MOLECULAR CHARACTERIZATION OF AFRICAN SWINE FEVER VIRUS IN SELECTED AREAS OF NORTHERN AND SOUTHERN TANZANIA DURING 2014 OUTBREAKS

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A DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN ONE HEALTH MOLECULAR BIOLOGY OF THE SOKOINE UNIVERSITY OF AGRICULTURE. MOROGORO, TANZANIA.

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ABSTRACT

African swine fever virus (ASFV) is a large double stranded DNA virus of *Asfarviridae* family that causes African swine fever (ASF), a highly contagious and fatal hemorrhagic disease of domestic pigs. African swine fever presents a serious threat to food security and livelihoods of pig-keeping farmers. The aim of this study was to diagnose and genotype ASFV in domestic pigs following reports of ASF outbreaks in northern and southern Tanzania during 2014. Fifteen tissue samples were collected from domestic pigs that died of hemorrhagic disease in Arusha, Makambako, Sumbawanga and Nkasi. Diagnosis of ASF was done by polymerase chain reaction (PCR) using PPA1/PPA2 primers while genotyping was done by amplification, nucleotide sequencing and phylogenetic analysis of the variable part of *B646L* gene and complete *E183L* gene using primers p72U/p72D and PPA89/PPA889, respectively. African swine fever virus was confirmed in domestic pigs that died of hemorrhagic disease in study areas. Phylogenetic analysis clustered ASFV from northern and southern Tanzania into genotypes X and II, respectively. Genotype X ASFV identified in present study in northern Tanzania was similar to previously reported ASFV in Moshi and Arusha during 2009 and 2013 outbreaks. Genotype II ASFV identified in southern Tanzania was 100% similar to other ASFV previously reported in southern Tanzania between 2010 and 2013, and to ASFV reported in Madagascar, Russia and other European countries. The present study shows persistence and geographical restriction of ASFV within Tanzania. It is recommended that quarantine and restriction of pig movement be strictly imposed, to prevent further spread of ASF within Tanzania.

DECLARATION

I, Pendo Vincent Mauya, do hereby declare to the Senate of Sokoine University of Agriculture that this dissertation is my own original work done within the period of registration and that it has neither been submitted nor being concurrently submitted in any other institution.

_____ _____

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The declaration is hereby confirmed;

_____ _____

Prof. Gerald Misinzo Date

(Supervisor)

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DEDICATION

I dedicate my dissertation work to my family and many friends. A special feeling of gratitude to my beloved parents, Vincent and Dayana Mauya for their kind co-operation and encouragement which helped me to complete this project. To my very special sisters Imani and Ester and my brother Mussa who have never left me alone.

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LIST OF ABBREVIATIONS AND ACRONYMS

ASF	African swine fever
ASFV	African swine fever virus
BLAST	Basic Local Alignment Search Tool
CPE	cytopathic effect
CSFV	Classical swine fever virus
CVR	conserved variable region
DNA	deoxyribonucleic acid
EFSA	European Food Safety Authority
ELISA	enzyme-linked immunosorbent assay
EMPRES	Emergency Prevention System
EU	European Union
FAO	Food and Agriculture Organization
FAT	fluorescent antibody test
x*g*	centrifugal force
g	gram
HAD	haemadsorption
IFA	indirect fluorescent antibody test
kbp	kilo base pair
MEGA	Molecular Evolution Genetics Analysis
OIE	Organizational International Epizootics (World organization for Animal Health)
PBS	phosphate- buffered saline
PCR	polymerase chain reaction
RNA	ribonucleic acid

RT-PCR	reverse transcription polymerase chain reaction
s	seconds
SACIDS	Southern African Centre for Infectious Disease Surveillance
WAHID	World Animal Health Information Database

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

African swine fever virus (ASFV) is a large double-stranded deoxyribonucleic (DNA) virus with 170-190 kilobase pairs (kbp), classified as sole member of *Asfarviridae* family (Dixon *et al*., 2005). It is responsible for a highly contagious and fatal disease of domestic pigs known as African swine fever (ASF), and represented a serious threat to swine industry in East Africa and the rest of the world (Atuhaire *et al*., 2013). Though first isolated in domestic pigs in 1921 in Kenya, the virus occurs naturally in both vertebrate and invertebrate sylvatic hosts throughout sub-Saharan Africa and is transmitted to domestic pigs when infected ticks of the *Ornithodoros moubata* complex feed on them (Penrith *et al*., 2004). A domestic pig cycle, which is apparently not reliant on the presence of the tick vector, is believed to occur in West, Central and East Africa (Penrith *et al*., 2004). African swine fever is endemic in most of sub-Saharan Africa, including the island of Madagascar; the highest incidence of disease being recorded between the equator and the northern Transvaal in southern Africa (Costard *et al*., 2009).

African swine fever is devastating for the pork industry, causing massive losses of animals due to mortality and stamping out and further economic loss from trade restrictions. African swine fever entered the European Union in 2014, with the first cases in Lithuania followed by Poland, Latvia and Estonia. The first detections in all of these EU member states were in wild boar found dead. African swine fever has recently emerged in several European countries, with cases often linked to the movement of native Eurasian wild boar (*Sus scrofa*) (Sanchez-Vizcaino *et al*., 2013). African swine fever was reintroduced into continental Europe via an incursion in Georgia in April 2007 from where it rapidly spread

into Armenia, affecting domestic pigs and wild boars (Sanchez-Vizcaino *et al*., 2013). African swine fever further expanded through wild boar populations around the Caucasus Mountains (WAHID, 2012; Sanchez *et al.,* 2013). The spread into Azerbaijan, Chechnya, the Russian Federation, Ukraine and Belarus caused large-scale epidemics in domestic pigs. Concurrent infection of domestic pig and wild boar populations has led to the persistence of ASF in many areas. Controlling ASF in Russia and the Caucasus region has proved to be extremely difficult, reflecting the complexity of regional sanitary, economic, environmental and sociocultural factors (Sanchez-Vizcaino *et al*., 2013). There are no vaccines and ASF is still on the move (Oura, 2014). Wild boars play an important role in the spread of ASF and potentially in its maintenance. It is difficult to eliminate ASF from wild boar populations once it has become endemic (Gogin *et al*., 2013). Contact between infected wild boar and domestic pigs on outdoor farms poses a risk of transmission. Movement of vehicle and staff, feeds contaminated with ASF are among the possible causes for infarm introduction of the disease.

Pig farming is one of the fastest growing livestock activities in Eastern, Western and Southern Africa and has become very attractive in countries in the subregions as a means of increasing food security, income generating and employment, however ASF has on several occasions hampered this benefits (Bastos *et al*., 2003). In Tanzania, the disease was first reported in 1914 (FAO, 2001). Several outbreaks of ASF occurred in 1962, 1987 and 1988; and major epidemics with high economic losses has occurred in Mbeya, Arusha and Kilimanjaro regions (OIE, 2008; Wambura *et al*., 2006). Other outbreaks occurred in different regions such as Dar es salaam and Morogoro in 2001, 2003 and 2004, Arusha in 2003, Kigoma (Kasulu and Kibondo Districts) in 2004, Mwanza in 2005, Turiani (Morogoro) and Dar es Salaam in 2008 and Longido (Arusha) in 2009 (Wambura *et al*.,

2006; Misinzo *et al.,* 2012a). In 2010, outbreaks of the disease occurred in Mbeya, Rukwa and Dar es Salaam regions (WAHID, 2012).

African swine fever is a frequently fatal disease of domestic pigs and the outbreaks of ASFV are still a great challenge for the swine industry in Africa. At present, there is no treatment or vaccine available, and control is based on rapid laboratory diagnosis, enforcement of strict sanitary measures and quarantine (Sanchez *et al*., 2009).

Different virus population dynamics, degrees of diversity and disease manifestations in susceptible hosts are exhibited in different epidemiological regions and outbreaks (Gallardo *et al*., 2011). Genotyping of ASFV isolates is vital in establishing the patterns of outbreaks for future control and eradication of the disease. For diagnosis of ASFV, the 5′ end of the *B646L* p72 gene is used as the target for PCR amplification because of high level sequence conservation within different ASFV of different genotypes (Aguero *et al*., 2003). Sequence analysis of variable genome regions has been extensively used for molecular epidemiological studies of ASFV isolates (Bastos *et al*., 2003). Historically, all ASFV p72 genotypes have been circulating in eastern and southern Africa (Costard *et al*., 2009). A combined p72–CVR approach has been successfully used to investigate the field heterogeneity causing recent and historical outbreaks in Eastern and Southern Africa (Thompson *et al*., 1994). Previous studies have demonstrated the value of full p54 gene sequencing for providing additional, intermediate resolution when typing ASFV viruses (Gallardo *et al*., 2009). By combining the three genes *B646L* (encoding for p72), *E183* (encoding for p54) and *pB602L* located within the central variable region (CVR), a high level resolution approach is achieved for viral discrimination/characterization (Wilkinson, 2000).

1.2 Problem Statement and Study Justification

African swine fever is highly contagious and fatal disease which can cause the mortality rates of up to 100% in domestic pigs of all ages during outbreaks, and hence impose a great challenge to swine industry in Tanzania. Previous studies showed that molecular characterization and genotyping of ASFV have been done in northern Tanzania and revealed different genotypes including IX, X, XV and XVI while in southern Tanzania, only genotype II has been reported (Wambura *et al*., 2006; Misinzo *et al*., 2011; Misinzo *et al*., 2012b). African swine fever outbreaks continue to be reported in major pig-producing areas in northern and southern Tanzania. In the present study, ASFV from northern and southern Tanzania were sequenced in order to understand whether the recent circulating strains of ASFV are similar to or differ from the previuos ones. These Results will contribute to the design of disease control strategies and policy formulation.

1.3 Objectives

1.3.1 General Objective

The main objective of this study was to genotype ASFV collected from domestic pigs in selected areas of northern and southern Tanzania during the 2014 outbreaks.

1.3.2 Specific Objectives

i. To establish the presence of ASFV in domestic pigs suspected to have ASF in Arusha, Makambako, Nkasi and Sumbawanga;
ii. To genetically characterize ASFV from domestic pigs in Arusha, Makambako, Nkasi and Sumbawanga;
iii. To establish the phylogenetic relatedness of ASFV of Arusha, Makambako, Nkasi and Sumbawanga with other ASFV at the GenBank.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 African Swine Fever Virus

2.1.1 Classification

African swine fever virus is a large DNA virus that used to belong to the family *Iridoviridae* but is now reclassified as the sole member of the genus *Asfivirus* of the family *Asfarviridae* (Dixon *et al*., 2005*)*. It is a complex icosahedral enveloped virus that shows many characteristics similar to *Iridovirus* and *Poxvirus* (Yáñez, 1995). The genome varies in length between 170 and 190 kbp depending on the virus isolate due to deletions and insertions occurring within the terminal regions of the genome and within a coding gene region within the central region of the genome termed the central variable region (CVR) (Lubisi, 2005; Sanchez-Vizcaino *et al*., 2009; Owolodun *et al.*, 2010; Dixon, 2012). African swine fever virus replicates in the cytoplasm of infected cells as other poxviruses and shares with the Poxvirus a similar genomic organization, like hairpins ends of the genome with inverted repeat sequences in terminal position (Sanchez-Vizcaino *et al*., 2009). Phylogenetic analysis made possible discrimination of the asfarviruses into a different group other than the group of poxviruses and iridoviruses (Raoult *et al*., 2004).

2.1.2 Virus Structure

Virus particles are organized as a complex multi-layers structure (Sanchez-Vizcaino *et al*., 2009). Has a linear, covalently close-ended double-stranded DNA genome (dsDNA), the virion consists of glycoprotein core structure with a diameter of 70-100 nm and is covered by a capsid with a diameter ranging from 172-191 nm (Lubisi, 2005; Blasco *et al*., 1989). The genome consist of a central conserved region (CCR) of which has about 125kb long and highly variable region located left and right ends having inverted complementary

tandem repeats of about 35kb and 25kb correspondingly (Wesley and Tuthill, 1984; Blasco *et al*., 1989).

2.1.3 Viral Pathogenesis

The virus inters the host through oral, respiratory tract or through biting then goes through the tonsil or dorsal pharyngeal mucosa and to the mandibular or retropharyngeal lymphnodes from there the virus spread through viremia (Sánchez-Vizcaíno *et al*., 2009; Plowright, 1994). In some cases the virus can go through bronchial, gastrohepatic or mesenteric lymph nodes. After its entrance the viremia is observed after three days (Costard *et al*., 2009). Injuries on endothelium lead to haemorrhges and disseminated intravascular coagulation (DIC). Hemorrhages,effusions and oedema are herald by thrombocytopenia, dysfibrinopaenia, coagulopathy and impaired vascular intergrity (Anderson *et al*., 1986; Lubisi, 2005). The incubation period is usually between 4-6 days (6-8 days in subacute cases) (Sánchez- Botija, 1982).

2.1.4 Virus Replication

Virus replication occurs in the macrophages and monocyte cells, the mode of entry of the virus is through receptor mediated endocytosis (Alcami *et al*., 1989) and is released from endocytic vesicles into the cytoplasm through fusion of the virus envelope with those of the endocytic vesicles (Valdeira *et al*., 1998). The replication cycle is mainly cytoplasmic but early replication has been described to occur in nucleus (Salas and Andres, 2012). The role of the nuclear stage is not well understood to date but at this stage the nuclear organization is affected near replication site (Salas and Andres, 2012).

There is expression of the enzymes required for DNA replication immediately following virus entry into the cytoplasm from partially uncoated core. Prior to the cyoplasimic phase

there is nuclear replication phase which occurs six hours post infection and diminishes to nearly zero after 12 hours post-infection (Dixon *et al*., 2012). Following the onset of DNA replication in the cytoplasm at about 6 hours post-infection a shift in pattern of virus gene transcription occurs (Salas *et al.,* 1986).

DNA replication is initiated by the introduction of a single strand nick in the genome near to one or both termini. The exposed 3′ OH group acts as a primer for DNA polymerase and DNA synthesis proceeds towards the genome termini. This generates an intermediate in which termini of nascent and template strands are self-complementary and fold-back to form a self-priming hairpin structure (Dixon, 2012).

A putative DNA primase (C962R) encoded by ASFV may play a role in initiation of DNA replication or lagging strand DNA synthesis suggestion for de novo replication (Salas *et al.,* 1986*).* The mature head to head concatemeric intermediates are resolved to unit length terminally cross-linked genomes, and packaged into mature virus particles in the cytoplasmic factory sites (Dixon, 2012).

2.2 Distribution of ASF in the World

Ornithodoros species are common in tropical and subtropical areas (Vial, 2009). Most species are found in Africa but they do also occur in North America, South America, Asia and Europe (Taylor *et al.,* 2007). Montgomery first described ASF in the 1920s in Kenya. Affected domestic pigs died in a characteristic hemorrhagic fever with a mortality that approached 100%. Pigs that got sick had been in close contact with wildlife suid species particularly warthogs. A virus was identified that did not cause clinical disease among the warthogs. African swine fever has since its first appearance been described in most sub-Saharan countries (Sánches-Botija, 1982; Wilkinson, 1984).

The first outbreak of ASF outside Africa was reported in 1957 in Portugal. The source of infection was waste containing pork from airplanes, which was fed to pigs near the airport in Lisbon. The disease was eradicated through testing and slaughter but new outbreaks in Lisbon occurred in the 1960s with further spread to Spain and the virus remained enzootic in the Iberian Peninsula until 1995 when extensive eradication programs were able to fight further spread. *Ornithodoros erraticus* was identified as a vector and reservoir for the virus (Sánches-Botija, 1982; Costard *et al.*, 2009).

Import of infected pork products has been the main source of ASF outbreaks in countries outside Africa. After introduction in an ASF free country, the disease is often transmitted through direct contact between animals and indirectly through contaminated vehicles and gears. Transmission also occurs when pigs are fed with infectious and not heat-treated pork products (Sánches-Botija, 1982). Sporadic outbreaks have been reported in many countries including Brazil, Cuba, Haiti, Dominican Republic, Malta, Italy, France, Belgium and the Netherlands. All European countries have been able to eradicate the disease except Sardinia (an Italian island in the Mediterranean) where ASF has remained endemic since its introduction in 1978. The traditional pig production in Sardinia includes free-range pigs with open grazing and small pig holding systems. This system includes contact with wild boars, which make eradication more complicated. In many countries these sporadic outbreaks of ASF have been confused with other swine diseases like Classical swine fever (CSF) and therefore for a time remained unnoticed. This has complicated identification of ASF and there are several examples where an outbreak has been going on for months before a correct diagnosis has been made (Costard *et al.*, 2009; Sanchez-Botija, 1982).

More recent outbreaks outside Africa were reported in 2007-2008 in the Caucasus region (Georgia, Armenia, and Azerbaijan) and Russia (Rowlands *et al.*, 2007). The first clinical cases in Georgia were seen before May 2007 but were not officially reported to the World

Organisation for Animal Health (OIE) until 5 June 2007 (FAO, 2008). Contaminated pork- and pork-products from international ships were most likely the sources of virus introduction. The infection could probably be established since most pigs in Georgia are kept on free range and have access to dumped waste. After introduction, the disease spread according to the main transport and marketing routes. The virus isolate belong to genotype II and has close relationship with strains from south east Africa (Mozambique, Madagascar and Zambia) (Rowlands *et al.*, 2007). The situation in the Caucasus region and Russia is not under control to date and further spread to Eastern Europe is likely (EFSA, 2010).

Today, ASF is, according to OIE one of the most important diseases in domestic pigs. It has major socioeconomic consequences, significant impact on the national trade in pigs and pig products and causes severe animal suffering. African swine fever virus is an OIE listed disease and outbreaks must be reported unconditionally (WAHID, 2012).

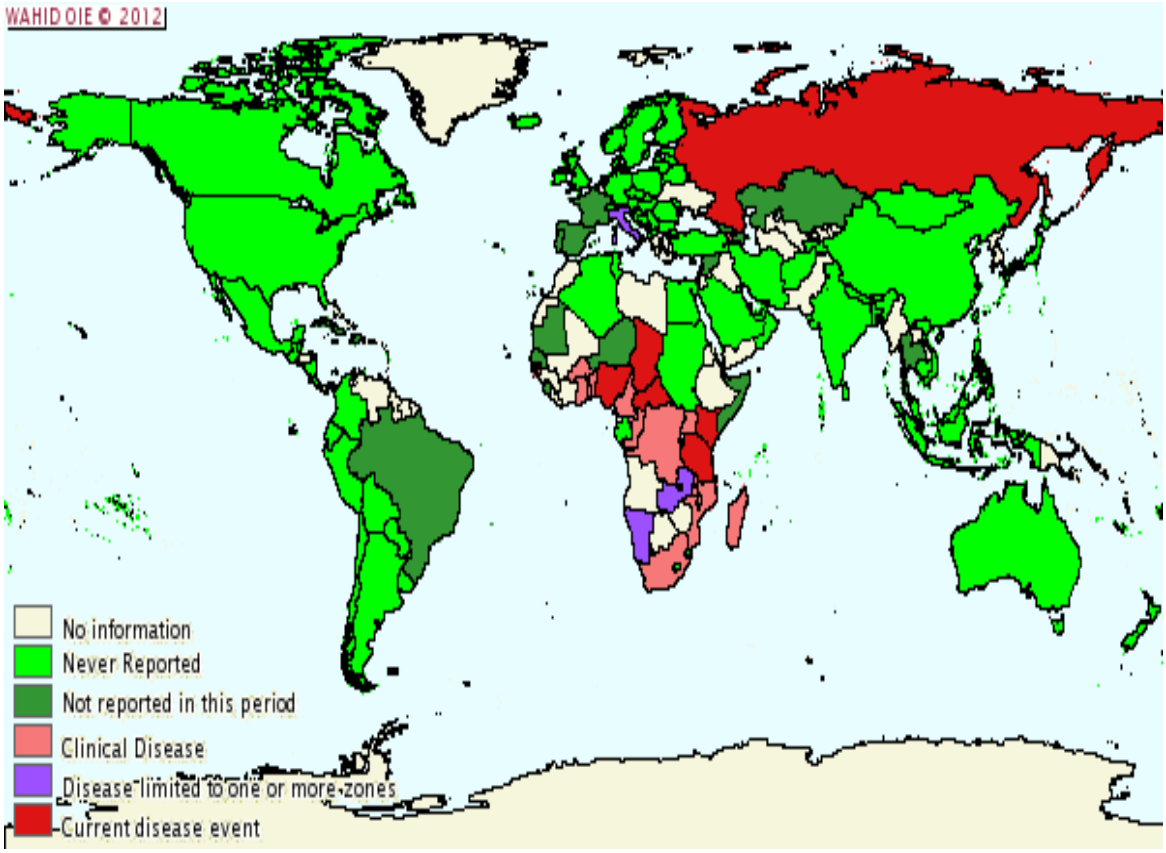

Figure 1: World distribution of African swine fever virus. Red colour indicate the areas where there is disease, purple the disease is limited to one or more zone, pink show the place where clinical disease observed, deep green the disease not reported in this period, pale green the disease never reported and white the disease information not reported (WAHID, 2012).

2.3 Epidemiology of ASFV

The virus originates from the African continent and the epidemiology of the disease is complex. In many countries south of Sahara the virus circulates in three different cycles: an ancient sylvatic cycle that involves soft ticks of the genus *Ornithodoros* and wild warthogs (*Phacochoerus africanus)*, a cycle between *Ornithodoros* and domestic pigs, and a domestic pig cycle that occurs in absence of ticks (Lubisi *et al.*, 2005). African swine fever virus can infect all members of the family suidae including domestic pigs, European and American wild boar, warthogs, bushpigs and giant forest hogs. Warthogs, giant forest hogs and bush pigs are together with the tick *Ornithodoros* considered natural reservoirs

and do not show clinical illness. Domestic pigs and wild boar on the other hand are susceptible to the virus and the mortality reaches up to 100% among naive animals (Penrith, 2004). The sylvatic cycle between wild warthogs and the soft tick of the species *Ornithodoros moubata* and *Ornithodoros porcinus* has been present in Africa for a very long time before the first introduction of the disease among domestic pigs in the 1920s. Warthogs are mainly found in eastern and southern Africa and are together with *Ornithodoros* species considered the major reservoir of ASFV.

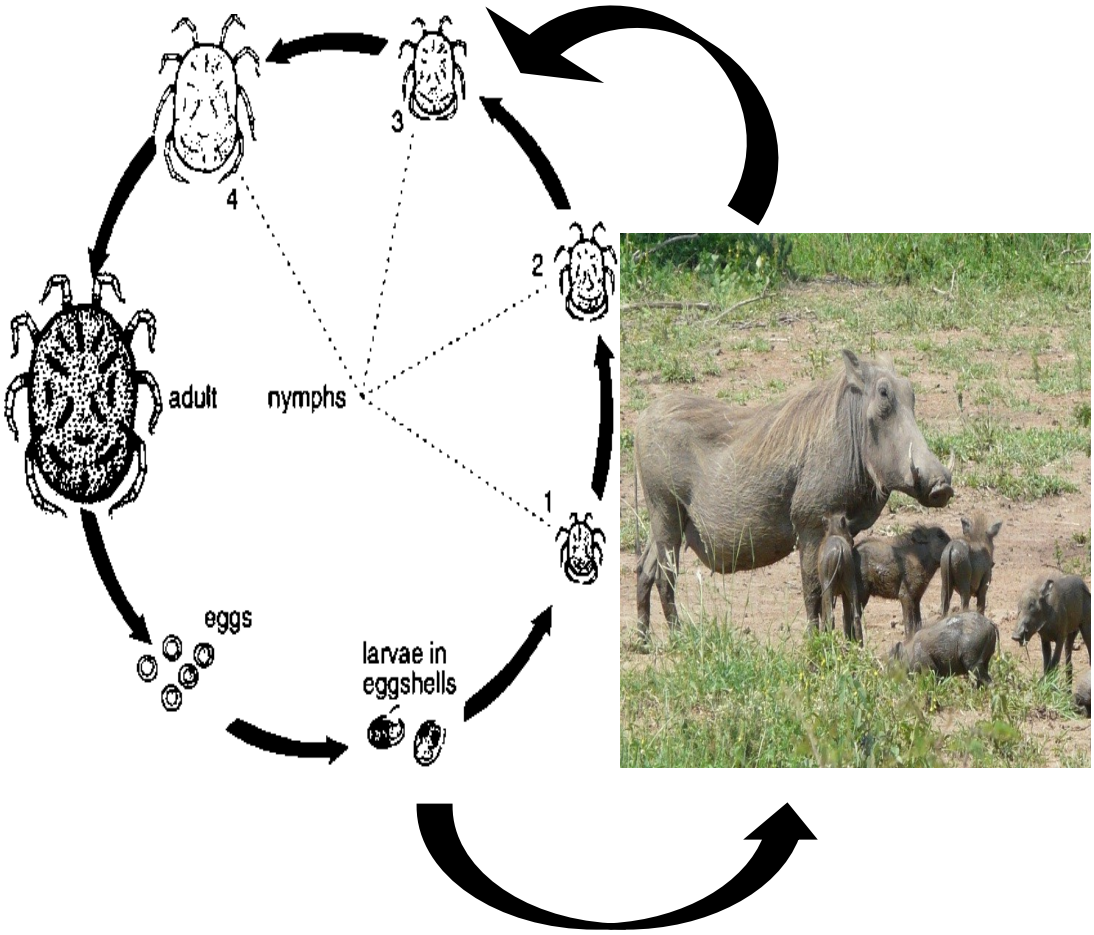

Figure 2: African swine fever virus circulating in the sylvatic cycle between warthog and *Ornithodoros* species. The virus is spread within the tick population sexually, transstadially and transovarian. *Ornithodoros* get infected when they feed on neonate viraemic warthogs (Modified from Vial, 2009).

2.4 Clinical Symptoms in Domestic Pigs

The clinical forms of African swine fever are divided into four groups, peracute, acute, sub-acute and chronic. In natural infection with ASFV, clinical signs appear approximately 5-15 days after infection. Clinical African swine fever cannot be separated from classical swine fever based on clinical signs. To establish which virus that is causing the disease laboratory methods are required (FAO, 2001).

2.5 Diagnostic Techniques

2.5.1 Identification of ASFV

Where ASF is suspected, the following samples should be sent to the laboratory: blood in anticoagulant (EDTA), spleen, lymph nodes, tonsil and kidney. These should be kept as cold as possible, without freezing, during transit. The diagnostic techniques include the following.

2.5.1.1 Haemadsorption Test

The haemadsorption (HAD) test (Malmquist and Hay, 1960) is based on the fact that pig erythrocytes will adhere to the surface of pig monocyte or macrophage cells infected with ASFV and that most virus isolates produce this phenomenon of haemadsorption. A positive result in the HAD test is definitive for ASF diagnosis. A very small number of ‘non-haemadsorbing’ viruses have been isolated, most of which are avirulent, but some do produce typical acute ASF. The test is carried out by inoculating blood or tissue suspensions from suspect pigs into primary leukocyte cultures, or into alveolar macrophages cell cultures, and also by preparing leukocyte cultures from the blood of pigs inoculated at the laboratory or from the blood of suspect pigs collected in the field.

2.5.1.2 Antigen Detection by Fluorescent Antibody Test (FAT)

The FAT, Bool (1969) can be used as an additional method to detect antigen in tissues of suspect pigs in the field or those inoculated in the laboratory. Positive FAT plus clinical signs and appropriate lesions can provide a presumptive diagnosis of ASF. It can also be used to detect ASFV antigen in leukocyte cultures in which no HAD is observed and can thus identify non-haemadsorbing strains of virus. It also distinguishes between the CPE produced by ASFV and that produced by other viruses, such as Aujeszky's disease virus or cytotoxic inoculums. However, it is important to note that in sub-acute and chronic disease, FAT has a significantly decreased sensitivity. This reduction in sensitivity may be related to the formation of antigen-antibody complexes in the tissues of infected pigs which block the interaction between the ASFV antigen and ASF conjugate (Sánchez-Vizcaíno, 2006).

2.5.1.3 Detection of Virus Genome by Polymerase Chain Reaction

PCR techniques have been developed, using primers from a highly conserved region of the genome, to detect and identify a wide range of isolates belonging to all the known virus genotypes, including both non haemadsorbing viruses and isolates of low virulence. The PCR techniques are particularly useful for identifying virus DNA in pig tissues that are unsuitable for virus isolation or antigen detection because they have undergone putrefaction, or when there is good reason to believe that virus may have been inactivated before samples are received in the laboratory.

PCR amplification by conventional PCR (Agüero *et al.,* 2003) can be performed. The ASFV primer set described in this procedure can be combined with a specific primer set for CSFV in a multiplex RT-PCR method that allows the simultaneous and differential detection of both virus genomes in a single reaction (Agüero *et al.,* 2003).

2.5.2 Serological Tests

Following infection antibodies may persist in recovered pigs for long periods, perhaps after infection, sometimes for life, and a number of tests are available for detecting these antibodies, although only a few of them have been developed for routine use in diagnostic laboratories (Escribano *et al.,* 1990; Pastor *et al.,* 1989). The most commonly used method is the ELISA (Wardley *et al.,* 1979; Sánchez-Vizcaíno *et al.,* 1982), which is suitable for examining either serum or fluid from the tissues. Confirmatory testing of ELISA-positive samples should be carried out in critical cases using an alternative test, such as the IFA test, immunoperoxidase staining or immunoblotting (Pastor *et al.,* 1989; Escribano *et al.,* 1990). Antibody is usually not detected in pigs infected with virulent ASFV as they die before it is produced. Antibodies are produced in pigs infected with low or moderately virulent ASF viruses, but these antibodies are not fully neutralizing.

2.5.2.1 Enzyme-Linked Immunosorbent Assay (ELISA)

The ELISA as described by Pastor *et al.,* (1990) is a direct test that can detect antibodies to ASFV in pigs that have been infected by viruses of low or moderate virulence. A highly sensitive and specific commercial ELISA kit based on a competition format that has been validated for use under different epidemiological situations is available. A cheaper alternative is to prepare a soluble antigen for use in an indirect ELISA. The procedure using this soluble antigen is described in a kit manual.

2.5.2.2 Indirect Fluorescent Antibody Test

This test should be used as a confirmatory test for sera from areas that are free from ASF and are positive in the ELISA, and for sera from endemic areas that give an inconclusive result in the ELISA (Pan *et al.,* 1974).

2.5.2.3 Immunoblotting Test

The technique is based on the antibody antigen binding principle, it combines the resolution of the gel electrophoresis with the specificity of the immunochemical detection. It is sensitive and very specific that can detect even week positive samples (Sanchez-Vizcaino *et al*., 2009; Lubisi, 2005).

2.6 Genotyping of ASFV

There are 22 genotypes of ASFV based on molecular phylogenetic analysis of the conserved vp72 protein (*B646L* gene). Genotype I, has been described in at least 24 countries in Europe, South America, the Caribbean and western and southern Africa, and the source of infection is most likely one of the western African countries (Bastos *et al*., 2003). Genotype I has previously been identified in eastern African sylvatic hosts such as bush pigs and ticks, which according to Lubisi *et al*. (2005) is notable since it only occurs among domestic pigs in western Africa. While genotypes V, X and XII were found in both domestic pigs, wild pigs and ticks, indicating that they occured as spill-over from the sylvatic cycle to domestic pigs. Genotypes XI, XIII and XIV appear to be completely associated with the sylvatic cycle since they all originate from ticks in warthog burrows. Genotypes II, VI, VII, IX and XVI on the other hand were only found among domestic pigs (Lubisi *et al.*, 2005). Genotype IX was found among domestic pigs in Uganda, Congo (Gallardo *et al.,* 2011) and Kenya (Gallardo *et al*., 2009). Genotype X has been isolated from domestic pig, ticks and warthogs in Uganda, Burundi, Tanzania and Kenya (Lubisi *et al.*, 2005; Boshoff *et al.*, 2007).

2.7 Control of ASF

Control of the disease can be done through quarantine of the infected area, with prevention of all movement of animals and animal products, establishment of a zone of surveillance

around the infected area, slaughter, with compensation of all affected and in-contact pigs after disease conformation, proper disposal of the carcasses and infected material by deep burial, disinfection of pig premises, detailed epidemiological investigation with tracing of possible sources of infection, watchful import policy for animals and animal products, proper disposal of waste food from aircraft or ships coming from infected countries, efficient sterilization of garbage and avoid contact between pigs and soft tick vectors or their habitats (OIE Manual, 2008; FAO, Manual, 2001; Penrith and Vosloo, 2009).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

The present study was conducted in northern and southern Tanzania. Tanzania is located on the Eastern side of the African continent. The country lies between latitudes 1º and 12º south of the Equator and 29º and 41º East of Greenwich. It is bordered with Kenya and Uganda to the north, Burundi, Rwanda and Democratic Republic of Congo to the west, Zambia, Malawi and Mozambique to the south and with the Indian Ocean on the east. Tanzania mainland area is 942 784 km^2. In Northern Tanzania, samples were collected from domestic pigs in Arusha at a farm in Sokoni area within Arusha municipality while in the Southern Tanzania samples were collected from Sumbawanga and Nkasi districts of Rukwa region, and Makambako district of Njombe region (Fig. 3).

3.2 Sampling and Tissue Processing

In the present study, tissue samples were obtained from domestic pigs after suspected outbreaks were reported. Tissue samples were collected at slaughter slabs/pens and were stored in sterile 50 ml Falcon tubes (Taizhou, China), packed into a cold box containing ice packs and transported to Sokoine University of Agriculture for Laboratory analyses. Upon arrival, tissue samples were homogenized in 1:10 phosphate-buffered saline followed by centrifugation to obtain tissue supernatant. The supernatant were placed in appropriately labeled cryovials (Taizhou, China) and stored at -80 ^oC until DNA extraction.

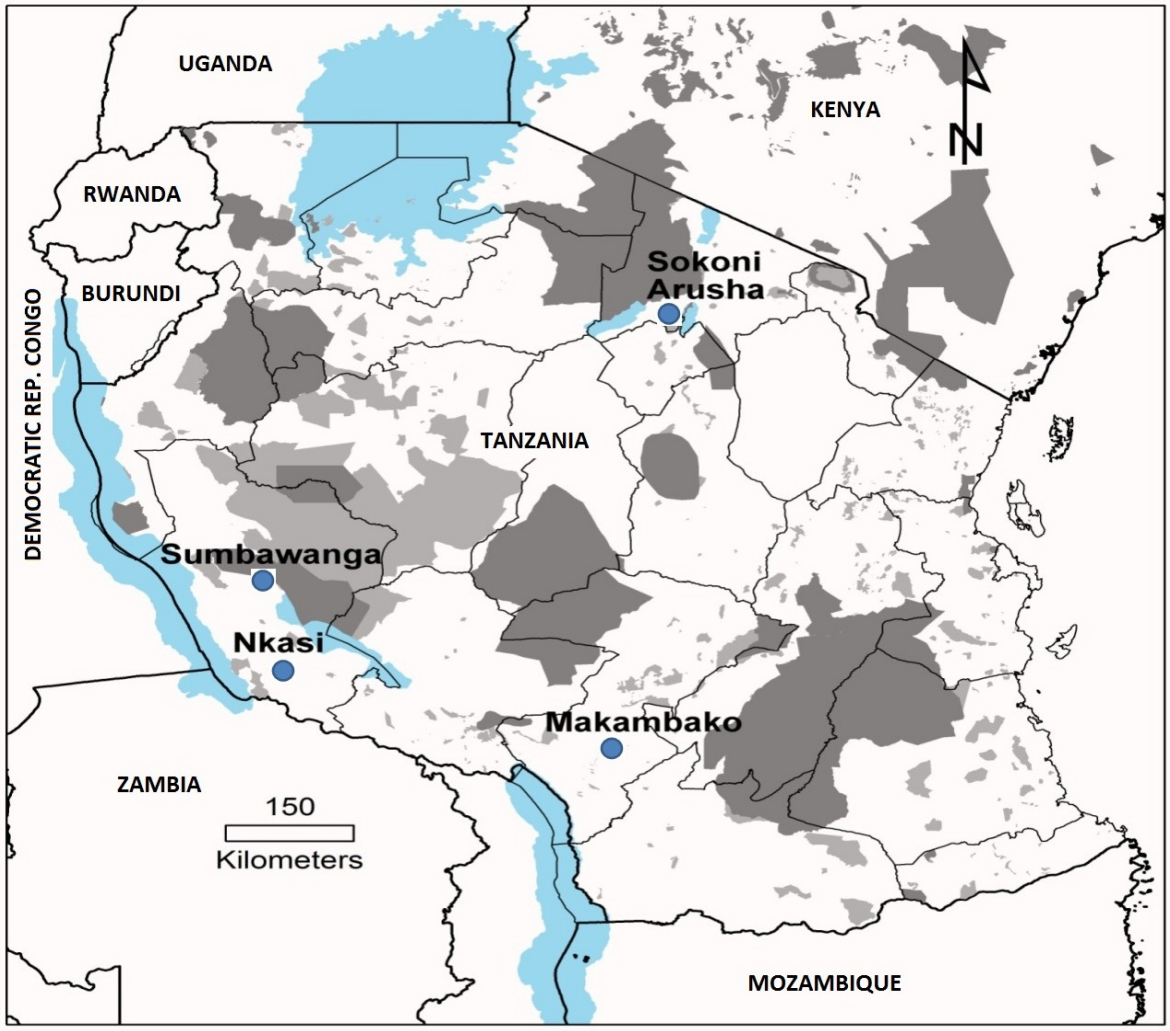

Figure 3: Sampling location for African Swine Fever. Map of Tanzania showing samplingareas where samples were collected from Sokoni area within Arusha municipality, Sumbawanga and Nkasi District in Rukwa Region and Makambako in Njombe Region. The locations from which samples from domestic pigs were collected are indicated with a blue circle on the map.

3.3 Diagnosis of ASFV in Pig Samples

Diagnosis of ASFV was made by the detection of ASFV genomic DNA from the extracted DNA from pig tissues

3.3.1 DNA Extraction and Storage

Extraction of DNA from tissue homogenate was performed using a viral RNA extraction mini kit (Qiagen, Hilden, Germany) following manufacturer's instructions. Briefly, 560 µl

of prepared AVL buffer was placed into a 1.5 ml micro centrifuge tube and 140 µl of homogenized tissue samples were added into the tube containing lysis buffer and mixed by pulse voltexing for 15 s. Thereafter samples were incubated at room temperature for 10 minutes and 560 µl of ethanol (100%) was added to the sample followed by vortexing for 15 s. The contents were centrifuged at 6,000 x*g* and the supernatant was passed through a Qiamp spin column to trap nucleic acids. Nucleic acids in the column were washed with washing buffers to remove PCR inhibitors. The spin columns were dried by high speed centrifugation followed by elution using elution buffer. Eluted DNA was stored at -20° C until PCR.

3.3.2 PCR Amplification of ASFV DNA

3.3.2.1 Diagnosis of ASFV using PCR

Diagnosis of ASFV was performed by amplification of the C-terminal region of p72 major capsid protein encoded by *B646L* gene using the primers PPA1/PPA2 (Table 2). The expected size of the PCR product was 257 bp. DNA amplification was done in a total volume of 20 µl using *Taq* polymerase (Thermo Scientific, Waltham, USA) as previously described by Aguero *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 62° C for 30 seconds, extension at 72° C for 1 minutes and incubation at 72° C for 10 minutes. The master mix composition for the diagnosis of ASFV is indicated in Table 1.

Table 1: Master mix composition for diagnosis and molecular amplification of ASFV

No	Component	Volume (µl)
1	2x PCR Buffer with *Taq* polymerase	12.5
2	10 µM forward primer (PPA1, P72U or PPA722)	0.5
3	10 µM reverse primer (PPA2, P72D, or PPA89)	0.5
4	Nuclease-free water	5.5
5	Extracted DNA template	1
	Total volume per reaction	20

3.3.2.2 Genotyping of ASFV

3.3.2.2.1 Partial Amplification of *B646L* gene (p72)

The C-terminal region of the *B646L* gene of ASFV was amplified using p72U and p72D primers as previously described by Bastos *et al*. (2003). The expected PCR product after PCR was 478 bp. 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. The master mix composition for p72 amplification indicated in Table 1 and set of primers indicated in Table 2.

3.3.2.2.2 Complete Amplification of the *E183L* gene (p54)

A full length of the *E183L* gene encoding the p54 protein was amplified in order to place the ASFV in major subgroups using the primers PPA722 and PPA 89 that amplify a 676 bp DNA fragment according to Gallardo *et al.* (2009). 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 57° C for 30 seconds, extension at 72° C for 1 minutes and incubation at 72° C for 10 minutes. The master mix composition for the p54 amplification is indicated in Table 1 and set of primers indicated in Table 2.

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Table 2: Set of primers used for diagnosis and genotyping of ASFV

Primer name	Nucleotide sequence 5'→3'	Target gene	Expected size (bp)
PPA1	AGTTATGGGAAACCCGACCC	*B646L*	257
PPA2	CCCTGAATCGGAGCATCCT	*B646L*	257
p72U	GGCACAAGTTCGGACATGT	*B646L*	478
p72D	GTACTGTAACGCAGCACAG	*B646L*	478
PPA 722	CGAAGTGCATGTAATAAACGTC	*E183*	676
PPA 89	TGTAATTTC ATTGCGCCACAAC	*E183*	676

3.4 Agarose Gel Electrophoresis of PCR Product

The total of 1.5 g of agarose gel was weighed and dissolved into 1X dissolving buffer, to make 1.5% agarose gel where all amplified products were visualized against the 100bp marker, and the sizes of different amplicons were visualized according to their sizes. Before loading, agarose gel was pre-stained with Gel Red (Biotium, Hayward, CA) and DNA was visualized using Gel documentation system (BioRad, Missouri, USA).

3.5 Sequencing of PCR products and Phylogenetic Analysis

3.5.1 Sequencing of PCR products

Sequencing of PCR products was done by using dideoxynucleotide cycle sequencing using a Big Dye Terminator kit v3.0 (Applied Biosystem Foster City, CA) following manufacturer's instruction. Primers used during amplification of p72, and p54 gene were used in subsequent sequencing. Thereafter fragments were resuspended in Hi-Di Formamide (Applied Biosystems, Foster city, CA). Before being separated on a 3500 Genetic Analyzer (Applied Biosystem, Foster city, CA).

3.5.2 Molecular Analyses

Visualization of sequencing chromatograms was done using Sequence Scanner v1.0 software (Applied Biosystem, Foster city, CA). Sequences of good quality were manually

assembled using a Notepad and ClustalW as implemented in MEGA 6.06 (Tamura *et al.,* 2011). Subsequently, aligned nucleotide sequences were used to construct phylogenetic trees with the Neighbor-Joining method using Kimura-2-parameter model at 1,000 bootstrap replications implemented in MEGA 6.06. Phylogenetic analysis was conducted by including some publicly available sequence data from GenBank, including the reference strains and the isolates to generate phylogenetic tree. Clustering patterns in the phylogenetic tree were used to place ASFV strains obtained in the present study into genotypes.

CHAPTER FOUR

4.0 RESULTS

4.1 Confirmatory Diagnosis of ASF

A total of four suspected ASF outbreaks were reported and investigated between September and December 2014, in northern Tanzania (Arusha) and southern Tanzania (Makambako, Sumbawanga and Nkasi) (Table 3). Spleen, liver and gastrohepatic lymphnodes collected from domestic pigs were tested for the presence of ASFV using PCR as previously reported by Aguero *et al*. (2003). Out of the 15 tissue samples collected from different domestic pigs, five tissue samples tested positive with diagnostic PCR using PPA1/2 primer (Table 3).

Table 3: Confirmatory diagnostic results for African swine fever virus (ASFV) in tissue samples collected from domestic pigs in northern and southern Tanzania after testing using polymerase chain reaction (PCR) as previously reported by Aguero *et al*. (2003)

Location (region)	Number of pigs tested	Number of ASF positive pigs
Sokoni (Arusha)	2	1
Makambako (Iringa)	7	1
Sumbawanga (Rukwa)	4	2
Nkasi (Rukwa)	2	1
Total	15	5

4.2 Postmortem Findings in Domestic Pig with ASF

The pathological findings observed in domestic pigs included enlarged and haemorrhagic gastrohepatic and renal lymph nodes, splenomegaly with necrotic foci, petechiation in kidneys, and oedema of the lungs.

4.3 Nucleotide Identity Based on BLASTn Search at GenBank

A total of four ASFV *B646L* (p72) gene nucleotide sequences were obtained from domestic pig sampled in Arusha (TAN/14/Arusha), Makambako (TAN/14/Makambako), Sumbawanga I for the Sumbawanga strain (TAN/14/Sumbawanga) and Nkasi (TAN/14/Nkasi). The results showed that TAN/14/Arusha was different from TAN/14/Makambako, TAN/14/Sumbawanga and TAN/14/Nkasi. Furthermore, the *B646L* (p72) gene nucleotide sequences obtained from Makambako, Sumbawanga and Nkasi were 100% identical. Nucleotide identity search at GenBank using BLASTn for TAN/14/ Arusha showed 100% similarity with genotype X ASFV, including ASFV previously reported in Tanzania, Uganda and Kenya (Table 4). However nucleotide identity search at GenBank using BLASTn with ASFV from Sumbawanga, Makambako and Nkasi showed 100% identity with genotype II ASFV isolate from Tanzania, Georgia and Madagascar (Table 5).

Table 4: Basic local alignment Search tool (BLAST) result for nucleotide sequence of TAN/14/Arusha from northern Tanzania against African swine fever virus nucleotide sequences at GenBank

Isolate	Country	Host species	Year-Outbreak	Town/Province	p72 gene Gene Bank accession number	% identity	Reference
TAN/13/Moshi	Tanzania	Pig	2013	Moshi	KF706352	100	Misinzo *et al*., 2010
Ken05/Tk1	Kenya	NK*	2005	NK*	KM111294	99	Bishop *et al*., 2015
Kn	Kenya	Pig	1966	Nairobi	AY578698	99	Zsak *et al*., 2005
Kenya 1950	Kenya	Pig	1950	Nairobi	AY261360	99	Bishop *et al*., 2015
UgH03	Uganda	Pig	2003	Hoima	FJ154428	99	Blanco *et al.,* 2006
Ken 06 Bus	0Kenya	NK*	2006	NK*	KM111295	99	Bishop *et al*., 2015
Complete genome	Uganda	NK*	1965	NK*	L27499	99	Yu,M., *et a*l., 1996
TAN/09/Longido	Tanzania	Pig	2009	Longido	JX262383	99	Misinzo *et al*., 2012
P72 Major capsid	NK*	NK*	NK*	NK*	L27498	97	Unpublished
Wb major	S.A	Tick	1987	Warmbaths	AY578707	96	Zsak *et al*., 2005

NK* = Not Known

Table 5: Basic local alignment Search tool (BLAST) result for nucleotide sequence of TAN/14/Makambako, TAN/14/Sumbawanga and TAN/14/Nkasi from southern Tanzania against ASFV nucleotide sequences at GenBank.

Isolate	Country	Host species	Year-Outbreak	Town/ Province	p72 gene GeneBank accession number	% Identity	Reference
TAN/12/Iringa	Tanzania	Pig	2012	Iringa	KF834193	100	Sikombe *et al*., 2012
p72 major capsid	Russia	Pig	2012	Krasnodar	KJ195685	100	Vlasova *et al*., 2014
Georgia 2007	Georgia	Pig	2007	NK*	AM999764	100	Chapman *et al*., 2010
TAN/10/ Kyela	Tanzania	Pig	2010	Kyela	JX391987	100	Misinzo *et al.,* 2012
NHV	NHV	Pig	1968	NK*	KM262845	99	Portugal *et al*., 2014
L60	Portugal	Pig	1960	NK*	KM262844	99	Portugal *et al*., 2014
BA71V	Spain	Vero cell	1972	Badajoz	U18466	99	Gonzalez *et al*., 1986
691-88	Russia	NK*	NK*	NK*	KJ671549	99	Malogolovkin *et al*., 2014
E75	Spain	NK*	NK*	NK*	FN557520	99	De Villiers *et al*., 2009
OUR88/3	Portugal	Pig	2007	NK*	AM12240	99	Chapman *et al*., 2007

NK* =Not Known

4.4 Phylogenetic Analysis Based on *B646L* (p72) Nucleotide Sequences

Nucleotide sequences of the C-terminal end of *B646L* (p72) gene were used in genotyping of ASFV after amplification and sequencing using p72U/D primers. The PCR amplification of ASFV on samples collected from domestic pigs in the present study using primers p72U/D produced an expected PCR product of approximately 476 bp (Fig. 4). The PCR products were sequenced and used in phylogenic analysis together with other ASFV belonging to genotype II and X (Table 5). All the Tanzanian viruses obtained in this study clustered into p72 genotype II and X together with some viruses previously isolated in other countries including Kenya, Uganda, Latvia, Lithuania, Madagascar, Estonia, Mozambique, Burundi and Ukraine.

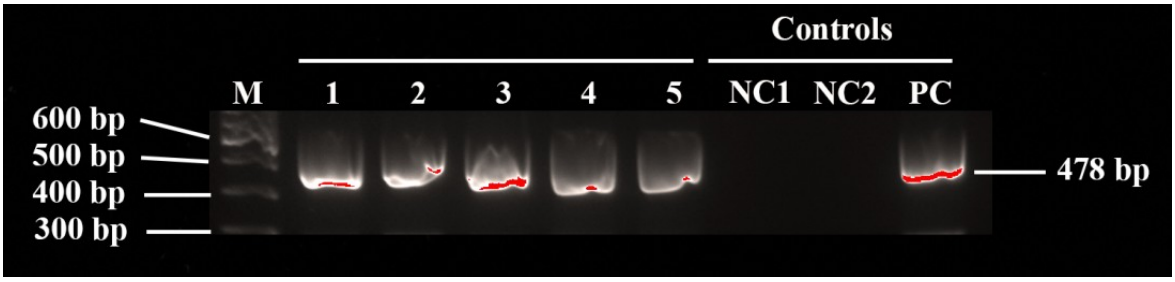

Figure 4: Partial amplification of the *B646L* (p72) gene of ASFV in five samples from Arusha(1), Makambako (2), Sumbawanga I (3), Sumbawanga II (4), and Nkasi (5) were amplified using p72U/D primers followed by agarose gel electrophoresis. A positive control (PC) ASFV from Ifakara was included plus a negative control during extraction (NC1) and PCR (NC2). An expected 478 bp fragment was observed in samples (1-5) from Northern and Southern Tanzania. The size of band were observed trough Marker (M).

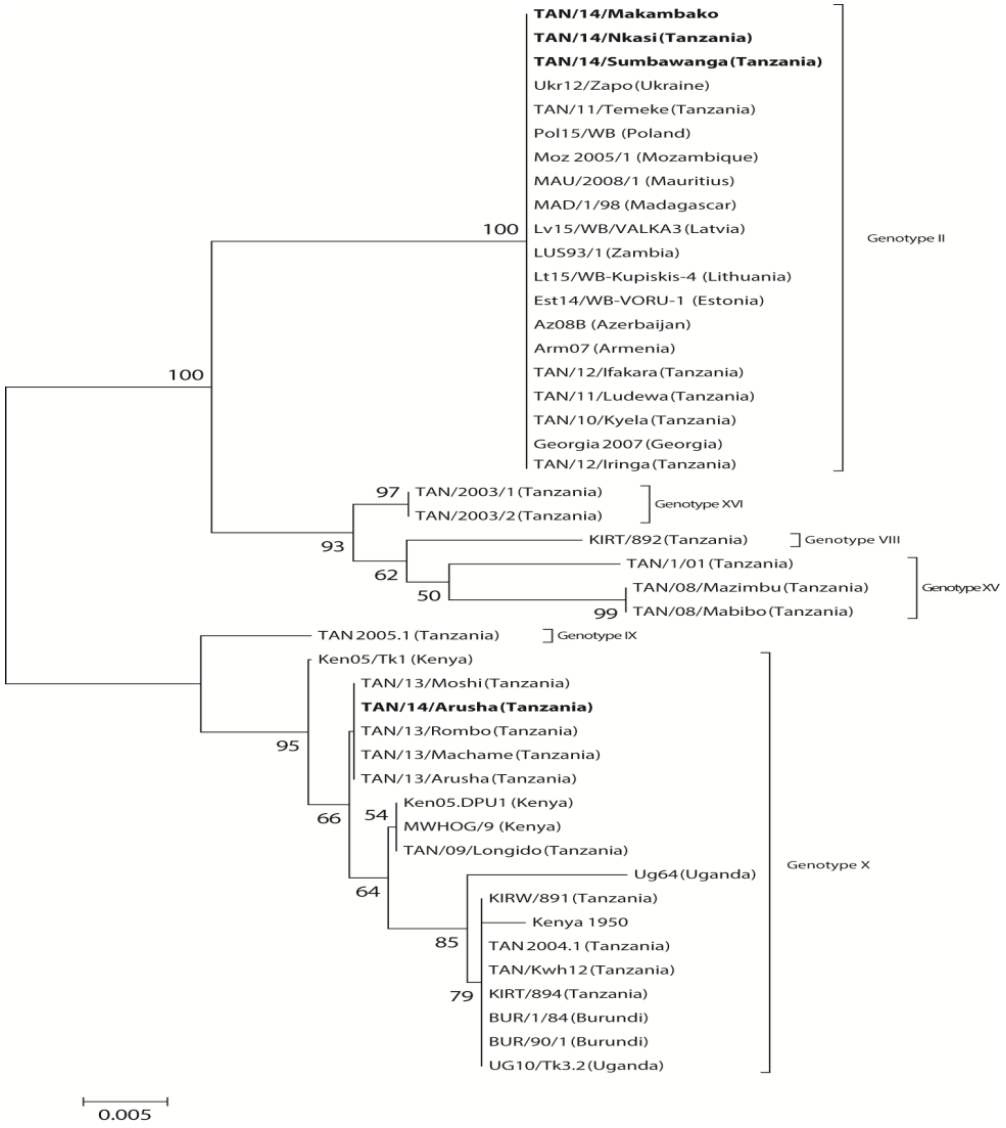

Figure 5: Phylogenetic tree of ASFV based on *B646L* (p72) gene nucleotide sequences. The phylogenetic tree was constructed using six representatives of genotypes circulating in Tanzania. ASFV from other countries belonging to genotype II and X were included in the analysis. The evolutionary history was inferred using the Minimum Evolution method (ME). The ME tree were searched using the close –Neighbour-Interchange (CNI) algorithm at a search level. A neighbour joining algorithm was used to generate the initial tree after 1,000 replicates. The samples in this study are bolded in black.

4.5 Phylogenetic Analysis Based on *E183L* (p54) Nucleotide Sequences

Nucleotide sequences of the full length of the *E183L* (p54*)* gene were used in genotyping of ASFV in major subgroups using the primers PPA722 and PPA 89 that amplified a DNA

fragment of approximately 676 bp (Fig. 6). The PCR products were sequenced and used in phylogetic analysis together with other ASFV belonging to genotype II and X. TAN/14/Arusha was identical with those isolate from Kenya, Uganda, and Burundi while the genotype II were identical to those from Georgia, Armenia, Azerbaijan, Belarus, Lithunia, Madagascar, Mozambique, Poland, Ukraine and Russia (Fig. 7).

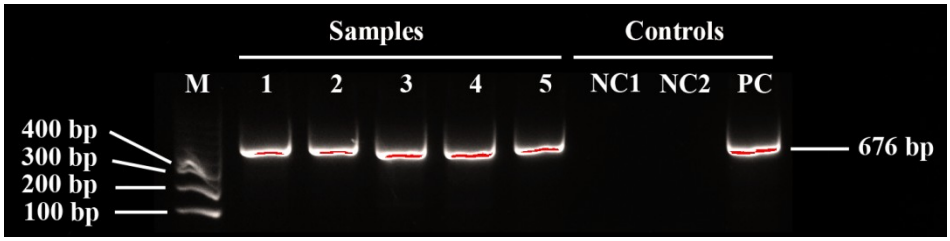

Figure 6: Full length amplification of the *E183L* (p54) gene of ASFV in five samples from Arusha(1), Makambako (2), Sumbawanga I (3), Sumbawanga II (4), and Nkasi (5) were amplified using p72U/D primers followed by agarose gel electrophoresis. A positive control (PC) ASFV from Ifakara was included plus a negative control during extraction (NC1) and PCR (NC2). An expected 676 bp fragment was observed in samples (1-5) from Northern and Southern Tanzania. The size of band were observed trough Marker (M).

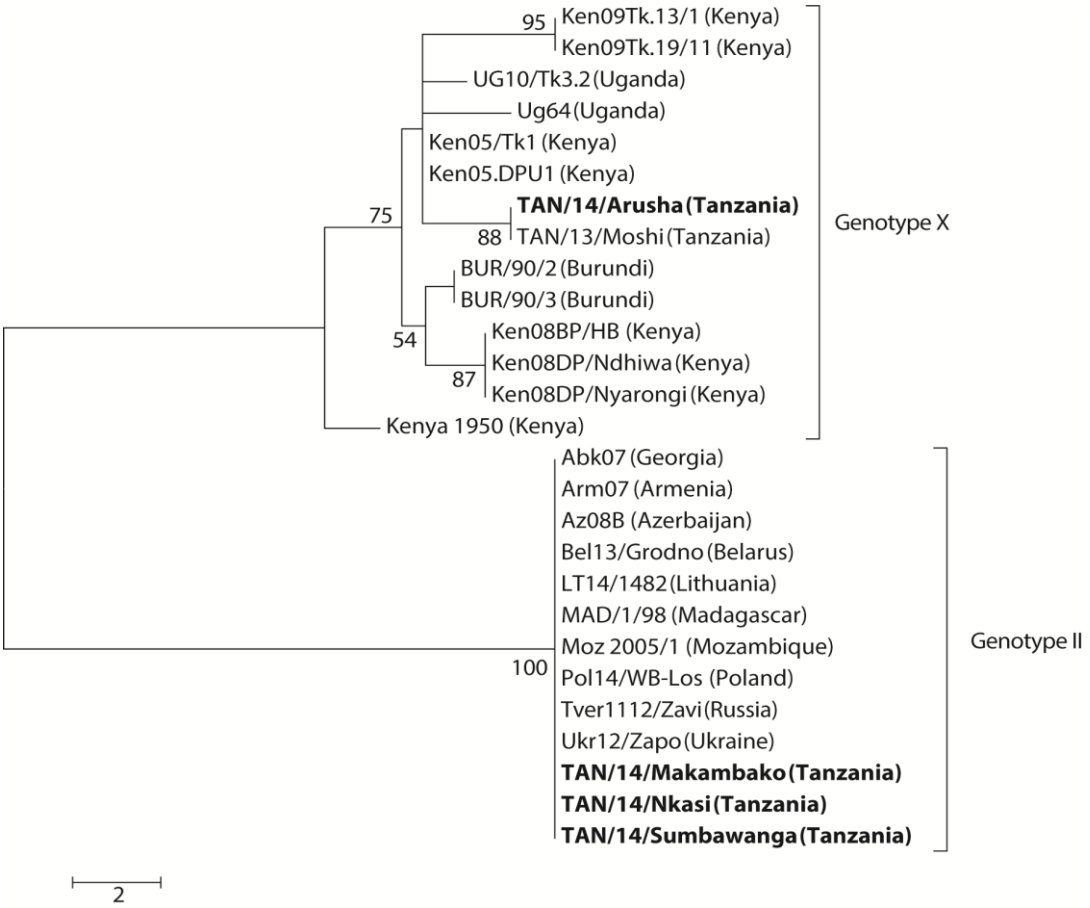

Figure 7: Phylogenetic tree of ASFV based on *E183L* (p54) gene nucleotide sequences. The phylogenetic tree was constructed using six representatives of genotypes circulating in Tanzania. ASFV from other countries belonging to genotype II and X were included in the analysis. The evolutionary history was inferred using the Minimum Evolution method (ME). The ME tree were searched using the close –Neighbour-Interchange (CNI) algorithm at a search level. A neighbour joining algorithm was used to generate the initial tree after 1,000 replicates. The samples in this study are bolded in black.

CHAPTER FIVE

5.0 DISCUSSION

In the present study, the presence of ASFV in dead domestic pigs suspected with ASF samples from Arusha, Sumbawanga, Makambako and Nkasi was investigated. The results of the present study confirmed the presence of ASFV in tissue samples obtained from domestic pigs that died of an haemorrhagic disease between August and December 2014 in Arusha, Sumbawanga, Makambako and Nkasi Tanzania. The confirmation of ASFV was performed by PCR using primers PPA1/PPA2 as previously described by Aguero *et al*. (2003). The confirmation of ASF in northern and southern Tanzania in 2014 shows persistent, re-infection or endemicity of ASFV circulation because the disease has been reported annualy in southern Tanzania between 2010 and 2013 and in northern Tanzania in 2009 and 2013 (Misinzo *et al*., 2012a; Misinzo *et al*., 2014).

Furthermore, the present study also investigated phylogenetic relationship of ASFV involved in ASF outbreaks in northern and southern Tanzania with other ASFV that caused previous outbreaks in Tanzania and elsewhere. African swine fever virus collected from Sumbawanga, Makambako and Nkasi (southern Tanzania) during 2014 clustered with genotype II ASFV. Genotype II ASFV have long been known to circulate in Zambia, Mozambique and Madagascar (Lubisi *et al*., 2007). In 2007, genotype II ASFV was possibly introduced from Madagascar into Georgia where by contaminated pork and pork-products from international ships were reported to be the most likely sources of virus introduction. The infection was probably established in pig's population in Georgia since pigs are kept on free range and have access to dumped waste (Rowlands *et al.*, 2007). After introduction of genotype II into Georgia, these ASFV have spread into other countries in the Caucasus and Russian region including Estonia, Poland, Ukraine, Latvia,

Armenia, Azerbaijan and Russia (Sanchez Viscaino *et al*., 2013). The spread of genotype II ASF within caucaus region, Russia and the European Union is thought to be due to transportation and bush pig migration (FAO, 2009). Through the spread and control and control of ASF in Caucasus region (Georgia, Armenia, and Azerbaijan) and Russia has been very difficult (Rowlands *et al.*, 2007). Similarly, after the introduction of genotype II ASFV in Tanzania in 2010 (Misinzo *et al*., 2012a), these viruses have persistently circulated in southern highland region including Iringa, Mbeya and Rukwa (Misinzo *et al*., 2012b). Therefore, the occurrence of ASF in Makambako, Nkasi and Sumbawanga found in the present study that is 100% identical to ASFV that have caused previous outbreak in Tanzania continues to show the persistence of this genotype II ASFV within this zone.

Similary, the results obtained in the present study show that ASFV from the 2014 Arusha outbreak belong to genotype X. African swine fever virus from Arusha were identical at the nucleotide level with ASFV from Rombo, Moshi and Arusha collected during 2013 outbreak with 100% nucleotide identity (Fig. 6) (Misinzo *et al*., 2014). Similar ASFV were reported in northern Tanzania in 2009 (Misinzo *et al.,* 2012b).This finding indicate that the same ASFV of 2009 and 2013 is still circulating or re-emerging frequently in Northern Tanzania. Genotype X ASFV have also been previously described to cause outbreaks in Kenya, Uganda and Burundi (Gallardo *et al*., 2008). It can be concluded that genotype X ASFV have a transboundary spread within eastern Africa due to horizontal transmission between pigs, this could be due to uncontrolled pig and pig product movements, swill feeding, and lack of implementation of biosecurity measures (Wambura *et al*., 2006; Misinzo *et al*., 2011, 2012b). The restriction of genotype II ASFV within southern Tanzania and genotype X within northern Tanzania has implication on the epidemiology of ASFV in Tanzania. The regional restriction of ASF could be due to absence of pig movements between southern and northern zones of Tanzania. Previous

report have shown that ASF outbreak in different zone of Tanzania spread to Dar es salaam due to marketing of pigs (Wambura *et al*., 2006, Misinzo *et al*., 2012a). The annual occurence of ASF outbreak within these major pig-producing regions could be linked to a number of epidemiological factors such as uncontrolled pig and pig product movements, swill feeding, and lack of biosecurity measures (Wambura *et al*., 2006; Misinzo *et al*., 2011, 2012b). Our personal communication with farmers has indicated the lack of knowledge of ASF. For instance, farmers continue to purchase stock (pigs) in areas with ongoing outbreaks due to the lower prices at times of outbreak. Slaughter of pigs with ASF continues in the presence of outbreak and pig butchers physically go to purchase pigs in farm and spread the disease unknowingly.

The Tanzania National Sample Census of Agriculture show that there are 1 584 411 pigs in Tanzania (URT, 2012). These pigs were more common in the southern region of Mbeya and Iringa however, with the highest densities were in Kilimanjaro and Dar es Salaam region. At the same time the annual growth of pig population between 1995 and 2008 was 10.2% (URT, 2012). The recurrence of ASF outbreaks in the major pig keeping regions in northern and southern Tanzania will undoubtedly reduce the growth of pig population in the country with a consequence that the poor farmers who depend on short cycle stocks for their livelihood will become more vulnerable and food security will be hampered. Illegal movement of pigs and swill feeding have been identified by Swai *et al* ., (2013) as major drivers for transmission of ASFV. It becomes necessary to avoid these activities to control ASFV in Tanzania.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

i. The present study confirmed the presence of ASFV in northern (Arusha) and southern Tanzania (Makambako, Sumbawanga, Nkasi) during 2014 outbreaks.

ii. Our findings show that the same genotypes X and II ASFV that circulated previously in northern and southern Tanzania respectively are still causing outbreaks, hence, it can be inferred that the virus is circulating persistently and recurrently within domestic pig cycles in these areas since 2010.

iii. African swine fever still poses the devastating deaths to pigs in Northern and other regions of southern highlands of Tanzania, since 2001 to date the disease has been circulating among pig farms making it to be endemic in these regions. This has serious implications in the livelihood of poor pig keeping farmers.

6.2 Recommendations

i. Quarantine and movement control have to be strictly imposed on all suspected or infected premises as soon as possible. No movement of pigs, pig products and other potentially infected materials should be allowed off the property until an investigation and diagnosis has been accomplished.

ii. Swill feeding is a high risk practice, as several diseases can be introduced into an otherwise healthy swine population. Pigs should not be fed swill that might contain

remains of pigs. It is recommended that farmers boil swill for 30 minutes and allow it to cool before feeding it to their pigs.

iii. Knowledge of the epidemiology of ASF and a full understanding of the evolution and spread of ASFV in this region require additional sequence analysis of ASFVs frequently reported in Nothern and Southern Tanzania.

iv. The Government especially Ministry of Livestock and Fisheries Development should ensure implementation of strategies for control of ASF in order to prevent the disease from reoccurring.

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