Development of a chromatographic lateral flow immunoassay for detection of African swine fever virus antigen in whole blood

by

Daniel Madden

B.S., Kansas State University, 2011
D.V.M., Kansas State University, 2016

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Diagnostic Medicine/Pathobiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2021

Approved by:

Major Professor
Dr. Jürgen A. Richt

Copyright

Abstract

African swine fever (ASF) is a highly lethal viral disease of domestic and wild pigs actively circulating in Africa, Europe, and Asia, causing millions of animal deaths and billions of dollars in economic losses. No effective vaccine exists for ASF, which limits control efforts to animal movement restrictions and culling affected herds. Rapid detection of African swine fever virus (ASFV) is essential for disease mitigation, and there is an unmet need for pen-side diagnostic tests with sufficient specificity and sensitivity. Using monoclonal antibodies targeting the ASFV protein p30, we developed a chromatographic lateral flow immunoassay (LFIA) for detection of ASFV in anticoagulated whole blood. The assay is field-deployable, requiring only water and providing results in 25 minutes. The LFIA was capable of detecting a genotype I and a genotype II strain of ASFV in EDTA blood from experimentally-infected pigs at varying time-points after challenge. Diagnostic sensitivity correlated with clinical disease severity, body temperature, and viral DNA levels, and was over 90% in animals with moderate to severe ASF-related symptoms. The LFIA also possessed a robust specificity of 98.7%, an important attribute for a rapid test deployed in outbreak scenarios where false positives and negatives can produce highly disruptive and wide-ranging countermeasures. Additional testing using field samples is required to further assess the viability of the assay as a diagnostic tool.

Table of Contents

List of Figures vi
List of Tables vii
Acknowledgements viii
Chapter 1 - Literature Review 1
African Swine Fever 1
Clinical Features 1
Transmission 2
Epidemiology 6
Control of ASF 7
African Swine Fever Virus 10
Taxonomy and Classification 10
Virion Structure 11
Infection and Pathogenesis 13
ASFV Protein p30 15
Diagnostic Assays for ASF 16
Molecular Detection 16
Serological Detection 18
Virus and Antigen Detection 22
Lateral Flow Immunoassays 24
Components of LFIAs 25
Applications for LFIAs 26
Purpose of Research 27
Figures and Tables for Chapter 1 28
Chapter 2 - Materials and Methods 32
Viruses and cells 32
Expression and purification of recombinant ASFV p30 33
Generation of monoclonal antibodies, screening by indirect immunofluorescence assay (IFA), and isotyping 34
Experimental infections and blood collection 35

Evaluation of whole blood samples by quantitative real-time PCR 37
LFIA testing of experimental samples 37
Statistical Analysis 38
Figures and Tables for Chapter 2 40
Chapter 3 - Results 42
Reactivity, specificity, and isotyping of anti-ASFV p30 monoclonal antibodies 42
Experimental challenge, clinical sampling, and qPCR 42
Sensitivity, specificity, and reproducibility of the LFIA 43
Figures and Tables for Chapter 3 47
Chapter 4 - Discussion and Conclusions 53
Figures for Chapter 4 58
References 59

List of Figures

Figure 1.1 Transmission cycles for ASFV 28
Figure 1.2 Distribution of ASFV genotypes in continental Africa and Madagascar 29
Figure 1.3 Diagram of a lateral flow immunoassay (LFIA) 30
Figure 2.1 Overview of LFIA components 40
Figure 3.1 IFA screening of monoclonal antibodies 47
Figure 4.1 p30 protein sequence alignment for Georgia 2007/1, E70, and Ken05/Tk1 ASFVs .. 58

List of Tables

Table 1.1 Diagnostic methods for ASF 31
Table 2.1 Overview of ASFV challenge experiments and sample collection 41
Table 3.1 Pre-challenge ASFV-negative samples tested by LFIA and qPCR 48
Table 3.2 Armenia 2007 post-challenge samples 49
Table 3.3 E70 and Ken05 post-challenge samples 50
Table 3.4 Pre- and post-challenge specificity and sensitivity for LFIA and qPCR 51
Table 3.5 Performance of the ASFV LFIA vs qPCR 52

Acknowledgements

I would first like to extend my thanks to my graduate mentor, Dr. Jürgen Richt, for giving me the opportunity to work in his laboratory and providing me with the resources and guidance needed to conduct my research. Working for him is far and away the best experience I have had in the College of Veterinary Medicine. I would also like to acknowledge my other graduate committee members. Dr. Bonto Faburay was the first person I worked with in a laboratory setting, and nearly every basic laboratory technique I learned by his teaching. Dr. Natasha Gaudreault taught me how to function competently in a BSL-3 laboratory setting and was always willing to answer my questions, no matter how trivial. Without these individuals, none of this work would have been possible.

I would also like to thank the entire Richt laboratory group for providing a collegial and professional work atmosphere and for their willingness to assist me with my work. I especially want to thank Dr. Chester McDowell for being an excellent co-worker and a better friend, and Dr. Jessie Trujillo for her willingness to critique my methods and guide my research.

The African swine fever viruses used in this research were generously provided by Dr. C. Gallardo at the European Union Reference Laboratory for African Swine Fever. The lateral flow immunoassay tests were provided by Silver Lake Research Corporation.

Finally, I would like to extend my sincerest thanks to my fiancé Dr. Bianca Libanori Artiaga and our two cats, Dr. Peanut McHandsomepants III and The Right Honourable Cashew von Booplesnoot. Without your support, I would not have made it through graduate school.

Chapter 1 - Literature Review

African Swine Fever

African swine fever (ASF) is a highly contagious viral disease of domestic pigs and wild boar caused by African swine fever virus (ASFV), which is actively circulating in large areas of Africa, Europe, and Asia. ASF outbreaks are severely disruptive to livestock production and trade, and are associated with significant economic losses in affected areas. The propensity for ASF to spread to new geographic areas, coupled with the lack of an available vaccine, make ASF a significant concern for domestic and global pork producers.

Clinical Features

Infection with ASFV can produce a wide array of clinical manifestations ranging from chronic or subclinical disease states to peracute hemorrhagic fever with mortality rates approaching 100%, depending on viral strain and host susceptibility (Blome et al., 2013). Virulent ASFV strains such as the currently spreading Eurasian genotype II isolates produce a rapidly progressive disease characterized by high fever, lethargy, digestive symptoms, erythema, cyanosis, respiratory distress, ataxia, and death (Blome et al., 2013; Gallardo et al., 2017). Hematological abnormalities consistent with disseminated intravascular coagulation (DIC) including petechiae, epistaxis, and prolonged clotting times, can be observed with highly virulent ASFV isolates (Villeda et al., 1993a+b; Villeda et al., 1995; Blome et al., 2013). European wild boar are highly susceptible to virulent genotype II ASFV isolates currently circulating in Eurasia, developing clinical disease with high mortality which mirrors that seen in domestic swine (Gabriel et al., 2011; Blome et al., 2012; Pikalo et al., 2020). ASFV strain diversity can also include low-virulence phenotypes which induce non-clinical or mild, chronic disease states. The non-hemadsorbing genotype I strains OUR T88/3 and OUR T88/4, isolated from *Ornithodoros*

erraticus ticks in Portugal, produced no obvious clinical signs in experimentally challenged pigs other than transient fever in one animal (Boinas et al., 2004). Experimental infection of pigs with another non-hemadsorbing genotype I isolate, NH/P68, resulted in infections with either no symptoms of illness or only a mild, chronic disease state characterized by delayed-onset mild transient fever, skin lesions, and joint swelling (Leitão et al., 2001). Naturally-resistant domestic swine have been previously reported in northern Mozambique based on the prevalence of circulating antibodies; however, this resistance was not inherited by offspring as documented in an experimental challenge study, suggesting factors other than genetics may be involved (Penrith et al., 2004). ASFV has been isolated from wild African suids including warthogs, bushpigs, and giant forest hogs. These animals do not develop apparent clinical disease with ASFV infection, even after experimental challenge with isolates virulent to domestic pigs (Thomson et al., 1980; Anderson et al., 1998). The epidemiological relevance of wild African swine species is discussed in the next section.

Transmission

ASFV is the only known DNA arbovirus and can be spread via multiple transmission cycles involving domestic pigs, wild suids, and soft tick vectors belonging to the *Ornithodoros* genus (Figure 1.1). The relative importance of these cycles is geographically variable and is dependent on factors including vector distribution, domestic and wildlife host density, and regional pork production and trade practices.

In Southern and Eastern Africa, ASFV is maintained in the environment through a sylvatic cycle involving transmission between *Ornithodoros porcinus porcinus* (also referred to as *O. moubata*) soft ticks and warthogs (*Phacochoerus africanus*) (Jori and Bastos, 2009; Costard et al., 2013; Jori et al., 2013). *O. porcinus porcinus* ticks harboring ASFV were first

identified in warthog burrows in the 1960s (Plowright et al., 1969). Soft ticks in warthog burrows become infected after feeding on juvenile warthogs with sufficiently high levels of viremia, and infected soft ticks transmit the virus to uninfected warthogs upon taking a bloodmeal. Warthogs remain asymptomatic after infection and there is little evidence to support direct transmission of the virus to other warthogs or domestic swine; however infected soft ticks can be physically carried by warthogs to farms, where they can subsequently transmit the virus to domestic pigs (Thomson et al., 1980; Thomson, 1985; Jori and Bastos, 2009; Costard et al., 2013). Within the tick life cycle, the virus is transmitted sexually, transovarially, and transstadially (Plowright et al., 1970; Plowright et al., 1974; Hess et al., 1989; Rennie et al., 2001; Kleiboeker and Scoles, 2001). *Ornithodoros* ticks live for many (up to 20) years and can maintain the virus for very long periods, making them an important reservoir of environmental ASFV persistence (Costard et al., 2013; Kleiboeker et al., 1998; Pérez-Sánchez et al., 1994; Plowright et al., 1969; Plowright et al., 1970). Other wild African suids are capable of being infected with ASFV. Bushpigs (*Potamochoerus larvatus*) can be infected with ASFV and the virus has been isolated from these animals, though its prevalence in this species appears to be significantly lower than in warthogs (Jori and Bastos, 2009). Bushpigs were shown to develop viral loads roughly 2-log lower than domestic pigs in blood, spleen, and tonsil after intramuscular challenge with a virulent ASFV isolate from Malawi, indicative of decreased susceptibility to the virus; this is consistent with the lack of clinical signs associated with ASFV infection in this species (Oura et al., 1998). Experimental transmission from infected bushpigs to domestic swine has been demonstrated for the virulent genotype VIII Malawi Lil20/1 ASFV strain but did not occur with the less virulent genotype I VIC T90/1 virus; transmission between bushpigs did not occur with either strain, and infected pigs did not transmit the virus by contact to uninfected bushpigs under experimental

conditions (Anderson et al., 1998). Infected bushpigs with sufficiently high viremia were able to transmit ASFV to *Ornithodoros* soft ticks allowed to feed on the animals in the same study (Anderson et al., 1998). However, bushpigs are nocturnal, do not burrow, and show lower population densities than warthogs, which restricts their potential interactions with domestic pigs and soft ticks, and they are not considered a major environmental risk for ASFV (Costard et al., 2013). While ASFV has also previously been isolated from a giant forest hog (*Hylochoerus meinertzhangeni*), the role of this species in the ecology of ASFV is negligible (Heuschele and Coggins, 1965; Jori and Bastos, 2009; Costard et al., 2013).

ASFV transmission can also occur between *Ornithodoros* soft ticks and domestic pigs, and has been documented in Africa, Madagascar, and the Iberian Peninsula (Haresnape et al., 1988; Haresnape et al., 1989; Kleiboeker et al., 1998; Boinas et al., 2004; Ravaomanana et al., 2010; Costard et al., 2013). *Ornithodoros erraticus* soft ticks, a species native to Portugal and Spain, were identified as a biological vector for ASFV in 1963, and *O. erraticus* ticks played an important role in the maintenance of the virus in the Iberian Peninsula; they were responsible for sporadic ASF outbreaks in the region as late as 1999 (Pérez-Sánchez et al., 1994; Basto et al., 2006a; Boinas et al., 2011; Costard et al., 2013; Boinas et al., 2014). Other *Ornithodoros* species, including the ones from the Americas, have been experimentally demonstrated to be competent for ASFV replication (Golnar et al., 2019). The highly virulent Georgia 2007/1 strain has been shown to replicate in experimentally-infected *O. erraticus* ticks (Diaz et al., 2012). However, there is little evidence supporting a major role for soft ticks in the current Eurasian epizootic; serological testing of wild boar in Germany did not reveal the presence of antibodies against *O. erraticus* antigen, suggesting widespread feeding on wild boar by these ticks is uncommon, and

soft ticks are uncommon in much of Central Europe and the Baltic (Pietschmann et al., 2016; Frant et al., 2017).

ASFV is highly infectious to domestic (*Sus scrofa domesticus*) and wild (*Sus scrofa*) pigs, and is readily transmitted within and between these species without its arthropod vector. ASFV can be spread between pigs by direct contact and indirectly through blood, feces, and body fluids from infected animals; evidence of limited aerosol transmission under experimental settings also exists (Costard et al., 2013; Guinat et al., 2016a+b; Olsen et al., 2017). Transmission efficiency is variable between high- and low-virulence strains (Gallardo et al., 2015; Guinat et al., 2016a+b). Wild boar provide an environmental reservoir for ASFV maintenance and geographic spread, and the virus has been isolated from these animals in Central and Eastern Europe, and recently as far west as Belgium and Germany (Costard et al., 2013; Linden et al., 2018; Cwynar et al., 2019; Sauter-Louis et al., 2020). Contact between wild boar and domestic swine occurs due to inadequate farm fencing and biosecurity. These features are common in small-scale swine operations that are widespread in parts of Eastern Europe and the Russian Federation, and this has played a significant role in the epidemiology and spread of ASFV in these areas as well as in Asia (Gogin et al., 2013).

Due to the environmental stability of ASFV, the virus is also capable of being efficiently transmitted indirectly via fomites, contaminated feed, and pork products (Costard et al., 2013). ASFV has been shown to remain stable in feed ingredients under conditions mimicking trans-Atlantic shipping, and infection of pigs via contaminated feed has been experimentally demonstrated (Dee et al., 2018; Stoian et al., 2019; Niederwerder et al., 2019). The illegal movement of pigs and pork products is a significant biosecurity threat and has been an important

factor in the emergence and spread of the virus in Europe and Asia (Gogin et al., 2013; Zhou et al., 2018).

Epidemiology

ASF was first recognized as a distinct viral disease of swine in 1921 following an outbreak in Kenya (Montgomery, 1921). ASF is endemic in a multitude of countries in Southern and Eastern Africa, as well as parts of West Africa (Costard et al., 2013; Jori et al., 2013; Penrith et al., 2013; Gaudreault et al., 2020). While the warthog-tick sylvatic cycle has been documented in Southern and Eastern Africa as a persistent environmental reservoir for the virus and a source of sporadic outbreaks, in West Africa there is little evidence to support a significant role for arthropod-borne transmission, and pig to pig transmission is the primary route by which the virus is maintained and spread (Penrith et al., 2009; Penrith et al., 2013).

Outside Africa, outbreaks of ASF have occurred in Europe, Asia, and the Americas (Costard et al., 2013). ASFV was first identified outside of Africa in Portugal in 1957, where a genotype I virus was found and subsequently spread throughout the Iberian Peninsula and into France, Belgium, The Netherlands, Italy, and Malta (Sánchez-Vizcaíno et al., 2013; Cwynar et al., 2019). In 1971, ASF was reported in Cuba, most likely after being introduced from Spain, and subsequently spread to multiple other Caribbean nations; by 1978, ASF was identified in Brazil (Costard et al., 2009). Subsequent eradication efforts were able to eliminate the disease from these areas, with the exception of the island of Sardinia, where the disease remains endemic to this day (Lyra et al., 1986; Costard et al., 2009; Moura et al., 2010; Costard et al., 2013). Eradication efforts in the 20th century in Spain and Portugal were complicated by the presence of the *O. erraticus* soft tick vector, which maintained the virus in the environment and was a source

of sporadic outbreaks (Pérez-Sánchez et al., 1994; Boinas et al., 2011; Boinas et al., 2014). Methods to control and mitigate the spread of ASF are discussed later.

In 2007, a highly virulent genotype II ASFV isolate emerged in the Republic of Georgia, likely as a result of feeding contaminated pork products to domestic pigs (Rowlands et al., 2008). Subsequently, ASF rapidly spread through the Caucasus region, the Russian Federation, and Eastern Europe, facilitated in part by the ubiquity of susceptible Eurasian wild boar in these regions, poor biosecurity practices on small backyard pig operations, and feeding of contaminated, untreated swill to pigs (Costard et al., 2013; Gogin et al., 2013; Oganesyan et al., 2013). ASFV has been detected in wild boar as far west as Germany and Belgium, and remains a significant threat to pork production in Western Europe (Linden et al., 2018; Sauter-Louis et al., 2020). Additionally, ASFV was identified in China, the world's largest pork producer, in 2018, ushering in a devastating new phase of this epizootic (Zhou et al., 2018). ASFV has rapidly spread through southern and eastern Asia and has been identified in Mongolia, Laos, Vietnam, Cambodia, Myanmar, North and South Korea, Indonesia, the Philippines, and Timor-Leste, inflicting catastrophic losses with millions of animal deaths occurring (Lu et al., 2020; Gaudreault et al., 2020).

Control of ASF

The lack of an available vaccine or treatment coupled with the high transmissibility of the disease means control and mitigation strategies for ASF revolve around prompt detection, restricting the movement of pigs and pork products, improving on-farm biosecurity practices, and culling affected animals and herds (Sánchez-Vizcaíno and Heath, 2019; Beltrán-Alcrudo et al., 2017). ASF is a World Organization for Animal Health (OIE) listed notifiable disease and should be reported promptly.

The legal and illegal movement of infected animals and contaminated pork products has been the major contributing factor to many past and ongoing outbreaks of ASF, including the current epizootic in Eurasia (Costard et al., 2013; Gogin et al., 2013; Oganesyan et al., 2013; Zhou et al., 2018; Lu et al., 2020). Consequently, culling of infected herds and strict isolation of herds in outbreak areas, along with restricting the movement of pork products and the practice of swill feeding which are potential sources of infection, are essential. These measures have been successful in controlling and eliminating past outbreaks in Western Europe, Brazil, and affected Caribbean islands (Penrith et al., 2013; Lyra et al., 1986; Moura et al., 2010). Shortly after the identification of ASF in Brazil in 1978, a robust and multi-faceted governmental response was initiated which rapidly communicated detection of the outbreak to neighboring countries and established prohibitions on animal movement, swill feeding, and events such as livestock fairs and exhibitions which could easily facilitate spread of the virus and disease endemicity. Compensation was paid to producers whose animals were culled, and special education and incentives for improving on-farm biosecurity and animal health practices were provided (Lyra et al., 1986; Moura et al., 2010). By 1984, ASF was successfully eradicated from Brazil (Moura et al., 2010). A similar method of culling combined with monetary compensation for producers was used in Western Europe following the emergence of ASF there in 1957, though complete eradication from the Iberian Peninsula took over 30 years due to the presence of vector-competent soft ticks (Penrith et al., 2013).

Improving farm biosecurity and animal health practices is important for ASF control, especially in areas where the complete eradication of the disease may not be possible due to tick and wildlife reservoirs. Adequate fencing serves as a barrier to contact between domestic pigs and wildlife, which has been a contributing factor to the ongoing epizootic in Eastern Europe

(Gogin et al., 2013). In areas where virus transmission by soft ticks is known to occur, adequate animal housing can reduce the likelihood of disease transmission. Shelters with earthen floors and crevices provide an environment where ticks can become established and thrive, and these should be avoided when possible. Additionally, pig housing should not be reused following an ASF outbreak, even if several years have passed, and contaminated housing should be burned. *Ornithodoros* ticks can be resilient to chemical acaricide treatment, and their long lifespan means they can survive in animal housing and transmit ASFV for substantial periods after becoming infected, even when pigs have not been present for years (Boinas et al. 2011; Penrith et al. 2013). ASFV is susceptible to multiple disinfectants such as sodium hypochlorite and quaternary ammonium compounds, which can minimize spread by fomites and mechanical vectors (Shirai et al., 2000; Krug et al., 2011). Feeding untreated swill, which is inexpensive and convenient, is a major biosecurity hazard and should be avoided.

Active cooperation by livestock owners is a necessary requirement for successful implementation of disease control efforts. Monetary compensation for owners of culled swine herds was a component of the successful ASF eradication efforts in Brazil and Western Europe. This strategy is useful because it 1) mitigates the severe economic devastation farmers suffer during outbreaks, and 2) encourages farmers to report incidents of suspected disease (Lyra et al., 1986; Moura et al., 2010; Penrith et al., 2013). Access to animal health resources and education of producers regarding disease prevention and recognition have also proven useful in combating ASF (Lyra et al., 1986; Moura et al., 2010). Importantly, authorities must take into account the different economic dynamics governing subsistence versus large-scale intensive animal agriculture, including factors such as access to loans for farm improvements, labor requirements,

and variability in available animal health services, and adapt disease countermeasures to fit these situations (Penrith et al., 2013).

African Swine Fever Virus

Taxonomy and Classification

ASFV is a large enveloped double-stranded DNA virus and the sole member of the genus *Asfivirus* in the *Asfarviridae* family, with a genome ranging in size from 170 to 190 kilobase pairs (kbp) depending on virus strain (Chapman et al., 2008; Dixon et al., 2013). ASFV is the only known DNA arbovirus, with *Ornithodoros* spp. serving as the arthropod vector. The ASFV genome encodes over 150 open reading frames (ORFs), though a substantial portion of the ASFV proteome is not structurally or functionally characterized (Dixon et al., 2013; Alejo et al., 2018; Keßler et al., 2018). Significant variability in genome size can exist between ASFV strains, primarily due to differences in the large multi-gene families (MGFs) within the viral genome (Chapman et al., 2008).

ASFV is classified into genotypes based on variability in a 478 base pair region coding for the carboxy-terminus of the viral p72 protein (Bastos et al., 2003), though other genetic elements have also been used to further discriminate between isolates (Nix et al., 2006; Gallardo et al., 2009a). Twenty-four genotypes of ASFV have been identified based on p72 sequence as of 2018 (Quembo et al., 2018). While ASFV is endemic throughout much of Africa, the greatest diversity of ASFV genotypes is seen in Southeast Africa, where pig-to-pig and sylvatic transmission cycles are present and overlap (Bastos et al. 2003; Lubisi et al. 2005; Boshoff et al. 2007; Penrith et al. 2013; Mulumba-Mfumu et al. 2019; Penrith et al. 2019; Bisimwa et al. 2020). All known ASFV genotypes can be found in this region, while genotype I ASFV is predominate in West Africa and genotype II in the island nation of Madagascar (Figure 1.2).

Outside Africa, genotype I ASFV remains endemic in the island of Sardinia. Genotype II viruses are responsible for the ongoing Eurasian epizootic which began in the Caucasus region in 2007 (Beltrán-Alcrudo et al., 2017). While p72 genotyping is commonly used in ASFV research, it is a poor predictor of strain virulence, and its utility is primarily limited to phylogenetic and epidemiologic analyses (Malogolovkin et al., 2015a; Beltrán-Alcrudo et al., 2017).

In addition to genotyping, ASFV can be classified into distinct serotypes based on hemadsorption-inhibition (HAI). Hemadsorption is mediated by the viral CD2-like protein (CD2v) and C-type lectin, and these proteins dictate serological specificity (Borca et al., 1994; Galindo et al., 2000; Malogolovkin et al., 2015b). A total of 8 serotypes have been identified by HAI. Multiple ASFV serotypes can exist within the same p72 genotype, different genotypes of viruses can be of the same serotype, and some serotypes are dominated by viruses of a specific p72 genotype (Malogolovkin et al., 2015a). Serotype-specific antibodies against CD2v and C-type lectin proteins have been shown to play a role in immune protection against homologous viruses in vaccine-challenge experiments using CD2v/C-type lectin chimeric virus constructs, suggesting these antigens are important targets for broadly-protective vaccine development efforts (Burmakina et al., 2016). Not all ASFV isolates possess the hemadsorbing phenotype, which is a limitation of classification by this method.

Virion Structure

ASFV is a large, enveloped virus with an icosahedral capsid averaging approximately 200nm in diameter (Carrascosa et al., 1984; Salas and Andrés, 2013). The virus is structurally complex. Previous studies of ASFV virions purified by Percoll gradient and resolved using 2D gel electrophoresis showed 54 discrete structural proteins, though a small number of these were identified as host-derived (Esteves et al., 1986). More recently, detailed analysis of purified

virions by mass spectrometry identified 68 ASFV polypeptides, along with 21 host cell proteins (Alejo et al., 2018). Protein expression within ASFV-infected host cells has also been evaluated by mass spectrometry, which identified 94 ASFV proteins, 28 of which were classified as structural (Keßler et al., 2018). In both of these recent studies, over 1/3 of the viral polypeptides identified were classified as having an unknown function, and much of the structural biology of ASFV remains to be elucidated.

Structurally, the ASFV virion consists of five distinct concentric regions, which are (outside to inside) the external envelope, viral capsid, inner envelope, core shell, and nucleoid region (Carrascosa et al., 1984; Salas and Andrés, 2013). The external envelope is derived from the host cell membrane during budding of the virus (Carrascosa et al., 1984). CD2v, the viral membrane protein involved in hemadsorption, has been firmly identified as being in the outer envelope (Borca et al., 1994; Alejo et al., 2018). The icosahedral capsid consists of roughly 2000 capsomeres and is primarily made of p72 protein, with protein p49 and PE120R being minor components (Carrascosa et al., 1984; Salas and Andrés, 2013; Alejo et al., 2018). p72 is an important viral antigen and is the target of several diagnostic assays, which will be discussed later. The inner membrane contains several viral proteins including p54, which is another antigen frequently targeted by serology, as well as p12, a protein involved in virus attachment which was originally believed to be located in the outer membrane (Carrascosa et al., 1993; Alejo et al., 2018). Below the inner membrane is the core shell, which is composed of a variety of proteins derived from proteolytic cleavage of the precursor polyproteins pp62 and pp220 (Simón-Mateo et al., 1993; Simón-Mateo et al., 1997; Andrés et al., 1997; Andrés et al., 2002). Polyprotein-derived core shell proteins include p5, p8, p14, p15, p34, p35, p37, and p150; together, these proteins are estimated to constitute roughly half of the total virion mass (Alejo et al., 2018). The

innermost region of the virus is the nucleoid, which contains the viral genome, DNA binding proteins, and multiple transcription factors (Salas and Andrés, 2013).

In addition to these structural elements, ASFV possesses an abundance of nonstructural proteins with a diverse array of functions involving genome replication and repair, transcription, nucleotide metabolism, host-cell interaction, and immune evasion (Dixon et al., 2013). Direct functional data for a significant portion of these proteins is unavailable and their putative role in ASFV biology is surmised from structural homology to other well-characterized proteins. Viral proteins which modulate host-cell responses are discussed more thoroughly in the next subsection.

Infection and Pathogenesis

ASFV primarily targets cells of the monocyte/macrophage lineage in swine (Gómez-Villamandos et al., 2013). Following infection by oral-nasal exposure, ASFV replicates in the tonsils and local lymphoid tissue then subsequently spreads via viremia, with significant viral titers in the blood; virus replication is associated with lesions in the spleen, liver, lungs, and other organs with substantial resident macrophage populations in later stages of the infection (Blome et al., 2013; Gómez-Villamandos et al., 2013). The precise mechanisms governing the tropism of ASFV for monocytes and macrophages are not entirely understood. While there is evidence of receptor-mediated virus uptake by macrophages, no specific cellular receptor for ASFV has been identified yet. Furthermore, non-selective uptake by macropinocytosis has been implicated in ASFV cell entry, and the virus is also capable of infecting cells outside the monocyte/macrophage lineage (Sánchez et al., 2013; Gómez-Villamandos et al., 2013; Alonso et al., 2013; Galindo and Alonso, 2017; Gaudreault et al., 2020). Fc-receptor mediated uptake has been hypothesized as a potential pathway for ASFV entry into macrophages based on

observations from vaccine-challenge experiments that induction of ASFV-specific antibodies can enhance infectivity *in vitro* and *in vivo*, and even cause an accelerated clinical course of disease in vaccinated animals suggestive of antibody-dependent enhancement of disease (Argilaguet et al. 2011; Blome et al. 2014; Sunwoo et al. 2019).

Infection of macrophages by ASFV triggers the release of large quantities of pro-inflammatory cytokines, which is an underlying mechanism for ASFV virulence. This pro-inflammatory cascade produces significant vascular endothelial disruption and consumption of clotting factors, resulting in coagulopathy and lesions characteristic of the hemorrhagic disease associated with virulent ASFV infections; importantly, differences in cytokine expression profiles between high-, moderate- or low-virulence strains have been observed (Blome et al., 2013; Gómez-Villamandos et al., 2013).

A number of ASFV proteins have been identified which modulate or counteract host immune responses and contribute to ASFV virulence. ASFV suppresses antiviral host responses through a variety of mechanisms, including the disruption of the cGAS-STING and toll-like receptor 3 signaling pathways to prevent type I interferon (IFN) production, by blocking production of immunoregulatory gene products at the transcriptional level, and by inhibiting apoptosis (Afonso et al., 2004; Dixon et al., 2013; de Oliveira et al., 2011; Golding et al., 2016; García-Belmonte et al., 2019; Gaudreault et al., 2020). The capacity of ASFV to suppress antiviral host responses is associated with virus virulence. For example, the cGAS-STING pathway resulting in IFN-β induction is inhibited in cells infected with the highly virulent Armenia 2007 virus but not with the attenuated NHV/P68 strain (García-Belmonte et al., 2019). MGF360 and MGF505/530 both contain genes associated with counteracting antiviral host responses, and deletions of genes within these multigene families has been shown to attenuate

certain virulent ASFV strains (O'Donnell et al., 2015; O'Donnell et al., 2016; Reis et al., 2016). MGF regions of the viral genome are highly variable between virulent and attenuated ASFV strains, suggesting that these immunomodulatory genes are significant contributors to the disease process and clinical outcomes associated with infection (Chapman et al., 2008).

ASFV Protein p30

p30 (also referred to as p32) is a membrane phosphoprotein encoded by the viral CP204L gene (Afonso et al., 1992; Prados et al., 1993). The protein is abundantly expressed early in the viral infection cycle, and can be detected in infected macrophages and extracellular culture supernatant as early as 2 hours post-infection (Tabarés et al., 1980; Afonso et al., 1992; Prados et al., 1993). p30 is also highly antigenic, and antibodies to the protein are among the first to be detectable in serum after ASFV infection, making it an attractive target for serological diagnostic assays (Tabarés et al., 1980; Pastor et al., 1989; Alcaraz et al., 1990; Pérez-Filgueira et al., 2006; Cubillos et al., 2013; Giménez-Lirola et al., 2016).

Previous studies have demonstrated that p30 is involved in viral entry into the cell. Treatment of Vero cell and porcine alveolar macrophage cultures containing bound but non-internalized ASFV with anti-p30 monospecific sera from immunized pigs showed anti-p30 sera treatment was able to block virus internalization by over 95%, suggesting p30 has a fundamental role in this process (Gómez-Puertas et al., 1996). Pre-treatment of macrophage cultures with recombinant p30 was also able to inhibit virus internalization in a dose-dependent manner, further supporting this hypothesis (Gómez-Puertas et al., 1998). Despite this, p30-specific antibodies alone are not sufficient to provide protection against ASFV challenge, suggesting virus internalization can also occur via p30-independent means (Gómez-Puertas et al., 1998; Escribano et al., 2013).

Diagnostic Assays for ASF

Diagnostic tests for ASF can be broadly classified into three categories: 1) molecular diagnostics for detection of ASFV DNA, 2) serological tests for detection of ASFV-specific antibodies, and 3) assays for detection of virus/antigen (Table 1.1). A variety of assays in each category are validated as fit for purpose by the OIE. However, limitations such as cost, equipment requirements, turn-around time, or inadequate performance are associated with currently available diagnostic tests, and no single assay possesses characteristics ideal for every situation.

Molecular Detection

PCR targeting ASFV DNA has proven to be a highly specific and sensitive method for detecting ASFV, and OIE-validated PCR assays have been developed in both conventional, gel-based and quantitative real-time formats. Real-time qPCR is considered the gold-standard test for the detection of ASFV in swine due to its superior specificity and sensitivity; these assays can be performed on a variety of sample types including blood, serum, swine oral fluids, culture supernatant, and organ and tissue homogenates, as well as poorly-preserved samples unsuitable for virus isolation and antigen detection (Beltrán-Alcrudo et al., 2017; Sánchez-Vizcaíno et al., 2019). PCR can also be used for detecting ASFV in *Ornithodoros* soft ticks, and is used in combination with DNA sequencing for genotyping of viral isolates (Bastos et al., 2003; Basto et al., 2006b).

Three validated PCR assays for ASFV detection are described in the *OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*, all of which target conserved portions of the viral *p72* gene and are capable of recognizing all known ASFV genotypes. The gel-based protocol possesses the advantage of simplicity in that it does not require a real-time thermocycler

(Agüero et al., 2003). Additionally, the ASFV-specific primer set for the gel-based, conventional assay can also be utilized in a one-step reverse transcription PCR (RT-PCR) assay for simultaneous detection and discrimination of ASFV and classical swine fever virus (CSFV), another high-consequence pathogen of swine which produces a clinically similar disease (Agüero et al., 2004). However, while the conventional PCR assay is considered suitable for a variety of uses, real-time qPCR shows greater sensitivity and is the OIE-recommended molecular detection method for ASFV for various applications including individual and population screening, disease surveillance, and confirmation of clinical cases (Sánchez-Vizcaíno et al., 2019). Two real-time qPCR assays are recommended by the OIE. The first utilizes a TaqMan hydrolysis probe and possesses significantly improved sensitivity over previously-recommended PCR assays, with a detection limit of 10-100 DNA copies, and was able to detect 25 different viral isolates representing 9 genotypes (King et al., 2003). The second assay uses a Universal Probe Library (UPL) and has the highest sensitivity of all ASFV PCR tests, with a detection limit between 4 and 18 DNA copies (Fernández-Pinero et al., 2013). A comparative analysis of molecular, serological, and antigen detection tests on experimental and field isolates from swine infected with European genotype II ASFV isolates revealed similar results, with the highest diagnostic sensitivity observed for the UPL qPCR, followed closely by the TaqMan-based real time qPCR assay, then conventional PCR. UPL qPCR also outperformed virus isolation using porcine blood monocytes, and all three PCR assays showed superior sensitivity when compared to a commercial p72 antigen detection ELISA, especially for samples with high cycle threshold (Ct) values, i.e. low virus loads (Gallardo et al., 2015). Additional TaqMan real-time qPCR assays which are validated but not outlined in the *OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* have been developed, including a multiplex qPCR assay for simultaneous

detection of CSFV, and commercial real-time qPCR kits are available from several manufacturers (Zsak et al., 2005; Tignon et al., 2011; Haines et al., 2013; Gallardo et al., 2019).

Conventional molecular diagnostic techniques are generally restricted to laboratory settings because of the space and electrical requirements to operate the machinery for nucleic acid extraction and thermocycling, and because PCR reagents often require cold storage (Gaudreault et al., 2020). The adaptation of molecular diagnostics to field-settings is an area of active research. The use of portable battery-powered thermocyclers and lyophilized reagents for real-time qPCR detection of ASFV have been demonstrated to be feasible, though assay performance is variable depending on sample quality and DNA extraction techniques; the portable thermocyclers are also not high-throughput (Liu et al., 2017; Liu et al., 2019; Wang et al., 2019). Isothermal DNA amplification strategies simplify the equipment requirements for portable molecular diagnostic systems. Linear isothermal amplification was shown to be specific but possess an analytical sensitivity at least 2 logs lower than real-time qPCR (Hjertner et al., 2005). Loop-mediated isothermal amplification (LAMP) for ASFV detection possesses a sensitivity approaching that of TaqMan-based real-time qPCR; however, optimization of reaction parameters and cutoff values is challenging for this assay type, and its use has therefore not been widely adopted (James et al., 2010; Oura et al., 2013). Other modalities, including cross-priming amplification (CPA) and recombinase polymerase amplification (RPA), have recently been investigated (Frączyk et al., 2016; Wang et al., 2017; Miao et al., 2019). However, none of these isothermal assays are currently validated for field use (Gallardo et al., 2019).

Serological Detection

Serology is a mainstay of ASF surveillance and eradication, with several tests validated and recommended for this purpose. The lack of an available ASF vaccine means ASFV-specific

antibodies are the result of current or prior infection or are maternally-derived. Furthermore, serological testing can possess advantages of speed, simplicity, and cost-effectiveness, depending on the assay format used (Cubillos et al., 2013; Beltrán-Alcrudo et al., 2017; Sánchez-Vizcaíno et al., 2019). The primary limitations of antibody testing are poor sensitivity with early-stage infections and an inability to distinguish between ongoing and past infection.

Antigens used for serological assays can be isolated from live virus preparations or produced via recombinant protein expression. Semi-purified viral p72 derived from MS cell cultures infected with ASFV was used as ELISA antigen after being identified as a highly immunogenic viral protein, and was a significant improvement over earlier crude antigen preparations (Tabarés et al., 1980; Tabarés et al., 1981). Subsequent evaluation revealed cytoplasmic soluble protein derived from ASFV-infected MS cells to be superior in identifying antibodies at earlier times post-infection, and this antigen preparation is utilized for the OIE-validated indirect ELISA and immunoblot/western blot protocols (Pastor et al., 1990; Sánchez-Vizcaíno et al., 2019). Acetone-fixed Vero and MS cells infected with ASFV are also utilized for the indirect fluorescent antibody test (IFAT) and indirect immunoperoxidase test (IPT), both of which are used as confirmatory assays (Botija, 1970; Pan et al., 1982; Sánchez-Vizcaíno et al., 2019). However, antigen preparations from live virus are laborious to make, necessitate high-containment laboratories to safely manufacture and inactivate, and require standardization between batches to compensate for variability in the production process (Cubillos et al., 2013). Consequently, a variety of assays which utilize recombinant proteins as antigens have been developed. Recombinant proteins can be manufactured in large quantities under standard laboratory conditions, and the homogeneity of these purified antigen preparations can improve assay consistency between lots; they also present a specifically-defined target for antibody

recognition and binding, which can improve diagnostic performance. A significant number of recombinant proteins have been evaluated for the ability to detect ASFV antibodies in both serum, oral fluids, and feces through ELISA and immunoblotting, including p30, p54, pp62, p72, and PB602L (Oviedo et al., 1997; Barderas et al., 2000; Gallardo et al., 2006; Pérez-Filgueira et al., 2006; Gallardo et al., 2009b; Cubillos et al., 2013; Nieto-Pelegrín et al., 2015; Giménez-Lirola et al., 2016; Kazakova et al., 2017). Additional antigenic components of ASFV have been identified, but their suitability as potential diagnostic targets has yet to be determined (Tabarés et al., 1980; Kollnberger et al., 2002).

ELISA is the most commonly used format for serological testing due to its speed, high throughput, cost-effectiveness, and excellent diagnostic performance (Beltrán-Alcrudo et al., 2017). In addition to the laboratory protocol for an indirect ELISA using cytoplasmic soluble antigen preparations, commercially manufactured and OIE-validated ELISAs utilizing semipurified p72 or recombinant versions of p30, p54, pp62, and p72 are available in indirect and competitive formats (Sánchez-Vizcaíno et al., 2019). ELISA is routinely used for disease surveillance, establishing population freedom from infection, and facilitating international movement of animals. Positive ELISA results should be validated by a second confirmatory test such as IFAT or IPT (Beltrán-Alcrudo et al., 2017; Sánchez-Vizcaíno et al., 2019). In addition to testing swine sera, the viability of using ELISAs to detect antibodies in other types of samples has also been investigated. Assays using semipurified p72 and recombinant p30 were able to recognize ASFV-specific antibodies in feces from domestic pigs experimentally challenged with the attenuated Ken05/TK1 strain at 9 days post-infection, though this result was only obtained from animals receiving a high challenge dose and was not obtained for all animals within this group (Nieto-Pelegrín et al., 2015). Additionally, a recombinant p30 ELISA was able to detect

antibodies within the oral fluids of pigs challenged with the low-virulence NHV/P68 strain at 8-12 days post-infection (Giménez-Lirola et al., 2016). Therefore, the ELISA platform is highly adaptable and potentially useful for other diagnostic samples that may be easier to obtain than serum.

While ELISAs remain the go-to method for routine ASFV serology, three other diagnostic assays for antibody detection are used. As mentioned above, the indirect fluorescent antibody test (IFAT) and indirect immunoperoxidase test (IPT) possess similar formats with different detection methods. Vero or MS cells are infected with a cell-adapted ASFV strain such as BA71v, then fixed with acetone and incubated with the questionable swine serum, after which the presence of bound antibody is determined using a peroxidase-conjugated (for IPT) or fluorescent dye-tagged anti-swine antibody (Botija, 1970; Pan et al., 1982). IFAT and IPT are recommended as confirmatory tests for ELISA-positive samples from areas presumed to be ASF-free, or for samples from ASF-endemic areas where ELISA results are inconclusive. The IPT has been demonstrated to be more sensitive than the OIE-recommended indirect ELISAs for samples collected at early times post-infection (Gallardo et al., 2015; Sánchez-Vizcaíno et al., 2019). Immunoblots or western blots, whereby ASFV antigen bound to a nitrocellulose membrane is probed with swine serum for ASFV-specific reactivity, is also useful for serological diagnosis. It has been demonstrated that antibodies in serum samples stored at room temperature or 37°C retain reactivity to immunoblots with cytoplasmic soluble ASFV antigen preparations longer than the OIE indirect ELISA, indicating this test may be preferable for poorly-preserved or low quality serum samples (Arias et al., 1993). Recombinant p54 constructs expressed in *E. coli* and baculovirus systems have been shown to provide simple interpretation of immunoblot results by eliminating assay background associated with cellular proteins from cell culture-derived ASFV

antigens (Alcaraz et al., 1995; Oviedo et al., 1997). Additionally, immunoblots using recombinant p30 have been able to identify ASFV-positive pigs and wild boar as early as 6-8 days post-infection (Kazakova et al., 2017). Despite this, IPT and IFAT are generally preferred as confirmatory tests.

There are two significant limitations associated with serology for ASF. First and foremost, all serological tests are plagued by poor sensitivity in detecting ASFV-positive animals in the days immediately following infection. Antibodies to ASFV antigens generally do not appear at detectable levels until 7-10 days post-infection, meaning serological tests are ineffective prior to this timeframe (Cubillos et al., 2013). The epizootic Eurasian ASFV isolates are highly virulent and rapidly lethal, with infected animals often succumbing to the disease before a detectable antibody response can be generated (Blome et al., 2012; Blome et al., 2013; Pikalo et al., 2020). Second, serological testing cannot reliably discriminate between ongoing infection and past disease, meaning a positive ELISA result cannot truly determine an individual animal's disease status without follow-up testing for ASFV antigen or ASFV genetic material. Nevertheless, serology remains a useful diagnostic methodology for widespread population surveillance and for detecting infections with low-virulence ASFV strains.

Virus and Antigen Detection

ASFV and viral antigens can be detected through methods involving infection of primary cell cultures and/or probing with ASFV-specific antibodies. Techniques which involve virus isolation and culturing can be highly specific, but these assays require specialized facilities, trained personnel, and can take over one week to complete, meaning their use is restricted to confirmatory testing in reference laboratories. Samples can also be evaluated directly for ASFV antigens by fluorescent antibody tests or through the use of commercially-available antigen

detection kits; however, these methods possess significant diagnostic limitations which restrict their use, and follow-up confirmatory testing is required.

ASFV can be isolated from blood and tissues which the virus infects. Following homogenization and centrifugation for clarification, supernatant from diagnostic tissue samples can be added to primary monocyte/macrophage cultures and the virus subsequently detected by two means: a hemadsorption (HAD) test and a fluorescent antibody test. The HAD assay is an OIE-validated confirmatory test, and hemadsorption is considered diagnostic for ASFV (Sánchez-Vizcaíno et al., 2019). In the HAD assay, cultures of swine peripheral blood monocytes or alveolar macrophages are inoculated with different dilutions of the tissue homogenate to be tested, then erythrocytes from defibrinated pig whole blood are added and cultures evaluated over the next 4-10 days for the adsorption of red blood cells onto the surface of ASFV-infected monocytes/macrophages (Enjuanes et al., 1976; Carrascosa et al., 2011; de León et al., 2013; Sánchez-Vizcaíno et al., 2019). This method is also useful for viral titration as it results in a semi-quantitative evaluation of tissue sample infectivity (Enjuanes et al., 1976; Carrascosa et al., 2011). HAD is mediated by the viral CD2v and C-type lectin proteins (Borca et al., 1994; Galindo et al., 2000). Not all ASFV isolates express the CD2v/C-type lectin proteins, and therefore the HAD test is not able to detect strains lacking CD2v; it is recommended that HAD negative results be confirmed using another test system. Additionally, HAD tests require a consistent supply of erythrocytes derived from anticoagulated pig blood, which may not always be available. To circumvent these potential limitations, virus isolation can also be performed by inoculating the diluted tissue samples onto porcine alveolar macrophage, peripheral blood monocyte, or bone marrow primary cell cultures; the cultures are fixed at 5-7 days post-infection, probed using a fluorescein isothiocyanate (FITC)-conjugated anti-ASFV antibody, and evaluated

with a fluorescence microscope (Sánchez-Vizcaíno et al., 2019). Regardless of the method used, virus isolation requires a significant amount of time and technical expertise, and work with live ASFV must occur in high-containment BSL-3 laboratories; this means that virus isolation assays are expensive and inefficient for screening large numbers of samples. Consequently, while virus isolation is a valuable diagnostic technique for ASF, its use is restricted to confirmatory testing and detailed characterization of laboratory samples.

In addition to virus isolation, clinical samples can also be screened directly for the presence of ASFV antigen. A fluorescent antibody test (FAT) targeting ASFV antigen can be performed directly on blood or tissue impression smears which are fixed and mounted on glass slides. This method possesses significantly reduced sensitivity especially for subacute and chronic ASF cases, possibly due to the presence of ASFV-specific antibodies in these pigs, which interfere with the binding of the fluorescent antibody probe (Sánchez-Vizcaíno et al., 2019). Assays for the capture and detection of ASFV antigen in blood samples have been described, and a sandwich ELISA and a lateral flow immunoassay (LFIA) using monoclonal antibodies targeting the p72 protein are commercially available. Data on these assays is limited but indicates poorer sensitivity than real-time PCR, especially for subacute and chronic ASF cases, as well as a higher potential for false positive results (Oura et al., 2013; Sastre et al., 2016).

Lateral Flow Immunoassays

Lateral flow immunoassays (LFIAs) are convenient diagnostic platforms for the detection of specific analytes, including antigen and antibody, within a liquid test system. LFIAs are characterized by their outward simplicity and ease of use – the sample is allowed to migrate through the test strip by capillary action, and binding of the analyte to a labeled antibody

conjugate produces (or inhibits) formation of a band in the detection area of the strip, allowing for a straightforward "yes/no" interpretation of test results which can be assessed by eye or using a dedicated reader (Koczula et al. 2016). LFIAs are commonplace point of care diagnostic test systems because they are easy to use, reliable, cost-effective, and a well-established technology (Wong and Tse, 2009).

Components of LFIAs

LFIAs generally consist of four basic structural components affixed to an inert backing strip (Figure 1.3): a membrane, a sample pad, an absorbent pad, and a conjugate pad (Koczula et al., 2016; Wong and Tse, 2009). The sample pad, located at the bottom of the test strip, functions to take up and evenly distribute the test sample toward the conjugate pad. The sample pad can physically filter out large particulates which may interfere with the assay, and frequently contains chemical additives including buffers and detergents to facilitate sample movement and interaction with the detection antibody. The conjugate pad contains the conjugated detection antibody in a stabilized form and is the site of interaction between analyte and detector. The absorbent pad, located at the top of the test strip, absorbs fluid through the membrane across the test strip and collects the sample. The membrane component of the strip contains immobilized substrate such as recombinant antigen or antibody that is capable of binding analyte-conjugate complexes, causing the formation of test bands in the detection window. The conjugated antibody used for detection is highly specific for a given analyte and generally of monoclonal antibody origin. Colloidal gold is the most commonly used label due to its chemical stability and intense color without the need for a development reaction (Koczula et al., 2016). Latex particles coupled to colored or fluorescent dyes, or other detection reagents, are also used for antibody labeling, depending on LFIA requirements (Wong and Tse, 2009).

Applications for LFIAs

LFIAs possess several desirable characteristics for a diagnostic platform. Their simple design means they are inexpensive to produce, straightforward to run, and generally stable under normal storage conditions. LFIAs provide results within minutes in an unambiguous format that is easy to interpret, making them well-suited to point-of-care applications (Koczula et al., 2016; Wong and Tse, 2009). However, several limitations are associated with the LFIA format. The simplicity of LFIA testing protocols means that only few, if any, procedural adjustments can be made. Strips have a maximum amount of fluid they are capable of testing before pads become saturated, and samples to be tested are necessarily restricted to these volumes; this also makes LFIA performance susceptible to inaccurate sample measurement and sample application. Furthermore, test samples must be liquid and possess a minimal amount of large particulates which could impede capillary action and affect test performance, meaning only certain kinds of diagnostic specimens can be screened by these assays (Koczula et al. 2016; Wong and Tse 2009).

LFIAs have been developed for a variety of veterinary infectious diseases such as brucellosis, influenza A, classical swine fever, African swine fever, foot-and-mouth disease, African trypanosomiasis, and bovine anaplasmosis (Nielsen et al. 2008; Ferris et al. 2009; Nielsen et al. 2009; Sastre et al. 2016; Sambandam et al. 2017; Pinto Torres et al. 2018; Sun et al. 2018; Zhu et al. 2019; Yang et al. 2020a+b). The portability and speed of the LFIA design makes it an attractive format for pen-side diagnostic tests, especially for diseases where rapid detection and diagnosis is critical.

Purpose of Research

ASF remains one of the most significant threats to the swine industry globally and in the United States; the ongoing outbreaks in Europe and Asia highlight the devastation brought by this disease to swine producers and the economy. Because no vaccine or treatment is available, prompt detection of the disease forms the bedrock of ASF mitigation strategies. The advent of pen-side diagnostic tests which can be administered by front-line veterinary field officers has been a significant benefit to animal health. Unfortunately, the currently available field-deployable diagnostic tests available for ASF carry significant performance limitations such as poor sensitivity (i.e. false negatives) in the case of serological ASF assays or an unacceptable rate of false positives for rapid ASFV antigen detection tests, which can be as devastating to producers as a false negative result. To address this need for a rapid and accurate pen-side ASF antigen test, we developed and evaluated a novel LFIA for ASFV antigen detection.

Figures and Tables for Chapter 1

Figure 1.1 Transmission cycles for ASFV

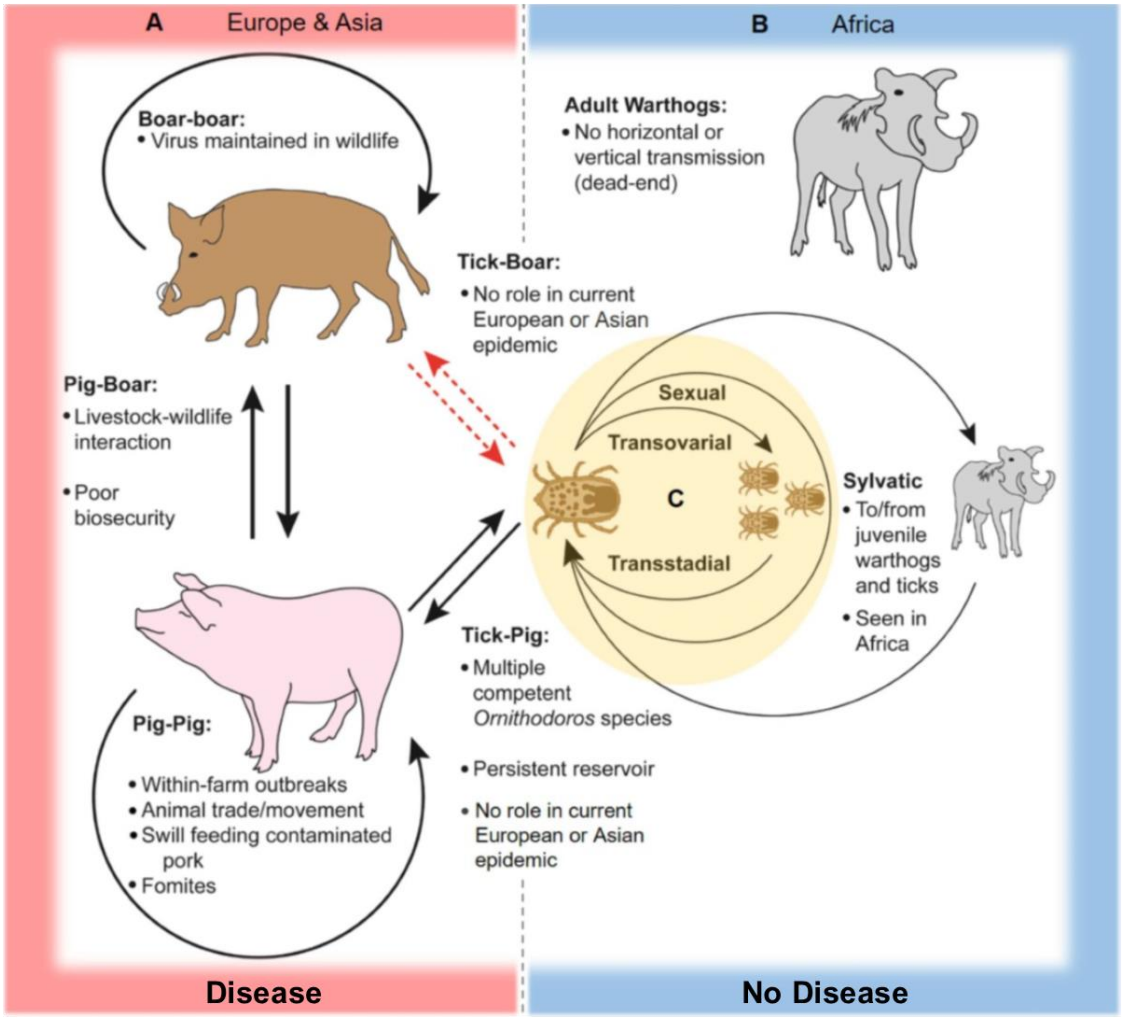

Figure legend: ASFV is propagated through multiple distinct cycles involving wildlife and domestic pigs which can overlap. **A)** In Europe and Asia, ASFV circulates in domestic pigs and wild boar, with high levels of mortality. ASFV is readily transmitted between pigs by direct contact and through contaminated pork products and fomites. Two-way transmission between domestic pigs and wild boar can occur at the livestock-wildlife interface, especially where there is inadequate farm biosecurity. Transmission between boar maintains the virus in the environment, and the widespread geographic distribution of wild boar facilitates spread of the disease. Historical outbreaks of ASF in the Iberian Peninsula were associated with two-way transmission between domestic pigs and *Ornithodoros erraticus* soft ticks, and multiple *Ornithodoros* species have been shown to be competent vectors. However, there is little evidence to support transmission between soft ticks and wild boar, or between soft ticks and domestic pigs, in the contemporary European and Asian epidemics. **B)** In Southern and Eastern Africa, in addition to transmission between pigs, a sylvatic cycle involving juvenile warthogs and *O. porcinus porcinus* soft ticks occurs and is a source of ASFV environmental persistence. Juvenile warthogs can become infected when soft ticks harboring the virus take a blood meal, and uninfected soft ticks become infected after feeding on viremic juvenile warthogs. Infected warthogs do not develop clinical disease. Adult warthogs do not maintain sufficiently high levels of viremia to transmit the virus and are considered dead-end hosts. **C)** Within the soft tick lifecycle, ASFV can be transmitted sexually, transovarially, and transstadially. *Ornithodoros* ticks have long lifespans and are capable of maintaining the virus for years. Figure adapted from Gaudreault, Madden *et al.*, 2020.

Figure 1.2 Distribution of ASFV genotypes in continental Africa and Madagascar

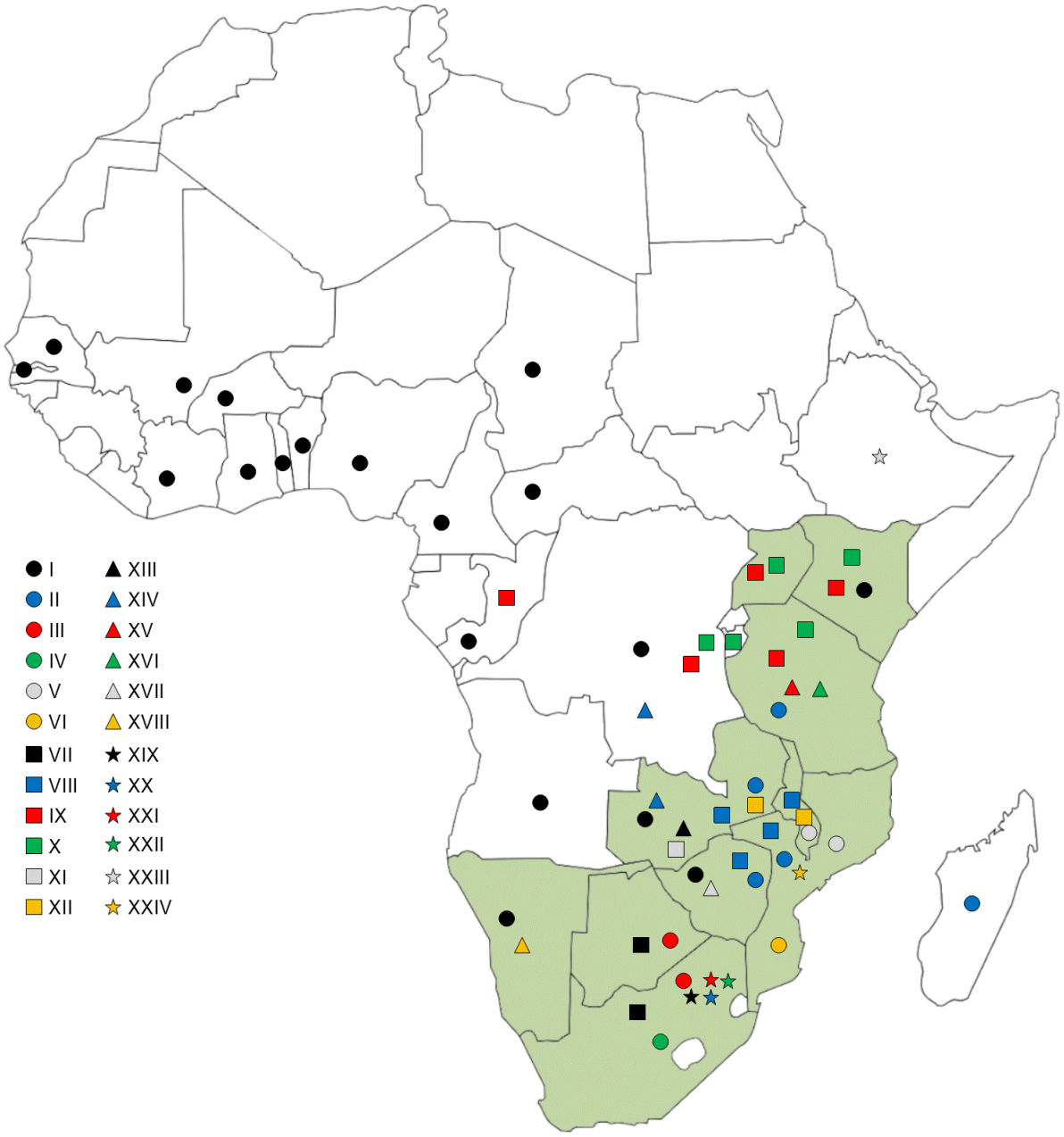

Figure legend: ASFV has been isolated from a majority of nations in Southern and Eastern Africa, as well as several West African nations. Genotype I viruses predominate in West Africa, where the virus is primarily maintained by transmission between domestic pigs with little evidence supporting a large role for wildlife in maintenance of the virus. The greatest genotypic diversity of ASFV is found in Southern and Eastern Africa where sylvatic and domestic pig transmission cycles overlap, and all 24 ASFV genotypes can be found in this region. Nations where the sylvatic transmission cycle between *Ornithodoros* soft ticks and warthogs is confirmed to exist are highlighted in green and include Botswana, Kenya, Malawi, Mozambique, Namibia, South Africa, Tanzania, Uganda, Zambia, and Zimbabwe. Genotype II ASFV is also present in the island nation of Madagascar.

Figure 1.3 Diagram of a lateral flow immunoassay (LFIA)

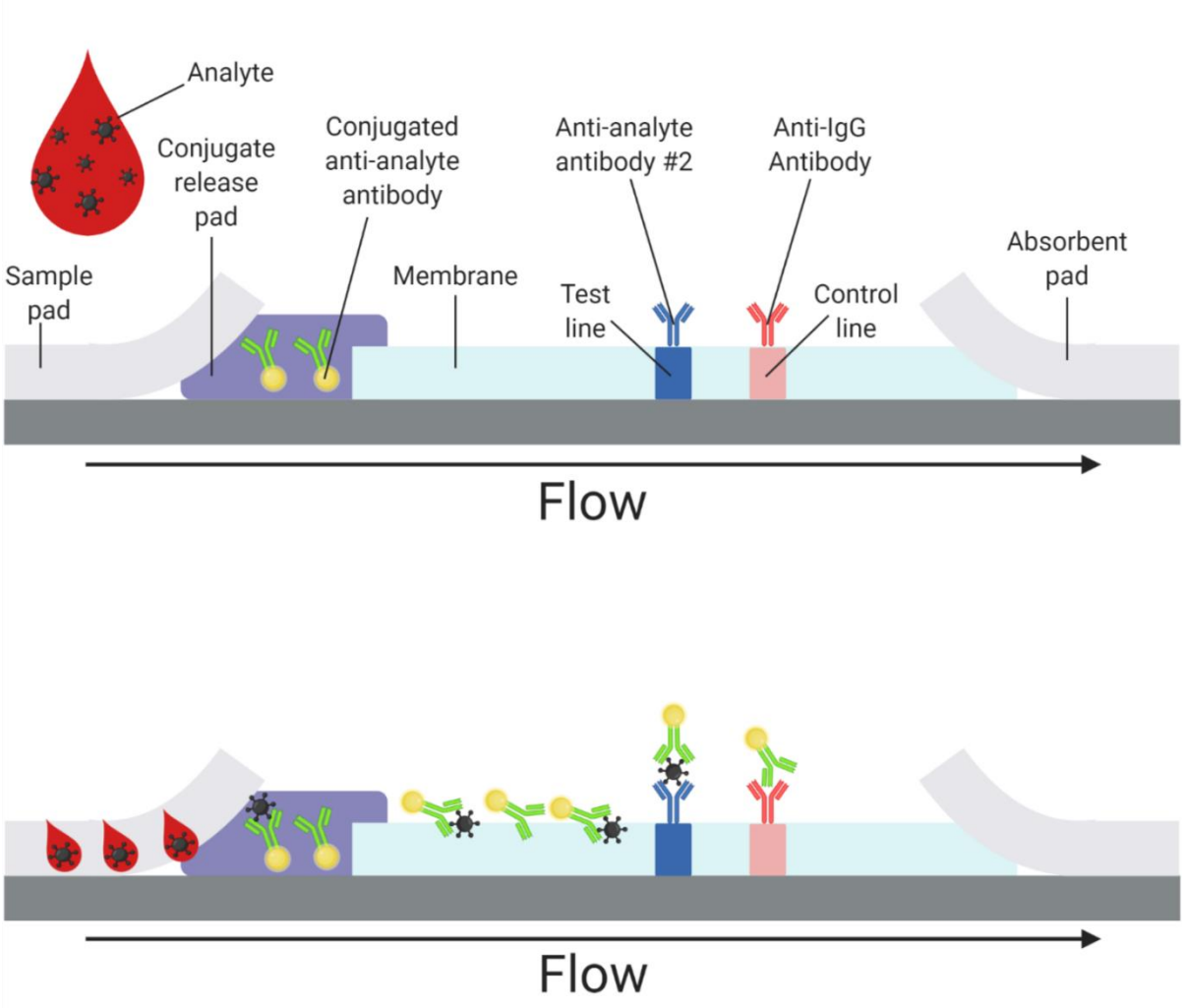

Figure legend: LFIAs consist of four general components 1) a sample pad, which takes up the test sample and directs it toward the conjugate pad and test membrane 2) a conjugate release pad containing analyte-specific antibody conjugated to a label such as colloidal gold 3) a test membrane with immobilized antibody attached at two regions, with one region containing antibody specific for the analyte at an epitope different than the one targeted by the conjugated antibody, and the second region containing anti-IgG antibody, and 4) an absorbent pad which pulls the sample across the membrane. **Bottom:** The test sample containing the analyte is taken up by the sample pad and directed to the conjugate release pad, where the conjugated antibody can bind the analyte. Conjugated antibody and antibody-analyte complexes are pulled across the membrane toward the absorbent pad by capillary action and interact with immobilized antibody at the test line and control line. The test line contains antibody targeting a second epitope on the analyte, allowing it to bind analyte-conjugated antibody complexes, resulting in the development of a band at the test line. The control line contains anti-IgG antibody which binds remaining conjugated antibody that is not immobilized at the test line, resulting in the development of a band which indicates the assay is functioning correctly.

Table 1.1 Diagnostic methods for ASF

Antibody Detection	Advantages	Disadvantages	Use	Comments
ELISA (antibody)	Inexpensive + high throughput Commercial + in-house formats Recombinant proteins for BSL-2 Good for detecting chronic ASF	Poor sensitivity for <7 DPI Cannot distinguish active vs past infection Requires virus/BSL-3 (in-house format) Variable quality between preparations	Surveillance, disease eradication, animal movement	Most commonly used Positive results should be confirmed
Immunoblot	May be better for poorly-preserved sera than ELISA Recombinant proteins for BSL-2	Lower throughput than ELISA Not commercially available Requires BSL-3 if using antigen from live virus	Confirmatory serological test	Use as alternative to IFAT/IPT for confirmatory testing
LFIA (antibody)	Field deployable Rapid results	Poorer sensitivity and specificity than ELISA	Preliminary testing, surveillance	Positive results should be confirmed
IFAT/IPT	High sensitivity/specificity	Requires virus culture/BSL-3 Requires fluorescence microscope (IFA)	Surveillance, disease eradication, animal movement, confirmatory test	Mainly used for confirmatory serological testing
Virus/Antigen Detection				
Virus isolation/hemadsorption (HAD)	Highly specific Multiple sample types HAD is definitive for ASFV	Slow (>7 days), technically challenging Uses primary cells and live virus (BSL-3) Cannot detect non-HAD strains	Confirmatory testing	Largely restricted to reference labs Useful for virus titration
Virus isolation/IFAT	Can detect non-HAD strains	Similar to HAD test	Confirmatory testing	Alternative to HAD test
Direct FAT	Easier than virus isolation Detection of virus in tissues	Lower sensitivity for subacute/chronic Requires fluorescence microscope	Confirmatory testing (positive results only)	Less preferable than virus isolation or PCR
ELISA (antigen)	Straightforward Gives evidence of active infection	Variable performance/reliability Poor performance with subacute/chronic	Preliminary testing	Not routinely recommended
LFIA (antigen)	Field deployable Rapid results Gives evidence of active infection	Poor performance with subacute/chronic Less reliable specificity Limited field data	Preliminary testing	Not routinely recommended
Genome Detection				
Real-Time PCR	Excellent sensitivity/specificity Multiple sample types Commercial and in-house	Requires expensive equipment	Surveillance, disease eradication, individual freedom from infection, animal movement, confirmatory testing	Gold-standard assay
Conventional PCR	Does not require real-time thermocycler	Poorer performance than real-time PCR Potential for cross-contamination	Surveillance, disease eradication, animal movement and freedom from infection	Less preferable than real-time PCR

IFAT/IPT = indirect fluorescent antibody test/indirect immunoperoxidase test; FAT = fluorescent antibody test; HAD = hemadsorption; DPI = days post-infection

Chapter 2 - Materials and Methods

Viruses and cells

Vero E6 cells obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) were used for the propagation of the cell-adapted ASFV genotype I strain BA71v. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 2mM L-glutamine and 4.5g/L glucose (Corning, Manassas, VA, USA), supplemented with 5% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA, USA) and 1x antibiotic-antimycotic solution (Gibco, Waltham, MA, USA). They were grown in a 37°C humidified incubator with 5% CO_2 atmosphere. For BA71v stock, sub-confluent cells were infected with BA71v at a multiplicity of infection (MOI) of 1 and incubated for 24 hours; afterwards, cells were lysed by two freeze-thaw cycles and culture supernatant clarified by spinning at 3,200 x G for 10 minutes to pellet cellular debris. The BA71v stock was titrated by $TCID_{50}$ assessing viral cytopathic effect (CPE) and stored at -80°C.

For experimental challenge of animals, three separate virus isolates were used - a highly virulent genotype II Armenia 2007 ASFV isolate, a highly virulent genotype I E70 ASFV isolate, and a genotype X Ken05/Tk1 ASFV isolate of moderate virulence. Viruses were obtained from the European Union Reference Laboratory for ASF [Centro de Investigación en Sanidad Animal, Instituto Nacional de Tecnología Agraria y Alimentaria (CISA-INIA), courtesy of Dr. C. Gallardo]. The E70 ASFV strain was initially isolated in 1970 from the spleen of an infected domestic pig in Spain and was passaged in swine buffy coat culture 6 times. The Ken05/Tk1 virus was originally isolated from a soft tick in central Kenya and passaged 3 times in swine buffy coat culture. Samples of the E70 and Ken05/Tk1 ASFV strains received from Spain were used directly for infection of pigs without additional propagation in cell culture. The Armenia

2007 ASFV isolate, isolated from the spleen of an infected pig, was initially passaged 6 times in swine buffy coat culture, and was subsequently propagated on primary porcine alveolar macrophages (PAMs) obtained via bronchoalveolar lavage as previously described by Carrascosa and coworkers (Carrascosa et al., 1982). All viruses were titrated in hemadsorbing units (HAUs) as described by Carrascosa and coworkers (Carrascosa et al., 1982). Viral stocks were stored at -80°C.

Expression and purification of recombinant ASFV p30

The sequence of the CP204L ORF coding for the ASFV protein p30 was obtained based on the Georgia 2007/1 ASFV genomic sequence (GenBank: FR682468.1) and was synthesized into the pUC57 plasmid by a commercial manufacturer (GENEWIZ, South Plainfield, NJ, USA). The full-length CP204L ORF was amplified by PCR using gene-specific primers containing a 5' CACC overhang within the forward primer and a CP204L ORF stop codon deletion in the reverse primer to allow expression of a c-terminal polyhistidine tag. The amplified PCR product was inserted into the pET101/D-TOPO vector (Invitrogen, Waltham, MA, USA) by TOPO® cloning (Invitrogen) then transformed into One Shot™ TOP10 chemically competent *E. coli* (Invitrogen). The proper gene sequence and orientation were confirmed by PCR and Sanger sequencing. Plasmid DNA was purified using a QIAprep Spin Miniprep Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. Purified plasmid DNA was then cloned into One Shot™ BL21 Star™ (DE3) chemically competent *E. coli* (Invitrogen) for recombinant protein expression.

For recombinant ASFV p30 expression, BL21 *E. coli* was grown in Miller's LB broth (Sigma-Aldrich, St. Louis, MO, USA) containing 100μg/mL ampicillin (Sigma-Aldrich) until reaching an O.D. of 0.6, upon which protein expression was induced by addition of 1mM

isopropyl β-d-1-thiogalactopyranoside (IPTG; Sigma-Aldrich). Cultures were grown for an additional 16 hours post-IPTG stimulation, after which bacteria were harvested by centrifugation. Recombinant fusion protein expression was confirmed by western blot using an anti-His (C-term) HRP monoclonal antibody (Invitrogen). Recombinant p30 was purified under native conditions by nickel affinity chromatography. Bacteria pellets were lysed by resuspending in lysis buffer consisting of 50mM sodium phosphate, 500mM sodium chloride, 10mM imidazole (Sigma Aldrich), 10% glycerol, pH 8.0 with benzonase (EMD Millipore, Burlington, MA, USA) added at 400U/g cell pellet. Suspensions underwent two freeze-thaw cycles and were sonicated using six 10-second bursts. Cellular debris was pelleted by high speed centrifugation (16,000xG for 20 minutes). Recombinant p30 protein was isolated from the supernatant by batch purification using Ni-NTA Superflow Resin (QIAGEN) with washes and elution using a step-wise imidazole gradient. Purified p30 was dialyzed against phosphate-buffered saline (PBS), pH 7.4 (Gibco), containing 150mM NaCl, 4mM EDTA, and 10% glycerol, and stored at -80°C for long-term. Recombinant p30 protein purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using NuPAGE 12% bis-tris gels (Invitrogen) and MOPS SDS running buffer (Invitrogen), with protein visualization via Coomassie blue staining using an eStain L1 Protein Staining System (GenScript, Piscataway, NJ, USA).

Generation of monoclonal antibodies, screening by indirect immunofluorescence assay (IFA), and isotyping

To generate monoclonal antibodies targeting the ASFV p30 protein, laboratory mice were immunized three times with recombinant p30 antigen, and 1 week after final vaccination splenocytes were isolated and fused to myeloma cells to generate hybridomas following standard protocols. Monoclonal antibodies were screened for specific reactivity to ASFV p30 protein by

IFA using infected cell cultures under BSL-3 laboratory conditions. In 96-well plates, Vero E6 cells were seeded at a density of $3.25x10^4$ cells/well in 100µL/well DMEM supplemented with antibiotic-antimycotic solution and 2.5% fetal bovine serum and allowed to attach for 2 hours in a 37°C humidified incubator with 5% CO2 atmosphere. Cells were infected with ASFV strain BA71v at a MOI of 0.01 or 0.05, with uninfected cells serving as negative controls. At 48 hours post-infection, cells were washed with PBS, pH 7.4, and fixed with 80% acetone (Sigma Aldrich) for 10 minutes at room temperature. Afterwards, two additional PBS washes were performed and 75µL/well serum-free hybridoma culture supernatant was added, and plates incubated overnight at 4°. The following morning, cells were washed three times with PBS containing 0.05% Tween-20 (Sigma-Aldrich) (PBS-T), and then 75µL/well PBS-T with 1% bovine serum albumin (Sigma-Aldrich) and 1:500 Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen) was then added. Plates were incubated for 1 hour at room temperature protected from light, then washed with PBS-T and counterstained with 75µL/well 300nM 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) substrate (Thermo Fisher Scientific, Waltham, MA, USA). Cells were then evaluated for specific fluorescence using an EVOS FL fluorescence microscope (Thermo Fisher Scientific). Monoclonal antibodies demonstrating ASFV-specific reactivity were isotyped using a murine Pro-Detect™ Rapid Antibody Isotyping Assay Kit (Thermo Fisher Scientific) following the manufacturer's instructions.

Experimental infections and blood collection

Thirty-one naive conventional outbred piglets 8-12 weeks of age were infected by intramuscular inoculation of ASFV strain Armenia 2007, E70, or Ken05/Tk1 at doses ranging from 16-360 HAUs as part of 7 separate animal experiments (Table 2.1). Anticoagulated (EDTA) whole blood was collected via jugular venipuncture at multiple days post-challenge

(DPC) and stored at -80°C until use. ASFV-negative whole blood samples from a total of 79 piglets 8-12 weeks of age, including pre-challenge (day 0) samples from the 31 infected animals listed in Table 2.1, were also collected. Pigs were monitored daily for rectal temperature and clinical disease by a veterinarian, and clinical scores were assigned to each animal by evaluating 8 clinical parameters on a scale of 0 (normal/absent) to 3 or 4 (most severe): liveliness (0 = normal; 1 = reduced liveliness but stands without help; 2 = sluggish and does not stand without help; 3 = dormant and refuses to stand with or without help), body shape (0 = normal; 1 = empty stomach/sunken flanks; 2 = empty stomach with indications of weight loss; 3 = wasting with visible ribs and vertebrae), respiratory function (0 = normal; 1 = increased respiratory rate; 2 = significantly increased respiratory rate and abdominal breathing; 3 = severe breathing difficulty including open mouth breathing, wheezing, or severe cough), neurological signs (0 = normal; 1 = stumbling or swaying gait that is quickly corrected; 2 = ataxia/paresis of hindquarters but able to walk; 3 = paralysis of hindquarters and inability to stand, or the presence of convulsions), skin lesions (0 = normal; 1 = erythema, or cyanosis over <10% of the body; 2 = cyanosis over 10-25% of the body or occasional skin bleeding; 3 = >25% cyanosis, large bruising or subcutaneous bleeding, ulceration/necrosis, and cold skin), digestive symptoms (0 = normal; 1 = diarrhea of <24 hours duration; 2 = diarrhea of >24 hours duration or occasional vomiting; 3 = bloody diarrhea or frequent vomiting), ocular/nasal discharge (0 = normal; 1 = thin discharge from nose and/or eyes without admixtures; 2 = thick discharge from nose and/or eyes without blood; 3 = bloody discharge from nose and/or eyes), and fever (0 = 37.8-40.5°C; 1 = 40.6-40.9°C at one time point; 2 = 40.6-40.9°C for two sequential days; 3 = ≥41.0°C; 4 = <37.8°C). Moribund animals and those with a total clinical score >16 were humanely euthanized by intravenous pentobarbital administration. All animal experiments and procedures were performed under high

containment BSL-3Ag conditions in the Biosecurity Research Institute at Kansas State University (KSU) following protocol evaluation and approval by KSU's Institutional Biosafety Committee (IBC, protocol #: 850, 1049, 1314) and Institutional Animal Care and Use Committee (IACUC, Protocol #: 3513, 3758, 4265).

Evaluation of whole blood samples by quantitative real-time PCR

Pre- and post-ASFV challenge, whole blood samples were evaluated for the presence of ASFV-specific genomic DNA by quantitative real-time PCR (qPCR) targeting the viral p72 gene. DNA was isolated from blood by magnetic bead extraction with MagAttract Suspension G beads (QIAGEN) and DNeasy Blood and Tissue Kit (QIAGEN) components using a KingFisher Duo Prime Purification System (Thermo Fisher Scientific) or taco™ Nucleic Acid Automatic Extraction System (GeneReach, Taichung City, Taiwan), with negative and positive extraction controls included with each run. Real-time PCR was performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using PerfeCTa FastMix II (Quantabio, Beverly, MA, USA) and primer and TaqMan probe sequences according to Zsak et al.. (2005). Negative controls for qPCR runs were molecular grade water, and positive amplification controls were quantified synthetic ASFV DNA (GenScript). Thermocycling parameters included an initial denaturation step at 95°C for 5 minutes, followed by 45 cycles of 95°C for 10 seconds then 60°C for 1 minute. A cycle threshold (Ct) of 35 was set as the cutoff for positive reactions. Viral DNA was calculated as copy number (CN) per milliliter whole blood (CN/mL) based on a standard curve generated from serial dilutions of the positive control DNA.

LFIA testing of experimental samples

LFIA test strips using anti-ASFV p30 monoclonal antibodies as antigen capture and conjugated detection antibodies were developed by Silver Lake Research Corporation and

provided by the manufacturer for testing. The assay consists of a reagent tube containing dried conjugate to which sample is added, and a strip-shaped dipstick through which the sample migrates and color development takes place (Figure 2.1a). Whole blood aliquots were thawed at room temperature, and 10μL blood was added to 200μL tap water in the LFIA reagent tubes. Tubes were briefly swirled to mix the ingredients and incubated for 5 minutes at room temperature, after which the tubes were swirled again and one LFIA test strip placed in each tube, allowing the sample to migrate up the strip. Strips were visually interpreted as positive or negative after 20 minutes (Figure 2.1b), and all results were photographed. All whole blood samples were tested in duplicate to evaluate concordance between test strip results. All manipulations involving infectious samples was performed under high containment BSL-3 laboratory conditions.

Statistical Analysis

Sensitivity and specificity of the LFIA were calculated by two methods: 1) using pre- and post-ASFV challenge as the determinant for true infection status, and 2) by using ASFV qPCR as the reference test for virus detection. LFIA results for each sample were interpreted as true positive (TP), true negative (TN), false positive (FP), and false negative (FN) for samples where both LFIA duplicate tests gave the same result (i.e. both positive or both negative), or as suspect when duplicates gave conflicting results (i.e. one positive and one negative). Specificity and sensitivity of the LFIA test were calculated as 100 x TN/(TN+FP) and 100 x TP/(TP+FN), respectively. Concordance between LFIA and ASFV qPCR results, and between LFIA test duplicates, was evaluated using two-by-two contingency tables using the VassarStats website. Cohen's unweighted kappa coefficient (κ) was calculated to assess the significance of agreement, with κ of 0.00 indicating poor agreement, 0.01-0.20 slight agreement, 0.21-0.40 fair agreement,

0.41-0.60 moderate agreement, 0.61-0.80 substantial agreement, and 0.81-1.00 almost perfect agreement (Landis and Koch, 1977).

Figures and Tables for Chapter 2

Figure 2.1 Overview of LFIA components

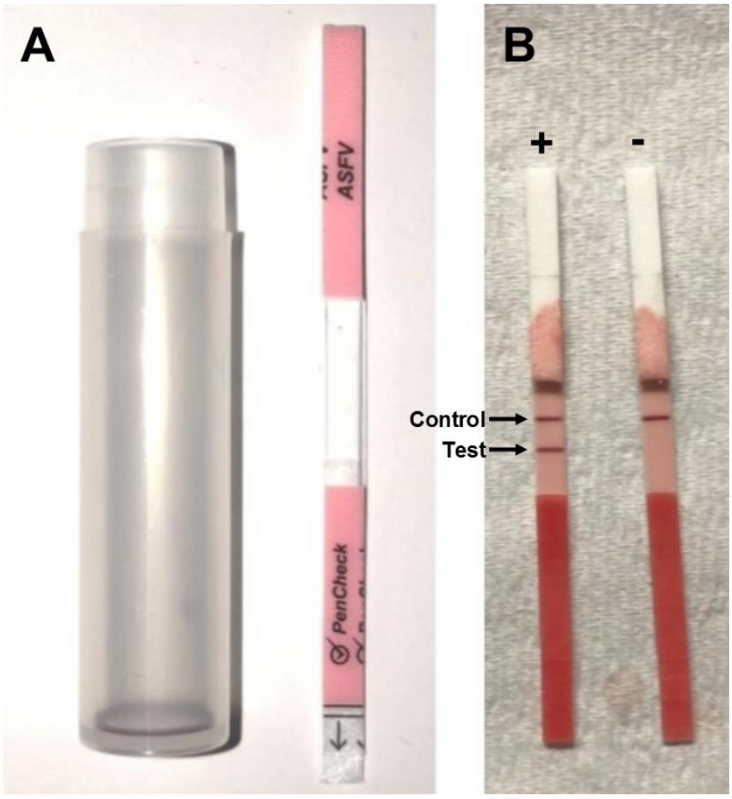

Figure legend: A) The anti-p30 LFIA consists of two components – a reagent tube containing dried conjugate, and a dipstick. Water and EDTA blood are added to the reagent tube and mixed, after which the dipstick is added to the tube and the sample allowed to migrate up the strip. **B)** Positive test results manifest as the development of two colored bands at the test line and the control line on the dipstick membrane, while negative results are indicated by the presence of only one band at the control line. No bands indicates an invalid test.

Table 2.1 Overview of ASFV challenge experiments and sample collection

Experiment #	Animal #	ASFV Strain	Dose (HAU)	Death (DPC)	Samples Tested (DPC)
#1	1-1	Arm07	160	10	0, 5, 7, 10
	1-2	Arm07	160	7	0, 5, 7
	1-3	Arm07	160	7	0, 5, 7
	1-4	Arm07	160	7	0, 5, 7
#2	2-1	Arm07	16	5	0, 5
	2-2	Arm07	16	5	0, 5
	2-3	Arm07	16	7	0, 5, 7
	2-4	Arm07	16	11	0, 5, 7, 10
	2-5	Arm07	16	8	0, 5, 7
#3	3-1	Arm07	360	7	0, 4, 5, 6, 7
	3-2	Arm07	360	7	0, 4, 5, 6, 7
	3-3	Arm07	360	8	0, 4, 5, 6, 7
	3-4	Arm07	360	8	0, 4, 5, 6, 7, 8
#4	4-1	Arm07	100	7	0, 5, 7
	4-2	Arm07	100	11	0, 5, 7, 10, 11
	4-3	Arm07	100	8	0, 5, 7, 8
#5	5-1	Arm07	100	7	0, 5, 7
	5-2	Arm07	100	8	0, 5, 7, 8
	5-3	Arm07	100	7	0, 5, 7
	5-4	Arm07	100	10	0, 5, 7, 10
	5-5	Arm07	100	9	0, 5, 7
#6	6-1	E70	360	7	0, 4, 6
	6-2	E70	360	7	0, 4, 6
	6-3	E70	360	7	0, 4, 7
	6-4	E70	360	8	0, 6
	6-5	E70	360	8	0, 4
#7	7-1	Ken05	360	8	0, 4, 8
	7-2	Ken05	360	8	0, 4, 8
	7-3	Ken05	360	8	0, 4, 6
	7-4	Ken05	360	7	0, 4, 6
	7-5	Ken05	360	5	0, 4, 5

HAU = hemadsorbing units Arm07 = Armenia 2007 DPC = days post-challenge

Chapter 3 - Results

Reactivity, specificity, and isotyping of anti-ASFV p30 monoclonal antibodies

Three monoclonal antibodies, designated 5C1, 2B8, and 1D8, showed specific reactivity against recombinant ASFV p30 antigen and against live virus cell cultures. IFA on Vero E6 cells infected with ASFV strain BA71v using these monoclonals showed highly specific fluorescence in the cytoplasm of ASFV-infected cells (Figure 3.1A-C), with minimal background reactivity observed in uninfected Vero E6 control cells (Figure 3.1E-H) and with infected cells which received no primary antibody treatment (Figure 3.1D). These antibodies were further characterized by isotyping, with monoclonals 5C1 and 1D8 belonging to the IgG1 subclass and monoclonal 2B8 to the IgG2b subclass, and all three possessing κ light chains (Figure 3.1). These three monoclonal antibodies were subsequently used to develop the LFIA.

Experimental challenge, clinical sampling, and qPCR

ASFV-negative whole blood samples were obtained from a total of 79 naïve piglets, including pre-challenge (0DPC) samples from the 31 animals subsequently infected with ASFV (Table 3.1). Clinical evaluation at the time of sampling showed the animals to be healthy and largely free of any signs of disease, with 74/79 piglets having a clinical score of 0, and the remaining 5 piglets having a clinical score of 1. Of the 5 piglets with a clinical score of 1, 4 had a mild fever of 40.6-40.7° and 1 showed mild lethargy, with no other symptoms present. ASFV genomic DNA was not detected by qPCR in any of these 79 whole blood samples.

All 21 piglets experimentally inoculated with the Armenia 2007 isolate of ASFV developed severe ASF with an overall trend of worsening clinical scores post-challenge until death (Table 3.2). Clinical signs were observable as early as 5DPC in some animals, with all piglets succumbing by 11DPC. Excluding post-mortem samples, the last sample obtained from

each piglet was associated with the highest clinical score observed for that animal, with the exception of animal 4-3 which displayed a significant drop in body temperature between 7DPC and euthanasia at 8DPC that resulted in a decreased fever score. qPCR values for EDTA blood samples follow a similar trend of progressively increasing CN/mL of ASFV genomic DNA beginning at 4-5DPC (Table 3.2). ASFV genomic DNA levels ranged from undetectable in five samples (1-1 5DPC, 2-4 5DPC, 2-4 7DPC, 3-4 4DPC, and 4-2 5DPC) to 1.39 x 10^9 CN/mL for sample 1-2 7DPC. As with clinical scores, higher qPCR values tended to be associated with samples obtained at a later time post-challenge. These results are consistent with the clinical course of infection with highly virulent Eurasian genotype II strains in domestic swine (Blome et al. 2013; Gallardo et al. 2017). Similar results were seen in the piglets challenged with E70 or Ken05 viruses (Table 3.3). Clinical signs emerged as early as 4DPC in these piglets, with all animals dying by 8DPC for either virus. qPCR values of >10^8 were seen in the last sample obtained before death for all animals challenged with E70 or Ken05.

Sensitivity, specificity, and reproducibility of the LFIA

A total of 79 pre-challenge and 74 post-challenge blood samples were evaluated by LFIA, with all samples being tested in duplicate and classified as LFIA positive (both duplicates return positive results), negative (both duplicates are negative), or suspect (one positive and one negative). Of the pre-challenge samples, 78/79 were negative by LFIA, including all samples from the 31 piglets subsequently challenged with ASFV (Table 3.1). While no pre-challenge sample tested positive in both LFIA duplicates, one sample (N-32) was classified as suspect because one LFIA strip showed negative for ASFV p30 antigen while the second LFIA strip showed positive. When samples with LFIA results classified as suspect are considered to be false positives, then the calculated specificity for the LFIA with pre-challenge samples is 98.7%

(Table 3.4). However, this value does not accurately reflect the suspect LFIA test result, which cannot be adequately classified into a discrete group as a false positive or false negative, and as a result this calculation will slightly underestimate actual specificity. If LFIA duplicates run on the same sample are considered as independent tests (N = 158) and all results classified as either positive or negative, then the calculated specificity of the LFIA for pre-challenge samples becomes 99.4%. The specificity of the ASFV qPCR was 100%, with no pre-challenge samples showing detectable levels of ASFV genomic DNA (Table 3.4).

Of the 74 post-challenge whole blood samples tested by LFIA, 56 were from animals infected with the Armenia 2007 ASFV strain, 8 were from animals infected with strain E70, and 10 from animals challenged with strain Ken05/Tk1 (Table 3.2 and 3.3). Of the Armenia 2007 post-challenge samples, 51/56 has detectable levels of ASFV genomic DNA with qPCR, for an overall Armenia 2007 post-challenge qPCR sensitivity of 91.1% (Table 3.2 and 3.4). In comparison, 31/56 Armenia 2007 post-challenge samples were LFIA positive on both test strips, 24/56 were negative, and 1 sample (3-4 7DPC) was classified as suspect, for an overall sensitivity of 55.4% when the suspect result is classified as a false negative (Table 3.2 and 3.4). Similar to the calculation for specificity, a result classified as suspect cannot be adequately categorized as a false negative, and this will produce a slight underestimate of diagnostic sensitivity. If all duplicates are considered independent tests (N = 112), sensitivity for post-challenge Armenia 2007 samples slightly increases to 56.3%. While the sensitivity of the LFIA for all Armenia 2007 post-challenge samples is low relative to qPCR, assay performance improves rapidly for animals with worse clinical disease. LFIA sensitivity is very poor (15.4%) for samples taken from asymptomatic animals with a clinical score of 0. However, sensitivity increases to 55.2% in animals with mild clinical signs (clinical score 1-6). If the suspect result

for sample 3-4 7DPC is considered a false negative, sensitivity is 90.9% for animals with moderate to severe symptoms (clinical score >6); this value rises to 95.5% if LFIA duplicates are considered independent tests (N = 22). Sensitivity for post-mortem Armenia 2007 samples is 100% (Table 3.4). A similar trend exists when evaluating results by fever score, with poor sensitivity (25.0%) seen in afebrile piglets that rises to 86.7% for animals with a body temperature of >41.0°C (fever score of 3) and 100% for animals with a temperature <37.8°C (fever score of 4). These results are consistent with those seen for qPCR, which also shows greater sensitivity for samples from animals with higher clinical and fever scores (Table 3.4).

Of the 8 post-challenge samples obtained from piglets challenged with ASFV strain E70, 6 showed positive results with both LFIA test strips and 2 were negative, for an overall sensitivity of 75% (Table 3.3 and 3.4). No E70 samples tested by LFIA showed suspect results. Similar to the Armenia 2007 samples, LFIA sensitivity was higher for samples taken from animals with significant clinical signs or fever than for asymptomatic and afebrile animals. qPCR was able to detect ASFV genomic DNA in all post-challenge E70 samples evaluated, for a post-challenge qPCR sensitivity of 100%. The LFIA was unable to detect ASFV p30 in any post-challenge samples from animals infected with strain Ken05/Tk1, despite all these samples showing high levels of ASFV genomic DNA on qPCR and all animals developing clinical ASF (Table 3.3 and 3.4).

Direct comparison between LFIA and qPCR results shows LFIA performance parallels viral DNA levels. Of the 84 blood samples (79 pre-challenge and 5 post-challenge) which were negative on qPCR, 83 tested negative by LFIA and 1 was considered suspect, indicating a specificity of 98.8% when the suspect result is classified as a false positive, or 99.4% when LFIA duplicates run on the same sample are considered independent tests (N = 168) (Table 3.5). The

sensitivity of the LFIA was poor for samples possessing low levels of ASFV DNA – no sample with $<10^7$ CN/mL ASFV DNA tested positive by LFIA for both Armenia 2007 and E70 samples. Of the 15 Armenia 2007 samples with ASFV DNA levels of 10^7-10^8 CN/mL, 7 were LFIA positive, 7 were negative, and 1 (3-4 7DPC) was suspect, likely due to the level of viremia being near the limit of detection for the assay, since LFIA sensitivity was greater than 95% for the 23 samples with Armenia 2007 ASFV DNA levels of 10^8-10^9, and both samples with $>10^9$ CN/mL were positive (Table 3.5). Similar performance was seen with E70 post-challenge samples. While the LFIA was unable to identify either sample with 10^7-10^8 CN/mL E70 genomic DNA, 6/6 samples with $>10^8$ CN/mL were identified as ASFV positive by LFIA, for a sensitivity of 100% in this category and an overall LFIA sensitivity of 75% for all qPCR-positive E70 samples (Table 3.5). Overall, moderate to substantial agreement was seen between qPCR and LFIA results for all samples, with $\kappa = 0.55$ (95% CI, 0.43-0.67). Since the LFIA strips do not react with Ken05/Tk1 ASFV, it is reasonable to exclude the Ken05/Tk1 post-challenge samples from this analysis; when this is done, the degree of agreement between LFIA and qPCR results becomes more substantial, with $\kappa = 0.65$ (95% CI, 0.53-0.78).

To evaluate the degree of agreement between LFIA strips run on identical test samples, all whole blood samples were tested with LFIA strips in duplicate and results compared between both strips. Out of a total of 79 pre-challenge blood samples, discordant LFIA results were observed only once with sample N-32 (Table 3.1). Similar performance was seen with the 74 post-challenge samples evaluated, with only one (3-4 7DPC) showing discordant results (Table 3.2). Overall, of the 153 blood samples tested, only two samples did not show matching results between LFIA duplicates, indicating near perfect agreement between LFIA duplicates, with $\kappa = 0.97$ (95% CI, 0.92-1.00).

Figures and Tables for Chapter 3

Figure 3.1 IFA screening of monoclonal antibodies

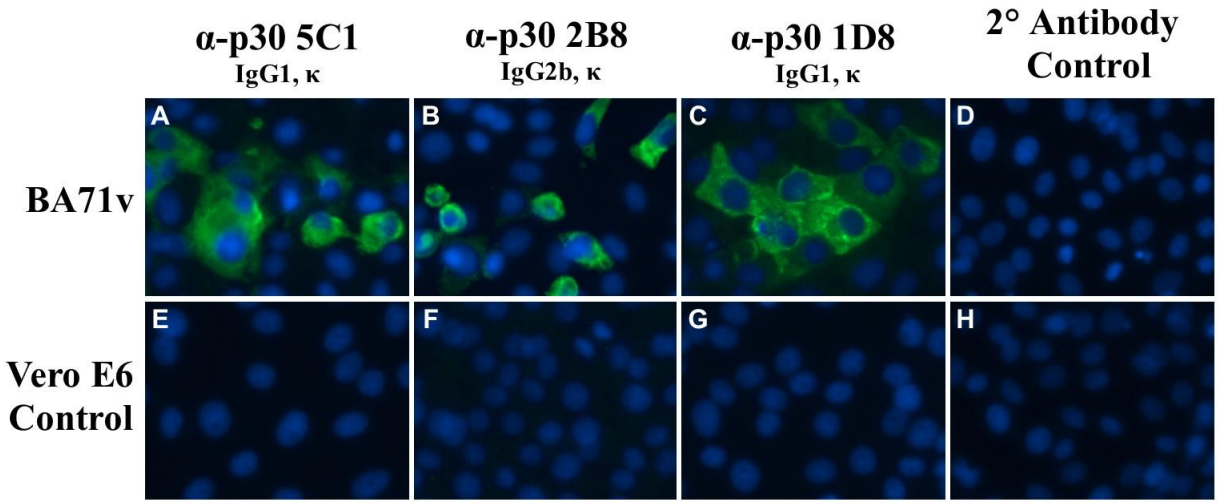

Figure legend: IFA using anti-p30 monoclonal antibodies on acetone-fixed Vero E6 cells revealed specific fluorescence associated with the cytoplasm of BA71v-infected cells (A-C) that is absent in wells containing uninfected control cells (E-H) or infected cells which were treated with the secondary detection antibody only (D). Isotyping results are listed below each monoclonal antibody. Monoclonal antibodies 5C1 and 1D8 belong to the IgG1 subclass, and monoclonal 2B8 to the IgG2b subclass. All 3 monoclonal antibodies possess κ light chains.

Table 3.1 Pre-challenge ASFV-negative samples tested by LFIA and qPCR (n=79)

Sample ID	qPCR CN/mL	LFIA (#1/#2)	Clinical Score	Comments	Sample ID	qPCR CN/mL	LFIA (1/2)	Clinical Score	Comments
1-1 0DPC*	ND	-/-	0		N-10	ND	-/-	0	
1-2 0DPC*	ND	-/-	0		N-11	ND	-/-	0	
1-3 0DPC*	ND	-/-	0		N-12	ND	-/-	0	
1-4 0DPC*	ND	-/-	0		N-13	ND	-/-	0	
2-1 0DPC*	ND	-/-	0		N-14	ND	-/-	1	Mild fever 40.6°
2-2 0DPC*	ND	-/-	0		N-15	ND	-/-	0	
2-3 0DPC*	ND	-/-	0		N-16	ND	-/-	0	
2-4 0DPC*	ND	-/-	0		N-17	ND	-/-	0	
2-5 0DPC*	ND	-/-	0		N-18	ND	-/-	0	
3-1 0DPC*	ND	-/-	0		N-19	ND	-/-	0	
3-2 0DPC*	ND	-/-	0		N-20	ND	-/-	0	
3-3 0DPC*	ND	-/-	0		N-21	ND	-/-	0	
3-4 0DPC*	ND	-/-	1	Mild fever 40.6°	N-22	ND	-/-	0	
4-1 0DPC*	ND	-/-	1	Slight lethargy	N-23	ND	-/-	0	
4-2 0DPC*	ND	-/-	0		N-24	ND	-/-	0	
4-3 0DPC*	ND	-/-	0		N-25	ND	-/-	0	
5-1 0DPC*	ND	-/-	0		N-26	ND	-/-	0	
5-2 0DPC*	ND	-/-	0		N-27	ND	-/-	0	
5-3 0DPC*	ND	-/-	0		N-28	ND	-/-	0	
5-4 0DPC*	ND	-/-	0		N-29	ND	-/-	1	Mild fever 40.6°
5-5 0DPC*	ND	-/-	0		N-30	ND	-/-	0	
6-1 0DPC*	ND	-/-	0		N-31	ND	-/-	0	
6-2 0DPC*	ND	-/-	1	Mild fever 40.7°	N-32	ND	-/+	0	LFIA suspect
6-3 0DPC*	ND	-/-	0		N-33	ND	-/-	0	
6-4 0DPC*	ND	-/-	0		N-34	ND	-/-	0	
6-5 0DPC*	ND	-/-	0		N-35	ND	-/-	0	
7-1 0DPC*	ND	-/-	0		N-36	ND	-/-	0	
7-2 0DPC*	ND	-/-	0		N-37	ND	-/-	0	
7-3 0DPC*	ND	-/-	0		N-38	ND	-/-	0	
7-4 0DPC*	ND	-/-	0		N-39	ND	-/-	0	
7-5 0DPC*	ND	-/-	0		N-40	ND	-/-	0	
N-1	ND	-/-	0		N-41	ND	-/-	0	
N-2	ND	-/-	0		N-42	ND	-/-	0	
N-3	ND	-/-	0		N-43	ND	-/-	0	
N-4	ND	-/-	0		N-44	ND	-/-	0	
N-5	ND	-/-	0		N-45	ND	-/-	0	
N-6	ND	-/-	0		N-46	ND	-/-	0	
N-7	ND	-/-	0		N-47	ND	-/-	0	
N-8	ND	-/-	0		N-48	ND	-/-	0	
N-9	ND	-/-	0						

ND = not detected

*pre-challenge sample from pigs listed in tables 2.1, 3.2, and 3.3

Table 3.2 Armenia 2007 post-challenge samples (n=56)

Sample ID	p72 qPCR CN/mL	LFIA (#1/#2)	Clinical Score	Fever Score	Other Signs	Comments
1-1 5DPC	ND	-/-	0	0	0	
7DPC	7.69E+03	-/-	2	0	2	
10DPC	6.35E+08	+/+	5	3	2	Euthanized
1-2 5DPC	4.53E+08	+/+	0	0	0	
7DPC	1.39E+09	+/+	5	0	5	Euthanized
1-3 5DPC	4.00E+08	+/+	2	2	0	
7DPC	7.09E+08	+/+	9	4	5	Euthanized
1-4 5DPC	1.51E+08	+/+	0	0	0	
7DPC	7.96E+08	+/+	10	4	6	Euthanized
2-1 5DPC	5.69E+08	+/+	5	2	3	Euthanized
2-2 5DPC	7.32E+08	+/+	7	3	4	Euthanized
2-3 5DPC	2.68E+08	-/-	2	2	0	
7DPC	1.28E+09	+/+	12	2	10	Euthanized
2-4 5DPC	ND	-/-	1	0	1	
7DPC	ND	-/-	0	0	0	
10DPC	1.89E+07	-/-	5	2	3	Death at 11DPC
2-5 5DPC	5.27E+07	-/-	1	1	0	
7DPC	9.05E+08	+/+	6	3	3	Death at 8DPC
3-1 4DPC	3.96E+05	-/-	0	0	0	
5DPC	9.37E+06	-/-	3	1	2	
6DPC	6.43E+07	+/+	6	3	3	
7DPC	4.10E+08	+/+	12	3	9	Euthanized
3-2 4DPC	6.46E+05	-/-	0	0	0	
5DPC	7.75E+07	-/-	2	0	2	
6DPC	5.08E+07	+/+	10	1	9	
7DPC	3.88E+08	+/+	N/A	N/A	N/A	Post-mortem
3-3 4DPC	2.94E+04	-/-	0	0	0	
5DPC	8.34E+06	-/-	4	1	3	
6DPC	5.09E+07	+/+	6	3	3	
7DPC	7.00E+08	+/+	11	0	11	Death at 8DPC
3-4 4DPC	ND	-/-	0	0	0	
5DPC	1.94E+05	-/-	0	0	0	
6DPC	4.36E+06	-/-	4	1	3	
7DPC	9.09E+07	+/-	7	0	7	Suspect LFIA
8DPC	3.82E+08	+/+	16	3	13	Euthanized
4-1 5DPC	1.35E+08	+/+	4	3	1	
7DPC	2.63E+07	+/+	N/A	N/A	N/A	Post-mortem
4-2 5DPC	ND	-/-	0	0	0	
7DPC	1.44E+05	-/-	0	0	0	
10DPC	7.47E+07	-/-	3	2	1	
11DPC	6.74E+07	+/+	5	2	3	Euthanized
4-3 5DPC	8.05E+05	-/-	0	0	0	
7DPC	3.23E+08	+/+	7	3	4	
8DPC	2.42E+08	+/+	3	0	3	Euthanized
5-1 5DPC	7.12E+07	+/+	2	2	0	
7DPC	1.48E+08	+/+	8	4	4	Euthanized
5-2 5DPC	1.09E+08	+/+	1	1	0	
7DPC	1.28E+08	+/+	5	3	2	
8DPC	2.52E+08	+/+	N/A	N/A	N/A	Post-mortem
5-3 5DPC	8.72E+07	+/+	3	3	0	
7DPC	2.16E+08	+/+	6	3	3	Death at 7DPC
5-4 5DPC	4.03E+03	-/-	0	0	0	
7DPC	1.86E+07	-/-	5	3	2	
10DPC	3.30E+07	-/-	6	3	3	Death at 10DPC
5-5 5DPC	3.38E+07	-/-	1	1	0	
7DPC	1.28E+08	+/+	4	3	1	Death at 9DPC

ND = not detectable; N/A = not applicable;

Other signs = liveliness, body shape, respiratory, neurological, skin, digestive, ocular/nasal

Table 3.3 E70 (n=8) and Ken05 (n=10) post-challenge samples

	Sample ID	p72 qPCR CN/mL	LFIA (#1/#2)	Clinical Score	Fever Score	Other Signs	Comments
E70	**6-1** 4DPC	9.96E+07	-/-	0	0	0	
	6DPC	7.32E+08	+/+	0	0	0	Death at 7DPC
	6-2 4DPC	7.92E+07	-/-	2	0	2	
	6DPC	5.45E+08	+/+	8	3	5	Death at 7DPC
	6-3 4DPC	2.31E+08	+/+	1	1	0	
	7DPC	4.55E+08	+/+	9	3	6	Euthanized
	6-4 6DPC	6.49E+08	+/+	3	3	0	Death at 8DPC
	6-5 4DPC	2.03E+08	+/+	0	0	0	Death at 8DPC
Ken05	**7-1** 4DPC	8.08E+06	-/-	0	0	0	
	8DPC	1.86E+07	-/-	6	0	6	Euthanized
	7-2 4DPC	3.37E+08	-/-	3	3	0	
	8DPC	1.16E+08	-/-	11	0	11	Euthanized
	7-3 4DPC	2.13E+08	-/-	3	3	0	
	6DPC	3.90E+08	-/-	6	3	3	Death at 8DPC
	7-4 4DPC	2.56E+08	-/-	0	0	0	
	6DPC	3.92E+08	-/-	2	0	2	Death at 7DPC
	7-5 4DPC	8.03E+07	-/-	4	3	1	
	5DPC	1.33E+08	-/-	11	3	8	Euthanized

Other signs = liveliness, body shape, respiratory, neurological, skin, digestive, ocular/nasal

Table 3.4 Pre- and post-challenge specificity and sensitivity for LFIA and qPCR

		LFIA					qPCR			
Challenge	**N**	**Pos.**	**Neg.**	**Suspect**	**Sen.**	**Spec.**	**Pos.**	**Neg.**	**Sen.**	**Spec.**
Pre	79	0	78	1	-	98.7%*	0	79	-	100.0%
Post-Arm07	56	31	24	1	55.4%*	-	51	5	91.1%	-
CS=0	13	2	11	0	15.4%	-	9	4	69.2%	-
CS=1-6	29	16	13	0	55.2%	-	28	1	96.6%	-
CS>6	11	10	0	1	90.9%*	-	11	0	100.0%	-
Dead	3	3	0	0	100.0%	-	3	0	100.0%	-
F=0	20	5	14	1	25.0%*	-	15	5	75.0%	-
F=1	7	2	5	0	28.6%	-	7	0	100.0%	-
F=2	8	5	3	0	62.5%	-	8	0	100.0%	-
F=3	15	13	2	0	86.7%	-	15	0	100.0%	-
F=4	3	3	0	0	100.0%	-	3	0	100.0%	-
Post-E70	8	6	2	0	75.0%	-	8	0	100.0%	-
CS=0	3	2	1	0	66.7%	-	3	0	100.0%	-
CS=1-6	3	2	1	0	66.7%	-	3	0	100.0%	-
CS>6	2	2	0	0	100.0%	-	2	0	100.0%	-
Dead	0	-	-	-	-	-	-	-	-	-
F=0	4	2	2	0	50.0%	-	4	0	100.0%	-
F=1	1	1	0	0	100.0%	-	1	0	100.0%	-
F=2	0	-	-	-	-	-	-	-	-	-
F=3	3	3	0	0	100.0%	-	3	0	100.0%	-
F=4	0	-	-	-	-	-	-	-	-	-
Post-Ken05	10	0	10	0	0.0%	-	10	0	100.0%	-

*Calculated sensitivity/specificity consider suspect results as false negative/false positive, respectively

CS = clinical score

F = fever score

Table 3.5 Performance of the ASFV LFIA vs qPCR

qPCR Results	N	LFIA Pos.	LFIA Neg.	LFIA Suspect	Sen.	Spec.
Neg	84	0	83	1	-	98.8%*
Pos - Arm07	51	31	19	1	60.8%*	-
$<10^5$	3	0	3	0	0.0%	-
10^5-10^6	5	0	5	0	0.0%	-
10^6-10^7	3	0	3	0	0.0%	-
10^7-10^8	15	7	7	1	46.7%*	-
10^8-10^9	23	22	1	0	95.7%	-
$>10^9$	2	2	0	0	100.0%	-
Pos - E70	8	6	2	0	75.0%	-
$<10^5$	0	-	-	-	-	-
10^5-10^6	0	-	-	-	-	-
10^6-10^7	0	-	-	-	-	-
10^7-10^8	2	0	2	0	0.0%	-
10^8-10^9	6	6	0	0	100.0%	-
$>10^9$	0	-	-	-	-	-

*Sensitivity/specificity consider suspect results as false negative/false positive

qPCR values in CN/mL whole blood

Chapter 4 - Discussion and Conclusions

ASFV rapid detection efforts are hindered by the limitations of currently available diagnostic assays. Serological tests for detection of ASFV-specific antibodies are available in a variety of formats; however, these assays show low sensitivity for early stage infections, and highly virulent ASFV strains frequently are lethal to infected pigs before a significant antibody response can be generated, which limits the utility of serological testing in identifying acute cases of ASF (Cubillos et al., 2013; Sánchez-Vizcaíno et al., 2019; Gaudreault et al., 2020). Laboratory-based virological detection methods such as virus isolation and fluorescent antibody testing require specialized facilities and experienced personnel which limits their utility, and PCR-based detection assays require sophisticated equipment and reliable access to clean laboratories and electricity (Oura et al., 2013; Sánchez-Vizcaíno et al., 2019; Gaudreault et al., 2020). Assays for viral antigen detection are attractive diagnostic tests for ASF because animals with severe clinical signs and which succumb from infection with highly virulent ASFV strains tend to have very high levels of virus in blood and tissues (Blome et al., 2013; Oura et al. 2013; Gallardo et al., 2017; Gaudreault et al. 2020). Commercial tests for the detection of the ASFV p72 antigen are available in ELISA and immunochromatographic strip formats. The published datasets for sensitivity and specificity of these assays are limited; however, poor sensitivity (84.3%) has been observed for the p72 antigen ELISA (Gallardo et al. 2015; Sastre et al., 2016; Sánchez-Vizcaíno et al., 2019).

In the present study, a chromatographic LFIA for the detection of the ASFV p30 antigen was developed and evaluated using a panel of whole blood samples from swine experimentally infected with various ASFV genotypes. The primary advantages of this newly developed LFIA are its speed and simplicity, coupled with an ability to reliably detect virus in clinically ill

animals with a very high degree of specificity. The p30-based LFIA is compact, does not require cold storage or any expensive or bulky reagents, and delivers results in 25 minutes, making it ideal for field use. Importantly, the ASFV LFIA could reliably identify samples from animals displaying moderate to severe clinical symptoms of ASF after experimental infection with virulent Armenia 2007 or E70 virus, with 12/13 samples from these animals testing positive and 1 sample showing suspect results, likely due to the level of virus in the sample being near the limit of detection for the assay (Table 3.4). The LFIA also showed a specificity of nearly 100% with pre-challenge samples from known ASFV-negative piglets (Table 3.4). Reliable specificity and sensitivity are essential for ASFV diagnostics because control methods such as quarantining and culling animals are severely disruptive economically, making false positives or false negatives a testing liability of significant concern. The high specificity of the LFIA allows for herd screening and testing of large numbers of animals because the probability of obtaining a false positive remains rather low even if large numbers of tests are performed. The sensitivity of the ASFV LFIA depends on clinical signs displayed by the animals: it correlates positively with the severity of clinical signs (Table 3.4). Currently, circulating Eurasian genotype II ASFV strains are both highly contagious and virulent, causing disease characterized by significant mortality and high levels of viremia in moribund and dying animals (Blome et al., 2013; Gallardo et al., 2017; Zhou et al., 2018). This allows the usage of a less sensitive field-deployable ASFV diagnostic assay based on antigen detection, because clinically ill animals have a high level of circulating virus antigen. Indeed, while the sensitivity of the ASFV LFIA was reduced when compared to qPCR for asymptomatic pigs or those displaying mild signs of ASF, the LFIA performance increased significantly with disease progression and reached sensitivity levels similar to qPCR (Table 3.4). Importantly, the ASFV LFIA could reliably

identify animals with high levels of circulating ASFV genomic DNA (Table 3.5). The ASFV LFIA sensitivity also increased with fever score (Table 3.4). Fever is an objective measurement which can be readily obtained in most settings, suggesting this clinical sign could be an important parameter to determine prior to testing.

The ASFV LFIA was evaluated using whole blood samples from piglets experimentally infected with three different ASFV isolates – the Armenia 2007 isolate (genotype II), the E70 isolate (genotype I), and the Ken05/Tk1 isolate (genotype X) (Table 2.1). Interestingly, the ASFV LFIA was unable to positively identify any samples from Ken05/Tk1-infected piglets, despite several of these samples coming from animals showing significant clinical signs of ASF and possessing ASFV genomic DNA levels of $>10^8$ CN/mL, a concentration associated with robust LFIA sensitivity for samples from Armenia 2007 and E70-infected animals (Table 3.3, 3.4, and 3.5). These results indicate the LFIA is unreactive with the Ken05/Tk1 p30 antigen. One likely explanation for this is that there are differences in the p30 protein between ASFV isolates from different geographic origins and different genotypes, i.e. between Arm07 and Ken05/Tk1. A similar phenomenon has been previously described for an indirect ELISA using baculovirus-expressed recombinant p30 to detect ASFV-specific antibodies. This ELISA, based on a recombinant ASFV p30 antigen derived from the genotype I E75 ASFV strain, showed significantly reduced sensitivity with serum samples from Uganda compared to samples from Nigeria and Spain (Pérez-Filgueira et al. 2006). Alignments of the p30 amino acid sequences from the Spanish ASFV E70 isolate with multiple Western, Southern, and Eastern African virus isolates showed 100% amino acid identity between E70 and the West African Benin 97/1 isolate, and 96-98% between E70 and multiple Southern African isolates; however, amino acid sequence identities between E70 and the Eastern African ASFV isolates Malawi Lil 20/1 1983 and Kenya

1950 were 91% and 85%, respectively, with the highest level of sequence variability associated with regions predicted to have high antigenicity (Pérez-Filgueira et al. 2006). Alignment of the p30 amino acid sequences for ASFV strains Georgia 2007/1 (Genbank: YP_009927217), a genotype II strain closely related to the Armenia 2007 ASFV, E70 (Genbank: AAL68656), and Ken05/Tk1 (Genbank: YP_009702826) using the Clustal Omega multiple sequence alignment program (EMBL-EBI, Cambridgeshire, UK) showed an amino sequence identity of 98% between Georgia 2007/1 and E70, but only 89% between Georgia 2007/1 and Ken05/Tk1, as well as a stretch of an additional 7 amino acid residues within the Ken05/Tk1 p30 that is not present in the Georgia 2007/1 and E70 proteins (Figure 4.1), supporting the hypothesis than the Ken05/Tk1 p30 may be antigenically distinct to a degree that would impede its recognition by monoclonal antibodies raised against a recombinant Georgia 2007/1 p30 protein such as those used for the ASFV LFIA. While such differences have implications for the utility of the p30-based ASFV LFIA in Eastern Africa, the epidemic ASFV isolates in Europe and Asia are genotype II isolates which are closely related to the Armenia 2007 ASFV isolate evaluated in this study.

Overall, the p30-based ASFV LFIA possesses robust specificity and is able to reliably identify genotype II Armenia 2007 and genotype I E70 ASFV-infected animals displaying moderate to severe clinical ASF. The LFIA is unable to identify Ken05/Tk1-infected piglets. Some limitations of the present study are related to the ASFV genotypes studied and the blood samples evaluated. All samples were gathered from experimental ASFV-infected animals challenged with genotypes I, II and X in laboratory settings. These animals were housed in high-containment facilities where exposure to other swine pathogens is minimal, and the potential for LFIA cross-reactivity with other infectious agents should be assessed. Additionally, the samples

evaluated were well-characterized and obtained and stored under ideal conditions which may not be applicable to field situations. Consequently, additional testing with field isolates and other viral genotypes will be needed to further evaluate the utility of this novel assay for ASFV diagnosis and control.

Figures for Chapter 4

Figure 4.1 p30 protein sequence alignment for Georgia 2007/1, E70, and Ken05/Tk1 ASFVs

```
Georgia 2007/1 MDFILNISMKMEVIFKTDLRSSSQVVFHAGSLYNWFSVEIINSGRIVTTAIKTLLSTVKY     60
E70            MDFILNISMKMEVIFKTDLRSSSQVVFHAGSLYNWFSVEIINSGRIVTTAIKTLLSTVKY     60
Ken05/Tk1      MDFILNISMKMEVIFKTDLRASSQVVFHAGSLYTWFSVEIINSGRIVTTAIKTLLSTVKY     60
               ********************:************.*************************

Georgia 2007/1 DIVKSARIYAGQGYTEHQAQEEWNMILHVLFEEETESSASS-------ENIHEKNDNETN     113
E70            DIVKSAHIYAGQGYTEHQAQEEWNMILHVLFEEETESSASS-------ESIHEKNDNETN     113
Ken05/Tk1      DIVRNARIYAGQGYTEQQAQEEWNMILHVLFEEETESTSSTSLESNHETNGHKADGHETN     120
               ***:.*:*********:**********************::*:        . *: :.:***

Georgia 2007/1 ECTSSFETLFEQEPSSEVPKDSKLYMLAQKTVQHIEQYGKAPDFNKVIRAHNFIQTIYGT     173
E70            ECTSSFETLFEQEPSSEEPKDSKLYMLAQKTVQHIEQYGKAPDFNKVIRAHNFIQTIHGT     173
Ken05/Tk1      ECTSSFETLFEQEPSSETPKDTKLYALAQKAVQHIEQYGKAPDFNKVIRAHNYIQTIYGS     180
               ***************** ***:*** ****:**********************:****:*:

Georgia 2007/1 PLKEEEKEVVRLMVIKLLKKK 194        100% Identity to Georgia 2007/1
E70            PLKEEEKEVVRLMVIKLLKKK 194         98% Identity to Georgia 2007/1
Ken05/Tk1      PLKEEEKEEVRLMVIKLLKKK 201         89% Identity to Georgia 2007/1
               ******** ************
```

Figure legend: The amino acid sequences of the p30 protein from Georgia 2007/1, E70, and Ken05/Tk1 ASFV isolates were aligned using the Clustal Omega program. The Georgia 2007/1 and E70 p30 amino acid sequences are both 194 residues long and share 98% identity with each other. The Ken05/Tk1 p30 amino acid sequence is 201 residues long, containing a stretch of 7 residues that is not present in either the Georgia 2007/1 or E70 p30 sequences. Ken05/Tk1 p30 only shares 89% identity with the Georgia 2007/1 p30. Notations regarding the alignment are in the bottom row. *Residue is conserved in all aligned sequences; :Residues are different but groups share strongly similar properties; .Residues are different but groups share weakly similar properties;

References

Afonso CL, Alcaraz C, Brun A, Sussman MD, Onisk DV, Escribano JM, Rock DL. 1992. Characterization of p30, a highly antigenic membrane and secreted protein of African swine fever virus. *Virology* 189:368-373.

Afonso CL, Piccone ME, Zaffuto KM, Neilan J, Kutish GF, Lu Z, Balinsky CA, Gibb TR, Bean TJ, Zsak L, Rock DL. 2004. African swine fever virus multigene family 360 and 530 genes affect host interferon response. *J Virol* 78:1858-1864.

Agüero M, Fernández J, Romero L, Sánchez Mascaraque C, Arias M, Sánchez-Vizcaíno JM. 2003. Highly sensitive PCR assay for routine diagnosis of African swine fever virus in clinical samples. *J Clin Microbiol* 41:4431-4434.

Agüero M, Fernández J, Romero LJ, Zamora MJ, Sánchez C, Belák S, Arias M, Sánchez-Vizcaíno JM. 2004. A highly sensitive and specific gel-based multiplex RT-PCR assay for the simultaneous and differential diagnosis of African swine fever and Classical swine fever in clinical samples. *Vet Res* 35:551-563.

Alcaraz C, De Diego M, Pastor MJ, Escribano JM. 1990. Comparison of a radioimmunoprecipitation assay to immunoblotting and ELISA for detection of antibody to African swine fever virus. *J Vet Diagn Invest* 2:191-196.

Alcaraz C, Rodriguez F, Oviedo JM, Eiras A, De Diego M, Alonso C, Escribano JM. 1995. Highly specific confirmatory western blot test for African swine fever virus antibody detection using the recombinant virus protein p54. *J Virol Methods* 52:111-119.

Alejo A, Matamoros T, Guerra M, Andrés G. 2018. A Proteomic Atlas of the African Swine Fever Virus Particle. *J Virol* 92:e01293-01318.

Alonso C, Galindo I, Cuesta-Geijo MA, Cabezas M, Hernaez B, Muñoz-Moreno R. 2013.

African swine fever virus-cell interactions: from virus entry to cell survival. *Virus Res* 173:42-57.

Anderson EC, Hutchings GH, Mukarati N, Wilkinson PJ. 1998. African swine fever virus infection of the bushpig (Potamochoerus porcus) and its significance in the epidemiology of the disease. *Vet Microbiol* 62:1-15.

Andrés G, Alejo A, Salas J, Salas ML. 2002. African swine fever virus polyproteins pp220 and pp62 assemble into the core shell. *J Virol* 76:12473-12482.

Andrés G, Simón-Mateo C, Viñuela E. 1997. Assembly of African swine fever virus: role of polyprotein pp220. *J Virol* 71:2331-2341.

Argilaguet JM, Pérez-Martín E, Gallardo C, Salguero FJ, Borrego B, Lacasta A, Accensi F, Díaz I, Nofrarías M, Pujols J, Blanco E, Pérez-Filgueira M, Escribano JM, Rodríguez F. 2011. Enhancing DNA immunization by targeting ASFV antigens to SLA-II bearing cells. *Vaccine* 29:5379-5385.

Arias M, Escribano JM, Sánchez-Vizcaíno JM. 1993. Persistence of African swine fever antibody reactivity on ELISA and immunoblotting assays. *Vet Rec* 133:189-190.

Barderas MG, Wigdorovitz A, Merelo F, Beitia F, Alonso C, Borca MV, Escribano JM. 2000. Serodiagnosis of African swine fever using the recombinant protein p30 expressed in insect larvae. *J Virol Methods* 89:129-136.

Basto AP, Nix RJ, Boinas F, Mendes S, Silva MJ, Cartaxeiro C, Portugal RS, Leitão A, Dixon LK, Martins C. 2006. Kinetics of African swine fever virus infection in Ornithodoros erraticus ticks. *J Gen Virol* 87:1863-1871.

Basto AP, Portugal RS, Nix RJ, Cartaxeiro C, Boinas F, Dixon LK, Leitão A, Martins C. 2006. Development of a nested PCR and its internal control for the detection of African swine

fever virus (ASFV) in Ornithodoros erraticus. *Arch Virol* 151:819-826.

Bastos AD, Penrith ML, Crucière C, Edrich JL, Hutchings G, Roger F, Couacy-Hymann E, G RT. 2003. Genotyping field strains of African swine fever virus by partial p72 gene characterisation. *Arch Virol* 148:693-706.

Beltrán-Alcrudo DG, AM; Gallardo, C.; Kramer, S. 2017. *African Swine Fever: Detection and Diagnosis - A Manual for Veterinarians, 19th ed*. Food and Agriculture Organization of the United Nations, Rome.

Bisimwa PN, Ongus JR, Tiambo CK, Machuka EM, Bisimwa EB, Steinaa L, Pelle R. 2020. First detection of African swine fever (ASF) virus genotype X and serogroup 7 in symptomatic pigs in the Democratic Republic of Congo. *Virology Journal* 17:135.

Blome S, Gabriel C, Beer M. 2013. Pathogenesis of African swine fever in domestic pigs and European wild boar. *Virus Res* 173:122-130.

Blome S, Gabriel C, Beer M. 2014. Modern adjuvants do not enhance the efficacy of an inactivated African swine fever virus vaccine preparation. *Vaccine* 32:3879-3882.

Blome S, Gabriel C, Dietze K, Breithaupt A, Beer M. 2012. High virulence of African swine fever virus caucasus isolate in European wild boars of all ages. *Emerg Infect Dis* 18:708.

Boinas F, Ribeiro R, Madeira S, Palma M, de Carvalho IL, Núncio S, Wilson AJ. 2014. The medical and veterinary role of Ornithodoros erraticus complex ticks (Acari: Ixodida) on the Iberian Peninsula. *J Vector Ecol* 39:238-248.

Boinas FS, Hutchings GH, Dixon LK, Wilkinson PJ. 2004. Characterization of pathogenic and non-pathogenic African swine fever virus isolates from Ornithodoros erraticus inhabiting pig premises in Portugal. *J Gen Virol* 85:2177-2187.

Boinas FS, Wilson AJ, Hutchings GH, Martins C, Dixon LJ. 2011. The persistence of African

swine fever virus in field-infected Ornithodoros erraticus during the ASF endemic period in Portugal. *PLoS One* 6:e20383.

Borca MV, Kutish GF, Afonso CL, Irusta P, Carrillo C, Brun A, Sussman M, Rock DL. 1994. An African swine fever virus gene with similarity to the T-lymphocyte surface antigen CD2 mediates hemadsorption. *Virology* 199:463-468.

Boshoff CI, Bastos AD, Gerber LJ, Vosloo W. 2007. Genetic characterisation of African swine fever viruses from outbreaks in southern Africa (1973-1999). *Vet Microbiol* 121:45-55.

Botija CS. 1970. [Diagnosis of African swine fever by immunofluorescence]. *Bull Off Int Epizoot* 73:1025-1044.

Burmakina G, Malogolovkin A, Tulman ER, Zsak L, Delhon G, Diel DG, Shobogorov NM, Morgunov YP, Morgunov SY, Kutish GF, Kolbasov D, Rock DL. 2016. African swine fever virus serotype-specific proteins are significant protective antigens for African swine fever. *J Gen Virol* 97:1670-1675.

Carrascosa AL, Bustos MJ, de Leon P. 2011. Methods for growing and titrating African swine fever virus: field and laboratory samples. *Curr Protoc Cell Biol* Chapter 26:Unit 26.14.

Carrascosa AL, Saastre I, González P, Viñuela E. 1993. Localization of the African swine fever virus attachment protein P12 in the virus particle by immunoelectron microscopy. *Virology* 193:460-465.

Carrascosa AL, Santarén JF, Viñuela E. 1982. Production and titration of African swine fever virus in porcine alveolar macrophages. *J Virol Methods* 3:303-310.

Carrascosa JL, Carazo JM, Carrascosa AL, García N, Santisteban A, Viñuela E. 1984. General morphology and capsid fine structure of African swine fever virus particles. *Virology* 132:160-172.

Chapman DAG, Tcherepanov V, Upton C, Dixon LK. 2008. Comparison of the genome sequences of non-pathogenic and pathogenic African swine fever virus isolates. *J Gen Virol* 89:397-408.

Costard S, Mur L, Lubroth J, Sanchez-Vizcaino JM, Pfeiffer DU. 2013. Epidemiology of African swine fever virus. *Virus Res* 173:191-197.

Costard S, Wieland B, de Glanville W, Jori F, Rowlands R, Vosloo W, Roger F, Pfeiffer DU, Dixon LK. 2009. African swine fever: how can global spread be prevented? *Philos Trans R Soc Lond B Biol Sci* 364:2683-2696.

Cubillos C, Gómez-Sebastian S, Moreno N, Nuñez MC, Mulumba-Mfumu LK, Quembo CJ, Heath L, Etter EM, Jori F, Escribano JM, Blanco E. 2013. African swine fever virus serodiagnosis: a general review with a focus on the analyses of African serum samples. *Virus Res* 173:159-167.

Cwynar P, Stojkov J, Wlazlak K. 2019. African Swine Fever Status in Europe. *Viruses* 11:310.

de León P, Bustos MJ, Carrascosa AL. 2013. Laboratory methods to study African swine fever virus. *Virus Res* 173:168-179.

de Oliveira VL, Almeida SC, Soares HR, Crespo A, Marshall-Clarke S, Parkhouse RM. 2011. A novel TLR3 inhibitor encoded by African swine fever virus (ASFV). *Arch Virol* 156:597-609.

Dee SA, Bauermann FV, Niederwerder MC, Singrey A, Clement T, de Lima M, Long C, Patterson G, Sheahan MA, Stoian AMM, Petrovan V, Jones CK, De Jong J, Ji J, Spronk GD, Minion L, Christopher-Hennings J, Zimmerman JJ, Rowland RRR, Nelson E, Sundberg P, Diel DG. 2018. Survival of viral pathogens in animal feed ingredients under transboundary shipping models. *PLoS One* 13:e0194509.

Diaz AV, Netherton CL, Dixon LK, Wilson AJ. 2012. African swine fever virus strain Georgia 2007/1 in Ornithodoros erraticus ticks. *Emerg Infect Dis* 18:1026-1028.

Dixon LK, Chapman DA, Netherton CL, Upton C. 2013. African swine fever virus replication and genomics. *Virus Res* 173:3-14.

Enjuanes L, Carrascosa AL, Moreno MA, Viñuela E. 1976. Titration of African swine fever (ASF) virus. *J Gen Virol* 32:471-477.

Escribano JM, Galindo I, Alonso C. 2013. Antibody-mediated neutralization of African swine fever virus: myths and facts. *Virus Res* 173:101-109.

Esteves A, Marques MI, Costa JV. 1986. Two-dimensional analysis of African swine fever virus proteins and proteins induced in infected cells. *Virology* 152:192-206.

Fernández-Pinero J, Gallardo C, Elizalde M, Robles A, Gómez C, Bishop R, Heath L, Couacy-Hymann E, Fasina FO, Pelayo V, Soler A, Arias M. 2013. Molecular diagnosis of African Swine Fever by a new real-time PCR using universal probe library. *Transbound Emerg Dis* 60:48-58.

Ferris NP, Nordengrahn A, Hutchings GH, Reid SM, King DP, Ebert K, Paton DJ, Kristersson T, Brocchi E, Grazioli S, Merza M. 2009. Development and laboratory validation of a lateral flow device for the detection of foot-and-mouth disease virus in clinical samples. *J Virol Methods* 155:10-17.

Frączyk M, Woźniakowski G, Kowalczyk A, Niemczuk K, Pejsak Z. 2016. Development of cross-priming amplification for direct detection of the African Swine Fever Virus, in pig and wild boar blood and sera samples. *Lett Appl Microbiol* 62:386-391.

Frant M, Woźniakowski G, Pejsak Z. 2017. African Swine Fever (ASF) and Ticks. No Risk of Tick-mediated ASF Spread in Poland and Baltic States. *J Vet Res* 61:375-380.

Gabriel C, Blome S, Malogolovkin A, Parilov S, Kolbasov D, Teifke JP, Beer M. 2011. Characterization of African swine fever virus Caucasus isolate in European wild boars. *Emerg Infect Dis* 17:2342-2345.

Galindo I, Almazán F, Bustos MJ, Viñuela E, Carrascosa AL. 2000. African swine fever virus EP153R open reading frame encodes a glycoprotein involved in the hemadsorption of infected cells. *Virology* 266:340-351.

Galindo I, Alonso C. 2017. African Swine Fever Virus: A Review. *Viruses* 9:103.

Gallardo C, Blanco E, Rodríguez JM, Carrascosa AL, Sanchez-Vizcaino JM. 2006. Antigenic properties and diagnostic potential of African swine fever virus protein pp62 expressed in insect cells. *J Clin Microbiol* 44:950-956.

Gallardo C, Fernández-Pinero J, Arias M. 2019. African swine fever (ASF) diagnosis, an essential tool in the epidemiological investigation. *Virus Res* 271:197676.

Gallardo C, Mwaengo DM, Macharia JM, Arias M, Taracha EA, Soler A, Okoth E, Martín E, Kasiti J, Bishop RP. 2009. Enhanced discrimination of African swine fever virus isolates through nucleotide sequencing of the p54, p72, and pB602L (CVR) genes. *Virus Genes* 38:85-95.

Gallardo C, Nieto R, Soler A, Pelayo V, Fernández-Pinero J, Markowska-Daniel I, Pridotkas G, Nurmoja I, Granta R, Simón A, Pérez C, Martín E, Fernández-Pacheco P, Arias M. 2015. Assessment of African Swine Fever Diagnostic Techniques as a Response to the Epidemic Outbreaks in Eastern European Union Countries: How To Improve Surveillance and Control Programs. *J Clin Microbiol* 53:2555-2565.

Gallardo C, Reis AL, Kalema-Zikusoka G, Malta J, Soler A, Blanco E, Parkhouse RM, Leitão A. 2009. Recombinant antigen targets for serodiagnosis of African swine fever. *Clin*

Vaccine Immunol 16:1012-1020.

Gallardo C, Soler A, Nieto R, Cano C, Pelayo V, Sánchez MA, Pridotkas G, Fernandez-Pinero J, Briones V, Arias M. 2017. Experimental Infection of Domestic Pigs with African Swine Fever Virus Lithuania 2014 Genotype II Field Isolate. *Transbound Emerg Dis* 64:300-304.

Gallardo C, Soler A, Nieto R, Sánchez MA, Martins C, Pelayo V, Carrascosa A, Revilla Y, Simón A, Briones V, Sánchez-Vizcaíno JM, Arias M. 2015. Experimental Transmission of African Swine Fever (ASF) Low Virulent Isolate NH/P68 by Surviving Pigs. *Transbound Emerg Dis* 62:612-622.

García-Belmonte R, Pérez-Núñez D, Pittau M, Richt JA, Revilla Y. 2019. African Swine Fever Virus Armenia/07 Virulent Strain Controls Interferon Beta Production through the cGAS-STING Pathway. *J Virol* 93:e02298-18.

Gaudreault NN, Madden DW, Wilson WC, Trujillo JD, Richt JA. 2020. African Swine Fever Virus: An Emerging DNA Arbovirus. *Front Vet Sci* 7:215.

Giménez-Lirola LG, Mur L, Rivera B, Mogler M, Sun Y, Lizano S, Goodell C, Harris DL, Rowland RR, Gallardo C, Sánchez-Vizcaíno JM, Zimmerman J. 2016. Detection of African Swine Fever Virus Antibodies in Serum and Oral Fluid Specimens Using a Recombinant Protein 30 (p30) Dual Matrix Indirect ELISA. *PLoS One* 11(9):e0161230.

Gogin A, Gerasimov V, Malogolovkin A, Kolbasov D. 2013. African swine fever in the North Caucasus region and the Russian Federation in years 2007-2012. *Virus Res* 173:198-203.

Golding JP, Goatley L, Goodbourn S, Dixon LK, Taylor G, Netherton CL. 2016. Sensitivity of African swine fever virus to type I interferon is linked to genes within multigene families 360 and 505. *Virology* 493:154-161.

Golnar AJ, Martin E, Wormington JD, Kading RC, Teel PD, Hamer SA, Hamer GL. 2019. Reviewing the Potential Vectors and Hosts of African Swine Fever Virus Transmission in the United States. *Vector Borne Zoonotic Dis* 19:512-524.

Gómez-Puertas P, Rodríguez F, Oviedo JM, Brun A, Alonso C, Escribano JM. 1998. The African swine fever virus proteins p54 and p30 are involved in two distinct steps of virus attachment and both contribute to the antibody-mediated protective immune response. *Virology* 243:461-471.

Gómez-Puertas P, Rodríguez F, Oviedo JM, Ramiro-Ibáñez F, Ruiz-Gonzalvo F, Alonso C, Escribano JM. 1996. Neutralizing antibodies to different proteins of African swine fever virus inhibit both virus attachment and internalization. *J Virol* 70:5689-5694.

Gómez-Villamandos JC, Bautista MJ, Sánchez-Cordón PJ, Carrasco L. 2013. Pathology of African swine fever: the role of monocyte-macrophage. *Virus Res* 173:140-149.

Guinat C, Gogin A, Blome S, Keil G, Pollin R, Pfeiffer DU, Dixon L. 2016. Transmission routes of African swine fever virus to domestic pigs: current knowledge and future research directions. *Vet Rec* 178:262-267.

Guinat C, Gubbins S, Vergne T, Gonzales JL, Dixon L, Pfeiffer DU. 2016. Experimental pig-to-pig transmission dynamics for African swine fever virus, Georgia 2007/1 strain. *Epidemiol Infect* 144:25-34.

Haines FJ, Hofmann MA, King DP, Drew TW, Crooke HR. 2013. Development and validation of a multiplex, real-time RT PCR assay for the simultaneous detection of classical and African swine fever viruses. *PLoS One* 8(7):e71019.

Haresnape JM, Wilkinson PJ. 1989. A study of African swine fever virus infected ticks (Ornithodoros moubata) collected from three villages in the ASF enzootic area of Malawi

following an outbreak of the disease in domestic pigs. *Epidemiol Infect* 102:507-522.

Haresnape JM, Wilkinson PJ, Mellor PS. 1988. Isolation of African swine fever virus from ticks of the Ornithodoros moubata complex (Ixodoidea: Argasidae) collected within the African swine fever enzootic area of Malawi. *Epidemiol Infect* 101:173-185.

Hess WR, Endris RG, Lousa A, Caiado JM. 1989. Clearance of African swine fever virus from infected tick (Acari) colonies. *J Med Entomol* 26:314-317.

Heuschele WP, Coggins L. 1965. Isolation of African swine fever virus from a giant forest hog. *Bull Epizoot Dis Afr* 13:255-256.

Hjertner B, Meehan B, McKillen J, McNeilly F, Belák S. 2005. Adaptation of an Invader assay for the detection of African swine fever virus DNA. *J Virol Methods* 124:1-10.

James HE, Ebert K, McGonigle R, Reid SM, Boonham N, Tomlinson JA, Hutchings GH, Denyer M, Oura CA, Dukes JP, King DP. 2010. Detection of African swine fever virus by loop-mediated isothermal amplification. *J Virol Methods* 164:68-74.

Jori F, Bastos AD. 2009. Role of wild suids in the epidemiology of African swine fever. *Ecohealth* 6:296-310.

Jori F, Vial L, Penrith ML, Pérez-Sánchez R, Etter E, Albina E, Michaud V, Roger F. 2013. Review of the sylvatic cycle of African swine fever in sub-Saharan Africa and the Indian ocean. *Virus Res* 173:212-227.

Kazakova AS, Imatdinov IR, Dubrovskaya OA, Imatdinov AR, Sidlik MV, Balyshev VM, Krasochko PA, Sereda AD. 2017. Recombinant Protein p30 for Serological Diagnosis of African Swine Fever by Immunoblotting Assay. *Transbound Emerg Dis* 64:1479-1492.

Keßler C, Forth JH, Keil GM, Mettenleiter TC, Blome S, Karger A. 2018. The intracellular proteome of African swine fever virus. *Sci Rep* 8:14714.

King DP, Reid SM, Hutchings GH, Grierson SS, Wilkinson PJ, Dixon LK, Bastos AD, Drew TW. 2003. Development of a TaqMan PCR assay with internal amplification control for the detection of African swine fever virus. *J Virol Methods* 107:53-61.

Kleiboeker SB, Burrage TG, Scoles GA, Fish D, Rock DL. 1998. African swine fever virus infection in the argasid host, Ornithodoros porcinus. *J Virol* 72:1711-1724.

Kleiboeker SB, Scoles GA. 2001. Pathogenesis of African swine fever virus in Ornithodoros ticks. *Anim Health Res Rev* 2:121-128.

Koczula KM, Gallotta A. 2016. Lateral flow assays. *Essays Biochem* 60:111-120.

Kollnberger SD, Gutierrez-Castañeda B, Foster-Cuevas M, Corteyn A, Parkhouse RME. 2002. Identification of the principal serological immunodeterminants of African swine fever virus by screening a virus cDNA library with antibody. *J Gen Virol* 83:1331-1342.

Krug PW, Lee LJ, Eslami AC, Larson CR, Rodriguez L. 2011. Chemical disinfection of high-consequence transboundary animal disease viruses on nonporous surfaces. *Biologicals* 39:231-235.

Landis JR, Koch GG. 1977. The measurement of observer agreement for categorical data. *Biometrics* 33:159-174.

Leitão A, Cartaxeiro C, Coelho R, Cruz B, Parkhouse RME, Portugal FC, Vigário JD, Martins CLV. 2001. The non-haemadsorbing African swine fever virus isolate ASFV/NH/P68 provides a model for defining the protective anti-virus immune response. *J Gen Virol* 82:513-523.

Linden A, Licoppe A, Volpe R, Paternostre J, Lesenfants C, Cassart D, Garigliany M, Tignon M, van den Berg T, Desmecht D, Cay AB. 2019. Summer 2018: African swine fever virus hits north-western Europe. *Transbound Emerg Dis* 66:54-55.

Liu L, Atim S, LeBlanc N, Rauh R, Esau M, Chenais E, Mwebe R, Nelson WM, Masembe C, Nantima N, Ayebazibwe C, Ståhl K. 2019. Overcoming the challenges of pen-side molecular diagnosis of African swine fever to support outbreak investigations under field conditions. *Transbound Emerg Dis* 66:908-914.

Liu L, Luo Y, Accensi F, Ganges L, Rodríguez F, Shan H, Ståhl K, Qiu HJ, Belák S. 2017. Pre-Clinical Evaluation of a Real-Time PCR Assay on a Portable Instrument as a Possible Field Diagnostic Tool: Experiences from the Testing of Clinical Samples for African and Classical Swine Fever Viruses. *Transbound Emerg Dis* 64:e31-e35.

Lu G, Pan J, Zhang G. 2020. African swine fever virus in Asia: Its rapid spread and potential threat to unaffected countries. *J Infect* 80:350-371.

Lubisi BA, Bastos AD, Dwarka RM, Vosloo W. 2005. Molecular epidemiology of African swine fever in East Africa. *Arch Virol* 150:2439-2452.

Lyra TM. 2006. [The eradication of African swine fever in Brazil, 1978-1984]. *Rev Sci Tech* 25:93-103.

Malogolovkin A, Burmakina G, Titov I, Sereda A, Gogin A, Baryshnikova E, Kolbasov D. 2015. Comparative analysis of African swine fever virus genotypes and serogroups. *Emerg Infect Dis* 21:312-315.

Malogolovkin A, Burmakina G, Tulman ER, Delhon G, Diel DG, Salnikov N, Kutish GF, Kolbasov D, Rock DL. 2015. African swine fever virus CD2v and C-type lectin gene loci mediate serological specificity. *J Gen Virol* 96:866-873.

Miao F, Zhang J, Li N, Chen T, Wang L, Zhang F, Mi L, Zhang J, Wang S, Wang Y, Zhou X, Zhang Y, Li M, Zhang S, Hu R. 2019. Rapid and Sensitive Recombinase Polymerase Amplification Combined With Lateral Flow Strip for Detecting African Swine Fever

Virus. *Front Microbiol* 10:1004.

Montgomery RE. 1921. On a form of swine fever occurring in British East Africa. *Journal of Comparative Pathology and Therapeutics* 34:159-191.

Moura JA, McManus CM, Bernal FE, de Melo CB. 2010. An analysis of the 1978 African swine fever outbreak in Brazil and its eradication. *Rev Sci Tech* 29:549-563.

Mulumba-Mfumu LK, Saegerman C, Dixon LK, Madimba KC, Kazadi E, Mukalakata NT, Oura CAL, Chenais E, Masembe C, Ståhl K, Thiry E, Penrith ML. 2019. African swine fever: Update on Eastern, Central and Southern Africa. *Transbound Emerg Dis* 66:1462-1480.

Niederwerder MC, Stoian AMM, Rowland RRR, Dritz SS, Petrovan V, Constance LA, Gebhardt JT, Olcha M, Jones CK, Woodworth JC, Fang Y, Liang J, Hefley TJ. 2019. Infectious Dose of African Swine Fever Virus When Consumed Naturally in Liquid or Feed. *Emerg Infect Dis* 25:891-897.

Nielsen K, Yu WL, Kelly L, Bermudez R, Renteria T, Dajer A, Gutierrez E, Williams J, Algire J, de Eschaide ST. 2008. Development of a lateral flow assay for rapid detection of bovine antibody to Anaplasma marginale. *J Immunoassay Immunochem* 29:10-18.

Nielsen K, Yu WL, Kelly L, Williams J, Dajer A, Gutierrez E, Ramirez Cruz G, Renteria T, Bermudez R, Algire J. 2009. Validation and field assessment of a rapid lateral flow assay for detection of bovine antibody to Anaplasma marginale. *J Immunoassay Immunochem* 30:313-321.

Nieto-Pelegrín E, Rivera-Arroyo B, Sánchez-Vizcaíno JM. 2015. First Detection of Antibodies Against African Swine Fever Virus in Faeces Samples. *Transbound Emerg Dis* 62:594-602.

Nix RJ, Gallardo C, Hutchings G, Blanco E, Dixon LK. 2006. Molecular epidemiology of

African swine fever virus studied by analysis of four variable genome regions. *Arch Virol* 151:2475-2494.

O'Donnell V, Holinka LG, Sanford B, Krug PW, Carlson J, Pacheco JM, Reese B, Risatti GR, Gladue DP, Borca MV. 2016. African swine fever virus Georgia isolate harboring deletions of 9GL and MGF360/505 genes is highly attenuated in swine but does not confer protection against parental virus challenge. *Virus Res* 221:8-14.

Oganesyan AS, Petrova ON, Korennoy FI, Bardina NS, Gogin AE, Dudnikov SA. 2013. African swine fever in the Russian Federation: spatio-temporal analysis and epidemiological overview. *Virus Res* 173:204-211.

Olesen AS, Lohse L, Boklund A, Halasa T, Gallardo C, Pejsak Z, Belsham GJ, Rasmussen TB, Bøtner A. 2017. Transmission of African swine fever virus from infected pigs by direct contact and aerosol routes. *Vet Microbiol* 211:92-102.

Oura CA, Edwards L, Batten CA. 2013. Virological diagnosis of African swine fever--comparative study of available tests. *Virus Res* 173:150-158.

Oura CA, Powell PP, Anderson E, Parkhouse RM. 1998. The pathogenesis of African swine fever in the resistant bushpig. *J Gen Virol* 79(6):1439-1443.

Oviedo JM, Rodríguez F, Gómez-Puertas P, Brun A, Gómez N, Alonso C, Escribano JM. 1997. High level expression of the major antigenic African swine fever virus proteins p54 and p30 in baculovirus and their potential use as diagnostic reagents. *J Virol Methods* 64:27-35.

Pan IC, Huang TS, Hess WR. 1982. New method of antibody detection by indirect immunoperoxidase plaque staining for serodiagnosis of African swine fever. *J Clin Microbiol* 16:650-655.

Pastor MJ, Arias M, Escribano JM. 1990. Comparison of two antigens for use in an enzyme-linked immunosorbent assay to detect African swine fever antibody. *Am J Vet Res* 51:1540-1543.

Pastor MJ, Laviada MD, Sanchez-Vizcaino JM, Escribano JM. 1989. Detection of African swine fever virus antibodies by immunoblotting assay. *Can J Vet Res* 53:105-107.

Penrith ML, Bastos AD, Etter EMC, Beltrán-Alcrudo D. 2019. Epidemiology of African swine fever in Africa today: Sylvatic cycle versus socio-economic imperatives. *Transbound Emerg Dis* 66:672-686.

Penrith ML, Thomson GR, Bastos AD, Phiri OC, Lubisi BA, Du Plessis EC, Macome F, Pinto F, Botha B, Esterhuysen J. 2004. An investigation into natural resistance to African swine fever in domestic pigs from an endemic area in southern Africa. *Rev Sci Tech* 23:965-977.

Penrith ML, Vosloo W. 2009. Review of African swine fever: transmission, spread and control. *J S Afr Vet Assoc* 80:58-62.

Penrith ML, Vosloo W, Jori F, Bastos AD. 2013. African swine fever virus eradication in Africa. *Virus Res* 173:228-246.

Pérez-Filgueira DM, González-Camacho F, Gallardo C, Resino-Talaván P, Blanco E, Gómez-Casado E, Alonso C, Escribano JM. 2006. Optimization and validation of recombinant serological tests for African Swine Fever diagnosis based on detection of the p30 protein produced in Trichoplusia ni larvae. *J Clin Microbiol* 44:3114-3121.

Pérez-Sánchez R, Astigarraga A, Oleaga-Pérez A, Encinas-Grandes A. 1994. Relationship between the persistence of African swine fever and the distribution of Ornithodoros erraticus in the province of Salamanca, Spain. *Vet Rec* 135:207-209.

Pietschmann J, Mur L, Blome S, Beer M, Pérez-Sánchez R, Oleaga A, Sánchez-Vizcaíno JM. 2016. African swine fever virus transmission cycles in Central Europe: Evaluation of wild boar-soft tick contacts through detection of antibodies against Ornithodoros erraticus saliva antigen. *BMC Vet Res* 12:1.

Pikalo J, Schoder ME, Sehl J, Breithaupt A, Tignon M, Cay AB, Gager AM, Fischer M, Beer M, Blome S. 2020. The African swine fever virus isolate Belgium 2018/1 shows high virulence in European wild boar. *Transbound Emerg Dis* 67:1654-1659.

Pinto Torres JE, Goossens J, Ding J, Li Z, Lu S, Vertommen D, Naniima P, Chen R, Muyldermans S, Sterckx YG, Magez S. 2018. Development of a Nanobody-based lateral flow assay to detect active Trypanosoma congolense infections. *Sci Rep* 8:9019.

Plowright W, Parker J, Peirce MA. 1969. African swine fever virus in ticks (Ornithodoros moubata, murray) collected from animal burrows in Tanzania. *Nature* 221:1071-1073.

Plowright W, Perry CT, Greig A. 1974. Sexual transmission of African swine fever virus in the tick, Ornithodoros moubata porcinus, Walton. *Res Vet Sci* 17:106-113.

Plowright W, Perry CT, Peirce MA. 1970. Transovarial infection with African swine fever virus in the argasid tick, Ornithodoros moubata porcinus, Walton. *Res Vet Sci* 11:582-584.

Prados FJ, Viñuela E, Alcamí A. 1993. Sequence and characterization of the major early phosphoprotein p32 of African swine fever virus. *J Virol* 67:2475-2485.

Quembo CJ, Jori F, Vosloo W, Heath L. 2018. Genetic characterization of African swine fever virus isolates from soft ticks at the wildlife/domestic interface in Mozambique and identification of a novel genotype. *Transbound Emerg Dis* 65:420-431.

Ravaomanana J, Michaud V, Jori F, Andriatsimahavandy A, Roger F, Albina E, Vial L. 2010. First detection of African Swine Fever Virus in Ornithodoros porcinus in Madagascar and

new insights into tick distribution and taxonomy. *Parasit Vectors* 3:115.

Reis AL, Abrams CC, Goatley LC, Netherton C, Chapman DG, Sanchez-Cordon P, Dixon LK. 2016. Deletion of African swine fever virus interferon inhibitors from the genome of a virulent isolate reduces virulence in domestic pigs and induces a protective response. *Vaccine* 34:4698-4705.

Rennie L, Wilkinson PJ, Mellor PS. 2001. Transovarial transmission of African swine fever virus in the argasid tick Ornithodoros moubata. *Med Vet Entomol* 15:140-146.

Rojo G, García-Beato R, Viñuela E, Salas ML, Salas J. 1999. Replication of African swine fever virus DNA in infected cells. *Virology* 257:524-536.

Rowlands RJ, Michaud V, Heath L, Hutchings G, Oura C, Vosloo W, Dwarka R, Onashvili T, Albina E, Dixon LK. 2008. African swine fever virus isolate, Georgia, 2007. *Emerg Infect Dis* 14:1870-1874.

Salas ML, Andrés G. 2013. African swine fever virus morphogenesis. *Virus Res* 173:29-41.

Sambandam R, Angamuthu R, Kanagaraj V, Kathaperumal K, Chothe SK, Nissly RH, Barry RM, Jayarao BM, Kuchipudi SV. 2017. An immuno-chromatographic lateral flow assay (LFA) for rapid on-the-farm detection of classical swine fever virus (CSFV). *Arch Virol* 162:3045-3050.

Sánchez EG, Quintas A, Pérez-Núñez D, Nogal M, Barroso S, Carrascosa Á L, Revilla Y. 2012. African swine fever virus uses macropinocytosis to enter host cells. *PLoS Pathog* 8(6):e1002754.

Sánchez-Vizcaíno JH, DL. 2019. African swine fever (infection with African swine fever virus), *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 8th ed*. Office International des Epizooties, Paris.

Sánchez-Vizcaíno JM, Mur L, Martínez-López B. 2013. African swine fever (ASF): five years around Europe. *Vet Microbiol* 165:45-50.

Sastre P, Gallardo C, Monedero A, Ruiz T, Arias M, Sanz A, Rueda P. 2016. Development of a novel lateral flow assay for detection of African swine fever in blood. *BMC Vet Res* 12:206.

Sauter-Louis C, Forth JH, Probst C, Staubach C, Hlinak A, Rudovsky A, Holland D, Schlieben P, Göldner M, Schatz J, Bock S, Fischer M, Schulz K, Homeier-Bachmann T, Plagemann R, Klaaß U, Marquart R, Mettenleiter TC, Beer M, Conraths FJ, Blome S. 2020. Joining the club: First detection of African swine fever in wild boar in Germany. *Transbound Emerg Dis* 00:1-9.

Shirai J, Kanno T, Tsuchiya Y, Mitsubayashi S, Seki R. 2000. Effects of chlorine, iodine, and quaternary ammonium compound disinfectants on several exotic disease viruses. *J Vet Med Sci* 62:85-92.

Simón-Mateo C, Andrés G, Almazán F, Viñuela E. 1997. Proteolytic processing in African swine fever virus: evidence for a new structural polyprotein, pp62. *J Virol* 71:5799-5804.

Simón-Mateo C, Andrés G, Viñuela E. 1993. Polyprotein processing in African swine fever virus: a novel gene expression strategy for a DNA virus. *EMBO J* 12:2977-2987.

Stoian AMM, Zimmerman J, Ji J, Hefley TJ, Dee S, Diel DG, Rowland RRR, Niederwerder MC. 2019. Half-Life of African Swine Fever Virus in Shipped Feed. *Emerg Infect Dis* 25:2261-2263.

Sun Z, Shi B, Meng F, Ma R, Hu Q, Qin T, Chen S, Peng D, Liu X. 2018. Development of a Colloidal Gold-Based Immunochromatographic Strip for Rapid Detection of H7N9 Influenza Viruses. *Front Microbiol* 9:2069.

Sunwoo SY, Pérez-Núñez D, Morozov I, Sánchez EG, Gaudreault NN, Trujillo JD, Mur L, Nogal M, Madden D, Urbaniak K, Kim IJ, Ma W, Revilla Y, Richt JA. 2019. DNA-Protein Vaccination Strategy Does Not Protect from Challenge with African Swine Fever Virus Armenia 2007 Strain. *Vaccines* (Basel) 7(1):12.

Tabarés E, Fernandez M, Salvador-Temprano E, Carnero ME, Sanchez-Botija C. 1981. A reliable enzyme linked immunosorbent assay for African swine fever using the major structural protein as antigenic reagent. *Arch Virol* 70:297-300.

Tabarés E, Martinez J, Ruiz Gonzalvo F, Sánchez-Botija C. 1980. Proteins specified by African swine fever virus. II. Analysis of proteins in infected cells and antigenic properties. *Arch Virol* 66:119-132.

Thomson GR. 1985. The epidemiology of African swine fever: the role of free-living hosts in Africa. *Onderstepoort J Vet Res* 52:201-209.

Thomson GR, Gainaru MD, Van Dellen AF. 1980. Experimental infection of warthos (Phacochoerus aethiopicus) with African swine fever virus. *Onderstepoort J Vet Res* 47:19-22.

Tignon M, Gallardo C, Iscaro C, Hutet E, Van der Stede Y, Kolbasov D, De Mia GM, Le Potier MF, Bishop RP, Arias M, Koenen F. 2011. Development and inter-laboratory validation study of an improved new real-time PCR assay with internal control for detection and laboratory diagnosis of African swine fever virus. *J Virol Methods* 178:161-170.

Villeda CJ, Gómez-Villamandos JC, Williams SM, Hervás J, Wilkinson PJ, Viñuela E. 1995. The role of fibrinolysis in the pathogenesis of the haemorrhagic syndrome produced by virulent isolates of African swine fever virus. *Thromb Haemost* 73:112-117.

Villeda CJ, Williams SM, Wilkinson PJ, Viñuela E. 1993. Consumption coagulopathy associated

with shock in acute African swine fever. *Arch Virol* 133:467-475.

Villeda CJ, Williams SM, Wilkinson PJ, Viñuela E. 1993. Haemostatic abnormalities in African swine fever a comparison of two virus strains of different virulence (Dominican Republic '78 and Malta '78). *Arch Virol* 130:71-83.

Wang A, Jia R, Liu Y, Zhou J, Qi Y, Chen Y, Liu D, Zhao J, Shi H, Zhang J, Zhang G. 2020. Development of a novel quantitative real-time PCR assay with lyophilized powder reagent to detect African swine fever virus in blood samples of domestic pigs in China. *Transbound Emerg Dis* 67:284-297.

Wang J, Wang J, Geng Y, Yuan W. 2017. A recombinase polymerase amplification-based assay for rapid detection of African swine fever virus. *Can J Vet Res* 81:308-312.

Wong R, Tse H. 2009. Lateral Flow Immunoassay doi:10.1007/978-1-59745-240-3. Humana Press, New York.

Yang F, Xiao Y, Chen B, Wang L, Liu F, Yao H, Wu N, Wu H. 2020. Development of a colloidal gold-based immunochromatographic strip test using two monoclonal antibodies to detect H7N9 avian influenza virus. *Virus Genes* 56:396-400.

Yang F, Xiao Y, Xu L, Liu F, Yao H, Wu N, Wu H. 2020. Development of an antigen-capture enzyme-linked immunosorbent assay and immunochromatographic strip based on monoclonal antibodies for detection of H6 avian influenza viruses. *Arch Virol* 165:1129-1139.

Zhou X, Li N, Luo Y, Liu Y, Miao F, Chen T, Zhang S, Cao P, Li X, Tian K, Qiu HJ, Hu R. 2018. Emergence of African Swine Fever in China, 2018. *Transbound Emerg Dis* 65:1482-1484.

Zhu M, Jia Y, Peng L, Ma J, Li X, Shi F. 2019. A highly sensitive dual-color lateral flow

immunoassay for brucellosis using one-step synthesized latex microspheres. *Analytical Methods* 11:2937-2942.

Zsak L, Borca MV, Risatti GR, Zsak A, French RA, Lu Z, Kutish GF, Neilan JG, Callahan JD, Nelson WM, Rock DL. 2005. Preclinical diagnosis of African swine fever in contact-exposed swine by a real-time PCR assay. *J Clin Microbiol* 43:112-119.