DETERMINATION OF OPTIMAL VIRAL DOSE, IMMUNOLOGICAL RESPONSE AND SURVIVAL RATE IN LOCAL AND EXOTIC PIGS EXPERIMENTALLY INFECTED WITH AFRICAN SWINE FEVER VIRUS

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE IN VETERINARY EPIDEMIOLOGY AND ECONOMICS

DEPARTMENT OF PUBLIC HEALTH, PHARMACOLOGY AND TOXICOLOGY

FACULTY OF VETERINARY MEDICINE

UNIVERSITY OF NAIROBI

2019

DECLARATION

This thesis is my original work and has not been presented for a degree in any other University

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DEDICATION

This thesis is dedicated to my family, my husband Felix Wandolo, children Sylvester, Esther, Samson and Jacob, University of Nairobi and to the entire ILRI team for their unwavering support.

ACKNOWLEDGEMENT

I am very grateful to the people who have played several roles in ensuring that I pursue my studies successfully.

I am grateful to God almighty for the favour granted to me during the whole period of study and writing this dissertation. It is my pleasure to express my sincere gratitude and appreciation to my supervisors Dr. Richard Bishop (ILRI), Dr. Edward Okoth (ILRI), Dr. Joshua Onono and Dr. Gabriel Aboge of University of Nairobi (UON) for their tireless efforts, guidance and support during the whole period of doing this work. Their constant advices, criticism, encouragement and provision of necessary tools during my work have seen my studies accomplished. My sincere gratitude extends to my colleagues who were there for me when conducting different activities of the research especially Cynthia Onzere for her help and endurance during laboratory work. Special appreciation goes to International Livestock Research Institute for allowing me to undertake the study in their precincts and the cooperation and tireless help they did to me during data collection. My sincere gratitude goes to University of Nairobi Library especially Ancietta Majau for taking her time to assist me. Moreover, I thank all participants who were involved in data collection for their cooperation. I am indebted to Richard Bishop and Felix Wandolo for funds provided to me during my study and funds for conducting this research.

TABLE OF CONTENTS PAGE

LIST OF TABLES PAGE

LIST OF FIGURES PAGE

LIST OF APPENDICES

LIST OF ABBREVIATIONS

ASF – African swine fever

ASFV – African swine fever virus

BSL 2 – Biosafety Level 2 Facility

CISA-INIA - Centro de Investigación en Sanidad Animal / Animal Health Research Center

CSF – Classical swine fever

Ct - Cut-off threshold value

DNA – Deoxyribonucleic Acid

Dpi – Days post inoculation

HAD – Haemadsorption units

ICDV – Icosahedral cytoplasmic deoxyribovirus

ILRI – International Livestock Research Institute

ODs – Optical Density values

PBMCs – Peripheral blood mononuclear cells

PCR – Polymerase chain reaction

RIA – Radioimmunoassay

RNA – Ribonucleic Acid

RPMI - Roswell Park Memorial Institute medium

VP – Viral Protein

ABSTRACT

Two genotypes of African swine fever virus were identified around East Africa based on molecular characterization of P72 and P54 genes. These are designated genotypes IX and X. whereas genotype IX has been associated with recent lethal ASF outbreaks in East Africa; genotype X has been associated with infections that apparently did not produce clinical responses, in south west Kenya. Biological and clinical characteristics of these viruses have not been studied in experimental infections. This study aimed to conduct an in vivo biological characterization of ASFV genotype IX isolate 1033 originating from an ASF outbreak in Western Kenya, that has been shown to be genetically closely related to many outbreak associated viruses in Kenya and Uganda isolated from both local and exotic breed pigs. The specific aim of the experiment was to find out the titration of the virus that was able to cause clinical disease following experimental infection as well as to study the clinical symptoms induced by the different dilutions in order to determine a useful challenge dose for vaccination research. Housed European breed and local free range domestic pigs were challenged with doses containing between $10^{-4}$and 10^{-5} HAD_{50} (Haemadsorbing units), of the highly virulent ASFV isolate 1033 by inoculation using the intramuscular route. Pigs were monitored for clinical progression and post mortem lesions and these were compared to previously describe experimental and natural infections. Samples were analyzed for virus titres, antibody and cellular responses in vitro. The statistical analyses were carried out using GraphPad Prism and Microsoft Excel.

The data obtained from the experiment gave the most effective dose for virus infection hence provided insight into the host response to infection by this particular isolate. For both exotic and local pigs, the clinical signs were characterized by loss of appetite, fever of between 39.6 and 41.4^{o}C, foul smelling diarrhoea, lateral recumbence and death. For the exotic, there were skin lesions characterized by cyanosis and haemorrhages. The main pathological lesions were characterized by generalized haemorrhages, coagulation disorders, oedema of the lungs and lymph nodes and gastroenteritis. The estimated optimal viral dose for the exotic pigs ranged between 10^{-6} to $10^{-7}HAD_{50}$/ml (titres) and $10^{-5.4}HAD_{50}$/ml for the local pigs. The mean optical density values for the immune response of exotic pig were 0.17 and 0.18 for the local pigs. Average survival time for the exotic pigs was 10 days post infection and 12 days post infection for the local pigs. The results achieved were aimed at providing basic information which will in future assist in vaccine search for ASF control and assist in developing guidelines for veterinarians regarding quarantine and control measures in naive pigs brought in and for outbreak control. Further research should be directed towards identifying the epitopes responsible for the increased survival of local pigs which can be used for vaccine development. A lot needs to be done on the African swine fever genome and the genetic material in the local pigs that enables partial survival to ASF infection should be identified and modified to see if the virulence of ASF will reduce or enable the exotic pigs survive ASF infection without clinical disease.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

African Swine Fever is a disease of the porcine characterized by generalized haemorrhages and high mortality rates which approaches 100% in affected farms (Penrith et al. 2013). It is caused by African swine fever virus (ASFV) of genus *Asfivirus* (Oura et al. 2013). The disease is closely related to classical swine fever in terms of clinical signs but differ epidemiologically and immunologically.

The virus has nucleoprotein structure which is 70 – 100 nm in diameter in mature virion and this is surrounded by icosahedral capsid which is 172 – 191 nm in diameter. The viral structure is enveloped by a lipid layer (Salas & Andrés 2013). There are attachment proteins; p12 and p24 which attaches on the extracellular regions of the virion while polyprotein products of protein pp220 (p150, p37, p34 and p15) are located in the core shells of the virion (Salas & Andrés 2013) and on the surface of the unenveloped intracellular virion, there is a capsid protein, p72. In addition, the African swine fever virus particle contains a DNA and other proteins (Salas & Andrés 2013). Furthermore, there are four enzymes encoded on the genome which play different roles (Lokhandwala et al. 2016).

The genome encodes multigene families (MGF) and this comprises of a larger portion of the genome. The MGF is located on the left terminus and right terminus and this renders the ASFV highly variable genetically (Escribano et al. 2013). Entry into the host cell is via receptor-mediated endocytosis. During the binding and entry process there is fusion of the virus envelope

and development of endocytotic pits hence virus release into the cytoplasm (Netherton & Wileman 2013). DNA reduplication first occurs in the nucleus of the infected cell and later continues in the cytoplasm (Howey et al. 2013) . Peak reduplication is achieved after eight hours(Escribano et al. 2013).

Once a pig is infected with ASFV, the virus penetrates and replicates in the lymphoid tissues especially the ones around the head and neck region. The virus then disseminates in the host systems through viraemia (Blome et al. 2013). African Swine Fever virus has predilection of antigen presenting phagocytic cells of the lymphoreticular system. It causes cytolysis in acute cases and hyperplastic changes in subacute cases. Pathological changes involve haemorrhages, oedema, thrombocytopaenia, effusions, impaired vascular integrity, coagulopathy and dysfibrinogenaemia (Gómez-Villamandos et al. 2013). As a result, the kidneys, lungs and skin of chronically infected pigs are compromised (Blome et al. 2013). Due to these processes, pigs develop fever of up to 42^{o}C and most of them die from infection.

Efforts towards production of ASF vaccine has been unfruitful, but new development in the bioinformatics platform provides hope that protection against this highly infectious disease may soon be a possibility (Kenah et al. 2016).

1.2 Problem Statement

African swine fever disease results in great economic losses in the country and globally thereby discouraging farmers from engaging in pig production. The disease is characterized by sporadic outbreaks leading to high morbidity and high mortality rates in affected pig herds. A series of experiments have been carried out using this isolate to establish its molecular characteristics (Okoth et. al, 2013). These studies resulted in understanding of the virus in terms of knowing the

genotype of this particular isolate as well as isolates around East Africa. This has also resulted to a better understanding on the epidemiology of the virus but there are no studies done on the immunological and biological characterization of the pathogen hence the need to carry out this project. This study was aimed at estimating the optimal viral dose, immunological response and survival rates of pigs of different genotypes with a focus on obtaining knowledge for developing a vaccine in the future.

1.3 Justification of the study

Frequent and sporadic ASF outbreaks have led to poor and decreased pork production in the country. There are challenges with the technology which is currently used for its control in Kenya. There is need to estimate the infective dose and immunological response and survival rates of swine infected with African swine fever virus and especially isolate (Bus.1033) that is involved in most ASF outbreaks in Kenya. This knowledge will assist in development of vaccine for the control of the disease. Initially, an experiment was performed entitled Biological and Immunological Characterization of African Domesticated Pigs Infected with an African Swine Fever Virus from Kenya (IACUC Reference number 2014.2.) in order to establish *in vivo* properties of ASFV 1033 and relate these to *in vitro* HAD_{50} titres. An experiment had been conducted at the Central Veterinary Laboratory Kabete pig facility in 2011 to compare responses to infection with an ASFV virus in pigs of Kenyan and European breeds (Ref: VN_IACUC-2011-ASF in vivo infection Ref 2011-04).

The results from the experiment in 2011 showed a level of tolerance to infection in local Kenyan pigs compared to pure landrace X large white crosses. This was indicated by a delayed onset of clinical signs and longer survival rates based on the experimental end point of 21 days. All exotic

bred pigs had died with classical ASFV symptoms on this timescale, whereas only three of 20 indigenous pigs had died. Given the differences in response observed in 2011, the current experiment was designed to build on the recent dose titration with a virulent genotype IX isolate (Bus.1033) that was isolated by ILRI as part of an AusAID-funded project in Busia district in 2013.The aim was to repeat the dose titration in indigenous pigs to see if the immunological response and survival rates differs from that which was observed in the European pigs. This was important since indigenous pigs represent a target for vaccination and experiments designed to evaluate protective immune responses. A second objective was to assess the reproducibility of the previous dose titration experiment in the European breeds. There appeared to identify a critical threshold between the response in pigs using a 10^{-4} HAD_{50} and 10^{-5} HAD_{50} of the stock virus (corresponding to HAD_{50} titres of approximately $10^{6}HAD_{50}$ and $10^{5}HAD_{50}$). This resulted in survival times of 8-9 or 16-24 days, respectively. It was important to check that this was reproducible in a repeat experiment.

1.4 General Objective

To investigate infective dose, immunological response and survival rates in porcine infected with the virus isolate 1033 under experimental conditions.

1.5 Specific Objectives

i. To estimate the infective dose of the virus isolate in pigs.

ii. To analyze immunological response of porcine infected with the virus isolate.

iii. To estimate the survival rate of porcine infected with the isolate.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 African Swine Fever

2.1.1 Aetiology

The disease is caused by African swine fever virus. It is arthropod-borne and capable of undergoing protein replication (Howey et al. 2013). It is a double stranded DNA virus (Salas & Andrés 2013). Whereas it is a DNA virus, the Classical swine fever virus is an RNA virus. The mature virion consists of a nucleoprotein core structure, 70 – 100 nm in diameter, surrounded by an icosahedral capsid of 172 – 191 nm in diameter, and a lipid containing envelope (Netherton & Wileman 2013). There are 22 genotypes of ASFV most of which are traced from the wildlife and each vary in terms of their virulence. In Kenya, most outbreaks are associated with genotype IX while genotype X was isolated from samples collected from pigs reared in Homa Bay (South West Kenya) in pigs which were not showing any clinical signs of the disease (Atuhaire et al. 2013).

2.1.2 Distribution

It was first described in Kenya as a disease affecting pigs, and it is distinct from the classical swine fever (Guinat et al. 2016). Following this discovery, the disease was later reported to occur in South African pig herds (Jori et al. 2013). Further discoveries were made in Angola in 1932 (Penrith et al. 2013). In South Africa and Kenyan farms, the disease outbreaks were linked to contacts between wild pig species and domesticated pigs, and therefore efforts to fight disease outbreaks was geared towards ensuring minimal or no interaction of wild swine from the domestic pigs (Penrith et al. 2013)

The disease was reported in Europe for the first time in 1957 in Portugal, with further outbreaks reported in 1960 from pigs which were apparently introduced into that country from Angola (Guinat et al. 2014). The second outbreak in Portugal was not quickly contained, and therefore it led to ASF rapid spread within Eastern Europe (Kalenzi Atuhaire et al. 2013). The disease later became endemic within the Iberian Peninsula, but the area was later clean from the disease (Costard et al. 2013). ASF became established in Italy on the island of Sardinia and later on it spread to Cuba where its eradication has been reported to be difficult. In 1977-78 there was a resurgence of ASF in the Iberian peninsula and this affected Latin America (Costard et al. 2013).

The cost of its eradication in the affected countries is considerably high and time consuming. European countries which had highly developed pig industries were seriously affected by the occurrence of ASF. This led to further studies in order to come up with a vaccine against the disease (Costard et al. 2013). But, vaccine development had already been initiated in Angola (Penrith et al. 2013). Apparently, this led to the production of low virulent virus in the process causing sub acute and chronic disease in pigs from connected farms and systems, hence the cases became complex to recognize. These low virulence viruses remained in circulation for longer periods in these pig populations.

Further research in ASF led to the discovery of the argasid ticks inhabiting the pig sties and pens. These ticks were capable of maintaining the virus and transmitting it to the soft ticks and the porcine (Jori et al. 2013). It was later observed in a study that the genus *Ornithodoros* could be found both in burrows and in *Kholas* in Southern and Eastern Africa. This was a different mode of transmission as compared to the spread of ASF in Angola (Penrith et al. 2013). There are situations where ASF virus infection has been endemic where warthogs did not occur (Jori et al.

2013). This has been the case in Malawi and Senegal (Dixon et al. 2013). The pig population in Cameroon got affected by ASF in 1981 when there was an outbreak (Penrith et al. 2013). Following these outbreaks, the disease later became endemic (Jori et al. 2013), and since the virus that caused the outbreak was similar to the isolates in Europe (Kalenzi Atuhaire et al. 2013).

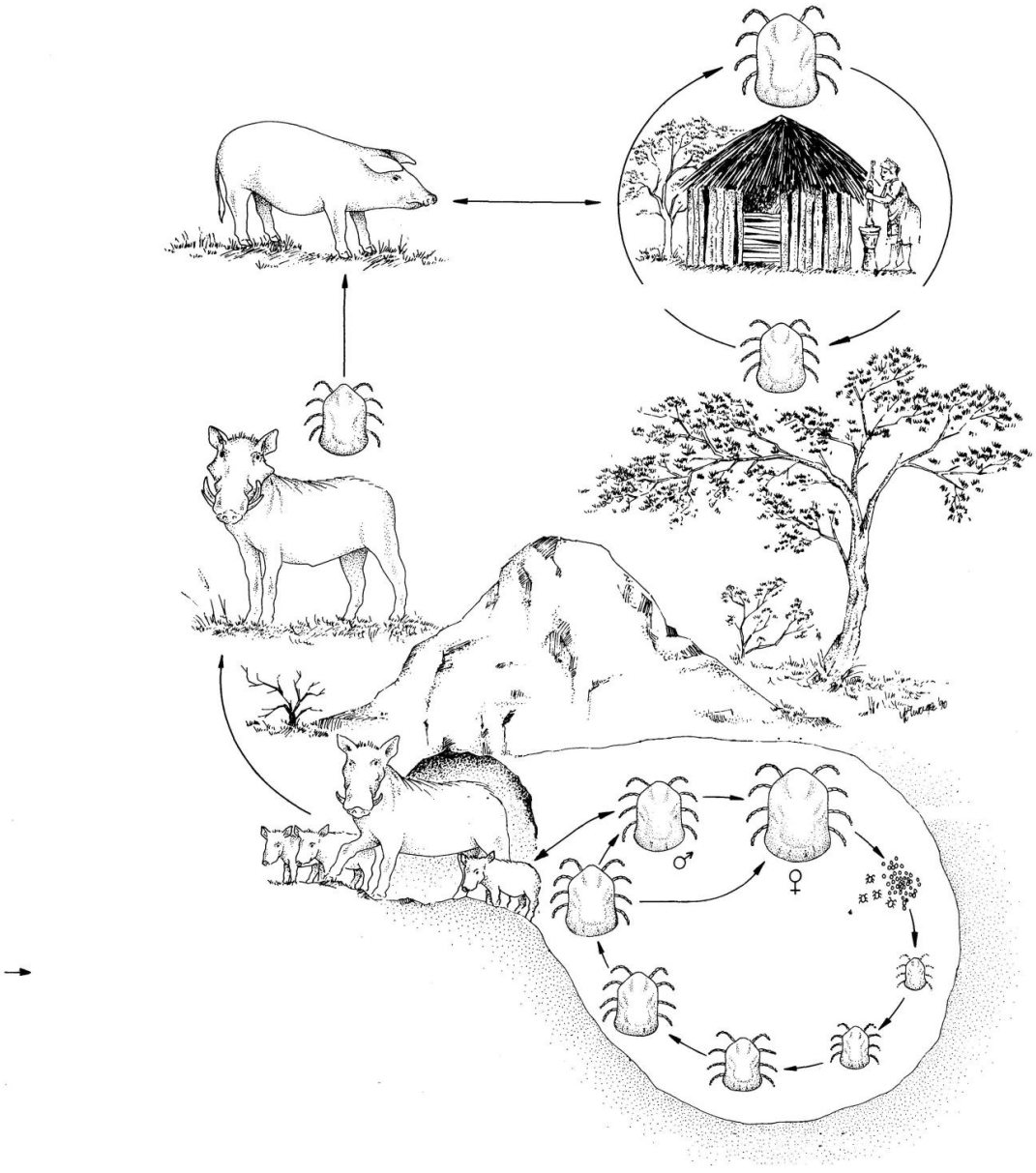

Figure 1: African swine fever virus transmission cycle (Penrith et al. 2012)

2.1.3 Transmission and Spread of African swine fever

A number of factors are involved in transmission of ASFV in the pigs and in the environment. The virus can spread as a result of contact between infected and clean pigs, bites from infected tampans (*Ornithodoros spp*.), pigs feeding on meat from affected pigs and by contact with infected objects (feed, bedding, clothes, equipment, footwear, vehicles) contaminated by virus-containing body fluids and wastes from infected pigs (Jori et al. 2013). It has been demonstrated that the natural hosts of the ASFV (warthogs) are unable to spread the virus directly (Jori et al. 2013). But, bush pigs are able to transmit virus to domestic pigs under experimental conditions (Jori et al. 2013). Also, ASFV can be spread through airborne route over short distances not exceeding two meters in experimental conditions (Costard et al. 2013).

There has been dramatic expansion of regions which are infected with African swine fever disease since 1994 (Costard et al. 2013). Most outbreaks have been linked to movement of infected pigs or pig products (Costard et al. 2013). Additionally, advanced molecular characterization has led to broader understanding and tracing origin of ASFV during outbreaks. Through advanced techniques it was demonstrated that unrelated ASFV (ASF virus of different genotypes) caused outbreaks in Tanzania in 2001, 2003 and 2004 but the exact source and route of infection was not established (Penrith et al. 2013).

Moreover, pigs which are reared extensively (free-ranging pigs); characterized by increased scavenging habits including feeding on meat from pigs that have died as a result of ASF disease as well as garbage containing remnants of infected pigs also causes exposure to infection with ASFV (Giménez-Lirola et al. 2016). Furthermore, people have also been reported to play a major

role in the spread of ASF due to poor habits of disposing infected carcasses (Costard et al. 2013). These infected carcasses are disposed in open areas where other pigs scavange, besides people moving from contaminated to uncontaminated areas without observing biosecurity measures and open boundaries involving unlicensed movement of animals and their products is also a major source of exposure (Jori et al. 2013). Literature have postulated that the ability of ASF virus to survive in a protein environment for long periods of time hence providing a good source of the virus especially pork from sick pigs, or those pigs that are found dead (Costard et al. 2013). The ASF virus can also survive in extreme temperatures, and therefore its inactivation require exposure to temperatures of over 60^0C for at least 30 minutes (Howey et al. 2013).

2.2 Socio-economic impact of ASF

African swine fever disease outbreak has resulted in catastrophic effects on pig production at household and at commercial levels (Gallardo M. C. et al. 2015). These have led to socio-economic problems thereby threatening food security. A typical case was experienced in Cameroon in 1982 in the Western province where 63% of the country's 1.1 million total pig population succumbed with 3,762 pig farmers reporting pig mortalities. As a result, majority of farm workers lost their jobs due to continued deaths that forced employers to reduce labour force. Pig eating ceremonies were completely abandoned leading to negative impact on the social aspect (Penrith et al. 2013).

ASF endemic in East Africa in 1989 led to high costs incurred during the control process (Atuhaire et al. 2013). This was in Zambia where 421,238 Zambian Kwacha was used in control measures in a commercial property (Costard et al. 2013). In Malawi, ASF outbreak in 1989 to

1990 led to deaths of 31,000 pigs (45%) out of 70,000 pigs' population as a result of mortalities and stamping out (M. C. Gallardo et al. 2015). Severe economic losses were also experienced during the control and eradication of ASF. Furthermore, designing models for the assessment of the economic impact of ASF outbreaks is reportedly costly especially during trade embargoes and eradication (Cardoso & Ferreira 2013).

2.3 Immunological Response

Vaccine development for African swine fever disease has been unfruitful due to extreme antigenic diversity (Giménez-Lirola et al. 2016). This has been demonstrated in several studies where the ASFV has been observed to show protective immunity. Pigs which recover from moderately virulent strains and pigs exposed to related virulent viruses have been reported to show long-term immunity. Challenge that is experienced with ASF virus infection is that these pigs rarely gain immunity with heterologous strains of the virus. As a result, there is cross-protective response but due to antigenic diversity among naturally occurring ASFV isolates vaccine development becomes a challenge. Cross-protection occurs in viruses of the same serogroups. The genotypes for ASFV are clearly define and therefore there is need to determine the serogroups so as to assist in vaccine research (C. Gallardo et al. 2015).

Most ASF outbreaks present as acute infections and in this form of the disease, the viral DNA will usually be detected and is usually high. This has been reported even in carcasses of pigs that die from acute ASF. Animals showing viraemia do not show any immune response as tested using by ELISA or by IPT (Cardoso & Ferreira 2013). Efforts have been made towards

development of live attenuated vaccines but due to safety concerns its use in field conditions is not yet recommended (Lacasta et al. 2015).

2.4 Infective Dose

Haemadsorption inhibition assay has been a key tool in determining the serological groups for ASFV as well as in determining the optimal viral dose (Dixon et al. 2013). The viral load capable of causing disease and to some extent mortality varies with the various genotypes since some viral strains are mild and some virulent. As a result there are different forms of the disease including peracute, acute, subacute and chronic forms (Blome et al. 2013). ASFV vary in virulence from extremely virulent strains to low virulent strains. The highly virulent strains causes upto 100% mortality while low virulent strains may lead to recovery, but result in life time carrier pigs which when introduced to ASF free area can cause a major outbreak of the disease (Giménez-Lirola et al. 2016).

2.5 Survival

Pigs infected with ASFV show different clinical forms and different outcomes depending on the isolate or strain (Gallardo et al. 2017). There are 22 genotypes of the ASFV but each varies in terms of virulence. Some infected pigs are presented with history of sudden death while others have notable symptoms typical of ASF then recovery; but others also show symptoms and succumb to infections. Those pigs that recover remain as carriers and may spread the disease in naïve pigs.

2.6 Control and Prevention of Africa swine fever Disease

Lack of vaccine and a wide range of genotypes causing ASF have led to heavy losses during the disease outbreak. Most farmers are also opting for other lucrative productions in order to prosper. To prevent ASF disease, a lot of measures have been put in to place to minimize losses during outbreaks(M. C. Gallardo et al. 2015). These include; ensuring no contact between warthogs and domestic pig, imposing quarantine so that infected pigs do not spread disease to the neighbourhood, stamping out the ill and in-contact pigs, prevention of movement of pigs and pig products through the infected areas and the application of chemical disinfectants (virkon) in infected swine habitats(Penrith et al. 2013). In 1990, there was successful eradication of ASF in Europe with the exception of Sardinia, the only ASF endemic place outside Africa (Escribano et al. 2013).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Design

This was randomized clinical trial. The experiment was carried out in an enclosed Biosafety Level 2 facility at the International Livestock Research Institute (ILRI). Twelve African indigenous breed of pigs from Homa Bay County in Western Kenya of both gender (male and female) were purchased from local farmers. These pigs were reared extensively by the owners, a system which is characterised by scavenging for food around villages. These pigs were over six months of age and they weighed above 50 kilograms. Twelve cross breed (Landrace X Large White) pigs were purchased from Uplands, a Farmer's Choice Limited farm located in Limuru, which is a quarantine station where exotic pigs are reared intensively for pork production. The pigs were of both male and female gender. They were above six months of age and weighed above 50 kilograms. For these two group of pigs there were 2 pigs which were used as donors to obtain blood for tissue culture for the experiments. These experimental pigs had no detectable level of African swine fever virus infection based on ASFV-specific polymerase chain reaction (PCR) and detectable antibody response using the routine serological test, INGENASA blocking ELISA. The pigs were tagged with eartags bearing different code numbers for ease of identification according to the standard serial identity of animals used in ILRI farms.

3.2 Ethical Statement

The experimental infection was performed at the secure animal facility (Biosafety Level 2 facility pig unit) at International Livestock Research Institute. The experiment was approved by

the ILRI Institutional Biosafety Committee (IBC) (Ref: VN_IACUC-2011-ASF in vivo infection Ref 2011-04).

3.3 Experimental design

The remaining twenty experimental pigs were divided into four groups, with each group comprising five pigs. There were two groups of local pigs and another two groups of exotic pigs. A preliminary experiment conducted at the director of veterinary services laboratories had shown that local breed of pigs survived experimental ASF infections, while the entire European breed of pigs died following experimental infection. Therefore in this experiment, it was hypothesized that all the ten controls would succumb during the period of the experiment.

3.4 Virus Preparation for Inoculation

Virus coded 1033 was isolated from the spleen of a pig sacrificed in the former Busia district during a longitudinal survey of ASF which was done by a team of researchers based at ILRI. The diagnostic specimen (spleen sample) was ASFV positive and was characterized and classified as a genotype IX ASFV isolate. Tissue homogenate was prepared from the spleen and passaged to obtain pure isolate of the 1033 ASF virus. The culture supernatant was subjected to alternate freeze-thawing process in order to break the cells and expose the virus. This virus was diluted ten-fold to the required doses used in the experiment. After a 21-day acclimatization period, the pigs were inoculated with 1 mL of virus at 10^{-4} 50% hemadsorbing doses (HAD_{50}) and 10^{-5} 50% hemadsorbing doses (HAD_{50}) in the different groups (Table 1).

Table 1: Arrangement of pigs in the pens as well as dose provided in the four groups

GROUP	BREED	NO. OF PIGS	PEN	DOSE IN HAD_{50}/mL
I	Local	5	D	10^{-4}
II	Local	5	A	10^{-5}
III	Exotic	5	C	10^{-4}
IV	Exotic	5	B	10^{-5}

3.5 Sample Collection for Analysis

A total number of 260 samples were collected which included 20 (5ml) whole blood, 60 (10ml) plain blood for harvesting serum, and 180 tissue samples. 20 mL of whole blood in heparin was harvested per pig for haemadsorption experiment, 20mL of whole blood in EDTA from each pig for use in virus detection, and 5mL of plain blood in order to harvest serum. All the samples were well labelled and transported in a cool box having ice parks to the laboratory for storage at -80^{o} C and further processing.

Table 2: Sampling Schedule

	Saturday	Sunday	Monday	Tuesday	Wednesday	Thursday	Friday
Day	**-2**	**-1**	**0**	**1**	**2**	**3**	**4**
EDTA (5ml)			X	X	X	X	X
Serum (10ml)			X				X
Heparin (20ml)			X				
	Saturday	Sunday	Monday	Tuesday	Wednesday	Thursday	Friday
Day	**5**	**6**	**7**	**8**	**9**	**10**	**11**
EDTA (5ml)	X	X	X		X		X
Serum (10ml)			X				X
Heparin (20ml)			X				
	Saturday	Sunday	Monday	Tuesday	Wednesday	Thursday	Friday
Day	**12**	**13**	**14**	**15**	**16**	**17**	**18**
EDTA (5ml)			X		X		X
Serum (10ml)			X				X
Heparin (20ml)			X				
	Saturday	Sunday	Monday	Tuesday	Wednesday	Thursday	Friday
Day	**19**	**20**	**21**	**22**	**23**	**24**	**25**
EDTA (5ml)			X				
Serum (10ml)			X				
Heparin (20ml)			X				
	Saturday	Sunday	Monday	Tuesday	Wednesday	Thursday	Friday
Day	**26**	**27**	**28**	**29**	**30**	**31**	**32**
EDTA (5ml)			X				
Serum (10ml)			X				
Heparin (20ml)			X				

Critically sick pigs with ASF symptoms were euthanized using a commercial Euthanasia Solution (Euthasol®) which was injected intravenous into jugular vein at a dose of 0.22 ml/kg IV. Post mortem was carried out and different tissue samples were collected. The organs included heart, lung, liver, spleen, kidney, tonsil, gastrohepatic lymph node, mesenteric lymph node and submandibular lymph node. These samples were packed in Falcon® tubes, properly labelled and taken to the laboratory for viral detection using real time Polymerase Chain Reaction (PCR) test.

3.6 DNA extraction from blood and tissues

Blood samples and tissue samples were kept at room temperature to thaw. Positive Extraction control and negative extraction control were included in the extraction process to ensure successful extraction process and to check for contamination. PEC was ASFV positive blood sample while NEC was sterile phosphate buffered saline (PBS) pH 7.0. Viral DNA was extracted from anticoagulated whole blood samples using a QIAamp® Viral RNA Mini Kit (QIAGEN protocol) using 200µL of blood per sample. For purification of DNA from tissues, a QIAamp® DNA Mini Kit (QIAGEN) was used according to the manufacturer's instructions (General Qiagen protocol pages 25 to 30). Subsequently, UPL (Universal Probe Library) real time PCR assay was performed according to the published protocol (Fernandez et. al 2012).

3.7 Determination of viral infective dose

Determination of infective dose was performed following protocols adopted from CISA-INIA, Spain (See appendix 10). In this procedure, all operations were performed under sterile conditions since contamination seriously affects the outcome. Between 500mls to 600mls of

heparinized blood was obtained intravenously from a donor pig. Following the PBMC (peripheral blood mononuclear cells) protocol (See appendix 10A); Peripheral Blood Mononuclear Cells (PBMCs) were harvested using a density gradient medium. These PBMCs are susceptible primary leukocyte of porcine origin. These were incubated at 37^{o}C and 5% CO_2 in RPMI 1640 media (Roswell Park Memorial Institute medium) for 4 days after which macrophages could be observed under microscope at a magnification of X40.

The macrophages were infected with African swine fever virus isolate 1033, then the media nourished with 10% porcine erythrocytes from donor pig. The virus was left to grow under special conditions for three days then observed under microscope to observe any haemadsorption. African swine fever virus has special protein known as CD2. It is this special protein on the cell wall of the virus that enables attachment of the red blood cells around the wall of the virus leading to Rosette formation hence haemadsorption (Penrith et al, 2012). Haemadsorption is characteristic of the African swine fever virus. It is this pure ASF 1033 isolate which was harvested, taken through a cycle of freeze-thawing to rupture the cells so as to expose the virus and then diluted in various proportions and used in the experiment (outlined in the Table 1). The end point of the virus titre was calculated using the Spearman-Karber method.

3.8 Determination of immune response in infected pigs using ELISA

Pig sera collected during the experiment was used to detect antibodies. The sera were brought to room temperature (allowed to thaw). Commercial kit was used following protocol provided by the manufacturer, INGEZIM PPA COMPAC K3 (INGENASA) → blocking ELISA assay which uses a monoclonal antibody (MAb) specific of VP73 ASFV protein.

3.9 Determination of survival rates of pigs

Of key interest was to determine if pigs inoculated with ASFV BUS1033 would survive or succumb. Also at which dose or dilution of the virus the pigs could succumb and if there was a difference in survival between the two breed of pigs. Survival functions were calculated for pig groups using Microsoft Excel. Comparisons were made within inoculated groups comparing doses and exposure length. This is a common approach (although not perhaps the most obvious) to base regression model on the proportional hazard assumptions. The exotic pigs were compared with the local pigs assuming that the ratio of the hazard for an individual on exotic to that for an individual on local remained constant over time. This applied even if the magnitude of hazards varied over time.

3.10 UPL Real-Time PCR Methodology

The primers, probe and Taqman Fast Advanced Mastermix were thawed on ice. These reagents were constituted to form a mastermix in a sterile 1.5mL eppendorf tube as outlined in the Qiagen protocol. The final volume required was based on the number of DNA samples assayed per run (cycle). 2.5L of DNA was added in clearly labelled smart cycler tubes; positive and negative reaction controls were included to check for contamination and ensure successful PCR run. The tubes were briefly centrifuged and placed in the Smartcycler (Cepheid) real-time PCR machine. The thermocycling profile included initial activation of DNA pool at 95^{o}C for three minutes followed by DNA denaturation at 95^{o}C for ten seconds and finally primer annealing at 58^{o}C for thirty seconds. DNA denaturation and primer annealing ran for 45 cycles with fluorescence acquisition being made in the FAM channel at the end of each PCR cycle. The generation of Cycle threshold (C_t) values was an indication of ASFV positive samples. The threshold cycle

(Ct) is the cycle number at which the fluorescent signal of the reaction crosses the threshold. The Ct is used to calculate the initial DNA copy number, because the Ct value is inversely related to the starting amount of the target (Real Time PCR Handbook).

3.11 Statistical analysis

GraphPad Prism and Microsoft Excel 2013 was used to analyze data obtained from the various tests. The Infective dose was expressed using haemadsorbing units which were achieved by using the Spearmann-Karber method. The wells in the plates used for culture which had haemardsorption (CPE) were counted and using a formula in the protocol, an approximate number of virus particles was achieved.Titer

= (X – d/2 + (d.S)) where;
X = log10 of lowest dilution (1)
d = log10 of dilution factor (1)
S = sum of proportion of HAD positive

Pig immune response to ASFV virus infection was determined using antibody titres. This was achieved after carrying out ELISA test and obtaining optical density values which were used to calculate percent positivity of sera samples collected at different days during the experiment. The day at which peak antibody titres was reached was observed in some pigs. For the survival curves, the experiment aimed at determining if pigs from the two breeds could survive upon infection by this particular isolate or if the pigs come down with the disease, how long it would take before pigs die. A survival curve was achieved by plotting the number of pigs which died and those that survived at different days during the experiment.

CHAPTER FOUR

4.0 RESULTS

4.1.1 Clinical signs observed in local infected pigs

The smallest pig in the group was 1052. The pig had signs of recumbence, reduced feed intake, weight loss from 4 dpi (days post infection) and foul-smelling watery diarrhoea. The highest temperature was recorded at 7 dpi which was 39.6^{o}C. Pig number 1052 generally appeared weak and was found dead at 8 dpi.

Two days later, 1055 died followed by 1054 and 1056 the following day. These pigs died after showing signs of depression, anorexia and intermittent fever. From 5 dpi, all Homa Bay pigs showed worsening clinical signs, which included depression, anorexia, and recumbence, accelerated and laboured breathing, diarrhoea and slight ataxia. On 14 dpi, all of the remaining Homa Bay pigs showed febrile temperature reactions (40.5^{o}C to 41.4^{o}C).

All the remaining three pigs (1059, 1074 and 1066) had to be euthanized due to severe dyspnea upon sampling. 1040 1058 and 1069 were in a moribund state and were therefore euthanized.

4.1.2 Clinical signs observed in exotic infected pigs

While the Local pigs showed clinical signs later, all exotic pigs (Kept in the same room but different pens as per dose group) responded earlier to the ASFV infection at 4 dpi. On that day 1097 developed fever for the first time but this was not accompanied by any other clinical signs. The following day other signs were noticed including severe depression, laboured breathing, anorexia, haemorrhages and congestion of extremities. Pig number PB005 had no fever but had clinical signs similar to 1097. All the exotic animals in the group inoculated 10^{-4}HAD50/mL of the virus developed similar signs with fever being recorded on the last days when the pigs were

terminally ill. Generally, the pigs were considered moribund after having fever of 40^{o}C and above for three consecutive days, complete loss of appetite for two days, recumbence and terminal dyspnoea.

4.2 Pathological Changes observed in dead pigs

The pathological changes were comparable among the sick pigs. The lesions observed had different severity. They involved; enlarged, hemorrhagic and oedematous lymph nodes (gastrohepatic lymph nodes were inflammed, the spleens were enlarged, the lungs were mottled and did not collapse, gall bladder oedema, haemorrhages in the kidneys and hemorrhages in the stomach. There were coagulation disorders in most of the pigs including large hematomas and generalized lymphocytosis. This was characterized by generalized abscessation of lungs as well as mottled appearance and adhesions of the lungs on the rib walls (Appendix 6).

4.3 Determination of infective dose (Haemadsorption)

Haemadsorption units' calculations used in the experiment gave an approximation of the viral load quantity enough to cause disease and at some point result in death. Although it is a crude method, infective dose was determined quantitatively by appreciating the intensity of haemadsorption seen per well which was later quantified using Spearman-Karber method.

One-tail test gave a p value of 0.02 while the two-tail test gave a p value of 0.03. Both statistical tests were significantly different between the viral titres obtained from the two groups of pigs.

Table 3: Virus titres and virus particles obtained from cell culture procedure

Virus	Donor	Virus titre (HAD_{50}/ml)	Virus particles (HAD_{50}/ml)
1033 Local	HB1052	5.40	1.59×10^{7}
1033 Local	HB1052	5.40	1.00×10^{8}
1033 Exotic	PB006	6.00	1.00×10^{10}
1033 Exotic	PB006	7.00	6.32×10^{8}

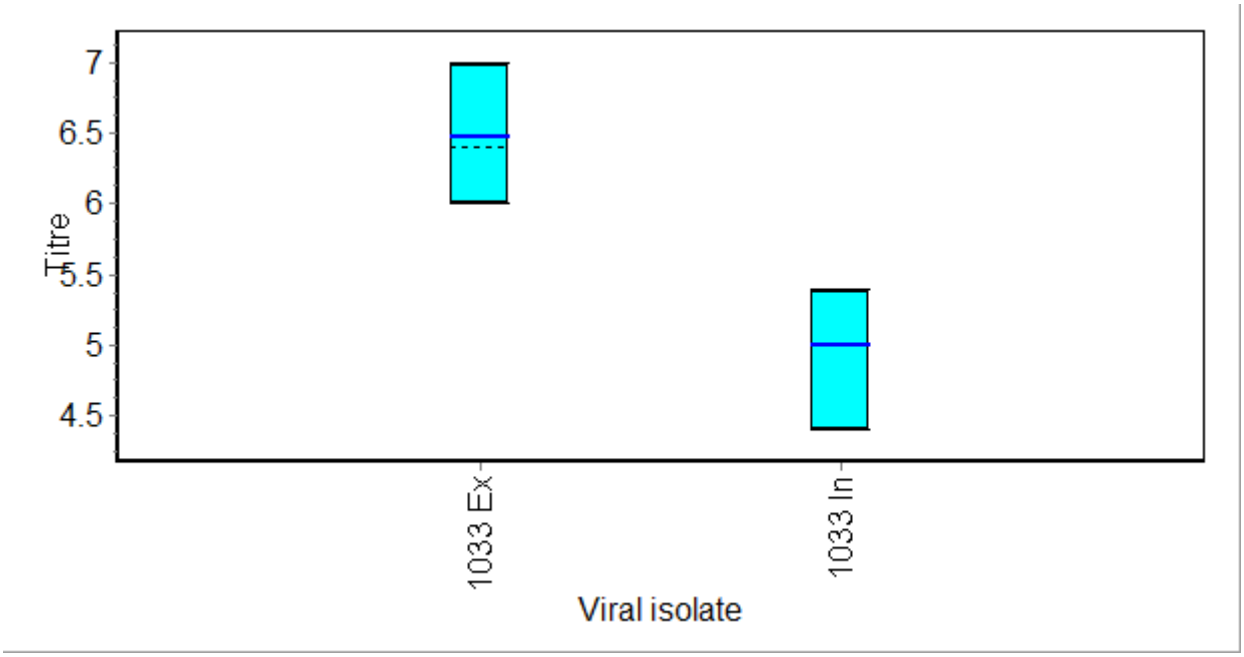

Figure 2: Box plot of virus titres obtained from the two categories/ groups of pigs

Table 4: Summary Statistical analysis of the Virus Titres obtained during haemadsorption procedure

t-Test: Two-Sample Assuming Equal Variances		
	1033 Ex	*1033 In*
Mean	6.47	5.07
Variance	0.25	0.33
Observations	3.00	3.00
Pooled Variance	0.29	
Hypothesized Mean Difference	0.00	
df	4.00	
t Stat	3.17	
P(T<=t) one-tail	0.02	
t Critical one-tail	2.13	
P(T<=t) two-tail	0.03	
t Critical two-tail	2.78	

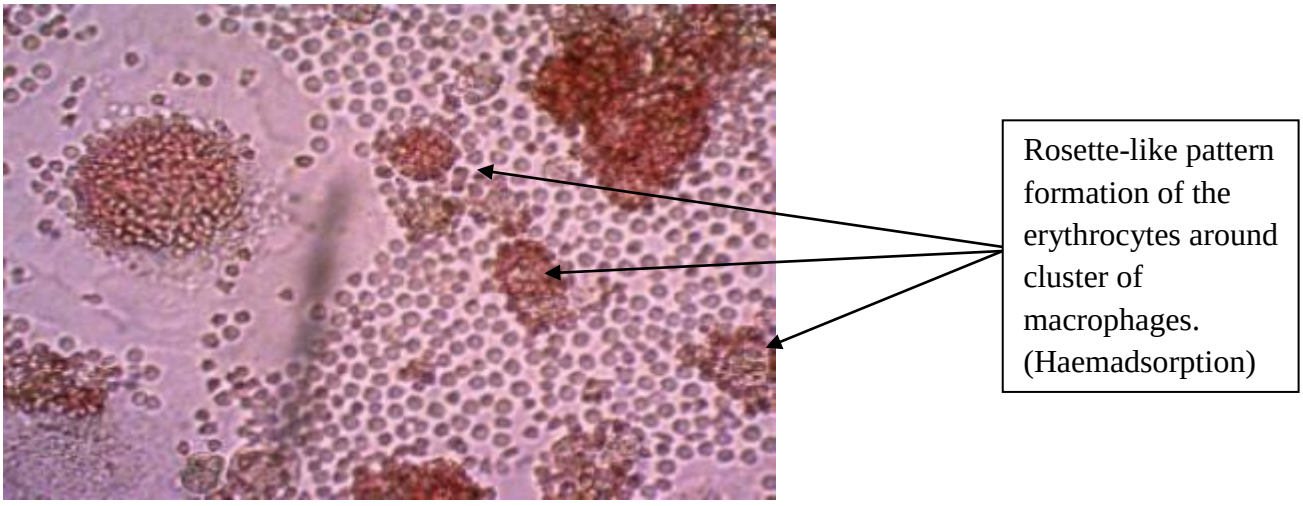

Figure 3: Photographic presentation of HAD Results obtained from the cell culture during virus growth

4.4 Immunological responses following Experimental Infection

Serum samples with optical density values lower than positive cut off were considered as positives to ASFV antibodies. Serum samples with OD values between both cut off were considered as doubtful hence it was recommended that re-testing be done one more time or apply a different technique to check the serum (IFI, Indirect ELISA, etc).

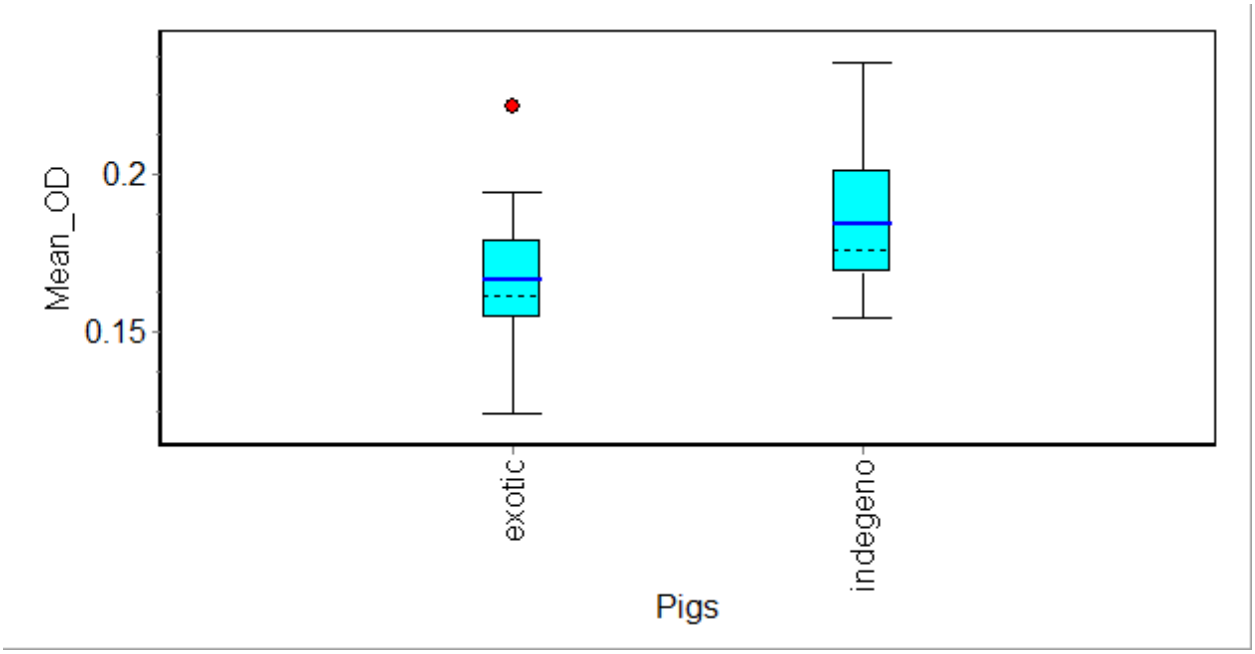

Figure 4: Box plot of comparative immunology results obtained during ELISA tests using pig sera samples from the two categories of pigs.

Table 5: Statistical Analysis of the Immune Responses (Antibody Responses) of the two categories of pigs

t-Test: Two-Sample Assuming Equal Variances		
	Mean OD X	***Mean OD Y***
Mean	0.1667	0.1842
Variance	0.0007	0.0006
Observations	10.0000	10.0000
Pooled Variance	0.0007	
Hypothesized Mean Difference	0.0000	
df	18.0000	
t Stat	-1.5225	
P(T<=t) one-tail	0.0726	
t Critical one-tail	1.7341	
P(T<=t) two-tail	0.1453	
t Critical two-tail	2.1009	

From the table, the p value in the one-tail test was 0.07 while the p value in the two-tail test was 0.14. Both values were above the critical p value of 0.05. Therefore, this means that there was no significant difference in the antibody response in the two categories of groups of pigs. This means that the null hypothesis was true. With only a few exceptions, most antibody detection techniques yielded negative results for the samples taken prior to inoculation and at the end of the trial. Eight sera samples gave positive results in the INGEZIM PPA COMPAC ELISA (Ingenasa), which detects p73-specific antibodies.

Three out of five exotic pigs (1094, 1096 and PB005) inoculated with ASFV1033 at dose $10^{-4}HAD_{50}$/mL were positive for ASFV antibodies at day 6. Exotic pig PB006 inoculated with ASFV1033 virus at dilution $10^{-5}HAD_{50}$/mL was also seropositive at day 6. These pigs died a few days later after showing some antibody response. Pigs 1094 died on day 8, 1096 died on day 7,

PB005 died on day 9 and PB006 died on day 10. This means the pigs died shortly after eliciting some antibody response towards ASFV.

Three out of five local pigs (1052, 1055 and 1058) inoculated with ASFV1033 at a dose of $10^{-4}HAD_{50}$/mL were positive for ASFV antibodies at day six. Also local pig identity 1069 inoculated with ASFV1033 virus at dilution $10^{-5}HAD_{50}$/mL was also seropositive at day 6. These local pigs survived at different times. Pig 1052 died on day 7, pig 1055 died on day 9 while 1058 and 1069 died on day 11.

Due to death within a few days after eliciting some antibody response, there was an association between the rate of seroconversion to time to death. On average the exotic pigs survived for 5.5 days which is approximately 6 days while the local pigs survived on average for 7 days' post infection. The pigs survived for a short time after eliciting antibody response. Therefore antibodies elicited could not confer protection for survival hence there was need to consider T-cell response. Seroconversion on average was observed at day 6 of the experiment in both local and exotic pigs.

4.5 Survival Time Analysis

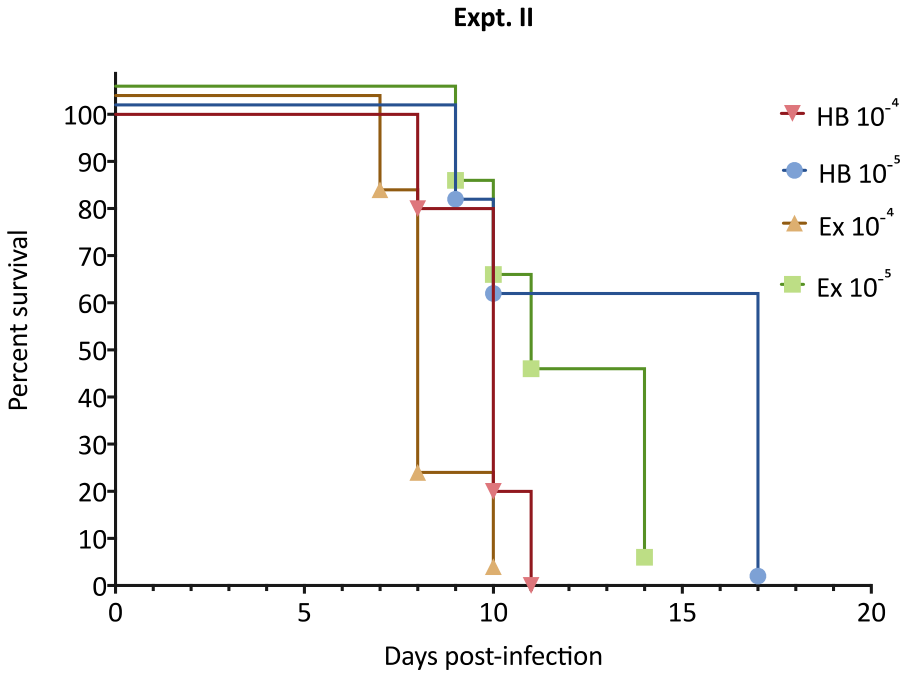

Figure 5: Percent survival in local and exotic breeds of pigs following ASF virus infection

Table 6: Table of survival time of individual pigs

Pig Id	Survival Time	Virus Infective Dose
1091	15	
1092	9	
1095	11	
1098	15	
PB006	10	10^{-5} HAD_{50}/mL
1059	17	
1074	17	
1069	10	
1066	17	
1040	9	$10^{-5}HAD_{50}$/mL
1093	10	
1094	8	
1096	9	
1097	7	
PB005	7	$10^{-4}HAD_{50}$/mL
1052	7	
1054	10	
1055	9	
1056	10	
1058	11	$10^{-4}HAD_{50}$/mL
Average Days of Survival	10.9	
Standard Deviation	3.3	

Table 7: Survival Analysis using Excel software

EXOTIC		LOCAL	
Mean	10.1	**Mean**	11.7
Standard Error	0.9	**Standard Error**	1.2
Median	9.5	**Median**	10
Mode	7	**Mode**	10
Standard Deviation	2.9	**Standard Deviation**	3.8
Range	8	**Range**	10
Minimum	7	**Minimum**	7
Maximum	15	**Maximum**	17
Count	10	**Count**	10

CHAPTER FIVE

5.0 DISCUSSION

The aim of this project was to give results on the virulence of the ASFV1033 strain among domestic pigs of different breeds (local and exotic) from two different production systems. Outcome showed that the porcine were susceptible to the recently identified ASFV1033 isolate from Western Kenya leading to acute and chronic form of the disease. Whereas three infected pigs succumbed on day 17, the other experimentally infected animals succumbed by day 15. The disease caused varied symptoms in pigs inoculated with highly diluted doses of the virus.

Both exotic and indigenous pigs manifested the same signs and symptoms of ASF regardless of the different strains found in different parts of the World. African swine fever has been misreported in place of other porcine diseases such as Salmonellosis, Swine Erysipelas, Trypanosomiasis, and Poisoning such as Warfarin poisoning. The average incubation period was 4 to 7 days for the inoculated pigs according to the blood samples run on ELISA test. ASFV was detected in blood of the experimentally infected pigs from the four groups even in pigs infected with low doses which included 10^{-6} to 10^{-8} HAD_{50}/mL doses (Ct values from PCR runs). Even though it is not yet understood why the results vary, 20 blood samples were processed and amplified to check for the presence of ASFV genome using the UPL qPCR assay. The results were consistence with the sensitivity of this assay. The time at which clinical signs started appearing and the time at which the virus could be picked in blood after infection did not vary much. This means under field conditions, clinical signs started showing immediately the pigs become infected. VP72, a capsid protein is highly conserved part of the ASFV genome hence most commonly used although VP54, VP30, CVR and TK were also used in this virus

characterization. VP72 is particularly important as it contains a neutralization site, is antigenically stable hence useful for viral diagnosis and has properties making it suitable for molecular epidemiological studies. Intramuscular route of infection was so far reliable even though there seemed to be slight variations when it came to shedding of the virus. There was a significant difference in the infectious period among the four groups. This difference in pathogenesis may be as a result of varied initial sites of multiplication as well as due to breed predisposition, causing effects to the pathways of ASFV shedding and excretion. As a result, data from the experiment could not be used as representative of the field ASFV infection. This therefore means further studies should be carried out to explore on the course of ASF disease infection in order to obtain better information.

Due to animal welfare rules provided in the ethical document, the experimental animals were humanely killed using Euthanaze® hence high possibility of having biased outcomes. There was similarity when it came to onset of clinical onset. ELISA results indicate that there was immune response in eight inoculated pigs. Initially, study on immune response was vital especially in controlling low virulent strains of ASF virus. In Western and Central Kenya, sporadic outbreaks have been occurring characterized by sudden death of a large population of pigs or animals developing high fever and dying after a couple of hours. In such cases, serum sample harvested from these animals do not show any immune response. Further tests need to be carried out to find out if within this short time there is any cytokine response since this current strain BUS1033 is not easily picked via ELISA. The ASF virus keeps on mutating time after time such that every time there is an outbreak you isolate the same genotype IX but a different more lethal strain. It mutates a small portion of the genome. Efforts to isolate genotype X in the current research study

have been unfruitful. There was difference in ASFV shedding among the Homa Bay pigs. This was as a result of individual susceptibility to infection that would determine efficient transmission. This experiment proved that a limited number of animals produced more specific results paving way for repeated animal sampling and environmental effects properly checked on compared to field condition. In all experiments, ASFV spread occurred, indicating that the disease spread at high rate in domestic pigs regardless of the breed. In field observations, there are minimal reports of pig deaths even in situations where BUS1033 strain is involved in outbreaks. This is because some pigs do not come down with ASF disease especially this isolate ASFV1033 which was obtained from a very healthy pig in Sigalame, Busia.

6.0 CONCLUSION

The indigenous pigs had a relatively longer survival rate/ days as compared to the exotic pigs. The estimated average viral titres in the two groups of pig were significantly different. Immunological responses to ASFV between the two groups of pigs were not significantly different.

7.0 RECOMMENDATION

The government should encourage local pig production by encouraging cross breeding local and exotic pigs so as to pave way for advanced genetic search. A lot needs to be done on the African swine fever genome.

The genetic material in the local pigs that enables partial survival to ASF infection should be identified and modified to see if the virulence of ASF will reduce or enable the exotic pigs survive ASF infection without clinical disease. All these can be embraced if genetic profiling is done especially for this particular genotype IX in order to cub the frequent and sporadic African swine fever outbreaks in the country because it will be a great platform for vaccine development.

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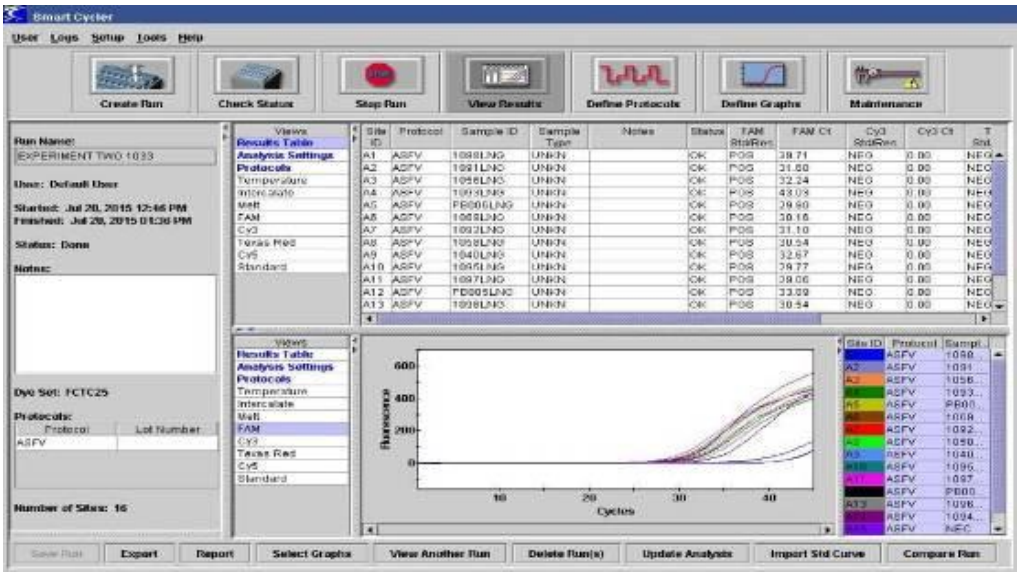
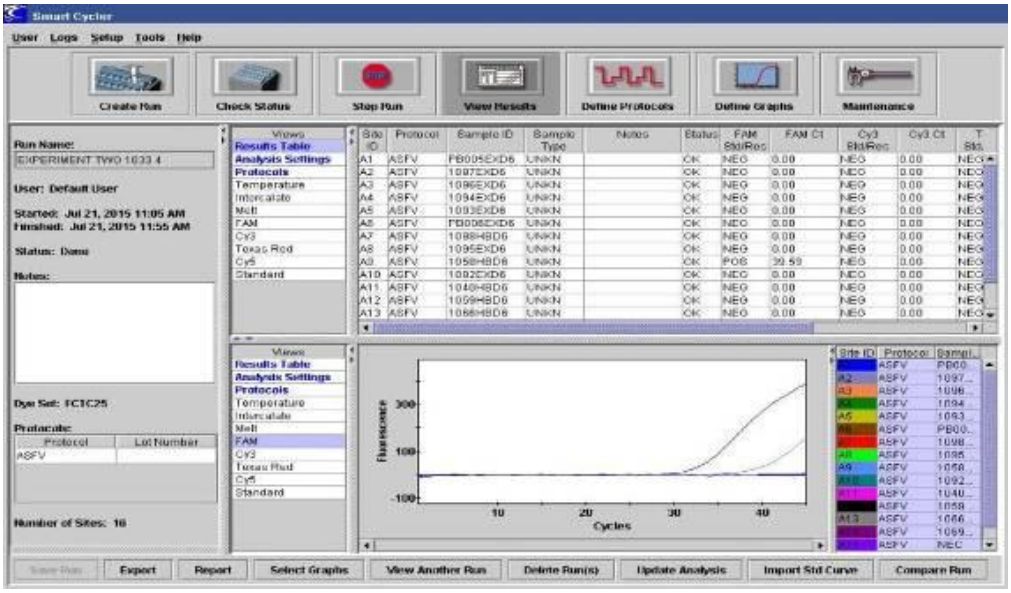
8.0 APPENDICES

Appendix 1: Sample Pictorial Presentations of Real Time PCR Results





Appendix 2: Plan of Comparative Experimental Challenge of Indigenous and European Pigs with Low Passage Virulent ASFV Virus stock

2541033 - Low (3) passage isolated from exotic pig alveolar macrophages from infected spleen.

~10^{10} HAD_{50} per ml in stock

Experimental Infection

Intramuscular inoculation of diluted stock virus (**A** - 10^{-4}, **B** - 10^{-5}) virus in 1ml

Animals

10 indigenous (Homabay) pigs and 10 exotic (European) domestic pigs

ASFV free/naïve

Age/Weight?

Groups:

I. **HB-A** 5 Homabay pigs – 10^{-4}
II. **HB-B** 5 Homabay pigs – 10^{-5}
III. **EU-A** 5 European pigs – 10^{-4}
IV. **EU-B** 5 European pigs – 10^{-5}

Clinical monitoring

Signs (Once Daily) – clinical scoring

Temperature (Once Daily)

Weight (Once)

Sampling

5 ml ETDA Blood for PCR/transcription analyses and complete blood counts (0, 1, 2, 3, 4, 5, 6, 7, 9, 11, 14, 16, 18, 21, 23, 25 and 28)

10ml Serum (0, 4, 7, 11, 14, 18, 21, 25 and 28) for serology/virus isolation

20ml Heparin Blood for PBMC isolation (0, 7, 14, 21 and 28)

Before euthanization/necropsy

5ml EDTA, 10ml Serum and 50ml Heparin blood (before euthanization)

Collection of tissue samples

Appendix 3: ELISA Results Showing Mean ODs

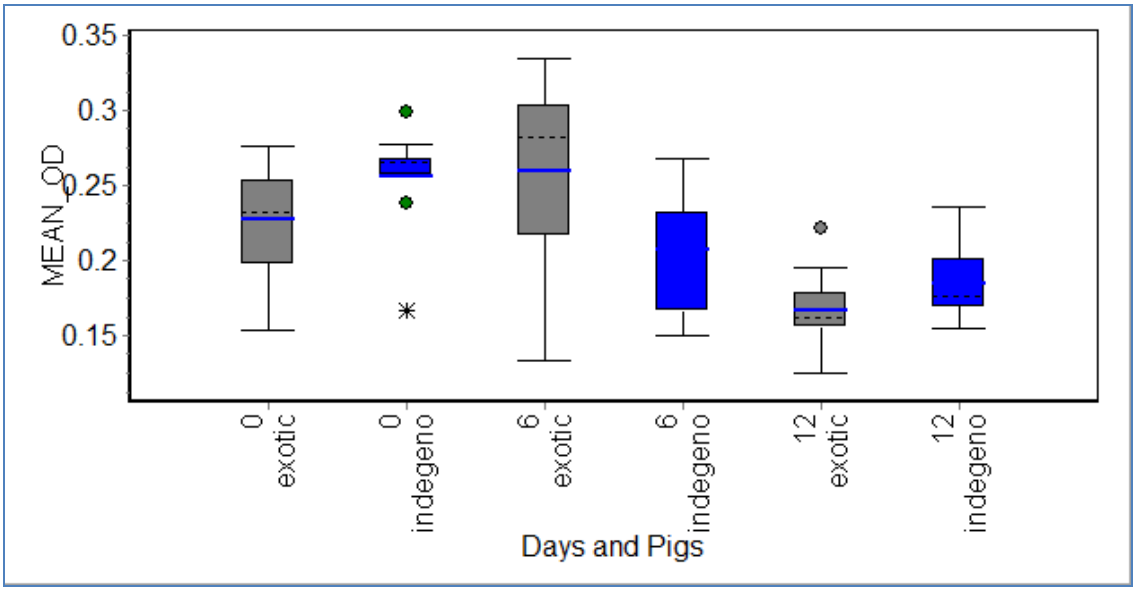
	ELISA RUN 1				
SAMPLE ID	**OD 1**	**OD 2**	**MEAN OD**	**% CUT OFF**	**INTERPRETATION**
1091/D0	0.269	0.283	0.276	-19.940476	NEGATIVE
1092/D0	0.223	0.236	0.2295	7.73809524	NEGATIVE
1095/D0	0.263	0.255	0.259	-9.8214286	NEGATIVE
1098/D0	0.241	0.226	0.2335	5.35714286	NEGATIVE
PB006/D0	0.252	0.237	0.2445	-1.1904762	NEGATIVE
1093/D0	0.214	0.296	0.255	-7.4404762	NEGATIVE
1096/D0	0.209	0.242	0.2255	10.1190476	NEGATIVE
1094/D0	0.108	0.199	0.1535	52.9761905	POSITIVE
1097/D0	0.138	0.256	0.197	27.0833333	NEGATIVE
PB005/D0	0.246	0.146	0.196	27.6785714	NEGATIVE
1059/D0	0.257	0.28	0.2685	-15.47619	NEGATIVE
1066/D0	0.241	0.277	0.259	-9.8214286	NEGATIVE
1040/D0	0.265	0.265	0.265	-13.392857	NEGATIVE
1055/D0	0.234	0.241	0.2375	2.97619048	NEGATIVE
1056/D0	0.267	0.271	0.269	-15.77381	NEGATIVE
1054/D0	0.26	0.27	0.265	-13.392857	NEGATIVE
1058/D0	0.177	0.156	0.1665	45.2380952	DOUBTFUL
1052/D0	0.365	0.149	0.257	-8.6309524	NEGATIVE
1069/D0	0.291	0.263	0.277	-20.535714	NEGATIVE
1074/D0	0.301	0.296	0.2985	-33.333333	NEGATIVE
1091/D6	0.276	0.354	0.315	-43.154762	DOUBTFUL
1092/D6	0.281	0.301	0.291	-28.869048	NEGATIVE
1095/D6	0.286	0.288	0.287	-26.488095	NEGATIVE
1098/D6	0.246	0.232	0.239	2.08333333	NEGATIVE
PB006/D6	0.141	0.125	0.133	65.1785714	POSITIVE
1093/D6	0.24	0.145	0.1925	29.7619048	NEGATIVE
1094/D6	0.284	0.149	0.2165	15.4761905	NEGATIVE
1096/D6	0.294	0.258	0.276	-19.940476	NEGATIVE
1097/D6	0.305	0.304	0.3045	-36.904762	NEGATIVE
PB005/D6	0.365	0.304	0.3345	-54.761905	POSITIVE
1059/D7	0.279	0.256	0.2675	-14.880952	NEGATIVE
1066/D7	0.22	0.246	0.233	5.6547619	NEGATIVE
1040/D6	0.233	0.156	0.1945	28.5714286	NEGATIVE
1055/D6	0.132	0.167	0.1495	55.3571429	POSITIVE
1056/D6	0.126	0.202	0.164	46.7261905	DOUBTFUL
1054/D6	0.247	0.206	0.2265	9.52380952	NEGATIVE

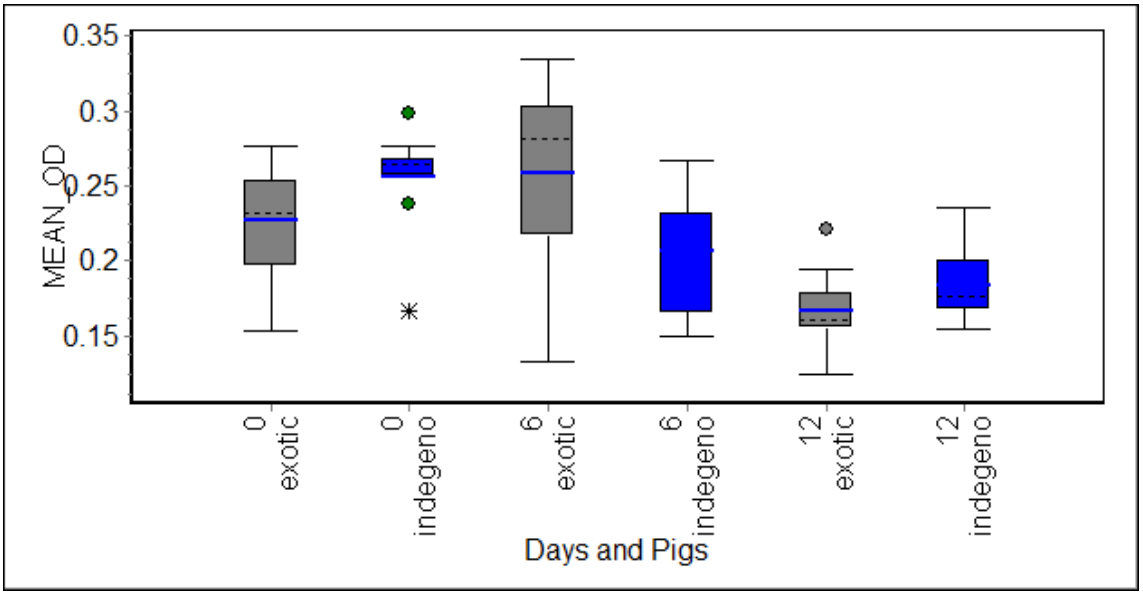
1058/D6	0.267	0.234	0.2505	-4.7619048	NEGATIVE
1052/D6	0.216	0.191	0.2035	23.2142857	NEGATIVE
1069/D6	0.225	0.193	0.209	19.9404762	NEGATIVE
1074/D6	0.201	0.129	0.165	46.1309524	DOUBTFUL
1091/D13	0.128	0.121	0.1245	70.2380952	POSITIVE
1091/D14	0.139	0.191	0.165	46.1309524	DOUBTFUL
1092/D9	0.156	0.158	0.157	50.8928571	POSITIVE
1095/D11	0.191	0.198	0.1945	28.5714286	NEGATIVE
1098/D13	0.155	0.154	0.1545	52.3809524	POSITIVE
1098/D14	0.142	0.142	0.142	59.8214286	POSITIVE
PB006/D10	0.146	0.169	0.1575	50.5952381	POSITIVE
1093/D10	0.197	0.146	0.1715	42.2619048	DOUBTFUL
1094/D8	0.213	0.229	0.221	12.797619	NEGATIVE
1096/D8	0.145	0.214	0.1795	37.5	NEGATIVE
1059/D17	0.138	0.206	0.172	41.9642857	DOUBTFUL
1066/D7	0.134	0.188	0.161	48.5119048	DOUBTFUL
1066/D17	0.171	0.178	0.1745	40.4761905	DOUBTFUL
1040/D9	0.202	0.188	0.195	28.2738095	NEGATIVE
1056/D10	0.162	0.194	0.178	38.3928571	NEGATIVE
1054/D10	0.142	0.167	0.1545	52.3809524	POSITIVE
1058/D11	0.163	0.174	0.1685	44.047619	DOUBTFUL
1069/D10	0.163	0.241	0.202	24.1071429	NEGATIVE
1074/D13	0.224	0.246	0.235	4.46428571	NEGATIVE
1074/D17	0.166	0.237	0.2015	24.4047619	NEGATIVE

ELISA RUN 2				
OD 1	**OD 2**	**MEAN OD**	**% CUT OFF**	**INTERPRETATION**
0.885	0.91	0.8975	-28.677331	NEGATIVE
0.849	0.831	0.84	-19.703472	NEGATIVE
0.879	0.825	0.852	-21.576278	NEGATIVE
0.843	0.777	0.81	-15.021459	NEGATIVE
0.794	0.792	0.793	-12.368318	NEGATIVE
0.843	0.815	0.829	-17.986734	NEGATIVE
0.649	0.826	0.7375	-3.7065938	NEGATIVE
0.292	0.745	0.5185	30.472103	NEGATIVE
0.236	0.797	0.5165	30.7842372	NEGATIVE
0.839	0.272	0.5555	24.69762	NEGATIVE
0.917	0.897	0.907	-30.159969	NEGATIVE
0.832	0.955	0.8935	-28.053063	NEGATIVE
0.904	0.923	0.9135	-31.174405	NEGATIVE
0.924	0.833	0.8785	-25.712056	NEGATIVE
0.847	0.944	0.8955	-28.365197	NEGATIVE
0.942	0.901	0.9215	-32.422942	NEGATIVE
0.308	0.358	0.333	59.4225517	POSITIVE
0.344	0.337	0.3405	58.2520484	POSITIVE
0.931	0.402	0.6665	7.37417089	NEGATIVE
0.965	1.181	1.073	-56.067109	POSITIVE
0.842	0.854	0.848	-20.952009	NEGATIVE
0.945	0.952	0.9485	-36.636754	NEGATIVE
0.931	0.825	0.878	-25.634023	NEGATIVE
0.814	0.714	0.764	-7.8423722	NEGATIVE
0.322	0.307	0.3145	62.3097932	POSITIVE
0.754	0.723	0.7385	-3.8626609	NEGATIVE
1.195	0.347	0.771	-8.934842	NEGATIVE
1.294	0.901	1.0975	-59.890753	POSITIVE
1.042	0.954	0.998	-44.362076	DOUBTFUL
1.025	0.968	0.9965	-44.127975	DOUBTFUL
0.831	1.031	0.931	-33.905579	NEGATIVE
0.595	0.742	0.6685	7.06203668	NEGATIVE
0.694	0.281	0.4875	35.3101834	NEGATIVE
0.335	0.649	0.492	34.6078814	NEGATIVE
0.281	0.54	0.4105	47.3273508	DOUBTFUL
0.738	0.654	0.696	2.77019118	NEGATIVE

0.915	0.695	0.805	-14.241124	NEGATIVE
0.789	0.618	0.7035	1.59968787	NEGATIVE
0.78	0.567	0.6735	6.28170113	NEGATIVE
0.639	0.321	0.48	36.4806867	NEGATIVE
0.691	0.385	0.538	27.4287944	NEGATIVE
0.357	0.705	0.531	28.5212641	NEGATIVE
0.356	0.346	0.351	56.6133437	POSITIVE
0.65	0.708	0.679	5.42333203	NEGATIVE
0.92	0.881	0.9005	-4.4233807	NEGATIVE
0.901	0.858	0.8795	-1.7693523	NEGATIVE
0.882	0.902	0.892	-3.3491311	NEGATIVE
0.849	0.835	0.842	2.9699842	NEGATIVE
0.854	0.797	0.8255	5.05529226	NEGATIVE
0.828	0.859	0.8435	2.78041074	NEGATIVE
0.672	0.819	0.7455	15.1658768	NEGATIVE
0.322	0.704	0.513	44.549763	DOUBTFUL
0.287	0.776	0.5315	42.2116904	DOUBTFUL
0.888	0.379	0.6335	29.3206951	NEGATIVE
0.813	0.873	0.843	2.8436019	NEGATIVE
0.944	1.019	0.9815	-14.660348	NEGATIVE
0.881	0.892	0.8865	-2.6540284	NEGATIVE
0.984	0.857	0.9205	-6.9510269	NEGATIVE
0.967	0.927	0.947	-10.300158	NEGATIVE
1.025	0.965	0.995	-16.366509	NEGATIVE

Appendix 4: Box Plot showing bthe mean ODs of ELISA Run 1 and Run 2

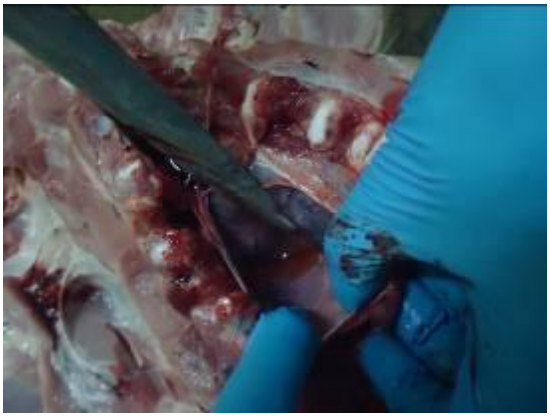




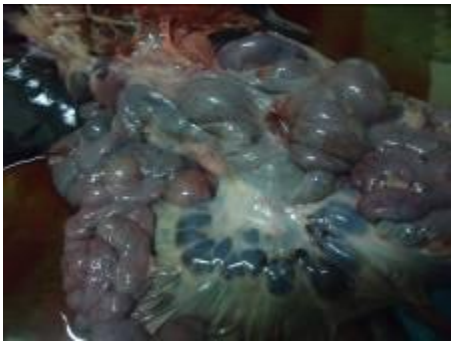
Appendix 5: Pictures showing Post Mortem lesions observed during the Experiment

Haemorrhages and congestion of extremities

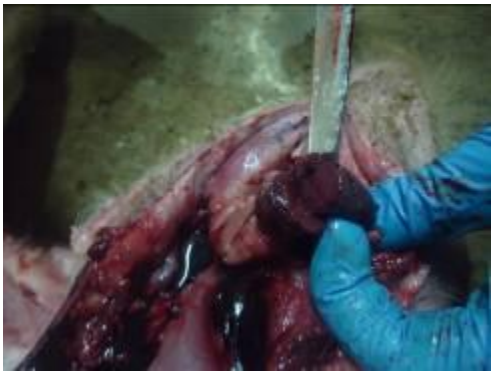
Huddling together is one of the initial signs



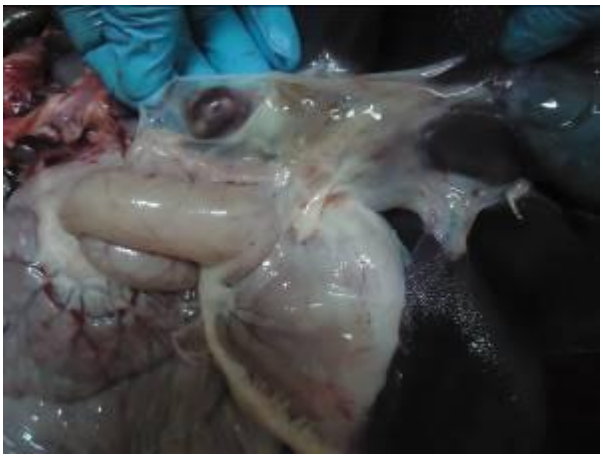
Blood-tinged fluid in the pericardium



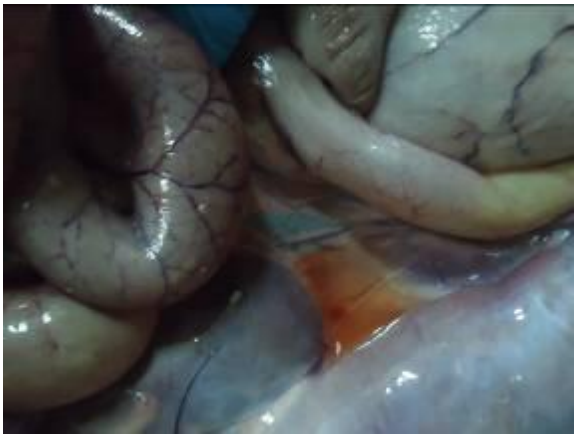
Enlarged and inflammed messenteric lymph nodes



Enlarged and inflammed submandibular lymph nodes

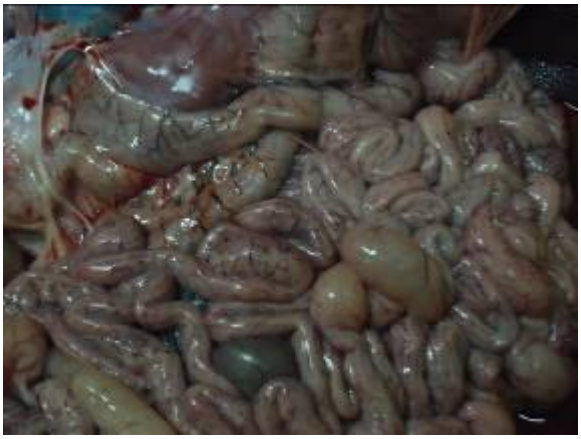


Enlarged and inflammed gatsrohepatic lymph node

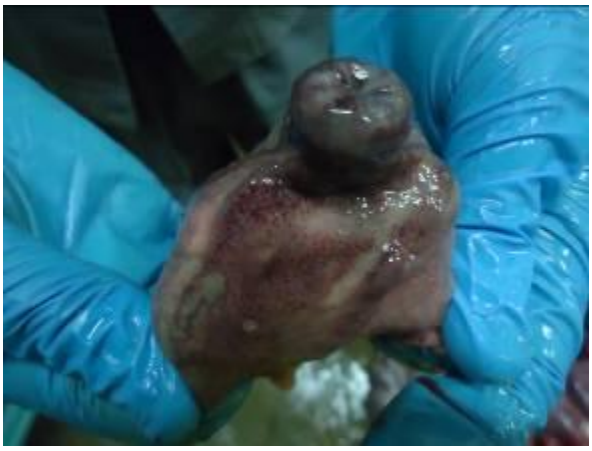


Serosanguinous fluid in the abdominal cavity

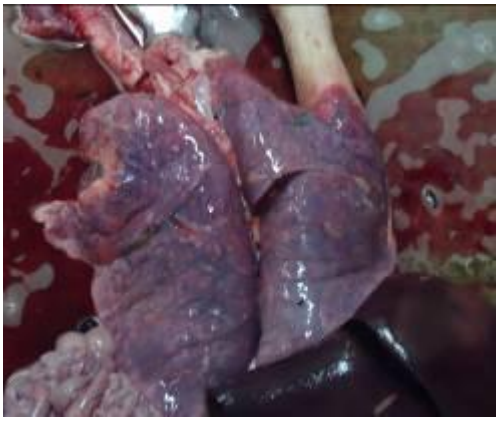
Enlarged spleen



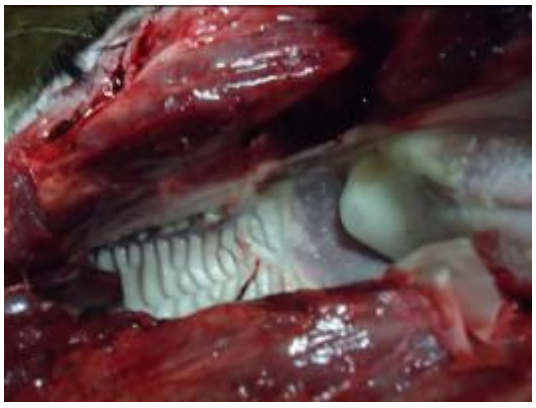
Congeted intestinal vessels



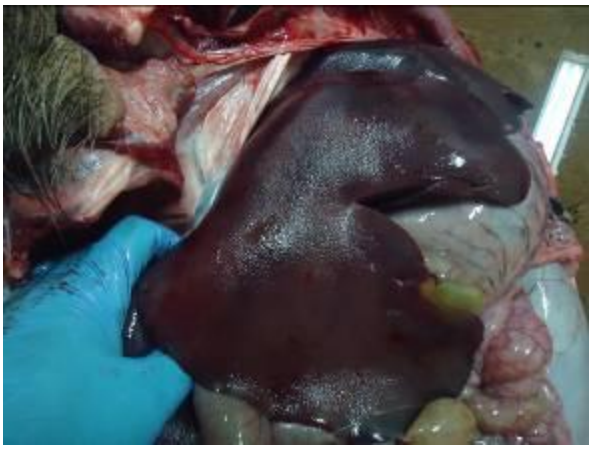
Inflammed and congested ileocaecal junction



Lungs are enlarged, do not collapse and trachea is full of froth.



Inflammed tonsils



Enlarged liver

Appendix 6: UPL PCR Results of Blood and Tissue Samples

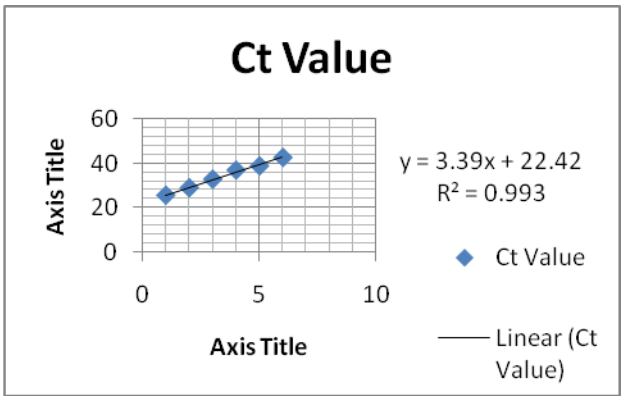


Figure 2: Ct Values of Different Concentrations of the ASFV1033 Virus

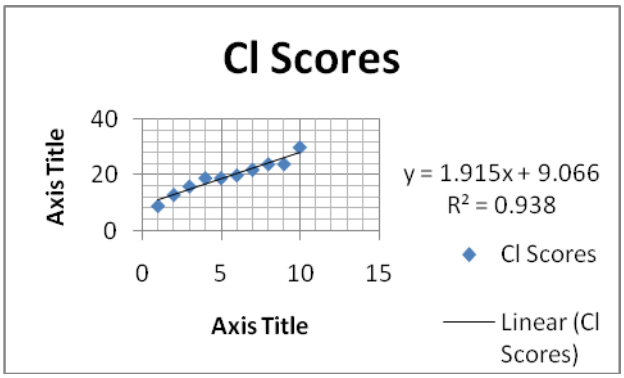


Figure 3: Clinical Score values of Exotic Pigs

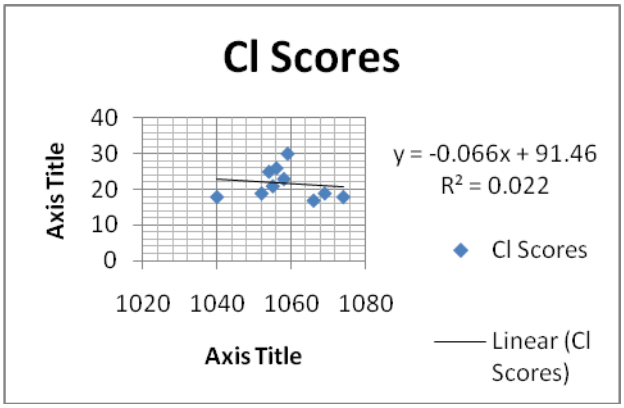


Figure 4: Clinical Score Values of Homa Bay/Local Pigs

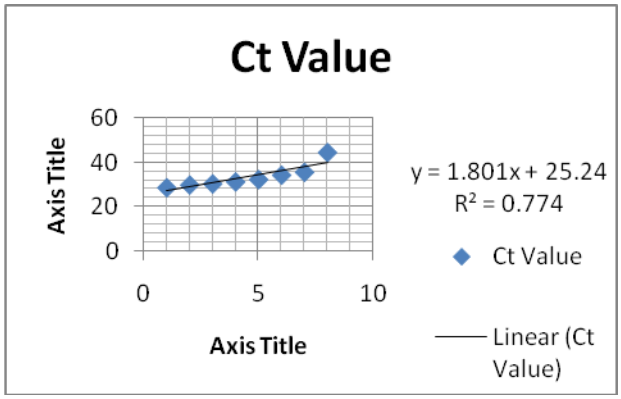


Figure 5: Ct Values for ASFV in Exotic Pigs Spleens

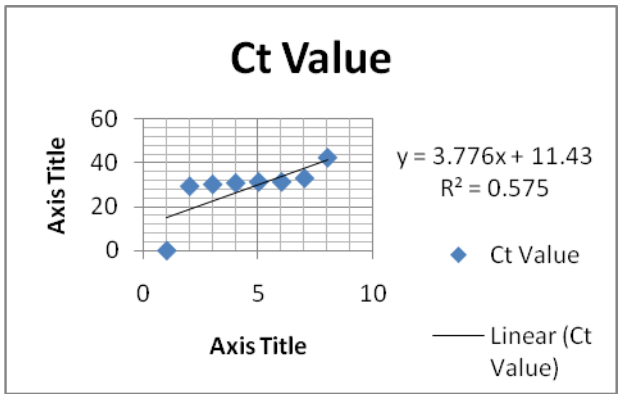


Figure 6: Ct Values of ASFV in Local Pigs Spleens

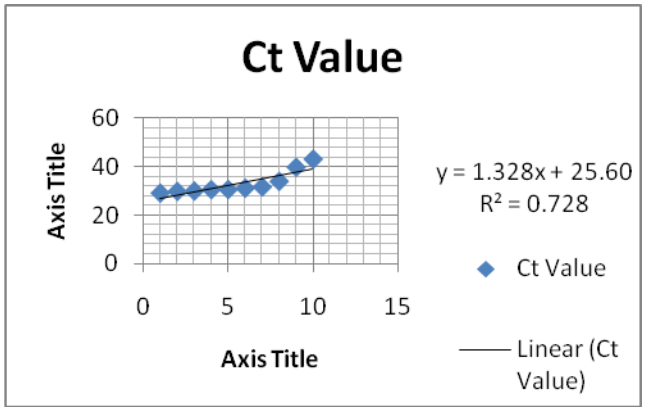


Figure 7: Ct values of Lung organs from Exotic Pigs

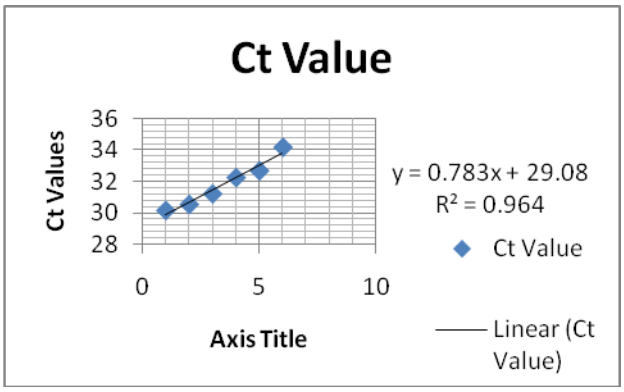


Figure 8: Ct Values of Lung Organs from Local Pigs

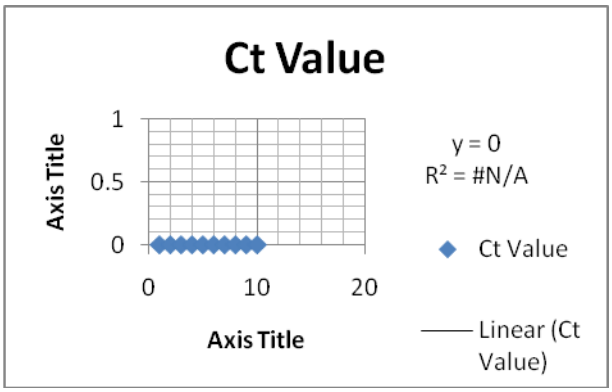


Figure 9: Ct Values EDTA Blood obtained from Exotic Pigs

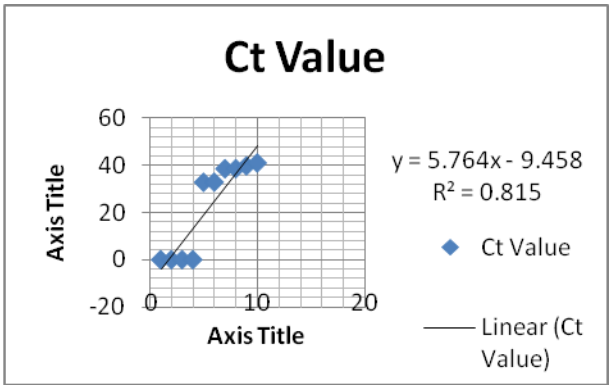


Figure 10: Ct Values of EDTA blood obtained from Local pigs

Appendix 7: Haemadsorption Culture Plate Layouts

Plate Infected with ASFV1033 at Dilution of $10^{-4}HAD_{50}/mL$

Dilution of virus		Infected Wells					Control Wells		
		1	2	3	4	5	6	7	8
10^{-1}	A	xx	xx	xx	xx	O	O	O	O
10^{-2}	B	xx	xx	xx	xx	O	O	O	O
10^{-3}	C	xx	xx	xx	xx	xx	O	O	O
10^{-4}	D	xx	xx	xx	xx	O	O	O	O
10^{-5}	E	xx	xx	xx	xx	O	O	O	O
10^{-6}	F	xx	xx	xx	xx	xx	O	O	O

Plate Infected with ASFV1033 at Dilution of $10^{-5}HAD_{50}/mL$

Dilution of virus		Infected Wells					Control Wells		
		1	2	3	4	5	6	7	8
10^{-1}	A	xx	xx	xx	xx	xx	O	O	O
10^{-2}	B	xx	xx	xx	xx	O	O	O	O
10^{-3}	C	xx	xx	xx	O	O	O	O	O
10^{-4}	D	xx	xx	O	O	O	O	O	O
10^{-5}	E	xx	xx	O	O	O	O	O	O
10^{-6}	F	xx	xx	O	O	O	O	O	O

Appendix 8: The Titers Calculated Using The Formula (Spearmann-Karber Method)

Titer

= (X – d/2 + (d.S)) where;

X = log10 of lowest dilution (1)

d = log10 of dilution factor (1)

S = sum of proportion of HAD positive

Counting of cells

10µl of the cells suspended in medium was added to 90µl of trypan blue (the dilution is 1/10). The viable cells were counted and the following calculations were made to achieve the required number of cells per well:

EXAMPLE:

If the number of cells counted were 194

Then:

194 x 10 x 10,000(Constant)/ml= 19.4 $x10^6$ cells/ml

Given the cells were suspended in 5 ml of medium then multiply the no. of cells/ml by 5

$19.4x10^6$ x 5= $97x10^6$ total cells.

Let's say one needs at least $15x10^6$ cells per well then;

$97x10^6/15x10^6$= ~6 wells

Appendix 9: Laboratory Standard Operating Procedures used During the Experiment

10.1 Virus Isolation Protocol

1. Cut the spleen into pieces
2. Place a sieve onto a sterile surface such as a petri dish or any other surface then place the tissue pieces onto the sieve.
3. Using a falcon tube top (falcon tube held upside down); crush the spleen on the sieve to allow the lysate to collect on the sterile surface.
4. Transfer the lysate into a sterile falcon tube.
5. Spin the lysate at 5000 rpm for 30 minutes and transfer the supernatant into a sterile falcon tube for initial virus passaging.

Virus isolation (passaging):

- To prepare the virus in bulk; use a 6 well culture plate
- Prepare porcine PBMCs using standard protocol
- Adjust cell concentration to 2 x 10^7/ml in complete RPMI (cRPMI) medium with 10% autologous serum [important for HAD], plate 3 mls/well (48-well flat bottom plate)
- Incubate at 37°C, 5% CO_2 for 2 days, remove unattached cells and add fresh cRPMI medium and incubate for another 2 days. Macrophages are now ready for infection with ASFV.
- Remove medium from the 4 day old macrophages (leave enough volume to keep cells covered to avoid drying) and infect with 150µl of the tissue lysate or virus culture from previous passage.
- Swirl the plate to spread the virus and incubate the plate at 37°C, 5% CO_2 for 1 hr for virus to infect macrophages.
- Add 3mls fresh cRPMI and incubate the plate at 37°C, 5% CO_2 for 24 hr.
- Add 60µls of autologous RBCs diluted 1 in 10 (i.e. 5ml RBCs topped up to 50 ml with sterile PBS) in the culture and incubate the plate at 37°C, 5% CO_2 for 2 - 4 days when hemadsorption is expected to be observed in the wells.
- Harvest the culture and transfer in clearly labeled 50 ml falcon tubes. Freeze and thaw alternatively in liquid nitrogen and 37°C water bath five times to release the virus from macrophages.

- Spin the tubes at 2500rpm for 30 minutes; transfer the supernatant into sterile clearly labeled falcon tubes and store accordingly for infections or next passaging.

10.1: Preparation of peripheral blood mononuclear cell (PBMC) by density gradient centrifugation

Procedure

Note: all operations are performed under sterile condition.

- Collect blood from the jugular vein into a syringe containing an equal volume of Alsever's solution and mix gently.
- Layer blood onto FicollPaque solution at 3:2 ratio and centrifuge at 2500 rpm for 30 minutes at room temperature, switch off the brakes of the centrifuge.
- Using a sterile pipette aspirate the PBMC from the interface, transfer to a sterile tube and top with warm Alsever's solution and centrifuge at 1800rpm for 10 minutes..
- Discard the supernatant and break the pellet. Add 3mls of Tris ammonium Chloride to lyse the RBCs and incubate this for 3min at room temperature. Top up with alseiver's solution and centrifuge at 1200 rpm for 10 min. This is the first wash.
- Discard the supernatant and break the pellet again, top up with alseiver's solution and centrifuge the PBMC at 1200 rpm for 10 minutes at room temperature. Repeat this once more to give a total of 3 washes. Or wash until the supernatant is clear and not cloudy. (Cloudiness is an indication of platelets).
- Resuspend the pellet of PBMC in medium of choice (preferably 5ml), depending on further use.

Preparation of complete medium

To prepare the medium; prepare a 10% RPMI medium with Fetal calf serum or autologous serum depending on the need. Penstrep, gentamycine and L-glutamine are also added onto the medium.

(Example: To prepare a total volume of 500ml medium, add 50ml of serum to 450ml RPMI 1640 then add 6ml each of Penstrep, gentamycine and L-glutamine to obtain complete medium)

Counting of cells

Take 10µl of the cells suspended in medium and add 90µl of trypan blue (the dilution is 1/10). Count the viable cells and make the following calculations:

EXAMPLE:

If the number of cells counted are 194

Then:

194 x 10 x 10,000(Constant)/ml= 19.4 $x10^6$ **cells/ml**

Given the cells were suspended in 5 ml of medium then multiply the no. of cells/ml by 5

$19.4x10^6$ x 5= $97x10^6$ total cells.

Let's say one needs at least $15x10^6$ cells per well then;

$97x10^6/15x10^6$= ~6 wells

Therefore to get enough media for 6 wells; top up the cells with medium to 18mls and distribute 3mls to each well of a 6 well plate.

Storage of cells

After counting the cells, take $5x10^6$ cells from the total cells, centrifuge the cells at 1200rpm for 10 minutes and discard the supernatant (medium). Add 300µl of RNA later to the pellet and store at -80°C.

Centrifuge the remaining cells at 1200rpm for 10 minutes and discard the supernatant. Break the pellet and add 750µl of 20% DMSO in FBS and 750 µl of FBS and mix gently. Cool cells in the lavender cool cell buckets at -80°C overnight and transfer the cells to liquid nitrogen the next morning.

After inoculation of the pigs with the various dilutions of the virus, whatever contents of the diluted virus is taken through laboratory procedure to determine if the virus present in it is viable. This done by checking the ability of the red blood cells to form a Rosette pattern around the virus particle. The process involves the following procedure:

10.2: Determination of ASFV HAD_{50} using swine macrophages

1. Prepare porcine PBMCs using standard protocol (Procedure above)
2. Adjust cell concentration to 2 x 10^7/ml in complete RPMI (cRPMI) medium with 10% autologous serum [important for HAD], plate 500 µls/well (48-well flat bottom plate)
3. Incubate at 37°C, 5% CO_2 for 2 days, remove unattached cells and add fresh cRPMI medium and incubate for another 2 days. Macrophages are now ready for infection with ASFV.

Virus Dilution:*Avoid aerosols at all costs.*

1. Mark 6 sterile 1.5 ml tubes starting with tube 1 as 10^{-1} and continue to 10^{-6}.
2. In each tube, add 0.9 mlscRPMI.
3. Add 0.1 ml of virus stock to the first tube (10^{-1}).
4. Mix by mild vortexing and *using a fresh pipette tip,* transfer 0.1 mls to tube 2 (10^{-2}).
5. Repeat above procedure till the last tube (10^{-6}). *Use fresh tip for each transfer*.
6. Remove medium from the 4 day old macrophages from step 3 above (leave enough volume to keep cells covered to avoid drying).
7. Starting with the lowest dilution (10^{-6}), transfer 0.050 mls (50 µl) of each virus dilution to each of the 5 wells (out of the 8 wells in each row) of a 48-well plate (leave 3 wells uninfected-add 500 µls fresh cRPMI). *Use fresh tip for each transfer*.
8. Swirl the plate to spread the virus and incubate the plate at 37°C, 5% CO_2 for 1 hr for virus to infect macrophages.
9. Add 500 µls fresh cRPMI and incubate the plate at 37°C, 5% CO2 for 24 hr.
10. Starting with the wells infected with the lowest dilution (10^{-6}), add 1.6 x 10^5 autologous RBCs in 20 µls and incubate the plate at 37°C, 5% CO2 for 2 - 4 days when hemadsorption is expected to be observed in the wells.

Determination of HAD_{50} titer:

1. Using the Spearman-Karber method the end-point of the virus titer is calculated. The titer is expressed as $\log^{10}$ HAD_{50} per 50 µl dose.
2. **Example**: Sample result from the plate after observation of hemadsorption;

10^{-1} dilution of the virus = 5 of 5 wells positive for HAD
10^{-2} dilution of the virus = 5 of 5 wells positive for HAD

10^{-3} dilution of the virus = 5 of 5 wells positive for HAD

10^{-4} dilution of the virus = 3 of 5 wells positive for HAD

10^{-5} dilution of the virus = 0 of 5 wells positive for HAD

10^{-6} dilution of the virus = 0 of 5 wells positive for HAD

Titer = (X - d/2 + [d • S]) where:

i. X = log10 of lowest dilution (1)
ii. d = log10 of dilution factor (1)
iii. S = sum of proportion of HAD positive, calculate below;

$\frac{5}{5}+\frac{5}{5}+\frac{5}{5}+\frac{3}{5}+\frac{0}{5} = 18/5 = 3.6$

b. Test sample titer = (1 - 1/2 + (1 • 3.6) = 4.1

The antilog of 4.1 = 12589 (for log antilog calculator check: http://ncalculators.com/number-conversion/anti-log-logarithm-calculator.htm)

Thus a dilution of 1:12589 is equivalent to 1 HAD_{50} per 50 µl.
If we are going to use 10^4 HAD_{50} to challenge pigs, then $12589/10^4 = 1.26$ will be the dilution factor of the stock virus.

NB: If HAD is observed in all the wells at the 10^{-6} dilution, repeat assay by first diluting an aliquot of the stock virus 1: 10^6 and then determine the HAD_{50} as above. Factor in the dilution factor to determine the virus titer in the stock.

10.3: ELISA WITH RECOMBINANT PROTEINS AS ANTIGEN

- Briefly, microtiter plates (Polysorbimmunoplates; Nunc) were incubated at 4°C overnight with 100 µl/well of recombinant proteins (9GL) or the negative control at a previously determined optimal concentration in coating buffer (0.1M carbonate buffer, pH 9.6).
- Take the 500 microlitre aliquot (saved prior to boost) and dilute an aliquot in the above buffer at 1:100; then add 100microlit of this in wells to coat the plates.
- The coated plates were washed four times with 200microl of phosphate-buffered saline (PBS; pH 7.5) containing 0.05% (vol/vol) Tween 20 (PBS-T) and used immediately or stored at 20°C until use.
- The plates were subsequently blocked with PBS (pH 7.5) containing 5% (wt/vol) skim milk (PBS-M) for 1 h at 37°C.
- The porcine sera were added at a dilution of 1:100 in PBS-M (These refer to sera collected from the trial animals) and incubated for 1 h at 37°C. Positive and negative

reference sera were included on each plate (Positive control: Use sera from the ASFV positive pigs from the field; Negative control is the pre-bleed sera from pigs.

- The plates were washed four times with PBS-T, and thegoat anti-swine HRP diluted 1:5,000 in PBS-M was added. The plates were incubated for 1 h at 37°C. Afterwards, the plates were washed as described above.
- 50 µl of o-phenylenediamine (Sigma) (not available; so we use TMB) was added to each well. After incubation for 20 min at room temperature, the reaction was stopped by the addition of 50µl of 3 N H2SO4 (stop soln in ingenasa kit), and the optical density (OD) was measured at a wavelength of 492 nm.
- Titers were expressed as the ratio between the ODs obtained for each sample against recombinant antigen-positive and negative control proteins.
- All serum samples with doubtful results (those with results in the cutoff interval) were considered positive.

Day 0 Date 28/10/2014

No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Group	**A**	**A**	**A**	**A**	**A**	**B**	**B**	**B**	**B**	**B**	**C**	**C**	**C**	**C**	**C**	**D**	**D**	**D**	**D**	**D**
Pig No.	**1040**	**1059**	**1066**	**1069**	**1074**	**1091**	**1092**	**1095**	**1098**	**PB006**	**1093**	**1094**	**1096**	**1097**	**PB005**	**1052**	**1054**	**1055**	**###**	**1058**
WBC (10^3/µl)	12.9	19.2	20.2	11.7	11.2	25.8	20.5	38.6	20.2	12.9	21	25.3	20.7	19.1	10.4	13.9	23.2	17	18	19.7
RBC (10^6/µl)	6.81	8.25	7.7	6.72	5.99	9.19	9.55	6.56	7.44	6.54	6.71	7.18	8.74	6.96	6.66	6.75	11.5	6.74	7.8	6.36
HGB (g/dl)	12.3	14.4	12.7	12.7	11.4	14.1	13.7	11.2	12.6	11.1	10.8	11.1	13	12	11.4	11.6	20.6	11.1	13	12
HCT (%)	44	49	43.3	40.8	38.3	51.2	49.6	39.9	44.3	39.1	38.8	39.4	45.5	43.2	40	37.7	70.1	38.7	47	42.8
MCV (fL)	64.6	59.4	56.2	60.7	63.9	55.7	51.9	60.8	59.5	59.8	57.8	54.9	52.1	62.1	60.1	55.9	61.1	57.4	60	67.3
MCH (pg)	18.1	17.5	16.5	18.9	19	15.3	14.3	17.1	16.9	17	16.1	15.5	14.9	17.2	17.1	17.2	17.9	16.5	17	18.9
MCHC (g/dl)	28	29.4	29.3	31.1	29.8	27.5	27.6	28.1	28.4	28.4	27.8	28.2	28.6	27.8	28.5	30.8	29.4	28.7	28	28
PLT (10^3/µl)	377	279	365	303	276	429	391	413	393	359	429	599	411	493	315	551	226	351	565	226
LYM (10^3/µl)	4.7	5	2.5	5.3	5.4	2.9	3.9	3.7	4.5	4	2.9	4.1	9.4	2.5	2.1	3.2	2.3	2.3	2.6	3.7
MO (10^3/µl)	0.3	0.4	0.4	0.2	0.3	0.5	0.6	0.6	0.6	0.5	0.5	0.6	0.2	0.4	0.1	0.3	0.5	0.4	0.4	0.1
EO (10^3/µl)	1.1	1.5	2.3	0.3	0.3	2.5	2.4	4.5	3.7	1.4	2.1	3.7	0.8	2.1	0.4	0.7	2.8	1.4	1.5	0.3
GR (10^3/µl)	6.8	12.3	15	5.9	5.2	19.9	13.6	29.8	11.4	7	15.5	16.9	10.3	14.1	7.8	9.7	17.6	12.9	13	15.6
RDW (%)	18	17.7	17.1	18	18.1	17.4	17.1	16.3	17.4	16.8	15.9	17.3	16.7	16	16	18	21.6	17.2	18	18.5
PCT (%)	0.3	0.27	0.32	0.2	0.17	0.32	0.28	0.38	0.35	0.3	0.34	0.42	0.27	0.45	0.35	0.3	0.18	0.29	0.4	0.19
MPV (fL)	8	9.5	8.8	6.7	6.3	7.5	7.1	9.1	9	8.4	8	7	6.6	9.1	11	5.5	7.9	8.2	6.7	8.5
PDW (%)	20.1	17.3	15.7	20.4	21.9	17.4	16.8	19.8	18.4	18.8	18.6	16.8	17.1	18.9	17.8	18.8	17.1	18.2	19	23.3
	15.67	12.5	6.25	26.5	18	5.8	6.5	6.17	7.5	8	5.8	6.833	47	6.25	21	10.7	4.6	5.8	6.5	37

Blood cell count: Comparative Pathogenesis of African swine fever virus isolate 1033 in Pigs

Day 6 Date 3/11/2014 6

No.	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
Group	**A**	**A**	**A**	**A**	**A**	**B**	**B**	**B**	**B**	**B**	**C**	**C**	**C**	**C**	**C**	**D**	**D**	**D**	**D**	**D**
Pig No.	**1040**	**1059**	**1066**	**1069**	**1074**	**1091**	**1092**	**1095**	**1098**	**PB006**	**1093**	**1094**	**1096**	**1097**	**PB005**	**1052**	**1054**	**1055**	**1056**	**1058**
WBC (10^3/µl)	6.6	22.4	21	3.3	10.7	17	7.3	13.9	18	11.9	14.1	17.4	11.8	13.4	10.8	8.9	13.6	10.4	7.6	5.9
RBC (10^6/µl)	8.65	7.08	7.54	6.51	5.56	6.99	6.97	6.63	7.17	5.94	6.08	7.14	8.17	6.14	6.31	7.28	6.62	6.83	7.11	5.25
HGB (g/dl)	16.9	12.3	12.8	12.2	10.5	10.6	10.3	11.2	11.9	9.7	9.9	11.1	11.8	10.4	10.8	11.8	11.5	11.1	11.2	9.8
HCT (%)	53.9	40.1	42	39.9	36.2	38.8	34.6	39.6	41.6	35.2	34.4	37.9	41.1	37.1	37.7	41.1	40.6	38.3	40.2	33.1
MCV (fL)	62.3	56.6	55.7	61.3	65.1	55.5	49.6	59.7	58	59.3	56.6	53.1	50.3	60.4	59.7	56.5	61.3	56.1	56.5	63
MCH (pg)	19.5	17.4	17	18.7	18.9	15.2	14.8	16.9	16.6	16.3	16.3	15.5	14.4	16.9	17.1	16.2	17.4	16.3	15.8	18.7
MCHC (g/dl)	31.4	30.7	30.5	30.6	29	27.3	29.8	28.3	28.6	27.6	28.8	29.3	28.7	28	28.6	28.7	28.3	29	27.9	29.6
PLT (10^3/µl)	241	373	449	210	315	365	118	294	439	282	45	416	297	252	390	425	363	343	432	115
LYM (10^3/µl)	1.3	6.5	4.9	1.6	4.9	7.1	2.5	5.2	8.2	3.4	4.7	4.2	5.5	3.9	3	3	5.2	3	1.3	0.8
MO (10^3/µl)	0.2	0.3	0.5	0.1	0.2	0.3	0.2	0.4	0.2	0.3	0.4	0.6	0.2	0.3	0.2	0.2	0.4	0	0.1	0.1
EO (10^3/µl)	0.4	0.5	0.4	0.3	0.8	0.7	0.5	1.3	0.7	0.6	1.4	0.7	1	0.9	0.5	0.7	0.7	0.2	1.1	0.5
GR (10^3/µl)	4.7	15.1	15.2	1.3	4.8	8.9	4.1	7	8.9	7.6	7.6	11.9	5.1	8.3	7.1	5	7.3	7.2	5.1	4.5
RDW (%)	17.9	16.3	17.2	17.9	19.7	18.2	17.4	16.2	17.7	17.5	16.7	18	17.4	16.3	16.3	17.8	22.4	17.8	17.4	18.3
PCT (%)	0.22	0.3	0.34	0.16	0.2	0.26	0.07	0.21	0.32	0.23	0.06	0.27	0.18	0.26	0.37	0.29	0.21	0.22	0.25	0.14
MPV (fL)	9.2	8.1	7.6	7.7	6.3	7.2	5.9	7.2	7.2	8.2	13.2	6.6	6	10.5	9.5	6.8	5.7	6.5	5.8	11.9
PDW (%)	20.3	16.3	16.1	20.1	21.4	16.8	18.5	20.4	17.4	19.2	17.1	17.6	16.3	18.9	20.4	18.8	17.7	18.1	19.6	20.9

Appendix 10: IACUC Form

IACUC - EXPERIMENTAL ANIMAL USE FORM

IACUC ref. no:

IACUC use only

This form is to be submitted to the IACUC mailbox (IACUCBOX@cgiar.org) after completing all sections and all endorsements (see Annex for instructions).

POINTS TO NOTE:

- This form is valid, unchanged, for one year.
- If minor changes are required then the **IACUC - EXPERIMENTAL ANIMAL USE MINOR AMENDMENT FORM** (see Annex for attachment – ***IACUCMinorAmendmentForm2009-2011***) should be completed and sent to the IACUC committee for consideration.
- The committee requires the form to be submitted to the IACUC committee at least two weeks before the start of the experiment to enable sufficient time for it to be approved.
- No experiment shall be approved unless the current file format (***IACUCForm2009-2011***) is used.
- On completion of filling in the Experimental Animal Request Form, ensure that the Risk Assessment Form (see Annex for attachment) is also filled, if required, and submitted to the Institute Biosafety Committee (IBC) committee.
- The ILRI IACUC committee follows international standards for animal care and use; the following sectionsare used to confirm that researchers have adequately considered the 3R's (Replacement, Reduction, Refinement) relating to the use animals in experiments.
- Only persons named on this form (section 3 – 5) shall be allowed to handle and/or carry out sampling of the experimental animals.
- Upon completion of the experiment, the PI shall complete the PI Report Back form attached and send it to the IACUC.

1. Title of experiment:	(Max. 30 words)
Clinical and immunological responses of West Kenya pigs to infection with defined titres of ASFV stock 1033	

2. Operating Project, location & grant code under which experiment falls:	e.g. BT02 NBO WEL004
BS03 NBO ESP004 ESP004H	

3. Principal Investigator (PI): (Person ultimately responsible for this experiment)	Dr Richard Bishop	Home Tel. Mobile Tel.	020 4223155 Mobile 0716 122701
4. Collaborators:	EU ASF Reference laboratory (CISA-INIA, Spain)		
5. Technicians/staff involved in animal sampling& handling:	Edward Okoth (ILRI) Gideon Ndambuki (ILRI) Victor Riitho (ILRI) Eunice Omondi (ILRI DVS)		

6. Overallobjectives of the experiment:	*(Provide numbered list)*
1. Clinical and immunological responses of indigenous Kenyan pigs to infection with defined titres of ASFV isolate 1033	

7. Background information:	*Give information that the reviewers can use to understand the experiment, include references to literature, past experiments, outcomes etc.* ***N.B. This should also include the overall justification for the experiment***

In order to establish *in vivo* properties of ASFV 1033 and relate these to*in vitro* HAD_{50} titres, we recently performed an experiment entitled BIOLOGICAL AND IMMUNOLOGICAL CHARACTERIZATION OF AFRICAN DOMESTIC PIGS INFECTED WITH AN AFRICAN SWINE FEVER VIRUS FROM KENYA (IACUC Reference number 2014.2.)An experiment was previously conducted at the central veterinary laboratory Kabete pig facility in 2011 to compare responses to infection with an ASFV virus in pigs of Kenyan and European breeds (Ref: VN_IACUC-2011-ASF in vivo infection Ref 2011-04).The results from the experiment in 2011 showed a level of tolerance to infection in local Kenyan pigs compared to pure landrace X large white crosses, as indicated by a delayed onset of clinical signs and longer survival rates based on the experimental end point of 21 days. All exotic bred animals had died with classical ASFV symptoms on this timescale, whereas only three of 20 Kenyan animals had succumbed. Given the differences in responseobserved in 2011,the current experiment is designed to build on the recent dose titration with a virulent genotype IX isolate (Bus.1033) that was isolated by ILRI as part of an AusAID-funded project in Busia district in 2013.The aim is to repeat the dose titration in indigenous pigs to see if this differs from that observed in the European animals. This is important since indigenous pigs represent a target for vaccination and experiments designed to evaluate protective immune responses. A second objective will be to assess the reproducibility of the previous dose titration experiment in the European breeds. Thereappeared to identify a critical threshold between the response in pigs using a 10^{-4} and $10^{-5}$of the stock virus (corresponding to HAD_{50} titres of approximately HAD_{50} 10^{6} and HAD_{50} $10^{5)}$. This resulted in survival times of 8-9 or 16-24 days, respectively. It will

be important to check that this is reproducible in a repeat experiment.

References

8. Work plan:

The experiment: Infection will be performed at the secure animal facility at ILRI (BSL2 pig unit). The experiment has been approved by the ILRI Institutional Biosafety committee (IBC).

We propose to infect tenindigenous pigs from Homa Bay district (in two separate groups of five each) infected with $10^6$and$10^5$$HAD_{50}$ units by the intramuscular(*i.m.*) route and ten weight-matched European control animals also in groups of five each.

In addition to monitoring of the clinical symptoms the following biological parameters will be measured:

- ASF PCR (real time and conventional) at days0, 1, 2, 3, 4, 5, 6, 7, 9, 11, 14, 16, 18, 21 and 28
- Complete blood counts at days 0, 1, 2, 3, 4, 5, 6, 7, 9, 11, 14, 16, 18, 21 and 28
- Serological responses at days0, 4, 7, 11, 14, 18, 21 and 28
- Nasal swabs at days 0, 1, 2, 3, 4, 5, 6, 7, 9, 11, 14, 16, 18, 21 and 28
- IFN-γ ELISpot responses from PBMC at days 0, 7, 14, 21 and 28
- Weights will be measured weekly
- Lesions and presence of virus in internal organs (spleen (most important for virus propagation), kidney, lymph nodes, tonsil, lung, liver, heart)following post mortem

The intramuscular (*i.m.*)infection protocol with ASFV has been used previously in the ILRI BSL2 pig facility.Dr. Okoth will perform the intramuscular inoculation (of which he has previous experience) together with Dr.. Omondi.

Sampling Timetable:

	Saturday	Sunday	Monday	Tuesday	Wednesday	Thursday	Friday
Day	**-2**	**-1**	**0**	**1**	**2**	**3**	**4**
EDTA (5ml)			X	X	X	X	X

Serum (10ml)			X				X
Heparin (20ml)			X				
	Saturday	Sunday	Monday	Tuesday	Wednesday	Thursday	Friday
Day	**5**	**6**	**7**	**8**	**9**	**10**	**11**
EDTA (5ml)	X	X	X		X		X
Serum (10ml)			X				X
Heparin (20ml)			X				
	Saturday	Sunday	Monday	Tuesday	Wednesday	Thursday	Friday
Day	**12**	**13**	**14**	**15**	**16**	**17**	**18**
EDTA (5ml)			X		X		X
Serum (10ml)			X				X
Heparin (20ml)			X				
	Saturday	Sunday	Monday	Tuesday	Wednesday	Thursday	Friday
Day	**19**	**20**	**21**	**22**	**23**	**24**	**25**
EDTA (5ml)			X				
Serum (10ml)			X				
Heparin (20ml)			X				
	Saturday	Sunday	Monday	Tuesday	Wednesday	Thursday	Friday
Day	**26**	**27**	**28**	**29**	**30**	**31**	**32**
EDTA (5ml)			X				
Serum (10ml)			X				
Heparin (20ml)			X				

9. Why are animals* required for this experiment?* *Including why THIS species*	*This short section refers to animal ethics considerations justifying why this research is being conducted on animals and cannot be carried out without their use (e.g. invitro).*
ASFV causes disease only in domesticpigs. There is no*invitro*model available for assessment of clinical,biological and immunologicalcharacteristics of the virus.	

10. Allocation of Animals to Experimental Groups:	*Amend table design if needed so experimental groups & allocations are clear*
Experiment groups(Code / Name)	**Number of animals**
Treatment groups (1) Ten (5 and 5)'indigenous' Kenyan pigs to be infected *i.m*with $10^6$and $10^5$$HAD_{50}$ units of ASFV stock Busia1033 (2) Ten control'exotic' European breed pigs to be infected with the same doses.	10 (5 and 5) indigenousanimals to be infected with 10^6 and $10^5$$HAD_{50}$ units of ASFV. 10(5 and 5) exotic animals to be infected with $10^6$and $10^5$$HAD_{50}$ units of ASFV
Total number of animals requested:	20

11. Justification for the number of animals:	*The aim is to minimise the number of animals required to achieve the experimental objectives. Include any sample size calculations.*
The titration experiment established that a dose of 10^6HAD50 units of the Busia 1033 stock was lethal for all three exotic pigs that received this dose of virus via the i.m. route of administration in 8 or 9 days. Therefore we expect that all five controls should succumb during the period of the experiment. A lower dose of $10^5$$HAD_{50}$ was notimmediatelylethal to the three pigs in the titration experiment, but the animals exhibited high temperatures (≥40°C) for a period of three days, and also mild external haemorrhaging on the ears, and were euthanized between days16-24.In view of the major difference in response in vivo between the two titres it will be important to repeat the experiment with an increased number of European breed animals. The severe response to the 10^6 HAD_{50} dose in the European breed animals with mortality in 8-9 days suggests that even if there is some degree of resistance or tolerance in the indigenous breeds at least the higher dose should induce a clinical reaction.	

12. Statistical design & analysis details:	*E.g. design details – factorial treatment structure, randomised etc& e.g. analysis details – analysis of variance, non-parametric test, transformation of data etc.*)

13. Specifics of animals required:			*(Complete sections relevant to the design and/or implementation of this experiment, add rows as needed, insert N/A if not relevant). * Age at start of experiment.*		
Species	**Breed/Strain**	**Sex**	**Age***	**Number**	**Other:** ***(specify)***
Indigenous Kenya pigs (*Sus scrofa*)	Ten African indigenous breed from Western Kenya	Both male and female	> 6 months	10	➢ 50 kg
Exotic domestic pigs (*Sus scrofa*)	TenLandrace x Large White from Farmer's Choice		>6 months	10	➢ 50 kg

14. Special animal health requirements:	(*E.g. anaplasma free, FMD vaccinated*)
The experimental animals should have no detectable ASFV infection based on ASFV-specificPCR or detectable antibody response using the routine serological test (INGENASA indirect ELISA).	

15. Proposed (future) commencement date:	15 October 2014	Duration:	28 days

16. Humane end-point for experimental animals:	This will be determined by Dr. Okoth, who has extensive experience of in vivo infection with ASFV,based on observationoftemperature and monitoring of the suite of clinical symptoms described below If two or more of the below conditions are observed in an animalconcurrently for24 hoursthen euthanasia will be implemented. Euthanasia is the only optionbecause there is no treatment currentlyavailable for the disease.

1. Extreme depression, coupled with a body temperature significantly above normal (≥40^{0}C for more eight hours). Non-responsive, or unconscious with no response to external stimuli such as handling or the toe-pinch withdrawal test.
2. Weight loss: Loss of 20% body weight. Weight loss will be characterized by cachexia and muscle wasting. Body condition scoring (1 – 5 with 1 being thin and 5 being very fat) will be used to validate weight loss using a baseline score at the beginning of the experiment.
3. Lack of appetite: Complete anorexia. This will be judged by monitoring of the pigs during feeding at all times during the experiment.
4. Weakness/inability to obtain feed or water: Inability to ambulate to reach food or water; lesions that interfere with eating or drinking: or reluctance to stand which persists for 48 hours.
5. Rapid clinical signs of haemorrhagic symptoms: Including haemorrhagic diarrhoea, haemorrhagic vomiting, etc.

17. Action to be taken at humane end-point:	*E.g. treat (if required) & return to farm/animal house, euthanize & incinerate, euthanize & use meat for human consumption etc.*
Euthanize and incinerate	

18. Action to be taken for surviving animals at the endpoint of the experiment:	*E.g. treat (if required) & return to farm/animal house (no treatment), euthanize & incinerate, euthanize & use meat for human consumption etc.*
Euthanize and incinerate	

19. Risk to non-experimental animals:	Yes
If yes, how will you deal with this risk:	Physical containment of the animals. Strict biosecurity protocols including decontamination of all liquid waste in holding tank (Sodium hypochlorite treatment) and incineration of solid waste (which will be secured in bags treated with Vircon) on a daily basis. Incineration of experimental animals at euthanasia orat the termination of the experiment. Biosecurity procedures to be adhered to by all scientists and other staff, including using of dedicated clothing and footwear, which will be changed on entry to and exit from the pig isolation facility. Locking of the isolation facility during non-working periods.
20. Minimum standard of holding facility:	Double Click and Select The experiment will be carried out at BSL2 Animal Isolation Unit at ILRI. The biosafety of the unit hasrecently been upgraded to allow performance of experiments using ASFV.

21. Routine procedures:		*Information needed for the animal handlers / farm staff*	
Weighing:	Yes	**Frequency:**	**Daily**
Temperature:	Yes	**Frequency:**	**Daily**
Feeding:	Yes	**Frequency:**	**Daily**
Watering:	Yes	**Frequency:**	**Daily**
Others:*(specify)* .	Double Click and Select		

22. SOPS: the new*SOP is available on this page*	*Reference from Work plan, insert new rows as needed.*
Number N/A	**Frequency Once (initial infection)**
9.5 Intramuscular injections (IACUC SOP manual 2)	Once during infections

23. Are there additional procedures for which SOPs are not available:	Yes

If YES*(add rows for multiple additional procedures)***:**

Name &Description:

Intramuscular inoculation

Additional information on SOP for intramuscular (i.m.) inoculation of pigs with ASFV:

- The animal will be held using a snout snare
- 1 ml of ASFV inoculum will be injected into the gluteal muscle of each pig using a 2 ml syringe and an 18G x 1½', 1.2mm x 38mm needle

Euthanasia of pigs

The animal will be held using a snout snare and the neck stretched well upwards. This is best achieved if the pig stands on all four legs. The snare will be placed behind the canine teeth so that it does not slip off or move rostrally towards the nasal cartilage, an area that is uncomfortable for pigs. The animal will try to move backwards and in that way tighten the rope sufficiently. It is therefore not necessary to prevent the pig from moving forwards. The correct needle puncture site is in the deepest point of the jugular groove formed between the medial sternocephalic and lateral brachiocephalic muscles. A commercial Euthanasia Solution (Sodium pentobarbital 390 mg + sodium phenytoin 50 mg/ml) (Euthasol®) will be injected IV into the jugular vein at a dose of 0.22 ml/kg IV (86 mg/kg pentobarbital)

Bleeding from the External Jugular of pigs

The animal will be held using a snout snare and the neck stretched well upwards while the pig is standing on all four legs. The snare will be placed behind the canine teeth so that it does not slip off or move rostrally towards the nasal cartilage, an area that is uncomfortable for pigs. The correct

needle puncture site will be located as described above and blood collected from the jugular vein using BD Vacutainer® needles (gauge x length: 21 x 1-1/2 inch) into 10 ml BD Vacutainer® glass serum tube and 4.5 ml 15% EDTA tubes (Becton, Dickinson and Company, United Kingdom).

Surgeon (if applicable**): Drs Edward Okoth and Eunice Omondi**

24. Does this experiment require the preparation of non-commercial products / materials?	No
If yes, a paragraph explaining the process and quality assurance standards should be sent for review by your OPL and Theme Director	

25. Use of potential Infectious / Hazardous Agents / Substances in animals:				*Indicate all that apply with a X in neighbouring column*
X	Infectious (including bacteria, viruses, parasites, prions)			Radioisotope
	Toxic chemicals			Carcinogens
	Other *(please specify)*:			Recombinant DNA and proteins

N.B. The IACUC will not approve protocols involving biohazardous substances without prior approval from the IBC(Institute Biosafety Committee).– APPROVAL ALREADY RECEIVED

26. Endorsements:		*Main signatures in the order below signify that all necessary obligations/requirements have been satisfied.*	
Signature of Principal Investigator *:	Richard Bishop	Date:	**07/10/2014**
Signature of Biometrician:	Jane Poole	Date:	
Signature of Project Leader	Richard Bishop	Date	
Signature of Operating Project Leader:	Steve Kemp	Date:	
Signature of DDG:	Steve Kemp	Date:	

* I certify that the animals to be used in this study will be used in accordance with ILRI regulations and standards for the care and used of animals. Moreover, I certify that pain or discomfort to animals will be limited to that which is unavoidable in the conduct of scientifically valuable research. To the best of my knowledge, the studies proposed do not unnecessarily duplicate any other in the published literature. In addition, I certify that the use of*invitro*, less invasive or painful, or other alternative techniques, has been considered. I have concluded that the species, number of animals and procedures to be used are the most appropriate for the proposed investigation. As the PI, I will be responsible for all work conducted under this protocol. Any revision to this protocol will be forwarded to the IACUC for review using the IACUC amendment form and revised protocol will not be implemented until IACUC approval has been obtained.

IACUC USE ONLY

Date received by IACUC:		**Received by:**	
Application number:			

IACUC review actions:	*(Reference can be to an e-mail, description of actions or edited version of form)*	
Reference:	**Requested by (name):**	**Action (what / by whom):**
Form approved after clarification on animal number. IACUC supposes all the safety on virus preparation and handling was addressed by the IBC.	IACUC	PI

IACUC FINAL APPROVAL:					
Signature:		**IACUC position:**		**Date:**	

ANNEX

The sign-off procedure is as follows

N.B. This process / chain should be indicated by FORWARDING e-mails through the process)

Principal Investigator (PI): This is be the person (researcher) who takes responsibility for ensuring that the experiment runs as stated in the protocol - and that animals are cared for as they should be. The PI should be the person who is touch with the field staff and can answer and explain all questions about the way the protocol is implemented and why. They will be based wherever the experiment is taking place.

↓

Biometrician: This is a named ILRI staff in the Research methods group. The current named biometrician is Jane Poole. The PI should discuss design of the experiment with the biometrician who will ensure that the protocol is statistically valid, that the experiment will provide the minimum data required to satisfy the stated research objective.

↓

Project Leader: This is the person who is directly responsible for the research project under which the PI is working and is likely to be the supervisor of the PI. In some cases PI and project leader will be the same person.

↓

Op Leader: Leader of the Operating Project (BT01, BT02, BT03) under which the experiment falls.

↓

Theme Director: Leader of the theme under which the OP and project fall.

Approval of Scientific Merit (Theme Director Approval Required):

The IACUC assumes that the proposed research has been reviewed and approved on the basis of scientific merit by the Theme Director, evidenced by their signed-off or his/her designee (in the "Endorsements" section).

Useful documents:

Biological Risk Assessment Form (submitted to the IBC committee):

Minor amendments form (to be used for amendments made to this form AFTER approval by the IACUC committee):

Principal Investigator (PI) Report-back Form (submitted to the IACUC committee after completion of the experiment):

Definitions:

The Three R's

- ***Replacement*** often means the use of an inanimate system as an alternative (e.g., a computer model or program,). It can also mean the replacement of more sentient animals with less sentient animals. It also includes the use of cell and tissue cultures.
- ***Reduction*** means a decrease in the number of animals used previously with no loss of useful information. This may be achieved by reducing the number of variables through good experimental design, by using genetically homogeneous animals or by ensuring that the conditions of the experiment are rigorously controlled.
- ***Refinement*** means a change in some aspect of the experiment that results in a reduction or replacement of animals or in a reduction of any pain, stress or distress that animals may experience. The establishment of early endpoints for intervention in a study that has the potential to cause pain or distress is an example of refinement.

Humane Endpoint - The humane endpoint is the point at which pain and/or distress is terminated, minimized or reduced by taking actions such as euthanatizing the animal; terminating a painful procedure; or giving treatment to relieve pain and/or distress