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# Detection and genetic characterization of sylvatic and outbreak African swine fever virus isolates in selected zones of Tanzania

Njau, Emma

NM-AIST

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# DETECTION AND GENETIC CHARACTERIZATION OF SYLVATIC AND OUTBREAK AFRICAN SWINE FEVER VIRUS ISOLATES IN SELECTED ZONES OF TANZANIA

**Emma Peter Njau**

**A Thesis Submitted in Fulfilment of the Requirements for the Award of the Degree of Doctor of Philosophy in Life Sciences of the Nelson Mandela African Institution of Science and Technology**

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## ABSTRACT

This thesis is about African swine fever (ASF), a severe disease of domestic pigs that is notifiable according to OIE. Virus strains circulating in Tanzania have been changing from time to time making surveillance studies imperative. This research aimed at understanding the African Swine Fever Virus (ASFV) setting in the sylvatic cycle devoid of domestic pigs and genetic characterization of the ASFV genotypes circulating in the domestic pig cycle by genome sequencing of the virus so as to provide information such as the relatedness of the virus strains for other studies. In the study, blood and tissue samples from outbreaks and from selected seven agro-ecological areas of Tanzania (Northern (n=58) Southern (n=18), Southern highlands (n=60), Coastal (n=30), Western (n=40), Central (n=40) and lake zone (n=56)) were screened for ASFV using antibody ELISA and PCR techniques. The study population included domestic cycle hosts (domestic pigs (n=302) and sylvatic cycle hosts (warthogs (n=9) and soft ticks (n=300)). Snowball sampling was applied for domestic pigs following outbreak notifications from the Ministry of Livestock and Fisheries for the areas that experienced outbreaks (Southern highlands, Lake Victoria, Coastal and Northern zones). Convenient sampling was implied for non-outbreak areas (Central, Western and Southern zones) and the Saadani National Park. Blood and tissue samples from domestic pigs (n=302), blood samples from warthogs (n=19) and whole ticks (n=300) from warthog burrows (n=5) were collected from the field and transported to the laboratory for analysis. Serum samples extracted from blood were analysed for the presence of ASFV antibodies. DNA was extracted from the tissues, ticks and whole blood samples for ASFV diagnosis. Representative positive samples were Sanger sequenced for genotyping and tissue cultured for whole genome sequencing. While using standard OIE recommended ASF diagnostic and genotyping primers, positive and negative controls were used in each process to ensure reliability and validity of the obtained results. ELISA results confirmed the exposure of warthogs to ASFV although none of the animals were found to be in active viremia. As opposed to warthogs, domestic pigs did not react positively to an ELISA test but they were in active viremia following a PCR test with ASFV primers. This study reported four ASFV genotypes currently present in Tanzania. Genotypes II, IX and X were reported from domestic pigs whereas genotype XV was reported from the ticks in the sylvatic cycle. This is the first study to report the presence of genotype XV from an ecosystem that has never recorded interaction with domestic pigs. The study also released the first full genome sequence of a genotype II ASFV strain from Africa (Tanzania/ Rukwa/2017/1) generated using Illumina sequencing (Deposited in the INSDC databases through the European Nucleotide Archive (ENA) under accession number LR813622). The reported strain was observed to have a 99.961 percentage identity with the updated Georgia

2007/1 reference isolate (FR682468.2), 99.960% identity with Polish isolate Pol16_29413_o23 (MG939586) and 99.957% identity with Chinese isolate ASFV-wbBS01 (MK645909.1). This gave a proposition on the relatedness of genotype II strains of the virus from the known geographical hotspot given that this was the most globally spread genotype. Findings from this study add an information on what was known regarding ASFV in the sylvatic cycle in the country, and as a global hotspot for ASF. The first ASF vaccine is likely to focus on genotype II as it is the most widespread genotype. This has an implication on the global efforts to control ASF while being able to follow the molecular changes of the virus from the hotspot areas. There is also a possibility that in Tanzania, ASFV is present in new areas and distributed widely than it was previously anticipated. Information on circulating genotypes and spatial distribution is paramount for devising control interventions in Tanzania.

## COPYRIGHT

## DECLARATION

I, Emma Peter Njau do declare to the Senate of the Nelson Mandela Africa Institution of Science and Technology that this Thesis is my own original work and that it has neither been submitted nor being concurrently submitted for degree award in any other institution.

Emma Peter Njau

Name and signature of Candidate Date

The above declaration is confirmed by:

Prof. Gabriel Mkilema Shirima

Name and Signature of Supervisor 1 Date

Prof. Lughano Jeremy Moses Kusiluka

Name and Signature of Supervisor 2 Date

Prof. Sarah Cleaveland

Name and Signature of Supervisor 2 Date

## CERTIFICATION

The undersigned certify that have read and hereby recommend for acceptance by the Senate of the Nelson Mandela African Institution of Science and Technology, a Thesis titled *"Detection and Genetic Characterization of Sylvatic and Outbreak African Swine Fever Virus Isolates in Selected Zones of Tanzania"* in Fulfillment of the Requirements for the Award of the Degree of Doctor of Philosophy in Life Sciences of the Nelson Mandela African Institution of Science and Technology.

Prof. Gabriel Mkilema Shirima

Name and Signature of Supervisor 1 Date

Prof. Lughano Jeremy Moses Kusiluka

Name and Signature of Supervisor 2 Date

Prof. Sarah Cleaveland

Name and Signature of Supervisor 3 Date

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international conferences and provision of some primers and expertise from the Defence Threat Reduction Agency (DTRA) funded ILRI-ASF project.

## DEDICATION

To GOD be all the Glory. This work is dedicated to my dad Dr. Peter Njau, my mum Mrs. Matilda Njau, my mum in love Mrs. Yusta Mushi and our sons Nathaniel, Jonathan and Ethan Mushi. A special space in its own sentence is for my dear husband Dr. James Richard Mushi.

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## LIST OF ABBREVIATIONS AND SYMBOLS

| | |
|---|---|
| ASF | African swine fever |
| ASFV | African swine fever virus |
| BLAST | Basic Local Alignment Search Tool |
| BMGF | Bill & Melinda Gates Foundation |
| Bp | Base pairs |
| BS | Broad sensitivity |
| BSA | Bovine Serum Albumin |
| CVR | Central variable region |
| DNA | Deoxy-ribonucleic acid |
| dNTP | Deoxynucleoside triphosphate |
| DPI | Days Post Infection |
| DS | Double stranded |
| DVO | District Veterinary Officer |
| EDTA | Ethylene Diamine Tetra Acetic Acid |
| ELISA | Enzyme Linked Immunosorbent Assay |
| ENA | European Nucleotide Archive |
| FAO | Food and Agriculture Organization of the United Nations |
| FAOSTAT | Food and Agriculture Organization Statistics Department |
| FAT | Fluorescent Antibody Test |
| gDNA | Genomic DNA |
| GPS | Geographical Positioning System |
| HAD | Haemadsorption |
| HS | High sensitivity |
| IBAR | Inter-African Bureau on Animal Resources |
| IFAT | Immunofluorescent Antibody Test |
| ILRI | International Livestock Research Institute |
| IU | Infectious Units |
| Kbp | Kilo base pairs |
| MEGA | Molecular Evolutionary Genetics Analysis |
| MGF | Multigene Families |
| MoI | Multiplicity of Infection |
| NCBI | National Centre for Biological Information |
| OD | Opaque Density |

| | |
|---|---|
| OIE | Office International des Epizooties |
| PAMs | Pulmonary Alveolar Macrophages |
| PBMCs | Peripheral Blood Mononuclear cells |
| PBS | Phosphate Buffered Saline |
| PCR | Polymerase Chain Reaction |
| PEHPL | Project for Enhancing the Health and Productivity of Livestock |
| PPA | Peste Porcine Africaine |
| QC | Quality control |
| QPCR | Quantitative Polymerase Chain Reaction |
| RPM | Rotations per minute |
| RPMI | Roswell Park Memorial Institute |
| S/P | Sample per Positive ratio |
| SNP | Saadani National Park |
| SSA | Sub-Saharan Africa |
| sWGA | Selective Whole Genome Amplification |
| TANAPA | Tanzania National Park Authority |
| TAWIRI | Tanzania Wildlife Research Institute |
| USA | United States of America |

# CHAPTER ONE

# INTRODUCTION

## 1.1 Background of the Problem

African Swine Fever (ASF) is an acute, severe haemorrhagic fever caused by a virus, the ASF virus (ASFV). The disease is highly contagious and in some virulent strains, mortality levels in the newly infected population are very high. To date, neither treatment nor vaccine has been proven to be effective against the disease although several vaccine trials are being carried out (Penrith, 2013). The ASFV is a large (170-190 kbp) double-stranded DNA virus that is known to be the only DNA virus causing haemorrhagic fever and being vectored by an invertebrate host (Ornithodoros tick), hence it is placed as a lone member in its family Asfarviridae and genus Asfivirus (Dixon *et al.,* 2013).

The ASF was reported for the first time in 1921 by Montgomery in Kenya (Montgomery, 1921). Prevention of the disease relies on strict biosecurity measures which are frequently not effectively applied, particularly in the endemic areas, resulting in the spread of the disease into areas where it has not previously been reported (Beltran-Alcrudo *et al.,* 2019; Costard *et al.,* 2009; Jurado *et al.,* 2018; Plut, 2018). Members of the swine family including but not limited to warthogs (*Phacochoerus africanus*), bush pigs (*Potamochoerus larvatus*) and giant forest hogs (*Hylochoerus meinertzhageni*) are known to be naturally infected by the virus. The ASFV is maintained in three cycles which are said to be overlapping, the first one being a sylvatic cycle involving a warthog and an argasid tick (*Ornithodoros moubata*); a cycle in domestic pigs involving *Ornithodoros porcinus* that lives in pig shelters and a cycle involving domestic pigs alone without involving ticks or wildlife (Arnot *et al.,* 2009). Furthermore, the virus has high genetic diversity in its natural hosts and 24 genomes have been identified based on the p72 gene (Bishop *et al.,* 2015).

The World Organization for Animal Health (OIE), considers ASF as a number one killer disease with the most serious constraints to the pig production industry within and outside Africa (OIE-Listed Diseases 2019). Despite the high turnover of pigs and benefits, the swine industry is constrained by poor husbandry practices and prevalent diseases such as African swine fever that cause massive losses in the east African region. Efforts towards containment of ASF and reduction of its effects, will have a direct effect on food security and economic status of farmers in sub-Saharan Africa.

Control of ASF is mainly focused on early laboratory diagnosis, quarantine and biosecurity (Costard *et al.,* 2009), which all have challenges especially in sub-Saharan Africa and specifically, on smallholder farmers in the region. To strengthen these control measures, it is important to understand how the virus is transmitted and maintained between the two prominent cycles, namely sylvatic and domestic cycles.

Twenty-four genotypes of the virus have been currently identified by sequencing of the p72/B646L gene of the virus (Bastos *et al.,* 2003; Galindo & Alonso, 2017). Most of these known genotypes can be associated with a particular geographical location especially in sub-Saharan Africa, where the virus almost certainly originated (Costard *et al.,* 2013). Among the 24 known genotypes, two of them namely genotype I and genotype II are of the major global concern as they have spread from Africa to other continents. Genotype II is of the high importance as it is the cause of an ASF pandemic currently encompassing much of eastern Europe, the Caucasus, the Baltic republics, Russia, China and parts of south-east Asia. The economic losses involved are huge, especially in China which ranks high in pig production, with majority of them reared in backyard or village systems (Food and Agriculture Organization of the United Nations [FAO], 2018; Ge *et al.,* 2018; Wen *et al.,* 2019). The socio-economic impact is often immense due to the loss of livelihoods for relatively poor small and medium scale pig keepers.

Multiple complete genome sequences of ASFV have now been determined with a strong bias towards p72-defined genotypes I and II. Many of the complete genomes are from isolates recovered from animals outside Africa and no study has yet described the full genome sequence of an African ASFV genotype II virus, despite the global importance of this genotype (FAO, 2018; Ge *et al.,* 2018). This study observed that apart from the presence of a sylvatic cycle, the epidemiological situation of ASF in Tanzania is more of a trans-boundary nature with some strains having already been reported in the neighbouring countries bordering Tanzania (Chang'a *et al.,* 2019; Fasina *et al.,* 2020). Although there was no evidence of any virus strain circulating in both domestic and sylvatic cycle, there is an association that is not clearly understood as outbreaks of the disease related to the genotype detected in the wild has been reported in Tanzania before (Misinzo *et al.,* 2011; Wambura *et al.,* 2006).

## 1.2 Statement of the Problem

The devastating effects of African swine fever in all areas where it has been reported qualifies the disease to be notifiable to the OIE. Despite all the efforts put into place, up to the moment

there is no cure or vaccine available in the market. There is a knowledge gap on the mechanism in which the disease is maintained in the absence of reported outbreaks. The role of the sylvatic cycle in disease outbreak has been studied in some countries (Bastos *et al.,* 2009, De Glanville *et al.,* 2014) but more is to be found of this role in Tanzania although some studies have demonstrated the direct relationship between the presence of the disease and the existence of a sylvatic cycle (Plowright *et al.,* 1969). In ASF endemic settings, the circulating genotypes have been changing from time to time posing difficulties in control interventions. The eastern part of Africa is known to be the geographical origin for most ASF genotypes including genotype II, however, there is no available full genome sequence of this genotype from Africa. This leads to a knowledge gap in the scientific community on the molecular characteristics of the genotypes from this part of the world. The current research has contributed towards bridging the gap by studying the currently circulating genotypes among and between the sylvatic and domestic ASFV cycles coupled with the first full genome sequence of ASFV genotype II.

## 1.3 Rationale of the Study

The knowledge on circulating ASFV genotypes in sylvatic and domestic cycle will shade light on the direct association between these cycles as well as the spill over points, a ruling that can help in control and prevention of ASFV outbreaks. Moreover, data derived from whole genome sequencing is vital in finding the smaller and larger variations between different strains of the same genotype. This will become handy in mediation of interventions such as vaccines and the use of advanced technologies e.g. CRISPR/Cas-9 technology as tools for ASF control in the near future.

## 1.4 Research Objectives

### 1.4.1 General Objective

This research aimed at identifying and characterizing the genetic profiles of sylvatic and outbreak ASFV circulating in selected zones of Tanzania so as to provide information useful for appropriate control interventions in Tanzania.

### 1.4.2 Specific Objectives

The specific objectives of the study were as follows:

(i) To identify ASFV strains circulating in both sylvatic and domestic cycles.

(ii) To establish genotypic relationship between outbreak and non-outbreak ASFV strains from warthogs, pigs and ticks.

(iii) To determine the whole genome sequence of a globally important ASFV strain circulating in selected areas of Tanzania

## 1.5 Research Questions

The present study attempted to answer the following research questions:

(i) Which ASFV strains are currently circulating in sylvatic and domestic cycles?

(ii) How genetically related are the ASFV strains from warthogs, pigs and ticks?

(iii) How can the whole genome sequencing of ASFV improve the understanding of ASF in Tanzania and globally?

## 1.6 Significance of the Research

The ASF is the most feared and devastating disease of pigs that has been creating despair and huge economic losses among smallholder farmers. Past experiences of economic ruins associated with repeated disease outbreaks are thought to be contributing to the slow pace of development of the subsector in Tanzania. Addressing the effects of African swine fever on pigs will have a direct contribution towards enhancing the health and productivity of pigs. To be able to prevent and control ASF effectively, there is a need to deeply understand the mechanisms of spread and maintenance of the virus in the past and present as well as prediction of future trends. By studying the relationship between sylvatic and domestic cycles, virus genomes associated with the disease as well as their relationship with both sylvatic and domestic cycles will be clearly identified. Isolation of the virus and sequencing of the complete genome of a globally important virus strain will also add to knowledge repository about this important disease and thereby, address existing knowledge gaps.

## 1.7 Delineation of the Study

This thesis is about African swine fever (ASF), and was aimed at understanding the African Swine Fever Virus (ASFV) setting in the sylvatic cycle devoid of domestic pigs and genetic characterization of the ASFV genotypes circulating in the domestic pig cycle by genome sequencing of the virus so as to provide information such as the relatedness of the virus strains for other studies.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 General Pig Production

A backbone of the economy of many developing countries especially in Africa, is agriculture including crop and livestock. In most African countries including Tanzania, the livestock sector does not only provide employment but is an important source of nutritious food. In farming activities, livestock have been a source of manure and manpower. Rapidly growing animals that have a high reproduction potential such as pigs have been a stable source of household income. However, disease burdens caused by viruses, bacteria, helminths and protozoa greatly hinder the growth and productivity of the livestock sector in Africa (Igbokwe & Maduka, 2018). This, in turn, leads to economic losses to pig farmers and reduces animal-source protein available to households, thus contributing to food and nutritional insecurity (Cappai *et al.*, 2018).

White meat production which includes pork and chicken meat has been given a priority by both the Tanzania Livestock Sector Analysis (2016/2017-2031/2032) and Tanzania Livestock Master Plan (2017/2018-2021/2022). According to Tanzania Livestock Master Plan (2017/2018-2021/2022), improvement on pig production will require controlling of ASF which is the major setback for the growth of this sector. Statistical reports showed that there has been a significant increase in number of live pigs in Tanzania from the year 2000 to date (Food and Agriculture Organization Statistics Department [FAOSTAT], 2020) as presented in Fig. 1.

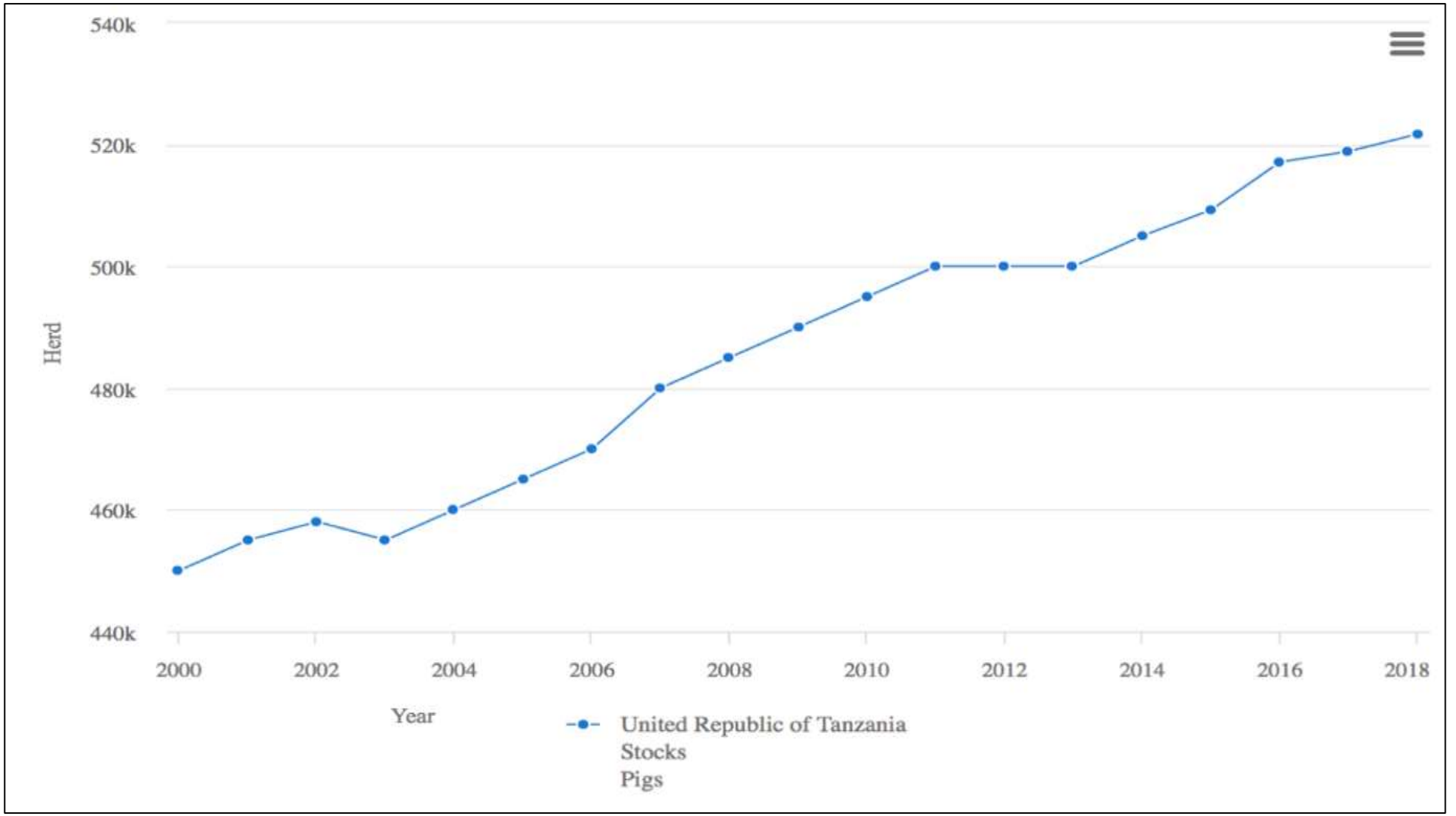



**Figure 1: A statistical representation of live pig production in Tanzania from the year 2000 to 2018 (FAOSTAT, 2020)**

According to the ILRI-AUIBAR Regional Report (Food and Agriculture Organization of the United Nations [FAO]; International Livestock Research Institute [ILRI]; African Union/ Interafrican Bureau for Animal Resources [AUIBAR], 2017), two main pig production systems are practiced in sub-Saharan Africa. These includes the extensive system, composed of small scale scavenging pigs and the semi-intensive system composed of small scale confined pigs. Information on pure breeds present in Tanzania has been recorded from fewer farms. The most common pig breeds are crosses within and between white breeds (Large White, Landrace, Welsh and Middle White) and coloured breeds (Wessex Saddleback, Essex, Large Black, Hampshire, Duroc, Tamworth and Gloucester Old Spots) that have been adapted to harsh environmental conditions scourges of various diseases and poor feeding (Wilson & Swai, 2014). The small-sized piggery has the lowest productivity with approximately six animals while the medium-sized farm has an average of 10 animals. Mortality rate of young pigs is observed to be 20% and 12% for small to medium village piggeries respectively with the piggery subsector having an annual growth rate of 7.7% compared to cattle which has 4.7% (MLF, 2017).

Generally, the pig industry has a potential for complementing and boosting household income above the poverty threshold within a short period of time given that pigs reproduce very rapidly as compared to other large mammals (Plate 1). When a statistical comparison is done between Tanzania and her immediate neighbours, the country's pig productivity is lagging behind all other nations with Uganda leading (FAOSTAT, 2020) as indicated in Fig. 2.

**Plate 1:** **A photo showing several piglets/farrowing sow as an indication of high productivity(Picture from Google images)**

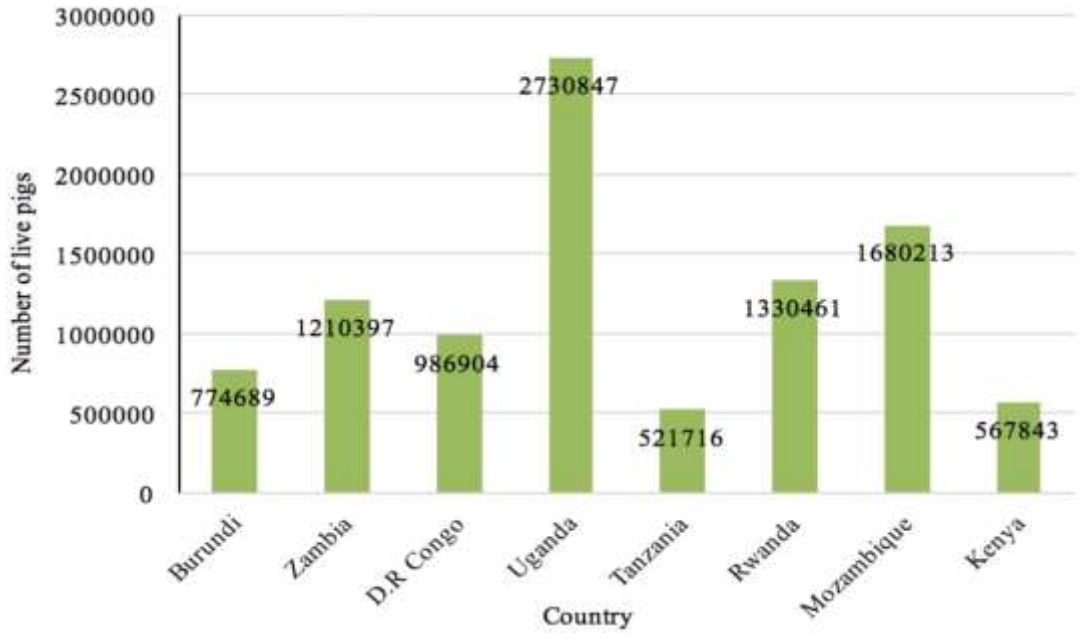



**Figure 2:** **Pig population comparison between Tanzania and neighbouring countries showing that Tanzania ranks low in pig production (FAOSTAT, 2020)**

## 2.2 The Genome and Genotypes of African Swine Fever Virus

The African swine fever virus (ASFV) (Fig. 3) causes a highly contagious and severe disease of domestic pigs posing serious threat to swine industry in the world, including East Africa (Penrith, 2009). The ASFV is a double-stranded, icosahedral, enveloped DNA arbovirus having a large size of 170 to 190 kilobase pairs (kbp) classified into the family *Asfarviridae*. The complete genome sequencing of this virus has identified approximately 160 genes which are organized in an almost similar manner to those of poxviruses (Dixon *et al.*, 2013).

The variation in the genome size is due to losses or gains of the multi gene families (MGF). Existing multigene families include MGF360, 110, 530, 300 and 100. Currently, 24 genotypes of the virus have been reported worldwide (Achenbach *et al.*, 2017). Five genotypes, namely; genotype II, IX, X, XV and XVI have been reported to be present in Tanzania (Mauya, 2015; Misinzo *et al.*, 2011; Misinzo *et al.*, 2014; Wambura *et al.*, 2006).

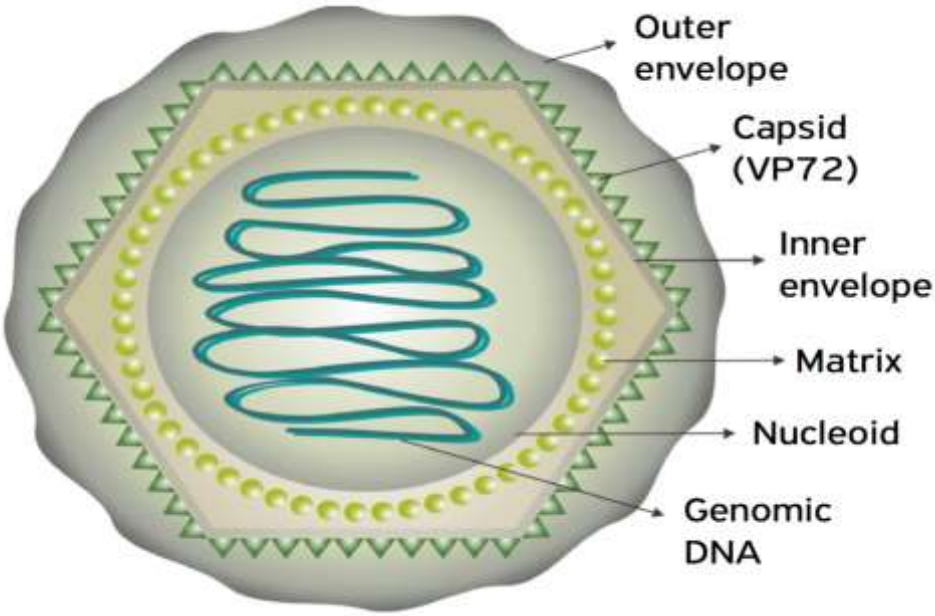



**Figure 3: Structure of an African swine fever virus displaying the important features including viral protein 72 (p72) that is used to characterize the virus into different genotypes (Freitas *et al.*, 2018)**

## 2.3 Epidemiology of African Swine Fever

African swine fever virus was firstly reported from domestic pigs in 1921 in Kenya (Fig. 4) after a retrospective recognition of the disease having occurred almost a decade earlier with further outbreaks reported in Portugal 1957 (Lubisi *et al.*, 2005).

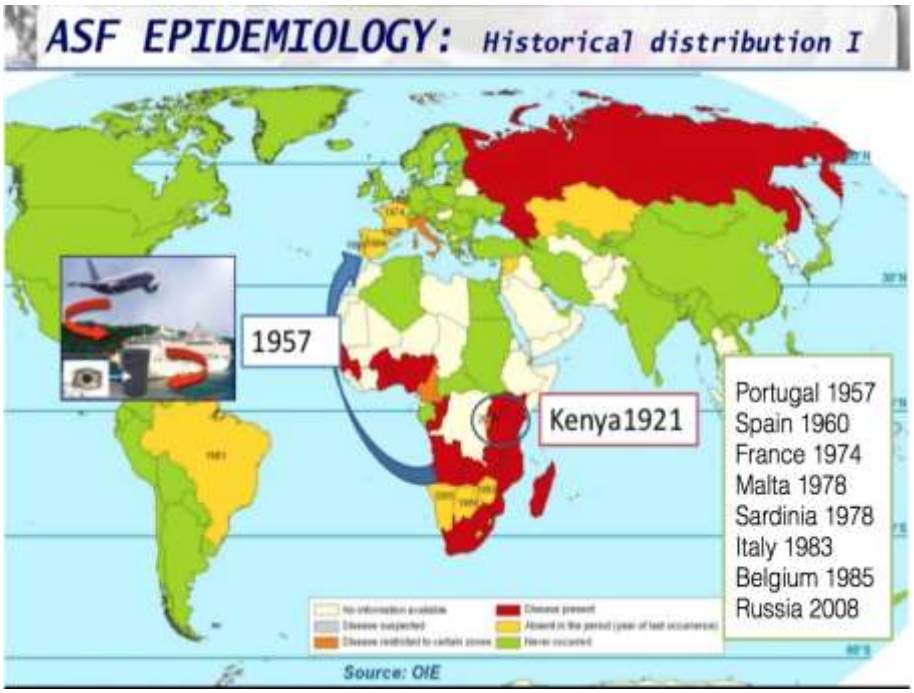



**Figure 4: A map showing the historical distribution of ASF (Office International des Epizooties [OIE], 2019)**

Since then, the sporadic outbreaks were reported in different countries inside and outside Africa including France, Belgium and Spain in 1980s (Costard *et al.,* 2009). The ASF was eradicated successfully from most of the European countries except in Sardinia, Italy where it has remained endemic to date (Costard *et al.,* 2013; Gallardo *et al.,* 2015).

In 2007, ASF was reported in Georgia thereby posing a possible involvement of other countries in the region (Beltrán-Alcrudo *et al.,* 2018). Between mid-2007 to date, ASF has been reported in the Caucasus, Russia, the Baltic republics, Czech Republic, Romania, Hungary, Bulgaria, Poland, Belgium, and most recently China, South Korea and Thailand (FAO, 2019). Despite significant efforts by FAO and other agencies to control its spread, the numbers of ASFV-infected animals are increasing rapidly in both domestic pigs and wild boar populations, with movements of the latter facilitating rapid geographical spread of the virus (Beltran-Alcrudo *et al.,* 2019). The disease has also spread to Mongolia and Vietnam within a short period of time (Bao *et al.,* 2019; Sánchez-Cordón *et al.,* 2018, OIE, 2018). The first outbreak that occurred in China in 2018 caused a huge economic loss to the country and to date, the effects and the outbreaks of the disease are still ongoing.

The ASF virus occurs naturally in vertebrate and invertebrate sylvatic hosts throughout sub-Saharan Africa (Atuhaire *et al.,* 2013). Twenty-four genotypes of the virus have been identified to date based on a p72 genetic region of the virus. Of the known ASFV genotypes, a large ASFV diversity has been found in the south-eastern region of Africa (Boshoff *et al.,* 2007; Lubisi *et al.,* 2005, 2007).

## 2.4 Molecular Epidemiology of African Swine Fever Virus in Sub-Saharan Africa

The last decade has seen a rapid increase in the pig production sector in Africa, and an equally rapid diagnosis of ASF and detection of ASFV, which have altogether provided a better improved epidemiological picture in disease-affected areas. The extensive molecular epidemiological studies of the ASFV genotypes circulating in Africa have shown a great diversity of isolates in the continent (Nix *et al.,* 2006). Some of these studies and the reported genotypes are listed in Table 1. The complexity of the ASF epidemiology has demonstrated the existence of 22 out of the known 24 genotypes in eastern and southern Africa based on the sequencing of the *p72* protein gene (Bastos *et al.,* 2003; Boshoff *et al.,* 2007).

The infection of susceptible animals can occur either through the sylvatic or the domestic cycles (Okoth *et al.,* 2013). In the sylvatic cycle, the ASFV circulates among wild reservoirs like bush pigs, warthogs and soft ticks (*Ornithodoros* spp.) (Costard *et al.,* 2009).

**Table 1: Some of the ASFV genotypes that have been reported in sub-Saharan Africa**

| Location | Genotype(s) | Sample source(s) | Year of study/collection | Reference(s) |
|---|---|---|---|---|
| Burundi | X | Domestic pigs | 2018 | Hakizimana *et al.* (2020) |
| Cameroon | I | Domestic pigs | 2010-2018 | Wade *et al.* (2019) |
| Cameroon | I | Domestic pigs | 2018 | Ngwa *et al.* (2020) |
| Democratic Republic of Congo (DRC) | IX | Domestic pigs | 2009 | Gallardo*et al.* (2011) |
| DRC | IX | Domestic pigs | 2016 | Patrick *et al.* (2020) |
| DRC | Not mentioned | Domestic pigs | 2011 | Patrick *et al.* (2019) |
| DRC | I, IX, XIV | Domestic pigs | 2005-2012 | Mlumba-Mfumu *et al.* (2017) |
| Ethiopia | XXIII | Domestic pigs | 2011 | Achenbach *et al.* (2017) |
| Ivory Coast | I | Domestic pigs | 2014 | Couacy-Hymann *et al.* (2019) |
| Ivory Coast | I | Domestic pigs | 2008-2013 | Kouakou *et al.* (2017) |
| Kenya | IX | Domestic pigs | 2006-2007 | Thomas *et al.* (2016) |
| Kenya | IX | Domestic pigs | 2007 | Gallardo *et al.* (2009 |
| Kenya | X, IX | Domestic pigs, soft ticks and warthogs | 2005 | Gallardo *et al.* (2011) |
| Kenya | IX, X | Domestic pigs | 2008-2009 | Okoth *et al.* (2013) |
| Kenya | IX, X | Soft ticks, Domestic pigs | 2006 | Bishop *et al.* (2015) |
| Kenya, Uganda | IX | Domestic pigs | 2011-2013 | Onzere *et al.* (2018) |
| Kenya-Uganda | IX | Domestic pigs | Not mentioned | Abworo *et al.* (2017) |
| Madagascar | Not mentioned | Soft ticks | 2007-2008 | Ravaomanana *et al.* (2010) |
| Madagascar, West Africa, and Mozambique | II, I, VIII | Domestic pigs | 2000 | Bastos *et al.* (2003) |
| Malawi | I, II | Domestic pigs | 2019 | Hakizimana *et al.* (2020) |
| Malawi | V, VIII | Warthogs, Domestic pigs | 1960 | Bastos *et al.* (2004) |
| Malawi, Mozambique, Zambia | VIII, V | Domestic pigs | 2001-2003 | Lubisi (2005) |
| Mauritius | II | Domestic pigs | 2007-2008 | Lubisi *et al.* (2009) |
| Mozambique | II, V, XXIV | Soft ticks | 2007 | Quembo *et al.* (2018) |
| Mozambique | II, VIII, V, VI | Domestic pigs | 1998 | Bastos *et al.* (2004) |
| Namibia | I and XVIII | Domestic pigs | 2018 | Molini *et al.* (2020) |

| Location | Genotype(s) | Sample source(s) | Year of study/collection | Reference(s) |
|---|---|---|---|---|
| Namibia | Not mentioned | Domestic pigs | 2018 | Samkange *et al.* (2019) |
| Nigeria | I | Domestic pigs | 2007-2015 | Luka *et al.* (2017) |
| Southern Africa | III, XX | Soft ticks | 1985-1987 | Arnot *et al.* (2009) |
| | III, XIX, XX, XXI | Soft ticks | 1987-1996 | Zsak *et al.* (2005) |
| | I, III, IV, VII, VIII, XIX, XX, XXI and XXII | Soft ticks, Warthogs | 1987-2003 | |
| | XIX, VII, XVII–XXII | Soft ticks | 1973-1999 | Boshoff *et al.* (2007) |
| Tanzania | II, IX, X | Domestic pigs | 2015-2017 | Yona *et al.* (2020) |
| Tanzania | XV | Soft ticks | 2017 | Peter *et al.* (2020) |
| Tanzania | II, IX | Domestic pigs | 2015 | Chang'a *et al.* (2019) |
| Tanzania | X | Domestic pigs | 2013 | Misinzo *et al.* (2014) |
| Tanzania | XV | Domestic pigs | 2008 | Misinzo *et al.* (2011) |
| Tanzania | X | Domestic pig | 2009 | Misinzo *et al.* 2012 |
| Tanzania | Not mentioned | Domestic pigs | 2001 | Wambura *et al.* (2006) |
| Uganda | IX | Domestic pigs | 2007 | Gallardo *et al.* (2011) |
| Uganda | IX | Domestic pigs | 2015 | Masembe *et al.* (2018) |
| Uganda | IX | Domestic pigs | 2015 | Mwiine *et al.* (2019) |
| Uganda | IX | Domestic pigs | 2010-2013 | Atuhaire *et al.* (2013) |
| Zaire (DRC) | I | Domestic pigs | 1974-1989 | Malogolovkin *et al.* (2015) |
| Zaire, South Africa | IV, XX | Domestic pigs, warthogs | 1977 – Zaire 1999 – S. Africa | Ndlovu*et al.* (2020) |
| Zambia | I | Domestic pigs | 2015 | Thoromo *et al.* (2016) |
| Zambia | I, II, XIV | Domestic pigs | 2013-2015 | Simulundu *et al.* (2018) |
| Zambia | II | Domestic pigs | 2017 | Simulundu *et al.* (2018) |
| Zambia | VIII | Domestic pigs | 1988 | Bastos *et al.* (2004) |
| Zambia, South Africa | I, III, XXII | Soft ticks | 1983 – Zambia 2008 – S. Africa | Ndlovu *et al.* (2020) |

| Location | Genotype(s) | Sample source(s) | Year of study/collection | Reference(s) |
|---|---|---|---|---|
| **Zimbabwe** | **II** | **Domestic pigs** | **2015** | **Van Heerden *et al.*, 2017** |

The domestic cycle, on the other hand, occurs when the virus is transmitted among domestic pigs independent of sylvatic hosts or arthropod vectors (Costard *et al.,* 2009). The transmission of ASFV from sylvatic to domestic hosts is assumed to occur when there is a spill over due to infected ticks or contaminated products that is fed on domestic pigs at the wildlife-livestock interface (Ogweng, 2017; Quembo *et al.,* 2018). This warthog-tick sylvatic cycle is linked to the diversity of the ASFV circulating in certain areas in sub-Saharan Africa (SSA), particularly where the wildlife-livestock interaction occurs (Bastos *et al.,* 2009; Penrith *et al.,* 2013). On the other hand, the continued spread of the virus in domestic pigs may be attributed to virus spill from wild boars or the properly established domestic transmission routes that involve the movement of infected pigs, pig products or fomites (Guinat *et al.,* 2016).

The complex epidemiological pattern of the ASFV is evident in many countries of SSA where outbreaks have been reported. The genetic characterization of ASFV using standardized genotyping procedures during the 2013-2015 outbreak in Zambia linked the outbreaks to ASFV genotypes I, II, and XIV (Simulundu *et al.,* 2018). In the study by Masembe *et al.* (2018) on the genotyping of five ASFV from domesticated swine in Uganda, four sequences were found to be similar and closely linked to the only established genome sequence of p72 genotype IX. In Kenya and Uganda, two ASFV genotypes, IX and X, have been observed to be dominant in domestic pigs, warthogs, and ticks (Gallardo *et al.,* 2009, 2011; Okoth *et al.,* 2013). Genotype I of the ASFV which is considered to be highly fatal was linked to the random but persistent outbreaks of ASF in both confined and unconfined small-scale Kenyan swine farms between 2005 and 2011 (Abworo *et al.,* 2017; Gallardo *et al.,* 2009, 2011). According to Quembo *et al.* (2018), Malawi seemingly has the highest number of genotypes VIII ASFV variants, many of which are common in its neighbours; Mozambique and Zambia, indicating the possibility of transboundary nature and the need for regional collaboration for successful containment of the disease. Some novel ASFV genotypes which have previously not been established in SSA have also been identified, for instance, genotype XXIV in Mozambique (Quembo *et al.,* 2018), and XXIII in Ethiopia (Achenbach *et al.,* 2017). Nevertheless, the most intricate and different blend of ASFV genotypes has been reported in the eastern and southern regions of Africa as seen in Fig. 5 where the transfer of the virus occurs via both sylvatic and domestic cycles (Quembo *et al.,* 2018).

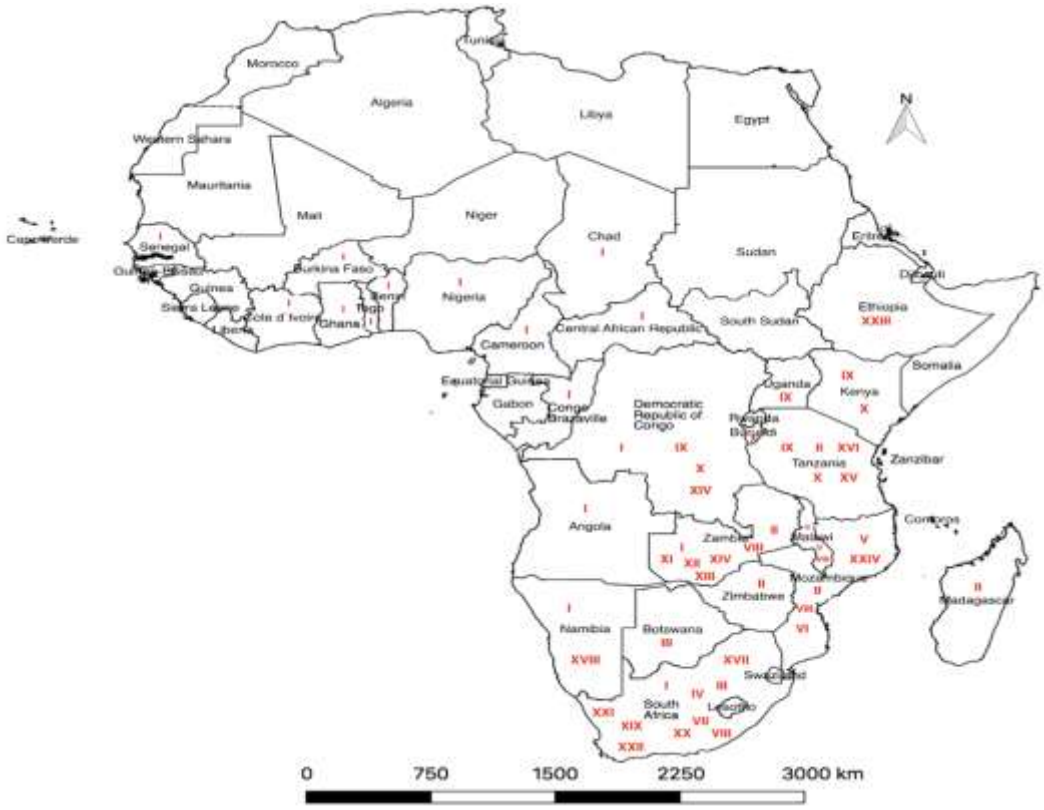



**Figure 5: A map showing the genetic diversity of ASFV p72 genotypes (written in red) in sub-Saharan Africa. The richest diversity is observed in the eastern and southern parts of Africa as compared to the western, central and northern parts**

The sylvatic cycle of the ASF in several countries in East and Southern Africa has been described in detail (Okoth *et al.,* 2013). While most of the ASFV genotyping studies in SSA have dwelled on the domestic cycle of transmission (Table 1), genotyping of ASFV in African warthogs has largely been overlooked, yet they are considered to be the main wild vertebrate hosts of the ASFV where the disease is endemic (Costard *et al.,* 2013). For instance, most of the surveillance studies on the circulating genotypes of ASFV in Tanzania have been limited to outbreaks in pig farms (Chang'a *et al.,* 2019; Misinzo *et al.,* 2011, 2014). Although other studies have attempted to establish the existence of ASFV genotypes in African warthogs and ticks (Bastos *et al.,* 2004; Gallardo *et al.,* 2011; Katale *et al.,* 2012; Ndlovu *et al.,* 2020; Peter *et al.,* 2020; van Rensburg *et al.,* 2020), they have not yet produced conclusive results. For instance, although seropositivity of ASFV was established in all sampled warthogs in the Serengeti ecosystem in Tanzania by Katale *et al.* (2012), the genetic makeup of the ASFV strains was not confirmed. Similarly, the serological evidence of ASFV was established at the wildlife-livestock interface of the Gorongosa National Park in Mozambique but little information is available on the presence or the genotypes of ASFV maintained in the wild hosts of the country's wildlife reserves. For the remaining parts of SSA, consistent data regarding the occurrence of sylvatic cycles are largely lacking (Quembo *et al.*, 2018).

Although warthogs are extensively distributed in the savannah parts of West Africa and the northern parts of Central Africa, the classical sylvatic cycle of ASF has not been demonstrated in these parts of SSA, probably because of the absence of *O. moubata* ticks in these regions (Etter *et al.,* 2011). As such, the transmission of ASFV in West Africa is purely independent of the sylvatic hosts (Hakizimana *et al.,* 2020), and most outbreaks are associated with the movement of infected pigs or pig products (Gallardo *et al.,* 2011). These contrasting epidemiological transmission patterns could be attributed to the genetic variability of the ASFV isolates from eastern and southern Africa that comprise 22 distinct genotypes as opposed to the high homogeneity in West African ASFV isolates of genotype I (Boshoff *et al.,* 2007; Lubisi *et al.,* 2005).

The rich genetic diversity of the ASFV is not only promoted by the sylvatic cycle but is also enhanced by the domestic cycle through the open borders and unrestricted movement of pigs and swine products. For instance, the transboundary spread of ASF in eastern Africa during outbreaks is mainly associated with the horizontal transfer of ASFV between pigs due to the unregulated movement of pigs and pig products across borders (Misinzo *et al.,* 2011, 2012; Wambura *et al.,* 2006). Several studies have documented the role played by cross-border pig movements concerning the outbreak of ASF in countries like Mozambique, Zambia, Tanzania, South Africa, Uganda and Kenya (Atuhaire *et al.,* 2013; Boshoff *et al.,* 2007; Gallardo *et al.,* 2009, 2011; Lubisi *et al.,* 2005; Misinzo *et al.,* 2011). For instance, during the 2010 ASF outbreak in Tanzania, genotype II of the virus was presumably introduced into the country from Malawi through cross-border transmission (Misinzo *et al.,* 2012). Similarly, the possibility of transboundary transfer of ASF in East Africa has been emphasized, particularly between Uganda and Kenya (Gallardo *et al.,* 2009, 2011). The ASF outbreaks in Kenya between 2006 and 2007 were due to a genetically similar virus to that isolated in Uganda around the same period (Gallardo *et al.,* 2011). Although the western and central parts of Africa have normally detected the existence of genotype I of the virus with low genetic variability, the dissemination of ASFV genotypes to western from eastern Africa has also been established (Boshoff *et al.,* 2007; Gallardo *et al.,* 2011; Lubisi *et al.,* 2005). This genotype has also been established in Kenya from the *p72* sequence of one isolate. Due to these trans-border transmissions, the ASFV genotypes initially believed to be specific to certain geographical locations have now been spread wider.

There is mounting evidence of the ability of apparently healthy pigs or persistently-infected animals to act as carriers of ASFV. Such carriers definitely contribute to the maintenance of

ASFV in pig production systems where clinical ASF outbreaks occur repeatedly. Studies in Uganda (Atuhaire *et al.,* 2013) and Kenya (Abworo *et al.,* 2017; Okoth *et al.,* 2013; Thomas *et al.,* 2016) have established ASFV sequences in healthy pigs, suggesting that less virulent strains could be circulating in pig populations in these countries. A separate study by Patrick *et al.,* (2020) also established the presence of ASFV in apparently healthy pigs in parts of the DRC. The disease is also increasingly becoming endemic in parts of south-eastern Africa in domesticated pigs that carry the virus but have gained resistance to it (Penrith *et al.,* 2004). Most investigations into the existence of ASFV in asymptomatic pigs have purely been surveillance studies and the genotypes of ASFV were not established. It is not known how the virus persists in endemic pig populations although it is assumed that survivors, sub-clinical and chronically-ill pigs all contribute to the maintenance of the virus (Gallardo *et al.,* 2015). Genotyping also suggests that the virus isolates associated with this condition can be of low or high virulence (Thomas *et al.,* 2016). Although the existence of long term carriers of ASFV has recently been disputed by some researchers (Stahl *et al.,* 2014, 2019), these studies provide evidence that isolates with reduced virulence could be circulating in SSA.

### 2.4.1 African Swine Fever Status in Tanzania

African swine fever was for the first time reported in Tanzania in 1914, followed by another reported outbreak in 1962. Major epidemics of ASF were reported in Tanzania between 1987 and 1988 although the disease is believed to have been present in the country longer than this stated periods (Wambura *et al.,* 2006). From the 2000s, Tanzania has been experiencing a number of frequent and persistent ASF outbreaks and now, the disease is considered to be endemic in many regions of the country with several outbreaks. Trends indicate the increase in both frequency and duration of the disease outbreaks from 2005 to 2013 (Misinzo *et al.*, 2014). The most frequently affected areas are in the northern (Kilimanjaro, Arusha) and southern highlands regions of Tanzania (Mbeya, Rukwa, Iringa, Katavi and Songwe) which include but not limited to Rombo, Babati, Longido, Kyela, Chunya, Ileje, Mbarali, Rungwe, Tukuyu and Ludewa districts. The disease has also been reported in Sumbawanga district in Rukwa region and Kilombero and Kilosa districts of Morogoro region (Chang'a *et al.,* 2019; Misinzo *et al.,* 2011, 2014). In all the affected regions and districts severe effects of the disease were recorded in domesticated pigs of both sex and all age groups. In Tanzania, the commonly circulating genotypes are genotype II, IX, X, XV and XVI according to p72 gene. Towards the end of 2015, an outbreak of the disease was reported in the Lake Victoria zone (Mwanza and Kagera regions) where it was last reported in 2005, some 10 years back (Misinzo *et al.,* 2011). The

isolates from Kagera and Mwanza were yet to be identified (Unpublished report from Veterinary Investigation Centre in Mwanza, 2015). However, the samples collected from Kahama district in Shinyanga region from the same zone were identified as genotype IX (Misinzo, *et al.,* 2011).

### 2.4.2 Transmission Pathways and the Role of Sylvatic Cycle in African Swine Fever Outbreaks

African swine fever virus is believed to be an ancient virus of the soft ticks (*Onithodoros moubata* complex group) which infected wild pigs for decades and the wild hosts that adapted to it display only mild or no symptoms (Dixon &Wilkinson, 1988; Haresnape *et al.,* 1988).
Wild pigs commonly found to be infected by ASFV include warthogs (*Phacochoerus africanus*), bush pigs (*Potamochoerus larvatus*), giant forest hogs (*Hylochoerus meinertzhageni*) and red river hogs (*Potamochoerus porcus*) (Anderson *et al.,* 1998; Thomson, 1985) but warthogs have been known to be the most significant vertebrate hosts of the sylvatic cycle. Three cycles are known to be responsible for the spread and maintenance of ASFV in Africa and beyond as hypothetically summarised in Illustration 1.

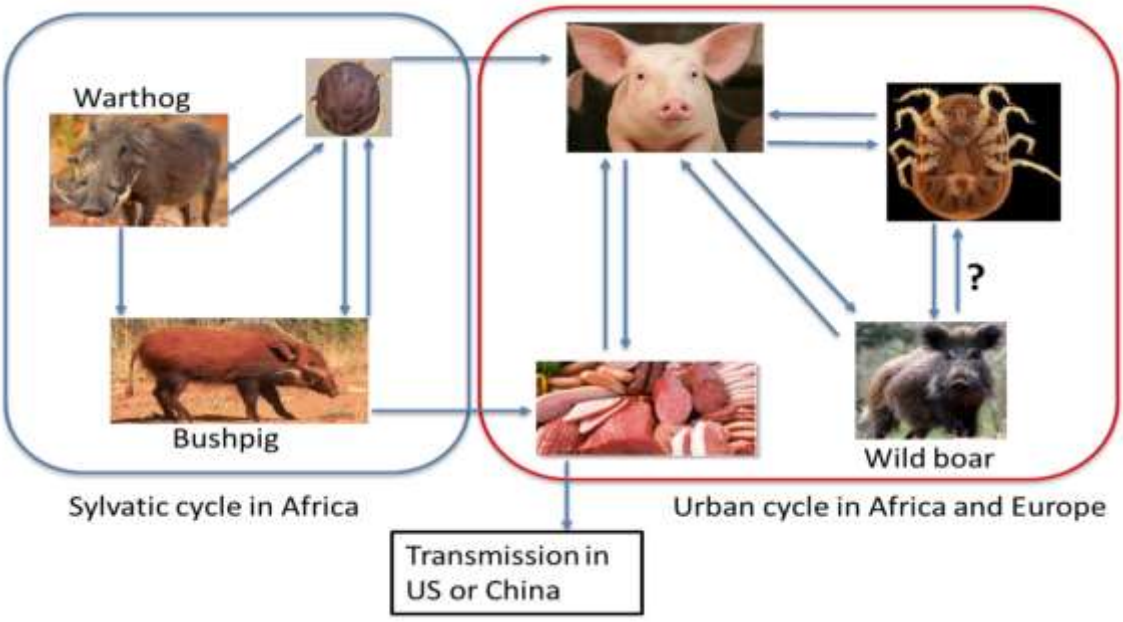



**Illustration 1: The transmission routes of African swine fever virus in different parts of the world (Petrovan, 2019)**

A sylvatic cycle between a tick vector belonging to the *Ornithodoros moubata* complex group (usually *Ornithodoros moubata porcinus*) and warthogs has been characterised in the past (Abworo, 2012; Anderson *et al.,* 1998; Bastos *et al.,* 2009; Lubisi *et al.,* 2005; Plowright *et al.,* 1969; Ravaomanana *et al.,* 2011). Bush pigs and giant forest hogs have also been found to be infected but their role in maintenance and transmission of the disease, if any, remains unknown.

The second cycle involves the tick vector and domestic pigs and the third, considered as the most important, is the pig-to-pig cycle through direct contact, also known as the domestic cycle. The wild boar (*Sus scrofa*) cycle is referred to as the fourth cycle that has been associated with ASF transmission in Europe and Asia (Bastos *et al.,* 2009).

A study conducted in warthogs in the Serengeti National Park in Tanzania revealed seropositivity in all the sampled animals although the responsible genotype was not identified (Katale *et al.,* 2012). Several species of *Ornithodoros* ticks have been identified and distributed worldwide but the most important species in the sylvatic cycle includes *O. moubata* and *O. moubata porcinus* (Haresnape *et al.,* 1988; Ravaomanana *et al.,* 2011). Several studies have been carried out in south-eastern Africa to identify the possible mechanisms by which ASF has been maintained in the absence of recorded outbreaks (Abworo *et al.,* 2017; Gallardo *et al.,* 2011; Lubisi *et al.,* 2005; Lubisi *et al.,* 2007; Penrith, 2013). Maintenance in the sylvatic cycle and in carrier pigs have both been suggested as mechanisms by which the virus can persist between outbreaks in East Africa. In Kenya, the same genotype of the virus had been reported from wild and domestic pig population in the same ecosystem, suggesting a possible connection of the domestic pigs with the sylvatic cycle (Abworo, 2012). However, most outbreaks occurring in the region have not been associated with ticks and wild hosts (Sanchez-Vizcaino *et al.,* 2015). A previous study done in 1968 at Kirawira area within Serengeti National Park in Tanzania provided evidence of a 15% infection prevalence of *Ornithodoros* ticks collected from warthog burrows (Plowright *et al.,* 1969). The tick's ability to maintain and spread the virus venereally, transovarially and transtadially has been described (Kleiboeker & Scoles, 2001). The importance of the sylvatic cycle and its hosts in sub-Saharan Africa and the Indian ocean islands has been discussed in details in a previous review (Jori *et al.,* 2013). The role of scavenging pigs, free-living hosts and pigs recovering from ASF in the epidemiology of the disease is still questionable although some studies have mentioned that they play a role in virus transmission (Probst *et al.,* 2019; Thomson, 1985).

## 2.5 Progression and Clinical Manifestations of African Swine Fever

Entry of the virus into pig cells is mostly via the tonsils closest to lymph nodes. The virus migrates to the tissue organs via the blood stream. Direct contact with infected pigs and contaminated fomites as well as consumption of swills from infected animals forms the main route of exposure (Guinat *et al.,* 2016). *Ornithodoros* ticks get infected with the virus when feeding on infected pigs; the virus replicates in their guts and migrate to salivary glands. Ticks infected with ASFV can bite pigs and directly introduce the virus into the blood acting as

vectors for the virus from an animal to another. Entry of the virus into pig cells is by endocytosis which can be clathrin or receptor-mediated as indicated in Fig. 6 (Galindo & Alonso, 2017; Rodrõ *et al.,* 1998).

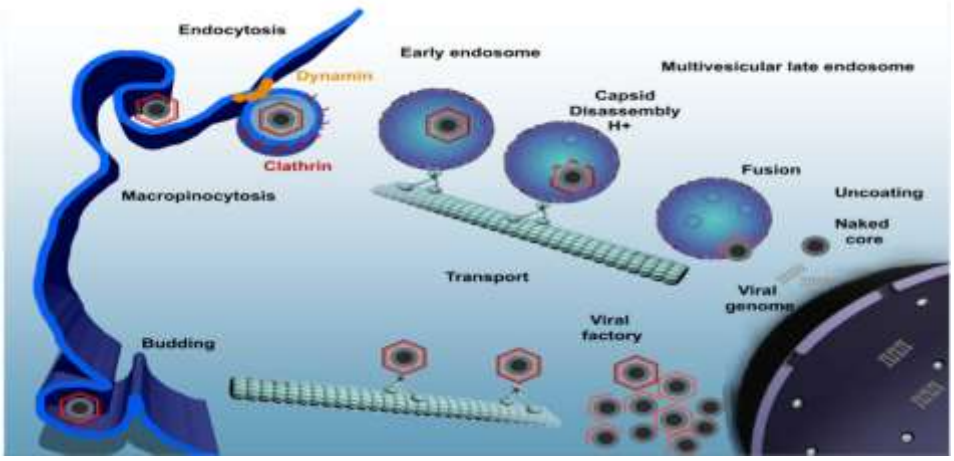



**Figure 6: Mechanism of ASFV entry into the animal cell, replication and release (Galindo & Alonso, 2017)**

When infecting a new herd, ASF is manifested by massive deaths of animals following high fever, loss of appetite, reduced movement and pigs clumping together. In the severe form of a disease, mortalities may occur in three days before other clinical signs are observed. However, appearance of the clinical signs may depend on viral genotype, animal breed, environmental conditions, incubation period and route of exposure (Jori & Bastos, 2009).

Petechial haemorrhages, mucoid diarrhoea and reddening of the skin around the ears, stomach and limbs are common in the less severe form of the disease (Plate 2) (Grzegorz *et al.,* 2016; Pikalo *et al.,* 2019). When a post-mortem examination is performed, haemorrhages are seen in internal organs including the lungs, lymph nodes, intestines, heart, kidney and the liver (Plate 3). The spleen increases in size and becomes darker than normal (Plate 3-c) (Sánchez-Cordón *et al.,* 2018).


**Plate 2: Clinical signs of ASF as observed in this study. An animal with fever and reddening of the ears and nose (2-a), Pinpoint haemorrhages in ear lobes (2-b), and Petechial heamorrhages on the skin surface (2-c)**

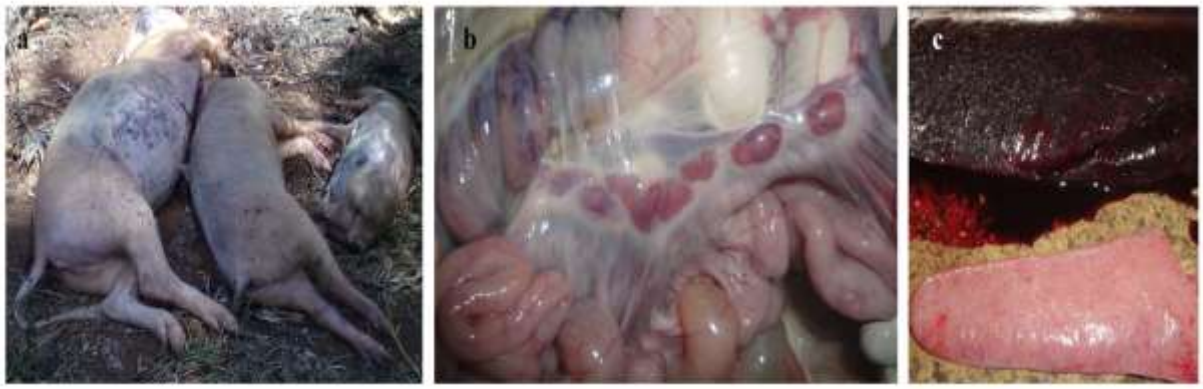


**Plate 3: Postmoterm features of ASF observed in this study with : (a)massive deaths of pigs (3-, (b) swollen lymph nodes and hemorrhages in the internal organs including intestines (3-b), and (c) enlarged spleen which is more darker in colour compared to a normal spleen**

## 2.6 Diagnosis of African Swine Fever

Several methods have been developed for the diagnosis of ASFV. Commonly used and OIE-recommended methods have been summarized in Table 2. The methods can be mainly categorized as those detecting host antibodies against the virus and those detecting the virus or its DNA. Antiviral antibodies may be detected long following an infection. Since there is no vaccine against the disease, presence of antibodies indicates exposure to the virus.

**Table 2: Summary of commonly used techniques for the diagnosis of African swine fever, their use, drawbacks and advantages**

| Virus Detection | Characteristics | Remarks |
|---|---|---|
| Haemadsorption test | The ASF virus is isolated from primary porcine macrophage cultures. ASFV is capable of infecting and replicating itself naturally in peripheral blood leukocyte cultures from pigs where, in addition to producing a cytopathic effect in the infected macrophages, it causes a characteristic effect of haemadsorption (HAD) prior to cell lysis. Under the microscope, it appears as rosettes of erythrocytes around the leukocytes. The haemadsorption technique is still the most sensitive and specific method of identifying ASFV, as none of the other swine viruses produce this effect. Despite the fact that haemadsorption is laborious to use and not as rapid as other diagnostic methods (with results taking 5–10 days), it is the technique of choice compared with other faster diagnostic | HAD is a technique currently used only in a few reference laboratories. HAD takes between 3 and 10 days to complete. |

| Virus Detection | Characteristics | Remarks |
|---|---|---|
| | methods, although it is important to bear in mind that some ASFV strains are non-haemadsorbing. In such cases, additional analyses of the cell sediment must be made, using the polymerase chain reaction technique or the fluorescent antibody test to confirm the presence of the virus. | |
| Direct immunofluorescence technique | The fluorescent antibody technique is based on the detection of viral antigens by staining cryostat sections or impression smears of tissues with fluorescein isothiocyanate (FITC)-conjugated anti-ASFV immunoglobulin. It is a very simple, rapid and sensitive method that can also be used on cell cultures infected with macerates of organs or tissues from suspect pigs. Under the microscope, infected cells display cytoplasmic inclusions that emit intense fluorescence. When the infection is advanced, the specific fluorescence can appear granular. Where the | FAT is a technique recommended only when polymerase chain reaction is unavailable or when there is insufficient experience with using PCR. It must not be forgotten that a negative result must be confirmed and it is recommended to carry out an antibody detection test in parallel. FAT takes 75 minutes to complete. |

| Virus Detection | Characteristics | Remarks |
|---|---|---|
| | infection is older than 10 days and antibodies have formed, these may block the conjugate and produce a false-negative result. For this reason, if FAT is the chosen technique, it must be used in parallel with an antibody detection test (indirect fluorescent antibody test, enzyme-linked immunosorbent assay or the immunoblotting test). | |
| Polymerase chain reaction (PCR) | PCR is a highly sensitive and specific technique that confirms the presence of the virus by amplifying the viral DNA present in the sample. The PCR technique uses primers from a highly conserved region of the genome to detect a wide range of known ASFV isolates, including haemadsorbing and non-haemadsorbing strains. It is currently used by reference laboratories for the virological diagnosis and confirmation of ASF. It can be used in both tissue samples and serum samples from | PCR is currently the most commonly used technique for etiological diagnosis but it calls for thorough training. PCR takes 5 to 6 hours to complete. |

| Virus Detection | Characteristics | Remarks |
|---|---|---|
| | animals with clinical signs, as it produces prolonged viraemia. The PCR technique can therefore be used to detect the virus in blood starting from the second day of infection and up to a period of weeks. | |
| Enzyme-linked immunosorbent assay (ELISA) | Techniques such as sandwich ELISA or immunodot blot have also been adapted for ASF, but are less commonly used because, even though they are extremely sensitive in the early phases of infection, this sensitivity diminishes drastically as from 9–10 days post-infection, as they may be blocked by antibodies, as described earlier in relation to FAT. | ELISA is not used routinely. It takes 3 hours to complete. |
| Indirect fluorescent antibody test (IFA) | IFA is a rapid technique with good sensitivity and specificity, where the specific antibodies present in the serum or exudates are made to react on a cell mat infected with the ASF virus. The reaction is displayed by adding iodinated protein A or a second fluorescein-labelled porcine anti-IgG antibody. | IFA is little used at present. There are no commercial reagents. It takes 2 hours to complete. |

| Virus Detection | Characteristics | Remarks |
|---|---|---|
| | Where positive samples are present on the cell mat, fluorescence appear at certain points close to the nucleus, which are the ASFV replication centres. | |
| ELISA | ELISA is the methodology used to carry out large-scale epizootiological and control studies. The ELISA technique currently used employs a soluble antigen containing most of the ASF virus proteins. This method is highly sensitive and specific, as well as being fast, easy and inexpensive. Recently, new ELISAs have been developed with non-infectious reagents, using the recombinant proteins p32, p54 and pp62 as viral antigens. These ELISAs are equally or more sensitive and specific than the current technique for analysing poorly conserved sera. | ELISA is currently the most commonly used technique, for which commercial diagnostic kits are also available. It takes 2 hours to complete. |

| Virus Detection | Characteristics | Remarks |
|---|---|---|
| Immunoblotting test | Immunoblotting is an immunoenzymatic technique whereby ASF viral proteins are transferred to nitrocellulose filters serving as the antigen strips on which the suspect serum is made to react, using protein A-peroxidase to detect specific antibodies. The immunoblotting technique is used to determine the reactivity of the antibodies present in the serum to different proteins induced specifically by the African swine fever virus. This characteristic, together with its high sensitivity and objectivity, makes immunoblotting the ideal serological diagnosis technique for confirming ASF. | No commercial diagnostic kits are available and the reagents are produced in some European Union and OIE Reference Laboratories. Immunoblotting is an excellent technique for serological confirmation in cases of doubt. It takes 3 hours to complete. |

**Sánchez-Vizcaíno (2010)**

## 2.7 Characterisation of African Swine Fever Virus

Multiple approaches have been used to characterize the ASF virus (Conley *et al.,* 2013). Molecular approaches have gained popularity and are now widely used for genotyping of both the virus and the mammalian host. These include biological markers as well as properties displayed by the virus in cultures. When cultured in the laboratory, cytopathic effects of infected cells and hemadsorption of red blood cells have been used to characterise the virus into either hemadsorbing or non-hemadsorbing strains (Rodríguez *et al.,* 2015). Infectivity and pathogenicity caused by the virus has also been used to characterise the virus as being highly, moderate or low pathogenic (de León *et al.,* 2013). Structural proteins, digestion by restriction endonuclease enzymes and the number of multigene families are also among different ways that have been used to study the virus (Dixon, 1986; Imbery & Upton, 2017; Misinzo *et al.,* 2014).

With the advancement in techniques including partial and whole genome sequencing, molecular techniques have taken over as the major approach to characterise the virus. Several regions of the virus have been used by scientists. The virus encodes 150 - 165 proteins with essential functions in its replication and evasion of host immune responses (Dixon *et al.,* 2013). A proteomic atlas of the ASFV has been provided by Alejo *et al.* (2018). Although about 54 of these proteins are structural (Dixon *et al.,* 2013), both structural and infection-related proteins have a role in modulating or regulating the host immune system evasion mechanisms such as the inhibition of host transcription factors that affect its replication (Reis *et al.,* 2017).

The ASFV genome is constituted of a conserved centre region of approximately 125 kb and two-variable ends that code for five multigene families (MGFs) that explains its variable genome size that ranges from 170 to 193 kb (Dixon *et al.,* 2013). Numerous MGFs are instrumental in determining the virulence of ASFV and the deletion of particular MGFs has resulted in attenuated phenotypes that facilitate immunity against challenges to its virulence (O'Donnell *et al.,* 2016). Although the critical role of MGFs in ASFV virulence has been demonstrated, the ability of the virus to develop antigenic variability to escape the host immune response is still unclear (Arias *et al.,* 2018). Likewise, the relationship of some genes in MGFs to host protection has not fully been described.

Currently, four different gene regions; *p72, p30, p54,* and *B602L* are commonly used in the identification of ASFV genotypes (Bastos *et al.,* 2004; Gallardo *et al.,* 2009, 2011; Nix *et al.,* 2006). The genotypic differentiation of ASFV genotypes is largely dependent on the

amplification and sequencing of the variable 3′-end of the *B646L* gene that encodes *p72* which is the main capsid protein (Boshoff *et al.,* 2007b). The sequencing of the *B646L* gene has defined 24 genotypes of the virus (I – XXIV) (Achenbach *et al.,* 2017; Arias *et al.,* 2018; Luka *et al.,* 2017). The detection of the novel genotype XXIV of the ASFV in Ethiopia (Achenbach *et al.,* 2017) suggests that there could be other ASFV genotypes yet to be revealed in SSA (Arias *et al.,* 2018). Analysis of the tandem repeats in the central variable region of the B602L gene (Gallardo *et al.,* 2009) or the intergenic region between the *I73R* and *I329L* genes at the right end of the genome (Gallardo *et al.,* 2014) can be used to distinguish closely-related ASFV isolates. The *B602L* gene is a particularly discriminative genetic marker whose sequencing has distinguished up to 31 subgroups of viruses with varying tetrameric amino acid repeats (Nix *et al.,* 2006). Many other gene regions like the *E183L, CP204L, and EP402R* encoding the *p54, p30,* and *CD2v* proteins, respectively have also proved valuable in the analysis of ASFV from various locations to trace its spread (Gallardo *et al.,* 2009, 2011). Studying of these regions of the virus is useful enough to enable tracking common sources of infection during an outbreak investigation and distinguishing viruses that are not of the same strain. This is particularly important in situations where the genotypes of the virus keep changing from time to time and at a particular time, different strains may be circulating in the same geographical area (Onzere *et al.,* 2018). Being a hotspot area for ASFV diversity, Tanzania has a complex epidemiological situation that requires constant survey for the virus detection through isolation, genotyping and sequencing of strains circulating in the country.

## 2.8 Prevention and Control of African Swine Fever

African swine fever being a trans-boundary animal disease (Beltran-Alcrudo *et al.,* 2019; Penrith, 2009), the focus for prevention and control includes the formal and informal interventions in national and international trade (Simulundu *et al.,* 2017). Even though quarantine measures are being practiced, disease introduction pathways are difficult to understand and use for risk estimation (Beltran-Alcrudo *et al.,* 2019). Moreover, although they are often put in place, biosecurity breaches have been often observed and reported (Chenais *et al.,* 2017; Nantima *et al.,* 2016). Evaluation of risk factors associated with occurrence or re-occurrence of the disease in an area has been used to provide information although such studies have not been practical enough to prevent the occurrence of the disease (Mur *et al.,* 2012; Martínez-López *et al.,* 2015). As has been the case with most animal diseases involving the wild and domesticated animals, methods such as vector control may require some modifications in the natural environment especially in the wild, rendering them difficult to implement (Artois,

2012). Most of the time, confirmation of ASF by detection of ASFV is done in OIE-approved laboratories (Oura *et al.,* 2013). These laboratories are often located several kilometres away from the outbreak areas that require shipment of samples over long distances, often using ground transport and thus delaying diagnosis. Although the zonal approved laboratories in the country provide remarkable technical capacity and acceptable biocontainment, it is however apparent that delays associated with remoteness hinder outbreak responses, the case that has been observed not only in animals but also in human viral diseases (Broadhurst *et al.,* 2016; Pollock &Wonderly, 2017).

Several studies have been undertaken in the past and more are ongoing in search for a good candidate vaccine for ASF. Techniques involved include adaptation of virus strains in cell culture to attenuate them although in these studies, such attenuated strains could not confer protection to the pigs upon exposure to virulent strains (Carlson *et al.,* 2016; Krug *et al.,* 2015; Sanford *et al.,* 2016). Deletion of genes associated with virulence has also been tried but the techniques were not reliable as the strains were able to regain the lost virulence gene when tested in the natural host or the dose difference between protective and lethal dose were too small and hence, unsafe for the animal (O'Donnell *et al.,* 2015, 2017). Techniques that have worked against other diseases such as di-codon deoptimization in which the virus is kept alive while reducing its virulence are still under trial (Steinaa *et al.,* 2019.)

# CHAPTER THREE

# MATERIALS AND METHODS

## 3.1 Description of the Study Area

Tanzania is the largest country in East Africa covering 945 200 km2 of which 60 000 km2 are occupied by water. It is constituted by mainland Tanzania and Zanzibar. Located on the southern part of the equator, Tanzania is bordered by the Indian Ocean and eight countries which are Kenya, Uganda, Rwanda, Burundi, Democratic Republic of Congo, Zambia, Malawi and Mozambique, positioning the country among the most bordered countries in Africa. The main economic activities of the country include agriculture, tourism, mining and fishing. Home to the best and biggest parks in Africa, Tanzania mainland is dominated by plains, useful for a vast of activities. Climatic conditions in the country are not extreme, mainly tropical with hot and humid coasts while the mountainous regions are cool. Long rains (March to May) and short rains (October to December) forms the two rainy seasons of the year with dry seasons in between (Shemsanga *et al.,* 2010).

A cross-sectional study was carried out to collect samples for the study between August 2016 and May 2017. For domestic pigs, snowball and convenient sampling strategies were used while targeting reported outbreaks and surveying the slaughter slabs and butcheries in the regions where no outbreaks were reported. Sampling for domestic pigs was carried out in selected regions of Tanzania (Fig. 7) with a representation of at least one region in each of the seven known agro-ecological zones. Five different regions of Tanzania reported outbreaks of ASF during the study period. The regions that had confirmed outbreaks were Manyara, Shinyanga, Morogoro, Rukwa and Mbeya. For the zones that no outbreak was reported, tissues were sampled at the butcheries and slaughter slabs. This decision was based on the experience from previous studies that have reported ASFV-positive samples in absence of a declared outbreak (Abworo *et al.,* 2017; Onzere *et al.,* 2018; Thomas *et al.,* 2016).

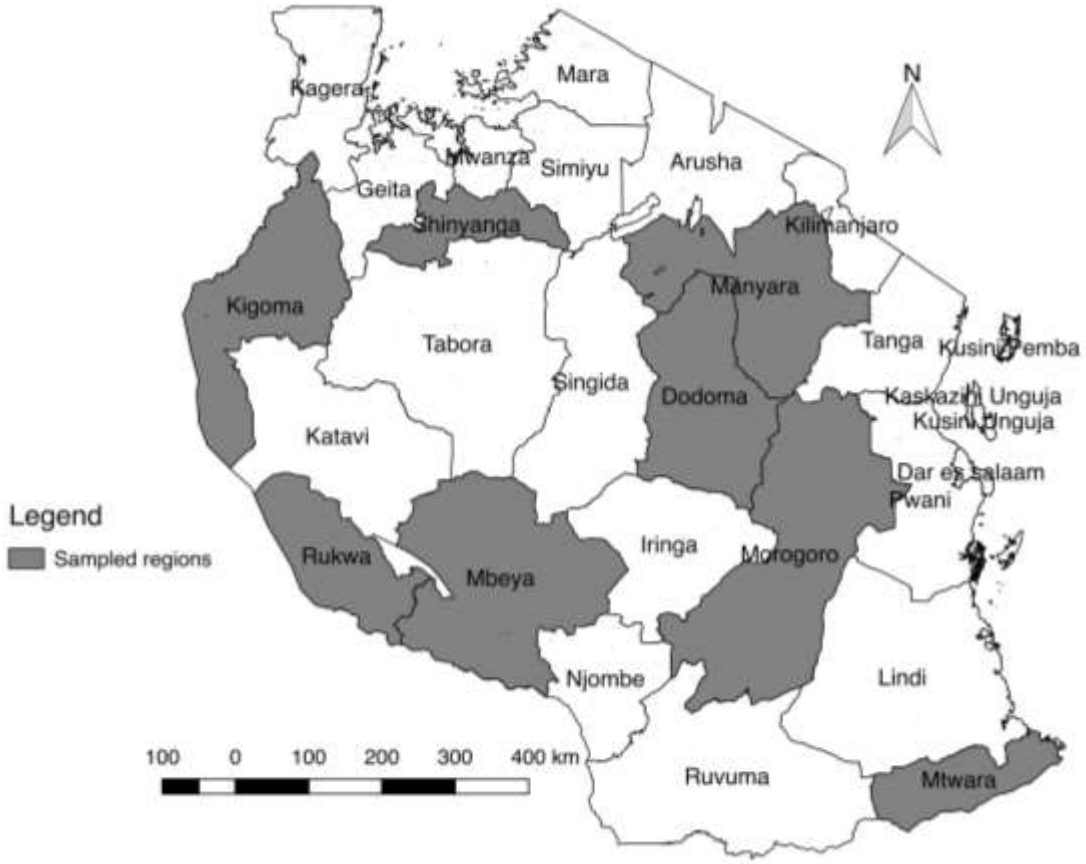



**Figure 7: Regions that pig samples were collected (shaded)**

For the sylvatic samples, sampling was conducted in Saadani ecosystem located in Tanga region on the north-eastern coast of Tanzania (Fig. 8). The Park is constituted by the former Mkwaja ranch, Zaraninge Forest Reserve and Saadani Game Reserve which, in 2005 were incorporated into the Tanzania national parks (Treydte *et al.,* 2005). Saadani National Park is about 1062 $km^2$ in size and it is the only Park with coastal-based wildlife conservation activities in Tanzania. The ecosystem falls into three districts of Handeni and Pangani in Tanga region and Bagamoyo in the Coast region. The distance from Saadani to the nearby major towns is 150 km to Bagamoyo, 200 km to Dar es Salaam, 75 km to Pangani and 40 km to Zanzibar. The Park is unfenced and is located between two villages, Mkwaja and Saadani, where major socio-economic activities including fishing, tour-guiding and salt-mining are carried out alongside conservation activities (Baldus *et al.,* 2001.).

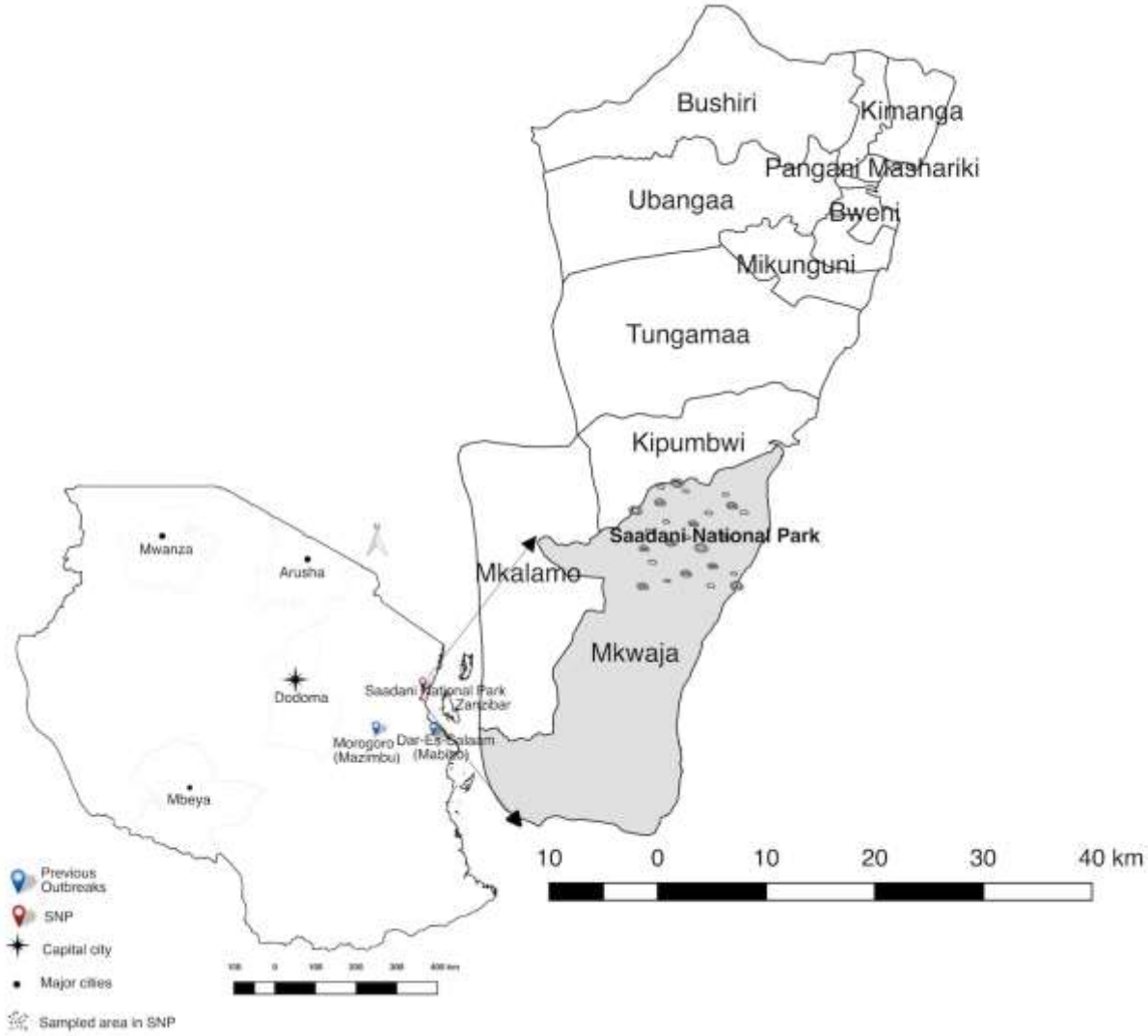



**Figure 8: A map of Tanzania showing the location of Saadani ecosystem where warthogs and ticks were sampled**

Saadani National Park has a coastal climate that is hot and humid, with the average temperature between 25 and 30 degrees Celsius throughout the year (Baldus *et al.,* 2001). The annual rainfall in the Park ranges between 500 and 1700 mm with a mean of 900 mm. The vegetation covering the Park includes mangrove forests around the Wami river to the Indian ocean with short and tall grass savannah and harsh black cotton plains in most areas (Cochard *et al.,* 2003). Domesticated animals observed in the study area include sheep, goats, cattle and chickens. The human population in Saadani village is dominated by Wazigua and Wadoe tribes who are mainly muslims and culturally consider any swine such as domestic pigs, warthogs or bush pigs as unclean animals. Any contact with them makes the person unclean, further explaining the absence of domestic pigs in the area. Thus, the Park was observed to have no interface with domestic pigs or pig farms and thus, suitable for studying a purely sylvatic host ASFV cycle. On the other hand, warthogs are very common in the area and frequently interact with human

dwellings. Thus, the Park was selected because it is an ecosystem with warthogs and *Ornithodoros* ticks but there is no interaction with domestic pigs.

## 3.2 Sampling Strategies

### 3.2.1 Sampling from Outbreak Areas

Outbreak investigation and notification was performed by the Ministry of Livestock and Fisheries through the zonal and regional veterinary officers. The field sampling team consisted of three veterinarians and one non-veterinarian scientist. Sampling was done following an official declaration of the outbreak by the district veterinary officer in charge and putting the area under quarantine. While in the field, biosafety and biosecurity measures to minimise the disease spread were strictly adhered to. Specific locations of the farms including GPS codes were recorded together with clinical signs observed in sick animals, sex, source and type of feed, who takes care of the pigs, other animal species located in the farm as well as the time in which the first detectable signs of the disease were observed by the farmer. Whole blood samples were collected using 10 mL vacutainers tubes (both plain and EDTA-tubes) from live sick pigs whereas, tissue biopsies such as liver, lung, kidney, spleen, mesenteric lymph nodes and heart were collected from dead pigs and placed in sterile 50 mL falcon tubes. All samples were transported in cool boxes with ice packs and were kept at -80ºC upon reaching the laboratory.

### 3.2.2 Sampling from Non-Outbreak Areas

In places where no outbreaks were reported/confirmed, samples were collected from slaughter slabs or butcheries. Tissue sections from spleen, lung, liver, mesenteric lymph node and kidney were collected and kept in a cool box containing ice to maintain a cold chain until they reached a refrigerator. Information on the sex of an animal and the location from which it came from was also recorded. Upon reaching the laboratory, samples were stored at -80ºC.

## 3.3 Sampling from the Sylvatic Cycle

Collection of study samples took place in the former Saadani Game Reserve which covers an area of around 200 $km^2$ while for logistic reasons excluded the two other components. This unique ecosystem captured the attention of this study because of the presence of warthogs, soft ticks and human beings in the absence of domesticated pigs.

### 3.3.1 Sampling of Warthogs

The field team was comprised of seven people. Animal handling was carried out by three veterinarians and two non-vet research scientists. Plate 4 shows some of the team members sampling warthogs in Saadani National park. Two park guards ensured the safety of staff and involved in sedating animals throughout the sampling processes.

**Plate 4: Photograph showing members of the team that carried out sampling activities in Saadani National Park**

Convenient sampling was done based on the road accessibility to vehicles and the allocated budget. Immobilisation of warthogs was done between 6 and 10 am when the warthogs were out of their burrows for feeding and later in the evening between 4 and 6 pm. Warthogs were darted from a vehicle or from a plain ground when the environment was safe using a compressed dart gun and plastic darts from Dan inject® (Daninject, DanWild LCC, Austin, Texas) loaded with a dosage of 0.04 mg/kg etorphine hydrochloride (M99®) (Novartis, Johannesburg) as an immobiliser at an approximate distance of less than 10 meters (Plate 2). Using a 16-gauge syringe, 10 mL of blood was collected from the jugular vein and immediately apportioned into a 5 mL plain- and 5 mL EDTA vacutainer tube. A total of 19 warthogs were sampled from Saadani National Park.

After blood collection, the animal was sprayed with a coloured wound spray, oxytetracycline (Alamycin®, Norbrook Laboratories, Ireland) at the needle injection site as disinfection and subsequent identification of a sampled animal to avoid re-sampling. Plate 5 shows a series of activities that took place while sampling warthogs in the Park. The immobilisation effects were reversed by intramuscular injection of 0.04 mg/kg diprenorphine hydrochloride (M5050®) (Norvatis, Johannesburg). Data on age group, sex and location of the animal were collected.

Immediately after collection, the samples in plain tubes were put in the rack for six hours to allow separation of the clot from serum. The serum was collected using disposable Pasteur pipettes and transferred into Eppendorf tubes. The blood in EDTA tubes and sera in Eppendorf tubes were immediately kept in a portable fridge at 4ºC until they reached the laboratory for longer storage at -80ºC while awaiting analysis.


**Plate 5:** **A series of activities for the sampling of warthogs in Saadani National Park where warthog darting using a gun is seen in (a). A serdated animal fell on the ground (b) followed by blood collection from a hind limb (c)**

### 3.3.2 Collection of Soft Ticks from Warthog Burrows

Since spotting all the burrows in the study area could require walking in the bush which was dangerous in the Park containing predators such as lions, only 12 were selected. Five active warthog burrows were identified following confirmation of the signs such as footsteps or presence of warthogs in the burrows prior sampling (Plate 6-b). Two days were dedicated for this activity where all the 12 burrows were visited and examined for the presence of ticks but were only found in the active burrows. For safety reasons, ticks were collected from the burrows when animals were out for feeding. The sand/soil was scooped from deep down the burrows using a long-handled spade (Plate 6-a), spread on a plastic sheet and exposed to strong sunlight for three minutes to stimulate movement and identification of ticks (Plate 6-c). The collected soft ticks (Plate 6-d) were placed into falcon tubes with perforated tops covered with a cloth material to ensure containment and ventilation. Once in the laboratory, ticks were preserved in 70% ethanol and kept in the refrigerator until analysis. Each burrow was considered a sampling stratum in which individual ticks were sampled units.


**Plate 6:** **Tick sampling activities from warthog burrows in Saadani national park. Active warthogs burrows were identified and sampled (a and b). Sand was exposed to the sun (c) to collect soft ticks (d)**

### 3.4 Preparation of Tissue Lysates/Suspensions

Among the tissue samples collected, from ASF-suspected cases, the spleens were the most preferred because it is known to have high concentration of the ASF virus (Thomson *et al.*, 1980). However, other tissue samples were collected, analysed in the laboratory and stored as tissue aliquots. Using sterile surgical blade, spleen samples were sliced into small pieces (~1 gram). Tissue slices were placed on sterile sieves positioned on top of sterile dishes prior to being crushed using a pestle allowing the debris to remain on the sieve and the supernatant to pass into the dish. The sieve was rinsed using 500 µl sterile PBS (pH 7.4) containing 5 µg/mL gentamycin sulphate (BioWhittaker, Walkersville, Maryland) and the PBS drained onto the dish. The tissue lysate in PBS was then pipetted out of the dish and transferred into a sterile 1.5 mL Eppendorf tube. The lysate was centrifuged at 14 000 rpm for 10 minutes to get rid of the tissue debris and the resultant supernatant was pipetted out and transferred into a sterile 1.5 mL Eppendorf tube and stored at -20ºC. All preparations for the tissue lysates were carried out in a biosafety cabinet while observing sterile conditions to avoid cross contamination between samples. The lysate obtained from this step was used for extraction of genomic DNA for ASFV validation, genotyping, selective whole genome amplifications, next generation sequencing and as an inoculum for virus isolation. These activities were carried out at the molecular biology laboratories based at BecA-ILRI, Nairobi.

### 3.5 Virus isolation on Peripheral Blood Mononuclear Cells

#### 3.5.1 Preparation of Peripheral Blood Mononuclear Cells

Virus isolation and cell culture activities were carried out at the Molecular biology laboratories based at BecA-ILRI, Nairobi. Although alveolar macrophages, Vero cells and immortalised cells have been employed to isolate ASFV (Carrascosa *et al.*, 2011). Peripheral blood mononuclear cells (PBMC) were used in this study.

Briefly, blood was collected intravenously from a donor European pig using a BD Vacutainer® needle (gauge x length: 21 x 1-1/2 inch). The blood was allowed to drain into 15 tubes of 10 mL BD Vacutainer® heparin-treated tubes. The blood from the tubes was pooled in a sterile beaker making a volume of 150 mL. This was distributed into six 50 ml BD Falcon® tubes at equal volumes of 25 mL and mixed with an equal volume (25 mL) of sterile PBS (pH 7.4). The diluted blood was layered onto Ficoll-Paque PLUS manufactured by GE Healthcare Life Sciences (Chicago, Illinois) at a ratio of 3:2, respectively prior to being centrifuged at 2500 rpm for 30 minutes to separate blood constituents (no brakes during deceleration). The PBMC

monolayer from each tube, visually seen as a fine white layer in the interphase between the red blood cells and the clear fluid, was pipetted out and transferred into a sterile 50 mL BD Falcon® tube; 1x PBS was added into the tube and the PBMCs were centrifuged at 1800 rpm for 10 minutes to pellet the cells. The supernatant was discarded, and residual red blood cells were lysed by incubating the pellet with 3 mL of Tris-buffered ammonium chloride solution (pH 7.2) for 10 minutes at room temperature. A minimal volume of sterile 1x PBS (pH 7.0) was then added onto the PBMCs in each tube prior to pooling them into a single 50 mL BD Falcon® tube and centrifuged at 1200 rpm for 10 minutes. The supernatant was discarded, and the cell pellet washed by addition of 50 mL PBS and spinning at 1200 rpm for 10 minutes. The washing step was repeated twice to get rid of platelets.

The PBMCs were suspended in 10 ml of Roswell Park Memorial Institute Medium (RPMI) 1640 manufactured by Sigma - Aldrich® (Reference No. R8758-500M) supplemented with 10% autologous serum, 5 µg/mL gentamycin sulfate (BioWhittaker, Walkersville, Maryland) and penicillin-streptomycin solution (ATCC® 30-2300™) manufactured by ATCC® at a final concentration of 50 IU/mL penicillin and 50 µg/mL streptomycin. The PBMC cells as seen in Plate 7 were counted using a haemocytometer.

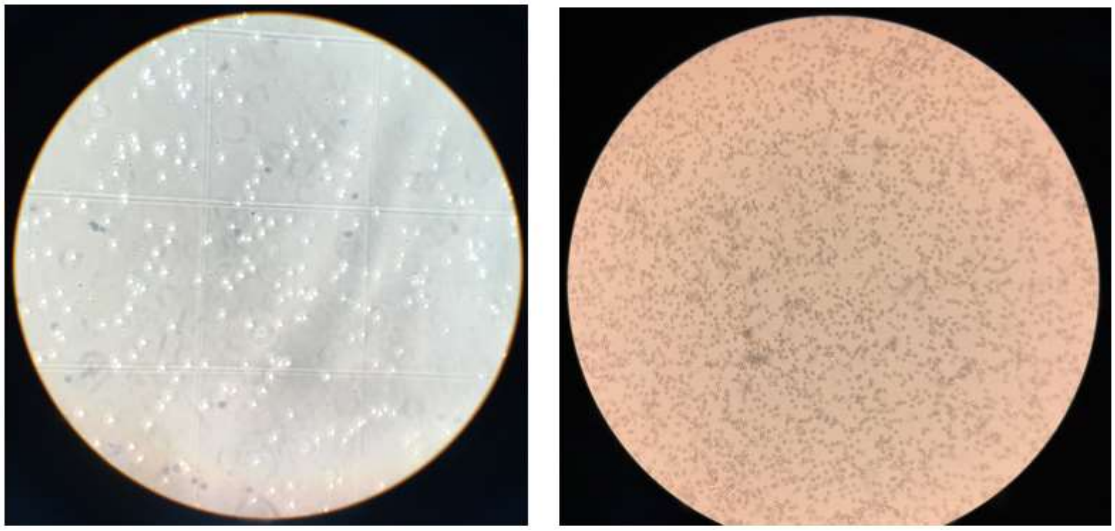


**Plate 7:** **Peripheral blood mononuclear cells after a harvest from pig blood. Live cells are shynny as seen on a haemocytometer (left). A good number of cells (right) was obtained for plating**

### 3.5.2 African Swine Fever Virus Isolation and Multiplication

Isolation of ASFV was conducted as previously described by Malmquist and Hay (1960). Briefly, 0.5 X 106 cells (obtained from section 3.5.1 above) in a 2 ml volume of the complete RPMI were seeded into each well of a 6-well Corning® Costar® TC-Treated culture plate

manufactured by Sigma-Aldrich®. The cells were incubated in the humidified chamber at 37°C and 5% $CO_2$ for three days to facilitate differentiation of mononuclear cells into macrophages. They were then inoculated with the thawed tissue suspensions (obtained from section 3.5 above) at a multiplicity of infection (MOI) of 1:10 and incubated for additional 24 hours (Thurber *et al.,* 2009). An aliquot of 50µl of 1% autologous red blood cells diluted in sterile PBS (pH 7.0) was added to each well and the plate was examined for evidence of haemadsorption over a 6-days period. To avoid virus modification following several passages while multiplying the number of viral particles, the ASFV isolates were blind-passaged once where the harvested culture was used as an inoculum to infect the fresh cell cultures.

### 3.5.3 Concentration and Purification of the Virus By Sucrose Gradient Ultracentrifugation

The harvested cells obtained from Section 3.5.2 above may burst by natural lysis and release viral particles in the culture medium. For the cells in which natural lysis did not occur, the viral particles are locked in the cytoplasm of the cells causing a need for engineered lysis such as freeze thawing to burst the cells and release viruses into the medium. A schematic illustration of virus particles release and purification is shown as adopted from a module in national programme on technology enhanced learning (NPTEL) in Illustration 2.

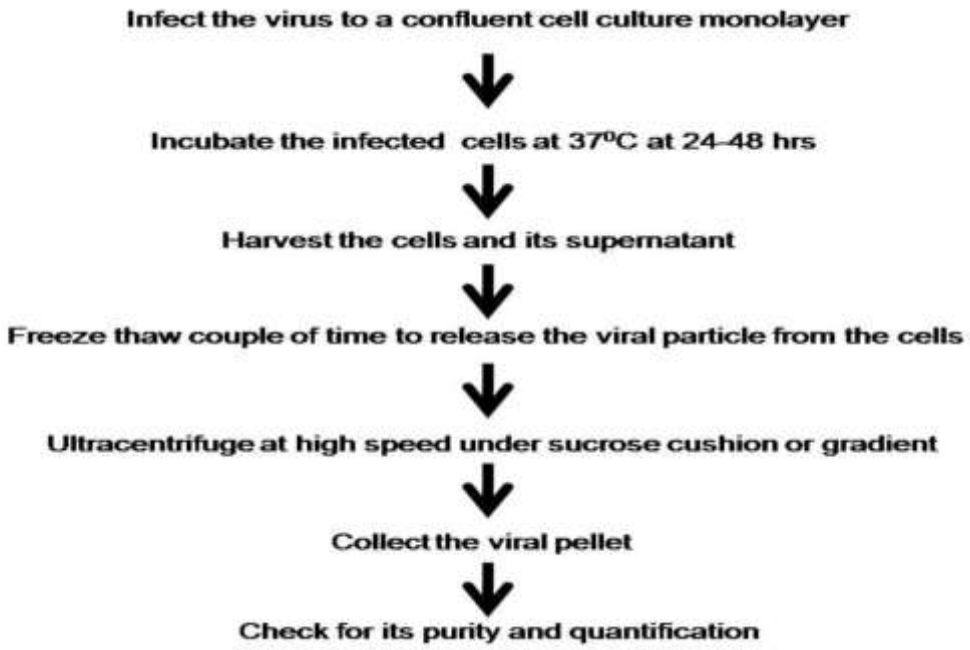



**Illustration 2: A schematic representation of stepwise procedures for releasing and purifying viruses from tissue culture**

Heat shock was used as the method of choice to release the viruses in the medium. In this process, the cells in a falcon tube were incubated in the water bath set at 37ºC for ten minutes followed by an immediate dip in liquid nitrogen for five minutes. This was followed by an immediate incubation in the water bath again for ten minutes and the processes were repeated three times. Purification of the cells to remove cell debris and most unwanted residues was done through 36% w/v sucrose cushion ultracentrifugation. Practically, tissue suspensions were thawed in water bath at 37ºC and vortexed for 60 seconds to dissolve any clumps. Centrifugation was performed at 950x g for three minutes at 4ºC. In addition, 7 mL of 36% sucrose were added in 10 mM Tris (pH 9) to a 12-ml thin-walled ultracentrifuge tube (#344059, 14x89 mm) (Beckman Coulter, Indianapolis). Furthermore, 5 mL of the virus suspension was layered onto the surface of the sucrose, slowly and carefully with a sterile transfer pipette without disturbing the sucrose layer. The tubes were then placed inside a bucket and balanced in a scale. Centrifugation was done at 30 000x g at 4ºC for 2 hours using the Optima XE-90 Ultracentrifuge in SW41 Ti rotor (Beckman Coulter, Indianapolis). After ultracentrifugation samples were removed, and the supernatant was carefully discarded. The pellet was re-suspended in 10 mM Tris (pH 9) in a total volume of 800 µl and the suspension was transferred in a 2 mL screw cap tube. The tube was sonicated at 57Hz for 1 minute and vortexed for 10 seconds. The virus was aliquoted in 200 µl volumes and stored in a -80ºC freezer until use. The obtained virus could be used as a seed to infect the new cell cultures in blind passages or in DNA extraction for genotyping and sequencing activities.

## 3.6 Extraction and quality control of Deoxy-Ribonucleic Acid

### 3.6.1 Deoxy-Ribonucleic Acid Extraction

Materials deployed for DNA extraction were the animal blood, tissue suspensions and harvested cell cultures depending on the activity to be performed. The 200 µl of material was used for DNA extraction using a DNeasy Blood and tissue kit (QIAGEN, GmbH-Germany) and performed according to the manufacture's protocol. For tick samples, total genomic DNA was extracted from ticks preserved in 70% alcohol using Qiagen DNeasy blood and tissue kit (QIAGEN, GmbH-Germany) with some modification on preparation of the ticks before extraction. Ticks were removed from 70% ethanol, dried on a bloating paper and placed in a clean 50ml falcon tube. The tube was topped with 25 mL MilliQ water and shaken slowly to avoid breakage of the ticks. Washing was repeated three times. Washed ticks were spread on a bloating paper and left to air dry for 15 minutes. Cleaned and dried ticks were put in 1.5 mL Eppendorf tubes. Using an empty Eppendorf tube, a small amount of liquid nitrogen was added

in the tube containing a tick and grounded using sterile plastic pestle (Bel-Art products, Wayne, USA) into a fine powder. Buffer ATL (180 µl) was added to the tick powder followed by 20 µl Proteinase K enzyme. Samples were incubated in a shaking incubator at 56ºC overnight to allow complete digestion of the samples. Quality and quantity of DNA was assessed using Nanodrop® and Qubit® spectrophotometry and verified by an agarose gel electrophoresis. DNA samples were aliquoted and stored at -20°C for further analyses.

### 3.6.2 Deoxy-Ribonucleic Acid Quality Control

Nanodrop spectrophotometer (ThermoFisher Scientific®, Waltham, Massachusetts) was the first quality control procedure to be performed once DNA was extracted or purified from the PCR product. Nanodrop spectrophotometer was loaded with 1µl of the sample and recorded. A good DNA gave a value of 1.7-2.0 as the ratio of absorbance between the wavelength at 260 nm and 280 nm (A260/A280), respectively. The Qubit® fluorometer was used to quantify the amount of intact DNA. Samples for qubit double stranded DNA high sensitivity (dsDNA HS) analysis were prepared according to the protocol supplied with the kit. Briefly, the Qubit® working solution was prepared by diluting the Qubit® dsDNA HS Reagent 1:200 in Qubit® dsDNA HS Buffer. For the standards, 190$\mu l$ of qubit working solution was added in the tube to which, 10 µl of standard was added. For the samples, 198 µl of the working solution was added followed by 2 µl of the sample. The tubes with 200 µl solution were vortexed and incubated at room temperature for two minutes and recorded. To visualize the obtained DNA an agarose gel electrophoresis was performed. For genomic DNA, a 1% agarose gel was prepared by dissolving 1 g of agarose powder in 100ml of 0.5x TAE buffer and run at 70 volts for 40 minutes. Lambda DNA of a known concentration was used as a positive control for genomic DNA. Good quality DNA remained intact on the well without movement. Intensity of the band was used to approximate the amount of DNA in the sample when compared to the lambda DNA. The PCR products where the DNA fragments were shorter, a 2% agarose gel was prepared by dissolving 2 g of agarose powder in 100ml of 0.5x TAE buffer and run at 70 volts for one hour to allow separation of the bands.

## 3.7 Laboratory Tests for African Swine Fever Virus

In order to address the study objectives, the first step was to identify the samples that were positive for ASFV. Two methods were deployed for diagnostic purposes in this study namely an antibody ELISA test and by PCR using ASFV specific primers.

### 3.7.1 Detection of African Swine Fever Virus Antibodies by indirect Enzyme Linked Immunosorbent Assay

The detection of ASFV antibodies utilised an indirect Enzyme-Linked Immunosorbent Assay (i-ELISA) (ID-VET, Grabels, France) that was performed on serum samples of both domestic pigs (n=100) and warthogs (n=19) according to the manufacturer's instructions. The test was considered valid if the optical density (OD) of positive control was more than 0.35 and the ratio between positive and negative control ODs was more than three. Samples were considered positive if the sample to positive control ratio (S/P%) was at least 40% (Cubillos *et al.,* 2013).

### 3.7.2 Detection of African Swine Fever Virus using Polymerase Chain Reaction

Conventional PCR using the OIE recommended primers PPA1 (5'AGTTATGGGAAACCCGACCC3') and PPA2 (5'CCCTGAATCGGAGCATCCT3') (OIE, 2013) was carried out on genomic DNA obtained from section 3.5 above as a confirmatory test for presence of ASFV. In summary, conditions for the PCR assay were as described by (Agüero *et al.*, 2003) where 4 µl of DNA, 12.5 µl of One Taq PCR buffer (Applied Biosystems) with the addition of 2.5 µl of $MgCl_2$, 0.5 µl concentrations of both primers (PPA-1/2) ten topped with 5 µl of PCR grade water to a total volume of 25 µl described previously [17]) and 0.625 U of Taq Gold polymerase (Applied Biosystems), in a total volume of 25 l. When the PPA-1/2 primer set was used, the reaction mixture was treated as follows: (a) incubated for 10 min at 95°C; (b) subjected to 40 cycles of PCR, with 1 cycle consisting of 15 s at 95°C, 30 s at 62°C, and 30 s at 72°C; and (c) incubated for 7 min at 72°C. When the OIE primer set was used, the reaction mixture was treated as follows: (a) incubated for 10 min at 95°C; (b) subjected to 35 cycles, with 1 cycle consisting of 30 s at 94°C, 30 s at 53°C, and 30 s at 72°C; and (c) incubated for 7 min at 72°C. Amplification products were analyzed by electrophoresis on a 2% agarose gel containing 0.5 g of ethidium bromide per mL.

For the positive samples, a size-based amplicon detection of a 257 bp region corresponding to the C-terminal region of the p72 major capsid protein encoded by the B646L gene (*VP72*) was detected on an agarose gel electrophoresis. The PCR reactions were performed in a total volume of 25 µL following standard PCR techniques.

### 3.7.3 Genotyping and genomic characterisation of African Swine Fever Virus

To characterize ASFV, samples that were positive for ASFV by using diagnostic primers PPA1 and PPA2 were subjected to genotyping PCR that targeted three polymorphic loci. These included the C-terminal region of B646L gene that encodes the major capsid protein p72 by using primers p72-U (5'GGCACAAGTTCGGACATGT3') and p72-D (5'GTACTGTAAC GCAGCACAG 3') which amplify a 478bp as suggested by Bastos *et al.,* (2003). DNA amplification was done at 95°C for 10 minutes as initial denaturation followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 48°C for 30 seconds, extension at 72°C for 1 minutes and incubation at 72°C for 10 minutes. Complete gene E183L that encodes the p54 ASFV protein which is critical in the recruitment of envelope precursors to the assembly site was amplified in order to place the ASFV in major subgroups using the primers PPA722 (5'-CGAAGTGCATGTAATAAACGTC-3') and PPA89 (5'-TGTAATT TCATT GCGCCACAAC-3') flanking a 676bp DNA fragment (Gallardo *et al.,* 2009). The DNA amplification was done at 95°C for 10 minutes as initial denaturation followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds, extension at 72°C for 1 minutes and a final extension of 72°C for 10 minutes. For the finer insight between the closely associated viruses, the variation and distribution of amino acid repeats was investigated using primers ORF9L-R and ORF9L-F (Gallardo *et al.*, 2009). An amplicon with an estimated size of between 300 and 350 bp was obtained following the same PCR conditions as for the p54 amplification. The pictorial presentation illustrates stepwise activities (Illustration 3).

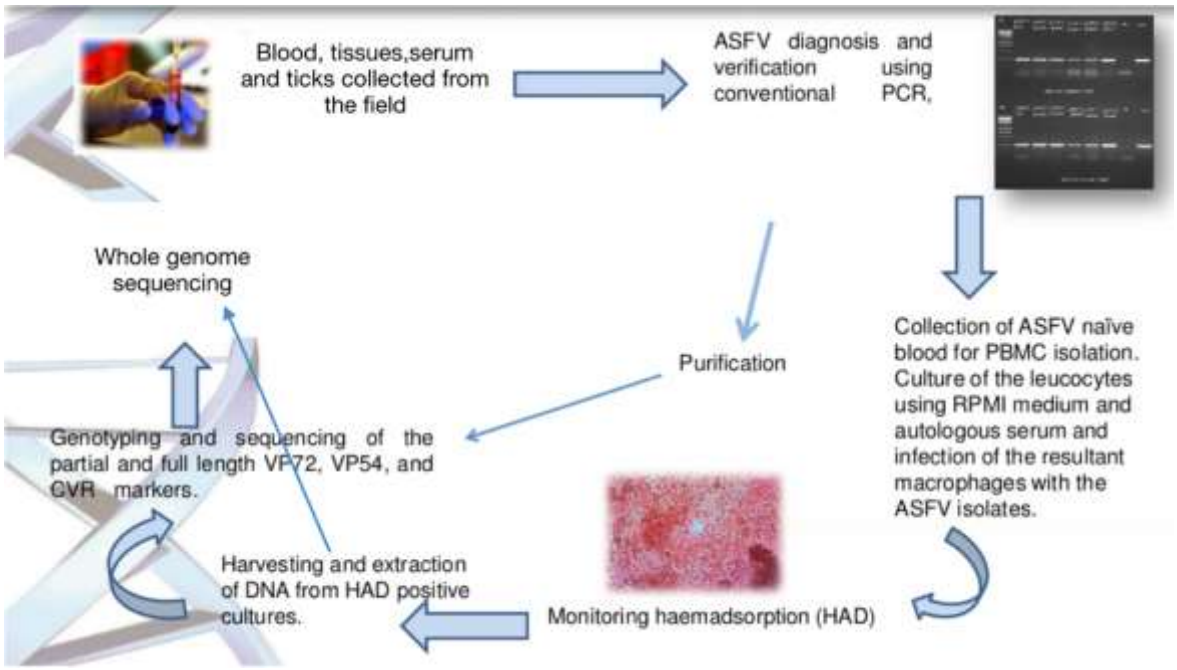



**Illustration 3: A series of activities for genotyping and sequencing of ASFV; from blood, tissue and ticks (Onzere, 2013)**

Amplification products of the expected size were purified using QIAquick PCR purification Kit (Qiagen, GmbH-Germany) following the manufacturer's recommendation and sent to Macrogen for Sanger sequencing (Seoul, South Korea). The flow of activities has been demonstrated in Illustration 4. In order to search for sequence similarity against other ASFV in GenBank, the obtained sequences were submitted to Basic Local Alignment Search Tool (BLAST) and sequences similar to them were downloaded with their respective accession number and used for comparison. Trimming, assembling and quality control were done using CLC main Work Bench version 7.8.1 software (Lubisi *et al.,* 2007; Misinzo *et al.,* 2011). A multiple alignment was created by using the ClustalW which included the sequences obtained from PCR products and equivalent regions from the majority of ASFV sequences available in GenBank. Three datasets were generated for phylogenetic analyses: The p72, Central Variable Region and P54 gene data sets. The phylogenetic tree was constructed by neighbour-joining method using Kimura-2-parameter option generated within MEGA 6.

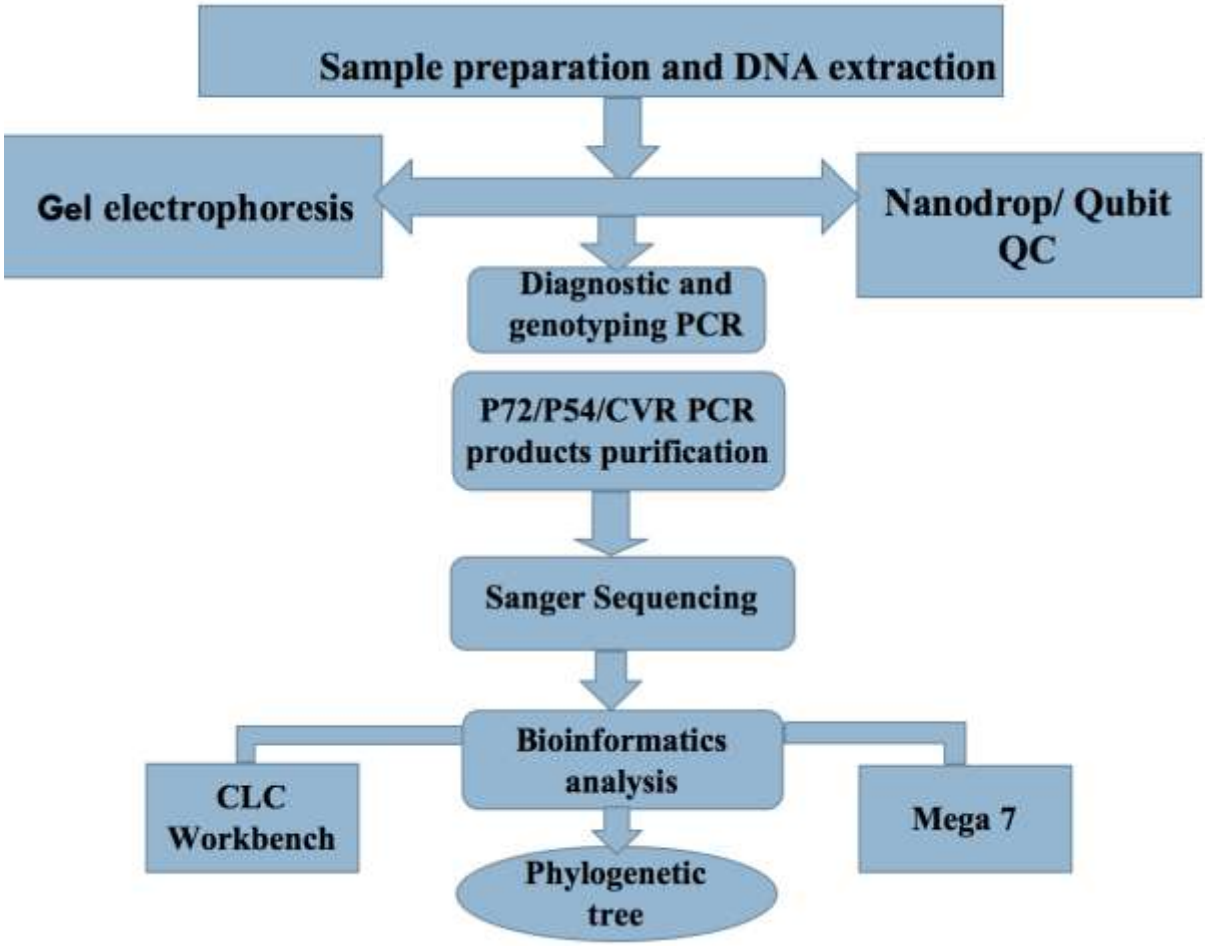



**Illustration 4: The flow of activities from DNA extraction to analysis of sequences**

### 3.7.4 Library preparation for viral Deoxy-Ribonucleic Acid

Preparation of DNA libraries, where fragments of DNA with variable lengths covering the whole genome were created and form an important step for genome sequencing. Library preparation was done as per the Nextera XT library preparation guide. In brief, tagmentation of genomic DNA, where the intact genomic DNA (gDNA) is fragmented into pieces using the Nextera transposomes followed by addition of tags with adapter sequences in their ends in a single step. The required volumes of 10 µL TD and 5 µL normalized gDNA were added to each well of a new Hard-Shell skirted PCR plate and pipetted to mix. This was followed by addition of 5 µL ATM to each well and pipetted to mix. Centrifugation was done at 280 × g at 20°C for 1 minute. The plate was placed on the pre-programmed thermal cycler and the tagmentation programme was run. When the sample reached 10°C, it was immediately removed from the machine and proceeded to the next step because the transposome is still active. Addition of 5 µL NT was done to each well and pipetted to mix. A centrifugation at 280 × g at 20°C for 1 minute was performed followed by an incubation at room temperature for 5 minutes. At this stage, the PCR plate contained 25 µL tagmented and neutralized gDNA, all of which used in the amplification step. Amplification of the tagmented DNA used a limited-cycle PCR programme that adds the Index 1, i7 (N), Index 2, i5(S), and full adapter sequences to the tagmented DNA from the previous step. The index adapters and Nextera PCR Master Mix were added directly to the 25 µL of tagmented gDNA from the previous step. Cleaning of libraries was done using AMPure XP beads to purify the library DNA and remove short library fragments.

To check for the quality which included the size and distribution of the libraries, 1µl of undiluted library was run on an Agilent Technology 2100 Bioanalyzer using a High Sensitivity DNA chip with outputs (Fig. 9). Typical libraries show a broad size distribution range of 250 to 1000 bp.

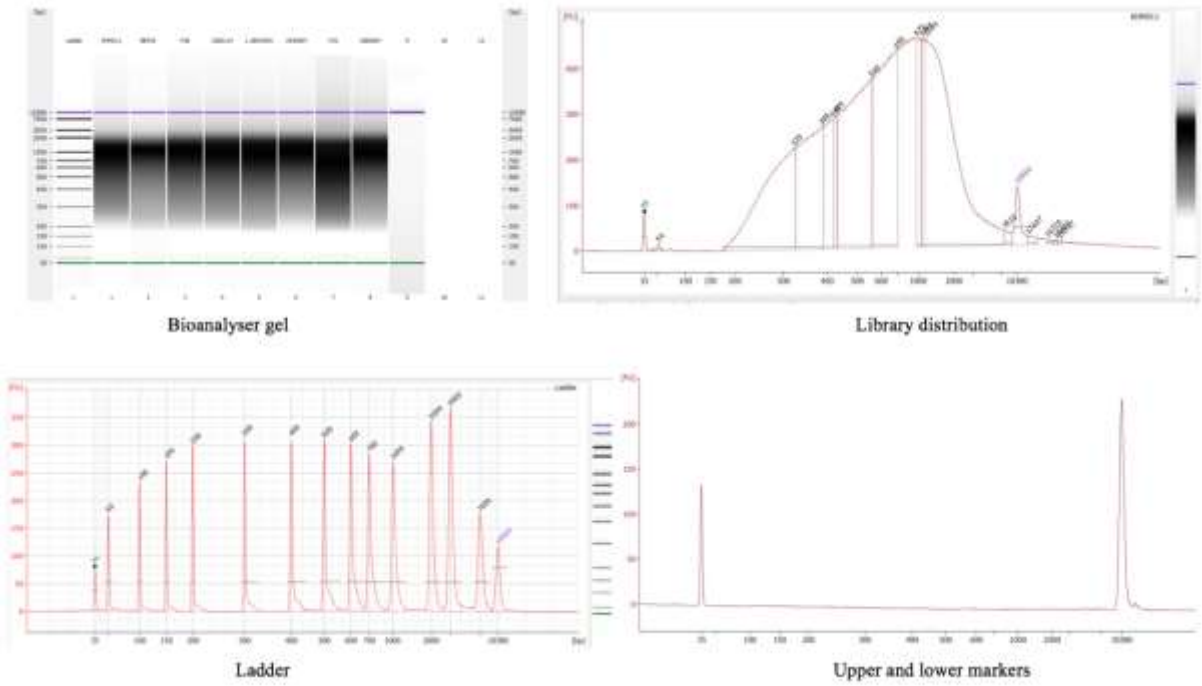



**Figure 9: Outputs from Bioanalyser 2100 machine showing the gel, ladder and library distribution located between the lower and upper markers**

### 3.7.5 Normalisation, Poolinga Sequencing of DNA libraries

Normalization of libraries was done to ensure an equal library representation in the pooled library. DNA library samples were normalized by diluting the sample with a sterile double distilled water to obtain a final concentration of 1ng of DNA in 1µL of the sample. Pooling libraries combined equal volumes of normalized libraries in a single tube. The flow cell C8NB7 used for the sequencing run was thawed at room temperature together with the sequencing reagents. Thawed reagents were pipetted to mix. A volume of 5 µL of the library was transferred into a new tube. The library was diluted to the loading concentration of the sequencing system using An Illumina MiSeq machine (M03021) for sequencing. The sequencing cycles run were 2*300 bp.

### 3.7.6 Nucleotide Sequencing and Phylogenetic Analysis

For the partial sequences, 4 µL of the PCR product were loaded into a 2% agarose gel for electrophoresis against a DNA ladder to assess for an expected DNA band. Samples that showed a band of expected size were purified by using a Qiaquick PCR purification kit according to the manufacturer's protocol. The purified products were transferred to Macrogen for Sanger sequencing (Seoul, South Korea). Sequences were cleaned, trimmed, cross-checked and contigs were formed by using CLC main workbench 7.9.1 software. The software was packed with necessary tools for analysis including BLAST, MUSCLE, CLUSTAL W

alignments and maximum likelihood phylogeny. Mega 7.0 software was used for construction of neighbour joining trees and FigTree v1.4.3 software was used for making better tree images. Reference sequences for analysis were downloaded directly from GeneBank (Nucleotide sequence NCBI). Sequences from one outbreak were identical hence, to make the phylogenetic trees less dense, two representatives from each outbreak were used.

For the whole genome sequence, a total of 54 public whole-genome ASFV sequences from Europe, Africa and Asia (attached as supplementary information) were downloaded from the servers European Nucleotide Archive (ENA) for the purpose of phylogenetic analysis and placement of the Tanzania/Rukwa/2017/1 isolate. After adding the Tanzania/Rukwa/2017/1 sequence, a multiple sequence alignment using mafft version 7.453 was generated (options --memsave --fft --maxiterate 2 --randomseed 148345). In order to trim the alignment from its gapped (> 10% gaps) and saturated (entropy h > 0.5) sites, BMGE v.1.12 (options -t DNA -g 0.1 -h 0.5 -b 10) was used which allowed a cleanup from 212,659 down to 169,170 sites suitable for phylogenetic inferences. FastTree version 2.1.11 (multi-threaded executable with OpenMP) was used to infer a phylogenetic tree using the GTR model of nucleotide evolution and a discrete gamma model with 20 rate categories (options -gtr -gamma -spr 4 -mlacc 2 -slownni for more exhaustive NNI steps during maximum-likelihood topology optimization).

### 3.7.7 Genome Assembly

The paired reads that were analyzed by the Illumina software as being part of the sample of interest were first trimmed using Trimmomatic (version 0.38) to remove adapter content and low-quality read ends. The exact trimming steps were as follows: ILLUMINACLIP: NexteraPE-PE.fa:2:30:12. LEADING:10. TRAILING:10 SLIDINGWINDOW:4:15 MINLEN:20. Out of the 3035,742 input read pairs, 92.90% survived as read pairs, 6.98% survived as forward only, 0.04% survived as reverse only, and 0.08% were dropped.

Bowtie2 version 2.3.4.1 was used to map the reads to a reference genomic dataset for the pig nuclear and mitochondrial genome. The reference encompasses 613 sequences corresponding to the (18+2) chromosomes and 593 scaffolds from the GCF_000003025.6 RefSeq assembly (*Sus scrofa* 11.1), together with a compendium of 327 complete mitochondrial sequences from diverse pig breeds, harvested through a GenBank. The mapping yielded overall alignment rates of 94.82% for paired reads and 91.44% for unpaired survivors (Individual reads that lost their pairs to an upstream step).

The reads that failed to align to the *Sus scrofa* reference were in a total of 473 890 pairs plus 72 928 unpaired reads. A unicycler version 0.4.7 was used, which on short-read data acts as a wrapper and enhancer for SPAdes (version 3.13.0). Results with or without the "-linear-seqs 1" option did not differ. All unmapped reads were used as the input to Unicycler (options '-1', '-2' and '-s' for paired reads 1, paired reads 2 and unpaired reads respectively). The best k-mer size for the assembly was 127, which yielded 505 contigs. After cleaning the graph and bridging contigs, the longest assembled segment obtained was of length 183 186 bp. It was the longest by far, since the second longest was of size 25 948 bp. As part of the Unicycler pipeline, all obtained segments were further polished by Pilon (version 1.23). The final assembly file contained 160 sequences. The resulting longest contig of length 183 186 bp is what is discussed here as the Tanzania/Rukwa/2017/1 full genome sequence.

### 3.7.8 Comparative Genomics

For comparative genomics analysis, reference genotype II whole genome sequences published in previous studies were retrieved from the European Nucleotide Archive. While all public genotype II sequences were closely related to the Tanzania/Rukwa/2017/1 sequence, the concentration was into three different geographical regions linked with the current pandemic of African swine fever virus: the most ancient genotype II isolate sampled outside of Africa, considered to be the index case of the current pandemic affecting Asia and Europe (Georgia 2007/1, formerly sequenced and published as FR682468.1; and most recently re-sequenced to produce the published sequence LR743116.1 – the one used here); the Polish strain Pol16_29413_o23 (MG939586.1, isolated in 2016 or 2017) and a Chinese strain ASFV-wbBS01 allegedly sampled in 2018 from a wild boar (MK645909.1). Further comparison of the Tanzania viral genome determined in this study was performed by mapping with the minimap2 program to the Georgia2007/1 isolate (LR743116.1).

### 3.7.9 Selective Whole Genome Amplification

This procedure used Phi29 polymerase which is an enzyme that amplifies DNA in a reaction using short oligonucleotide probes as primers. The sWGA reaction was performed in 0.2 mL PCR tubes. The reaction (50 µL total volume) containing 5ng of template DNA, 1× Bovine Serum Albumin (New England Biolabs, Massachusetts), 1mM dNTPs (New England Biolabs, USA), 2.5 µM of each amplification primer, 1× Phi29 reaction buffer (New England Biolabs, Massachusetts), and 30 units of Phi29 polymerase (New England Biolabs, Massachusetts), was placed in a PCR machine programmed to run a “stepdown” protocol consisting of 35°C for

5 min, 34°C for 10 min, 33°C for 15min, 32°C for 20 min, 31°C for 30 min, 30°C for 16h then heating at 65°C for 15min to inactivate the enzymes prior to cooling to 4°C.

# CHAPTER FOUR

# RESULTS AND DISCUSSION

## 4.1 Results

### 4.1.1 Detection of African Swine Fever Virus in a Sylvatic Cycle Devoid of Domestic Pig Involvement

The parts of the results focusing on warthogs, ticks and the virus detected in them are being presented in this sub-section. Sixteen out of nineteen (84%) warthogs were found positive for antibodies against ASFV using antibody ELISA with majority having OD above 50% indicating strong positivity (Fig. 10). Although 92% of the seropositive warthogs were adults, majority (86%) were females of which 82% were seropositive for ASFV (Table 3). However, when blood samples from the immobilised warthogs were tested by PCR, none of the 19 collected samples amplified for the presence of the viral DNA. This finding indicated that none of the sampled warthogs was in an active viremia state during the study period.

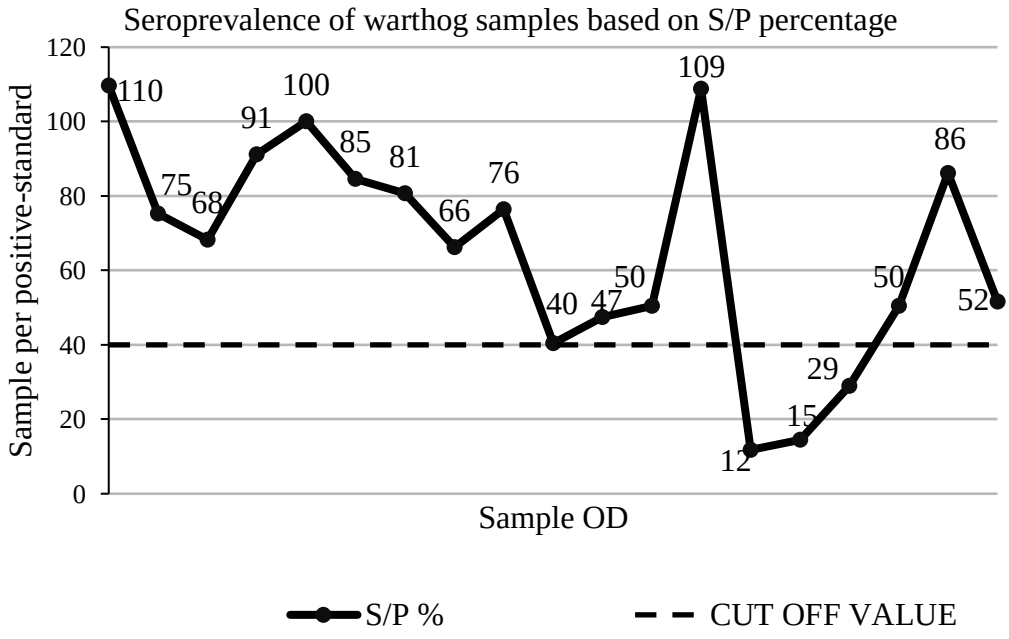



**Figure 10: The distribution of sample OD with reference to the S/P cut-off at 40%**

**Table 3:** **ELISA seropositive based on sex and age category of warthogs from Saadani National Park**

| Sex | ASFV antibody-ELISA | | | | Total |
|---|---|---|---|---|---|
| | Adult warthogs | | Juvenile warthogs (<4 months) | | |
| | Positive | Negative | Positive | Negative | |
| Male | 2 | 1 | 2 | 0 | 5 |
| Female | 9 | 0 | 3 | 2 | 14 |
| Total | 11 | 1 | 5 | 2 | 19 |

### (i) Identification and Classification of Ticks

A minimum of 12 and a maximum of 48 ticks large enough to be independent samples were recovered from one burrow. Although it was not possible to identify the ticks down to their genus level through microscopic examination, features as referenced from Saari *et al.* (2019) including leathery and wrinkled dorsal surface covered with nodules, lack of eyes, no rigid scutum and mouth not visible from above were typical for the *O. moubata* complex group (Plate 8). Nymph ticks were differentiated from the adults by the lack of a genital pore observed in the ventral view.

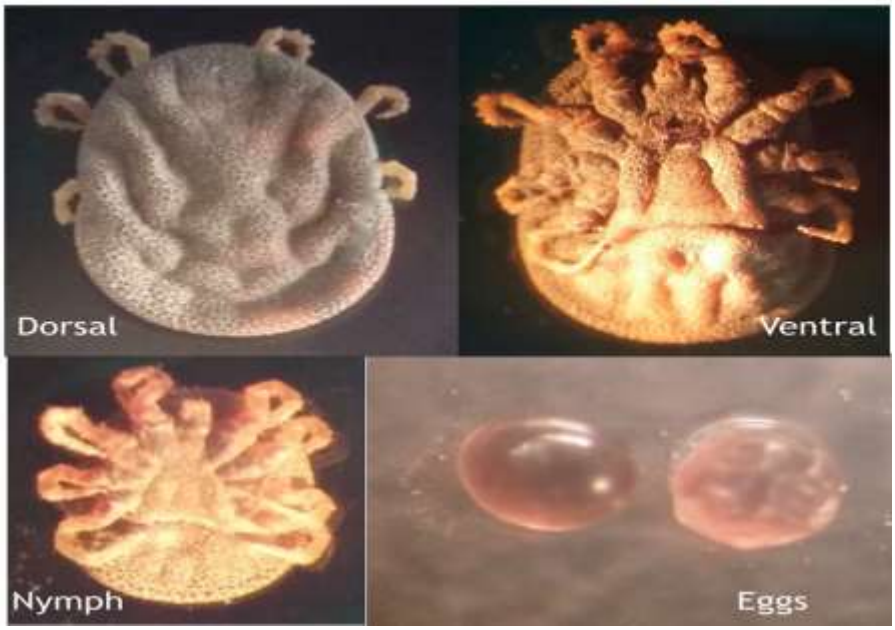



**Plate 8:** **Images showing features that are typical for ticks of the *Ornithodoros* complex group (Taken under 20x magnification of a compound microscope). The wrinkled surface and lack of visible mouth parts in dorsal view, as well as lack of a genital pore in nymph that is seen in the ventral view of an adult tick were some of the observed features**

Large ticks (5-8 mm) from each burrow were treated as an individual sample while small ticks (less than 5 mm in size) were pooled together. Actual size of ticks in mm was measured by a ruler as seen in Plate 9. A total of 111 large ticks were characterised individually.


**Plate 9:** **Actual size of ticks collected from warthog burrows. The ticks are measured in mm using a ruler**

**(ii) Diagnosis of African Swine Fever Virus from tick samples using the Polymerase Chain Reaction**

A total of 20 out of 111 (18%) ticks analysed individually were found positive to ASFV by conventional PCR (Table 3). An association study was also performed to relate the feeding status of the ticks with their ASFV status. It was found that majority of the analysed ticks (62%) had a blood meal prior collection. Of the infected ticks, 55% had a blood meal in their guts (Table 4).

**Table 4:** **Descriptions of ticks collected from warthog burrows indicating their feeding status and overall prevalence as detected by PCR**

| Burrow | Ticks | Engorged ticks | PCR positive | |
|---|---|---|---|---|
| | | | Engorged | Total ASFV-positive Prevalence %) |
| 1 | 48 | 31 | 5 | 9 (18.7%) |
| 2 | 20 | 15 | 2 | 2 (10.0%) |
| 3 | 17 | 4 | 0 | 3 (17.6%) |
| 4 | 14 | 10 | 3 | 3 (21.4%) |
| 5 | 12 | 9 | 3 | 3 (25.0%) |
| **Total** | **111** | **69** | **13** | **20** |

### 4.1.2 Genotyping of Viral DNA from Ticks

Following the detection of ASFV in ticks, it was necessary to determine the genotype to which the virus belongs. One positive tick sample per burrow (N=5) was sequenced for the p72, p54 and CVR regions.

**(i) Sequencing p72 (B646L gene)**

Phylogenetic analysis of the p72 region of the virus showed clustering of the Saadani sequences obtained in this study with sequences from 2008 ASF outbreaks in Tanzania (Figure 11), with strong support for the sequences being of genotype XV origin (Misinzo *et al.*, 2011).

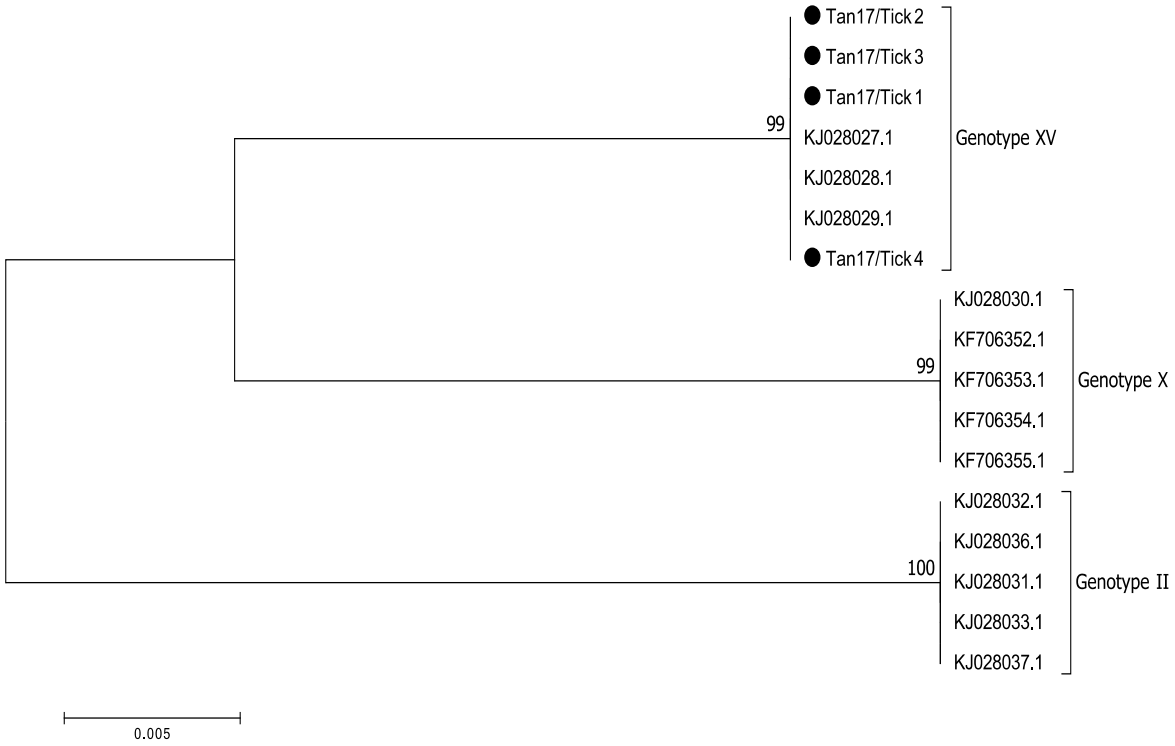



**Figure 11: p72 (B646L) phylogenetic tree showing the relationship between sequences from this study (•) with reference sequences from previous Tanzania ASF outbreaks (including sequences from 2008 that clustered under genotype XV)**

**(ii) Sequencing p54 (E183L gene)**

Sequence analysis of E183L gene (encoding the antigen p54) was used to differentiate between closely related ASF viruses of the same genotype due to the highly conservative nature of this region. Sequences obtained from this study clustered under the same genotype XV as those from 2008 ASF outbreaks in Tanzania at 100 bootstraps (Fig. 12).

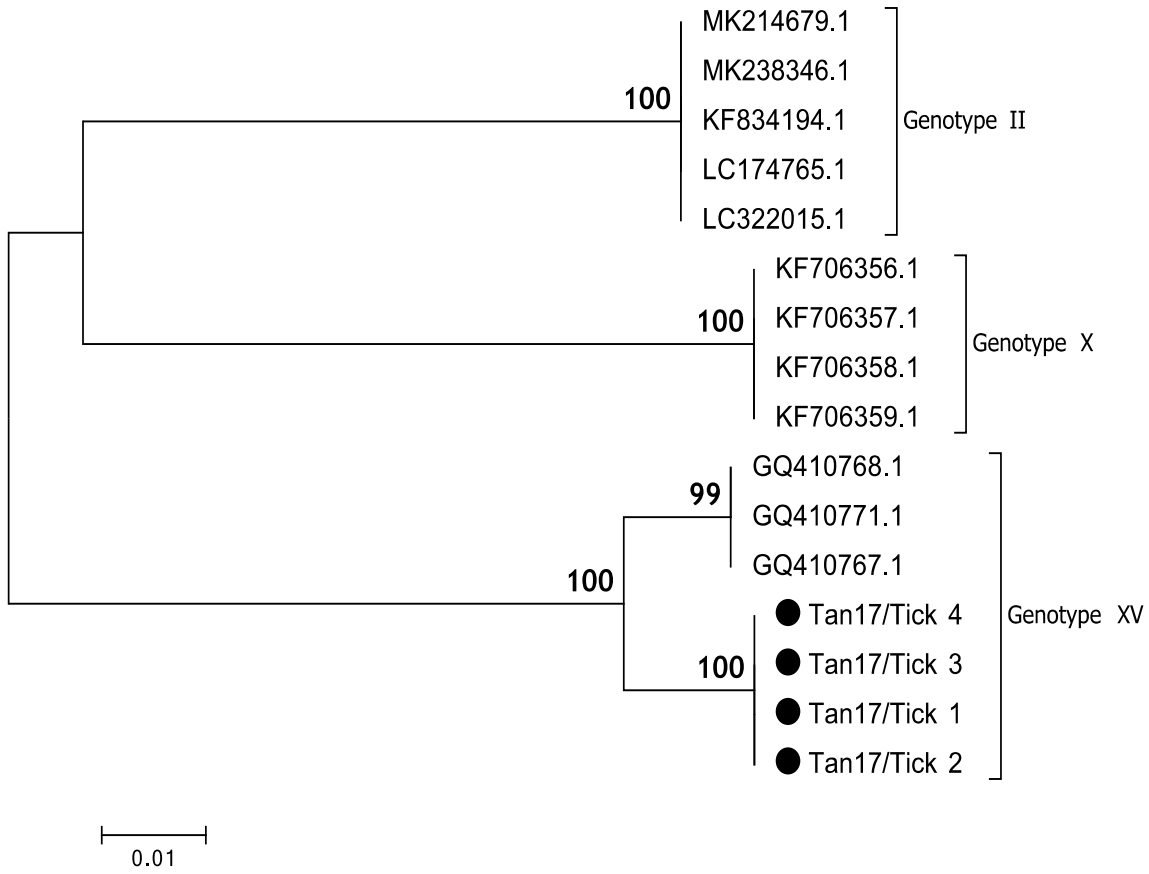



**Figure 12: Phylogenetic tree of p54 (E183L) DNA sequences from ticks (●) and reference sequences for genotypes II, X and XV retrieved from GenBank**

### (iii) Sequencing the Central Variable Region (B602L gene)

Comparison of the central variable region (CVR) was done with sequences from the 2008 outbreak. Sequences from this study belonged together with the references at a bootstrap value of 89 but sub clustered differently (Fig. 13). In an in-depth analysis, amino acids from the region were analysed by counting the tetrameric amino acid repeats known to exist in the CVR of ASFV isolates. The amino acid repeats known to be present in ASFV isolates are CAST/CVST/CTST (denoted as A), CADT/CTDT (B), GAST/GANT (C), CASM (D), CANT (F), CTNT (G), NEDT (M), NVDT/NVGT/ NVNT (N), NANI/NADI/NASI (O), RAST (H), SAST (S), NVNT (T), NAST/NADT/NANT (V) and SADT/ SVDT (W) (Nix *et al.,* 2006; Boshoff *et al.,* 2007; Lubisi *et al.,* 2007).

The results (Table 5) indicate that although the sequences clustered together under genotype XV, they did not resemble in the amino acid sequences suggesting they belong to different branches.

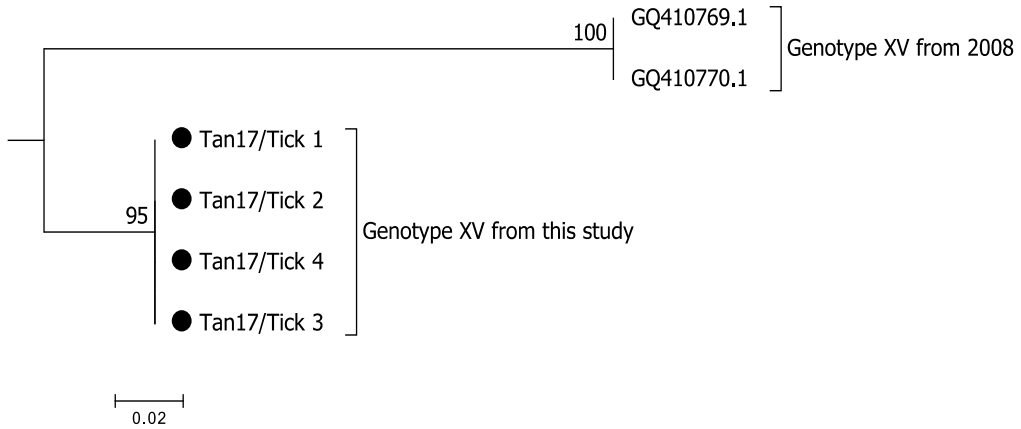



**Figure 13:** **Bootstrap relationship for the B602L CVR region for genotype XV sequences from ticks (•) and the 2008 Tanzania outbreak references from GenBank**

**Table 5:** **Tetrameric repeats of amino acid sequences obtained from the CVR of a genotype XV ASFV collected from Saadani (shorter) and from 2008 outbreak (longer)**

| ASFV Isolate | Tetrameric repeat | Number of repeats |
|---|---|---|
| Tan17/Tick 1-4 | AVUAVUVVAVVVVAVVUV | 18 |
| Mazimbu and Mabibo | AVUAVUVAVVUAVUVAVUVAVVUAVVUUUXV | 31 |

### (iv) Molecular characterization and phylogenetic study of African Swine Fever Virus isolates from Tanzania mainland (2016-2017) African Swine Fever Virus

A cross-sectional study for ASFV exposure in domestic pigs involving the seven agro-ecological zones of the mainland Tanzania was carried out in the period between August 2016 and May 2017.

#### *Screening of domestic pig samples for African Swine Fever Virus*

Samples collected from the various agro-ecological zones (Fig. 7) were screened by using an antibody ELISA kit (ID-VET, Grabels, France) and OIE recommended diagnostic primers PPA1 and PPA2. However, ASFV antibodies were not detected in sera collected from the sampled domestic pigs, a finding that will be elaborated later under the discussion section. A total of 302 samples were collected and 84 (28%) of them were confirmed positive for ASFV by PCR (Table 6). The DNA extracted from domestic pig samples was found to be of satisfactory quality and quantity (Plate 10) when analysed by the gel electrophoresis and nanodrop techniques. The samples were confirmed to be positive following an amplification of a 257bp size band following an agarose gel electrophoresis of the PCR product (Plate 11).

**Table 6: A summary of PCR positive samples and the detected genotypes from different zones of Tanzania in 2016-2017**

| Year | Zone | Sampling location | | Type of samples | Status when sampling | Number and samples tested | Number of positive samples | Genotypes |
|---|---|---|---|---|---|---|---|---|
| | | Region | District | | | | | |
| 2017 | Northern | Manyara | Babati | Blood Tissues | Reported outbreak | 508 | 4 | X |
| 2016 | Southern highlands | Mbeya | Mbalali, Mbozi, Chunya, Mbeya Urban, Ileje | Tissues | Endemic area | 30 | 25 | II |
| 2017 | Coastal | Morogoro | Kilosa, Mvomero | Tissues | Reported outbreak | 30 | 23 | II |
| 2016 | Southern | Mtwara | Masasi, | Tissues | No outbreak reported | 18 | 0 | - |
| 2017 | Western | Kigoma | Kasulu, Kigoma Urban | Tissues | No outbreak reported | 40 | 0 | - |
| 2017 | Central | Dodoma | Dodoma Urban | Tissues | No outbreak reported | 40 | 0 | - |
| 2017 | Southern highlands | Rukwa | Mpanda, Sumbawanga | Tissues | Reported outbreak | 30 | 28 | II |
| 2016 | Lake zone | Shinyanga | Kahama Urban | Blood Tissues | Reported outbreak | 506 | 4 | IX |


**Plate 10: Genomic DNA obtained after extraction from animal tissues as analysed by agarose gel electrophoresis. Lambda DNA was used as a standard**

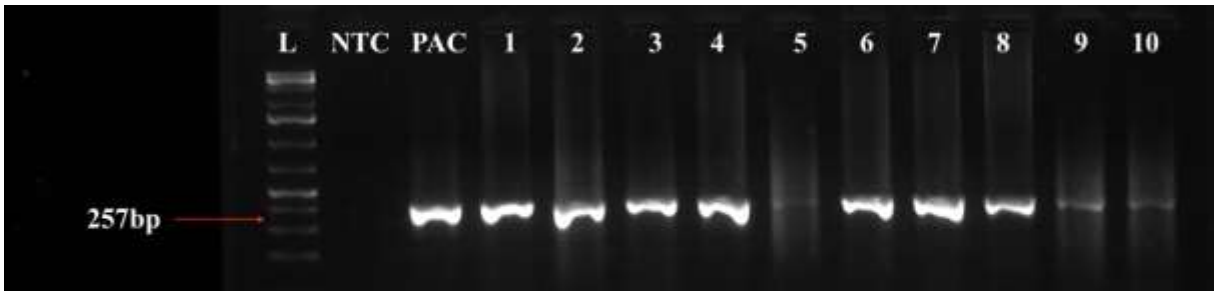
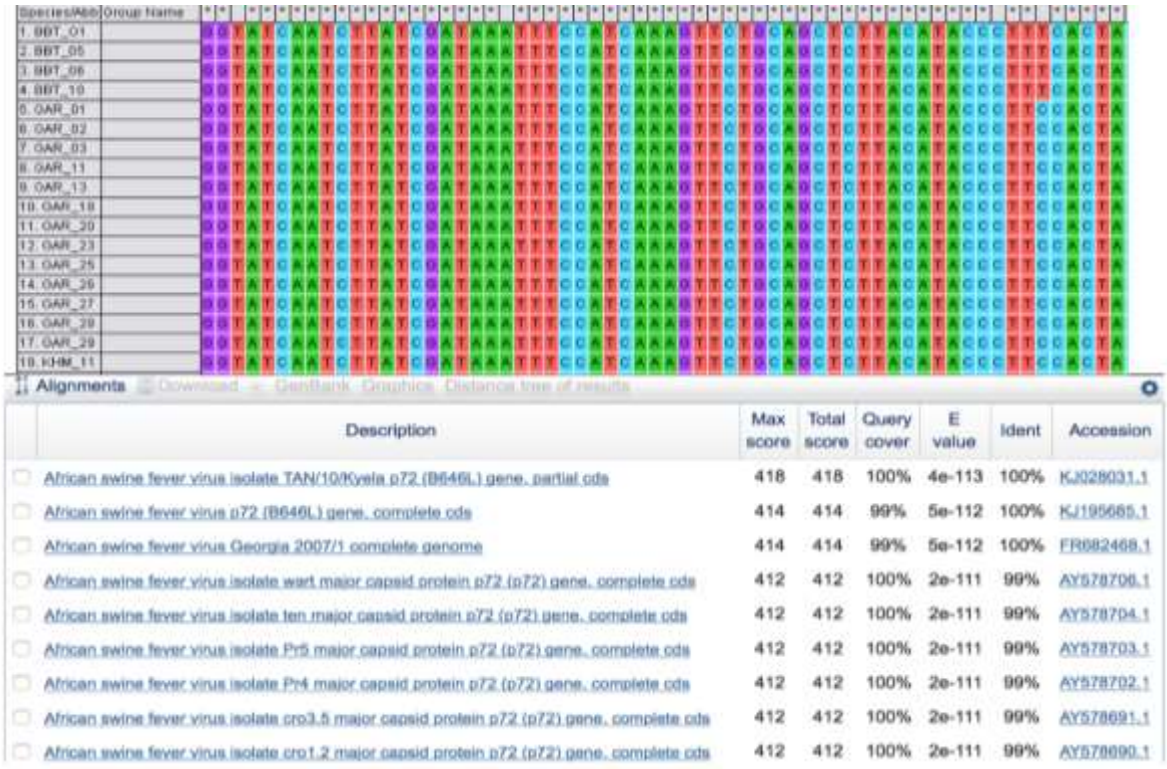



**Plate 11:** **An image showing amplification of a 257bp size band obtained from agarose gel electrophoresis as a confirmation for ASFV. L (lader), NTC (Negative Control), PAC (Positive Amplification Control) and 1-10 represent the tested samples**

***P72 Gene Analysis Revealed the Virus Genotypes***

To be able to group the ASF virus, the region coding for B606L, the p72 was used to characterize the viruses from the ASFV positive samples into the currently known 24 genotypes. A total of 52 samples were subjected to partial sequencing. The obtained sequences comprised of genotypes II, IX and X (Table 6 above). These genotypes are distributed across zones with genotype X detected in the northern zone whereas, genotype IX detected in the Lake zone and II in the Eastern and South-west regions of Tanzania (Fig. 14). Further analysis confirmed that samples collected from the same outbreak originated from the same virus genotype (Fig. 15.) These findings confirm that there was no mixed infection or co-infection of viruses of different genotypes within one outbreak. Following clustering against reference genotypes on phylogenetic tree, the three genotypes of the viruses clustered separately from each other (Fig. 16).


**Figure 14:** **A colour coded map showing the distribution of ASFV genotypes identified from domestic pigs in mainland Tanzania**



Alignments

| Description | Max score | Total score | Query cover | E value | Ident | Accession |
|---|---|---|---|---|---|---|
| African swine fever virus isolate TAN/10/Kyela p72 (B646L) gene, partial cds | 418 | 418 | 100% | 4e-113 | 100% | KJ028031.1 |
| African swine fever virus p72 (B646L) gene, complete cds | 414 | 414 | 99% | 5e-112 | 100% | KJ195685.1 |
| African swine fever virus Georgia 2007/1 complete genome | 414 | 414 | 99% | 5e-112 | 100% | FR682468.1 |
| African swine fever virus isolate wart major capsid protein p72 (p72) gene, complete cds | 412 | 412 | 100% | 2e-111 | 99% | AY578706.1 |
| African swine fever virus isolate ten major capsid protein p72 (p72) gene, complete cds | 412 | 412 | 100% | 2e-111 | 99% | AY578704.1 |
| African swine fever virus isolate Pr5 major capsid protein p72 (p72) gene, complete cds | 412 | 412 | 100% | 2e-111 | 99% | AY578703.1 |
| African swine fever virus isolate Pr4 major capsid protein p72 (p72) gene, complete cds | 412 | 412 | 100% | 2e-111 | 99% | AY578702.1 |
| African swine fever virus isolate cro3.5 major capsid protein p72 (p72) gene, complete cds | 412 | 412 | 100% | 2e-111 | 99% | AY578691.1 |
| African swine fever virus isolate cro1.2 major capsid protein p72 (p72) gene, complete cds | 412 | 412 | 100% | 2e-111 | 99% | AY578690.1 |

**Figure 15: Sequences from similar outbreaks aligned together indicating that they were of the same genotype**

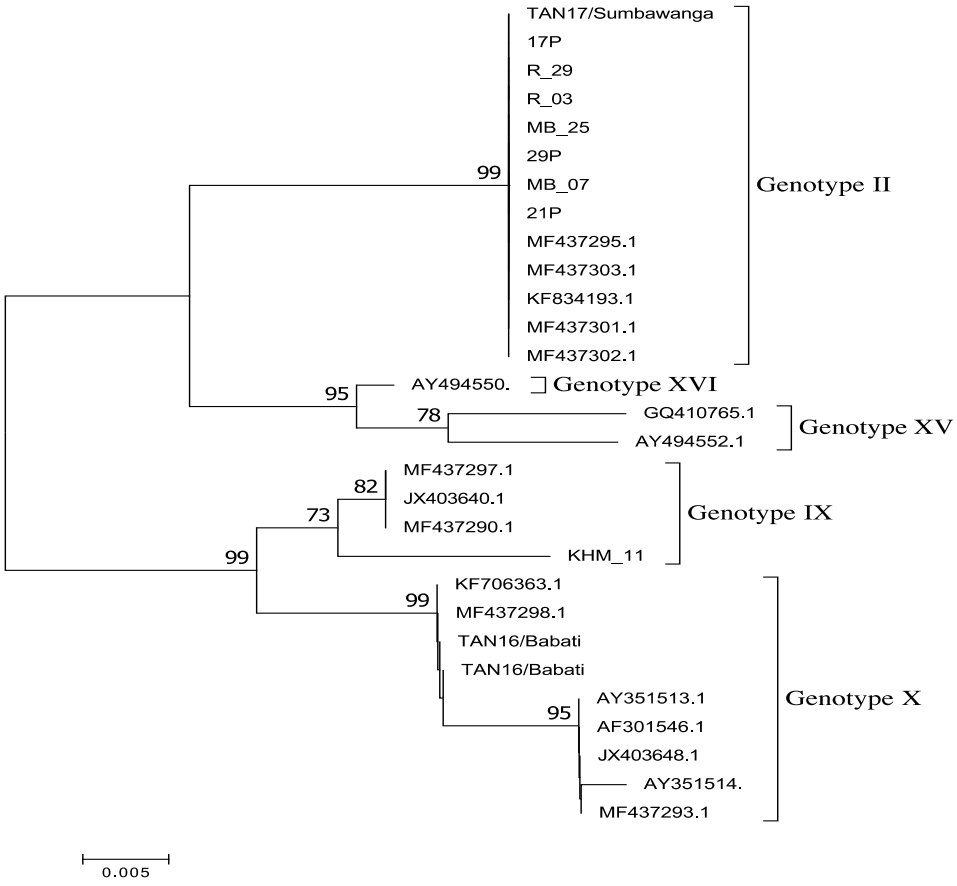



**Figure 16: p72 (B646L) phylogenetic tree showing the relationship between ASFV sequences from this study with reference sequences from previous outbreaks in Tanzania**

### *p54 gene phylogeny*

The PCR amplification of the p54 region produced a 676 bp band on an agarose gel. Sequencing of this region gives additional information for viruses that are closely related. Isolates from this study were compared with p54 ASFV sequences retrieved from GenBank (Fig. 17). The ASFV positive samples that were of the same genotype produced an identity at the nucleotide level when aligned together for the p54 region (Fig. 18).

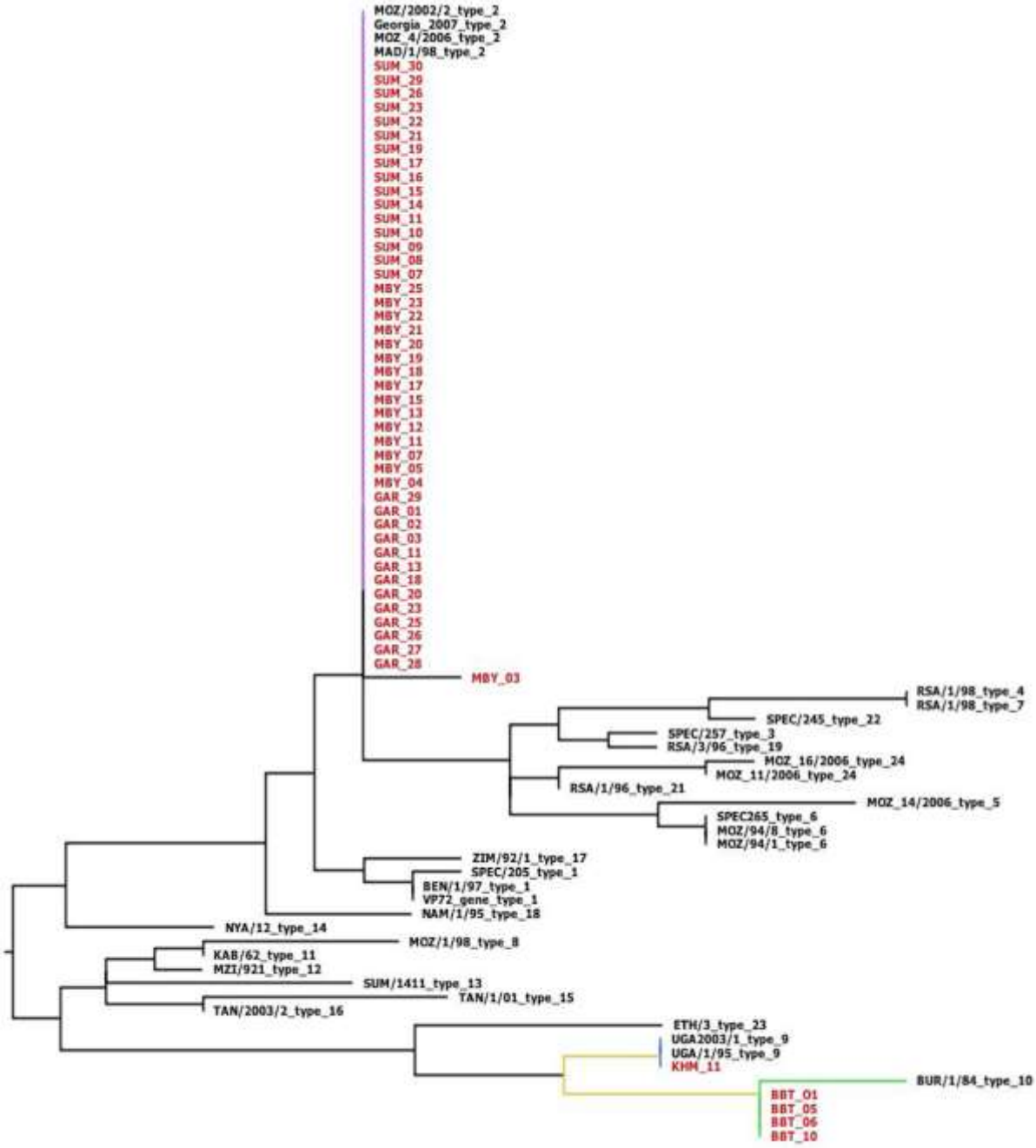



**Figure 17:** **Phylogenetic tree of p54 (E183L) sequences showing the clustering of this region in different subgroups as determined by references retrieved from GenBank**

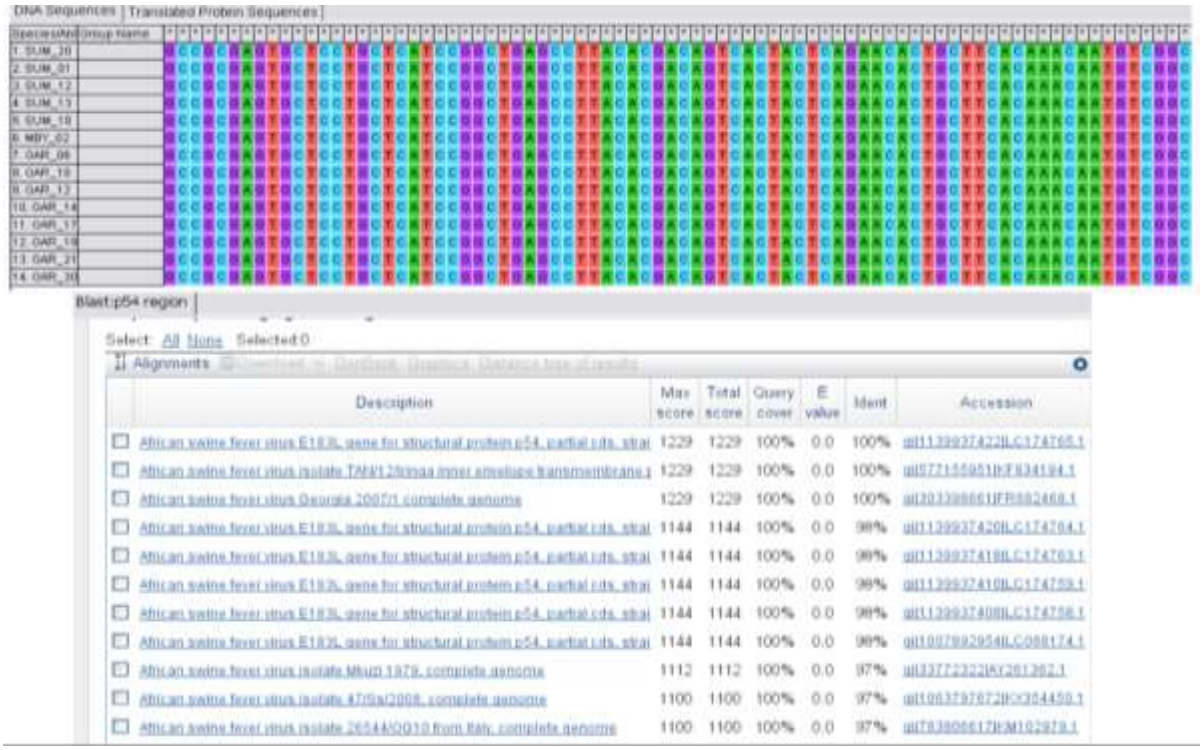



**Figure 18: Using a MEGA software, alignment of sequences belonging in the same genotype resulted into 100% identity**

### (v) Comparison of Different Methods for Molecular Amplification and Purification of African Swine Fever Virus for Whole Genome Sequencing

To achieve the objective on whole genome sequencing, assessment of the best technique to be used was done. Different methods have been used in the past to obtain close to pure viral nucleic acids. These includes but not limited to virus isolation on different media, selective whole genome amplification and virus ultracentrifugation to concentrate the virus from materials with high viral titres. Even though promising results have been obtained from these techniques, different laboratories have been reporting different results leading to the need for standardization of the techniques that give the best results. In this section, results obtained from various techniques utilized in a level three laboratory in search of a standard method for isolation and purification of ASFV for whole genome sequencing work are presented.

#### *Virus Enrichment on Cell Culture*

African swine fever virus enrichment was done on peripheral blood mononuclear cells (PBMCs) lines. Fresh cells were prepared and infected in the laboratory and the presence of viruses on the cells was analysed by haemodsorption using homologous RBCs whereby, haemaglutination was observed (Plate 12).

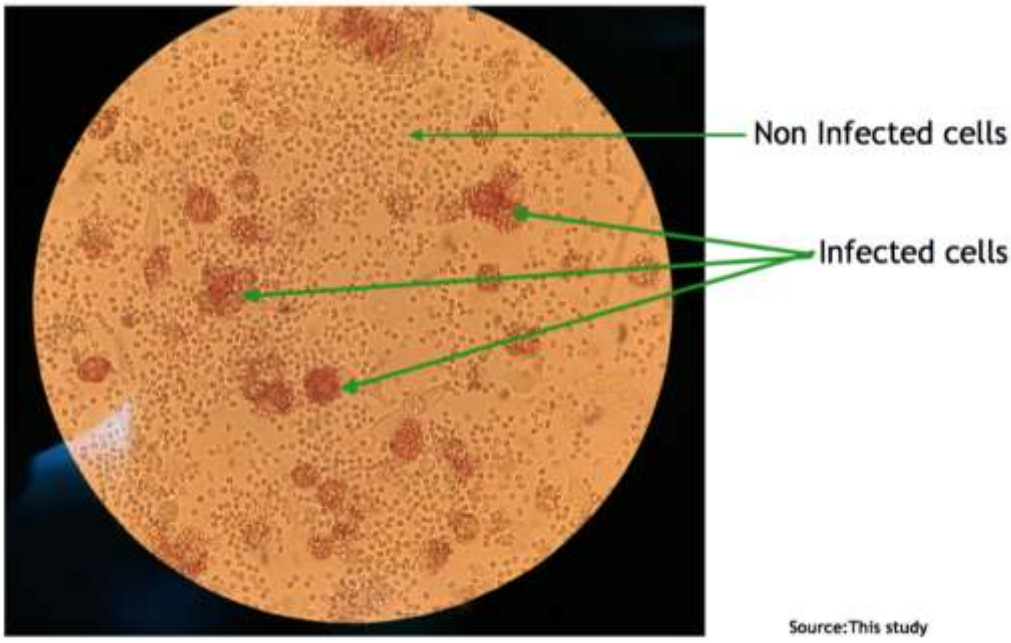



**Plate 12:** **Hemaglutination due to ASFV infection in a cell culture appearing as rosettes as observed under 40x objective light microscope**

### (vi) Comparison of DNA concentration and quality of the libraries for the three techniques utilized

To determine how the different techniques performed, a comparison of the yield obtained was done where the libraries prepared for sequencing were compared against tissue culture as a standard. Quality and quantity of the libraries were checked by a bioanalyser 2100 as described in the methodology. The overall observation indicated that sWGA had the highest yield in qubit concentration (Table 7). Selective whole genome amplification technique resulted into the highest detected gDNA (Fig. 20) as compared to raw tissue samples (Fig. 21). However, upon sequencing, there were contamination from non-ASFV genomes because the used enzymes and primers could amplify most other genomes found in the sample. The resulting chromatograms from the bioanalyser (Fig. 22) and the gel gave the same results.

**Table 7:** **Qubit concentration for libraries (ng/µl) for the three sets prepared by different enrichment methods**

| S/N | Sample | Tissue (T) | Cell Culture (C) | sWGA(S) |
|---|---|---|---|---|
| 1 | 15R | 3.04 | 6.62 | 9.28 |
| 2 | BBT01 | 0.753 | 0.890 | 3.77 |
| 3 | KHM011 | 2.27 | 2.35 | 6.90 |
| 4 | 15M | 1.90 | 9.36 | 10.5 |
| 5 | 30P | 3.43 | 4.05 | 4.91 |
| 6 | 15P | 2.20 | 3.52 | 8.53 |

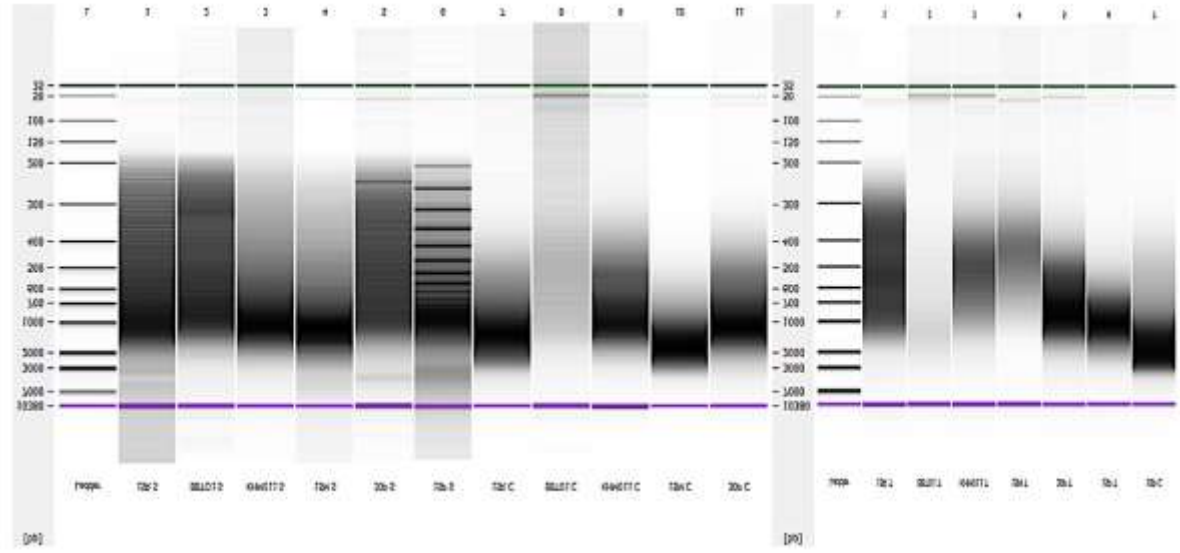


**Figure 19:** **Bioanalyser gel showing the quality and distribution of NextEra XT libraries**

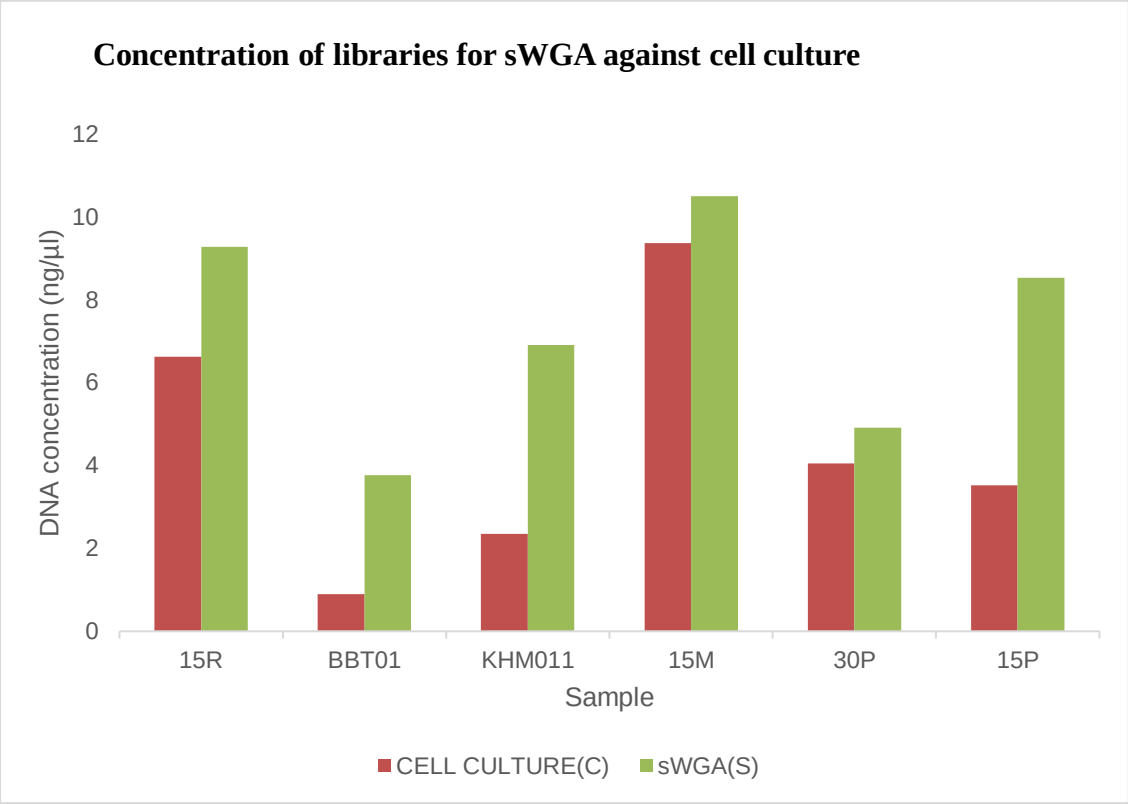



**Figure 20: Comparison of the gDNA yield between cell culture and sWGA**

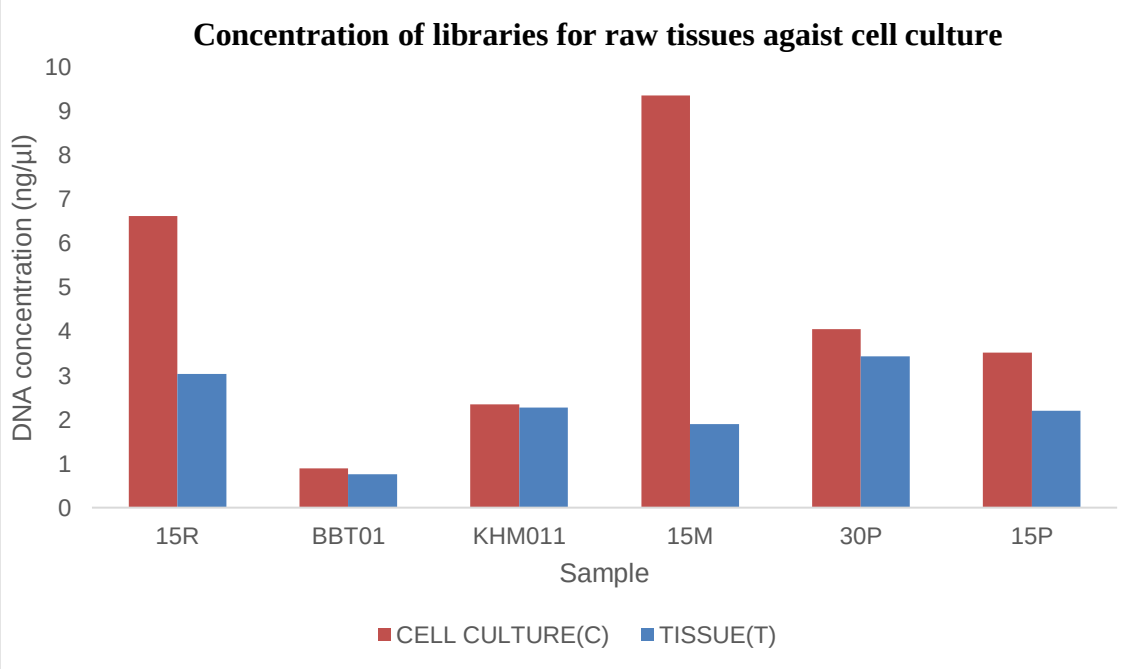



**Figure 21: Comparison of the gDNA yield between raw tissue lysates and cell culture**

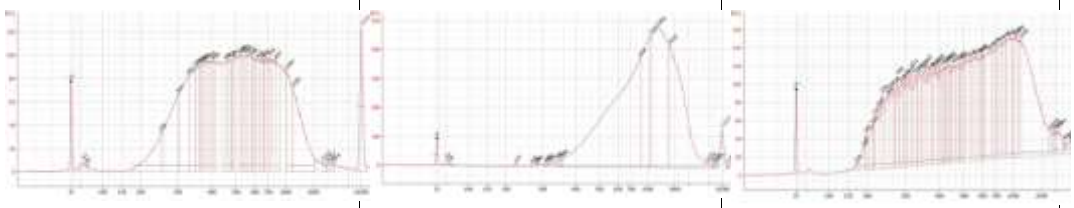
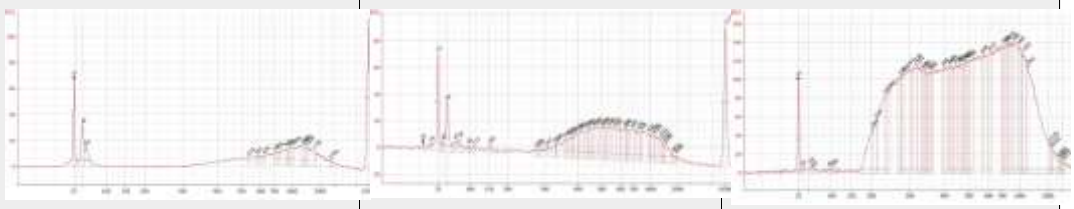
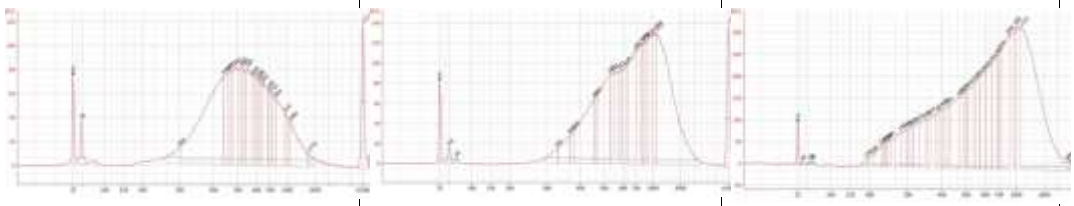
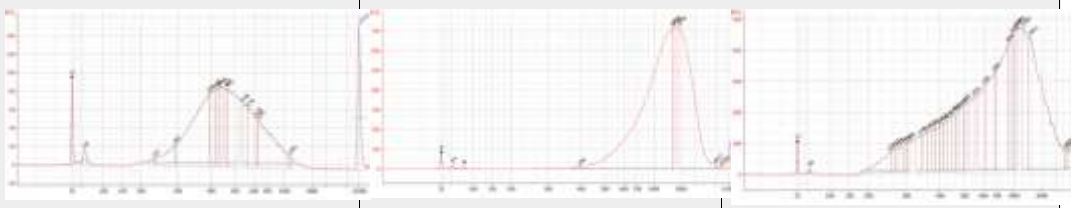
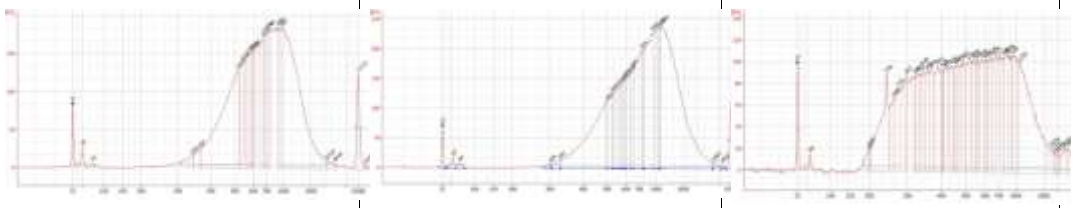
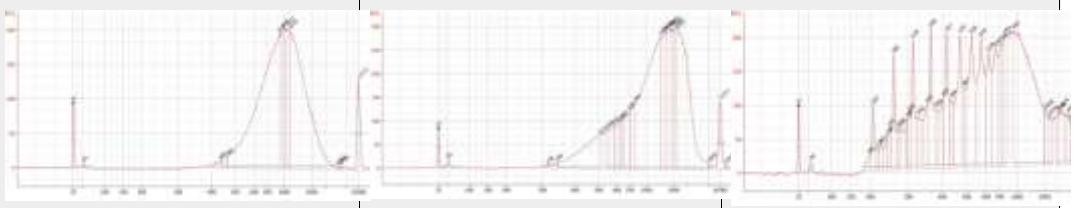
| Sample | Tissue (T) | Cell Culture (C) | sWGA (S) |
|---|---|---|---|
| 15R |  | | |
| BBT01 |  | | |
| KHM011 |  | | |
| 15M |  | | |
| 30P |  | | |
| 15P |  | | |

**Figure 22: Chromatograms showing variations in terms of size and distribution of DNA libraries obtained following the three different enrichment approaches**

For the sample with a high virus titre, read yield was 1.30 Gbp out of the reads that passed through filters. It was observed that the reads from this sample came with a very high GC

content peaking at around 67%, while *Sus scrofa* is known to be around a median GC content of 41.5% and ASFV is around 38.5% to 39.7% GC content. For the samples that went through selective whole genome amplification, the total yield obtained was 1.53Gbp of the reads that passed through filters. Of the reads, 61.66% pairs survived as pairs following trimmomatic quality trimming and were used in the analysis. The proportion of reads aligning to the pig genome was significantly lower than expected with only 53.33% of the paired reads having survived the read quality trimming by trimmomatic mapping to the pig genome. The GC content was of the expected normal distribution around 40%, with two separate and sharp peaks observed at 45% and 54%, respectively in a non-normal distribution. Following a BLAST search, the majority of the reads appeared to match those of *Neospora caninum*. A reconstruction of a full genome proved failure with the longest ASFV scaffold being of size 9,575bp. Most scaffolds had highest identity with the strains Georgia 2007/1, Georgia 2008 /2 and Odintsovo_02/14 (Chapman *et al.*, 2011; Farlow *et al.*, 2018). There were 48 distinct scaffolds of the size above 1kbp that mapped to ASFV.

For a sample prepared through cell culture, the total yield obtained was 1.83 Gbp which was a reasonable amount of the reads passing the Illumina filters. The assembly produced a contig of length 183 186 bp that aligned very well (99.94% identity on a stretch of length 167 558 bp) with a recently sequenced isolate from Poland, Pol16_29413_o23 (Mazur-Panasiuk *et al.*, 2019).

### 4.1.3 The First Complete Genome Sequence of African Swine Fever Virus Genotype II Isolate from Africa

#### (i) Genomic Sequence Procedures

Several complete genotype II sequences have been produced from other continents. Despite East Africa being considered the hotspot of ASFV, none of these full genome sequences are directly reported from Africa. In this section, the first complete genotype II sequence of a Tanzanian isolate collected in 2017 in Rukwa region (Fig. 23) generated using next generation sequencing technology following virus amplification in cell culture was reported.

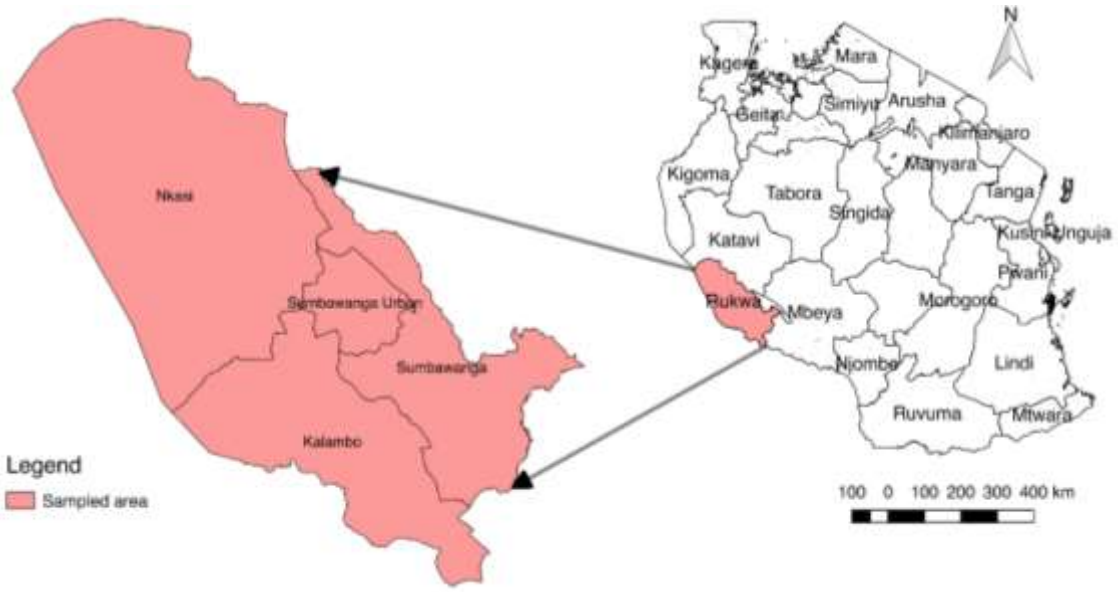



**Figure 23:** **The map indicating the location within Tanzania where domestic pig samples were collected and whole genome sequencing performed**

***Isolation of African Swine Fever Virus in Peripheral Blood Mononuclear Cells***

To be able to study the whole viral genome, virus propagation on cell cultures was the first activity to ensure that enough viral genome is obtained. Matured cells were infected at the MOI of 1:10 with the virus suspension made from grinding the spleen tissue in PBS. The strain used in this experiment was haemadsorbing, hence the presence of infection was observed following formation of rosette structures following addition of homologous red blood cells in an infected culture (Plate 13).

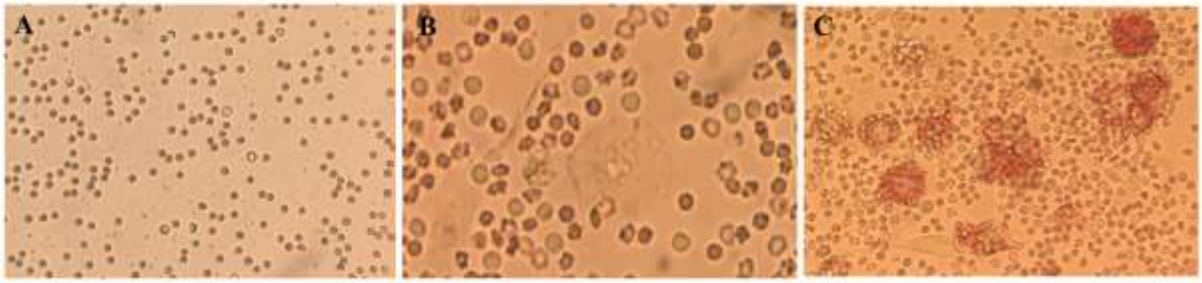



**Plate 13:** **Virus isolation on PBMCs showing freshly isolated PBMCs (A), non-infected mature PBMCs used as control (B), infected mature PBMCs (C) with hemadsorption observed at 4dpi**

The amplicon also showed an amplification of a 257 bp band (Similar to that observed in Plate 11) when run on agarose gel electrophoresis, which was the expected fragment size for ASFV positive samples in accordance to the primers used. Three inoculums obtained from the field were subjected to this method although only one sample yielded a full genome sequence when the assembly process was complete.

***NextEra XT library preparation for ASFV sequencing***

For the sequencing activity to be achieved, an important step for next generation sequencing is library preparation. The libraries were prepared using a NextEra library preparation kit as described before. The quality of the library generated was assessed on a Bioanalyser 2100 (Agilent Technology, California). Both the gel and the graphs showed a fragment size distribution ranging between 200 and 2000 bp as shown in Fig. 24A and B. The library was distributed between the markers in Fig. 24C as expected in the protocol.

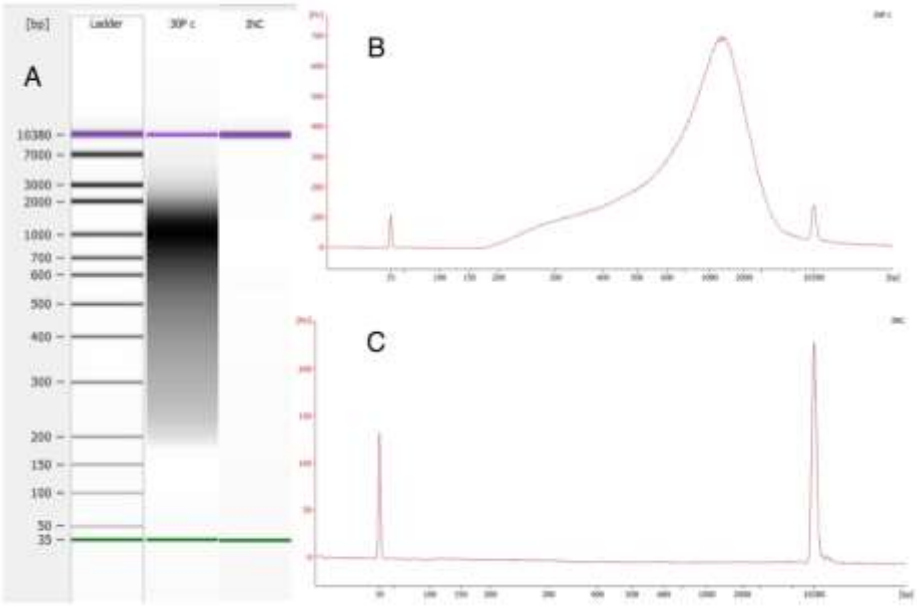



**Figure 24: Bioanalyser results for the prepared libraries with the gel showing library size (A), library distribution between the lower and upper library markers (B) and library markers (C)**

**(ii) Phylogenetic tree and ASFV whole genome analysis**

The phylogeny established on genome-wide sequence data undoubtedly places the Tanzania/Rukwa/2017/1 strain as a member of the genotype II of African swine fever virus, a monophyletic clade that is highly conserved as displayed in Fig. 25 (with the exception of Estonia 2014 / LS478113 which displays some amount of divergence). This further analyze the position of Tanzania/Rukwa/2017/1 with respect to other genotype II viruses, 29 genotype II sequences including ours were gathered, plus an outgroup sequence from genotype I (isolate 74/Ss/2008 from Sardinia). Alignment and trimming were done using BMGE with options -t DNA -g 0.0 -h 0.4 -b 10 and then, inferred a phylogenetic tree with FastTree resulting into cladogram (Fig. 26). This analysis showed a substantial well-supported signal suggesting that Tanzania/Rukwa/2017/1 is among the set of available full-genome sequences of genotype II, the closest to the phylogenetic divergence point (the most recent common ancestor) between

genotypes I and genotypes II: as the only genotype II genome from Africa, Tanzania/Rukwa/2017/1 is logically close to the centre of diversity for ASFV.

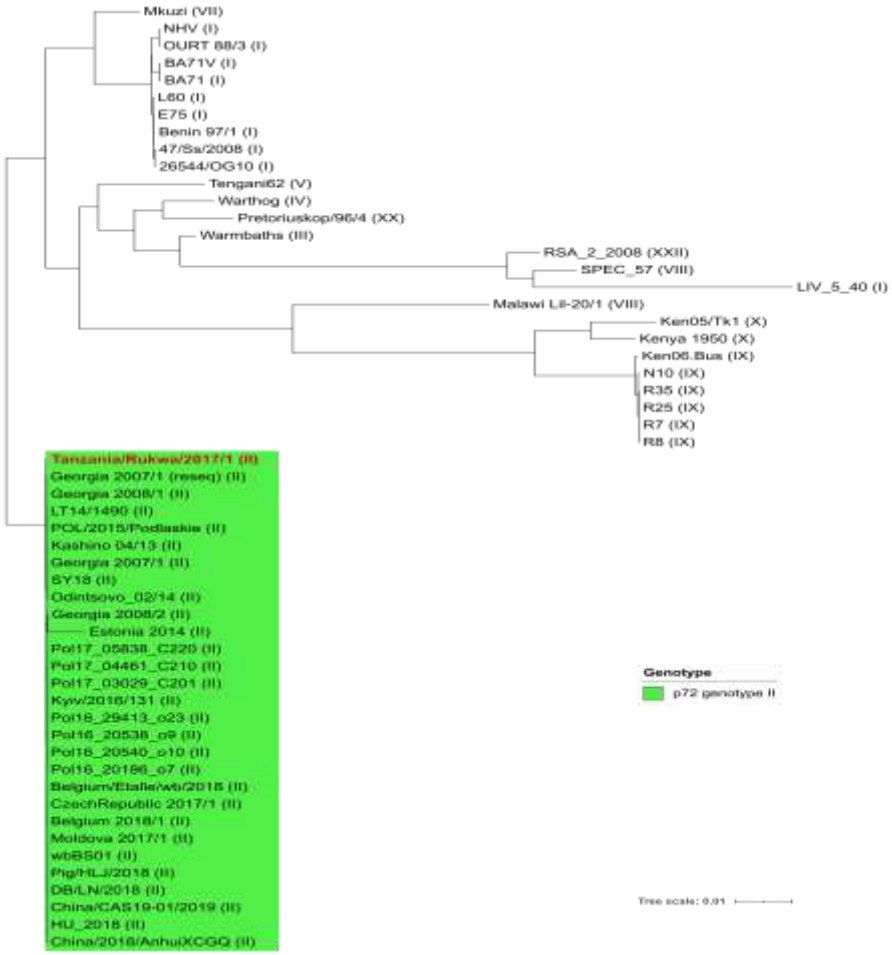



**Figure 25: Phylogenetic tree showing the relationship between the genotype II sequence from this study (Red) and genotype II sequences retrieved from NCBI**

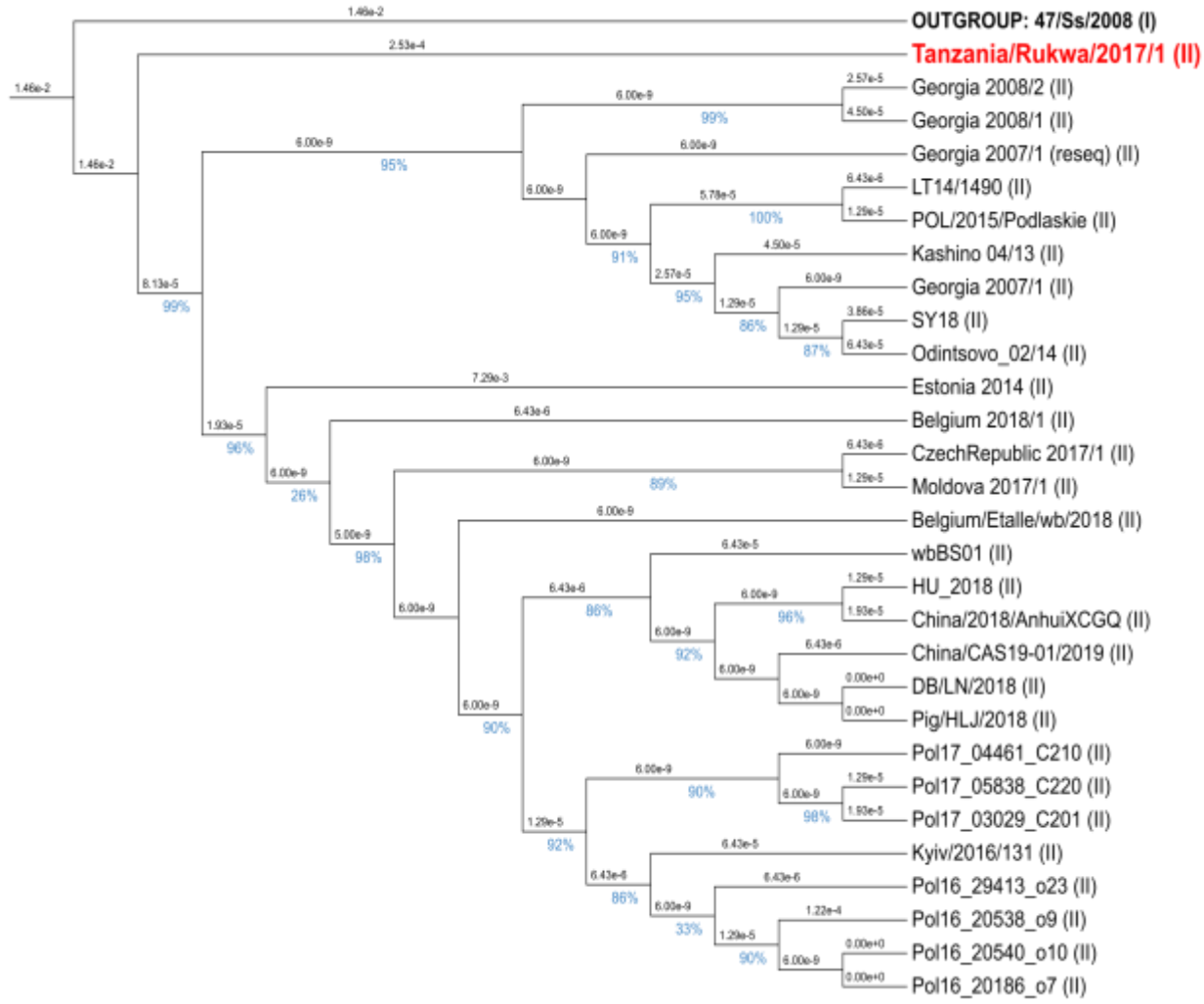



**Figure 26: A maximum-likelihood phylogenetic tree of 29 complete genotype II genomes rooted with a genotype I outgroup sequence from Sardinia**

The tree is represented as a cladogram instead of a phylogram for the sake of legibility. Branch lengths, expressed in number of expected substitutions per site, are displayed in scientific notation above the branches. Local Shimodaira-Hasegawa (SH-like) statistical branch supports calculated by FastTree are displayed as percentages in blue colour below the branches. There is high support (99%) attributed to the branch that makes Tanzania/Rukwa/2017/1 (red) basal to the clade of all genotype II sequences.

### (iii) Comparative Genomics

To be able to find out whether the current genotype II was similar or different from other genotype II sequences, there was alignment of the genome obtained from this study with some known annotated genotype II sequences. Two of the references were closely related to the sequence from this study. These are the Polish strain Pol16_29413_o23 (MG939586.1) and a Chinese strain ASFV-wbBS01 (MK645909.1) Further comparison of the isolated genome was

done by mapping with minimap2 to the Georgia2007/1 isolate (FR682468.1) generating the circus plot in Figure 27. The observed mapping was similar to the one obtained by Bowtie2 on ASFV-wbBS01 (MK645909.1).

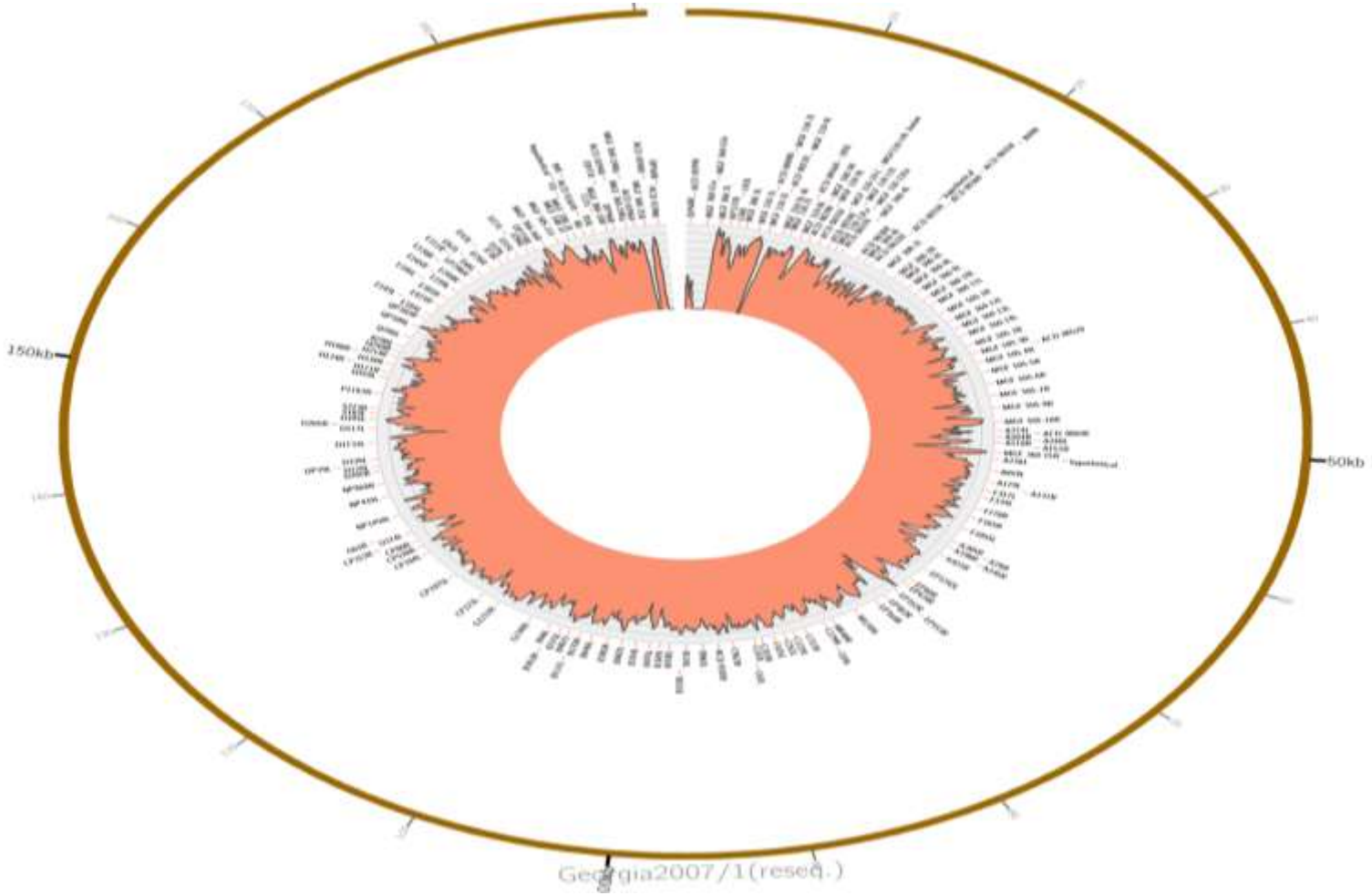



**Figure 27:** **Circos plot representing the mapping of reads from the Tanzania/Rukwa/2017/1 genome to the recently corrected Georgia 2007/1 (FR682468.2) strain. The plot shows log read depth values. The inner wheel gives approximate locations for the open reading frames**

## 4.2 Discussion

### 4.2.1 Detection of African Swine Fever Virus in a Sylvatic Cycle Devoid of Domestic Pig Involvement

The findings of this study add to the understanding of the sylvatic cycle of African swine fever in Tanzania. The findings generated are consistent with previous studies which demonstrated that the sylvatic cycle plays a vital role in the maintenance and spread of the ASF virus in eastern Africa (Anderson *et al.,* 1998; Bastos *et al.,* 2009; Okoth *et al.,* 2012; Plowright *et al.,* 1969) and southern Africa region including Malawi, Zambia and Mozambique (Anderson *et al.,* 1998; Boshoff *et al.,* 2014; Gallardo *et al.,* 2011; Haresnape *et al.,* 1988; Quembo *et al.,* 2018; Stahl *et al.,* 2014). In addition to the existing knowledge, the current exploration was on a pure sylvatic cycle in which domestic pigs were excluded, unlike the earlier studies on the role of the sylvatic hosts and vectors in Tanzania that focused on interactions between sylvatic hosts and domestic pigs (Plowright *et al.,* 1969; Bastos *et al.,* 2009; Jori *et al.,*2009).

In this study, ASFV infections were detected in ticks suggesting that ASFV is circulating within a tick-warthog cycle in Tanzania in the absence of potential transmission to and from domestic pigs. The present findings augment findings reported in several other studies indicating an independently-maintained sylvatic ASFV cycle (Abworo, 2012; Anderson *et al.,* 1998; Stahl *et al.,* 2014). The finding that 69% of the collected ticks had a blood meal in their gut, of which 55% were ASFV-positive, further support existence of the sylvatic cycle. Given that *Ornithodoros* ticks are known to be host-specific and the fact that they were collected inside the warthog burrows highly suggests that the ticks had fed on warthogs. The Saadani ecosystem had a unique interaction in which wild warthogs have adapted into mingling with Saadani villagers and domesticated animals such as goats; finding shelter and protection from around the village. In such a situation, if in future domestic pigs are reared within or in proximities, a spill over effect might occur resulting into ASF outbreaks.

The detection of antibodies but not ASFV nucleic acid in warthog blood is consistent with other studies which showed that warthogs can clear or maintain the virus at an undetectable level by conventional PCR in their blood (Jori *et al.,* 2009; Thomson, 1985). This could explain the levels of seropositivity in warthogs in this study devoid of viral detection reported. A high viremia may be observed in warthog piglets that spend more time in the burrows during early stages of life (Dellen, 1980) but the current study could not capture very young piglets below

four months of age as they were not available. Available piglets ranged between four to six months of age (Table 3) and they were sampled while in grazing areas and none was viremic.

The ASF virus is maintained in the ticks transovarially and transstadially (Kleiboeker & Scoles, 2001), which explains the observation that seven out of twenty non-engorged ticks were PCR positive for ASFV. High levels of exposure among warthogs, therefore, may explain how this biological system is efficient in maintaining and transmitting the virus in the sylvatic ecosystem with a low prevalence of infected ticks. The current study could not show a clear association between sex and age of warthogs with ASFV exposure status. This can partly be due to the limited sample size that did not provide for adequate representation of different age groups and sex (Table 3); and partly because none of the animals in any category was found in active viremia.

The p72 references from GenBank and the sequences from this study were highly similar, and they clustered together under genotype XV with the bootstrap value of 99 (Fig. 11). Upon a BLAST search of the tick sequences, there was 100% identity with the Tanzanian genotype XV references previously reported in outbreaks (Misinzo *et al.*, 2011, 2014; Yona, 2017). Narrowing down to the p54 region that is more conserved at the strain level, the BLAST search returned a 95% identity with other genotype XV sequences from Tanzania and the sequences clustered under different branches of the same subtree (Fig. 12). An analysis of the CVR showed variations in both numbers of the tetrameric amino acid repeats and their sequences. Despite a strong bootstrap support of 89% that the sequences belonged to the same group, a phylogenetic tree showed a difference in sub-clustering of the sequences from this study with those reported in the 2008 outbreak (Misinzo *et al.*, 2011; 2014). The first seven tetrameric repeats were identical in both the references and the sequences from this study showing a level of relatedness within the sequences. Sequences from this study appeared to be shorter, with 18 tetrameric repeats as compared to the references that had 31 repeats (Table 5). This study could not give a clear conclusion of whether such variations would result in a more or less virulent strain of the virus.

Based on the findings emanating from the close relationship between sequences from this study and those of 2008 outbreak, several hypotheses can be derived. One hypothesis is that the virus which caused an outbreak in 2008 might have found its way into the wild, became maintained in the sylvatic cycle and underwent some genetic modifications. The virus in the sylvatic cycle, may have spilled over into the domestic pigs through poaching or illegal hunting of warthogs and caused an ASF outbreak which was not been reported in the past. Alternatively, the sylvatic

cycle in Saadani could have been an uninterrupted cycle that maintained itself for many years independent of any other interactions. Generally, these results implicate genotype XV as potentially infective to warthogs as evidenced by seropositivity in warthog sera (Fig. 10). Although the current study cannot make any inference about the virulence of this specific sylvatic strain to domestic pigs, it may suggest a consideration that any genotype XV strain can result in ASF outbreaks. Further research on the directional flow of ASFV between the sylvatic and domestic cycles, its virulence and the role that wild hosts and vectors play in modifying the virus warrant further studies.

This study recommends a comparison between the whole virus genomes of genotype XV from ticks in Saadani National Park with whole genome sequences of isolates from 2008 outbreaks. This could provide more information on virus evolution and transmission dynamics. Studies on the virulence of genotype XV are needed to assess its potential for local and global spread and risks thereof.

### 4.2.2 Enzyme Linked Immunosorbent Assay Test, Molecular Characterization and Phylogenetic Study of African Swine Fever Virus Isolates from Tanzania Mainland (2016-2017)

#### (i) Enzyme Linked Immunosorbent Assay Test on Domestic Pig Sera Samples

An antibody ELISA test was carried out to diagnose domestic pig sera as previously mentioned under 3.7.1. Unlike the observation of high seroprevalence in warthog sera, none of the domestic pig samples was positive for antibody ELISA. From the literature, naturally infected hosts that survive infection usually develop antibodies against ASFV from 7 to 10 days post infection. The detection of specific antibodies against ASFV should therefore be performed for the diagnosis of subacute and inapparent forms of the disease (Cubillos *et al.,* 2013). In this study, sera samples were collected from notified outbreaks where animals were sick within three to five days after infection. This means that the animals were sampled way before they could develop any antibodies against ASFV. On the same note, domesticated pigs that contracted ASF died within three to seven days post infection. This could explain why the domestic pigs were ELISA negative but the PCR results were positive.

### (ii) Polymerase Chain Reaction Test, Molecular Characterization and African Swine Fever Virus Genotyping

Previous studies have reported a number of different ASFV genotypes in Tanzania including genotype X, XV, XVI that seemed to be dominant in the last decade (Misinzo *et al.,* 2011, 2014; Yona, 2017). Currently, genotypes IX and II are encountered, with genotype II being the most spread around Tanzania and the world, raising a special interest. It is the genotype that has escaped from Africa on several occasions to different parts of Europe such as in Georgia in 2007. In 2018, this specific genotype was first reported in Asia where it was responsible for a loss of millions of pigs resulting into the worst ever reported economic loss due to ASF (Bao *et al.,* 2019; FAO, 2018; Ge *et al.,* 2018). Phylogenetic studies of genotype II sequences from this study with those retrieved from NCBI revealed that the sequences clustered under the same genotype. Both p72 and p54 phylogenetic trees clustered the sequences in the same group as evidenced in Fig.16 and Fig. 17 respectively. Recruitment criteria for discrimination of the sequences based on CVR required the sequences of the same genotype that were obtained from different outbreaks in different geographical locations. Genotype II strains were the most widespread in the country being reported from three different agro-ecological zones (Table 6) thus qualified for this analysis. The obtained results could be used for the tracing of outbreak origins and finding a connection between them. Most of the outbreaks were located near areas known to be associated with marketing of live pigs or pig products and close to the main roads, the risk factors previously reported by Fasina *et al.,* (2020). Providentially, all positive non-outbreak samples were of a genotype II origin. However, there were no differences observed at this level between the genotype II strain from outbreak and that originating from non-outbreak areas. Taking a closer look at Fig. 17, samples abbreviated by ‘MBY’ were collected from a non-outbreak area but they clustered together with ‘GAR’ and ‘SUM’ abbreviate samples which were collected from outbreaks. Figure 18 also shows that the samples from the two groups were identical at the genotype level. These findings could be suggestive of the endemic nature of ASFV genotype II in the southern highlands of Tanzania. There is also an observation that some domestic pigs are asymptomatic to ASF in that region although more evidence needs to be gathered.

## 4.2.3 Comparison of Different Methods For Molecular Amplification and Purification of African Swine Fever Virus for Whole Genome Sequencing

The selective whole genome amplification technique proved to be effective in a study where it amplified a Plasmodium genome for sequencing (Oyola *et al.,* 2016) while tissues with high

virus titres were also used successfully in another study (Masembe *et al.,* 2018). It was observed that the reads from a high virus titre sample came with a very high GC content peaking at around 67%, while *Sus scrofa* is known to be around a median GC content of 41.5% and ASFV is around 38.5% to 39.7% GC content (Granberg *et al.,* 2016). Upon further analysis, 0.89% of the paired reads having survived the read quality trimming by trimmomatic mapped to the pig genome. An analysis based on random BLASTing of reads from that sample gave an overwhelming majority of reads allegedly from various strains of *Stenotrophomonas maltophilia* and from *Sphingomonas* sp. LK11 (Hou *et al.,* 2012). The literature reported that *Stenotrophomonas maltophilia* has been demonstrated to co-occur in pigs with the swine influenza A virus (Powell and Straub, 2018). The GC contents of 66.7% and 66.3% for the two reference strains of *S. maltophilia*, respectively K279a and RR-10 has also been reported (Chapman *et al.,* 2011; Farlow *et al.,* 2018). This is consistent with what was found as GC content in the reads.

Efforts to reconstitute any meaningful ASFV genome from this sample proved failure with only a scaffold of length 598bp being highly similar to Georgia 2007/1, a genotype II strain (99.83% identity on that small stretch of DNA). From this finding, it was concluded that sequencing directly from a tissue lysate was quick and easy but not reliable for the recovery of whole genome of the virus. A sample prepared through cell culture was run along the samples prepared from the two other methods. Because cell culture is known to be the gold standard, performance from other techniques was to be judged based on how better they were as compared to virus isolation on cell culture.

Total yield obtained was 1.83 Gbp which was a reasonable amount of the reads passing the Illumina filter. The assembly produced a contig of length 183,186 bp that aligned very well (99.94% identity on a stretch of length 167 558 bp) with a recently sequenced isolate from Poland, Pol16_29413_o23 (Mazur-Panasiuk *et al.,* 2019). The isolate clustered tightly with the rest of the genotype II covering the Asian and European continents (Gallardo *et al.,* 2018) from Georgia to the more recent outbreaks in Russia, Eastern Europe and China as shown in the phylogenetic tree in Fig. 24. This was a good indicator that there was a definitive genotype II virus in this sample. Using the cell culture technique, which is a gold standard for virus isolation, it was possible to generate a full ASFV genome from the 1.83 Gbp length.

Virus isolation in tissue culture remains a gold standard for confirmation of viruses in samples. Given that the virus used in this study has hemadsorption properties, a combination of virus isolation and hemadsorption was used both as a confirmatory test for the samples, and to

increase the virus titre and reduce host genome contamination (Leland & French, 1988). This procedure provided sufficient viral DNA to assemble a complete genome, excluding the terminal repeats, as a single contig, using only a medium throughput Illumina MiSeq machine. In future, deep sequencing of specific loci using a higher throughput platform such as the Illumina Hiseq, would provide information on micro variation that may be present at low levels within genomes.

### 4.2.4 The First Complete Genome Sequence of African Swine Fever Virus Genotype II Isolate from Africa

Genotype II p72 group ASFV is the most geographically widespread of the twenty-four viral genotypes that are currently known (Gallardo *et al.*, 2015; Sánchez-Cordón *et al.,* 2018). The genotype II virus that was detected in Georgia in 2007 subsequently spread rapidly through the Caucasus into Russia and was then disseminated both East and West, though animal and product trade as well as increased movements by people resulting in an ongoing disease pandemic spanning a vast region from Eastern Europe to China. Based on genotyping and whole genome sequencing, the Georgia 2007 virus is believed to have originated in south-eastern Africa, probably Mozambique or Madagascar (Rowlands *et al.,* 2008, Chapman *et al.,* 2011). Some data exists suggesting that p72 genotype II viruses may be actively transmitted from the warthog sylvatic cycle to domestic pigs via Ornithodoros ticks at the wildlife-domestic pig interface as was the case in Mozambique (Quembo *et al.,* 2017). Genotyping of genotype II viruses circulating in Zambia was consistent with recent introduction of these viruses from Tanzania (Simulundu *et al.,* 2017). A complete picture of the dynamics and evolution of ASFV genotype II in the endemic areas of Africa comprising Mozambique, Zambia and Tanzania will require determination of additional complete genome sequences from both current and archived samples.

The Tanzanian whole genome determined in this study is closely related to genotype II viruses derived from Georgia 2007, as indicated by the clustering of the Tanzanian genome together with these viruses in a maximum likelihood phylogenetic tree constructed from all available complete genomes (Fig. 25). Genotype II from Tanzania exhibits a high level of similarity over the entire length of the genome with the recently re-sequenced Georgia 2007 (Forth *et al.,* 2018) as seen in Figure 26, a Polish genotype (Mazur-Panasiak, 2019) and a Chinese isolate, originating from a wild boar. The Tanzania/Rukwa/2017/1 virus isolate was collected at around 1270 km away from Beira (Mozambique), 1920 km from Maputo (Mozambique) and 2000 km from Antananarivo (Madagascar). These are not as many kilometres from the plausible south-

eastern African source of the virus that was introduced to Georgia through a pork product (Rowlands *et al.*, 2008). Detailed analysis indicates that, although similar to other genotype II viruses, Tanzania/Rukwa/2017/1 shows greater molecular divergence from Georgia 2007/1 and other group II genomes recently derived from the reference, than the members of the Georgia 2007 group differ from one another. This presumably relates to the fact that the mutations in Tanzania/Rukwa/2017/1 are likely to have accumulated over a longer timescale than the 11 years that have elapsed since the introduction of ASFV to Georgia in 2007. It is unknown whether the transboundary Georgia 2007 virus inoculum was clonal or already contained more than one genotype.

The complete genome sequencing of genotype II (Tanzania/Rukwa/2017/1) showed many similarities concluding on its genotype II nature beyond doubt when placed with other genotype IIs around the world. Some few differences due to mutation have been observed in Tanzania/Rukwa/2017/1, for example, the codon TGG (tryptophan) in wbBS01 is mutated to encode arginine (AGA) in Tanzania/Rukwa/2017/1. Additionally, MGF110-3L is truncated at the 5' end. Within MGF110-4L, there are also SNPs, specifically T/C at position 8160 and C/A at position 8204. In the fused MGF 110 protein MGF110-5L-6L, a deletion of three nucleotides (ATG) across a codon boundary in Tanzania/Rukwa/2017/1, corresponding to positions 8670 to 8672 in wbBS01 was observed. In respect of the multicopy gene families located at the 3' end of the genome, MGF360-21R is almost entirely deleted from the Tanzania/Rukwa/2017/1, but it is present in Georgia 2007/1 and the Polish and Chinese viruses derived from it over the last ten years. On an applied note, the observation of molecular divergence between Tanzania/Rukwa/2017/1 and Georgia 2007/1, together with the viruses recently derived from the latter, suggests that live attenuated vaccines derived from Georgia/2007/1 may not always cross-protect against ASFV genotypes derived from different regions of Africa, as it has been observed for phylogenetically distant ASFV strains within the ASFV p72 genotypes (Lopez *et al.*, 2020). The majority of the mutations separating Tanzania/Rukwa/2017/1 from other genotype II viruses are inserts/deletions (indels) rather than SNPs, although the latter are also present. This pattern has been noted previously when available ASFV type II genomes were compared (Zhu *et al.*, 2016). The reason for this mode of genome evolution being more prevalent is not fully understood. However, the virus encodes a proof-reading enzyme 3' to 5' exonuclease, which possibly represents an adaptation to survival in the oxidizing environment of the macrophage and may contribute to the relative rareness of SNPs as reviewed by Dixon *et al.* (2013). At the same time, the virus also encodes an unrestrained DNA ligase, which may facilitate the generation of indels through non-homologous end joining (Dixon *et al.*, 2013).

In the case of the length and copy number of genes within the five terminal multicopy gene families, it may be the case that some gene copies are not essential for virus survival. However, loss of certain open reading frames in the multicopy families has been associated with reduction of virulence in domestic pigs, including the recent genotype II isolates from wild boar and domestic pigs (Zani *et al.,* 2018; O'Donnell *et al.,* 2018) as; reviewed in Dixon *et al.* (2013). Moreover, some genes of known function are entirely conserved throughout the genotype II group of viruses, for example p30 (CP204L) is 100% conserved, with no SNPs. This gene was shown to be key to viral replication (Hübner *et al.,* 2018). The p72 major surface protein gene (B646L) is also 100% conserved between Tanzania/Rukwa/2017/1 and MK645909. This is an important finding given that most available primers used for diagnosis (PPA1 and PPA2 primers) and genotyping (e.g. p72-U and p72-D primers) are designed from this region of the virus. This observation has an important implication in disease control as the highly conserved region may be a good target for a vaccine.

# CHAPTER FIVE

# CONCLUSION AND RECOMMENDATIONS

## 5.1 Conclusion

The principal findings of this thesis include the information on the genotypes that are currently circulating in mainland Tanzania, the release of the first full genome sequence of genotype II origin from Africa and information on genotypic relationship between outbreak and non-outbreak ASFV strains from warthogs, pigs and ticks.

From the first objective which was to identify ASFV strains circulating in both sylvatic and domestic cycles, four different genotypes have been observed to be circulating in Tanzania during the study period. These are genotypes II, IX, X and XV. While genotypes II, IX and X were observed in the domestic pig circle, genotype XV was observed in the sylvatic cycle. Focusing on the sylvatic cycle in Saadani, genotype XV has been observed in absence of domestic pigs, probably indicating that the genotype is native to the country. The study suggests a further investigation of genotype XV in other areas of the country as this genotype has only been reported in Tanzania. These country specific genotypes should be taken into account in vaccine development.

The distribution of genotypes II, IX and X in Tanzania enabled this study to meet the second objective which was to establish genotypic relationship between outbreak and non-outbreak ASFV strains from warthogs, pigs and ticks. The genotypes of ASFV that are currently reported in domestic pigs (genotype II, IX and X). The observation that the country's peripheries were more affected by ASF than the centre cemented on the transboundary nature of ASF.The dynamics of the disease flow between Tanzania and her neighbours is not very clear and further investigation is recommended.

From the final objective which was the complete genome sequence of genotype II (Tanzania/Rukwa/2017/1), a light has been shed on the relationship between the Tanzanian genotype two and those from outside Africa. Further sequencing of other genotype II strains from sub-Saharan Africa is highly recommended. This will provide more information on the variations within and between genotypes found in Africa. Availability of other complete sequences from other genotype II endemic African countries such as Malawi, Madagascar and Zambia will contribute much into uncovering more information on the African genotype II viruses.

## 5.2 Recommendations

From the first objective which was to identify ASFV strains circulating in both sylvatic and domestic cycles,further investigation of genotype XV in other areas of the country as this genotype has only been reported in Tanzania. These country specific genotypes should be taken into account in vaccine development.

The observation that the country's peripheries were more affected by ASF than the centre cemented on the transboundary nature of ASF.The dynamics of the disease flow between Tanzania and her neighbours is not very clear and further investigation is recommended.

Further sequencing of other genotype II strains from sub-Saharan Africa is highly recommended as will provide more information on the variations within and between genotypes found in Africa.

## REFERENCES


Abworo, E. O., Onzere, C., Amimo, J. O., Riitho, V., Mwangi, W., Davies, J., Blome, S., & Bishop, R. P. (2017). Detection of African swine fever virus in the tissues of asymptomatic pigs in smallholder farming systems along the Kenya–Uganda border: Implications for transmission in endemic areas and ASF surveillance in East Africa. *Journal of General Virology*, 98(7), 1806-1814.

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

Achenbach, J. E., Gallardo, C., Nieto-Pelegrín, E., Rivera-Arroyo, B., Degefa-Negi, T., Arias, M., Jenberie, S., Mulisa, D. D., Gizaw, D., Gelaye, E., Chibssa, T. R., Belaye, A., Loitsch, A., Forsa, M., Yami, M., Diallo, A., Soler, A., Lamien, C. E., & Sánchez-Vizcaíno, J. M. (2017). Identification of a New Genotype of African Swine Fever Virus in Domestic Pigs from Ethiopia. *Transboundary and Emerging Diseases*, *64*(5), 1393–1404. https://doi.org/10.1111/tbed.12511

Alejo, A., Matamoros, T., Guerra, M., & Andrés, G. (2018). A Proteomic Atlas of the African Swine Fever Virus Particle. *Journal of Virology*, *92*(23), e01293-18. https://doi.org/10.1128/JVI.01293-18

Anderson, E. C., Hutchings, G. H., Mukarati, N., & Wilkinson, P. J. (1998). African swine fever virus infection of the bushpig (*Potamochoerus porcus*) and its significance in the epidemiology of the disease. *Veterinary Microbiology*, *62*(1), 1–15. https://doi.org/10.1016/S0378-1135(98)00187-4

Arias, M., Jurado, C., Gallardo, C., Fernández-Pinero, J., & Sánchez-Vizcaíno, J. M. (2018). Gaps in African swine fever: Analysis and priorities. *Transboundary and Emerging Diseases*, *65*(S1), 235–247. https://doi.org/10.1111/tbed.12695

Arnot, L. F., Toit, J. T., & Bastos, A. D. S. (2009). Molecular monitoring of African swine fever virus using surveys targeted at adult Ornithodoros ticks: A re-evaluation of Mkuze Game Reserve, South Africa. *Onderstepoort Journal of Veterinary Research*, *76*(4), 385–392. https://doi.org/10.4102/ojvr.v76i4.22

Artois, M. (2012). The Role of Wildlife in the Control of Domestic Animal Diseases. *Commission régionale Europe*. https://www.google.com

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), 1-8.

Baldus, R. D., Roettcher, K., & Broska, D. (2001). *An Introduction to Tanzania's Future 13th National park.* https://scholar.google.com

Balyshev, V. M., Vlasov, M. E., Imatdinov, A. R., Titov, I. A., Morgunov, S. Y., & Malogolovkin, A. S. (2018). Biological properties and molecular-genetic characteristics of African swine fever virus isolated in various regions of Russia in 2016–2017. *Russian Agricultural Sciences*, 44(5), 469-473.

Bao, J., Wang, Q., Lin, P., Liu, C., Li, L., Wu, X., Chi, T., Xu, T., Ge, S., Liu, Y., Li, J., Wang, S., Qu, H., Jin, T., & Wang, Z. (2019). Genome comparison of African swine fever virus China/2018/Anhui XCGQ strain and related European p72 Genotype II strains. *Transboundary and Emerging Diseases*, *66*(3), 1167–1176. https://doi.org/10.1111/tbed.13124

Barongo, M. B., Bishop, R. P., Fèvre, E. M., Knobel, D. L., & Sematimba, A. (2016). A Mathematical Model that Simulates Control Options for African Swine Fever Virus (ASFV). *Plos One*, *11*(7), e0158658. https://doi.org/10.1371/journal.pone.0158658

Bastos, A. D. S., Arnot, L. F., Jacquier, M. D., & Maree, S. (2009). A host species-informative internal control for molecular assessment of African swine fever virus infection rates in the African sylvatic cycle Ornithodoros vector. *Medical and Veterinary Entomology*, *23*(4), 399–409. https://doi.org/10.1111/j.1365-2915.2009.00828.x

Bastos, A. D. S., Penrith, M. L., Macome, F., Pinto, F., &Thomson, G. R. (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*(3), 169–182. https://doi.org/10.1016/j.vetmic.2004.09.003

Bastos, A. D. S., Penrith, M. L., Crucière, C., Edrich, J. L., Hutchings, G., Roger, F., Couacy-Hymann, E., & Thomson, G. (2003). Genotyping field strains of African swine fever

virus by partial p72 gene characterisation. *Archives of Virology*, *148*(4), 693–706. https://doi.org/10.1007/s00705-002-0946-8

Beltran-Alcrudo, D., Kukielka, E. A., De Groot, N., Dietze, K., Sokhadze, M., & Martinez-Lopez, B. (2018). Descriptive and multivariate analysis of the pig sector in Georgia and its implications for disease transmission. *PLoS One*, 13(8), e0202800.

Beltran-Alcrudo, D., Gallardo, M. A. A. C., Kramer, S. A., Penrith, M. L., Kamata, A., & Wiersma, L. (2017). *African Swine Fever: Detection and Diagnosis (No. 19)*. Food and agriculture Organization of the United Nations (FAO). https://scholar.google.com

Beltran-Alcrudo, D., Falco, J. R., Raizman, E., & Dietze, K. (2019). Transboundary spread of pig diseases: The role of international trade and travel. *BMC Veterinary Research*, 15(1), 1-14.

Bishop, R. P., Fleischauer, C., De Villiers, E. P., Okoth, E. A., Arias, M., Gallardo, C., & Upton, C. (2015). Comparative analysis of the complete genome sequences of Kenyan African swine fever virus isolates within p72 genotypes IX and X. *Virus Genes*, *50*(2), 303–309. https://doi.org/10.1007/s11262-014-1156-7

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), 45–55. https://doi.org/10.1016/j.vetmic.2006.11.007

Broadhurst, M. J., Brooks, T. J. G., & Pollock, N. R. (2016). Diagnosis of Ebola Virus Disease: Past, Present, and Future. *Clinical Microbiology Reviews*, *29*(4), 773–793. https://doi.org/10.1128/CMR.00003-16

Cappai, S., Rolesu, S., Coccollone, A., Laddomada, A., & Loi, F. (2018). Evaluation of biological and socio-economic factors related to persistence of African swine fever in Sardinia. *Preventive Veterinary Medicine*, *152*, 1–11.

Carlson, J., O'Donnell, V., Alfano, M., Velazquez Salinas, L., Holinka, L. G., Krug, P. W., Gladue, D. P., Higgs, S., & Borca, M. V. (2016). Association of the Host Immune Response with Protection Using a Live Attenuated African Swine Fever Virus Model. *Viruses*, *8*(10), 291. https://doi.org/10.3390/v8100291

Carrascosa, A. L., Bustos, M. J., & De Leon, P. (2011). Methods for Growing and Titrating African Swine Fever Virus: Field and Laboratory Samples. In J. S. Bonifacino, M. Dasso, J. B. Harford, J. Lippincott-Schwartz, and K. M. Yamada (Eds.), *Current Protocols in Cell Biology*. John Wiley and Sons, Inc. https://doi.org/10.1002/0471143030.cb2614s53

Chang'a, J. S., Mayenga, C., Settypalli, T. B. K., Achenbach, J. E., Mwanandota, J. J., Magidanga, B., Cattoli, G., Jeremiah, M., Kamigwe, A., Guo, S., Kalabi, D., Mramba, F., & Lamien, C. E. (2019). Symptomatic and asymptomatic cases of African swine fever in Tanzania. *Transboundary and Emerging Diseases*, *66*(6), 2402–2410. https://doi.org/10.1111/tbed.13298

Chapman, D. A. G., Darby, A. C., Silva, M. D., Upton, C., Radford, A. D., & Dixon, L. K. (2011). Genomic Analysis of Highly Virulent Georgia 2007/1 Isolate of African Swine Fever Virus. *Emerging Infectious Diseases*, *17*(4), 599. https://doi.org/10.3201/eid1704.101283

Chenais, E., Sternberg-Lewerin, S., Boqvist, S., Liu, L., LeBlanc, N., Aliro, T., Masembe, C., & Ståhl, K. (2017). African swine fever outbreak on a medium-sized farm in Uganda: Biosecurity breaches and within-farm virus contamination. *Tropical Animal Health and Production*, *49*(2), 337–346. https://doi.org/10.1007/s11250-016-1197-0

Conley, Y. P., Biesecker, L. G., Gonsalves, S., Merkle, C. J., Kirk, M., & Aouizerat, B. E. (2013). Current and Emerging Technology Approaches in Genomics: Technology Approaches in Genomics. *Journal of Nursing Scholarship*, *45*(1), 5–14. https://doi.org/10.1111/jnu.12001

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

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

Couacy-Hymann, E., Kouakou, K. V., Achenbach, J. E., Kouadio, L., Koffi, Y. M., Godji, H. P., Adjé, K. E., Oulaï, J., Pell-Minhiaud, H. J., & Lamien, C. E. (2019). Re-emergence

of genotype I of African swine fever virus in Ivory Coast. *Transboundary and Emerging Diseases*, *66*(2), 882–896. https://doi.org/10.1111/tbed.13098

Cubillos, C., Gómez-Sebastian, S., Moreno, N., Nuñez, M. C., Mulumba-Mfumu, L. K., Quembo, C. J., Heath, L., Etter, E. M. C., Jori, F., Escribano, J. M., & Blanco, E. (2013). African swine fever virus serodiagnosis: A general review with a focus on the analyses of African serum samples. *Virus Research*, *173*(1), 159–167.

De León, P., Bustos, M. J., & Carrascosa, A. L. (2013). Laboratory methods to study African swine fever virus. *Virus Research*, *173*(1), 168–179.

Dixon, L. K. (1986). *The Structure and Function of the African Swine Fever Virus Genome.* https://scholar.google.com

Dixon, L. K., & Wilkinson, P. J. (1988). Genetic Diversity of African Swine Fever Virus Isolates from Soft Ticks (*Ornithodoros moubata*) Inhabiting Warthog Burrows in Zambia. *Journal of General Virology*, *69*(12), 2981–2993. https://doi.org/10.1099/0022-1317-69-12-2981

Dixon, L. K., Chapman, D. A., Netherton, C. L., & Upton, C. (2013). African swine fever virus replication and genomics. *Virus Research*, 173(1), 3-14.

Etter, E. M., Seck, I., Grosbois, V., Jori, F., Blanco, E., Vial, L., Akakpo, A.J., Bada-Alhambedji, R., Kone, P., & Roger, F.L. (2011). Seroprevalence of African swine fever in Senegal, 2006. *Emerging Infectious Diseases*, 17(1), 49.

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

FAO. (2018). *African Swine Fever threatens People's Republic of China: A Rapid Risk Assessment.* https://scholar.google.com

FAO;ILRI;AU-IBAR. (2017). *Regional Strategy for the Control of African Swine Fever in Africa* https://scholar.google.com.

Farlow, J., Donduashvili, M., Kokhreidze, M., Kotorashvili, A., Vepkhvadze, N. G., Kotaria, N., & Gulbani, A. (2018). Intra-epidemic genome variation in highly pathogenic

African swine fever virus (ASFV) from the country of Georgia. *Virology Journal*, *15*(1), 190. https://doi.org/10.1186/s12985-018-1099-z

Fasina, F. O., Kissinga, H., Mlowe, F., Mshang'a, S., Matogo, B., Mrema, A., Mhagama, A., Makungu, S., Mtui-Malamsha, N., Sallu, R., Misinzo, G., Magidanga, B., Kivaria, F., Bebay, C., Nong'ona, S., Kafeero, F., & Nonga, H. (2020). Drivers, Risk Factors and Dynamics of African Swine Fever Outbreaks, Southern Highlands, Tanzania. *Pathogens*, *9*(3), 1-18. https://doi.org/10.3390/pathogens9030155

Freitas, T. R. P., & Lyra, T. M. (2018). Molecular studies on African swine fever virus from Brazilian isolates. *Arquivos do Instituto Biológico*, 85, 1-8.

Gallardo, C., Ademun, A. R., Nietro, R., Nantima, N., Arias, M., MArtin, E., Pelayo, V., & Bishop, R. P. (2011). Genotyping of African swine fever virus (ASFV) isolates associated with disease outbreaks in Uganda in 2007. *African Journal of Biotechnology*, *10*, 3488–3497.

Gallardo, C., Anchuelo, R., Pelayo, V., Poudevigne, F., Leon, T., Nzoussi, J., Bishop, R., Pérez, C., Soler, A., Nieto, R., Martín, H., & Arias, M. (2011). African swine fever virus p72 genotype IX in domestic pigs, Congo, 2009. *Emerging Infectious Diseases*, *17*(8), 1556–1558. PubMed. https://doi.org/10.3201/eid1708.101877

Gallardo, C., Fernández-Pinero, J., Pelayo, V., Gazaev, I., Markowska-Daniel, I., Pridotkas, G., Nieto, R., Fernández-Pacheco, P., Bokhan, S., Nevolko, O., Drozhzhe, Z., Pérez, C., Soler, A., Kolvasov, D., & Arias, M. (2014). Genetic variation among African swine fever genotype II viruses, eastern and central Europe. *Emerging Infectious Diseases*, *20*(9), 1544–1547. PubMed. https://doi.org/10.3201/eid2009.140554

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

Gallardo, C., Nurmoja, I., Soler, A., Delicado, V., Simón, A., Martin, E., Perez, C., Nieto, R., &Arias, M. (2018). Evolution in Europe of African swine fever genotype II viruses from highly to moderately virulent. *Veterinary Microbiology*, *219*(January), 70–79. https://doi.org/10.1016/j.vetmic.2018.04.001

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

Ge, S., Li, J., Fan, X., Liu, F., Li, L., Wang, Q., Ren, W., Bao, J., Liu, C., Wang, H., & Liu, Y. (2018). Molecular characterization of African swine fever virus, China, 2018. *Emerging Infectious Diseases*, 24(11), 2131.

Gómez-Puertas, P., Rodríguez, F., Oviedo, J. M., Brun, A., Alonso, C., & Escribano, J. M. (1998). The African Swine Fever Virus Proteins p54 and p30 Are Involved in Two Distinct Steps of Virus Attachment and Both Contribute to the Antibody-Mediated Protective Immune Response. *Virology*, *243*(2), 461–471.

Granberg, F., Torresi, C., Oggiano, A., Malmberg, M., Iscaro, C., Mia, G. M. D., & Belák, S. (2016). Complete Genome Sequence of an African Swine Fever Virus Isolate from Sardinia, Italy. *Genome Announcements*, 4(6), e01220-16.

Grzegorz, W., Magdalena, F., Krzysztof, N., & Zygmunt, P. (2016). Selected aspects related to epidemiology, pathogenesis, immunity, and control of African swine fever. *Journal of Veterinary Research*, *60*(2), 119–125.

Guinat, Claire, Gogin, A., Blome, S., Keil, G., Pollin, R., Pfeiffer, D. U., & Dixon, L. (2016). Transmission routes of African swine fever virus to domestic pigs: Current knowledge and future research directions. *Veterinary Record*, *178*(11), 262–267.

Hakizimana, J. N., Kamwendo, G., Chulu, J. L. C., Kamana, O., Nauwynck, H. J., & Misinzo, G. (2020). Genetic profile of African swine fever virus responsible for the 2019 outbreak in northern Malawi. *Research Square*, 2020, 1–17.

Hakizimana, J. N., Nyabongo, L., Ntirandekura, J. B., Yona, C., Ntakirutimana, D., Kamana, O., Nauwynck, H., & Misinzo, G. (2020). Genetic Analysis of African Swine Fever Virus From the 2018 Outbreak in South-Eastern Burundi. *Frontiers in Veterinary Science*, *7*, 871. https://doi.org/10.3389/fvets.2020.578474

Haresnape, J. M., Wilkinson, P. J., & Mellor, P. S. (1988). Isolation of African swine fever virus from ticks of the Ornithodoros moubata complex (Ixodoidea: Argasidae) collected within the African swine fever enzootic area of Malawi. *Epidemiology and Infection*, *101*(1), 173–185.

Hou, D., Bi, Y., Sun, H., Yang, J., Fu, G., Sun, Y., Liu, J., & Pu, J. (2012). Identification of swine influenza A virus and Stenotrophomonas maltophilia co-infection in Chinese pigs. *Virology Journal*, 9, 169. https://doi.org/10.1186/1743-422X-9-169

Igbokwe, I. O., & Maduka, C. V. (2018). Disease burden affecting pig production in Nigeria: Review of current issues and challenges. *Revue d'élevage et de Médecine Vétérinaire Des Pays Tropicaux*, *71*(1–2). https://doi.org/10.19182/remvt.31290

Imbery, J., & Upton, C. (2017). *Organization of the Multigene Families of African Swine Fever Virus*. https://scholar.google.com

Galindo, I., & Alonso, C. (2017). African swine fever virus: A review. *Viruses*, 9(5), 103.

Jori, F., Vial, L., Penrith, M. L., Pérez-Sánchez, R., Etter, E., Albina, E., Michaud, V., & Roger, F. (2013). Review of the sylvatic cycle of African swine fever in sub-Saharan Africa and the Indian ocean. *Virus Research*, *173*(1), 212–227.

Jori, F., & Bastos, A. D. (2009). Role of wild suids in the epidemiology of African swine fever. *EcoHealth*, 6(2), 296-310.

Jurado, C., Martinez-Aviles, M., De La Torre, A., Štukelj, M., De Carvalho Ferreira, H. C., Cerioli, M., Sánchez-Vizcaíno, J. M., & Bellini, S. (2018). Relevant measures to prevent the spread of African swine fever in the European Union domestic pig sector. *Frontiers In Veterinary Science*, 5, 77.

Katale, B. Z., Fyumagwa, R. D., Mdaki, M. L., & Hoare, R. (2012). Prevalence of African swine fever virus in Warthogs in the Serengeti ecosystem, Tanzania. *Research Opinions in Animal and Veterinary Sciences*, *2*(5), 339–343.

Kolbasov, D., Titov, I., Tsybanov, S., Gogin, A., & Malogolovkin, A. (2018). African Swine Fever Virus, Siberia, Russia, 2017. *Emerging Infectious Diseases*, *24*(4), 796–798. https://doi.org/10.3201/eid2404.171238

Kouakou, K. V., Michaud, V., Biego, H. G., Gnabro, H. P. G., Kouakou, A. V., Mossoun, A. M., Awuni, J. A., Minoungou, G. L., Aplogan, G. L., Awoumé, F. K., Albina, E., Lancelot, R., & Couacy-Hymann, E. (2017). African and classical swine fever situation in Ivory-Coast and neighboring countries, 2008–2013. *Acta Tropica*, *166*, 241–248. https://doi.org/10.1016/j.actatropica.2016.10.027

Krug, P. W., Holinka, L. G., O'Donnell, V., Reese, B., Sanford, B., Fernandez-Sainz, I., Gladue, D. P., Arzt, J., Rodriguez, L., Risatti, G. R., & Borca, M. V. (2015). The Progressive Adaptation of a Georgian Isolate of African Swine Fever Virus to Vero Cells Leads to a Gradual Attenuation of Virulence in Swine Corresponding to Major Modifications of the Viral Genome. *Journal of Virology*, *89*(4), 2324–2332. https://doi.org/10.1128/JVI.03250-14

Leland, D. S., & French, M. L. V. (1988). Virus Isolation and Identification. In E. H. Lennette, P. Halonen, F. A. Murphy, A. Balows, & W. J. Hausler (Eds), *Laboratory Diagnosis of Infectious Diseases Principles and Practice* (pp. 39–59). Springer New York. https://doi.org/10.1007/978-1-4612-3900-0_3

Lopez, E., van Heerden, J., Bosch-Camós, L., Accensi, F., Navas, M. J., López-Monteagudo, P., Argilaguet, J., Gallardo, C., Pina-Pedrero, S., Salas, M. L., Salt, J., & Rodriguez, F. (2020). Live Attenuated African Swine Fever Viruses as Ideal Tools to Dissect the Mechanisms Involved in Cross-Protection. *Viruses*, *12*(12), 1474. https://doi.org/10.3390/v12121474

Lubisi, B. A., Bastos, A. D. S., Dwarka, R. M., & Vosloo, W. (2005). Molecular epidemiology of African swine fever in East Africa. *Archives of Virology*, *150*(12), 2439–2452. https://doi.org/10.1007/s00705-005-0602-1

Lubisi, B. A., Dwarka, R. M., Meenowa, D., & Jaumally, R. (2009). An Investigation into the First Outbreak of African Swine Fever in the Republic of Mauritius. *Transboundary and Emerging Diseases*, *56*(5), 178–188. https://doi.org/10.1111/j.1865-1682.2009.01078.x

Lubisi, B. A., Bastos, A. D. S., Dwarka, R. M., & Vosloo, W. (2007). Intra-genotypic resolution of African swine fever viruses from an East African domestic pig cycle: A combined p72-CVR approach. *Virus Genes,* 35(3), 729-735.

Luka, P. D., Achenbach, J. E., Mwiine, F. N., Lamien, C. E., Shamaki, D., Unger, H., & Erume, J. (2017). Genetic Characterization of Circulating African Swine Fever Viruses in Nigeria (2007–2015). *Transboundary and Emerging Diseases*, *64*(5), 1598–1609. https://doi.org/10.1111/tbed.12553

Malogolovkin, A., Burmakina, G., Titov, I., Sereda, A., Gogin, A., Baryshnikova, E., & Kolbasov, D. (2015). Comparative analysis of African swine fever virus genotypes and serogroups. *Emerging Infectious Diseases*, 21(2), 312.

Martínez-López, B., Perez, A. M., Feliziani, F., Rolesu, S., Mur, L., & Sánchez-Vizcaíno, J. M. (2015). Evaluation of the risk factors contributing to the African swine fever occurrence in Sardinia, Italy. *Frontiers in Microbiology*, 6, 314.

Masembe, C., Sreenu, V. B., Da Silva, F. A., Wilkie, G. S., Ogweng, P., Mayega, F. J., Muwanika, V. B., Biek, R., Palmarini, M., & Davison, A. J. (2018). Genome sequences of five African swine fever virus genotype IX isolates from domestic pigs in Uganda. *Microbiology Resource Announcements*, 7(13), e01018-18.

Mauya, P. V. (2015). *Molecular Characterization of African Swine Fever Virus in Selected Areas of Northern and Southern Tanzania during 2014 outbreaks* [Doctoral Dissertation, Sokoine University of Agriculture]. https://scholar.google.com

Mazur-Panasiuk, N., Woźniakowski, G., & Niemczuk, K. (2019). The first complete genomic sequences of African swine fever virus isolated in Poland. *Scientific Reports*, 9(1), 1-9.

Misinzo, G., Jumapili, F., Ludosha, M., Mafie, E., Silialias, J., Mushi, R., Viaene, W., & Doorsselaere, J. (2012). Genotyping of African swine fever virus from a 2009 outbreak in Tanzania. *Research Opinions in Animal and Veterinary Sciences*, *2*(5), 334–338.

Misinzo, G., Kwavi, D. E., Sikombe, C. D., Makange, M., Peter, E., Muhairwa, A. P., & Madege, M. J. (2014). Molecular characterization of African swine fever virus from domestic pigs in northern Tanzania during an outbreak in 2013. *Tropical Animal Health and Production*, *46*(7), 1199–1207. https://doi.org/10.1007/s11250-014-0628-z

Misinzo, G., Magambo, J., Masambu, J., Yongolo, M. G., Van Doorsselaere, J., & Nauwynck, H. J. (2011). Genetic Characterization of African Swine Fever Viruses from a 2008 Outbreak in Tanzania. *Transboundary and Emerging Diseases*, *58*(1), 86–92.

Misinzo, G., Gwandu, F. B., Biseko, E. Z., Kulaya, N. B., Mdimi, L., Kwavi, D. E., Sikombe, C. D., Makange, M., & Madege, M. J. (2014). Neisseria denitrificans restriction endonuclease digestion distinguishes African swine fever viruses of eastern from southern African origin circulating in Tanzania. *Research Opinions in Animal and Veterinary Sciences*, 4(4), 212-217.

Misinzo, G., Kwavi, D. E., Sikombe, C. D., Makange, M., Peter, E., Muhairwa, A. P., & Madege, M. J. (2014). Molecular characterization of African swine fever virus from domestic pigs in northern Tanzania during an outbreak in 2013. *Tropical Animal Health and Production*, 46(7), 1199-1207.

Mlumba-Mfumu, L. K., Achenbach, J. E., Mauldin, M. R., Dixon, L. K., Tshilenge, C. G., Thiry, E., Moreno, N., Blanco, E., Saegerman, C., Lamien, C. E., & Dillo, A. (2017). Genetic Assessment of African Swine Fever Isolates Involved in Outbreaks in the Democratic Republic of Congo between 2005 and 2012 Reveals Co-Circulation of p72 Genotypes I, IX and XIV, Including 19 Variants. *Viruses*, *9*, 31.

Molini, U., Mushonga, B., Settypalli, T. B. K., Dundon, W. G., Khaiseb, S., Jago, M., Cattoli, G., & Lamien, C. E. (2020). Molecular characterization of African swine fever virus from outbreaks in Namibia in 2018. *Transboundary and Emerging Diseases*, *67*(2), 1008–1014. https://doi.org/10.1111/tbed.13399

Mur, L., Martínez-López, B., & Sánchez-Vizcaíno, J. M. (2012). Risk of African swine fever introduction into the European Union through transport-associated routes: returning trucks and waste from international ships and planes. *BMC Veterinary Research*, 8(1), 1-12.

Mwiine, F. N., Nkamwesiga, J., Ndekezi, C., & Ochwo, S. (2019). Molecular Characterization of African Swine Fever Viruses from Outbreaks in Peri-Urban Kampala, Uganda. *Advances in Virology*, *2019*, 1463245. https://doi.org/10.1155/2019/1463245

Nantima, N., Davies, J., Dione, M., Ocaido, M., Okoth, E., Mugisha, A., & Bishop, R. (2016). Enhancing knowledge and awareness of biosecurity practices for control of African swine fever among smallholder pig farmers in four districts along the Kenya–Uganda border. *Tropical Animal Health and Production*, *48*(4), 727–734. https://doi.org/10.1007/s11250-016-1015-8

Ndlovu, S., Williamson, A. L., Malesa, R., Van Heerden, J., Boshoff, C. I., Bastos, A. D., Heath, L., & Carulei, O. (2020). Genome sequences of three African swine fever viruses of genotypes I, III, and XXII from South Africa and Zambia, isolated from ornithodoros soft ticks. *Microbiology Resource Announcements*, 9(10), e01376-19.

Ngwa, N., Abouna, A., Zoli, A. P., & Attili, A. R. (2020). Epidemiology of African Swine Fever in Piggeries in the Center, South and South-West of Cameroon. *Veterinary Science*, *7*, 123.

Nix, R. J., Gallardo, C., Hutchings, G., Blanco, E., & Dixon, L. K. (2006). Molecular epidemiology of African swine fever virus studied by analysis of four variable genome regions. *Archives of Virology*, *151*(12), 2475–2494. https://doi.org/10.1007/s00705-006-0794-z

O'Donnell, V., Holinka, L. G., Gladue, D. P., Sanford, B., Krug, P. W., Lu, X., Arzt, J., Reese, B., Carrillo, C., Risatti, G. R., & Borca, M. V. (2015). African Swine Fever Virus Georgia Isolate Harboring Deletions of MGF360 and MGF505 Genes Is Attenuated in Swine and Confers Protection against Challenge with Virulent Parental Virus. *Journal of Virology*, *89*(11), 6048–6056. https://doi.org/10.1128/JVI.00554-15

O'Donnell, V., Holinka, L. G., Sanford, B., Krug, P. W., Carlson, J., Pacheco, J. M., Reese, B., Risatti, G. R., Gladue, D. P., & Borca, M. V. (2016). African swine fever virus Georgia isolate harboring deletions of 9GL and MGF360/505 genes is highly attenuated in swine but does not confer protection against parental virus challenge. *Virus Research*, *221*, 8–14. https://doi.org/10.1016/j.virusres.2016.05.014

O'Donnell, V., Risatti, G. R., Holinka, L. G., Krug, P. W., Carlson, J., Velazquez-Salinas, L., Azzinaro, P. A., Gladue, D. P., & Borca, M. V. (2017). Simultaneous Deletion of the 9GL and UK Genes from the African Swine Fever Virus Georgia 2007 Isolate Offers Increased Safety and Protection against Homologous Challenge. *Journal of Virology*, *91*(1), e01760-16. https://doi.org/10.1128/JVI.01760-16

Ogweng, P. (2017). *The Role of the Bushpigs in the Epidemiology of African Swine Fever at the Wildlife-Livestock Interface in Uganda* [Masters Dissertation]. Makerere University. https://scholar.google.com/schola

OIE-Listed diseases. (2019). *OIE - World Organisation for Animal Health*. http:// www. oie. int/ en/ animal-health-in-the-world/oie-listed-diseases-2019/

Okoth, E., Gallardo, C., Macharia, J. M., Omore, A., Pelayo, V., Bulimo, D. W., Arias, M., Kitala, P., Baboon, K., Lekolol, I., Mijele, D., & Bishop, R. P. (2013). Comparison of African swine fever virus prevalence and risk in two contrasting pig-farming systems

in South-west and Central Kenya. *Preventive Veterinary Medicine*, *110*(2), 198–205. https://doi.org/10.1016/j.prevetmed.2012.11.012

Omambia, C. S., & Gu, Y. (2010). The cost of climate change in Tanzania: impacts and adaptations. *Journal of American Science*, 6(3), 182-196.

Onzere, C. (2013). *Project Presentation*. https://scholar.google.com

Onzere, C. K., Bastos, A. D., Okoth, E. A., Lichoti, J. K., Bochere, E. N., Owido, M. G., Ndambuki, G., Bronsvoort, M., & Bishop, R. P. (2018). Multi-locus sequence typing of African swine fever viruses from endemic regions of Kenya and Eastern Uganda (2011–2013) reveals rapid B602L central variable region evolution. *Virus Genes*, *54*(1), 111–123. https://doi.org/10.1007/s11262-017-1521-4

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

Oyola, S. O., Ariani, C. V., Hamilton, W. L., Kekre, M., Amenga-Etego, L. N., Ghansah, A., Rutledge, G. G., Redmond, S., Manske, M., Jyothi, D., Jacob, C. G., Otto, T. D., Rockett, K., Newbold, C. I., Berriman, M., & Kwiatkowski, D. P. (2016). Whole genome sequencing of Plasmodium falciparum from dried blood spots using selective whole genome amplification. *Malaria Journal*, *15*(1), 597. https://doi.org/10.1186/s12936-016-1641-7

Patrick, B. N., Dieudonne, W. S., Théophile, N. M., Ahadi, B. B., Espoir, B. B., Gustave, N. M., & Bajope, K. K. B. (2019). Evidence of African Swine Fever Virus in Pigs Slaughtered at Muhanzi Municipal Abattoir in Bukavu City, Eastern of Democratic Republic of Congo. *International Journal of Microbiology and Biotechnology*, *4*(1), 1–7.

Patrick, B. N., Machuka, E. M., Githae, D., Banswe, G., Amimo, J. O., Ongus, J. R., Masembe, C., Bishop, R. P., Steinaa, L., Djikeng, A., & Pelle, R. (2020). Evidence for the presence of African swine fever virus in apparently healthy pigs in South-Kivu Province of the Democratic Republic of Congo. *Veterinary Microbiology*, *240*, 108521. https://doi.org/10.1016/j.vetmic.2019.108521

Penrith, M. L., Thomson, G. R., Bastos, A. D. S., Phiri, O. C., Lubisi, B. A., Macome, F., Pinto, F., Botha, B., & Esterhuysen, J. (2004). An investigation into natural resistance to African swine fever in domestic pigs from an endemic area in southern Africa. *Revue Scientifique et Technique (International Office of Epizootics)*, *23*(3), 965–977.

Penrith, M. L., Vosloo, W., Jori, F., & Bastos, A. D. S. (2013). African swine fever virus eradication in Africa. *African Swine Fever Virus*, *173*(1), 228–246.

Penrith, M. L. (2013). History of'swine fever'in Southern Africa: historical article. *Journal of the South African Veterinary Association*, 84(1), 1-6.

Penrith, M. L. (2009). African swine fever. *Onderstepoort Journal of Veterinary Research*, 76(1), 91-95.

Peter, E., Machuka, E., Githae, D., Okoth, E., Cleaveland, S., Shirima, G., Kusiluka, L., & Pelle, R. (2021). Detection of African swine fever virus genotype XV in a sylvatic cycle in Saadani National Park, Tanzania. *Transboundary and Emerging Diseases*, 68(2), 813-823.

Petrovan, V. (2019). *Antigenic Characterization of African Swine Fever Virus (ASFV) p30 and p54 Proteins*. https://scholar.google.com

Pikalo, J., Zani, L., Hühr, J., Beer, M., & Blome, S. (2019). Pathogenesis of African swine fever in domestic pigs and European wild boar: Lessons learned from recent animal trials. *Virus Research*, *271*, 197614. https://doi.org/10.1016/j.virusres.2019.04.001

Plowright, W., Parker, J., & Peirce, M. A. (1969). African Swine Fever Virus in Ticks (Ornithodoros moubata, Murray) collected from Animal Burrows in Tanzania. *Nature*, *221*(5185), 1071–1073. https://doi.org/10.1038/2211071a0

Pollock, N. R., & Wonderly, B. (2017). Evaluating Novel Diagnostics in an Outbreak Setting: Lessons Learned from Ebola. *Journal of Clinical Microbiology*, *55*(5), 1255–1261. https://doi.org/10.1128/JCM.00053-17

Powell, J. D., & Straub, T. M. (2018). Advances and Remaining Challenges in the Study of Influenza and Anthrax Infection in Lung Cell Culture. *Challenges*, 9(1), 2, 1-10.

Probst, C., Gethmann, J., Amler, S., Globig, A., Knoll, B., & Conraths, F. J. (2019). The potential role of scavengers in spreading African swine fever among wild boar. *Scientific Reports*, *9*(1), 1–13. https://doi.org/10.1038/s41598-019-47623-5

Quembo, C. J., Jori, F., Vosloo, W., & Heath, L. (2018). Genetic characterization of African swine fever virus isolates from soft ticks at the wildlife/domestic interface in Mozambique and identification of a novel genotype. *Transboundary and Emerging Diseases*, *65*(2), 420–431. https://doi.org/10.1111/tbed.12700

Ravaomanana, J., Jori, F., Vial, L., Pérez-Sánchez, R., Blanco, E., Michaud, V., & Roger, F. (2011). Assessment of interactions between African swine fever virus, bushpigs (*Potamochoerus larvatus*), Ornithodoros ticks and domestic pigs in north-western Madagascar: ASFV and its Potential Reservoir Hosts in Madagascar. *Transboundary and Emerging Diseases*, *58*(3), 247–254. https://doi.org/10.1111/j.1865-1682.2011.01207.x

Ravaomanana, J., Michaud, V., Jori, F., Andriatsimahavandy, A., Roger, F., Albina, E., & Vial, L. (2010). First detection of African Swine Fever Virus in Ornithodoros porcinus in Madagascar and new insights into tick distribution and taxonomy. *Parasites and Vectors*, *3*(1), 115. https://doi.org/10.1186/1756-3305-3-115

Reis, A. L., Netherton, C., & Dixon, L. K. (2017). Unraveling the Armor of a Killer: Evasion of Host Defenses by African Swine Fever Virus. *Journal of Virology*, *91*(6), e02338-16. https://doi.org/10.1128/JVI.02338-16

Rodríguez, J. M., Moreno, L. T., Alejo, A., Lacasta, A., Rodríguez, F., & Salas, M. L. (2015). Genome Sequence of African Swine Fever Virus BA71, the Virulent Parental Strain of the Nonpathogenic and Tissue-Culture Adapted BA71V. *Plos One*, *10*(11), e0142889. https://doi.org/10.1371/journal.pone.0142889

Saari, S., Näreaho, A., & Nikander, S. (2019). Chapter 9: Arachnida. In S. Saari, A. Näreaho, & S. Nikander (Eds.), *Canine Parasites and Parasitic Diseases* (pp. 187–228). Academic Press. https://doi.org/10.1016/B978-0-12-814112-0.00009-X

Samkange, A., Mushonga, B., Mudimba, D., Chiwome, B. A., Jago, M., Kandiwa, E., Bishi, A. S., & Molini, U. (2019). African Swine Fever Outbreak at a Farm in Central Namibia. *Case Reports in Veterinary Medicine*, *2019*, 3619593. https://doi.org/10.1155/2019/3619593

Sánchez-Cordón, P. J., Montoya, M., Reis, A. L., & Dixon, L. K. (2018). African swine fever: A re-emerging viral disease threatening the global pig industry. *The Veterinary Journal*, *233*, 41–48. https://doi.org/10.1016/j.tvjl.2017.12.025

Sánchez-Vizcaíno, J. M. (2010). *Early Detection and Contingency Plans for African Swine Fever*. https://scholar.google.com

Sanford, B., Holinka, L. G., O'Donnell, V., Krug, P. W., Carlson, J., Alfano, M., Carrillo, C., Wu, P., Lowe, A., Risatti, G. R., Gladue, D. P., & Borca, M. V. (2016). Deletion of the thymidine kinase gene induces complete attenuation of the Georgia isolate of African swine fever virus. *Virus Research*, *213*, 165–171.

Simulundu, E., Chambaro, H. M., Sinkala, Y., Kajihara, M., Ogawa, H., Mori, A., Ndebe, J., Dautu, G., Mataa, L., Lubaba, C. H., Simuntala, C., Fandamu, P., Simuunza, M., Pandey, G. S., Samui, K. L., Misinzo, G., Takada, A., & Mweene, A. S. (2018). Co-circulation of multiple genotypes of African swine fever viruses among domestic pigs in Zambia (2013–2015). *Transboundary and Emerging Diseases*, *65*(1), 114–122.

Simulundu, E., Sinkala, Y., Chambaro, H. M., Chinyemba, A., Banda, F., Mooya, L. E., Ndebe, J., Chitanga, S., Makungu, C., Munthali, G., Fandamu, P., Takada, A., & Mweene, A. S. (2018). Genetic characterisation of African swine fever virus from 2017 outbreaks in Zambia: Identification of p72 genotype II variants in domestic pigs. *Onderstepoort Journal of Veterinary Research*, *85*, 1–5.

Simulundu, Edgar, Lubaba, C. H., van Heerden, J., Kajihara, M., Mataa, L., Chambaro, H. M., Sinkala, Y., Munjita, S. M., Munang'andu, H. M., Nalubamba, K. S., Samui, K., Pandey, G. S., Takada, A., & Mweene, A. S. (2017). The Epidemiology of African Swine Fever in "Nonendemic" Regions of Zambia (1989-2015): Implications for Disease Prevention and Control. *Viruses*, *9*(9). 236. https://doi.org/10.3390/v9090236

Stahl, K., Ogweng, P., Okoth, E. A., Aliro, T., Muhangi, D., LeBlanc, N., Atimnedi, P., Berg, M., Bishop, R.P., Rasmussen, H. B., & Masembe, C. (2014). Understanding the dynamics and spread of African Swine fever at the wildlife livestock interface: Insights into the potential role of the bushpig *Potamochoerus larvatus. Suiform Soundings*, 3, 24-29.

Ståhl, K., Sternberg-Lewerin, S., Blome, S., Viltrop, A., Penrith, M. L., and Chenais, E. (2019). Lack of evidence for long term carriers of African swine fever virus: A systematic review. *Virus Research*, *272*, 197725. https://doi.org/10.1016/j.virusres.2019.197725

Steinaa, L., Bishop, R. P., Okoth, E. A., Svitek, N., & Riitho, V. (2016). Pig vaccines and diagnostics for African swine fever in Uganda. *Livestock and Fish Brief*, 26, 1-4.

Štukelj, M., & Plut, J. (2018). A Review of African Swine Fever: Disease that is Now a Big Concern in Europe. *Contemporary Agriculture*, *67*(2), 110–118. https://doi.org/10.2478/contagri-2018-0016

Thomas, L. F., Bishop, R. P., Onzere, C., Mcintosh, M. T., Lemire, K. A., de Glanville, W. A., Cook, E. A. J., & Fèvre, E. M. (2016). Evidence for the presence of African swine fever virus in an endemic region of Western Kenya in the absence of any reported outbreak. *BMC Veterinary Research*, *12*(1), 192. https://doi.org/10.1186/s12917-016-0830-5

Thomson, G. R. (1985). *The Epidemiology of African swine fever: The Role of Free-living Hosts in Africa*. https://scholar.google.com

Thomson, G. R., Gainaru, M. D., &Dellen, A. F. V. (1980). *Experimental Infection of Warthog (phacochoerus Aethiopicus) with African Swine Fever.* https://scholar.google.com

Thoromo, J., Simulundu, E., Chambaro, H. M., Mataa, L., Lubaba, C. H., Pandey, G. S., Takada, A., Misinzo, G., & Mweene, A. S. (2016). Diagnosis and genotyping of African swine fever viruses from 2015 outbreaks in Zambia. *Onderstepoort Journal of Veterinary Research*, *83*, 1–5.

Treydte, A. C., Edwards, P. J., & Suter, W. (2005). Shifts in native ungulate communities on a former cattle ranch in Tanzania. *African Journal of Ecology*, *43*(4), 302–311. https://doi.org/10.1111/j.1365-2028.2005.00573.x

Van Heerden, J., Malan, K., Gadaga, B. K., & Spargo, R. M. (2017). Reemergence of African Swine Fever in Zimbabwe, 2015. *Emerging Infectious Diseases*, *23*(5), 860–861. PubMed. https://doi.org/10.3201/eid2305.161195

Van Rensburg, L. J., Etter, E., Heath, L., Penrith, M. L., & Van Heerden, J. (2020). Understanding African swine fever outbreaks in domestic pigs in a sylvatic endemic

area: The case of the South African controlled area between 1977–2017. *Transboundary and Emerging Diseases*, 67(6), 2753-2769. https://doi.org/10.1111/tbed.13632

Wade, A., Achenbach, J. E., Gallardo, C., Settypalli, T. B. K., Souley, A., Djonwe, G., Loitsch, A., Dauphin, G., Ngang, J. J. E., Boyomo, O., Cattoli, G., Diallo, A., & Lamien, C. E. (2019). Genetic characterization of African swine fever virus in Cameroon, 2010–2018. *Journal of Microbiology*, *57*(4), 316–324. https://doi.org/10.1007/s12275-019-8457-4

Wambura, P. N., Masambu, J., & Msami, H. (2006). Molecular Diagnosis and Epidemiology of African Swine Fever Outbreaks in Tanzania. *Veterinary Research Communications*, *30*(6), 667–672. https://doi.org/10.1007/s11259-006-3280-x

Wen, X., He, X., Zhang, X., Zhang, X., Liu, L., Guan, Y., Zhang, Y., & Bu, Z. (2019). Genome sequences derived from pig and dried blood pig feed samples provide important insights into the transmission of African swine fever virus in China in 2018. *Emerging Microbes and Infections*, *8*(1), 303–306. https://doi.org/10.1080/22221751.2019.1565915

Wilson, R. T., & Swai, E. S. (2014). *Pig Production in Tanzania: A Critical Review*. *32*(1), 46–53. http://www.tropicultura.org/text/v32n1/46.pdf

Yona, C. M., Vanhee, M., Simulundu, E., Makange, M., Nauwynck, H. J., & Misinzo, G. (2020). Persistent domestic circulation of African swine fever virus in Tanzania, 2015–2017. BMC *Veterinary Research*, 16(1), 1-13.

Zsak, L., Borca, M. V., Risatti, G. R., Zsak, A., French, R. A., Lu, Z., Kutish, G. F., Neilan, J. G., Callahan, J. D., Nelson, W. M., & Rock, D. L. (2005). Preclinical Diagnosis of African Swine Fever in Contact-Exposed Swine by a Real-Time PCR Assay. *Journal of Clinical Microbiology*, 43(1), 112. https://doi.org/10.1128/JCM.43.1.112-119.2005

# APPENDICES

## Appendix 1: Data availability

The full genome assembly generated from this study has been deposited in the INSDC databases through the European Nucleotide Archive (ENA) under accession number LR813622. The ENA URL for this genome is http://www.ebi.ac.uk/ena/data/view/LR813622. The study ID pertaining to the present publication is PRJEB38524, the sample ID for the sequenced isolate is ERS4590719 and the assembly accession is GCA_903819505. Partial sequences from this study are openly available at NCBI at https://www.ncbi.nlm.nih.gov/genbank with GenBank accessions MT396736-MT396747 under the GenBank BankIt submission ID: 2168824.

The data that support the findings of this study are openly available in NCBI at https://www.ncbi.nlm.nih.gov/genbank

GenBank accessions produced from this study for partial sequences are
-MT396736, MT396737, MT396738, MT396739, MT396740, MT396741, MT396742, MT396743, MT396744, MT396745, MT396746, MT396747 under the GenBank BankIt submission ID: 2168824.

The full genome assembly generated from this study has been deposited in the INSDC databases through the European Nucleotide Archive (ENA) under accession number LR813622.

The ENA URL is http://www.ebi.ac.uk/ena/data/view/LR813622.
The study ID pertaining to the present publication is PRJEB38524, the sample ID for the sequenced isolate is ERS4590719 and the assembly accession is GCA_9038195

## RESEARCH OUTPUTS

### (i) Publication

Njau, E. P., Domelevo Entfellner, J. B., Machuka, E. M., Bochere, E. N., Cleaveland, S., Shirima, G. M., Kusiluka, L .J., Upton, C., Bishop, R. P., Pelle, R., Okoth, E. A. (2021). The first genotype II African swine fever virus isolated in Africa provides insight into the current Eurasian pandemic. *Scientific Reports*, *11*(1), 1-13.

Njau, E. P., Machuka, E. M., Cleaveland, S., Shirima, G. M., Kusiluka, L. J., Okoth, E. A., & Pelle, R. (2021). African swine fever virus (ASFV): Biology, genomics and genotypes circulating in sub-Saharan Africa. *Viruses*, *13*(11), 2285.

Peter, E., Machuka, E., Githae, D., Okoth, E., Cleaveland, S., Shirima, G., Kusiluka, L., Pelle, R., (2021) Detection of African swine fever virus genotype XV in a sylvatic cycle in Saadani National Park, Tanzania. *Transboundary and Emerging Diseases*, *68*(2), 813-823.

### (ii) Poster Presentantion