THE PATHOGENESIS OF AFRICAN SWINE FEVER: FURTHER CHARACTERIZATION OF INFECTION MODELS AND TISSUE DYNAMICS

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ABSTRACT

THE PATHOGENESIS OF AFRICAN SWINE FEVER: FURTHER CHARACTERIZATION OF INFECTION MODELS AND TISSUE DYNAMICS

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African swine fever virus (ASFV) causes a reportable and often fatal disease of domestic pigs and wild boars. The current absence of effective prophylaxis or countermeasures makes it a significant agricultural and economic hazard within affected and neighboring regions. Despite extensive ASF research, key elements of the virus-host interaction have yet to be fully explained. Contributing to this knowledge gap is the lack of congruence in infection models amongst investigators. An infection/challenge model that closely simulates natural infection while using precise, measurable inoculation methods, and providing reproducible and quantifiable results is imperative for successful ASFV research and countermeasure development. This dissertation first compares the clinical, viral and shedding dynamics of four modes of infection: Intramuscular (IM), Intranasopharyngeal (INP), Intraoropharyngeal (IOP), and Direct contact (DC), which are commonly used in ASFV investigation. This determines if different inoculation routes/dosages alter the pathogenesis of ASFV in swine and which route most closely resembles natural infection. While developing a standard infection model, a secondary aim of the project was to further clarify the events of infection by detailed characterization of the tissues and cells at different stages of disease. This was investigated in a bipartite approach, both *in vivo* and *in vitro*, utilizing virologic and molecular detection methods, as well as immunomicroscopic detection and localization of ASFV antigen alongside cellular markers of interest.

Comparison of infection routes revealed that INP simulated natural infection while maintaining reproducibility and ease of control of variables. Examination of an extensive array

of postmortem tissue samples revealed low and somewhat route-dictated detection of ASFV at previremic stages of infection, and systemic dissemination of the virus once viremia occurred. Microscopic analysis of tissues indicated that infected cells were distributed within interfollicular regions of lymphoid tissue and the interstitium of non-lymphoid tissue. Immunomicroscpic characterization determined that cells in which ASFV antigen was detected were predominately of monophagocytic origin, with high but variable expression of CD172a, CD163 and lower yet noteworthy expression of CD203a and sialoadhesin.

This dissertation presents a comprehensive examination of ASF challenge models, necessary for the prospective standardization of live-animal ASF research. Furthermore, the distinctive characterization of ASFV-infected tissue and cells contribute to the growing understanding of the microenvironment required for the disease. Knowing what tissues and cell types are infected by ASFV can contribute to understanding the molecular or immunologic pathways involved, which viral genes/proteins are necessary for cellular infection. Ultimately this can lead to better targeting of prophylaxis or treatments for ASF.

This work is dedicated to my loving parents, sister, and brother-in-law, without whose constant love and support this endeavor would not have been possible. To Dr. Leland McLaughlin, who introduced me to the world of veterinary medicine. To Vivian and Adah, who motivated me spiritually. And to a host of friends, family members, colleagues, and lab mates who helped and supported me along the way.

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PREFACE

The work contained within this PhD dissertation was performed at Plum Island Animal Disease Center within the Foreign Animal Disease Research Unit, Agricultural Research Service, United States Department of Agriculture under an agreement with the College of Veterinary Medicine, Department of Pathobiology and Diagnostic Investigation at Michigan State University. Chapters 2 and 3 were originally generated as separate manuscripts for publication in scholarly, peer- reviewed journals. Minor modifications from their original form were performed in order to provide continuity of the dissertation as a complete body of work. However, some portions, such as material and methods, have been repeated.

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KEY TO ABBREVIATIONS

ASF- African swine fever

ASFV- African swine fever virus

CD- Cluster of differentiation

C_t- Cycle threshold

C_tTE- Cycle threshold titer equivalent

DC- Direct Contact

DIC- Disseminated intravascular coagulopathy

DNA- Deoxyribonucleic acid

DPC- Days post contact

DPI- Days post inoculation

ELISA- Enzyme-linked immunosorbent assay

FAT- Fluorescent antibody technique

FMDV- Foot and Mouth Disease virus

HAD- Hemadsorption assay

HAD_{50}- 50% Hemadsorbing dose

IHC- Immunohistochemistry

IL- Interleukin

IM- Intramuscular

IN- Intranasal

INP- Intranasopharyngeal

IO- Intraoroal

IOP- Intraoropharyngeal

mAB- Monoclonal antibody

MGF- Multigene family

MHC- Major histocompatibility complex

MIF- Multichannel immunofluorescence

MPS- Monophagocytic system

NK- Natural Killer cells

OCT- Optimum Cutting Temperature compound

ON- Combined Oronasal

ORF- Open reading frame

PBMC- Peripheral blood mononuclear cells

PBS- Phosphate buffered saline

PCK- Pancytokeratin

PI3K- Phosphoinositide-3-kinase

qPCR- Quantitative real-time Polymerase Chain Reaction

RPMI- Roswell Park Memorial Institute medium

SD- Standard deviation

SEM- Standard error of the mean

SLA- Swine leukocyte antigen

Sn- Sialoadhesin

SWC- Swine workshop cluster

VI- Virus isolation

VP- Viral protein

vWF- von Willebrand factor

CHAPTER 1:

Introduction

Erin B. Howey

Introduction

African swine fever virus (ASFV) is a large (approximately 170-190 kilobase pairs) double stranded DNA virus (genus *Asfarvirus*). A member of the family *Asfarviridae,* ASFV is the only DNA arbovirus (Tulman et al., 2009). It is responsible for the contagious hemorrhagic disease entity, African swine fever (ASF), which occurs in domestic pigs and European wild boars resulting in up to 100% mortality (Gómez-Villamandos et al., 2013).

Presently, there is no effective treatment or prophylaxis for ASF (Sánchez-Vizcaíno et al., 2015). Control of the disease is dependent on the rapid recognition of infected animals, laboratory confirmation, euthanasia of all infected and exposed animals, and stringent disposal and disinfection methods. The significant economic cost in monitoring for or eradicating disease outbreaks has caused the devastation of the swine industry in affected countries where outbreaks occur and constant exhaustion of the economy in countries where the disease is endemic (Costard et al., 2009; Penrith, 2009). While the pathogenesis of ASF has been thoroughly investigated for almost a century, detailed information regarding the virus-host interaction has yet to be established. Further research into the host interactions with ASF is essential to develop methods that may prevent the disease or at least reduce its economic impact.

African swine fever virus

The icosahedral ASFV virion has a diameter of 200 nm and is composed of over 50 polypeptides (Tulman et al., 2009). Intracellular virions contain an 80 nm virion core that is composed of the viral genome and enclosed by a dense protein core shell that is comprised of viral protein 150 (vp150)(Dixon et al., 2013b). Two lipid bilayers surround the core and are likely derived from the endoplasmic reticulum of the host cell. Surrounding this lipid bilayer is

the capsid, which provides the icosahedral structure of the virion and is composed of the structural proteins, vp72 and vpE120R (Tulman et al., 2009). Extracellular virions also posses an outer envelope, derived from the host cell plasma membrane during budding. VP12, the viral attachment protein, and the CD2-like protein both localize within this outer envelope (Borca et al., 1994; Carrascosa et al., 1993). Both intracellular and extracellular virions are infectious (Tulman et al., 2009).

The ASFV genome is 170-190 kb and consists of a central conserved region, central variable region, left and right terminal genomic regions, and terminal inverted repeat sequences. The ASFV genome contains 151 to 167 open reading frames (ORFs) and encodes complimentary structural and membrane proteins, as well as proteins which play a role in different stages of development of the virus within infected cells (Dixon et al., 2013b; Tulman et al., 2009). Over 100 viral proteins including structural proteins, glycoproteins and phosphoproteins have been identified. At least 28 structural viral proteins (vp) for ASFV have been identified (Tabares et al., 1980) as well as over 100 virus induced proteins within infected macrophages. The terminal genomic regions contain tandem arrays of five different multigene families (MGF) (Tulman et al., 2009). These include MGF 360, MGF 110, MGF 300, MGF 530/505 and MGF 100. Gain or loss of these members results in length variation of the genomic DNA. Their presence or loss has been associated with changes in virulence of several strains (Neilan et al., 2002; O'Donnell et al., 2015). Each end contains a partially paired sequence of A-T rich polynucleotides closed by hairpin loops, similar to poxvirus.

Viral entry and morphogenesis

Virus replication and systemic circulation primarily occurs within cells of the monophagocytic system (MPS) (Gómez-Villamandos et al., 2013; Gómez-Villamandos et al.,

2003; Maurer and Griesemer, 1958). ASFV enters host cells via receptor-mediated endocytosis (Alcami et al., 1990). Nonetheless, a definitive receptor has not been uncovered. Saturable binding sites may also mediate virus entry into target cells (Alcami et al., 1990; Carrascosa et al., 1999). Successful infection requires both entry into the cell, as well as the correct cellular components and pathway activation to ensure proper genome replication and virion production.

The virus is first absorbed into clathrin-coated pits, which are invaginations within the host cell plasma membrane. This process is mediated via vp54 and dynamin, a cellular GTP-ase involved in the final separation of the endocytic vesicle from the plasma membrane (Galindo et al., 2015; Hernaez and Alonso, 2010). Other cellular components, such as membrane cholesterol and actin filaments, also contribute to proper endosome structure and appear indispensable to ASFV entry (Muñoz-Moreno et al., 2015; Tulman et al., 2009). Cytoskeletal microtubules are involved in transportation of the virus through the cytoplasm. Other pathways of viral entry into the cell include micropinocytosis (Sanchez et al., 2012) and phagocytosis (Basta et al., 2010). However, it is not known if these alternate entry methods result in successful infection. Additional cellular pathways involved in viral entry include the Na^+/H^+ exchange, which functions both in micropinocytosis/ endocytosis, as well as Phosphoinositide-3-kinase (PI3K) involved in internalization, cell cycle progression, cytoskeletal rearrangements and membrane trafficking (Galindo et al., 2015; Muñoz-Moreno et al., 2015; Sanchez et al., 2012). After entry, virions are associated within cellular endosomes. As endosomes mature, they move closer to the nucleus and undergo changes in lipid and protein expression. In addition, the environment within the endosome becomes acidic. ASFV depends on this acidic environment in order to undergo decapsidation, virion core disassembly and penetration into the host cell cytoplasm (Muñoz-Moreno et al., 2015).

ASFV transcription has four phases: immediately-early, early, intermediate and late and starts immediately upon viral entry (Rodriguez and Salas, 2013). ASF virions posses a DNA-dependent RNA polymerase and do not require host cellular enzymes to initiate transcription. Early transcription is thought to occur during the nuclear phase of the viral replication; however, the mechanism is yet to be fully identified. Immediate early transcripts are expressed transiently during early replication (approximately 3 hours post infection) but are absent once replication ceases. Intermediate transcription requires established DNA synthesis, but similar to the immediate early phase, is transient between 4-8 hours post infection. This phase produces transcription factors necessary for late gene expression. The late transcription phase requires DNA replication and *de novo* protein synthesis to be already underway (Tulman et al., 2009).

ASFV DNA replication consists of both an early nuclear and late cytoplasmic phase (Tulman et al., 2009). Enzymes within the viral core, activated during decapsidation and viral core disassembly, result in the expression of immediate early and early genes before viral replication begins (Muñoz-Moreno et al., 2015). It is hypothesized that a DNA polymerase is responsible for initial replication of partial ASFV sequences within the nucleus before being transported to the cytoplasm via vp37 for full viral genome replication (Alonso et al., 2013; Muñoz-Moreno et al., 2015). It is here that perinuclear viral factories are formed, supported again by microtubules, and viral replication is completed. Within viral factories, viral morphogenesis occurs (Salas and Andres, 2013). During this time, there is general down regulation of host protein synthesis and drastic rearrangement of cytoplasmic organelles (Netherton and Wileman, 2013; Tulman et al., 2009).

Again utilizing microtubules, assembled virions migrate away from virus factories to the host plasma membrane. Mature virions are released from the cell via budding, resulting in the addition of the plasma membrane derived outer membrane (Tulman et al., 2009).

Epidemiology and economic impact

ASF was first described by R. Montgomery in 1921 in Kenya and distinguished as a disease entity separate from Classical swine fever (Montgomery, 1921). Several reports followed, tracking ASF through South Africa (1928), and Angola (1932)(Sánchez Botija, 1982). In Europe, ASF was first detected in Portugal in 1957 and again in the 1960's most likely after having been introduced from Angola. This latter occurrence led to a rapid spread of the virus throughout Europe including Spain, France, Italy, Sardinia, Malta, Belgium, and the Netherlands (Penrith and Vosloo, 2009). In the 1970's and 80's, ASF extended from Spain to the Americas. Outbreaks in the Caribbean first began in Cuba in 1970, with a secondary outbreak in 1981. ASF was then detected in the Dominican Republic and neighboring country of Haiti in 1978. Within South America, ASF was detected in Brazil in 1978 (Sánchez Botija, 1982; Sánchez-Vizcaíno et al., 2015). Over the course of 30 years, vigorous eradication programs removed ASF from European and American continents, with the exception of the island of Sardinia (Mur et al., 2016). Since 1994, ASF has remained endemic in previously exposed parts of Africa such as Mozambique and Kenya with new introduction into Cote d'Ivoire, Nigeria, Togo, Ghana, Burkina Faso, Tanzania, and Zambia (Ayoade and Adeyemi, 2003).

In 2007, a highly virulent strain of ASFV entered Georgia through the port of Poti, purportedly via contaminated food waste, and resulted in the now endemic spread throughout the region, including Armenia (2007), Azerbaijan (2008), Iran (2009), Ukraine (2012), Belarus

(2013), and several parts of the Russian federation (Costard et al., 2013c; Rowlands et al., 2008). Recently ASFV has breached the borders of the European union, via the migration of infected wild boar, to enter several countries including Poland, Lithuania, Latvia, and Estonia (Sánchez-Vizcaíno et al., 2015). These recent outbreaks have reignited research in both the transmission and pathogenesis of ASF in order to control spread of the disease and its impact on affected countries (Ayoade and Adeyemi, 2003; Sánchez Botija, 1982; Sánchez-Vizcaíno et al., 2015; Sánchez-Vizcaíno, 2012).

Although newer strains appear to have high virulence, all strains of ASFV pose a significant threat when introduced into new regions. Highly virulent isolates result in high mortality rates in a brief timeframe, and due to their short disease course, lead to a rapid loss of livestock before the disease can be controlled. Low virulence isolates cause fewer clinical signs, as well as milder lesions that can easily be misdiagnosed as other diseases. This prolongs the recognition of ASFV within an affected population and allows insidious spread before proper diagnosis is achieved. In addition, less virulent strains may not be detected with certain diagnostic assays such as the hemadsorption assay, further delaying detection. Another major concern with low virulent isolates is that pigs that survive overt disease remain infected, thus representing a constant source of infection through shedding of the virus or via infected meat if slaughtered (Mebus and Dardiri, 1979, 1980).

Costs to control and/or eradicate ASF within a region or country are significant. In Cuba, eradication of the disease was only achieved through destruction of the entire native pig population followed by the purchase of replacement stocks of pigs. This cost the country an estimated total of 9.4 million dollars (USD). Eradication efforts in Spain during the 1960's resulted in an excess of 92 million dollars spent (Costard et al., 2009). In conjunction with

eradication costs include the export restriction of livestock or pork products commonly placed on affected countries resulting in further loss of income.

Methods of viral detection

Several assays are used in the investigation of African swine fever virus. In a diagnostic setting, techniques fall within four distinct categories: Detection of infectious virus, detection of nucleic acid, antigen detection, and antibody detection. Virus and antigen detection methods are utilized to identify and confirm the presence of ASFV during field outbreaks, while serological tests identifying circulating antibodies are used to monitor the ongoing presence of the virus in endemic areas or in outbreaks involving strains of low virulence (OIE, 2012). In the research setting, a combination of these assays may be used depending on the goals of the research.

Because ASF is a transboundary disease, handling, manipulation and storage of live virus or samples presumably infected with ASFV is restricted to select laboratory facilities throughout the world. These laboratories meet strict biological safety criteria in order to prevent accidental release of virus outside of the controlled laboratory environment and into susceptible animal populations. The resulting limitations on ASFV handling and restricted availability of tests and/or reagents has hindered the development and validation of new assays for clinical or research use (Oura et al., 2013).

Diagnosis of ASF first relies of the recognition of clinical symptoms and inclusion of the disease within differentials. ASF can be clinically confused with other porcine diseases, especially in early stages of outbreaks where only individual animals have been sick or found dead and the possibility of herd related illness has not been recognized. For example, skin hyperemia can be observed in classical swine fever (CSF) and swine erysipelas. Renal petechiae

and splenomegaly can also be observed in cases of CSF, porcine dermatitis and nephropathy syndrome (PDNS), bacterial septicemia. and highly pathogenic forms of porcine reproductive and respiratory syndrome (PRRS) (OIE, 2012; Sánchez-Vizcaíno et al., 2015). Thankfully, an array of diagnostic tools is available for rapid diagnosis of the disease.

Virus Detection: Virus isolation and hemadsorption assay

Hemadsorption properties of ASFV, the process of attachment of red blood cells to the surface of infected macrophages/monocytes (de Leon et al., 2013; OIE, 2012), was first reported by Hess, et al in 1960 (Hess, 1960). Hemadsorption due to ASFV infection has been observed both as a natural phenomenon *in vivo* (Sierra et al., 1991) as well as in cultured primary porcine macrophages *in vitro* (Hess, 1960; Malmquist and Hay, 1960). This is in unique contrast to hemadsorption observed with select paramyxoviruses, porcine hemagglutinating encephalomyelitis virus, or pseudorabies virus, which is only observed in inoculated cultures of immortal cell lines (monkey kidney or canine kidney cell lines) to which red blood cells of other species (chicken, human, mouse, rat or hamster) are added (Young, 2009). This property is linked to ASF genes ORF EP402R and ORF EP153R, and a CD2-like protein (Borca et al., 1994; Galindo et al., 2000; OIE, 2012). Hemadsorptive properties of ASFV do not directly correlate with virulence, however the unique characteristic has been harnessed into a useful diagnostic assay.

Primary porcine macrophages are first harvested either via peripheral blood or lung lavage from a naïve pig, then purified and seeded onto tissue culture plates. These cultured cells are inoculated with titrated samples and incubated for at least 24 hours. Washed and diluted red blood cells, from the same pig from which the macrophages are derived, are then added to the

inoculated cultures. The cell cultures are monitored daily for attachment of red blood cells to the surface of infected macrophages, also known as rosette formation and consistent with hemadsorption (Carrascosa et al., 2011; OIE, 2012). Based on these results, the amount of virus within a given sample can be quantified.

Evidence of as few as one cell demonstrating hemadsorption within a culture confirms the presence of ASFV, making this test highly sensitive (Enjuanes et al., 1976). This specificity and sensitivity of hemadsorption assay (HAD) has made it the preferred method of detection and the gold standard in ASFV diagnostics (OIE, 2012). The HAD assay is the primary method of virus detection both in field samples as well as in live animal inoculation studies. Micromethodization techniques, eliminating the need of large amounts of buffy coat used in the original procedure, made the test less labor intensive and efficient. This transformation to a high-throughput method allows rapid high volume testing (Enjuanes et al., 1976; Greig, 1975). While highly sensitive and specific, the hemadsorption assay does have drawbacks. Inoculated cultures require examination for up to 10 days to confirm the presence of virus (King et al., 2003; OIE, 2012; Zsak et al., 2005). This is a drawback in an outbreak setting where crucial, time-dependent decisions on the movement restriction or culling of animals rely on laboratory confirmation of ASFV. The hemadsorption assay is less likely to detect chronic ASF due to the low amount of virus present during this state. Several non-hemadsorbing strains have been discovered (Coggins et al., 1968; Gonzague et al., 2001). In addition, cytotoxicity or presence of pseudorabies virus within the inoculum can result in cytopathic effects and inhibit hemadsorption properties of cultured cells. Because of these issues, supernatant from negative samples must undergo up to three subpassages within fresh cultures and confirmatory testing by other methods such as fluorescent antibody testing or PCR (OIE, 2012). The slow turnaround

time and repeated handling of samples, which could result in cross contamination, decreases this assay's usefulness. Because of these shortcomings, additional methods of detection have been developed to run in tandem with HAD.

Several established cell lines, such as monkey derived Vero, and COS-1 cells, or porcine derived cell lines such as immortalized swine pulmonary alveolar macrophages **(**IPAM), and wild boar lung cells (WSL) are sensitive to ASFV(Carrascosa et al., 2011). These cell lines can be used to isolate and quantify virus in cases where large quantities of cells are needed or when large amounts of virus must be produced. In addition, such cell lines can be used to quantify virus within a sample similar to the HAD assay; however, these methods instead evaluate cytopathic effects or plaque formation. The positive aspects of usage of these cell lines is the ready availability of large volumes of homogenous cells. Certain non-hemadsorbing isolates of ASFV have been adapted to Vero and COS-1 cells which allows further research in characterizing these strains, but restricts their usage to only these cell lines. In addition, it is difficult to directly compare the quantified values derived from plaque assays or measured cytopathic effect to those derived from the hemadsorption assay (Carrascosa et al., 2011; de Leon et al., 2013).

Antigen detection: Fluorescent antibody technique and immunomicroscopy

The use of fluorescent antibody technique (FAT) to detect intracellular ASFV antigen within tissue smears or sections has been described in several reports including time-course studies (Botija, 1970; Boulanger et al., 1966; Boulanger et al., 1967b; Colgrove, 1968, 1969; Heuschele et al., 1966). This assay utilizes fluorescein isothiocyanate (FITC)-conjugated anti-ASFV antibodies applied to acetone fixed samples, which are then evaluated microscopically. Detection of ASFV antigen in leukocyte cultures has proven this technique useful in identifying

non-hemadsorbing strains of ASFV (OIE, 2012; Oura et al., 2013). However, FAT is only used as a supportive diagnostic technique for a presumptive diagnosis, not as a stand alone test. The necessary requirement of frozen tissues/ cryosectioning to perform this technique limits the study of ASF by this method to biosafety level 3 laboratories (Ballester et al., 2010) making it of no use as a field assay. In addition, the formation of antibody-antigen complexes commonly formed in subacute and chronic cases of ASF may block interaction of ASFV antigen with the conjugated antibody, thus resulting in false negatives (Oura et al., 2013).

Most studies of ASF use FAT *in vitro* via cell culture. However, it may be used to localize the virus within different cell types or organs collected from infected pigs. This has helped in detection of virus replication within specific cell types: leukocytes (Carrasco et al., 1996b; Nunes et al., 1975), kidney cells (Gómez-Villamandos et al., 1995c; Greig et al., 1967), and endothelial cells (Wilkinson and Wardley, 1978). However, the application of these findings for pathogenesis purposes are limited due to the fact that the involvement of some cell types has not been demonstrated *in vivo* or has not been observed via time course study. In addition, the identity of immunopositive cells within these studies has been established only by general visual morphology.

Other immunomicroscopic methods such as, chromogen based immunohistochemistry (IHC) have been utilized for ASF research but has concentrated on specific tissues such as the spleen and lymph node (Fernandez et al., 1992a; Fernández de Marco et al., 2007; Gregg et al., 1995; Mìnguez et al., 1988; Perez et al., 1994; Rodriguez et al., 1996b).

Co-localization with cell specific antibodies to confirm the cell type in question has not been documented. The recent capability of applying multiple fluorescent labels to one tissue section via confocal microscopy or multichannel immunofluorescence (MIF) enhances the

accuracy of virus localization to specific cell types making this technique valuable in future pathogenesis studies.

DNA detection: Polymerase chain reaction and in situ hybridization

Polymerase chain reaction (PCR) techniques have been developed and prove to be both sensitive and rapid means of ASFV nucleic acid detection (Aguero et al., 2003; Aguero et al., 2004; Basto et al., 2006; Fernández-Pinero et al., 2012; Giammarioli et al., 2008; King et al., 2003; McKillen et al.; Tignon et al., 2011; Zsak et al., 2005). The techniques use primers from highly conserved regions of the genome, commonly vp72, to detect and identify a wide range of isolates from known genotypes, as well as from different hosts (domestic pigs, wild suids and ticks). Both hemadsorbing and non-hemadsorbing strains can be detected, as well as strains of differing virulence (Zsak et al., 2005). Tissue homogenates, cell supernatants, blood, serum, and secretory samples are all easily tested by PCR, even degraded tissues or samples where live virus may have been inactivated due to less than ideal handling (OIE, 2012). The presence of inhibitors (e.g. heme or leukocyte-derived DNA within blood samples) can result in false negatives, therefore sensitivity of the assay may vary dependent on type of sample analyzed (Zsak et al., 2005). Differing sensitivity between assays has also been observed, therefore only three have been validated for diagnostic purposes by the OIE (OIE, 2012; Oura et al., 2013).

For ASF, PCR assays have been designed for two distinct purposes. First, PCR has been used as a rapid confirmatory method for field outbreaks (Aguero et al., 2003; King et al., 2003; Zsak et al., 2005). Both gel –based assays for qualitative diagnosis or quantitative real time PCR for quantification of viral DNA within a sample are available. More recently, multiplexed PCR assays to screen for ASFV in conjunction with other viral porcine diseases have been developed (Aguero et al., 2004; Giammarioli et al., 2008; Wernike et al., 2013). Secondly, PCR techniques

are utilized to further genotype isolates, leading to better epidemiological and virulence categorization.

PCR has not yet been utilized for elucidation of the pathogenesis of ASFV within tissues in a time-course driven or comprehensive approach (OIE, 2012). In addition, PCR may be used for early detection of the virus and tissue localization; however, this method does not permit exact cellular localization.

While the reports of in situ hybridization (ISH) for the detection of ASFV nucleic acids are few (Ballester et al., 2010; Oura et al., 1998b), it proves a useful resource. The technique allows visual detection of the presence of the viral genome within tissues. ISH may be used in with formalin fixed tissues, allowing research to extend to the BSL-2 laboratory. ISH can incorporate up to two probes, allowing virus localization and identification of the cell type involved (Oura et al., 1998b).

Antibody detection: Serological assays

Specific ASFV antibodies in serum, secretory fluids or tissues can be detected by variety of methods such as enzyme-linked immunosorbent assay (ELISA) and immunoblotting (Gallardo et al., 2012; Mur et al., 2013; OIE, 2012).

These techniques are valuable in identifying the dynamics of the humoral immune response, especially in subclinical or recovered pigs. Antibodies can be detected as early as 1 week post infection and can persist for extended periods of time (Muñoz-Moreno et al., 2015). Conversely, peracute and acute forms of the disease, in which pigs die before antibodies are produced, can not be examined by this assay. These tests only detect antibody production and not viral replication, which is necessary to localize the virus in the early pathogenesis of the disease. Despite being useful in subclinical cases, ELISAs have a decreased sensitivity in samples from

subacute or chronic cases due to formation of antibody-antigen complexes and an overall lower sensitivity in comparison to PCR (Oura et al., 2013). In addition, false positives can occur resulting in required secondary confirmation by immunoblotting techniques (Cubillos et al., 2013). Therefore in the research setting, these serological methods are used in conjunction with other forms of virus detection to give a more comprehensive picture of viral pathogenesis at different stages of disease (OIE, 2012).

Conventional ELISAs utilize antigens for viral protein vp72 derived from infected monkey kidney cells (Vero or MS cell lines) (Cubillos et al., 2013). Recently, commercially available ELISA kits have been developed, creating a more accessible, affordable, and standardized method of antibody detection. Newer antigen detection methods utilize recombinant viral proteins (e.g. vp72, vp54, vp30) expressed in baculovirus or bacteria such as E. coli, thereby eliminating the requirement of virus infected cells to produce the antigen. These newer assays appear highly sensitive in comparison to the conventional ELISAs. However, there is demonstrable variation in sensitivity when analyzing isolates from geographically different regions (Cubillos et al., 2013).

Only recently have ELISAs been applied in time-course transmission studies to clarify the pathogenesis. Unfortunately sera collected throughout these studies, which separately examined low and high- virulent strains circulating within the current European outbreak, were negative by commercial ELISA and secondary confirmation by immunoblotting (Guinat et al., 2014; Pietschmann et al., 2015).

Transmission

Naturally occurring ASFV is transmitted through three main cycles: 1) Sylvatic cycle, 2) Tick-pig cycle, and 3) Domestic cycle. During the first outbreaks in Kenya and South Africa, contact between wild pigs (e.g. warthogs and bushpigs) and domestic pigs were found to be key in disease transmission (Detray, 1963; Montgomery, 1921). However, warthogs (Phacochoerus africanus), bushpigs (Potamochoerus porcus), giant forest hogs (Hylochoerus spp.) and peccaries (Pecari spp.) remain asymptomatic, with low levels of viremia insufficient for direct transmission of the virus to domestic pigs. Research revealed that argasid soft ticks (Ornithodoros spp., especially O. moubata and O. erraticus) act as reservoirs and vectors in the transmission of ASFV from warthogs to pigs. Ticks found within warthog burrows infect young warthogs, born free of infection, early in life. These young warthogs develop low viremia, which then results in infection of ticks that feed on viremic piglets. Within the tick, viral replication occurs within the midgut and is essential for maintenance of the virus. The virus is sustained in this sylvatic cycle between the tick and warthog, as well as within the tick population itself (trans-ovarialy, trans-stadialy, and horizontally) (Burrage, 2013; Wilkinson, 1986). In the Tick-pig cycle, domestic pigs are exposed to infected ticks when foraging on land inhabited by warthogs, or when warthogs are killed and brought back to villages for slaughter. Two potential routes of infection by ticks are described: deposition of the virus through salivary fluids into a feeding lesion and/or exposure through coxal fluids secreted shortly after feeding (Kleiboeker and Scoles, 2001; Oura et al., 1998b). Tick infection and excretion rates vary widely between strains (Wilkinson et al., 1988).

Both sylvatic and tick-pig cycles played a major role in the regional outbreaks in South and East Africa, as well as in the 1960's epizootic in Portugal and Spain. However, there is little

implication of the sylvatic cycle with previous outbreaks in South America and the Caribbean or with the present Eastern European spread (Gogin et al., 2013). These occurrences have been linked to the transportation of infected domestic pigs into those countries, or pig consumption of illegally discarded waste or pork products contaminated with ASFV (Costard et al., 2013a; Gogin et al., 2013). ASFV stays viable in pork products, despite freezing, cooking or smoking, for up to 3-4 months (Farez S., 1997; McKercher et al., 1978; Wilkinson, 1986). This point draws attention to the important fact that once ASFV is established within a domestic pig population, it is spread by means other than tick transmission.

Domestic pigs shed the virus through oronasal and urogenital secretions, blood, urine, and feces (Greig and Plowright, 1970). Transplacental transmission does not occur (Sánchez Botija, 1982). Therefore, direct contact between naïve and infected pigs is the main mode of transmission within a herd. Aerosolized ASFV can travel short distances (2-3 meters) which may facilitate transmission between pens, but does not contribute to long distance spread of the disease (Wilkinson et al., 1977). It is not known what anatomical site aerosolized virus is generated from, nor has it been examined if secreted viruses from specific anatomic sites facilitate infection during contact more than others (e.g. nasal secretions versus oral secretions). Comparatively, aerosolized virus in Classical Swine Fever is strongly associated with virus excretion in feces (Weesendorp et al., 2009); whereas aerosolized Foot and mouth disease virus in pigs, which does contribute to long distance transmission, is due to abundant amplification and secretion via oropharyngeal fluids and exhaled virus (Alexandersen and Donaldson, 2002; Alexandersen and Mowat, 2005).

Indirect transmission via fomites can introduce ASFV from farm to farm (Costard et al., 2013c). European wild boars (*Sus scrofa ferus*) are comparably susceptible to the disease, and

shed the virus in sufficient quantities for transmission. Hence, direct contact between domestic pigs and wild boar perpetuates disease spread in endemic Sardinia and has been associated with the current Europe outbreak (Blome et al., 2013; Gabriel et al., 2011). Production system preferences such as open herds, outdoor facilities, and free-range farming facilitate this route of transmission.

Viral replication sites

The mononuclear phagocytic system (MPS) is the principal site of viral replication and is considered the ultimate target of ASFV (Colgrove et al., 1969; Fernandez et al., 1992a; Sánchez-Vizcaíno et al., 2015). This involves not only circulating monocytes, but resident macrophages within lymphoid tissues, interstitial macrophages within the renal and hepatic parenchyma (Gómez-Villamandos et al., 1995b), as well as interstitial and alveolar macrophages within the lungs (Carrasco et al., 1996a).

The porcine monocyte/macrophage population expresses a heterogeneous array of cellular surface antigens, which can differ based on stage of maturation, function and stimulation (Bullido et al., 1997; Chamorro et al., 2005). In swine, five different clusters of differentiation specific to myeloid origin have been recognized including: CD14, CD16, CD172a, CD163 and CD203a (Piriou-Guzylack and Salmon, 2008). Further categorization has been performed based primarily on the varied combination of expression of CD172a, CD163, CD14 and SLA II to correlate with four major stages of maturation (Chamorro et al., 2005). While all monophagocytic cells express CD172a, a member of the signal regulatory protein family (SIRP) and myeloid cell marker, it has been suggested that apparent subpopulations differ in their susceptibility to ASFV infection.

Viral replication in other leukocytes, including megakaryocytes and neutrophils, within the bone marrow has also been reported, but is considered of less importance for the progression of the disease (Gómez-Villamandos et al., 1997a; Gómez-Villamandos et al., 1996; Gómez-Villamandos et al., 1997b). Although viral particles have been observed within lymphocytes (Wardley et al., 1977), based on current knowledge, viral replication does not occur within lymphoid cells (Carrasco et al., 1997b; Carrasco et al., 1996b; Gómez-Villamandos et al., 2003).

Stages of ASF pathogenesis

The stages of ASF pathogenesis are not as clearly defined in the literature as in other diseases; however, it appears to follow the same sequential order as most viral diseases with phases of pre-viremia, viremia and post-viremia.

Pre-viremia

Little is known regarding the events of infection before the onset of viremia. What is known is derived from a small collection of transmission studies which examine domestic pigs euthanized at set time points post inoculation in order to document the onset of lesions and the time course for detection of virus in organs affected (Colgrove et al., 1969; Greig, 1972; Heuschele, 1967; Plowright et al., 1968; Wilkinson and Donaldson, 1977). Virus could be detected via virus isolation within select organs prior to the detection within blood. However, different routes of inoculation, different virus strains and dosages used in these studies have resulted in conflicting results. Oral inoculation of newborn pigs using ASFV-Hinde WH II at $10^{7\text{-}7.5}$ 50% hemadsorbing doses (HAD_{50}) resulted in the detection of virus within the tonsil (presumably tonsil of the soft palate), submandibular lymph nodes, and parotid lymph nodes as early as 8 hours post infection (Colgrove et al., 1969). Following intranasal inoculation with

ASFV-Tengani at 10^5 HAD_{50}, virus was located within the submandibular lymph nodes, mediastinal lymph nodes, and tonsil at 24 hours post inoculation (Heuschele, 1967). A second intranasal study utilized ASFV-Tengani at a higher concentration of 10^7 HAD_{50}, demonstrated virus within the retropharyngeal lymph nodes and nasal mucosa at 16 hours post inoculation (Plowright et al., 1968). Following transmission through direct contact, virus could be detected within the submandibular lymph nodes, parotid lymph nodes, retropharyngeal lymph nodes, tonsil and retropharyngeal mucosa at 48 hours post contact (Greig, 1972). Deeper visceral lymph nodes and other lymphoid organs (e.g. intestinal Peyer's patches) were occasionally collected in theses studies, but did not appear to be involved in the early phase of disease when examined (Colgrove et al., 1969; Plowright et al., 1968).

Contrastingly, an aerosol exposure study located virus within the bronchial lymph nodes and spleen on the day of exposure (Wilkinson et al., 1977). This suggests that droplet size of aerosolized virus may facilitate bypassing oronasal mucosa or tonsillar sites and transit of virus to bronchial lymph nodes and other visceral organs via initial entry through the lungs. Droplet size of aerosolized ASFV has yet to be examined.

The above studies demonstrate that, despite variations in approach, the oronasal mucosal surfaces, tonsillar tissues, and lymph nodes of the head and neck are involved in early infection and replication of ASFV in domestic pigs. The fact that lymph nodes are most consistently involved suggests that, although virus must gain entry into the body by breaching the mucosal surfaces, these surfaces may serve as mere transit points. From there, the virus likely travels via lymphatics or vasculature to the lymph nodes where infection is then established. An alternative hypothesis is that initial infection does occur in mucosal or tonsil tissues, but not in quantities detectable by the diagnostic methods currently applied. All studies listed above used either HAD

assay, FAT or both. Application of more current viral or antigen detection methods (e.g. PCR) may clarify the role of specific organs in the pre-viremic phase of ASF.

Furthermore, early studies failed to examine select tissues that may play a role in virus entry. Tonsils of the soft palate were examined in a subset of the above studies (Colgrove et al., 1969; Greig, 1972; Heuschele, 1967); however, lingual tonsil, nasopharyngeal tonsil and paraepiglottic tonsil were commonly omitted from the tissue selection. The close anatomical association of these tissues to oro- and nasopharyngeal mucosal surfaces, where inoculum would be in contact, makes virus entry through these sites equally probable and worthy of examination.

What transpires at these early anatomical sites of infection at the cellular level has not been determined. Whether the virus first enters by breaching an intact mucosal surface, by transient infection of mucosal epithelium or by being taken up by antigen presenting cells is unknown. Despite the fact that principal viral replication within the MPS was recognized early on in research of ASF, only recently have the pathways mediating virus entry into these monocytes/ macrophages been partially established (Galindo et al., 2015; Hernaez and Alonso, 2010; Muñoz-Moreno et al., 2015).

Viremia

Viremia is defined as the period of hematological presence and spread of the virus. The viremic stage is noted as early as 2 days post infection (Heuschele, 1967; Mebus and Dardiri, 1979; Plowright et al., 1968; Wilkinson et al., 1981) or 1-2 days before pyrexia occurs (Greig and Plowright, 1970). Depending on virulence of the strain involved, once viremic, clinical presentation of ASF can vary.

Onset of viremia commonly marks detection of virus throughout all tissues (Colgrove et al., 1969; Greig, 1972; Greig and Plowright, 1970; Mebus and Dardiri, 1979, 1980; Mebus et al.,

1978; Wilkinson and Donaldson, 1977; Wilkinson et al., 1977; Wilkinson et al., 1981), as well as a cascade of biochemical responses as discussed in later sections. In addition to the principal replication sites discussed above, virions have been located by electron microscopy within endothelial cells (Gómez-Villamandos et al., 1995b; Sierra et al., 1989), hepatocytes (Sierra et al., 1987), interstitial fibroblasts, smooth muscle cells of the spleen (Carrasco et al., 1997a), renal tubular epithelial cells and glomerular mesangial cells (Gómez-Villamandos et al., 1995d). However, these cell types are not thought to be main locations of replication. Viral replication causes cytopathic effects (CPE) in affected cells such as cytoplasmic clearing at the site of viral replication, margination of chromatin, nuclear rounding, presence of cytoplasmic vacuoles, cellular swelling and loss of intercellular junctions (Gómez-Villamandos et al., 1997a; Gómez-Villamandos et al., 1995d; Gómez-Villamandos et al., 1997b; Hervas et al., 1996). Most investigators accept the theory that systemic spread of ASFV likely occurs through hematogenous circulation of infected monocytes, but this has never been verified.

Post-viremia

Most pigs die while still in a viremic state. However, limited field and experimental studies involving low virulence strains have shown recovery within a subset of pigs and cessation of viremia around 35-55 days post inoculation (Anderson, 1986; Hamdy and Dardiri, 1984; McVicar, 1984). Recovered pigs often resist challenge with a homologous strain; but stress or exposure to a heterogeneous strain of ASFV can result in activation of any residual virus and recrudescence (Hamdy and Dardiri, 1984; Malmquist, 1963; Mebus and Dardiri, 1980). Both scenarios may lead to clinical signs and death, or shedding of the virus in numbers sufficient enough to spread infection.

Clinical manifestations of disease

In domestic pigs, clinical signs can vary depending on strain virulence (high, moderate and low). There are 4 major clinical categories: peracute, acute, subacute and chronic forms (Detray, 1963). In general, the virus has an incubation period, defined as the time point of likely infection to onset of clinical symptoms, of 2-19 days during natural infection, with an accelerated incubation time (2-5 days) in experimentally infected pigs (Wilkinson et al., 1981). During a typical outbreak in swine herds, the disease initially affects only a few pigs and spreads through the herd in a matter of days to weeks (depending on the virulence of the strain). In both acute and chronically infected pigs, the virus is excreted throughout the entire course of the disease (Ayoade and Adeyemi, 2003). Patterns of disease differ between African and European outbreaks and can be attributed to the virulence of the strains involved. While African outbreaks are predominately acute and peracute, European outbreaks are more chronic (Sánchez Botija, 1982; Wilkinson, 1986). Other factors: including the number of animals involved, and environmental conditions, such as sanitation, also affect the course of the disease.

Peracute form

Highly virulent strains are responsible for peracute forms of ASF causing death 1-4 days after infection (Sánchez-Vizcaíno et al., 2015). This form often results in death with no additional clinical signs most likely due to vascular compromise and shock. Mortality is commonly 100% (Sánchez Botija, 1982).

Acute form

Both high and moderately virulent strains can result in acute forms of ASF at 3-8 and 11-15 days post infection, respectively (Sánchez-Vizcaíno et al., 2015). The acute form may last up to

seven days and is characterized by high fever (40-42 ºC) within 3-7 days post infection, decreased appetite at 2-3 days prior to pyrexia, recumbence, staggered gait, muscle tremors, and dyspnea and generalized hemorrhages. Mucoid to bloody nasal discharge, conjunctivitis, and mucoid to bloody diarrhea are common. Hyperemia of the ears, mouth, and ventrum are severe and extensive. Pigs with acute ASF typically die from shock. Mortality associated with the acute form is 90-100% (Sánchez Botija, 1982; Sánchez-Vizcaíno, 2012).

Subacute and Chronic forms

Subacute and chronic forms of ASF had not been reported until the spread of ASFV into Europe, which coincided with the emergence of less virulent strains (Mebus and Dardiri, 1979; Mebus et al., 1978). Subacute forms, caused by moderately virulent strains, may last up to 70 days and involve mild illness and intermittent fever (Moulton and Coggins, 1968). These pigs show clinical signs similar to acute cases for approximately 6-7 days, however by day 11, clinical signs subside and affected pigs gain normal appetite, and leukocyte counts. The mortality rate with subacute ASF ranges from 30-70%. Recovered pigs can go on to show no clinical signs yet still shed virus up to 6 weeks post infection (Detray, 1963; Sánchez-Vizcaíno et al., 2015). Abortion is common in pregnant sows with subacute ASF. Delivered piglets often have a high mortality (Wardley et al., 1983a).

Alternatively, pigs who survive subacute infection may go on to display chronic forms of the disease (de Carvalho Ferreira et al., 2012; Wilkinson, 1984). The chronic form lasts for 2-15 months and consists of intermittent fever, anorexia, joint swelling, keratitis, coughing, diarrhea and occasional vomiting (Ayoade and Adeyemi, 2003; Detray, 1963). Cutaneous ulceration and necrosis are commonly observed (Sánchez Botija, 1982). Chronic ASF may resemble and is

often confused with Classical Swine Fever, resulting in delayed detection and allowing further spread of the disease (Mebus and Dardiri, 1980; Penrith et al., 2004).

Subclinical animals

Subclinical/carrier infection has been associated with strains of low virulence, as well as vaccination attempts with a live attenuated vaccine, as seen during 1962 in the Iberian Peninsula. These pigs are apparently healthy with no clinical signs. Subclinical animals are found to shed the virus for up to 16 months post infection; however, lifetime carriers have not been documented (de Carvalho Ferreira et al., 2012; McVicar, 1984; Wilkinson, 1984, 1986). ASFV antibodies are present for up to 6 months. Virus distribution in tissues of subclinically infected animals is often irregular and in low concentrations (Mebus and Dardiri, 1980). This is thought to be due to virus persistence within resident macrophages (Anderson, 1986). Researchers have not uncovered what factors determine carrier state or the carrier's ability to transmit disease.

Clinical disease in wild boars

In wild boars, ASF clinically mirrors the peracute form of the disease. Animals present with depression, diarrhea and occasional epistaxis (Gabriel et al., 2011). Classic signs such as hemorrhagic diarrhea are not observed and cutaneous hyperemia cannot be evaluated due to skin pigmentation. Convulsions and ataxia are commonly seen before death (Blome et al., 2013). Chronic or carrier forms have not been observed in wild boars (Blome et al., 2012).

For consistent classification of experimentally reproduced forms of ASF, a simplified clinical grading scale (mild, moderate, severe) was proposed which essentially reflects the peracute, acute, and subacute forms of the disease based on six main categories: body temperature, body condition, behavior, skin, digestive system, and respiratory system (Galindo-

Cardiel et al., 2013). For chronically-infected pigs, a more extensive grading system based on ten categories was established to record the more subtle changes somewhat specific to these forms (e.g. body wasting, and stiff joints) (de Carvalho Ferreira et al., 2012). Still a more standardized grading system has yet to be adopted across ASF research. For example, a grading system that could encompass all forms of the disease and take in consideration the day-to-day variability of some clinical signs would be useful. In addition, neither grading system takes into account hypothermia commonly observed in pigs right before going into shock or pigs that die spontaneously without showing clinical signs and therefore would be scored as 0. Such standardization would facilitate comparisons between virus strains and infection models.

Pathologic findings

While gross and histologic lesions have been well described under both natural and experimental conditions, the reported sequence of the occurrence of these lesions has varies according to different studies. While the spleen has been reported as the first organ affected in many *in vivo* studies, tonsil has also been suggested as the primary site of viral infection. This incongruence is likely due to strain virulence, route of inoculation, and method of detection. Many studies that use intramuscular inoculation reported spleen as the first organ to be affected (Childerstone et al., 1998; Oura et al., 1998b) while oral, nasal and aerosol inoculation studies reported tonsils and/ or lymph nodes of the head and neck as the primary sites of viral infection (Colgrove et al., 1969; Heuschele, 1967; Plowright et al., 1968; Wilkinson and Donaldson, 1977).

Principal gross lesions consist of generalized hemorrhages, splenomegaly and hemorrhagic, enlarged lymph nodes (Gómez-Villamandos et al., 2003; Sánchez-Vizcaíno et al.,

2015). These changes can vary in severity depending of the form of disease. Splenic changes range from moderate splenomegaly in chronic ASF to a markedly enlarged, hemorrhagic and friable spleens within acute disease. Splenic infarcts are common in subacute infection (Sánchez-Vizcaíno et al., 2015).

Lymphadenomegaly is common in all forms of ASF. In conjunction with enlargement, lymph nodes display vascular chances ranging from moderate congestion to diffuse hemorrhage and engorgement, often resembling hematomas (Galindo-Cardiel et al., 2013; Sánchez-Vizcaíno et al., 2015). These changes may be generalized or affect select nodes. Lymph nodes of the head and neck are first to be involved (Carrasco et al., 1997b). Gastrohepatic and renal lymph nodes are prominent commonly within late stages of disease (Gómez-Villamandos et al., 2003).

Hemorrhage within the renal cortex, ranging from petechiation to ecchymosis, is a common and almost pathognomonic lesion associated with ASF (Gómez-Villamandos et al., 1995c; Wilkinson et al., 1981). Peri-renal edema also occurs with subacute forms. Hemorrhage of the urinary bladder is sometimes observed along the mucosal surface. These changes do not often result in hematuria (Galindo-Cardiel et al., 2013; Sánchez-Vizcaíno et al., 2015).

Within the heart, ecchymoses of the epicardial and endocardial surfaces, as well as coronary vessels occur (Ayoade and Adeyemi, 2003; Wilkinson et al., 1981). Hydropericardium is found within subacute ASF, whereas chronic ASF may result in a fibrinous pericarditis (Sánchez-Vizcaíno et al., 2015).

Lungs are firm and non-collapsing, and foam or fluid occupies the trachea and large bronchi. Pulmonary congestion, and edema are variable. Fibrinous pleuritis and interstitial to bronchopneumonia occur in chronically affected pigs (Galindo-Cardiel et al., 2013; Sánchez-Vizcaíno et al., 2015).

Hepatic congestion with a prominent lobular pattern is seen within all forms of ASF at varying degrees of severity. Gallbladder distension and gallbladder edema are common (Galindo-Cardiel et al., 2013; Sánchez-Vizcaíno et al., 2015). Less common lesions include pancreatic hemorrhage and necrosis.

Hemorrhagic lesions are less likely within chronic forms of ASF. However, secondary bacterial infections are common (Sánchez-Vizcaíno et al., 2015). General ascites, and hydropericardium, are common findings with this form of the disease (Sánchez-Vizcaíno et al., 2015). Other lesions associated with chronic ASF include arthritis and meningoencephalitis (Detray, 1963).

Within aborted fetuses, lesions include petechiae of multiple organs and anasarca (Ayoade and Adeyemi, 2003). Abortion is associated with subclinical ASF but appears to be due to stress of the infection on the sow, and not abortogenic properties of the virus. ASFV has not been isolated in tissues collected from aborted fetuses (Schlafer et al., 1984; Schlafer and Mebus, 1984, 1987).

The principal histologic change is the proliferation and subsequent depletion of cells of monophagocytic origin within multiple organs, such as Kupffer cells in the liver or alveolar and interstitial macrophages in the lungs (Fernandez et al., 1992b). This is accompanied by vascular changes such as congestion, hemorrhage and thrombosis. Hemorrhage, due to endothelial damage, is noted days before viral replication is detected ultrastructurally (Gómez-Villamandos et al., 2003). Massive loss of lymphocytes within the spleen, tonsils, lymph nodes, and thymus is the result of apoptosis, as discussed in a later section (Gómez-Villamandos et al., 2003; Gómez-Villamandos et al., 1995a; Oura et al., 1998a).

Hematological changes

Reported hematological changes have been variable and appear dependent on the form of infection, differences between strains and host factors, e.g. age. Severe lymphopenia (particularly B lymphocytes), thrombocytopenia, and monocytosis are commonly observed with ASFV infections (Blome et al., 2013; Ramiro-Ibanez et al., 1997; Wardley and Wilkinson, 1977). These changes are due to ASFV-mediated apoptosis of lymphocytes and prolonged survival and stimulation of monocytes and macrophages. Transient neutrophilia followed by neutropenia has been reported in some cases (Genovesi et al., 1988). There are no changes within eosinophil numbers. Thrombocytopenia has been noted despite unimpaired thrombocytopoiesis and megakaryocyte hyperplasia (Genovesi et al., 1988). Hemorrhages are commonly found within the lymph nodes, spleen and skin, but may involve other organs. Despite these hemorrhages, anemia is not a prominent feature of the disease (Blome et al., 2013). Hypergammaglobulinemia may be observed in chronic forms of ASF (Ayoade and Adeyemi, 2003; Wardley et al., 1983a).

Host immune response

The immune response to ASF infections is associated with variations in strain virulence and subsequent clinical forms (Sánchez-Vizcaíno et al., 1981). Humoral immunity within infected pigs is functional and, with cellular mediated immunity, collectively contributes to the immunologic response to ASFV, albeit ineffectively. Due to the rapid disease course, pigs infected with highly virulent ASFV strains do not produce measurable antibodies, whereas infection with low to moderately virulent ASFV strains results in the production of large amounts of immunoglobulins with antibody production being reported as early as 6 dpi (Sánchez-Vizcaíno et al., 1981). Transplacental transmission of maternal antibodies from carrier

sows to fetal pigs has been documented, however, antibodies disappeared within 3 months after birth (Sánchez Botija, 1982). Piglets born to sows with circulating ASFV antibodies do not survive challenge with similar ASFV strains at 6-7 months of age (Penrith et al., 2004).

The presence of significant neutralizing function of theses antibodies, necessary to prevent infection, is widely debated (De Boer, 1967; De Boer et al., 1969; Escribano et al., 2013; Gomez-Puertas et al., 1996; Neilan et al., 2004). Antibody-mediated immune responses to ASFV infection do appear to occur. Wardley et al. demonstrated *in vitro* (Wardley, 1982; Wardley et al., 1983a) and *in vivo* (Wardley et al., 1985) that passively transferred pooled antisera, which had no neutralizing activity, could partially protect pigs against virus challenge at 140, 370 or 693 days post infection. This was demonstrated by a decrease in pyrexia and viremia. It is hypothesized that antibody dependent cell cytotoxicity may be the mechanism for protection (Martins et al., 1993; Wardley and Wilkinson, 1985).

ASFV infected pigs maintain a competent immune response when exposed to a different virus. Convalescent pigs, that survived infection with an attenuated ASFV strain and subsequently challenged with Foot and Mouth disease virus (FMDV) one month post initial inoculation, were able to mount an immune response and develop neutralizing antibodies specific to the challenge virus despite the inability to develop antibodies to ASFV(De Boer, 1967). Furthermore, ASFV was demonstrated to interfere with FMDV infection in pigs initially inoculated with ASFV and challenged with FMDV 3 days later (Gregg et al., 1995). Within this study, all pigs developed clinical disease or lesions consitent with ASF and/or became viremic. In contrast, only a small subset ASFV/FMDV infected pigs showed FMD lesions, viremia or neutralizing antibodies. Protection via interferon production or competitive infection and

depletion of interdigitating dendritic cells were hypothosized as possible causes of this interference (Gregg et al., 1995).

A decrease in complement levels occurs in chronic forms of ASF (Wardley et al., 1985); (Slauson and Sánchez-Vizcaíno, 1981) and is likely due to the formation of immune complexes within the kidney, lungs and skin, not decreased production (Blome et al., 2013). A functional decrease in complement has not been examined.

Specific host cell types are pivotal in the immune response to ASFV. ASFV results in the activation of $CD8^+$ lymphocytes, which are shown to have a major role in countering infection and modulating severity of clinical disease (Oura et al., 2005; Ramiro-Ibanez et al., 1997). Utilizing Cytotoxic T cell lysis assays, subsets of $CD8^+$ lymphocytes derived from pigs infected with a low virulence strain (ASFV-NHV), lysed infected macrophages inoculated with the same strain (Martins et al., 1993). Lysis of infected cells is mediated via the classical pathway of complement activation with ASFV, a characteristic different from cell lysis induced by other viruses, which is mediated by the alternate pathway. This is thought to be due to properties of the virus, not the host, however, this theory has yet to be resolved (Wardley and Wilkinson, 1985).

Natural killer (NK) cells, which are known to target virus-infected cells through innate immune mechanisms, are also implicated in the host response to ASFV. However, their role is less defined. Non-virulent ASFV strains result in increased Natural Killer cell activity, whereas infection with virulent strains show minimally increased or decreased activity. While this suggests a correlation between virulence and NK cell function, decreased activity is likely due to decreased numbers of circulating cells. Increased temperature has been documented to reduce Natural Killer cell activity *in vitro* (Norley and Wardley, 1983). Regardless, both responses appear transient, occurring around 3-7 days post infection but gradually returning to or near

normal during late stages of infection (Leitao et al., 2001; Norley and Wardley, 1983) (Takamatsu et al., 2013). The decrease in activity does not appear to correlate specifically with the onset of viremia or pyrexia.

The current understanding of the ASFV- associated immune response has lead to valiant yet ultimately unsuccessful efforts in vaccine creation. Attempts to vaccinate pigs against ASFV using conventional methods of vaccine production have not been successful in conveying complete protection. Killed virus, such as glutaraldehyde-fixed, infected cells, does result in a milder form of disease with reduced fever, viremia and anorexia when challenged with a homologous strain but does not result in immunity (Detray, 1963). Experiments using detergent-treated infected porcine alveolar macrophages did not reduce disease severity or produce antibodies in pigs vaccinated with the prepared cells and then challenged 28 days later with the same strain (Forman et al., 1982). Classically attenuated strains, created via subpassage in tissue culture, have shown somewhat effective protection against homologous strains but not heterogeneous strains of ASFV (King et al., 2011; Lacasta et al., 2015). Detray also attempted to vaccinate naïve pigs using hyper-immune anti-ASFV serum derived from convalescent pigs. This resulted in acute death when serum, which was purified but not inactivated, was given; or a carrier state when the serum was inactivated (Detray, 1963).

As discussed in earlier sections, several viral genes and proteins have been identified as essential to ASFV infectivity. Antibodies against viral proteins 54 and 72, as well as vp30 have shown to significantly inhibit virus attachment and internalization, respectively (Gomez-Puertas et al., 1996). Subunit vaccines targeting these essential viral genes have been developed, yet only offer a delay in onset of the disease and have no effect on disease severity or outcome (Neilan et al., 2004).

More recently, genetic engineering methods of vaccine production have been applied in ASF research. Infection with ASFV strains that feature a variety of genetic attenuations or deletions of nonessential viral genes have, at best, resulted in partial protection against homologous viruses challenge (Abrams et al., 2013; Dixon et al., 2013a; Lewis et al., 2000; McVicar, 1984; Mulumba-Mfumu et al., 2015; O'Donnell et al., 2015).

Immune modulation and apoptosis

Few reports describe specific cytokine levels in ASFV infected pigs (Ramiro-Ibanez et al., 1997; Salguero et al., 2008; Salguero et al., 2002); therefore, a standard and reproducible cytokine profile of ASFV-infected pigs has yet to be documented. Changes in inflammatory cytokine levels appear to be dependent on the strain involved and often reflect the clinical form of disease. In general, tumor necrosis factor alpha (TNF-α), interleukins (IL) 1 and 6, and interferons (IFN) alpha and beta increase during early phase of infection when pyrexia and viremia are first detected (Gomez del Moral et al., 1999; Salguero et al., 2002; Takamatsu et al., 2013). This is consistent with general characteristics of acute inflammation in which endothelial cells become activated, diapedesis of circulating leukocytes begins, and clotting and complement cascades are initiated. This combination of cytokines is also consistent with the known febrile response (Zachary and McGavin, 2012). The increase in TNF-α contributes to cellular apoptosis, increased vascular permeability and disseminated intravascular coagulopathy in ASF infection (Gomez del Moral et al., 1999). Increases in type 1 IFNs are likely an attempt to prevent further viral infection of host cells and to activate NK cells and macrophages. In acute infections, expression of the IL-2 receptor is decreased (Ramiro-Ibanez et al., 1996). This suggests that ASFV may modulate cellular IL-2 receptor expression in order to prevent lymphocyte development and activation. IL-1, a versatile component of both innate and adaptive immune

response, decreases by 3 days post infection. Interleukin-4, major histocompatibility complex class I (MHC I), and interferon gamma appear unaffected (Ramiro-Ibanez et al., 1997).

Specific ASFV genes are known to modulate the host anti-viral response via direct control of host gene transcription or expression (Sanchez et al., 2013). The best understood example is ASFV gene A238L which produces a protein that is similar to IκB, yet nonfunctional. Through competitive binding, A238L blocks NFκB translocation, resulting in inhibition of the synthesis of multiple pro-inflammatory cytokines, thereby dampening the immune response. A238L also prohibits the activity of calcineurin, resulting in inhibition of the NFAT transcription factor family. This leads to downregulation of cyclooxygenase-2 and ultimately inhibition of prostacyclin production, possibly contributing to DIC, and inflammation (Tulman et al., 2009). Experimental strains of ASFV which lack A238L result in an increase in TNF-α and IL-1 in infected pigs (Salguero et al., 2008). Less virulent, non-hemadsorbing strains appear to cause increased production of TNF-α, IL-1, IL-6, IL-12, IL-15 and IL-10 in porcine macrophage cultures in comparison to highly virulent strains, however the A238L encoding gene was identical in the compared strains (Gil et al., 2003).

Apoptosis of lymphocytes and destruction of lymphoid tissue including lymph nodes, spleen, and thymus is a prominent feature of ASF resulting in clinical lymphopenia and a visual decrease in lymphocytes in lymphoid tissues examined microscopically. The distinction between apoptosis and necrosis of cells during ASFV infection has been made in studies utilizing terminal deoxynucleotidyl transferase (TdT)-mediated dUTP end labeling technique (TUNEL) or by ultrastructural examination (Carrasco et al., 1996d; Gómez-Villamandos et al., 1995a; Oura et al., 1998a). The modification of multiple pathways, including cytokine production from virus

activated macrophages, is thought to control lymphocyte apoptosis during infection. However, the underlying pathogenesis is not fully resolved.

Viral genes encoding proteins similar to p21, IAP, and Bcl-2 block apoptosis and encourage survival of infected macrophages (Afonso et al., 1996; Oura et al., 1998a). Concurrently, these activated and ASFV infected macrophages secrete cytokines, specifically TNF-α, which may indirectly induce apoptosis in nearby lymphocytes. This is supported by increased expression of TNF-α at sites of lymphocyte apoptosis. In addition, supernatant from infected cells has demonstrated the ability to induce apoptosis in cultured, non-infected lymphocytes *in vitro*. This ability was then blocked by the addition of anti-TNF-α antibodies (Tulman et al., 2009).

Within infected cells *in vitro*, pro-apoptotic proteins Bax, p53 and caspase-3 are increased in expression, particularly at late stages of infection. This suggests that ASFV may directly activate several apoptotic pathways within infected macrophages at late stages of disease (Granja et al., 2004). Ultimately, it appears that ASFV attempts to destroy lymphocytes, thereby crippling their contribution to both cellular and humoral immune responses; all the while temporarily prolonging the survival of macrophages in order to allow sufficient virus replication and circulation.

Knowledge gaps

There has been considerable effort to better understand the pathogenesis of ASF. Despite these efforts, significant knowledge gaps concerning the virus-host interaction remain. The key element of ASF pathogenesis is the tropism of ASFV for monophagocytic cells. Yet not all types of monophagocytic cells become infected. Factors such as designated cell

receptors that would account for this discriminant tropism have not yet been identified. An improved understanding of viral mechanisms has been documented in *in vitro* models, for example clathrin-dependent virus entry into cells. However, researchers have not yet translated these *in vitro* described characteristics of ASF *in situ*. Are these characteristics specific to functional or phenotypic subsets of macrophages? Do these characteristics affect tropism of virus infection or replication to specific organs? Thus our understanding of ASFV interactions with different cell types within their natural tissue environment remains limited. A comparative examination of the distribution of ASFV in tissues and cell types both in a spatial and temporal manner would be necessary to to develop a consistent narrative of how ASFV first enters the host, which tissues and cells are primarily infected and where the main viral replication does take place through the course of disease. Some of the current gaps in our knowledge are the result of uncontrollable variation in host response and disease manifestations among research animals. Others are due to a lack of consistent and robust animal models amongst different studies. In addition, novel molecular pathology techniques have yet to be applied to ASF research.

Dissertation scope

The work described herein aims to clarify two main areas of ASFV research. First, as described above, there is a lack of standardization amongst ASFV infection models. Variation both between and within different routes of infection, as well as variation in dosages, viral strains, and grading of lesions has limited the comparability of results between live-animal studies. This is especially relevant in the time course examination of primary infection sites. Chapter 2 compares four swine models commonly used in ASFV inoculation studies and

examines survival times, clinical signs, viral shedding, and pathology for each model at varying dosages. Our goal is to generate a baseline of outcome expectations by controlling for variables. Ultimately, we want to produce a reliable challenge model that accurately mirrors the natural course of disease in a controlled fashion and can be used to generate quantifiable results.

The second aim of this dissertation is to examine the detection of ASFV within tissues at varying stages of disease. Chapter 3 identifies viral dynamics within tissues by quantifying infectious virions and viral DNA. Distribution of virus within tissues, as well as infected cell types will be characterized microscopically to better understand ASFV pathogenesis. A better understanding of the tissues and cell types that are initially infected by ASFV is vital to identify the molecular pathways of ASFV infection: which viral or cellular genes/proteins are necessary for initial cellular entry, and which genes/proteins should be altered or deleted in modified strains used for vaccine development.

The final chapter of the dissertation summarizes and discusses the obtained results, together with limitations and future perspectives for continued research within the specific area.

To conclude, this detailed investigation will result in an optimized simulated natural infection model that will allow for further characterization of ASFV pathogenesis and development of novel therapeutic and prophylactic modalities.

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CHAPTER 2:

Pathogenesis of highly virulent African swine fever virus in domestic pigs exposed via intraoropharyngeal, intranasopharyngeal, and intramuscular inoculation, and by direct contact with infected pigs.

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Abstract

To investigate the pathogenesis of African swine fever virus (ASFV), domestic pigs (n=18) were challenged with a range (10^2 – 10^6 50% hemadsorbing doses (HAD_{50})) of the highly virulent ASFV-Malawi strain by inoculation via the intraoropharyngeal (IOP), intranasopharyngeal (INP), or intramuscular (IM) routes. A subsequent contact challenge experiment was performed in which six IOP-inoculated donor pigs were allowed to have direct contact (DC) with six naïve pigs for exposure times that varied from 24-72h. All challenge routes resulted in clinical progression and postmortem lesions similar to those previously described in experimental and natural infection. The onset of clinical signs occurred between 1-7 days post inoculation (dpi) and included pyrexia with variable progression to obtundation, hematochezia, melena, moribundity and death with a duration of 4-11 days. Viremia was first detected between 4-5 dpi in all inoculation groups whereas ASFV shedding from the nasal cavity and tonsil was first detected at 3-9 dpi. IM and DC were the most consistent modes of infection, with 12/12 (100%) of pigs challenged by these routes becoming infected. Several clinical and virological parameters were significantly different between IM and DC groups indicating dissimilarity between these modes of infection. Amongst the simulated natural routes, INP inoculation resulted in the most consistent progression of disease across the widest range of doses whilst preserving simulation of natural exposure and, therefore, may provide a superior system for pathogenesis and vaccine efficacy investigation.

Introduction

African swine fever (ASF) is a highly contagious, often fatal, transboundary disease of domestic and wild suids caused by ASF virus (ASFV) (Tulman et al., 2009). It is the sole member of the *Asfarviridae* family and is a large, double stranded DNA virus with a 170-190 kb genome. Due to the lack of a commercial or experimental vaccine or treatment, large economic losses are associated with ASFV outbreaks (Sánchez-Vizcaíno et al., 2012). Such an event occurred in 2007, when ASFV was introduced into the Republic of Georgia which has subsequently spread to Russia, Armenia, Azerbaijan, Ukraine, and most recently to Belarus (WAHID, 2013), with the range of the virus progressively extending west towards Europe (Costard et al., 2013b).

In domestic swine, it is generally accepted that under natural conditions, ASFV is primarily transmitted via direct contact with excreted viral particles through nuzzling and/or ingestion (Sánchez-Vizcaíno et al., 2012; Wardley et al., 1983b). However, the detailed mechanism of this mode of transmission has yet to be explicitly defined. Arthropod-borne transmission via *Ornithodoros spp* ticks, which is a relevant means of transmission of ASFV within the wild suid population and from wild suids to domestic swine, is a minor method of transmission between domestic pigs (Arzt et al., 2010).

An extensive portion of *in vivo* ASF research has utilized the intramuscular (IM) route of inoculation (Ballester et al., 2010; Carrasco et al., 1997b; Carrasco et al., 1996c; Childerstone et al., 1998; Fernández de Marco et al., 2007; Gómez-Villamandos et al., 1997a; Gómez-Villamandos et al., 1998; Gómez-Villamandos et al., 1995a; Gómez-Villamandos et al., 1995d; Gómez-Villamandos et al., 1997b; Oura et al., 1998b; Salguero et al., 2004). This method results in highly reproducible clinical disease. However, since IM inoculation does not result in viral

interaction with the oral and upper respiratory mucosal surfaces, it bypasses many of the innate defense mechanisms the virus would normally encounter through natural infection. Therefore, it may be a suboptimal inoculation method to examine the early pathogenesis and previremic stages of disease. Similarly, these limitations make IM challenge, an unnatural model for vaccine assessment.

The conventional pathogenesis of ASF has been investigated *in vivo* using simulated natural routes of inoculation including intranasal (IN) (Greig, 1972; Greig and Plowright, 1970; Heuschele, 1967; Plowright et al., 1968), intraoral (IO) (Boulanger et al., 1967a; Colgrove et al., 1969), combined oronasal (ON) (McVicar, 1984; Mebus and Dardiri, 1979, 1980; Mebus et al., 1978) and direct contact/aerosol (DC) (Wilkinson and Donaldson, 1977; Wilkinson et al., 1977; Wilkinson et al., 1981). Within all of the studies noted, the main route of virus detection was through virus isolation and was usually performed only on the blood, serum or a limited number of tissues. Few have focused on virus detection within secretions (de Carvalho Ferreira et al., 2012; Greig and Plowright, 1970; McVicar, 1984). While these reports have contributed to current understanding of the viral dynamics and pathogenesis of ASF, they fail to capture some specific information, which may contribute to develop the next generation of rationally designed countermeasures. Specifically, confirmation of the primary site(s) of infection and elucidation of virus-host interactions during early pathogenesis would facilitate the process of developing and validating effective prophylaxis. Furthermore, variability in methodologies including virus strain, dose, and route of challenge has confounded comparison between studies. The importance of standardizing experimental models including the description and interpretation of clinical and pathologic findings in experimental ASF has recently been emphasized (Galindo-Cardiel et al., 2013). The goal of such standardization is to facilitate comparison of studies between research

groups for the purpose of advancing ASF vaccine development whilst avoiding redundancy of research activities.

In the current study, we compared four routes of challenge of domestic pigs with ASFV in order to characterize route- and dose-dependent viral dynamics and the transmission of ASFV via direct contact. This was done to determine how different means of exposure/inoculation alter the pathogenesis of ASFV in swine and to determine which route most closely simulates natural infection with optimum experimental reproducibility.

Materials and methods

Virus

The highly pathogenic ASFV isolate Malawi Lil-20/1 was originally isolated from *Ornithodoros* sp. ticks during a field outbreak in Malawi in 1983 and obtained from L. Dixon (Institute of Animal Health, Pirbright Laboratory, Woking, Surrey, United Kingdom) (Borca et al., 1998). The stock virus was passaged once in primary porcine macrophages derived from heparinized swine blood. The tissue culture supernatant was then stored as 10^8 50% hemadsorbing dose (HAD_{50}) aliquots at -70 ºC.

The inoculum was prepared by initially diluting stock virus 10-fold in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco; Invitrogen, NY) containing 1% Antibiotic/Antimycotic (100X) (10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of Fungizone®) (Gibco; Invitrogen, NY) and subsequently performing additional dilution to achieve the desired viral dose in 2ml of media.

Animals

Conventional castrated male Yorkshire pigs from a herd demonstrated to be free of

porcine reproductive and respiratory syndrome virus were used for all experiments. Animals were dewormed and vaccinated for common porcine pathogens including porcine circovirus 2 prior to arrival at our facility. Pigs were approximately 3-4 months old and weighed 22-27kgs upon arrival. All animal procedures were performed following Protocol 225-10-R approved by the Plum Island Animal Disease Center Institutional Animal Care and Use Committee (IACUC), which ensures ethical and humane treatment of experimental animals. The animal experiments were carried out in BSL-3 Ag isolation rooms at the Plum Island Animal Disease Center. Animals were kept within the housing facilities 7 days prior to the start of the experiments to allow for acclimation to the new environment. All animals were fed daily and had unlimited access to water.

All animals except for donors in the DC study were allowed to survive until terminal disease. Severely moribund animals were humanely euthanized by intravenous injection with 85.8 mg/kg of sodium pentobarbital. Animals without clinical disease were humanely euthanized at the end of the study as described above.

Study design

Direct inoculation studies

Experiments were performed to compare three routes of direct inoculation. Eighteen pigs were used in these experiments. Six pigs were assigned to each of the three inoculation route groupings: Intramuscular (IM), Intranasopharyngeal (INP) and Intraoropharyngeal (IOP). Inoculation route groupings were further subdivided into three inoculation dosage groups: low dose 10^2 HAD_{50} (n=2), mid dose 10^4 HAD_{50} (n=2) and high dose 10^6 HAD_{50} (n=2). IM–inoculated pigs received a 2 mL injection of inoculum within the right semimembranosus

muscle. Pigs assigned to INP inoculation were sedated with an intramuscular injection of Telazol (3mg/kg), Ketamine (8mg/kg), Xylazine (4mg/kg), placed in sternal recumbency and 2 mL inoculum was instilled through the nares into the nasopharynx via a 15 gauge silicon catheter (Abbott Laboratories, IL). Pigs assigned to IOP inoculation received 2mL of the inoculum perorally via syringe with an attached 6-inch sterile metal cannula. Animals were sedated, placed in dorsal recumbency and 2 mL of the inoculum was deposited in the ventral portion of the soft palate and palatine tonsil.

Direct contact (DC) transmission studies

Additional experiments were performed to characterize ASFV transmission from IOP-inoculated pigs and viral dynamics in pigs challenged by direct contact. Donor (n=6) and naïve pigs (n=6) were initially housed in two separate rooms. Donor pigs were inoculated via the intraoropharyngeal method as described above with 2 mL of inoculum at a dose of 10^5 HAD_{50} of the same ASFV Malawi Lil-20/1 described above. Upon detection of pyrexia (rectal temperature greater than or equal to 40ºC) in at least 4 donor pigs (6 days post inoculation (dpi)), contact pigs were transferred into the donor room and allowed to comingle with inoculated pigs. Feed was withheld for approximately 4 hours to encourage contact between pigs. After 24 hours (1 day post contact (dpc)), two contact animals were removed and isolated into a separate room and two donor animals were euthanized to maintain the donor/naïve pig ratio. This process was repeated at 48 and 72 hours (2 and 3 dpc). Contact-exposed animals were then monitored for onset and progression of clinical disease.

Clinical evaluation

Incidence proportion of ASF was calculated for each dose/route combination as [(number

of successfully infected pigs for that dose and route) ÷ (all pigs subjected to that dose and route)]. For all studies, clinical evaluations were performed on all animals until death or termination of the experiment. Clinical signs used to characterize progression of ASF are listed in table 1. These clinical signs were assigned a numerical value based on severity and significance. The sum of the scores of all clinical signs present for each pig was recorded daily.

Table 1. Clinical signs and scoring value for ASF

Characteristic	Score	
Behavior and Mentation:		
	0	Normal, alert, responsive
	1	Mildly obtunded. Slightly reduced liveliness, stands up unassisted, resists restraint or rectal thermometer
	2	Obtunded. Reluctant to stand but will do so when assisted; decreased resistance to restraint or rectal thermometer
	3	Intermittent ataxia, disorientation, can still stand/walk or Will not stand/walk even when assisted, still conscious
	4	Moribund. Nonambulatory, unconscious/nonresponsive
Neurologic signs:		
	0	Normal
	2	Unambiguous neurologic signs (eg. convulsions, seizures)
Defecation:		
	0	Normal to mildly soft stools
	1	Profuse watery diarrhea, +/- mild hematochezia or melena
	2	Severe to marked hematochezia/ Melena
Body Temperature:[a]		
	0	38-39.9°C
	1	Temperature greater than or equal to 40°C at any point of study
	2	Temperature greater than or equal to 40°C for at least 2 subsequent days
	3	Temperature greater than or equal to 41°C
	4	Temperature less than 38°C

[a]Temperature scores are never reduced

Sample collection and processing

Sample collection including whole blood with EDTA, clotted blood for the collection of serum, swab specimens from the nasal cavity and superficial scrapings from the tonsils of the soft palate were performed on the day of inoculation at 0 days post inoculation (dpi) followed by sample collection every other day on all animals until death or termination of the experiment. Pigs were manually restrained for sample collection. Whole blood and serum were collected via the jugular vein. Tonsil scrapings were collected with a sterile spoon by gently scraping along the surface of the tonsil of the soft palate, exfoliating superficial epithelial cells while making sure to not disrupt the mucosa. Nasal swabs were collected bilaterally using sterile cotton swabs extended just caudal to the alar fold. Following collection, nasal swabs and tonsil scrapings were immediately immersed in 1 mL of DMEM with 5% antibiotic/ antimycotic; then, all samples were transferred on ice to the laboratory for processing. All samples were transferred to cryovials and stored at -70ºC until they were analyzed by virus isolation (VI).

Necropsies were performed as soon as possible following euthanasia or natural death. Tissues collected during postmortem examination included: tonsils of the soft palate, lingual tonsil, nasal tonsil, dorsal soft palate (rostral and caudal), dorsal nasopharynx, caudal nasal turbinate, epiglottis, trachea, bronchial mucosa, lung, thymus, liver, spleen, kidney, adrenal gland, thyroid, pancreas, small intestine, large intestine, bone marrow, and lymph nodes including: retropharyngeal, submandibular, hilar, gastrohepatic, renal, inguinal and popliteal. Urine was also taken via direct aspiration from the urinary bladder at the time of necropsy. Tissue samples collected were placed in cryovials as described above and cryomolds, embedded in Optimal Cutting Temperature (OCT) medium (Tissue-Tek O.C.T. compound, Sakura Finetek,CA) and then frozen in liquid nitrogen. A detailed description of tissues from these

animals will appear in Chapter 3.

Virus isolation and titration

Blood, serum, nasal swabs, tonsil scrapings, urine (postmortem only) and tissues from infected pigs were screened for ASFV by virus isolation and titration (VI) on primary porcine blood macrophage cell cultures prepared from defibrinated swine blood as previously described (Zsak et al., 2005). Presence of virus was determined by identification of infected cells by rosette formation (hemadsorption) and titers expressed as hemadsorbing dose (HAD_{50}) calculated by the Spearman-Karber method (Mahy, 1996).

Immunomicroscopy

Microscopic localization of the virus in tissues positive for ASFV via VI was performed via immunohistochemistry (IHC). Multichannel immunofluorescence (MIF) was used to detect virus in combination with other cellular markers in order to further determine/characterize phenotypes of infected cells. All primary antibodies were extensively tested prior to this study in sections of tissue from ASFV infected or non-infected pigs, using IHC, to ensure specificity.

For IHC and MIF, OCT-embedded tissue samples were cryosectioned onto electrostatically charged glass slides and fixed for 10 minutes in acetone at –20 °C. Slides were blocked for 2 hours at 20 °C with Phosphate buffered saline with tween (PBST) containing 6% mixed serum and 2% powdered non-fat milk. A mouse monoclonal primary antibody 1D9, targeting the ASFV VP30 (Cuesta-Geijo et al., 2012; Galindo et al., 2012) (kindly provided by Javier Dominguez Juncal, Departamento de Biotecnología, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)), was diluted 1:250 in blocking buffer and applied to

tissue sections that were then incubated for 20 hours at 4 °C.

For IHC, specific anti-ASFV immunoreactivity was detected using a commercial micropolymer alkaline phosphatase detection system (Mach 3 AP kit; Biocare, CA) as per manufacturers' recommendation with an alkaline phosphatase substrate (Vector Red; Vector Laboratories, CA). Slides were counterstained with Gill's hematoxylin and cover slipped using routine methods. A duplicate serial section of each tissue screened was treated with a mouse monoclonal anti-foot and mouth disease virus (FMDV) antibody (10GA4, targeting FMDV O1-Brugge (Arzt et al., 2009) serving as a negative control.

Multichannel immunofluorescence was performed similarly as described for IHC, except ASFV antigen detection was performed in combination with primary antibodies with specificity for host cellular markers including pancytokeratin (180059, Zymed, Invitrogen, NY) for epithelium, CD163 and CD172a for macrophages (MCA 2311, and MCA2312GA, respectively AbD Serotec UK). After incubation of primary antibodies for 20 hours at 4 °C, isotype-specific secondary antibodies labeled with fluorescent dyes (AF 350, 488, 594, and 647, AlexaFluor; Molecular Probes Inc., OR) were applied and slides were incubated for 1 hour at 37 °C. Images were obtained using a Nikon Eclipse 90i multichannel epi-fluorescent microscope equipped with 350, 488, 594, and 647 excitation filter cubes and a digital camera.

Statistical analyses

All statistical analyses were performed using R statistical program (R core Team, 2013), employing different R packages (explained in detail below). Statistical significance was considered as $p<0.05$. All data reported herein are not statistically significant unless explicitly stated.

Survival analysis

Duration of survival for each pig was calculated as the total number of days from inoculation/exposure to natural death or euthanasia due to moribundity. Survival functions were calculated for pigs belonging to the IM, INP, IOP and DC groups and compared through a Cox proportional hazard regression model using the package "Survival" for R software (Therneau, 2013). Multiple comparisons were made: 1) between inoculation/infection routes (IM, INP, IOP and DC), 2) within inoculated groups (IM, INP and IOP) comparing type of inoculation route and dose, 3) within each dose (comparing different inoculation routes), 4) within each route (comparing doses), 5) within the DC pigs (comparing exposure length). Survival was not calculated for donor pigs (IOP donor group) from the contact experiment since these pigs were euthanized at predetermined times regardless of clinical disease.

Clinical signs, viremia and shedding

For all clinical and virological parameters examined, the mean +/- standard deviation (SD) for each infection group/dose combination was calculated using data values collected per individual animal per time point. The effect of the inoculation/infection route used to establish infection was compared across groups (both within the direct inoculation studies and between direct inoculation and contact studies) for the outcome variables present in Tables 2 and 3. These comparisons were made using regression analysis, using a linear model, considering the 4 types of inoculation/infection route as categorical explanatory variables: intramuscular (IM), intranasopharyngeal (INP), intraoropharyngeal (IOP) and direct contact (DC). A linear model was also used to assess the effects of different exposure levels applied in the pigs infected by direct contact, with exposure as explanatory variable (with 3 exposure levels - 1 day, 2 days and 3 days) and variables present in Tables 2 and 3 as outcome. Linear model assumptions were

confirmed through visual inspection of the residual plots. The statistical power of these experiments was analyzed retrospectively using the package "pwr" (Champely, 2012). The study did not have enough power to test for possible interactions of type of inoculation route and dose used for inoculation. The power of the analysis using only the explanatory variable type of inoculation/infection route (determining differences between inoculation/infection groups) varied from 0.58 to 0.31, depending on the number of missing values.

Results

Direct inoculation studies

Inoculation and infectivity

Successful infection of individual pigs was determined by the presence of characteristic clinical signs combined with detection of ASFV within the blood components (viremia). In the IM group, all animals regardless of dose were successfully infected (2/2 high dose, 2/2 mid dose, and 2/2 low dose (100% incidence)). In the INP and IOP groups, incidence was 100% within the mid (2/2) and high dose (2/2) groups; however, amongst low dose animals, 1/2 pigs in the INP group and the entire (0/2) IOP group did not become infected (low dose incidence = 50%, 0% respectively).

Survival

The IM-inoculated pigs had the shortest overall survival (mean 8.5 ±1.8 dpi, n=6) (Table 2, Figure 1A, Figure 2), however, no statistically significant differences were found between the IM, INP and IOP groups in the Cox proportional hazard analysis. IM-inoculated pigs also had the shortest survival following onset of pyrexia (mean 4.3± 1.9 days, n=6). Mean survival after

pyrexia was longest within the low dose group (6± 0.0 days, n=2) and equal within the mid and high dose groups (3.5± 2.1 days, n=2 per group). Amongst the INP-inoculated pigs, overall mean survival was 10.3 ±1.9 dpi with 5.5 ± 1.8 days survival following the onset of pyrexia. Survival was shortest within the high dose group (9.0± 2.8 days, n=2) compared to the mid and low dose groups (11.0± 1.4 days, n=2 per group). Overall survival within the intraoropharyngeal groups was the longest compared to all other routes with a mean of 10.5 ±2.4 dpi (n=6). Survival following onset of pyrexia was 4.5 ± 2.1 days; however, the number of days to onset of pyrexia was the longest (5.8 ± 1.3 dpi).

The dose used for inoculation had a significant effect on the pig's survival, with pigs inoculated with the low dose (10^2 HAD_{50} ASFV) having a significantly longer survival (p=0.02) than pigs inoculated with a higher dose (10^6 HAD_{50} ASFV). However, when groups were compared within the same dose (i.e. across route), there were no statistical differences between the different inoculation routes.

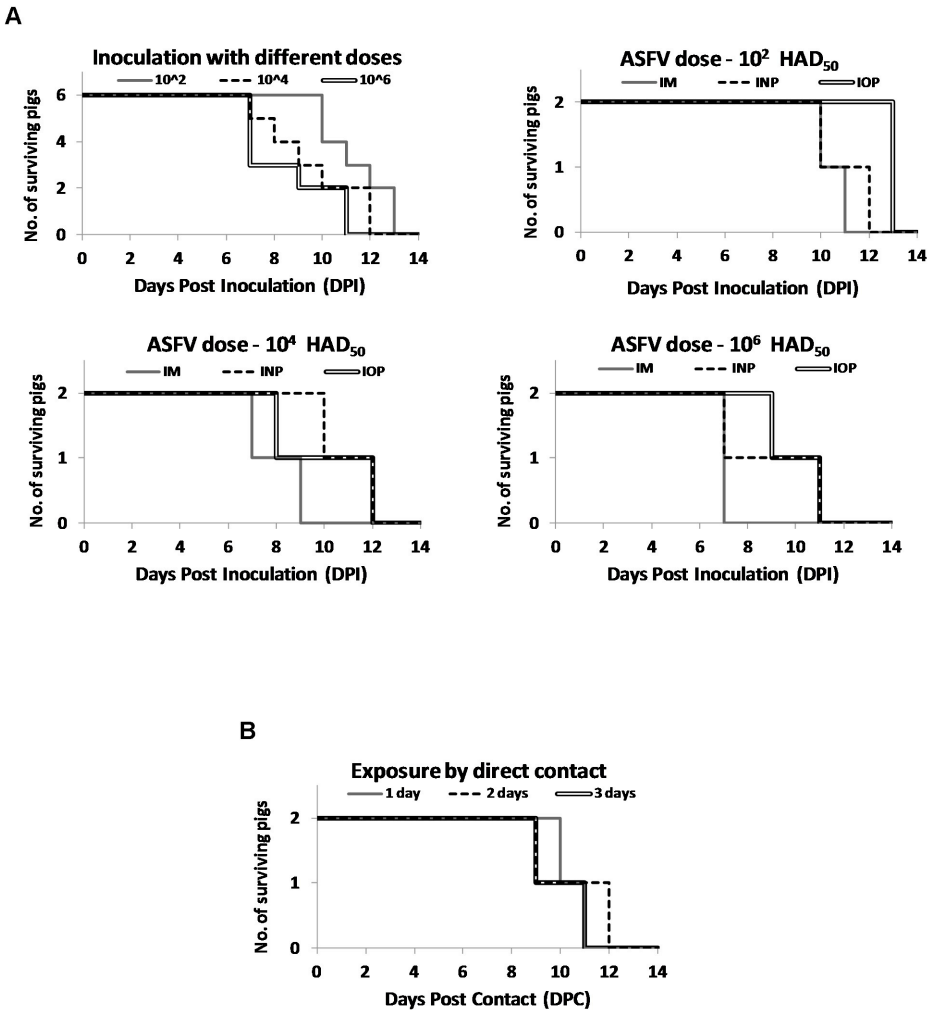

Figure 1. Descriptive survival analyses for ASFV-exposed pigs. Survival analyses relating dose/exposure length to survival duration within the direct inoculation studies (A) and direct contact study (B).

Table 2. Clinical characteristics of pigs infected with ASFV by different routes and dosages

Group		No. infected/ total no.	Total days survival (±SD)	Pyrexia	Clinical signs	
				Days to onset (±SD)	Days to onset (±SD)	Maximum clinical score (±SD)
IM	**Overall**	6/6	8.5 (±1.8)[c]	5.2 (±1.2)[c]	4.5 (±1.2)[c]	6.3 (±1.4)[c]
	10^2 (n=2)	2/2	10.5 (±0.3)	5.5 (±0.7)	5.5 (±0.7)	7.0 (±1.4)
	10^4 (n=2)	2/2	8.0 (±1.4)	5.5 (±0.7)	5.0 (±0.0)	6.5 (±2.1)
	10^6 (n=2)	2/2	7.0 (±0.0)	4.5 (±2.1)	3.0 (±0.0)	5.5 (±0.7)
INP	**Overall**	5/6	10.3 (±1.9)[c]	5.0 (±2.7)[c]	2.6 (±2.5)[c]	6.2 (±2.8)[c]
	10^2 (n=2)	1/2	11.0 (±1.4)	7.0 (±0.0)[a]	7.0 (±0.0)[a]	9.0 (±0.0)[a]
	10^4 (n=2)	2/2	11.0 (±1.4)	7.0 (±0.0)	1.5 (±0.7)	5.0 (±1.4)
	10^6 (n=2)	2/2	9.0 (±2.8)	2.0 (±0.0)	1.5 (±0.7)	6.0 (±4.2)
IOP	**Overall**	4/6	10.5 (±2.4)[c]	5.8 (±1.3)[c]	4.3 (±2.4)[c]	4.8 (±4.0)[c]
	10^2 (n=2)	0/2	13.0 (±0.0)	–	–	–
	10^4 (n=2)	2/2	10.0 (±2.8)	6.5 (±0.7)	3.5 (±3.5)	6.5 (±5.0)
	10^6 (n=2)	2/2	8.5 (±0.7)	5.0 (±1.4)	5.0 (±1.4)	3.0 (±0.0)
DC	**Overall**	6/6	10.3 (±1.2)[c]	5. (±1.8)[c]	5.0 (±1.9)[c]	6.8 (±2.0)[c]
	1dpc (n=2)	2/2	10.5 (±0.7)	5.0 (±0.0)	4.5 (±0.7)	7.5 (±0.7)
	2dpc (n=2)	2/2	10.5 (±2.1)	3.5 (±2.1)	3.5 (±2.1)	8.0 (±1.4)
	3dpc (n=2)	2/2	10.0 (±1.4)	7.0 (±0.0)	7.0 (±0.0)	5.0 (±2.8)
IOP DONOR	**Overall**	4/6	–[b]	3.6 (±0.6)	4.0 (±1.7)	4.0 (±2.5)

[a] One animal affected within group. [b] Survival not calculated due to predetermined euthanasia. [c] No statistical difference between group overall values (IM, INP, IOP and DC). ⁻ Not detected. [SD] Standard deviation.

Clinical signs

For all route-dose combinations, the most consistent and commonly earliest detected clinical sign was pyrexia ranging from 40 - 41.9 ºC. Diarrhea, hematochezia and melena were common in all groups during the late stages of infection, except the contact experiment donors. Less consistent clinical signs included obtundation, oculonasal discharge, coughing, and dermal hyperemia which were often transient and variable throughout all groups. Terminal stage ASF was characterized by moribundity including a rapid and substantial decrease in body temperature, lack of response to stimulus, inability to stand or ambulate, and occasionally, seizure-like behavior. There were no statistically significant differences ($p>0.05$) between the IM, INP, IOP and DC routes for total days of survival, days to onset of pyrexia and clinical signs, and maximum clinical score (Table 2).

Amongst the IM-inoculated pigs, the onset of clinical signs was shortest within the high dose group (Table 2, Figure 2) including pyrexia (4.5± 2.1 days) and obtundation (6.0± 0.0 days) which occurred in all pigs (n=6). Melena/hematochezia was observed in one animal from the mid and high dose groups, with the shortest onset (5.0± 0.0 days) within the high dose. The overall highest mean clinical score was 6.3± 1.4 with the highest mean clinical score observed within the low dose group (n=2). The IM-inoculated pigs, had significantly earlier onset of obtundation and onset of melena/hematochezia ($p<0.05$) in comparison to every group, or in comparison to the DC and INP routes individually.

The lowest overall number of days to the onset of clinical signs was amongst the INP-inoculated pigs (mean 2.6 ±2.5 dpi, 5/6 pigs). However, onset within the low dose group was substantially longer (7 days) than the mid and high dose groups (1.5± 0.7 days) (Figure 2D-F). The lowest overall number of days to onset of pyrexia was amongst the INP-inoculated pigs

(mean 5.0 ± 2.7 dpi, n=2) with the high dose group having the shortest onset (2.0± 0.0 days, n=2) compared to the low (1/2 pigs) and mid dose (2/2 pigs) groups (7.0± 0.0 days). Obtundation and melena/hematochezia occurred in 4 out of 6 pigs with respective means to onset of 9.5 ±1.0 and 10.0± 0.8 days. The mean maximum clinical score amongst the INP-inoculated pigs was 6.2± 2.8 with the highest clinical score occurring within the low dose group (9.0± 0.0).

The mean maximum clinical score for the IOP groups was 4.8± 4.0 (4/6 pigs) with the lowest clinical score found within the high dose group. Clinical signs consistent with ASF were not observed in pigs from the low dose group. Onset of pyrexia occurred within the mid and high dose groups at 6.5 ± 0.7 and 5.0 ±1.4 days respectively (n=2 per group). Obtundation and melena/hematochezia were detected only in one mid dose IOP-inoculated animal starting at 10 and 11 dpi respectively.

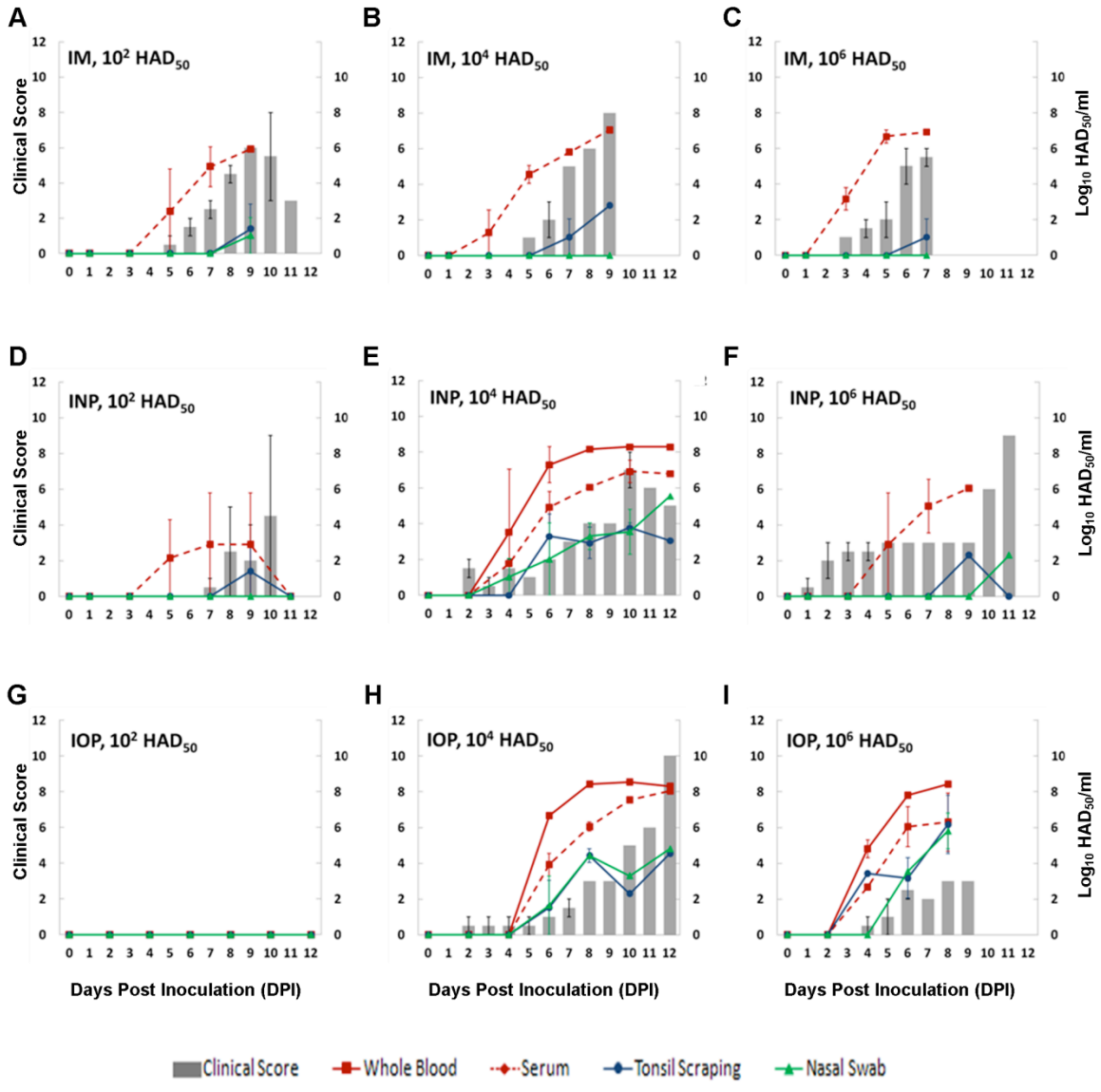

Figure 2. Relationship between clinical score and virus shedding in pigs inoculated IM (A-C), INP (D-F), and IOP (G-I) with ASFV Malawi at 10^2, 10^4 and 10^6 HAD_{50}. Lines indicating viral titer in whole blood, serum, tonsil swabs and nasal swabs are expressed on the right Y-axis. The daily clinical score (bars) are expressed on the left Y-axis

Viremia and ASFV shedding

The IM-inoculated pigs (n=6) had the shortest overall period to onset of viremia (mean 4.3± 1.6 dpi) and number of days of viremia (5.2 ±1.3 days) (Table 3). Amongst the IM-inoculated pigs, the low dose group had both the longest time before onset and longest duration of viremia (Figure 2A, Table 3). The highest mean titer of ASFV in serum was detected within the high dose group (6.9 ±0.2 $log_{10}HAD_{50}$/mL). Onset to detection of ASFV from the tonsil was shortest within the high dose group. Nasal shedding was only detected in 1 pig within the low dose IM group which occurred at 9 dpi. There was an overall mean of 4.0 ±1.6 days from onset of viremia to detection of ASFV in secretions. IM-inoculated pigs had significantly different results in comparison with pigs infected by DC. Specifically these pigs had longer days to onset of tonsil shedding, and lower maximum ASFV titers in tonsil and nasal swabs (Table 3).
The overall number of days to onset of viremia was the longest in the INP groups at 5.4 ±1.1 dpi (5/6 pigs). The number of days of viremia averaged 5.6 ±1.7 days with the high dose group having the shortest length of viremia (Figure 2C, Table 3). The overall mean maximum serum/whole blood titer was 7.0± 1.2 $log_{10}HAD_{50}$/mL. Tonsillar ASFV was detected in 4 out of 6 INP-inoculated animals with a mean onset of 7.5 ± 1.7 days and a max titer of 3.3± 1.0 $log_{10}HAD_{50}$/mL. Nasal shedding was detected in 3 out of 6 animals with a mean onset of 7.7 ±3.5 dpi and a max titer of 4.2 ±1.7 $log_{10}HAD_{50}$/mL. The mean difference between the onset of viremia and onset of ASFV detection in secretions was 1.5± 1.9 days. In comparison to DC-exposed pigs, the INP-inoculated pigs had significantly longer days to onset of tonsil shedding (for INP), and significantly lower maximum titers in tonsil.

Within the IOP groups, overall onset of viremia was detected at 5.0 ±1.2 dpi with overall duration of viremia for 5.3 ± 1.7 days for 4 out of 6 pigs inoculated (Table 3). The high dose

group had both the shortest onset to viremia and shortest duration. The mean maximum titer from whole blood was 8.5 ±0.1 $log_{10}HAD_{50}$/mL. Tonsil and nasal shedding was detected within all infected animals (4/6) with a mean onset of shedding at 5.5± 1.9 and 6.5 ±1.0 dpi respectively. The mean maximum titers for tonsil and nasal swabs for IOP-inoculated pigs were 5.3±1.7 and 5.2 ±1.1 $log_{10}HAD_{50}$/mL. The period between onset of viremia and viral shedding was the shortest of all routes at 0.5±1.2 days. There were no statistical significant differences in any of the outcome variables between the IOP and the DC groups.

Table 3. Virologic parameters of pigs infected with ASFV by different routes and dosages

Group		No. infected/ total no.	Viremia			Shedding			
			Days to onset (±SD)	Duration (±SD)	Maximum Titer (±SD)[bcg]	Days to onset of tonsil shedding (±SD)	Maximum Titer (±SD)[b]	Days to onset of nasal shedding (±SD)	Maximum Titer (±SD)[b]
IM	**Overall**	6/6	4.3 (±1.6)[e]	5.2 (±1.3)[e]	6.5 (±0.6)[c]	8.0 (±1.2)[e]	2.4 (±0.4)[e]	9.0 (±0.0)[ae]	2.1 (±0.0)[ae]
	10^2 (n=2)	2/2	6.0 (±1.4)	5.5 (±0.7)	6.1 (±0.0)[c]	9.0 (±0.0)[a]	2.8 (±0.0)[a]	9.0 (±0.0)[a]	2.1 (±0.0)[a]
	10^4 (n=2)	2/2	4.0 (±1.4)	5.0 (±2.8)	6.4 (±0.9)[c]	8.0 (±1.4)	2.4 (±0.5)	–	–
	10^6 (n=2)	2/2	3.0 (±0.0)	5.0 (±0.0)	6.9 (±0.2)[c]	7.0 (±0.0)[a]	2.1 (±0.0)[a]	–	–
INP	**Overall**	5/6	5.4 (±1.1)[e]	5.6 (±1.7)[e]	7.0 (±1.2)	7.5 (±1.7)[e]	3.3 (±1.0)[e]	7.7 (±3.5)[e]	4.2 (±1.7)[ef]
	10^2 (n=2)	1/2	5.0 (±0.0)[a]	6.0 (±0.0)[a]	5.8 (±0.0)[a]	9.0 (±0.0)[a]	2.8 (±0.0)[a]	–	–
	10^4 (n=2)	2/2	5.0 (±1.4)	7.0 (±0.0)	8.3 (±0.0)[c]	6.0 (±0.0)	4.0 (±0.7)	6.0 (±2.8)	5.2 (±0.5)
	10^6 (n=2)	2/2	6.0 (±1.4)	4.0 (±1.4)	6.3 (±0.4)	9.0 (±0.0)[a]	2.3 (±0.0)[a]	11.0 (±0.0)[a]	2.3 (±0.0)[a]
IOP	**Overall**	4/6	5.0 (±1.2)[e]	5.3(±1.7)[e]	8.5 (±0.1)	5.5 (±1.9)[ef]	5.3 (±1.7)[f]	6.5 (±1.0)[e]	5.2 (±1.1)[f]
	10^2 (n=2)	0/2	–	–	–	–	–	–	–
	10^4 (n=2)	2/2	6.0 (±0.0)	5.0 (±2.8)	8.6 (±0.0)	7.0 (±1.4)	4.4 (±0.5)	7.0 (±1.4)	4.7 (±0.2)
	10^6 (n=2)	2/2	4.0 (±0.0)	5.5 (±0.7)	8.4 (±0.2)	4.0 (±0.0)	6.2 (±2.3)	6.0 (±0.0)	5.8 (±1.4)
DC	**Overall**	6/6	5.2 (±1.5)[e]	6.2 (±1.7)[e]	8.8 (±0.3)	3.5 (±2.4)[f]	5.2 (±0.8)[f]	7.7 (±0.6)[e]	4.7 (±0.1)[f]
	1dpc (n=2)	2/2	4.0 (±1.4)	7.5 (±2.1)	8.7 (±0.2)	1.5 (±0.7)	4.7 (±0.5)	8.0 (±0.0)	4.6 (±0.4)
	2dpc (n=2)	2/2	5.0 (±1.4)	6.5 (±0.7)	8.8 (±0.4)	4.0 (±2.8)	5.8 (±0.0)	7.5 (±0.7)	4.7 (±0.2)
	3dpc (n=2)	2/2	6.5 (±0.7)	4.5 (±0.7)	8.9 (±0.5)	5.0 (±2.8)	5.2 (±1.2)	7.5 (±0.7)	4.8 (±0.0)
IOP DONOR	**Overall**	4/6	4.8 (±2.2)	–[d]	7.4 (±3.2)	2.8 (±1.5)	4.1 (±1.2)	6.5 (±1.3)	4.6 (±1.3)

[a] One animal affected within group. [b] Log10HAD50/mL. [c] All titers derived from whole blood except [c] below (serum derived). [d] Duration of viremia not calculated due to predetermined euthanasia. Group overall values (IM, INP, IOP and DC) with a common superscript ([e] or [f]) are not significantly different ($p>0.05$). [g] No statistical differences were calculated for this group. [–] Not detected. [SD] Standard deviation

Direct contact experiment

Inoculation and infectivity

For the IOP-inoculated donor pigs from the contact experiment, the overall incidence of ASFV infection was 66% (4/6 pigs), identical to the incidence in IOP-inoculated animals from the direct inoculation studies. Within DC groups, all animals, regardless of exposure time, were successfully infected (incidence = 100%) (Table 2).

Survival

All donor animals were euthanized between 7-9 dpi in accordance with the predetermined scheme to maintain 1:1 ratio with contact pigs. Naïve pigs were exposed to IOP-inoculated donors for 1, 2, or 3 days. Overall mean survival for all three exposure groups (n=6) was 10.3±1.2 dpc with the longest onset of survival following onset of pyrexia (5.9± 2.0 days) compared to direct infection routes (Table 2, Figure 1B). Animal exposed to donors for 3 dpc had the shortest overall survival compared to the 1 dpc and 2 dpc-exposure groups. However, there were no statistical differences in the survival according to the Cox proportional analysis between different exposure durations.

Clinical signs

Among the IOP-inoculated donors, the mean number of days to onset of pyrexia was 3.6 ± 0.6 dpi. The mean duration to onset of clinical signs was 4.0± 1.7 dpi (Table 2). Two animals were pyrexic for 1-2 days but their temperature returned to within normal limits before euthanasia. These two pigs did not become viremic nor was shedding detected. One pig was not pyrexic by the time of euthanasia, but was viremic and shed virus nasally on the day of euthanasia (8 dpi). Four out of six pigs were obtunded with a mean onset of 4.0 ±2.5 dpi. Donor animals did not have melena or hematochezia.

Amongst the DC-exposed pigs, onset of clinical signs began with pyrexia that was detected first within the 2dpc-exposure group (3.5± 2.1 dpc, n=2) followed by the 1dpc-exposure (4.5± 0.7 dpc, n=2) and 3dpc-exposure (7.0± 0.0 dpc, n=2) groups. Obtundation was detected within 5 out of 6 pigs with a mean onset of 10.6± 1.1 dpc. Melena/hematochezia was detected in 2 of 6 pigs with a mean onset of 11.5± 0.7 dpc. The DC-exposed pigs had the highest overall maximum clinical score (6.8± 2.0); with the highest score in the 2dpc group followed by the 1dpc and 3dpc groups.

Viremia and ASFV shedding

The mean detection of onset of viremia occurred within 4 out of 6 of the donor group at 4.8± 2.2 dpi with the mean maximum whole blood titer of 7.4± 3.2 $log_{10}HAD_{50}$/mL. The onset of tonsil shedding was detected prior to viremia in 2 pigs. Onset of tonsil and nasal shedding was detected at 2.8± 1.5 and 6.5± 1.3 dpi respectively. Maximum titers for tonsil and nasal swabs were 4.1± 1.2 and 4.6± 1.3 $log_{10}HAD_{50}$/mL respectively (Figure 3A, Table 3). The difference between onset of viremia and shedding averaged 1.3± 0.6 days.

Amongst contact-exposed pigs, mean onset of viremia was 5.2± 1.5 dpc and was detected first within the 1dpc-exposure group (4.0 ±1.4 dpc, n=2) followed by the 2dpc and 3dpc-exposure groups (5.0 ±1.4 and 6.5 ±0.7 dpc, n=2 per group). The overall mean highest whole blood titer was 8.8 ±0.3 $log_{10}HAD_{50}$/mL, which was the highest amongst all routes (Table 3). Tonsillar detection of ASFV occurred prior to viremia in 4 pigs. The mean onset of tonsil and nasal shedding was detected at 3.5± 2.4 and 7.7± 0.6 dpc respectively, with mean maximum titers of 5.2 ± 0.8 and 4.7± 0.1 $log_{10}HAD_{50}$/mL. The difference between onset of viremia and shedding averaged 1.7± 1.7 days.

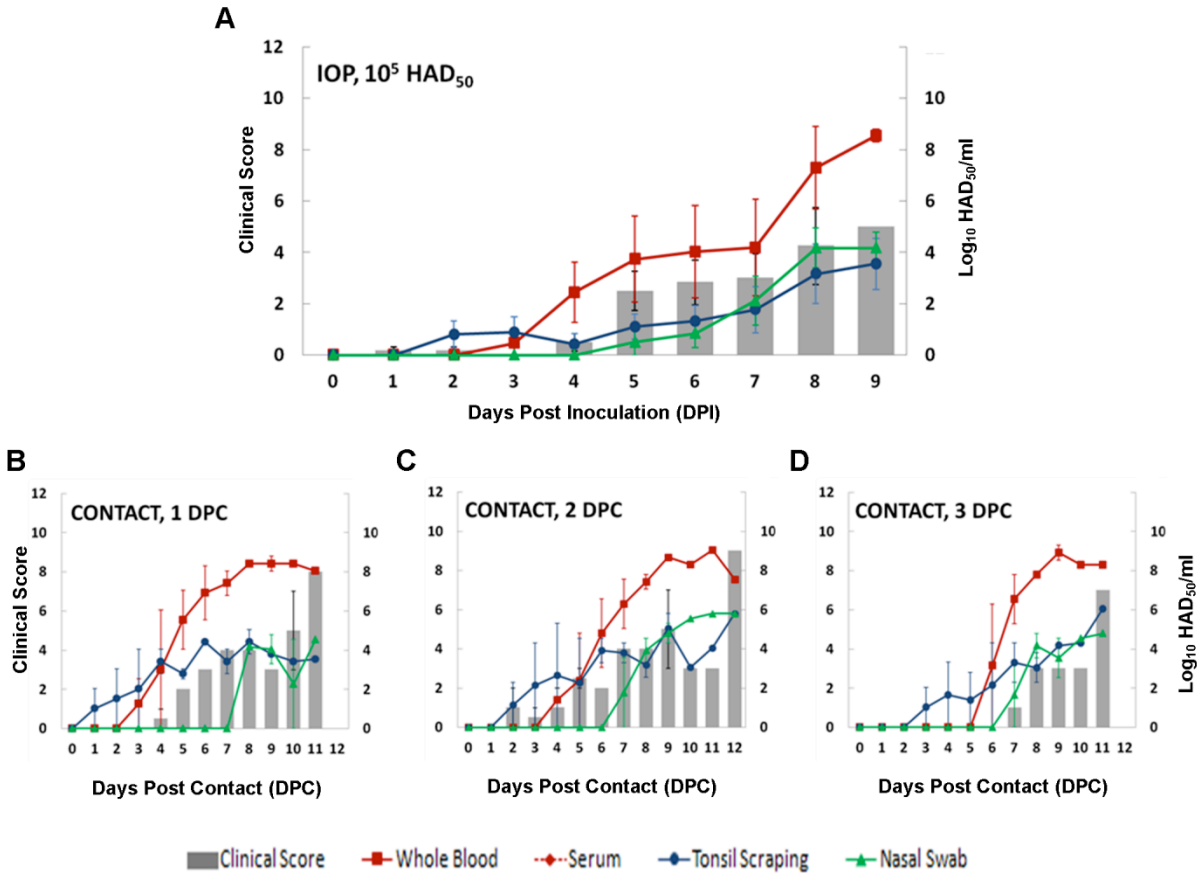

Figure 3. Relationship between clinical score and virus shedding in donor pigs inoculated IOP (A) with ASFV Malawi at 10^5 HAD_{50} and pigs exposed via Direct Contact (DC) for 24 (B), 48 (C) and 72 hours (D). Lines indicating viral titer n whole blood, serum, tonsil swabs and nasal swabs are expressed on the right Y-axis. The daily clinical score (bars) are expressed on the left Y-axis.

Postmortem lesions

All four routes produced comparable gross and histologic lesions in pigs at terminal stages of disease. The most consistent lesion amongst pigs was enlarged and often hemorrhagic lymph nodes. The most severe lymphadenomegaly commonly involved the gastrohepatic lymph

nodes. Splenomegaly ranged from mild to severe enlargement. Renal lesions varied from mild cortical petechia to renomegaly with diffuse hemorrhage and congestion.

Histologically, lymphoid organs of all animals examined had perifollicular regions expanded by cellular debris, extensive hemorrhage, and distinct fragmented nuclear remnants, most consistent with lymphocyte apoptosis (Figure 4A and 4B). Lymphoid follicles were still identifiable but less distinct compared to normal lymphoid architecture in naïve pigs.

Immunohistochemical localization of ASFV antigens

Examination of the distribution of virus and its association with specific cellular markers was performed on tissues collected from pigs of each inoculation route. Within lymphoid tissues of pigs with fulminant ASF, there was extensive cell-associated labeling with the anti-ASFV-VP30 antibody. Localization was predominately within interfollicular regions with few immunopositive cells within follicles (Figure 4C). Within the tonsil of the soft palate, labeling was most prominent surrounding tonsillar crypts, with few labeled cells within the tonsillar crypt epithelium. This VP30-positive labeling did not colocalize with pancytokeratin.

Within all positive tissues, immunoreactive cells were morphologically consistent with large mononuclear cells (interpreted as macrophages). Combined multichannel immnuofluorescence demonstrated that cells immunopositive for ASFV were also commonly positive for monocyte/macrophage cellular markers such as CD163 (Figure 4D-G) and CD172a (not shown).

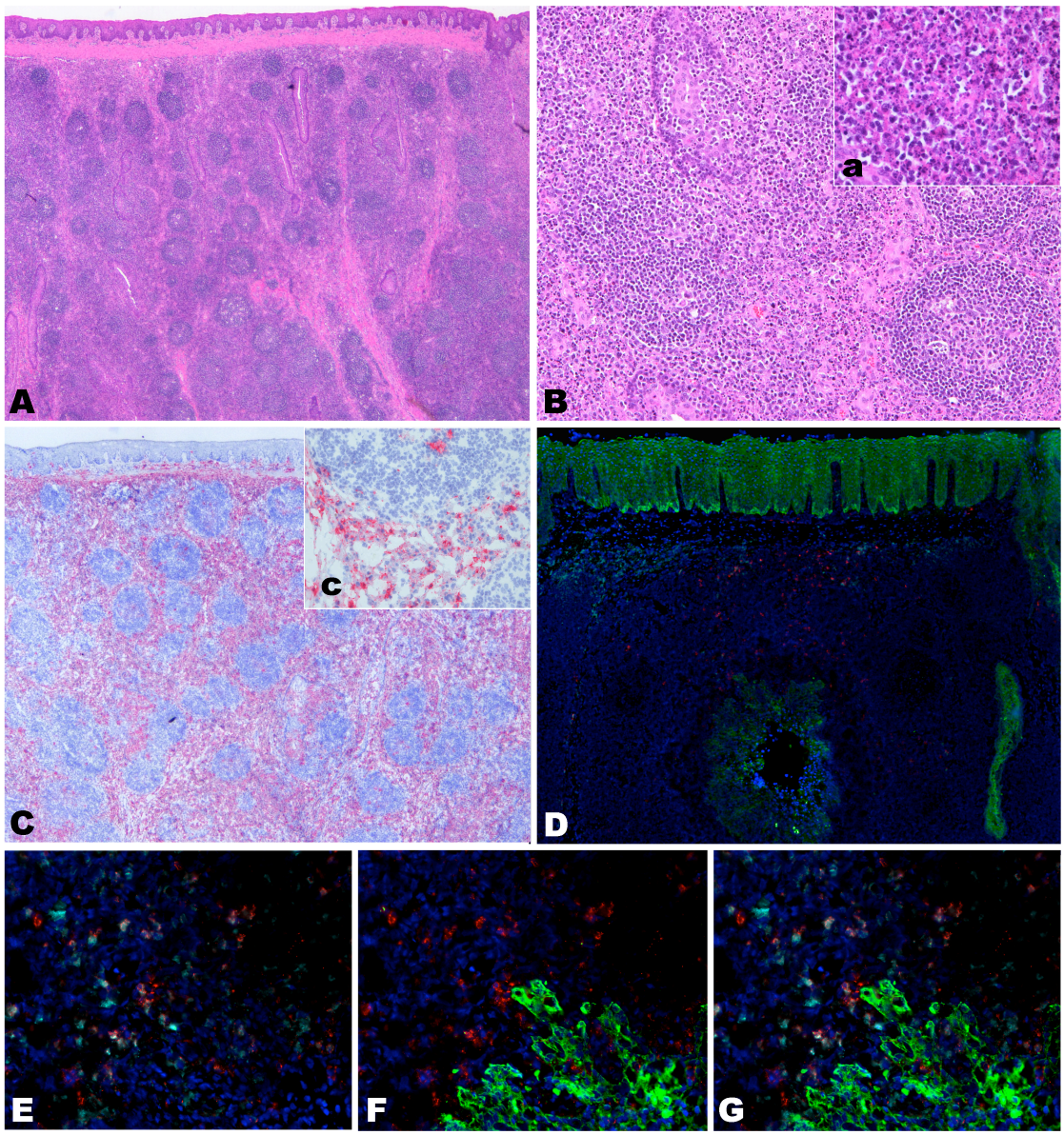

Figure 4. Histopathology of lesions from pigs infected with ASFV- Malawi. Palatine tonsil, animal #38, INP ASFV-Malawi 10^2 HAD50/mL; Lymphoid tissues including tonsils (A-B), lymph nodes, and spleen displayed loss of lymphocytes and karyorrhectic debris. Scale bars: A 500um, B 100um Immunohistochemical staining (C) via Alkaline phosphatase polymer kit with anti-ASFV VP30 (1D9). Positive labeling extensively throughout interfollicular regions. Scale

Figure 4. (cont.) bar 500um. Inset (c), region of interest at higher magnification. Multichannel immunofluorescence (D-G). D. Low magnification four channel image containing Anti-ASFV (red), pancytokeratin (green), macrophage marker CD163 (aqua) and nuclear staining with DAPI (blue). Scale bar 500um E. Three channel image containing Anti-ASFV (red), leukocyte marker macrophage marker CD163 (aqua) and nuclear staining with DAPI (blue). F. Three channel image containing Anti-ASFV (red), pancytokeratin (green), and nuclear staining with DAPI (blue). G. Four channel merged image with colocalization of Anti-ASFV (red) and leukocyte marker macrophage marker CD163 (aqua), pancytokeratin (green), and nuclear staining with DAPI (blue) Scale bar E-G 100um.

Discussion

The goal of this investigation was to evaluate dose- and route-dependent effects on the clinical course of ASF and viral dynamics of ASFV within domestic pigs. This work serves to clarify, and compare and contrast previous conclusions derived from a collection of pathogenesis studies whose work is not directly comparable due to variability of virus strain, dose, inoculation technique, and detection methods. In addition, this work contributes to the establishment of challenge model systems for ASF which closely simulate natural infection. The goals of the model systems were to achieve modes of challenging pigs that were 1) consistent with previous accounts of ASF, 2) consistent across experimental subjects, 3) compatible with the current understanding of natural routes of direct pig-to-pig transmission, 4) allowed control of dose and timing of challenge, and 5) provided natural engagement of host immune defenses. Recent studies have led towards standardization of clinical scoring systems for ASF (de Carvalho Ferreira et al., 2012; Galindo-Cardiel et al., 2013), and the current work demonstrates adaptation of these systems where appropriate.

Four routes of challenge (IM, IOP, INP, DC) resulted in a similar clinical course of ASF that was consistent with previously described field cases (Ayoade and Adeyemi, 2003; Detray, 1963; Sánchez Botija, 1982) and experimental models (Boulanger et al., 1967a; Colgrove et al., 1969; Greig, 1972; Greig and Plowright, 1970; Heuschele, 1967; McVicar, 1984; Mebus and Dardiri, 1979, 1980; Mebus et al., 1978; Plowright et al., 1968; Wilkinson and Donaldson, 1977; Wilkinson et al., 1977; Wilkinson et al., 1980). Clinical signs did not vary substantially according to route of inoculation. The greatest consistency was achieved similarly by IM and DC challenge which both had 100% incidence of infection. However, these systems suffer from unnatural delivery of virus (IM) and inability to precisely control dose and timing of challenge (DC). All pigs challenged via DC became infected with ASFV regardless of length of exposure, thereby demonstrating that only 24 hours of exposure to shedding animals is required for infection with ASFV. This finding is consistent with the previous works that demonstrated transmission after 24 hours (Greig and Plowright, 1970) or 6 hours (Ekue et al. 1989) of exposure to donor pigs. However, other studies have concluded that the quantity of virus shed 24 hours following pyrexia was inadequate to ensure infection in pigs exposed via direct contact (Greig and Plowright, 1970; Plowright et al., 1968). INP inoculation had higher incidence whilst maintaining a natural exposure route and allowing control of dose and timing of challenge. Additionally, the shortest time to onset of clinical signs was observed within the INP groups, however this difference was not statistically significant.

Precise determination of pig infectious dose 50% ([PID_{50}], i.e. the minimum dose necessary to infected 50% of pigs) for the distinct inoculation routes was not possible due to logistical constraints, which dictated some aspects of experimental design. However, the data herein suggests that the required dose of ASFV to establish infection is lowest for IM,

intermediate for INP, and highest for IOP delivery of ASFV to domestic swine. Specifically, low dose inoculation (10^2HAD$_{50}$) incidence proportions were 100% (IM), 50% (INP), and 0% (IOP). Yet, at 10^4 HAD$_{50}$ all routes had 100% incidence of infection. This is consistent with previous studies that have demonstrated low ASFV IM dose (lower than 10^5 TCID$_{50}$) (Pan et al., 1984; McVicar et al., 1984) and high IOP dose (PID$_{50}$ higher than 10^3 TCID$_{50}$) (de Carvalho Ferreira et al., 2012; Greig, 1972; McVicar, 1984). Additionally, Maurer et al demonstrated that a minimal infectious dose of 10^5 HAD$_{50}$ was necessary to infect pigs orally with ASFV (Maurer and Griesemer, 1958). The precise manner by which specific routes of inoculation of ASFV correlate with variable PID$_{50}$ via distinct pathogenesis mechanisms remains to be elucidated.

The route-specific clinical and virological characteristics described herein are likely to be the result of distinct route-determined, unique pathogenesis events in early ASFV infection. Pigs inoculated IM had the shortest mean survival duration; however the difference was not statistically significant. Shortest onset to viremia amongst IM-inoculated pigs was statistically significant. This may be due to more direct access to the vascular system associated with deposition within the muscle as compared to contact with intact mucosa as occurred with simulated natural challenge routes. Furthermore, the finding of several statistically significant differences in the viremia and shedding parameters between the intramuscular and the direct contact route suggests that the intramuscular route is a poor simulator of natural infection. As a result, inoculation via IM should be avoided in experimental studies trying to mimic a natural course of disease. In contrast, both the IOP and INP inoculated pigs had comparable (i.e. not significantly different) results compared to the DC exposed pigs suggesting that IOP and INP are more likely to closely simulate natural infection.

The implied superior infectivity of ASFV via INP inoculation as compared to IOP was

reflected in slightly higher incidence proportion and delayed onset of pyrexia and longer survival time seen among INP-inoculated pigs in comparison with all successfully IOP-inoculated pigs (including donors in DC experiment). However, IOP led to significantly higher shedding titers in the tonsil compared with INP inoculation. This effect may have been due to anatomic and physiological factors currently under investigation in our laboratory. IOP inoculation directly deposits the inoculum along the tonsil of the soft palate. While this tonsil has been documented as an early site of viral replication (Heuschele, 1967; Plowright et al., 1968), to our knowledge, specific virus-host interactions in ASFV primary infection *in vivo* have never been explicitly described. Thus, the lower incidence of infection by the IOP route compared to INP-inoculation could be explained by lack of establishment of systemic disease subsequent to primary infection within the tonsil of the soft palate and other oropharyngeal tissues. Since low-dose INP-inoculated pigs had a higher incidence of successful infection than IOP, the nasopharynx may provide more permissive conditions for establishing systemic ASFV infection. INP inoculation targets nasopharyngeal tissues, including nasal tonsil, dorsal soft palate and walls/roof of the nasopharynx. The data presented in this study suggest that sites targeted by INP inoculation, or other tissues in the respiratory tract, may provide important portals for ASFV infection.

Increased inoculation dose of ASFV was associated with a significant decrease in survival duration of pigs. Animals inoculated with 10^6 HAD_{50}, regardless of route, had a decreased mean number of days to onset of clinical signs (pyrexia, obtundation, hematochezia/melena) and survival compared to the mid and low dosage groups. The number of days to onset of viremia and shedding was decreased compared to mid and low doses as well, suggesting that a higher viral dose resulted in overwhelming primary replication and faster systemic dissemination of the virus. Paradoxically, the high-dose pigs had lower mean maximum

clinical scores because of the rapidity of progression to death before the full severity of disease had occurred. Thus, maximum clinical score does not (in itself) describe the severity of disease but must be evaluated in context with duration of survival, virological characteristics, and pathologic findings.

During the clinical phase of infection, ASFV was variably detected in whole blood, serum and tonsil and nasal swabs. Amongst all challenge routes, viremia and shedding commonly either coincided with the onset of pyrexia or it occurred 2 days before or after pyrexia, which is in agreement with other studies (Greig and Plowright, 1970, McVicar, 1984). The DC-exposed pigs had the highest mean ASFV titer within whole blood. Throughout the course of this study whole blood samples had titers of almost 2 $\log_{10}$ higher than serum samples. This is consistent with previous studies that has described greater number of mature virus particles associated with erythrocytes and within circulating monocytes in whole blood, in comparison to free virions within serum (Gómez-Villamandos et al., 2013).

Detection of ASFV within secretions (tonsil scraping and nasal swabs) varied across infection routes and dosage groups. ASFV titers in tonsil scrapings and nasal swabs were generally quite similar, suggesting that virus was released from both regions and/or virus quantities equilibrate between nasal and oral secretions. One noteworthy exception to this trend was amongst DC-exposed pigs wherein ASFV was consistently detected in tonsil scrapings (for 1-6 days) prior to detection in nasal swabs. Across all three inoculation routes, initial detection of ASFV shedding occurred at the same time, or subsequent to, virus detection in whole blood and serum. However, within the contact challenge study, ASFV was detected in tonsil scraping samples 1-4 days prior to detection in blood of IOP-inoculated donors and DC-exposed pigs. This indicates that, under these distinct conditions, it was uniquely possible to detect local viral

replication and shedding within the oral cavity prior to systemic dissemination. This is consistent with previous studies in which ASFV could be detected within oral and pharyngeal swabs 1-2 days prior to viremia and pyrexia (Greig and Plowright, 1970) or as early as 24 hours post inoculation (hpi) in lymphoid and respiratory tract tissues (Heuschele, 1967; Plowright et al., 1968).

Gross and histologic changes in pigs in the late stages of disease were similar regardless of route of infection and were consistent with postmortem findings described in field and experimental cases of ASF. Systemic hemorrhages affecting the kidney and heart and pulmonary congestion and edema are similar to previously described lesions (Detray, 1963). Amongst individual pigs, severity of lesions varied from mild to severe based upon recently published recommendations for standardization of pathologic findings for ASF (Galindo-Cardiel et al., 2013). The hallmark depletion and apoptosis of lymphocytes throughout the lymphoid tissue with subsequent infiltration of macrophages commonly described for ASF (Gómez-Villamandos et al., 2003) was observed histologically within this study.

Immunohistochemical visualization of ASFV antigen was demonstrated in tonsil of the soft palate of pigs in terminal stages of ASF, further confirming the presence and characterizing the distribution of virus within tissues. Immunoreactive cells were large mononuclear cells and colocalization with monocyte/macrophage cellular antigen suggested that the predominate infected cell type is of monocytic origin. Colocalization of ASFV with CD163a supports previous suggestions that this molecule may serve as an important ASFV receptor (Alonso et al., 2013; Sánchez-Torres et al., 2003). Additionally, this is consistent with numerous reports indicating monocytotropism of ASFV (Detray, 1963; Tulman et al., 2009; Wardley and Wilkinson, 1978).

Conclusions

The current study has demonstrated that four modes of challenging pigs with ASFV result in similar ASF syndromes with slight variations. The IM route may be advantageous for the study of late stage ASF events; however, several significant differences from DC indicated this is not an optimal system to simulate natural infection. DC would be preferable when simulating a natural manner of transmission was utmost priority; however, this system is expensive due to the inclusion of additional (donor) animals in each experiment and does not allow control of dose quantity and timing. The two simulated natural manners of inoculation, IOP and INP, allowed precise control of timing and dose of ASFV delivery and had similar incidence of successful infection of pigs. Overall, the slightly lower minimum infectious dose and slightly higher incidence of infection subsequent to INP inoculation in these experiments suggest that INP may be the preferred route of inoculation for pathogenesis and vaccine challenge studies of pigs with ASFV. Further validation of these techniques with larger sample sizes would contribute to stronger conclusions regarding the differences between the infection systems described herein.

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CHAPTER 3:

Tissue and cellular tropism of highly virulent African swine fever virus in domestic pigs

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Abstract

The anatomic sites and cellular characteristics of viral infection and replication in the pathogenesis of African swine fever virus (ASFV) in domestic pigs were investigated through a series of controlled inoculation experiments. Numerous tissues from multiple pigs at various stages of infection were examined by virus isolation (VI), qPCR, and, immunomicroscopy. In viremic animals, abundant ASFV and viral DNA were detected within all tissues examined with the greatest viral loads detected in spleen and bone marrow. Sensitivity of detection was similar by VI and qPCR. Early immunomicroscopic detection of ASFV antigen was achieved at 2 days post infection (dpi) in the spleen of one intramuscular (IM) inoculated pig prior to detectable viremia and at 4 dpi early in the viremic phase within the lungs and gastrohepatic lymph nodes of one intranasopharyngeal (INP)-inoculated pig. In all tissues examined, ASFV antigen was detected with high percentage colocalization with cell markers CD172a and CD163 indicating viral tropism for cells of monocyte/macrophage lineage. In lymphoid tissues, distribution of ASFV was predominantly interfollicular (paracortical). In lungs, viral antigen was localized to $CD172a^{+}$ cells within alveolar septa, suggesting tropism for resident tissue macrophages or pulmonary intravascular macrophages, but not alveolar macrophages. In all tissues with epithelial component, ASFV colocalized with a low proportion of cytokeratin-positive cells indicating weak tropism of the virus for epithelial cells.

Introduction

African Swine Fever (ASF) is a lethal, transboundary disease of wild and domestic suids caused by ASF virus (ASFV). Replication of the virus within cells of the mononuclear phagocytic system (MPS), including circulating monocytes, inflammatory macrophages and resident macrophages, has been thoroughly described (reviewed in (Gómez-Villamandos et al., 2013; Gómez-Villamandos et al., 2003). Although viral replication has been documented principally in the MPS, the mechanisms by which ASFV first enters tissues and cells has not been determined.

The early in vivo pathogenesis of ASFV has been investigated in few comprehensive time-course studies of experimentally infected pigs followed by tissue collection and virus isolation (VI) (Colgrove et al., 1969; Greig, 1972; Heuschele, 1967; Plowright et al., 1968; Wilkinson and Donaldson, 1977). Wilkinson and Donaldson demonstrated recovery of ASFV from the spleen and bronchial lymph node in one pig exposed to ASFV-KWH/12 on the day of indirect contact exposure (Wilkinson and Donaldson, 1977). Colgrove et al. detected ASFV-Hinde WH II at 8 hours post infection (hpi) in the tonsil (tonsil of the soft palate presumably), submandibular and parotid lymph nodes in previremic newborn pigs (Colgrove et al., 1969). The same study demonstrated low titer viremia and isolation of virus at 8 hpi from the lungs, nasopharynx, and medial retropharyngeal lymph nodes in pigs 15 days to 5-9 months old. These studies demonstrate the importance of tonsils, oropharyngeal mucosa, lungs and regional lymph nodes as early replication sites during simulated natural infection and suggest that tissue-specific predilection in early pathogenesis may be dependent on several factors (e.g. mode of inoculation, virus strain, and pig age).

However, several potentially relevant anatomic sites of the upper respiratory and

gastrointestinal tracts were not examined in the studies described above, including the nasopharyngeal tonsil, lingual tonsil, and paraepiglottic tonsil. In fact, most time-course studies examined limited range of tissues; thus, the full representation of relevant infection sites may not be complete.

Cell-specific tropism of ASFV within tissues has been investigated via transmission electron microscopy (TEM)(Carrasco et al., 1997b; Gómez-Villamandos et al., 1997a; Gómez-Villamandos et al., 1997b; Sierra et al., 1990), in-situ hybridization (ISH)(Ballester et al., 2010; Oura et al., 1998b), immunohistochemistry (IHC) (Fernandez et al., 1992a; Fernández de Marco et al., 2007; Gregg et al., 1995; Mìnguez et al., 1988; Perez et al., 1994; Rodriguez et al., 1996a) and immunofluorescence assays (FA)(Boulanger et al., 1967b; Greig, 1972; Heuschele et al., 1966). Using FA, ASFV was detected within the tonsil, submandibular lymph node, bronchial lymph node, and lung at 30 hours post infection, within the spleen and liver at 36 hpi and within all tissues examined by 48 hpi from pigs (Colgrove et al., 1969). Infected cells were described as morphologically consistent with resident macrophages and reticular cells in early pathogenesis, but also included lymphocytes at later stages.

Two studies have used localization of virus in combination with cellular markers to further characterize the infected cells. Oura et al (Oura et al., 1998b) used a combination of ISH and double-marker IHC to detect viral protein, DNA, and RNA in conjunction with a cellular marker for macrophages (mAb C4). In that study, virus was detected in macrophages of the splenic red pulp, liver, gastrohepatic lymph node and tonsil as early as 2 dpi. Minguez et al.(Mìnguez et al., 1988) used double labeling IHC to identify ASFV infected cells within the spleen and lymph nodes (cervical, retropharyngeal, mesenteric and renal) in conjunction with cellular markers for T lymphocytes (CD4 and CD8) and macrophages (CD172). Positive labeling

for ASFV was identified within macrophages; however, not all macrophages contained virus and not all cells containing virus were macrophages. The virus was not found within labeled T lymphocytes.

Several additional studies have performed detailed examination of ASFV interaction with specific tissues including but not limited to tonsil of the soft palate (Fernández de Marco et al., 2007; Gómez-Villamandos et al., 1997b), lymph nodes (Carrasco et al., 1997b; Gregg et al., 1995; Salguero et al., 2002) and spleen (Carrasco et al., 1997a; Salguero et al., 2002); however, variability in inoculation technique, methods of cellular identification and lack of time course examination in some of these works fail to clarify the full role of these tissues/cells in the pathogenesis of ASF.

The goal of the current study was to further our understanding of ASF pathogenesis through examination of the distribution and viral loads of ASFV in numerous tissues of domestic pigs exposed to ASFV by different routes: intramuscular (IM), intranasopharyngeal (INP) and direct contact (DC). Virus localization at different stages of infection was performed by VI, qPCR, and immunomicroscopy, utilizing simultaneous localization of cell markers to characterize cellular phenotypes in infected tissues.

Materials and methods

Virus and animals

Experimental design has been described in detail previously in Chapter 2 (Howey et al., 2013). All animal procedures were performed following a protocol approved by the Plum Island Animal Disease Center Institutional Animal Care and Use Committee (IACUC), which ensured ethical and humane treatment of experimental animals. Briefly, castrated male Yorkshire pigs,

approximately 3-4 months old and 22-27kgs were separated in groups by dose (10^2, 10^4, and 10^6 HAD_{50}) and inoculation route (intramuscular, intranasopharyngeal, and direct contact). Intraoropharyngeal inoculated pigs were sampled but negative via VI and qPCR (Appendix 1). All pigs were inoculated with the highly pathogenic ASFV isolate, Malawi Lil-20/1. Pigs were euthanized at predetermined time points or allowed to progress to fulminant ASF as per predetermined study goals.

Sample collection and processing

All animals were necropsied immediately following euthanasia or natural death. An extensive number of tissues were collected during postmortem examination to ensure thorough inspection of possible sites of infection and included: tonsil of the soft palate, lingual tonsil, nasopharyngeal tonsil, dorsal soft palate (rostral and caudal), dorsal nasopharynx, caudal nasal turbinate, epiglottis, trachea, bronchial mucosa, lung, thymus, liver, spleen, kidney, adrenal gland, thyroid, pancreas, small intestine, large intestine, bone marrow, and lymph nodes including: retropharyngeal, submandibular, hilar, gastrohepatic, renal, inguinal and popliteal. Minor variation of the number of tissues collected was based upon stage of infection.
All tissue samples were thoroughly rinsed with tap water and divided into subsections: three 50 mg sections were placed into individual 2ml screw cap cryovials and frozen in liquid nitrogen; additinal sections were oriented into cryomolds, embedded in Optimal Cutting Temperature (OCT) medium and then frozen in liquid nitrogen. Both cryovials and cryomolds were then stored at -70º C until processed.

Virus isolation and titration

Tissues from infected pigs were screened for ASFV via VI using primary porcine blood macrophage cell cultures prepared from defibrinated swine blood as previously described (Zsak

et al., 2005). Briefly, heparin-treated swine blood was incubated at 37°C for 1 hour to allow sedimentation of the erythrocyte fraction. Mononuclear leukocytes were separated by flotation over a Ficoll-Paque (Pharmacia) density gradient (specific gravity, 1.079). The monocyte/macrophage cell fraction was cultured in plastic Primaria tissue culture flasks (Falcon; Becton Dickinson Labware) for 24 hours. Adherent cells were then detached from the flasks and reseeded into Primaria 96-well dishes at a density of 1x 10^7 cells per ml for use 24 hours later.

Tissue samples were thawed and macerated by tissue lyser bead beater (Qiagen, Valencia, CA). For all samples, serial ten-fold dilutions were created. These dilutions were then inoculated into the macrophage-containing plates with the addition of washed red blood cells from the pig from which the macrophages originated. Cultures were incubated at 37°C and examined daily for rosette formation (hemadsorption). Virus titers were calculated using the Spearman-Karber method (Mahy, 1996) and expressed as $Log_{10}HAD_{50}$/g.

ASFV DNA detection via qPCR

After maceration, 125 µl of each tissue sample was transferred to a 96-well plate (Thermo Scientific, Waltham, MA) containing lysis/binding solution. DNA was extracted using Ambion's MagMax-96 Viral RNA Isolation Kit (Ambion, Austin, TX) on a King Fisher-96 Magnetic Particle Processor (Thermo Scientific, Waltham, MA). Once extracted, 2.5 µl of DNA was analyzed in a total reaction volume of 25µl by quantitative real-time polymerase chain reaction (qPCR) on the ABI 7500 system (Applied Biosystems, Austin, TX) using the AgPath-ID™ One-Step RT-PCR Reagents (Applied biosystems), UPL probe #162 (Roche cat no. 04694490001) and primer set designed based on the DNA sequence of the VP72 coding genome region (Fernández-Pinero et al., 2012). Cycling conditions excluded the 10 minute (50 C) RT

step in order to prevent amplification of RNA but included a 15 minute (95 C) denaturing step linked to a 45-cycle PCR (95 C for 15 seconds and 60 C for 60 seconds) which generated a fluorogenic signal in samples with positive results. Samples with cycle thresholds (C_t) less than 40 were considered positive.

In order to generate a standard curve, serial dilutions (10^{-1}, $10^{-1.5}$, 10^{-2}, $10^{-2.5}$, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}) were generated from a high titer tissue (spleen) sample, previously titrated in porcine macrophage culture with titers expressed in $\log_{10}HAD_{50}$/mg. DNA was extracted using the above method and tested via qPCR to determine the end point dilution at which a positive amplification signal could be detected (Figure 5). The dilution curve was performed and analyzed with each PCR assay performed. These calibration points were used to derive a linear relationship between C_t values and viral titer from which the standard curve was estimated. The formula relating viral titers with DNA concentration was applied to PCR results (C_t values) obtained from each run, and DNA amounts were expressed as C_t titer equivalents (C_tTE).

Statistical differences between results obtained by PCR (C_t titer equivalent) and determined by VI (viral titer) were tested using a Wilcoxon signed rank test (a non-parametric test), comparing two non-normal distributions, with significance being set at $p<0.05$. The level of agreement between the tests was also assessed using a Cohen's kappa coefficient, an agreement statistic (Cohen, 1960).

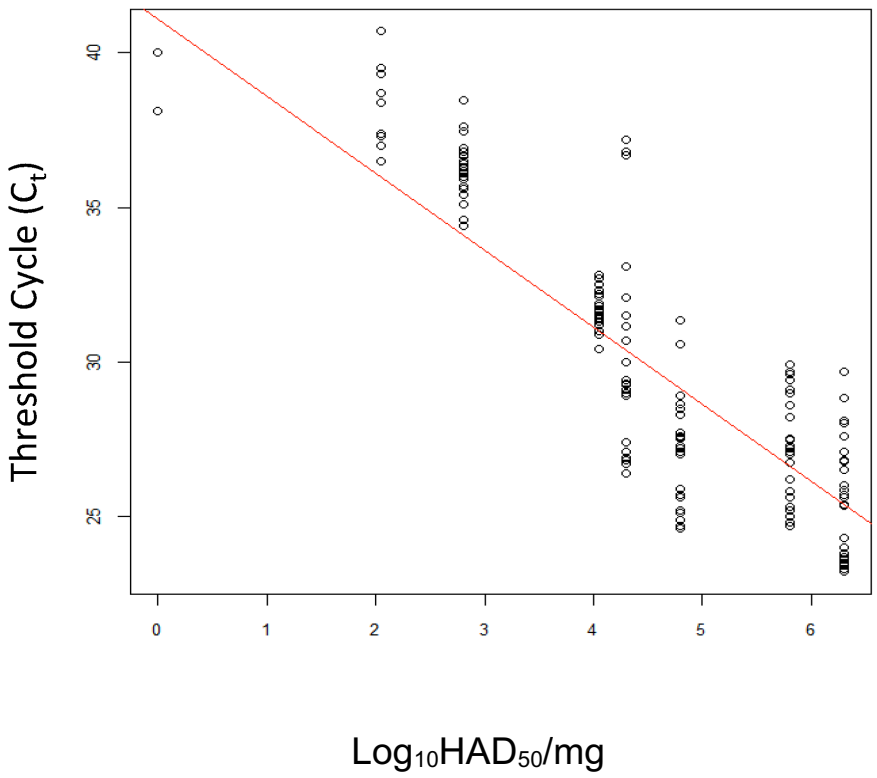

Figure 5. Standard calibration curve for the ASFV qPCR assay. Sensitivity of the ASFV qPCR assay was calculated by using extracted DNA of serial dilutions (10^{-1}, $10^{-1.5}$, 10^{-2}, $10^{-2.5}$, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}) generated from a high titer tissue (spleen) sample, concurrently titrated in porcine macrophage culture with titers expressed in $log_{10}HAD_{50}$/mg. Dilutions were included and analyzed with each PCR assay performed. An inverse linear logarithmic relationship between C_t values and viral titers was determined (r^2= 0.8446).

Immunomicroscopy

Further localization of ASFV in tissues that were positive by VI was performed via immunohistochemistry (IHC). Multichannel immunofluorescence microscopy (MIF) was used to simultaneously detect ASFV in combination with other cellular markers in order to further determine/characterize phenotypes of infected cells.

OCT-embedded tissue samples for IHC and MIF were cryosectioned at 4 µm thickness onto electrostatically charged glass slides, fixed for 10 minutes in acetone at –20 °C, and then air-dried at room temperature. Slides were blocked for 2 hours at 20 °C with PBST containing 6% mixed serum and 2% powdered non-fat milk. A mouse monoclonal primary antibody 1D9, targeting the ASFV VP30 (Cuesta-Geijo et al., 2012; Galindo et al., 2012), was diluted in blocking buffer, and applied to tissue sections that were then incubated for 24 hours at 4 °C.

For IHC, specific anti-ASFV immunoreactivity was detected using a commercial kit (Mach 3 AP kit, Biocare). An alkaline phosphatase chromagen substrate (Vector Red, Vector Laboratories) was applied and allowed to develop for approximately 7 minutes. Slides were then counterstained with Gill's hematoxylin and cover slipped using routine methods. A duplicate serial section of each tissue screened was treated with a mouse monoclonal anti-foot and mouth disease virus (FMDV) (10GA4, targeting FMDV O1-Brugge) (Arzt et al., 2009) antibody served as a negative control. Additional negative control tissue sections from a naïve pig were also included. Discernible cell-associated red chromagen detection within the tissue of interest with concurrent absence of chromagen detection within negative control slides was considered positive for the presence of ASFV VP30 antigen. Positive labeling was visualized by light microscopy and imaged with a digital camera.

MIF was performed similarly as described for IHC except combinations of monoclonal antibodies (listed in Table 4) were applied. After incubation of primary antibodies for 24 hours at 4 °C, secondary antibodies corresponding to the primary antibody isotypes and labeled with fluorescent dyes (AF 350, 488, 594, 647; AlexaFluor, Invitrogen, CA) were applied and sections were incubated for 1 hour at 37 °C.

Table 4. Monoclonal Antibodies used in this study

Target	Clone	Isotype	Dilution	Source	Labeling characteristics
ASFV p30 (1D9)		IgG2a	1:250	INIA, courtesy of Javier Dominguez Juncal	IC, dense granular
ASFV p72			1:10	APHIS-USDA	IC, dense granular
CD203a	PM18-7	IgG1	1:100	LifeSpan BioSciences	IC, dense granular
CD163	2A10/11	IgG1	1:50	AbD Serotec	IC, fine granular
Sialoadhesin (Sn)	3B11/11	IgG1	1:100	AbD Serotec	IC, fine granular
CD172a	DH59B	IgG1	1:100	Washington State University	IC, fine granular
CD172a	74-22-15A	IgG2b	1:100	Washington State University	IC, fine granular
SLA II- DQ (MHC II)	K274.3G8	IgG1	1:50	AbD Serotec	IC, globular
CD44	BAG40A	IgG3	1:200	Washington State University	
Pancytokeratin (PCK)	3856	Rabbit	1:200	Invitrogen	IC, homogenous
Cytokeratin 18 (CK18)	KS-B17.2	IgG1	1:50	Sigma-Aldrich	IC, homogenous
Von Willebrand Factor (vWF)		Rabbit	1:500	Dako	IC, globular

IC, Intracytoplasmic

Images were obtained using a Nikon Eclipse 90i multichannel light and epi-fluorescent microscope equipped with 350, 488, 594, and 647 excitation filter cubes. Serial sections of tissues, which proved of special interest (particularly tonsil of the soft palate, lingual and nasopharyngeal tonsils, soft palate, pharyngeal mucosa, lymph nodes of the head and neck, and spleen) were processed repeatedly to ensure that fully representative tissue sections were examined for each organ.

Results

Virus detection within tissues via Virus Isolation and qPCR

Comparison of titers determined by VI and titer equivalents by qPCR demonstrated that qPCR detection often coincided with VI, indicating similar sensitivity and specificity for both tests. The high agreement level (kappa=0.94) also indicated strong consensus between tests. Interestingly, although detection parameters were very similar for both techniques, titers determined by VI were statistically different from C_t titer equivalents (C_tTE) determined by qPCR ($p=0.02$) (Tables 5-7). Also, qPCR titer equivalents were generally higher than true VI titers, with 63% of non-negative values having higher values on qPCR than VI.

Table 5. Tissue-Specific Distribution of ASFV in Intramuscular (IM) Inoculated Pigs[a]

	1DPI			2DPI			Late term		
	P#1			P#2			P#3		
	Euthanized			Euthanized			Euthanized- 9DPI		
Final blood/serum titer	0			0			7.05		
	VI	PCR	C_t Titer Equivalent[b]	VI	PCR	C_t Titer Equivalent[b]	VI	PCR	C_t Titer Equivalent[b]
Lingual Tonsil	0.0	NEG	0.0	0.0	NEG	0.0	4.55	30.7	4.6
Tonsil of the Soft Palate	0.0	NEG	0.0	0.0	NEG	0.0	5.55	24.8	6.3
Dorsal Soft Palate (Rostral)	0.0	NEG	0.0	0.0	NEG	0.0	6.05	26.5	5.8
Dorsal Soft Palate (Distal)	0.0	NEG	0.0	0.0	NEG	0.0	4.3	31.2	4.5
Posterior Nasal Turbinates	0.0	NEG	0.0	0.0	NEG	0.0	5.3	28.9	5.1
Nasopharyngeal Tonsil	0.0	NEG	0.0	0.0	NEG	0.0	6.3	25.5	6.1
Dorsal Nasal Pharynx	0.0	NEG	0.0	0.0	NEG	0.0	6.8	22.9	6.9
Ventral Epiglottis	0.0	NEG	0.0	0.0	NEG	0.0	NC	NC	NC
Proximal Trachea	0.0	NEG	0.0	0.0	NEG	0.0	NC	NC	NC
Tracheal Bifurcation	0.0	NEG	0.0	0.0	NEG	0.0	NC	NC	NC
Proximal Middle Lung	0.0	NEG	0.0	0.0	NEG	0.0	6.55	24.7	6.4
Middle Middle Lung	0.0	NEG	0.0	0.0	NEG	0.0	5.55	27.0	5.7
Distal Middle Lung	0.0	NEG	0.0	0.0	NEG	0.0	5.8	24.4	6.4
Retropharyngeal Lymph Node	0.0	NEG	0.0	0.0	NEG	0.0	6.3	22.5	7.0
Submandibular Lymph Node	0.0	NEG	0.0	0.0	NEG	0.0	6.05	22.9	6.9
Liver	0.0	NEG	0.0	0.0	33.5	3.8	6.05	22.6	6.9
Spleen	0.0	NEG	0.0	2.3	33.6	3.8	7.55	22.9	6.9
Hilar Lymph Node	0.0	NEG	0.0	0.0	NEG	0.0	5.8	25.9	6.0
Thyroid	0.0	NEG	0.0	0.0	NEG	0.0	NC	NC	NC
Kidney	0.0	NEG	0.0	0.0	NEG	0.0	6.05	27.3	5.6
Thymus	0.0	NEG	0.0	0.0	NEG	0.0	NC	NC	NC
Adrenal Gland	0.0	NEG	0.0	0.0	NEG	0.0	NC	NC	NC
Pancreas	0.0	NEG	0.0	0.0	NEG	0.0	NC	NC	NC
Cerebellum	0.0	NEG	0.0	0.0	NEG	0.0	NC	NC	NC
Cerebrum	0.0	NEG	0.0	0.0	NEG	0.0	NC	NC	NC
Bone Marrow	0.0	NEG	0.0	0.0	NEG	0.0	7.05	18.9	8.0
Heart- Left Ventricle	0.0	NEG	0.0	0.0	NEG	0.0	4.3	27.2	5.6
Gastrohepatic Lymph Node	0.0	NEG	0.0	0.0	NEG	0.0	6.3	24.5	6.4
Renal Lymph Node	0.0	NEG	0.0	0.0	NEG	0.0	7.3	24.9	6.3
Inguinal Lymph Node	0.0	NEG	0.0	0.0	NEG	0.0	6.05	26.8	5.8
Peyer's Patch	0.0	NEG	0.0	0.0	NEG	0.0	NC	NC	NC
Ileum	0.0	NEG	0.0	0.0	NEG	0.0	NC	NC	NC
Large Intestine	0.0	NEG	0.0	0.0	NEG	0.0	NC	NC	NC
Popliteal Lymph Node	0.0	NEG	0.0	0.0	NEG	0.0	6.55	24.0	6.6
Pharyngeal Diverticulum	NC	NC	NC	NC	NC	NC	NC	NC	NC
Prescapular Lymph Node	0.0	NEG	0.0	0.0	NEG	0.0	6.05	25.3	6.2

[a]DPI, days post inoculation; DPC, days post contact; VI, virus isolation; PCR, real-time polymerase chain reaction; NEG indicates PCR negative; NC indicates sample not collected. PCR values shown are the means of two replicates. Negative tissues indicate that DNA quantity was below the detection threshold, corresponding to cycle threshold (C_t) greater than 40; Viral titer corresponds to $Log_{10}HAD_{50}$/ml or $Log_{10}HAD_{50}$/mg of tissue. [b]Statistical difference compared to VI titer (p= 0.02).

Table 6. Tissue-Specific Distribution of ASFV in Intranasopharyngeal (INP) Inoculated Pigs[a]

	1DPI			2DPI			4 DPI			Late term		
	P#4			P#5			P#6			P#7		
	Euthanized			Euthanized			Died[c]			Euthanized- 10 DPI		
Final blood/serum titer	0			0			3.05			5.8		
	VI	PCR	C_t Titer Equivalent[b]	VI	PCR	C_t Titer Equivalent[b]	VI	PCR	C_t Titer Equivalent[b]	VI	PCR	C_t Titer Equivalent[b]
Lingual Tonsil	0.0	NEG	0.0	0.0	NEG	0.0	NC	NC	NC	4.55	33.2	3.9
Tonsil of the Soft Palate	0.0	NEG	0.0	0.0	NEG	0.0	3.05	38.4	2.4	4.55	27.5	5.5
Dorsal Soft Palate (Rostral)	0.0	NEG	0.0	0.0	NEG	0.0	3.3	NEG	0.0	4.55	33.2	3.9
Dorsal Soft Palate (Distal)	0.0	NEG	0.0	0.0	NEG	0.0	NC	NC	NC	4.3	31.3	4.4
Posterior Nasal Turbinates	0.0	NEG	0.0	0.0	NEG	0.0	NC	NC	NC	5.55	32.4	4.1
Nasopharyngeal Tonsil	0.0	NEG	0.0	0.0	NEG	0.0	3.55	35.4	3.2	6.3	23.7	6.6
Dorsal Nasal Pharynx	0.0	NEG	0.0	0.0	NEG	0.0	3.3	38.0	2.5	5.55	28.8	5.2
Ventral Epiglottis	0.0	NEG	0.0	0.0	NEG	0.0	NC	NC	NC	NC	NC	NC
Proximal Trachea	0.0	NEG	0.0	0.0	NEG	0.0	NC	NC	NC	NC	NC	NC
Tracheal Bifurcation	0.0	NEG	0.0	0.0	NEG	0.0	NC	NC	NC	NC	NC	NC
Proximal Middle Lung	0.0	NEG	0.0	0.0	NEG	0.0	3.8	31.4	4.4	6.3	24.5	6.4
Middle Middle Lung	0.0	NEG	0.0	0.0	NEG	0.0	3.55	29.9	4.9	5.8	24.1	6.5
Distal Middle Lung	0.0	NEG	0.0	0.0	NEG	0.0	3.3	29.2	5.1	6.05	28.0	5.4
Retropharyngeal Lymph Node	0.0	NEG	0.0	0.0	NEG	0.0	3.05	36.7	2.9	5.55	26.3	5.9
Submandibular Lymph Node	0.0	NEG	0.0	0.0	NEG	0.0	2.3	36.6	2.9	6.55	26.8	5.8
Liver	0.0	NEG	0.0	0.0	NEG	0.0	NC	NC	NC	6.3	23.2	6.8
Spleen	0.0	NEG	0.0	0.0	NEG	0.0	NC	NC	NC	6.8	24.5	6.4
Hilar Lymph Node	0.0	NEG	0.0	0.0	NEG	0.0	NC	NC	NC	5.3	24.8	6.3
Thyroid	0.0	NEG	0.0	0.0	NEG	0.0	NC	NC	NC	NC	NC	NC
Kidney	0.0	NEG	0.0	0.0	NEG	0.0	NC	NC	NC	5.55	29.5	4.9
Thymus	0.0	NEG	0.0	0.0	NEG	0.0	NC	NC	NC	NC	NC	NC
Adrenal Gland	0.0	NEG	0.0	0.0	NEG	0.0	NC	NC	NC	NC	NC	NC
Pancreas	0.0	NEG	0.0	0.0	NEG	0.0	NC	NC	NC	NC	NC	NC
Cerebellum	0.0	NEG	0.0	0.0	NEG	0.0	NC	NC	NC	NC	NC	NC
Cerebrum	0.0	NEG	0.0	0.0	NEG	0.0	NC	NC	NC	NC	NC	NC
Bone Marrow	0.0	NEG	0.0	0.0	NEG	0.0	NC	NC	NC	6.8	24.5	6.4
Heart- Left Ventricle	0.0	NEG	0.0	0.0	NEG	0.0	NC	NC	NC	4.8	32.3	4.2
Gastrohepatic Lymph Node	0.0	NEG	0.0	0.0	NEG	0.0	3.55	37.1	2.8	6.55	26.3	5.9
Renal Lymph Node	0.0	NEG	0.0	0.0	NEG	0.0	NC	NC	NC	6.8	27.7	5.5
Inguinal Lymph Node	0.0	NEG	0.0	0.0	NEG	0.0	NC	NC	NC	5.3	29.7	4.9
Peyer's Patch	0.0	NEG	0.0	0.0	NEG	0.0	NC	NC	NC	NC	NC	NC
Ileum	0.0	NEG	0.0	0.0	NEG	0.0	NC	NC	NC	NC	NC	NC
Large Intestine	0.0	NEG	0.0	0.0	NEG	0.0	NC	NC	NC	NC	NC	NC
Popliteal Lymph Node	0.0	NEG	0.0	0.0	NEG	0.0	NC	NC	NC	6.05	27.6	5.5
Pharyngeal Diverticulum	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
Prescapular Lymph Node	0.0	NEG	0.0	0.0	NEG	0.0	NC	NC	NC	5.05	26.9	5.7

[a]DPI, days post inoculation; DPC, days post contact; VI, virus isolation; PCR, real-time polymerase chain reaction; NEG indicates PCR negative; NC indicates sample not collected. PCR values shown are the means of two replicates. Negative tissues indicate that DNA quantity was below the detection threshold, corresponding to cycle threshold (C_t) greater than 40; viral titer corresponds to $Log_{10}HAD_{50}$/ml or $Log_{10}HAD_{50}$/mg of tissue. [b]Statistical difference compared to VI titer (p= 0.02). [c]Died due to non-ASF related trauma.

Table 7. Tissue-Specific Distribution of ASFV in Direct Contact (DC) Infected Pigs[a]

	Late term P#8 (1DPC) Euthanized- 10DPC			Late term P#9 (2DPC) Euthanized- 9DPC			Late term P#10 (3DPC) Euthanized- 11DPC		
Final blood/serum titer	8.55			8.55			8.3		
	VI	PCR	C_t Titer Equivalent[b]	VI	PCR	C_t Titer Equivalent[b]	VI	PCR	C_t Titer Equivalent[b]
Lingual Tonsil	4.8	29.3	5.0	5.3	23.3	6.7	4.8	27.1	5.6
Tonsil of the Soft Palate	4.8	30.0	4.8	6.05	22.3	7.0	5.8	20.6	7.5
Dorsal Soft Palate (Rostral)	4.55	31.9	4.3	5.05	26.4	5.9	2.8	24.3	6.5
Dorsal Soft Palate (Distal)	4.05	26.7	5.8	4.55	25.7	6.1	5.8	25.6	6.1
Posterior Nasal Turbinates	5.05	30.1	4.8	5.55	24.2	6.5	6.55	25.4	6.1
Nasopharyngeal Tonsil	4.8	24.5	6.4	5.8	23.6	6.6	6.55	24.1	6.5
Dorsal Nasal Pharynx	4.05	32.5	4.1	4.55	27.9	5.4	5.55	23.5	6.7
Ventral Epiglottis	4.3	30.0	4.8	4.8	27.0	5.7	4.55	22.1	7.1
Proximal Trachea	4.05	31.2	4.5	4.3	26.0	6.0	4.55	25.2	6.2
Tracheal Bifurcation	4.3	28.1	5.4	4.55	26.6	5.8	5.55	26.1	5.9
Proximal Middle Lung	6.05	24.5	6.4	5.05	25.3	6.2	4.8	25.1	6.2
Middle Middle Lung	2.8	24.2	6.5	6.05	23.3	6.8	6.8	21.5	7.3
Distal Middle Lung	6.3	25.0	6.3	6.3	25.4	6.1	5.8	24.1	6.5
Retropharyngeal Lymph Node	5.8	29.2	5.1	5.3	25.0	6.3	6.05	26.5	5.8
Submandibular Lymph Node	5.8	24.1	6.5	6.3	22.0	7.1	6.8	22.8	6.9
Liver	6.3	19.4	7.9	6.8	19.1	8.0	6.05	19.9	7.7
Spleen	7.3	18.3	8.2	6.8	22.6	7.0	7.55	21.3	7.3
Hilar Lymph Node	5.05	26.1	6.0	6.55	23.5	6.7	6.55	22.8	6.9
Thyroid	5.05	27.6	5.5	5.3	25.3	6.2	5.05	26.0	6.0
Kidney	6.55	24.5	6.4	6.55	31.9	4.3	5.8	25.9	6.0
Thymus	5.3	23.4	6.7	5.8	23.7	6.6	5.8	26.7	5.8
Adrenal Gland	5.8	22.4	7.0	6.3	25.2	6.2	5.55	22.8	6.9
Pancreas	4.3	32.2	4.2	6.3	27.3	5.6	4.3	31.0	4.5
Cerebellum	5.05	29.9	4.8	5.55	29.6	4.9	5.55	30.8	4.6
Cerebrum	4.8	31.7	4.3	5.3	30.1	4.8	6.3	26.9	5.7
Bone Marrow	7.05	19.2	7.9	6.55	22.8	6.9	7.05	21.8	7.2
Heart- Left Ventricle	5.05	25.0	6.2	4.8	27.2	5.6	4.8	26.3	5.9
Gastrohepatic Lymph Node	6.05	23.4	6.7	6.55	26.2	5.9	7.05	24.4	6.4
Renal Lymph Node	6.05	25.3	6.2	7.05	24.9	6.3	7.3	26.0	6.0
Inguinal Lymph Node	4.8	26.4	5.9	5.55	26.3	5.9	7.05	24.9	6.3
Peyer's Patch	4.55	26.8	5.7	5.05	23.3	6.8	4.8	28.9	5.1
Ileum	5.05	28.7	5.2	3.8	27.2	5.6	4.8	29.5	5.0
Large Intestine	4.05	28.4	5.3	3.8	24.8	6.3	5.3	25.7	6.1
Popliteal Lymph Node	4.8	27.7	5.5	6.3	22.1	7.1	6.55	29.7	4.9
Pharyngeal Diverticulum	5.05	23.6	6.7	5.3	27.3	5.6	5.3	22.8	6.9
Prescapular Lymph Node	5.05	25.2	6.2	6.05	21.7	7.2	6.8	25.8	6.0

[a]DPI, days post inoculation; DPC, days post contact; VI, virus isolation; PCR, real-time polymerase chain reaction; NEG indicates PCR negative; NC indicates sample not collected. PCR values shown are the means of two replicates. Negative tissues indicate that DNA quantity was below the detection threshold, corresponding to cycle threshold (C_t) greater than 40; viral titer corresponds to $Log_{10}HAD_{50}$/ml or $Log_{10}HAD_{50}$/mg of tissue. [b]Statistical difference compared to VI titer (p= 0.02).

Early pathogenesis

ASFV was not detected within tissues of animals euthanized at 1 dpi (Tables 5-7). The only detection of ASFV in a pre-viremic pig occurred at 2 dpi within the spleen of one intramuscularly inoculated animal with a titer of 2.3 $log_{10}HAD_{50}$/mg and DNA detection by qPCR at 3.8 C_tTE (Table 5). A similar quantity ASFV DNA was detected in the liver of this animal with no detection of ASFV. ASFV was detected in tissues of one early viremic, asymptomatic INP-inoculated pig which died due to non-ASF related trauma at 4 dpi. In this animal, similar, moderate quantities of ASFV (titers ranging from 2.3-3.8 $log_{10}HAD_{50}$/mg and C_tTE ranging from 2.4-5.1) were detected within the tonsil of the soft palate, dorsal soft palate, nasopharyngeal tonsil, dorsal nasopharynx, lung, retropharyngeal lymph node, submandibular lymph node, and gastrohepatic lymph node.

Late pathogenesis

ASFV and viral DNA were detected within all tissues examined in pigs at late stages of disease regardless of route of infection (Tables 5-7). Viral titers in late stage tissues ranged from 4.3 – 7.55 log10HAD50/mg with CtTE values of 4.1-8.2. The highest titers /CtTE values commonly were detected within the spleen (mean titer 7.2±0.4 log10HAD50/mg, mean CtTE value 7.2±0.7) and bone marrow (mean titer 6.9±0.2 log10HAD50/mg, mean CtTE value 7.3±0.7). ASFV detection was consistently high in draining lymph nodes of the head, neck and thorax, including retropharyngeal (mean titers/ CtTE of 5.8±0.4/ 6.02±0.7), submandibular (6.3±0.4/ 6.64±0.5), prescapular (5.8±0.6/ 6.26±0.8) and hilar (5.85±0.7/ 6.38±0.4) lymph nodes. Comparatively, visceral lymph nodes (i.e. gastro-hepatic, and renal lymph nodes) commonly contained more infectious virus with mean titers of 6.5±0.4 and 6.9±0.5log10HAD50/mg and

mean C_tTE values of 6.26±0.4 and 6.06±0.3, respectively. ASFV and viral DNA were routinely detected within the lingual tonsil, tonsil of the soft palate and nasopharyngeal tonsil with mean titers of 4.8±0.3, 5.3±0.6 and 5.9±0.7 $\log_{10}$HAD50/mg and titer equivalents of 5.16±1.1, 6.22±1.1, and 6.34±0.3 respectively. Amongst tonsil tissues, 50% of the animals had highest titers and titer equivalents in nasopharyngeal tonsil. In the remaining animals, values for this tissue were similar to that of the lingual tonsil or tonsil of the soft palate. Lingual tonsil and dorsal soft palate consistently had the lowest titers and highest C_t values.

Histologic changes of tissues infected with ASFV

Architecture and distribution of all cell phenotypes examined were similar amongst tonsil of the soft palate, nasopharyngeal tonsil, paraepiglottic tonsil and lingual tonsil with the exception of the lack of identifiable tonsil crypts within the lingual tonsil. Therefore, in some passages to follow, these tissues will be referred to collectively as tonsillar tissue.
Lymphoid tissues including tonsillar tissues, lymph nodes, and spleen maintained normal architecture (Figures 6 A, 6 D, 6 G and 6 J); however, there was a consistent decrease in lymphocyte quantities and expansion of the interfollicular regions with varying degrees of edema, hemorrhage (Figure 6 G), and within the spleen, necrosis (Figure 6 J). In some pigs, perifollicular regions were diffusely infiltrated by large mononuclear cells with moderate to abundant cytoplasm, consistent with macrophages. Within both perifollicular and follicular regions, nuclear debris, suggestive of lymphocyte apoptosis was observed. Within mucosal tissues, varying degrees of mononuclear infiltrate was observed within the superficial submucosa occasionally in combination with poorly defined lymphoid follicles (Figure. 7 A). Lungs contained varying degrees of mononuclear infiltrate that expanded the alveolar interstitium

(Figure 7 D). This was occasionally accompanied by vascular congestion, hemorrhage and/or edema. Sections of liver contained peri- and intralobular mononuclear infiltrates with occasional single cell hepatocyte necrosis (Figure 7 G). In addition, two pigs that died spontaneously had grossly visible areas of hepatocellular necrosis indicated by tan, slightly depressed, well demarcated, irregularly shaped regions on the visceral (Figure 8 A) and cut surfaces. Histologically, these areas were defined by extensive regions of hepatocellular necrosis with no apparent zonality, cellular debris and infiltration of macrophages and lymphocytes (Figure 8 B).

In pigs that died spontaneously from ASF, there were variable gross and histologic changes suggestive of multifocal myocardial infarction distributed throughout most of the ventricular myocardium (Figure 8 C). Grossly these lesions were well demarcated, pale tan foci which were dry and similarly pale on cut surface (Figure 8 C). Histologically, there were multiple microvascular thrombi of small vessels with adjacent degeneration of myocytes and mild infiltrates of small mononuclear cells (Figure 8 D). Throughout the remainder of tissues examined, minimal to mild mononuclear interstitial infiltrates were present.

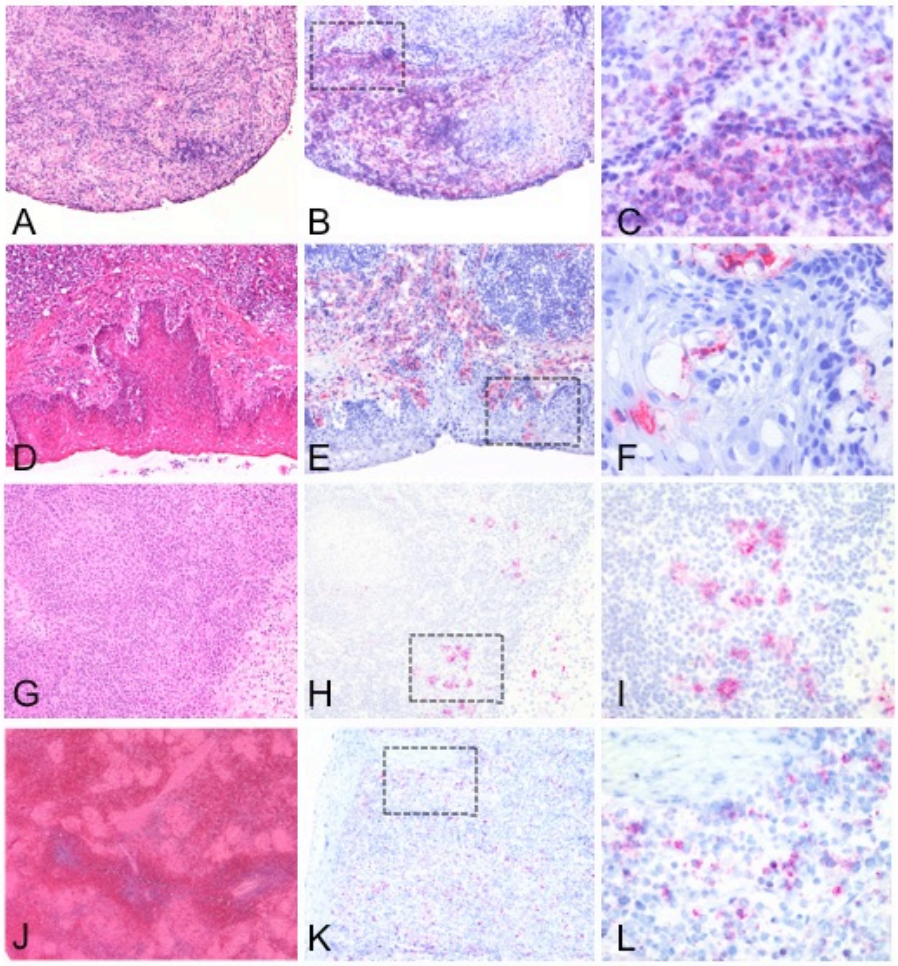

Figure 6. Pig. Light microscopy and immunohistochemical distribution of viral antigen in lesions from clinical ASFV infected pigs. A-C. Nasopharyngeal tonsil. A. Low magnification showing decreased follicular and perifollicular lymphocytes. Hematoxylin and eosin (HE). B. Low magnification showing ASFV+ cells (red) within submucosal and perifollicular regions, and occasionally within the crypt epithelium. Immunohistochemical (IHC) staining with anti-ASFV VP30 (1D9). C. High magnification of cells positive for ASFV surrounding and within the crypt epithelium. IHC. D-F. Tonsil of the soft palate. D. Low magnification showing decreased perifollicular lymphocytes and submucosal mixed cellular infiltrates. HE. E. Low magnification showing ASFV+ cells within submucosal and perifollicular regions, and

Figure 6. (cont'd) occasionally within the crypt and surface epithelium. IHC. F. High magnification of cells positive for ASFV within the epithelium. IHC. G-I. Gastrohepatic lymph node. G. Low magnification showing decreased follicular and perifollicular lymphocytes. HE. H. Low magnification showing ASFV+ cells (red) within perifollicular regions. IHC. I. High magnification of cells positive for ASFV. J-L. Spleen. J. Low magnification showing diffuse necrosis throughout the red pulp. Hematoxylin and eosin (HE). K. Low magnification showing ASFV+ cells within perifollicular regions adjacent to a smooth muscle trabeculae, Immunohistochemical (IHC) staining with anti-ASFV VP30 (1D9) L. High magnification of cells positive for ASFV. IHC.

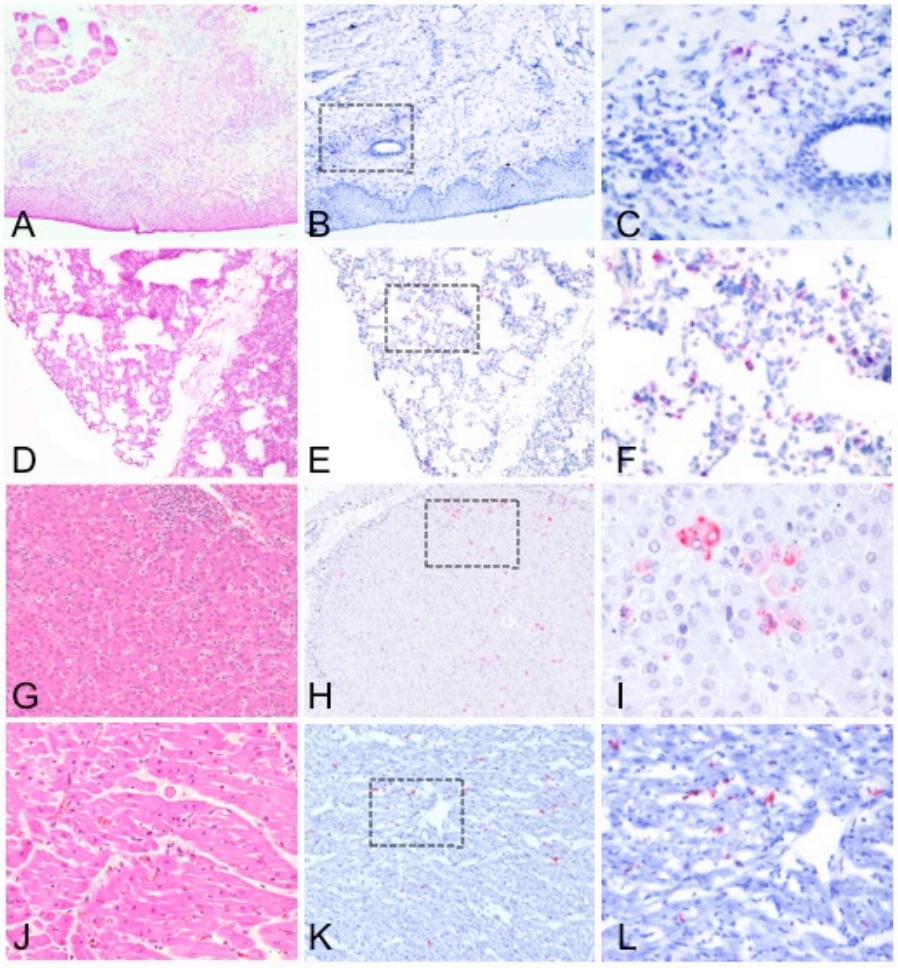

Figure 7. Pig. Light microscopy and immunohistochemical distribution of viral antigen in lesions from clinical ASFV infected pigs. A-C. Dorsal soft palate. A. Low magnification showing scattered submucosal infiltrates of lymphocytes and macrophages. HE. B. Low magnification showing rare ASFV+ cells within the submucosa. IHC. C. High magnification of cells positive for ASFV. IHC. D-F. Lung. D. Low magnification showing increased cellularity within the alveolar interstitium. HE. E. Low magnification showing ASFV+ cells within the interstitium of alveolar septae. IHC. F. High magnification of cells positive for ASFV. IHC. G-I. Liver. G. Liver with inflammatory infiltrates. HE. H. Low magnification showing scattered ASFV+ cells within hepatic lobules (red). IHC. I. High magnification of ASFV+ hepatocytes and sinusoidal macrophages. IHC. J-L. Heart. J. Myocardium with normal myocytes and scattered

Figure 7. (cont'd) interstitial cells. HE. K. Low magnification showing ASFV+ cells within the interstitium of the myocardium. IHC. L. High magnification of cells positive for ASFV. IHC.

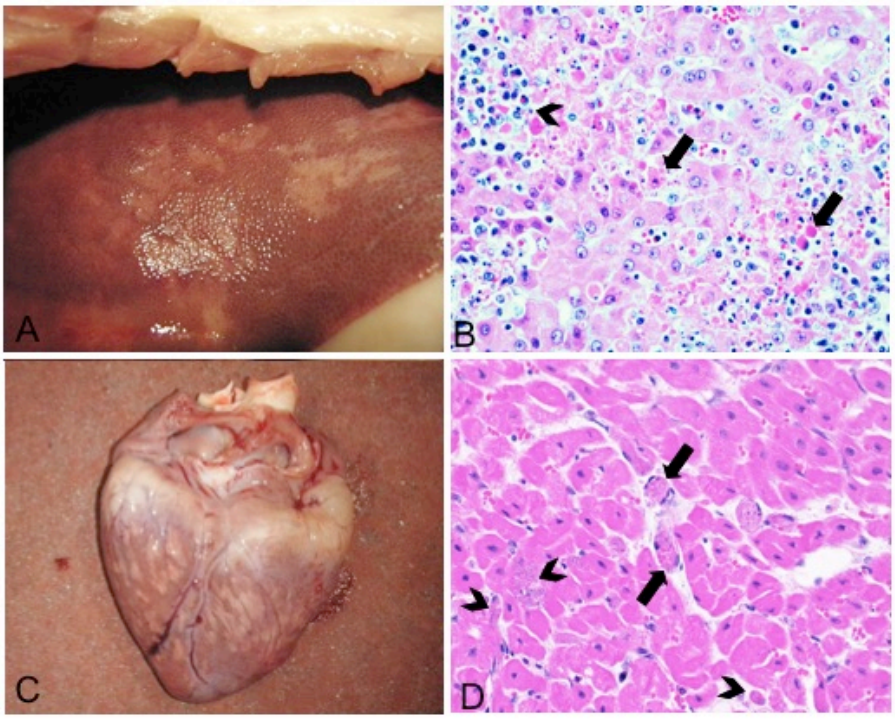

Figure 8. Novel lesions from ASFV infected pigs. A. Liver, Gross pathology. Multifocal random hepatic necrosis. B. Liver, light microscopy. Light microscopy showing random hepatocellular necrosis (arrows), cellular debris and mixed inflammatory mononuclear cell infiltrates (arrowheads). Hematoxylin and eosin (HE). C. Heart, Gross pathology. Multifocal myocardial necrosis of the left and right ventricles. D. Heart, light microscopy. Myocardium with vascular thrombosis (arrows), individual myocyte necrosis and mineralization (arrowheads).HE.

Immunomicroscopic localization of ASFV antigens

In all instances of immunolocalization, cells immunoreactive for ASFV had diffuse, granular cytoplasmic chromagen or fluorophore staining. The majority of the cells were large with moderate to abundant cytoplasm and no clear architectural association with adjacent cells, consistent with monophagocytic origin. ASFV antigen was localized in tissues of 2 pigs at early, pre-clinical stages of infection and in all tissues of all pigs examined at late stages of infection.

Within pharyngeal mucosal tissues including the dorsal soft palate, ventral soft palate and dorsal nasopharynx, ASFV-positive cells were distributed within the superficial submucosa with occasional cells within the surface epithelium (Figures 7 B-C).

Within the livers of infected pigs, distribution of histopathologic changes, and ASFV antigen varied dependent on stage of infection. In early stages of disease, individual or small clusters of ASFV+ cells were present within sinusoids (consistent with sinusoidal macrophages), but also in periportal regions of inflammatory infiltrates or occasional hepatocytes (Figures 7 H-I). In some pigs, the late stage of ASFV infection resulted in submassive hepatocellular necrosis with diffuse ASFV antigen within these regions in cells that were morphologically and phenotypically consistent with either hepatocytes, Kupffer cells or infiltrating macrophages (Figure 14 B). ASFV antigen was not detected within the biliary epithelium.

Within the heart, there was immunolocalization of ASFV in the infarcted regions observed grossly and histologically, however morphological and phenotypic characterization of affected cells was inconclusive. Within non-infarcted regions of late stage infected pigs; ASFV + cells were occasionally dispersed within the interstitium (Figures 7 K-L).

At late stages of infection, ASFV was localized to large mononuclear cells consistent with resident macrophages in all tissues examined. These ASFV+ cells were detected

interspersed with parenchymous cells, within connective tissue stroma, and perivascularly/intravascularly associated with small-caliber vessels.

In order to characterize cellular phenotypes of infected cells, distribution of ASFV antigen was examined in conjunction with variations of host cell markers by simultaneous MIF. In all tissues and stages of infection, ASFV predominantly colocalized to cells expressing markers consistent with myelomonocytic origin including CD172a, and CD163. Some tissue-specific variation in colocalization occurred as described below.

Early pathogenesis

The earliest immunohistochemical detection of ASFV antigen coincided with the early detection by VI and qPCR in the spleen of one IM-inoculated pig at 2dpi (Figures 9 A-B). Within this spleen, there was a single focus of several ASFV + cells in a perifollicular region adjacent to a smooth muscle trabecula. ASFV colocalized with CD172a, but not with Sn, CD21, CD3, vWF, or actin (Figures 10 A-B). At 4 DPI, ASFV antigen was detected in the lungs and gastrohepatic lymph node of one INP inoculated pig (Figures 9 C-F). In this pig, ASFV antigen was localized within perifollicular regions of the lymph node (Figures 9 C-D) and within the alveolar interstitium of the lungs (Figures 9 E-F). Relatively few ASFV+ cells were present in these tissues, with patchy distribution.

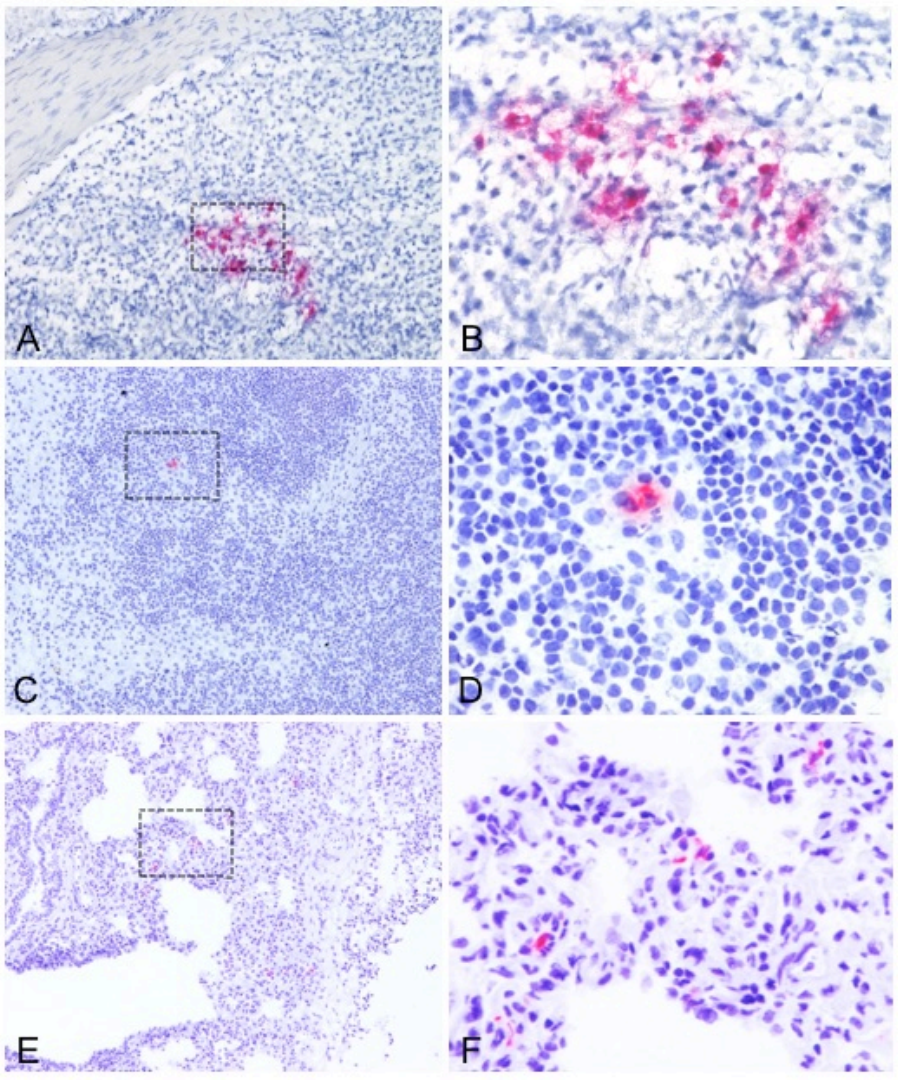

Figure 9. ASFV antigen detection in preclinical ASFV infected pigs. Immunohistochemical (IHC) staining with anti-ASFV VP30 (1D9). A-B. Spleen A. Low magnification showing a focal cluster of ASFV+ cells within the red pulp adjacent to a smooth muscle trabeculae, B. High magnification of cells positive for ASFV. C-D. Gastrohepatic lymph node C. Low magnification showing a focal positive cell within the perifollicular region. D. High magnification of ASFV positive cell. E-F. Lung E. Low magnification showing rare ASFV+ cells within the interstitium of alveolar septae. F. High magnification of cells positive for ASFV.

Late pathogenesis

In the late stage of infection, lymphoid regions of tonsils, spleen, and lymph nodes of all pigs examined had similar, abundant ASFV antigen with minor variation of distribution. In all cases, viral antigen was abundant in classical perifollicular (T lymphocyte) regions with substantial, but not exclusive, sparing of lymphoid follicles Figures 6 B-C, E-F, H-I and K-L). In perifollicular regions, extensive ASFV antigen was predominantly within extensive groups or clusters of cells, whereas follicles typically had 0-3 individual ASFV+ cells (Figures 11 A-B). In tonsillar tissues, within crypt epithelium and crypt lumens, multiple individual and small clusters of ASFV+ cells were interspersed and closely associated with epithelial cells (Figures 6 B-C, 6 E-F, 11 A-B, and 12 B). Fewer ASFV-immunoreactive cells were identified within the surface epithelium and superficial submucosa.

In all lymphoid tissues examined, markers for cells of monophagocytic origin CD163, CD172a, and Sn had similar distribution with minor variation in quantity (Figures 11 A-B). In nasopharyngeal tonsil, tonsil of soft palate, and spleen, CD172a was the most abundantly detected cell marker in these tissues. Cells expressing CD172a also had the highest proportional detection of ASFV antigen (Figure 11 A). In both tonsils, a moderate proportion of CD172a-expressing cells were ASFV-infected (Figure 11 A). In lymphoid tissues, CD163 was somewhat less abundant. Cells expressing CD163 also had slightly lower proportional detection of ASFV antigen (Figures 11 B and 14A). Additional monophagocytic cell markers Sn and CD203a had similar distribution but substantially lower quantity than CD163 and CD172a; ASFV rarely colocalized with these cells (not shown).

ASFV distribution was also examined in the conjunction with epithelial and vascular cell markers. Anti-pancytokeratin (PCK) immunoreactivity delineated the surface and tonsil crypt

epithelium within tonsil tissues. Within tonsil tissues, cells immunoreactive for ASFV were often observed distributed within surface and crypt epithelium (Figures11 A-B, 12 B); however, these cells were only rarely concurrently ASFV+PCK+. Few cells immunoreactive for CK18 (M-cells) were distributed solely within the epithelium of the tonsil crypts. CK18+ASFV+ colocalization was not observed in any tissue (Figure 12 B). Von Willebrand factor-immunoreactive cells (vWF+) were consistently morphologically associated with vessel or sinus linings within all tissues examined and are thus interpreted as vascular endothelium. ASFV+ cells were often observed in close proximity to vWF+ cells, and in rare instances there was colocalization of ASFV+vWF+ (Figures 13 B and 14 B).

Cells expressing CD3 were distributed within perifollicular zones distinctly sparing follicles within lymph nodes and tonsil tissues. Cells immunoreactive for CD21 were almost exclusively distributed within lymphoid follicles within lymphoid and tonsil tissues (Figures10 A and 12 A). While ASFV+ cells were observed adjacent to CD21+ and CD3+ cells, cells with concurrent immunoreactivity with either of the two antibodies with the ASFV antigen minimal to absent.

Within the lungs of pigs at late stages of infection there were abundant individual ASFV+ cells within the alveolar septa distributed diffusely throughout the lungs (Figures 7 E-F and 13 A-B). ASFV+ cells were also distributed throughout areas of bronchus associated lymphoid tissue (BALT). Pulmonary cells contaiing ASFV antigen were rarely CD172a+, PCK+, or vWF+ (Figures 13A-B). However CD172a+ cells had the highest proportion of being ASFV+. ASFV antigen was rarely detected in cells morphologically consistent with alveolar macrophages within alveolar spaces. These cells were variably CD203a+, however colocalization with ASFV antigen was not detected (Figure 13A). ASFV was not detected within bronchiolar epithelium or pleural

surfaces. Colocalization with ASFV antigen was not observed for CD21 or CD3 within pulmonary tissues.

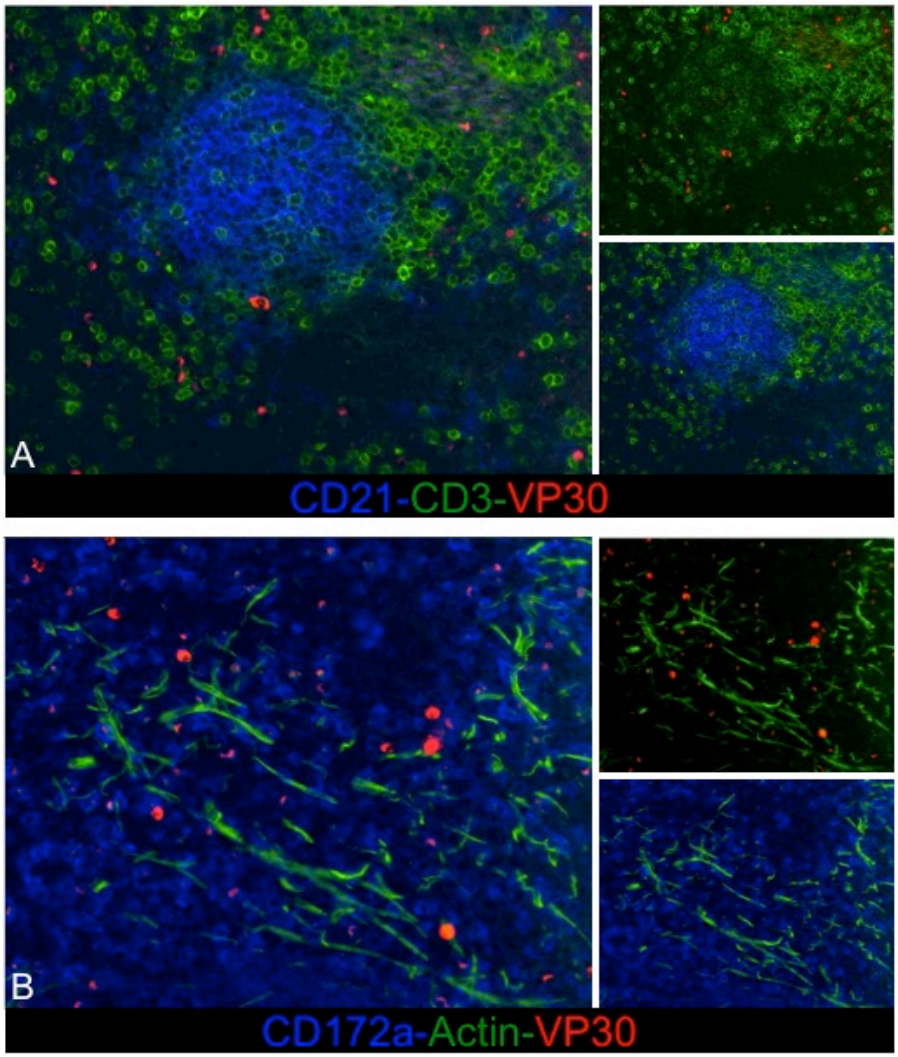

Figure 10. Phenotypic characterization of spleen from ASFV infected pigs using Multichannel Immunofluorescence. Color coded antibody combinations described below each panel. A. Spleen. ASFV antigen distribution in relation to T lymphocytes (CD3+) and B lymphocytes (CD21+). Large merged image showing a lack of colocalization of ASFV antigen with CD3 or CD21. B. Spleen. ASFV antigen distribution in relation to macrophages (CD172a+) and supporting reticulo-endothelial cells (Actin+). Large merged image showing colocalization of ASFV antigen with CD172a+ cells. Viral antigen did not colocalize with actin.

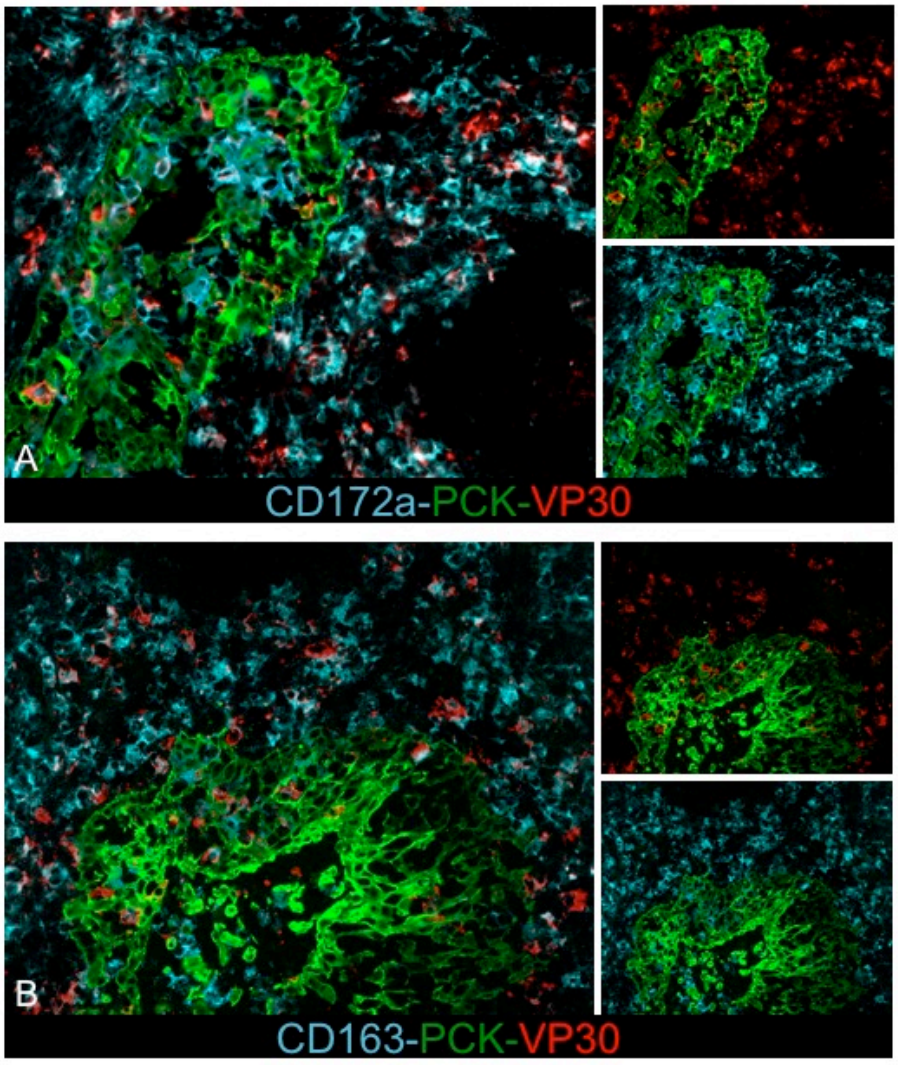

Figure 11. Phenotypic characterization of tonsillar tissue from ASFV infected pigs using Multichannel Immunofluorescence. Color coded antibody combinations described below each panel. A. Tonsil of the soft palate. ASFV antigen distribution in relation to macrophages (CD172a+) and crypt epithelium (pancytokeratin+). Large merged image showing colocalization of ASFV antigen with both CD172a+ and pancytokeratin. B. Tonsil of the soft palate. ASFV antigen distribution in relation to macrophages (CD163+) and crypt epithelium (pancytokeratin+). Large merged image showing colocalization of ASFV antigen with both CD163+ and pancytokeratin.

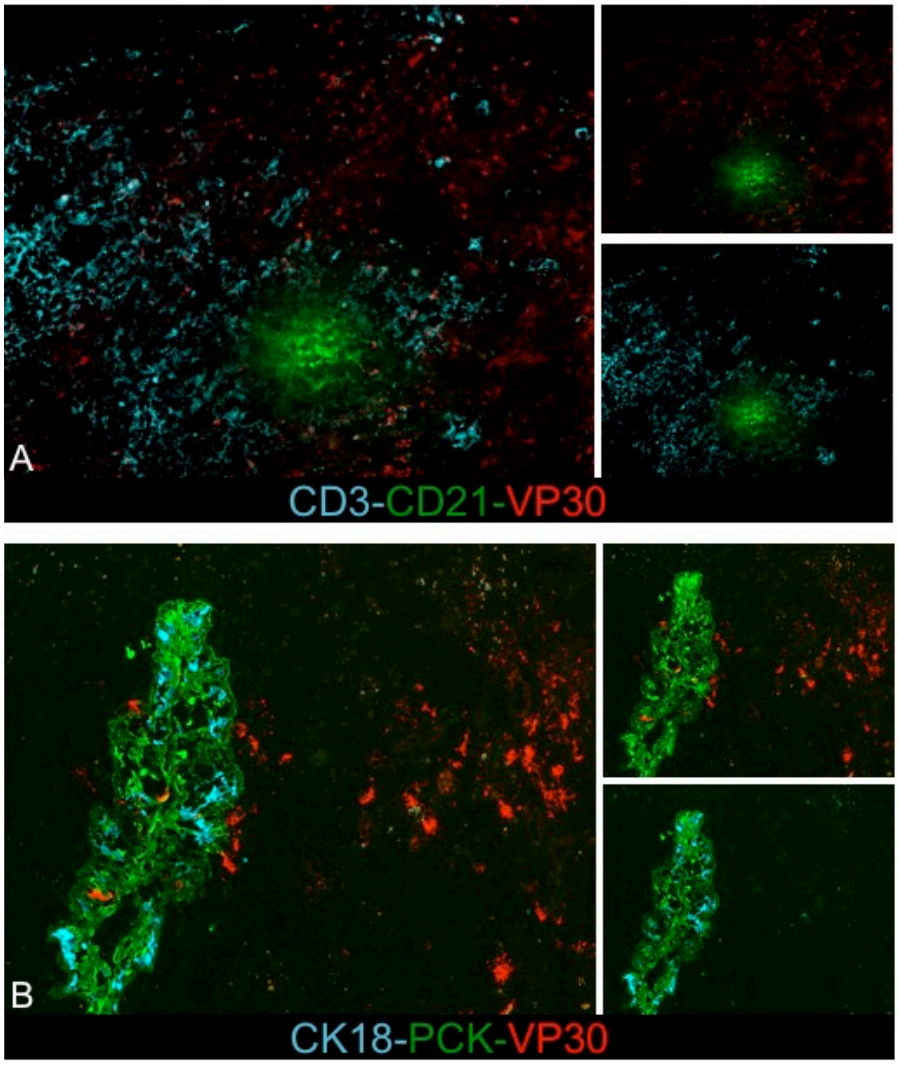

Figure 12. Phenotypic characterization of tonsillar tissue from ASFV infected pigs using Multichannel Immunofluorescence. Color coded antibody combinations described below each panel. A. Nasopharyngeal tonsil. ASFV antigen distribution in relation to T lymphocytes (CD3+) and B lymphocytes (CD21+). Large merged image showing a colocalization of ASFV antigen with CD3 but not CD21. B. Tonsil of the soft palate. ASFV antigen distribution in relation to M cells (Cytokeratin 18+) and crypt epithelium (pancytokeratin+) cells. Large merged image showing colocalization of ASFV antigen with pancytokeratin but not cytokeratin 18.

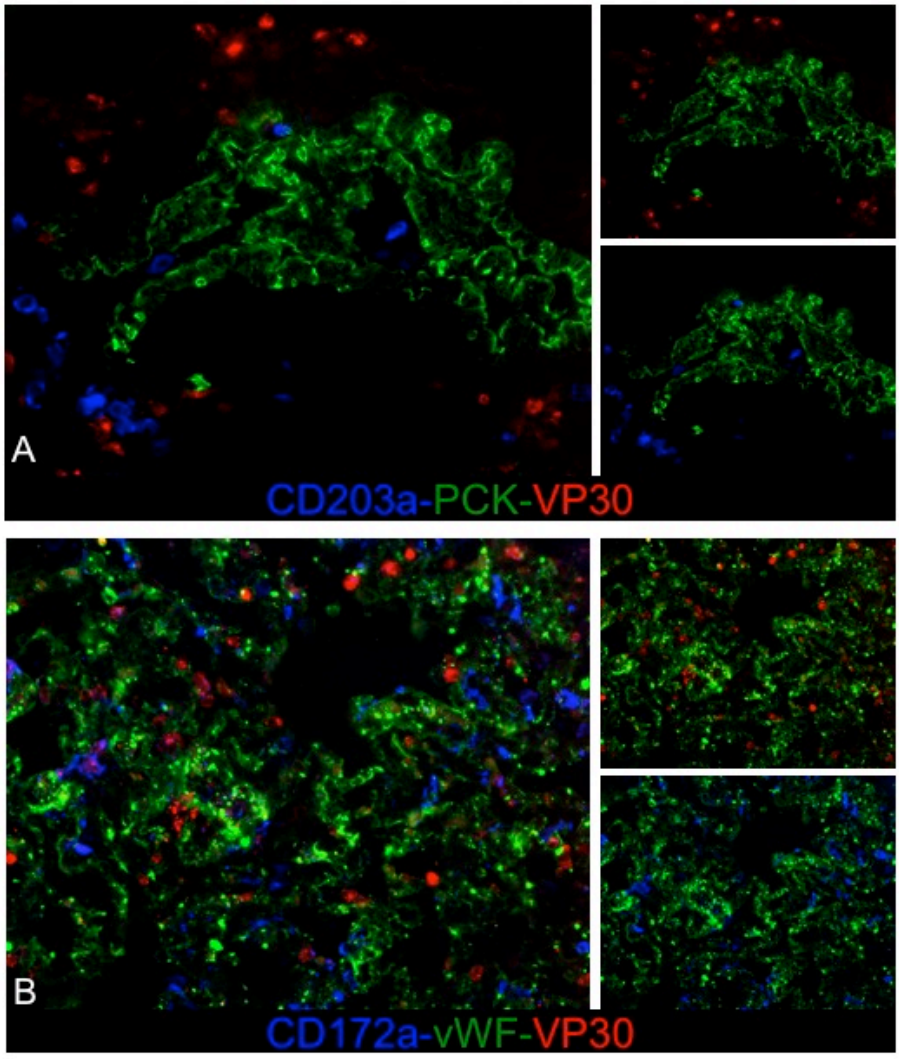

Figure 13. Phenotypic characterization of lung from ASFV infected pigs using Multichannel Immunofluorescence. Color coded antibody combinations described below each panel. A. Lung. ASFV antigen distribution in relation to alveolar macrophages (CD203a+) and bronchiolar epithelium (pancytokeratin) cells. Large merged image showing a lack of colocalization of ASFV antigen with either CD203a or pancytokeratin. B. Lung. ASFV antigen distribution in relation to alveolar capillaries (vWF+) and alveolar interstitial macrophages (CD172a). Large merged image showing colocalization of ASFV antigen with both CD172a+ and vWF.

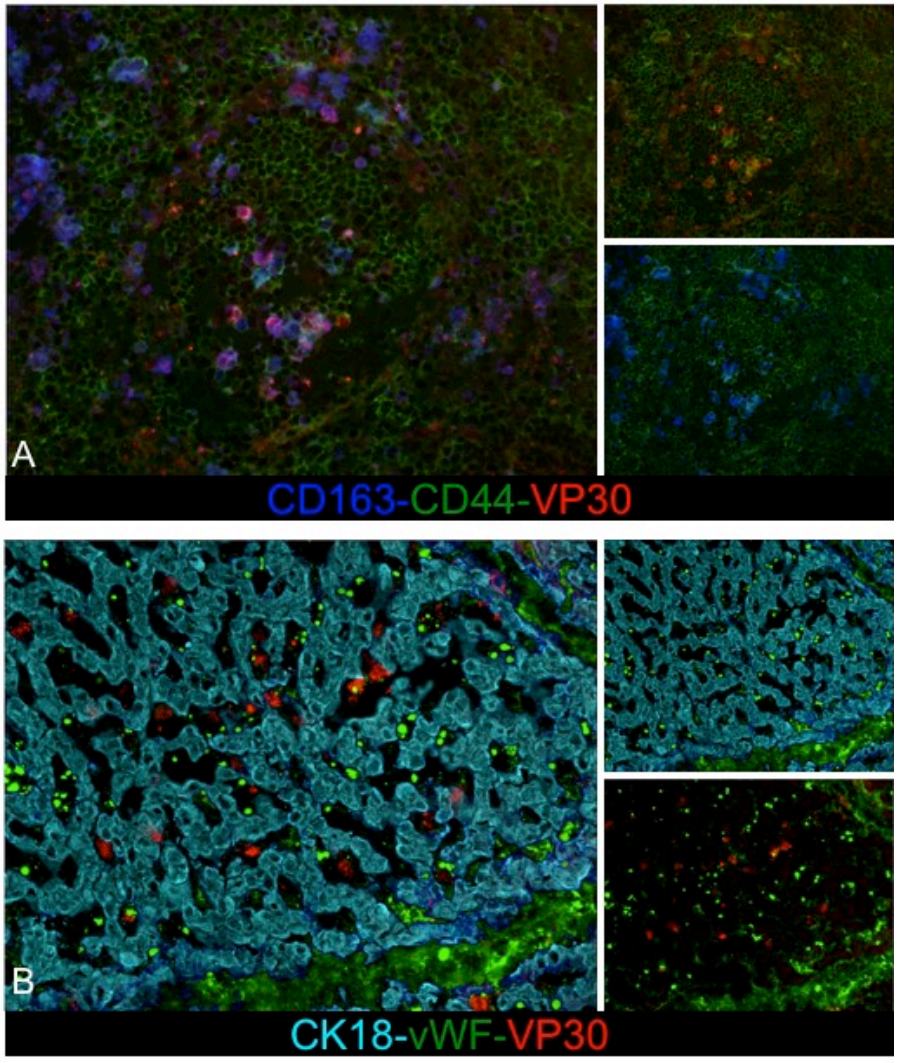

Figure 14. Phenotypic characterization of lymph node and liver from ASFV infected pigs using Multichannel Immunofluorescence. Color coded antibody combinations described below each panel. A. Gastrohepatic lymph node. ASFV antigen distribution in relation to macrophages (CD163+) and leukocyte marker (CD44). Large merged image showing colocalization of ASFV antigen with both CD163 and CD44. B. Liver. ASFV antigen distribution in relation to hepatocytes (cytokeratin 18+) and sinusoidal endothelium (vWF). Large merged image showing colocalization of ASFV antigen with occasional cytokeratin 18+ and vWF.

Discussion

The work described herein provides various novel insights to virus-host interactions in domestic pigs infected with ASFV. Highly detailed tissue-level and cell-specific characterization of the distribution of virulent ASFV is described in manners that have not previously been reported. Quantitation of viral loads was compared in numerous tissues by the two most common detection techniques: virus isolation and qPCR. Cellular tropism was examined by immunomicroscopy including markers for phenotypes of cells of monophagocyte, lymphocyte, epithelial, and vascular lineages.

Tissue localization and comparison of methods of high-throughput screening

The purpose of performing initial screening of tissues using both qPCR and virus isolation with detection by hemadsorption was twofold. The primary goal was to compare efficacy and utility of the techniques as high-throughput screening methodologies; the ancillary goal was to use the compiled comparative data to investigate novel aspects of ASFV pathogenesis. Sensitivity for both assays was similar, particularly for early time points of infection. However, translation of qPCR-generated C_t values to titer equivalents demonstrated a statistical significance between actual VI titers and the translated titers. Translated titers were commonly higher by a difference of 0.5 – 1 $\log_{10}$. This difference may be attributable to biological as well as technical factors. The basis for differential biological detection derives from the fact that the qPCR assay detects a single target DNA sequence whereas VI-positivity requires the presence of infectious virions. Thus qPCR would be expected to detect every virion-packaged DNA target plus all the targets which are incomplete or complete but unassembled. This would lead to higher translated titer equivalents compared to “true titers”. The technical explanation for the difference is related to the VI titration methodology, which results in a

relatively high limit of detection (LOD is 2.05 $log_{10}HAD_{50}$/mg) and discrete titer intervals. C_t titer equivalents, although derived from a VI standard curve, allow for continuous quantitation and lower theoretical limits of detection. By comparison, the lowest tissue titer at which ASFV antigen could be localized immunomicroscopically was 2.3 $log_{10}HAD_{50}$/mg; however, detection parameters of IHC were not compared quantitatively to VI and qPCR.

The only previremic event detected in the pathogenesis of ASF was at 2 dpi in the spleen and liver of one pig inoculated intramuscularly. Despite extensive screenings, ASFV was not localized by any technique to mucosal sites, tonsils, or primary draining lymph nodes prior to the onset of viremia. Once viremia occurred, ASFV rapidly disseminated and was generally detectable in every tissue examined. This contrasts various earlier studies which demonstrated early detection in retropharyngeal lymph node and nasal mucosa at 16 hpi (Plowright et al., 1968), mandibular and mediastinal lymph node and tonsil at 24 hpi (Heuschele, 1967), or mandibular, parotid and lateral retropharyngeal lymph nodes, tonsil, retropharygeal mucosa and spleen at 48 hpc (Greig, 1972). The limited detection at early stages within the current may be due to various differences in study design and virus strains. However, likely contributory factors include the use of macroscale titration technique utilizing larger quantities input of tissue by earlier studies and some studies use of young pigs with immature immune systems (Colgrove et al., 1969).

The early, previremic detection of ASFV in the spleen suggests a role as a site of viral amplification in early pathogenesis. The localization to CD163+ and CD172a+ cells implicates these cells as critical targets of infection. The manner by which ASFV translocated to the spleen in this animal and the events that led to establishment of viremia remain undetermined.

In the late stages of ASF, viral loads were consistently highest in the lymphohemopoetic

tissues with highest titers in bone marrow, spleen, and visceral lymph nodes. This is consistent with previous works, which have demonstrated ASFV's tropism for these tissues(Gómez-Villamandos et al., 2013; Gómez-Villamandos et al., 2003; Maurer and Griesemer, 1958). This may be due to a large quantity of susceptible resident cells and a predilection by infected cells to traffic to these tissues. The mechanism of the commonly reported predilection of ASFV for visceral lymph nodes remains undetermined. Hemorrhagic lymphadenopathy of gastrohepatic and renal lymph nodes are considered near-pathognomonic lesions for ASF (Sánchez-Vizcaíno et al., 2015).

Gastro-hepatic lymph nodes run along the hepatic vein and artery and drain the liver, gallbladder, stomach, a portion of the respiratory diaphragm and head of pancreas and duodenum in. Renal lymph nodes are located adjacent to the renal artery and primarily draining the renal cortex and subcapsular space. Both locations drain areas of filtration of the systemic circulation as well as organs which also have high viral titers. This may contribute to high viral loads entering these lymph nodes and causing predilection.

Tonsillar tissues typically had lower viral loads than the non-epithelial lymphoid tissues despite abundant microscopic localization of ASFV in tonsillar lymphoid regions. This is likely due to a dilution effect based on the current finding that the epithelial and stromal regions of tonsils support scant ASFV replication. Amongst tonsils examined, the nasopharyngeal tonsil frequently contained higher amounts of ASFV and viral DNA as compared to other tonsil sites, regardless of the route of inoculation. This suggests that the nasopharyngeal tonsil may be more important than the tonsil of the soft palate in the pathogenesis of ASFV particularly considering the permissiveness of pigs to ASFV infection via the nasopharyngeal inoculation (Howey et al., 2013).

Immunomicroscopic detection of ASFV in concert with cellular markers

In the current study, a titer of approximately 2.3 $log_{10}HAD_{50}$/mg was necessary before antigen could be definitively detected by immunohistochemistry or immunofluoresence. Although improvements in sensitivity of immunomicroscopy have occurred over the years, similarly inferior limits of detection compared to virus titration have been described in previous studies (Colgrove et al., 1969; Greig, 1972; Heuschele, 1967). Thus, the minimum threshold of ASFV for detection by immunohistochemistry is greater than the quantity required for detection by VI or qPCR.

Within all tissues and timepoints examined there was consistent predominance for localization of ASFV to cells of the mononuclear phagocytic system as is consistent with several previous studies (Colgrove et al., 1969; Fernandez et al., 1992a; Fernández de Marco et al., 2007). In comparison to the above mentioned studies, the present study is the first to further characterize ASFV infected cells beyond morphology or single immunologic staining by using a simultaneous combination of multiple cellular markers with ASFV antigen. By doing so, this study 1) gives further evidence for the *in-vivo* involvement of monophagocytic cells during ASFV infection, 2) demonstrates the heterogeneity of infected monophagocytic cells within the tissues of interest, and 3) explores how heterogeneity may contribute to the pathogenesis and tropism of ASFV infection.

In all tissues examined CD172a or CD163 had the highest prevalence of colocalization with ASFV. In pigs, CD172a is a relatively broad marker of myeloid cells including neutrophils, circulating monocytes, macrophages and dendritic cells (Alvarez et al., 2000). CD163 is a member of the scavenger receptor family located on circulating monocytes as well as inflammatory and resident tissue macrophages and has been shown to increase expression in

porcine macrophages upon activation and maturation (Sánchez et al., 1999). CD163 has previously been described as a putative receptor for ASFV (Sánchez-Torres et al., 2003) although recent works suggest that the presence of CD163 does not significantly affect macrophage permissiveness to infection (Lithgow et al., 2014).

In the current study, not all CD163 or CD172a positive cells were simultaneously positive for ASFV nor were all ASFV positive cells simultaneously positive for the two markers. This suggests that while cells with these phenotypes appear to be highly permissive to ASFV infection, these markers are not required for entry nor does their presence guarantee infection. Other factors which may affect or work in tandem with cellular expression, may influence permissiveness including cellular maturity and cytokine expression.

In the current study, ASFV colocalized minimally with Sn and CD203a. Sn (sialoadhesin), like CD163, is a member of the scavenger receptor family and is located on circulating monocytes as well as inflammatory and resident macrophages (Alvarez et al., 2014). This receptor is thought to contribute to viral entry in other monocytotropic porcine diseases (e.g. porcine reproductive and respiratory syndrome virus) (Van Gorp et al., 2008; Welch and Calvert, 2010) functioning either as coreceptor, or as an alternate to CD163. Lack of extensive colocalization of Sn and ASFV immunoreactivity in this study suggests that Sn+ macrophages are not involved in the pathogenesis of ASFV.

CD203a (SWC9) is a marker for alveolar macrophages and a subset of mature tissue macrophages (Chamorro et al., 2000; Dominguez et al., 1998). The low number of CD203a+ASFV+ cells suggests that the subpopulations of tissue macrophages which express CD203a are not as permissive to ASFV infection as other macrophage subpopulations. This indicates that alveolar macrophages, in comparison with alveolar interstitial and intravascular

macrophages, are not major targets for ASFV *in vivo* infection in pulmonary tissues. While alveolar macrophages have been used *ex vivo* for virus isolation and flow cytometry analysis of ASFV(Alcami et al., 1990; Bullido et al., 1997; Chamorro et al., 2000; Forman et al., 1983), the present findings support that within the lungs *in vivo*, intravascular and interstitial macrophages are principal targets of ASFV (Carrasco et al., 1996a; Fernandez et al., 1992b; Sierra et al., 1990).

The observed distribution of CD3+ and CD21+ cells within lymphoid tissues is consistent with expected localization of T and B lymphocytes respectively. The lack of concurrent ASFV colocalization with the respective lymphocyte markers indicates that T and B lymphocytes are not major targets of ASFV infection/replication. This is consistent with previous literature which establishes that although ASFV can replicate within lymphocytes, replication is limited in comparison to virus replication within macrophages and that the effects upon lymphocytes and lymphoid tissues represent an indirect effect upon lymphocytes (Oura et al., 1998a; Takamatsu et al., 1999; Wardley and Wilkinson, 1977; Wardley et al., 1977).

Cell-associated ASFV was infrequently identified interspersed with epithelial cells of mucosal surfaces and endothelial cells lining small vessels. In most instances, identification of these cells was most consistent with MPS cells transmigrating or resident within mucosal or vessel surfaces. However, in some instances there was colocalization with anti-VWF (endothelial cells) or anti-pancytokeratin (epithelial cells). Colocalization with CK18, an M-cell marker in pigs (Gebert et al., 1994) was never observed. Infection of endothelial cells by ASFV has been described previously although true replication is variable and thought to not be a major source of viral replication (Gómez-Villamandos et al., 1995a; Sierra et al., 1989). Infection of epithelial cells by ASFV has not been reported.

Conclusions

The work described herein has provided novel insights to virus host interactions of ASFV. Most findings are consistent with previous reports on tissue and cellular tropism of this virus. It is hoped that the anatomic locations and detailed phenotypic characterizations of ASFV-infected cells, particularly distinct sub-populations of monophagocytic cells will advance the quest for vaccine development, by elucidating which cells of the natural host must be protected. Specifically, these findings will be critical for future work aimed at establishing the functionalities of ASFV-susceptible cells in order to target and monitor these cells responses to countermeasures products. Further investigation of these findings may be pursued by validation with other strains of ASFV *in vivo* and through investigation of the cellular trends *ex vivo* in primary macrophage cultures. Such studies are already underway in the investigators' laboratory.

APPENDIX

Table 8. Appendix 1- Tissue-Specific Distribution of ASFV in Intraoropharyngeal (IOP) Inoculated Pigs[a]

	2DPI			3DPI			3DPI			4DPI			5DPI			6DPI		
	P#11			P#12			P#13			P#14			P#13			P#14		
	Euthanized			Euthanized			Euthanized			Euthanized			Euthanized			Euthanized		
Final blood/serum titer	0			0			0			0			0			0		
			C_t Titer			C_t Titer			C_t Titer			C_t Titer			C_t Titer			C_t Titer
	VI	PCR	Equivalent[b]	VI	PCR	Equivalent[b]	VI	PCR	Equivalent[b]	VI	PCR	Equivalent[b]	VI	PCR	Equivalent[b]	VI	PCR	Equivalent[b]
Lingual Tonsil	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0
Tonsil of the Soft Palate	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0
Dorsal Soft Palate (Rostral)	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0
Dorsal Soft Palate (Distal)	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0
Posterior Nasal Turbinates	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0
Nasopharyngeal Tonsil	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0
Dorsal Nasal Pharynx	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0
Ventral Epiglottis	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0
Proximal Trachea	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0
Tracheal Bifurcation	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0
Proximal Middle Lung	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0
Middle Middle Lung	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0
Distal Middle Lung	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0
Retropharyngeal Lymph Node	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0
Submandibular Lymph Node	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0
Liver	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0
Spleen	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0
Hilar Lymph Node	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0
Thyroid	0.0	NEG	0.0	0.0	NEG	0.0	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
Kidney	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0
Thymus	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0
Adrenal Gland	0.0	NEG	0.0	0.0	NEG	0.0	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
Pancreas	0.0	NEG	0.0	0.0	NEG	0.0	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
Cerebellum	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0
Cerebrum	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0
Bone Marrow	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0
Heart- Left Ventricle	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0
Gastrohepatic Lymph Node	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0
Renal Lymph Node	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0
Inguinal Lymph Node	0.0	NEG	0.0	0.0	NEG	0.0	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
Peyer's Patch	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0
Ileum	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0
Large Intestine	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0
Popliteal Lymph Node	0.0	NEG	0.0	0.0	NEG	0.0	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
Pharyngeal Diverticulum	NC	NC	NC	NC	NC	NC	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0
Prescapular Lymph Node	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0	0.0	NEG	0.0

[a]DPI, days post inoculation; DPC, days post contact; VI, virus isolation; PCR, real-time polymerase chain reaction; NEG indicates PCR negative; NC indicates sample not collected. PCR values shown are the means of two replicates. Negative tissues indicate that DNA quantity was below the detection threshold, corresponding to cycle threshold (C_t) greater than 40; Viral titer corresponds to $Log_{10}HAD_{50}$/ml or $Log_{10}HAD_{50}$/mg of tissue. [b]Statistical difference compared to VI titer (p= 0.02).

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CHAPTER 4:

Conclusions

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Conclusions

The main focus of the present dissertation was to examine aspects of the pathogenesis and pathology of experimentally induced ASF, studied by a combination of virologic, molecular and microscopic techniques in pigs challenged by different routes of infection.

Summary of findings

Four routes of challenge (IM, IOP, INP, DC) were examined. All routes resulted in a similar clinical course of ASF, consistent with previous described accounts within the literature. Gross and histologic changes in pigs in the late stages of disease were similar regardless of route of infection and were consistent with postmortem findings described in field and experimental cases of ASF. For all three inoculation routes, initial detection of ASFV shedding occurred at the same time, or briefly following onset of viremia. In contrast, shedding within DC infected pigs was detected prior to viremia.

A singular live-animal infection model ideal for use for all ASFV research was not defined. Instead, minor variations between routes suggest that during experimental planning, the chosen route of infection should be dependent on the goals of research. INP route should be chosen for the examination of early pathogenesis of the disease, specifically for the time course evaluation of immune response or infection sites. DC would be preferable when simulating a natural manner of transmission of ASFV, particularly for vaccine challenge experiments. DC is also ideal when study designs are not limited by animal numbers or time restrictions. The IM route may be beneficial for the study of late stage ASF events, when maximum incidence of infection is necessary.

It would be erroneous to assume that the model outcomes described within this

dissertation are reflective of all strains of ASFV. However, this work sets the foundation for a standardized approach to in vivo research, which would facilitate intra-strain or cross strain comparisons.

Quantitative PCR and hemadsorption were used for initial screening of tissues for ASFV and were found to be similar in sensitivity. Pre-viremic detection of virus was observed within the spleen and liver of a single IM inoculated pig. Virus was detected within all tissues at late stages of disease regardless of inoculation route. Novel aspects of this study include the first comprehensive time-course examination of tissues derived from ASFV infected animals using qPCR.

Lesions, such as myocardial infarction and necrosis, and multifocal hepatic necrosis described herein have not been extensively described in ASF literature. ASFV antigen was detected within both lesions by IHC. Myocardial lesions were most commonly associated with pigs that died spontaneously and histologically were associated with vascular thrombosis. This finding reaffirms the accepted theory that the terminal pathogenesis of ASF is disseminated intravascular coagulation. Hepatocellular necrosis was only grossly visible in the most severe cases but histologically ranged from individual hepatocellular necrosis to submassive necrosis with varying associated inflammation. While both lesions were not routinely observed in all infected animals, their presence, in conjunction the presence of viral antigen does expand the range of possible lesions associated with ASF. While the pathologic lesions described in this dissertation represent an acute form of ASF derived from a highly virulent strain, it would be worthy of investigation to examine if these novel liver and/or heart lesions manifest with other strains or forms of the disease.

This is also the first study to examine individual tonsillar tissues as anatomically distinct

regions within the scope of ASFV infection. By doing so, a higher detection of virus and viral DNA within the nasopharyngeal tonsil in comparison to tonsil of the soft palate or lingual tonsil was observed. This may represent a different role or predilection of the nasopharyngeal tonsil within the pathogenesis compared to other tonsillar tissues.

Immunomicroscopic characterization revealed infected cells within several tissues to be of monophagocytic origin, consistent with the literature. It is worthy to note that, within the lungs, alveolar interstitial and intravascular macrophages appear to be the preferred cellular subtypes for infection when compared to alveolar macrophages. This finding, along with the immunomicroscopic characterization suggests that subtypes within the phenotypically heterogeneous population of the MPS may be more permissive to infection than others. ASFV infected cells most consistently stained with CD172a, a broad monophagocytic cell marker. However not all CD172a+ cells were simultaneously positive for ASFV VP30, nor were all ASFV positive cells simultaneously positive for the marker. Within more specific subsets of monophagocytic cells, ASFV VP30 commonly colocalized with CD163, while Sn^+ and CD203a+ subsets were less likely to be ASFV VP30 positive. When examining non- monophagocytic cells types, ASFV antigen was not associated with B and T lymphocytes, M cells, or vascular endothelium. ASFV did colocalize with cells of epithelial origin, which may represent transit of the virus either out of, or into the mucosal surface.

Limitations of project

An inability to consistently detect virus within tissues at pre-viremic stages of disease was the largest limitation of this project. A few factors could have contributed to this obstacle. First, due to theoretically low amounts of virus during this phase of infection, lack of detection

could simply be due to sampling an area of an organ where replication has yet to occur. Secondly, early detection of the virus in the tissues may still be beyond the threshold of detection of current screening methods. Sensitivity of both high throughput methods (qPCR and VI) proved similar. In addition, a titer of approximately 2.3 $\log_{10}HAD_{50}$/mg was necessary before antigen could be definitively detected via immunomicroscopy. Some hypothesized alternatives could be returning to the macroscale hemadsorption technique which utilizes a large volume (1 gram) of tissue. In small trials of this technique, performed in conjunction with other projects, 1-2 $\log_{10}HAD_{50}$/g could be detected within the nasal and lingual tonsil of pigs inoculated IN with 10^4 HAD dose and euthanized at 2 and 4 days post infection prior to onset of clinical disease or viremia (data not shown). However, this technique proved to be both labor and resource intensive, making it no longer a viable method for high throughput analysis nor feasible to utilize within the scope of this project. Possible immunomicroscopic alternatives include in-situ hybridization however, this detection method has been minimally used in ASFV research and has not been evaluated specifically in low titer tissues.

Utilizing multichannel immunofluorescence to characterize ASFV infected cells *in situ* was a technique that has never before been applied in ASF research. This was a novel approach to a better classification of infected cells types, while examining their temporal, spatial and tissue-specific distribution. A limitation to this approach was the somewhat restricted availability of monoclonal antibodies for cellular markers that were: 1) reactive in pigs, yet were 2) of differing immunoglobulin isotypes that could be used in combination with each other for histologic examination. In addition, some cell types of interest are not well characterized phenotypically in pigs and therefore only non-specific antibodies are available to identify them (i.e. porcine dendritic cells). The evolving understanding of the phenotypic heterogeneity of

monocytes and macrophages may be the key to fully understanding permissiveness of infection subsequently identifying mechanisms by which to block infection; however, this aspect was merely touched on due to these restrictions.

Future aims

Ultimately, all research is performed to build upon a growing knowledge base in order to solve a question or problem. This work builds upon the understanding of the virus-host interaction of ASFV at both the tissue and cellular level. Nevertheless, additional ASFV pathogenesis research must be performed in order to make definitive conclusions. Future directions of the project would include further examination of early time points of infection of inoculated pigs in order to screen tissues for first detection of virus. Based on the high incidence of infection and uniformity of clinical disease, as described within chapter 2, INP inoculation would be the optimum model to carry out such experiments. Modifications in detection methods, as described above, could be incorporated, dependent on the scale of the experiment or number of animals involved.

Further investigation of the phenotypic characteristics of ASFV infected monophagocytic cells would require not only further immunomicroscopic examination, but the incorporation of flow cytometric analysis and functional assays. Novel approaches may include the analysis of tissue- or blood- derived PBMCs from infected pigs during the course of infection, or cultured PBMCs infected at various stages of maturation. This employs a different perspective on MPS phenotypes during infection, by longitudinally examining the stages of clinical infection, or the effects of maturation in a cross-sectional manner. A preliminary experiment was performed using a longitudinal approach to phenotypic characterization by utilizing MIF examination of

ASFV and mock-infected blood derived PBMCs over time (data not shown). Morphologic and phenotypic changes over time within mock infected cells corresponded to the routine differentiation of blood monocytes into mature macrophages, as described within the literature. Similar to tissues, there were correlations of CD163 and CD172a expression with ASFV VP30 detection however, ASFV infection was not strongly confined to one phenotype. As the general understanding of porcine MPS expands, this knowledge should likewise be applied to ASFV research.