MAKERERE UNIVERSITY

PREVALENCE AND GENOTYPING OF AFRICAN SWINE FEVER VIRUS IN APPARENTLY HEALTHY PIGS IN MASAKA, MUKONO AND KAMULI DISTRICTS IN UGANDA

BY

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2011/HD17/2777U

A DISSERTATION SUBMITTTED TO THE DIRECTORATE OF RESEARCH AND GRADUATE TRAINING AS PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTERS OF SCIENCE IN MOLECULAR BIOLOGY OF MAKERERE UNIVERSITY

SEPTEMBER 2015

DECLARATION

I **Joyce Akol** declare that this work is my original work and has never been submitted to this university or any other institution of higher learning for any award.

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DEDICATION

This work is dedicated to my parents, Mr. John Francis Esegu and Mrs. Mary Akello Esegu, my brothers Olinga C, Otule M, Omulala S, Esegu M, sisters Adeke B, Alupo J, my sister-in-law Kongai J and my dear son Aginya E.J, for the encouragement, spiritual, financial and moral support accorded to me during my studies.

ACKNOWLEDGEMENT

I would like to express my gratitude to the International Livestock Research Institute-Smallholder Pig Value Chain Development (ILRI-SPVCD) project Uganda for funding my research work. I am forever grateful to my supervisors; Associate Professor. Charles Masembe, Dr. Michel Dione and Dr. Denis Muhangi for their patience; tireless guidance, professional commitment, influence and guidance that has helped me finish my work. I would like to express my thanks to Dr. Yona Baguma at the National Crops Resources Research Institute (NaCRRI) for granting me the opportunity to study and work concurrently. I also appreciate the Madhvani group for financially supporting me with tuition fees for my first year. I am entirely indebted to all my lecturers for the knowledge imparted which has improved me as a whole. I also extend my thanks to the District Veterinary Officers(DVOs) of Masaka, Mukono and Kamuli districts together with the local council members and field support staff for their contribution to the study and Dr. Edward Okoth who guided me with data analysis. To my friends Martin Chamai, Joseph Kungu and Doreen Buhwa thank you so much for the academic, financial and moral support. Last but not least, I appreciate my parents, brothers; sister and son for bearing with my absence, you all are my biggest inspiration. Finally to God for His provident love and mercy that has enabled me to complete this work.

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LIST OF ABBREVIATIONS

ASF	African swine fever
ASFV	African swine fever virus
CVR	Central variable region
DVO	District Veterinary Officer
EDTA	Ethylene Diamine Tetra acetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
GIS	Geographical Information System
ILRI	International Livestock Research Institute
LC	Local council
NAADS	National Agricultural Advisory Services
NGO	Non-Governmental Organization
OD	Optical Density
OR	Odds Ratio
PC	Positive Control
RR	Rural-Rural
RT-PCR	Real time Polymerase Chain Reaction
SPVCD	Smallholder Pig Value Chain Development

ABSTRACT

African swine fever (ASF) is a viral hemorrhagic disease associated with death in infected pigs. African swine fever virus (ASFV) is a DNA virus that circulates in blood and lymphoid system of the pigs causing disease. There are various reports on ASF outbreaks in the country with a few confirmed in apparently healthy pigs which pigs show no signs of infection. Therefore a survey of apparently healthy pigs was undertaken to show the extent they habour the antibodies and antigen of ASFV and later determine the genetic diversity of the virus ASF in Kamuli, Mukono and Masaka districts of Uganda using serological, molecular and genotyping techniques. In total 1,192 blood and sera samples were collected and analyzed. All the pigs tested except one (1/1192) were negative for (ASFV) and none for antibodies indicating that ASFV causes a paracute / acute infection in Ugandan pigs with rare detection of virus or antibodies in apparently healthy pigs. Therefore chronically infected pigs are unlikely to be important in the epidemiology of ASF. The positive pig in Kamuli district was infected with genotype IX, the most common circulating ASFV genotype in Uganda. With one positive pig for ASFV, it was not possible to authoritatively associate predictors of infection with disease in tested pig farms. It is thus recommended that these predictors of infection with ASFV are studied in future ASF outbreak areas where the virus or antibodies in pigs may occur in high prevalence.

Key words: African swine fever virus, apparently healthy pigs, Serology, PCR, Sequencing

CHAPTER ONE

INTRODUCTION

1.1 Background

Uganda has the largest and rapidly growing pig production industry in Eastern Africa and its pig population has risen from 0.19 to 3.2 million in the past 3 decades (UBOS, 2013). This increase is estimated to be about 70% in Uganda that has the highest per capita pork consumption in East Africa (UBOS, 2008). Pig production is mainly dominated by the rural free-range smallholder systems ; however, other systems such as the intensive and semi-intensive also exist but are more common in the urban and peri-urban areas (Muhanguzi *et al.*, 2012).

Despite the increase in pig production in the country, this industry faces a lot of constraints like: poor housing, high feeding costs and disease burden (Dione *et al.*, 2014). ASF is important socio-economically to the farmers; because it can lead to very high mortality and morbidity in infected pigs. These "apparently healthy pigs" on farms are rarely tested for presence of antibodies and ASFV because these tests are quite expensive. The apparently healthy pigs are pigs which show no signs of disease but may harbor disease causing pathogens.

Epidemiological studies undertaken to understand the disease in Uganda have hinted at possible presence of the virus in domestic pigs without clinical signs of ASF (Tejler, 2012: Atuhaire *et al.*, 2013). These chronically infected pigs may spread the virus for long since they may have acquired resistance or adapted to the condition, hence the continued outbreaks that lead to income losses and unemployment in the country (Atuhaire *et al.*, 2013). However, other factors like: consumption of contaminated swill, concentrate, pasture, sharing feeding utensils and the use of the same breeding

boars may precipitate viral infection amongst pigs (Fasina *et al.,* 2012). Exchange of pigs among farmers is another avenue for viral spread in the pig population communities (Costard *et al.,* 2012). These pigs need to be investigated for pathogen presence using rapid and reliable diagnostic techniques.

This study was therefore designed to determine the sero and antigen status of ASFV in "apparently healthy" pigs in Masaka, Mukono and Kamuli districts in Uganda.

1.2 Statement of the problem

Smallholder pig farmers face a serious challenge of ASF. Recent studies have shown presence of the virus in some apparently healthy pigs mainly at the slaughter slabs (Tejler, 2012:Muwonge *et al.,* 2012:Atuhaire *et al.,* 2013). Inaddition, various activities along the pig value chain like; movement of pigs from farm to farm and from farm to market; movement of extension service providers from farm to farm without protective wear and disinfection expose the pigs to viral infection (Nantima *et al.,* 2015). At the farm level, there is lack of proper disease surveillance, quarantine, protective wear and disinfection of personnel and equipment (Fasina *et al.,* 2012). Subsequently, farmers normally borrow boars for mating and restock their farms with pigs from within the community without determining their health status also poses a risk of infection (Muhangi *et al.,*2015). With the lack of routine diagnostics in the smallholder farmers and high disease prevalence as reported by farmers without confirmation in Masaka, Mukono and Kamuli districts (Dione *et al.,* 2014), there was need to detect presence of antibodies and ASFV in the apparently healthy pigs on farm in Mukono, Masaka and Kamuli districts.

1.3 General objective

The general objective was to assess the healthy status of the apparently healthy pigs to ASF antibodies and antigen in Masaka, Mukono and Kamuli districts.

1.3.1 Specific objectives

1. To determine the antibody and ASFV DNA status in apparently healthy pigs in the districts of Mukono, Masaka and Kamuli.
2. To genotype the detected ASFV from the apparently healthy pigs.

1.4 Research questions

1. What is the sero and antigen status of ASFV in apparently healthy pigs in the above districts?
2. What are the circulating genotypes of ASFV in apparently healthy pigs in the above districts?

1.5 Justification of the study

Several households depend on the livestock industry like piggery for improved livelihood. ASF poses a major challenge to the pig industry in Uganda where the disease is endemic. With the disease being ranked first in Masaka, Mukono and Kamuli districts (Dione *et al.,* 2014), there was need to confirm presence of ASFV and antibodies in the apparently healthy pigs in these same districts. Determining the sero and antigen status of the ASFV in apparently healthy pigs and further characterization of the virus will help understand the molecular epidemiology of the disease in Uganda. This information once generated, will be communicated and used to better inform the currently available prevention and control strategies for ASF.

CHAPTER TWO

LITERATURE REVIEW

2.1 Pig production systems in Uganda

The pig production industry in Uganda has experienced a population increment of about 70% in the last decade (UBOS, 2013). The livestock sector contributes 3% GDP to the government of Uganda (MAAIF, 2011). Pig farming provides improved livelihood to the farmers mainly in rural areas since the costs incurred are not high. The pigs are easily sold off by farmers to cater for school fees, medical bills and other family requirements (Ouma *et al.*,2014). The pig feces are used as manure to improve soil fertility; further improving agriculture which is the backbone of this country. The breeds of pigs reared are dependent on the farmers' perceptions and include, land race, yorkshire, large white, cambrough (exotics), cross breed (hybrid) and local breed (indigenous) (Tatwangire, 2013). The exotic and cross breeds mature fast. On the contrary, the local breeds are assumed to be disease resistant and easier to manage since minimal attention is required.

Uganda generally has three pig management systems which are; intensive, semi-intensive and extensive systems (Tatwangire, 2013). The intensive pig system involves housing the pigs in pens and providing them with feeds and water (Mutetikka, 2009; Pezo & Waiswa, 2012; Ouma *et al.*, 2013; Dione *et al.,* 2014). These enclosed houses are constructed depending on the availability of resources as raised or flat cemented floor. In the semi-intensive system pigs are housed but occasionally left to roam outside posing a risk to viral infection (Mutetikka, 2009). Finally, in the extensive system the pigs are left to roam around the area looking for food and water, with little effort required for management hence such pigs are at a very high risk of viral infection.

Farmers in the rural area mainly practice the extensive system since the majority have limited resources for developing structure and maintaining the pigs (Dione *et al.,* 2014). Although the extensive system is inexpensive, other farmers complain of crop destruction by roaming pigs (Pezo, D. & Waiswa, 2012). Most of the peri-urban and urban pig farmers construct pig shelters using timber, concrete, galvanized iron and partition the herd for easy management (Tatwangire, 2013). In the rural areas however, majority of farmers construct pig shelters using local materials like mud, bamboo, wood, grass and banana leaves which are less durable and expensive since these materials need to be replaced with time.

2.2 African swine fever

African swine fever is a haemorrhagic viral disease in pigs which is responsible for high mortality rates thus affecting the pig industry in Sub-Saharan Africa (Dixon & Takamatsu, 2012). The disease was first described in Kenya in 1920's, and ever since then, it has spread to other parts of the world (Montgomery, 1921).

2.2.1 Aetiology

The causative agent of ASF is a double stranded, icosahedral DNA virus (Dixon *et al.,* 2000). This disease was first described by Montgomery 1921 in Kenya and there are 22 known genotypes (I-XXII) of ASFV circulating world-wide (Boshoff *et* al., 2007 and Bastos *et al.,* 2003). Although some studies suggest both genotypes IX and X to be present in Uganda, most recent studies indicate genotype IX is more prevalent (Atuhaire *et al.,* 2014).

2.2.2. Viral Structure and Replication

The ASFV is organized in complex multi-layer structures consisting of an 80 nm core structure, a 30nm nucleiod, surrounded by a 50nm lipid layer and protein icosahedral capsid (Carrascosa *et al.*, 1985). The diameter is estimated to be about 170-190nm and has an estimated size of 175-215nm. The Viral DNA genome is about 170-193kbp in length. The variations in length are due to losses or gain of members of multi-gene families which include 100, 110, 300, 360, 505/530 and p22 (Dixon *et al.*, 2012).The external envelope, although not necessary for infectivity, contains CD2v (EP402R), the only glycoprotein involved in virus haemadsorption (Ruiz-Gonzalvo *et al.,* 1996; Rowlands *et al.,* 2009) . Viral entry into the porcine host mainly is through the tonsils to the closest lymph nodes, blood where it is carried to tissue organs. The pig may be bitten by infected ticks during feeding and introduce the virus directly into the blood stream of the pig. Upon entry into the soft tick, the virus replicates in different cell types, and in the mononuclear phagocytic system of pigs and bush pigs (Carriloo *et al.,* 1994; Karalyan et al., 2012). Viral entry into the host cell is through receptor–mediated endocytosis although recent studies suggest that viral entry may be through clathrin dependent endocytosis and micropinocytosis (Sánchez *et al.,* 2012; Alonso *et al.,* 2013). The major proteins involved in macropinocytosis are capsid protein p72, p54 and p12. These proteins are responsible for binding and entry into the cytoplasm (Etter *et al.,* 2011). Although immature viral DNA is observed in the nucleus and mature viral DNA in the cytoplasm, both DNAs are responsible for the mature cross-linked viral DNA present in infected cells (Dixon *et al.,* 2012). The early phase of viral replication occurs in the nucleus with peak DNA replication occurring for the first six hours and then decreases to zero in the next 12hours (Dixon *et al.,* 2012). Viral assembly takes place in the cytoplasm, since the cytoplasm contains viral structural proteins, viral DNA and many membranous

materials required for ASFV assembly (Rojo *et al.,* 1999). Attachment proteins p12 and p24 are found on the external membrane of the extracellular particles, while p150, p37, p34 and p14 proteins are localized in the virus core (Alcami *et al.,* 1990; Suárez, Salas, & Rodríguez, 2010). The Vp72 protein (B646L gene) is the main component of the viral capsid while p54 (E183L gene) is the most important integral membrane protein and both proteins are used in virus genotyping (Cobbold&Wileman, 1998). The inner viral envelope is very complex and contains many viral proteins with trans-membrane domains (Sun *et al.,* 1996). The ASFV particles contain different enzymes for replication, mRNA polyadenylation, methylation and capping. Clumping of DNA polymerase to DNA is facilitated by DNA polymerase type B and protein E301R. The C962R gene may be important for DNA repair at the replication fork, although more studies should be undertaken to verify this (Dixon *et al.,* 2012). Synthesis of mRNA in the cytoplasm is possible without host RNA polymerase II, because the virus possesses all enzymes and factors required for transcription and translation process.

2.2.3 Pathology

Pigs infected with ASFV show varying forms of the disease; peracute, acute, subacute and chronic forms. The variations in the forms of the disease are associated with virulence of the viral strain and immunological status of the pigs (Wilkinson, 2000). The peracute form is linked with thrombocytopenia mainly in the stomach resulting in prolonged bleeding and death of the pig. Acute and subacute forms of the disease are characterized with severe vascular changes, haemorrhage in different organs, mucosal nasal discharge and nasal haemorrhage, renal petechiae, diffuse haemorrhage in the lymph nodes and erythema. Erythema is easily seen in the white pigs. Oedema of the gall bladder, perirenal oedema and pulmonary oedema is evident in infected pigs.

2.2.4 Clinical Signs

The appearance of symptoms in pigs depends on the incubation period of the virus, the viral genotype, animal breed, environment, pig production system and viral load in the pig (Jori & Bastos, 2009). There are a number of clinical symptoms observed amongst which include; the typical and acute forms that affect the lympho-reticular endothelial cells, high fever, anorexia, dullness, reddened skin particularly on the ears, lower legs and ventral abdomen (mainly observed in exotic pigs), abdominal pain, nasal discharge, vomiting, constipation, bloody diarrhea and abortion in pregnant sows. Pigs that present with these signs normally die between three to 14 days after infection (Mebus, 1988). Unfortunately, pigs that usually recover from this state become carriers of the virus and spread the virus to naïve pigs through contaminated faeces, fluids and exhalation. Sub-acute forms of the disease caused by less virulent strains of the virus display with wavering fever, swollen and painful joints, cardiac damage, moist cough and difficulty in breathing(Costard *et al.*, 2012). Subsequently, these pigs continue to exist for weeks to several months, with their role in disease transmission not fully investigated. The chronic form of the disease may be seen in survivors and is characterized by emaciation, stunted growth, and haemorrhagic necrosis of the skin, bony protuberances and deep ulceration.

2.2.5 Epidemiology of ASF

The transmission cycle of ASF Mainly occurs in three cycles: the sylvatic, tick and pig –pig cycle, with few studies showing the existence of transmission from the wild life to the domestic pigs in Africa mainly in Uganda (Jori *et al.*, 2013).

2.2.5.1 Sylvatic cycle

Warthogs, possibly bush pigs and forest hogs are known vertebrate hosts of ASFV. The virus is transmitted to the ticks as they feed on these vertebrate hosts. Eventually, the infected ticks transmit the virus through bites to naïve warthogs and pigs. The ASFV replicates in the bush pigs, although their involvement in viral transmission to the pigs and soft ticks is not well understood (Jori & Bastos, 2009). A recent study by Ståhl *et al.*,(2014) gives an insight of the probable involvement of bush pigs in the transmission of ASFV to pigs and warthogs. Studies on bush pigs are limited since bush pigs are not easily monitored as a result of their few numbers and their nocturnal nature.

2.2.5.2 Tick-pig cycle

The soft ticks are reservoirs and the only known arthropod vectors for the ASFV. The ASFV replicates in the midgut and spreads to the coxal and salivary gland in the ticks. The virus is then transmitted to the pigs as the ticks feed (Kleibeker *et al.,* 1999; Ticks *et al.,* 2007). Notably, these ticks can remain infectious for about 15months without any blood meal and some studies have detected presence of the virus in soft ticks found in pig pens emptied four years ago (Ravaomanana *et al.,* 2010).

2.2.5.3 Pig to pig cycle

This cycle sometimes is referred to as the indirect transmission of ASFV in pigs. The virus is spread to pigs through contaminated feeds, fomites, vehicles, clothes and poor farming systems (Penrith, 2009). Transmission of ASFV by aerosol may occur since the virus normally occurs during the dry season. The contaminated aerosol may facilitate the spread of the virus between herds that are near each other. The ASFV can remain viable in pig secretions, products, bi-products and decayed blood

for about 11 days which may aid in the viral spread (Penrith, 2009). Outbreaks in Tanzania were previously linked to movement of infected pigs and pork products (Misinzo *et al.*, 2009),which factors were responsible for disease outbreaks in Uganda (Muhangi *et al.*, 2014). Important to note is the presence of ASFV without presence of a sylvatic cycle, mainly in West Africa and some parts of Uganda (Jori *et al.*, 2013).The pig cycle could be the main attribute of ASFV transmission in Uganda, although pigs grazing close to forests are at a high risk of infection because wildlife and ticks colonize them when looking for food (Björnheden, 2011).

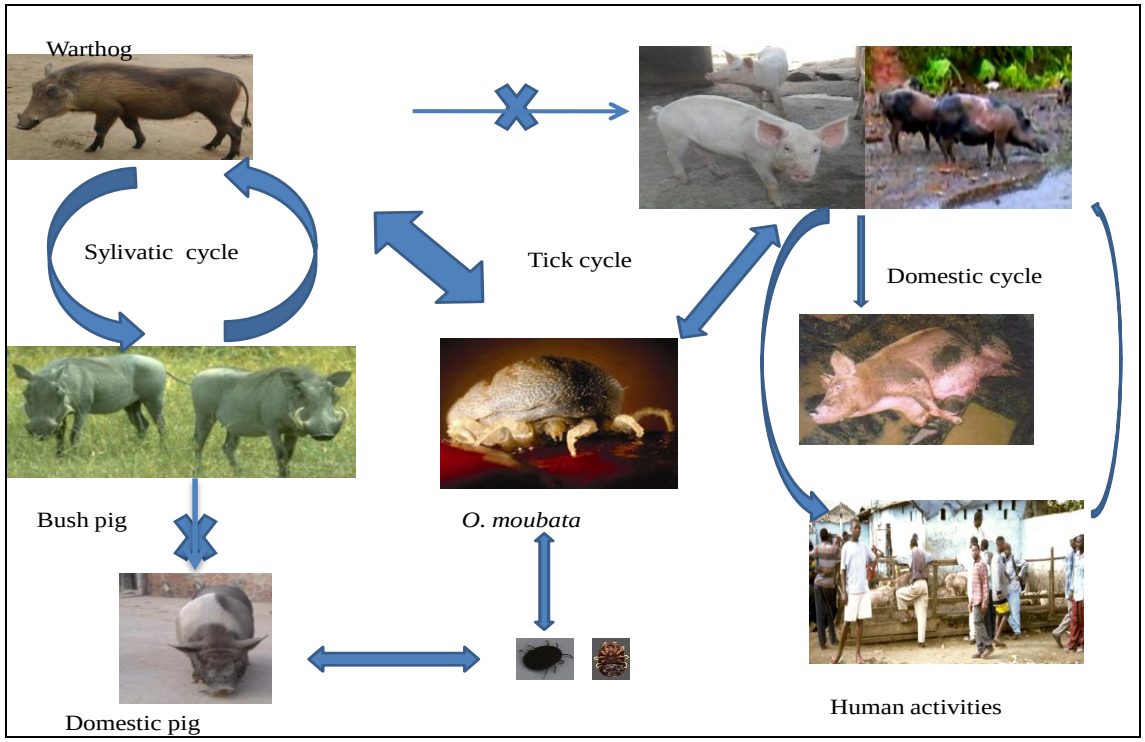

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Figure 1; Transmission cycles of the African swine fever virus

2.3 Burden of ASFV in Uganda

ASF is endemic in the country with over 300 outbreaks reported between 2001 and 2012 (Atuhaire *et al.*, 2013) which were responsible for several pig deaths hence affecting the farmers and economy at large. There are continuous rampant outbreaks in Uganda with a number of serological and molecular epidemiology studies on ASFV undertaken (Atuhaire et al., 2013). In Uganda, a study carried out in Mubende showed a 0.2% seroprevalence at slaughter slabs (Muwonge*et al.,* 2012) and in Gulu a 50% presence of ASFV was observed (Tejler, 2012) during outbreak investigations. However, in Busia there was no presence of ASFV antibodies using the OIE serological prescribed tests during the 2007 outbreak since the virus has no neutralizing antibodies (Gallardo *et al.*, 2011).

2.4 The ASFV Genome and Genotyping

The ASFV genome is encoded by five multigene families and about 50 polypeptides. Structural or function proprieties were identified in 113 proteins after complete sequencing of one Spanish isolate (Yanez *et al.,* 1995). The presence /absence of some of these proteins in some viral isolates are responsible for the heterogeneity in ASFV. Classification and understanding the virus isolates can be determined through genetic studies. The PCR-sequencing method used for genetic studies provides accurate explanation of major genotype nucleotide sequences of the viral genome (Bastos *et al.,* 2003). In depth genetic characterization of the central variable region (CVR) of the 9RL open reading frame (ORF) shows intra-genotypic relation of the various isolates (Bastos *et al.,* 2004). The molecular epidemiology of ASF in East African showed sixteen vp72 genotypes existed after phylogenetic analysis of the conserved 404bp region of the C-terminal end of the vp72 gene (Lubisi *et al.,* 2005). Phylogenetic analysis of four variable regions of 41 isolates of the vp72 genotype

produced 16 new sub-groups (Nix *et al.,* 2006). Phylogenetic analysis of the CRV which varies in size from 300-500bp and has a 132-bp direct repeat, analyses the number and composition of tandem tetramers in a given isolate (Irusta *et al.,* 1996). Combined vp72 and CVR phylogenetic analysis permits intra genotyping of ASFV isolates in pigs. Molecular characterization of the p72, p54 and the CRV of the identified viral isolates showed genotype IX to be circulating in Uganda (Gallardo *et al.,* 2011; Atuhaire *et al.,* 2013). The genotype IX is present both in pigs and wild suids in areas around national parks, forests, neighboring communities and areas far away from wildlife (Jori *et al.,* 2013). However, regions far away from these national parks complain of pig deaths due to ASFV, therefore it is important to identify other factors linked to disease transmission apart from the ticks and wild suids which are known to be reservoirs.

2.5 Laboratory diagnosis of ASFV

Isolation and diagnosis of ASFV is possible using various techniques; both serological and molecular methods as recommended by OIE, 2008.

2.5.1 Haemadsorption virus isolation (HAD)

The test involves the adherence of pig erythrocytes to surface infected pig monocytes or macrophages with ASFV (Malmquist& Hay 1960). This test is suitable for only virus adsorbing isolates and not the non-adsorbing isolates that lack the CD2v protein responsible for absorbing red blood cells to infected virus cell. The suspected blood or tissue suspension is cultured in primary porcine bone marrow (PBM) cells, primary leukocyte cultures or into alveolar macrophage cells as described by Malmquist& Hay (1960). Although the test is sensitive, it is laborious, time consuming and not

suitable for ‘non- haemadsorbing’ ASFV (OIE, 2008). Therefore, the test is normally performed as a confirmatory test on positive samples from ELISA and PCR.

2.5.2 Detection of antibodies against ASFV

There are a variety of tests available for the detection of antibodies against ASFV. These tests are able to detect antibodies; produced immediately after infection and also antibodies in pigs that have recovered normally referred to as “apparently healthy”. These apparently healthy pigs are normally infected with less or non-virulent isolates and positive pigs are easily detected through serological tests (OIE, 2012). Antibody ELISAs detect IgM for recent infection and IgG in chronically infected or apparently healthy pigs (Reis *et al.,* 2007). These antibodies are present and detected in serum and fluids from tissues. The commonly used ELISA techniques are immunofluorescence antibody test and immunoblotting. Immunofluorescence involves the use of cell cultures or tissue sections from infected pigs stained and viewed under a light microscope (OIE, 2012). Immunoblotting is able to detect weak positive samples since viral proteins that induce specific antibodies are placed on antigen strips. The ELISAs are more effective than HAD because both virulent and avirulent strains can be detected using non-infectious soluble antigens (Wardley *et al.,* 1979). Pigs infected with virulent ASFV normally do not produce antibodies but kill the pigs within a short time before an immune response is activated. Therefore these tests are performed in combination with tests that detect viral presence in the infected pigs.

2.5.3 Detection of viral particle

The virus in body fluid mainly in blood can be detected after DNA extraction. A wide range of isolates, both haemadsorbing and non haemadsorbing can be amplified. There are various viral detection techniques like: polymerization chain reaction (PCR), Real-time PCR, and the loop

amplification mediated polymorphism (LAMP) (OIE, 2008). These techniques involve the use of species specific primer sets to amplify a conserved region of the ASFV genome, thermal stable enzyme, dNTPs, Magnesium chloride, distilled water and the extracted DNA template. The LAMP method is very sensitive, specific and requires a single temperature for the reaction as compared to the other amplification method with pre-set condition reactions at different temperatures (Hjertner *et al.,* 2005). This makes LAMP cheaper and ideal for viral detection in developing countries and in areas where the disease is endemic and a single temperature required for amplification (Notomi *et al.,* 2000). The Realtime PCR is more sensitive than the other techniques because very small quantities of viral DNA in a sample can be detected. Realtime PCR requires less time to attain results as compared to the others. In addition both qualitative and quantitative results are determined on a computer, thus no need of running a gel (Gallardo *et al.,* 2012). On the other hand, conventional PCR is advantageous in that viral DNA can be amplified, size of the genotype determined and the purified products sent for sequencing (Aguero *et al.,* 2003; Steiger *et al.,* 1992; Bastos *et al.,* 2003).

2.6 Risk factors for African swine fever transmission in Uganda

The pig farmers, pig traders and family members normally do not adhere to biosecurity methods of decontamination of personal protective wear with a disinfectant before and after visiting the farm hence a risk for the disease transmission. The pig traders/middle men move from farm to farm looking for pigs to sale; and transport these pigs on motorcycles and lorries without decontamination (Aliro *et al.,*2012) . The pigs are sold very fast during or when farmers suspect disease oubreak (Muwonge *et al.,* 2012: Muhangi *et al.,* 2014). They are sold cheaply or slaugherted immediately without inspection. At the slaughter slab, there are insufficient or no facilities for waste disposal of blood, offals and used water. Therefore, the pork obtained may be contaminated and is sold in the butcheries

or roasted/fried by the road sides, restaurants and bars (Costard *et al.*, 2012). The risk factors for disease transmission need to be investigated, understood and communicated to farmers, stakeholders and policy markers to contain and prevent disease outbreaks in the country.

2.7 ASF control measures

ASF being endemic in Southern and East Africa has proved difficult to eradicate. Most of the farmers in the pig sector are subsistence farmers with limited finances available to invest on sanitary biosafety measures; hence they expose themselves to high risks of infection (Tatwangire A., 2013). These risks predispose farmers to ASFV outbreaks and cause enormous losses in the house-hold and country at large. The lack of restriction of live animal movement within a country, transboundary and transcontinental, free-ranging and farm visits, facilitates disease spread (Costard *et al.*, 2009). Training of farmers, veterinary and livestock producers on cause and transmission of disease creates disease awareness; thus controlling the spread (OIE, 2010). Regular spraying with acaricides on tick infested herds or complete abandonment of previously infested places prevents survival and breeding ground for ticks that may harbor the virus (Costard *et al.*, 2012). In areas with frequent outbreaks contact of infected faeces with other animals, birds, pigs, chicken and humans can result in transmission to healthy pigs, therefore proper disposal of this waste prevents viral spread since the virus remains viable for 60 days in the excreta (Penrith, 2009). Quarantine for a minimum of 30 days is important in addition to cleaning, disinfecting clothes, foot wear and isolation units since animals excreta are regarded infectious (Penrith, 2009). Restaurant waste and kitchen scraps that provide high energy and protein as growth requirements for pigs, are heated to 100ºC for 1 hour to destroy the virus before feeding the pigs (OIE, 2010). Improperly buried infected carcasses, when exhumed are potential sources of infection. The control strategy for ASFV transmission largely relies on early

rapid disease diagnosis, implementing strict biosecurity measures and good communication among all parties involved during and after outbreaks (Oura *et al.,* 2013).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study area

A cross-sectional survey was conducted from April to August 2013 in the districts of Masaka, Mukono and Kamuli. These districts were selected based on Step 1: Geographical targeting using GIS characterization and spatial analysis to select potential sites, pig population density of >20 heads/Km^2 and poverty (people living on <1.25$/day) levels of >50%. The population density in Masaka and Mukono was > 50 head/ Km^2 while Kamuli was between 5-10 head/ Km^2. All the above districts had a 60-70% population of people living on <1.25$/day.

3.3 Study sites

The study sub-counties and villages were selected using pig population data at sub-county level from the Livestock Census data of 2008. For each district, 4-6 sub-counties were purposively selected basing on pig population density. Within each selected sub-county, two to three villages were randomly selected. In this study, 23 villages out of the 35 were selected purposively across the three districts (Table 1). The number and choice of villages was based on financial resources availability and other activities taking place in the same villages, to minimise farmer fatigue (Ouma, *et al.*, 2014).

Table 1; Districts, sub counties and villages visited

District	Sub-county	Village
Mukono	Kkingo	Kisoso
		Ssenya
	Kimanya-kyabakuza	Kijjabwemi
		Kyabakuza
	Katwe-Butego	Butego
		Kyamuyimbwa
	Nyendo-Ssenyange	Ssenyange A
	Kabonera	Kikalala
	Kyanamukaka	Kanoni-Bukunda
		Lukindu
Kamuli	Bugabula	Baluboinewa
	Butansi	Bukyonza
	Kitayunjwa	Ntansi
	Namwendwa	Isingo A
		IsingoB
Mukono	Goma	Misindye
	Mukono TC	Kitete
		Joggo
	Kyampisi	Ddundu
		Kyoga
	Ntenjeru	Bugoye/Kabira
		Kazo/Kalagala
		Nsanja/Gonve

The map of Uganda with the study sites in different colours

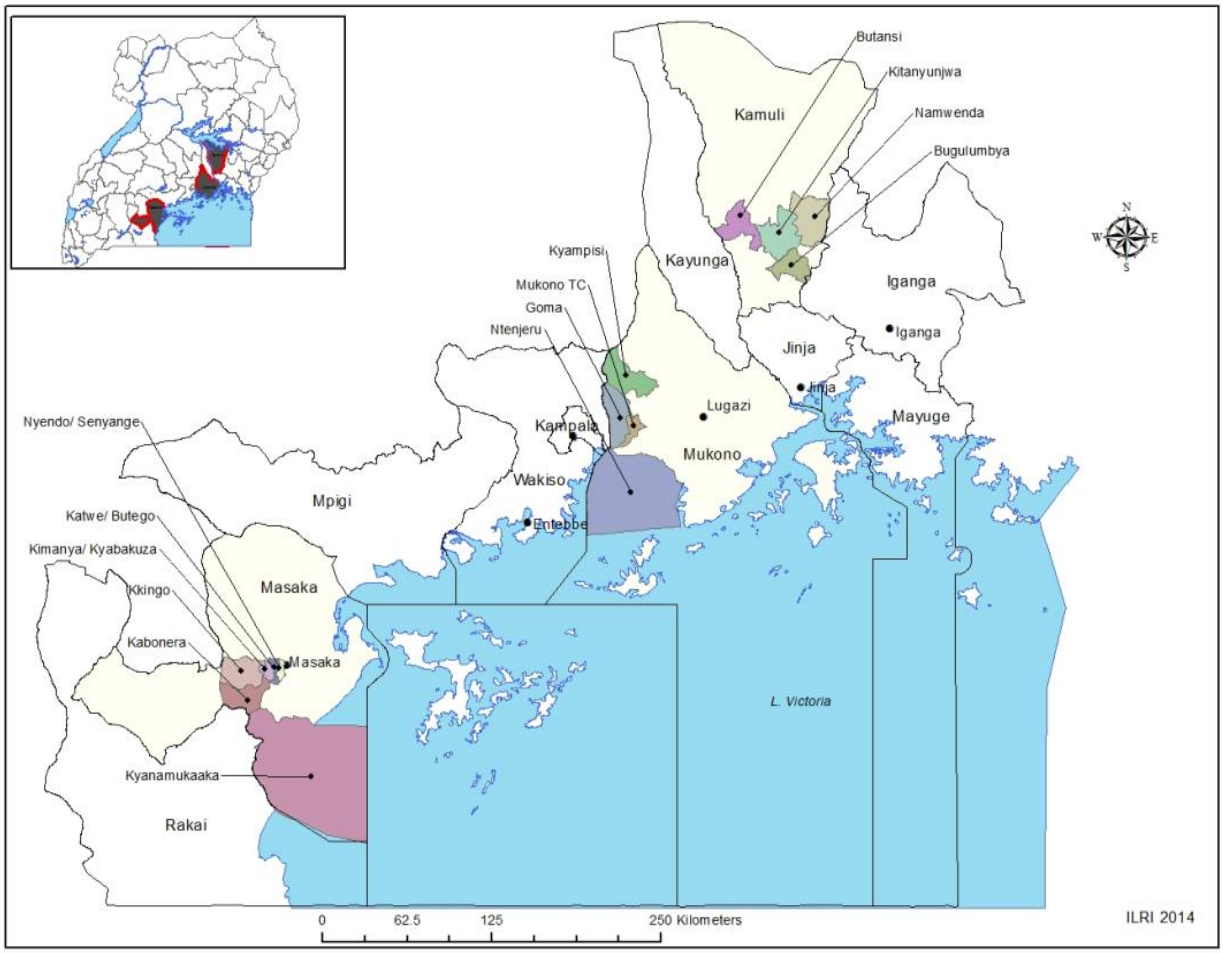

Figure 2 Map of Uganda showing study sites, with districts and Sub-counties

3.4 Sample size determination

The sample size was calculated using the formula adopted from Thrusfield (2007) as follows: $n = [Z^2P(1-P)]/d^2$ Where: n is the required sample size; Z is the multiplier from a standard normal distribution (1.96) at a probability level of 0.05; P is the estimated prevalence which is most conservatively estimated to be 50% considering that there is no reliable prevalence data for African swine fever per district and d is the desired precision for the estimate (+/- 5%). A sample size of 384

pigs was required for the study in each district. A total of 375, 408, and 402 pigs were sampled in Masaka, Kamuli and Mukono, respectively. The actual number of pigs sampled in Masaka was lower than the required sample size because of the harsh working conditions experienced due to heavy rain on some days. More pigs in Kamuli and Mukono were sampled because they were available.

3.5 House-hold and pig selection

A list of all pig keeping house-holds was generated in each village. The study house-holds were then randomly selected using computer-generated random numbers. The inclusion criteria were as follows: pigs older than three months since we assumed they were exposed, not weak or emaciated, not a pregnant sow or a sow with a litter under two months old to were not considered to avoid any losses of pigs and at less than 2 months, it was difficult to collect the blood sample. In each house-hold, one pig fulfilling the inclusion criteria was randomly selected for blood collection. A pig bio-data form and a structured questionnaire were administered to the owner of the pigs.

3.6 Sample collection

The farmers were first briefed on the background of the study before signing the consent forms. One pig randomly selected from each farm was restrained using a snare. Subsequently, whole blood was collected from the jugular vein of the pig using sterile vacutainer needles into sterile vacutainer tubes. The plain vacutainers were used to obtain serum after centrifugation at 3000rpm for 10 minutes and used for ASFV antibody detection. Whole blood was collected in EDTA-coated vacutainers and used for PCR. These tubes were labeled immediately with the farmer (identity) ID; the district and house-hold number e.g. M001. The tubes then were put in a cold box before transportation to the laboratory for analysis. Both whole blood and serum were put into two separate cryovials and stored at -80ºC

until analyses. At the farm, biosecurity measures like: disinfection of all personnel, materials used, gumboots and vehicles with 70% Virkon diluted in a pump sprayer was done before movement to the next farm to prevent virus spread.

3.7 ASF DNA detection, characterization and antibody detection

This study used both antibody and viral DNA detection methods simultaneously as recommended by the OIE for diagnosis of ASF. Antibody analysis on the harvested serum and DNA extraction from whole blood was performed at the Molecular Genetics Laboratory at Makerere University, College of Agriculture and Environmental Sciences. Molecular detection of the virus on the extracted DNA samples was performed using UPL Realtime PCR at the International Livestock Research Institute (ILRI) Nairobi.

3.7.1 The ASFV antibody detection

Blood collected in the plain vacutainer tubes was centrifuged at 3000 rmp for 10 minutes to separate the blood clot from serum. The supernatant (serum) was then harvested into two cryovials and stored at -80ºC. A commercial blocking enzymatic immunoassay kit from Ingenasa (Ingezim 11.PPA.K3, ingenasa, Madrid, Spain) was used for ASFV antibody detection. This test is very sensitive and specific (OIE, 2008). The wells in the plates were pre-coated with a purified protein extract from the virus (VP73). Protein VP73 is a major structural protein from the ASFV which is the most antigenic and suitable for diagnosis (OIE,2008). Before use, the serum stored at -80ºC was left to thaw to room temperature together with the ELISA kit reagents (INGEZIM COMPAC 11.PPA.K3) except the enzyme conjugate. All reagents used in this process were purchased with the kit, except for absolute alcohol used to dilute some reagents. Both samples and controls were diluted directly in the 96 well

plates in 1:1(50µl of sample serum+50µl of diluent) with the diluent supplied in the kit. The plates were then sealed and incubated at 37ºC for 1 hour. The plate contents were later emptied and washed. The plates were then washed 4 times by pipetting 300µl of washing solution (supplied in the kit) to each well. The washing solution used was diluted according to the manufacturer's protocol, and after the last wash, the plate was turned over on an absorbent paper. Specific conjugate of 100µl was prepared according to the manufacturer's protocol and added to each well, sealed and incubated for 30 minutes at 37ºC. After incubation, the plates were once more washed 5 times using the wash solution. Then 100µl of substrate was added to each well and the plate was left at room temperature for 15 minutes. Finally, 100µl of stop solution was added to each well. The optical density (OD) value was read at 450nm using an ELISA reader (Multiskan EX, Vantaa Finland) with the help of Ascent software (www.ascent.software.html). The negative and positive cut off values were obtained from both the mean OD values of the negative (NC) and positive (PC) controls. The test was valid if the OD of the NC was at least 4 times higher than the OD of the PC according to the manufacturer's protocol:

$$\frac{\text{OD NC}}{\text{OD PC}} = \geq 4$$

The calculation of the cut off was important in the classification of samples as negative, ambiguous and positive. The cut off value was calculated using the equation:

Positive Cut Off= NC-[(NC-PC X0.5]

Negative Cut Off=NC-[(NC-PC X0.4]

Interpretation of results:

Sera with optical density lower than positive Cut Off were considered as positive sera to ASFV antibodies while, sera with optical density higher than Negative Cut Off were considered as negative sera. Sera with optical density between the positive and negative Cut Off were considered as doubtful sera and was re-run to ascertain the true status of the sample.

3.7.2 Total genomic extraction

Total genomic DNA was extracted from whole blood samples. The whole blood samples were left to thaw to room temperature before extraction using the DNAeasy Blood and Tissue Purification kit (QIAGEN, Hilden, Germany). A volume of 200µl whole blood was pipetted into a 2ml eppendorf tube. The proceeding steps were performed according to the manufacturer's instructions. Later, 5µl of the DNA extract was run on a 2% agarose gel containing ethidium bromide which intercalates with DNA in an electrophoresis tank for 20 minutes. The product of electrophoresis was visualized under ultra violet light

3.7.3 Real time PCR

A commercial real time PCR kit was purchased which contained all the reagents required to detect presence of ASFV from the extracted DNA samples. The kit that was used constituted: Universal Probe Library (UPL#162), specifically designed primer set ASF-VP72 F1 (CCCAGGRGATAAAATGACTG) and ASF-VP72-R-1(CACTRGTTCCCTCCACCGATA) to detect a conserved sequence of ASFV DNA, and Taqman polymerase enzyme. The UPL ASFV Real time PCR master mix was constituted according to the manufacture's protocol as shown in table 2 below. A volume of 22.5µl of the master mix was drawn into each SmartCycler tube (www.cepheid.com). Afterward, 2.5µl of the extracted DNA from each sample was added to make a

25µl volume for real-time PCR. Besides this, positive and negative controls were set before running the samples in an automated real-time SmartCycler (Cepheid Inc., Sunnyvale, California). The programme was run according to the set conditions; activation of DNA polymerase at 95ºC for 3minutes, DNA denaturation at 95ºC for 10 seconds, primer annealing and elongation at 58ºC for 30seconds. The SmartCycler was set for 45 cycles with a fluorescence collection in the FAM channel at the end of each cycle.

Table 2; Shows the Real time PCR reaction mix

Master mix reagents	Volume 1x(Reaction 25µl)	Reactions N	Final Concentration
PCR water	7.75µl		
Master mix 2x	12.5µl		1X
VP72-F 20µM	1µl		0.8µM
VP72-R 20µM	1µl		0.8µM
Taqman probe10µM	0.25µl		0.25Mm
Master mix volume	22.5µl		
2.5µl of template DNA was added to each smart cycler tube			

3.7.2.3 Genotyping of ASFV

Conventional PCR was performed on the single sample that was positive in the real-time PCR. This PCR reaction constituted primers amplifying different regions of the viral DNA as shown in table 3 as previously defined (Bastos *et al.*, 2003). Before the amplification process, 2μl of the DNA template was added to 23μl of prepared PCR master mix in a 0.2ml reaction tube to make 25μl as previously descried by (Bastos *et al.*, 2003). The master mix contained 16.375 μl ddH_2O, 2.5μl of PCR buffer 10x, 2.5μl of $Mgcl_2$ 25Mm, 0.5μl dNTP's10Mm, 0.5μl primer forward and primer reverse for each set, 0.125μl of Taq Gold 5U/μl and 2 μl of template DNA for a single reaction. The reaction was run as follows; activation of Taq Gold DNA polymerase for 10 min at 95ºC, DNA denaturation for 30

seconds at 95ºC, annealing of primer at 52ºC for 1 minute and elongation for 1 minute at 72ºC for 35 cycles and one final cycle of elongation for 10 minute at 72ºC and hold at 4ºC. The PCR products were then run on a 2% agarose gel using a 1 kb lambda DNA/ EcoRI + Hind III marker and visualized under UV light to obtain a 478bp, 676bp and 400-600bp region (Bastos *et al.*, 2003) .

Table 3; Sets of primer sequences used for base pair amplification of ASFV DNA

Primer	**Sequence**	**Expected amplicon (bp)**
p72-U p72-D	5'-GGCACAAGTTCGGACATGT-3' 5'GTACTGTAACGCAGCACAG3'	478
PPA89 PPA722	5'TGTAATTTCATTGCGCCACAAC3' 5'CGAAGTGCATGTAATAAACGTC3'	676
CVR1 CVR2	5'ACTTTGAAACAGGAAACWAATGATG3' 5'ATATTTTGTAATATGTGGGCTGCTG3'	400-600

3.7.5 Purification of PCR Products

The PCR products were purified to remove excess oligonucleotides primers, dNTPs and enzymes. The process involved addition of 5 volumes of Buffered PBS to 1 volume of PCR sample. The mixture was then placed in a 2ml QIA quick spin column provided in the kit and centrifuged for I minute to bind the DNA in sample to the column. The flow-through was discarded and the QIA quick column was placed back into the same tube. Buffer PE 750µl was added to the QIA quick column and centrifuged for 1 minute to wash DNA bound on the column. The flow through was discarded and the QIA quick column placed in a clean 1.5ml microcentrifuge tube. The bound DNA was eluted after addition of 50µl of elution buffer to the center of the QIA quick membrane and centrifugation for 1 minute. The purified PCR product was analyzed on a 2% gel, where 1 volume of loading dye

was added to 5 volumes of purified DNA. This solution was mixed by pipetting up and down before loading the gel and then electrophoresis was run.

3.7.6 Sequencing of purified PCR Products

The purified PCR products from the gel were sent for sequencing to determine the base sequence arrangement of the p72 and p54 coding regions of the amplified ASFVs' genome. Specific sets of primers both forward and reverse targeting the p72 and p54 regions were used for amplification, dNTPs, ddNTPs and DNA polymerase. The cycle sequencing involved the use of the Big Dye Terminator version 3.1 kit (Applied Bio system) which later was run on an automated DNA sequencer (ABI Prism® 3700) by Macrogen in the United Kingdom.

3.7.7 Analysis of sequences

Sequences in ABI format were imported to CLC main work bench 6.8.1 for editing. The forward and reverse sequences of each region were visualized and later aligned to generate consensus sequences in Bioedit (Hall, 1999). Before production of a consensus sequence, quality control was performed which involved trimming of the first 10 bases and the last 300 bases of both the forward and reverse sequences. The trimming was done to improve on the quality of reads. Consensus sequences generated were used as query (sequences of interest) and subjected to BLAST (Basic Local Alignment Search Tool) on (http://blast.ncbi.nlm.nih.gov) to produce homologous sequences to ASFV from previous studies available in the gene bank. The default settings in the gene bank were used to compare the query sequence against the sequences in the gene bank. Similar sequences were downloaded into MEGA 6 (www.megasoftware.net) and aligned with MUSCLE in the same software as described by Tamura *et al.,* (2013).

3.7.8 Phylogenetic analysis

The current isolate together with downloaded sequences from the gene bank (http://blast.ncbi.nlm.nih.gov) were aligned using MEGA 6 .The Kimura 2-parameter model which had the lowest BIC number (Bayesian Information Criterion) was determined in MEGA 6 and used to estimate the best phylogenetic trees (Tamura *et al,* 2013).

3.8 Ethical consideration

This study was part of a large funded program implemented by the International Livestock Research Institute and permission was sought from the Uganda National Council of Science and Technology(approval is 1477, appendix 2). Permission before sample collection from owners and managers of the facilities was attained after signing the consent form. This study was approved by the College of Veterinary Medicine, Animal production and Biosecurity COVAB Higher degrees committee (VAB/REC/13/102).

CHAPTER FOUR

RESULTS

4.1 Demographic characteristics

A total of 1192 pigs were sampled in the three districts of Uganda. Majority of the respondents in the survey were male (68%) as compared to female (32%).

4.1.2 Characteristics of pig production systems

The pig production system in the three districts varied. More farmers kept the cross breed of pigs (mixed colours), while the highest percentage of the local breed pigs (black in colour) was in Mukono as shown in table 5.The extensive management system characterized by free movement of pigs was practiced in Masaka and Kamuli districts, with Masaka having the highest (81.2%) number of farmers. Regarding the feed type, most farmers fed their pigs on all feed types with exception of Mukono who never use swill, pasture and commercial feeds as shown in table 5

Table 4; Characteristics of production system

Animal characteristics	Categories	Kamuli	Masaka	Mukono
Breed type	Exotic	30	34	36
	Local	35	40	25
	Cross	27	32	41
Management system	Extensive	18.8	81.2	0
	Intensive	6.1	60.2	33.7
	Sem-intensive	52.4	11.4	36.2
Feed type	Crop residue	79.7	17.1	3.2
	Swill	85.7	14.3	0
	Pasture	92.3	7.7	0
	Combination(all above)	23.7	37.2	39.1
	Commercial	72.7	27.3	0

4.2 Antibody detection of ASF

None of the 1197samples from apparently healthy pigs screened for the presence of ASF antibodies was positive using Antibody ELISA. One sample from Mukono was doubtful (optical density of 0.98275 between the positive cut off of 0.6065 and negative cut off of 1.1514), but on subsequent analysis it was confirmed negative.

4.3 Molecular detection of ASFV

4.3.1 Detection of ASFV using Real time PCR

In this analysis, 1 out of 1192 samples was positive for ASFV. This sample was from Kamuli district, Butansi sub-county, Ntansi village. The sample had a Ct value of 25.37 while the positive control had a Ct value of 21.33.

4.3.2 Conventional amplification of ASFV

The p72, p54 and the central variable region of ASFV were amplified using different sets of primers as shown in the gel picture below figure 1.

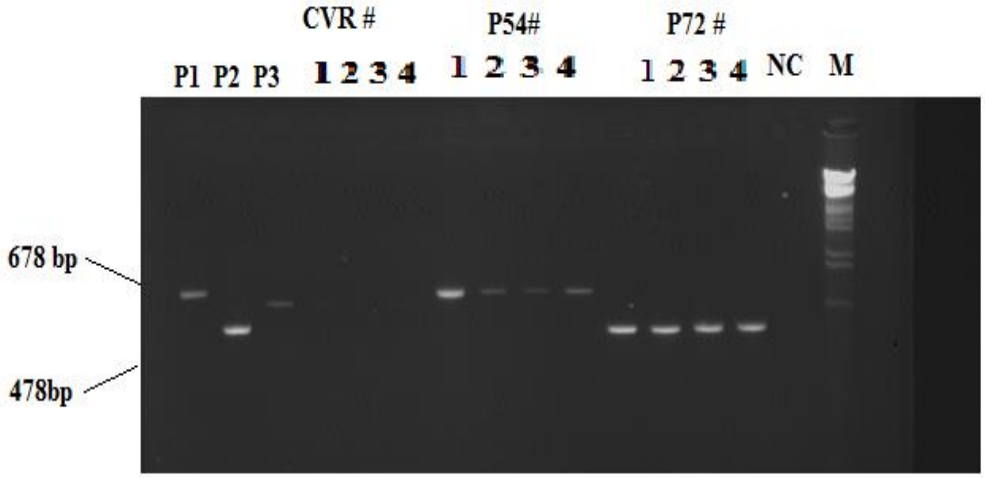

Figure 3 shows each sample ran with the primers amplifying different regions of the ASFV genome.P1-P3 is the positive control with primers amplifying different size segments; P54 (678bp) and P72 (478bp). The samples are 1,2,3,4 and NC is the negative control and M the ladder 1 Kb.

4.4 Sequencing

4.4.1 Sequence Alignment of the p72 and p54

Sequences that were downloaded using BLAST (htt://blast.st-vancbi.nl.gov/Blast.cgi) (Tamur *et al.*, 2013) were between 80-100% similarities with the query sequence and had E values between 0 and 3e-137. The query sequence was (ASF KAMULI 2013) obtained from this study as shown in table 5 below.

Table 5; Sequences downloaded from NCBI, region amplified, country of origin and genotype

Accession numbers and abbreviation	Region	Country of origin	Genotype
KC112561 Ken10/KAKFAI	p72	Kenya	IX
KC112563.1 Ken10/Kis028	p72	Kenya	IX
KC909904.1 Ug12Kampala4	p72	Uganda	IX
KC990902.1 Ug10 Amuru	p72	Uganda	IX
KC990898.1 Ug10Moyo2	p72	Uganda	IX
KC990895.1 Ug11 Mpigi	p72	Uganda	IX
KC990892.1 Ug10Kumi	p72	Uganda	IX
KC990890.1 Ug12Kabale	p72	Uganda	IX
ASF KAMULI 2013	p72	Uganda	IX
FJ154429.1 Ug03H.2	p72	Uganda	IX
FJ154433.1 Ug03P.6	p72	Uganda	IX
GQ477138.1 UG07.Wak	p72	Uganda	IX
GQ477140.1 UG07Wak3	p72	Uganda	IX
AY351549.1 MWHOG/3	p72	Georgia	X
AY351565.1 MWHOG/9	p72	Georgia	X
FJ174383.1 ug64	p72	Uganda	X
AF449472.1 BUR/90/1	p72	Burundi	X
AY538726.1 MOZ-77/98	p72	Mozambique	II
KC990889.1 Ug12 Kampala4	p54	Uganda	IX
ASF KAMULI 2013	p54	Uganda	IX
KC990886.1 Ug10 Amuru	p54	Uganda	IX
KC990882.1 Ug10 Moyo 2	p54	Uganda	IX
KC990880.1 Ug10 Tororo	p54	Uganda	IX
KC112574.1Ken11/Kaksp	p54	Kenya	IX
KC112573.1 Ken11/Thik P06	p54	Kenya	IX
KC112570.1Ken10/Kis028	p54	Kenya	IX
KC112568.1Ken10 KAKFA1	p54	Kenya	IX
GQ477149.1 UG 07 Mukono	p54	Uganda	IX
GQ477148.1 UG 07.Wak4	p54	Uganda	IX
GQ477146.1 UG07 Wak2	p54	Uganda	IX
FJ174432.1 Ug03H.1	p54	Uganda	IX
KC990888.1 Ug13.Busia2	p54	Uganda	IX
FJ174430.1 Ug64	p54	Uganda	X
JN590915.1 Ken08DP/Ndhiwa	p54	Kenya	X
JN590916.1 Ken08DP/Nyarongi	p54	Kenya	X
GQ410767.1 TAN98MAZIMBU	p54	Tanzania	XV
GQ410763.1 TAN08MABIBO	p54	Tanzania	XV

Sequence alignment of the downloaded p72 and p54 had gaps at various regions, when compared with the ASF KAMULI 2013 obtained from this study as shown in fig 4 below.

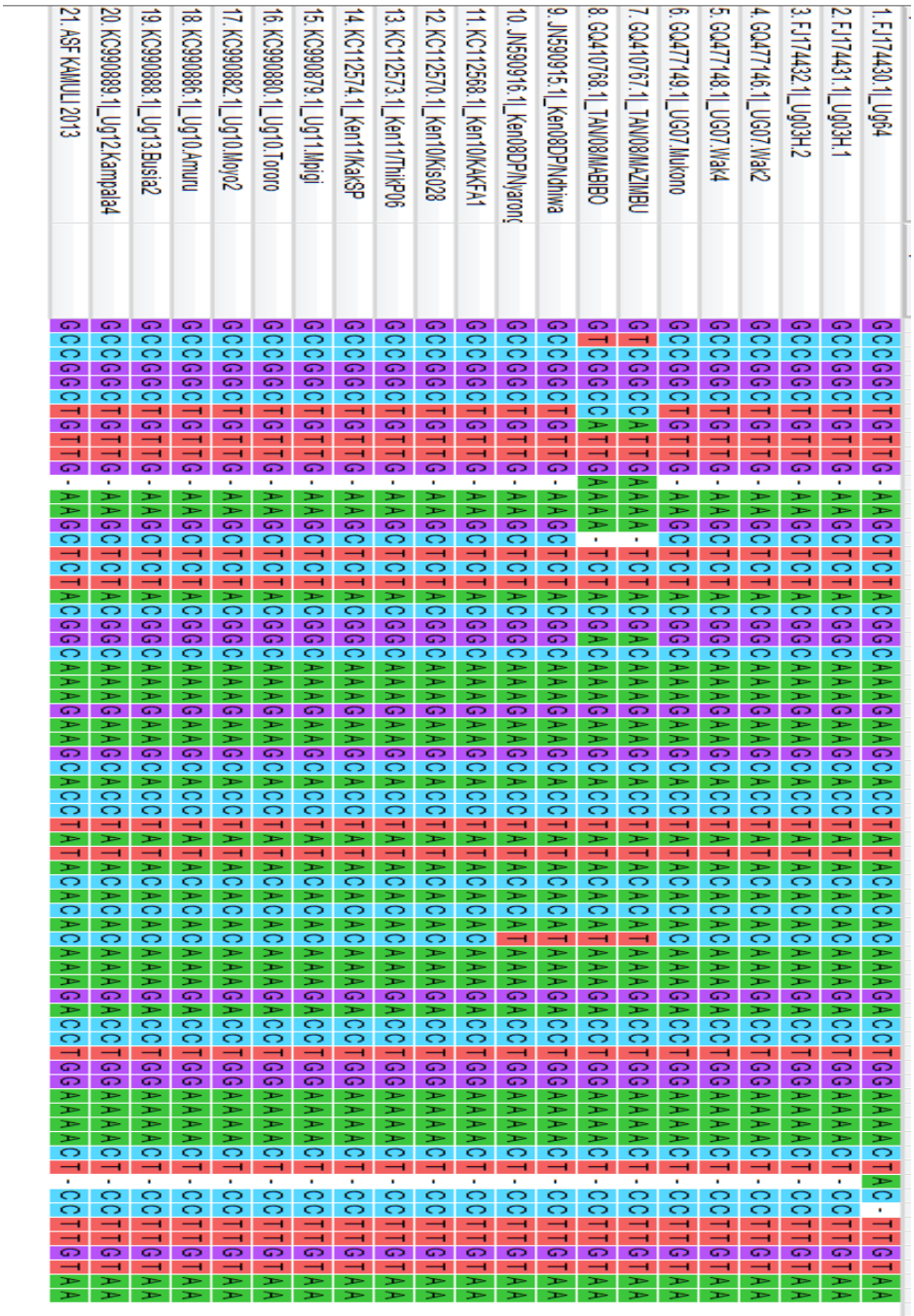

Figure 4; Sequence alignment of the P54 region of the different isolates. The gaps indicate missing bases which could be associated with mutations in those areas over time.

4.4.2 Phylogenetic analysis of the p72 and P54 regions

The downloaded p72 sequences after alignment with the query (ASF KAMULI 2013) and comparison with 14 isolate sequences representing genotype II, X and IX showed the ASF KAMULI 2013 belonged to genotype IX. The query sequence clustered with previous isolates from Uganda and some from Kenya responsible for outbreaks as seen in fig—below.

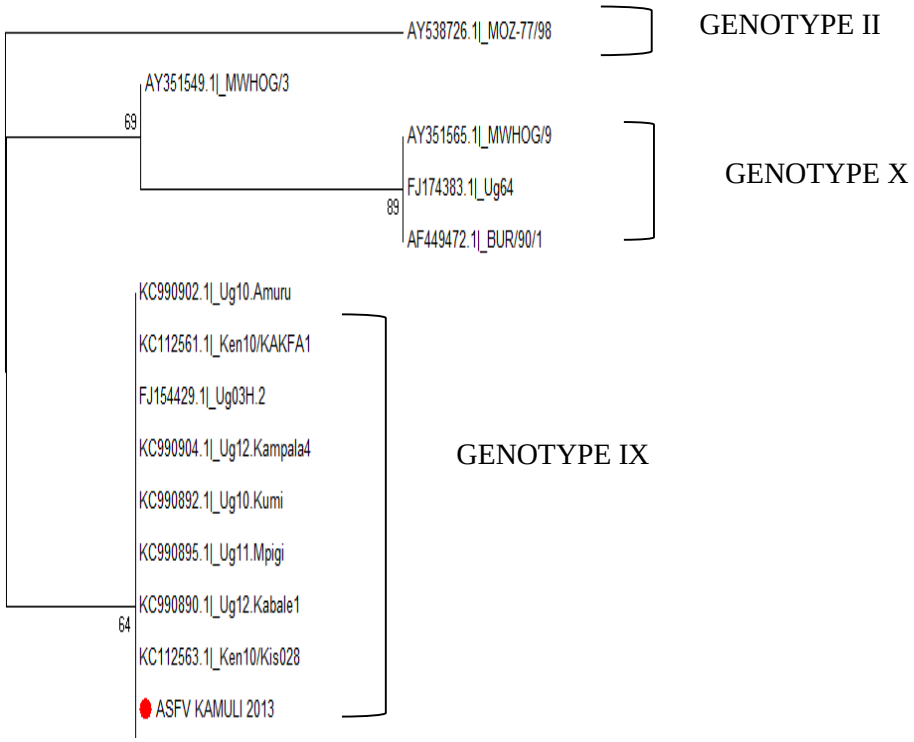

Figure 5; Phylogenetic tree base of the C-terminal end of the p72 sequences (ASF KAMULI-p-72) and other isolates. The Neighbor-joining model was used for evolutionary history; the evolutionary distances were computed using kimura 2-parameter and the bootstrap test was set at 1000 replicates. The analysis was comprised of 14 nucleotide sequence.

Sequences of the P54 also showed the ASF KAMULI

Analysis of this region is important in the molecular epidemiological studies of the various virus isolate, since more information from the sequences is captured (Bastos *et al.*, 2003). The ASF KAMULI 2013 clustered with previous Ugandan and Kenyan isolates belonging to genotype IX.

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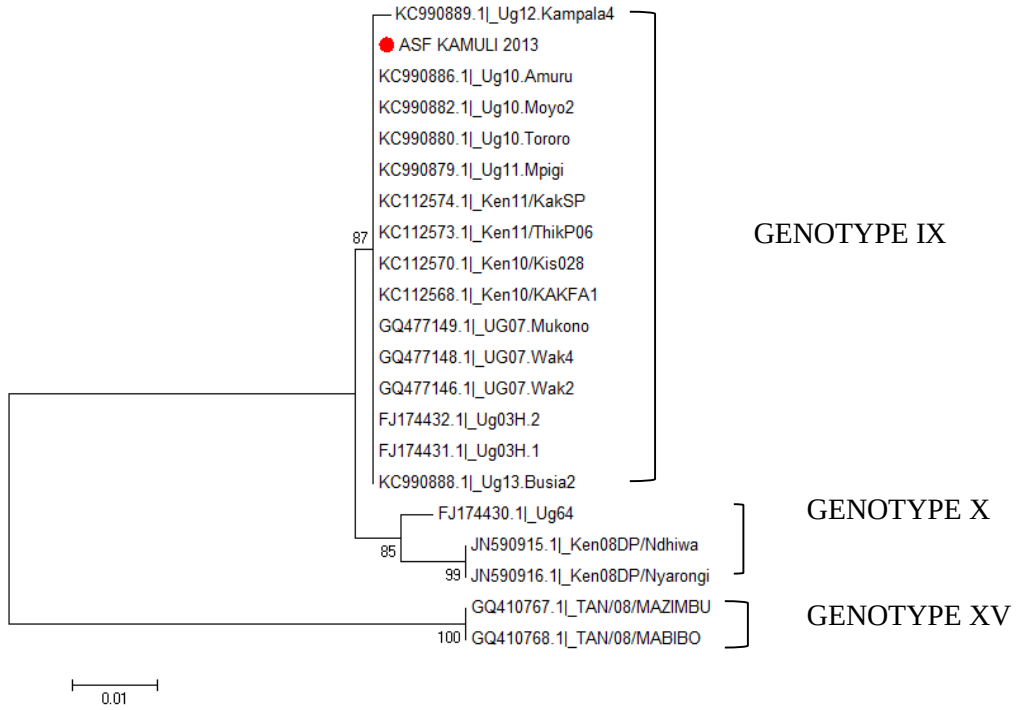

Figure 6; Phylogenetic tree base of the full length P54 protein of one isolate from this study (ASF-KAMULI 2013) with other sequences from Uganda, Kenya and Tanzania. The Neighbor-joining model was used for evolutionary history; the evolutionary distances were computed using kimura 2-parameter and the bootstrap test was set at 1000 replicates.

CHAPTER FIVE

DISCUSSION

This was a cross-sectional study carried out in the districts of Masaka, Mukono and Kamuli to determine the antibody and antigen status in apparently healthy pigs. A large sample size of 1192 blood and serum samples was collected from pigs in the different geographical locations. Only 1 sample out of 1192 was positive for ASFV using the molecular diagnostic tests.

The production systems practiced in these districts were mainly the extensive system involving tethering and free ranging highest in Kamuli. This finding is in conformity with (Dione *et al.,* 2014), where the extensive system is thought to be the cheapest since little effort in terms of time, feed and labour is required from the farmer.

All serum samples screened in the study were non-reactive to indirect ELISA indicating absence of detectable levels of serum antibodies to ASFV. In comparison to previous studies, these findings are different from Atuhaire *et al.*,(2013), who reported 11.5% presence of ASFV antibodies in slaughter slabs in Kampala and also with Tejler, (2012) who reported 0.5% presence in Gulu district. However, the finding from this study are similar to Muhangi *et al.* (2014) from Masaka and Rakai districts who also never detected ASF antibodies in apparently healthy pigs.

This may be attributed to the fact that farmers sell off their animals immediately they hear of ASF outbreaks, but suspected case are collected, sold, poled together for slaughter hence increased chance of the antibody detection in the slaughter slabs (Atuhaire *et al.,* 2013). There is also a possibility that there was no recent infection and outbreaks when the sampling was done. These results are in agreement with findings from Gallardo *et al.*,(2011) and Okoth *et al.,* (2012) who also did not detect antibodies using the indirect ELISA kit (Ingezim 14HSK3, ingenasa, Madrid, Spain). This failure to

detect circulating antibodies could be as a result of the pigs dying before seroconverting as this could have been an early stage infection (OIE, 2012). Other factors like disease tolerance due to different host factors, and presence of avirulent strains may not induce antibody responses to the virus thus the absence of antibodies (Costard *et al.,* 2009). The samples in this study might have been collected during post infection or after outbreaks when pigs were wiped out with disease or sickly pigs sold for slaughter.

Molecular diagnosis using UPL # 162-Real-time PCR showed viral presence in one of the samples collected in this study. The UPL # 162 Real-time PCR is a more sensitive and specific method for detection of minute volumes of the virus in domesticated pigs, wild porcine and ticks (Fernández-Pinero *et al.,* 2012). Isolates from previous studies on apparently healthy pigs were sequenced and genotype IX was confirmed to be circulating in Uganda (Atuhaire *et al.,* 2013; Gallardo *et al.,* 2011). Coincidently, the isolate sequenced from this study belongs to genotype IX. This confirms persistence of genotype IX in Uganda which is implicated in previous and recent outbreaks in the country (Atuhaire *et al.,* 2013; Gallardo *et al.,* 2011).

These findings also suggest possible seasonality of ASF occurrence. Sampling was conducted during the rainy season, though it has been observed that majority of the outbreaks occur during the dry season Atuhaire *et al.,* (2013) possibly associated with times pigs are allowed to roam freely. In addition, the ASF KAMULI 2013 isolate clusters with previous isolates of genotype IX responsible for 2010-2012 outbreaks in Uganda and 2010 -2011 Kenya (Atuhaire *et al.,* 2013; Gallardo *et al.,* 2011) . This shows there is a possibility of pig movements between borders (Kenya-Uganda) and within country (Uganda) since this ASF KAMULI 2013 was introduced because farmer had not experienced any outbreaks before (Atuhaire *et al.,* 2013). Most of the farmers interviewed were

knowledgeable of the clinical signs and symptom, hence they would sell off their pigs immediately on hearing or noticing these clinical signs and symptoms thus a further explanation of the low prevalence of ASF observed (Chenais *et al.*, 2015). Finally from this study, where one sample was positive for ASFV indicates ASFV is highly pathogenic and is associated with acute and paracute disease, thus the chronic carriers are unlikely to be important in the epidemiology of ASF. This one sample was positive for ASFV but seronegative which implies long time carriers may not be associated with disease transmission in these apparently healthy pigs in areas with no reports on previous outbreaks. From this study, only one pig was positive for ASFV and so it was not possible to identify predicators for ASF in the three districts.

Limitations

There were various limitations encountered during the survey which included the:

- Most of the farmers often got bored when answering the questionnaires, because they were long, consuming the farmers' time to perform other tasks.
- Some farmers had sold their animals to traders and this resulted in looking for other interested farmers having animals.
- There was also a problem of farmer identification which was time consuming, because most female farmers were known by their children's names and not by their own or husband's names.
- The CVR of the virus was not amplified in this study because the sample was exhausted, since a lot of optimization of the PCR was required. Therefore difference in the tetrameric repeats in the p72 region was not determined as this provides high level resolution for viral discrimination.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The findings from this study show a very low prevalence of ASFV like those in the literature which indicates that ASFV is paracute / acute and is rarely detected in apparently healthy pigs. This indicates that chronically infected pigs are not likely to be important in ASFV epidemiology.

The commonly circulating virus in Uganda is genotype IX.

6.2 Recommendations

The following recommendations are made from the study

1. Longitudinal surveillance systems should be designed to understand the epidemiology of ASFV which is dynamic. These systems should be periodically monitored and evaluated to identify risk factors responsible for the disease's continued existence.
2. A risk-based approach should be formulated on biosecurity measures to check on farmers' adherence and implementation on these measures in the country during and outside outbreaks.

REFERENCES

Aguero, M., Fernandez, J., Romero, L., Sanchez Mascaraque, C., Arias, M., & Sanchez-Vizcaino, J. M. (2003). Highly sensitive PCR assay for routine diagnosis of African swine fever virus in clinical samples. *Journal of Clinical Microbiology*, *41*, 4431–4434.

Alcami, A., Carrascosa, A.L., Vinuela, E. (1990). Interaction of African swine fever virus with macrophages. *Virus Research*, *17*, 93–104.

Aliro, T., Tejler, E., Muhangi, D., Nyanabo, S., Boqvist, A., Ademun Okurut, A., Masembe, C., Emanuelson, U., Stahl, K. (2012). Spatio-temporal dynamics of African swine fever in Gulu district, northern Uganda. EPIZONE 6th Annual Meeting "viruses on the move".

Alonso, C., Galindo, I., Cuesta-Geijo, M. A., Cabezas, M., Hernaez, B., & Muñoz-Moreno, R. (2013). African swine fever virus-cell interactions: from virus entry to cell survival. *Virus Research*, *173*(1), 42–57. https://doi.org/10.1016/j.virusres.2012.12.006

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

Atuhaire, D. K., Afayoa, M., Ochwo, S., Mwesigwa, S., Okuni, J. B., Olaho-Mukani, W., & Ojok, L. (2013). Molecular characterization and phylogenetic study of African swine fever virus isolates from recent outbreaks in Uganda (2010-2013). *Virology Journal*, *10*(1), 247. https://doi.org/10.1186/1743-422X-10-247

Bastos, A.D., Penrith, M.L, Cruciere, C., Edrich, J.L., Hutchings, G., Roger, F., Couacy-Hyman, E., Thomson, G. . (2003). Genotyping field strains of African swine fever virus by partial p72 gene characterisation. *Archives of Virology*, *148*, 693–706.

Bastos, A.D., Penrith, M.L., Macome.F., Pinto, F., Thomson, G. (2004). Co-circulation of two genetically distinct viruses in an outbreak of African swine fever in Mozambique: no evidence for individual co-infection. *Veterinary Microbiology*, *103*, 169–182.

Björnheden, L. (2011). A study of domestic pigs , wild suids and ticks as reservoirs for African swine fever virus in Uganda Sveriges lantbruksuniversitet A study of domestic pigs , wild suids and ticks as reservoirs for African swine fever virus in Uganda.

Boshoff, C. I., Bastos, a D. S., Gerber, L. J., & Vosloo, W. (2007). Genetic characterisation of African swine fever viruses from outbreaks in southern Africa (1973-1999). *Veterinary Microbiology*, *121*(1–2), 45–55. https://doi.org/10.1016/j.vetmic.2006.11.007

Carrascosa, A.L., Val, M.D., Santaaren, J.F., Vinuela, E. (1985). Purification and properties of African swine fever virus. *Journal of Virology*, *54*, 337–344.

Carriloo, C., Borca, M.V., Afonso, C.L., Onisk, D.V., Rock, D. L. (1994). Long-term persistent infection of swine monocytes/macrophages with African swine fever virus. *Virology Journal*, *68*, 580–583.

Chenais, E., Boqvist, S., Emanuelson, U., Ouma, E., Dione, M., & Aliro, T. (2015). Knowledge , Attitudes and Practices Related to African Swine Fever Within Smallholder Pig Production in Northern Uganda, *2011*, 1–15. https://doi.org/10.1111/tbed.12347

Cobbold,C &Wileman, T. (1998). The major structural protein of African swine fever virus p73, is packaged into large structures,indicative of viral capsid or matrix precursors, on the endoplasmic reticulum. *Journal of Virology*, *72*(6), 5215–5223.

Costard, S., Mur, L., Lubroth, J., Sanchez-Vizcaino, J. M., & Pfeiffer, D. U. (2012). Epidemiology of African swine fever virus. *Virus Research*, 1–7. https://doi.org/10.1016/j.virusres.2012.10.030

Costard, S., Wieland, B., de Glanville, W., Jori, F., Rowlands, R., Vosloo, W., … Dixon, L. K. (2009). African swine fever: how can global spread be prevented? *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *364*(1530), 2683–96. https://doi.org/10.1098/rstb.2009.0098

Dione, M. M., Ouma, E. a, Roesel, K., Kungu, J., Lule, P., & Pezo, D. (2014). Participatory assessment of animal health and husbandry practices in smallholder pig production systems in three high poverty districts in Uganda. *Preventive Veterinary Medicine*, *117*, 565–576. https://doi.org/10.1016/j.prevetmed.2014.10.012

Dixon, L.K., Costa, J.V., Escribano, J.M., Rock, D.L., Vinuela, E., Wilkinson, P. J. (2000). Family Asfarviridae. *In Virus Taxonomy,7th Report of the ICTV*, 159–165.

Dixon, L. K., Chapman, D. a G., Netherton, C. L., & Upton, C. (2012). African swine fever virus replication and genomics. *Virus Research*, *173*(1), 3–14. https://doi.org/10.1016/j.virusres.2012.10.020

Dixon, L. K., & Takamatsu, H. (2012). African swine fever virus: current situation and prospects for control. *Pig Journal*, *67*, 11–17. Retrieved from http://www.pigjournal.co.uk

Etter, E. M. C., Seck, I., Grosbois, V., Jori, F., Blanco, E., Vial, L., … Roger, F. L. (2011). Seroprevalence of African swine fever in Senegal, 2006. *Emerging Infectious Diseases*, *17*(1), 49–54. https://doi.org/10.3201/eid1701.100896

Fasina, F. O., Agbaje, M., Ajani, F. L., Talabi, O. A., Lazarus, D. D., Gallardo, C., … Bastos, A. D. S. (2012). Risk factors for farm-level African swine fever infection in major pig-producing areas in Nigeria, 1997-2011. *Preventive Veterinary Medicine*, *107*(1–2), 65–75. https://doi.org/10.1016/j.prevetmed.2012.05.011

Fernández-Pinero, J., Gallardo, C., Elizalde, M., Robles, A., Gómez, C., Bishop, R., & Heath, L., Couacy-Hymann, E., Fasina, F.O., Pelayo, V., Soler, A., Arias, M. (2012). Molecular diagnosis of African swine fever by a new real-time PCR using Universal Probe Library. *Transboundary and Emerging Diseases*. Retrieved from http://dx.doi.org/10.1111/j.1865-1682.2012.01317

Gallardo, C., Ademun, A. R., Nieto, R., Nantima, N., Arias, M., Pelayo, V., & Bishop, R. P. (2011). Genotyping of African swine fever virus (ASFV) isolates associated with disease outbreaks in Uganda in 2007, *10*(17), 3488–3497. https://doi.org/10.5897/AJB10.1439

Gallardo, C., Anchuelo, R., Pelayo, V., Poudevigne, F., Leon, T., Nzoussi, J., … Arias, M. (2011). African swine fever virus p72 genotype IX in domestic pigs, Congo, 2009. *Emerging Infectious Diseases*, *17*(8), 1556–8. https://doi.org/10.3201/eid1708.101877

Gallardo, C., Elizalde, M., Robles, A., & Go, C. (2012). Molecular Diagnosis of African Swine Fever by a New Real-Time PCR Using Universal Probe Library, 1–11.

https://doi.org/10.1111/j.1865-1682.2012.01317.x

Gallardo, C., Okoth, E., Pelayo, V., Anchuelo, R., Llorente, A., Nieto, R., … Bishop, R. P. (2011). African swine fever viruses with two different genotypes , both of which occur in domestic pigs , are associated with ticks and adult warthogs , respectively , at a single geographical site, 432–444. https://doi.org/10.1099/vir.0.025874-0

Hall, T. A. (1999). Bioedit a user friendly biological sequence alignment editor and analysis program for windows 95/98/NT. *In Nucleic Acids Symposium*.

Hjertner, B., Meehan, B., McKillen, J., McNeilly, F., Belák, S. (2005). Adaptation of an Invader assay for the detection of African swine fever virus DNA. *Journal of Virological Methods 124*, 1–10.

Irusta, P. M., Borca, M. V, Kutish, G. F., Lu, Z., Caler, E., Carrillo, C., & Rock, D. L. (1996). Amino acid tandem repeats within a late viral gene define the central variable region of African swine fever virus. *Virology*, *220*(1), 20–27. https://doi.org/10.1006/viro.1996.0281

Jori, F., & Bastos, A. D. S. (2009). Role of wild suids in the epidemiology of African swine fever. *EcoHealth*, *6*(2), 296–310. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/19915917

Jori, F., Vial, L., Penrith, M. L., Pérez-Sánchez, R., Etter, E., Albina, E., … Roger, F. (2013). Review of the sylvatic cycle of African swine fever in sub-Saharan Africa and the Indian ocean. *Virus Research*, *173*(1), 212–27. https://doi.org/10.1016/j.virusres.2012.10.005

Karalyan, Z., Zakaryan, H., Sargsyan, K., Voskanyan, H., Hakobyan, L., Abroyan, L., … Karalova, E. (2012). Pathology of porcine peripheral white blood cells during infection with African swine fever virus. *BMC Veterinary Research*, *8*, (28 February 2012)-(28 February 2012). Retrieved from <Go to ISI>://CABI:20123095929

Kleibeker, S.B., Scoles, G.A., Burrage, T.G., Sur, J. (1999). African swine fever virus replication in the midgut epithelium is required for infection of Ornithodoros ticks. *Journal of Virology*, *73*(10), 8587–8598.

Lubisi, B. A., Bastos, A. D. S., Dwarka, R. M., & Vosloo, W. (2005). Molecular epidemiology of African swine fever in East Africa, 2439–2452. https://doi.org/10.1007/s00705-005-0602-1

Malmquist, W.A & Hay, D. (1960). Hemaadsorption and cytopathic effect produced by African swine fever virus in swine bone marrow and buffy coat cultures. *AM J Vet Res*, *21*, 104–108.

Mebus, C. A. (1988). Afircan swine fever. *Advances in Virus Research*, *35:*, 251–269.

Misinzo, G., Jumapili, F., Ludosha, M., Mafie, E., & Silialis, J. (2009). Genotyping of African swine fever virus from a 2009 outbreak in Tanzania. *Research Opinions in Animal &Veterinary Sciences*, 334–338.

Montgomery, R. E. (1921). On a form of African swine fever occuring in the British East Africa (Kenya colony). *Journal of Comparative Pathology and Therapeutics*, *34*, 159–191, 243–265.

Muhangi, D., Masembe, C., Berg, M., Ståhl, K., & Ocaido, M. (2014). Practices in the pig value chain in Uganda; implications to African swine fever transmission. *Livestock Research for Rural Development*, *26*((5).).

Muhangi, D., Masembe, C., Emanuelson, U., Boqvist, S., Mayega, L., Ademun, R. O., … Ståhl, K. (2015). A longitudinal survey of African swine fever in Uganda reveals high apparent disease

incidence rates in domestic pigs , but absence of detectable persistent virus infections in blood and serum. 1–11. https://doi.org/10.1186/s12917-015-0426-5

Muhanguzi, D., Lutwama, V., & Mwiine, F. (2012). Factors that influence pig production in Central Uganda - Case study of Nangabo Sub-County, Wakiso district. *Veterinary World*, *5*(6), 346. https://doi.org/10.5455/vetworld.2012.346-351

Mutetikka, D. (2009). A Guide to pig Production at Farm level,Makerere University, Kampala-Uganda. *Fountain Publishers, Kampala*.

Muwonge, A., Munang'andu, H. M., Kankya, C., Biffa, D., Oura, C., Skjerve, E., & Oloya, J. (2012). African swine fever among slaughter pigs in Mubende district, Uganda. *Tropical Animal Health and Production*, *44*(7), 1593–8. https://doi.org/10.1007/s11250-012-0112-6

Nantima, N., Ocaido, M., Ouma, E., Davies, J., Okoth, E., & Bishop, R. (n.d.). Risk factors associated with occurrence of African swine fever outbreaks in smallholder pig farms in four districts along the Uganda-Kenya border. https://doi.org/10.1007/s11250-015-0768-9

Nix, R.J., Gallardo, C., Hutchings, G., Blanco, E., Dixon, L. K. (n.d.). Molecular epidemiology of African swine fever virus studied by analysis of four variable genome regions. *Archives of Virology*, *151,* 2475–24.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., & Hase, T. (2000). Loop-mediated isothermal amplification of DNA. *Nucleic Acids Research*, *28*(12), E63. https://doi.org/10.1093/nar/28.12.e63

O.I.E. (2010). Early detection and contingency plan for African swine fever, 139–147.

OIE. (2008). Chapter 2.8.1. African swine fever. In Manual of diagnostic tests and vaccines for terrestrial animals 2008. Office International des Epizooties, Paris, France. http://www.oie.int/eng/ normes/mmanual/2008/pdf/2.08.01_ASF.pdf.

OIE. (2012). African swine fever. In: Manual of Diagnostic Tests and vaccine for Terrestrial Animals. Office Internatinal des Epizooties, Paris, France. (Chapter 2.8.1,NB: Version adopted in May 2012).

Okoth,E.,Gallardo,C.,Macharia,J.M.,Omore,A.,Pelay,V.,Bulimo,D.W.,Arias,M.,Kitala,P.,Baboon, K.,Lekolol, I.,Mijele, D.,Bishop, R. . (n.d.). Comparison of African swine fever virus prevalence and risk in two constrating pig-farming systems in South-West and Central Kenya. *Preventive Veterinary Medicine*, *110*(2), 198–205.

Ouma, E., Dione, M., Lule, P., Pezo, D., Marshall, K., Roesel, K., Mayega, L., Kiryabwire, D., Nadiope, G and Jagwe, J. (2014). *Smallholder pig value chain assessment in Uganda : results from producer focus group discussions and key informant interviews*. Nairobi, Kenya.

Ouma, E., Dione, M., Lule, P., Roesel, K., & Pezo, D. (2013). Characterization of smallholder pig production systems in Uganda: Constraints and opportunities for engaging with market systems. *Livestock Research for Rural Development*, *26*(3).

Oura, C. a L., Edwards, L., & Batten, C. a. (2013). Virological diagnosis of African swine fever--comparative study of available tests. *Virus Research*, *173*(1), 150–8. https://doi.org/10.1016/j.virusres.2012.10.022

Penrith, M.-L. (2009). African swine fever. *The Onderstepoort Journal of Veterinary Research*,

76(1), 91–95. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/19967933

Pezo, D. & Waiswa, C. (2012). Farming Systems Perspectives:Lessons for managing health risks in smallholder pig systems.Paper presented in the South-South Symposium"Managing Risks in Emerging Pork Markets". Hano, Viet Nam 23-25 Apirl 2012; International Livestock Research Institute(ILRI) and Makerere University Kampala, Uganda.

Ravaomanana, J., Jori, f., Andriatsimahavandy, A.,Roger,F., Albina, E., Vial, L. (2010). First detection of African swine fever virus in Ornithodoros porcinus in Madagascar and new insights into tick distribution and taxonomy. *Parasites and Vectors*, *3*, 115.

Reis, A.L., Parkhouse, R.M., Penedos, A.R., Martins, C., Leitao, A. (2007). Systematic analysis of longitudinal serological responses of pigs infected experimentally with African swine fever virus. *General Virology*, *88:*, 2426–2534.

Rojo, G., Chamorro, M., Salas, M.L., Vinuela, E., Cuezva, J.M., Salas, J. (1999). Replication of African swine fever DNA in infected cells. *Virology*, *257*, 524–536.

Rowlands, R. J., Duarte, M. M., Boinas, F., Hutchings, G., & Dixon, L. K. (2009). The CD2v protein enhances African swine fever virus replication in the tick vector, Ornithodoros erraticus. *Virology*, *393*(2), 319–328. https://doi.org/10.1016/j.virol.2009.07.040

Ruiz-Gonzalvo, F., Rodriguez, F., Escribano, J. M. (1996). Functional and Immunological properties of the baculovirus-expressed hemagglutinin of African swine fever virus. *Virology*, *218*, 285–289.

Ståhl.M.K., Ogweng.P., Okoth. E., Aliro.T, Muhangi.D., LeBlanc.N, Atimnedi.P., B. M., & Bishop.R.P., Rasmussen.H.B, Masembe. C. (2014). Understanding the dynamics and spread of African swine fever virus at the wildlife-livestock interface: insights into the potential role of the bushpig, Potamochoerus larvatus. *Newsletter of the IUCN / SSC Wild Pig, Peccary and Hippo Specialist Groups*, *13*(1).

Steiger Y., Ackermann M., M. C., & U., K. (1992). Rapid and biologically safe diagnosis of African swine fever virus infection by using polymerase chain reaction. *Journal of Clinical Microbiology*, *30*, 1–8.

Suárez, C., Salas, M. L., & Rodríguez, J. M. (2010). African swine fever virus polyprotein pp62 is essential for viral core development. *Journal of Virology*, *84*(1), 176–87. https://doi.org/10.1128/JVI.01858-09

Sun, H., Jenson, J., Dixon, L.K., Parkhouse, M. E. (1996). Characterisation of the African swine fever virion protein,j18L. *Virology Journal*, *77*(5), 941–946.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution*, *30:*, 2725–2729.

Tatwangire A. (2013). *Sucesses and failures of institutional innovations to improve access to services,input and output markets for smallholder pig production system and value chains in Uganda.* Retrieved from Review Report. www.livestockfish.cgiar.org

Tejler, E. (2012). Outbreaks of African swine fever in domestic pigs in Gulu district , Uganda Outbreaks of African swine fever in domestic pigs in Gulu district , Uganda.

Thrusfield, M. (2007). Veterinary Epidemiology, 3rd edition.

Ticks, S., Vial, L., Wieland, B., Jori, F., Etter, E., Dixon, L., & Roger, F. (2007). Fever Virus DNA in, *13*(12), 1928–1931.

UBOS. (2008). *A summary report of the national livestock census*. Entebbe, Uganda.In Anonymus(eds.) Kampala.

Uganda Bureau of Statistics. (2013). 2013 Statistical Abstract, 1–264. Retrieved from www.ubos.org

Wardley, R.C., Abu, Elzein, E.M.E., Crowther, P.J. (1979). A solid- phase enzyme linked immunosorbent assay for the detection of African swine fever virus antigen and antibody. *Journal of Hygiene (Cambridge)*, *83*, 363–369.

Wilkinson, P. J. (2000). African swine fever In: Manual of Standards for Diagnostic Tests and Vaccines, fourth ed. Office International des Epizooties, Paris.

Yanez, R.J., Rodriguez, J.M., Nogal, M.L., Yuste, L., Enriquez, C., Rodriguez, J.M., Vinuela, E. (1995). Analysis of the complete nucleotide sequence of African swine fever virus. *Virology*, *208*(1), 249–278.