Evaluation of cross protection by an attenuated African swine fever virus isolate against heterologous challenge

By

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"For the love of science"

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Abbreviations

AMC	Alveolar Macrophage Culture
ARC	Agricultural Research Council
ASF	African swine fever
ASFV	African swine fever virus
BC	Blood Macrophage Culture
BDK	Iron dextran 10%
BMC	Bone Marrow Macrophage Culture
CSF	Classical swine fever
CSFV	Classical swine fever virus
CVR	Central variable region
DIC	Disseminated intravascular coagulation
DL	Detection limit
Dpc	Days post challenge
Dpi	Days post inoculation
Dpv	Days post vaccination
EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme Lynked Immunosorbent Assay
FMD	Foot and mouth disease
g	relative centrifugal force (rcf)

GH	Gastro-hepatic
HAD	Haemadsorption
HAD_{50}	Median tissue culture haemadsorption ($TCID_{50}$)
HLD	Hold
HPLC	High Performance Liquid Chromatography
Ig	Immunoglobulin
IL	Interleukin
IM	Intramuscular
i.u.	International units
INF	Interferon
Ln(n)	Lymph node(s)
Med.	Mediastinal
Mes.	Mesenteric
ml	Millilitre
NSPCA	National Society for the Prevention of Cruelty to Animals
OD	Optical density
OIE	Office International des epizooties (World Organization for Animal Health)
ORF	Open reading frame
OVI	Onderstepoort Veterinary Institute
p72	protein 72

PCR	Polymerase Chain Reaction
PPA	PCR directed to vp72 coding region of ASFV genome
PRRS	Porcine reproductive and respiratory syndrome
rpm	rotations per minute
RT-PCR	real time PCR directed to vp72 coding region of ASFV genome
TAD	Transboundary animal disease
TADP	Transboundary animal diseases programme
$TCID_{50}$	Median tissue culture infective dose (HAD_{50})
TNF	Tumour necrosis factor
UV	Ultraviolet
VP	Virus particle
vp	viral protein
w/v	weight/volume
%	Percentage

Thesis Summary

African Swine Fever Virus (ASFV) is an *Asfivirus* and is the only member of the family *Asfarviridae*. It manifests as a disease that varies from acute to sub-acute or chronic forms. A true carrier state in domestic pigs is unknown but chronically affected individuals may carry and spread the virus for extended periods. African Swine Fever (ASF) is a socio-economically important disease characterized by high morbidity and mortality affecting the livelihood of many small to big scale farmers and seriously compromising international trade. Strategic measures to control this disease are by physical containment and culling in outbreak situations. There is no vaccine available. Nevertheless, every pork producer should ideally be actively involved in having biosecurity measures in place to avoid contamination and contacting their veterinary services in case of suspicion of ASF to have appropriate samples analysed. Official veterinary services must be equipped with proper diagnostic tools in order to provide a quick response.

The sensitivity of currently available diagnostic tests at the Transboundary Animal Diseases Programme, Onderstepoort Veterinary Institute was analysed in order to report the best technique available. Sensitivity to ASF virus infection and therefore diagnostic potential of cell primary cultures as bone marrow macrophages, blood macrophages and alveolar macrophages was done via comparison of titre results from inoculations of ASFV SPEC 257 as control, and ASFV MOZ 1/98. In addition, molecular detection of specific DNA fragments within the viral genome were compared using five different PCRs. Bone marrow macrophage cultures and blood macrophage cultures were the most reliable cells whereas alveolar macrophages more often showed contamination. Results show that PPA PCR and real time PCR detected the highest diluted samples, thus the lowest concentration of virus, in both trials done with ASFV MOZ 1/98 and ASFV SPEC 257.

In addition, animal trials were performed by inoculating domestic pigs with four different ASFV isolates of varying pathogenicity. These viruses were all from distinct geographic origins. Non-virulent ASFV OURT 3/88 and high virulent ASFV BENIN 1/97 were previously described and used as reference viruses. ASFV MOZ 1/98, suspected of having high virulence and ASFV MKUZE, which was thought to

be of low virulence were included in this study to provide further information on the pathological and clinical outcome of the disease as well as measuring viral replication in various organs and blood. The study showed that ASFV MKUZE was of intermediate virulence, whilst ASFV MOZ 1/98 was highly virulent with a high mortality rate. Results confirmed the inadequacy of ASFV MKUZE to act as vaccine opposed to ASFV OURT 3/88.

Following this, a potential vaccine by use of attenuated Portuguese ASFV OURT 3/88 tested against virulent heterologous challenge with a strain now known with certainty to cause acute ASF, the isolate ASFV MOZ 1/98 collected from a diseased pig in Mozambique. Domestic commercial pigs where submitted to either one or two vaccinations before challenge. Viral load in blood and tissue samples was higher in unvaccinated animals and higher in single vaccinated than in pigs vaccinated twice. However, acute ASF afflicted all groups with severe clinical signs and post-mortem lesions. Although it did not confer total immunity it was determined that pigs vaccinated with European attenuated ASFV OURT 3/88 acquired partial protection against challenge with virulent southern Africa ASFV MOZ 1/98.

by

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Aims of the study

The aims of this study were to compare the diagnostic sensitivity of three different primary cell lines for ASF virus isolation and five PCR protocols. Furthermore, domestic pigs were infected in order to access the pathogenic effects caused by inoculations of an isolate from Mozambique, ASFV MOZ 1/98, and another from South Africa, ASFV MKUZE. Comparisons with standard non-virulent ASFV OURT 3/88 and a highly virulent isolate ASFV BENIN 1/97 served as control. Finally, a second animal trial was performed to evaluate the ability of fully attenuated European strain ASFV OURT 3/88, to protect domestic pigs against challenge with a highly virulent heterologous African strain, ASFV MOZ 1/98.

Chapter 1

Literature review

1.1. Introduction

African swine fever (ASF) is an often-fatal disease of domestic pigs caused by the African swine fever virus (ASFV), which is classified in the genus *Asfivirus* and as the only member of the family *Asfarviridae*. ASFV is a large icosahedral shaped particle and its genome is a double-stranded DNA molecule ranging in size from 170 to 190 kbp (Dixon *et al.*, 2004). DNA sequence data confirm that ASFV shares characteristics with the *Poxviridae* and *Iridoviridae* families of viruses (Stasiak *et al.*, 2003).

This disease may manifest in different forms according to the virulence of the isolate in question. The virulent form is characterized by a haemorrhagic outcome, high infectiousness and accountable for rapid losses at every level of pig production as it causes a great number of deaths in a short time. Moderately virulent isolates are responsible for less severe clinical signs and a longer course of disease therefore tending towards subacute to chronic ASF classification. Non-virulent strains of ASFV, however, do not cause any signs of disease except for induction of antibodies.

Furthermore, natural hosts harbour ASFV asymptomatically in a sylvatic cycle (King *et al*., 2011). Wild suids and soft ticks may carry the virus, and are often located near areas where small-scale pig farmers are established. Subsistence farming methods where pigs forage in the bush generate an imminent risk of introduction of new isolates in farming sites where animals are prone to developing disease thus replicating infection to other producers by indirect or direct contact. This is especially true for the majority of enzootic countries where there is constant circulation of the virus in the surrounds of domestic pig locations (reviewed by Penrith *et al*., 2004a).

ASF is a transboundary animal disease (TAD) and it is recognized as top priority by the Food and Agricultural Organization (FAO) and World Organization for Animal Health (OIE), along with diseases like foot-and-mouth disease, Rift Valley fever, classical swine fever, rinderpest, peste des petits ruminants, contagious bovine pleuropneumonia and the highly pathogenic avian influenza. TADs are a serious risk to the world's livestock production and food security whilst compromising international trade. Thus, in the past decades, public health authorities in industrialized countries faced an increasing number of food safety issues especially as world trade has been motivating the globalization of food and feed traffic that unfortunately presents new risks. The disease is a severe impediment in developing countries. Although pig production in Africa comprises less than one per cent of the world's pork, it is of inestimable importance at local level in many countries. This is especially true where forested regions predominate since cattle generally do not thrive in a free ranging system there. Pork is one of the major sources of animal protein due to their efficient conversion of low-grade nutritional sources into high quality protein at a reasonable price. Furthermore, in some areas, pigs are kept for religious and cultural practices and they may serve as monetary reservoir in many cases. Therefore, considering the devastating effects of ASF the disease is a major setback for pork production both for large scale producers as well as for subsistence farmers. The situation is especially serious in sub-Saharan Africa where there is extensive evidence for endemicity of the disease in many countries (reviewed by Penrith *et al.,* 2004a; Domenech *et al.*, 2006; Reis *et al.*, 2007).

1.2. Epidemiology

1.2.1. History and current state of ASF

ASF was first described in Kenya in 1907 as reported by Montgomery (1921), referring to the disease as East African Swine Fever. However, it seems that ASFV has been in Africa long before the first official reports of the disease. Since it does not affect its natural or indigenous hosts it only caused mortalities when Portuguese missionaries and colonists brought domestic breeds of pigs to sub-Saharan Africa about 500 years ago (reviewed by Penrith *et al.*, 2004a).

In South Africa the earliest record of the disease dates back to 1926 (Steyn 1932) which occurred in the northern area of the country in the Limpopo province. In this case, contact between warthogs (*Phacochoerus africanus*) and domestic pigs occurred. In 1933, two outbreaks were reported in the Cape allegedly because infected pigs were imported from the former province of Transvaal. Outbreaks followed until 1939. Numerous cases also happened between 1951 and 1962 and between 1973 and 1975 in the northern parts of the country (Pini & Hurter 1975). Transboundary movement of infected pigs from Namibia to South Africa was responsible for the 1951 outbreaks. The later occurrences of ASF in South Africa are associated with warthog presence. Pigs reared in a free-range system are exposed to the natural host of the virus and have greater risk of becoming infected. Still in southern Africa, ASF was observed for the first time in Angola in 1932 and in Mozambique in 1954 (Mendes 1994; reviewed by Penrith *et al*., 2004a). Subsequently, there were reports from most countries of Central and southern Africa. In 1994, Kenya reported the disease for the first time after an apparent absence of 30 years. In 1997/98 Madagascar reported the presence of the virus in its territory for the first time (reviewed by Penrith *et al*., 2004a). Kenya suffered ten outbreaks between 2006 and 2007.

In 1994, the disease was causing fatalities in pork production in Mozambique but it had never been reported south of the Save river, where it had not been present before. For a while, this area was very important since it was the only economically active region of the country because most of the roads and railway networks were unavailable and criminal attacks constantly occurred. The reason behind the 1994 outbreak in Mozambique south of the Save River was due to the end of the civil war in 1992. As successful negotiations for peace took place, the country slowly rebuilt and connectivity restored. Consequently, ASF expanded to the southern part of the country via a longitudinal road corridor linking the north and south via infected meat, pork products, live animals, or even iatrogenic dissemination (Penrith *et al.*, 2007). The occurrence of ASF in Mozambique is reflected in the biannual reports to OIE on WAHID but outbreaks are no longer reported immediately as ASF is endemic. The continuity of these outbreaks is also indicated in the reports book of the Mozambique national institute for animal research (Instituto de Investigação Animal – IIAM) as recently as 2011 and

diagnosed by with fluorescent antibody test (FAT) on organ samples of suspicious cases.

In West Africa, Senegal first reported the disease to OIE in 1978, although unpublished reports indicate that the disease was already present in southern Senegal and Guinea Bissau as early as 1959. More recently in 1996 an outbreak in Ivory Coast initiated a pandemic in West Africa with subsequent serious outbreaks happening in Benin, Togo and Nigeria in 1997 and Ghana in 1999 (reviewed by Penrith *et al*., 2004a).

ASF reached Europe via Portugal in the late fifties as it quickly established in the Iberian Peninsula. Outbreaks followed in many European countries as well as Cuba, the Dominican Republic, Haiti, and Brazil that are presently free of the disease except for the island of Sardinia in Italy (reviewed by Penrith *et al*., 2004a). The infectiousness of and devastation caused by ASFV were again demonstrated when recent outbreaks occurred in countries never before afflicted such as Georgia, Azerbaijan, Armenia, Mauritius and Russia.

In OIE report (World Animal Health org., 2012), Nigeria is endemic; outbreaks in Tanzania have been resolved but ongoing in Russia, Chad, Central African Republic, and Kenya. In 2009 Cape Verde reported clinical disease and as recent as 2011, clinical disease was also demonstrated in Benin, Burkina Faso, Cameroon, Democratic republic of the Congo and Republic of the Congo, Ghana, Guinea-Bissau, Kenya, Madagascar, Malawi, Mozambique, Nigeria, Russia, Tanzania, Togo and Uganda. In South Africa, a recent infection was detected outside the ASF control zone in 2011.

1.2.2. Maintenance and transmission of the virus

1.2.2.1. Sylvatic cycle

ASFV causes a persistent but asymptomatic infection in its natural hosts, warthogs, bushpigs (*Potamochoerus porcus and Potamochoerus larvatus*), and the soft ticks of the *Ornithodoros moubata* complex (Walker 1933; reviewed by Penrith *et al.*, 2004a). This complex includes *O. porcinus domesticus* and *O. porcinus porcinus* from Africa, found in domestic premises and in large animal

burrows (Walton 1979). These natural hosts act as reservoirs of the virus. Findings also indicated that the viraemic stage of warthogs occurs only in neonatal individuals still confined to their burrows until they are approximately four weeks of age. Circulation of virus in the sylvatic cycle occurs between wild suids at this early age and the *O. porcinus porcinus* soft ticks present in their burrows that temporarily feed on the newborns (reviewed by Penrith *et al*., 2004a). Furthermore, ticks may transmit the virus amongst themselves by three different means: transtadially, transovarially and transexually from male to female. Although *O. porcinus* spend most of their lives in burrows, they may exit the burrow while still attached to the wild suidae increasing the chances of getting close to domestic premises and eventually finding a susceptible domestic pig in order to replicate and disseminate the disease into pork production (Plowright *et al.*, 1969). In the Iberian Peninsula, *O. erraticus*, which inhabited pig shelters, proved a competent vector for ASFV (Sanchez-Botija 1963).

1.2.2.2. Domestic cycle

In southern Africa, the introduction of new virus into commercial domestic pigs occurs from transmission of ASF from its natural reservoirs in the wild. It results primarily through contact between domestic pigs and argasid ticks. The soft-shelled eyeless ticks may be transferred to domestic pig surrounds by adult warthogs during the short while they attach for feeding when wild pigs forage near domestic habitation.

There have been situations that suggest the possibility of resistant domestic pigs being reservoirs of ASFV and maintaining the domestic cycle by becoming a long-term virus supply for new outbreaks. In 1954, in Angola where there was no known association of the domestic swine with warthogs, free-ranging domestic pigs that roamed around the villages acted as a reservoir for the virus. Another example occurred in Tete province in northern Mozambique where it appears that a local breed of domestic pigs may also act as carriers. Serological tests show that these pigs have been infected but never appeared symptomatic. According to evidence, it seems this is not a common event since domestic pigs that are true carriers are unreported so far. Nonetheless, these asymptomatic pigs may infect others until

up to two months after becoming exposed (reviewed by Penrith *et al*., 2004a; Penrith *et al.*, 2007).

Experimental evidence also suggests that other possible options of virus transmission from the sylvatic to the domestic cycle may be from direct contact of bushpigs with domestic pigs (Anderson *et al.*, 1998) and from eating infected warthog tissue. Once ASFV has entered the domestic cycle, it circulates in domestic pigs independently of wild suids or ticks by a few different means. The most common mechanism of maintenance is by direct contact between infected and susceptible animals where virus excreted in the urine, faeces, ocular, and nasal discharge infects new individuals. Virus dissemination by indirect contact is also common, for example transport of pigs in a vehicle not properly decontaminated and clothing, footwear or any other instruments without the proper sanitation. Veterinarians may cause involuntary indirect transmission by iatrogenic infection with needles, blades, or other material. Feed is also a common form of domestic infection. Pig products can circulate very broadly to different locations, cities, and countries, mostly for human consumption but remains may go to domestic pigs and infect these animals (reviewed by Penrith *et al*., 2004a; Domenech *et al.*, 2006). The recent outbreak in Georgia in 2007 may have been due to importation of contaminated meat from southern Africa. Phylogenetic analysis of the circulating isolate showed there was a close relation to isolates belonging to genotype II, as are the viruses circulating in Mozambique, Madagascar and Zambia hence a raising suspicion of the intercontinental transportation of infected pork from east Africa (Rowlands *et al*., 2008).

In the manufacturing of partially cooked ham or dried salami and pepperoni sausages the curing period appears to eliminate ASFV (McKercher *et al.*, 1978). However, depending on the processes involved, pork products may contain infectious virus that can significantly contribute to international spread of the disease. Furthermore, mechanical transmission by the stable fly (*Stomoxys calcitrans*) can happen within 24 h after ingestion of the virus rendering another mean of transmission that may require added investment for more biosafety measures in pigsties with commercial pigs (Mellor *et al.*, 1987).

1.2.3. Control Strategies

Given the virus's infectivity and its severe forms of clinical manifestations, strict sanitary rules and procedures should apply to the herds at risk of infection. Unfortunately, their application implies an added investment. Subsistence farmers are generally not keen or financially prepared to consider the implementation of such measures as they adopt a free ranging small scale production system and are therefore particularly at risk. Since these producers rely heavily on pig keeping for being able to render low cost, high quality dietary proteins, there will be food security issues as outbreaks occur. Control measures for prevention of ASF presently do not inspire as much confidence as the use of effective vaccines would. Therefore, sanitary biosafety rules are the only option to reduce the risk of an outbreak.

Since there is still a lack of a vaccine against ASF, in the event of an outbreak, procedures involve movement restriction of pigs and their products by setting up a quarantine area and stamping out policy of all infected and in-contact individuals. Thereafter, carcasses must have proper disposal through incineration or deep burial and disinfection of premises. Premises that had ASF should remain empty for a quarantine period and if soon to be repopulated after the outbreak it should first go through a stage repopulated with a small number of sentinel animals. For appropriate disinfection/sterilization of ASFV contaminated material or areas it is important to keep the following information in mind: ASFV is highly resistant to low temperatures and it is heat inactivated by 56°C for 30 minutes. It is inactivated by exposure to pH of less than 3.9 or higher than 11.5. It is susceptible to ether and chloroform and it may be inactivated by 8/1000 sodium hydroxide (30 minutes), hypochlorite's (2.3% chlorine for 30 minutes), 3/1000 formalin (30 minutes), 3% ortho-phenylphenol and iodine compounds. Furthermore, ASFV can remain viable for long periods in blood, faeces, and tissues (reviewed by Penrith *et al.*, 2004a; reviewed by Penrith & Vosloo, 2009, OIE Manual 2005).

The existence of a sylvatic cycle amongst wild suids and soft ticks is an important factor for endemicity of the disease because on occasion the virus may affect domestic pork production. Strict control measures must be applied so as to avoid

contact between domestic pigs and all wildlife reservoirs. This approach appears to be effective, as outbreaks tend to be sporadic and locally restricted in southern African countries that apply strict zoosanitary measures (Boshoff *et al.*, 2007). However, in some locations ASFV harbours in domestic pigs (Penrith *et al.,* 2004b). In this case, successful prevention requires community involvement and improved husbandry (Penrith *et al.*, 2007).

1.2.4. Molecular epidemiology

The ASFV genome encodes five multigene families and more than 50 polypeptides. Complete genome sequencing of one Spanish isolate revealed that structural or functional properties were present in 113 viral proteins (Yáñez *et al.*, 1995). Some of these proteins correspond to known virulence factors that modulate virus-host interactions and may or not be present in the DNA of various isolates evidencing the heterogeneity existent in ASFV. Genetic studies offer an important insight for the modern understanding and classification of a virus. A retrospective analysis of the source and spread of historical outbreaks can produce important insights for epidemiological investigations and the source of outbreaks and therefore contribute to the planning of the most appropriate measures. For ASF, this type of studies were previously only achieved by genomic characterization methods such as restriction fragment length polymorphism analysis as performed by Dixon and Wilkinson (1988) for the characterization of ASFV from *O. moubata* ticks present in burrows from Zambia. However, recently the PCR-sequencing method permits more rapid and accurate description of the major genotype nucleotide sequences of the viral genome (Bastos *et al.*, 2003) and the genetic characterization of the central variable region (CVR) of the 9RL open reading frame (ORF) studies the intra-genotypic relationships of the outbreak isolates (Bastos *et al.*, 2004).

There is a relative heterogeneity amongst isolates involved in outbreaks in southern and East Africa. According to Lubisi *et al.* (2005), in a study of the molecular epidemiology of ASF in East Africa, sixteen vp72 genotypes were consistently recovered (I-XVI) through the phylogenetic analysis of a homologous 404 bp region that corresponds to the C-terminal end of the vp72 gene. Nix *et al.*

(2006) subdivided 41 isolates of ASFV (previously constituting the same group) by phylogenetic analysis through comparison of four variable regions and originated 16 new sub-groups. Another PCR used in phylogenetic analysis is the CVR PCR directed at the central variable region (CVR) of ASFV genome contained within the 9-RL open reading frame (ORF). The CVR varies in size within the ORF from 300 to 500 bp and contains a 132-bp direct repeat. Identifying and grouping ASFV isolates is done by analysis of the number and composition of tandem tetramers in the CVR of a given isolate (Irusta *et al.*, 1996). Phylogenetic analysis through the combined vp72-CVR approach in pig outbreaks in southern Africa between 1973 and 1999 revealed the presence of 14 distinct vp72 genotypes. Six of them are novel (genotypes XVII-XXII), eight were country specific, and the remaining six had a trans-boundary distribution. The study concluded that the genotype XIX virus had an extended field presence in South Africa between 1985 and 1996 (Boshoff *et al*., 2007). The vp72-CVR combination allows resolution of intragenotypic relations of ASFV strains circulating in domestic pigs in East Africa. It resulted in seven CVR lineages from viruses that were previously resolved into only two vp72 genotype VIII lineages (Lubisi *et al.*, 2007).

1.2.5. Vaccines

Although there is still not an efficient vaccine commercially available for ASF, research to develop a prophylactic vaccine is ongoing and results seem promising. Passively transferring ASFV antibodies into pigs led to protection of domestic swine against lethal infection with a homologous strain. Humoral activity is involved in protection against this disease since antiviral antibodies represent an important component of the homologous protective immune response to ASFV (Onisk *et al.*, 1994). However, animals immunized with baculovirus-expressed vp30, vp54, vp72, and vp22 and thereafter challenged with a homologous virulent isolate did not show resistance against ASF although a slight delay to onset of clinical disease was noted (Neilan *et al.*, 2004). Furthermore it has been shown that protection against virulent inoculums of ASFV may be acquired by pigs previously inoculated with certain non-pathogenic isolates (Lewis *et al*., 2000; Leitão *et al*., 2001; Oura *et al*., 2005) and by resistance in animals that survived infection by a certain isolate and thereafter challenged with homologous and

heterologous strains (King *et al*., 2011). In the majority of cases there is a lack of heterologous protection among strains of ASFV, causing a big setback in the development of a vaccine (reviewed by Kleiboeker, 2002). Genomic variations that account for genotype classification inherent in ASFV isolates according to DNA sequence and related to various geographic locations provoked different immune reactions in pigs. These represent a challenge in the creation of a vaccine that may work in several regions of the world. Considerations on the actual circulating isolates of each region must be included in propositions of vaccines based on attenuated strains.

1.3. Pathogenesis

Within Africa, the form of ASF most commonly observed is acute disease derived from infection by pathogenic strains. Morbidity and mortality may reach up to 100% especially in the acute and sub-acute stages. The incubation period after infection varies between five to 15 days from direct contact exposure or less than five days after an infectious tick bite (reviewed by Kleiboeker, 2002).

According to the virus in question, the character of the disease may change when it becomes endemic in domestic pigs. Both moderately virulent and low virulent isolates may be involved in such areas. Virulence factors coded on ASF isolate genomes that allow evasion of host defence mechanisms determine strains that cause either no relevant pathology or severe affliction according to presence or absence of such genes. Thus, the behaviour of each ASFV isolate varies.

The tonsil, respiratory and digestive tracts are incriminated as routes of entrance of the virus. Depending on the route of infection, ASFV is present in the pharyngeal tonsils and mandibular lymph nodes but it may occur first in bronchial or gastric lymph nodes. The mandibular lymph nodes are the site of primary replication. The subsequent spread of the virus in the organism is haematogenous (Greig 1972, reviewed by Penrith *et al*., 2004a). Greig and Plowright (1970) have demonstrated that after direct contact infection, virus is present in tonsilar swabs as early as one or two days before the onset of pyrexia. Pigs infected by highly virulent isolates die very quickly (peracute) with lung oedema being often the immediate cause of death in these cases. Animals suffering from acute ASF

generally die showing low or no antibody production (reviewed by Kleiboeker, 2002). In subacute to chronic cases, pneumonia is frequent and leads to serofibrinous pleuritis with pleural effusions and adhesions. Extensive serofibrinous pericarditis and adhesions cause some animals to develop cardiac insufficiency that may result in intermandibular oedema. Oedematous swelling of the joints in the limbs because of joint and tendon sheath effusions may also appear and cause serious discomfort. Although sometimes joints may not be swollen, there is clinical and histological evidence of arthritis. Furthermore, in acute cases it is generally accepted that the destruction of macrophages contributes importantly to the impaired haemostasis because it generates the release of active substances (mediators) like enzymes, cytokines (IL-1, IL-6 and TNF-α) complement factors and arachidonic acid metabolites (Penrith *et al*. 2004; Salguero *et al.*, 2005). The widespread effusion and haemorrhage in acute ASF is because of increased vascular permeability. It results from the effect of mediators in endothelial cells and the activation of the clotting cascade that causes a disseminated intravascular coagulation (DIC) due to the liberation of prostaglandin E_2 from the infected macrophage. The release of cytokines precipitates the apoptosis of both T and B-lymphocytes causing a marked lymphopenia that develops consistently in virulent cases of ASF (Villeda *et al.*, 1993; Oura *et al.*, 1998a; Salguero *et al.* 2005). Childerstone *et al*. (1998) demonstrated decreased stimulation of splenocytes in pigs infected with various strains of ASFV with consequent affected proliferation of $CD4^+$ and $CD8^+$ T cells. Another interesting feature of ASF is the formation of immune complexes of ASF antigen and antibody that contribute to the onset of thrombocytopenia by aggregation of platelets and consumption of platelets due to coagulopathy (reviewed by Penrith *et al*., 2004a).

1.3.1. Virus replication

The ASFV and viruses from the families *Poxviridae*, *Iridoviridae*, *Phycodnaviridae,* and *Mimiviridae* share the common feature of replication at least partially in the cytoplasm. This common aspect groups them in a superfamily named the nucleo-cytoplasmic large DNA virus family (Iyer *et al.*, 2006). Replication takes place in the cytoplasm and the virions are associated with the ribosomes (Gómez-Villamandos *et al.*, 1997). However, examination of the ultrastructural localization

of ASFV DNA, showed existence of nuclear and cytoplasmic stages in its synthesis. Virus-specific DNA sequences are disttinguishable in the nucleus of infected Vero cells early in the synthesis of the viral DNA and are later found exclusively in the cytoplasm. An electron microscopic autoradiography of ASFV-infected macrophages showed that the nucleus is a site of viral DNA replication in the early phase of the process (Rojo *et al.*, 1999). ASFV assembly occurs in the cytoplasmic viral factories that contain viral structural proteins, viral DNA, and amorphous membranous material used to produce viral envelopes. The double membrane seen in the viral envelope derives from the mechanism of involvement of the virus by the endoplasmic reticulum cisternae (Rouiller *et al.*, 1998).

The genome encodes 150 open reading frames, and 50 viral proteins incorporated in the viral particle (Esteves *et al.*, 1998). Approximately 35% of the mass of the virion is provided by vp72 constituting the major capsid protein, and 25% are structural proteins derived from the polyprotein vp220 (vp150, vp37, vp34 and vp14) (Andrés *et al.*, 1997). Intracellular mature virions travel by microtubule-mediated transport to the plasma membrane and emerge by budding, becoming the infectious extracellular enveloped virions in Vero cells (Breese & Pan 1978).

Enveloped mature virions are present within membrane indentations of erythrocytes (Carrasco *et al.*, 1996). During viraemia, the virus is associated with red blood cells and peripheral leukocytes with up to 90 % of the circulating virus being associated to the erythrocytes. Although viral replication may also occur in other cell types like endothelial cells, pericytes, glomerular mesangial cells, renal collecting duct epithelial cells, hepatocytes, neutrophils and megakaryocytes, it does not seem to contribute significantly to the outcome of the disease (Penrith *et al*., 2004b). Replication of the virus DNA occurs primarily in reticular cells, monocytes, and macrophages. ASFV shows a predilection for the antigen-presenting cells of the macrophage-mononuclear system penetrating into these cells after adsorption to protein membrane receptors on the cell surface (Carrasco *et al.,* 1996; Penrith *et al*., 2004b). Mature viral particles were observed in invaginations of the lymphocyte membrane and mature virions in the cytoplasm. Replication does not seem to occur in these cells though because incomplete virions and virus replication sites are not present (Carrasco *et al*., 1996).

1.3.2. Virulence factors

At least half of the genes encoded in the ASFV genome have an important role for virus survival and transmission. These genes modulate virus manipulation of the host response to infection in order to circumvent elimination. Understanding these genes may be useful in the discovery of novel immunomodulatory drugs (Dixon *et al.*, 2004).

The ability of ASFV to penetrate host defence systems is due to encoded proteins in its genome that are involved in modulating the host response to infection. The A238L encoded protein has potent immunomodulatory function. CD2v resembles the host CD2 protein, which is expressed on T cells and NK cells and causes adsorption of red blood cells around virus infected cells and extracellular virus particles. The absence of this gene in some isolates renders these non-haemadsorbing viruses. Expression of the CD2v protein aids virus dissemination in pigs and the protein has a role in impairing bystander lymphocyte function. Two ASFV proteins, an IAP, and a Bcl2 homologue inhibit apoptosis in infected cells facilitating progeny virions. The ASFV genome also encodes the product vp21 that inhibits apoptosis of infected macrophages allowing them to tolerate productive viral replication (Dixon *et al.,* 2004). In contrast to apoptotic inhibition, Hernáez *et al.* (2004) reported that vp54-encoded protein is responsible for apoptosis of ASFV infected cells. A timely controlled cell death is appropriate for virus dissemination in the organism.

1.4. Immunology

ASF is generally responsible for high mortality and few pigs recover from virulent disease. Individuals that recover may be resistant to homologous exposure although it does not mean it remains protected to heterologous isolates since ASFV differ in the specificity of immune responses induced. As mentioned before, the virus has predilection for replicating in cells of the monocyte/macrophage lineage, which causes detrimental effects in the immune system of infected pigs (Dixon *et al.,* 2004). These cells necrotize as a direct effect of infection, which compromises antigen presentation. In addition, apoptosis of lymphocytes B and T,

and modulation of swine leukocyte antigens occur. Apoptosis of lymphocytes is due to happen because of the release of cytokines from infected macrophages (reviewed by Penrith *et al.*, 2004a).

Warthogs and bushpigs are resistant to ASF and therefore usually show levels of virus replication lower than in domestic pigs. However, in susceptible hosts ASFV has mechanisms to evade immune defences. Immune suppression in domestic pigs relates to release of viral protein vp36 by infected macrophages as well as a reduction in expression of class I and II major histocompatibility antigens. The product of ORF 8-DR (structurally and functionally similar to CD2) has an immunosuppressive role (reviewed by Penrith *et al.*, 2004a). Kollnberger *et al.* (2002) characterized fourteen ASFV serological immunodeterminants. These 14 independent viral proteins stimulated antibody production in domestic pigs. Six encode in the open reading frames and are designated B602L, C44L, CP312R, E184L, K145R, and K205R. Another five are structural proteins like A104R, vp10, vp32, vp54 and vp72 and three are non-structural as RNA reductase, DNA ligase and thymidine kinase (Kollnberger *et al.,* 2002). Serological and cellular components are important in ASFV immunity. Sera from pigs infected experimentally had Ig isotype antibody responses evaluated against 12 viral proteins. Total IgG was present against viral proteins E183L/p54, K205R, A104R, and B602L. IgM was only present to the protein K205R and there was an IgG1 response to all of the proteins reflecting that maybe a dominant Th2-controlled immune response is present in ASF. It is suggested that total IgG response to the A104R protein might be an indicator that this response is somehow involved in protection because it was higher in asymptomatic than in chronically infected pigs (Reis *et al*., 2007).

Evidence indicates that cellular and cellular-based immune responses are important for ASFV infected pigs survival. Pigs experimentally infected with a low virulent isolate developed cytotoxic T lymphocytes specific against ASFV and increased natural killer cell activity. These individuals survived a second challenge with a highly virulent strain (reviewed by Kollnberger *et al.*, 2002; reviewed by Gil *et al.*, 2008). In work done by Jenson *et al.* (2000), using induced resistant pigs, $CD8^+$ T lymphocytes recognised various products obtained from sonication of DNA

of an ASFV isolate. Oura *et al.* (2005) showed significant importance of $CD8^+$ lymphocytes in protection of pigs against challenge with a virulent isolate while previously exposed to non-virulent for induction of immunity. Gil *et al.* (2008) related the importance of $CD8^+$ lymphocytes and showed a positive interaction of cytokines in cellular processes. These cytokines (IFNα, TNFα and IL12p40) were elevated according to quantification determined by ELISA in sera of pigs experimentally inoculated with low virulent virus when compared to inoculation by the highly pathogenic strain.

1.5. Diagnostics

Aspects involving epidemiology data, clinical signs and lesions allow recognition of acute ASF. The clinical presentation and post-mortem lesions are similar to a few other diseases, however not many cause such high mortality amongst pigs of all ages especially if it is manifested acutely and consequently having a short survival period. Unfortunately, in pigs that die peracutely, they often do so before either clinical signs or significant lesions have developed making field diagnosis more challenging. Nevertheless, acute ASF can be easily mistaken for CSF (hog cholera) and for bacterial septicaemias like acute erysipelas (*Erysipelothrix rhusiopathiae*), septicaemic pasteurellosis and salmonellosis (reviewed by Penrith *et al*., 2004a; OIE manual 2005; OIE manual 2012). These diseases have lower mortality. Infection with *Trypanosoma suis* is characterized by high mortality in pigs within a short period, with signs of febrile disease and therefore should be included in differential diagnosis. Differential confirmation is by observation of the protozoa in blood smears. A few intoxications can be confused with ASF, as they may be responsible for widespread haemorrhage and high mortality. Difficulty in differentiating ASF is evident when infection prevails for longer and consequently develops subacute, chronic, or even subclinical forms (reviewed by Penrith *et al*., 2004a).

1.5.1. Clinical signs

The survival time, which is defined as the period from the onset of pyrexia to death, is predominantly short for virulent African strains of the virus, varying

between two and nine days in more than 90% of cases, with a mean of 5 days. The disease can manifest in a variety of clinical signs that range from hyperacute, acute to a subacute or a chronic form. Rectal temperatures reach 41ºC to 42ºC within 48 hours of the initial rise and in the hyperacute form, death may occur during this time and consequently while many clinical signs are yet to develop. The hyperacute form of ASF is common in African outbreaks and very few animals recover. In the acute form, there is observation of listlessness, partial or complete anorexia, swaying gait, incoordination, convulsions and muscular tremors, paresis, congestion and cyanosis of the skin of the tail, limbs, ears, snout, and abdomen. Haemorrhages are common in the skin. Animals usually crowd together and they may have a disinclination to stand. Respiration can become shallow and rapid and present an accelerated heart rate. Mucopurulent ocular and nasal discharges are often present and in recumbent animals with severe lung oedema, there is profuse, watery or frothy, bloodstained nasal discharge. Vomiting is common and some animals become constipated, with bloodstained mucus on the faeces while others develop watery diarrhoea or dysentery, with fresh or blackened blood stuck on to the perineum and tail. Generally, gestating sows often abort early in the course of the disease but all stages of pregnancy are affected. Towards the end of acute disease, rectal temperature falls rapidly to sub-normal levels, followed by coma and death (reviewed by Penrith *et al*., 2004a).

As time passes and the pig is still alive, ASF progresses to subacute and after three to four weeks, it presents irregular remittent fever, anorexia, and consequent loss of condition. Pigs may develop cough, dyspnoea, and joint swelling. Some individuals become longer-term survivors (chronic form). It is common to find circular necrotic lesions in the skin of these animals and to see emaciation (Leitão *et al.*, 2001; reviewed by Penrith *et al*., 2004a).

Haematology analysis during the acute stage reveals white blood cell count as reduced or normal total leukocyte count, but differential counts reveal lymphopenia and neutrophilia with a left shift. However, in the subacute or chronic disease, the leukocyte count normalizes maybe owing to increased haematopoiesis (Villeda *et al.*, 1993; reviewed by Penrith *et al*., 2004a).

1.5.2. Gross Pathology

Gross pathology of the peracute form is less clear than the following stages as it does not show many lesions. However, it may show skin flushing of extremities and the ventral abdomen especially in white-skinned pigs. General congestion of organs with fluid exudation into body cavities and possibly fibrin strands on organ surfaces may be present. The acute form shows evident reddening to purplish cyanosis of the extremities and the ventral surface. Frank haemorrhages may be present. Tear marks with mucopurulent ocular exudates may be evident. Mucopurulent nasal discharge or bloody froth oozing from nostrils is indicative of severe lung oedema and haemorrhage. During necropsy the opening of the carcass usually reveals accumulation of bloody fluid in body cavities, and widespread haemorrhage in organs and on the parietal surfaces. Petechiae and/or ecchymoses are usually evident in the renal cortex, as well as on the capsule of the spleen. Generally, organs appear congested. The spleen may be markedly enlarged and darkly congested and infarcts are occasionally evident at the margins. Lymph nodes, in particular those of the head and the gastrointestinal tract (the hepatogastric and mesenteric lymph nodes) are markedly swollen and haemorrhagic. The lungs do not collapse in an open thorax. These present congested to haemorrhagic and the interlobular septa have accumulation of fluid. Cut surfaces are filled with froth, which may be bloodstained. The gastrointestinal tract may appear normal to severely congested, and haemorrhages are usually present in the gastric mucosa. Contents of the intestines are generally fluid and sometimes bloodstained, and the rectum may contain hard faeces covered with bloody mucus. Pigs that survive longer (chronic form) can exhibit severe interstitial pneumonia with mottled lungs that may be covered by fibrinopurulent exudates and swollen joints with accumulation of turbid fluid inside. Generally, the carcasse of chronically affected individuals is thin with the appearance of having a long hairy coat. Ulcerative non-healing sores may be present over bony points. Other lesions of chronic ASF include fibrinous pericarditis and interstitial pneumonia and enlarged and firm lymph nodes (reviewed by Penrith *et al*., 2004a).

1.5.3. Tissue sampling

When there is suspicion of an ASF outbreak, samples should go to the laboratory to screen for ASFV. For this purpose blood in anticoagulant (ethylene diamine tetra-acetic acid (EDTA)), spleen, tonsil, kidney, lymph nodes are the appropriate target tissues for sampling. These samples should be properly identified and packaged (not allowing leakages) and kept as cold as possible during transport, but without being frozen. Since it is not always possible to have cooling conditions for transport, samples should be in 50% glycerosaline, although it may reduce the likelihood of virus identification (reviewed by Penrith *et al*., 2004a; OIE manual 2005).

1.5.4. Histopathology

The histopathology of the acute stage presents with fibrinoid vasculitis observed especially in the lymphoid organs, and severe karyorrhexis of macrophages in lymphoid tissues is evident. In the spleen, the Schweiger-Seidel sheaths are destroyed because of macrophage necrosis. Infiltration of monocytes and macrophages, often karyorrhectic and usually perivascular, occurs in different organs including the tonsils, skin, peri-ocular tissues, red pulp of the spleen, lymph nodes, gastro-intestinal mucosa, lung, liver and brain, and less commonly in the oral and pharyngeal mucosa, kidneys and adrenal glands. Individual organs reveal haemorrhages and other lesions that result from processes caused by ASFV replication in host cells, mainly macrophages. Serous fluid accumulates in alveoli and septa or there is infiltration of mononuclear cells into alveolar septa. There is infiltration of mononuclear cells and eosinophils in portal tracts of the liver and necrosis of single hepatocytes. Lesions in the kidneys vary from haemorrhage to acute fibrinous glomerulonephritis, accompanied by varying degrees of tubular degeneration and necrosis, and protein casts. In pigs that survive for a few days, non-suppurative meningoencephalitis is usually present. The chronic form manifests by showing thickened pericardium and epicardium with granulation tissue covered by a fibrinous exudate and infiltrated by mononuclear cells. Lung septa show infiltration of mononuclear cells. Focal accumulations of mononuclear cells in alveoli become necrotic and surrounded by infiltrating mononuclear cells

including plasma cells. These foci may present as mineralized and walled off by fibrosis. Articular swellings happen because of granulation tissue infiltrated by mononuclear cells. Infiltration of mononuclear cells in the leptomeninges and around blood vessels in the brain, in particular the brain stem, is a constant finding in chronic ASF. Chronic changes in the lymph nodes result from proliferation of reticular cells that often obliterate the sinuses, and occupy and enlarge the germinal centres (reviewed by Penrith *et al*. 2004a).

1.5.5. Virus isolation

Virus isolation can be performed using a haemadsorption test (HAD) which is definitive for the diagnosis of ASF. Malmquist and Hay (1960) described a method of ASFV cell culture by use of blood macrophages and bone marrow macrophages in detail. Zhang *et al*. (2006) reported on viral isolation via macrophages extracted via lavages of lung alveolus. It is a cytopathogenesis test to detect infectious virus and carried out by inoculating suspect blood or tissue suspensions, especially of spleen and lymph nodes, into surviving porcine monocyte or macrophage cultures derived from peripheral blood, bone marrow, or lung washings. The adsorption of several layers of erythrocytes to infected cells usually becomes visible in one to five days, depending on the quantity of infectious virus present. To demonstrate weak haemadsorption, which may be only one or two cells thick a subculture applies. Although most of the non-haemadsorbing viruses are avirulent, some may also cause acute ASF. Whenever this type of virus is involved, the macrophage cultures undergo cytolysis after two to seven days and the diagnosis is confirmed using methods for detection of viral antigen (such as immunofluorescence) or DNA (e.g. PCR) (reviewed by Penrith *et al*. 2004a; OIE manual 2005).

1.5.6. Detection of viral antigens

Various techniques are available to detect viral antigens in pigs. All are of decreasing value from seven to 12 days following onset, when antibodies begin to appear. Direct immunofluorescence is commonly used especially in remote areas without access to high tech options. It is based on observation via UV microscope of antigen or antibody on impression smears or cryostat sections of tissues or inoculated cell cultures fixed on glass slides and stained with fluorescein

isothiocyanate. Other tests that may be used for antigen detection are the complement fixation test, immunodiffusion using hyperimmune pig serum and lymph node, liver or kidney tissue, ELISA, and radio-immuno-assay. However, these techniques do not have the sensitivity of the HAD in terms of detection of virus that is still infectious and not just traces of its presence (reviewed by Penrith *et al*. 2004a). Indirect sandwich ELISA has been shown to detect antigens of ASFV via use of polyclonal and vp72 monoclonal antibodies (Hutchings *et al*., 2006).

1.5.7. Detection of viral DNA

The detection of viral DNA is by use of PCR that was developed using primers from highly conserved regions of the genome. This tool allows the detection and identification of a wide range of isolates belonging to all the known virus genotypes, including non-haemadsorbing viruses that are hard to detect by virus isolation. It is a highly sensitive and specific test and useful in detection of viral DNA in pig tissues that are unsuitable for virus isolation or antigen detection because they have undergone putrefaction. It is valuable if the virus has become inactivated during transport or at any time before samples arrive in the laboratory. A variety of PCRs are currently being used for different purposes such as detecting viral protein 72 gene DNA via the vp72 diagnostic PCR (Wilkinson 2000) as well as for genome sequencing and phylogenetic analysis by a wider amplification of the same vp72 gene designated the vp72 epidemiology PCR (Bastos *et al.,* 2003). The CVR PCR is for phylogenetic analysis of ASF isolates (Bastos *et al.,* 2004). There are other PCR methods including viral protein 30 (vp30) gene directed PCR (Perez-Filgueira *et al.,* 2006), viral protein 54 gene (vp54) PCR (Gallardo *et al.*, 2009) and the PPA PCR (Aguero *et al.,* 2003). The nested PCR targets the vp72 gene region, is extremely sensitive, and finds its application in detecting low levels of ASFV DNA especially in ticks (Basto *et al.*, 2006). The real time quantitative analysis PCR (King *et al.*, 2003), is directed to vp72 as well. It is useful in analysis of virus load in various samples by setup of a standard curve via dilutions of plasmid containing ASFV vp72 gene.

1.5.8. Detection of antibodies against ASFV

Recovered pigs may have antibodies for long periods after infection. There is a variety of tests available for detection of these antibodies but only a few of routine use in diagnostic laboratories. The most commonly used is the ELISA, which is suitable for examining either serum or fluid from tissues but confirmatory testing of ELISA-positive samples should be carried out using alternative tests, such as the immunofluorescence antibody test, immunoperoxidase staining or immunoblotting. Pigs infected with virulent ASFV do not produce antibodies, since death occurs suddenly before an immune response can emerge. Both the immunoeletrophoresis test and ELISA are for large-scale screening of sera. The ELISA is more sensitive for detecting individual positive sera and used extensively as part of eradication programs. Although the method used depends on staff and facilities, when pigs are infected with non-virulent isolates or those of low virulence, serological tests are good to detect positive animals (OIE manual, 2012). Antibody detection ELISA determines IgM for recent infections or IgG for cases of chronically affected pigs. IgM was found in a majority of pigs from seven days post inoculation (dpi) and IgG was detected 14 dpi (Reis *et al.*, 2007).

1.6. Problem statement

Sensitive diagnostic tests are important for accurate detection of infectious agents and general investigation of ASF. Thus, diagnostic tools with high sensitivity are obviously better for both research and diagnostic services since low levels of virus may go undetected with some tests. This is especially true for wrongly preserved samples with degraded concentrations of virus. Motivated by a need to evaluate a few options for detection of ASFV, a number of the currently available tests were analysed for sensitivity by comparison of the respective detection limits using ten-fold dilutions of virus. The aim was to have grounds for recommending the most appropriate tests to use for various applications at Transboundary Animal Disease Programme (TADP) in ARC/OVI.

Certain viruses presently circulating in southern Africa were of unknown post-infection outcome in commercial domestic pigs. In order to get more information

on viruses that were collected in within the region but for which no experimental data were available a trial was set to obtain pathological findings and reveal the behaviour of both ASFV MKUZE and ASFV MOZ 1/98. There are various genotypes of ASFV more or less virulent to pigs depending on the virulence factors encoded in its genome. The animal trial aimed to have control groups that served for comparison with the effects of well-known high virulent virus ASFV BENIN 1/97, and non-virulent virus previously used as vaccine ASFV OURT 3/88.

ASFV quickly spreads to neighbouring areas where pigs are present. Outbreaks are generally characterized by high socio-economic impact and without a vaccine, the disease tends to be problematic thus motivating the search for effective means of control. Although biosafety procedures are of irrefutable significance, the search for vaccine that would protect against currently circulating viruses is crucial. The project therefore wanted to look into possibilities for a vaccine, and a challenge model was set. The cross-protection of the isolate ASFV OURT 3/88, a European strain, was evaluated against a circulating southern African isolate ASFV MOZ 1/98.

1.7. Benefits arising from the study

The sensitivity of various diagnostic assays was determined by comparing detection limits of viral dilutions inoculated into three different cell types and tested with various PCRs. A recommendation was made as to the most appropriate diagnostic setup for ASFV detection for routine application during the research outputs of the project and evaluating samples from the animal trials in which virus load could be low.

The first pig trial described pathological findings and demonstrated viral presence in a variety of tissue samples including blood over time of two isolates previously not assessed in these terms (ASFV MKUZE and ASFV MOZ 1/98). These were poorly understood viruses currently circulating in South Africa and Mozambique respectively. This work has provided new insights into the pathogenicity and clinical outcome of infection with such viruses.

The second pig trial was to test the cross-protection potential of a virus that does not cause symptomatology and may work as vaccine. In this case, it was by inoculation with ASFV OURT 3/88 and challenge with ASFV MOZ 1/98. A positive outcome of this project may lead to the creation of a prophylactic measure against ASF and help areas where pig production is important as an effective source of good quality animal protein.

1.8. Aims of this study

- To compare the diagnostic sensitivity of different diagnostic tests:
    - Using three cell types for virus isolation
    - And five PCR protocols
- To access the outcome of infection in domestic pigs with an isolate from Mozambique (ASFV MOZ 1/98) and another from South Africa isolated from ticks in the control zone (ASFV MKUZE) and compare it to that caused by non-virulent ASFV OURT 3/88 and highly virulent isolate ASFV BENIN 1/97.
- To evaluate the ability of a fully attenuated European strain, ASFV OURT 3/88, to protect pigs against challenge with a highly virulent African strain, ASFV MOZ 1/98.

Chapter 2

Materials and Methods

2.1. Comparison of various diagnostic assays

The sensitivity of a number of ASFV detection tests was determined and compared. These included virus isolation also designated here as the haemadsorption test (HAD) done by using three different cell culture types (bone marrow, blood and alveolar macrophages) and various PCRs (see below). The virus isolation assays were evaluated on ten-fold dilutions (neat to 10^{-8}) of two live viruses. Dilutions were inoculated onto three 96 well plates each seeded with a different type of cell in order to determine which produces the highest titre. DNA extraction was done on a fraction of the dilutions shortly after preparation and the DNA stored at -80°C until used for analysing detection limits of a number of PCRs. Each experiment was repeated six times to validate the detection limit or sensitivity of both these sets of tests (virus isolation and PCRs).

2.1.1. Viruses

The viruses used were the ASFV SPEC 257 and the ASFV MOZ 1/98 stored as stocks in the TADP laboratory at -80°C. Both these isolates have haemadsorbing capacity noted by the microscopic observation of haemadsorption of red blood cells to macrophages. ASFV SPEC 257 was obtained from a tick in 1985 in Thabazimbi, South Africa (TADP submission records) and ASFV MOZ 1/98 from an outbreak in domestic pigs in Mozambique in 1998 (Bastos *et al*., 2004). The isolate from South Africa has previously been extensively adapted to cell culture at TADP and therefore it was appropriate to serve as a positive control whilst monitoring cell quality in this investigation. It passaged 12 times in blood macrophages and it presented a final titre of 1×10^6 $TCID_{50}$/ml. ASFV MOZ 1/98 was adapted to cells by passaging six times in blood macrophage cultures in order to have more of this virus isolate available for research or diagnostics. It presented a titer of 1×10^4 $TCID_{50}$/ml.

2.1.2. Cell culture

HAD requires primary pig leukocyte cultures. Thus, monocytes/macrophages were acquired from peripheral blood and bone marrow as described in Malmquist and Hay (1960) with a few modifications and from the lungs according to Zhang *et al*. (2006). These cultures were blood macrophage culture (BC), bone marrow macrophage culture (BMC) and alveolar macrophage culture (AMC). Cells were harvested during post-mortems of six to eight-week-old Landrace x Large White piglets euthanized by intracardiac injection of Sodium Pentobarbitone (200 to 400 mg/kg). A constant fresh cell source was obtained from piglets sacrificed weekly at ARC/OVI – TADP. Euthanasia went according to ARC/OVI ethics committee approval and as part of the diagnostics department's routine procedures for collection of primary cells for use in virus isolation.

2.1.2.1. Blood leukocyte cell culture

Blood was collected into a one litre sterile container with one ml Heparin Sodium (5000 i.u/ml) from exsanguinations via incision of the axillary blood vessels after euthanasia. In order to inhibit microbial and fungal proliferation in the culture, an antimicrobial and antifungal cocktail consisting of 1 ml of 4% Benzylpenicilin (SIGMA®), 1 ml of 6.4% Streptomycin sulphate (SIGMA®), 1 ml of 7.5% Neomycin-sulphate (Merck) and 1 ml of 5 mg Amphotericin B (Bristol-Myers Squibb) was added to the blood. Iron dextran 10% (BDK) was added to the solution in a 1:10 ratio. Thereafter, 100 ml aliquots were introduced in Erlenmeyer flasks and left to incubate for 20 minutes at 37˚C until sediments formed. The white blood cell rich supernatant was collected into a 250 ml plastic centrifuge bottle (Beckman®) and diluted 1:1 with wash buffer (phosphate buffered saline (PBS), 0.4% Penicillin, 0.64% Streptomycin, 0.75% Neomycin and 10% normal bovine serum (NBS)). Thereafter, washing ensued by centrifugation at 1073 g (Beckman® model J-6B) for four minutes at room temperature and discarding the supernatant. This process was repeated three times and the resultant sediment from the last washing step re-suspended in 100 ml 0.82% ammonium chloride and centrifuged as before. Subsequently there was addition of wash buffer and one last washing step. The white blood cell sediment was re-suspended in growth medium (42% Earle's medium (Appendix A), 0.4% Hepes (UniLab) (Appendix B), 13% swine serum,

0.52% penicillin, 0.832% streptomycin and 0.975% neomycin). Volumes of 100 µl were aliquoted in 96 well plates (maxisorp, NUNC™) at 37˚C, 4.2% CO_2 and the medium was changed after 48 hours and replaced with fresh growth medium containing 0.5% red blood cells. Red blood cells were aliquoted from the initial blood collection from the pig referred to above and subjected to a washing process with PBS. Thereafter, washing was performed by mixing equal amounts of blood and PBS, and centrifuging in order to produce sediment and discarding the supernatant. This process was done twice and packed red blood cells were stored at 4˚C until introduced into the cultures along with new growth media.

2.1.2.2. Bone marrow leukocyte cell culture

Bone marrow leucocytes were extracted from both femoral bones collected from the euthanized pig. These bones were cleared of flesh and cartilage, introduced in 300 ml of wash buffer (Appendix E), and incubated for 20 minutes at 37˚C. Subsequently, bone-cutting forceps facilitated bone fragmentation into small pieces collected into a one litre conical recipient containing 250 ml of wash buffer, incubated at 37˚C and gently shaken for 90 minutes. Butter muslin was placed over funnels to filter the crushed pieces of bone and to collect a cell rich solution into 250 ml plastic centrifuge bottles (Beckman®). Centrifugation followed at 300 g (Beckman® model J-6B) for 15 minutes at room temperature and the supernatant was removed. The remaining sediment was re-suspended in 0.82% ammonium chloride solution and centrifuged at 300 g (Beckman® model J-6B) for 15 minutes. Afterwards, two washing steps with wash buffer were carried out followed by suspension of the sediment in growth medium. One hundred microlitres of the cell rich suspension were introduced into 96 well plates (NUNC™) and incubated with 4.2% CO_2 at 37˚C. Medium was changed after 48 hours and replaced by fresh growth medium containing 0.5% red blood cells.

2.1.2.3. Lung cells

Alveolar leucocytes were obtained from both lungs of each piglet. The alveoli were washed with growth medium (95% RPMI-1640 media (SIGMA®), 5% foetal bovine serum (GIBCO®) and a 0.5% combination of antimicrobial and antimycotic (GIBCO®) that contains 10,000 units of penicillin (base), 10 g of streptomycin

(base), and 25 g of amphotericin B/ml utilizing penicillin G (sodium salt), streptomycin sulphate, and amphotericin B in 0.85% saline). The lungs were held at the larynx by a clamp suspended on a stand inside a laminar flow cabinet. Obstructive or possibly contaminating tissue was removed at the opening of the trachea, the entrance site for the media. Washes were performed by introducing 50 ml of growth media at a time and palpating the lungs. The wash fluid resultant from the first lavage was discarded and the fluid collected during the next four washes into a 250 ml sterile bottle (Scott Duran). The wash fluid should appear foamy after the lavages. The resultant cells were transferred to a reagent reservoir (NUNC^{TM}) and introduced into 96 well plates (NUNC^{TM}) by multichannel pipettes at volumes of 100 µl per well and followed by incubation in the 4.2% CO_2 incubator at 37°C. Two days after the cells were seeded they attached to the plates and media was changed for fresh growth media with 0.5% of red blood cells as referred to above.

2.1.3. Haemadsorption assays

Once cell cultures had been incubating for two days and the media changed, the plates were returned to the 4.2% CO_2 incubator at 37°C for 2 hours so that the cells would settle down until virus inoculation. Viruses were inoculated at 50 µl volume per well per column (8 wells) and left to incubate for four days, at which time plates were read. Both isolates used in this study are capable of causing haemadsorption and rosettes should form within 4 days of incubation. Ten-fold dilutions of each isolate (ASFV MOZ1/98 and ASFV SPEC 257) were prepared in RPMI-1640 media (SIGMA®), foetal bovine serum (GIBCO®) and a combination of antibiotics and antimycotic (GIBCO®). Each dilution was distributed in separate columns on the plate (from neat virus in column one to dilution 10^{-8} in column nine) whilst the remainder of the 96-well (NUNC^{TM}) plate (columns ten to twelve) was inoculated with media alone to serve as negative control. Scoring was done according to identification of positive wells by observation of rosettes and titre calculated via the Spearman-Karber method (Karber, 1931). These tests were repeated six times.

Karber formula:

$\log TCID_{50} = Xa + D (Sp-0.5)$

Xa= - (lowest dilution factor with all positive wells)

D= (log dilution factor)

Sp= (sum of the proportion of positive tests beginning at Xa)

2.1.4. DNA extraction

DNA extraction was done according to Boom *et al.* (1990), using a silica/guanidium-based method on every 200 µl aliquots of each dilution (Figure 2.1). One ml of lysis buffer (Appendix C) was added to 40 µl of silica (Appendix D) and dissolved by vortexing. Subsequently 940 µl was added to each aliquot, vortexed, and left to stand for five minutes at room temperature inverting the tubes at every one-minute interval. These tubes were vortexed once again and centrifuged at 20000 g for 15 seconds and the resultant supernatant discarded in a 10 M NaOH bottle (waste bottle). Thereafter, 900 µl of wash buffer (Appendix E) was added and vortexed followed by a short spin. The supernatant was discarded again into the waste bottle. Subsequently a wash was made with 900 µl of ethanol (70%) and a final one with 900 µl of acetone. Once the acetone supernatant was discarded, the tubes were left open in a dry bath at 56°C for 20 minutes in order to dry the silica pellets. Fifty µl of 1 X TE (Appendix F) was then added to the dried silica pellet and the tubes were vortexed and incubated with the lids closed for two minutes at 56°C on the heating block. Finally, after centrifugation for ten minutes, the supernatant was transferred to a clean tube and stored at -80°C until use in the PCR assays.

2.1.5. PCR assays

Various PCRs were tested for their detection limits on the extracted DNA via identification of the faintest visible band present for the respective dilution. Various PCRs were tested that target the vp72 gene region, *viz*. the PPA PCR (Table 2.1), the p72 PCR recommended in OIE manual 2005 for ASFV detection (Table 2.2) nested PCR (Table 2.3) and the Taqman® real time PCR (RT-PCR) which has recently been recommended in the OIE manual 2012 (Table 2.4). The nested PCR

is composed of two runs done in succession with the second being with primers nested to the DNA fragment resultant from the preceding reaction. The CVR PCR (Table 2.5) was also tested and its primers were designed to amplify the central variable region situated in the 9RL open reading frame. These assays were repeated 6 times for statistical analysis.

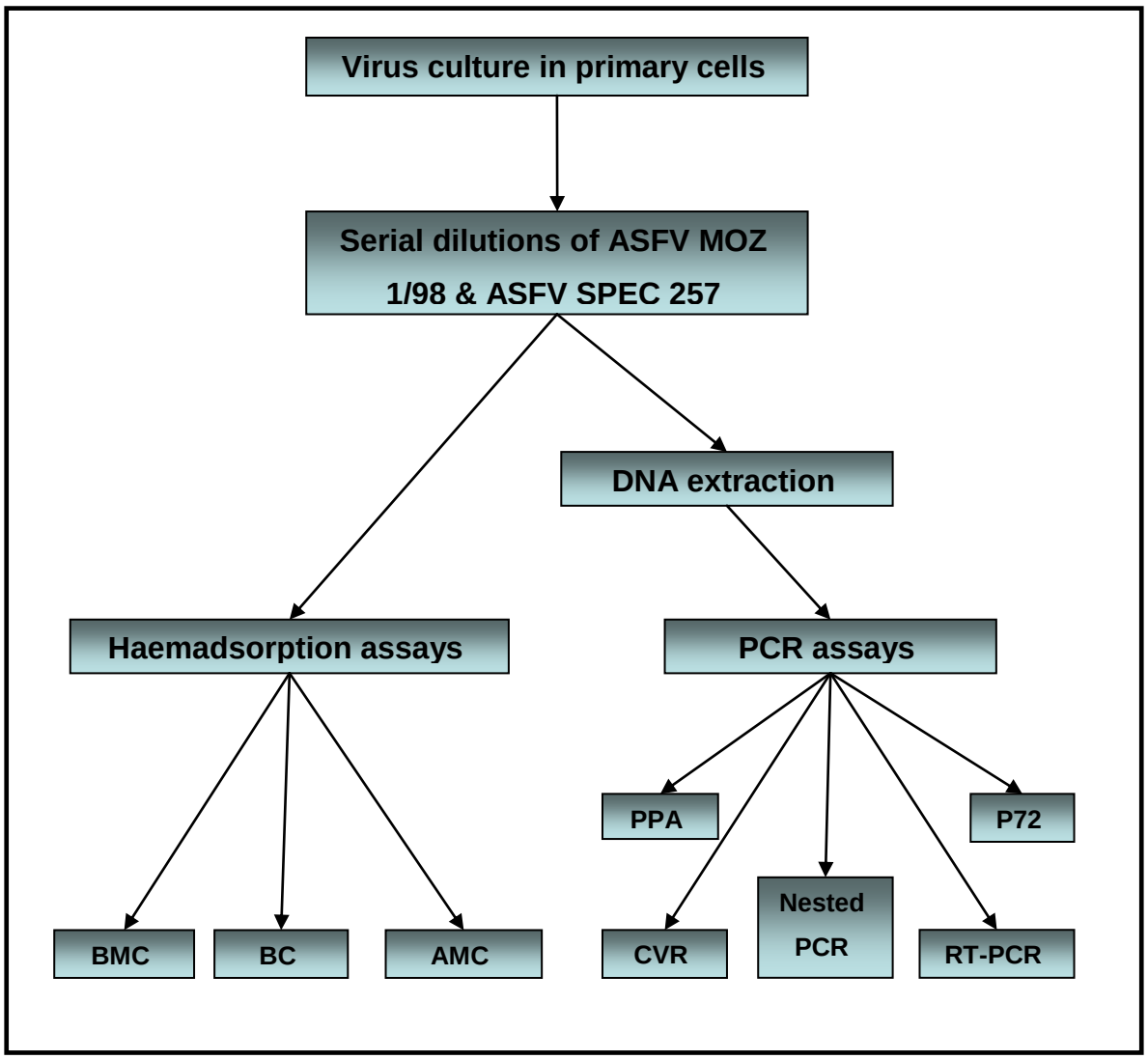


Figure 2.1 Layout used to assess the analytical sensitivity of diagnostic tests using three different cell lines and five PCR assays on serial dilutions of both ASFV SPEC 257 and ASFV MOZ 1/98.

The respective PCR primers, probe and cycling conditions utilised are described in Table 2.1 to Table 2.5.

Table 2.1 Primers and cycling conditions for PCR described in Aguero *et al.* (2003), designated here as PPA PCR.

p72 PPA PCR	
Primer sense	5’ AGT TAT GGG AAA CCC GAC CC 3’
Primer anti-sense	5’ CCC TGA ATC GGA GCA TCC T 3’
Cycling conditions	HLD: 95˚C (5 min) 40 cycles: 95˚C (15 sec), 62˚C (30 sec), 72˚C (30 sec) / HLD 72˚C (7 min) and 4˚C (∞)
Mastermix	HPLC H_20: 17 µl Kapa ready mix: 25 µl Primer sense: 2.5 µl Primer anti-sense: 2.5 µl DNA: 3 µl
Product size	257 bp

HLD: Hold; HPLC: Ultrapure water.

Table 2.2 Primers and cycling conditions for PCR described in Wilkinson (2000).

p72 PCR	
Primer sense	5’ ATG GAT ACC GAG GGA ATA GC 3’
Primer anti-sense	5’ CTT ACC GAT GAA AAT GAT AC 3’
Cycling conditions	1 HLD: 94˚C (5 min) 35 cycles: 94˚C (1 min), 53˚C (1 min), 72˚C (1 min) / HLDs 72˚C (10 min) and 4˚C (∞)
Mastermix	HPLC H_20: 17 µl Kapa ready mix: 25 µl Primer sense: 2.5 µl Primer anti-sense: 2.5 µl DNA: 3 µl
Product size	278 bp

HLD: Hold; HPLC: Ultrapure water.

Table 2.3 Primers and cycling conditions described in Basto *et al.* (2006). It is composed of two PCR reactions by two sets of primers.

p72 nested PCR		
	1st Round	2nd Round (nested primers)
Primer sense	5' GAC GCA ACG TAT CTG GAC AT 3'	5'-TAC TAT CAG CCC CCT CTT GC-3'
Primer anti-sense	5' TTT CAG GGG TTA CAA ACA GG-3'	5' AAT GAC TCC TGGGAT AAA CCA T 3'
Cycling conditions	1 HLD: 95˚C (3 min) 35 cycles: 95˚C (30 sec), 60˚C (30 sec), 72˚C (30 sec) / 2 HLD 72˚C (10 min) and 4˚C (∞)	
Mastermix	HPLC H_20: 9.5 µl Kapa ready mix:12.5 µl Primer sense: 1 µl Primer anti-sense: 1 µl DNA: 1 µl	HPLC H_20: 9.5 µl Kapa ready mix: 12.5 µl Primer sense: 1 µl Primer anti-sense: 1 µl DNA: 1 µl
Product size	370 bp	243 bp

HLD: Hold; HPLC: Ultrapure water.

Table 2.4 Primers, probe and cycling conditions for the real time PCR described in King *et al.* (2003).

p72 real time PCR	
Primer sense	5' CTG CTC ATG GTA TCA ATC TTA TCG A 3'
Primer anti-sense	5' GAT ACC ACA AGA TC(AG) GCC GT 3'
Probe	probe: 5'-6-carboxy-fluorescein (FAM)]-CCA CGG GAG GAA TAC CAA CCC AGT G-3'-6-carboxy-tetramethyl-rhodamine (TAMRA)
Cycling conditions	1 repeat at 50˚C for 2 min / 1 repeat at 95˚C for 10 min / 40 repeats at 95˚C for 15 sec and at 60˚C for 1 min
Mastermix	HPLC H_20: 9.75 µl TaqMAN master MIX: 12.5 µl PROBE: 0.25 µl Primer sense: 0.25 µl Primer anti-sense: 0.25 µl DNA: 2 µl

HLD: Hold; HPLC: Ultrapure water.

Table 2.5 Primers and cycling conditions for the PCR reaction described in Bastos *et al*. (2004).

9RL open reading frame of the central variable region CVR PCR	
Primer sense (FLR)	5' TCG GCC TGA AGC TCA TTA G 3'
Primer anti-sense (FLF)	5' CAG GAA ACT AAT GAT GTT CC 3'
Cycling conditions	1 HLD: 94˚C (5 min) / 33 cycles: 94˚C (30 sec), 53˚C (30 sec), 72˚C (30 sec) / HLDs 72˚C (7 min) and 4˚C (∞)
Mastermix	HPLC H_20: 17 µl Kapa ready mix: 25 µl FLR: 2.5 µl FLF: 2.5 µl DNA: 3 µl
Product size	500 bp

HLD: Hold; HPLC: Ultrapure water.

2.1.5.1. Agarose gels

Agarose gels for electrophoresis were prepared by dissolving multipurpose agarose, Agarose MP (Roche, Mannheim, Germany), in 1 X TAE buffer (40 mM Tris-acetate, 2 mM EDTA; pH 8), with the addition of 3 µl ethidium bromide 10 mg/ml (Promega) to every 100 ml of heated agarose solution as described by Ausubel *et al.* (1987). Gels were set at 2% (w/v) agarose for the nested PCR products and at 1% (w/v) for all other PCR types in this experiment except in the case of the real time PCR. Detection limits were according to the dilution that corresponds to the last dilution visible band observed in the gel by UV fluorescence on a transilluminator (Alphaimager® HP). Band size was determined according to a 100 bp ladder (Promega, Madison, USA) running parallel along with the samples at 100 volts for 15 minutes in an electrophoresis tank (Labnet, Inc.). For the real time PCR, detection limits where determined by Ct values crossing a threshold line set at 0,000605 for all analyses. The threshold line was set according to mid height of the quantification curves of the standard plasmids.

2.2. Animal experiments

The pathogenicity studies consisted of two different trials done by inoculation of domestic pigs with ASF viruses. The first one was designated Virulence trial which evaluated the pathology resultant from infection with different isolates of ASFV and the second one as Vaccination trial for the cross-protection analysis of a first 'infection' with a non-virulent strain prior to inoculation with a virulent virus. Both these animal trials were done according to ethical guidelines described below.

2.2.1. Ethical consideration and approval

Animal experiments were conducted strictly according to animal welfare ethics. The ARC/OVI Ethics Committee and the Faculty of Veterinary Science of the University of Pretoria Ethics Committee approved this project (see Appendix A). End-points were pre-determined on a score sheet recommended by the National Society for the Prevention of Cruelty to Animals (NSPCA) in which animals that scored higher than 10 had to be euthanized (see Appendix B). Therefore, to minimize discomfort, all individuals that reached this point were euthanized when deemed necessary.

2.2.2. Facilities

Experiments took place in the TADP stables, which are bio-safety level 3 buildings and are equipped with a negative pressure system with Hepa filters and sealed doors in each compartment therefore containing viruses solely inside each room. Stables sizes varied between 36 m^2 to 72 m^2 allowing comfortable space for movement and cleaned twice daily, fresh water was available *ad libitum* and feed was provided daily in the form of concentrate pellets.

2.2.3. Virus inoculums

Viruses used were:

- Non-virulent isolate ASFV OURT 3/88 obtained from *Ornithodoros erraticus* tick in the Alentejo province in Portugal (Boinas, 1995), passaged in BC;

- Highly virulent isolate collected from a domestic pig in Benin, named ASFV BENIN 1/97 (Chapman *et al.*, 2008), passaged in BC;
- South African non-haemadsorbing virus designated as ASFV MKUZE, isolated from an *O. porcinus* tick in the Mkuze game reserve and of unknown pathology (Thomson, 1985), passaged in BC;
- Suspected virulent ASFV MOZ 1/98 isolate collected in the province of Tete in Northwest Mozambique in 1998 from an acutely infected pig (Penrith *et al.,* 2004b), passaged in BC.

Virus isolates were cultured and titrated in BC cells. Titres of these inoculums were adjusted by further culturing or diluting in growth medium to 10^4 HAD_{50}/ml. Growth medum was prepared by mixing 95% RPMI-1640 media (SIGMA®), 5% foetal bovine serum (GIBCO®), 0.5% combination of antimicrobial and antimycotic (GIBCO®) that contained 10,000 units of penicillin (base), 10,000 µg of streptomycin (base), and 25 g of amphotericin B/ml utilizing penicillin G (sodium salt), streptomycin sulphate, and amphotericin B in 0.85% saline.

All inoculums were sterilized using 0.22 µm non-pyrogenic bacterial filters (Millex®GP) before infection and tested according to the European Pharmacopoeia (2005), and on the universal bacteria 16S rRNA PCR (Standard quality control methods implemented at TADP) ruling out possible bacterial contamination. The inoculums were confirmed for the presence of ASF virus using the p72 PCR.

2.2.4. Experimental animals

The animals utilised in this research were female domestic Landrace x Large White pigs that weighed around 35 kg, aged approximately 3 months at the time of inoculation. These pigs came from the Animal Production Unit in ARC that abides by bio-safety rules, situated in an ASF free region, and tested negative to ASF, FMD, PRRS, and CSF. They entered the high containment facility at TADP at least one week prior to challenge to allow for habituation to the new environment. For two weeks before the start of the trial pigs were under quarantine observation. Pigs identified as shown in Table 2.6.

Table 2.6 Identification of individuals numerically and by inoculation.

Virulence Trial		Vaccination Trial		
Inoculation	Pig number	Inoculation	Pig number	Group
OURT 3/88	1 2 3	2 x OURT 3/88 1 x MOZ 1/98	1 2 3 4 5 6	1
MOZ 1/98	1 2 3			
MKUZE	1 2 3	1 x OURT 3/88 1 x MOZ 1/98	1 2 3 4 5 6	2
BENIN 1/97	1 2 3	1 x MOZ 1/98	1 2 3	3

2.2.5. Virulence trial design

The virulence trial consisted of infections with a different virus isolate of ASFV per group of three pigs and it was designed to serve as controls to provide background information for the immunization trial that followed. Thus, the pathology of four different isolates of ASFV was the subject of evaluation for virulence in naïve domestic pigs. Three individuals were inoculated with each virus separately, leading to 12 infected animals in total. Three non-inoculated pigs were euthanized and blood, sera and organ samples collected to serve as negative controls. Animals in each group were randomly allocated to different stables. Each group was kept in a separate room and a qualified veterinarian monitored the trials daily.

On the same day, all groups of pigs had an intramuscular (IM) injection of 1 ml of each live virus at 10^4 HAD_{50}/ml in the left hind leg. After inoculation, examination of animals was performed twice daily for presence of clinical signs. Temperatures were recorded every morning until death or termination based on ethical

considerations. Furthermore, all individuals were subjected to collection of blood and sera scheduled as described in Table 2.7 to evaluate sero-conversion and viraemia. Animals were examined by necropsy after either euthanasia or death. During post-mortems, lesions and organ samples (see 2.2.9) were collected.

Table 2.7 Virulence Trial data collection time schedule.

Days	-2	0	3	5	7	14	20
	Pre-bleed	Infection					Termination
Sera	x	x	x	x	x	x	x
Blood	x	x	x	x	x	x	x
Rectal ˚C	Daily						
Clinical signs	Daily						

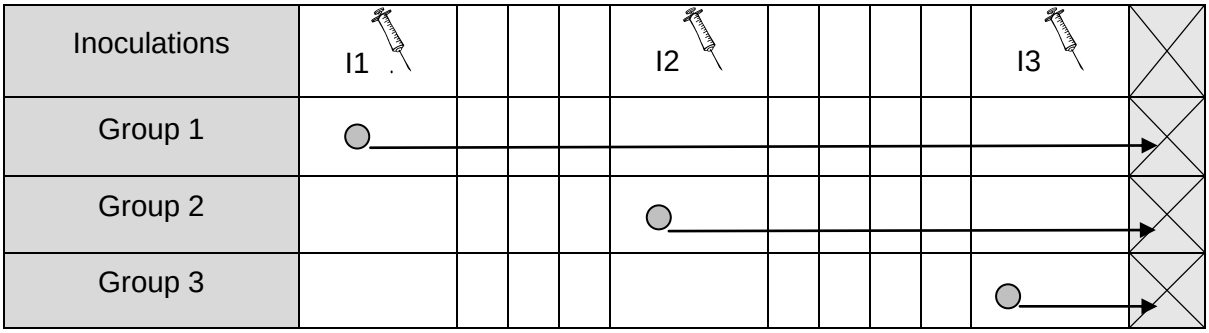
x: collection date

2.2.6. Vaccination trial design

Immunization trials were designed to test the cross-protection potential of a non-virulent isolate of ASFV (ASFV OURT 3/88) against a virulent southern African isolate from Mozambique (ASFV MOZ 1/98) in domestic pigs. Fifteen domestic naïve pigs were divided randomly into three groups. Group 1, consisting of six animals, received inoculations with 10^4 HAD_{50}/ml of non-virulent isolate ASFV OURT 3/88 at day 0 and day 21 and was challenged with ASFV MOZ 1/98 at day 49. All six animals in Group 2 were inoculated once with ASFV OURT 3/88 and challenged 28 days later. The third group of three animals (control group) functioned as control for the pathogenicity of virulent ASFV MOZ 1/98. Vaccinations were staggered to allow the challenge inoculation to occur simultaneously in all groups. The inoculation timeline is demonstrated in Table 2.8.

Table 2.8 Vaccination trial lay out.

Days	0				21					49	T



I1: First inoculation with ASFV OURT 3/88; I2: Second inoculation with ASFV OURT 3/88; I3: Challenge with ASFV MOZ 1/98; T: Termination.

Inoculations of suspended viral isolates and subsequent monitoring were as explained in 2.2.4. Furthermore, collection of blood, temperature, and clinical signs carried out according to Table 2.9.

Table 2.9 Vaccination trial data collection time schedule

Days		0	3	5	7	10	14	21	24	26	28	31	35	49	52	54	56	60
Group 1	Blood	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	
Group 2	Blood							x	x	x	x	x	x	x	x	x	x	x
Group 3	Blood													x	x	x		
Rectal ˚C		Dailly																
Clinical signs		Daily																

x: collection date

2.2.7. Recording of clinical signs

Animals were under clinical observation until termination, and from the onset of apparent disease, clinical signs were vigilantly recorded. Routine temperature measurement was performed daily by means of physical immobilisation and noting of clinical signs such as behavioural discrepancies like reduced appetite or water intake, depression, posture, huddling together, lethargy, lesions on the skin, blood

flow in conjunctiva or vaginal mucosa, disease related secretions, stools and urine appearance, temperature and tremors by simple observation.

2.2.8. Blood and sera samples

Pigs were restrained for blood and sera collection by chemical sedation via injection of Azaperone 40 mg/ml (1-2 ml / 20 kg). The collection schedule for the virulence trial is in Table 2.7 and for the vaccination trial in Table 2.9. Blood and sera were obtained from the anterior vena cava and collected into anticoagulant BD Vacutainer® tubes with liquid K_3EDTA and BD Vacutainer® tubes with clot activator and gel for serum separation. A duplicate sample from every blood collection was stored at -80ºC. Sera were aliquoted and stored at 4ºC until analysis.

2.2.9. Post-mortem procedures and sampling

At time of death, post-mortems were performed on every pig in order to register the macroscopic pathological findings. Necropsies were performed according to standard techniques with external physical examination and internally by opening the carcass. Tissue sample collection ensued during internal examination and in agreement with a pattern from less possible contamination loci to more potential infectious tissues. Skin from the tip of the ear; tonsils; lungs; heart; mediastinal lymph nodes; spleen; liver; kidney; gastro-hepatic lymph node; mesenteric lymph nodes; gastric and intestinal mucosa; necrotic skin wounds and bladder were collected in 4.5 ml cryotube vials (Nunc ™) for storage at -80ºC for virus load analysis.

2.2.10. Antibody detection

Serum samples were centrifuged at 6000 g in an Eppendorf centrifuge 5702 (Hamburg, Germany) for 10 minutes and the liquid phase was used for the serological analysis by ELISA. Serological antibody detection was performed by means of the blocking ELISA Ingezim PPA Compaq (INGENASA, Madrid, Spain) according to the kit protocol.

All reagents reached room temperature before starting the test except for the conjugate. Fifty µl of serum was introduced into each well in addition to the test samples; positive and negative controls were included in duplicate. The plates were coated with antigen that consists of an ASFV vp72 purified protein extract. The remaining wells were filled with 50 µl of sera samples arranged in duplicate. The plate was incubated for one hour at 37ºC. Washing ensued four times. Subsequently, 100 µl specific conjugate consisting of monoclonal antibody against vp72 conjugated with peroxidase was introduced to each well and incubated for 30 minutes at 37ºC. Afterwards, another washing procedure was repeated five times. Substrate was added to every well and incubated at room temperature for 15 minutes. Finally, stop solution was added to block the colorimetric reaction and the plate was read on a 450 nm filter and sample status assigned according to the following:

Positive cut off = NC - ((NC-PC) x 0.5)

Negative cut off = NC - ((NC-PC) x 0.4)

NC= OD of Negative Control Serum
PC= OD of Positive Control Serum
OD= Optical Density

Serum samples with an OD lower than the positive cut off were considered positive to ASFV antibody and negative if higher than negative cut off sera. When these values were between both cut offs then it was taken as ambiguous or uncertain results.

2.2.11. DNA extraction of blood samples

DNA extraction from blood samples was done within 48 hours post-collection according to the protocol for isolation of nucleic acids from mammalian whole blood in the High Pure PCR Template Purification Kit (Roche, Mannheim, Germany). Briefly, binding buffer and proteinase K were added to 200 µl of whole blood and incubated for 10 minutes at 70ºC. Thereafter, isopropanol was added and the sample was introduced into a high filter tube supplied in the kit. Subsequently, five centrifugations with addition of buffers between each spin

according to the kits protocol was done in an Eppendorf centrifuge 5424 (Hamburg, Germany). The flow-through product was retained in collection tubes in the last step of elution of the DNA with 200 µl pre-warmed elution buffer at 70ºC. The final product was stored at -20ºC awaiting real time PCR analysis.

2.2.12. DNA extraction of organ samples

DNA extraction from organ samples was done according to the protocol for isolation of nucleic acids from mammalian tissue by utilizing the High Pure PCR Template Purification Kit (Roche, Mannheim, Germany). Tissue samples were cut and weighed and 25-50 mg of sample added to a nuclease-free 1.5 ml microcentrifuge tube. Maceration of sample material was done with sterile plastic pointers that adequately fit in 1.5 ml Eppendorf tubes containing the organ along with the addition of tissue lysis buffer and proteinase K. Total digestion of the macerate ensued by incubation at 55ºC for 1 hour. Subsequently all steps involved in extraction, elution, and storing are as described in 2.2.11.

2.2.13. Real time PCR on blood and organ samples

The Taqman® (New Jersey, USA) real time PCR (King *et al.*, 2003) described in Table 2.4, was utilised for quantification of ASFV present in DNA extracted product from blood and tissue material that were collected during both trials. The quantification via real time PCR requires the setup of a standard curve through predefined dilutions of plasmid pGEMp72TM in duplicate in order to set parameters for the determination of DNA load in each sample.

2.2.14. Statistical analyses

The program GraphPad Prism Version 5.02, 2008 was used to analyse the survival rates and compare the levels of viral DNA in blood and tissue samples detected during the experiments.

Chapter 3

Comparison of various diagnostic assays

3.1. Introduction

Sensitive and accurate detection of ASFV is important in order to provide a timely laboratory diagnosis of the disease and to determine the presence of virus and viral nucleic acid for research purposes. This study aimed to compare a number of in house and published tests that are currently in use, including cell culture diagnosis and molecular detection of ASFV. These were: HAD via use of leucocytes from three different organs in the pig and various PCRs (Figure 2.1). Viruses used here were previously adapted to cell culture: ASFV MOZ 1/98 grown six times in BC with a titre of 1 x 10^4 $TCID_{50}$/ml, and ASFV SPEC 257 grown for 12 times in BC with a titre of 1 x 10^6 $TCID_{50}$/ml. The sensitivity of these assays was determined using serial 10-fold dilutions of each viral suspension by either noting the resulting titres achieved in HADs or by the respective detection limits visualised in agarose gels by electrophoresis of every PCR amplicon that was obtained.

3.2. Results

3.2.1. Haemadsorption assays

The diagnostic sensitivity experiment using HAD assays with three different types of cells was performed in order to determine the most reliable and sensitive primary cell culture for virus growth and detection. Reliability and sensitivity were determined comparing the corresponding titres obtained from the scoring of rosettes present in each dilution and by repeatability of results over time.

All HAD assays were examined according to efficiency of the cell culture in terms of being able to score the respective titres of each viral inoculation accurately and repetitively. Since these were both haemadsorbing viruses, when all steps were performed correctly and without contaminations or other negative external factors affecting titrations, there should be a haemadsorption reaction (Figure 3.1) in the cell cultures. Therefore, rosettes were microscopically detected throughout the

viral suspension serial ten-fold dilution inoculated along columns in plates. In some situations, however, plates were considered unreadable because of the lack of formation of rosettes and/or contamination that could be physically observed by a change in medium colour and by observing contaminants under the microscope.

Results of titres in each weekly ten-fold dilution of both ASFV MOZ 1/98 and ASFV SPEC 257 inoculated on the three cell cultures are shown in Table 3.1 and Table 3.2. Initially cell cultures were compared by using both viruses for ten repetitions. Analysis on cultures of the same cell types using ASFV SPEC 257 was further done totalling 24 repetitions of inoculations (Table 3.3 and Table 3.4). The latter analysis was also useful for the weekly routine monitoring of cell quality (cell quality control) by the ASF diagnostics department for HAD testing on incoming samples of unknown status and suspicious of ASF.

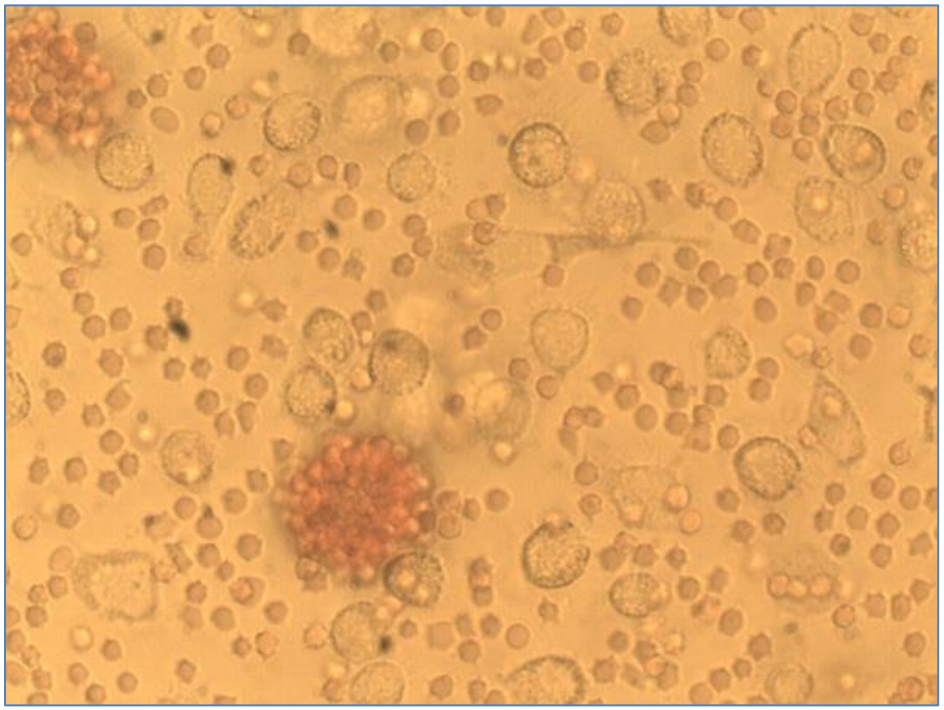


Figure 3.1 Image of haemadsorption of red blood cells to macrophages in blood cultures inoculated with ASFV MOZ 1/98.

Table 3.1 HAD cell culture titres comparison using ASFV MOZ 1/98 and ASFV SPEC 257 (determined 96 hours post inoculation by the Spearman Karber method)

Assay	Culture	ASFV MOZ 1/98	ASFV SPEC 257
		$TCID_{50}$	$TCID_{50}$
1	BMC	4.7×10^{3}	1.1×10^{6}
	BC	4.7×10^{3}	3.6×10^{6}
	AMC	2.7×10^{4}	3.6×10^{6}
2	BMC	6.3×10^{3}	3.6×10^{8}
	BC	1.5×10^{3}	1.5×10^{3}
	AMC	----------	----------
3	BMC	4.7×10^{4}	6.3×10^{7}
	BC	1.5×10^{2}	----------
	AMC	4.7×10^{3}	1.1×10^{7}
4	BMC	2.7×10^{5}	2.7×10^{4}
	BC	6.3×10^{2}	4.7×10^{6}
	AMC	----------	----------
5 (1)	BMC	2×10^{6}	2×10^{7}
	BC	$4{,}7 \times 10^{5}$	$6{,}3 \times 10^{6}$
	AMC	$1{,}5 \times 10^{5}$	$6{,}3 \times 10^{6}$
6 (2)	BMC	$1{,}5 \times 10^{5}$	$4{,}7 \times 10^{6}$
	BC	$1{,}1 \times 10^{5}$	$8{,}4 \times 10^{5}$
	AMC	----------	----------
7 (3)	BMC	----------	$6{,}3 \times 10^{6}$
	BC	----------	$8{,}4 \times 10^{4}$
	AMC	----------	----------
8 (4)	BMC	----------	$1{,}1 \times 10^{6}$
	BC	----------	$1{,}5 \times 10^{7}$
	AMC	----------	----------
9 (5)	BMC	$1{,}1 \times 10^{5}$	2×10^{6}
	BC	$1{,}1 \times 10^{5}$	3.6×10^{6}
	AMC	----------	----------
10 (6)	BMC	3.6×10^{5}	$1{,}1 \times 10^{7}$
	BC	2×10^{4}	$6{,}3 \times 10^{4}$
	AMC	----------	----------

---: Unreadable; BMC: Bone marrow culture; BC: Blood culture; AMC: Alveolar macrophage culture; numbers in brackets correspond to the dilutions of virus that are repeated in Table 3.1 and Table 3.3, and were used in the PCR assays as well.

For ASFV MOZ 1/98 and ASFV SPEC 257, both causing haemadsorption, BMC and BC were more reliable as is demonstrated in Table 3.1 and Table 3.2. The cultures had better results for both repeatability and sensitivity with BMC and BC often obtaining higher titres than AMC. However, there were statistically no significant differences between BMC and BC for ASFV MOZ 1/98 over the first eight observations (repeats of inoculations with ASFV MOZ 1/98 presented in Table 3.1; $p = 0.17$ on the paired t test). The same applies to ASFV SPEC 257 over 24 repeats of inoculations with ASFV SPEC 257 (demonstrated in Table 3.1 and Table 3.3; $p = 0.32$ on the paired t test). AMC results were not included in the statistical analysis, as there were too many omissions.

Table 3.2 HAD cell culture efficacy comparison of ASFV MOZ 1/98 and ASFV SPEC 257

Culture	ASFV MOZ 1/98		ASFV SPEC 257	
	Readable	The number of times the highest titre was obtained amongst cell cultures	Readable	The number of times the highest titre was obtained amongst cell cultures
BMC	8/10 (80%)	7	10/10 (100%)	8
BC	8/10 (80%)	3	9/10 (90%)	4
AMC	3/10 (30%)	1	3/10 (30%)	1

Table 3.3 HAD titres for ASFV SPEC 257 cultures (determined 96 hours post inoculation by the Spearman Karber method).

Assay	ASFV SPEC 257	
	Cell culture	$TCID_{50}$
1	BMC	1.1×10^{6}
	BC	3.6×10^{6}
	AMC	3.6×10^{6}
2	BMC	3.6×10^{8}
	BC	1.5×10^{3}
	AMC	----------
3	BMC	6.3×10^{7}
	BC	----------
	AMC	1.1×10^{7}
4	BMC	2.7×10^{6}
	BC	8.4×10^{6}
	AMC	4.7×10^{6}

5	BMC	2.7×10^4
	BC	4.7×10^6
	AMC	----------
6	BMC	1.5×10^7
	BC	1.5×10^7
	AMC	8.4×10^6
7	BMC	4.7×10^8
	BC	----------
	AMC	----------
8	BMC	3.6×10^6
	BC	2.7×10^6
	AMC	----------
9	BMC	1.2×10^6
	BC	2.7×10^4
	AMC	2.7×10^6
10	BMC	----------
	BC	1.5×10^4
	AMC	----------
11	BMC	----------
	BC	1.5×10^4
	AMC	----------
12	BMC	----------
	BC	1.5×10^5
	AMC	----------
13	BMC	1.5×10^4
	BC	6.3×10^5
	AMC	----------
14	BMC	1.1×10^5
	BC	6.3×10^2
	AMC	----------
15 (1)	BMC	2×10^7
	BC	$6{,}3 \times 10^6$
	AMC	$6{,}3 \times 10^6$
16 (2)	BMC	$4{,}7 \times 10^6$
	BC	$8{,}4 \times 10^5$
	AMC	----------
17 (3)	BMC	$6{,}3 \times 10^6$
	BC	$8{,}4 \times 10^4$
	AMC	----------

18 (4)	BMC	$1,1 \times 10^6$
	BC	$1,5 \times 10^7$
	AMC	----------
19 (5)	BMC	2×10^6
	BC	3.6×10^6
	AMC	----------
20 (6)	BMC	$1,1 \times 10^7$
	BC	$6,3 \times 10^4$
	AMC	----------

---: Unreadable; BMC: Bone marrow culture; BC: Blood culture; AMC: Alveolar macrophage culture; numbers in brackets correspond to the dilutions of virus that are repeated in Table 3.1 and Table 3.3, and were used in the PCR assays as well.

According to Table 3.4 and Table 3.3, BMC and BC were almost equally reliable types of cell cultures given the highest number of scored or readable plates and highest titres acquired.

Table 3.4 HAD cell culture efficacy comparison of all ASFV SPEC 257 titrations performed

Culture	Readable	The number of times the highest titre was obtained amongst all cell cultures
BMC	21/24 (87.5%)	17
BC	21/24 (87.5%)	13
AMC	8/24 (33.3%)	6

3.2.2. PCR sensitivity trials

The sensitivity analysis of various PCRs commonly used in the detection of ASFV was based on the same principles as above in relation to reliability. Sensitivity was analysed according to detection limits (DL) obtained in each run by observing the faintest visible DNA band in the agarose gel electrophoresis that corresponded to a certain log value of the ten-fold dilutions for both ASFV MOZ 1/98 and ASFV SPEC 257 and virus copy number was determined by real time PCR. DNA extractions were performed on the same day the virus dilutions were prepared in order to avoid skewed results due to loss of concentration of the virus over time. Detection limits were analysed on agarose electrophoresis gels at 2% for the ns

PCR and 1% on all others as product genome segments differ in size and consequently migrate at different rates in the gel. Photographs were taken to catalogue gels (Appendix A). Figure 3.2 to Figure 3.9 shows gel photographs of the PCR products obtained from p72, CVR, PPA, nested and real time PCRs from the second repeat of ten-fold dilutions (neat to 10^{-8}) for both ASFV MOZ 1/98 and ASFV SPEC 257.

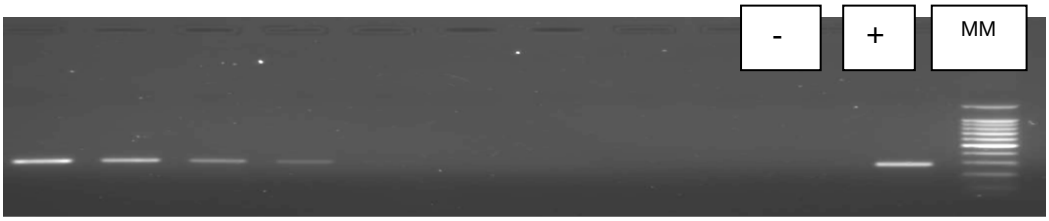


Figure 3.2. p72 PCR of dilution from week 2 of ASFV MOZ 1/98

Figure 3.3. CVR PCR of dilution from week 2 (ASFV MOZ 1/98)

Figure 3.4 PPA PCR of dilution from week 2 (ASFV MOZ 1/98)

Figure 3.5. Nested PCR of dilution from Week 2 (ASFV MOZ 1/98)

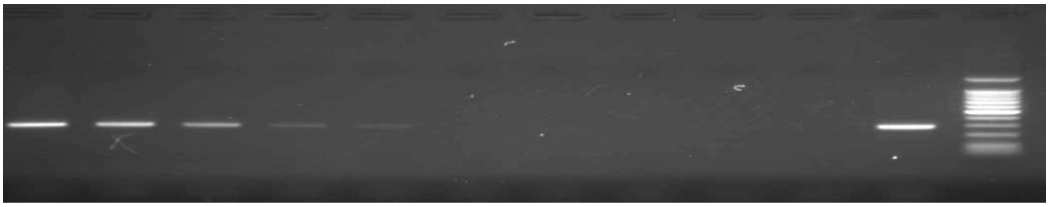


Figure 3.6. P72 PCR of dilution from week 2 (ASFV SPEC 257)

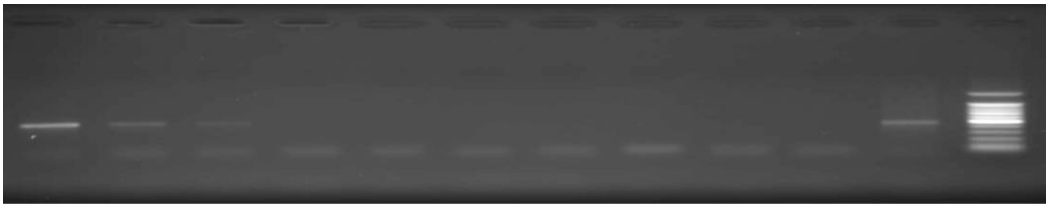


Figure 3.7. CVR PCR of dilution from week 2 (ASFV SPEC 257)

Figure 3.8. PPA PCR of dilution from week 2 (ASFV SPEC 257)

Figure 3.9 Nested PCR of dilution from week 2 (ASFV SPEC 257)

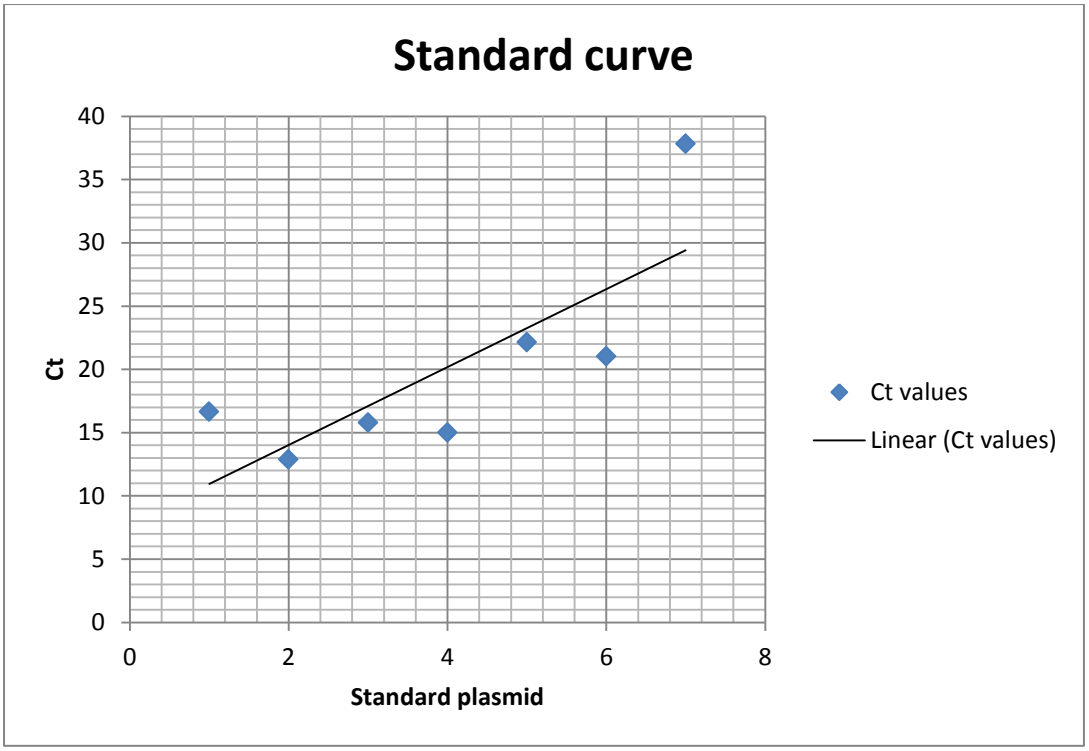


Figure 3.10 Standard curve of real time PCR from standard dilutions of pGEMp72TM for determination of detection limits by use of ASFV MOZ 1/98.

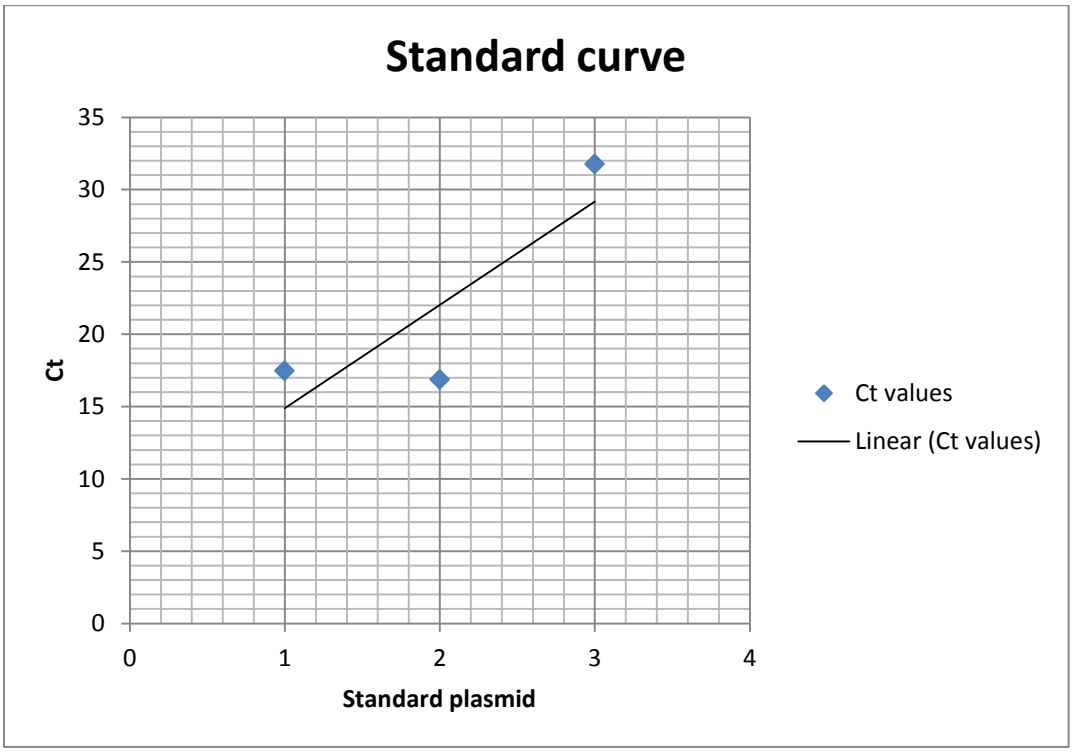


Figure 3.11 Standard curve of real time PCR from standard dilutions of pGEMp72TM for determination of detection limits by use of ASFV SPEC 257.

A Taqman real time PCR run includes a plasmid with standard sets of concentration included along diluted repeats in order to generate a single standard curve that validates each run (Figure 3.10 and Figure 3.11). Here the plasmid used was pGEMp72TM. It is intended to generate a standard curve from the known concentrations of plasmid dilutions that control the quality of the run and allow quantification of samples that are being analysed.

The concentration of virus is given as virus particle per ml (vp/ml) for each limit dilution that was detected for every PCR in the trial (Table 3.5 and Table 3.6). These were determined by the resultant dilution point that was considered as detection limit for each test and subtracted to the initially known titre of either ASFV MOZ 1/98 or ASFV SPEC 257.

Table 3.5 Virus particle per ml (VP/ml) of detection limits (DL) on PCRs by using ten-fold dilutions of ASFV MOZ 1/98 (BC passage 6) at starting titre of 1×10^4 $TCID_{50}$/ml.

Test number	PCR	CVR	p72 PCR	PPA	Nested	Real time
1	DL	10^{-2}	10^{-4}	10^{-5}	10^{-1}	10^{-3}
	VP/ml	10^{2}	10^{0}	10^{-1}	10^{3}	10^{1}
2	DL	10^{-1}	10^{-3}	10^{-4}	10^{-1}	10^{-4}
	VP/ml	10^{3}	10^{1}	10^{0}	10^{3}	10^{0}
3	DL	10^{0}	10^{0}	10^{-1}	10^{0}	10^{-1}
	VP/ml	10^{4}	10^{4}	10^{3}	10^{4}	10^{3}
4	DL	10^{-3}	10^{-1}	10^{-1}	10^{-1}	10^{-1}
	VP/ml	10^{1}	10^{3}	10^{3}	10^{3}	10^{3}
5	DL	10^{-2}	10^{-3}	10^{-4}	10^{-1}	10^{-3}
	VP/ml	10^{2}	10^{1}	10^{0}	10^{3}	10^{1}
6	DL	10^{-1}	10^{-3}	10^{-1}	10^{0}	10^{-4}
	VP/ml	10^{3}	10^{1}	10^{3}	10^{4}	10^{0}

Based on the results obtained using the Kruskal-Wallis and Dunn's Multiple Comparison Test, there were no significant differences between the various PCR assays since P value was 0.919, when testing ASFV MOZ 1/98 dilutions.

Table 3.6. Virus particles per ml (VP/ml) of detection limits (DL) on PCRs by using ten-fold dilutions of ASFV SPEC 257 (BC passage 12) at a starting titre of 1 x 10^6 $TCID_{50}$/ml.

Test number	PCR	CVR	p72 PCR	PPA	Nested	Real time
1	DL	10^{-2}	10^{-4}	10^{-5}	10^{-3}	10^{-3}
	VP/ml	10^{4}	10^{2}	10^{1}	10^{3}	10^{3}
2	DL	10^{-2}	10^{-4}	10^{-5}	10^{-3}	10^{-3}
	VP/ml	10^{4}	10^{2}	10^{1}	10^{3}	10^{3}
3	DL	10^{-2}	10^{-4}	10^{-3}	10^{-4}	10^{-3}
	VP/ml	10^{4}	10^{2}	10^{3}	10^{2}	10^{3}
4	DL	10^{-1}	10^{-2}	10^{-5}	10^{-3}	10^{-3}
	VP/ml	10^{5}	10^{4}	10^{1}	10^{3}	10^{3}
5	DL	10^{-2}	10^{-3}	10^{-3}	10^{-2}	10^{-3}
	VP/ml	10^{4}	10^{3}	10^{3}	10^{4}	10^{3}
6	DL	10^{-2}	10^{-4}	10^{-6}	10^{-3}	10^{-3}
	VP/ml	10^{4}	10^{2}	10^{0}	10^{3}	10^{3}

The Kruskal-Wallis test showed significant differences (P=0.0011) between the various assays. The Dunn's Multiple Comparison Test showed that the significant differences were between the CVR and p72 PCR assays and between CVR and PPA PCR assays for ASFV SPEC 257 dilutions.

3.3. Discussion

The recognition of an ASF outbreak includes the observation of clinical signs in the field as well as laboratory testing for confirmation. In order to design a proper ASF control and eradication program, reliable tests are required. Ideally, cell culture proves the presence of live virus in the submitted sample and determines if it has haemadsorbing capacity when these are grown in cultures with red blood cells added. For non-haemadrorbing isolates, titrations are determined by detection of cytopathic effects on cells (Malmquist & Hay, 1960). PCR tests, however, generally have better sensitivity than cell cultures as it detects lower quantities of virus and can be used on samples that are inappropriately stored rendering the virus dead (Kong *et al.,* 2001; Sauer *et al.,* 2005; Flekna *et al.,* 2007). Furthermore, via PCR analysis results may be achieved quicker because of the minimum two-day incubation period that ASFV cell cultures require. Nevertheless, there are serological tests such as ELISA and IFA used in laboratories that do not use molecular tests and where there are not enough biosafety measures in place to be able to grow virus in order to produce a virus isolation test on cell cultures. A disadvantage of the serological techniques is they may not detect current infections. For example, acute ASF does not allow enough time for antibody production to take place in domestic pigs because they die early during course of disease. Note that IgM is used to detect sick animals and it is produced during acute infections but it was found in a majority of pigs only from seven dpi, which may be too late already. IgG is related to cases of subacute disease as it is detected as late as 14 dpi (Reis *et al.*, 2007). Therefore, none of these antibody detection tests would provide the most accurate laboratory diagnosis in case of an outbreak situation.

The haemadsorption assays tested three types of cell cultures for ASF virus isolation (AMC, BC and BMC), (SOP3, 5 and 6 TADP, ARC-OVI). By simple observation of results of this study, AMC was demonstrated to be the least reliable as it had less readable repetitions, was more susceptible to contamination, and had lower titres for both cultured viruses. This type of culture was characterized by many omissions of results in both ASFV MOZ 1/98 and ASFV SPEC 257 cultures, clearly showing it to be the less reasonable test culture to use in diagnostics. It is

important to keep in mind that the process of harvesting alveolar macrophages is prone to bacterial contamination from lung tissue since it is not such a sterile source as are the other two types of cell used for culture. BC and BMC, however, were very resilient in the sense that results were reliable and uncontaminated most of the time, facilitating observation of rosettes. The cell density typically seen in BMC made it less comfortable to read than BC but titres scored equally high in general. However, it should be noted that both viruses used in this study were previously adapted to blood culture by various passages and this may affect results.

Simple statistical analysis with paired t test revealed there were no significant differences between BMC and BC on the repetitions done with either ASFV MOZ 1/98 for eight observations, and also not for ASFV SPEC 257 over 24 observations. AMC results were not analysed because results had too many unreadable plates. Contamination was confirmed by change in the colour of media and bacterial presence via direct observation on the microscope of granulation inside macrophages resulting from phagocytosis, and for detection of movement of the same.

Furthermore, PCR sensitivity trials compared detection limits of each test used and virus copy number was determined for the corresponding dilutions of the viral suspension that indicated a limit of detection. The viruses used were the same as for the previous haemadsorbing assays as well as the serial dilutions corresponding to six repeats done in cell culture. Coincidentally, the same repeats that did not show results (three and four), apparently due to contamination of ASFV MOZ 1/98 suspension, are the same ones that strangely showed low detection limits for every PCR by using the same serial dilutions of such an isolate. Statistical analysis of these data indicated no significant differences between the various PCR assays ($P = 0.919$) when testing by use of the Kruskal-Wallis and Dunn's Multiple Comparison Test, probably due to interference with contaminations.

In relation to evaluation of detection limits of the various PCRs by use of ASFV SPEC 257 isolate the Kruskal-Wallis test showed significant differences ($P=0.0011$) amongst the various assays. The Dunn's Multiple Comparison Test

showed that the significant differences in terms of detection limits for each were between the CVR and p72 PCR assays and between CVR and PPA PCR assays.

By looking at results individually and comparing limits of dilution that were identified via a DNA band on agarose, PPA PCR detected the highest diluted samples, thus the lowest concentration of virus, in both trials done with ASFV MOZ 1/98 and ASFV SPEC 257. According to Aguero *et al*., 2003 PCR with primers PPA1 and PPA2 (referred to here as PPA PCR) when compared to the p72 PCR for their sensitivity ended up with similar results. Detection limits were equal but the last visible band photographed on agarose gel was brighter for PPA PCR, indicating it is slightly more sensitive than p72 PCR, which is recommended in the OIE manual of 2005 for diagnosis of ASFV. Only recently, the ASF OIE manual has been updated and it actually recommends PPA PCR over the mentioned p72 PCR (OIE manual 2012). Results in this experiment proved the same but by a log value difference between the dilutions to which each was able to detect. PPA PCR was shown to be more sensitive. King *et al.* (2003) analysed sensitivity of real time PCR compared to the p72 PCR used in this study as well and reached the same conclusion as the present work. By testing a battery of samples of various ASFV isolates in serial 10-fold dilutions, the author was able to demonstrate the capacity of real time PCR to find viruses in diluted suspensions, hence where a smaller quantity of virus is present. CVR PCR utilized in this trial gave similar results to the method that was originally intended for differentiation of viruses of the same genotype by sequence determination of the central variable region (CVR) of the ASFV genome (Bastos *et al.,* 2004). It is also compared to the aforementioned PCRs in the same way and it was shown to be the less sensitive test in this study. Recently, the OIE manual 2012 recommended the use of Taqman real time PCR (King *et al.*, 2003) for molecular detection of ASFV or the PPA PCR (Aguero *et al.*, 2003), both directed to the encoded protein p72. The manual also recommends use of the multiplex PCR that is similar to the previous one because it is combined with another set of primers that allows differentiation between CSFV and ASFV by processing at the same time (Aguero *et al.*, 2004).

Chapter 4

Virulence trial of four geographically distinct ASF isolates by inoculation into domestic pigs

4.1. Introduction

Animal infectivity trials were conducted since there is a lack of knowledge on the pathogenesis of certain ASFV isolates collected in southern Africa. The aim was to find a strain that would be suitable as a potential vaccine candidate and another pathogenic isolate from Southern Africa to function as challenge virus to test the expected immunizing capacity of the candidate vaccine. Ideally, the vaccine strain should be from Southern Africa and fully attenuated in order to be used as live vaccine.

Furthermore, the information generated here on the behaviour of certain ASFV isolates upon inoculation of commercial Landrace x Large White domestic pigs, such as general clinical observations, disease progression and specific lesions linked to viral presence in tissue samples will increase background knowledge on ASF, benefiting future clinical and genetic research.

Four strains were utilized in this study of which two have been previously studied. These are the European avirulent isolate ASFV OURT 3/88 (Boinas, 1995) and West African ASFV BENIN 1/97 which is known to be highly virulent and capable of causing acute ASF (Chapman *et al.*, 2008). The remaining two isolates are both from Southern Africa; a strain of unknown virulence designated ASFV MKUZE (Thomson, 1985) and ASFV MOZ 1/98 suspected to be highly virulent since it was collected from an acutely diseased pig (Penrith *et al.*, 2004b).

4.2. Results

Infections were done by a single intramuscular inoculation with one of the isolates (ASFV OURT 3/88; ASFV BENIN 1/97; ASFV MKUZE; ASF MOZ 1/98) in each

group of three domestic pigs, allowing observation of the pathological development post infection as explained in detail in chapter 2.

4.2.1. Preparation of inoculum

The haemadsorbing viruses ASFV MOZ 1/98 and ASFV BENIN 1/97 were grown on cell culture to confirm formation of rosettes and to determine the titre. For inoculation in pigs it was previously determined that a titre of 10^4 HAD_{50}/ml was adequate for intramuscular inoculation of domestic pigs (Penrith *et al.,* 2004b; Oura *et al.*, 1998a). This titre was achieved by either diluting viral suspensions in RPMI media or growing the isolates in pig primary cell culture as explained in 2.1.2. The inoculum or viral suspension was prepared to a volume of 1 ml with a titre of 1 x 10^4 HAD_{50}/ml. The other two isolates (ASFV OURT 3/88 and ASFV MKUZE) do not have haemadsorbing capacity thus rendering it difficult to determine their titre and therefore to manipulate concentration. In order to confirm the presence of ASFV in all four inoculums the PCR developed by Wilkinson (2000), directed at the vp72 gene, was done on a sample of each viral suspension. The products of each virus are shown in Figure 4.1 on a 1% agarose gel with a negative and positive control and a 100 bp molecular marker. Although concentration could not be determined from such DNA fragments observed in the gel, the intensity of the bands indicated ASFV OURT 3/88 and ASFV MKUZE suspensions were sufficient to perform inoculations when compared to the haemadsorbing viruses bands that appeared with lighter luminescence but set to a titre of 10^4 HAD_{50}/ml via cell culture.

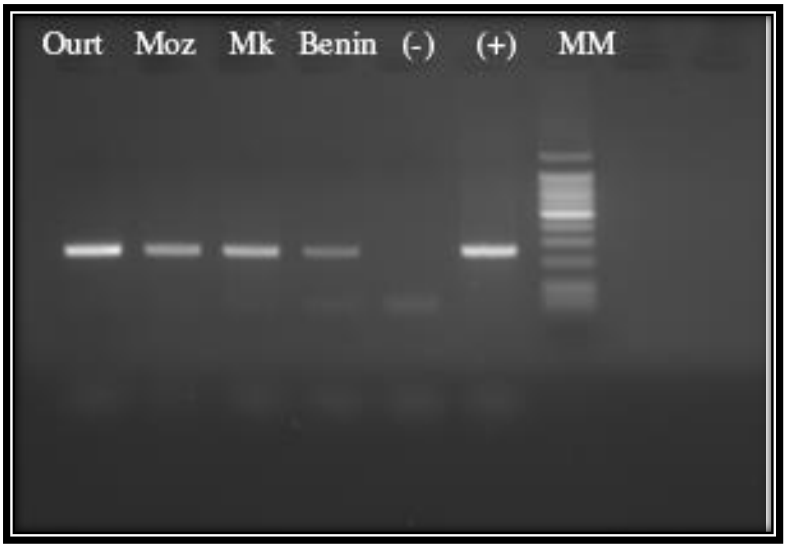


Figure 4.1 Diagnostic p72 PCR of inoculums ASFV OURT 3/88 as Ourt; ASFV MOZ 1/98 as Moz; ASFV MKUZE as Mk; ASFV BENIN 1/97 as Benin; (-) negative control; (+) positive control; MM: 100 bp molecular marker.

4.2.2. General outcome of infections in domestic pigs

Table 4.1 summarises the general outcome of the infections referred to as days post infection (dpi) of the start of clinical signs, the detection of viraemia in blood by real time PCR, sero-conversion using ELISA and date of death or euthanasia.

All pigs survived infection asymptomatically with ASFV OURT 3/88 until termination of the experiment at day 19. At day seven, one of the pigs had sero-converted. By 14 dpi, all three pigs were sero-positive. There was no viraemia detected in any of these pigs throughout the trial as determined by PCR.

The group infected with ASFV BENIN 1/97 showed clinical signs four dpi and viraemia three dpi using PCR. By seven dpi, sera were negative for ASF antibodies and since all three pigs reached animal welfare scoring for termination, euthanasia occurred at day seven.

Pigs inoculated with ASFV MKUZE were all showing fever at seven dpi although it may be due to the infected swelling in the hind leg, because of repeated needle trauma (see below). These animals only showed viraemia by 18 dpi and by 14 dpi, every animal had sero-converted. Note that both inoculations done with ASFV

OURT 3/88 and ASFV MKUZE continued for 19 and 18 days after infection respectively. Pigs were either not showing symptoms (ASFV OURT 3/88) or not too severe (ASFV MKUZE). The trials therefore continued for longer than that of ASFV MOZ 1/98 and ASFV BENIN 1/97. By the time of euthanasia, there were enough data rendering it unnecessary to continue submitting the pigs to confinement and discomfort.

The group of pigs infected with ASFV MOZ 1/98 registered the beginning of clinical signs as early as two dpi, viraemia at three dpi and rapidly died by six dpi without sero-converting.

Table 4.1 General characteristics of disease caused in pigs by ASF strains isolated from ticks and domestic pigs

Strain	Days post infection				
	Clinical Signs	Viraemia	Sero-conversion		Death
OURT 3/88	ND	ND	7 ø	14 øø	19*
MKUZE /78	7	18	7 (ND)	14 øø	18*
MOZ 1/98	2	3	-----	-----	6
BENIN 1/97	2	3	7 (ND)	-----	7

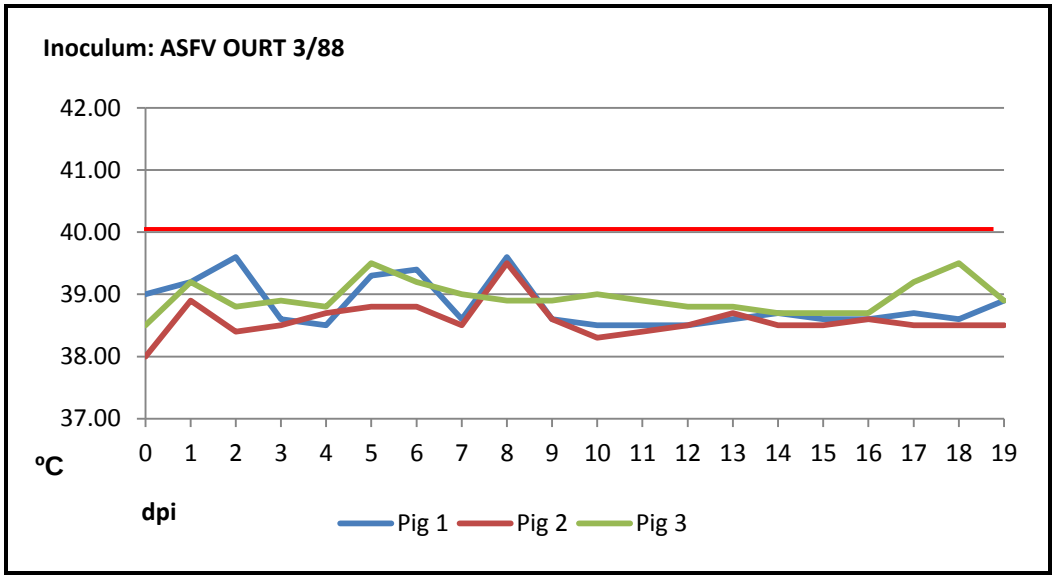
ND not detected; * Animals euthanized in the end but did not reach NSPCA score; ø one animal was positive; øø all pigs are sero-positive; -----: no samples collected.

Individual rectal temperatures of infected pigs are in Figure 4.2a) to Figure 4.2d). The three domestic pigs belonging to each inoculation group were numbered as Pig 1, Pig 2, and Pig 3. Fever was defined as rectal temperature above 40.0°C. The body temperature threshold to determine fever was set to compensate for the unwanted overheating these animals commonly show when they are stressed.

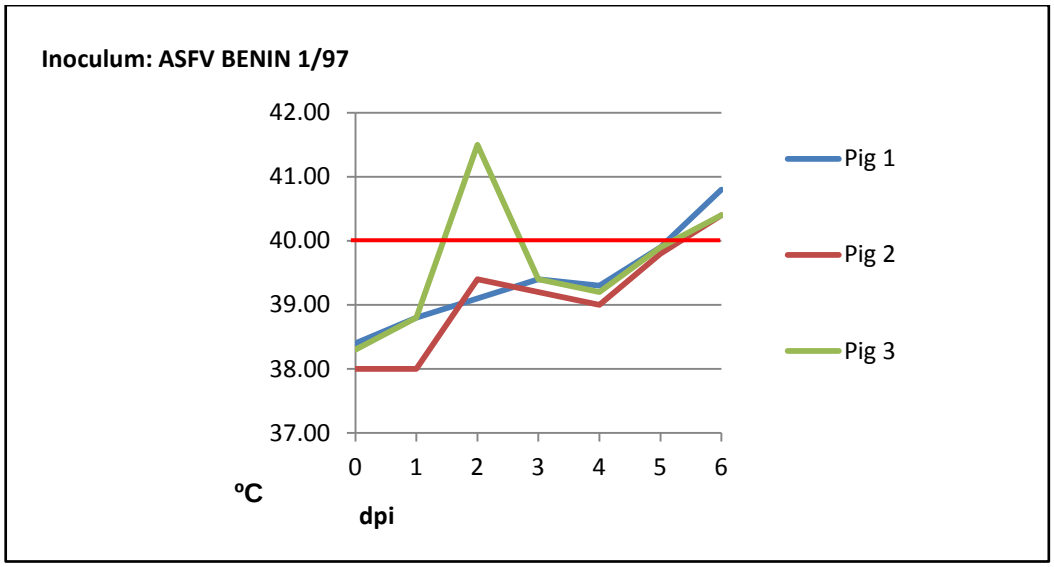
Analysis of the rectal temperature data chart reveals that infection with isolate ASFV OURT 3/88 did not produce any significant rise in temperature throughout the entire duration of the trial (Figure 4.2a). The group inoculated with ASFV BENIN 1/97, which is known to manifest ASF in the acute form (like the Mozambican isolate), had every pig's temperature entering the fever range (above 40.0°C) only at six dpi (Figure 4.2b) although a transient rise to 41.5°C in Pig 3

was observed at two dpi. Animals subjected to infection with ASFV MKUZE showed fever with temperatures rising above 40.0°C from five dpi in Pig 3, six dpi in Pig 2 and seven dpi in Pig 1 (Figure 4.2c). In contrast, ASFV MOZ 1/98 inoculation resulted in two animals (Pig 1 and Pig 3) reaching temperatures of 40.0°C as soon as two dpi and by three dpi all animals registered 40.0°C or above (Figure 4.2d).

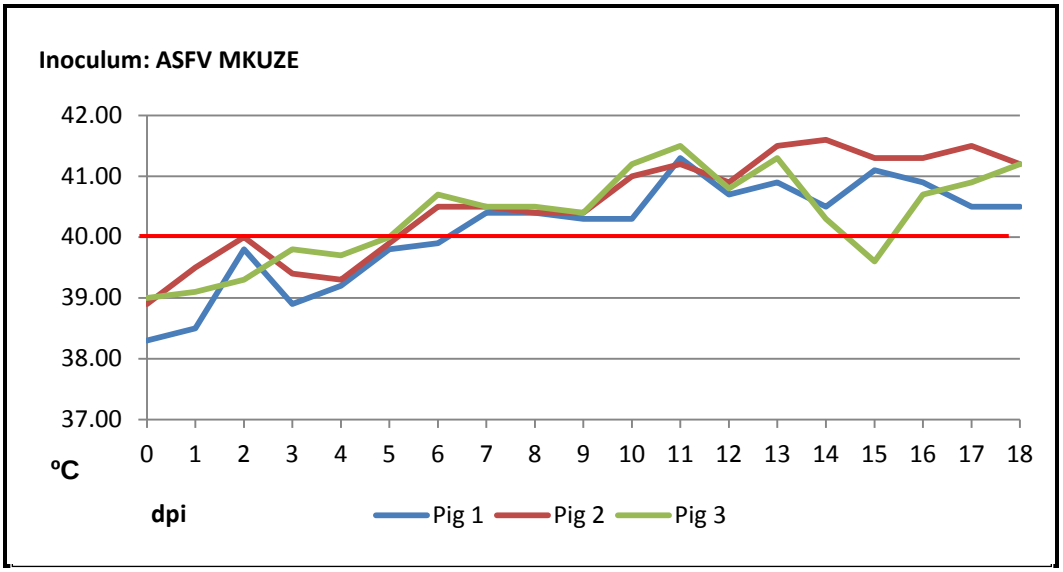
a)



b)



c)



d)

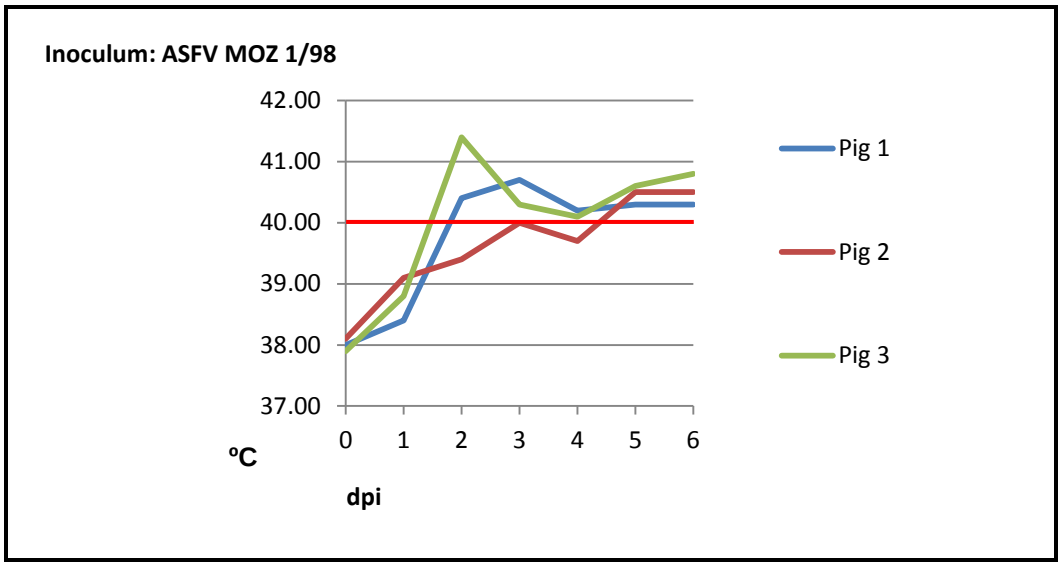


Figure 4.2 Rectal temperatures (°C) of three pigs per group (coulored lines) at days post infection (dpi) with a) ASFV OURT 3/88; b) ASFV BENIN 1/97; c) ASFV MKUZE and d) ASFV MOZ 1/98 until termination or death. Fever threshold (40.0°C) is indicated as a horizontal red line.

4.2.3. Clinical signs

Daily observation of pigs noted the clinical progression of every individual (Table 4.2). Animals that were infected by ASFV OURT 3/88 did not show clinical signs for the duration of the trial therefore being confirmed as asymptomatic. Inoculation with ASFV MKUZE manifested a clinically moderate outcome and according to clinical signs and duration of disease, defined as a chronic type. Although the

three pigs in this group developed fever and complications derived from the excess needle trauma to muscles in the hind leg, these animals did not require ethics intervention until final euthanasia at termination of the trial. In contrast, groups that were infected with ASFV MOZ 1/98 and ASFV BENIN 1/97 showed typical, acute ASF. Both isolates manifested clinically as acute since animals died or were selected for termination as soon as seven dpi. Two of the pigs in the ASFV BENIN 1/97 inoculation group reached the NSPCA scoring system threshold that qualified them for humane euthanasia at day seven (Pig 2 and Pig 3). In all others that died without chemical termination (all three pigs in ASFV MOZ 1/98 group and Pig 1 in ASFV BENIN 1/97 group), it happened overnight before their having qualified for euthanasia. Clinical signs and time relation of disease progression were consistently similar for both these isolates.

Clinical signs in the high virulent inoculum groups (ASFV MOZ 1/98 and ASFV BENIN 1/97) started by pigs showing serous ocular discharge from two dpi which lasted throughout the remaining days progressing to mucopurulent ocular discharge in Pig 2 of the ASFV BENIN 1/97 infected group by seven dpi. From five to seven dpi all pigs in ASFV MOZ 1/98 group showed evident congestion in sclera, conjunctiva, periorbital area, ears, snout, and body in general. They presented as lethargic, showing slow reaction to touch, off feed and with tremors. Animals in this group died overnight from six to seven dpi and Pig 3 was showing bloody froth protruding from the nostrils when found dead in the morning. In the ASFV BENIN 1/97 group, congestion in the sclera, conjunctiva, periorbital area, ears, snout, and body in general showed at day five. By day six, bloody diarrhoea, huddling together, tremors as well as serious lethargy and innapetence could be seen in every pig. While Pig 1 died overnight, from six to seven dpi, the remaining Pig 2 and Pig 3 stopped eating by morning, qualifying for humane euthanasia.

Complications due to needle trauma briefly referred to above in the group of suidae inoculated with ASFV MKUZE may be related to severe inflammation that these animals developed in the hind leg at the inoculation site and if not directly related to effects of the ASF strain, could have compromised the study. Swelling was identified from eight dpi although it is not described in Table 4.2. Also at eight dpi, all pigs showed fever and serous ocular discharge. As days passed the

swelling increased and consequent ulceration of the skin accompanied by progressively worse lameness that led to evident reluctance to move, and it may have been the origin of the fever presented in all pigs at eight dpi. Thus, this could be derived from an infection at the site where needle trauma was intense because of the various IM injections given for different purposes (inoculation or sedation). Pigs infected by ASFV OURT 3/88 showed mild swelling at the site of inoculation. Measures were taken to minimize discomfort and to prevent compromising further analyses of results in the MKUZE inoculated group. Therefore, hydro massage to the affected area was being done daily and an anti-inflammatory (phenylbutazone) administered at 11, 14, and 15 dpi in order to limit inflammation as well as to decrease fever and pain. From 10 dpi, these pigs began to show diarrhoea and by the termination date, congestion in the ears and tail, huddling together and showing a slow reaction to touch while very depressed were the clinical signs recorded. Once these pigs were euthanized, post-mortems were done and circular necrotic lesions in the skin were observed in Pig 1 and Pig 3. According to Leitão *et al.* (2001), this type of lesion in the skin has been described in cases of chronic ASF. Kleiboeker *et al.* (2002) included this lesion in an ASF pathogenesis review as a pathological finding of chronically affected pigs.

Table 4.2 Clinical signs of ASFV inoculations in the virulence trial pigs

Clinical signs: serous ocular discharge (π) & muco-purulent ocular discharge (ππ); congestion (c) & cyanotic congestion (cc) of ears (e), snout (s), tail (t), periorbital (o) or body in general (b), conjunctiva (cj) and sclerae (sc), e.g. congestion of snout (cs); pinpoint haemorrhages on the skin (ħ); diarrhoea (D); bloody diarrhoea (Dħ); tremors (¥); huddling together (Δ); lethargy/apathy/depressed/slow reaction to touch (‡); prostration (†); fever above 40 °C (F); off-feed (ø); euthanized (E); Died (×).

Pigs / Days	OURT 3/88	BENIN 1/97			MKUZE			MOZ 1/98		
	1; 2; 3	1	2	3	1	2	3	1	2	3
0										
1										
2		Π	Π	F, π		F		F, π	Π	F, π
3		Π	Π	Π				F, π	F, π	F, π
4		Π	Π	Π				F, π	Π	F, π
5		π, ‡, ce, cs, cpo, csc,ct	π, ‡, ce, cs, cpo, csc, ct	π, ‡, ce, cs, cpo, csc, cb, Dħ			F	F, π, ‡, ce, cs, cpo, csc, cb,	F, π, ‡, ce, cs, cpo, csc, cb	F, π, ‡, ce, cs, cpo, csc, cb
6		F, π, ‡, Dħ, Δ	F, π, ‡, Dħ, Δ, ¥	F, π, ‡, Dħ, Δ, ¥		F	F	F, π, ‡, ce, cs, csc, ccj, ø	F, π, ‡, ce, cs, csc, ccj, cb, ¥, ø	F, π, ‡, ce, ¥, ø
7		X	ππ, Dħ, ø, Δ, ‡, E	π, Dħ, ø, Δ, ‡, E	F, π	F, π	F, π	×	×	×
8					F, π	F, π	F, π			
9					F, π	F, π	F, π			
10					F, π	F, π	F, D, π			
11					F, π	F, π	F, D, π			
12					F, π	F, π	F, D, π			
13					F, π	F, D, π	F, D π			
14					F, D, π	F, D, π	F, D, ce, π			
15					F, D, π	F, D, π	F, D, ce, π			
16					F, D, π	F, D, π	F, D, ce, π			
17					F, D, ce, ct, ‡, Δ, π	F, D, ‡, Δ, π	F, D, ce, ct, ħ, ‡, Δ, π			
18					E	E	E			
19	E									

4.2.4. Serology

To identify antibody production in the different groups of pigs, sera were collected from every animal two days before inoculation (day -2), and at days 0, five, seven, 14 and 18 (for ASFV MKUZE group only) or 19 dpi (for ASFV OURT 3/88 group only). Optical density (OD) values where obtained from such sera via the use of the INGENASA blocking ELISA as described in 2.2.10.

Naturally, given the course of infection, fewer samples were collected from groups inoculated with acute strains. Both groups of pigs (inoculated with ASFV MOZ 1/98 or ASFV BENIN 1/97) had collections from days -2, 0, and five but also day seven for Pig 2 and Pig 3 that were still survivors of infection with ASFV BENIN 1/97. Antibodies were not detected in any of the animals subjected to infection by these viruses throughout the survival period (Table 4.3). Three pigs showing acute ASF in the group inoculated by ASFV MOZ 1/98 and one in ASFV BENIN 1/97 infected group died overnight by six dpi. The remaining two individuals in the latter group were severely ill and were euthanized at seven dpi.

Sera from animals in groups inoculated by the moderately virulent and non-virulent viruses that caused chronic disease like ASFV MKUZE or asymptomatic like ASFV OURT 3/88 started to show antibodies at seven dpi to 14 dpi and thereafter every individual in both these groups was considered positive (Table 4.3).

Table 4.3 ELISA OD values on sera of the Virulence Trial pigs

Days post inoculation	Virus isolate	OD levels for each pig		
		1	2	3
- 2 (two days before inoculation)	OURT 3/88	1.3865	1.4755	1.4645
	BENIN 1/97	1.474	1.389	1.38
	MKUZE	1.426	1.425	1.4105
	MOZ 1/98	1.684	1.422	1.4215
0	OURT 3/88	1.356	1.4105	1.5135
	BENIN 1/97	1.4535	1.466	1.407
	MKUZE	1.3925	1.426	1.441
	MOZ 1/98	1.464	1.431	1.385
5	OURT 3/88	1.321	1.416	1.376
	BENIN 1/97	1.5995	1.601	1.565
	MKUZE	1.4995	1.476	1.397
	MOZ 1/98	1.527	1.578	1.6085
7	OURT 3/88	1.268	1.1615	0.8985
	BENIN 1/97	1.4485	1.4835	Death
	MKUZE	1.212	1.1805	1.3615
	MOZ 1/98	Death	Death	Death
14	OURT 3/88	0.7015	0.694	0.5595
	MKUZE	0.722	0.888	0.807
18	MKUZE	0.8115	0.835	0.5455
19	OURT 3/88	0.544	0.6235	0.626
Negative controls	**(-) 1; (-) 2; (-) 3**	1.4185	1.3845	1.592

Positive cut off = 1.048 / Negative cut off =1.2316 >> until 14 dpi, Pig 1 in ASFV OURT 3/88 group (light shade).

Positive cut off = 0.9915 / Negative cut off = 1.1623 >> after 14 dpi, Pig 2 in ASFV OURT 3/88 group (dark shade).

OD = optical density

4.2.5. Gross pathology

After death or humane termination of each pig, post-mortems were performed and suitable tissue samples collected in context with previously reported pathology of the disease (Plowright *et al.*, 1994; reviewed by Penrith *et al.,* 2004a; Boinas *et al*., 2004). These were stored for further analysis of virus load in organ tissues by real time PCR quantification. Abnormal findings during external examination and inside the open carcasses were noted in order to relate it to clinical signs, indirect antibody ELISA and virus loads.

Post-mortem lesions are presented separately for each group. Table 4.4 refers to lesions detected during necropsy of pigs in the group inoculated by ASFV OURT 3/88. There were no significant signs identified at post-mortem that could be related to the inoculation of a pathogenic strain at the date of euthanasia, which occurred 19 dpi. During necropsy only a few mild alterations were noted in some organs. Scattered ecchymotic haemorrhages were seen in stomach of Pig 1 as well as mild swelling in the mesenteric lymph nodes. There was mild congestion in the tonsils of Pig 2 and mild swelling in spleen of Pig 3. The most curious finding, however, was the bilateral cysts in the kidneys in Pig 1 and unilateral in Pig 3.

Table 4.4 Post-mortem of domestic pigs inoculated with ASFV OURT 3/88

Pig number		**1**	**2**	**3**
Internal examination				
Lymphadenopathy	Tonsils	-	c	-
	Mediastinal lnn	-	-	-
	Gastro-hepatic lnn	-	-	-
	Mesenteric lnn	s	-	-
	Renal lnn	-	-	-
Viscera	Spleen	-	-	s
	Kidneys (general)	Bilateral Cyst (2cm)	-	Unilateral Cyst (5cm)
	Stomach (general)			
	Non-glandular	‡	-	-
	Glandular	‡	-	-
Date of euthanasia (dpi)		19	19	19

No alterations: (-); Congestion (mild): c; Ecchymotic haemorrhages (few): ‡; Swelling (mild): s.

Post-mortem findings in pigs infected with virulent ASFV BENIN 1/97 are described in Table 4.5. These were complex but coherent with the expected pathogenic outcome of acute ASF virus infection. The alterations noted correlated to the severity of clinical signs detected during the trial and of a generalised haemorrhagic syndrome observed at death. One of the pigs (Pig 1) died overnight from six to seven dpi and the remaining two were euthanized as per ethical agreement on day seven. Pigs 2 and 3 presented congestion in ears, snout, periorbital area, limbs, and tail. Some distinct findings were ocular discharges noted as mucopurulent in Pig 1 and Pig 2; there was foam in the oral cavity of Pig 1, blood in stools of Pig 1 and Pig 3, and swollen joints in Pig 2 and Pig 3. There was a common haemorrhagic lymphadenopathy in all individuals, haemorrhages in heart, kidneys, pancreas, small and large intestine and mesentery, congestive splenomegaly and hepatomegaly. Pig 1 was the only individual at post-mortem internal examination that presented with haemothorax and haemopericardium, froth and blood in cut sections of the lung with severe interlobular oedema.

Table 4.5 Post-mortem of domestic pigs inoculated with ASFV BENIN 1/97.

Pig number		**1**	**2**	**3**
External examination				
Cyanotic congestion of the skin	Ears	-	cc	cc
	Periorbital	-	-	cc
	Snout	-	cc	cc
	Tail	-	cc; †	-
	Limbs	-	cc	-
Ocular discharge		ττπ	ττπ	π
Foam in oral cavity		++	-	-
Stools adhered to perineum		++	-	++
Blood in stools		++	-	++
Swollen joints		-	+++	+
Internal examination				
Haemodynamic disorders	Haemothorax	+++	-	+
	Haemopericardium	+	-	-
	Haemoperitoneum	-	-	+
Lymphadenopathy	Tonsils	ΔΔΔ; sss	ΔΔΔ; sss	ΔΔΔ; sss
	Mediastinal lnn	ΔΔΔ; sss	ΔΔΔ; sss	sss
	Gastro-hepatic lnn	ΔΔΔ; sss	ΔΔΔ; sss	ΔΔΔ; sss
	Mesenteric lnn	ΔΔΔ; sss	ΔΔΔ; sss	ΔΔΔ; sss
	Renal lnn	ΔΔΔ; sss	ΔΔΔ; sss	ΔΔΔ; sss

Viscera	Lungs (general)	sss	•••	•••
	Froth in cut sections	+++	-	-
	Blood in cut sections	+++	-	-
	Interlobular oedema	+++	-	-
	Heart (general)			
	Left auricle/atrium	†	-	-
	Right auricle/atrium	†	-	-
	Left ventricle	†	-	-
	Right ventricle	†	-	-
	Spleen	sss	ss	sss
	Liver	sss	ss	sss
	Kidneys (general)	-	ss	
	Renal cortex	††	††	‡‡
	Renal medulla	††	††	‡‡
	Pancreas	-	ΔΔ	-
	Stomach (general)	-	-	c
	Non-glandular	-	-	c
	Glandular	†	-	c
	Small intestine	††;cc	cc	ΔΔ
	Large Intestine	cc	-	ΔΔ
	Mesentery	cc	cc	c
Survival period (dpi)		6	7	7

No alterations: (-); Mild: +; Moderate: ++; Severe: +++; Serous ocular discharge (π) and mucopurulent ocular discharge (ππ); Consolidation (Severe or extensive) •••; Congestion (mild): c; Congestion (moderate): cc; Haemorrhagic congestion (moderate): ΔΔ; Haemorrhagic congestion (severe): ΔΔΔ; Petechial haemorrhages (few): †; Petechial haemorrhages (multifocal or diffuse): ††; Ecchymotic haemorrhages (diffuse): ‡‡; Swelling (mild): s; Swelling (moderate): ss; Swelling (severe): sss.

Infection of domestic pigs with the tick isolate from Mkuze, South Africa (ASFV MKUZE) presented an outcome of moderate severity. Table 4.6 describes lesions found in this group in post-mortems done after euthanasia, at 18 dpi. The most important finding was the round ulcerative necrosis found in the skin of affected individuals, which is consistent with cases of chronic ASF (Figure 4.3) (Leitão *et al.*, 2001; Kleiboeker, 2002).

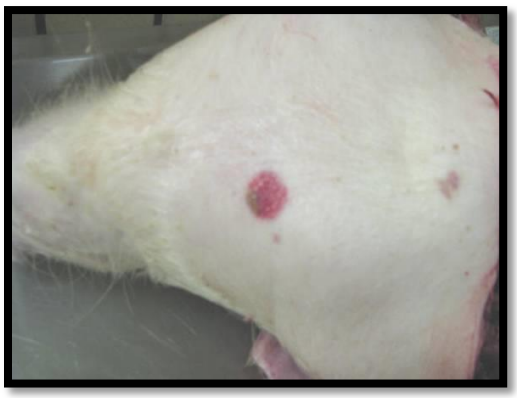


Figure 4.3 Local circular necrosis on skin of Pig 3 from group inoculated with ASFV MKUZE.

Other significant lesions found were in the glandular stomach, as extensive ulcers in Pig 3 and few petechial haemorrhages in Pig 1 and Pig 3, and sloughing of mucosa in the large intestine of Pig 2. In Pig 3, there was moderate swelling in tonsils, mesenteric lymphnodes, spleen, and liver. Partial lung consolidation occurred in all three pigs. Oedematous swelling in the left hind leg in all pigs in this group was seen at the IM inoculation site.

Table 4.6 Post-mortem of domestic pigs inoculated with ASFV MKUZE

Pig number		**1**	**2**	**3**
External examination				
Stools adhered to perineum		+	+	+
Soft stools		+	+	+
External lesions on the skin		u	u	uu
Swollen joints		-	Left tibiometatarsal joint (+++)	-
Internal examination				
Lymphadenopathy	Tonsils	-	-	ss
	Mesenteric lnn	-	Δ; s	ss
Viscera	Lungs (general)	••	•	•
	Froth in cut sections	-	+	-
	Spleen	-	-	ss
	Liver	-	-	ss
	Kidneys (general)			
	Renal cortex	†	†	†; c
	Stomach (general)			
	Glandular	ΔΔ	c	†; uuu
	Small intestine	c	c	c
	Large Intestine		sloughing off mucosa (+)	-
Date of euthanasia (dpi)		18	18	18

No alterations: (-); Mild: +; Moderate: ++; Severe: +++; Consolidation (localized) •; Consolidation (diffuse): ••; Congestion (mild): c; Haemorrhagic congestion (mild): Δ; Haemorrhagic congestion (moderate): ΔΔ; Petechial haemorrhages (few): †; Swelling (mild): s; Swelling (moderate): ss; Swelling (severe): sss. Ulcerative necrosis (mild): u; Ulcerative necrosis (multifocal or diffuse): uu; Ulcerative necrosis (extensive or severe): uuu.

Inoculation with ASFV MOZ 1/98 resulted in a virulent acute form of ASF (Table 4.7). Like the isolate from Mkuze, pathology of this virus was unknown to date. Pigs in this group died overnight from day six to seven after inoculations. They showed congestion and cyanotic congestion in ears, periorbital area, snout, tail, limbs, and ventrum. A particular finding in Pig 3 was blood coming out from the nostrils (epixtasis), when found dead together with Pig 1 and Pig 2 on day six after inoculation (Figure 4.4).

Figure 4.4 Epistaxis observed in Pig 3 from group inoculated with ASFV MOZ 1/98 after overnight death at six to seven dpi

Internal examination revealed generalised lymphadenopathy, haemothorax, and haemoperitoneum in all animals. Haemopericardium was seen only in Pig 2. Froth and blood in cut sections of the lung and interlobular oedema were present in Pig 2 and Pig 3. Haemorrhage was found in the heart of Pig 1. Congestive splenomegaly and hepatomegaly were noted in every pig. Haemorrhage was observed in kidneys of all pigs and in stomach of Pig 1, small intestine in Pig 2 and Pig 3 and large intestine in Pig 3 only. Congestion was detected in the glandular portion of Pig 2 stomach and in the mesentery of all. A peculiar finding during internal post-mortem analysis was the circular necrotic ulcers observed in the mucosa of the ileum in Pig 1.

Table 4.7 Post-mortem of domestic pigs inoculated with ASFV MOZ 1/98

Pig number		1	2	3
External examination				
Cyanotic congestion of the skin	Ears	ccc	cc	cc
	Periorbital	ccc	cc	cc
	Snout	ccc	cc	-
	Tail	ccc	-	cc
	Limbs	ccc	-	cc
	Ventrum	ccc	-	-
	Dorsum	ccc	-	-
Ocular discharge		π	π	π
Epixtasis		-	-	+++
Internal examination				
Haemodynamic disorders	Haemothorax	+	+	+
	Haemopericardium	-	+	-
	Haemoperitoneum	+	+	+
Lymphadenopathy	Tonsils	ΔΔΔ; sss	ΔΔΔ; sss	ΔΔΔ; sss
	Mediastinal lnn	ΔΔΔ; sss	ΔΔΔ; sss	ΔΔΔ; sss
	Gastro-hepatic lnn	ΔΔΔ; sss	ΔΔΔ; sss	ΔΔΔ; sss
	Mesenteric lnn	ΔΔΔ; sss	ΔΔΔ; sss	ΔΔΔ; sss
	Renal lnn	ΔΔΔ; sss	ΔΔΔ; sss	ΔΔΔ; sss
Viscera	Lungs (general)	•••	sss	sss
	Froth in cut sections	-	+++	+++
	Blood in cut sections	-	+++	+++
	Interlobular oedema	-	+++	+++
	Heart (general)	†	Ss	ss
	Spleen	s	sss	sss
	Liver	ss	sss	sss
	Kidneys (general)		sss	S
	Renal cortex	‡‡	‡‡; ††	‡‡; ††
	Renal medulla	‡‡	‡‡; ††	‡‡; ††
	Stomach (general)	†	-	-
	Non-glandular	†	-	-
	Glandular	†	cc	†
	Small intestine	ulcers in ileum	†	††
	Large Intestine	-	-	††
	Mesentery	C	cc	cc
Survival period (dpi)		6	6	6

No alterations: (-); Mild: +; Moderate: ++; Severe: +++; Serous ocular discharge (π); Consolidation (Severe or extensive) •••; Congestion (mild): c; Congestion (moderate): cc; Cyanotic congestion (extensive or severe): ccc; Haemorrhagic congestion (mild): Δ; Haemorrhagic congestion (moderate): ΔΔ; Haemorrhagic congestion (severe): ΔΔΔ; Petechial haemorrhages (few): †; Petechial haemorrhages (multifocal or diffuse): ††; Ecchymotic haemorrhages (few): ‡; Ecchymotic haemorrhages (difuse): ‡‡; Swelling (mild): s; Swelling (moderate): ss; Swelling (severe): sss.

4.2.6. Viral load analysis

Blood and organ samples were subjected to analysis with the Taqman real time PCR for quantification of ASFV DNA. Blood was harvested at specified time points and tissue samples (organs) at post-mortem. Samples were frozen at -80°C for subsequent DNA extraction and amplification of the vp72 gene. This approach allowed comparison between the pigs in the same group subjected to the same infection and between groups inoculated with different isolates. The aim was to evaluate the development of viraemia over time and virus organ tropism by quantification of viral load in every blood and tissue sample involved.

4.2.6.1. Pigs infected with ASFV OURT 3/88

There was no evidence of ASFV DNA in blood and organ samples of pigs infected with ASFV OURT 3/88 via detection by the Taqman real time PCR.

4.2.6.2. Pigs infected with ASFV BENIN 1/97

Pigs subjected to inoculations with ASFV BENIN 1/97 developed viraemia quickly after infection and in every pig from three dpi until death or termination at day seven. See results in Table 4.8.

Table 4.8 ASFV quantification (virus/ml) of blood samples from ASFV BENIN 1/97 group.

Dpi	**Pig number**		
	1	**2**	**3**
Pre-bleed (day -2)	Negative	Negative	Negative
Day 0 (Infection)	Negative	Negative	Negative
1	Negative	Negative	Negative
3	6.13×10^5	5.91×10^5	5.76×10^5
5	1.39×10^6	2.36×10^6	2.33×10^5
7	Death	4.09×10^6	4.68×10^6

The ASFV BENIN 1/97 group had the majority of organs positive to ASFV by real time PCR. Pig 1 showed all samples positive except the lungs, Pig 2 presented

heart, kidneys and intestine as negative and Pig 3 was negative for tonsil, and both mediastinal and mesenteric lymphnodes (Table 4.9).

Table 4.9 ASFV quantification (virus/ml) of tissue samples from ASFV BENIN 1/97 group.

Organ	**Pig number**		
	1	**2**	**3**
Skin	2.13×10^5	1.55×10^5	2.94×10^5
Tonsil	1.58×10^6	1.38×10^4	Negative
Lungs	Negative	6.88×10^2	1.55×10^5
Heart	2.69×10^4	Negative	3.41×10^4
Mediastinal lnn	2.68×10^5	2.72×10^4	Negative
Spleen	4.39×10^6	3.09×10^4	1.89×10^5
Liver	3.04×10^6	2.44×10^3	2.65×10^5
Kidneys	1.27×10^5	Negative	1.08×10^5
Gastro-hepatic lnn	3.72×10^6	8.33×10^5	2.94×10^5
Mesenteric lnn	8.88×10^5	1.91×10^5	Negative
Stomach	4.83×10^4	6.54×10^4	2.42×10^4
Intestine	3.29×10^5	Negative	1.41×10^4

Figure 4.5 shows that the organ samples most affected after infection with ASFV BENIN 1/97 inoculum were in order of decreasing concentration of virus, the gastro-hepatic lymph nodes, spleen, liver, tonsil, mesenteric lymphnodes, skin, intestine, mediastinal lymphnodes, kidneys, lungs, stomach, and heart.

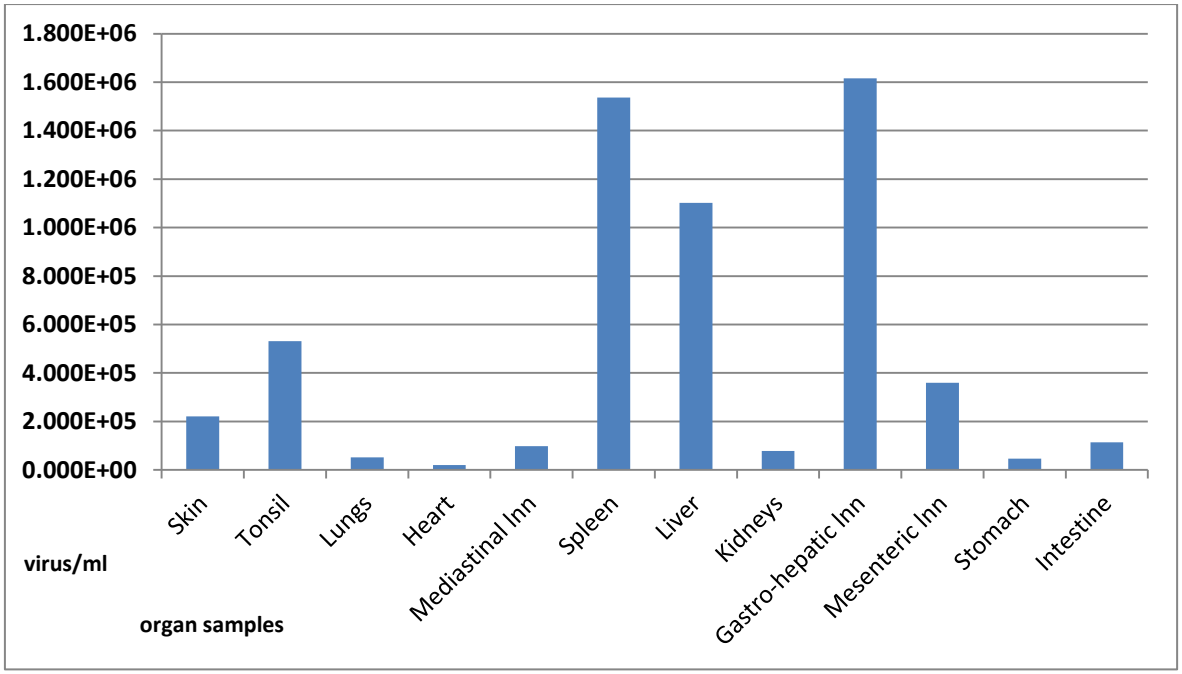


Figure 4.5 Viral load (virus/ml) averages of each organ sample from three pigs inoculated with ASFV BENIN 1/97

4.2.7.3. Pigs infected with ASFV MKUZE

Pigs in the group inoculated by ASFV MKUZE did not develop viraemia until the last day of the trial at 18 dpi. At this point viraemia in blood of Pig 2 was 2.86 x 10^4 virus/ml and in Pig 3 as a 1.24 x 10^4 virus/ml (Table 4.10).

Table 4.10 ASFV quantification (virus/ml) of blood samples from ASFV MKUZE group.

Dpi	Pig number		
	1	2	3
Pre-bleed (Day -2)	Negative	Negative	Negative
Day 0 (Infection)	Negative	Negative	Negative
...	Negative	Negative	Negative
18	Negative	2.86 x 10^4	1.24 x 10^4

Shading: Inoculation day; (...): mid collection days negative to PCR at 1, 3, 5, 7, 10, 14, 24, 26, 28, 31 and 33 dpi.

Virus load in organs collected from pigs subjected to ASFV MKUZE inoculum are in Table 4.11. Pig 2 was positive in the skin, tonsil, mediastinal lymphnodes, and liver.

Pig 3 was positive in the skin, tonsil, spleen, and liver. In contrast, organs were all negative to the presence of the virus for Pig 1.

Table 4.11 ASFV quantification (virus/ml) of post mortem samples from ASFV MKUZE group.

Organ	**Pig number**		
	1	**2**	**3**
Skin	Negative	1.69×10^3	7.25×10^3
Tonsil	Negative	4.72×10^2	2.17×10^2
Lungs	Negative	Negative	Negative
Heart	Negative	Negative	Negative
Mediastinal lnn	Negative	2.27×10^3	Negative
Spleen	Negative	Negative	4.35×10^2
Liver	Negative	1.68×10^3	5.39×10^2
Kidneys	Negative	Negative	Negative
Gastro-hepatic lnn	Negative	Negative	Negative
Mesenteric lnn	Negative	Negative	Negative
Stomach	Negative	Negative	Negative
Intestine	Negative	Negative	Negative

A graphic representation of the average virus load results of organ samples obtained from all three pigs in this group are shown in Figure 4.6. The skin was the tissue sample that revealed the highest average of virus load, followed by mediastinal lymphnodes, liver, tonsil and spleen. The remaining organ samples were all negative by real time PCR quantification.

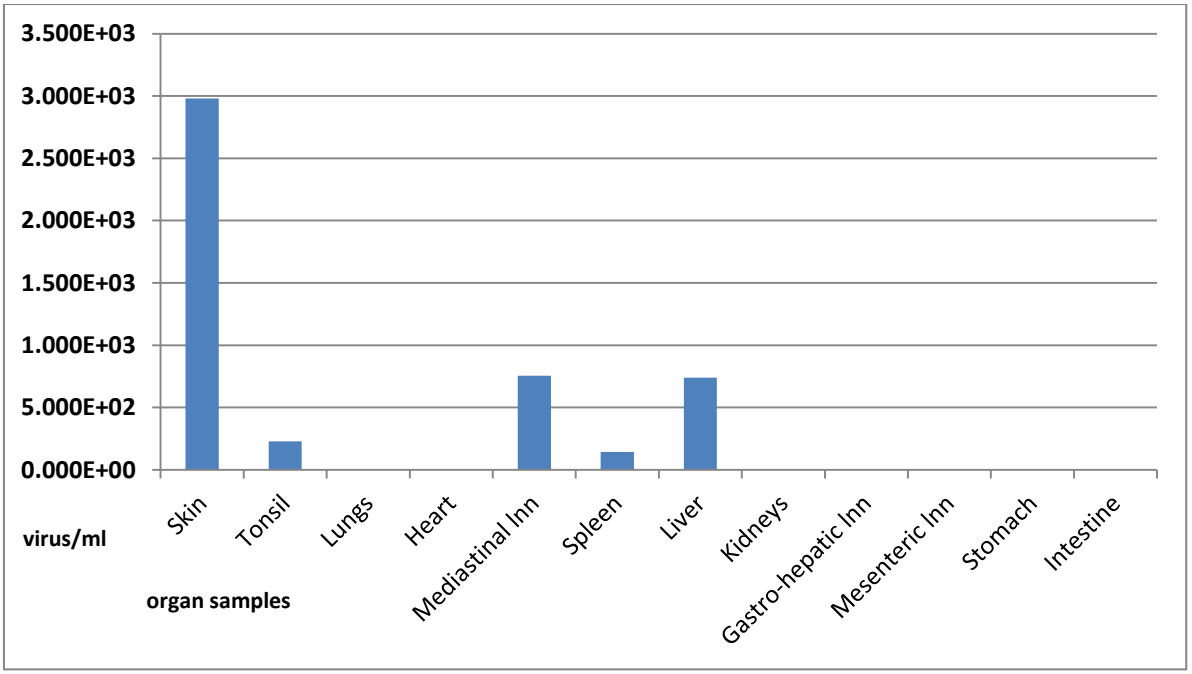


Figure 4.6 Viral load (virus/ml) averages of each organ sample from three pigs inoculated with ASFV MKUZE

At post-mortem of the ASFV MKUZE group, a sample from the swollen area in the hind leg was collected from every pig in order to screen for other infectious agents that may have caused the swelling. It was found to contain *Mycoplasma hyosynoviae*, in Pig 1 and Pig 2 and *Fusobacterium necrophorum, Porphyromonas gingivicanis* and *Lactobacillus* (Table 4.12) as determined by an in house PCR (Standard quality control methods implemented at TADP).

Table 4.12 Bacterial identification from samples: Skin and muscle at lesion site

Pigs	Bacterial identification
Pig 1	*Mycoplasma hyosynoviae*
Pig 2	*Mycoplasma hyosynoviae*
Pig 3	*Fusobacterium necrophorum; Porphyromonas gingivicanis; Lactobacillus*

4.2.7.4. Pigs infected with ASFV MOZ 1/98

Pigs rapidly developed viraemia in the ASFV MOZ 1/98 infected group. Real time PCR on DNA extracted samples from blood collected at three dpi revealed every pig to be positive. Showing 2.72 x 10^5 virus/ml for Pig 1, 1.01 x 10^6 virus/ml for Pig 2 and 1.06 x 10^5 virus/ml for Pig 3 (Table 4.13), higher than pigs infected with ASFV

MKUZE at 18 dpi (2.86 x 10^4 virus/ml for Pig 2 and 1.24 x 10^4 virus/ml for Pig 3). At five dpi, virus load in Pig 1 increased to 1.35 x 10^6 virus/ml and oddly in Pig 2 decreased to 1.06 x 10^5 virus/ml. In Pig 3 it reduced to 7.21 x 10^4 virus/ml. Every pig in the ASFV MOZ 1/98 inoculation group had positive blood samples on real time PCR at days three and five while at day seven they were all found dead in the morning. Furthermore the virus load of blood samples had similar quantitative results compared to what was found for ASFV BENIN 1/97. Virus proliferation in these individuals was considerably faster which is consistent with the severe pathology caused by both virulent inoculums.

Table 4.13 ASFV quantification (virus/ml) of blood samples from ASFV MOZ 1/98 group

Dpi	**Pig number**		
	1	**2**	**3**
Pre-bleed (Day -2)	Negative	Negative	Negative
Day 0 (Infection)	Negative	Negative	Negative
1	Negative	Negative	Negative
3	2.72 x 10^5	1.01 x 10^6	1.06 x 10^5
5	1.35 x 10^6	1.06 x 10^5	7.21 x 10^4
7	Death	Death	Death

In Pig 1, all collected organs were positive while in Pig 2 kidneys were negative, and in Pig 3 the heart and intestine were negative (Table 4.14).

Table 4.14 ASFV quantification (virus/ml) of post mortem samples from ASFV MOZ 1/98 group.

Organ	**Pig number**		
	1	**2**	**3**
Skin	2.55 x 10^4	9.59 x 10^4	3.45 x 10^3
Tonsil	1.59 x 10^5	2.98 x 10^6	4.31 x 10^4
Lungs	7.66 x 10^4	3.7 x 10^5	2.47 x 10^6
Heart	4.8 x 10^2	4.61 x 10^3	Negative
Mediastinal lnn	7.77 x 10^4	2.2 x 10^5	2.19 x 10^5
Spleen	8.33 x 10^5	8.22 x 10^5	4.46 x 10^4
Liver	8.36 x 10^5	5.72 x 10^6	2.2 x 10^5

Kidneys	3.94×10^3	Negative	2.2×10^5
Gastro-hepatic lnn	3.65×10^5	1.31×10^6	2.67×10^6
Mesenteric lnn	4.42×10^5	8.55×10^4	7.32×10^5
Stomach	3.2×10^3	6.13×10^5	7.73×10^3
Intestine	4.01×10^3	4.09×10^2	Negative

On average the organs that became most affected after infection with ASFV MOZ 1/98 were in the following order from highest to lowest viral load detected by real time PCR: liver, gastro-hepatic lymph nodes, followed by tonsils and lungs, spleen, mesenteric lymphnodes, stomach, mediastinal lymphnodes, kidneys, skin, heart and finally the intestine (Figure 4.7).

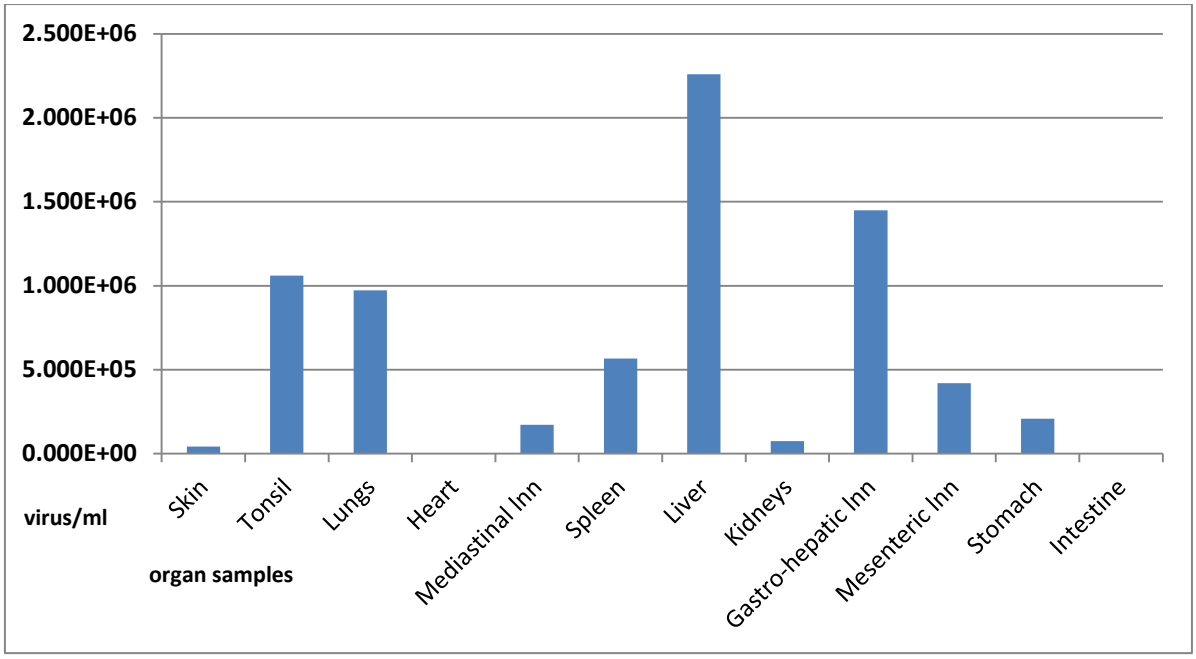


Figure 4.7 Viral load (virus/ml) averages of each organ sample from three pigs inoculated with ASFV MOZ 1/98

Comparisons of the real time PCR data as virus load averages per group of pigs and potential virus tropism between pigs subjected to ASFV BENIN 1/97 (Figure 4.5), ASFV MKUZE (Figure 4.6) and ASFV MOZ 1/98 (Figure 4.7) are summarised in Table 4.15. Virus load averages are in order of highest quantification of virus to lowest for every tissue sample. The results show that ASFV MKUZE inoculations resulted in concentrations of virus in tissue 10^3 virus/ml while ASFV MOZ 1/98 and ASFV BENIN 1/97 was detected as high as 10^6 virus/ml.

Table 4.15 Ranking of averages of virus load in organ samples from groups inoculated by ASFV MKUZE, ASFV MOZ 1/98 and ASFV BENIN 1/97.

Rank	Mkuze	Virus/ml	Benin 1/97	Virus/ml	Moz 1/98	Virus/ml
1	Skin	2.98×10^3	GH lnn	1.62×10^6	Liver	2.26×10^6
2	Med. Lnn	7.57×10^2	Spleen	1.54×10^6	GH lnn	1.45×10^6
3	Liver	7.40×10^2	Liver	1.10×10^6	Tonsils	1.06×10^6
4	Spleen	1.45×10^2	Tonsils	5.31×10^5	Lungs	9.72×10^5
5	Tonsils	2.30×10^2	Mes. lnn	3.60×10^5	Spleen	5.67×10^5
6			Skin	2.21×10^5	Mes. lnn	4.20×10^5
7			Intestine	1.14×10^5	Stomach	2.08×10^5
8			Med. lnn	9.84×10^4	Med. lnn	1.72×10^5
9			Kidneys	7.83×10^4	Kidneys	7.46×10^4
10			Lungs	5.19×10^4	Skin	4.16×10^4
11			Stomach	4.60×10^4	Heart	1.70×10^3
12			Heart	2.03×10^4	Intestine	1.47×10^3

Med.: Mediastinal; GH: Gastro-hepatic; Mes.: Mesenteric

4.3. Discussion

The various inoculations administered to a small number of pigs manifested differently in each group according to the specific ASF virus strain. These were classified as causative agents of asymptomatic ASF like the ASFV OURT 3/88, chronic ASF for the ASFV MKUZE, and of acute ASF in the case of ASFV MOZ 1/98 and ASFV BENIN 1/97.

The isolate from Portugal, ASFV OURT 3/88 is described in literature as non-virulent and non-haemadsorbing (Boinas 1995, Boinas *et al.*, 2004). In contrast, there were not any reports to date about the effects of infection in domestic pigs with ASFV MKUZE. Furthermore, analysis of the pathological outcomes confirmed what was suspected of the isolate from Mozambique (ASFV MOZ 1/98). This correlates with previous reports that the high virulence of this virus caused acute ASF in piglets that were descendants of a local domestic breed supposedly with some level of resistance to ASF and in Landrace cross breed domestic pigs as a group control for that trial (Penrith *et al.*, 2004b). The virus ASFV BENIN 1/97 was previously known to be highly pathogenic and causative of acute ASF (Chapman *et al.*, 2008) hence it was used here to function as virus of high virulence. The outcome of the latter inoculation was as expected and served as a good comparison against ASFV MOZ 1/98 since both developed similar lesions and were characterized as causing an acute viral haemorrhagic syndrome.

Considering that swine are prone to increased body temperature especially when manipulated by physical restraint, the fever threshold was set at a rectal temperature of 40.0°C to avoid false positives. This is a common approach for temperatures obtained from pigs (Carpintero *et al.*, 2007). Thus, the ASFV OURT 3/88 inoculated group never showed abnormally elevated temperatures and in the ASFV MKUZE group, all pigs had fever at seven dpi until termination at 18 dpi. In contrast, all three pigs of the ASFV MOZ 1/98 group manifested fever by three dpi rendering them as the quickest individuals to reach fever threshold after inoculation. Although ASFV BENIN 1/97 is causative of acute ASF in domestic pigs, fever was detected considerably later, at six dpi. Results were consistent with literature comparisons for the designations of non-virulent ASFV OURT 3/88 strain according to Boinas, 1995;

moderately virulent and causative of chronic disease ASFV MKUZE, which was previously unknown; virulent and causative of acute disease ASFV MOZ 1/98 and ASFV BENIN 1/97 as reviewed by Penrith *et al*., 2004a and Carpintero *et al.,* 2007.

Viral load analysis by the use of vp72 Taqman real time PCR quantification revealed different concentrations of ASFV in the blood as well as in the collected organ samples from the four groups of domestic pigs. The non-pathogenic ASFV OURT 3/88 never revealed presence of virus either in blood or in any tissue sample collected at post-mortem. ASFV MKUZE DNA was detected via the Taqman real time PCR in blood on the last day of the trial and in organ samples of Pig 2 and Pig 3 (Pig 1 did not reveal viraemia nor was it positive to the presence of ASFV in organs). Virus load in organ samples was detected at a concentration of virus lower than for the acute ASF causing agents, ASFV MOZ 1/98 and ASFV BENIN 1/97. The latter isolates caused acute haemorrhagic syndromes and lead to animals dying or being euthanized as soon as six and seven dpi respectively for each group and with clinical signs and post-mortem lesions similar for both groups. In these pigs viraemia was detected at three dpi and had higher virus load compared to ASFV MKUZE group at 18 dpi. Viral quantification was also higher in both pathogenic strains (ASFV MOZ 1/98 and ASFV BENIN 1/97).

Pigs infected with ASFV OURT 3/88 never showed clinical signs, post-mortems clear of ASF gross lesions and real time PCR virus load analysis was negative in all blood collections and organ samples. However, just as in the ASFV MKUZE infected group, sero-conversion was first detected at seven dpi and by 14 dpi, they were all positive which is similar to findings in work done by Boinas *et al.*, 2004 on ASV OURT 3/88. The most curious finding in ASFV OURT 3/88 group of animals was the bilateral cysts in the kidneys in Pig 1 and unilateral in Pig 3. Although there was not any analysis done on these cysts beyond detection at post-mortem it is probable they were not because of ASFV inoculation, but rather a congenital defect. ASFV OURT 3/88 DNA was not detected in blood or in any tissue sample collected at post-mortem. These results are consistent with the asymptomatic behaviour by the absence of clinical signs (including fever) and lesions at necropsy shown before (Boinas, 1995; Boinas *et al.*, 2004). PCR results obtained from this group of pigs confirmed observations in terms of lack of clinical signs, negative post-mortems, and

IMP testing. Therefore, except for the sero-conversion in all individuals by 14 dpi, there was no other indication of infection by ASFV.

For ASFV MKUZE, DNA was detected via the Taqman real time PCR in blood towards the end of the trial. Leitão *et al*., 2001 demonstrated viraemia in domestic pigs late during infection with a non-pathogenic and non-haemadsorbing isolate. Virus load in blood and organ samples were at lower concentration than for ASFV MOZ 1/98 and ASFV BENIN 1/97. Pigs inoculated with ASFV MKUZE revealed a clinically mild form of ASF defined here as chronic disease according to previous characterizations on ASF (Leitão *et al.*, 2001: Kleiboeker, 2002; Penrith *et al.*, 2004b). Infections with this isolate revealed viraemia only at 18 dpi by real time PCR testing which was the last day of the trial for this group. It remains unclear whether these animals may have developed evident ASF disease if they lived longer. However since they had developed antibodies there might have been a certain level of protection acquired against ASF. It could be informative to repeat this inoculation and leave the animals for longer to verify further progression of the infection.

Nevertheless, clinical signs noted in the ASFV MKUZE inoculation group of pigs appeared from eight dpi and may relate to severe inflammation at the injection site area on the pig's hind legs with marked swelling that later on caused ulceration of the skin along with lameness. This lesion was not tested for the presence of ASFV DNA but curiously, this was the only time that high virus loads were found in the skin of the pigs and could indicate a tropism of this virus. A tissue sample of this swollen area in the hind leg revealed bacterial infection. Pig 1 and Pig 2 in this group tested positive for *Mycoplasma hyosynoviae* infection (Table 4.13). *Mycoplasma* arthritis disease (*Mycoplasma hyosynoviae* infection) is usually sudden in onset, the first signs being reluctance to rise at feeding time. In the case of this infection, temperature may be normal or slightly elevated. Pig 3 was positive to *Fusobacterium necrophorum, Porphyromonas gingivicanis* and *Lactobacillus.* According to Langworth (1977), *Fusobacterium necrophorum* causes foot rot in pigs, especially when kept on concrete, although individuals had no lesions in the muscular upper region of the hind leg.

Naturally, suspicion may arise in relation to the inoculum sterility but every viral suspension passed through non-pyrogenic bacterial filters of 0.22 µm (Millex®GP)

before infecting pigs. In addition, it was tested on agar plates and broths according to the European Pharmacopoeia (2005), as well as on a 16S rRNA PCR (Standard quality control methods implemented at TADP) for the early detection of bacterial contaminants. Intramuscular injections in pigs that weigh more than 10 kg should ideally be in the neck. The hind leg is for individuals not old enough for application in the neck muscles, as in piglets. The severe swelling that developed was a probable cause for the observed clinical signs such as fever, lameness, ocular discharges, diarrhoea, and depression. Adverse effects of repeated needle trauma (due to sedations for blood and sera collection) associated with bacterial infection may have compromised disease progression analysis of ASFV MKUZE inoculation. Naturally, given the high virus load detected by real time PCR in the skin, virus tropism for this organ is evident hence the most probable cause for the lesions that were seen associated with secondary bacterial infection. Nonetheless, this isolate was causative of chronic ASF when considering the general observations. Towards termination, two of these pigs showed spots of localized circular necrosis on the skin, which is a characteristic lesion of chronic ASF reported before by Leitão *et al.* (2001).

Animals started to sero-convert from seven dpi and by 14 dpi all ASFV MKUZE infected pigs had reacted positive to ASFV antigen on ELISA. In this trial, the organs showing the highest ASF virus load were the skin, tonsils and liver. Although the three pigs in this group developed fever, and a few complications derived from the excess needle trauma to muscles in the hind leg, these animals did not require ethics intervention until as late as 18 dpi.

The outcome of ASFV MOZ 1/98 infection in domestic pigs resembled that of ASFV BENIN 1/97 in the sense it had similar time progression of symptoms until death and it presented as a general haemorrhagic syndrome. A similar pattern of lesions when infected with the highly virulent ASFV BENIN 1/97 and ASFV MOZ 1/98 was observed. In both of these infections, animals only lived as long as six to seven dpi. Viraemia as determined by real time PCR started from three dpi. Clinical signs developed around the same period but in the ASFV MOZ 1/98 inoculated group they were observed one day earlier than for ASFV BENIN 1/97. Nevertheless, both viruses had a similar clinical pattern with the exception of a more severe lung

affliction in ASFV MOZ 1/98 group and bloody diarrhoea seen in the pigs infected with ASFV BENIN 1/97. According to Carrasco *et al*., 1996, acute ASF causes formation of alveolar oedema in the lungs of afflicted animals and by fibrin microthrombi in septal capillaries. Therefore, taking into consideration the results seen in the temporal progression of the disease and the characteristic clinical signs and post-mortem lesions observed, these two strains were recognised as causative agents of acute ASF in agreement with other descriptions of ASF (reviewed by Penrith *et al.*, 2004a). In another trial (King *et al.*, 2011) where naive pigs were challenged with ASFV BENIN 1/97, these survived longer than our control group since they lived up to eight dpc.

ELISA results did not indicate antibody response in these animals. Moderately virulent viruses elicit increased antibody responses compared to virulent strains (Fernandez, 1992). Previous studies indicate that neutralising antibody response in ASFV challenged domestic pigs tends to appear at 10-12 or 12-14 days after exposure (Wardley *et al.*, 1987). Since individuals affected by acute disease have a shorter survival time they do not reach the point of measurable antibody production hence it is not detected by ELISA which was the case here for ASFV BENIN 1/97 and ASFV MOZ 1/98 challenged groups. Detection of IgM is indicated for recent infections and IgG in chronic cases. According to Reis *et al.*, 2007, IgM is present in a majority of pigs from seven dpi and IgG from 14 dpi. In the present experiment, ELISA aimed to detect IgG.

As previously described by Oura *et al*. (1998a), domestic pigs inoculated with ASFV presented initial high levels of virus replication in the spleen rapidly followed by escape of the virus and high levels in other lymphoid organs. Similarly in the present study viral quantification evaluated in tissue samples from infected pigs revealed the spleen, tonsils and either gastro-hepatic, mediastinal or mesenteric lymphnodes as organs with the highest quantity of ASFV DNA. Interestingly, the skin revealed high virus load in animals inoculated with ASFV MKUZE raising the possibility of slightly different viral tropism in cases of chronic ASF.

ASFV MOZ 1/98 caused epixtasis in Pig 3 and at post-mortem it was determined it had the most severe lung pathology along with Pig 2 compared to Pig 1 and coinciding with the higher virus load in lungs for Pig 3 followed by Pig 2 and then Pig

1. Although lesions were observed in ASFV BENIN 1/97 pigs, virus load in lung tissue was higher with ASFV MOZ 1/98 inoculation. The virulent isolates were causative of acute disease by development of generalized haemorrhagic lymphadenopathy seen in necropsies of pigs inoculated with ASFV MOZ 1/98 and ASFV BENIN 1/97. Interestingly, the viral load quantification detected similar values for each type of lymphnodes in pigs for both inoculations. The absence of ASFV from the intestine in the ASFV MKUZE group of pigs contradicts the severe diarrhoea the animals presented. Furthermore, whilst in ASFV MOZ 1/98 group the intestine viral load was the lowest, for the ASFV BENIN 1/97 it was higher which relates well to the bloody diarrhoea observed in all animals in this group.

Coggins *et al,* 1968, and Vigario *et al.*, 1974 reported that viruses lacking the ability to cause haemadsorption generally tend to be non-virulent or of reduced virulence due to the deletion of the gene encoding the CD2v protein. Deletion of CD2v protein gene from the genome of a virulent ASFV with haemadsorbing ability delays the onset of viraemia and dissemination of viruses within infected pigs (Borca *et al.,* 1998) and was previously linked to natural attenuation of ASFV. Nevertheless, other authors have found that certain non-haemadsorbing viruses cause ASF (Thomson *et al.*, 1979; Leitão *et al.*, 2001). In our study, phenotypic characterization of the ASFV OURT 3/88 and ASFV MKUZE isolates *in vitro* revealed that they lack the ability to cause haemadsorption (HAD) or bind red blood cells. Thomson, 1985 and Boinas *et al.*, 2004 have previously described ASFV MKUZE and ASFV OURT 3/88 isolates as non-haemadsorbing viruses. Evidence presented here demonstrates that although ASFV MKUZE is non-haemadsorbing there was no indication that it is a non-virulent isolate and it is classified as moderately virulent.

A non-fatal infection of domestic pigs from a specific isolate could provide some level of protection against inoculation with other ASFV virulent strains (Lewis *et al.*, 2000; Boinas *et al*., 2004). Similarly, there is a report of domestic pigs that acquired protective immunity through inoculation of a non-haemadsorbing and non-fatal isolate prior to challenge with a known highly virulent strain (Leitão *et al*., 2001). Furthermore, there is resistance to ASF in certain endemic regions in which individuals may be repeatedly in contact with local strains of ASFV and therefore

may have acquired immunity against the pathological effects of the disease (Nsalambi, 1987; Mendes, 1994; Penrith *et al*., 2004b).

Based on the outcome of this trial and background on the strains involved, two of the isolates were selected for further vaccine research: ASFV OURT 3/88 as candidate vaccine and ASFV MOZ 1/98 as challenging virus. Results obtained from the trial in the present study confirmed the non-pathogenic outcome of inoculation with ASFV OURT 3/88 and it determined that it is able to induce antibody production in domestic pigs. According to Boinas *et al.*, 2004, inoculations of pigs with non-virulent isolates of ASF, including ASFV OURT 3/88, were characterised by causing sporadic viraemia and sero-conversion. Pigs were inoculated with ASFV OURT 3/88, and subsequently challenged with a Portuguese pathogenic virus isolated from ticks (ASFV OURT 88/1). The challenge virus did not cause clinical signs other than intermittent fever in one of the animals, and did not produce either viraemia or death. In addition, King *et al.* (2011) demonstrated cross protection by utilizing both Portuguese isolates ASFV OURT 3/88 (non-virulent) and ASFV OURT 1/88 (virulent) as sequential first and boost vaccination respectively and thereafter challenging them with geographically distinct viruses from Africa and revealing some degree of protection.

The ASFV MKUZE isolate from Kwazulu-Natal in South Africa (Thomson, 1985) has potential for use as live vaccine because it seemed avirulent and therefore ideal as strain for a possible vaccine with the addition that it is from within the region of southern Africa. Unfortunately, according to the present results it proved to be inadequate since it is too virulent to function as live vaccine.

Hoping for possible immunization of pigs, ASFV OURT 3/88 was introduced in the following vaccination trial given the antibody production induced by this isolate and the confirmed lack of symptomatology after infection. The trial included vaccination of pigs with this non-virulent virus against a subsequent challenge with virulent southern African isolate, ASFV MOZ 1/98, actually circulating and devastating farmers in southern Africa.

Chapter 5

Vaccination trial in domestic pigs by single and double immunization with ASFV OURT 3/88 and challenge with ASFV MOZ 1/98

5.1. Introduction

Although current ASF control measures contribute to efforts in containing or avoiding transmission of the virus, history shows that in order to efficiently contain outbreaks or completely eradicate a highly contagious disease, the additional use of vaccines is beneficial. There is no vaccine commercially available, that will efficiently protect domestic pigs against ASF. However, there have been many attempts to find one and results are motivating further research. Boinas *et al*. (2004) has demonstrated that pigs infected with certain non-virulent ASF viruses were resisted or had a delay in clinical signs of up to 14 dpi when subsequently subjected to infection with pathogenic strains. One such strain that provided some level of protection was the attenuated European ASFV OURT 3/88 collected from domestic pigs in the Alentejo province in Portugal (Boinas, 1995). According to King *et al.* (2011), ASFV OURT 3/88 belongs to genotype I. In this study, the ability of such a non-virulent isolate to induce protection against viruses of higher virulence was under evaluation in a pig trial composed of various groups of animals with a geographically distinct challenge virus (ASFV MOZ 1/98). The isolate ASFV MOZ 1/98 is genotype VIII according to Boshoff *et al*. (2007). Both of these viruses were previously included in the virulence trial in domestic pigs described in Chapter 4. The clinical outcome of both isolates had therefore previously been determined, so they could thus function as control groups for the experiment in terms of potential pathological manifestation of each strain.

5.2. Results

The vaccination trial was composed of two groups of six domestic pigs crossbred between Largewhite and Landrace. These were inoculated with non-virulent ASFV OURT 3/88 (Genotype I) once or twice before challenge with virulent and heterologous ASFV MOZ 1/98 (Genotype VIII). In Group 1, vaccination was at day 0 followed by a booster 21 days later followed by challenge after 49 days. Group 2 was subjected to a single vaccination and challenged 28 days later. A third group, consisting of three pigs, was inoculated with ASFV MOZ 1/98 without prior vaccination and designated as the control group (Table 2.8).

Both single and double vaccinations regime failed to confer total protection against subsequent challenge with a highly virulent isolate. All vaccinated pigs presented with clinical signs as soon as one day post challenge (dpc). In addition, viraemia was detectable by three dpc with the strain from Mozambique and eventually every animal succumbed to infection with death occurring within 11 dpc (Table 5.1).

5.2.1. Clinical outcome

To monitor the health of domestic pigs the approach was the same as for the virulence trial described in Chapter 4. In Group 1, Pig 1 rapidly died at five dpc, Pig 2, Pig 3 and Pig 6 died seven dpc while Pig 4 survived until eight dpc. Interestingly, Pig 5, which had been showing fever since before challenge, presented with less severe symptoms and was therefore euthanized at nine dpc for welfare reasons (loneliness not ideal for pigs in containment) but not because it qualified for termination. In Group 2, Pig 1 and Pig 5 died overnight at five dpc and Pig 6 was euthanized at six dpc since it reached the animal welfare score (10 or more). Pig 3 died by seven dpc, and by 11 dpc Pig 4 died and Pig 2 was euthanized for welfare reasons. Finally, in the control group Pig 1 and Pig 2 died at four dpc and Pig 3 at five dpc.

Table 5.1 Characteristics of disease caused in pigs vaccinated with a live attenuated strain (ASFV OURT 3/88) following challenge with virulent ASFV MOZ 1/98.

Groups	No of pigs	Days post challenge (dpc)			
		Fever	Clinical Signs	Viraemia	End point
Group 1	6	2[1]	3	3	5-9
Group 2	6	1-4	3-4	3	5-11
Group 3 (control group)	3	2[2]	3	3	4-5

[1]: Except Pig 5 that showed Fever since before challenge

[2]: Except Pig 1 that never showed Fever

The percentage survival of each group per days alive in the study is represented next in (Figure 5.1).

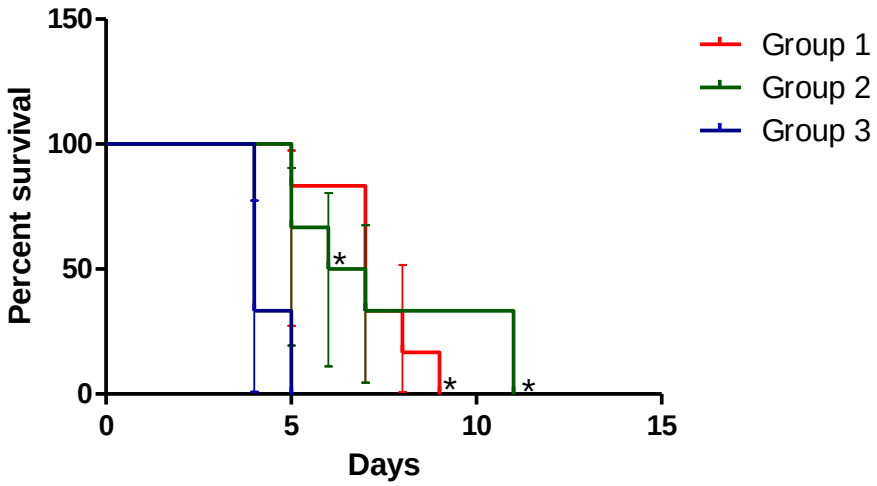


Figure 5.1 Survival graph indicating the percentage survival for Groups 1-3. Error bars indicate the 95% confidence intervals. * = indicates an animal that was euthanised

The log-rank (Mantel-Cox) test indicated that there was significant difference (P = 0.0196) between the survival rates of Groups 1 and 2 compared to Group 3. However, there was no significant difference between the survival rates of groups 1 and 2 (P = 0.6548; Mantel-Cox test). The median survival time for Group 1 was 7 days, and for Group 2, 6.5 days with a ratio of 1.077 (95% CI 0.7544 – 1.399) while

for Group 3 the median survival time was 4 days. The hazard ratio between Groups 1 and 2 was 1.387 (95% CI 0.3306 to 5.818).

5.2.2. Rectal temperatures

Rectal temperatures were monitored every morning for the duration of the trial. In Group 1, temperatures slightly above 40.0°C were recorded until 14 days post vaccination (dpv) after first inoculation, but returned to normal. Only one pig (5) had fever 23-24 days after the second vaccination. The rest showed normal temperatures until two days after challenge with the virulent isolate from Mozambique (Figure 5.2). At this point, pigs became seriously ill and high rectal temperatures were measured, close to 42.0°C in some cases.

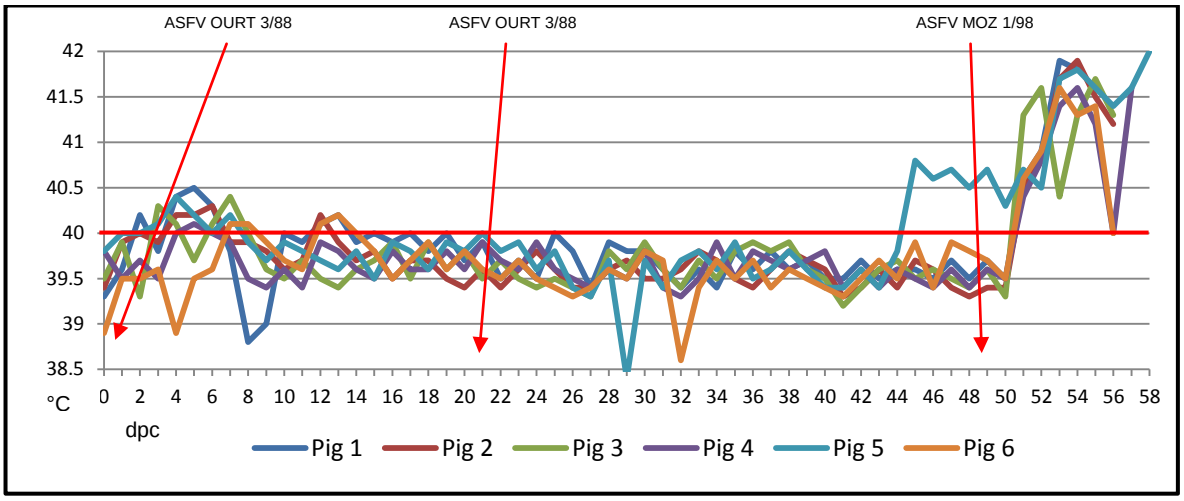


Figure 5.2 Rectal temperature (°C) of pigs in Group 1 (coloured lines). Vaccination with ASFV OURT 3/88 occurred 0 and 21 days and challenge with ASFV MOZ 1/98 at day 49 (indicated by arrows). Fever threshold (40.0°C) is shown as a horizontal red line.

Group 2 was subjected to a single immunization with ASFV OURT 3/88 and was challenged with ASFV MOZ 1/98 28 days later (Figure 5.3). Four of the six pigs in this group (Pig 1, Pig 2, Pig 5 and Pig 6), showed a significant rise in temperature from 9-21 dpv but returned to normal. However, by three to four dpc, all pigs had developed temperatures above 41.0°C. There was a typical rise approaching 42.0°C similar to animals in Group 1.

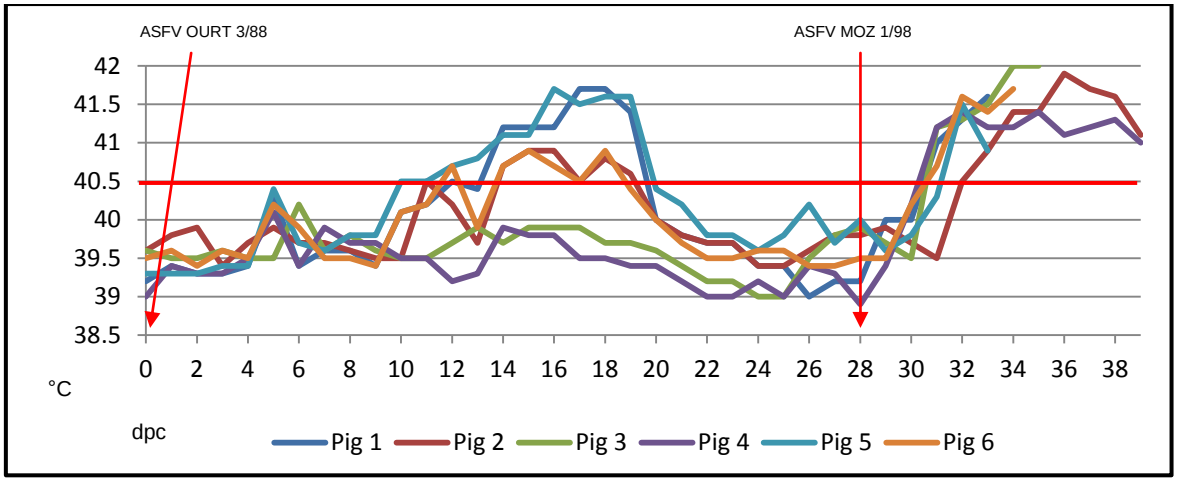


Figure 5.3 Rectal temperatures (°C) of pigs in Group 2 (coloured lines). Vaccination with ASFV OURT 3/88 occurred at day 0 and challenge with ASFV MOZ 1/98 at day 28 (indicated by arrows). Fever threshold (40.0°C) is indicated as a horizontal red line.

The control group was subjected to ASFV MOZ 1/98 challenge without vaccination (Figure 5.4). Two animals had slightly elevated temperatures by two dpc. Interestingly, Pig 1 never manifested fever although clinical signs were severe and similar to the rest and to previous results reported in Chapter 4.

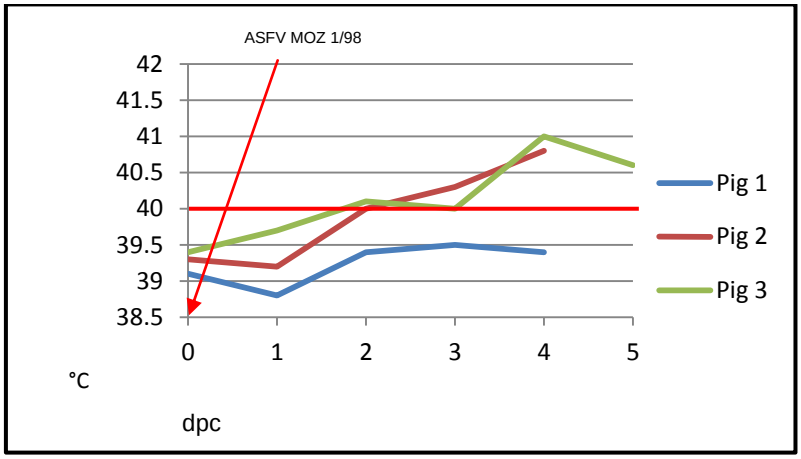


Figure 5.4 Rectal temperatures (°C) of pigs (coloured lines) in control group challenged with ASFV MOZ 1/98 (indicated by arrow) in days post challenge (dpc). Fever threshold (40.0°C) is indicated as a horizontal red line.

5.2.3. Clinical signs

A full description of clinical signs is given in Table 5.2. Analogous to previous findings (Chapter 4), there were no clinical signs after vaccination with ASFV OURT

3/88. At three days after challenge with ASFV MOZ 1/98, every pig in both Groups 1 and 2 manifested as clinically sick. Clinical signs detected in the aforementioned groups where similar to the control group. However, animals in the latter group presented a faster debilitation process since two of them died four dpc and the remaining one the next day. Symptoms were equally severe in all regardless of past immunizations.

The majority of pigs started out by showing serous ocular discharge, fever, and congestion in the skin. As a few days passed (one to four dpc) debilitation was evident and clinical signs of ASF continued to appear. There was serious depression, slow reaction to touch, diarrhoea, huddling together, tremors and eventually they stopped eating and became prostrated.

Table 5.2 Clinical signs of ASFV inoculations of candidate vaccine trials from time of challenge with ASFV MOZ 1/98

Clinical signs: serous ocular discharge (π) & muco-purulent ocular discharge (ππ); congestion (c) & cyanotic congestion (cc) of ears (e), snout (s), tail (t), periorbital (o) or body in general (b), conjunctiva (cj) and sclerae (sc), e.g. congestion of snout (cs); diarrhea (D); tremors (¥); huddling together (Δ); cough or barks (ĉ); lethargy/apathy/depressed/slow reaction to touch (‡); prostration (†); fever above 40.0°C (F); off-feed (ø); euthanized (E); Died (×).

Days	Group 1						Group 2						Control group		
	(1)	(2)	(3)	(4)	(5)	(6)	(1)	(2)	(3)	(4)	(5)	(6)	(1)	(2)	(3)
0					F										
1					F		F								
2	F	F	F	F	F	F	F			F		F		F	F
3	F,π	F,π	F,π	F,π	F,π	F,π	F,π	π	F,π	F,π	F,π	F,π	π,‡,	F,π,‡,	F,π,‡
4	F,π,‡,ce, ccj,csc	F,π,‡	F,π,‡	F,π,‡	F,π	F,π,‡	F,π,‡	F,π,‡	F,π,‡,ce,co, ccj,csc	F,π,‡	F,π,‡	F,π,‡	π,‡,ce,co, ×	F,π,‡,ce,co, ×	F,π,‡,ce,co
5	F,π,‡,ce, ccj,csc, ×	F,π,‡,Δ	F,π,‡,Δ	F,π,‡,Δ.ĉ	F,π	F,π,‡,Δ	F,π,‡,ce, co,cs,cb, ×	F,π,‡, Δ	F,π,‡,Δ,ce,co, cs,cb	F,π,‡,Δ	F,π,‡,Δ,ř, †,ce,co,ct, ×	F,π,‡,Δ,ř,ce, cs,cb,ĉ			F,ππ,‡,ce, co,cs, ø, ×
6		F,π,‡,Δ	F,π,‡,Δ	F,π,‡,Δ	F,π	F,π,‡,Δ		F,π,‡,Δ	F,π,‡,Δ,D	F,π,‡,Δ		F,π,‡,Δ,ř, ce,cs,cb,ĉ, ø,E			
7		F,‡,ce,ct,ø,×	F,‡,ce,ct,D,ø,×	F,‡,cect	F,π ,‡	F,‡,D, π, ×		F,π,‡,Δ	F,π,‡,Δ,ce,cs,D,×	F,π,‡,Δ					
8				F,‡,Δ,ø,×	F,π ,‡			F,π,‡,Δ		F,π,‡,Δ					
9					F,ø,‡,E			F,ππ,‡,Δ		F,ππ,‡,Δ					
10								F,ππ,‡,Δ		F,ππ,‡,Δ					
11								F,ππ,‡,Δ,ø,¥,E		F,ππ,‡,Δ,ø,¥,×					

5.2.4. Gross pathology

During post-mortem, tissue samples were stored for further analysis and all lesions noted. Selection of samples was according to previously described ASF pathology (Penrith *et al*., 2004b; Plowright *et al.*, 1994) and quantified for ASF virus in tissue material as before (Chapter 2, Boinas *et al*., 2004; King *et al*., 2011).

Internal and external post-mortem examinations indicated that lesions were similar to observations noted before. The most common external lesions included congestion and cyanotic congestion in ears, periorbital area, snout, tail, limbs, and ventrum. Internally we found severe generalised enlargement and haemorrhages in lymph nodes. Other lesions were: congestive splenomegaly, hepatomegaly; blood stained froth from nostrils, trachea and cut lung surface; severe interlobular oedema of the lungs; excess yellowish fluid in the thorax, pericardial sac and peritoneum; oedema and petechiation of gall bladder walls; congestion and haemorrhages of the mucosa of the stomach (fundus and pylorus) and small intestines; petechiation of both renal cortex and medulla; and congested urinary bladder mucosa. Furthermore, lesions of pigs in Group 1 (Table 5.3), Group 2 (Table 5.4) and control group (Table 5.5) were comparable since the result was severe acute pathological manifestation characterized by a haemorrhagic syndrome among generalized lymphadenopathy, which all indicates to they were caused by the same challenge virus, ASFV MOZ 1/98.

In Group 1, Pig 5 demonstrated a less severe pathological outcome noted during post-mortem examination. There were no haemorrhages observed in this individual and it was euthanized for ethical reasons other than disease discomfort. Pig 2 involved in Group 2 showed considerably fewer lesions than the rest of the animals of the same group and specifically did not present haemorrhages. Therefore, it lived longer than the other pigs in all groups.

Table 5.3 Post-mortem of domestic pigs in Group 1

Pig number		1	2	3	4	5	6
External examination							
Cyanotic congestion of the skin	Ears	ccc	c	ccc	ccc	cc	-
	Periorbital	ccc	-	ccc	ccc	-	-
	Snout	ccc	-	ccc	ccc	-	-
	Tail	ccc	c	ccc	ccc	-	-
	Limbs	ccc	-	ccc	ccc	-	-
	Ventrum	ccc	-	ccc	ccc	-	-
	Dorsum	-	-	ccc	ccc	-	-
	Lateral plane	-	-	ccc	ccc	-	-
Ocular discharge		ππ	ππ	ππ	ππ	ππ	ππ
Stools adhered to perineum		-	-	+	-	-	-
External wounds						+	
Internal examination							
Haemodynamic disorders	Hydrothorax	-	-	-	+	-	-
	Hydropericardium	-	-	-	+	-	-
Lymphadenopathy	Tonsils	Δ	ΔΔ; ss	ΔΔ; ss	ΔΔ; ss	c	ΔΔ; ss
	Mediastinal lnn	ΔΔ; sss	ΔΔΔ; sss	ΔΔΔ; sss	ΔΔΔ; sss	-	ΔΔΔ; sss
	Gastro-hepatic lnn	ΔΔ; sss	ΔΔΔ; sss	ΔΔΔ; sss	ΔΔΔ; sss	c	ΔΔΔ; sss
	Mesenteric lnn	ΔΔΔ; sss	ΔΔΔ; sss	ΔΔ; ss	Δ; s	-	ΔΔΔ; sss
	Renal lnn	ΔΔΔ; sss	ΔΔΔ; sss	ΔΔΔ; sss	ΔΔΔ; sss	-	ΔΔΔ; sss
Viscera	Diaphragm	-	-	-	-	-	‡‡
	Lungs	sss	ss	sss	sss	••	sss; ••
	Froth in cut sections	+++	++	+++	+++	-	+++
	Blood in cut sections	+++	-	+++	+++	-	-
	Interlobular oedema	+++	++	+++	+++	-	-
	Heart						
	Left auricle/atrium	††	††; ‡‡	††; ‡‡	†††; ‡‡‡	-	‡‡
	Right auricle/atrium	††	††; ‡‡	††; ‡‡	†††; ‡‡‡	-	‡‡
	Left ventricle	††	-	-	-	-	‡‡
	Right ventricle	††	-	-	-	-	‡‡
	Spleen	s	ss	s	ss	s	sss
	Liver	s	‡	-	s	-	ss
	Gall bladder	-	†	-	‡‡	-	‡‡‡
	Kidneys						
	Renal cortex	†††	†††	†	‡‡‡	-	†††; ‡‡‡
	Renal Cortico-medullar junction	-	-	-	‡‡‡	-	-
	Renal medula	†††	†††	‡	‡‡‡	-	-
	Renal pelvis						†††; ‡‡‡
	Subcapsular haemorrhage	-	-	-	++	-	+++

	Stomach						
	Non-glandular	-	††	-	-	-	††; cc
	Glandular	†; c	††; cc	††; cc	‡; c	-	††; cc
	Small intestine	†††; ‡‡‡	-	‡‡	††; cc	-	††; ‡‡
	Large Intestine	†††; ‡‡‡	-	‡‡; cc	††; cc	-	††; ‡‡
	Mesentery						
	Urinary bladder	‡‡	††	‡‡	-	-	†
	Mucopurulent dischage	-	+	+	-	-	-
Date of death/euthanasia (dpc: Days after MOZ 1/98)		5	7	7	8	9	7

No alterations: (-); Mild: +; Moderate: ++; Severe: +++; Serous ocular discharge (π) and mucopurulent ocular discharge (ππ); Consolidation (diffuse): ••; Congestion (mild): c; Congestion (moderate): cc; Cyanotic congestion (extensive or severe): ccc; Haemorrhagic congestion (mild): Δ; Haemorrhagic congestion (moderate): ΔΔ; Haemorrhagic congestion (severe): ΔΔΔ; Petechial haemorrhages (few): †; Petechial haemorrhages (multifocal or diffuse): ††; Coalescent petechial haemorrhages: †††; Ecchymotic haemorrhages (few/): ‡; Ecchymotic haemorrhages (difuse): ‡‡; Coalescent ecchymotic haemorrhages: ‡‡‡; Swelling (mild): s; Swelling (moderate): ss; Swelling (severe): sss.

Table 5.4 Post-mortem of domestic pigs in Group 2

Pig number		1	2	3	4	5	6
External examination							
Cyanotic congestion of the skin	Ears	ccc	cc	ccc	ccc	ccc	ccc
	Periorbital	ccc	-	-	ccc	-	ccc
	Snout	ccc	-	ccc	ccc	ccc	ccc
	Tail	ccc	cc	ccc	ccc	ccc	ccc
	Limbs	ccc	-	ccc	ccc	-	ccc
	Ventrum	ccc	-	-	ccc	-	ccc
	Dorsum	ccc	-	-	ccc	-	ccc
	Lateral plane	-	-	-	ccc	-	ccc
Ocular discharge		ттт	ттт	ттт	ттт	ттт	ттт
Epixtasis		+	-	-	-	-	-
Stools adhered to perineum		-	-	+	-	-	-
External wounds		-	+	-	-	+++	-
Internal examination							
Haemodynamic disorders	Hydrothorax	+	-	-	-	++	
	Haemothorax	-	-	-	+	-	
	Hydropericardium	+	-	-	-	+++	
	Haemopericardium	-	-	-	+++	-	
	Hydroperitoneum	-	-	+	-	-	
Lymphadenopathy	Tonsils	Δ; s	Δ; s	ΔΔ; ss	Δ; s	ΔΔ; ss	ΔΔ; ss
	Mediastinal lnn	ΔΔΔ; sss	-	ΔΔΔ; sss	Δ; s	ΔΔΔ; sss	ΔΔΔ; sss
	Gastro-hepatic lnn	ΔΔΔ; sss	Δ; s	ΔΔΔ; sss	ΔΔΔ; sss	ΔΔΔ; sss	ΔΔΔ; sss
	Mesenteric lnn	ΔΔΔ; sss	-	ΔΔΔ; sss	ΔΔΔ; sss	Δ; s	ΔΔ; ss
	Renal lnn	ΔΔΔ; sss	Δ; s	ΔΔΔ; sss	ΔΔΔ; sss	ΔΔΔ; sss	ΔΔ; ss
	Inguinal lnn	-	+	-	-	-	-
Viscera	Diaphragm	-	-	‡	-	‡	
	Lungs	sss; ††; ••	•••	sss; •	sss; •••	sss; •	ss; ••; †; ‡
	Froth in cut sections	+++	-	+++	+++	+++	++
	Blood in cut sections	+++	-	+++	+++	-	++
	Interlobular oedema	+++	-	+++	+++	+++	++
	Heart						
	Left auricle/atria	†	-	††	†	-	-
	Right auricle/atria	†	-	††	†	-	-
	Left ventricle	†	-	††	‡	†; ‡	-
	Right ventricle	†	-	††	‡	†; ‡	-
	Spleen		ss	ss	sss	sss	s
	Liver	s	-	ss	ss	-	
	Gall bladder	‡‡; †††	-	‡‡	ss; ‡‡‡	-	
	Kidneys						
	Renal cortex	†††	-	††; ‡‡	‡	††	‡
	Renal cortico-medullar junction	†††	-	††; ‡‡	‡	c	‡

	Renal medula	-	-	-	‡	-	-
	Renal pelvis	-	-	††; ‡‡	‡	‡‡	-
	Subcapsular Haemorrhage	-	-	-	++	-	-
	Pancreas	-	-	s; cc	-	-	
	Stomach						
	Non-glandular	-	-	††	-	-	
	Glandular	cc	-	††; cc	†††; cc; uu	c	†; c
	Small intestine	††	-	††; cc	††	-	††
	Large Intestine	‡‡‡	-	††	†	-	††
	Mesentery	-	-	-	-	cc	-
	Urinary bladder	††; cc	-	cc	†; c	‡‡	‡
Death/euthanasia (in dpc)		5	11	7	11	5	6

No alterations: -; Mild: +; Moderate: ++; Severe: +++; Serous ocular discharge (π) and mucopurulent ocular discharge (ππ); Consolidation (localized) •; Consolidation (diffuse): ••; Consolidation (Severe or extensive) •••; Congestion (mild): c; Congestion (moderate): cc; Cyanotic congestion (extensive or severe): ccc; Haemorrhagic congestion (mild): Δ; Haemorrhagic congestion (moderate): ΔΔ; Haemorrhagic congestion (severe): ΔΔΔ; Petechial haemorrhages (few): †; Petechial haemorrhages (multifocal or diffuse): ††; Coalescent petechial haemorrhages: †††; Ecchymotic haemorrhages (few): ‡; Ecchymotic haemorrhages (difuse): ‡‡; Coalescent ecchymotic haemorrhages: ‡‡‡; Swelling (mild): s; Swelling (moderate): ss; Swelling (severe): sss; Ulcerative necrosis (multifocal or diffuse): uu

Table 5.5 Post-mortem of domestic pigs in Group 3

Pig number		1	2	3
External examination				
Cyanotic congestion of the skin	Ears	cc	cc	ccc
	Periorbital	cc	cc	ccc
	Snout	cc	cc	ccc
	Tail	-	-	ccc
	Limbs	-	-	ccc
	Ventrum	-	-	ccc
Ocular discharge		ππ	ππ	ππ
External wounds		++	++	-
Internal examination				
Haemodynamic disorders	Hydrothorax	-	-	+
	Haemothorax	+	+	-
	Haemoperitoneum	+	+	-
Lymphadenopathy	Tonsils	ΔΔ; ss	ΔΔ; ss	ΔΔΔ; sss
	Mediastinal lnn	ΔΔ; ss	Δ; s	Δ; s
	Gastro-hepatic lnn	ΔΔΔ; sss	ΔΔΔ; sss	ΔΔΔ; sss
	Mesenteric lnn	ΔΔΔ; sss	ΔΔΔ; sss	ΔΔΔ; sss
	Renal lnn	ΔΔΔ; sss	ΔΔΔ; sss	ΔΔΔ; sss
Viscera	Lungs	ss; cc	ss; cc	sss
	Froth in cut sections	-	+++	+++
	Blood in cut sections	-	-	-
	Interlobular oedema	-	-	+++
	Heart	s	s	
	Left auricle/atria	††	-	-
	Right auricle/atria	††	-	-
	Left ventricle	††	-	††; ‡‡
	Right ventricle	††	††	††; ‡‡
	Spleen	s	-	-
	Liver	-	ss	ss
	Kidneys			
	Renal cortex	‡	-	††
	Renal cortico-medullar junction	‡	-	-
	Renal medulla	-	-	-
	Renal pelvis	-	-	††
	Stomach			
	Glandular	ΔΔ	-	cc
	Small intestine	††	††	‡
	Large Intestine	††	††	†††
	Mesentery	c	c	c
	Urinary bladder	‡	††	-
Date of death (in dpc)		4	4	5

No alterations: -; Mild: +; Moderate: ++; Severe: +++; Mucopurulent ocular discharge:ππ; Congestion (mild): c; Congestion (moderate): cc; Cyanotic congestion (extensive or severe): ccc; Haemorrhagic congestion (mild): Δ; Haemorrhagic congestion (moderate): ΔΔ; Haemorrhagic congestion (severe): ΔΔΔ; Petechial haemorrhages (few): †; Petechial haemorrhages (multifocal or diffuse): ††; Coalescent petechial haemorrhages: †††; Ecchymotic haemorrhages (few): ‡; Ecchymotic

haemorrhages (difuse): ‡‡; Coalescent ecchymotic haemorrhages: ‡‡‡; Swelling (mild): s; Swelling (moderate): ss; Swelling (severe): sss.

5.2.5. Viral load analysis

As in the virulence trial, viral quantification was carried out on blood samples collected at set dates during the trials (stipulated in Table 5.6) and organ tissues harvested at necropsy. Both blood and organ samples were appropriately stored after collection until DNA extraction. Subsequently, purified DNA samples were subjected to the vp72 Taqman real time PCR.

5.2.5.1 Viraemia

None of the vaccinated pigs in both Groups 1 and 2, demonstrated viraemia by real time PCR after ASFV OURT 3/88 inoculation, except Pig 4 in Group 1 that had a transient reaction at 35 dpv (14 days after the second vaccination). Besides this exception, ASFV DNA in blood only showed after challenge with ASFV MOZ 1/98. In both Groups 1 and 2, samples were positive in one pig each as soon as three dpc. In the control group virus was present as soon as three dpc in all individuals.

In Group 1 Pig 5 never showed any viral DNA in the blood (Table 5.6) whilst Pig 1 was positive as soon as three dpc. By five dpc, all the remaining pigs demonstrated viraemia. Pig 1 manifested a significant increase from three to five dpc (4.09×10^2 to 1.15×10^5 virus/ml), while the virus load levels in the rest of the pigs in the group remained stable after challenge.

Table 5.6 ASFV quantification (virus/ml) of blood samples from pigs in Group 1.

Dpv	Pig number					
	1	2	3	4	5	6
0	Negative	Negative	Negative	Negative	Negative	Negative
...	Negative	Negative	Negative	Negative	Negative	Negative
21	Negative	Negative	Negative	Negative	Negative	Negative
...	Negative	Negative	Negative	Negative	Negative	Negative
35	Negative	Negative	Negative	5.43×10^2	Negative	Negative
49	Negative	Negative	Negative	Negative	Negative	Negative
52	4.09×10^2	Negative	Negative	Negative	Negative	Negative
54	1.15×10^5	1×10^4	1.31×10^4	1.23×10^4	Negative	4.46×10^4
56	Death	2.25×10^4	1.41×10^4	8.59×10^3	Negative	1.82×10^4
60	-----	Death	Death	Death	Negative	Death

Shading: Inoculation days; (...): mid collection days negative to PCR at 3, 5, 7, 10, 14, 24, 26, 28, 31 and 33 dpi; -----: no data.

In Group 2, the earliest positive individual was Pig 3 demonstrating viraemia as soon as three dpc and with a significant increase from three dpc (2.05×10^3 virus/ml) to five dpc (1.03×10^5 virus/ml). By five dpc, four pigs in this group were showing viraemia while Pig 4 only became positive at seven dpc and the remaining Pig 2 turned viraemic as late as 11 dpc (Table 5.7).

Table 5.7 ASFV quantification (virus/ml) of blood samples from pigs in Group 2.

Dpv	Pig number					
	1	2	3	4	5	6
0	Negative	Negative	Negative	Negative	Negative	Negative
...	Negative	Negative	Negative	Negative	Negative	Negative
28	Negative	Negative	Negative	Negative	Negative	Negative
31	Negative	Negative	2.05×10^3	Negative	Negative	Negative
33	1.74×10^4	Negative	1.03×10^5	Negative	3.71×10^4	8.59×10^3
35	Death	Negative	Death	5.43×10^2	Death	Death
39	-----	3.66×10^3	-----	Death	-----	-----

Shading: Inoculation days; (...): mid collection days negative to PCR at 3, 5, 7, 10, 14, 24 and 26 dpi; -----: no data.

Pigs in the control group were all positive as soon as three dpc (with ASFV MOZ 1/98) and revealed high virus loads. Pig 3 survived one day longer than the rest in the group and an increase in blood virus load was noted from three to five dpc (Table 5.8).

Table 5.8 ASFV quantification (virus/ml) of blood samples from pigs in Group 3.

Dpc	**Pig number**		
	1	**2**	**3**
0	Negative	Negative	Negative
3	9.55×10^5	2.04×10^5	4.35×10^4
5	Death	Death	2.27×10^6

Shading: Inoculation days.

Virus load in Groups 1 and 2 was significantly statistically different at day 5 compared to Group 3 but there was no significant difference between Groups 1 and 2 using a two-way ANOVA with repeated measures.

5.2.5.2 Virus load in organs

All organ samples collected from Pigs 1 and 6 in Group 1 were positive for viral DNA, while Pig 5 that showed no viraemia had DNA present only in the lungs and gastro-hepatic lymph nodes (Table 5.9). The intestine samples were positive in only two of the six pigs, while Pig 1 had > 10^5 virus/ml in the spleen, liver, and gastro-hepatic lymphnodes.

Table 5.9 ASFV quantification (virus/ml) of post mortem samples from pigs in Group 1.

Organ	**Pig number**					
	1	**2**	**3**	**4**	**5**	**6**
Skin	2.3×10^4	4.16×10^3	2.9×10^4	5.46×10^2	Negative	5.35×10^3
Tonsil	6.36×10^1	1.62×10^3	Negative	Negative	Negative	2.66×10^2
Lungs	2.79×10^4	1.72×10^4	Negative	2.03×10^4	1.21×10^4	5.54×10^2
Heart	1.23×10^3	6.73×10^3	1.64×10^3	3.72×10^2	Negative	5.32×10^1

Mediastinal lnn	1.42×10^4	4.98×10^3	2.51×10^2	2.57×10^2	Negative	6.02×10^1
Spleen	3.25×10^5	6.43×10^4	Negative	9.07×10^3	Negative	1.18×10^3
Liver	3.39×10^5	3.49×10^3	4.42×10^2	1.1×10^3	Negative	9×10^2
Kidneys	3.1×10^4	7.03×10^3	Negative	4.83×10^2	Negative	8.25×10^1
Gastro-hepatic lnn	1.84×10^5	1.87×10^2	Negative	4.76×10^2	2.72×10^1	2.23×10^3
Mesenteric lnn	7.17×10^3	4.57×10^3	9.11×10^1	1.82×10^3	Negative	4.8×10^2
Stomach	2.65×10^3	1.18×10^3	1.18×10^2	2.03×10^2	Negative	2.44×10^1
Intestine	2.97×10^3	Negative	Negative	Negative	Negative	1.64×10^2
Bladder	1.26×10^2	1.23×10^4	2.35×10^3	5.58×10^2	Negative	1.38×10^2

In Group 1, the organs most affected as determined by average virus load in the different tissue samples were in the following order from high to low: spleen followed by liver, gastro-hepatic lymphnodes, lungs, skin, kidneys, mediastinal lymphnodes, mesenteric lymphnodes, bladder, heart, stomach, intestine, and tonsils (Figure 5.5.).

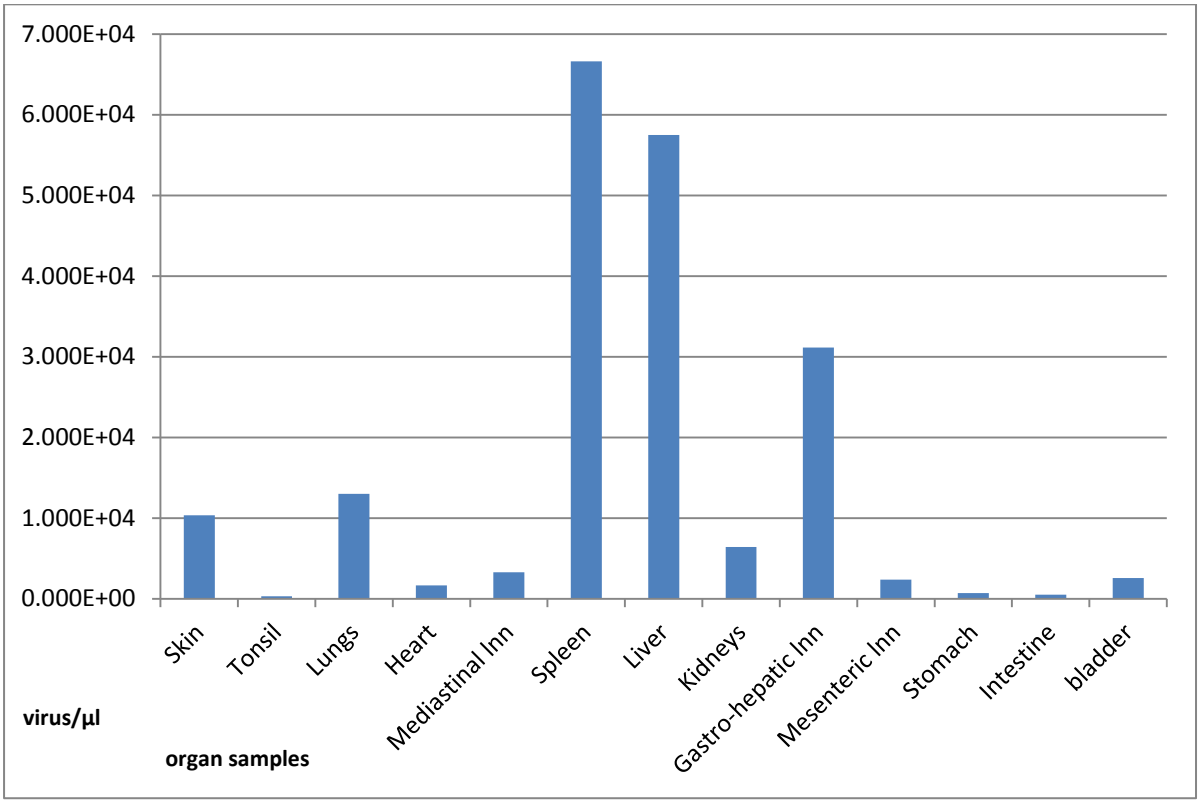


Figure 5.5 Viral load (virus/ml) averages of each organ sample from six pigs in Group 1.

Table 5.10 summarises the results obtained from organs collected in Group 2. Four pigs (Pig 1, Pig 3, Pig 4, and Pig 6) had ASF DNA in all collected organs. Pig 2 was the last in Group 2 to become viraemic and had fewer organs affected since only the

skin, tonsil, lungs, mediastinal lymphnodes, and intestine were positive and with virus load less than 10^3 virus/ml. Furthermore, Pig 5 was negative on tonsil and mesenteric lymphnodes.

Table 5.10 ASFV quantification (virus/ml) of post mortem samples from pigs in Group 2.

Organ	Pig number					
	1	2	3	4	5	6
Skin	8.59×10^4	2.44×10^2	1.63×10^4	8.66×10^3	8.62×10^3	6.1×10^4
Tonsil	1.13×10^5	2.4×10^2	1.22×10^5	6.91×10^1	Negative	1.66×10^3
Lungs	1.83×10^5	1.13×10^3	1.09×10^5	7.96×10^3	2.18×10^4	9.37×10^4
Heart	2.18×10^2	Negative	2.72×10^4	1.01×10^3	2.29×10^3	4.01×10^3
Mediastinal lnn	5.58×10^2	5.24×10^2	2.1×10^5	1.25×10^2	6.02×10^2	2.03×10^3
Spleen	2.19×10^5	Negative	2.58×10^4	5.35×10^4	3.29×10^3	1.04×10^5
Liver	3.32×10^5	Negative	1.35×10^5	1.82×10^4	2.68×10^3	3.79×10^5
Kidneys	3.14×10^4	Negative	1.13×10^5	5.95×10^3	1.14×10^3	8.92×10^3
Gastro-hepatic lnn	1.95×10^4	Negative	1.12×10^5	7.88×10^3	2.32×10^3	8.59×10^4
Mesenteric lnn	3.94×10^3	Negative	1.11×10^5	1.65×10^3	Negative	3.94×10^3
Stomach	6.25×10^3	Negative	1.42×10^4	5.28×10^3	1.27×10^3	8.74×10^3
Intestine	5.54×10^3	2.18×10^2	5.32×10^4	3.98×10^2	1.37×10^2	1.1×10^3
Bladder	4.28×10^3	Negative	1.09×10^5	7.1×10^2	1.21×10^2	1.48×10^3

Organ sample from pigs in Group 2 with the highest average of virus load was the liver followed by the lungs, spleen, tonsil, gastro hepatic lymphnodes, mediastinal lymphnodes, skin, kidneys, mesenteric lymphnodes, bladder, intestine, stomach, and the heart (Figure 5.6).

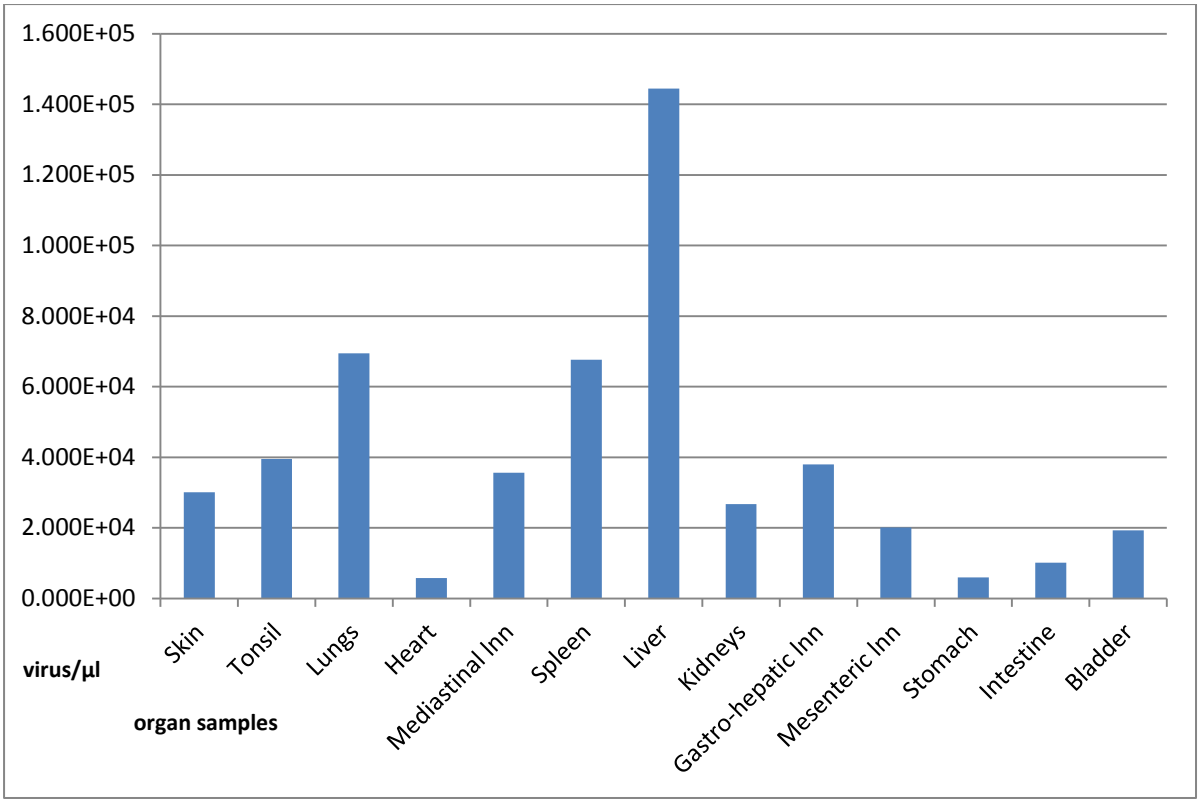


Figure 5.6 Viral load (virus/ml) averages of each organ sample from six pigs in Group 2

Organ samples from the three pigs in the control group were mostly positive for viral DNA except liver and kidney in Pig 1 and intestine in Pig 2 (Table 5.11).

Table 5.11 ASFV quantification results (virus/ml) using realtime PCR of post mortem samples from pigs in control group (group 3).

Organ	Pig number		
	1	2	3
Skin	2.97×10^4	1.77×10^4	1.43×10^4
Tonsil	4.16×10^5	2.84×10^2	5.06×10^5
Lungs	1.31×10^6	8.44×10^4	5.39×10^5
Heart	3.48×10^3	4.8×10^4	1.67×10^4
Mediastinal lnn	1.58×10^5	1.12×10^5	1.91×10^4
Spleen	7.99×10^5	3.94×10^5	8.14×10^5
Liver	Negative	1.71×10^5	1.33×10^6
Kidneys	Negative	6.95×10^4	1.69×10^5
Gastro-hepatic lnn	1.64×10^4	4.98×10^4	1.19×10^6
Mesenteric lnn	4.13×10^4	1.79×10^3	1.1×10^5

Stomach	1.23×10^5	3.09×10^4	1.06×10^5
Intestine	2.1×10^4	Negative	5.43×10^4
Bladder	5.13×10^4	7.7×10^3	4.61×10^4

The organ with the highest average of virus load in the control group was the spleen. Subsequently in decreasing order of average of quantified virus obtained per sample were spleen, lungs, liver, gastro-hepatic lymph nodes, tonsil, kidneys, mediastinal lymph nodes, stomach, mesenteric lymph nodes, bladder, intestine, heart, and skin (Figure 5.7).

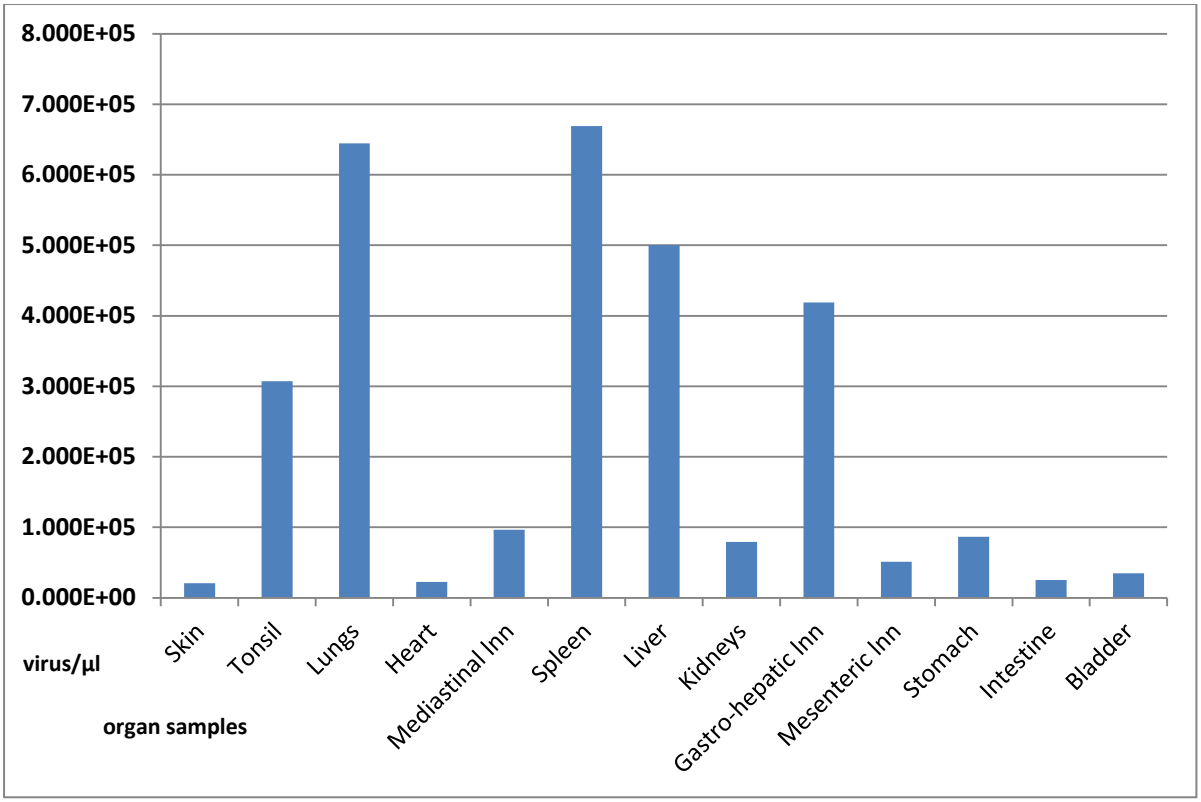


Figure 5.7 Viral load (virus/ml) averages of each organ sample from three pigs in Group 3

The average virus load ranking is represented in Table 5.12 for each tissue sample collected from pigs in their respective groups. It indicates highest average virus load to the lowest. Since there was no indication of virus in organ samples by PCR after infection with ASFV OURT 3/88, it is safe to assume these results were a measurement of ASFV MOZ 1/98 present in the collected organs as a consequence of the challenge inoculation.

Table 5.12 Ranking of the average virus load in organ samples from highest to lowest concentration of virus in Groups 1, 2 and 3.

Rank	Group 1	Virus/ml	Group 2	Virus/ml	Group 3	Virus/ml
1	Spleen	6.66×10^4	Liver	1.44×10^5	Spleen	6.69×10^5
2	Liver	5.75×10^4	Lungs	6.94×10^4	Lungs	6.44×10^5
3	GH lnn	3.12×10^4	Spleen	6.76×10^4	Liver	5.00×10^5
4	Lungs	1.30×10^4	Tonsil	3.95×10^4	GH lnn	4.19×10^5
5	Skin	1.03×10^4	GH lnn	3.79×10^4	Tonsil	3.07×10^5
6	Kidneys	6.43×10^3	Med. lnn	3.56×10^4	Kidneys	7.95×10^4
7	Med. lnn	3.29×10^3	Skin	3.01×10^4	Med. Lnn	9.64×10^4
8	Bladder	2.58×10^3	Kidneys	2.67×10^4	Stomach	8.66×10^4
9	Mes. lnn	2.36×10^3	Mes. lnn	2.01×10^4	Mes. lnn	5.10×10^4
10	Heart	1.67×10^3	Bladder	1.93×10^4	Bladder	3.50×10^4
11	Stomach	6.96×10^2	Intestine	1.01×10^4	Intestine	2.51×10^4
12	Intestine	5.22×10^2	Stomach	5.96×10^3	Heart	2.27×10^4
13	Tonsils	3.25×10^2	Heart	5.79×10^3	Skin	2.06×10^4

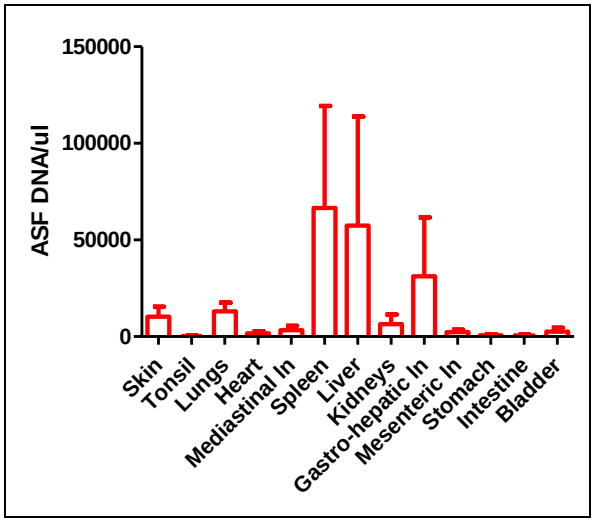
Med.: Mediastinal; GH: Gastro-hepatic; Mes.: Mesenteric;

In addition Mean, SD (standard deviation), SEM and %CV (covariance) were calculated for the viral organ loads per group. These statistical results are in Table 5.13.

Table 5.13 Viral organ loads results per group with Mean, SD (standard deviation), SEM and %CV.

	Group 1				Group 2				Group 3			
	Mean	SD	SEM	%CV	Mean	SD	SEM	%CV	Mean	SD	SEM	%CV
Skin	10342.67	12445.09	5080.688	120.3277	30120.67	34846.33	14225.95	115.6891	20566.67	8090.323	4670.95	39.33707
Tonsil	324.9333	642.7614	262.4062	197.8133	39494.85	60492.57	24695.99	153.1657	307428	269774.1	155754.2	87.75197
Lungs	13009	11114.43	4537.446	85.43644	69431.66	71784.44	29305.87	103.3886	644466.7	619569.4	357708.5	96.13676
Heart	1670.867	2564.599	1046.993	153.4892	5788	10594.58	4325.221	183.044	22726.67	22863.69	13200.36	100.6029
Mediastinal lnn	3291.367	5684.62	2320.736	172.713	35639.83	85421.19	34873.05	239.679	96366.66	70757.35	40851.78	73.42513
Spleen	66591.66	129024.4	52673.98	193.7545	67598.34	83544.61	34106.95	123.5897	669000	238275	137568.2	35.6166
Liver	57488.67	137917.2	56304.44	239.9	144480	171570.2	70043.23	118.7501	500333.3	723581.6	417760	144.6199
Kidneys	6432.583	12348.22	5041.138	191.9636	26735	43778.23	17872.39	163.7488	79500	84942.63	49041.65	106.8461
GH lnn	31153.37	74883.97	30571.25	240.372	37933.33	48449.79	19779.54	127.7235	418733.3	668145.3	385753.8	159.5634
Mesenteric lnn	2355.183	2917.656	1191.128	123.8823	20088.33	44572.46	18196.63	221.8823	51030	54757.24	31614.11	107.304
Stomach	695.9	1054.985	430.6959	151.6001	5956.667	5170.883	2111.004	86.80833	86633.34	49009.22	28295.49	56.57085
Intestine	522.3333	1200.9	490.2653	229.9106	10098.83	21215.31	8661.115	210.0769	25100	27381.2	15808.54	109.0884
Bladder	2578.667	4842.761	1977.049	187.8009	19265.17	43988.97	17958.42	228.3342	35033.33	23813.72	13748.86	67.97446

a)



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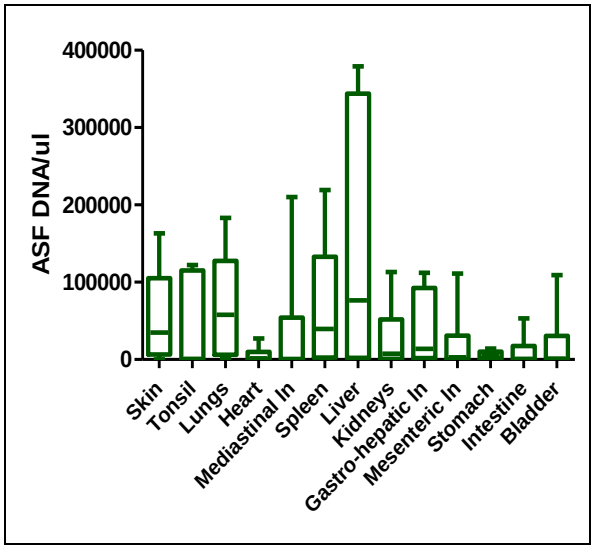

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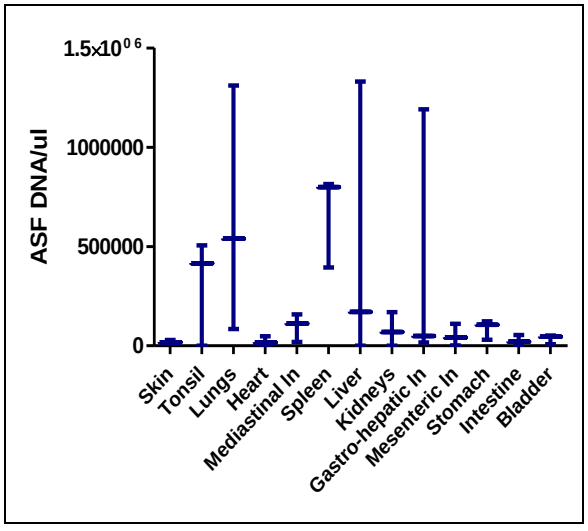

Figure 5.8 Bar charts indicating the mean and SD of the different organs for each group (a: red = Group 1; b: green = Group 2; c: blue = Group 3)

From Figure 5.8a - c the mean and SD of each group is demonstrated graphically. Furthermore, when data from virus load in organ samples were run in a two way ANOVA analysis there was statistical difference between lungs ($P<0.001$), spleen ($P<0.001$), liver ($P<0.001$) and gastro-hepatic lymphnodes ($P<0.01$) when comparing Groups 1 and 3 (control group). There was also a statistical difference between Groups 2 and 3 (control group) in the lungs ($P<0.001$), spleen ($P<0.001$), liver ($P<0.05$) and gastro-hepatic lymphnodes ($P<0.01$). However, there were no significant differences between virus load in Groups 1 and 2 ($P>0.05$).

5.3. Discussion

All domestic pigs submitted to the scheduled inoculations behaved similarly in the sense that either none of the animals vaccinated with OURT 3/88, with either one or two inoculations, showed any symptoms indicative of ASF infection. However, clinical signs appeared a few days after challenge with virulent ASFV MOZ 1/98 and all animals died. Although there was no significant difference in the virus loads in the various organs between the two vaccinated groups (P>0.05), there were significant differences from the unvaccinated group, especially in the lungs, spleen, liver and gastro-hepatic lymphnodes. The pathological alterations detected in the pigs during virulent ASF disease account for the lesions seen in acute disease in which domestic pigs usually die within 5 - 7 days after infection showing generalized lymphadenopathy and an acute haemorrhagic syndrome (Oura *et al.*, 1998a; Oura *et al.*, 1998b; Penrith *et al.*, 2004b). Common lesions that may be detected during necropsy in either acute or subacute infections are diffuse haemorrhages in lymph nodes, spleen, kidneys, liver and other organs (Fernandez, 1992; Gomez-Villamandos *et al.*, 1995; Penrith *et al.*, 2004b). Often death is due to hypovolaemic shock induced by pathogenic mechanisms directly related to the virulence of the strain causing disease (Mebus, 1988; Fernandez, 1992; Villeda *et al.*, 1993). In the current study, pigs developed clear signs of disease only after challenge with ASFV MOZ 1/98, and death occurred between four to 11 dpc in all groups involved. None of the vaccinated pigs in Groups 1 or 2 demonstrated viraemia by PCR after ASFV OURT 3/88 infection, except Pig 4 in Group 1 that strangely showed a transient reaction at 35 dpv. Necropsies revealed that the majority of animals died suddenly because of internal haemorrhage and consequent hypovolaemic shock and the majority showed haemorrhagic and congestive lymphadenopathy, except for Pig 5 in Group 1 and Pig 2 in Group 2.

The median survival times (where fractional survival equals 50%) for Groups 1 and 2 were similar, as was the hazard ratio, indicating no significant difference in survival rates between the double and single vaccination regimes. However, despite the fact that all pigs died in the end, there was a significant difference (P = 0.0196) between the survival rates of the vaccinated and unvaccinated groups.

ASFV DNA did not show in blood samples after inoculation with ASFV OURT 3/88 alone. Blood samples of one pig in each of Groups 1 and 2 gave positive results on the real time PCR as soon as three days after submitting them to challenge with the pathogenic strain (ASFV MOZ 1/98). In contrast, all pigs in the control group presented viraemia at this point, suggesting that non-immunized animals developed ASF more rapidly. The earliest general onset of viraemia in the control group and the quantity of virus detected compared to other groups relates to the survival period of pigs from the point when they were challenged until termination. Individuals in both groups vaccinated with ASFV OURT 3/88 had longer survival periods (5 to 9 dpc; 5 to 11 dpc respectively in Group 1 and Group 2) than control group pigs (4 to 5 dpc in Group 3). However there was no statistical significant difference in survival time between the single (Group 2) and the boosted vaccinated (Group 1) animals.

In the present study, it was interesting to note that Pig 5 in Group 1 demonstrated a less severe pathological outcome noted throughout the entire trial and it survived longer than the other pigs in the same group and showed less clinical signs. During post-mortem examination no haemorrhages were observed in this individual. Euthanasia of this animal was for ethical reasons other than disease discomfort. Interestingly, it showed fever before inoculation with ASFV MOZ 1/98 and this persisted until termination. Viral load analysis indicates Pig 5 never developed viraemia and had few organ tissue samples positive on real time PCR (lungs and gastro hepatic lymphnodes). Pig 2 in Group 2 demonstrated more subtle signs of disease since it was the last to show fever, viraemia was detected considerably later than the rest (11 dpc) and fewer lesions were observed at post-mortem. It did not present haemorrhages while the rest did and fewer organ samples were positive. All pigs in the control group developed homologous pathologies but strangely, Pig 1 did not show fever.

Tonsils showed the lowest virus load in Group 1 whilst in Group 2 and 3, copy number of virus ranked amongst the highest in tonsils. Virus load in liver was very high in the majority of animals. Furthermore the bladder, which was not included in the preceding virulence trial, showed virus load up to 10^5 virus/ml in some pigs except in Pig 5 from Group 1 and Pig 2 from Group 2. These did not develop severe

pathology and infection was not present in many other organs as shown in Table 5.9 and Table 5.10.

The isolate designated ASFV MKUZE (Thomson, 1985) included in Chapter 4 was considered as a potential vaccine candidate because it induced antibodies, did not cause serious pathology and did not kill the pigs up to 18 dpi at which time they were euthanized. However, it seemed to cause chronic ASF (see chapter 4). Results of the virulence trials suggest that although the ASFV strain isolated from ticks in KwaZulu-Natal (ASFV MKUZE) displayed reduced pathogenesis, it is not sufficiently avirulent for use as a live vaccine. In contrast, ASFV OURT 3/88, which is a fully attenuated strain, did not induce any clinical signs, pathological lesions or viraemia. Nevertheless, sera tested positive to ASFV antibodies at seven dpi, which is an indication of viral replication. This isolate was an appropriate candidate to test for its cross-immunisation potential against a virulent southern African ASF isolate (ASFV MOZ 1/98). These viruses are different genotypes ASFV OURT 3/88 (genotype I) and ASFV MOZ 1/98 (genotype VIII). Whatever vaccine against ASF is developed, it should prevent viral replication so that effects of the virus on the reticulo-endothelial system avoid consumption coagulopathy of the infected individual (Wardley *et al.*, 1985).

The immunological mechanisms involved in the defence of pigs against virulent ASF are still undergoing research in order to define appropriate prophylactic measures. In a study done by Neilan *et al.*, 2004 neutralizing antibodies to proteins involved in ASFV attachment, vp30, vp54 and vp72, did not protect pigs from ASFV challenge but it did delay the onset of disease. Antibodies against vp72 were detected in pigs inoculated with ASFV OURT 3/88 in the virulence trial (chapter 4). Furthermore, ASFV OURT 3/88 can boost the production of antibody against ASFV in domestic pigs without causing apparent disease (Boinas *et al*., 2004). Pigs that were immunised with live attenuated ASF viruses engineered with deletions of specific ASFV virulence genes showed immunity when challenged with homologous parental virus (Lewis *et al.*, 2000). According to Lewis *et al*. (2000), Leitão *et al*. (2001) and Oura *et al*. (2005), full or partial protection can develop against infection with more pathogenic strains, in pigs previously infected with a non-pathogenic ASFV isolate. King *et al.* (2011), reported on a similar attempt to immunize domestic pigs.

Although first vaccination was with non-virulent ASF OURT 3/88, booster vaccination was different (ASFV OURT 1/88, virulent strain). Challenge virus was either the virulent ASFV BENIN 1/97 (used in chapter 4 as reference virus) or an isolate from Uganda collected in 1965. They were able to induce immunity against such strains since most pigs did not become sick nor showed viraemia. The present study is similar but booster vaccination (Group 1) was with the same virus as the first inoculation (ASFV OURT 3/88), before virulent challenge with a geographically distinct and pathogenic isolate (ASFV MOZ 1/98).

According to Pan and Hess, 1984 an ASF isolate is a heterogeneous virus population consisting of clones that have different biological characteristics. The virulence of an isolate is according to the virulence of the predominant clones and not by the quantity of virus administered. They concluded that immune pigs were not necessarily resistant to homologous or heterologous challenge exposure. In the current study, although single and boosted vaccination of susceptible pigs with ASFV OURT 3/88 did briefly delay the initial onset of disease and lowered virus concentration in certain organs, it had no effect on disease development, progression, or outcome. The virus used here was isolated from ticks in Europe and is genetically distinct from strains typically circulating in southern Africa, thus being a heterologous strain of ASFV. Since infection with attenuated strains confers protection to pigs against homologous challenge it is conceivable that partial protection could be attained following vaccination with an African attenuated strain, genetically closer to the current circulating southern Africa isolates. Results described here are consistent with previous reports suggesting that in some cases, European ASFV isolates are attenuated and better adapted to domestic pigs than African field strains (Ordas-Alvarez & Marcotegui 1987; Mebus 1988). In light of this, efforts to identify naturally attenuated African strains should continue. Alternatively, attenuated viruses based on African strains could potentially be developed *in vitro* by site directed mutagenesis or deletion of immunomodulatory genes.

Chapter 6

Conclusions

The various methods tested for the detection of ASFV that involve cell cultures and PCRs are important for ASF research and diagnosis. Cell cultures allow virus growth from positive blood or organ samples like spleen, liver, and lymphnodes. It detects viable virus in animals and ticks. Alternatively, PCR testing may be more sensitive because it allows detection of DNA from ASFV at lower concentration and if the isolate is no longer viable in blood or tissue, where a diagnosis is important.

In the present study, cell cultures revealed different titres and in comparison, bone marrow macrophage cultures (BMC) seemed to be the most reliable followed closely by blood macrophage culture (BC) and lastly, alveolar macrophage cultures (AMC) with few readable or reliable results. However, P values proved there were no statistically significant differences between BMC and BC. The AMC results were not integrated in the statistical analysis for the reason that there were too many contaminated plates that therefore were excluded from evaluation. Contamination seemed to especially affect AMC cell cultures. Although BMC showed high cell density which may complicate detection of rosettes, it did not compromise titrations.

The PCR trial results showed no significant differences between the various PCR assays ($P = 0.919$) when testing the ASFV MOZ 1/98 serial ten-fold dilutions made before DNA extractions. In contrast when testing dilutions done from ASFV SPEC 257 suspension there were significant differences detected between the Central variable region PCR (CVR) and p72 PCR assays and between CVR and PPA PCR assays.

Results from the virulence trial in chapter 4 enabled conclusions to be drawn about the pathology of previously unknown ASFV MKUZE and ASFV MOZ 1/98. It was clear that ASFV MKUZE is moderately virulent and causes chronic ASF whilst ASFV MOZ 1/98 is virulent and responsible for acute ASF. Furthermore, this trial confirmed what was expected from reference viruses ASFV OURT 3/88 and ASFV BENIN 1/97.

Since ASF has a sylvatic cycle where virus can remain undetected until contact with susceptible domestic pigs, the availability of a vaccine to control the disease is very important. Threats to commercial pig production are constantly evident with the potential for new outbreaks by spill over of the agent from the wild reservoir to the domestic cycle. In addition, the failures of some governments to compensate farmers when culling is done to contain the disease encourages illegal movement and allows continuous new outbreaks because infected live pigs and contaminated meat are sold in local informal markets without sanitary inspection.

Despite extensive efforts to develop a vaccine, there is still not a product on the market that protects pigs against haemorrhagic acute ASF. In the present study, the isolate used as potential vaccine (ASFV OURT 3/88) showed statistically significant protection when inoculated into domestic pigs subsequently submitted to challenge with southern African ASFV isolate, ASFV MOZ 1/98. This is true for the difference in survival rates of group 1 and group 2 when compared to group 3 ($P = 0.0196$). The same does not apply between the survival rates of groups 1 and 2 ($P = 0.6548$). In conclusion however, both single and double vaccination regimes failed to confer total protection against subsequent inoculation with a highly virulent isolate since all pigs showed symptoms from one-day post challenge (dpc) with ASFV MOZ 1/98. Viraemia was detectable by three dpc, and animals reached the end-point within eleven dpc. Nevertheless, although the vaccination regimes seemed not to have worked entirely, it did result in lower virus load concentrations in the organs and blood of the pigs involved. This indicates there is a potential for the development of live attenuated virus vaccines for protection of domestic commercial pigs against ASF. A more attenuated isolate than ASFV MKUZE from within the same region should be trialled in order to determine whether it confers protection from virulent isolates in the area.

Chapter 7

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