Epitope mapping of African swine fever virus p72 capsid protein using polyclonal swine sera and monoclonal antibodies

by

Mallory Elizabeth Phillips

A.S., Butler Community College, 2012
B.S., Kansas State University, 2014

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Diagnostic Medicine and Pathobiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2016

Approved by:

Major Professor
Raymond R. R. Rowland

Copyright

Abstract

African swine fever is a hemorrhagic disease of domestic pigs caused by African swine fever virus (ASFV), a double-stranded DNA virus and the only member of the family *Asfarviridae*. The structure of this multilayer virion contains more than 34 proteins including the protein p72 which is the major capsid protein. A single conformational neutralizing epitope has been identified on p72, but information on the other antigenic regions (epitopes) is lacking. The objective of this study was to identify p72 epitopes using polyclonal swine sera and a panel of monoclonal antibodies with the ultimate goal being the development of a blocking ELISA assay for the detection of anti-ASFV antibodies. The segment of the p72 protein from amino acids 1 to 345 was divided into five overlapping fragments which were then commercially synthesized. These fragments were cloned into the pHUE expression vector and transformed into *Escherichia coli* competent cells. The recombinant proteins were expressed *in vitro*, purified, and used as antigens in indirect ELISAs and western blots to test monoclonal antibodies and polyclonal swine sera. The monoclonal antibodies were produced against the p72 protein based on the ASFV Georgia/07 strain. The polyclonal sera were obtained from pigs immunized with a defective alphavirus replicon particle, RP-sHA-p72, expressing a recombinant protein composed of the extracellular domain of the ASFV HA protein together with the whole p72 protein. The polyclonal sera reacted to p72 in two distinct regions: between amino acids 1 and 83 and between amino acids 250 and 280. The anti-p72 reactive monoclonal antibodies reacted with p72 in three regions: between amino acids 100 and 171, amino acids 180 and 250, and amino acids 280 and 345. Fine mapping with oligopeptides allowed for the identification of six different linear epitopes. Among the monoclonal antibodies selected for blocking assay development, two have been shown to be promising candidates for further evaluation using sera from ASFV-infected pigs.

Table of Contents

List of Figures

List of Tables

Acknowledgements

I am deeply grateful for the support from all of the individuals who helped me complete my thesis. I would especially like to express my appreciation to my major professor, Dr. Bob Rowland, for offering me the opportunity to conduct research in his laboratory, for teaching me critical thinking, scientific writing, and how to become an independent researcher. I would like to thank my committee members, Dr. Bill Wilson and Dr. Steve Dritz, for their wisdom, expertise, and patience.

I would like to express my gratitude to the members of the Rowland lab for all of their support, advice, and help. I am particularly grateful to Maria V. Murgia, Cathy Troupe, and Vlad Petrovan for all of their knowledge and technical advice.

This project was funded by the Kansas National Bio and Agro-Defense Facility Transition Fund and the Kansas Bioscience Authority through a matching grant to Kansas State University's Center of Excellence for Emerging and Zoonotic Animal Diseases. The monoclonal antibody development project was funded by the U.S. Department of Homeland Security under IAA Award No. HSHQDC-12-X-00122.

Chapter 1 - African Swine Fever Virus Literature Review

African swine fever

African swine fever (ASF) is a highly contagious and lethal viral disease of swine that has had a significant socioeconomic impact in the developed and developing world, making it a global animal health priority (Agüero et al., 2003; Sanchez-Vizcaino & Neira 2012). Although the disease is not currently present in the United States (OIE, 2013), it is crucial that the United States is able to detect the presence of ASFV so that the negative impacts that have occurred elsewhere during outbreaks of ASFV are minimized should such an outbreak occur in this country (Oura, Edwards, & Batten, 2013). Currently, however, there are no diagnostic assays available in the United States to test for the presence of ASFV. A contributing factor for the lack of diagnostic assays to test for the virus is the limited information available regarding the antigenic binding sites, or epitopes, on the major viral proteins. Determining the locations on these proteins to which antibodies are able to bind, a process known as epitope mapping, would facilitate the development of serological diagnostic assays that could be used to test for the presence of this significant swine pathogen should it be imported into the United States.

African swine fever virus (ASFV), the causative agent of ASF, is a large icosahedral DNA virus of about 193 kilobase pairs (kbp) with a conserved central region of approximately 125 kilobases (kb) and variable ends (Dixon et al., 2005). The disease can occur in various forms, ranging from highly lethal to subclinical infections, resulting in up to 100% morbidity in naïve pigs of all breeds and ages. Mortality rates vary between 0% and 100% depending on the viral isolate; health, age, and other factors affecting the host; the dose; and the route of exposure to the virus (Costard, Mur, Lubroth, Sanchez-Vizcaino, & Pfeiffer, 2013).

ASF affects members of the *Suidae* family with no evidence suggesting zoonosis. It infects a range of domestic and wild suids and, depending on the species, can cause disease or result in an asymptomatic carrier state in the animal (Costard et al., 2013). Species affected include the domestic pig (S*us scrofa domesticus*), Eurasian wild boar (*Sus scrofa*), warthogs (*Phacochoerus aethiopicus*), bush pigs (*Potamochoerus porcus*), and giant forest hogs (*Hylochoerus meinertzhageni*) (Costard et al., 2013; Kleiboeker, 2008). Warthogs and bush pigs are known to be wildlife reservoirs for the virus in Africa. Additionally, the argasid soft tick of the genus *Ornithodoros* can serve as vectors for transmission of the virus in Africa (Anderson, Hutchings, Mukarati, & Wilkinson, 1998; Burrage, 2013; Penrith, Vosloo, Jori, & Bastos, 2013).

ASFV was first documented in domesticated swine in Kenya in 1921. Sanchez-Botija describes evidence for the first known cases of ASFV infection outside of Africa. This occurred in Portugal in 1957 as a result of waste from airline flights being fed to pigs in areas with close proximity to the Lisbon airport. This outbreak was successfully eradicated but soon after more outbreaks occurred in additional European countries. Since the 1960s, outbreaks have occurred in Italy, Spain, Malta, Belgium, and the Netherlands (as cited in Costard et al., 2009; Oura, Powell, Anderson, & Parkhouse, 1998). In the 1970s, the virus then spread to South America and the Caribbean but was eradicated from these areas by the death or depopulation of infected pigs. In 2007, ASFV was introduced into the Caucasus region via the Republic of Georgia and spread into Eastern Europe where it has become endemic (Costard et al., 2013). Since that time, the disease has spread rapidly among the domestic and wild pig populations causing widespread disease throughout this vast region and extending from Russia to the edges of Eastern Europe. Outbreaks continue to be reported periodically outside of the continent of Africa, causing great economic losses in the affected countries. Eradication has been achieved in most of these cases,

although it remains endemic in Sardinia, Italy, and most of sub-Saharan Africa (Sánchez-Vizcaíno & Neira , 2012). Currently ASFV is present in the African countries of Togo, Kenya, Burundi, Mali and South Africa and outside of Africa in Sardinia, Poland, Lithuania, Russia, and the Ukraine (Arzt, White, Thomsen, & Brown, 2010; OIE, 2013).

Increases in pig populations in Eastern Asia as well as continued increases in trade activity have led to concerns that ASFV could spread further across Europe and Asia (Costard et al., 2013). Spread of the virus across Eurasia and into Europe could allow the virus to be carried from Europe into North America via existing intercontinental commerce. The United States has been one of the top world pork import and export countries, and it is crucial that the country protects its own swine industry from this devastating viral disease. Because the introduction of ASF into the United States would have an enormous economic impact similar to what has occurred in other countries, the potential of introducing ASF into the country is a risk that needs to be mitigated (Center for Food Security and Public Health, College of Veterinary Medicine, Iowa State University, 2015).

Based on the impact that ASF would have on pork production and trade, as well as the costs of eradicating the disease should the virus appear in the United States, it has been estimated that the net benefit of preventing the introduction of ASF into the country is approximately $4.5 billion (Costard et al., 2009). Since there is no effective vaccine or preventative measures available to combat ASF, the current outbreaks in Eastern Europe have created interest in developing rapid and specific diagnostic assays for surveillance of the virus (Dixon, Chapman, Netherton, & Upton, 2013; Gallardo et al., 2015; Oganesyan et al., 2013).

Clinical signs

Pigs infected with ASF exhibit numerous clinical signs as well as gross pathology that are similar to those observed in animals suffering from other diseases. These diseases include classical swine fever (CSF), salmonellosis, erysipelas, porcine dermatitis and nephropathy syndrome (PDNS), and porcine reproductive and respiratory syndrome (PRRS). As a result, it can be difficult to distinguish ASF from these other diseases by either clinical or post-mortem examination (King et al., 2003; OIE, 2012; Oura, Edwards, & Batten, 2013; United States Animal and Plant Health Inspection Service, 1998). Therefore, the only way to differentiate infection with ASFV from other swine diseases is through diagnostic tests.

The clinical presentation of ASFV varies from hyperacute, with almost 100% mortality after 4 to 7 days post-infection (dpi), to chronic disease, with most animals surviving and becoming carriers of the disease. Infection with different viral isolates results in these varying degrees of illness (Blome, Gabriel, & Beer, 2013; Martínez-López et al., 2015). Hyperacute and acute disease are the most severe forms of ASF. Acute disease is characterized by a 3- to 5-day incubation period followed by a high fever and rapid death within 5 to 10 days from initial infection. Hyperacute disease has a more rapid onset when compared to acute disease. The chronic form is more insidious; clinical manifestations of disease develop more slowly and mortality rates are not as high but hallmarks of the disease ultimately appear. Subacute disease presents as an intermediate level of illness between what is seen in the hyperacute or acute forms and the chronic form. Similarly, the time required for clinical manifestations to develop is also intermediate in nature for subacute disease. The subacute and chronic forms are more difficult to recognize when compared to acute or hyperacute disease since the symptoms are less severe and mortality rates are lower. Furthermore, if subacute or chronic forms of ASF occur in an area

where the virus has never been detected, the lack of experience of farmers and veterinarians with ASFV infection can make this disease more difficult for them to detect. These different forms of ASF are seen with varying frequency in the regions where the virus has been detected. For example, within the continent of Africa, acute ASF is seen more frequently than the less severe forms of infection. In recent outbreaks elsewhere, the most predominant forms of ASF have been the subacute or chronic forms of the disease. (Kleiboeker, 2008; Oura et al., 2013; United States Animal and Plant Health Inspection Service, 1998).

Infection can produce a wide range of symptoms including fever, anorexia, cyanosis of the skin, vomiting, diarrhea, tachycardia, tachypnea, arthritis, incoordination, and death. Abortion may also occur in pregnant sows (Kleiboeker, 2008; OIE, 2012). Extensive hemorrhages occur predominately in the lymph nodes, kidneys, and heart. Edema, pericarditis, splenomegaly, and hyperemia of the intestines are also observed (Galindo-Cardiel et al., 2013). The extent and nature of the symptoms and gross lesions will vary depending upon the virulence of the strain, host factors, and the course of the disease (Blome et al., 2013; Gómez-Villamandos, Bautista, Sánchez-Cordón, & Carrasco, 2013).

ASFV isolates have shown a preference for infecting cells of the mononuclear phagocytic system. The virus replicates primarily in monocytes and lymphocytes and attacks other myeloid lineage cells as well, including leukocytes and dendritic cells. These antigen presenting cells (APCs) have critical roles in the immune system (Blome et al., 2013; Muñoz-Moreno, Galindo, Cuesta-Geijo, Barrado-Gil, & Alonso, 2015; Takamatsu et al., 2013). Infection with more virulent isolates of the virus results in increased viral replication and accumulation of the virus in tissue compared to less virulent isolates. High levels of virus have been detected in the spleen, lymph nodes, liver, kidney, lungs, and tonsils. Specific macrophage types demonstrated to be

infected with ASFV include red pulp macrophages, extrafollicular macrophages of gastrohepatic and submandibular lymph nodes, Kupffer cells in the liver, pulmonary intravascular macrophages, and extrafollicular macrophages of the tonsils (Oura et al., 1998).

In Europe, wild boar and feral pigs have been shown to be as susceptible as domestic pigs to ASFV infection (Correia, Ventura, & Parkhouse, 2013; Galindo-Cardiel et al., 2013; Jori & Bastos, 2009). Some animals, such as warthogs and bush pigs, become infected but are asymptomatic; they do not develop the typical clinical signs and symptoms (Burrage, 2013; Kleiboeker, 2008). High rates of morbidity and mortality, along with rapid spread of the virus, are usually seen when ASFV is introduced into a geographical region or pig population previously devoid of the virus. Conversely, morbidity and mortality rates have been reported to be lower in regions where ASFV is endemic (Costard et al., 2013).

Transmission

ASF spreads rapidly in pig populations through direct animal-to-animal transmission via the oronasal route or indirect contact via fomites including vehicles, feed, and equipment. Additionally, the argasid soft ticks of the genus *Ornithodoros* can serve as vectors for transmission of ASFV, creating the infection cycle that maintains the virus in Africa via *O. moubata*, although infection with ASFV can also be fatal for these ticks (Burrage, 2013; Hess, Endris, Lousa, & Caiado, 1989; Kleiboeker & Scoles, 2001). Animals who exhibit subclinical disease, are chronically infected, or have recovered from infection are likely to contribute to the persistence of the virus in areas where it is endemic. These animals may also contribute to sporadic outbreaks and spread of ASFV into areas where it had not previously existed. This is thought to be due to the fact that animals can remain infected for several weeks and, during that time, are able to spread the virus to other pigs through direct or indirect contact (Costard et al.,

2013). The virus is shed from infected animals and can be found in all excretions and secretions. Once shed, the virus can survive outside of the host for several days which is another contributing factor for the spread of disease (Montgomery, 1921; United States Animal and Plant Health Inspection Service, 1998).

Pig production plays a major role in providing food security within the continent of Africa and throughout the world (Thomson, 1985). Different types of farms such as commercial pig units, small and semi-commercial units, and partially to fully free-range farms have considerably different biosecurity measures. This range in biosecurity is an important contributing factor to the spread of ASF and other diseases (Mokoele et al., 2015). Another factor contributing to the spread of ASFV is the feeding of uncooked garbage (swill) to pigs. ASFV remains infectious for long periods of time in uncooked pork products, such as blood and tissue. For example, ASFV has been shown to survive in natural sausage casings for 97 days (Arzt et al., 2010; Costard et al., 2009; United States Animal and Plant Health Inspection Service, 1998; Wieringa-Jelsma et al., 2011). The ability of the virus to survive in pork products for such a long period of time facilitates introduction of the virus into new areas. Additionally, transmission of aerosolized virus is suggested to occur over short distances, depending on the time the particles remain in the air and biological decay (de Carvalho Ferreira, Weesendorp, Quak, Stegeman, & Loeffen, 2013; Gallardo et al., 2011).

It has been shown that *Ornithodoros* ticks infected with ASFV are able to carry the virus for 6 months or longer and it may persist for years. When the ticks are present in the environment where infected animals had previously existed, these ticks could infect healthy animals when swine are reintroduced to the farm following culling of the herd (Hess et al., 1989; Plowright, Parker, & Peirce, 1969; United States Animal and Plant Health Inspection Service,

1998). *Ornithodoros erraticus* was first identified as a vector and reservoir for ASFV in Spain by Sanchez-Botija in the 1960s. Subsequently Plowright et al. showed the importance of other *Ornithodoros* species, including the *Ornithodoros moubata* complex, in Africa (as cited in Burrage, 2013; Costard et al., 2009, 2013; Rennie, Wilkinson, & Mellor, 2001). There are tick species present in North America (*O. coriaceus, O. turicata,* and *O. parkeri*) that have been experimentally shown to be able to carry ASFV (Costard et al., 2009; Sanchez-Vizcaino & Neira, 2012). This is concerning because if ASFV was to appear in North America, these ticks could serve as a vector for spread of the virus among uninfected animals.

Infection of the tick helps to maintain the virus in Africa by the way of the naturally occurring cycle between warthog, bushpig, and tick (Burrage, 2013; Costard et al., 2013). When an infected tick bites a previously uninfected warthog, the virus replicates in the animal and a low level of viremia develops. Although low, this level of viremia is sufficient to infect other ticks that in turn feed on the hog. Domestic pigs are then infected when they are bit by the infected ticks (Burrage, 2013). Warthogs, thought to be the original vertebrate host of ASF, are widely distributed throughout Africa. There are a multitude of factors making the warthog the most important vertebrate reservoir for ASFV; living in burrows that are also inhabited by *Ornithodoros* ticks, the behavior of young animals, and both direct and indirect contact with domestic pigs are a few of these factors (Costard et al., 2013).

Transstadial, transovarial, and sexual transmission of ASFV has been demonstrated in these ticks, although these factors are dependent upon the species of tick and the strain of the virus (Mellor & Wilkinson, 1985). The *Ornithodoros* ticks are long lived which contributes to the ability of the tick colonies to maintain ASFV for several years. Transovarial transmission of ASFV has been shown to occur from ticks of the *O. moubata* complex, allowing for the survival

of the virus within the tick population in the absence of infected animals. ASFV has also been shown to replicate within tick eggs. The likelihood that ASFV will be maintained within the tick population increases when ticks have a regular supply of blood meals; however, if not reintroduced to the virus, the ticks will eventually clear the infection (Plowright, Perry, & Greig, 1974; Rennie et al., 2001). It is important to note that although the *Ornithodoros* tick can serve as a vector for ASFV, the tick is not a required intermediate vector for the transmission of ASFV. Viral spread occurs more commonly via other mechanisms of infection such as direct contact between infected and uninfected animals

There is international interest in preventing this virus from entering other countries. Poor biosecurity, together with the extensive commercial trade, keeps ASFV-free countries such as the United States at a constant risk of having the disease introduced (Agüero et al., 2003). A large population of pigs is a risk factor contributing to the probability that a country may become infected with ASFV (Penrith et al., 2013). Because of the presence of potential tick vectors and feral pig populations in North America along with the fact that the United States is one of the top world pork producers, proper biosecurity practices are vital for the prevention of the virus entering the country.

Control

ASFV is a notifiable disease; if the virus is suspected, it should be reported immediately. A quick response and prevention of further infection is key for containing any outbreaks in ASFV-free regions (OIE, 2012). In countries where ASFV is endemic, successful eradication programs are based on the diagnosis of ASFV, the culling of pigs that are infected or have possibly been in contact with the disease, safe carcass disposal, sanitation, disinfection, animal movement controls and quarantines, and the prevention of domestic pig contact with wild pigs

and infected ticks. Eradication is difficult and can take decades to accomplish due to the presence of reservoirs for the virus in the wild. This process is even more difficult when tick vectors are present. Furthermore, eradication efforts are expensive due to costs associated with proper disposal of carcasses and those costs involved with repopulating a farm (Cubillos et al., 2013; Scott, 1965).

Currently, there is no effective vaccine available with which to combat ASFV; there is ongoing research for vaccine development (Bastos et al., 2003; Zsak, Onisk, Afonso, & Rock, 1993). Experimentally, classically attenuated vaccines using the non-virulent Portuguese isolate OURT88/3 and virulent isolate OURT88/1 have been shown to be efficient at controlling homologous challenge but were limited against heterologous challenge (King et al., 2011; Lacasta et al., 2015; Oura, Denyer, Takamatsu, & Parkhouse, 2005). It has been suggested that the outbreaks in the Iberian Peninsula between 1960 and 1995 were a consequence of the use of live attenuated vaccines (Costard et al., 2013). Although this type of vaccine has been demonstrated to induce a protective immune response, the use of live attenuated vaccines, especially in regions where the virus is not present, has been limited due to their infectious nature (Lacasta et al., 2015).

As mentioned, ASF infection can be maintained in an area through the activity of *Ornithodoros* ticks. In areas where ticks played a key role in the establishment and maintenance of ASFV infections, the risk of infection decreased following the elimination of the tick population. Therefore, control measures for ASF need to include methods to eradicate ticks from environments, such as the use of acaricides (Costard et al., 2013).

Decontamination of possible fomites and the premises where infected animals had been housed reduces the risk of spreading the virus to additional animals who have not been infected

with ASFV. Proper disinfection is crucial for the prevention of ASFV spread and can be achieved through a wide range of techniques. The virus is highly resistant in the environment and has been found to survive for 2 to 3 months under most environmental conditions. It has been found to survive for 3 months in ham, 6 months in bone marrow, and 18 months in blood meal. ASFV has also been shown to survive for 11 days in feces at room temperature and at least 1 month in contaminated pig pens (Carillo, 2014; Food & Agriculture Organisation of the United Nations, n.d.). ASFV is stable over a wide pH range from a pH of 4 to a pH of 10. Furthermore, many common disinfectants are ineffective against ASFV. Sodium hypochlorite (an oxidizing agent), a 1% citric acid solution, alkalis, iodine, and quaternary ammonium compounds have been reported to destroy the virus and are the disinfectants of choice for decontaminating ASFV (Krug, Larson, Eslami, & Rodriguez, 2012; Krug, Lee, Eslami, Larson, & Rodriguez, 2011; Shirai, Kanno, Tsuchiya, Mitsubayashi, & Seki, 2000). A few examples of these disinfectants include CaviCide® and Virkon™ S. CaviCide®, an alkali, is a non-corrosive cleaner that can be used as an effective disinfectant for ASFV. The alcohols in the compound break down the lipoprotein complexes and penetrate the cell membrane to cause irreversible damage to the inside of the cell (Crawford, Yu, Keegan, & Yu, 2000; Severs & Lamontagne, 2002). A 1% solution of Virkon™ S, an oxidizing agent, is recommended for cleaning and disinfecting swine units as well as agricultural equipment and instruments. This disinfectant has been proven effective, although it can be expensive and difficult to use on farms (Amass, Ragland, & Spicer, 2001; Herńndez, Martró, Matas, Martín, & Ausina, 2000).

The United States Department of Agriculture (USDA) prohibits the import of live pigs and uncooked pork products from those regions infected with ASFV to prevent the introduction of ASF into the United States (United States Animal and Plant Health Inspection Service, 1998).

As another precaution, the feeding of uncooked garbage to pigs has also been prohibited in some countries (Scott, 1965). Unprocessed meat must be heated to a minimum of 70 ˚C for 30 minutes to inactivate ASFV; 30 minutes at 60 ˚C has been found sufficient for the inactivation of serum and body fluids (Center for Food Security and Public Health, College of Veterinary Medicine, Iowa State University, 2015). In a study by Wieringa-Jelsma *et al*., (2011) both sodium chloride salt and phosphate supplemented salt were also found to be capable of inactivating ASFV within 48 hours.

African swine fever virus

The causative agent of ASF is African swine fever virus (ASFV) which belongs to the genus *Asfivirus* and is the only member of the family *Asfarviridae* (Dixon et al., 2013). The *Asfarviridae* family is included in the nucleocytoplasmic large DNA virus (NCLDV) superfamily, order Megavirales. Members of NCLDV include *Poxviridae, Iridoviridae, Ascoviridae, Phycodnaviridae, Mimiviridae*, and *Marseilleviridae*. Poxviruses and asfarviruses infect vertebrate animals whereas mimiviruses, phycodnaviruses, and Marseilleviruses infect diverse unicellular eukaryotes. The NCLDVs share a common origin and undergo crucial parts of their replication within the cytoplasm of the infected cells (Colson et al., 2013). Most members of this group have an icosahedral morphology, with the exception of the *Poxviridae* family. Despite the different morphologies of ASFV and poxvirus, their genome structures are the same and they both have the ability to regulate their gene expression by temporal control of transcription (Rodríguez & Salas, 2013; Salas & Andrés, 2013).

ASFV is a large enveloped virus that has an average diameter of approximately 200 nm (Kleiboeker, 2008). The viral genome of ASFV consists of a linear, double-stranded DNA molecule that ranges in length from 170 to 193 kilobase pairs (kbp) depending on the specific

viral isolate. The genome encodes for more than 150 polypeptides, at least 50 of which result in production of the structural proteins that comprise the different domains of the viral particle. High antigenicity has been shown in 14 of these polypeptides. Some of the viral genes code for the enzymes required for transcription and viral replication while others are involved in virus-host interactions, such as permitting the virus to evade host cell defenses and altering host cell function to aid in the survival of the virus (Dixon et al., 2013; Epifano, Krijnse-Locker, Salas, Rodríguez, & Salas, 2006; Kollnberger, Gutierrez-Castañeda, Foster-Cuevas, Corteyn, & Parkhouse, 2002). There are many genes for which the function remains unknown although it has been suggested that many of these are involved with the ability of the virus to survive and grow in the tick (Burrage, 2013).

The dense 80 nm virion core is composed of a nucleoid, which contains the viral genome, and the nucleoproteins p10 and pA104R. This core is enclosed by the core shell that contains several major components of the virus particle including p35, p15, p150, p37, p34, and p14 (Andrés, Alejo, Salas, & Salas, 2002; Salas & Andrés, 2013). Surrounding the core are two lipid bilayers. These bilayers form the inner envelope and, outside of this envelope, is an icosahedral capsid (Cubillos et al., 2013). Constituents of the inner envelope include p54, p17, pE248R, and p12. The capsid structure is composed of p72, pE120R, and pB438L (Figure 1.1). The virus exits from the host cells by budding through the plasma membrane and, in this way, obtains an outer envelope which contains the proteins p24, CD2v, p30, and p12 (Paulino Gómez-Puertas et al., 1998; Salas & Andrés, 2013).

Capsid protein p72

The gene B646L codes for a protein with a molecular weight of 72 kDa that is expressed late in infection (Dixon et al., 2013; López-Otín, Freije, Parra, Méndez, & Viñuela, 1990;

Pitcher, Dixon, & Turner, 1992). This protein, p72, comprises about 32% of the total protein mass of the virion (Bastos et al., 2003). It is involved in virus attachment (Dixon et al., 2013) and is a major immunogen in natural infection (Tabares, Fernandez, Salvador-Temprano, Carnero, & Sanchez-Botija, 1981). In 1993, Zsak *et al*. (1993) identified that p72 contains a highly conserved neutralizing epitope. This epitope was shown to be conformational in a study by Borca and colleagues (1994). The correct folding and assembly of p72 in the virion requires a virus-encoded chaperone protein encoded by gene B602L. B602L prevents the capsid protein from aggregating before it is delivered onto the capsid shell. B602L and p72 then dissociate after folding but before the assembly of p72 into the virion (Cobbold, Windsor, & Wileman, 2001; Dixon et al., 2013). Yu *et al.* (1996) performed amino acid sequence comparison of p72 from a Ugandan strain from 1965, a Dominican Republic strain (DR-2), and two European strains (BA71V and E70) and found p72 to be highly conserved among them (Figure 1.2). This finding was supported by a subsequent study performed by de Villiers *et al.* (2010) in which mostly synonymous mutations were found in the nucleotide alignment of p72 from 11 ASFV isolates. Because of the antigenic stability of p72, it has been suggested that this protein would be a good candidate for use in the development of a diagnostic test for ASF (López-Otín et al., 1990). This study focuses on the N-terminal region of p72 due to this high conservation among isolates (Figure 1.3).

Sequences of the variable genome regions of ASFV have been analyzed and used for genotyping different ASFV isolates. This is a valuable tool to distinguish between viruses that are causing outbreaks. Differentiation of the genomes by nucleotide sequence analysis can be accomplished by comparing the C-terminal end of p72 (Figure 1.4). This process has been used to identify 23 distinct ASFV genotypes (Achenbach et al., 2016; Bastos et al., 2003; Costard et

al., 2013). Complete viral discrimination between isolates can be achieved by combining the partial sequencing of p72, full sequencing of p54, and sequencing of the central variable region (CVR) of B602L (Atuhaire, Afayoa, Ochwo, Mwesigwa, Okuni, et al., 2013; Carmina Gallardo, Mwaengo, et al., 2009).

Antibodies to capsid protein p72

Since there is no vaccine available for ASF, detection of antibodies to ASF is indicative that the animal had a previous infection with the virus. Pigs that survive natural infection produce antibodies to this virus between 7 and 10 days post infection. These antibodies persist for long periods, making them appropriate markers for diagnosis of the disease (Atuhaire, Afayoa, Ochwo, Mwesigwa, Mwiine, et al., 2013; Gallardo, Reis, et al., 2009). Protein p72 is one of the first viral proteins responsible for the induction of anti-ASFV antibodies after natural infection (Cubillos et al., 2013). It was demonstrated that antibodies to p72 neutralize infection of virulent ASFV isolates in Vero cell cultures and swine macrophages (Zsak et al., 1993) by preventing virus attachment (Gómez-Puertas et al., 1996) and in turn inhibiting virus replication.

Along with the production of neutralizing antibodies to p72, the amino acid sequence of p72 is highly conserved among isolates making it a good candidate for serological diagnosis. Mapping the neutralization epitope, or epitopes, on the p72 protein using recombinant p72 protein will be useful for structure-function analysis, the clarification of important biological processes, and for the development of serological assays (Cubillos et al., 2013; Yu et al., 1996).

Epitope mapping

Epitope mapping is performed to identify the recognition sites of the antigen (epitopes) to which antibodies bind. Understanding the specific interaction between the antibodies and epitopes provides the foundation for the development of vaccines and serological-based assays

(Gershoni, Roitburd-Berman, Siman-Tov, Freund, & Weiss, 2012). B-Cell epitope mapping uses a series of overlapping synthetic peptides to identify linear antigenic determinants recognized by a particular antibody through an immunoassay, such as an ELISA. Using overlapping fragments ensures that epitopes are not missed by being cut at the fragment junctions (Heuzenroeder, Barton, Vanniasinkam, & Phumoonna, 2009). Epitope mapping makes it possible to identify and differentiate conformational and continuous epitopes. The structure of a conformational epitope depends on the intact conformation of the native protein. A continuous epitope is a linear peptide fragment of a protein that is able to bind to anti-protein antibodies (Sundberg, 2009; Van Regenmortel, 2009). The simplest way to identify desired epitopes is to isolate a monoclonal antibody that will bind to it. The "gold standard" for epitope mapping is the co-crystallization of the antigen-antibody complex followed by x-ray diffraction and analysis. A second approach to epitope mapping uses nuclear magnetic resonance (NMR) which gives a picture of the antigen-antibody complex in solution. Other means of epitope mapping include computational docking, binding analyses, alanine scanning mutagenesis (ASM), and saturating mutagenesis (Gershoni et al., 2012).

Previous studies have found that proteins p72, p54, and p30 of ASFV have immunogenic epitopes, although there is limited information available on the specific epitopes that anti-p72, p54, and p30 antibodies recognize (Neilan et al., 2004). One epitope in p54 was shown to be a DNA-binding domain (Escribano, Galindo, & Alonso, 2013)and a conformational-dependent neutralizing epitope was identified on p72 (Borca et al., 1994; Zsak et al., 1993).

Diagnostic tests

Since ASF is a high-consequence pathogen, there are detrimental consequences of an ASFV-positive test result not only to the farmer but also to the region and country. Due to the

absence of a commercially available vaccine and the lack of a pen-side test for ASF, the correct diagnosis of the disease is the only method to help with outbreak surveillance and to completely eradicate ASFV from affected countries (Barderas et al., 2001; Perez et al., 2011). This results in a strong need for a highly sensitive and specific diagnostic test for the rapid detection of ASFV. It is essential to implement control measures in a timely manner to prevent virus spread and to differentiate ASF from the other swine diseases that have similar clinical symptoms (Alonso, 2013; Oura et al., 2013).

Diagnostic tests for ASF can be classified into two groups, those that detect the virus and those that detect anti-viral antibodies. The detection and identification of ASFV-specific antigens, viral DNA, or antibodies from samples is considered a positive diagnosis of ASFV (Oura et al., 2013). The OIE currently recommends multiple serological and virological diagnostic tests for the detection of the ASFV virus, its antigens, and its genome using the following: hemadsorption tests (HAD), polymerase chain reaction (PCR) assays, radioimmunoassays (RIA), fluorescent antibody tests (FAT), indirect fluorescent antibody (IFA), immunoblotting (IB), and enzyme-linked immunosorbent assays (ELISA). Samples that can be tested for ASFV include blood preserved in an anticoagulant, spleen, lymph nodes, liver, and tonsil (Crowther, Wardley, & Wilkinson, 1979; OIE, 2012; Oura et al., 2013). Other assays such as immunocytochemistry and *in situ* hybridization have been described in disease pathogenesis studies but are not ideal for routine diagnosis due to the need for specialized equipment and the difficulty in interpreting the results (Hutchings & Ferris, 2006; Mao, Javois, & Kent, 1994).

OIE approved virological detection methods include assays such as PCR, FAT, and HAD. HAD is the “gold-standard” assay for ASFV diagnosis. It is a virus isolation technique that can be used to detect ASFV in peripheral blood leukocytes due to the ability of ASFV to

induce hemadsorption of the pig erythrocytes to the surface of infected monocytes or macrophages. A positive HAD test result can be obtained within 24 hours and is considered a conclusive diagnosis for ASFV. However, not all ASFV isolates are hemadsorbing and some will give negative results for a HAD test, resulting in the need for other diagnostic assays such as FAT. Other disadvantages for HAD tests include the requirement of primary cell cultures and the length of time of up to 6 days required to confirm a negative result.

The "gold standard" test for the detection of the ASFV genome is PCR. The OIE recommends two validated PCR procedures, a conventional PCR and a real-time TaqMan® PCR. These methods use primer pairs and probes that target the coding region of the capsid protein p72. (OIE, 2012). There is also a loop-mediated isothermal amplification (LAMP) PCR assay that has been used for ASFV detection, although it is not recommended by the OIE (James et al., 2010). Although PCR is a highly sensitive diagnostic method with the ability to detect virus in deteriorated samples, it generally requires expensive instruments for accurate thermocycling (OIE, 2012; Oura et al., 2013).

Using rapid, sensitive, and specific serodiagnostic procedures could contribute to the eradication of ASFV in affected regions. Serological assays are the most commonly used diagnostic assays for detection of antibodies to ASFV in subacutely or chronically infected animals. However, they are not useful to test for ASFV in pigs with acute disease because these animals usually die prior to developing antibodies to ASFV. In addition, serological assays do not require expensive equipment and facilities and have a relatively low cost to run. In endemic regions, serological assays have been found useful for the detection of antibodies in samples from animals that survive infection since antibodies to ASFV persist for long periods of time. Serological assays approved by the OIE include IFA, IB, and ELISA. It has been found that

these tests are best when used in combination with other assays, especially when the techniques use crude antigens (Cubillos et al., 2013; Gallardo et al., 2015; OIE, 2012; Oura et al., 2013).

The ELISA is used to detect ASF antibodies in pigs infected with low or moderately virulent viruses. The OIE-approved ELISAs for anti-ASFV antibody detection are based on the use of antigens obtained using live virus. This has several disadvantages including complications in achieving standardization of assays and risks associated with the handling of the virus as this requires the use of a biosafety level 3 (BSL-3) facility (Cubillos et al., 2013; Gallardo, Reis, et al., 2009; OIE, 2012). The ELISA is one of the most widely used techniques for the diagnosis of subacute and asymptomatic carriers of ASFV and was developed to assist in rapid diagnosis and to supplement the traditional HAD test for ASFV (Alcaraz, Diego, Pastor, & Escribano, 1990; Cubillos et al., 2013; Hutchings & Ferris, 2006). Although using an ELISA allows for the rapid screening of a large number of samples for the presence of virus or the antibody recognizing it, a disadvantage to this assay is that inconclusive results generally have to be confirmed by another assay (Alcaraz et al., 1990; Cubillos et al., 2013; Gallardo, Reis, et al., 2009; OIE, 2012). Using a standard ELISA that uses live virus for the production of ASFV antigens is ideal as a screening tool due to the high sensitivity of the assay. Disadvantages to the standard ELISA include the amount of false positive reactions obtained with field sera and the difficulty of standardizing the assay among laboratories. The use of recombinant proteins in place of conventional methods using live virus are alternative systems developed to resolve some of these issues. The use of recombinant proteins in diagnostic assays, such as the ELISA, offers a safe and reproducible alternative by providing a simpler interpretation of the test results, increasing assay specificity by reducing false positive reactions, avoiding using infectious virus, and allowing for the

standardization of antigen production (Barderas et al., 2001; Cubillos et al., 2013; Tabares et al., 1981).

IFA is used as a confirmatory test for inconclusive ELISA results for sera from endemic areas and for a positive ELISA result for sera from areas that are considered free of the disease. IB is a very specific assay that enables a more objective interpretation of results. It is used to confirm results with individual sera and is also used as an alternative confirmatory assay to IFA. These tests are based on the use of recombinant proteins which meet all biosecurity measures, providing accuracy and enabling a standardized diagnostic interpretation. IB is considered the "gold standard" assay recommended by the OIE as a confirmatory test for anti-ASFV antibody detection (Cubillos et al., 2013; Gallardo, Reis, et al., 2009; Muñoz-Moreno et al., 2015).

Previously, protein p72 has been used in the development of ELISA-based diagnostic assays. Freije *et al.* (1993) developed an indirect ELISA test using recombinant p72 expressed in *E.coli.* Moreover, a study by Vidal *et al.* (1997) showed that a sandwich ELISA using monoclonal antibodies produced against p72 had high sensitivity and specificity against homologous sera. Cubillos *et al.* (2013) also showed that an indirect sandwich ELISA, based on ASFV recombinant protein p30, is an effective assay for the diagnosis of ASFV. It was recommended that another diagnostic test be used in combination with the ELISA due to the decreased sensitivity compared to the HAD test and the possibility that the ELISA may not detect low concentrations of the virus.

In Europe, there are currently three ELISA kits commercially available for the detection of ASFV antibodies. One is an indirect ELISA by Svanova that targets protein p30. The other two assays use the major capsid protein p72 alone and in combination with other proteins as a target. The first is an IDvet multi-antigen indirect ELISA for the detection of monoclonal

antibodies against p30, pp62, and p72. The second is an Ingenasa blocking ELISA based on the use of monoclonal antibodies against p72 (Gallardo et al., 2015). There is also an Ingenasa antigen-capture assay based on the use of monoclonal antibodies to p72 for virus detection.

Blocking ELISA

As mentioned previously, ASFV is a high consequence pathogen and an assay with high specificity is needed. A blocking ELISA is able to give this high specificity. The basic principle of this assay lies in the ability of the test sera to block the binding of a monoclonal antibody to the target antigen. It is important that the test sera and the monoclonal antibodies react to the same viral epitopes (Hasan et al., 2016). By epitope mapping p72, we will be able to determine which monoclonal antibody could best compete with known positive serum samples for antigen binding in order to create a better blocking ELISA. Shown in figure 1.5 is a blocking ELISA scheme. In an blocking ELISA, the plate is coated with the antigen and the sample is added. If antibodies are present in the sample, they will bind to the antigen. The monoclonal antibody is then added to the plate, and if the antibodies are bound to the antigen, they will block the binding of the monoclonal antibodies. In blocking ELISA with direct detection, the monoclonal antibody is already labeled with the enzyme. In our pilot assay, we did not have a labeled monoclonal antibody, therefore in order to visualize the results, an HRP-labeled anti-mouse monoclonal antibody was then added to the plate followed by the addition of the substrate. A light blue or clear well indicates a positive result, whereas a negative result will be a dark blue.

Blocking ELISAs have been developed for the detection of *Mycoplasma hyopneumonia,* hepatitis E virus (HEV), and avian influenza virus (AIV), as well as other pathogens (Chen et al., 2016; Henriques et al., 2016; Liu, 2014; Yang et al., 2016). Chen *et al.* (2016) compared a traditional ELISA to a blocking ELISA that tested for anti-HEV antibodies in pig serum. Their

results show the blocking ELISA seems to provide the same sensitivity and a higher specificity than the other assay. They found that blocking ELISAs based on the use of monoclonal antibodies could decrease the non-specific binding and help to improve the specificity of detection of antibodies in serum. Yang *et al.* (2016) and Henriques *et al.* (2016) compared different blocking ELISAs to the gold standard hemagglutination inhibition test (HI) for the detection of AIV and found a high correlation between the two assays and again, sensitivity and specificity were increased when compared to other ELISA assays. Furthermore, Liu *et al.* (2014) developed a blocking ELISA the for *M. hyopneumoniae* which had both high sensitivity and high specificity. A few advantages of using monoclonal antibodies for the detection of anti-ASFV antibodies includes a high specificity and an unlimited supply of a standardized reagent. Overall, using a blocking ELISA has the potential advantage of being a high throughput, rapid, sensitive, and specific method for the detection of anti-ASFV antibodies.

Alphavirus vectors

The polyclonal serum used in this study was from pigs immunized with an alphavirus based vector expressing p72. Alphavirus-based vectors are single-cycle replication-deficient recombinant replicon particles (RP) that can express one or more protein of interest. The alphavirus genome is divided into two open reading frames (ORFs). The first ORF encodes the nonstructural proteins and the second ORF encodes the virus structural proteins, the capsid and envelope proteins. In the alphavirus-based vector, these proteins are replaced by the heterologous genes of interest. Then the replicon, together with the helper RNA coding for the capsid and envelope proteins, are co-electroporated into Vero cells. After 24 hours, the replicon particles are harvested. The use of alphaviruses in vaccine development is due to their ability to infect a broad range of animals without inducing symptoms; their low seroprevalence reduces interference with

immune response to the protein of interest. Advantages of using RPs include the ability to quickly obtain high titer virus preparations and not having the need for a helper virus to package the recombinant alphavirus RNAs. It has been shown that these vectors created good vaccines (Lundstrom, 2014; Rayner, Dryga, & Kamrud, 2002).

Purpose

There is limited information on the immunogenic epitopes on the major capsid protein p72, and in the U.S. there are no commercially available tests for ASFV. The purpose of this study was to map the epitopes of ASFV major capsid protein p72 using polyclonal swine sera and a panel of monoclonal antibodies. The ultimate goal of this research is the development of a blocking ELISA for the detection of anti-ASFV antibodies.

Tables and figures

Figure 1.1 Virion structure of the African swine fever virus

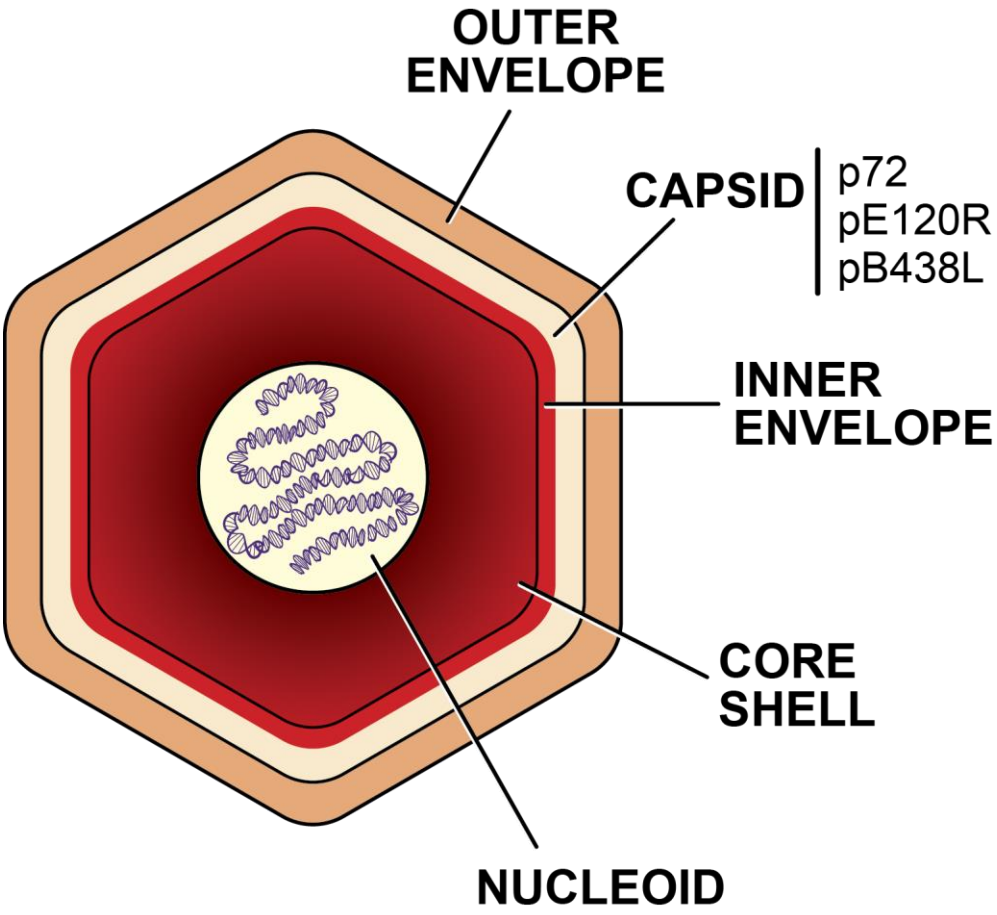

Figure 1.1 Virion structure of the African swine fever virus. ASFV is a multilayer virus with an icosahedral structure. The layers consist of a central nucleoid containing the DNA surrounded by a core shell. Outside this core shell is the inner envelope and surrounding the inner envelope is the capsid containing pE120R, pB438L, and major capsid protein p72. The virus obtains an outer envelope when budding through the cell membrane. ©2016. Mal Rooks Hoover, CMI.

Figure 1.2 Amino acid sequence alignment of the p72 whole protein

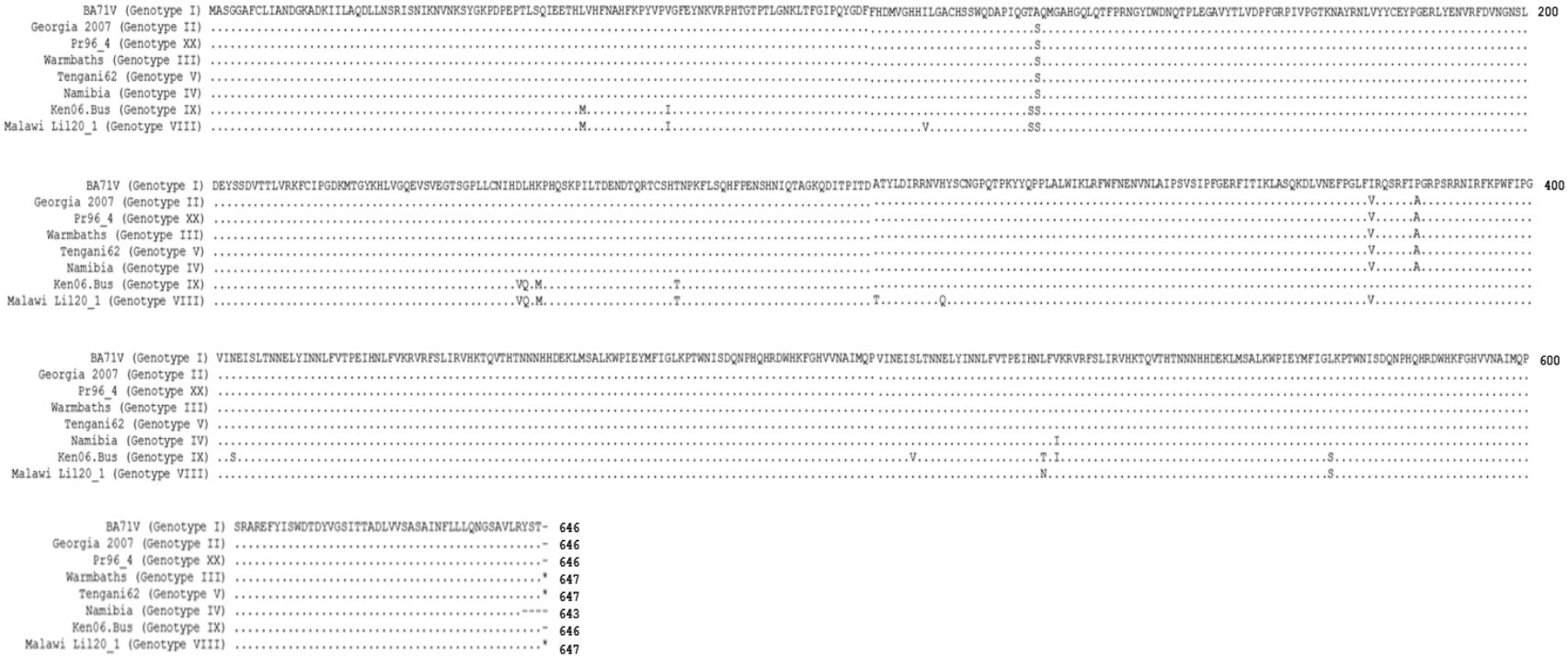

Figure 1.2 Amino acid sequence alignment of the p72 whole protein. An amino acid alignment of the p72 whole protein from amino acid 1 to 646 of a sample of 8 genotypes. Dots indicate conservation of the amino acids in these locations of the sequence.

Figure 1.3 Amino acid sequence alignment of the N-terminus of the p72 protein

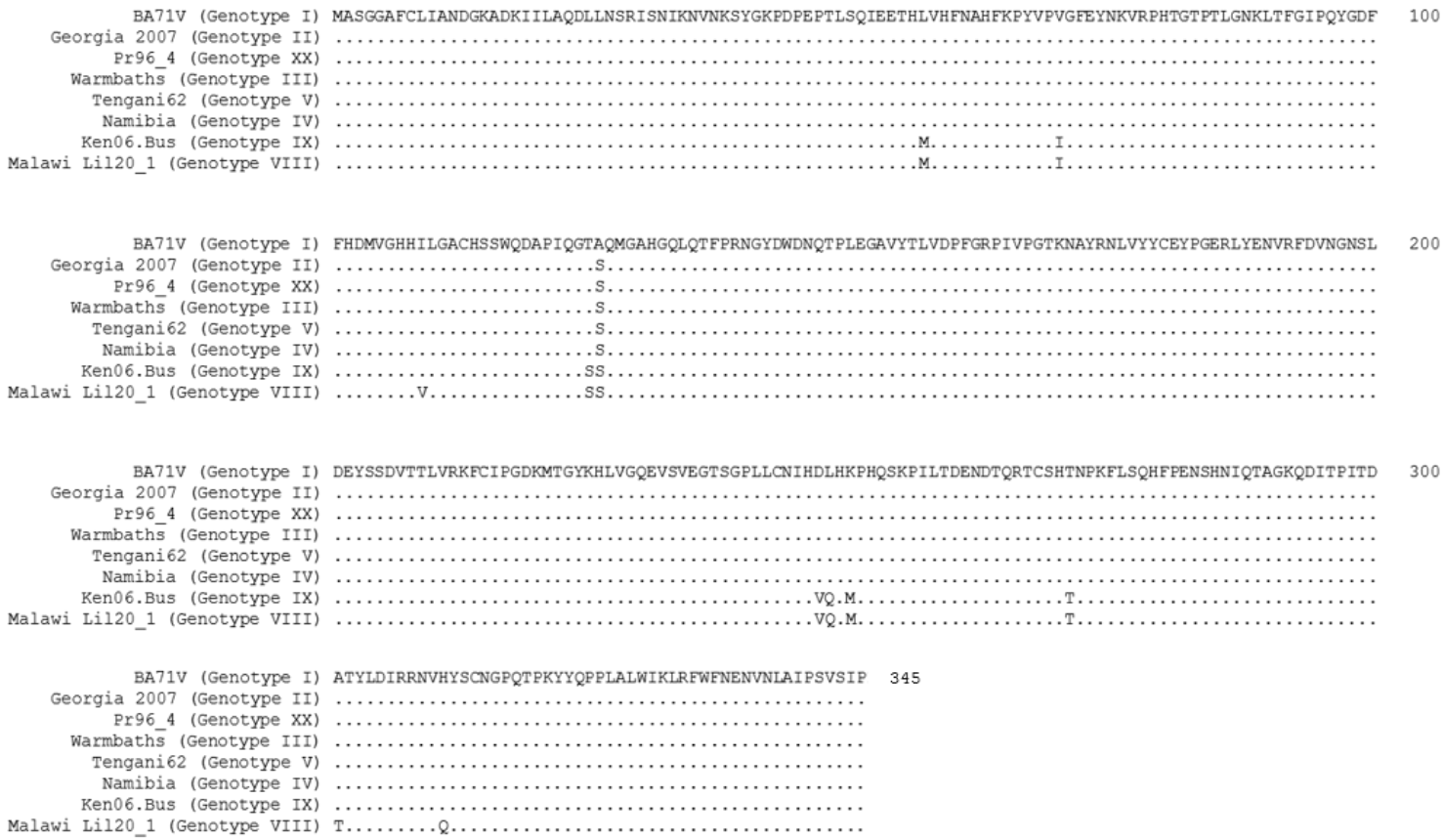

Figure 1.3 Amino acid sequence alignment of the N-terminus of the p72 protein. An alignment of the N-terminal amino acid sequence alignment of the first 345 amino acids of 8 different genotypes. Dots indicate the same amino acid.

Figure 1.4 Nucleotide sequence alignment of the C-terminus of the p72 protein

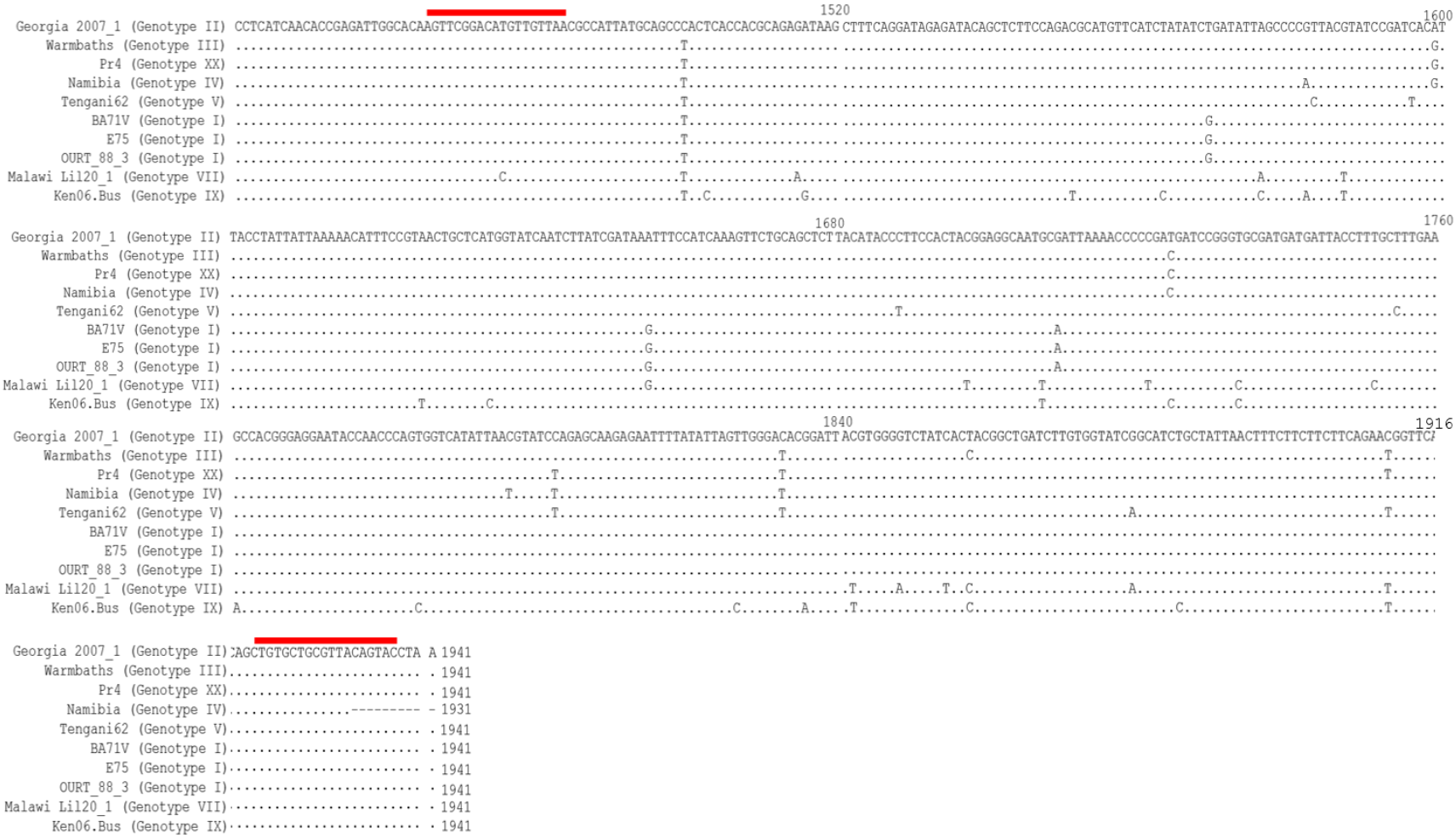

Figure 1.4 Nucleotide sequence alignment of the C-terminus of the p72 protein. Shown on the left are isolates of 7 of the 23 different genotypes of ASFV. The C-terminus region of the p72 protein is the variable region that has been used to differentiate the 23 different genotypes. Dots indicate the same nucleotide. The red lines represent the forward and reverse primers used for genotyping.

Figure 1.5 Illustration of the blocking ELISA assay

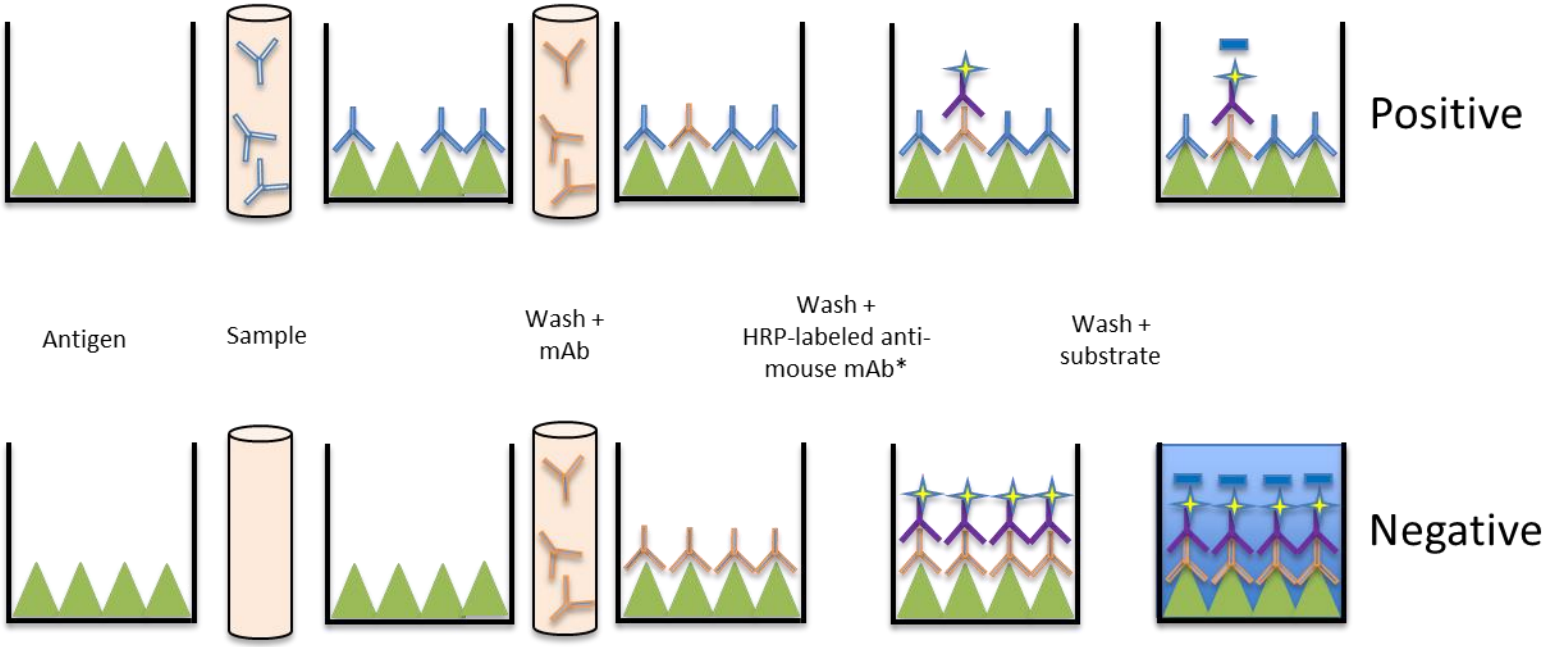

Figure 1.5 Illustration of the blocking ELISA assay. Antigen is bound to the plate. When the sample is added, if antibodies are present they will bind. When the monoclonal antibody is added, the antibodies from the serum will block the binding of the monoclonal antibody to the antigen. An HRP-labeled anti-mouse monoclonal antibody is then added to visualize the results. When the substrate is added, a positive sample will be light blue or clear, whereas a negative sample would be a strong blue.

Chapter 2 - Materials and Methods

Cloning, expression, and purification of recombinant ASFV p72 polypeptides

Cloning of overlapping fragments of the ASFV p72 protein

The ASFV p72 protein, based on the BA71V strain, was divided into five overlapping fragments from amino acid (aa) 1 to 345: Fragment 1 (aa 1-100), Fragment 2 (aa 130 -250), Fragment 3 (aa 180-280), Fragment 4 (aa 250-345), and a fifth fragment referred to as P6 (aa 83-171). Moreover, the p72 region between amino acids 180 and 250 and between amino acids 280 and 345 was divided further into Fragment A (aa 180-225), Fragment B (aa 205-250), Fragment C (aa 280-320), and Fragment D (aa 300-345) (Figure 2.1). The nucleotide sequences of all of the fragments were commercially synthesized. The fragments were cloned into the pCR2.1® TOPO® cloning vector (Invitrogen) following the manufacturer's instructions. Briefly, an adenine nucleotide was added to the 3' end of the fragments creating a 3' A overhang. The reaction mixture used to create these overhangs consisted of 5 µl of DNA (10 ng/µl), 1X polymerase buffer, 1 µm dATP, and 5 U *Taq* DNA polymerase (New England Biolabs, Inc.) in a total volume of 20 µl. This mixture was incubated at 72 ˚C for 15 minutes. The cloning reaction consisted of 4 µl of DNA with 3' A overhangs, 1 µl of the pCR® 2.1-TOPO® vector, and 1 µl of a salt solution. The reaction was incubated at room temperature for 5 minutes. *E. coli* chemically competent cells, either TOP10F' (Invitrogen) or NEB 10-beta (New England Biolabs, Inc.), were then transformed with 2 µl of the product from the TOPO® cloning reaction. After a 30-minute incubation on ice, the cells were heat-shocked at 42 ˚C for 30 seconds followed by an incubation period on ice for 5 minutes. Finally, 200 µl of super optimal broth with catabolite repression (SOC) medium (New England Biolabs, Inc.) was added to the cells, followed by a 1-hour incubation at 37 ˚C in a shaking incubator. The cells were then inoculated onto

Luria-Bertani (LB) agar containing ampicillin and incubated overnight at 37 ˚C. Colonies were selected from the plates, transferred into LB media with ampicillin, and grown overnight at 37 °C in a shaking incubator. The plasmid DNA was purified from the selected colonies using the PureYield™ Miniprep Kit (Promega), following the manufacturer's instructions. The plasmids were then analyzed for the presence of the target insert by double restriction enzyme digestion using *Sac*II and *Eco*RI High-Fidelity restriction enzymes. For the restriction enzyme digestion reaction, 1 µg of purified plasmid DNA was mixed with 2 µl CutSmart Buffer (New England Biolabs, Inc.), 20 U of *Eco*RI-HF (New England Biolabs, Inc.), 20 U of *Sac*II (New England Biolabs, Inc.) and nuclease-free water to make up a final volume of 20 µl. The reaction was then incubated for 1 hour at 37 ˚C followed by an enzyme inactivation step with incubation at 65 ˚C for 20 minutes. Products of the restriction enzyme digest were separated via agarose gel electrophoresis. This was accomplished by preparing 1% agarose gels containing ethidium bromide, loading the digestion products into the wells of the gels, and running the gel for 30 minutes at 100 volts. The fragments were visualized by examining the gel under UV light.

One of the clones containing the target band was used for subcloning into the pHUE expression vector (Figure 2.2a). The plasmid containing the target insert was subjected to double restriction digestion as described above, the target band was gel purified using Wizard® SV Gel and PCR Clean-Up system (Promega), following the manufacturer's instructions. Purified DNA was then ligated into the pHUE expression vector by mixing 1 µl of 10X T4 DNA Ligase buffer, 50 ng of vector DNA, purified DNA at 1:3 ratio (vector:DNA), 1 µl of T4 DNA Ligase and nuclease-free water up to 20 µl. The reaction mixture was incubated at room temperature for 30 minutes and transformed into TOP10F' or NEB 10-beta *E. coli* competent cells (New England Biolabs, Inc.), as described above. The clones were screened, as described above. One

clone containing pHUE with the target band was selected and the plasmid was used to transform BL21 (DE3) *E. coli* chemically competent cells (New England Biolabs, Inc.), following manufacturer's instructions. Briefly, 20 µl of BL21 (DE3) chemically competent cells were transformed with 2 µl of the pHUE plasmid. The cells were incubated on ice for 30 minutes, then heat shocked for 30 seconds at 42 ˚C, followed by incubation on ice for 5 minutes. SOC medium (200 µl) was added to the cells followed by incubation in a shaking incubator at 37 ˚C for 1 hour. The cells were then added to 7 ml LB media with ampicillin (Fisher Scientific), returned to the shaking incubator, and incubated overnight at 37 ˚C. The resulting culture was then used for protein expression and a bacterial stock was prepared and stored at -80 ˚C for future use.

Protein expression

For the purpose of recombinant protein expression, BL21(DE3) *E. coli* cells, containing the pHUE plasmid with one of the p72 fragments, were grown in LB media at 37 ºC until the optical density (OD600) reached between 0.4 and 0.6. Protein expression was induced by the addition of Isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM. After addition of IPTG, the bacterial cells were grown for an additional 4 hours and then harvested by centrifugation 4000 x g for 10 minutes. Aliquots of 1 ml were taken hourly starting before the addition of IPTG. The aliquots were pelleted and suspended in 100 µl of SDS sample buffer (sodium dodecyl sulfate with Tris-HCl pH 6.8, glycerol, β-mercaptoethanol, ethylenediaminetetraacetic acid (EDTA) and bromophenol blue) for analysis by SDS-PAGE. Prior to gel electrophoresis, the aliquots collecting during the protein expression were boiled for 5 minutes before loading into the gel. Electrophoresis was performed at 150 V for approximately 45 minutes. The gel was washed with warm double distilled water three times for 1 minute each and was stained for 10 minutes using Coomassie blue staining (SimplyBlue^{TM} SafeStain by

Novex). After staining, the gel was washed again using warm double distilled water. A picture of the gel was acquired using the UVP GelDoc-It™ Imaging System.

Purification under native conditions

Proteins were purified using a PrepEase® Histidine-tagged Protein Purification Kit (USB). The pellet obtained from the protein expression protocol was suspended in 1X lysis, equilibration and wash (LEW) buffer (50 mM sodium dihydrogen phosphate and 300 mM sodium chloride, pH 8.0), with 10 µl/ml of EDTA-free protease inhibitor cocktail (PIC) (THERMO scientific), and 1 mg/ml of lysozyme. After incubation on ice for 30 minutes, sonication was performed using the Model 3000 Ultrasonic Homogenizer (Biologics, Inc.) for 3 minutes at 40% power. The bacterial lysate was then centrifuged at 10,000 x g for 30 minutes. The supernatant was filtered with a 0.45-µm PVDF syringe filter (Fisher) to eliminate any insoluble material. Next, gravity flow, affinity chromatography was performed by the addition of the filtered lysate to a Ni-IDA mini column to capture the 6X Histidine-tagged polypeptides. After three 2-ml washes with 1X LEW buffer, the protein was eluted three times with volumes of 1.5 ml, 1.5 ml, and 2 ml in 1X elution buffer (50 mM sodium dihydrogen phosphate, 300 mM sodium chloride and 250 mM Imidazole, pH 8.0). The elutions were stored at 4 ˚C until used in western blots and ELISAs. The purification was confirmed by SDS-PAGE and western blot with anti-histidine tag monoclonal antibody.

Protein purification using detergent

This protocol used the detergent Sarkosyl (sodium lauroyl sarcosinate) in CAPS (3-[Cyclohexylamino]-1-propanesulfonic acid) buffer (CAPS/Sarkosyl). The protocol for protein purification using CAPS/Sarkosyl was the same as the “native” protocol through the initial centrifugation step. The pellet post-centrifugation was suspended in 0.5 M CAPS/0.3% Sarkosyl

buffer (pH 8.0) at a concentration of 10-15 mg of pellet per milliliter of buffer. The suspended pellet was subjected to four freeze-thaw cycles with temperatures at -80 ºC and 37 ºC, respectively. The bacterial lysate was then centrifuged at 10,000 x g for 30 minutes at 4 ºC. The supernatant was filtered with a 0.22-µm filter and purified using Ni-IDA column. The purification using the Ni-IDA columns was as described above with the exception of the wash and elution buffers that were used. The wash buffer was 1X LEW with 0.5 M CAPS/0.3% Sarkosyl, while the elution buffer was 1X Elution buffer with 0.5 M CAPS/0.3% Sarkosyl.

Purification under denaturing conditions

The protocol for purification under denaturing conditions was the same as the "native" protocol through the initial centrifugation step. After centrifugation of the bacterial lysate, the supernatant was discarded and the pellets were suspended in 1X LEW buffer with 8M urea. The suspended pellets were incubated on ice for 30 minutes followed by sonication for 3 minutes at 40% power and then centrifugation at 10,000 x g for 30 minutes. Ni-IDA column purification was carried out the same as previously described in the "native" protocol with the exception of using 1X LEW and 1X elution buffers combined with 8M urea.

Polyclonal swine serum

The polyclonal swine serum samples were obtained from pigs immunized with an alphavirus-based replicon particle expressing the extracellular domain of the HA protein (based on the E75 strain) together with the p72 whole protein (based on the BA71V strain).

Monoclonal antibodies

The panel of 29 monoclonal antibodies were produced by our collaborators at the Foreign Animal Disease Diagnostic Laboratory (FADDL) at Plum Island Animal Disease Center (PIADC). The monoclonal antibodies were produced against a truncated p72 protein (from

amino acids 20 to 303) based on a current ASFV strain, Georgia/07, and expressed in baculovirus. This protein was used for mouse immunizations and also as an antigen in the indirect ELISA for screening the monoclonal antibodies. The monoclonal antibodies were screened at FADDL using indirect ELISA and immunofluorescence assay (IFA).

Synthesized oligopeptides

Overlapping 15-mers, coupled with ovalbumin, were commercially synthesized by 21st Century Biochemicals (Table 2.1). These oligomers were made corresponding to the areas of reactivity of the monoclonal antibodies.

Indirect ELISA

A 96-well flat-bottom plate (Costar) was coated with 100 µl of protein diluted to 4 µg/ml in carbonate coating buffer (CCB) and incubated for one hour at 37 ºC. The plate was washed three times with 0.05% Tween 20 in PBS (PBS-T) and then blocked by adding 100 µl of 10% goat serum in PBS (PBS-GS) to each well and incubated for one hour at 37 ºC. The plate was frozen at -20 ˚C if the assay was to be done at a later time. After washing the plate three times with PBS-T, the primary antibodies were added. Substances used as the primary antibody source were polyclonal swine serum from two pigs immunized with RP-sHA-p72 (Pig #5 and Pig #9, Day 57), a negative-control serum sample, and a panel of monoclonal antibodies. Two-fold serial dilutions in PBS-GS were done across the 96-well plate for each of these then the plate was incubated at 37 ˚C for 1 hour. The plate was washed three times with PBS-T before the secondary antibody was added. The secondary antibodies used were goat anti-swine IgG conjugated to horse radish peroxidase (HRP) (ICN Biomedicals Inc.), diluted 1:2000 in PBS-GS, for the polyclonal sera and goat anti-mouse IgG conjugated to HRP (ICN Biomedicals Inc.), diluted 1:500 in PBS-GS, for the monoclonal antibodies. The plate was washed three times with

PBS-T then 100 µl of ABTS® Microwell Peroxidase Substrate colorimetric reagent was added to visualize the reaction. After incubation at room temperature for 20 minutes, 100 µl of 1% SDS in double-distilled water was added to stop the reaction. Absorbance values were read at 405 nm and 650 nm. The absorbance of the sample was obtained by subtracting absorbance reading A650 from absorbance reading A405. Fine mapping was then performed by indirect ELISA using overlapping 15-mers coupled with ovalbumin as the antigen and following the above protocol.

Western blotting

Western blots were performed using purified elutions from each of the five fragments and the p72 whole protein. The protein fragments were separated in a 12% SDS-PAGE gel by electrophoresis done at 150 V for 45 minutes. The proteins were then blotted into a 0.2 µm PVDF membrane (Amersham) as follows: the SDS-PAGE gel was rinsed three times for 5 minutes each in PBS-T on a shaking platform; the membrane was activated by submerging it in 100% methanol followed by rinsing it in double-distilled water for 10 minutes on a shaking platform; and the gel, filter papers and PVDF membrane were soaked for 20 minutes in 1X transfer buffer (glycine, tris base, SDS, double-distilled water and methanol). The blot apparatus was assembled and the electrophoretic transfer was done using a Mini Trans-Blot® Electrophoretic Transfer Cell (BIO-RAD, Hercules, CA) following the manufacturer's instructions. After transfer, the membrane was blocked using 5% non-fat dry milk in PBS (NFDM) or protein-free buffer and incubated overnight at 4 ˚C. Following the blocking step, the membrane was washed three times with PBS-T, the first time for 15 minutes followed by two additional wash steps for 5 minutes each. The primary antibody was then added to the membrane and incubated for 1 hour at room temperature. The primary antibody sources used were

anti-histidine monoclonal antibody, diluted 1:1000 in NFDM; polyclonal swine sera from two pigs immunized with RP-sHA-p72 at day 0 and day 57, diluted 1:300 in protein-free blocking buffer (G-Biosciences); and 29 monoclonal antibodies, diluted 1:100 each in NFDM. The incubation was followed by three washes with PBS-T as described above. An HRP-labeled secondary antibody was then added and the membrane was incubated for 1 hour at room temperature on a shaking platform. The secondary antibodies used were goat anti-mouse IgG conjugated to HRP that was diluted 1:500 in NFDM when mouse monoclonal antibodies were used as the primary antibody and goat anti-swine IgG conjugated to HRP that was diluted 1:3000 in protein-free blocking buffer when polyclonal swine serum was used as the primary antibody. The reaction was visualized by using the CN/DAB Substrate Kit (Thermo Scientific Pierce) by adding the substrate to the membrane and incubating it on a shaking platform for 5 to 10 minutes at room temperature until color developed. The reaction was stopped by rinsing the membrane with double-distilled water. A picture of the membrane was acquired using the UVP GelDoc-It^{TM} Imaging System.

Determining monoclonal antibody half-maximum values

Five anti-p72 reactive monoclonal antibodies were further tested to determine their half-maximum values, the concentration at which 50% of the monoclonal antibody reacts with its antigen. This was accomplished by performing indirect ELISAs using the ASFV p72 whole protein as the antigen. The protocol described above was again used but with the following modifications: two-fold dilutions of the selected p72 monoclonal antibodies were made starting from the 1:200 dilution; and the secondary antibody was goat anti-mouse IgG conjugated to HRP (ICN Biomedicals, Inc.) at a dilution of 1:4000.

Blocking ELISA with indirect detection

A 96-well flat-bottom plate (Costar) was coated with 100 µl of ASFV p72 whole protein diluted to 4 µg/ml in CCB, leaving the last row without protein, and then incubated for 1 hour at 37 ºC. The plate was washed three times with PBS-T and then blocked by adding 100 µl of 10% PBS-GS to each well. The plate was incubated for 1 hour at 37 ˚C. Following incubation, the plate was again washed three times with PBS-T. Polyclonal swine serum from two pigs immunized with RP-sHA-p72 (Pig #5 and Pig #9, Day 57) and a negative-control serum were added undiluted to the first well of each column, doubling dilutions were performed using PBS-GS, and the plate was incubated at 37 ˚C for 1 hour. The plate was washed three times with PBS-T and then the monoclonal antibodies were added. The monoclonal antibodies were used at dilutions corresponding to their half-maximum absorbance values then the plate was incubated at 37 ˚C for 30 minutes. The plate was washed three times with PBS-T after which the secondary antibody, goat anti-mouse IgG conjugated to HRP (ICN Biomedicals, Inc.), was added at a dilution of 1:4000 in PBS-GS and incubated at 37 ˚C for one hour. The plate was washed three times with PBS-T then 100 µl of ABTS® Microwell Peroxidase Substrate colorimetric reagent was added to each well to visualize the reaction. After incubation at room temperature for 20 minutes, 100 µl of 1% SDS in double-distilled water was added to the wells to stop the reaction. The absorbance of the sample was obtained by subtracting measurements read at 405 nm and 650 nm.

Tables and figures

Figure 2.1 ASFV p72 overlapping fragment scheme

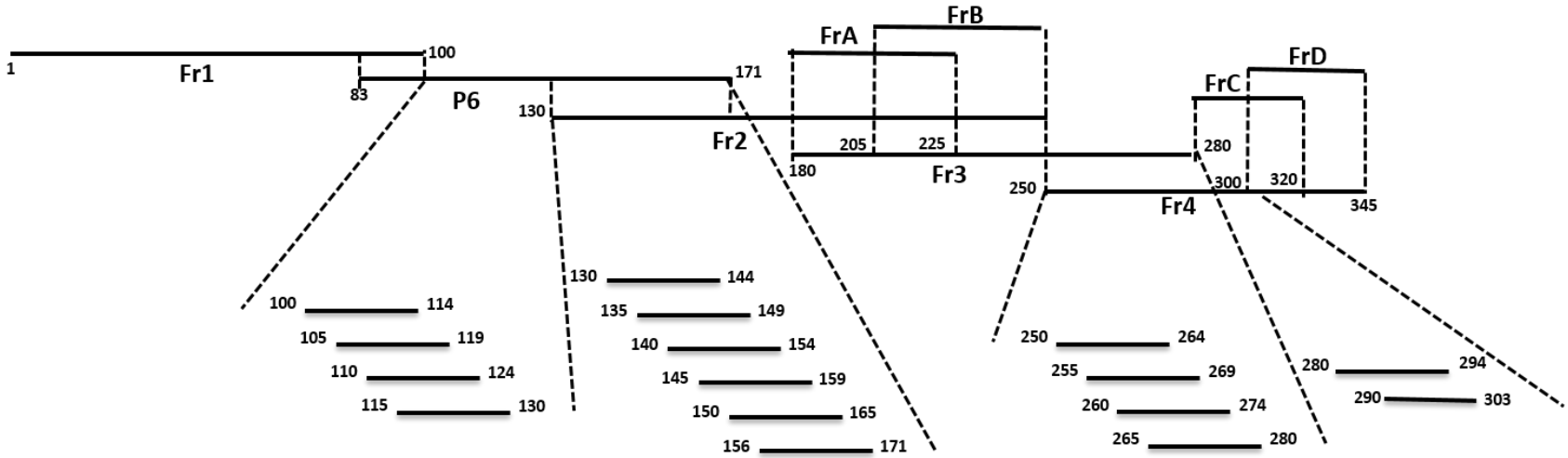

Figure 2.1 ASFV p72 overlapping fragment scheme. ASFV p72 (based on BA71V) from amino acid 1 to 345 was divided into fragments 1, P6, 2, 3, and 4. The region from amino acid 180 to 250 was divided further into Fragment A (FrA) and Fragment B (FrB) and the region from amino acid 280 to 345 was divided further into Fragment C (FrC) and Fragment D (FrD). Sixteen overlapping oligopeptides are shown below from amino acid 100 to 171 and amino acid 250 to 303.

Figure 2.2 The pHUE expression vector

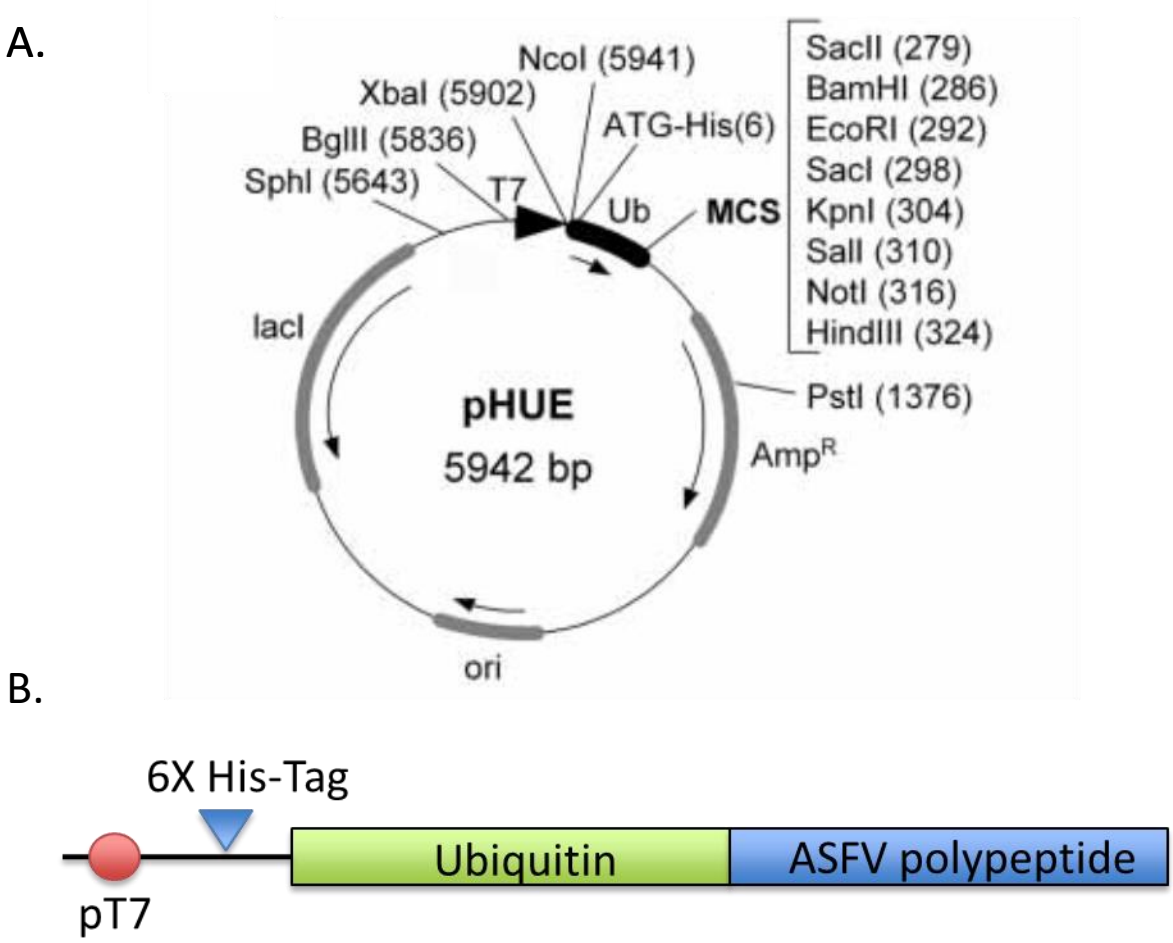

Figure 2.2 The pHUE expression vector. A. Plasmid map of the pHUE expression vector showing the T7 promoter, ubiquitin, and restriction enzyme recognition sites (Catanzariti, Soboleva, Jans, Board, & Baker, 2004). The protein sequences were inserted between restriction enzyme recognition sites for *Sac*II and *Eco*RI. **B.** A histidine-ubiquitin-ASFV fusion protein.

Table 2.1 Oligopeptides and their corresponding amino acids

Oligopeptide	Amino Acids
I	100-114
II	105-119
III	110-124
IV	115-130
V	130-144
VI	135-149
VII	140-154
VIII	145-159
IX	150-165
X	156-171
XI	250-264
XII	255-269
XIII	260-274
XIV	265-280
XV	280-294
XVI	290-303

Table 2.1 Oligopeptides and their corresponding amino acids. Sixteen overlapping 15-mers coupled with ovalbumin used for the fine mapping of ASFV major capsid protein p72.

Chapter 3 - Results

Production of ASFV p72 overlapping fragments in *Escherichia coli*

Fragments were initially cloned into the pCR® 2.1-TOPO® cloning vector (Invitrogen) and inserted into NEB 10-beta cells. Agarose gel electrophoresis of the PCR products was performed. Subsequent analysis of the gels revealed a single band of the correct size for all of the ASFV p72 fragments (data not shown). The rationale for using pCR® 2.1-TOPO® as a shuttle vector include the following: the creation of 5'-A and 3'-T overhangs for the direct ligation of the PCR products obtained from amplification with *Taq* polymerase, the speed and efficiency of ligation, the presence of *Eco*RI and *Sac*II recognition sites flanking the PCR product insertion site for easy scanning of clones, inclusion of both kanamycin and ampicillin resistance genes for selection in *E. coli,* and the ability to perform blue/white colony screening. Inserts were then digested from the pCR® 2.1 vector with the *Sac*II and *Eco*RI enzymes and inserted in frame into the *Sac*II and *Eco*RI sites of the pHUE vector. These two sites were selected due to the nucleotide sequence of the fragments being devoid of the recognition sequences for the *Sac*II and *Eco*RI restriction enzymes.

The pHUE *E. coli* expression vector was utilized because the products of expression with this vector include a 6X histidine tag and ubiquitin. The 6X histidine tag allows for simple affinity column purification and the fusion of cloned peptides to ubiquitin increases the bacterial expression and solubility of the peptides. Successful cloning of the fragments into the pHUE expression vector is shown by the agarose gel electrophoresis of the double restriction enzymatic digestion reactions with the restriction enzymes *Sac*II and *Eco*RI which reveals a single band of the correct size for all p72 fragments (Figure 3.1).

To express fusion proteins of ubiquitin with the p72 fragments, the pHUE vector containing the fragments was initially transformed into BL21 (DE3) *E. coli* competent cells (Invitrogen). This cell line was selected for its capability to express proteins from the T7 promoter efficiently. In order to optimize protein expression, aliquots were taken immediately prior to induction with IPTG as well as every hour thereafter for a total of 4 hours. After 4 hours of incubation with IPTG, SDS-PAGE analysis showed peak expression of all products with each fragment and the p72 whole protein being of the correct size (Figure 3.2). Fragments 2, 3, P6, A, B, C, and D were purified using the manufacturer's protocol for native conditions. Not all fragments were soluble under native conditions which led to the conclusion that these proteins were forming insoluble inclusion bodies inside the *E. coli* bacterial cells. Fragment 4 was purified using CAPS/Sarkosyl, and by using the strong denaturant urea, Fragment 1 and the p72 whole protein were purified using the protocol for purification under denaturing conditions. Overall, transformation, expression, and purification of each fragment and the p72 whole protein resulted in a band of the predicted molecular weight, along with other proteins co-purified with the target band (Figure 3.3). Each fragment and the p72 whole protein reacted positively when tested on a western blot against the anti-histidine monoclonal antibody. This antibody recognizes the 6X histidine tag and confirmed the purification of the ASFV p72 proteins (Figure 3.4).

Monoclonal antibody screening

The monoclonal antibodies were produced and screened at FADDL by ELISA using baculovirus-expressed p72 as well as by IFA on ASFV Lisbon-infected cells. As shown in table 3.5, all of the 29 monoclonal antibodies recognized the p72 protein by ELISA. On the other hand, only 6 of the 29 monoclonal antibodies recognized the native form of the protein in the ASFV-infected cells. Furthermore, the isotype of each monoclonal antibody was determined and

the majority of them were of the IgG1 kappa (IgG1κ) isotype, although four were a mixture of two different clones. In addition, in our laboratory, all 29 of the monoclonal antibodies were tested against the p72 protein expressed in *E. coli.* Of these, 27 out of 29 had positive results. The remaining two monoclonal antibodies were not considered further in this study (Table 3.5).

Mapping of ASFV p72 epitopes using polyclonal serum and monoclonal antibodies

Polyclonal serum samples from two pigs, Pig #5 and Pig #9, immunized with the RP-sHA-p72 construct were reacted against the ASFV p72 whole protein and the p72 fragments on both ELISA (Figure 3.5) and western blot (Figure 3.6). The polyclonal sera reacted to the p72 regions between amino acids 1 and 83 and between amino acids 250 and 345. The reactivity of the polyclonal sera for both ELISA and western blot is summarized in Table 3.1. A panel of 29 monoclonal antibodies produced against a truncated p72 protein, from amino acid 20 to 303, was also reacted on ELISA (Figure 3.7) and western blot (Figure 3.8) against the p72 whole protein and fragments. As stated previously, we did not consider the 2 monoclonal antibodies that did not react with the p72 whole protein for further mapping. The other 27 monoclonal antibodies tested for mapping reacted to the p72 regions between amino acid 100 to 170 and amino acid 180 to 303 (Table 3.2). The reactivity of the monoclonal antibodies can be divided into five distinct groupings: Group 1, from amino acid 100 to 130; Group 2, from amino acid 130 to 180; Group 3, from amino acid 180 to 250; Group 4, from amino acid 250 to 280; and Group 5, from amino acid 180 to 303 (Table 3.3). Five monoclonal antibodies (7C5, 9G6, 9G11, 5B10, and 81) reacted with two different protein regions. Three monoclonal antibodies (7C5, 9G6, and 9G11) reacted by ELISA and western blot with the region between amino acid 100 and 130 and cross-reacted to the region between amino acid 280 and 303 by ELISA. Monoclonal

antibody 5B10 reacted with the epitope between amino acids 160 and 165 and cross-reacted on ELISA with the p72 region between amino acids 280 to 303. Also, monoclonal antibody 81 reacted to the regions between amino acids 130 and 171 as well as between amino acids 250 and 280.

Based on the identification of those monoclonal antibody reactive regions, fine mapping was performed using overlapping 15-mers coupled with ovalbumin. The oligomers were reacted against the monoclonal antibodies by ELISA, and six linear epitopes were identified (Table 3.4). One of the monoclonal antibodies that reacted to the oligomers, monoclonal antibody 81, recognized two different epitopes (amino acid 165 to 170 and amino acid 250 to 255). Figure 3.9 illustrates a summary scheme of the reactivity of the polyclonal sera against the p72 fragments together with the reactivity of the monoclonal antibodies against the p72 fragments and oligomers.

Blocking ELISA with indirect detection

Of the 27 anti-p72 reactive monoclonal antibodies tested, five were chosen for the development of a blocking ELISA. The monoclonal antibodies were chosen based on two characteristics: their recognition of the native form of the protein as shown by IFA on ASFV-infected Vero cells, and their recognition of a p72 conserved area as shown by the comparison of the N-terminal amino acid sequences of a representative sample of eight isolates belonging to different genotypes (Figure 3.10). For the development of the blocking assay, polyclonal sera from pigs #5 and #9 and the selected monoclonal antibodies were used. The monoclonal antibodies were used at a dilution corresponding to their absorbance half-maximum as determined by indirect ELISA (Figure 3.11), while the polyclonal sera were used undiluted and then two-fold serially diluted. Two out of five monoclonal antibodies tested (monoclonal

antibodies 85 and 23) in the blocking ELISA produced clear results together with the polyclonal sera used undiluted (Figure 3.12). The mean was calculated for each monoclonal antibody using the absorbances of three wells without serum. To determine the cutoffs for positive and negative results, two times the standard deviation was added or subtracted from the mean. An absorbance above the negative cutoff was considered a negative result and an absorbance below the positive cutoff was considered a positive result. The points that exist between these cutoffs require further testing. Monoclonal antibody 23 is one of the monoclonal antibodies with a mixed isotype; it will need to be subcloned before it can be used further in the development of the blocking assay.

Tables and figures

Figure 3.1 Double restriction enzyme digestion of the pHUE plasmid containing p72 fragment inserts

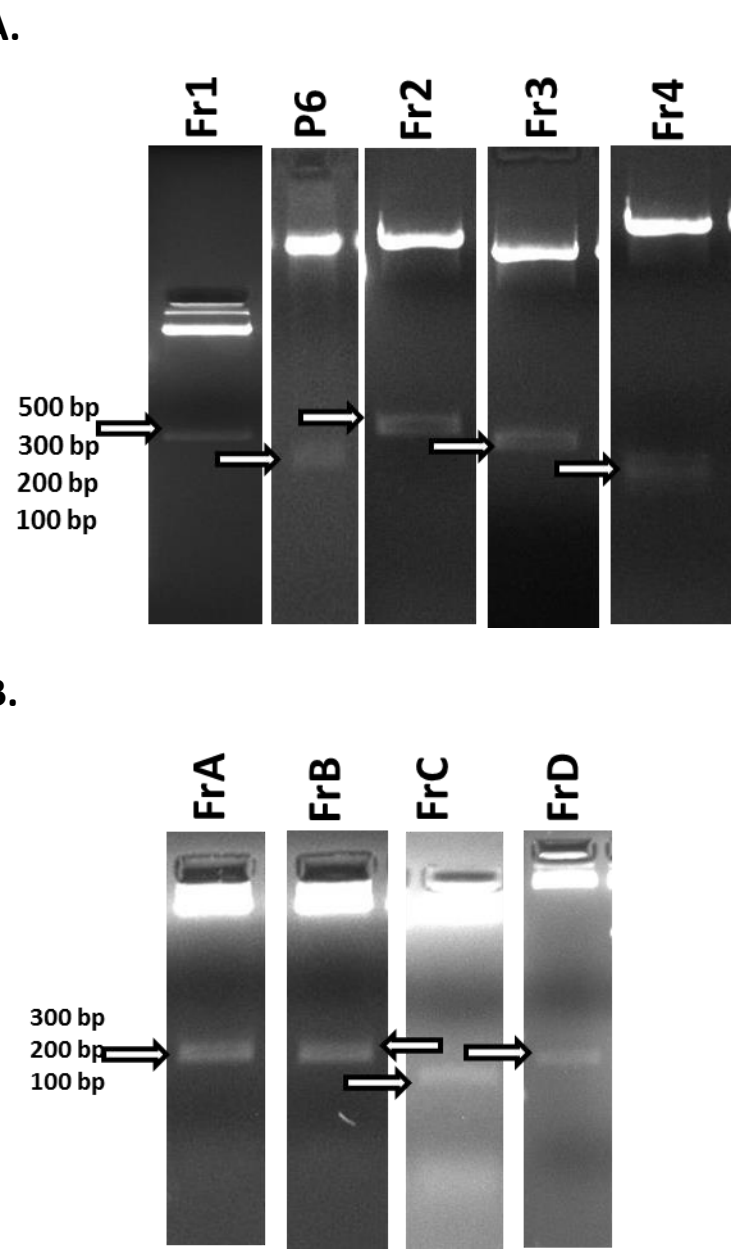

Figure 3.1 Double restriction enzyme digestion of the pHUE plasmid containing p72 fragment inserts. Successful cloning of ASFV p72 fragments. **A.** Fragment 1 (Fr1) is 300 base pairs (bp); fragment P6 is 264 bp; fragment 2 (Fr2) is 360 bp; fragment 3 (Fr3) is 300 bp; and fragment 4 (Fr4) is 285 bp. **B.** Fragment A (FrA) is 148 bp; fragment B (FrB) is 145 bp; fragment C (FrC) is 133 bp; and fragment D (FrD) is 145 bp.

Figure 3.2 Protein expression

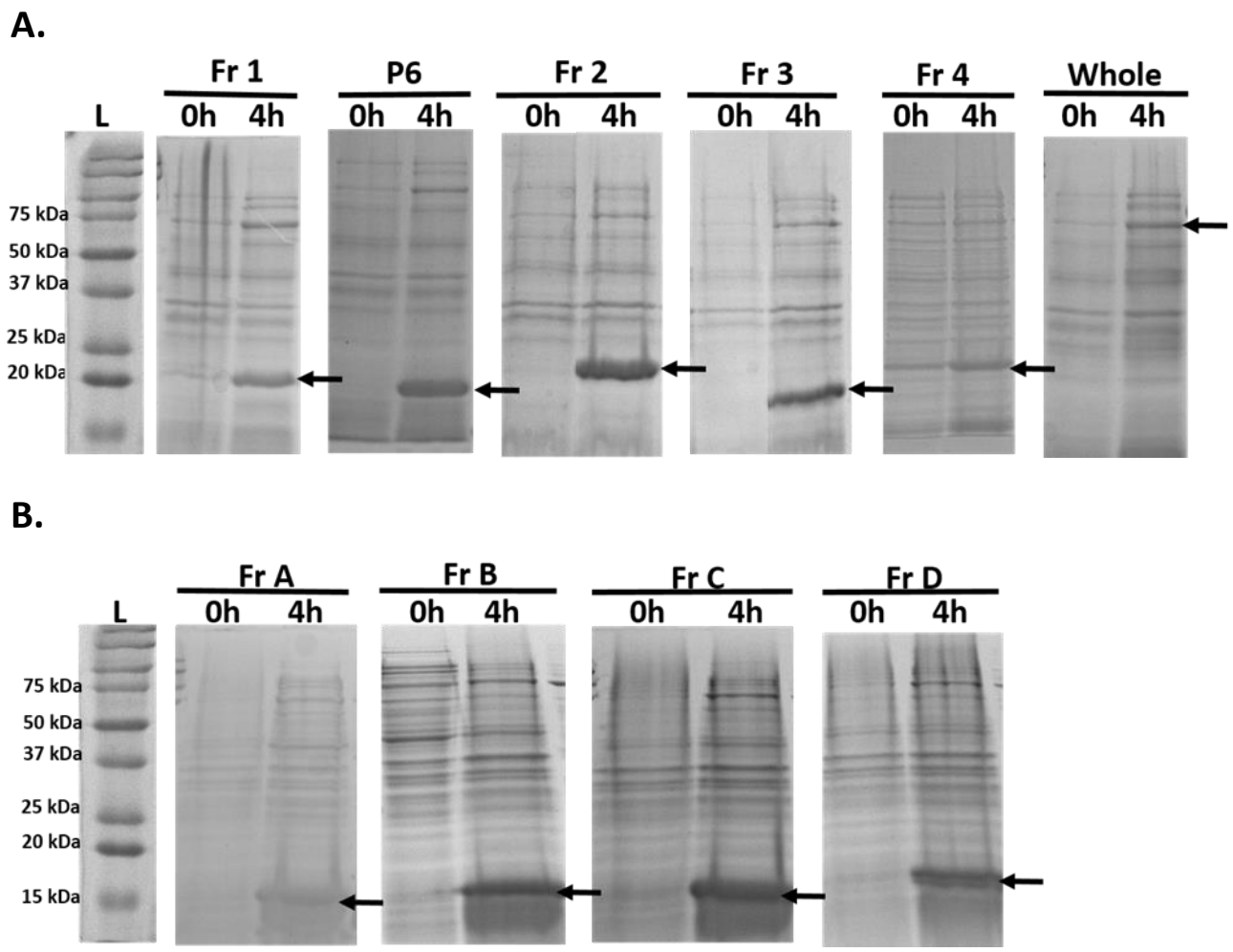

Figure 3.2 Protein expression. The ladder is the standard molecular marker, Kaleidoscope (Bio-Rad). 0h is before induction with IPTG, 4h is 4 hours after induction with IPTG. **A.** Expressions of ASFV p72 fragments 1-4, P6, and p72 whole protein. **B.** Expressions of ASFV p72 fragments A-D.

Figure 3.3 Protein purification

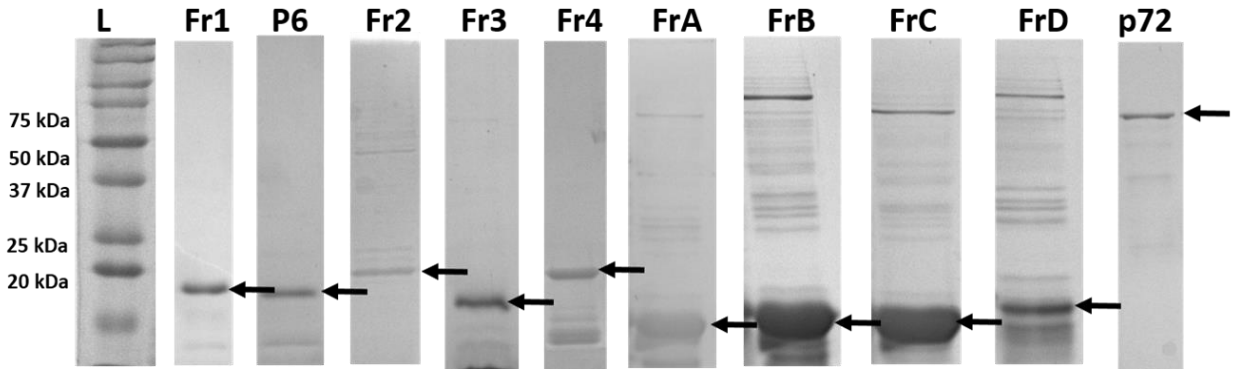

Figure 3.3 Protein purification. The ladder is the standard molecular marker, Kaleidoscope (Bio-Rad). The first elution of the purifications of ASFV p72 fragments 1-4, fragment P6, fragments A-D, and p72 whole protein. A band of the correct molecular weight is seen for each of the fragments and whole protein as indicated by the arrows.

Figure 3.4 p72 Western blot with anti-histidine monoclonal antibody

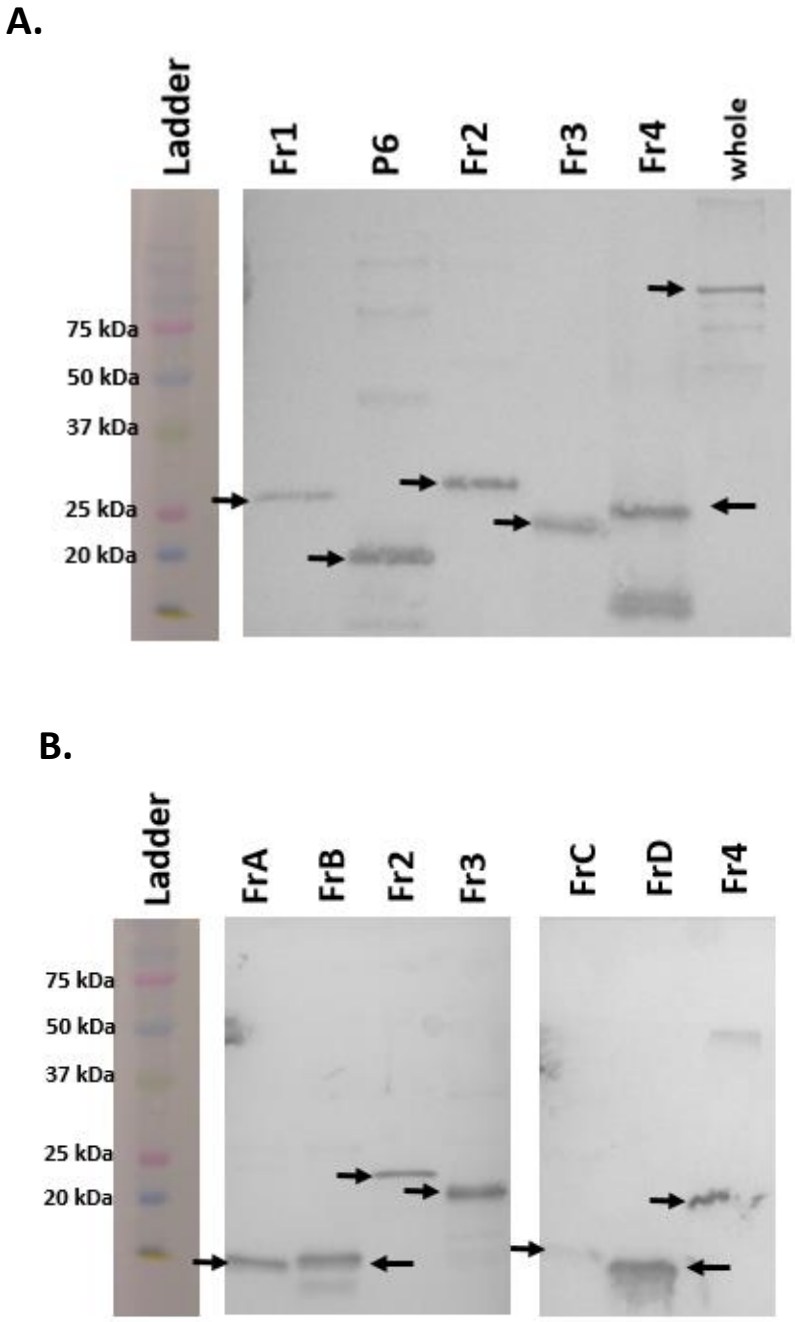

Figure 3.4 p72 Western blot with anti-histidine monoclonal antibody. The ladder is the standard molecular marker, Kaleidoscope (Bio-Rad) **A.**. Fragments 1, P6, 2, 3, 4, and the p72 whole protein were tested on western blot against anti-histidine monoclonal antibody. Fragment 1 (Fr1), P6, and fragment 4 (Fr4) are 20 kDa. Fragment 2 (Fr2) is 23 kDa. Fragment 3 (Fr3) is 21 kDa. The p72 whole protein is 75 kDa. **B.** Fragments A and B were tested on western blot against an anti-histidine monoclonal antibody along with fragments 2 and 3 as a positive control. Fragment A (FrA) and fragment B (FrB) are 15 kDa. Fragments C and D were tested on western blot against an anti-histidine monoclonal antibody along with fragment 4 for a positive control. Fragment C (FrC) and fragment D (FrD) are both 15 kDa and fragment 4 (Fr4) is 20 kDa.

Figure 3.5 p72 ELISA with polyclonal sera at 1:160 dilution

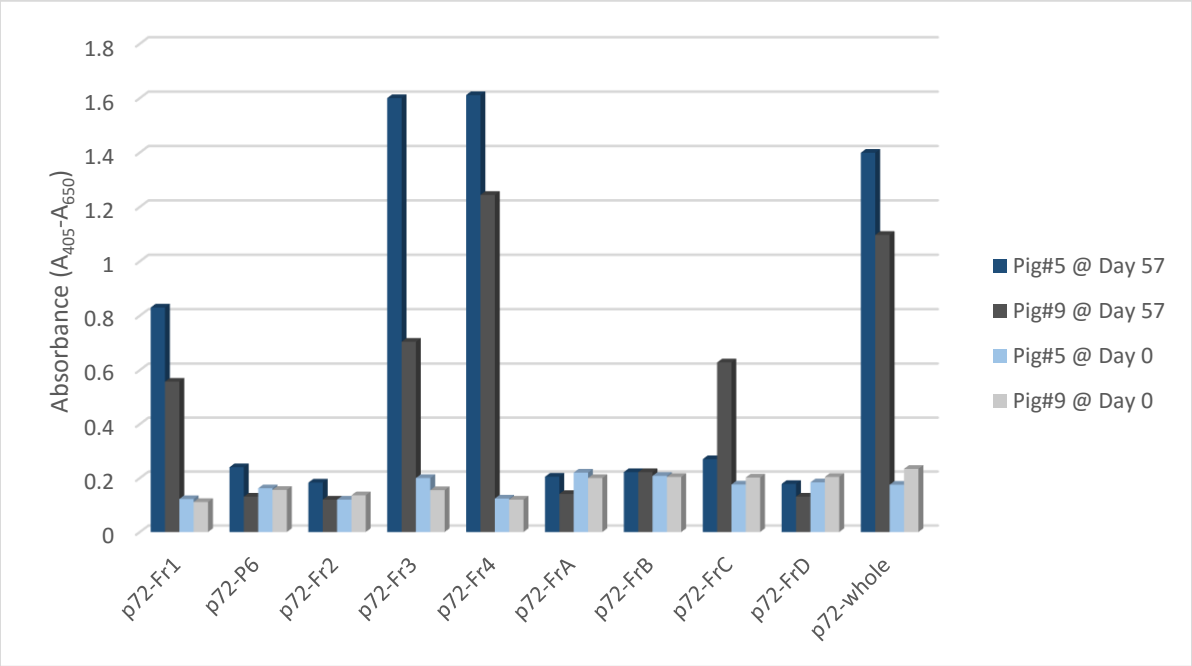

Figure 3.5 p72 ELISA with polyclonal sera at 1:160 dilution. The ASFV p72 fragments and p72 whole protein were tested on an ELISA against the polyclonal swine sera from pigs #5 and #9 at day 0 (negative control sera) and at day 57.

Figure 3.6 p72 Western blot with polyclonal swine sera

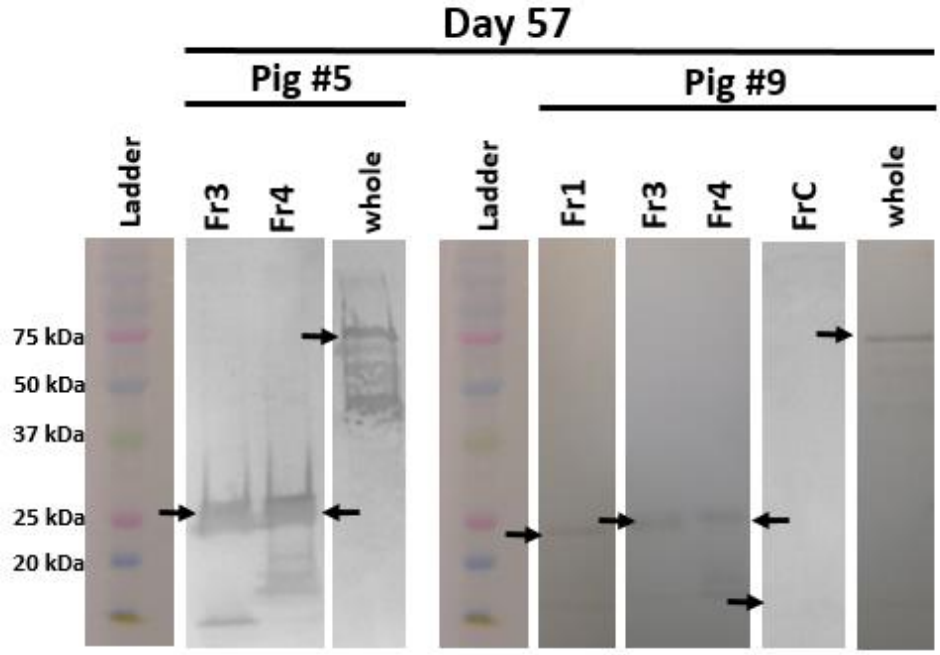

Figure 3.6 p72 Western blot with polyclonal swine sera: The ladder is the standard molecular marker, Kaleidoscope (Bio-Rad). Only the p72 reactive fragments are shown in the picture. Pig# 5 and pig# 9 are the sources of the polyclonal sera at day 57 used in the assay. Arrows indicate the bands corresponding to the p72 fragments and p72 whole protein.

Table 3.1 Polyclonal epitope mapping summary table for western blot and ELISA

A.

Polyclonal Serum	Antigen: *E. coli* expressed recombinant proteins											
	p72-Fr1 (aa 1-100)		p72-P6 (aa 83-171)		p72-Fr2 (aa 130-250)		p72-Fr3 (aa 180-280)		p72-Fr4 (aa 250-345)		p72 whole protein (aa 1-646)	
	ELISA	WB	ELISA	WB	ELISA	WB	ELISA	WB	ELISA	WB	ELISA	WB
Pig #5	+	-	-	-	-	-	+	+	+	+	+	+
Pig #9	+	+	-	-	-	-	+	+	+	+	+	+

B.

Polyclonal Serum	Antigen: *E. coli* expressed recombinant proteins							
	p72-FrA (aa 180-225)		p72-FrB (aa 205-250)		p72-FrC (aa 280-320)		p72-FrD (aa 300-345)	
	ELISA	WB	ELISA	WB	ELISA	WB	ELISA	WB
Pig #5	-	-	-	-	-	-	-	-
Pig #9	-	-	-	-	+	+	-	-

Table 3.1 Polyclonal epitope mapping summary table for western blot and ELISA. A. Fragments 1, P6, 2, 3, 4, and whole protein. **B.** Fragments A, B, C, and D. For both figures A and B, a reaction is indicated with (+) and no reaction is indicated with (-).

Figure 3.7 p72 ELISA with monoclonal antibodies and negative control serum at 1:16 dilution

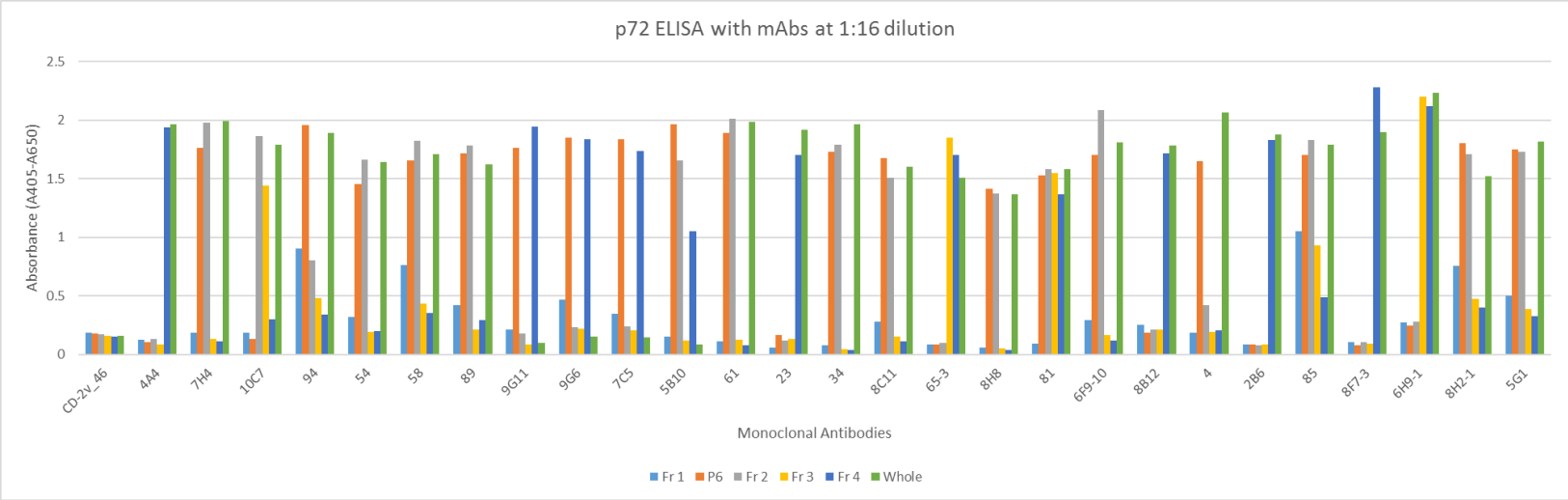

Figure 3.7 p72 ELISA with monoclonal antibodies and negative control serum at 1:16 dilution. CD-2v_46 is the negative control monoclonal antibody that was used.

Figure 3.8 Western blot of p72 with monoclonal antibodies

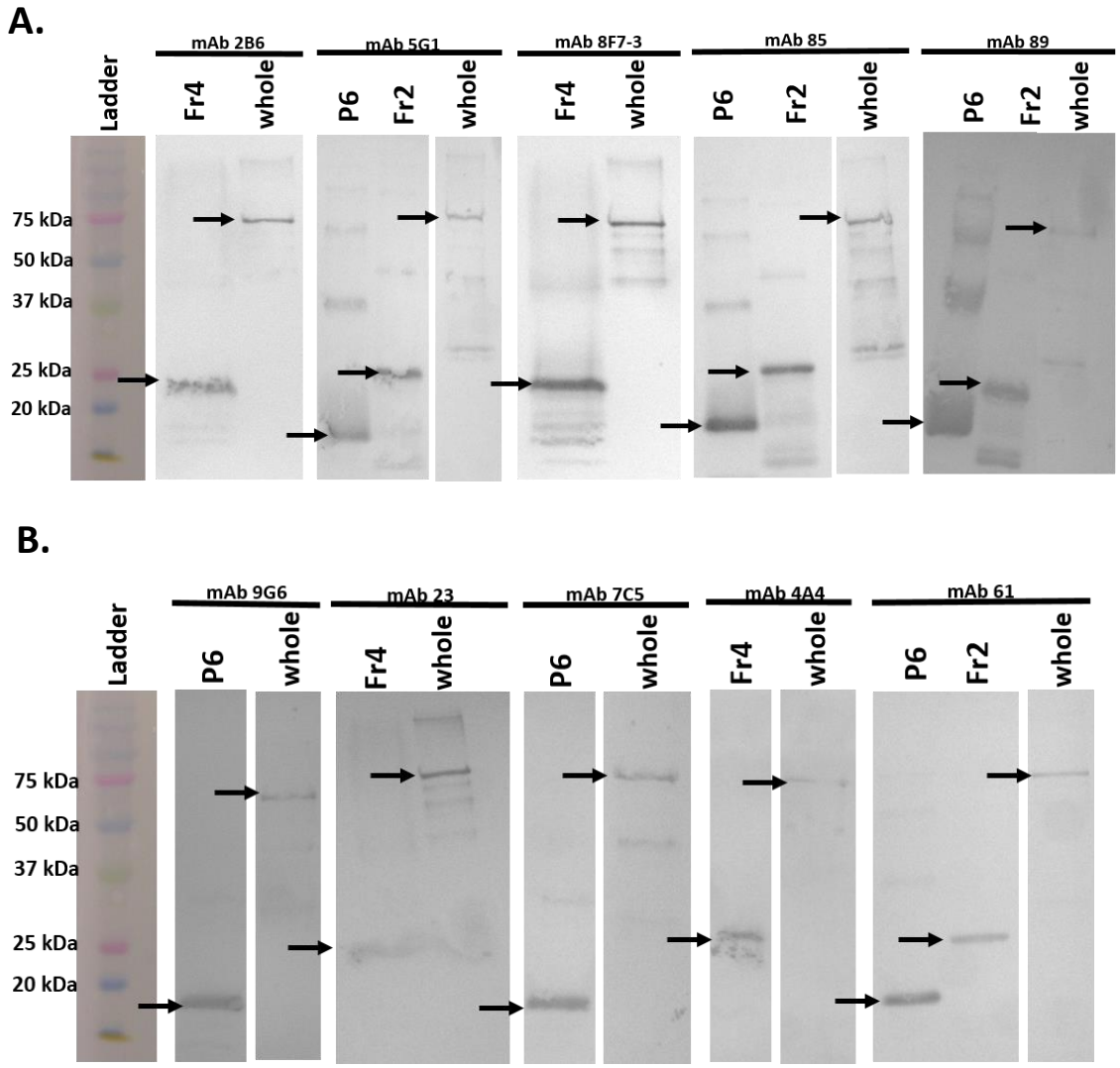

Figure 3.8 Western blot of p72 with monoclonal antibodies: As described in Materials and Methods, the ASFV p72 fragments were tested against a panel of monoclonal antibodies. In figures A-E (C, D, and E are on following page), the ladder is the standard molecular marker, Kaleidoscope (Bio-Rad). **A.** Monoclonal antibodies 2B6, 5G1, 8F7-3, 85, and 89. **B.** Monoclonal antibodies 9G6, 23, 7C5, 4A4, and 61. (Continued on the following page)

Figure 3.8. Western blot of p72 with monoclonal antibodies (continued)

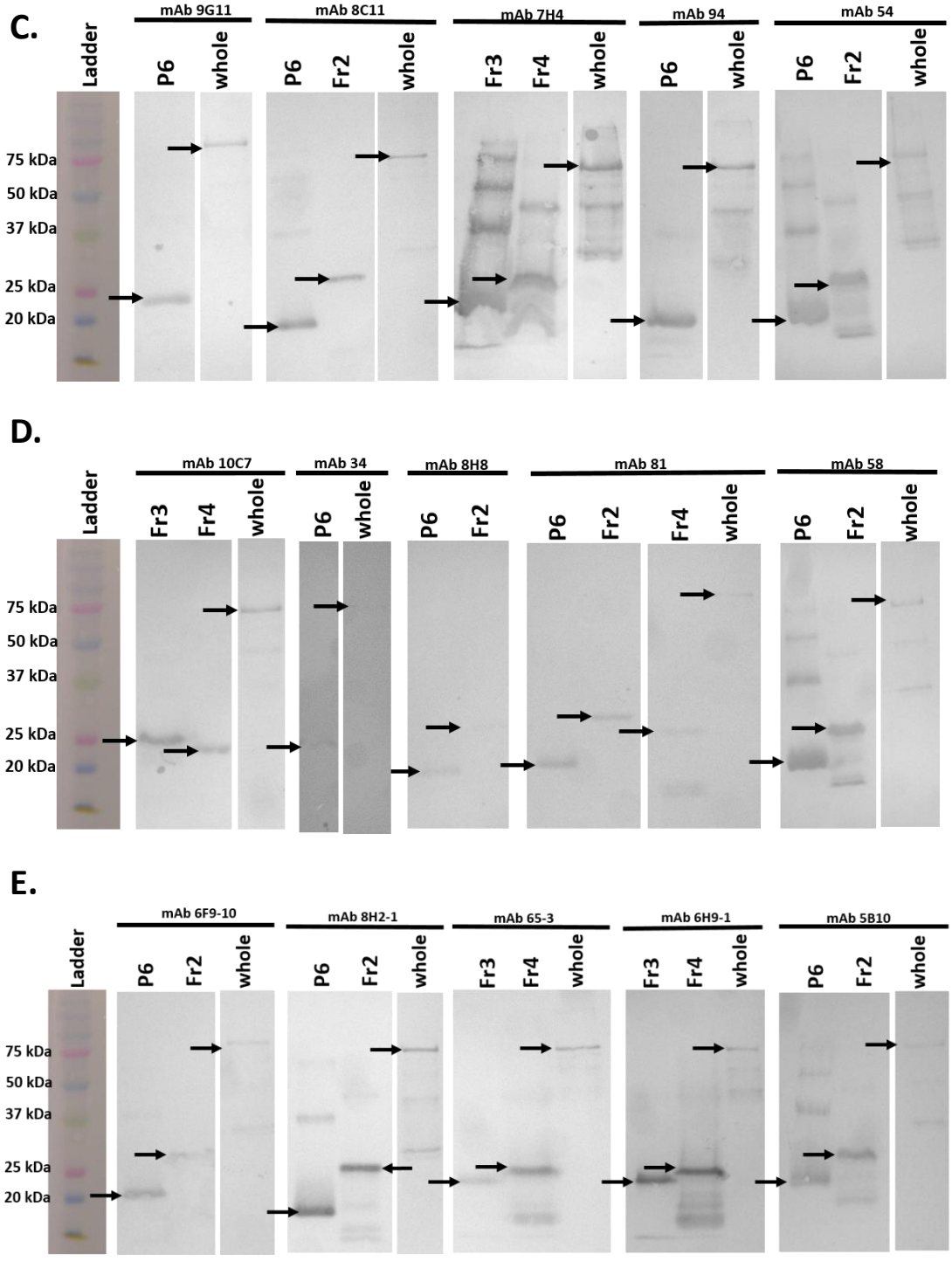

Figure 3.8 Western blot of p72 with monoclonal antibodies (continued): C. Monoclonal antibodies 9G11, 8C11, 7H4, 94, and 54. **D.** Monoclonal antibodies 10C7, 34, 8H8, 81, and 58. **E.** Monoclonal antibodies 6F9-10, 8H2-1, 65-1, 6H9-1, and 5B10.

Table 3.2 Monoclonal antibody epitope mapping summary for western blot and ELISA

p72 mAbs	Antigen: *E.coli* expressed recombinant proteins									
	p72-P6 (aa 83-171)		p72-Fr2 (aa 130-250)		p72-Fr3 (aa 180-280)		p72-Fr4 (aa 250-345)		p72 whole protein (aa 1-646)	
	ELISA	WB	ELISA	WB	ELISA	WB	ELISA	WB	ELISA	WB
10C7	-	-	+	+	+	+	-	-	+	+
23	-	-	-	-	-	-	+	+	+	+
2B6	-	-	-	-	-	-	+	+	+	+
34	+	+	+	-	-	-	-	-	+	+
4	+	-	-	-	-	-	-	-	+	-
4A4	-	-	-	-	-	-	+	+	+	+
54	+	+	+	+	-	-	-	-	+	+
58	+	+	+	+	-	-	-	-	+	+
5B10	+	+	+	+	-	-	+	-	-	+
5G1	+	+	+	+	-	-	-	-	+	+
61	+	+	+	+	-	-	-	-	+	+
7C5	+	+	-	-	-	-	+	-	-	+
7H4	+	+	+	+	-	-	-	-	+	+
81	+	+	+	+	+	-	+	+	+	+
85	+	+	+	+	-	-	-	-	+	+
89	+	+	+	+	-	-	-	-	+	+
8B12	-	-	-	-	-	-	+	-	+	-
8C11	+	+	+	+	-	-	-	-	+	+
8H8	+	+	+	+	-	-	-	-	+	-
94	+	+	-	-	-	-	-	-	+	+
9G11	+	+	-	-	-	-	+	-	-	+
9G6	+	+	-	-	-	-	+	-	-	+
65-3	-	-	-	-	+	+	+	+	+	+
6F9-10	+	+	+	+	-	-	-	-	+	+
6H9-1	-	-	-	-	+	+	+	+	+	+
8F7-3	-	-	-	-	-	-	+	+	+	+
8H2-1	+	+	+	+	-	-	-	-	+	+

Table 3.2 Monoclonal antibody epitope mapping summary for western blot and ELISA. A positive reaction is indicated with (+) and no reaction is indicated with (-).

Table 3.3 Monoclonal antibody groupings

Group 1	
(amino acid 100-130)	
4	9G11
7C5	9G6
94	
Group 2	
(amino acid 130-171)	
7H4	54
58	5B10
5G1	61
61	81
85	89
8C11	8H8
6F9-10	34
8H2-1	
Group 3	
(amino acid 180-250)	
10C7	
Group 4	
(amino acid 250-280)	
65-3	6H9-1
81	
Group 5	
(amino acid 280-345)	
4A4	8B12
8F7-3	23
2B6	7C5
9G11	9G6

Table 3.3 Monoclonal antibody groupings.

Table 3.4 Oligomer reactivity with anti-p72 reactive monoclonal antibodies

	Antigen: *E.coli* expressed recombinant proteins	Antigen: synthesized oligomers
p72 mAbs	**Mapping Location**	**Epitope recognized**
10C7	180-250	N.D.
23	280-345	280-289
2B6	280-345	280-289
34	130-171	160-165
4	100-130 (ELISA only)	No reactivity
4A4	250-345	295-303
54	130-171	160-165
58	130-171	160-165
5B10	130-171; 280-345 (ELISA only)	160-165 (No reactivity with overlapping oligomers between aa 280 and 303)
5G1	130-171	N.D.
61	130-171	160-165
7C5	100-130; 280-345 (ELISA only)	No reactivity
7H4	130-171	160-165
81	130-171; 260-280 (ELISA only); 280-345 (WB only)	250-255 and 160-165
85	130-171	160-165
89	130-171	160-165
8B12	250-345 (ELISA only)	295-303
8C11	280-345 (ELISA only)	160-165
8H8	130-171	165-171
94	100-130	No reactivity
9G11	100-130; 280-345 (ELISA only)	No reactivity
9G6	100-130; 280-345 (ELISA only)	No reactivity
65-3	250-280	275-280
6F9-10	130-171	160-165
6H9-1	250-280	275-280
8F7-3	280-345	280-289
8H2-1	130-171	160-165

Table 3.4 Oligomer reactivity with anti-p72 reactive monoclonal antibodies. The anti-p72 monoclonal antibodies reacted with *E. coli* expressed recombinant proteins mapping region compared with the epitopes recognized by monoclonal antibodies when reacted to synthetic oligomers. N.D. = Not done.

Figure 3.9 Epitope mapping summary scheme

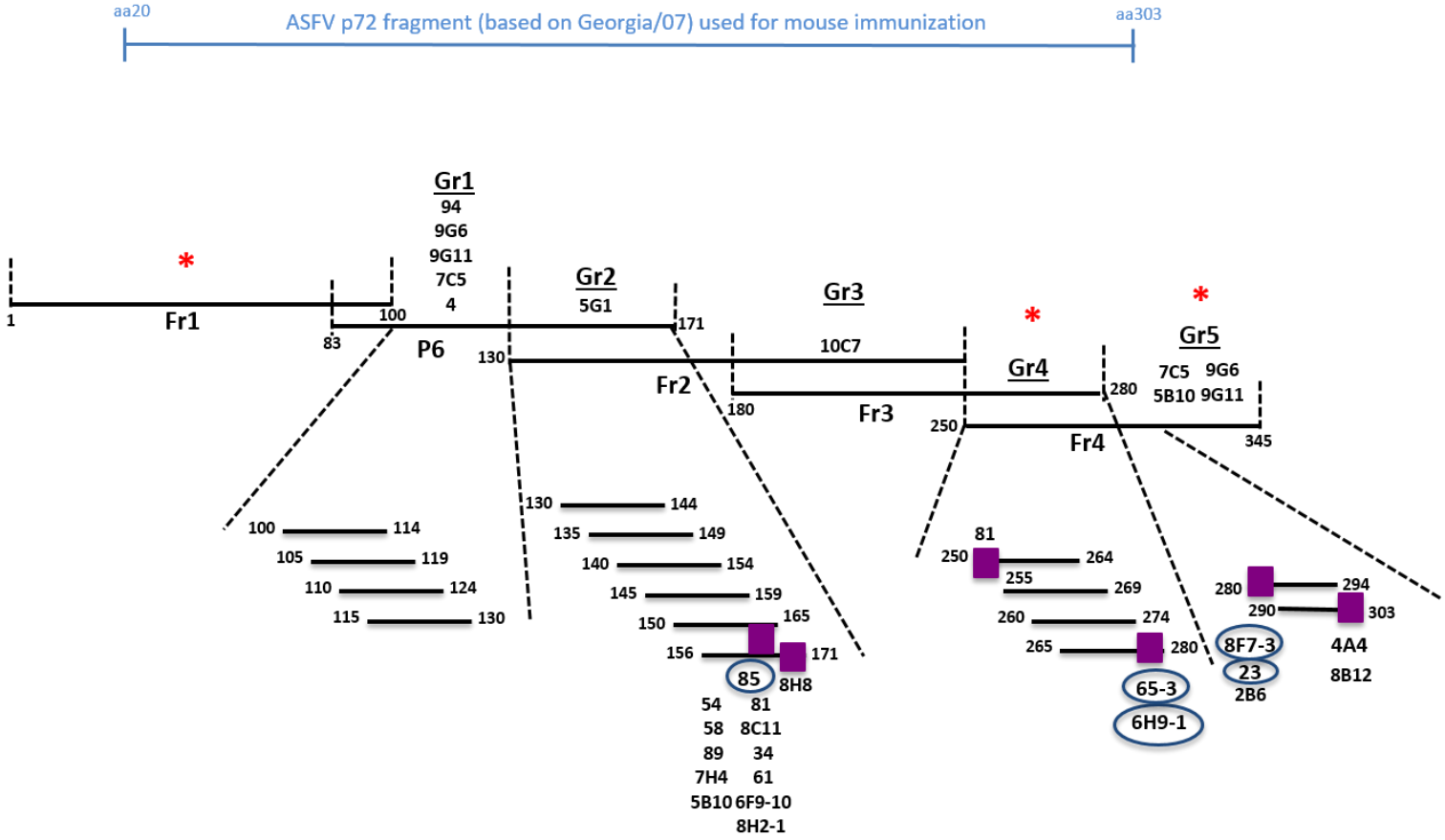

Figure 3.9 Epitope mapping summary scheme. The blue line, from amino acid 20 to amino acid 303, is the ASFV p72 fragment (based on the Georgia/07 strain) used for mouse immunization for the monoclonal antibodies. Numbers 1 to 345 represent amino acid positions of ASFV p72 (based on BA71V). Polyclonal sera reactivity to the p72 fragments is shown as the red asterisks and monoclonal antibody reactivity to the p72 fragments is shown in black. Oligopeptides are the black lines illustrated below with reactivity to the monoclonal antibodies in purple. Monoclonal antibodies selected for further testing in the blocking ELISA are circled in blue.

Table 3.5 Monoclonal antibody screening results

mAbs	Isotype	Baculovirus-Expressed p72	Lisbon-Infected Vero cells	*E.coli*-Expressed p72 whole protein	
		ELISA	IFA	ELISA	WB
10C7	IgA-κ	+	-	+	+
23	IgG1-κ /λ	+	+	+	+
2B6	IgG1-κ /λ	+	-	+	+
34	IgG1-κ	+	-	+	+
4	IgG1-κ	+	-	+	-
4A4	IgG1-κ	+	+	+	+
54	IgG1-κ	+	-	+	+
58	IgG1-κ	+	-	+	+
5B10	IgG1/IgG3-κ	+	-	-	+
5G1	IgG1-κ	+	-	+	+
61	IgG1-κ	+	-	+	+
6B1	IgG1-κ	+	-	-	-
6C7	No Reactivity	+	-	-	-
7C5	IgG1-κ	+	-	-	+
7H4	IgG1-κ	+	-	+	+
81	IgG1-κ	+	-	+	+
85	IgG1-κ	+	+	+	+
89	IgG1-κ	+	-	+	+
8B12	IgG1/IgM-k	+	-	+	-
8C11	IgG1-κ	+	-	+	+
8H8	IgG1-κ	+	-	+	-
94	IgG1-κ	+	-	+	+
9G11	IgG1-κ	+	-	-	+
9G6	IgG1-κ	+	-	-	+
65-3	IgG1-κ	+	+	+	+
6F9-10	IgG1-κ	+	-	+	+
9H9-1	IgG1-κ	+	+	+	+
8F7-3	IgG1-κ	+	+	+	+

Table 3.5 Monoclonal antibody screening results. Monoclonal antibodies were screened by ELISA using baculovirus-expressed p72 and screened by IFA in ASFV-infected cells. Furthermore, the isotype of each monoclonal antibody was determined and tested against the protein based on BA71V expressed in *E.coli.*

Figure 3.10 Monoclonal antibody reactivity to the N-terminal amino acid sequence alignment of the p72 protein

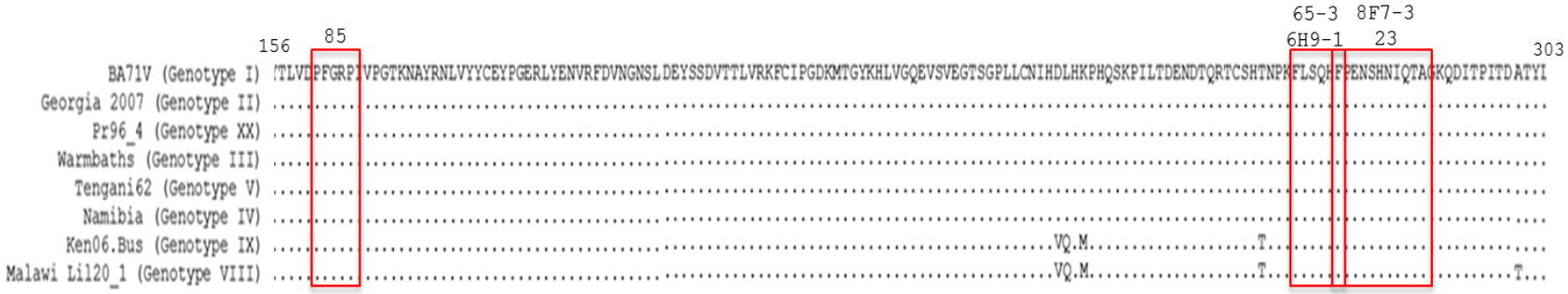

Figure 3.10 Monoclonal antibody reactivity to the N-terminal amino acid sequence alignment of p72. Conservation of the reactive epitopes to the monoclonal antibodies selected for further development of a blocking ELISA (85, 65-3, 6H9-1, 8F7-3, and 23). Monoclonal antibody 85 reacted to the epitope from amino acid 160 to 165, monoclonal antibodies 65-3 and 6H9-1 reacted to the epitope from amino acid 275 to 280, and monoclonal antibodies 8F7-3 and 23 reacted to the epitope from amino acid 280 to 290.

Figure 3.11 Determination of monoclonal antibody half-maximum values

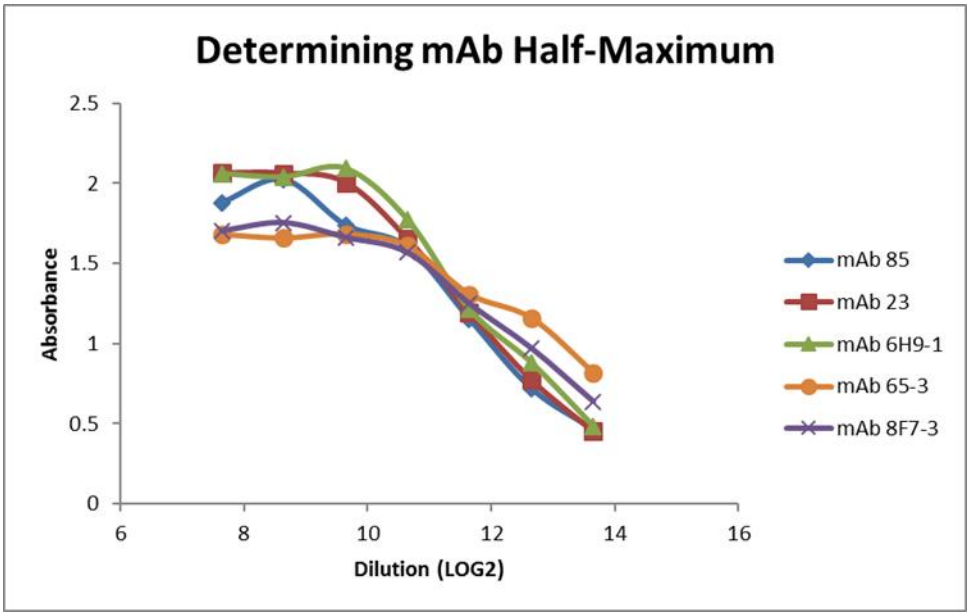

Figure 3.11 Determination of monoclonal antibody half-maximum values. The monoclonal antibodies used for the blocking ELISA were used corresponding to their half-maximum absorbance as determined by indirect ELISA.

Figure 3.12 Blocking ELISA with indirect detection

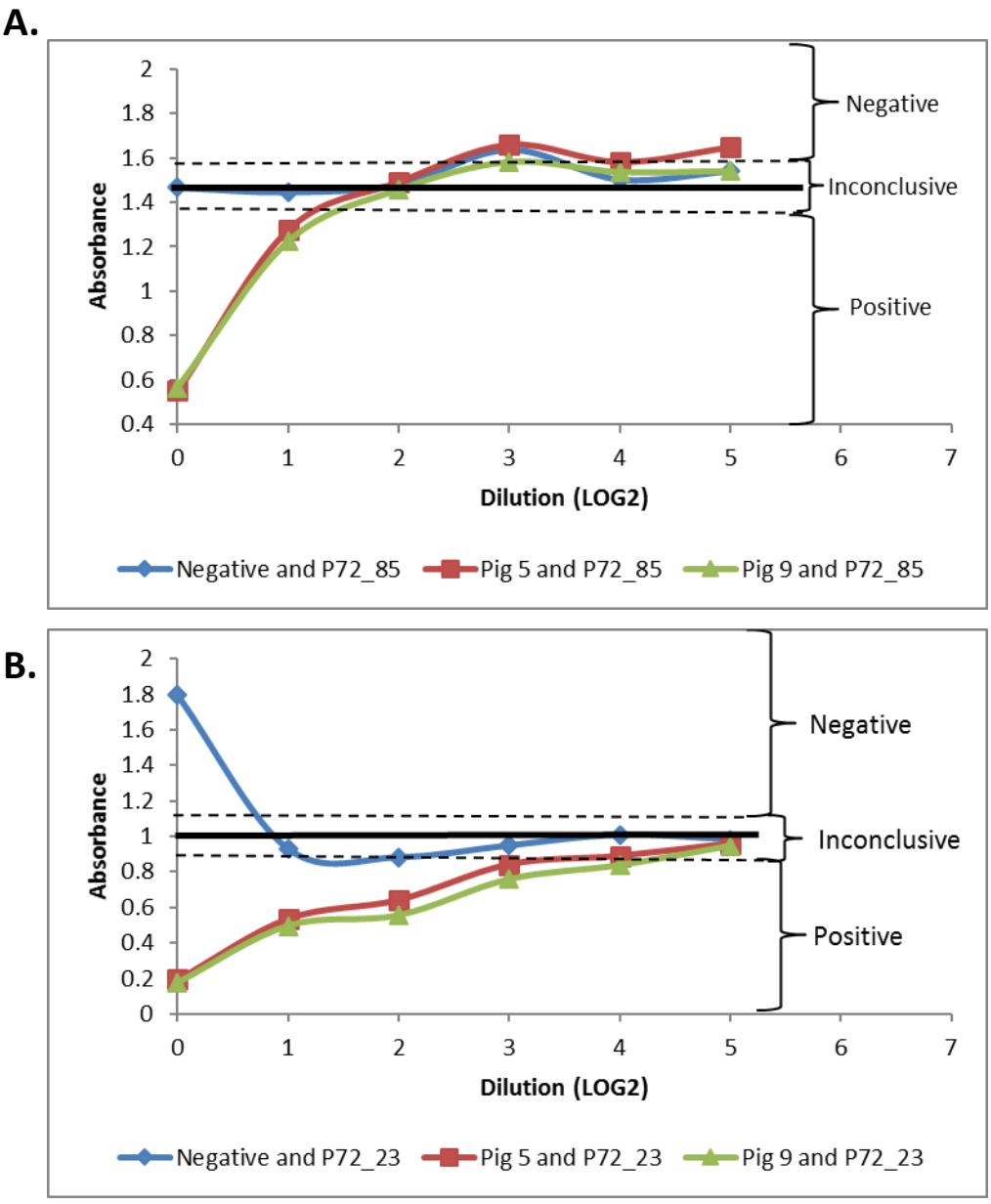

Figure 3.12 Blocking ELISA with indirect detection: A. Monoclonal antibody 85 reactivity against two positive serum samples, Pig #5 and Pig #9, and a negative control serum. **B.** Monoclonal antibody 23 against two positive serum samples, Pig #5 and Pig #9, and a negative control serum. Negative control serum reactivity is in blue, Pig #5 reactivity is in red, and Pig #9 reactivity is shown in green on both graphs. Positive and negative cutoffs were determined by the addition or subtraction of two standard deviations from the mean of three wells with no serum.

Chapter 4 - Discussion and Conclusion

Since there are no commercially available diagnostic assays for ASFV in the United States and limited information on the immunogenic epitopes on the major capsid protein p72, this project focused on the epitope mapping of p72 for the development of a blocking ELISA.

We were successful in expressing the p72 whole protein and the overlapping polypeptides as p72-ubiquitin fusion proteins. The ubiquitin fused to the proteins served to increase the solubility and expression of the proteins (Catanzariti et al., 2004). Although we used an *E. coli* expression plasmid containing ubiquitin, the p72 whole protein, fragment 1, and fragment 4 remained insoluble. The results of the p72 expression are in accordance with a previous study where the p72 whole protein was expressed in *E. coli* and found to be insoluble (Freije et al., 1993). It was found that 8M urea was needed for the purification of fragment 1 and the p72 whole protein. The insolubility of fragment 1 could possibly be explained by the presence of multiple hydrophobic amino acid residues within the sequence. The formation of insoluble inclusion bodies can be the result of the over-expression of recombinant proteins in *E. coli* (Singh & Panda, 2005). The solublilization and recovery from the inclusion bodies is complex and protein specific but can be accomplished by using a detergent such as Sarkosyl or the strong denaturant urea. The successful use of Sarkosyl for purification of many proteins that form insoluble inclusion bodies has previously been reported (Frankel, Sohn, & Leinwand, 1991; Singh & Panda, 2005). This method was used for the purification of fragment 4.

Through epitope mapping, this study has identified multiple areas of antigenic reactivity on the ASFV major capsid protein, p72. The amino acid alignment comparison between BA71V and Georgia/07 shows only one amino acid difference at position 126 in the first 345 amino acids of p72. The single amino acid difference did not have any effect on the reactivity of the

monoclonal antibodies to p72. This is in accordance with previous comparisons of isolates which demonstrated that ASFV p72 is highly conserved among them (de Villiers et al., 2010; Yu et al., 1996).

Zsak *et al.* (1993) identified a highly conserved neutralizing epitope and Borca *et al.* (1994) determined that this epitope, which is located between amino acid 393 and 442, is conformational. After testing the 27 monoclonal antibodies for the fine mapping of p72, we identified six linear epitopes upstream of the previously identified conformational epitope. These six epitopes were determined to be linear due to their ability to recognize very small epitopes when tested on western blot. Of the 27 monoclonal antibodies tested, five monoclonal antibodies were found to have reactivity with multiple regions on p72. Although the double reactivity of monoclonal antibody 5B10 can be explained by the fact that it was shown to have a mixed isotype, it is unclear why the other monoclonal antibodies reacted to two different regions.

There is a definite need for a reliable diagnostic assay for ASFV. This is largely due to the fact that no commercial vaccine for ASFV is currently available. The rapid serodiagnosis of ASFV could contribute to the complete eradication of the disease in certain regions affected by the virus and could also help prevent spread of the disease into countries that are currently unaffected by it. The use of monoclonal antibodies specific to p72 allows for the development of a sensitive, specific, and reproducible blocking ELISA and helps to ensure the highly specific nature of the test system. Also, because recombinant protein p72 is used in the assay, there is no need to work with live virus which eliminates the requirement for performing the assay in a BSL-3 facility. During the development of a blocking ELISA, five of the 27 monoclonal antibodies tested were selected based on their ability to recognize the native form of the whole protein as well as a conserved area within the sequence. The results of the pilot blocking ELISAs

with the selected anti-p72 monoclonal antibodies and sera from pigs immunized with defective alphavirus particles expressing sHA-p72 show two promising candidates out of the five that were selected, monoclonal antibodies 85 and 23. These monoclonal antibodies will be used for further evaluation using sera from ASFV-infected pigs. However, anti-p72 monoclonal antibody 23 is not a pure monoclonal antibody as it was determined to have a mixed isotype of IgG1-k/λ. This antibody will need to be subcloned prior to being used further in the development of a blocking ELISA.

ASFV is a high-consequence pathogen and therefore, the development of a tool for the rapid and reliable detection of ASFV is needed. This research allows for future development of such an assay as well as for other research endeavors using the antigenic regions of major capsid protein p72.

Chapter 5 - References

Achenbach, J. E., Gallardo, C., Nieto-Pelegrín, E., Rivera-Arroyo, B., Degefa-Negi, T., Arias, M., … Sánchez-Vizcaíno, J. M. (2016). Identification of a New Genotype of African Swine Fever Virus in Domestic Pigs from Ethiopia. *Transboundary and Emerging Diseases*, n/a-n/a. https://doi.org/10.1111/tbed.12511

Agüero, M., Fernández, J., Romero, L., Mascaraque, C. S., Arias, M., & Sánchez-Vizcaíno, J. M. (2003). Highly Sensitive PCR Assay for Routine Diagnosis of African Swine Fever Virus in Clinical Samples. *Journal of Clinical Microbiology*, *41*(9), 4431–4434. https://doi.org/10.1128/JCM.41.9.4431-4434.2003

Alcaraz, C., Diego, M. D., Pastor, M. J., & Escribano, J. M. (1990). Comparison of a Radioimmunoprecipitation Assay to Immunoblotting and ELISA for Detection of Antibody to African Swine Fever Virus. *Journal of Veterinary Diagnostic Investigation*, *2*(3), 191–196. https://doi.org/10.1177/104063879000200307

Alonso, C. (2013). African swine fever virus conquers new territories. *Virus Research*, *173*(1), 1–2. https://doi.org/10.1016/j.virusres.2013.01.019

Amass, S. F., Ragland, D., & Spicer, P. (2001). Evaluation of the efficacy of a peroxygen compound, Virkon S, as a boot bath disinfectant, *9*(3), 121–123.

Anderson, E. C., Hutchings, G. H., Mukarati, N., & Wilkinson, P. J. (1998). African swine fever virus infection of the bushpig (Potamochoerus porcus) and its significance in the epidemiology of the disease. *Veterinary Microbiology*, *62*(1), 1–15.

Andrés, G., Alejo, A., Salas, J., & Salas, M. L. (2002). African Swine Fever Virus Polyproteins pp220 and pp62 Assemble into the Core Shell. *Journal of Virology*, *76*(24), 12473–12482. https://doi.org/10.1128/JVI.76.24.12473-12482.2002

Arzt, J., White, W. R., Thomsen, B. V., & Brown, C. C. (2010). Agricultural Diseases on the Move Early in the Third Millennium. *Veterinary Pathology Online*, *47*(1), 15–27. https://doi.org/10.1177/0300985809354350

Atuhaire, D. K., Afayoa, M., Ochwo, S., Mwesigwa, S., Mwiine, F. N., Okuni, J. B., … Ojok, L. (2013). Prevalence of African swine fever virus in apparently healthy domestic pigs in Uganda. *BMC Veterinary Research*, *9*, 263. https://doi.org/10.1186/1746-6148-9-263

Atuhaire, D. K., Afayoa, M., Ochwo, S., Mwesigwa, S., Okuni, J. B., Olaho-Mukani, W., & Ojok, L. (2013). Molecular characterization and phylogenetic study of African swine fever virus isolates from recent outbreaks in Uganda (2010–2013). *Virology Journal*, *10*, 247. https://doi.org/10.1186/1743-422X-10-247

Barderas, M. G., Rodríguez, F., Gómez-Puertas, P., Avilés, M., Beitia, F., Alonso, C., & Escribano, J. M. (2001). Antigenic and immunogenic properties of a chimera of two immunodominant African swine fever virus proteins. *Archives of Virology*, *146*(9), 1681–1691.

Bastos, A. D. S., Penrith, M.-L., Crucière, C., Edrich, J. L., Hutchings, G., Roger, F., … R Thomson, G. (2003). Genotyping field strains of African swine fever virus by partial p72 gene characterisation. *Archives of Virology*, *148*(4), 693–706. https://doi.org/10.1007/s00705-002-0946-8

Blome, S., Gabriel, C., & Beer, M. (2013). Pathogenesis of African swine fever in domestic pigs and European wild boar. *Virus Research*, *173*(1), 122–130. https://doi.org/10.1016/j.virusres.2012.10.026

Borca, M. V., Irusta, P., Carrillo, C., Afonso, C. L., Burrage, T., & Rock, D. L. (1994). African swine fever virus structural protein p72 contains a conformational neutralizing epitope. *Virology*, *201*(2), 413–418. https://doi.org/10.1006/viro.1994.1311

Burrage, T. G. (2013). African swine fever virus infection in Ornithodoros ticks. *Virus Research*, *173*(1), 131–139. https://doi.org/10.1016/j.virusres.2012.10.010

Carillo, C. (2014). Organisms of concern but not foodborne or confirmed foodborne: African Swine Fever Virus. In *Encyclopedia of Food Safety* (Vol. 2, pp. 241–244). Academic Press.

Catanzariti, A.-M., Soboleva, T. A., Jans, D. A., Board, P. G., & Baker, R. T. (2004). An efficient system for high-level expression and easy purification of authentic recombinant proteins. *Protein Science : A Publication of the Protein Society*, *13*(5), 1331–1339. https://doi.org/10.1110/ps.04618904

Center for Food Security and Public Health, College of Veterinary Medicine, Iowa State University. (2015). African Swine Fever. Retrieved from http://www.cfsph.iastate.edu/Factsheets/pdfs/african_swine_fever.pdf

Chen, Y., Zhao, Q., Liu, B., Wang, L., Sun, Y., Li, H., … Zhou, E.-M. (2016). A Novel Blocking ELISA for Detection of Antibodies against Hepatitis E Virus in Domestic Pigs. *PloS One*, *11*(3), e0152639. https://doi.org/10.1371/journal.pone.0152639

Cobbold, C., Windsor, M., & Wileman, T. (2001). A virally encoded chaperone specialized for folding of the major capsid protein of African swine fever virus. *Journal of Virology*, *75*(16), 7221–7229. https://doi.org/10.1128/JVI.75.16.7221-7229.2001

Colson, P., De Lamballerie, X., Yutin, N., Asgari, S., Bigot, Y., Bideshi, D. K., … Raoult, D. (2013). “Megavirales”, a proposed new order for eukaryotic nucleocytoplasmic large

DNA viruses. *Archives of Virology*, *158*(12), 2517–2521. https://doi.org/10.1007/s00705-013-1768-6

Correia, S., Ventura, S., & Parkhouse, R. M. (2013). Identification and utility of innate immune system evasion mechanisms of ASFV. *Virus Research*, *173*(1), 87–100. https://doi.org/10.1016/j.virusres.2012.10.013

Costard, S., Mur, L., Lubroth, J., Sanchez-Vizcaino, J. M., & Pfeiffer, D. U. (2013). Epidemiology of African swine fever virus. *Virus Research*, *173*(1), 191–197. https://doi.org/10.1016/j.virusres.2012.10.030

Costard, S., Wieland, B., de Glanville, W., Jori, F., Rowlands, R., Vosloo, W., … Dixon, L. K. (2009). African swine fever: how can global spread be prevented? *Philosophical Transactions of the Royal Society B: Biological Sciences*, *364*(1530), 2683–2696. https://doi.org/10.1098/rstb.2009.0098

Crawford, L., Yu, Z.-J., Keegan, E., & Yu, T. (2000, November 1). A Comparison of Commonly Used Surface Disinfectants. Retrieved September 22, 2016, from http://www.infectioncontroltoday.com/articles/2000/11/a-comparison-of-commonly-used-surface-disinfectan.aspx

Crowther, J. R., Wardley, R. C., & Wilkinson, P. J. (1979). Solid-phase radioimmunoassay techniques for the detection of African swine fever antigen and antibody. *The Journal of Hygiene*, *83*(2), 353–361.

Cubillos, C., Gómez-Sebastian, S., Moreno, N., Nuñez, M. C., Mulumba-Mfumu, L. K., Quembo, C. J., … Blanco, E. (2013). African swine fever virus serodiagnosis: a general review with a focus on the analyses of African serum samples. *Virus Research*, *173*(1), 159–167. https://doi.org/10.1016/j.virusres.2012.10.021

de Carvalho Ferreira, H. C., Weesendorp, E., Quak, S., Stegeman, J. A., & Loeffen, W. L. A. (2013). Quantification of airborne African swine fever virus after experimental infection. *Veterinary Microbiology*, *165*(3–4), 243–251. https://doi.org/10.1016/j.vetmic.2013.03.007

de Villiers, E. P., Gallardo, C., Arias, M., da Silva, M., Upton, C., Martin, R., & Bishop, R. P. (2010). Phylogenomic analysis of 11 complete African swine fever virus genome sequences. *Virology*, *400*(1), 128–136. https://doi.org/10.1016/j.virol.2010.01.019

Dixon, L. K., Escribano, J. M., Martins, C., Rock, D. L., Salas, M. L., & Wilkinson, P. J. (2005). Asfarviridae. In C. Fauquet, M. A. Mayo, J. Maniloff, U. Desselberger, & L. A. Ball (Eds.), *Virus Taxonomy: VIIIth Report of the ICTV* (1st ed., pp. 135–143). London, UK: Elsevier Academic Press. Retrieved from https://www.elsevier.com/books/virus-taxonomy/fauquet/978-0-12-249951-7

Dixon, Chapman, D. A. G., Netherton, C. L., & Upton, C. (2013). African swine fever virus replication and genomics. *Virus Research*, *173*(1), 3–14. https://doi.org/10.1016/j.virusres.2012.10.020

Epifano, C., Krijnse-Locker, J., Salas, M. L., Rodríguez, J. M., & Salas, J. (2006). The African swine fever virus nonstructural protein pB602L is required for formation of the icosahedral capsid of the virus particle. *Journal of Virology*, *80*(24), 12260–12270. https://doi.org/10.1128/JVI.01323-06

Escribano, J. M., Galindo, I., & Alonso, C. (2013). Antibody-mediated neutralization of African swine fever virus: myths and facts. *Virus Research*, *173*(1), 101–109. https://doi.org/10.1016/j.virusres.2012.10.012

Food & Agriculture Organisation of the United Nations. (n.d.). AHP Disease Manual. In G. Garner, P. Saville, & A. Fediavsky (Eds.), *African Swine Fever*. http://lrd.spc.int/ext/Disease_Manual_Final/a120__african_swine_fever.html.

Frankel, S., Sohn, R., & Leinwand, L. (1991). The use of sarkosyl in generating soluble protein after bacterial expression. *Proceedings of the National Academy of Sciences of the United States of America*, *88*(4), 1192–1196.

Freije, J. M., Muñoz, M., Viñuela, E., & López-Otín, C. (1993). High-level expression in Escherichia coli of the gene coding for the major structural protein (p72) of African swine fever virus. *Gene*, *123*(2), 259–262.

Galindo-Cardiel, I., Ballester, M., Solanes, D., Nofrarías, M., López-Soria, S., Argilaguet, J. M., … Segalés, J. (2013). Standardization of pathological investigations in the framework of experimental ASFV infections. *Virus Research*, *173*(1), 180–190. https://doi.org/10.1016/j.virusres.2012.12.018

Gallardo, C., Anchuelo, R., Pelayo, V., Poudevigne, F., Leon, T., Nzoussi, J., … Arias, M. (2011). African Swine Fever Virus p72 Genotype IX in Domestic Pigs, Congo, 2009. *Emerging Infectious Diseases*, *17*(8), 1556–1558. https://doi.org/10.3201/eid1708.101877

Gallardo, C., Mwaengo, D. M., Macharia, J. M., Arias, M., Taracha, E. A., Soler, A., … Bishop, R. P. (2009). Enhanced discrimination of African swine fever virus isolates through nucleotide sequencing of the p54, p72, and pB602L (CVR) genes. *Virus Genes*, *38*(1), 85–95. https://doi.org/10.1007/s11262-008-0293-2

Gallardo, C., Nieto, R., Soler, A., Pelayo, V., Fernández-Pinero, J., Markowska-Daniel, I., … 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. *Journal of Clinical Microbiology*, *53*(8), 2555–2565. https://doi.org/10.1128/JCM.00857-15

Gallardo, C., Reis, A. L., Kalema-Zikusoka, G., Malta, J., Soler, A., Blanco, E., … Leitão, A. (2009). Recombinant Antigen Targets for Serodiagnosis of African Swine Fever. *Clinical and Vaccine Immunology : CVI*, *16*(7), 1012–1020. https://doi.org/10.1128/CVI.00408-08

Gershoni, J. M., Roitburd-Berman, A., Siman-Tov, D. D., Freund, N. T., & Weiss, Y. (2012). Epitope Mapping. *BioDrugs*, *21*(3), 145–156. https://doi.org/10.2165/00063030-200721030-00002

Gómez-Puertas, P., Rodríguez, F., Oviedo, J. M., Brun, A., Alonso, C., & Escribano, J. M. (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*(2), 461–471. https://doi.org/10.1006/viro.1998.9068

Gómez-Puertas, P., Rodríguez, F., Oviedo, J. M., Ramiro-Ibáñez, F., Ruiz-Gonzalvo, F., Alonso, C., & Escribano, J. M. (1996). Neutralizing antibodies to different proteins of African swine fever virus inhibit both virus attachment and internalization. *Journal of Virology*, *70*(8), 5689–5694.

Gómez-Villamandos, J. C., Bautista, M. J., Sánchez-Cordón, P. J., & Carrasco, L. (2013). Pathology of African swine fever: the role of monocyte-macrophage. *Virus Research*, *173*(1), 140–149. https://doi.org/10.1016/j.virusres.2013.01.017

Hasan, N. H., Ebrahimie, E., Ignjatovic, J., Tarigan, S., Peaston, A., & Hemmatzadeh, F. (2016). Epitope Mapping of Avian Influenza M2e Protein: Different Species Recognise Various Epitopes. *PLoS ONE*, *11*(6). https://doi.org/10.1371/journal.pone.0156418

Henriques, A. M., Fagulha, T., Barros, S. C., Ramos, F., Duarte, M., Luís, T., & Fevereiro, M. (2016). Development and validation of a blocking ELISA test for the detection of avian influenza antibodies in poultry species. *Journal of Virological Methods*, *236*, 47–53. https://doi.org/10.1016/j.jviromet.2016.07.006

Herńndez, A., Martró, E., Matas, L., Martín, M., & Ausina, V. (2000). Assessment of in-vitro efficacy of 1% Virkon against bacteria, fungi, viruses and spores by means of AFNOR guidelines. *The Journal of Hospital Infection*, *46*(3), 203–209. https://doi.org/10.1053/jhin.2000.0818

Hess, W. R., Endris, R. G., Lousa, A., & Caiado, J. M. (1989). Clearance of African swine fever virus from infected tick (Acari) colonies. *Journal of Medical Entomology*, *26*(4), 314–317.

Heuzenroeder, M. W., Barton, M. D., Vanniasinkam, T., & Phumoonna, T. (2009). Linear B-Cell Epitope Mapping using Enzyme-Linked Immunosorbent Assay for Libraries of Overlapping Synthetic Peptides. In *Epitope Mapping Protocols* (2nd ed., pp. 137–144). Humana Press.

Hutchings, G. H., & Ferris, N. P. (2006). Indirect sandwich ELISA for antigen detection of African swine fever virus: comparison of polyclonal and monoclonal antibodies. *Journal of Virological Methods*, *131*(2), 213–217. https://doi.org/10.1016/j.jviromet.2005.08.009

James, H. E., Ebert, K., McGonigle, R., Reid, S. M., Boonham, N., Tomlinson, J. A., … King, D. P. (2010). Detection of African swine fever virus by loop-mediated isothermal

amplification. *Journal of Virological Methods*, *164*(1–2), 68–74. https://doi.org/10.1016/j.jviromet.2009.11.034

Jori, F., & Bastos, A. D. S. (2009). Role of Wild Suids in the Epidemiology of African Swine Fever. *EcoHealth*, *6*(2), 296–310. https://doi.org/10.1007/s10393-009-0248-7

King, D. P., Reid, S. M., Hutchings, G. H., Grierson, S. S., Wilkinson, P. J., Dixon, L. K., … Drew, T. W. (2003). Development of a TaqMan® PCR assay with internal amplification control for the detection of African swine fever virus. *Journal of Virological Methods*, *107*(1), 53–61. https://doi.org/10.1016/S0166-0934(02)00189-1

King, K., Chapman, D., Argilaguet, J. M., Fishbourne, E., Hutet, E., Cariolet, R., … Takamatsu, H.-H. (2011). Protection of European domestic pigs from virulent African isolates of African swine fever virus by experimental immunisation. *Vaccine*, *29*(28), 4593–4600. https://doi.org/10.1016/j.vaccine.2011.04.052

Kleiboeker, S. B. (2008). African Swine Fever. In *Foreign Animal Diseases* (7th ed., pp. 111–116). United States Animal Health Association.

Kleiboeker, S. B., & Scoles, G. A. (2001). Pathogenesis of African swine fever virus in Ornithodoros ticks. *Animal Health Research Reviews / Conference of Research Workers in Animal Diseases*, *2*(2), 121–128.

Kollnberger, S. D., Gutierrez-Castañeda, B., Foster-Cuevas, M., Corteyn, A., & Parkhouse, R. M. E. (2002). Identification of the principal serological immunodeterminants of African swine fever virus by screening a virus cDNA library with antibody. *The Journal of General Virology*, *83*(Pt 6), 1331–1342. https://doi.org/10.1099/0022-1317-83-6-1331

Krug, P. W., Larson, C. R., Eslami, A. C., & Rodriguez, L. L. (2012). Disinfection of foot-and-mouth disease and African swine fever viruses with citric acid and sodium hypochlorite

on birch wood carriers. *Veterinary Microbiology*, *156*(1–2), 96–101. https://doi.org/10.1016/j.vetmic.2011.10.032

Krug, P. W., Lee, L. J., Eslami, A. C., Larson, C. R., & Rodriguez, L. (2011). Chemical disinfection of high-consequence transboundary animal disease viruses on nonporous surfaces. *Biologicals*, *39*(4), 231–235. https://doi.org/10.1016/j.biologicals.2011.06.016

Lacasta, A., Monteagudo, P. L., Jiménez-Marín, Á., Accensi, F., Ballester, M., Argilaguet, J., … Rodríguez, F. (2015). Live attenuated African swine fever viruses as ideal tools to dissect the mechanisms involved in viral pathogenesis and immune protection. *Veterinary Research*, *46*. https://doi.org/10.1186/s13567-015-0275-z

Liu, D. (2014). *Manual of Security Sensitive Microbes and Toxins*. CRC Press.

López-Otín, C., Freije, J. M., Parra, F., Méndez, E., & Viñuela, E. (1990). Mapping and sequence of the gene coding for protein p72, the major capsid protein of African swine fever virus. *Virology*, *175*(2), 477–484.

Lundstrom, K. (2014). Alphavirus-Based Vaccines. *Viruses*, *6*(6), 2392–2415. https://doi.org/10.3390/v6062392

Mao, S.-Y., Javois, L. C., & Kent, U. M. (1994). Overview of Antibody Use in Immunocytochemistry. In *Immunocytochemical Methods and Protocols* (2nd ed., Vol. 115, p. 3). Totowa, NJ: Humana Press. Retrieved from http://laboratorium.dp.ua/books/Immunocytochemical-Methods-and-Protocols-2nd.pdf

Martínez-López, B., Perez, A. M., Feliziani, F., Rolesu, S., Mur, L., & Sánchez-Vizcaíno, J. M. (2015). Evaluation of the risk factors contributing to the African swine fever occurrence in Sardinia, Italy. *Frontiers in Microbiology*, *6*. https://doi.org/10.3389/fmicb.2015.00314

Mellor, P. S., & Wilkinson, P. J. (1985). Experimental transmission of African swine fever virus by Ornithodoros savignyi (Audouin). *Research in Veterinary Science*, *39*(3), 353–356.

Mokoele, J. M., Janse van Rensburg, L., van Lochem, S., Bodenstein, H., du Plessis, J., Carrington, C. A. P., … Fasina, F. O. (2015). Overview of the perceived risk of transboundary pig diseases in South Africa. *Journal of the South African Veterinary Association*, *86*(1), 1197.

Montgomery, R. E. (1921). On A Form of Swine Fever Occurring in British East Africa (Kenya Colony). *Journal of Comparative Pathology and Therapeutics*, *34*, 159–191. https://doi.org/10.1016/S0368-1742(21)80031-4

Muñoz-Moreno, R., Galindo, I., Cuesta-Geijo, M. Á., Barrado-Gil, L., & Alonso, C. (2015). Host cell targets for African swine fever virus. *Virus Research*, *209*, 118–127. https://doi.org/10.1016/j.virusres.2015.05.026

Neilan, J. G., Zsak, L., Lu, Z., Burrage, T. G., Kutish, G. F., & Rock, D. L. (2004). Neutralizing antibodies to African swine fever virus proteins p30, p54, and p72 are not sufficient for antibody-mediated protection. *Virology*, *319*(2), 337–342. https://doi.org/10.1016/j.virol.2003.11.011

Oganesyan, A. S., Petrova, O. N., Korennoy, F. I., Bardina, N. S., Gogin, A. E., & Dudnikov, S. A. (2013). African swine fever in the Russian Federation: spatio-temporal analysis and epidemiological overview. *Virus Research*, *173*(1), 204–211. https://doi.org/10.1016/j.virusres.2012.12.009

OIE. (2012). African Swine Fever. In *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (7th ed., pp. 1067–1079).

OIE. (2013). World Animal Health Information Database (WAHIS Interface). Disease Information Database. Retrieved September 16, 2016, from http://www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/Diseasedistributionmap

Oura, C. A. L., Denyer, M. S., Takamatsu, H., & Parkhouse, R. M. E. (2005). In vivo depletion of CD8+ T lymphocytes abrogates protective immunity to African swine fever virus. *Journal of General Virology*, *86*(9), 2445–2450. https://doi.org/10.1099/vir.0.81038-0

Oura, C. A. L., Edwards, L., & Batten, C. A. (2013). Virological diagnosis of African swine fever--comparative study of available tests. *Virus Research*, *173*(1), 150–158. https://doi.org/10.1016/j.virusres.2012.10.022

Oura, C. A., Powell, P. P., Anderson, E., & Parkhouse, R. M. (1998). The pathogenesis of African swine fever in the resistant bushpig. *Journal of General Virology*, *79*(6), 1439–1443. https://doi.org/10.1099/0022-1317-79-6-1439

Penrith, M.-L., Vosloo, W., Jori, F., & Bastos, A. D. S. (2013). African swine fever virus eradication in Africa. *Virus Research*, *173*(1), 228–246. https://doi.org/10.1016/j.virusres.2012.10.011

Perez, T., Gallardo, C., Nieto, R., De Mia, G., Vela, C., Bishop, R., … Sanz, A. (2011). Development and preliminary validation of a pen-side test based on the use of vp72 protein for ASFV antibody detection, 206.

Pitcher, L. J., Dixon, L. K., & Turner, P. C. (1992). A sequence comparison of the VP72 gene of African swine fever virus. *Biochemical Society Transactions*, *20*(1), 30S.

Plowright, W., Parker, J., & Peirce, M. A. (1969). African swine fever virus in ticks (Ornithodoros moubata, murray) collected from animal burrows in Tanzania. *Nature*, *221*(5185), 1071–1073.

Plowright, W., Perry, C. T., & Greig, A. (1974). Sexual transmission of African swine fever virus in the tick, Ornithodoros moubata porcinus, Walton. *Research in Veterinary Science*, *17*(1), 106–113.

Rayner, J. O., Dryga, S. A., & Kamrud, K. I. (2002). Alphavirus vectors and vaccination. *Reviews in Medical Virology*, *12*(5), 279–296. https://doi.org/10.1002/rmv.360

Rennie, L., Wilkinson, P. J., & Mellor, P. S. (2001). Transovarial transmission of African swine fever virus in the argasid tick Ornithodoros moubata. *Medical and Veterinary Entomology*, *15*(2), 140–146. https://doi.org/10.1046/j.1365-2915.2001.00282.x

Rodríguez, J. M., & Salas, M. L. (2013). African swine fever virus transcription. *Virus Research*, *173*(1), 15–28. https://doi.org/10.1016/j.virusres.2012.09.014

Salas, M. L., & Andrés, G. (2013). African swine fever virus morphogenesis. *Virus Research*, *173*(1), 29–41. https://doi.org/10.1016/j.virusres.2012.09.016

Sanchez-Vizcaino, J.M., & Neira, M.A. (2012). African Swine Fever Virus. In *Diseases of Swine*. John Wiley & Sons.

Scott, G. R. (1965). Prevention, control and eradication of African swine fever. *Bulletin - Office International Des Épizooties*, *63*(5), 751–764.

Severs, Y. D., & Lamontagne, M.-C. (2002). A literature review of disinfectants: Effects when used by CF divers in cleaning rebreather sets. Defence R&D Canada-Toronto.

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. *The Journal of Veterinary Medical Science / the Japanese Society of Veterinary Science*, *62*(1), 85–92.

Singh, S. M., & Panda, A. K. (2005). Solubilization and refolding of bacterial inclusion body proteins. *Journal of Bioscience and Bioengineering*, *99*(4), 303–310. https://doi.org/10.1263/jbb.99.303

Sundberg, E. J. (2009). Structural Basis of Antibody-Antigen Interactions. In *Epitope Mapping Protocols* (2nd ed., pp. 23–36). Humana Press.

Tabares, E., Fernandez, M., Salvador-Temprano, E., Carnero, M. E., & Sanchez-Botija, C. (1981). A reliable enzyme linked immunosorbent assay for African swine fever using the major structural protein as antigenic reagent. *Archives of Virology*, *70*(3), 297–300. https://doi.org/10.1007/BF01315136

Takamatsu, H.-H., Denyer, M. S., Lacasta, A., Stirling, C. M. A., Argilaguet, J. M., Netherton, C. L., … Rodríguez, F. (2013). Cellular immunity in ASFV responses. *Virus Research*, *173*(1), 110–121. https://doi.org/10.1016/j.virusres.2012.11.009

Thomson, G. R. (1985). The epidemiology of African swine fever: the role of free-living hosts in Africa. *The Onderstepoort Journal of Veterinary Research*, *52*(3), 201–209.

United States Animal and Plant Health Inspection Service. (1998). *African swine fever :a foreign threat to U.S. hogs.* [Washington, D.C.?] : U.S. Dept. of Agricutlure, Animal and Plant Health Inspection Service. Retrieved from http://hdl.handle.net/2027/umn.31951d01665759r

Van Regenmortel, M. H. V. (2009). What is a B-Cell Epitope? In *Epitope Mapping Protocols* (2nd ed., pp. 3–20). Humana Press.

Vidal, M. I., Stiene, M., Henkel, J., Bilitewski, U., Costa, J. V., & Oliva, A. G. (1997). A solid-phase enzyme linked immunosorbent assay using monoclonal antibodies, for the detection of African swine fever virus antigens and antibodies. *Journal of Virological Methods*, *66*(2), 211–218.

Wieringa-Jelsma, T., Wijnker, J. J., Zijlstra-Willems, E. M., Dekker, A., Stockhofe-Zurwieden, N., Maas, R., & Wisselink, H. J. (2011). Virus inactivation by salt (NaCl) and phosphate supplemented salt in a 3D collagen matrix model for natural sausage casings. *International Journal of Food Microbiology*, *148*(2), 128–134. https://doi.org/10.1016/j.ijfoodmicro.2011.05.010

Yang, J., Dai, X., Chen, H., Teng, Q., Li, X., Rong, G., … Li, Z. (2016). Development of blocking ELISA for detection of antibodies against H9N2 avian influenza viruses. *Journal of Virological Methods*, *229*, 40–47. https://doi.org/10.1016/j.jviromet.2015.12.011

Yu, M., Morrissy, C. J., & Westbury, H. A. (1996). Strong sequence conservation of African swine fever virus p72 protein provides the molecular basis for its antigenic stability. *Archives of Virology*, *141*(9), 1795–1802.

Zsak, L., Onisk, D. V., Afonso, C. L., & Rock, D. L. (1993). Virulent African swine fever virus isolates are neutralized by swine immune serum and by monoclonal antibodies recognizing a 72-kDa viral protein. *Virology*, *196*(2), 596–602. https://doi.org/10.1006/viro.1993.1515