-Tagged

Protein Purification Kit (USB) following the manufacturer's instructions. Specificity of the recombinant protein was verified in western blot using anti-His TAG mAb (Clone J099B12; Biolegend, San Diego, CA).

2.4 Expression of p30 fragments in eukaryotic expression system. ASFV p30 was codon-optimized for expression in mammalian cells, and synthesized by Integrated DNA Technologies (IDT, Coralville, IA). To map p30 epitopes recognized by monoclonal antibodies, p30 gene fragments (Figure 5C) were cloned into pEGFP-C3 vector (Clontech Laboratories, Mountain View, CA) using standard molecular cloning method. These p30 fragments were designed based *in silico* B-cell linear epitope prediction of p30 [Figure 3.5B; (Larsen et al., 2006)].

2.5 Monoclonal antibody production. Monoclonal antibodies against p30 were produced as previously described (Fang et al., 2006; Li et al., 2012). Briefly, 6-8 weeks old BALB/C mice were immunized with 50-100 μg/mouse of purified p30 protein mixed with an equal volume of incomplete Freund's adjuvant. Mice were immunized intraperitoneally three times at a two-week interval. The mice were euthanized three days after the final immunization, the splenocytes were collected and fused with NS1 myeloma cells. After fusion, cells were cultured in 24-well plates in HAT selection media (Cat# 21060-017, Gibco, Life Technologies, Thermo Fisher Scientific Inc., Pittsburgh, PA). Culture supernatants were initially screened for p30-specific antibodies by immunofluorescence assay (IFA) on Vero cells, which were infected with a defective alphavirus replicon particle expressing p30 of BA71V strain (RP-30). Hybridoma clones producing p30-specific antibodies were sub-cloned into the single cells clones (monoclones). They were further

screened by IFA on RP-30-infected Vero cells; and the p30 mAb positive hybridoma clones were confirmed by IFA using ASFV-infected Vero and swine macrophages (see below).

2.6 Immunofluorescence assay (IFA). For IFA test on RP-30-infected cells, Vero cells at 70~80% confluent in 96-well plates were infected with RP-30 (1.89 x10^9 RP/ml) at 1:400 dilution. At 24 hours post infection (hpi), cells were fixed with ice-cold methanol for 30 min at -20°C. Cell monolayers were incubated with undiluted hybridoma culture supernatants (100 µl/well) overnight at 4°C. The secondary antibody Alexa-Fluor-488 goat anti-mouse IgG (Thermo Fisher Scientific, Waltham, MA) diluted 1:400 in PBS was used for detection. Nuclear was counterstained with 4', 6-diamidino-2-phenylindole (DAPI). The plate was checked under EVOS FL fluorescence microscope (Thermo Fisher Scientific, Waltham, MA).

For IFA test on ASFV-infected cells, Vero cells were infected with ASFV strain BA71V, while porcine alveolar macrophages were infected ASFV Georgia/07. At 48 hpi, cell monolayers were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. Cells were permeabilized by incubation with 0.1% Triton X-100 for 5 min and incubated with anti-p30 mAb followed by incubation with Alexa-Fluor-488 conjugated goat anti-mouse IgG (Thermo Fisher Scientific, Waltham, MA). Nuclear staining with DAPI was performed. The plate was observed under EVOS FL fluorescence microscope (Thermo Fisher Scientific, Waltham, MA).

For epitope mapping, IFA test was performed using Vero cells transfected with pEGFP-C3 constructs (Figure 3-5C) containing p30 gene fragments. At 24 hours post transfection (hpt), cell monolayers were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. After being permeabilized with 0.1% Triton X-100 for 5 min, cells were incubated with anti-p30 mAb followed by incubation with Alexa-Fluor-594 conjugated goat anti-mouse IgG (Thermo

Fisher Scientific, Waltham, MA). Nuclear staining with DAPI was performed. The plate was checked under EVOS FL fluorescence microscope (Thermo Fisher Scientific, Waltham, MA).

2.7 Western blot analysis. Western blot was performed using Vero cells infected with RP30. Briefly, RP-30-infected cells were harvested at 36 hpi with Pierce IP Lysis Buffer (Thermo Fisher Scientific, Waltham, MA) containing Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, MO). Cell debris was removed by centrifugation at 15,000g for 15 min at 4 °C. Cell lysates were mixed with Laemmli sample buffer (4X) and boiled at 95 °C for 5 min. After being separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS- PAGE), proteins were transferred onto a nitrocellulose membrane. The membrane was blocked overnight with 5% skim milk at 4 °C, and then incubated with anti-p30 mAb at an appropriate dilution at room temperature for 1 h. After washing with 1xPBS (containing 0.05% TWEEN 20; PBST), the membrane was further incubated with the secondary antibody, IRDye® 800CW Goat anti-Mouse IgG (H + L) (LI-COR Biosciences, Lincoln, NE) for 1 h at room temperature. After washing with PBST, the target proteins were visualized using a digital image system (Odyssey infrared imaging system; LI-COR Biosciences, Lincoln, NE). The expression of housekeeping gene GAPDH was detected as a loading control.

2.8 Immunoprecipitation assay. Immunoprecipitation assay was performed using Vero cells infected with RP30. At 36 hpi, cell lysates were harvested with IP lysis/wash buffer supplemented with Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, MO). Cell debris were removed through centrifugation at 12,000 g for 15 min at 4 ^{o}C. The cell lysate was subjected to immunoprecipitation with anti-p30 mAb using Pierce™ Classic Magnetic IP/Co-IP Kit (Thermo

Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. The expression of p30 in RP-30-infected cells was detected by western blot analysis with another mAb against p30.

2.9 Immunohistochemistry on ASFV-infected tissues. Tissues from pigs infected with ASFV strain Georgia 2007 were fixed in formalin and embedded in paraffin. They were subsequently sectioned at 4 μm onto positively charged slides. Slides were stained using the Leica Bond-Max autostainer with the Polymer Refine Red Detection kit (Cat# DS9390, Leica Biosystems Inc., IL), and then retrieved with Proteinase K for ten min at room temperature. The anti-p30 monoclonal antibodies (47-3, 62-35, and 142-4), diluted 1:100 in Bond Primary Antibody Diluent (Leica Biosystems, Tris-buffered saline), were added to the slides and incubated for 15 min at room temperature. Polymerization was performed with Polymer-AP α-Rabbit (Leica Biosystems Inc.) for 25 min at room temperature. The reaction was visualized using Fast Red chromogen and slides were counterstained with hematoxylin. Slides were observed under Nikon Eclipse 501 microscope (Nikon Corporation, Japan) and pictures were taken at magnifications of 10 X. The slides were also counterstained with hematoxylin and eosin (HE).

3.0 Indirect ELISA. Purified recombinant protein of p30 (full-length or fragments) was coated (4 ug/ml) on the flat bottom polystyrene plate at 100ul/well in carbonated coating buffer (pH 9.6). The plate was incubated for 1 h at 37°C followed by three washes with PBST (1xPBS containing 0.05% Tween 20). The plate was then blocked for 1 h at 37°C using 10% goat serum in PBS (PBS-GS). After washing with PBST, 100 μl of anti-p30 mAb or swine serum diluted 1:400 was added to each well and incubated for 1 h at 37°C, followed by 1h incubation at 37º C with HRP-conjugated goat anti-mouse IgG (ICN Biomedical) diluted at 1:2000 in PBS-GS or

HRP-conjugated goat anti-pig IgG (KPL Sera Care) diluted 1:4000 in PBS-GS. The HRP activity was measured using an ABTS 1-Component Microwell Peroxidase Substrate kit (KPL). The reaction was stopped by adding 1% SDS and the absorbance was measured at 405-650 nm. A non-related monoclonal antibody was used as negative background control. For swine serum, 0 DPI or DPV were included as background control.

3.1 Sequence analysis and disordered protein prediction analysis. To investigate the degree of conservation of the identified regions recognized by our mAbs, we selected twelve ASFV representative strains corresponding to circulating or historic genotypes. The GeneSilico MetaDisorder server (Kozlowski and Bujnicki, 2012) was used to predict the presence of intrinsically disordered regions on p30. The p30 amino acid sequence used for this analysis was based on BA71V strain (GenBank Acc # NC_001659.2).

3.2 Proteolytic assay and freeze thaw cycles. A 10 μg of TPCK Trypsin (Roche, Mannheim, Germany) was used to digest 100 μg p30(aa 101-204) in 1M KCl and 200 mM HEPES (pH 7.4) at 37˚C for different time points (0, 10, 15, 20 minutes). After incubation, reactions were stopped by the addition of an equal volume of 4×sodium dodecyl sulfate (SDS) loading buffer with β-mercaptoethanol and heating to 100°C for 5 min. Another 100 μg of p30 (aa 102-204) was frozen at − 80 °C for an hour, followed by thawing at room temperature. Finally, the samples treated with TPCK trypsin and freeze-thaw cycles were analyzed by western blotting with mAb 62-35 or Penta His antibody, as described above.

3.3 Half maximum calculation and development of a blocking ELISA assay. The three monoclonal antibodies were further tested to evaluate the possibility of using in a blocking ELISA (bELISA) format. First, we calculated their half-maximum values, by performing indirect ELISA as described previously. Next, we tested three sets of serum samples: first set, collected at 57 days post vaccination (dpv) from 10 pigs immunized with RP-p30, the second set represented by serum collected at 17 days post infection (DPI) from pigs challenged with low virulent OURT 88/3 and finally, a set of samples from pigs vaccinated with adenovirus expressing a cocktail of ASFV antigens (including p30). Briefly, serum samples were added undiluted in the first well of each column, then doubling dilutions were performed in PBS-GS. After the incubation, plates were washed 3 X times with PBST, then blocking antibodies were used at dilutions corresponding to their half-maximum absorbance values, then the plate was incubated at 37℃ for 30 minutes. Optimal dilutions of serum and mAbs were determined using a checkerboard titration test and the percentage of blocking was calculated using the formula: 100 - [(OD serum sample*100/OD background control)]. All the ELISA plates contained negative control serum collected at 0 dpv or dpi.

3. Results

3.1. Generation of monoclonal antibodies against ASFV p30. To produce the p30 antigen for mouse immunization, synthetic gene corresponding to CP204L gene sequence of BA71V strain was cloned and expressed as His-tagged recombinant protein in *Escherichia coli*. Recombinant p30 protein formed inclusion bodies. A strong denaturant (urea) was used to solubilize the protein and a protein-refolding step was performed. The purity of the recombinant protein was evaluated using sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel electrophoresis

followed by Coomassie blue staining. As shown in Figure 3.1A, the His-tagged p30 migrated to its predicted size (25 kDa). The identity of the protein was confirmed by Western blot analysis with anti-His antibody (Figure 3.1B).

To generate the anti-p30 mAb, mice were immunized with the p30 recombinant protein. After mouse splenocytes were fused with NS-1 myeloma cells, supernatants from the resulting hybridoma cells were screened by immunofluorescence assay (IFA) using Vero cells infected with recombinant alphavirus replicon particles expressing p30 protein (RP-30) (Figure 3.2A). Initial hybridoma screening yielded 3 primary clones of hybridoma. They were subcloned and 31 monoclones were obtained. One mAb from each primary clone, mAb #47-3, #62-35, and #142-4, were selected for further characterization (Table 3.1). Isotype analysis revealed that all three mAbs possessed an IgG1 heavy chain combined with a kappa light chain.

Using the cell lysate of RP-30 infected Vero cells, this panel of mAbs was tested on their reactivity in western blot analysis and immunoprecipitation. In the western blot, all three mAbs specifically detected a protein band just above the 25 kDa protein marker, which corresponded to the predicted size of p30 protein; as we expected, this band was not detected in mock-infected cells (Figure 3.2B). Consistently, these mAbs also detected p30 protein in immunoprecipitated proteins from RP-30 infected Vero cells (Figure 3.2C).

3.2. Reactivity of anti-p30 monoclonal antibodies in ASFV-infected cells and tissues. We further confirmed the reactivity of this panel of mAbs in ASFV-infected cells. IFA was performed on porcine alveolar macrophages (PAMs) infected with ASFV strain Georgia/2007. The cells were fixed and incubated with mAb 47-3, 62-35, and 142-4. As shown in Fig. 3.3, all three mAbs specifically recognized the ASFV-infected cells, in contrast all the cells with the

DAPI nuclear staining (Figure 3.3A-C). This result was further confirmed in Vero cells infected with cell culture adapted strain BA71V (Figure 3.3D). All three mAbs showed strong fluorescent signals on BA71V-infected Vero cells, while no fluorescent signal was detected on non-infected cells, indicating these mAbs specifically recognized the viral antigen in ASFV-infected cells.

The ability to detect antigen in paraffin-embedded tissues is an important diagnostic application and creates the opportunity to screen for ASFV in archived tissues. For this experiment, we used paraffin-embedded thin sections from pigs experimentally infected with ASFV Georgia/2007 (Popescu et al., 2017). Both mAbs 47-3 and 62-35 showed positive results for the presence of ASFV in lung, tonsil, spleen and lymph nodes; whereas, tissues from mock-infected pigs were negative. The strongest reactivity was obtained with mAb 47-3. Representative IHC result for mAb 47-3 is shown in Figure 3.4.

3.3. Epitope mapping using anti-p30 mAbs, vaccinated and infected serum. To map the epitope of each mAb, we initially tested the antibodies on Western blot for their reactivity against four large polypeptide fragments expressed from *E. coli* (Figure 3.5A; Suppl. 1): the full-length 204 amino acids (aa) of p30 protein, and three overlapping fragments, p30 (1-100 aa), p30 (101-204 aa) and p30 (50-150 aa). The results show that all three antibodies recognized the full-length recombinant protein. This is consistent with the antibody response generated by infected or vaccinated pigs (Figure 3.6). The mAb 47-3 recognized the N-terminal half of the protein, p30 (1-100), whereas mAbs 62-35 and 142-2 reacted with the C-terminal half of the protein, p30 (101-204). Additional N-terminal truncates were made to narrow down the epitope region of mAb 47-3: p30 (24-60 aa), p30 (40-80 aa), and p30 (61-100 aa). The result showed that the peptide p30 (61-100 aa) was recognized by the mAb 47-3, indicating a recognition of a relatively

short linear epitope located within the 40-aa of p30 (61-100 aa) peptide sequence. Using the same approach, four overlapping C-terminal truncates were constructed, including p30 (111-160 aa), p30 (161-204 aa), p30 (91-130), p30 (143-182 aa). Interestingly, none of these fragments reacted with mAbs 62-35 and 142-2. We further confirmed the result using peptide ELISA. Recombinant protein of full-length p30 or each of p30 truncates was used as the antigen to coat on the ELISA plate, and mAbs 62-35 and 142-2 were tested on ELISA. The result was consistent with that of Western blot (data not shown); therefore, we hypothesized that these two mAbs recognized a larger conformational epitope.

To test this hypothesis and confirm our previous data, we performed IFA to test the reactivity of these mAbs on a panel of p30 truncates in eukaryotic expression system (Figure 3.5C). The advantage of using the eukaryotic system is that eukaryotic cells have all the components necessary for the protein post translational modifications, which are predicted for the C-terminus of p30 (Prados et al., 1993). The p30 fragments were designed based *in silico* B-cell linear epitope prediction (Figure 3.5B). Each truncated p30 peptide was constructed as an EGFP-tagged fusion protein and expressed in Vero cells (Figure 3.5C). IFA results consistently showed that mAb 47-3 recognized the epitope located with 61-93 aa of the protein, while both mAbs 62-35 and 142-2 only recognize the large truncate of p30 (120-204 aa) (Figure 3.5C-D). The epitope recognized by mAbs 62-35 and 142-2 seems to be complicated. The IFA result suggests that the 120-204 aa region contains a conformational epitope, but western blot analysis with denatured peptide suggests that 101-204 aa region contains a linear epitope (Figure 3.5A). Therefore, the region recognized by mAbs 62-35 and 142-2 might use an unusual folding mechanism when the antibody-antigen interaction occurs. In order to understand this abnormal phenomenon, we used the GeneSilico MetaDisorder server, which was previously used to predict

regions of intrinsic disorder on viral proteins (Wang et al., 2016). The results revealed that p30 contains a predicted disorder region between aa 91-143 (Figures 3.6 and 3.9). This region is enriched in glutamic acid residues and is highly hydrophilic, which are two of the main characteristics of an intrinsically disordered protein (IDP).

Another propriety that is characteristic to IDP's is the high immunogenicity due to the exposed hydrophilic residues. Therefore, we compared our results with a previous study done by our group (Murgia et al., 2018). We pursued the hypothesis that p30 antigen might be recognized by the immune system and therefore we could elucidate the abnormal behavior described previously. Therefore, based on published data we compared our epitope mapping results with the following: sera collected at 0 DPI from pigs vaccinated pigs only (RP-30), sera collected at 17 DPI from pigs infected with OURT88/3 only, and sera from RP-30 + OURT88/3 prime-boosted pigs collected at 17 DPI. As shown in Figure 3.6, serum from all groups reacted with the whole p30 (1-204), whereas fragment p30(1-100) possessed the least reactivity. Most of the reactivity was directed towards p30(50-150) and p30(101-204) suggesting that the immunodominant region is likely located in the region of amino acids 101-150 of p30. The greatest effect of RP-30 priming was found in the recognition of the p30(1-100) fragment. As shown in Figure 3.6B, antibody activity was significantly higher in the RP-30 + OURT88/3 prime-boosted pigs when compared to either the RP-30-only or OURT88/3-only group. Taken together, these results show that priming with RP-30 prior to infection with OURT88/3 expanded the recognition of minor epitopes located near the N-terminal end of p30.

Finer mapping was conducted using the same set of overlapping oligopeptides that was shown in Figure 3.5 A. However, this time reactivity was detected as compared with our initial mapping with the anti p30 mAbs. The greatest amount of activity was found in the recognition of

two overlapping oligopeptides, p30(91-130) and p30(111-160) (Figure 3.7A). Interestingly, reactivity was greatest for the OURT88/3-only and RP-30 + OURT88/3 prime-boosted pigs. As noted by the authors, the main effect of RP-30 priming with the OURT88/3 boost was found in the recognition of the p30(61-110) oligopeptide with 9 of 10 pigs in the RP-30 + OURT88/3 prime-boosted group recognized this region, compared to 1 of 10 and 3 of 6 pigs for the RP-30-only and OURT88/3-only group, respectively (Figure 3.7B). Therefore, we can conclude that the region recognized 61-93 contains a linear epitope with an important immunological function.

3.3 Freeze thaw/trypsin treatment. In our WB experiment we observed that mAb 62-35 reacted just with fragment 101-204. Since the transfer buffer we used contained SDS, it is unlikely that the protein was renatured during blotting. Moreover, using the software GeneSilico metadisorder predicted that C-terminal part of p30 is an intrinsically disordered protein, between residues 91-143 (Figure 3.8). The intrinsically disordered proteins have a high proportion of hydrophilic and solvent-accessible residues (Wang et al., 2016). However, the results obtained after freeze thaw cycles or trypsin treatments (no difference between p30 101-204 and a non-related protein, GFP) were inconclusive and were not included further in the discussion.

3.4 Sequence analysis. To determine whether these epitope regions are conserved among different genotypes of ASFV, we analyzed p30 sequences from 19 genotypes (Figure 3.9). The amino acid alignment on the mAb 47-3 epitope region identified a total of 7 aa difference. One single aa difference (His_{66} to Arg_{66}) between Georgia/07 and BA71V, did not impair the binding of the mAb to its targeting epitope. It is likely other ASFV strain with an Arg at that position (belonging to genotypes III, IV, V, VI, VII, VIII, XX) could be recognized by mAb 47-3. On the

other hand, mAbs 62-35 and 142-2 recognized a larger region located between aa 120-204, regions which is also shown to be immunogenic. There are several different residues scattered through this region in different ASFV strains. ASFV strains in genotype XX and XXI has the same change in aa 139 (E to V) as that in Georgia/07, which did not affect the mAb recognition in this region. Whether the other aa changes in genotypes III-XIX affect the binding of the mAb needs to be further analyzed.

3.4 Application of anti-p30 mAb in ELISA development. To evaluate the feasibility of using this panel of anti-p30 mAbs in serological test, we developed a blocking ELISA (bELISA) for detecting the host antibody response in ASFV-infected pigs.

To develop the bELISA, four sets of serum samples were used as the known positive-testing sample population (n=65). The first set of 33 samples were from pigs immunized with RP-p30 (BA71V strain, Genotype I), the second set of 22 samples were from pigs infected with ASFV strain OURT 88/3 (Genotype I, identical with BA71V), the third set of 8 samples from pigs immunized with Adenovirus cocktail (including p30, based on Georgia/07, Genotype II) and the last set represented by 1 sample from a pig challenged with Georgia/07 (Genotype II). The known negative-testing sample population contains 129 samples from non-infected pigs or collected at 0 DPI or DPV. Initially, the optimal dilution of p30 antigen was determined so that the mAb generated an optical density (OD) of approximately 1 in the absence of a competitor (Figure 3.10). The bELISA test conditions were further optimized using a checkerboard format using all three mAbs. Interestingly, only mAb 142-4 showed blocking activity against all serum samples that we tested. Thus, this mAb was used for the bELISA initial validation.

Next, we investigated the antibody response kinetics in pigs infected with OURT 88/3 (Figure 3.11). ROC analysis was performed to analyze the bELISA results obtained with the positive- and negative-testing sample populations in order to determine an optimized cutoff that maximizes both the diagnostic specificity and diagnostic sensitivity of the assays. A two-graph ROC plot for the bELISA was generated. An optimized cutoff at 49.44 % of inhibition (PI) was calculated. A diagnostic sensitivity of 98.5 % and a diagnostic specificity of 99.2 % were calculated for the bELISA based on anti-p30 mAb 142-4 (Figure 3.12).

4. Discussion

ASFV infection can have a deleterious effect on swine production, causing excessive morbidity and mortality in domestic pigs. Current outbreaks of ASFV in China and some European countries pose the potential pandemic threat to global swine industry. Since no vaccine and treatment are available for ASFV, the only strategy to control the disease is to quarantine, isolate and eliminate the infected animals in order to stop the spreading of the disease. High levels of biosecurity measures, including surveillance along with rapid diagnostics are critical to identify infected animals; therefore, sensitive and specific diagnostic reagents and assays are urgently needed.

Monoclonal antibody (mAb) is a key reagent for diagnostic detection of viral infection. In this study, we produced a panel of mAbs against the p30 protein of ASFV. Three mAbs (47-3, 62-35, 142-4) were selected for further characterization and assay development. These mAbs showed good sensitivity and specificity in IFA, ELISA, Western blot and immunoprecipitation. They were determined to recognize both ASFV strains that we tested in infected cells, including viruses of genotype I (BA71V) and genotype II (Georgia/2007). Results of the IHC on Georgia/

2007 infected tissues demonstrated that mAb 47-3 is a good reagent for the detection of ASFV antigen in formalin-fixed tissues. The mAb could detect virus in all tested tissues (lung, tonsil, spleen, lymph nodes) at 4 days post infection, and remarkably, before the appearance of lesions. This is consistent with a previous study that p30 antigen was detected in tonsil at 5 days post-infection (Dixon et al., 2017). Due to the propriety of p30 to induce neutralizing antibodies we also performed a virus neutralization assay as described in Chapter 2. However, the mAbs did not show any neutralizing activity when tested individually or combined (data not shown).

The exact epitopes recognized by this panel of anti-p30 mAbs need to be further studied. Our preliminary epitope mapping identified that mAb 47-3 recognizes an epitope located in the region between aa 61 to 93. This region is partially conserved (1 aa difference) among at least 7 genotypes, suggesting that the mAb 47-3 could be able to detect at least 7 genotypes of ASFV. Interestingly, the region contains aa 61-93 was also recognized by the serum antibody generated from ASFV-infected pigs (Murgia et al., 2018). Moreover, using the vaccination strategy of incorporating priming with an Alphavirus-expressing p30 antigen followed by boosting with an attenuated live virus, the major effect of the prime boost was enhanced recognition of an epitope covered by the peptide sequence 61–110, suggesting this region contains the epitope with important immunological function (Murgia et al., 2018). The major conclusion that could be drawn is that the antibody response might be different during natural infection and immunization, therefore creating a potential viral escape mechanism.

In contrast, mAbs 62-35 and 142-4 only recognized a large polypeptide fragment at the C-terminal end (120-204 aa) that contains a region (91-143 aa) predicted to be intrinsically disorder (IDP). A similar phenotype was observed in the nucleocapsid of porcine epidemic diarrhea virus (PEDV), demonstrating that a larger intrinsically disordered region contains the

epitope targeted by a mAb (Wang et al., 2016). The 120-204 aa region appears possessing characteristics of IDP. In SDS-PAGE analysis, the p30 fragment covering amino acids 50-150 showed aberrant migration at a higher molecular weight than the predicted size (Supplemental figure). Such phenomenon was also observed in Ebola virus nucleoprotein (NP). The C-terminus of NP also contains a highly acid region that was predicted to be disordered. In SDS-PAGE analysis, NP showed aberrant migration with apparent molecular mass larger than that of predicted size (Shi et al., 2008). However, the protease and freeze-thaw treatments of recombinant fragments 101-205 were not conclusive (data not shown). Structural studies would aid in identifying the structure of the p30 protein, resolving this issue. Another property related to the IDS is the highly immunogenic nature of the C-terminal region, which could be due to the exposure of acidic residues. A recent animal study revealed that the C-terminal part of p30 is immunodominant, and the region recognized by ASFV-infected pigs was determined to be predominantly located between aa 111-130 (Murgia et al., 2018). Another interesting observation in our study is that within this low structural order but highly antigenic region of p30, a higher degree of variability exists between different ASFV isolates (Perez-Filgueira et al., 2006). The variability does not affect the prediction of disordered regions for isolates belonging to both genotype I and II. However, the implications of disordered regions in the function of ASFV p30 protein needs to be further studied. Future study is needed to identify the specific mAb binding site, which would allow developing genotype-specific diagnostic assays, as well as assays applicable to genetically diversified field strains. Serology tests are commonly used diagnostic tools for detection of viral infection and disease surveillance.

The current serological assays approved by the World Organization for Animal Health (OIE) are using live virus as antigen, which involves high containment facilities and select agent

permits (OIE Manual; 2012). To overcome this problem several serological assays were developed using recombinant ASFV proteins expressed in *E.coli* or baculovirus. Despite achieving higher sensitivity, one of the disadvantages continues to be the number of false positive results obtained with field sera, therefore a second confirmatory test is required (Cubillos et al., 2013). The mAb-based bELISA can provide the similar level of sensitivity but higher level of specificity when compared with traditional ELISA (Chen et al., 2016). Therefore, in the case of ASF free areas, like the US, it is necessary to have high specificity of the assay. In this study, we developed a bELISA using the mAb 142-4. With the available serum samples from pigs immunized with RP-p30 (based on BA71V, Genotype I) or adenovirus cocktail (based on Georgia/07, Genotype II) and from pigs infected with OURT 88/3 (Genotype I), Georgia/07 (Genotype II), our bELISA showed reasonable sensitivity (98.5) and high specificity (99.2), suggesting this assay could be used to detect antibody responses from pigs infected by ASFV of both genotypes I and II. Antibody response from kinetics revealed an early response (7 DPI), as compared with other serological assays developed, which identified an antibody response at 8 DPI. However, due to the limited availability of our serum samples, this assay needs to be further validated using large numbers of samples (including oral fluids) from pigs infected by genetically diversified ASFV strains.

5. Conclusion. In summary, we generated a panel of anti-p30 mAbs; these mAbs were characterized on three important properties: (i) reactivity on ASFV-infected cells with BA71V, Georgia/2007 strains; (ii) immunohistochemical analysis in tissues from pigs infected with Georgia/2007 strain; (iii) specific epitope region recognized by a specific mAb and ASFV infected and vaccinated pigs and (iv) application in development of serological assays. This panel of mAbs and mAb-based diagnostic assays will be important tools to aid in ASF disease control and prevention.

Acknowledgements

This project was funded by the Kansas National Bio and Agro-Defense Facility Transition Fund and the Kansas Bioscience Authority through a matching grant to Kansas State University's Center of Excellence for Emerging and Zoonotic Animal Diseases. The anti-p30 monoclonal antibody development project was supported by the Kansas State National Bio and Agro-defense Facility Transitional Fund (to R. R. R. Rowland) and Kansas State University College of Veterinary Medicine research startup fund (to Y. Fang).

We would like to thank Dr. Baker for the contribution of the pHUE expression vector. Also, we would like to thank Dr. Jerome Nietfeld for the IHC analysis.

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Table 3-1 Summary of anti-p30 mAbs generated in this study.

mAb	Isotype	IFA	IHC	IP/WB	ELISA	Epitope region
47-3	IgG1 lambda	+	+	+	+	aa 61-93
62-35	IgG1 lambda	+	+/-	+	+	aa 120-204
142-4	IgG1 lambda	+	+/-	+	+	aa 120-204

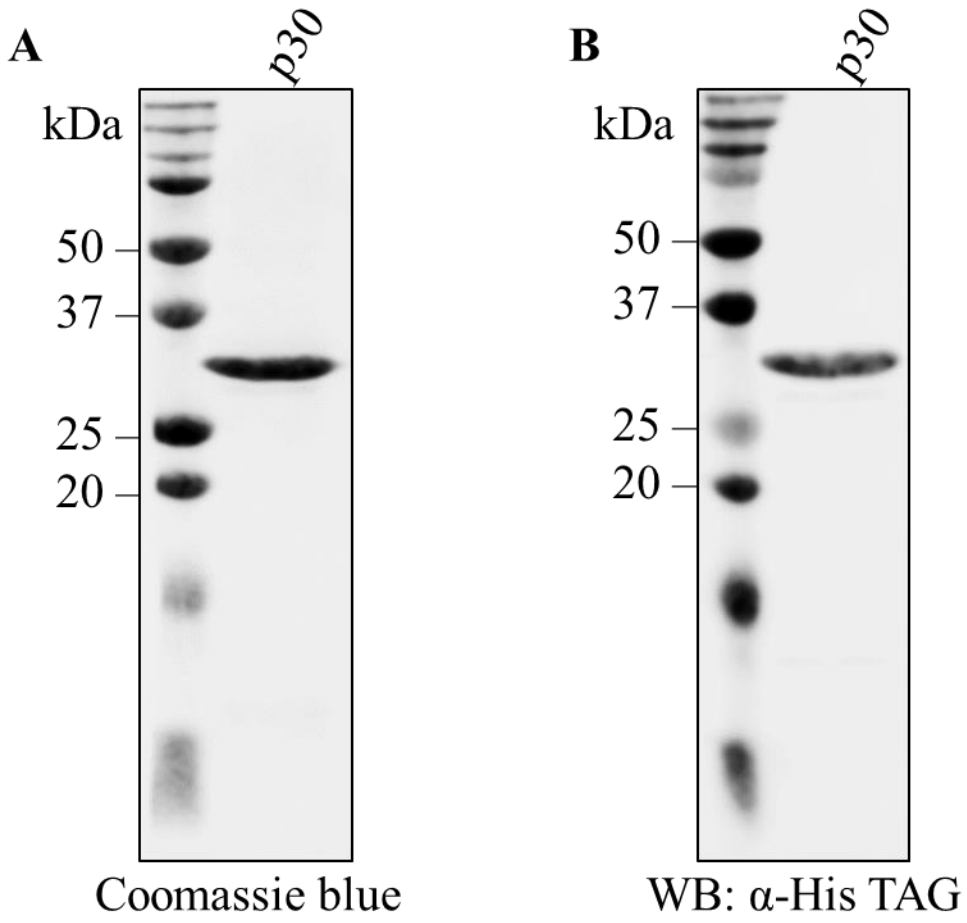


Figure 3-1. ASFV p30 antigen production. (A) SDS-PAGE of His-tagged p30 recombinant protein preparation, followed by Coomassie blue staining. (B) Western blot analysis using anti-His TAG mAb. For both panels A and B, the left lane shows the molecular weight marker, while the right lane shows p30 protein.

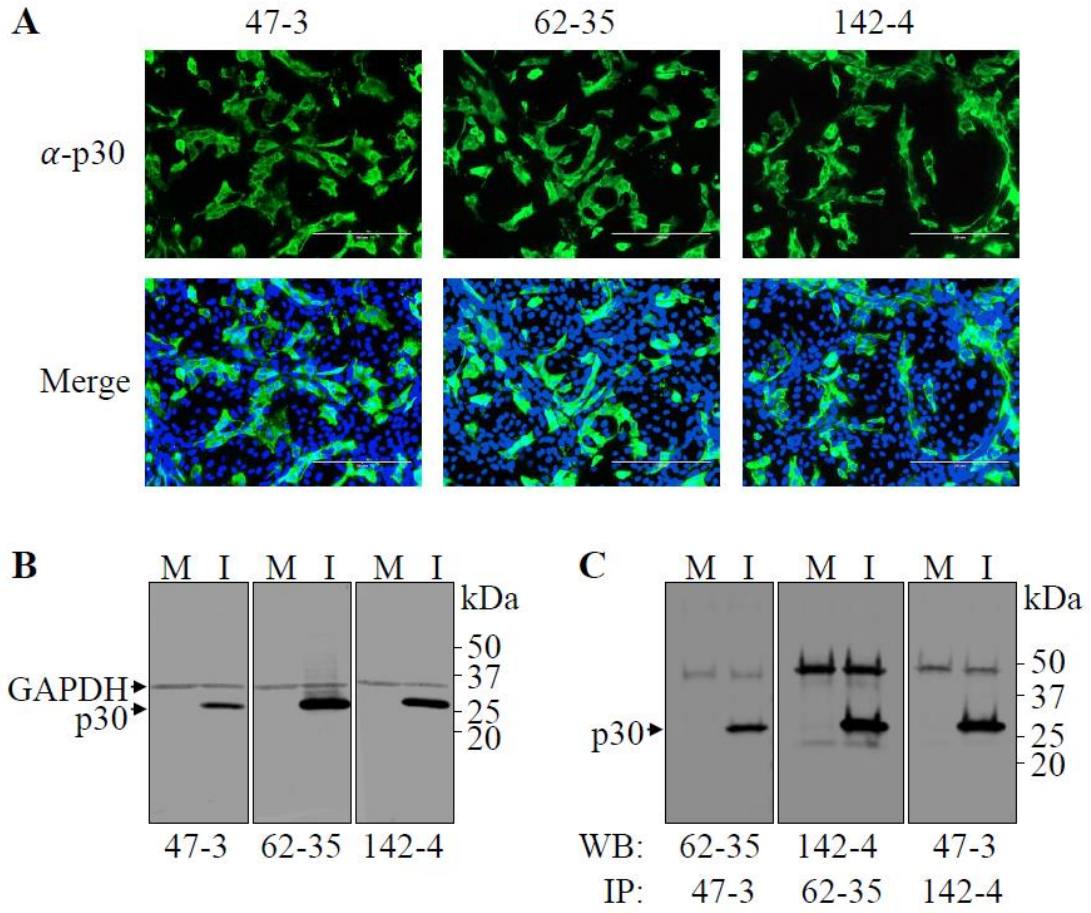


Figure 3-2. Detection of p30 expression in Vero cells infected with recombinant alphavirus replicon particles expressing p30 (RP30). (**A**) Immunofluorescent assay detection of p30 expression. RP30 infected cells were fixed at 36 h post infection. Cells were incubated with p30-specific mAbs listed on the top of each panel and stained with Alexa-Fluor-488 conjugated goat anti-mouse IgG (green). Cells were counterstained with DAPI (blue). (**B**) Western blot detection of p30 expression. RP30 infected cells were harvested at 36 h post infection, and western blot analysis was performed using the p30-specific mAbs as indicated on the bottom of each panel. (**C**) Immunoprecipitation analysis of RP-30 infected cell lysate with p30-specific mAbs. For each panel, a set of two mAbs were used. IP: mAb used to precipitate the proteins from cell lysate; WB: mAb used to detect the p30 protein in the membrane after immunoprecipitation. For both 2B and 2C panels, the size of the protein is labeled on the right side of the panel. M: Mock-infected cell lysate; I: RP30 infected cell lysate.

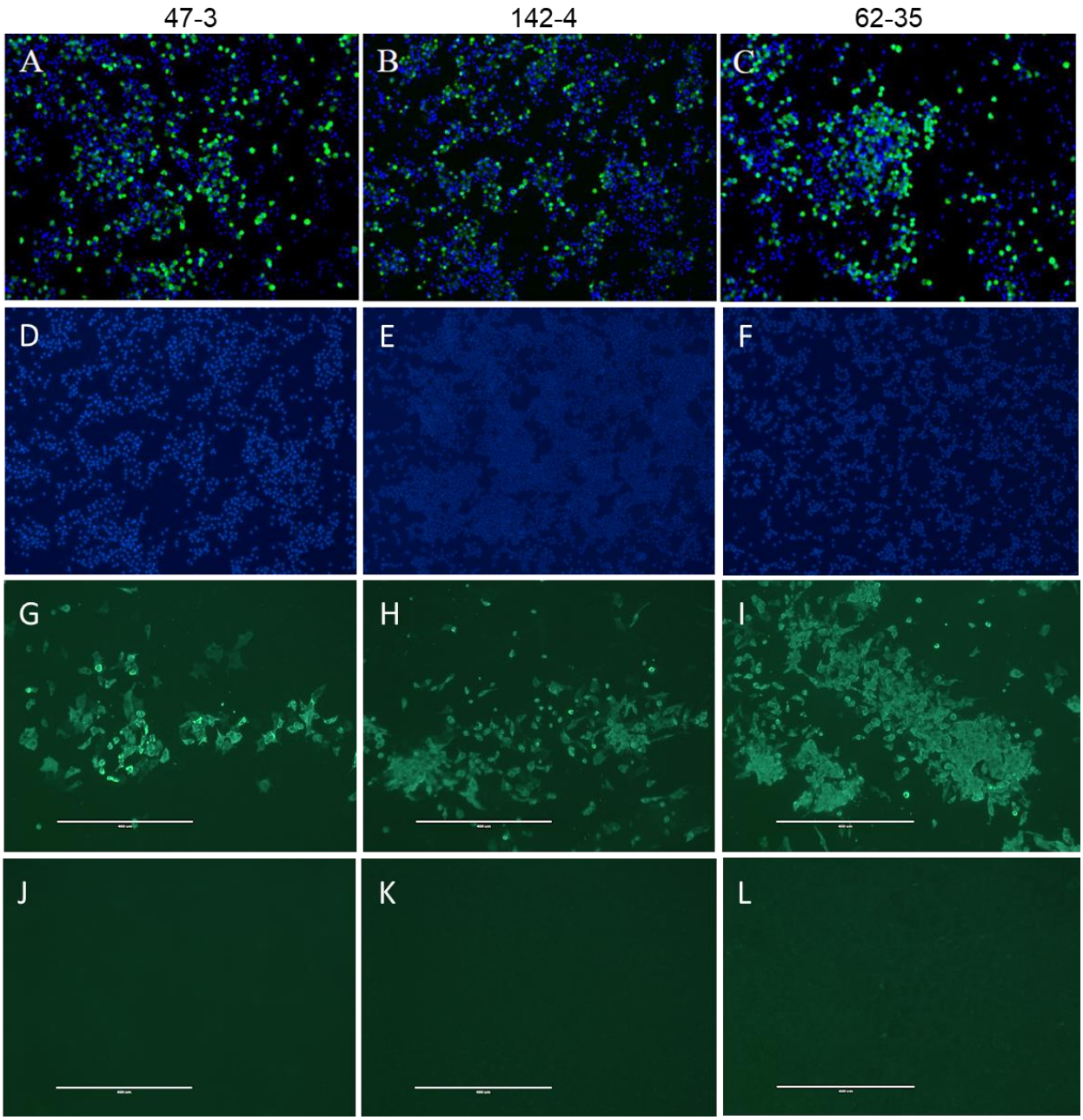


Figure 3-3 Immunofluorescent assay detection of ASFV in virus-infected cells. (A-C) Porcine alveolar macrophages (PAMs) were infected with ASFV strain Georgia/2007. **(D-F)** Mock-infected PAMs. Cells were fixed and incubated with an anti-p30 mAb as indicated and stained with Alexa-Fluor-488 conjugated goat anti-mouse IgG (green). Cell nuclei were counterstained with DAPI (blue). **(G-I)** Vero cells were infected with ASFV strain BA71V**. (J-L)** Mock-infected Vero cells. Cells were fixed and stained with an anti-p30 mAb as the primary antibody and Alexa-Fluor-488 conjugated goat anti-mouse IgG as the secondary antibody**.**

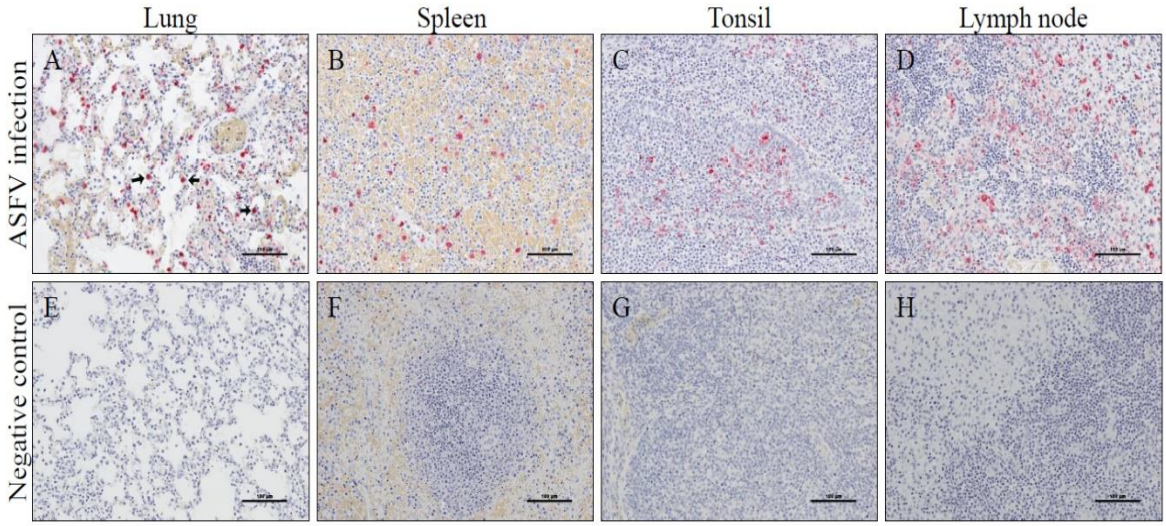


Figure 3-4. Immunohistochemical analysis of tissue samples. (**A-D**) Tissue samples from pigs infected with ASFV strain Georgia/2007. (**E-H**) Tissue samples from negative control pig. Immunohistochemistry (IHC) was performed using thin section of paraffin-embedded tissues, including lung (**A, E**), spleen (**B, F**), tonsil (**C, G**) and lymph node (**D, H**). Tissue sections were incubated with primary mAb 47-3 and polymerization was performed with Polymer-AP α-mouse. Colors were developed using Fast Red chromogen and slides were counterstained with hematoxylin. Bar = 100 µm.

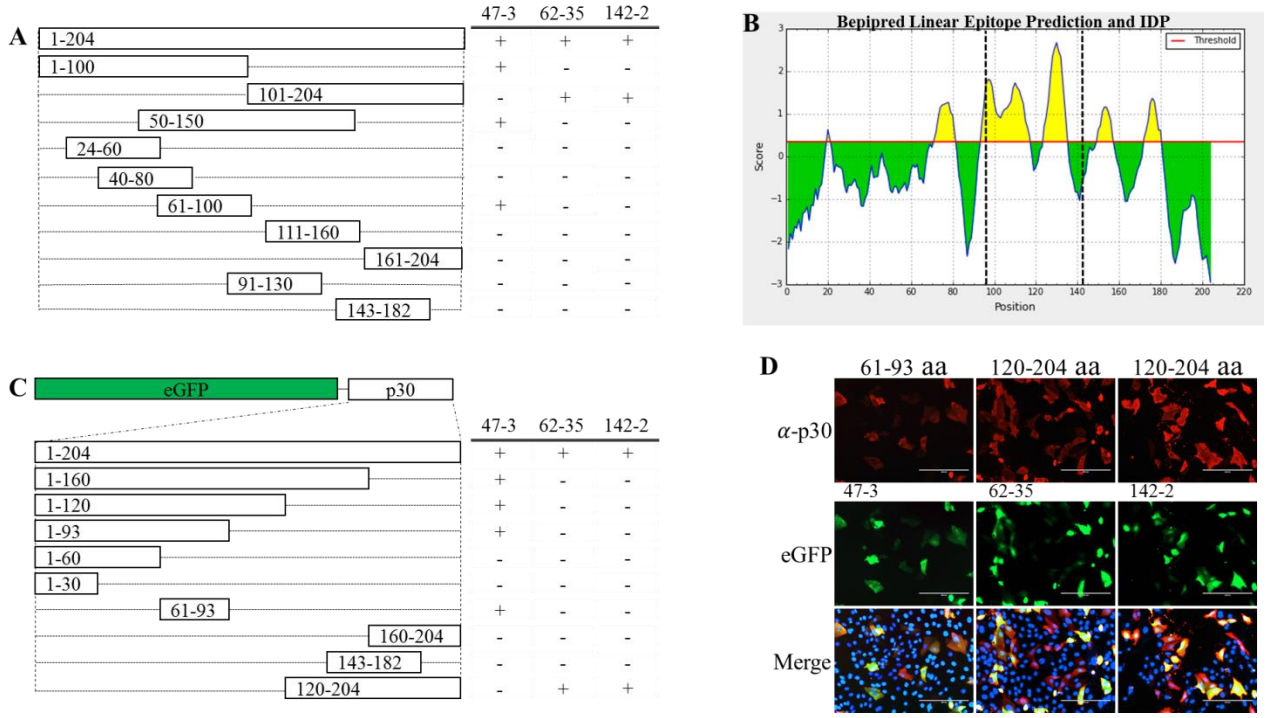


Figure 3-5. Epitope mapping of p30 monoclonal antibodies. (A) A schematic diagram of *E. coli* expressed constructs used as antigens in western blot for epitope mapping **(B)** Antigenic regions of p30 was predicted by in silico analysis of Bepipred Linear Epitope Prediction. The immunogenic regions were highlighted with yellow color and the region predicted to contain an intrinsically disordered region is marked with dashed vertical bars. **(C)** A schematic diagram of constructs expressing EGFP-p30 fusion proteins and a summary of epitope mapping by IFA. White bars denote truncated p30, whereas dash lines denote truncation regions. **(D)** p30 fragments recognized by monoclonal antibodies (47-3, 62-35, and 142-2) determined by IFA. The EGFP-p30 fusion proteins detected by monoclonal antibodies and Alexa-Fluor-594 conjugated goat anti-mouse IgG (red fluorescence) Vero cells, and green fluorescence signal (EGFP) indicated the expression of fusion proteins.

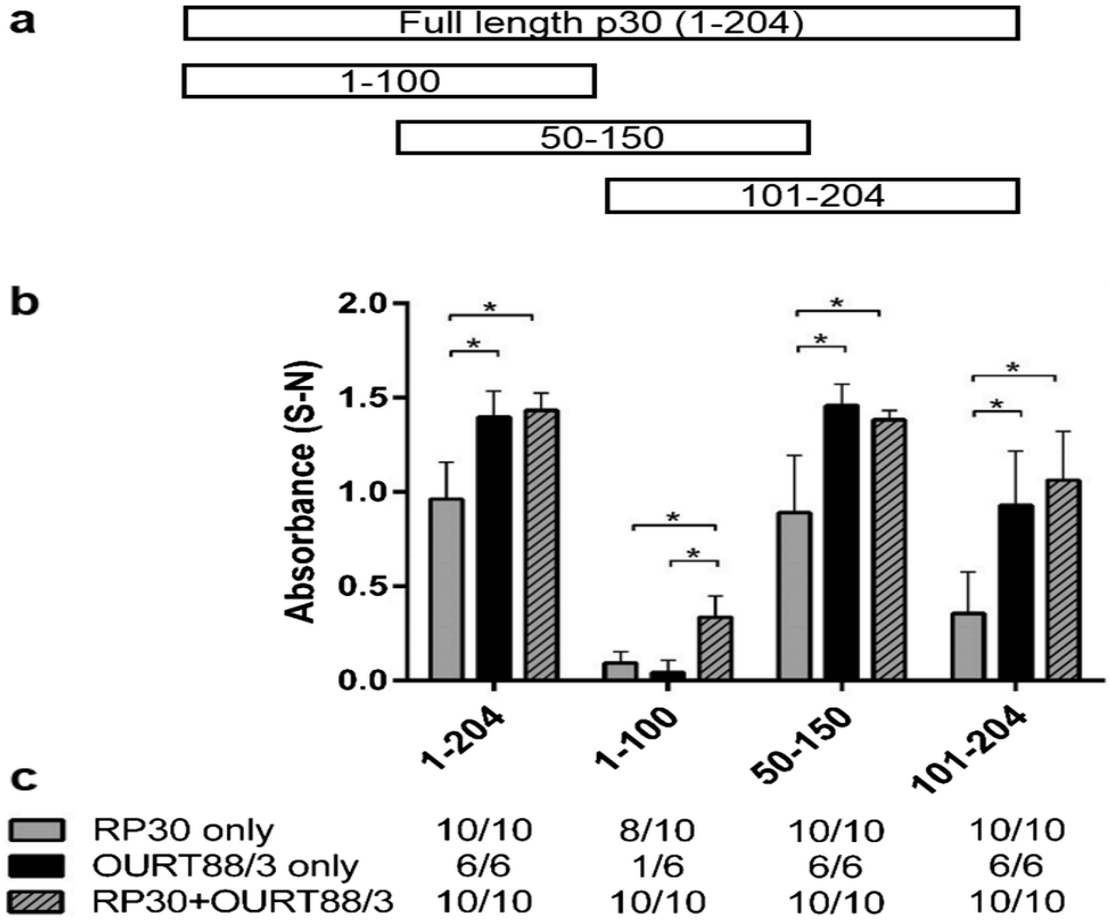


Figure 3-6. Epitope mapping using infected and vaccinated pig serum. (A) Pig sera were tested at 1:400 by ELISA against p30 whole protein or overlapping polypeptides; corresponding amino acids are indicated. The RP30 + OURT88/3 and OURT88/3-only groups represent sera collected at 17 DPI. The RP30-only group represents sera collected at 0 DPI from pigs primed two times with RP30 but not yet boosted with OURT88/3. Absorbance values are shown as sample (S) minus negative control mean (N) for each fragment. Significant differences between group means for each fragment (*) were calculated by two-way ANOVA with Tukey's multiple comparison test with a 95% confidence interval in GraphPad Prism version 6. **(B)** The number of reactive over total pigs from each group is shown for each oligopeptide. Values ≥ 0.05 indicate reactivity (Taken from Murgia et. al 2018)

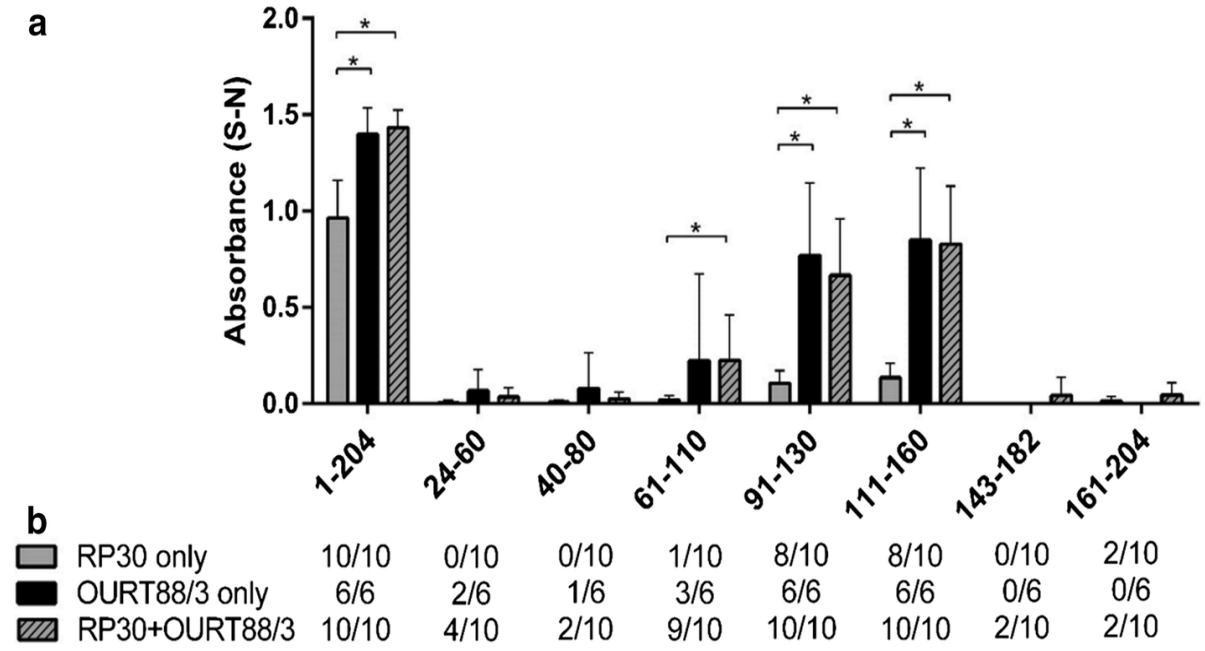


Figure 3-7 Fine mapping of p30 recombinant fragments using infected and vaccinated serum. (A) The RP30 + OURT88/3 and OURT88/3-only groups represent sera collected at 17 DPI. The RP30-only group represents sera collected at 0 DPI from pigs primed two times with RP30 but not yet boosted with OURT88/3. Absorbance values are shown as sample (S) minus negative control mean (N) for each fragment. Significant differences between group means for each fragment (*) were calculated by two-way ANOVA with Tukey's multiple comparison test with a 95% confidence interval in GraphPad Prism version 6. **(B)** The number of reactive over total pigs from each group is shown for each oligopeptide. Values ≥ 0.05 indicate reactivity (Taken from Murgia et al., 2018)

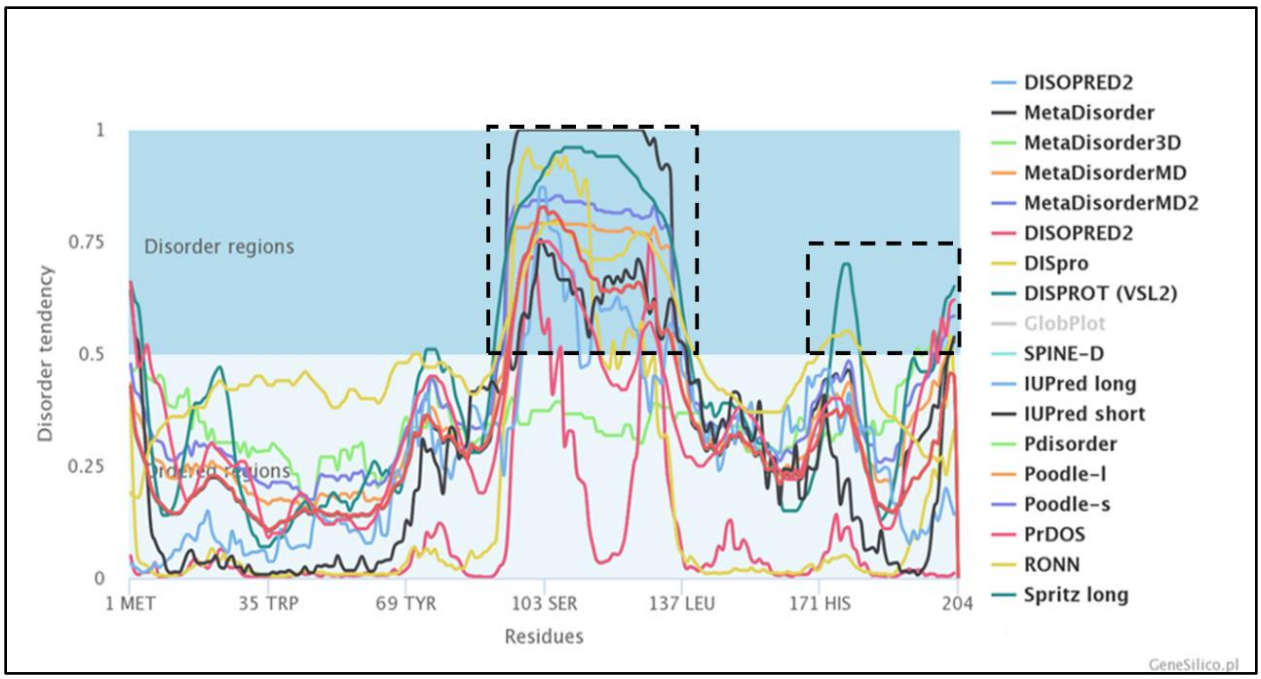


Figure 3-8 The disorder plot of p30 based on BA71V strain was predicted using the GeneSilico MetaDisorder server. The x-axis shows the residues from 1 to 204, and the y-axis shows the disordered tendency ranging from 0 to 1. All residues with a disorder probability of more than 0.5 were considered as being disordered.

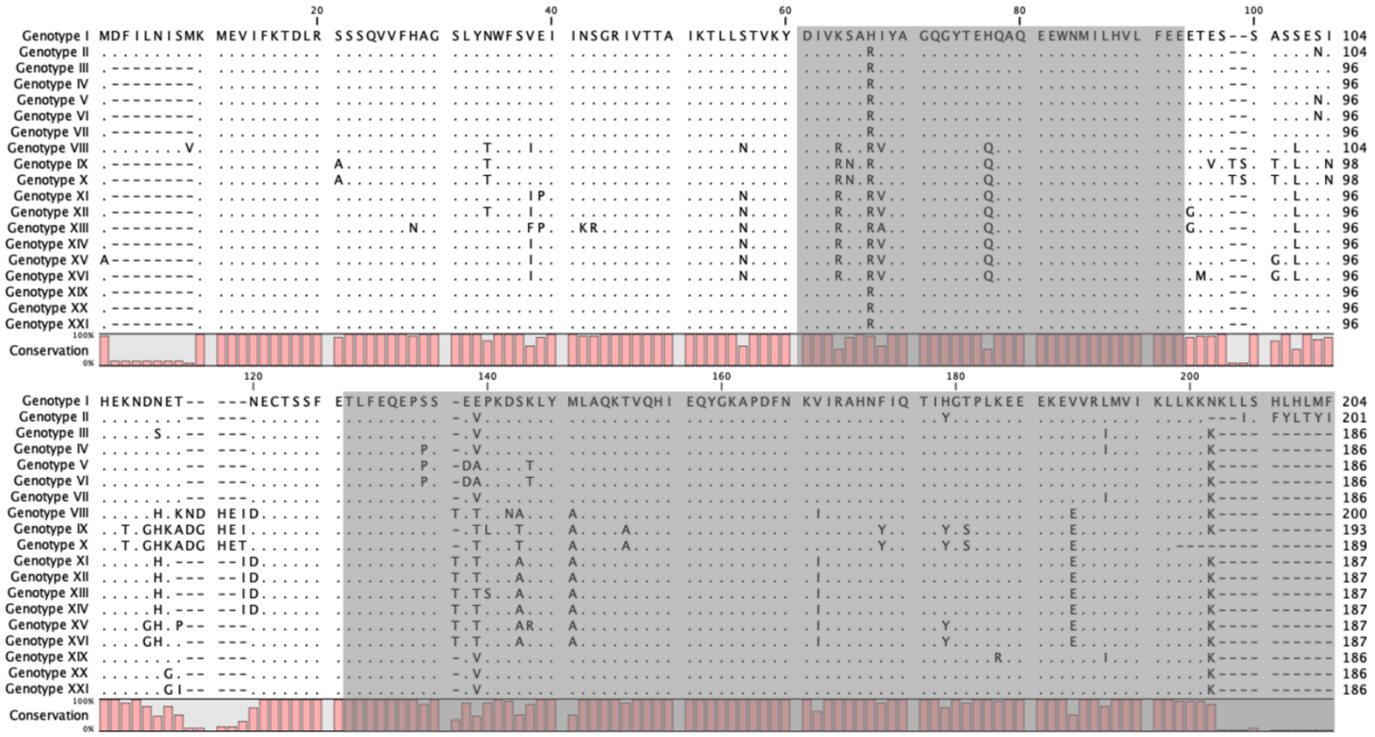


Figure 3-9 Sequence alignment of p30 proteins of 19 ASFV genotypes. Sequence alignment was performed with CLC Genomics Workbench 11 (QIAGEN, Hilden, Germany). Matching residues are denoted with dot, whereas gap regions are denoted with dash line. The numbers on the top indicate position on p30 of Genotype I, and the coordinate of amino acid in the alignment is specified on the right terminus for each sequence. Epitope regions of amino acid 61~93 recognized by ASFV RP-30+OURT 88/3 and monoclonal antibody 47-3 and amino acid 120~204 recognized by monoclonal antibodies 62-35 and 142-2 are highlighted with grey color. The GenBank accession numbers of 19 ASFV p30 protein sequences are: Genotype I (BA71V; U18466.2), Genotype II (Georgia 2007/1; FR682468.1), Genotype III (SPEC/257; EU874265.2), Genotype IV (RSA/04/3; EU874308.1), Genotype V (MOZ/1960; EU874309.1), Genotype VI (SPEC/265; EU874264.2), Genotype VII (SPEC/154; EU874291.2), Genotype VIII (PHW/88/1; EU874257.2), Genotype IX (KEN/05/1; EU874301.1), Genotype X (ken09Tk.13/1; HM745382.1), Genotype XI (KAB/62; EU874289.1), Genotype XII (MZI/92/1; EU874288.2), Genotype XIII (SUM/1411; EU874287.1), Genotype XIV (NYA1/2; EU874302.1), Genotype XV (TAN/01/1; EU874303.2), Genotype XVI (TAN/03/1; EU874304.1), Genotype XIX (RSA/96/2; EU874281.2), Genotype XX (RSA/95/4; EU874295.1), and Genotype XXI (RSA/96/1/P; JQ745031.1).

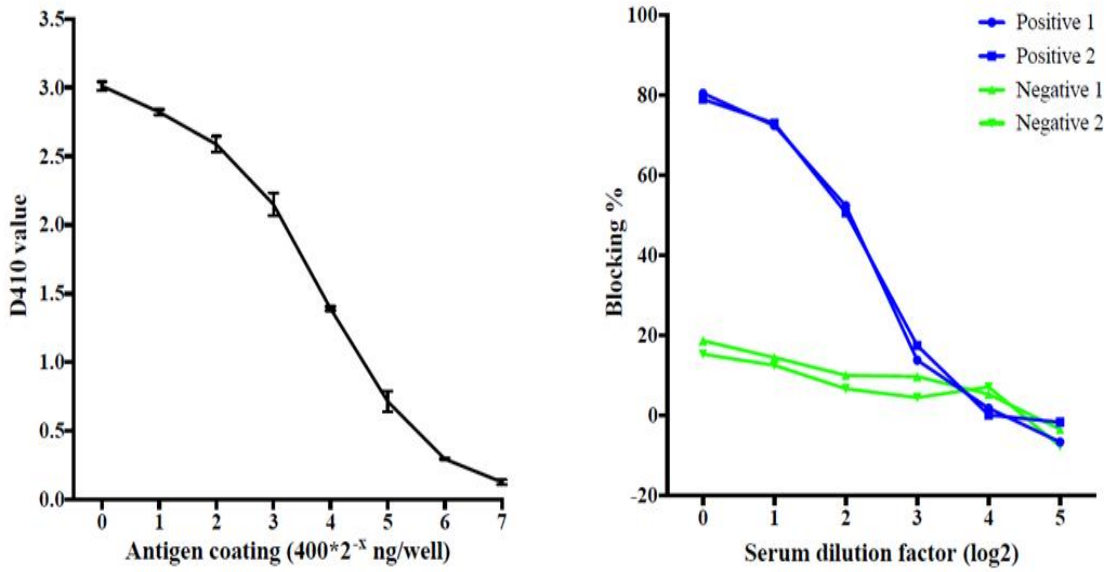


Figure 3-10 Optimization of bELISA conditions using mAb 142-4. Left graph represents a checkerboard format to adjust antibody dilution and protein concentration. Right graph shows the optimization of the assay using different dilutions of serum samples (2 from vaccinated pigs at 0 DPV and 55 DPV).

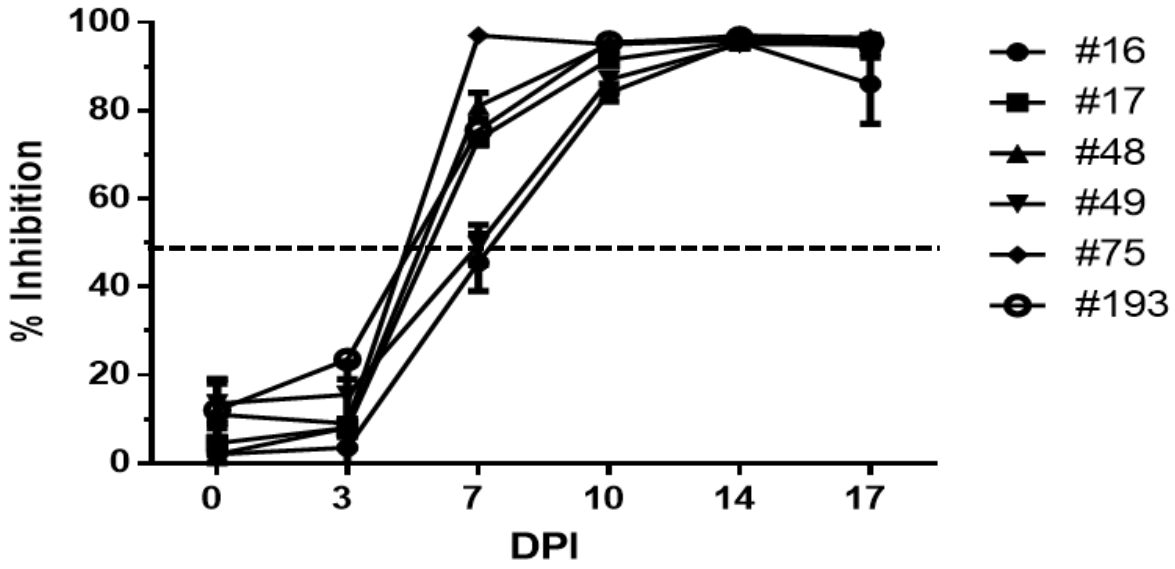


Figure 3-11 Antibody response kinetics from pigs challenged with low virulent ASFV strain (OURT 88/3). Results are presented as PI and the negative-positive cutoff (dashed line) was calculated based on the average of 0 DPI samples ± 3 SD. All the animals were positive by 10 DPI and an early antibody response could be detected at 7 DPI.

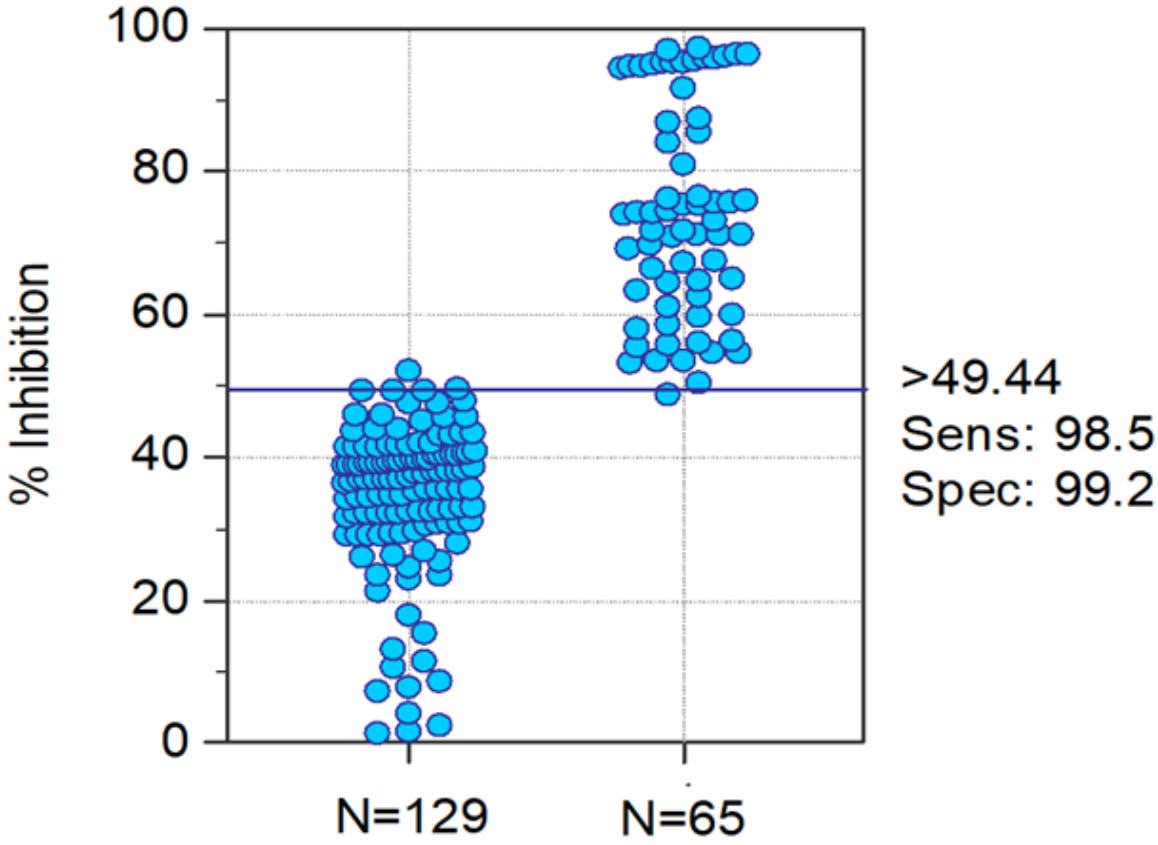
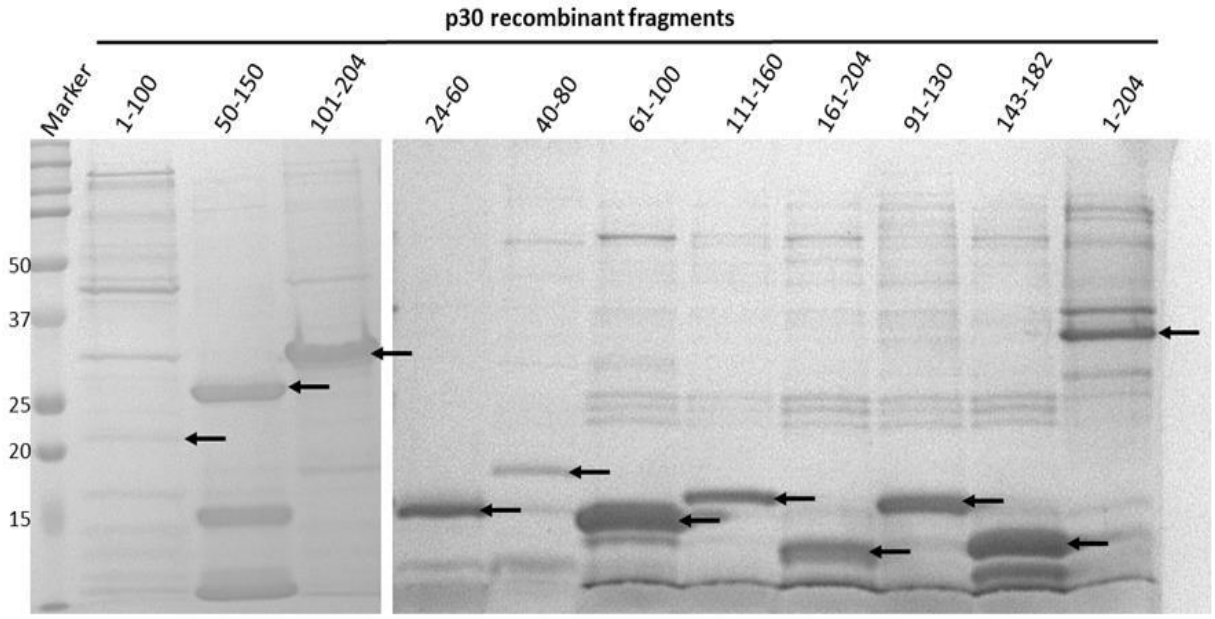


Figure 3-12 Development of bELISA assay using serum samples from known positive and known negative animals. A total of 194 serum samples with known serological status were assayed for establishing a cut off value for the assay.



Supplemental 1 SDS page analysis of recombinant p30 fragments produced in *E. coli*. The p30 fragments and whole protein position in the gel are indicated by the arrows.

Chapter 4 - Discussion

African swine fever is the most important foreign animal disease (FAD) that currently threatens swine production worldwide. Since it was reintroduced in Republic of Georgia, ASF has spread throughout Europe and Southeast Asia (China, Mongolia, Cambodia), causing billions of US dollars in economic losses and more than 2 million animals killed. The most significant cause of this recent geographical spread is believed to be due to the illegal movement of animals, trade, and contaminated pork products. Due to the complex architecture of the virus, genetic complexity and a basic understanding of the immune response during infection, no commercial vaccine is currently in sight.

Under those circumstances, diagnostic tests used in surveillance programs represent the first line of defense against ASF. A wide variety of diagnostic tests have been developed to detect either viral antigen or specific antibodies. However, there is room for improvement when it comes to sensitivity and specificity. As summarized in this thesis, there are very few diagnostic kits for ASFV detection currently validated and serological tests for large scale diagnostics are still not being implemented. Moreover, ELISA systems should be developed for alternative non-invasive sample matrices (like urine, oral fluids) that could also be used for wild/feral pigs.

To improve the current available tests, we developed a set of mAbs against ASFV p30 and p54 that can be incorporated in modified genotype-specific ELISA tests for detection of anti-ASFV antibodies. The selected antigens utilized for mice immunization were based on the reference genomes BA71V (Genotype I, p30 antigen) and Georgia/07 (Genotype II, p54 antigen). Genotype I strain is adapted to grow in Vero cells, but is identical with other Genotype I strains used to monitor antibody response (Reis et al., 2007). Genotype II is currently the strain present in Eastern Europe and Asia. Monoclonal antibodies offer significant advantages, such as

safety (i.e. absence of residual virus compared to hyperimmune sera), specificity, and unlimited availability because hybridoma-secreting mAbs can be grown indefinitely (Greenfield, 2014). The latter characteristic is important in the development of diagnostic assays. In fact, it allows for the standardization of reagents, overcoming one of the drawbacks of the use of hyperimmune sera, which is seen in the variability between lots (Fafetine et al., 2013).

To address our proposed end goal to generate next generation ELISA assay, we performed epitope mapping analysis. The knowledge of the mAbs' binding sites would allow the identification of conserved and variable epitopes. Canonically, epitopes for mono- and polyclonal antibody recognition can be divided into two main groups: linear and conformational forms. Linear epitopes are formed by a continuous sequence of amino acids, while conformational epitopes consist of amino acid sequences that are discontinuous in the protein but are brought together upon three-dimensional protein folding. It has been showed that 90% of B cell-epitopes are conformational epitopes that result from the antigen presentation process. However, it is particularly difficult to detect conformational epitopes due to the native conformation of the viral protein and the use of X-ray crystallography that can locate the antibody-antigen interaction is highly laborious and expensive (Potocnakova et al., 2016). Therefore, our approach was to map linearized epitopes on p30 and p54 using ELISA, and denaturating Western blot assay. Based on those approaches we managed to identify a partially conserved linear epitope located on the N-terminus of p30 between amino acids 61-93. Moreover, the recognition of this epitope is enhanced after vaccination and challenge, implying that it might serve an important immunological function. The highly immunogenic region recognized by our mAbs and infected pigs will need to be further analyzed. We did find some abnormal behavior on SDS PAGE analysis during the hybridoma screening and therefore we

hypothesized that this region has proprieties associated with intrinsically disordered proteins. This is the first time an IDP is described for ASFV, although we could not prove one of the proprieties of IDP, namely cold tolerance and protease susceptibility. Further sophisticated analysis should be performed in order to answer that question.

Conversely, we managed to map five linear epitopes on the p54 protein. This protein has been previously described as a good candidate for antibody detection, a target of neutralizing antibodies, and a virus attachment protein, involved in the intracellular transport of the virus. Despite these functions or potential applications in the disease control, there is limited information describing functional domains or antigenic regions, except for the aa sequence which binds to the cellular light chain dynein. Our results identified one epitope localized between aa 118-127 in a variable region, which apparently is also highly immunogenic, being recognized by sera from ASFV-infected pigs. More, it seems that region 93-107 might contain a neutralization epitope. Unfortunately, we couldn't characterize the p54 response after a prime boost approach as described for p30 due to the low immunogenicity of the alphavirus construct.

This information can then be exploited for the design of diagnostic assays able to detect a wide range of isolates (conserved epitopes), or to distinguish between them (variable epitopes). Furthermore, our mAbs are valuable tools for studying ASF disease pathogenesis, as shown by their use as reagents in immunohistochemistry (IHC) for the detection of ASFV in paraffin-embedded tissues. The panel of monoclonal antibodies against p30 was used for developing a blocking ELISA making in undiluted samples. Since the initial antibody response against every viral infection is initiated by IgM or IgA, this assay can detect a broad repertoire of isotypes, since it relies on species specific secondary antibodies. Initial validation with our archived serum samples revealed a high specificity of the assay. This characteristic is desired in surveillance

programs and as a requirement for reference diagnostic laboratories. Moreover, we addressed the initial three steps recommended by the OIE manual for development of diagnostic assays: 1) Definition of the intended purpose(s) 2) Optimization 3) Standardization 4) Repeatability 5) Analytical sensitivity 6) Analytical specificity 7) Thresholds (cut-offs) 8) Diagnostic sensitivity and 9) Diagnostic specificity. Future studies will go towards a robust validation of this assay following OIE manual and in integration into multiplexing platforms.

This body of work, using multiple, robust analyses, provides evidence that monoclonal antibodies can be adapted to be used not only as unlimited diagnostic reagents, but also as a mean to explain some unique viral and immunological ASFV mechanisms. This work supports the continuous need of diagnostic innovation to protect the US economy from ASF introduction and can also be adopted in endemic regions to facilitate eradication of the disease.