MOLECULAR EPIDEMIOLOGICAL STUDY OF FOOT-AND-MOUTH DISEASE VIRUS IN TANZANIA

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ABSTRACT

Foot-and-mouth disease (FMD) is endemic in Tanzania since it was first reported in 1954. Foot-and-mouth disease is caused by FMD virus (FMDV) that presents in seven serotypes. The FMD outbreaks that occur almost each year are caused by different serotypes in the country. With its large area of 945 000 km^2 and its climate which varies from tropical to temperate, large number of livestock including 25.8 million cattle (Ministry of Agriculture, Livestock and Fisheries, 2016) and high density of wild animals, it is very difficult to control the spread of this economically important viral disease. This is compounded by the lack of the comprehensive studies that could indicate the spatial distribution of genetically divergent FMDV field strains. This study was conducted to investigate the distribution of FMDV strains circulating in the country, with the main focus on the molecular epidemiology of FMDV in endemic settings of Tanzania using the molecular approach and field epidemiological data. The overriding research question for this study was "what is the contribution of genetic variability of FMDV to the occurrence of FMD in Tanzania?" The general objective of this study was to determine the epidemiological information and molecular and genetic diversity of circulating FMDV field strains in Tanzania. A cross sectional study design was used for this research in a total of 361 (n=361) epithelial tissue samples from tongue and interdigital space of feet were purposively collected from FMD suspected cases/outbreaks in different geographic areas of Tanzania. Laboratory analysis of samples collected between 2008 and 2013 was performed at the Centre for Infectious Diseases and Biotechnology (CIDB) and Faculty of Veterinary Medicine, Sokoine University of Agriculture using the optimized protocols adopted by the FAO World Reference Laboratory for Foot-and-Mouth Disease (WRLFMD). A real-time RT-PCR was used to screen epithelial samples to ascertain the presence of the FMDV genome in the sample. Conventional RT-PCR employing serotype-

specific oligo-nucleotides (primers) was used to amplify genome fragments of FMDV in the vesicular epithelium. Two assays real-time RT-PCR and antigen detection ELISA were evaluated for their specificity and sensitivity as tests of choice for detection of FMDV from field samples. The genetic characterization of 56 field samples was performed by VP1 sequencing and phylogenetic analysis using Neighbour joining method. A sensitivity result for rRT-PCR assay was 86.6% (n= 26) and specificity was 70.0% (n= 21) while for Antigen-ELISA had a sensitivity of 70.0% (n= 21) with a specificity of 70.0% (n= 21). These results were still moderate as most of the time Antigen-ELISA can give positive results with only about 70-80% of epithelial suspensions that contain virus due to its low sensitivity. The phylogenetic analysis of type O revealed that all samples (n= 39) clustered into the EA-2 topotype while, serotype A (n=12), viruses were classified into one major genetic clade (G-I) within topotype AFRICA and SAT1 viruses (n= 5) were placed within the topotype 1 (NWZ). For SAT1 the nucleotide differences in VP1 coding region was between 15-20.0%; the nucleotide number of composition was 663 and the average nucleotide match was from 645-660 with the other Tanzanian strains. The molecular epidemiological results from this study indicated the increase of genetic variability of FMD viruses that are independently maintained within Tanzania and the east African region. These findings suggest that outbreaks that took place during the last six years were scattered throughout the country/region and there is suggestive evidence of the history of introductions, exchange and spread of FMDV within Tanzania and its neighbouring border countries. Phylogenetic analysis of complete VP1sequences revealed evidence for the presence of distinct strains within the country and historical genetic relationship of some strains within the Great Lakes countries of Africa.

This study provides a ‘valuable pilot’ report on genetic characterization, epidemiological situation and phylogeography of FMDV circulating in Tanzania from 2008-2013. Also, due to gradual increase of the amount of diversity within Tanzanian strains (e.g. serotype

O) it is advisable that the future study should be based on molecular evolution of FMDV with the main focus of understanding the rates of nucleotide substitution. Further studies are required to investigate the possible sources of FMDV, transmission ability of field strains and maintenance of viruses in Tanzania. Therefore, further studies should be done to ascertain the possible sources of FMDV and how it is maintained in these endemic settings.

DECLARATION

I, Raphael Samwel Sallu, do hereby declare to the Senate of the Sokoine University of Agriculture that this dissertation is my 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 above declaration if confirmed

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DEDICATION

This work is dedicated to my late Cousin Joseph Kihiyo Mtoi. Rest in Peace (R.I.P).

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LIST OF ABBREVIATIONS AND SYMBOLS

°C	Degree Centrigrade
AUG	Adenine, Uracil, Guanine
BDSL	Biological Diagnostic Supplies Limited
BME	β-mercaptoethanol
BPB	bromophenol blue
CA	cytosine adenine
cDNA	Complementary Deoxyribonucleic acid
CFE	Cytopathic effect
CIDB	Centre for Infectious Diseases and Biotechnology
CTAB	Cetyl trimethylammonium bromide
dATP	deoxyadenosine 5'-triphosphate
dCTP	deoxycytidine 5'-triphosphate
DNA	deoxyribose nucleic acid
dNTPs	deoxynucleoside 5'-triphosphates
EA-2	East Africa-2
EDTA	ethylenediaminetetraacetate
ELISA	Enzyme Linked Immunosorbent Assay
EMCV	Encephalomyocarditis virus
EtBr	ethidium bromide
EtOH	ethanol
EUFMD	European Union Commission for Foot- and- Mouth Disease
FAM	6-carboxy-fluorescine (Blue)
FAO	Food and Agriculture Organization of the United Nations
Fig.	Figure

FMD	Foot-and-Mouth disease
FMDV A	Foot-and-mouth disease virus serotype A
FMDV O	Foot-and-mouth disease virus serotype O
FMDV SAT1	Foot-and-mouth disease virus serotype SAT1
FMDV SAT2	Foot-and-mouth disease virus serotype SAT2
FMDV SAT3	Foot-and-mouth disease virus serotype SAT3
FMDV	Foot-and-mouth disease virus
g	gram(s)
GT	guanine thymine
h	hour(s)
HEX	hexachloro-6-carboxy-flourescent (Green)
HTST	High Temperature Short Time
IC	Internal control
IRES	internal ribosomal entry site
kb	kilobases
LAMP	Loop Mediated Amplification Polymerase Chain Reaction
LFD	Lateral flow device
L^{pro}	Leader protease
mA	milli Amperes
min	minute(s)
ml	millilitre(s)
MoWLD	Ministry of Water and Livestock Development
MW	molecular weight
MKUKUTA	National Strategy for Growth and Reduction of Poverty
NED	6-carboxy-X-rhodamine (Yellow/Black)
ng	nanogram(s) = 10^{-9} gram

nm	nanometer(s) = 10^{-9} meter
NSP	Non structural protein
OD	optical density
ODx	Optical density at x nm
OIE	World Organization for Animal Health
ORF	Open reading frame
PBS	Phosphate Buffered Saline
PCP	Progressive Control Pathway
PCR	Polymerase Chain Reaction
Pmol	Picomole
PV	Polio virus
RGD	Tripeptide arginine-glycine-aspartic acid
RNA	Ribonucleic acid
rpm	rounds per minute
rRT-PCR	Real-Time Reverse Transcription Polymerase Chain Reaction
RT	Reverse transcription
RT-LAMP	Reverse Transcription Loop Mediated Isothermal Amplification Polymerase Chain Reaction
SAT	Southern African Territories
Sec	second(s)
STE	Sodium Tris-EDTA (also TEN)
SUA	Sokoine University of Agriculture
Tab	Table
TAE	Tris-acetate EDTA (buffer)
TBE	Tris-borate EDTA
TE	Tris-EDTA (buffer)

TVLA	Tanzania Veterinary Laboratory Agency
U	unit(s) of enzyme
UTR	Untranslated Region
UV	ultraviolet
V	volts
VI	Virus isolation
VIC	tetrachloro-6-carboxy-fluorescine (green)
VNT	Virus Neutralization Test
VP1	Viral Protein 1
VP2	Viral Protein 2
VP3	Viral Protein 3
VP4	Viral Protein 4
µg	microgram(s) = 10^{-6} gram
µl	microlitre(s) = 10^{-6} litre

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Foot-and-mouth disease virus (FMDV) belongs to the genus *Aphthovirus,* family *Picornaviridae* and causes Foot-and-mouth disease (FMD) a highly infectious disease that affects domestic and wild cloven-hoofed animal species (Bastos *et al*., 2003; Quian *et al.,* 2003). The virus exists in seven serotypes, whereby protection from one serotype does not confer immunity against the other six. Foot-and-Mouth Disease is highly contagious and that the virus has high antigenic diversity that makes the disease difficult to control. The disease causes significant economic losses due to deaths of young animals, decreased productivity, and trade sanctions against livestock and livestock products from infected regions. The potential for high economic loss is exemplified by the devastating 2001 FMD epidemic in the United Kingdom that resulted in a total cost of over £5 billion (Knight-Jones and Rushton, 2013).

The term 'topotype' is used to reflect the presence of genetically and geographically distinct evolutionary lineages (Samuel and Knowles, 2001); for example, the serotype South African Territories1 (SAT1) can be grouped into eight topotypes I to VIII (Appendix 1). This is based on nucleotide differences (within Viral Protein1 (VP1) coding sequence) of up to 15% (Samuel and Knowles, 2001). The serotype SAT1 topotype III is found in Tanzania, Zambia, Malawi, Kenya and Zimbabwe according to the study done by Vosloo *et al.,* (2002). SAT2 viruses appear to be particularly diverse, with the largest number of topotypes, whilst serotype C, probably as a result of being the rarest serotype in the continent, has the fewest (Bastos *et al.,* 2001; Knowles *et al*., 1998; Reid *et al*., 2001; Sangare *et al*., 2001, 2001, 2002b; Vosloo *et al.,* 1996). Topotype 'richness' in Africa is

thus summarized as SAT2>SAT1=A>O>SAT3>C and furthermore, topotypes diversity does not appear to be influenced by serotype prevalence which is broadly described by SAT2>O>A>SAT1>SAT3>C (Vosloo *et al.,* 2002).

FMD serotypes show that six of the seven serotypes of FMD (O, A, C, SAT1, SAT2, SAT3) have occurred in Africa, while Asia contends with four serotypes (O, A, C, Asia 1), and South America with only three (O, A, C). Periodically, there have been incursions of types SAT1 and SAT2 from Africa into the Middle East (Donaldson, 1999; Valarcher *et al.,* 2004; FMD Homepage – Maps, 2006). This disease is endemic in most countries of sub-Saharan Africa (Vosloo *et al.,* 2002) and is commonly reported in Southern African Development Community (SADC) countries and efforts to control to date have not been very successful. This is mainly due to the inadequate knowledge on the actual FMDV serotypes and topotypes circulating in these countries. The knowledge of the serotypes and topotypes is a prerequisite for effective regional disease control strategies.

The epidemiology of FMD on the African continent is influenced by two different patterns, *viz*, a cycle in which wildlife ungulates plays a role in maintaining and spreading the disease to other susceptible domestic animals and a cycle that is maintained within domestic animals and that is independent of wildlife. In southern Africa, the former cycle predominates due to the presence of African buffalo (*Syncerus caffer*), the only wildlife species for which long-term maintenance of FMDV has been described (Hedger *et al.,* 1973). In SADC region (Angola, Botswana, Democratic Republic of Congo DRC, Lesotho, Madagascar, Malawi, Mauritius, Mozambique, Namibia, Seychelles, South Africa, Swaziland, Tanzania, Zambia and Zimbabwe) both cycles probably occur, while in West Africa, due to the absence of significant numbers of wildlife hosts the virus is believed to be maintained primarily within the domestic animal cycle. In southern Africa,

a number of countries have been able to control FMDV by separating infected buffalo from livestock and by limited use of vaccination. Both (Vosloo *et al*., 2004, 2002 and Batho *et al*., 2004), conducted an FMD emergency audit on Southern Africa and observed that Africa has the greatest diversity of FMD serotypes.

Based on the prevalence data from (Rweyemamu *et al.,* 2008b), the distribution of FMDV serotypes and topotypes is due to factors such as animal movement patterns, impact of wildlife and the farming systems. Lack of movement control within countries and across international borders for both wildlife and domestic animals aggravates the problem, and gives credence to the fact that FMD will remain a problem on the continent for the foreseeable future. The intense trading of animals and animal products within Africa and their export to other regions, make this continent a major source and reservoir of FMDV sometimes providing new variants which impact a wider region (Jori et al., 2009).

From the epidemiological surveillance done by Rweyemamu *et al.,* 2008b it has been observed that Tanzania is in the Great Lakes cluster which comprises of the countries of East African Community (EAC) (i.e. Tanzania, Kenya, Uganda, Rwanda and Burundi) plus the eastern part of the Democratic Republic of Congo (DRC). This region not only has large livestock populations but also the highest concentration of wildlife in the world. Farming is dominated by agro-pastoral communities and is characterized by communal grazing and migrations. Eastern DRC is heavily dependent on trade of livestock from Uganda, Tanzania, Rwanda and Burundi. This Great Lakes cluster probably contains the most complicated FMD situation in the world. The cluster probably contains several FMD primary endemic foci. Five serotypes (O, A, C, SAT1 and SAT2) are endemic in this cluster and the sixth serotype (SAT3) isolated in wildlife (African buffalo) in Uganda in 1970 (Hedger *et al.,* 1973), has not been isolated from the livestock in the Great Lakes cluster except for a recent report from Uganda of SAT3 from an long horned ankole calf

(Dhikusooka *et al*., 2015). According to Sangula *et al*., 2011and Jamal and Belsham, (2013) serotype C has not been identified elsewhere since it was last isolated in Kenya 2004.

In Tanzania the common bovine FMDV serotypes observed are SAT1, SAT2, A and O (Fig. 1). These serotypes were identified by c-ELISA Antigen during the period of seven years 2002-2009 (Kasanga *et al*., 2012). The serotypes were observed from the following regions namely; Kagera (type O), Kilimanjaro (SAT 2), Morogoro (SAT 2), Pwani (SAT1 and SAT2), Rukwa (SAT2), Singida (SAT2) and Tabora (SAT1) (Kasanga *et al.,* 2014, Kasanga *et al.,* 2012; Kivaria, 2003). The geographical distribution of FMDV serotypes from (1967-2009), is shown in Fig.1 (Kasanga *et al.,* 2014).

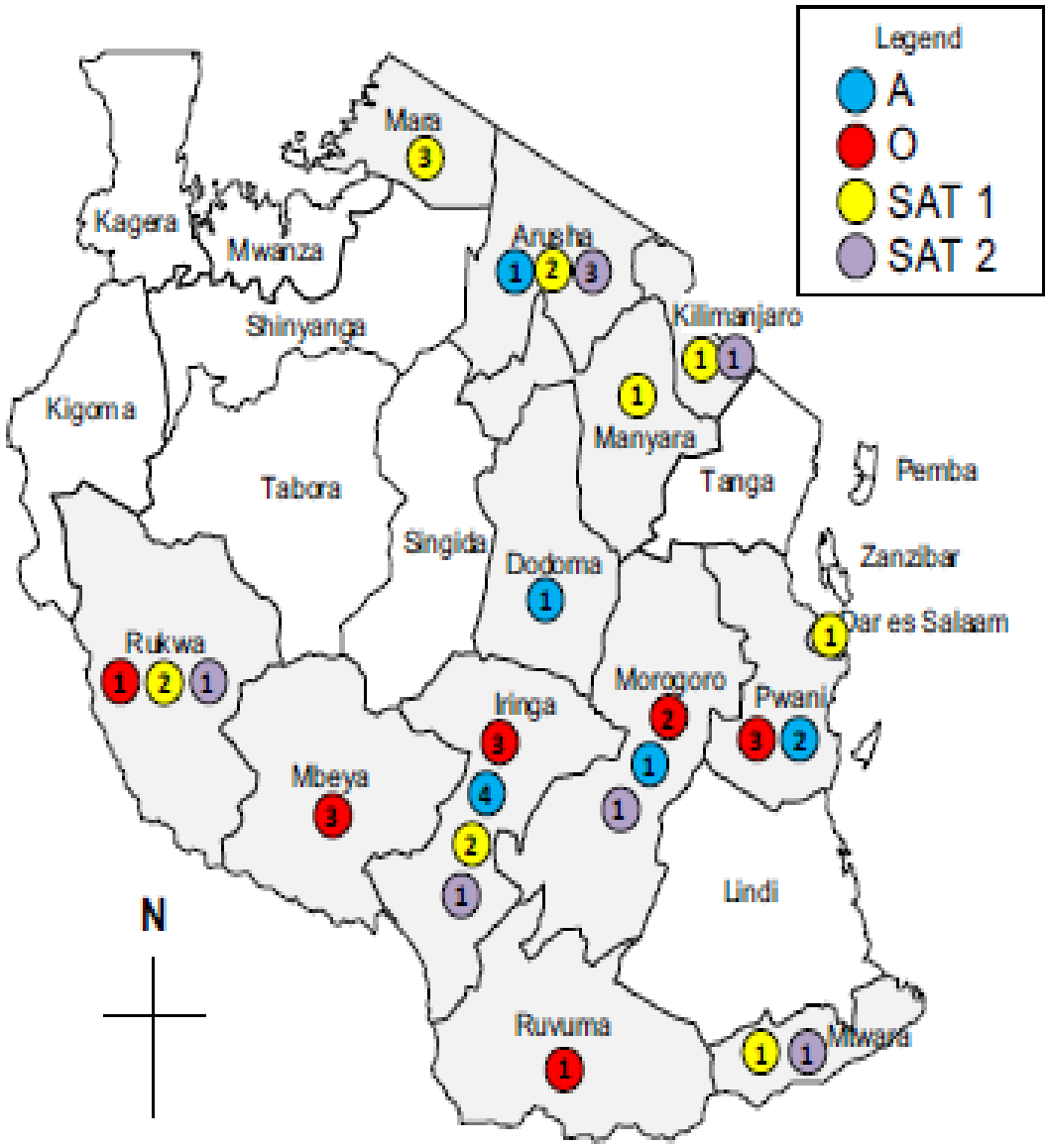

Figure 1: Map of Tanzania showing location of the 48 virus isolates (1967-2009), Source: Kasanga *et al.*, 2014.

The previous efforts to control FMD yielded futile results, as there are reported cases where there was occurrence of the disease after the animals have been vaccinated. Also the use of quarantine and culling strategies are very expensive to execute and difficult due to geographical landscape of the country. According to Paton *et al.,* 2009, control of FMD needs early warning and immediate epidemic response that will include a rapid typing of

FMDV isolates. This is very important to identify suitable vaccines and detection of the antigenic shift which is not done in most of the third world countries including Tanzania.

The intention of this study was to investigate the genetic variability of FMDV in relation to the endemicity of FMD in Tanzania. The study was based on the application of the molecular and epidemiological data to determine the distribution, phylogeography of the circulating FMDV in livestock within Tanzania. The outcome of this research could provide information necessary for recommendation of proper disease control measures in Tanzania and neighbouring countries.

1.2 Problem Statement and Justification

FMD is still a major global animal health problem and is a severe constraint to international trade on livestock and livestock products. Although FMD has been eradicated in Europe, North America, parts of South America, Australia and some island regions of Asia, it is still prevalent in many countries in Africa, Asia and South America. Between 2002 and 2010 the numbers of FMD outbreaks have occurred in the Sub-Saharan region and increased in frequency and in some cases outbreak have persisted for longer periods than expected up to 24 months (SADC-TADs, 2010 report). The reasons for this situation are complex and of multifactorial nature; this is due to the highly infectious nature of the FMDV and the compounding economic constraints following an outbreak of the disease.

The Sub-Saharan region is facing a fundamental dilemma in the context of FMD control and elimination strategies due to persistence of particular genetic subtypes (topotypes) as observed over the last 40 years (Kitching, 2000; Knowles and Samuel, 2003). Also, there is no any control programme that includes these countries; this is due to differences in economy and social development. There are significant gaps in knowledge; particularly

with regards to the manner in which the viruses persist, spread, and evolved and on how to stimulate a better immune response through vaccination (Paton *et al.,* 2009). Considering that, there is inadequate information on the existence of specific serotypes and topotypes circulating in different specific locations of this region. There is need for surveillance and characterization of the virus. In this study, the VP1 coding region sequences were used to identify the four serotype and genetic relationship between the viruses. VP1 is among the four structural proteins and is exposed on the capsid surface (Acharya *et al*., 1998; Domingo *et al.,* 2002) and it plays an essential role in forming the virus particles.

The VP1 protein is highly polymorphic and carries the virus major neutralizing antigenic sites (Kitson *et al*., 1990; Thomas *et al.,* 1988). Over the years, the partial or complete VP1 coding sequence has been used in the molecular epidemiological investigation, the development of genetically engineered vaccines, and the establishment of diagnostic methods as well as to trace origin and the spread of FMDV and also for typing and subtyping of the virus (Beck and Strohmaier, 1987; Ruiz-Jarabo *et al*., 2000). So the partial and full VP1 nucleotide sequences are the preferred region for comparison of FMDV isolates.

The disease status in the country is rampant as for the period of my study (2010-2013) 654 cases were reported (MLFD annual report, 2013). The efforts to control FMD in Tanzania are not very effective due to the lack of appropriate molecular epidemiological and baseline surveillance data. This is due to the fact that the molecular characteristics and spatiotemporal distribution of recent FMDV strains have not been consistently studied. This data will provide FMDV diversity information in the region that could help in making tailored vaccines. As vaccine strain selection for emerging FMDV endemic countries can be addressed through antigenic and genetic characterisation of recently

circulating viruses (Bari *et al.,* 2014). Control by vaccination is very difficult due to the genetic, antigenetic variations between the existing subtypes and also the information gap concerning the ability to predict the performance of vaccines. Therefore, vaccination can be effective in one area but not in another despite of using the same strain of virus due to inadequate knowledge about antigenicity, temporal and spatial dynamics of the virus that could direct vaccine matching. Improvement of laboratory and the field test could also be used in generating reliable information that can be utilized to control the occurrence of FMD and its spread to the new area within the country.

The key research questions of the current study were:

1. What are the genetic characteristics of circulating FMDV in endemic settings of Tanzania?
2. Are the FMDV subtypes restricted to specific geographical location in Tanzania or is there a more wide spread occurrence from neighbouring countries?
3. What is the sensitivity and specificity of Antigen-ELISA and Real time RT-PCR in detection of FMDV?

1.3 Objectives

1.3.1 General Objective

To determine molecular characteristics and generate epidemiological data/information that could be used to study and analyse the genetic diversity of FMDV in endemic settings of Tanzania.

1.3.2 Specific Objectives

i. To determine the sensitivity and specificity of rRT-PCR and Antigen-ELISA.
ii. To determine the genetic diversity and phylogenetic relationships of the currently circulating FMD viruses using the virus structural protein (VP1) coding sequence.
iii. To examine the spatiotemporal distribution of FMDV serotypes and subtypes in Tanzania.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Historical perspective and economic importance of FMD

Foot-and-mouth disease is a highly contagious disease affecting cloven-hoofed animals such as cattle, buffalo, pigs and small ruminants (Alexanderson *et al*., 2003). The first written description of FMD probably occurred in 1514, when Fracastorius described a similar disease of cattle in Italy (Fracastorius, 1546). Almost 400 years later, in 1897, Loeffler and Frosch, (1897) demonstrated that a filterable agent caused FMD. This was the first demonstration that a disease of animals was caused by a filterable agent and ushered in the era of virology. Also this can be found in a book from the Italian physician Hieronymi Fracastorii (Wright, 1930) in which, he described a cattle disease similar to the signs manifested by animal suffering from FMD. Since then, several indications of FMD can be found in the literature (Thalmann and Nockler, 2001).

For Tanzania, since its first documentation in 1927 and first isolation of the virus in 1954, many FMD outbreaks have occurred across different areas. It is the most important viral disease that is endemic and most important transboundary animal diseases (TADs) in Tanzania (Swai *et al.,* 2009). Of the seven known FMDV serotypes, four (O, A, SAT1 and SAT2) have been previously identified and reported in Tanzania (Mlangwa, 1983; Rweyemamu and Loretu, 1972; Rweyemamu *et al*., 2008b; Swai *et al*., 2009; Vosloo *et al*., 2002).

FMD ranks as one of the most economically important infectious diseases of animals according to the World Organisation for Animal Health (OIE). FMD has very serious direct and indirect economic effects. Direct economic effects include loss of productivity

in terms of meat and milk, loss of draught power, loss of weight, retardation of growth, delayed conception and abortion, death in calves and lambs (James and Rushton, 2002). Apart from the impact of FMD on animal health, the disease has been described by Rweyemamu and Leforban (1999) as the most important constraint to international trade in animals and animal products, which restricts trade in a south-to-north direction (Rweyemamu and Leforban, 1999).

The severity of the disease depends on the virus strain and the type of animal affected, and sequelae are found to be more important than the clinical disease (Woodbury, 1995; Kitching and Hughes, 2002). For example, from the second half of the 19th Century, on the account of the increase of animal trade due to development of new transport routes (O'Rourke and Williamson, 2002), the disease became more important and started to cause severe economic losses. According to Picado *et al*., (2010), FMD has had a great economic impact on Tanzania's livestock sector, with over 16 million heads of cattle in 2002–2003 (FAO, 2003).

Thus, with this current status of FMD endemicity, it will be impossible for Tanzania to participate in international livestock trade between countries according to OIE regulations (OIE, 2013). The emphasis on FMD control is to enable the country to get FMD free status so as to create international markets that will help reduce poverty in FMD endemic countries like Tanzania (Perry and Rich, 2007). These, animal diseases, in particular transboundary animal diseases (TADs) such as FMD, severely constrain the development of competitive livestock enterprises in developing countries (Perry and Sones, 2007; Knight-Jones and Rushton, 2013).

2.2 Control of FMD

Since the beginning of the 20th Century, FMD has been of considerable concern to many countries, and outbreaks or the fear of disease incursions have led to the establishment of institutes to investigate methods to control the disease. The choice of control policy adopted by a given country depends on its FMD status and the risks of incursions of the disease (Ahl *et al*., 1991).

The world distribution of this one disease is almost a mirror image of the world-wide global economic structure with the high-income, industrialized countries being generally free from FMD, while the disease is persistently endemic in low-income countries suffering from food deficits (Rweyemamu and Astudillo, 2002). Rweyemamu and Astudillo (2002) suggested that, effective and sustained control of FMD at any geographical level could only be achieved through inclusive area-wide programmes rather than through isolated individual farm interventions, unless animals are housed under a high level of bio-security.

The success of any FMD control campaign ultimately depends on the abundant supply of vaccine of the appropriate strain composition and proven potency, adequate vaccine coverage, rapid vaccine development, overall planning and management by a well-resourced veterinary service, and the involvement and cooperation of the livestock farmers (Rweyemamu and Garland, 2006; Maree *et al.,* 2014). Vaccines for the control of FMD in endemic regions are mostly used for mass prophylactic application. Such vaccines are multivalent to provide protection against multiple serotypes, and should have a potency of at least 3 potential doses (PD_{50}) per dose (Rweyemamu *et al.,* 2008b). This approach is compatible with the Progressive Control Pathway for FMD control (PCP-FMD) (FAO-EuFMD-OIE, 2011), which is designed to guide countries in the planning and

management of efforts to increase the level of control of FMD from the early stages up to the point where an application to the OIE for official recognition of freedom from FMD (with or without vaccination) may be successful and sustainable. The PCP-FMD is implemented both in FMD free areas as to maintain their OIE free status and in endemic settings. The current inactivated vaccines have proven effective in reducing clinical disease in FMD-endemic areas and have been critical to the success of FMD control programs in South America and Europe (Brown, 2003).

2.2.1 Control of FMD incursion into FMD-Free Countries or Zones

In countries free of FMD that have naive livestock populations, great attention is paid to reducing the possibility of incursions of the virus. These include border and import controls of animals and the products from other countries. Most declared FMD free countries are banking on stamping out, sanitation and restrictive quarantine as the first approach for controlling this disease (Paton *et al*., 2009). The current FMD vaccine is an inactivated whole-virus preparation that is formulated with adjuvant prior to use in the field (Maree *et al.,* 2014). A number of countries have established vaccine banks which contain concentrated antigen stored in the gaseous phase of liquid nitrogen (Doel, 2003). Antigen stored under these conditions is stable for a longer period of time than formulated vaccine (Doel and Pullen, 1990).

Vaccine banks contain antigen against a number of virus serotypes and provide member countries with an almost immediate source of vaccine. A recent report by Doel (2003) provides an in-depth review of the history, production, and utilization of inactivated FMD vaccines. In case of an out-break in FMD free countries stamping out that implied the killing and destruction of all infected animals and their immediate susceptible contacts is applied and is followed by thorough cleaning and disinfection of the affected premises. Farmers are compensated for their loss of livestock. The compensation is mostly done in

developed countries but very rarely in developing countries due to economic hardships. For example, the first country to apply this method was Britain in 1892 with a substantial program for FMD control (Sutmoller *et al.,* 2003). The decision was made to eradicate every outbreak by 'stamping-out'. In FMD free countries, stamping-out has is the first option to eradicate the disease. As a first line of defence it is often quite successful, at least if the disease has not yet spread too widely and if the density of livestock in the area is relatively low.

In FMD free countries the control of FMD by quarantine is practiced where farmers are advised to keep movements of susceptible animals, people and vehicle to an absolute minimum. This includes: to limit the movement of people between buildings as much as possible; to place foot dips at all entrances, service and feed delivery points; to only allow cleaned and disinfected vehicles to visit the farm; to only allow essential visitors; to limit contact with other peoples, livestock, other keepers of livestock or with people who have had dealings with livestock such as abattoir workers, pig catchers, veterinarians or other farm inspectors; to ensure clean boots and clothing is worn on entrance; to ensure that hands are cleaned and disinfected on entrance; and to control dogs, cats, rats and other small animals that may spread the disease. Therefore, the control of the disease in endemic areas has a potential to reduce the risk of incursions into disease-free regions (Grunman and Baxt, 2004).

2.2.2 Control FMD in endemically affected Countries or Zones

The FMD is endemic in Asia, Africa, South America and Middle East (Gleeson *et al*., 2003; Kitching, 1998; Knowles and Samuel, 2003). Some countries within these regions are free from the disease where occasionally FMDV can cause sporadic outbreaks. Foot-and-mouth disease is endemic in most of sub-Saharan Africa including Tanzania, and is

considered to be one of the most widely distributed TADs in the world (OIE and FAO Reports, 2003). FMD is highly contagious and can spread rapidly through close contact between animals, as well as on contaminated equipment, clothing and footwear, on contaminated material such as hay and feed, or on contaminated raw meat that is fed to susceptible animals.

FMD is listed by the OIE in their notifiable disease list due to its ability of rapid spread regionally and internationally. Sub-Saharan Africa is endowed with an abundance of wildlife, which has been conserved within national parks and game reserves (Chardonnet *et al*., 2002; Maree *et al*., 2014). Effective control and prevention of FMD relies largely on the implementation of strategies such as physical separation of wildlife and livestock, repeated vaccination of cattle herds exposed to wildlife, control of animal movements, and careful assessment of the risk of FMDV introduction into disease-free areas (Thomson *et al.,* 2003; Bruckner *et al.,* 2002; Jori *et al.,* 2009, Maree *et al*., 2014).

The accurate diagnosis of FMDV infection is of utmost importance for the eradication and control of the disease in endemic regions (Maree *et al*., 2014). The initial diagnosis of FMD is normally based on clinical signs, but this can easily be confused with other vesicular diseases (Remond *et al*., 2002).

For example, in Tanzania most of the vaccines are cocktail of serotype A, O, SAT1 and SAT2. But, this selection of FMD vaccine candidates is complicated by the wide spectrum of genetic and antigenic variability of the FMDV and the continuous emergence of new mutants from populations that escape the host immune response (Haydon *et al*., 2001; Domingo and Holland, 1988; Holland *et al.,* 1982). More studies are needed to assess whether this vaccination strategy of using multiple serotypes would be effective in

endemic regions of Africa for SAT serotypes and when applied to the field and to find out whether the production process would be economically viable (Maree *et al.,* 2014).

Additionally, individuals would end-up sourcing vaccines without the knowledge of the current circulating strains in their region, leading to a poor vaccine match (Maree *et al.,* 2014). This often leads to no or ineffective control in FMD endemic African regions. The development of new vaccines against FMD in endemic countries in Africa should therefore take into account the ecosystem-based synchronization as FMD control strategies employed in these regions (Rweyemamu and Astudillo, 2002). Following an FMD outbreak in Africa, most of the veterinary authorities enforce quarantines to restrict livestock and livestock product movement as the first control measure. Movement restrictions are necessary to control the potential spread of the disease.

In FMD endemic countries vaccination is used as a first approach to control outbreaks, however, should be based on a proper evaluation of epidemiological and risk factors for each individual contact farm. It was suggested that, there is a great need for risk-based surveillance to be able to determine primary endemic areas and factors that influence disease dissemination, to assist the design of targeted, area-wide, or ecosystem-based disease control strategies, as African regions embark on the PCP-FMD.

2.3 Taxonomy and Classification of FMDV

Foot-and-mouth disease virus (FMDV) is a member of the picornavirus family. The picornaviruses are currently divided into nine genera; FMDV is the prototype aphthovirus (Fig.2). Other well-known picornaviruses include poliovirus (PV, an enterovirus), human rhinoviruses and encephalomyocarditis virus (EMCV, a cardiovirus). Picornavirus particles are roughly spherical (about 30 nm in diameter) and are comprised of 60 copies

of four different virus-encoded capsid proteins, VP1 (1D), VP2 (1B), VP3 (1C) and VP4 (1A) together with a single copy of the viral RNA genome. Each picornavirus has a single-stranded RNA genome of positive polarity (about 8 kb in length) and the genomic RNA is infectious (Belsham and Bostock, 1988).

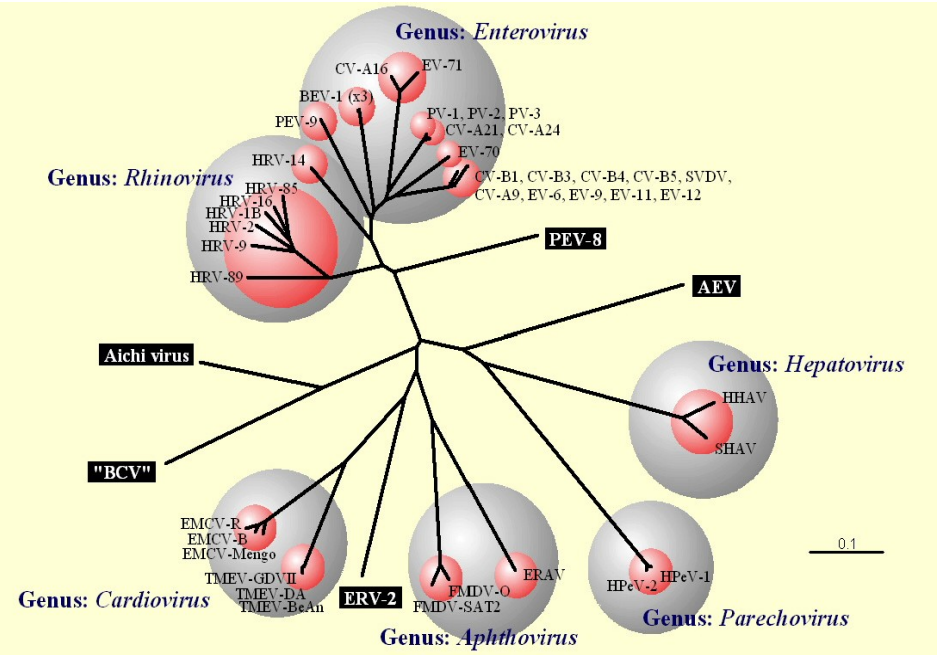

Figure 2: Taxonomy of *Picornaviridae*. Source: Knowles *et al.,* 2008

2.3.1 Serotypes

The grouping is based on the nucleotide sequence of the VP1 (1D) structural protein of the capsid (Knowles and Samuel, 2003; Marquardt and Adam, 1990). The VP1 gene is important due to its role for antigenicity, immunity three line of defence and serotype specificity (Jackson *et al*., 2003). These serotypes show some regionality but, are not distributed equally around the world while the O serotype is most common. Within serotypes there are multiple topotypes that are usually related to geographical region of the disease occurrence or subtype. Many studies have been undertaken previously to characterise and group viruses of each serotype to better understand the geographical

movement of FMDV and identify the source of new outbreaks (Knowles and Samuel, 2003).

2.4 Foot-and-Mouth Disease virus structure and Genome organization

The genome of FMDV is over 8000 bases in length and encapsidated in an icosahedral structure of outer protein (Jackson *et al*., 2003; Mason *et al*., 2003a). The FMDV (open reading frame) ORF can be divided into four regions based on the presence of cleavage sites; L^{pro}, structural protein (P1) and non-structural proteins (P2 and P3), (Robertson *et al*., 1985; Rueckert and Wimmer, 1984). The L^{pro} contains two in-frame AUG initiation codons that encode two L proteins, Lab and Lb. The P1 region of the genome encodes the four structural proteins, 1A (VP4), 1B (VP2), 1C (VP3) and 1D (VP1) that make up the outer coat of virus (Fig. 3).

The outer protein coat of FMDV, also known as the capsid is made up of 60 copies each of the four structural proteins. VP4 protein is internally in contact with RNA while the other three proteins are on the surface. These four proteins assemble to form a protein sub-unit or protomer and later five protomers join to form a pentamer. Twelve pentamers join up enclosing a strand of RNA to create a virus particle called provirion. The P2 region encodes three non-structural (NSP) proteins namely 2A, 2B and 2C and the P3 region encodes another three NSP, 3A, $3C^{pro}$ and $3D^{pol}$ protein. In addition, FMDV has two untranslated regions (UTRs) at both ends of the genome.

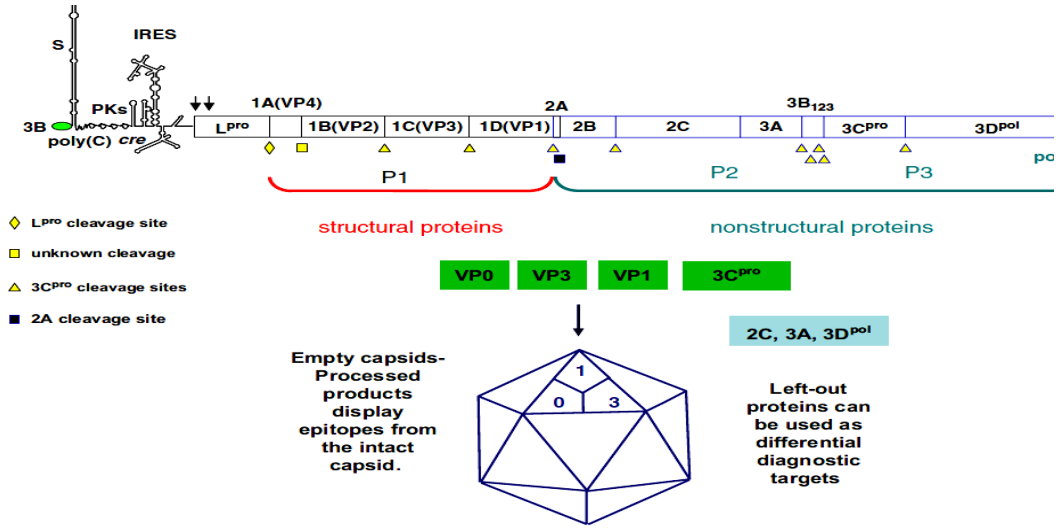

Figure 3: **Structure and Genome Organization of FMDV. Source: Belsham, (1993).**

The 5'UTR region consists of the S-fragment, poly C, pseudoknot, cre structure and internal ribosome entry site (IRES) which is over 1300 bases (Forss *et al*., 1984). At the other end of the FMDV genome is the 3'UTR region with a poly-A tract (Fig. 3) (Dorsch-Hasler *et al*., 1975). The structural proteins form the capsid of the virion and with the exception of VP4, are surface exposed (Acharya *et al*., 1989). VP1 is involved in the host cell interaction via the Arg-Gly-Asp (RGD) sequence dependent integrins and heparin sulphate proteoglycan receptors (Neff *et al.,* 1998; Jackson *et al*., 2000; Jackson *et al.,* 2007). The nonstructural proteins are involved in replicatory and other biological functions (Grubman and Baxt, 2004; Moffat *et al.,* 2005).

While the functions of some of the nonstructural proteins are less well understood, the 2B protein is known to be involved in membrane rearrangements required for viral RNA replication and capsid assembly while 3A and 3B are reported to play a role in virulence and host range (Nunez *et al*., 2001; Pacheco *et al.,* 2003). The 3C protease is responsible for most of the proteolytic cleavage in the viral polyprotein and RNA replication while 3D forms the core subunit of the RNA-dependent RNA polymerase (Forss *et al*., 1984; Vakharia *et al.*, 1987).

2.4.1 Antigenic Diversity

Antigenic diversity among ribonucleic acid (RNA) viruses occurs as a result of rapid mutation during replication and recombination/or reassortment between genetic materials of related strains during co-infections (Mumford, 2007). Antigenic variability is common in many picornaviruses, but, including FMDV this variation differs in extent and is not equal in all picornaviruses, e.g. there are three polio serotypes and more that hundred rhinovirus serotypes (Domingo *et al.,* 2001; Duarte *et al.,* 1994).

The molecular basis of antigenic variation in FMDV has been extensively studied and it is well known that FMDV exhibits a high degree of genetic and antigenic variation (Domingo *et al*., 2002). As with other RNA viruses such as influenza viruses, this high level of variation is attributable to the error-prone replication of viral RNA and the inadequacy of a proof-reading mechanism associated with the viral replicase (Domingo & Holland, 1997; Steinhauer *et al.,* 1987). Antigenic variation in the field increases with time and most probably results from immunologic pressure placed on the virus by either the infected or vaccinated host species (Domingo, *et al*., 2003; Haydon *et al*., 2001).

Recombination, spontaneous mutation and migration are the three major forces driving the molecular evolution of all viruses. The spontaneous mutation leads to a statistical effect called the genetic drift. FMDV is thought to evolve mainly through genetic drift, due to the high error-prone nature of its RNA polymerase (Domingo *et al*., 2006). Recombination plays an important role in FMDV evolution and occurs mainly in the gene region coding for the non-structural proteins (Haydon *et al*., 2004; Haydon and Woolhouse, 1998; Tosh *et al*., 2002b). The evolution of the structural proteins, especially VP1, which plays a major role in virus entry, seems to be mainly shaped by genetic drift (Domingo *et al*., 2005b, Domingo *et al*., 2005c; Domingo, 2006). The 1D encoded structural proteins, VP1,

is surface exposed and contains the major antigenic determinants of the virus. VP1 alone is known to elicit neutralizing antibodies and contains two immunogenic sites at amino acid position 140-160 (the G-H loop) and at residue 200-213 (the C-terminus region). Variation at critical amino acid residues within the G-H loop and C-terminus regions contributes to the antigenic variation of the virus (Sobrino *et al*., 2001).

In addition, VP1 contains a highly conserved sequence called the RGD (Arginine-Glycine-Aspartate) motif that is involved in cell attachment (Leippert *et al.,* 1997). The RGD is a motif found in a number of cell attachment proteins and recognized by some member of the intergrin family. Most, but not all, strains of FMDV have conserved this motif (Belsham, 1993). It has been suggested that this region represents the cell attachment site for FMDV and indeed this motif appears exposed in the reduced form of the virus when the βG- βG loop is resolved. Heparan sulphate has an important role in cell entry by FMDV. The binding site is a shallow depression on the virion surface, located at the junction of the three major capsid proteins, VP1, VP2 and VP3. Two pre-formed sulphate-binding sites control receptor specificity (Fry, 1999). Residue 56 of VP3, an arginine in this virus, is critical to this recognition, forming a key component of both sites.

These features together with the high levels of variation make VP1 the gene of choice to study genetic relationship between isolates. For example, since the first report by Beck and Stromeier (1987), gene sequences have been used routinely for molecular epidemiology studies of FMD virus occurring in different regions throughout the world, including Africa (Vosloo *et al*., 1992; Dawe *et al*., 1994; Vosloo *et al* 1995; Knowles *et al*., 1998; Bastos *et al.,* 2000, 2001; Sangare *et al.,* 2001).

2.4.2 Physico-chemical properties of the FMD viruses

The virus is sensitive to an acidic and alkaline environment, high temperature (>50°C) and UV light (Forss *et al*., 1984). However, under certain circumstances and temperate conditions it can survive in the environment such as in contaminated fodder for up to one month. Furthermore, it is relatively insensitive to cold; survives longest in conditions of low temperatures and can survive in uncooked meat for long periods, particularly if the meat is quick-frozen, which reduces production of acid in the meat.

At optimal pH (7.2 - 7.6) FMD virus could survive in the following manner; year at 4°C; 8 - 10 weeks at 22°C; 10 days at 37°C; less than 30 minutes at 56°C. At least 90% of the deactivation of virus after: 3 seconds at 61 °C, 20 seconds at 55 °C, one hour at 49 °C, seven hours at 43 °C, 21 hours at 37 °C, 11 days at 20 °C, 18 weeks at 4 °C. At 56 °C for 30 minutes "is sufficient to destroy most strains"(Forss *et al*., 1984). "If protected by mucus or faeces and shielded from strong sunlight, picornaviruses are relatively heat stable at usual ambient temperatures." In milk, because virus shed from infected mammary glands is incorporated into milk micelles and fat droplets, the virus is partially protected against heating and a portion of the viral population may be viable after pasteurization at 72°C for 15 seconds and even after heating at 88°C for 50 seconds. High temperature short-time (HTST) pasteurization at 72 °C for 15 seconds (as mandated by the Pasteurized Milk Ordinance of the Food and Drug Authority (USA), 2003) in a flow pasteurizer eliminated up to 6 $\log_{10}$ FMDV in skimmed or whole milk, as shown by cell culture, but residual viral infectivity could be detected by inoculation into steers. Increasing the time for which the milk was heated up to 36 seconds, or heating up to 80 °C or 95 °C, did not entirely eliminate infective virus from whole milk as shown by inoculation of steers (Forss *et al*., 1984).

2.4.3 Replication of the FMDV

The virus particle (25-30 nm) has an icosahedral capsid made of protein, without an envelope outer layer, containing a single strand of ribonucleic acid with a positive encoding of its genome. When the virus comes in contact with the membrane of a host cell, it binds to a receptor sites and triggers a folding-in of the membrane. Once the virus is inside the host cell, the capsid dissolves, and the RNA gets replicated, and translated into viral proteins by the host cell's ribosomes using a cap-independent mechanism driven by the internal ribosome entry site element. The synthesis of viral proteins includes 2A 'cleavage' during translation (Belsham, 1995). They include proteases that inhibit the synthesis of normal cell proteins, and other proteins that interact with different components of the host cell. The infected cell ends up producing large quantities of viral RNA and capsid proteins, which are assembled to form new viruses. After assembly, the host cell lyses (bursts) and releases the new viruses (Martinez-Salas *et al*., 2008a).

2.5 Diagnosis of FMD

Due to the rapidity of spread of FMD and the serious economic consequences that can arise from an outbreak, prompt, sensitive and specific laboratory diagnosis and identification of the serotype of the viruses involved in disease outbreaks is essential for designing control regime (Jamal and Belsham, 2013; King *et al*., 2012 ; Kasanga *et al* 2014b). Diagnosis of FMD can be done normally based on clinical signs, but this can easily be confused with other vesicular diseases such as swine vesicular stomatitis, malignant catarrhal fever (MCF), rinderpest (Remond *et al*., 2002). Detection of FMDV specific antibodies can also be used and antibodies to viral nonstructural protein can be used as indicators of infection irrespective of vaccination status (OIE, 2009).

An essential component of any disease control strategy, are diagnostic assays that can rapidly confirm the initial clinical determination of infection (Jamal and Belsham, 2013). The rapid and accurate diagnosis of FMD is of utmost importance to the control of any infection given the extreme contagiousness of the causative virus (Maree *et al*., 2014). The vesicular diseases such as, swine vesicular disease, vesicular stomatitis, and vesicular exanthema of swine cause vesicular lesions in swine and cattle cannot be distinguished from those caused by FMD clinically (Bachrach, 1968). In addition, FMDV infection of sheep and goats can be difficult to detect clinically (Donnelly *et al*., 2001; Geering, 1967). Sensitive diagnostic assays are also necessary to distinguish vaccinated from infected or convalescent animals.

2.5.1 Clinical signs

Traditionally, diagnosis of FMD was based on clinical signs that are characterised by an acute febrile condition and the formation of vesicles in the mouth and on the feet. The resultant pain leads to weakness and inappetance. Following a period of initial pyrexia of 40ºC which can last for one to two days, a variable number of vesicles develop on the tongue, hard palate, dental pad, lips, gum, muzzle, coronary band, interdigital spaces, teat and snout in pigs (Alexandersen *et al.*, 2003). This picture is happening regularly in most countries in sub-Saharan Africa; but, these signs often go unnoticed in real field conditions, but for pastoralists have good knowledge of the diseases. Frequently, it is the authorities lack of response or lack of proper epidemio-surveillance system that affect reporting of this disease by the famers to the relevant veterinary authorities to verify clinical symptoms is a main problem which is due to poor communication and infrastructure. Hence, it is vital that the recognition of signs of the disease by the farmer is promptly conveyed to relevant veterinary authorities to verify clinical symptoms, and suspect samples should be sent to the reference laboratory for confirmation (Maree *et al.,* 2014).

In the plate 1, cattle were showing typical clinical signs of FMD, such as ulcerations on epithelial tissues of gums, tongue and froth coming out of mouth.

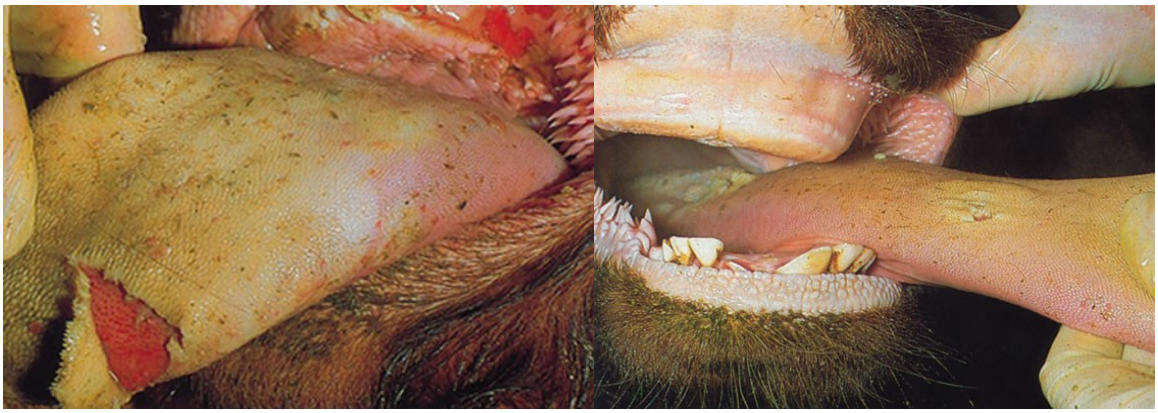

Plate 1: Cattle showing the typical FMD clinical signs. Source: Current study.

2.5.2 Laboratory diagnosis

Diagnosis of FMD is by virus isolation or by the demonstration of FMD viral antigen or nucleic acid in samples of tissue or fluid. Detection of virus-specific antibody can also be used for diagnosis, and antibodies to viral non-structural proteins (NSPs) can be used as indicators of infection, irrespective of vaccination status (OIE, 2013). Laboratory diagnosis in most of the sub-Saharan Africa including Tanzania is very challenging, this is due to fact that most of the samples received by laboratory can be of poor quality due to an ineffective cold-chain and long transport periods (Maree *et al*., 2014). Universal existing diagnostic techniques for the detection of FMDV are based on identification of the infectious agent, detection of viral antigen by ELISA, molecular detection of viral antigen and detection of FMDV specific antibody. In sub-Saharan Africa, the accurate laboratory diagnosis of FMD is only carried out at specialized laboratory (Regional, National and Reference laboratories e,g. FAO Reference Laboratory for FMD at the Pirbright).

2.5.2.1 Identification of the agent

i) Virus isolation

Diagnosis of FMDV infection can also be demonstrated by isolating the virus by cell cultures (OIE, 2012; OIE, 2008). The cell cultures should be examined for cytopathic effect (CPE) for 48 hours. If no CPE is detected, the cells should be frozen and thawed, used to inoculate fresh cultures and examined for CPEs for another 48 hours. Some field viruses may require several passages before they become adapted to mice (Skinner, 1960). In the case of oral pharyngeal fluids, pre-treatment with an equal volume of chloro-fluoro-carbons may improve the rate of virus detection by releasing virus from immune complexes. All the mentioned techniques are only in use in specialized laboratories, although simplified systems for potential field-use are under development (Callahan *et al*., 2002).

ii) Immunological Methods

a) Enzyme-linked immunosorbent assay (ELISA)

The preferred procedure for the detection of FMD viral antigen and identification of viral serotype is the ELISA (Ferris & Donaldson, 1992; Roeder & Le Blanc Smith, 1987). This is an indirect sandwich test in which different rows in multiwell plates are coated with rabbit antisera to each of the seven serotypes of FMDV. These are the 'capture' sera. The ELISAs are blocking- or competition-based assays that use serotype-specific polyclonal antibodies (PAbs) or MAbs, are quicker to perform and are not dependent on tissue culture systems and the use of live viruses. ELISA is able to detect viral antigens and confirm the clinical diagnosis and identify the FMDV serotypes (Ferris *et al.,* 1988).

b) Lateral flow device (LFD)

Another antigen detection method that has been developed is the "lateral flow device" (LFD) which has been evaluated and shown to be pan-reactive to all FMDV serotype

except for serotype SAT2. Since this technique is easy and rapid, it has the potential to be used for pen-side diagnosis for FMD suspected outbreak (Ferris *et al.,* 2009). This field test can be used in some countries including Tanzania. The LFD is based on FMDV antigen detection, which is easy to use and can be utilized on the farm to reduce the time required for transport and laboratory diagnosis. The detection of FMDV antigens by direct application of vesicular fluids and epithelial suspensions from animals of an infected farm may reduce the chances of diagnostic error arising from nonspecific reactions. Data from the field illustrates the potential for the LFD to be used in locations close to animals to provide rapid support to veterinarians in their clinical assessment of suspected FMD cases (King *et al.,* 2012). Also the simplicity and stability of the LFD may be important features for FMD diagnosis in sub-Saharan African countries.

c) Complementary fixation test

Complement fixation test was the earliest laboratory test used to diagnose FMD. This test was later replaced by ELISA due to its low sensitivity, specificity and difficulty in interpretation of its results due to pro and anti-complement activities.

iii) Nucleic acid recognition methods

Molecular methods, such as conventional reverse transcription-polymerase chain reaction (RT-PCR) assays have been developed and also can provide serotype-specific results (Reid *et al*., 1999). The RT-PCR has been shown to be a useful tool for the diagnosis of FMD as it offers the advantages of fast, sensitive and reliable diagnosis. The presence of viral genomic material can be detected using RT-PCR assays. A variety of RT-PCR methods have been reported in recent years for the early detection of FMDV RNA in epithelium, cell culture isolates and other tissues using universal primers for all seven serotypes (Meyer *et al*., 1991).

However, the number of samples that can be analyzed simultaneously with this technique is limited and this approach may not be able to cope with samples that might be received during an epidemic. RT-PCR can be used to amplify genome fragments of FMDV in diagnostic materials including epithelium, milk, serum and probang samples (Amarel *et al*., 1993). RT combined with real-time PCR has sensitivity comparable to that of virus isolation and automated procedures enhance sample throughput (Reid *et al*., 2003; Reid *et al*., 2001). Therefore real-time RT-PCR (rRT-PCR) was developed and has been shown to have high sensitivity and specificity for the detection of FMDV genomes of all seven serotypes (Reid *et al*., 2002, King *et al*., 2007, 2006). This assay has been used on a large number of tissues samples, serum samples, swab samples and tissue culture supernatants. RT-PCR can be used to amplify genome fragments of FMDV in diagnostic materials including epithelium, milk, serum and Oral pharyngeal (OP) samples.

Serotyping primers have also been developed (Vangrysperre & De Clercq, 1996). Simplified RT-PCR systems for potential field use are under development (Callahan *et al.,* 2002). Other approaches, like loop-mediated isothermal amplification (LAMP), have been developed, which enable the tests to be conducted in the field using inexpensive tools. RT-LAMP amplifies specific nucleotide sequences at a constant temperature and thus does not require a thermocycler. The assay is based on the principle of DNA amplification by an autocycling strand displacement reaction. The assay is performed using a set of two specially designed inner primers and two outer primers and a DNA polymerase with high strand displacement activity (Notomi *et al.,* 2000). The molecular epidemiology of FMD is based on the comparison of genetic differences between viruses. Dendrograms showing the genomic relationship between vaccine and field strains for all seven serotypes based on sequences derived from the 1D gene (encoding the VP1 viral protein) have been published (Knowles and Samuel, 2003; http://www.wrlfmd.org/).

2.5.2.2 Serological tests

Serological tests for the detection of antibodies against FMD viruses irrespective of the vaccination status have been applied in some studies (Berger *et al*., 1990). Although these tests were serotype specific, they were tedious to use for screening purposes especially in areas where FMD is endemic. Serological tests for FMD are of two types; those that detect antibodies to viral structural proteins (SP) and those that detect antibodies to viral non-structural proteins (NSPs).

a) Virus neutralization test (VNT)

The virus neutralization test (VNT) is currently considered as the "gold standard" for detection of antibodies to structural proteins of FMDV and is a prescribed test for import/export certification of animals/animal products (OIE, 2012). However, as various primary cells and cell lines with variable degrees of sensitivities are used in the VNTs, they are more prone to variability than other serological tests (Jamal and Belsham, 2013). Furthermore, VNT is slower, subject to contamination and requires restrictive bio-containment facilities in contrast to other serological tests which can use inactivated viruses as antigens.

The test was found to be as sensitive as the conventional antigen ELISA for the detection of FMDV in epithelial suspensions tested and had an equivalent 100% sensitivity on the cell culture supernatants of FMDV serotypes O, A, C and Asia-1.

b) Solid-phase competitive ELISA

The method described (Paiba *et al.,* 2004) can be used for the detection of antibodies against each of the seven serotypes of FMDV. As an alternative to guinea-pig or rabbit antisera, suitable Monoclonal antibodies (MAbs) can be coated to the ELISA plates as

capture antibody or peroxidase-conjugated as detecting antibody (Brocchi *et al.,* 1990). A commercial kit is available for serotype O with a different format but similar performance characteristics (Chenard *et al.,* 2003).

c) Liquid phase blocking ELISA

Antigens are prepared from selected strains of FMDV grown on monolayers of BHK-21 cells. The unpurified supernatants are used and pre titrated in a two fold dilution series but without serum. The final dilution chosen is that which, after addition of an equal volume of diluents, gives an absorbance on the upper part of the linear region of the titration curve (optical density approximately 1.5). Phosphate Buffered Saline (PBS) containing 0.05% Tween 20 and phenol red indicator is used as a diluent (PBST). The other reagents used in the test are the same as those in the solid-phase blocking ELISA.

d) Structure and Non structural protein (NSP) antibody test

Serological tests to detect FMDV non-structural proteins (NSP) as well as FMDV structural protein of serotype O have been developed (Chenard *et al*., 2003; Sorensen *et al*., 2005). The demonstration of specific antibodies to structural proteins in non-vaccinated animals is indicative of prior infection with FMDV and this can be achieved by this technique (Crowther and Abu, 1979).This is particularly useful in mild cases or where epithelial tissue cannot be collected. Tests for antibodies to some NSPs of FMDV are useful in providing evidence of previous or current viral replication in the host, irrespective of vaccination status. NSPs, unlike structural proteins, are highly conserved and therefore are not serotype specific and as a consequence, the detection of these antibodies is not serotype restricted. The NSP test can be used to indicate the FMD infection for discrimination of infected animal from vaccinated ones (De Diego *et al*., 1997; Mackay *et al*., 1998; Sorensen *et al*., 1998; Shen *et al*., 1999; Bruderer *et al*., 2004; Clavijo *et al*., 2004a).

2.6 Epidemiology of FMD

Currently, Europe, North and Central America, Greenland, Australasia and Oceania are FMD-free (Fig. 4). The current global burden of FMDV infection is maintained within three continental reservoirs in Asia, Africa, and South America, which can further be subdivided into seven major virus pools of infection (Brown, 2003; Tully and Fares, 2008). Even though there are some common features in the spread of FMD, each of the seven serotypes, or even their variations (topotypes), has a different way of transmission, clinical appearance and species tropism (Kitching, 2005). The SATs serotypes are normally restricted to sub-Saharan Africa (Vosloo *et al*., 1996). Types O and A are the world most distributed, occurring in many parts of Africa, southern Asia, the Far East and South America. Type C appears to have become confined to the Indian sub-continent and Asia 1 normally only occurs in southern Asia (Knowles and Samuel, 2003), but this serotype has not been seen since 2004 (Sangura *et al*., 2011; Jamal and Belsham, 2013). The seven FMDV serotypes cluster into type-specific lineages (topotypes) when comparing either nucleotide or amino acid sequences. These are genetically grouped and based on their geographic origin and this has led to their being referred to as topotypes (Knowles and Samuel, 2003).

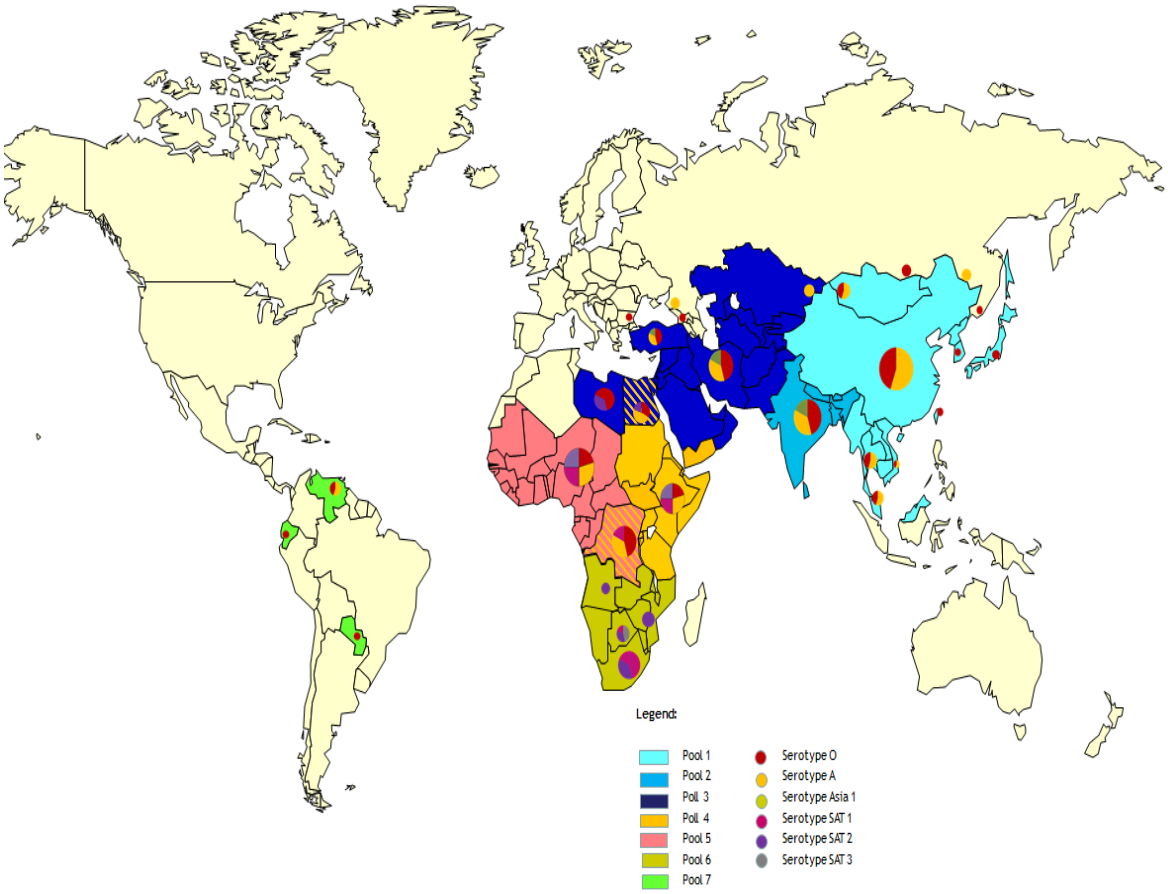

Figure 4: Foot-and-mouth disease virus pools distribution, 2011-2013. Source: European Commission for the control of FMD.

The serotypes of FMDV are not distributed uniformly around the world. The serotype O, A and C viruses have had the widest distribution and have been responsible for outbreaks in Europe, America, Asia and Africa. The disease has been present in almost every part of the world where livestock are kept. More than 100 countries including Asian, African and South American are still affected by FMD and distribution of the disease roughly reflects economic development where more developed countries have eradicated the disease.

Based on genetic and antigenic analyses, FMDVs throughout the world have been sub-divided into seven regional pools (Di Nardo *et al*., 2011; Sumption *et al*., 2012). Certain countries share viruses belonging to two different pools, for example, Egypt and Libya

(Fig. 4). Egypt and Libya are indicated as being in multiple pools, since they have evidence of FMDV that have originated from two or more pools in the recent past (4 years). Virus circulation and evolution within these regional virus pools result in changing needs for appropriate vaccine selection (Belsham and Jamal, 2013).

In Africa, the FMD serotypes are not uniformly distributed, and each serotype results in different epidemiological pattern (Maree *et al.,* 2014). Based on the genetic characterization of the virus and antigenic relationship of FMDV in Africa, the virus distribution has been divided into three virus pools: namely pool 4 covering East and North Africa, with predominance of serotypes A, O, SAT1, SAT2 and SAT3 serotypes (Maree *et al.,* 2014).

The disease is known to be endemic in Tanzania and is widely distributed in many parts of the country with at least four serotypes being found. The four serotypes that were detected during the period of from 2003 to 2010 were O, A, SAT 1 and SAT 2 (Kasanga *et al.,* 2012). The serotypes O, A, SAT 1 and SAT 2 are associated with the recent FMD outbreaks in different areas of Tanzania. Studies conducted by others have reported the existence of serotypes O and SAT 2 in Tanzania to be as far back as in 1950s with the detection of SAT 1 for the first time in 1971 (Rweyemamu and Loretu, 1972, 1973). From the previous studies it is indicated that types O and SAT 2 are old in Tanzania and serotype SAT 2 was detected almost throughout the country (Kasanga *et al.,* 2012). Serotype O is widespread in the Northern, Southern, Western and Eastern zones. The topotype distribution of FMDV serotypes for O, A, SAT1 and SAT2 in Tanzania, is for serotype O (VI), A (III), SAT1 (III) and SAT2 (IV) (Rweyemamu *et al.,* 2008b).

Recent changes in the political and economic growth have resulted in increased movement of livestock across international borders as taking into considerations that Tanzania has wide and porous borders with its neighbour countries. The movement of pastoralist with their herds (traditional animal movements) searching for pasture and water also contributed a lot in the transmission of the disease and spread of serotypes/genotypes to other areas. The livestock market system where the animals from different places are gathered together for one or two days and sharing the same facilities is also a contributing factor for the disease transmission.

Transmission from the wildlife and livestock interface is the big challenge because in these two ecosystems the animals are sharing the grazing and watering grounds. The transmission from carrier animals to susceptible hosts, under field conditions, has so far only been shown for Africa buffaloes (*Syncerus caffer*) to cattle and impala (*Aepyceros melampus*) (Bastos *et al*., 2000; Dawe *et al*., 1994).

The most common route of introduction of FMD into a country free of FMD has been the illegal use of contaminated swill feed in cattle (Hartnett *et al*., 2007) while the movement of infected cattle, goats and other livestock is the major cause of disease spread. The transmission via aerosols can also occur, depending on weather conditions and the characteristics of virus survival and dissemination (Alexandersen *et al*., 2002a; Alexandersen and Donaldson, 2002; Donaldson, 1972; Donaldson *et al*., 1987; Seller and Closter, 2008).

2.7 Molecular Epidemiology of FMD

Globally, there are seven immunologically distinct serotypes of FMDV (O, A, C, Asia 1, SAT1, SAT2 and SAT3) and the cumulative incidence of FMDV serotypes show that six

of the seven serotypes of FMD (O, A, C, SAT1, SAT2, and SAT3) have occurred in Africa (Valarcher *et al.,* 2004; Donaldson, 1999). The epidemiology of FMD in Africa has been reviewed by Vosloo *et al.,* 2002: Vosloo *et al.,* 2004; Rweyemamu *et al.,* 2008b; Paton *et al.,* 2009) and Tanzania in particular (Kivaria and Kapaga, 2002; Kivaria, 2003). Also, Batho (2003) conducted an FMD emergency audit on southern Africa. In another study by Rweyemamu *et al*. (2008a), it has been already remarked Africa has the greatest diversity of FMD serotypes. For Africa, the FMDV serotypes are not uniformly distributed, and each serotype results in different epidemiological patterns (Maree *et al*., 2014).

Generally, the current FMD situation in eastern Africa and other countries in the Great Lake zone, which includes Tanzania is the most complicated FMD situation in the world (Rweyemamu *et al.,* 2008b) and yet very little is known about the evolutionary forces which contribute to the complexity of the epidemiology of the disease in this region. The transmission and maintenance of FMD in this region is complex, as farming practices, trade, and high concentration of wildlife contribute to the maintenance and spread of the virus (Maree *et al*., 2014). Also, farming is dominated by agro-pastoral and pastoral communities and is characterized by communal grazing and migrations.

Previous studies on some of the serotypes in East Africa have alluded to the complex epidemiology and genetic diversity of the viruses (Sahle, 2004). The Southern African Development Community (SADC) is endemic with FMDV SATs, but some SADC countries, with the exception of Zimbabwe and Mozambique, are free from FMD and meet the conditions of the OIE for zonal or country freedom from FMD without vaccination (Maree *et al*., 2014).

Foot-and-mouth disease is endemic in Tanzania, with outbreaks occurring almost each year in different parts of the country. Currently four known serotypes (A, O, SAT1 and

SAT2) are circulating within the country and it is speculated that are shared with neighbouring countries. For example, it was reported that, isolates of serotypes A, O, SAT1, and SAT2 from Tanzania and Kenya (2004–2009) were genetically related (Kasanga *et al.*, 2014; Kasanga *et al.,* 2012). Therefore, understanding the epidemiology of a disease is essential for the formulation of the most effective control strategies.

2.8 Phylogeography of Pathogens and its Relevance to the Epidemiology of FMD

Phylogeography simply means the phylogenetic analysis of organismal data in the context of the geographic distribution of organism (Yoon *et al*., 2011). As Avise (1987) conceived it, "phylogeography is the phylogenetic analysis of geographically contextualized genetic data for testing hypotheses regarding the causal relationship among geographic phenomena, species distribution, and the mechanisms driving speciation". Breakthroughs in DNA sequencing technology in 1980's revolutionized evolutionary biology, and out of this revolution emerged what has become a highly influential discipline known as phylogeography.

The combination of molecular biology, epidemiology and population genetics in the science of molecular epidemiology is a powerful tool to develop control strategies for infectious disease (Klein, 2007). Understanding the geographical position and nature of FMDV genetic diversity is critical for improving control strategies and vaccine design, and such diversity is the reliable source of information about the virus' epidemic. In this study, the application of molecular techniques is used to locate the geographically position of FMDV genotype in endemic settings of Tanzania.

The phylogeography study combined with phylogenetic, geographical and epidemiological information can be applied to investigate the spatial distribution and diversity of FMDV. This enables the understanding of the past geographical spread of the

infection, shedding light on the tangle of demographic, social and biological factors that have given rise to current patterns of diversity, and elucidating previously unrecognized routes of ongoing transmission (Avise, 1987). Evolutionary, phylogenetic and coalescent-based analyses of pathogen genomes are now established tools in molecular epidemiology, and especially relevant for recent epidemics with limited historical surveillance data (Pybus *et al*., 2001; Worobey *et al*., 2008).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

This research study was done in four different geographical zones of Tanzania (North-Western, North- Eastern, Southern highlands and South-Western). Tanzania is located in the East Africa, and lies between Latitude 5.6° South of the equator and longitude 36.3° East of Greenwich Meridian. FMDV samples of the three serotypes (O, A and SAT1) were selected considering a spatial temporal and geographic distribution representation from the virus collection at the Centre for infectious Disease and Biotechnology. These selected samples were collected from cattle with clinical signs between 2008 and 2013 consisted of mainly vesicular epithelial tissue from the field (Plate. 1). Collection of the samples followed the OIE guidelines and all the bio-data such as the location where the animals were sampled Geographical Positioning system (GPS), name of the area, farmer, type of animal, age and sex. Additionally, also type of the farming system practiced by farmer for examples zero grazing, range was observed, pastoralist system.

3.2 Samples, Sample Collection, Preparation and Storage

Samples (n= 361) used in this study were tissue epithelia from FMD suspected cattle purposely obtained from all endemic zones of Tanzania from the field outbreaks (Fig. 5). The epithelia samples were taken from affected muzzle, gums, tongue and interdigital spaces and were preserved in a 50/50 mixture of PBS and glycerol at pH of 7.2 and stored at -80^{o}C. Before the epithelium tissues were ground, drying was done using the blotted dry absorbent to reduce glycerol content and weighed. An epithelial suspension was prepared by grinding the sample in sterile mortar and pestle with sterile sand. The suspension was centrifuged at 224 xg for 10 minutes and the supernatant collected for genomic based and serological analysis.

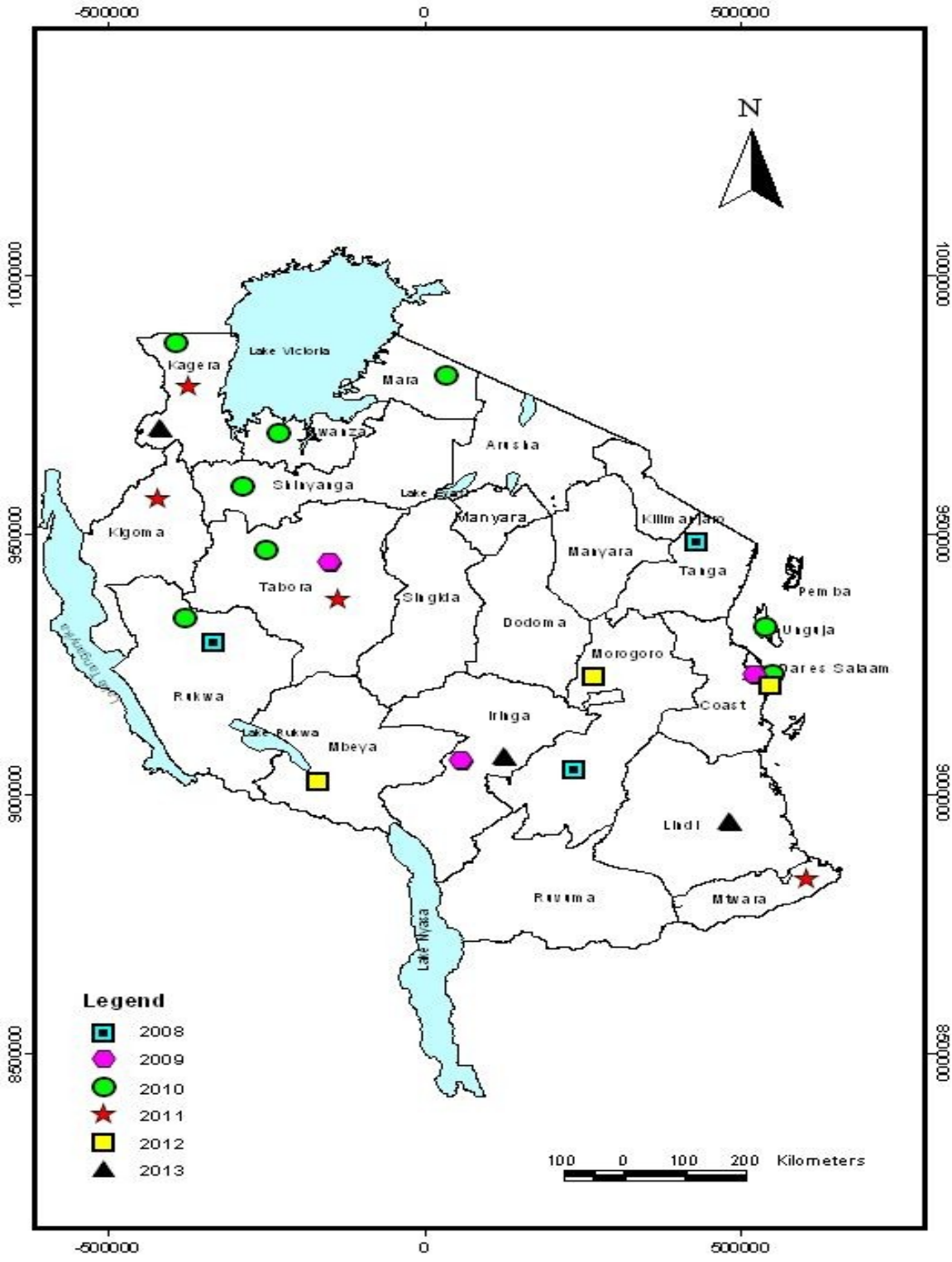

Figure 5: Geographic location indicating where the FMD samples were obtained and years of the outbreak from 2008-2013. Source: Current study

3.3 Sensitivity, Specificity and Molecular survey of FMDV

The aim of conducting this study was to evaluate the specificity and sensitivity of these two methods (Ag-ELISA and rRT-PCR) and the application of molecular survey in detecting FMDV from the field samples. The strategy that was employed in this study conformed to the OIE/FAO World Reference Laboratory for Foot-and-Mouth Disease (WRLFMD) standards. Therefore, molecular screening were combined with sequence analysis to study genetic correlations between field isolates and helped to determine molecular epidemiology of FMDV as per (Reid *et al.,* 1999; Knowles and Samuel, 1994; Knowles and Samuel, 2003).

3.3.1 RNA extraction

The RNA extraction was done directly from the 361 samples recovered from vesicular epithelial tissue suspension using QIAamp® Viral RNA kit (Qiagen, Hilden, Germany) using protocol as described by the manufacturer (Appendix vi). In addition to samples, RNA extraction was done to positive (serotype O Kenya vaccine strain) and negative control used was (epithelial tissue from tongue of FMD free animal).

3.3.2 Real Time RT-PCR.

The detection for the presence of FMDV genome in the samples was done using the rRT-PCR assay that targeted the 3DR coding region using reverse, forward primers and probe (Tab. 1) as described previously by (Callahan *et al.,* 2002).

Table 1: The 3D forward and reverse primers together with 3D probe used in rRT-PCR Assay

Primer	Oligo name	Sequence (5'-3')	Working conc.
Forward Primer	Callahan 3DF	5' – ACT ggg TTT TAC AAA CCT gTg A – 3'	10 pmol/µl
Reverse Primer	Callahan 3DR	5' – gCg AgT CCT gCC ACg gA – 3'	10 pmol/µl
Taqman probe	Callahan 3DP	6-FAM 5' – TCC TTT gCA CgC CgT ggg AC – 3' TAMRA	5 pmol/µl

The stock solution concentration for the primers and probes was 100 µM (100 pmol/µl). This concentration was reduced to the working solution of 10 µM (10 pmol/µl) by taking 10 µl of the stock and 90 µl of nuclease free water (Qiagen) mixed well and stored at -20°C. The master mix kit used in this assay was Invitrogen Superscript III Platinum One Step rRT-PCR System (Cat. No. 11732-020) with the final volume per reaction as 20µl plus 5µl from each template RNA. The reaction mix as per one reaction was as follows; 2x reaction mix 12.5 µl, nuclease free water 1µl, Primer 3DF 2 µl, (10 µM), Primer 3DR 2 µl (10 µM), Probe 3DP 1.5 µl (10 µM), ROX 1:10 pre-diluted Superscript III RT 0.5 µl, Platinum Taqmix µl.

The master mix was prepared in designated master mix room inside the biological biosafety cabinet (Forma class II, 2A, Thermo-Electron Corporation), Then the mixture was dispensed in a fast optical 96-wells reaction plate with barcode 0.1 µl (MicroAmp®-PCR compatible DNA/RNA/RNase free). Each sample was mixed with the master mix plus known positive and negative amplification controls. The plate was sealed with the optic adhesive film (MicroAmp®-PCR compatible DNA/RNA/RNase free). After mixing, the plate was sealed and centrifuged at 1107xg (Eppendorf series 5810R) for 3 minutes to remove all the bubbles.

Before running the assay the machine used (Applied Biosystems 7500 Fast Real-Time PCR System) was calibrated. The calibration was on ROI, background, optical, dye (CY3,CY5, FAM, JOE, NED, ROX, SYBR, TAMRA, TEXAS RED, VIC) and instrument verification. The RT-PCR regime was as follows, 30 minutes for 60°C (reverse transcriptase step), 10 minutes at 95°C (inactivation reverse transcriptase/activation DNA polymerase), 15 seconds for 95°C (denaturation) and 1 minute at 60°C (annealing and elongation) the two last steps went for 50 cycles. Measurement of fluorescence was taken at the end of second step at stage 3. The results interpretation was as follows, samples with Ct < 32: positive, samples with 32 < Ct < 50: ambiguous, retest and samples with no Ct as negative (Fig.6). The cycle threshold value (Ct value) was fixed automatically from the machine software (7500 software v2.0.5).

3.3.3 Conventional RT-PCR

This reaction was performed in 40 suspected epithelium samples that were positive for FMDV genome using rRT-PCR (Reid *et al.,* 2001). The 50 µl reaction mix (total volume) including: 1.5 µl of each primer, 1µl enzyme, 2µl $MgCl_2$, 1µl dNTPs, 5µl Buffer 10x, 6µl extracted RNA and 32µl DEPC water. The primer sequence for forward was 5' – gCC Tgg TCT TTC CAg gTC T – 3' and for reverse 5' – CCA gTC CCC TTC TCA gAT C – 3'. The thermal profiles used for amplification of the VP1 sequence of various serotypes were as follows: FMDV O and Asia1: 42°C for 30 min, 94°C for 5 min, 35 cycles of 94°C for 60 s, 60°C for 60 s, and 72°C for 90 s, followed by a final extension of 72°C for 5 min. Conditions were the same for the other serotypes, except that extension temperatures were 55°C for A, C, 50°C for SAT 1, SAT2 and SAT 3. Temperature cycling was carried out using the GeneAmp® PCR systems 9700 (Applied Biosystems, Foster City, USA). Standard 2.0% (w/v) agarose gel (SeaKem® LE Agarose) was prepared by dissolving 3.0g agarose in 150 ml 1x TBE electrophoresis buffer (0.04M Tris-Borate, 0.001M EDTA, pH

8.0) in Erlenmeyer flask. The mixture was heated in a hot plate or microwave to allow the agarose to dissolve and form a gel. The gel was allowed to cool to about 20°C before adding 3 µL of ethidium bromide (1 µg/ml).

The gel was then poured into a horizontal gel tray fitted with appropriate combs. After about 40 min of gel polymerization, the combs were carefully removed and the tray immersed in an electrophoresis tank containing electrophoresis buffer (1 x TBE). Then 3 µL of DNA products of each individual sample was mixed with 2µL of bromophenol blue dye diluted 6x (3:1 water: 6 x dye, 50Mm EDTA, 50mM Nacl, 50% Glycerol) and then loaded into separate lanes (slots) of the submersed agarose gel. The samples were run at 120 volts for 45 min. After the run, the gel was removed and photographed under UV light using a video capture system (Molecular Imager® Gel Dox XR System 170-8170 with Flowgen IS 1000; Bio-Rad, Seoul, Korea).

3.3.4 Indirect Sandwich Antigen Capture ELISA (Ag-ELISA)

This test is based on a standard indirect sandwich ELISA techniques to determine the presence of FMDV antigens in tissue samples as described by Roeder and Le Blanc Smith (1987: Ferris and Dowson, 1988). Rabbit antisera specific for the different serotypes of FMDV are passively adsorbed to polystyrene microwells. With the addition of test sample, antigen (if present) is trapped by the immobilized antibodies. Specific guinea pig anti-FMDV detecting antibodies are detected by means of the rabbit anti-guinea pig Ig conjugated to horse radish peroxidise. With the addition of substrate/chromogen solution, a colour product develops which may be measured and interpreted with respect to the antigen content of the test sample. Indirect Sandwich Antigen Capture ELISA was done to confirm the results obtained from tests like rRT-PCR. This Ag-ELISA test was done following the manufacturer bench protocol procedures Biological Diagnostic Supplies Limited (BDSL).

For this test 30 samples (Tab. 2) were prepared and stored in 0.04 M PBS at -90 to -50°C and the other lot were stored at -30 to -5°C after the addition of equal volume of sterile glycerol (50%, v/v). Calculations of mean background was done for each plate by adding the OD (492 nm) value of 5 and 6 wells of each row (serotype) and dividing by 2. These OD values were due to the reagents and not to a specific reaction between antigen and antisera. A mean corrected OD value of >0.1 above background indicated a positive results and the serotype was read. Those values that were close to 0.1 were treated with caution and retested.

3.3.5 Determination of Sensitivity and Specificity of two assays

The aim of conducting this study was to evaluate the specificity and sensitivity of these two methods (Ag-ELISA and Real-Time RT-PCR) in detecting FMDV from the 30 selected field samples of serotype O (Tab. 3). The strategy that was employed in this study conformed to the OIE/FAO World Reference Laboratory for Foot-and-Mouth Disease (WRLFMD) standards. (Tab.2) and tables 3a, 3b, 3c and 3.d illustrate how the sensitivity for both tests was obtained. The table 3a has two columns which indicated the actual condition of the subjects, diseased or non-diseased.

The rows indicate the results of the test, positive or negative. Sensitivity is the probability that a test will indicate 'disease' among those with the disease and can be calculated as, Sensitivity: A/(A+C) × 100, whereas A is true positive and C is a false negative. Specificity is the fraction of those without disease who will have a negative test result and can be calculated as, Specificity: D/(D+B) × 100, whereas, D is true negative and B true negative. Both Sensitivity and Specificity are characteristics of the test and population does not affect the results.

3.4 Molecular Epidemiology of FMDV serotype O, A and SAT1

The objective of the present study was to determine the molecular characteristics of the FMDV and to use the phylogeography and phylogenetic parameters to determine the spatial distribution of FMDV and possible sources of FMDV that causes endemicity situation in the country. Thirty-nine serotype O (Accession number: KJ947808-KJ947825), 12 serotype A (Accession number: KJ947812-KJ947822) and five serotype SAT1 (Accession number: KJ947812-KJ947822) field samples were collected from 2008 to 2013 as per Fig.4 and Appendix 6.

3.4.1 RNA Amplification

One-step reverse-transcription polymerase chain reaction (RT-PCR) was carried out using the Qiagen One-step RT-PCR kit (Cat. no. 210210) as per manufacture's recommendations. In this study, VP1 primers were used (O-1C244F/O-1C272F and EUR-2B52R for O, SAT1, SAT3-P1-1222F/SAT-2B208R and A-1C612F/EUR-2B52R) as shown in Tab. 2. The thermal profiles used for amplification of the VP1 sequence of various serotypes were as follows: FMDV O and Asia1: 42°C for 30 min, 94°C for 5 min, 35 cycles of 94°C for 60 s, 60°C for 60 s, and 72°C for 90 s, followed by a final extension of 72°C for 5 min. Conditions were the same for the other serotypes, except the extension temperatures were 55°C for A, C, 50°C for SAT 1, SAT2 and SAT 3. Temperature cycling was carried out using the GeneAmp® PCR systems 9700 (Applied Biosystems, Foster City, USA). These conditions as described by (Knowles *et al*., 2005, 2009). The PCR products were cleaned up using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) as described in the manufacture's to remove unincorporated primers and free nucleotides prior to nucleotide sequencing.

Table 2: The detailed information of the VP1 primers used in the study

Primer identity	Primer Sequence	Molecular weight	Final Concentration
C-1C536F	5' TAC AGG GAT GGG TCT GTG TGT 3'	7440	10nmol.
EUR-2B52R	5' GAC ATG TCC TGC ATC TGG TTG AT 3'	7928	10nmol.
O-11C244F	5' GCA GCA AAA CAC ATG TCA AAC 3'	7901	10nmol.
A-1C612F	5' TAG CGC CGC CAA AGA CTT TCA 3'	6455	10nmol.
As1-1C530F	5' CCA CRA GTG TGC ARG GATGG T 3'	6840	10nmol.
SAT1-1C559F	5' GTG TAT CAG ATC ACA GAC ACA CA 3'	7026	10nmol.
SAT3-P1-222F	5' ATT CTG CAT TTC ATG TAC AC 3'	6051	10nmol.
SAT2-1C445	5' TGG GAC ACM GGI YTG AAC TC 3'	7281	10nmol.

3.4.2 Direct sequencing

The purified PCR fragments were directly amplified using BigDYE® Terminator v3.1 Cycle Sequencing Kit (ABI, Warrington, UK). Sequencing PCR fragments were separated using an automated dideoxynuclotide cycle sequencing DNA sequencer to obtain the complete VP1 sequences. For sequences editing and assembling, the chromatograms obtained for each individual reaction using forward or reverse primers were edited manually to avoid the misreading of peak dyes using SEQUENCHER 4.8 computer software (Gene Code Corporation, USA) and assembled into contigs. The consensus nucleotide sequences were exported to BioEdit computer program and manually aligned.

3.4.3 Phylogenetic analysis of FMDV VP1 sequences

The PCR products were directly sequenced on both strands to obtain the complete VP1 sequences, which were compared with the other relevant FMDV VP1 sequences within the same serotypes. Computer-assisted comparisons of the nucleotide sequences were made to find the similarities of nucleotides sequences in National Centre for Biotechnology Information (NCBI-http://www.ncbi.nlm.nih.gov/), using BLASTN search program. Also for further sequences comparisons other sequences resource used was done

using FASTA search from European Bioinformatics Institute (EBI: http:// www.ebi.ac. uk/service/) maintained by European Molecular Biology Laboratories (EMBL).

Nucleotide sequences that encoded amino acid from the VP1 gene were translated to the (deduced) amino acid sequences by the EditSeq (DNAstar, Madison, USA). To compare with in-group sequences, additional sequences from the GenBank were used for the VP1 region. Alignments of VP1 sequences were done using Clustal W algorithm method (MegAlign; DNAstar, Madison, USA) and Bio-Edit 7.0 (Hall, 1999). The analysis done was phylogeny reconstruction with a scope of all selected taxa. These alignments were used to construct distance matrices by using the Kimura 2-parameter nucleotide substitution model (d: Transitions + Transversions) in the program MEGA 5.1. Midpoint-rooted neighbour-joining trees were then constructed with MEGA 5.1 software. The robustness of the tree topology was assessed with 1,000 bootstrap replicates and value ≥ 70% were shown in (Fig. 8, 9 and 10) as previously described (Tamura *et al*., 2011). The rate and pattern among sites were uniform rates and patterns among lineages were homogenous. The pairwise deletion was used for gaps, missing data treatment and the codons included were 1^{st} +2^{nd} + 3^{rd} + Noncoding.

CHAPTER FOUR

4.0 RESULTS

4.1 Quantification of FMDV genome

The results of the rRT-PCR assay were assessed by the Ct value. The negative Ct value for any test and control sample considered Ct of ≥50.0 and was selected as the positive/negative cut-off Ct values. The results from primer combinations as per (Table 2) indicated that 50.13% of samples (*n* = 181) were positive for FMDV genome by Real-time RT-PCR with Ct values <32. The samples with Ct value <20 were 65 (39.9%), Ct value between 21-25 were 51 (28.17%), Ct of 25-32 were 34 (18.78%), Ct value ≤ 32 to <40 were 31 (17.12%) and negative samples with no Ct value were 180 (50.00%) (Fig. 6).

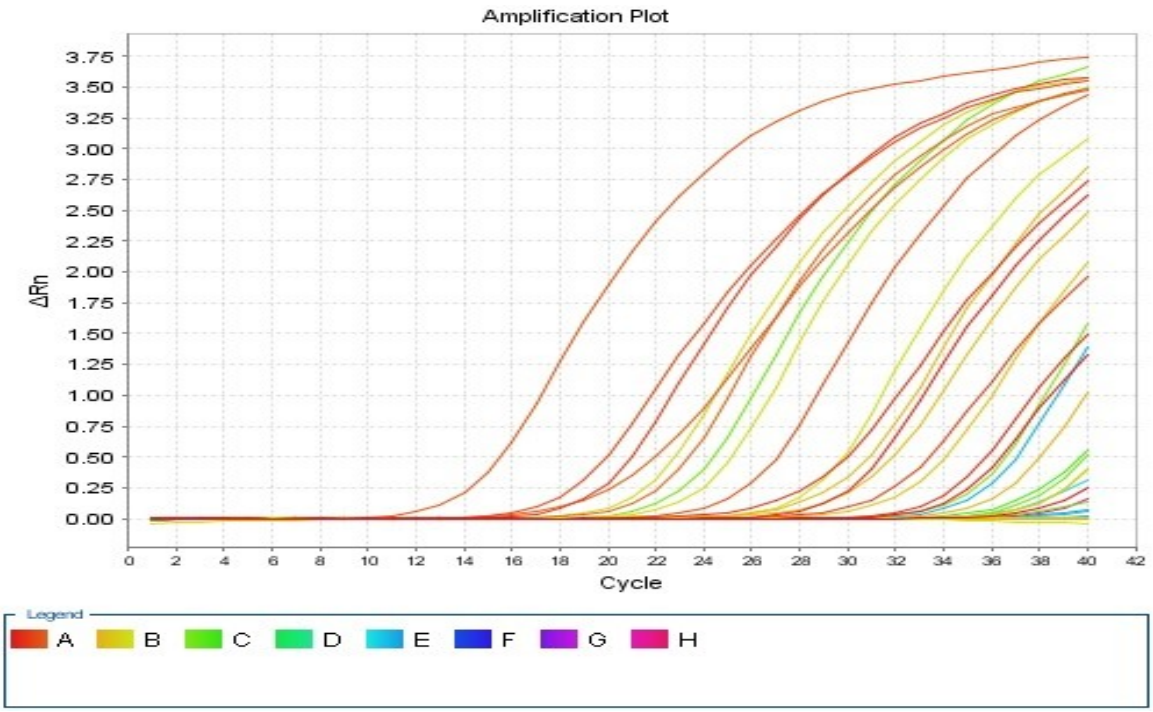

Figure 6: Results based on the Ct value obtained from sample screening using rRT-PCR assay. Source: CIDB-TVLA Lab. 2011.

In this current study positive samples for FMDV were found in Tabora, Serengeti, Musoma, Kahama, Mwanza, Rorya, Iringa, Mbeya, Rukwa and Pwani region, which amounts to the majority of the country. But, the study revealed that the FMDV is spreading fast in Tanzania, as it was found in Nzega, Mtwara, Tabora, Kibondo, Dar Es Salaam and Sumbawanga. In the previous study (Kasanga *et al.,* 2012), the FMDV was only reported in regions such as Iringa, Morogoro, Pwani, Dar Es Salaam and Dodoma. According to Rweyemamu and Loretu (1972), FMDV was regularly reported in the Northern, Northern-lake and Central zones of Tanzania up to 1971. With this current study, it seems that FMDV is reappearing and spreading contrary to the previous reports (Kasanga et al.,2012). Also, another finding from this screening indicated that application of rRT-PCR can be used as a valuable tool along with traditional procedures in rapid detection of FMDV genome in field samples.

4.2 CONVENTIONAL RT-PCR

Conventional RT-PCR was used to compliment the results obtained from Real-time RT-PCR. In addition, molecular typing of the FMDV genome positive samples using serotype specific primers revealed the existence of several serotypes; serotype SAT1 (17.54%; n = 60), serotype A (28.65%, n = 98), serotype O (28.65%, n = 98) and SAT2 (28.07%; n = 96). In certain circumstances the samples which were diagnosed as FMD virus positive differed between the conventional RT-PCR assays and rRT-PCR, for example, rRT-PCR detected FMD viral RNA in 10 samples which had been evaluated as negative by the conventional RT-PCR assays.

4.3 Sensitivity and Specificity for rRT-PCR and Ag-ELISA

In this study sensitivity for rRT-PCR was 86.6% and specificity was 70.0% this is supported by Ferris and Dowson (1988) and as per (Appendix 8).

Table 3a and 3b: Illustrate the formula and calculations involved in getting specificity and sensitivity of rRT-PCR Assay.

3a.

	Disease		
Test	Present	Absent	Total
Positive	A (True positive)	B (False positive)	True test positive
Negative	C (False negative)	D (True negative)	True test negative
Total	True Diseased	True Non Diseased	Total

3b.

	Disease		
Test	Present	Absent	Total
Positive	26	9	35
Negative	4	21	27
Total	30	30	

Sensitivity: A/(A+C) × 100, as A= 26 and C=4, therefore 26/(26+4)x100= 86.6%

Specificity: D/(D+B) × 100, as D=21 and B=9, therefore 21/(21+9)x100=70.0% (Tab. 3a and 3b).

The results from Ag ELISA were taken from colour change and the Optical Density (OD) of 492 nm obtained from reader (Multi Scan, Thermo, USA). The colour development from strong to very weak resulted from five-fold dilution series of each of the control inactivated antigens on plate.

Table 4c and 4d: Illustrate the formula and calculations involved in getting specificity and sensitivity of Ag-ELISA Assays

3c.

	Disease		
Test	Present	Absent	Total
Positive	A (True positive)	B (False positive)	True test positive
Negative	C (False negative)	D (True negative)	True test negative
Total	True Diseased	True Non Diseased	Total

3d.

	Disease		
Test	Present	Absent	Total
Positive	21	9	30
Negative	9	21	30
Total	30	30	

Sensitivity: A/(A+C) × 100, as A= 21 and C=9, therefore 21/(21+9)x100= 70.0%

Specificity: D/(D+B) × 100, as D=21 and B=9, therefore 21/(21+9)x100=70.0% (Tab. 3c and 3d).

The sensitivity test for Ag-ELISA was 70.0% which is still moderate as most of the time Ag-ELISA can give positive results with only about 70-80% of epithelial suspensions that contain virus due to a lack of sensitivity. Specificity was 70.0% that is also moderate value for detecting the true negative.

4.4 Molecular Epidemiology of FMDV Serotypes A, O and SAT1

The main focus of this study was to determine the spread of FMDV serotypes A, O and SAT1 in Tanzania for the period of six years (2008-2013) Tab.9. The nucleotide and amino acid sequences of the VP1 coding region (639 bp) of the Tanzanian FMDV serotypes O, A and for SAT1 (663 bp) were subjected to phylogenetic analysis to determine their genotypes and genetic relationships with other African strains. Genetic

characterization based on the VP1 (1D) region of FMDV is widely used because of its significance for virus attachment and entry, protective immunity, and serotype specificity (Burman *et al.,* 2006; Jackson *et al.*, 2003).

4.4.1 Phylogenetic analysis of FMDV serotype O

The results in this study revealed that FMDV serotype O is the most widely spread serotype and is it almost spread in the entire country. It is also the most responsible FMDV serotype for the regular outbreaks each year and its lineages showed great genetic heterogeneity. The FMDV type O sequences acquired differed, by roughly 3.0% nucleotide identity, from most closely related sequences retrievable from GenBank. As presented in (Fig.7), the phylogenetic analysis it becomes clear and evident that all recently sequenced FMD serotype O viruses from Tanzania belong to the East Africa-2 (EA-2) topotype (Appendix 1). For all O isolates the total numbers of comparisons were 3955 and minimum numbers of nucleotide comparisons were 600 within the gene length of 639. The FMDV serotype O genetic analysis based on the complete VP1 coding sequences revealed that isolates O/TAN-CVL-2012-0321, O/TAN-CVL-2012-0318, O/TAN-CVL-2010-0019, O/TAN-CVL-2010-0017, O/TAN-CVL-2011-0108, O/TAN-CVL-2011-0106, O/TAN-CVL-2009-0040, O/TAN-CVL-2011-0362, 0364, 0366 and O/TAN-CVL-2010-0004 shared high levels of nucleotide and deduced amino acid (identity at 99.8–99.9% and 99.9–100%, respectively (Appendix ii). These viruses were clustered together (A cluster) with other viruses from neighboring countries like Zambia (O/ZAM/3/2010, O/ZAM/4/2010, O/ZAM/1/2010 and O/ZAM/P3/2010), Democratic Republic of Congo (O/COD/1/2010, O/COD/3/2010, O/COD/4/2010), Mali (O/MAL/1/98), Uganda (O/UGA/3/2002), Kenya (O/KEN/5/2002) (Fig. 7), (Appendix 2). All viruses studied from the outbreaks in Southern highlands of Tanzania showed a limited degree of variation in the VP1 gene, with values of over 99.3% genetic relatedness among them.

On the other hand, the heterogeneity among Tanzanian FMDV serotype O seemed to be maintained by the survival of the different isolates in the field. For example (Fig. 7), it is evident that Southern highland regions (Mbeya, Iringa and Rukwa) are predominantly a reservoir of FMDV serotype O (Tab. 9) as the virus circulating in that region was identical to the isolates (O/TAN-CVL-2010-0109, O/TAN-CVL-2010-0004, O/TAN-CVL-2010-0019, O/TAN-CVL-2012-318 and O/TAN-CVL-2012-321, O/TAN-CVL-2013-0362, O/TAN-CVL-2013-0364, O/TAN-CVL-2013-0366) that caused most of outbreaks in places like Dar Es Salaam, Kagera, Mtwara, and Coastal regions.

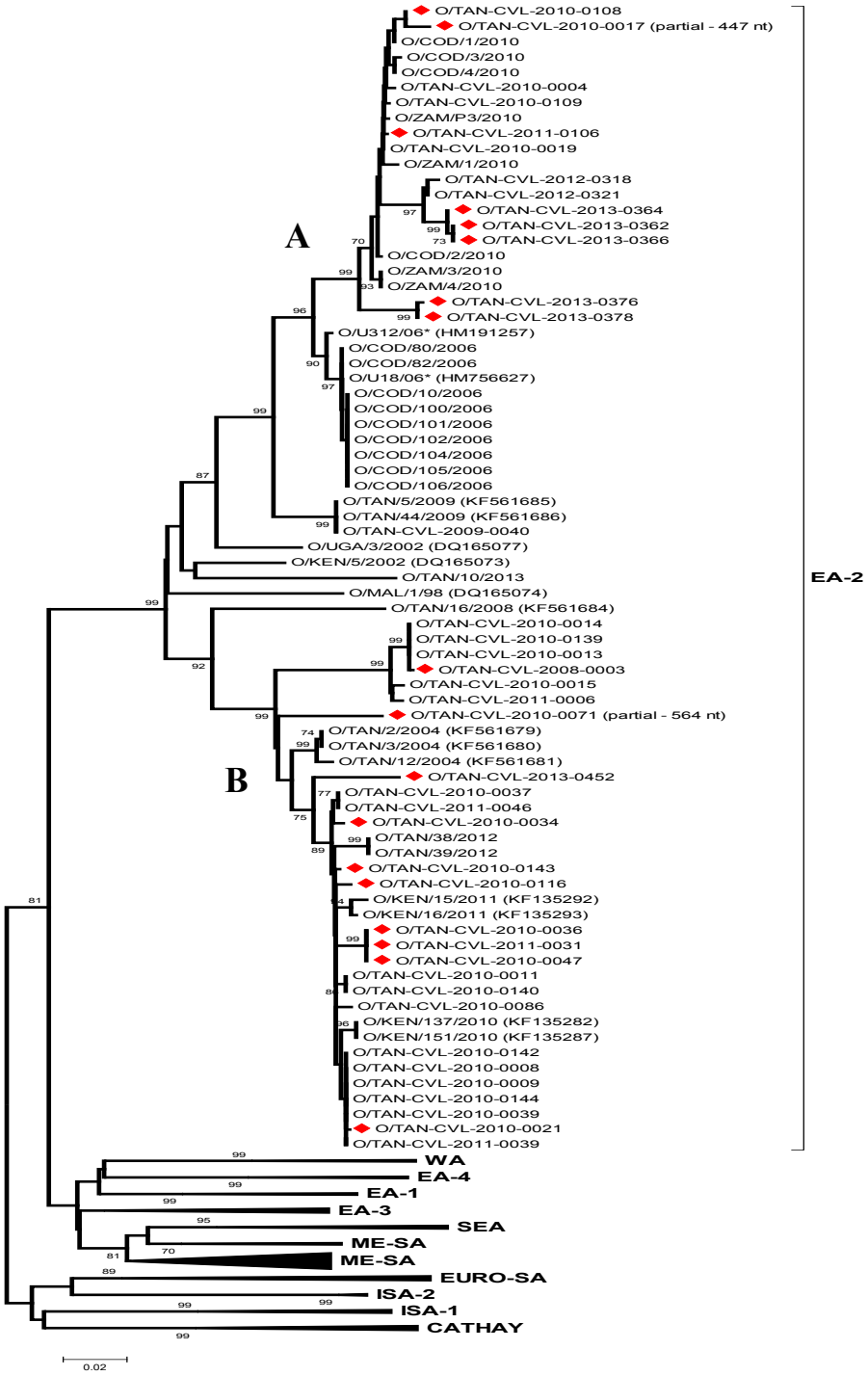

Figure 7: Phylogenetic tree showing the relationships of FMDV type O isolates in Tanzania. Bootstrap values of 70% or above are shown near the major nodes.

4.4.2 Phylogenetic analysis of serotype A

The complete nucleotide sequence of the VP1-coding region was determined for 12 FMDV serotype A viruses obtained from epithelial samples in 13 regions which are FMD endemic (Fig.11).

The A FMDV isolates were classified into one major topotype G-I AFRICA (Fig. 8) and (Appendix 1). For all isolates the total numbers of comparisons were 1540 and minimum numbers of nucleotide comparisons were 600 within the gene length of 639 except for sample 374 with partial VP1 of 493 (Fig. 8). When compared with the available sequences from viruses circulating in Tanzania for the past six years showed the most closely related reference viruses were from neighboring countries such as Kenya (A/KEN/42/66, A21/Lumbwa/KEN/64) and Uganda (A/UGA/13/66) with relatedness value of 87 to 86% . As the nucleotide (nt) and amino acid (aa) comparisons showed the present circulating viruses shared about 92% to 100% nt and aa.

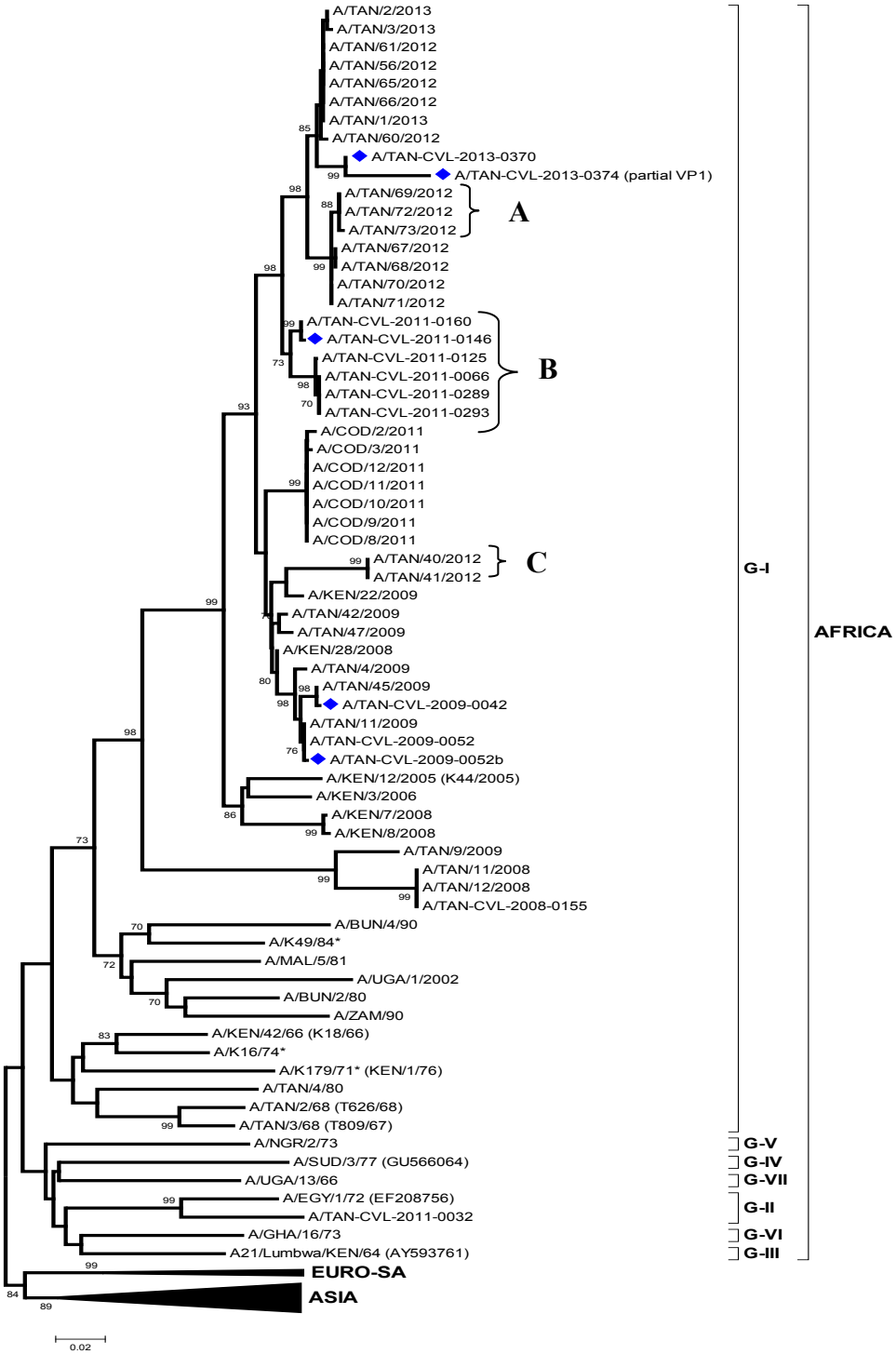

Figure 8: Midpoint-rooted neighbour-joining tree showing the relationships between the FMD serotype A virus isolates with other contemporary and reference viruses. Bootstrap support values above 99% are shown near the major nodes.

Table 4: FMDV viruses that are closely related to Tanzanian viruses obtained from phylogenetic analysis of serotype A topotype Africa and Strain–G1

Virus	Related virus	No. Nucleotide composition	. of Nucleotide Match	% Identity	% difference
A-TAN-CVL-2008-0155	A/TAN/11/2008	639	639	100.0	0.0
A-TAN-CVL-2009-0052	A/TAN/11/2009	639	639	100.0	0.0
A-TAN-CVL-2011-0066	A/TAN-CVL-2011-0289	639	639	100.0	0.0
A-TAN-CVL-2011-0125	A/TAN-CVL-2011-0066	639	638	99.8	0.1
A-TAN-CVL-2011-0160	A/TAN-CVL-2011-0125	639	630	98.5	1.4
A-TAN-CVL-2011-0289	A/TAN-CVL-2011-0293	639	639	100.0	0.0
A-TAN-CVL-2011-0293	A/TAN-CVL-2011-0066	639	639	100.0	0.0
A-TAN-CVL-2009-0042	A/TAN/45/2009	639	639	100.0	0.0
A-TAN-CVL-2009-052b	A/TAN/11/2009	639	639	100.0	0.0
A-TAN-CVL-2013-0370	A/TAN/1/2013	639	631	98.7	1.2
A-TAN-CVL-2013-0374	A/TAN-CVL-2012-0374	493	477	96.7	3.2
A-TAN-CVL-2011-0146	A/TAN-CVL-2011-0160	639	639	100.0	0.0

In this study, the most closely related viruses differ from 1.25 to 5.92% that we can say they are related with few divergences. The strains A /TAN-CVL-2013-0370 and A/TAN-CVL-2013-374 came from recent outbreak of year 2013 and came from western part of Tanzania (Kagera) that are closely related by 99% (Fig. 8) and (Tab. 4 and 5).

Table 5: Viruses that were mostly closely related reference viruses from neighbouring countries for serotype A topotype Africa and strain–G1

Name of the virus	Reference virus	No. Nucleotide composition	No. of Nucleotide Match	% Identity	difference
A-TAN-CVL-2008-0155	A/KEN/42/66 (K18/66)	639	518	81.0	18.9
A-TAN-CVL-2009-0052	A/KEN/42/66 (K18/66)	639	551	86.2	13.7
A-TAN-CVL-2011-0066	A/KEN/42/66 (K18/66)	639	545	85.2	14.7
A-TAN-CVL-2011-0125	A/KEN/42/66 (K18/66)	639	544	85.1	14.8
A-TAN-CVL-2011-0160	A/KEN/42/66 (K18/66)	639	543	84.9	15.0
A-TAN-CVL-2011-0289	A/KEN/42/66 (K18/66)	639	545	85.2	14.7
A-TAN-CVL-2011-0293	A/KEN/42/66 (K18/66)	639	545	85.2	14.7
A-TAN-CVL-2009-0042	A/KEN/42/66 (K18/66)	639	546	85.4	14.5
A-TAN-CVL-2009-052b	A/KEN/42/66 (K18/66)	639	551	86.2	13.7
A-TAN-CVL-2013-0370	A/KEN/42/66 (K18/66)	639	536	83.8	16.1
A-TAN-CVL-2013-0374	A/KEN/42/66 (K18/66)	493	401	81.3	18.6
A-TAN-CVL-2011-0146	A/KEN/42/66 (K18/66)	639	543	84.9	15.0

4.4.3 Phylogenetic analysis of serotype SAT1

For the phylogenetic analysis of SAT1 (Fig. 9), it can be concluded that all FMD type SAT1 viruses are isolates from Tanzania. The numbers collected were very few and just in two regions (Morogoro and Dar Es Salaam) (Table 6 and 7) as there were only a few outbreaks due to SAT1. From the phylogenetic analysis these isolates were placed within the topotype 1NWZ (Appendix 1) and (Fig. 9), the nucleotide differences in VP1 coding region of 15-20%. The nucleotide number of composition was 663 and the average nucleotide match was from 645-660 with the unknown strain. All bootstrap of 70% and above are shown (Fig. 9) and number of bootstrap replications of 1000.

For all SAT1 isolates the total numbers of comparisons were 431 and minimum numbers of nucleotide comparisons were 600 within the gene length of 663. The isolates number SAT1/TAN-CVL-2012-0359 and SAT1/TAN-CVL-2012-0361 are 100% identical and were obtained in the same outbreak in the same area in the Morogoro region (Fig. 10, 11). The five SAT1 isolates used in this study SAT1/TAN-CVL-2012, SAT1/TAN-CVL-2012-0354,SAT1/TAN-CVL-2012-0355, SAT1/TAN-CVL-2012-0359, SAT1/TAN-CVL-2012-0360 and SAT1/TAN-CVL-2012-361(Fig. 9) are clustered together in a group with isolates SAT1/TAN/11/2012, SAT1/TAN/23/2012, SAT1/TAN/25/2012 and SAT1/TAN/27/2012 obtained from the Northern part of Tanzania (Fig. 10; Tab.8). The number of nucleotide match ranges between 659 to 663 and the percentage identity ranges between 99.5% to 100.0% with nucleotide difference between 0.0 to 0.15 % (Tab. 5 and 6).

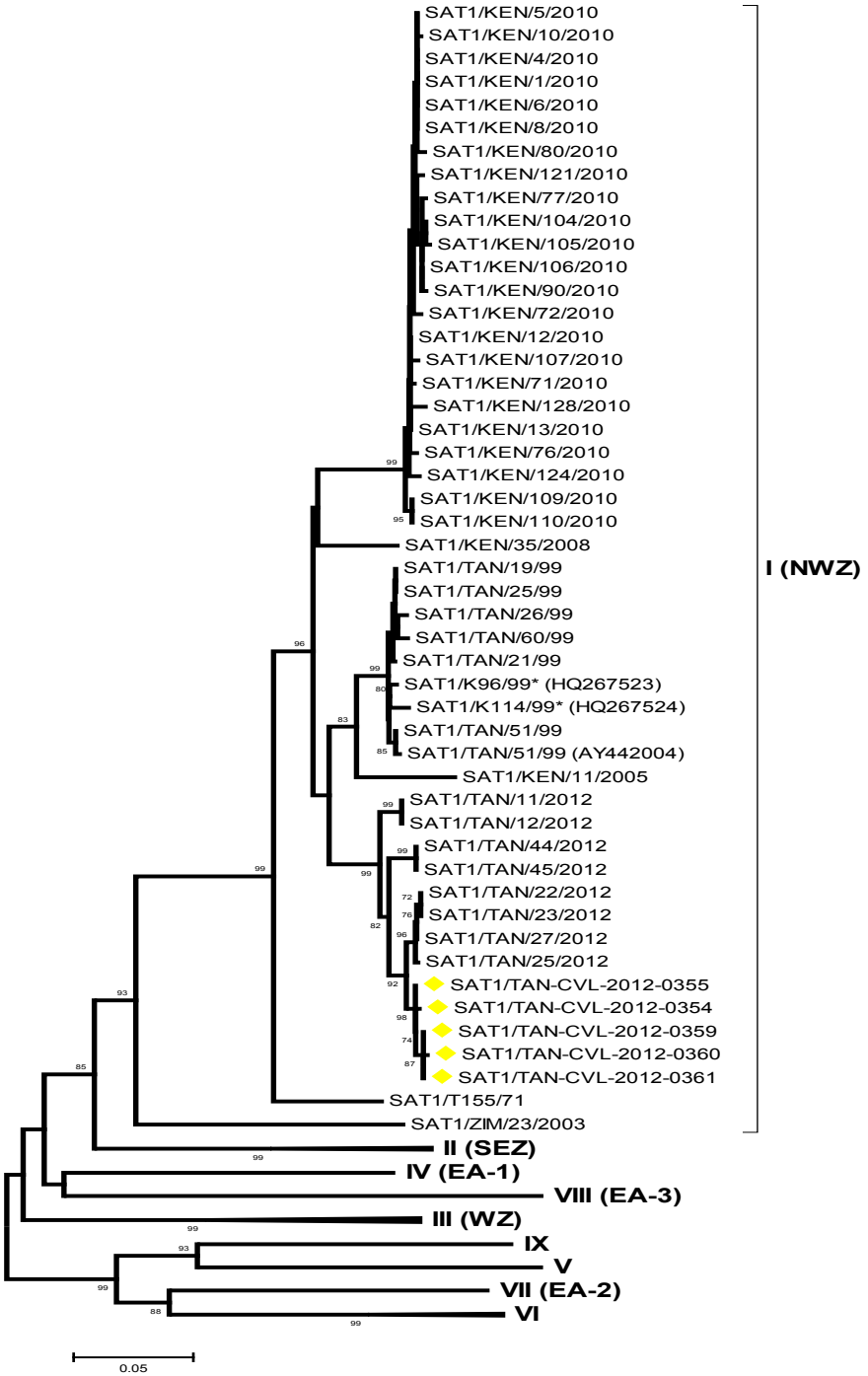

Figure 9: Midpoint-rooted neighbor-joining tree (based on the complete virus protein [VP] 1 coding sequence) showing the relationships between the FMDV serotype SAT1 isolates from Tanzania and other contemporary and reference viruses.

Table 6: Viruses that were most closely related reference viruses of topotype I (NWZ) for SAT1 used in this study.

ıs Name	Related virus	No. of nucleotide composition	. of nucleotide match	% Identity	%Difference
SAT1/TAN-CVL-2012-0354	SAT1/T155/71	663	591	89.1	10.8
	SAT1/ZIM/23/2003	663	538	81.1	18.8
SAT1/TAN-CVL-2012-0355	SAT1/TI55/71	663	592	89.2	10.7
	ZIM03-23	663	539	81.3	18.7
SAT1/TAN-CVL-2012-0359	SAT1/T155/71	663	590	88.9	11.0
	ZIM03-23	663	537	81.0	19.0
SAT1/TAN-CVL-2012-0360	SAT1/T155/71	663	589	88.8	11.1
	ZIM03-23	663	537	81.0	19.0
SAT1/TAN-CVL-2012-0361	SAT1/T155/71	663	590	88.9	11.0
	ZIM03-23	663	537	81.0	19.0

Table 7: Most closely related viruses from SAT I (NWZ) topotype and unnamed strain

Virus Name	Related virus	Nucleotide comp	↓Nucleotide match	% Identity	⅟%Difference
SAT1/TAN-CVL-2012-0354	T1/TAN-CVL-2012-0355	663	662	99.8	0.1
	T1/TAN-CVL-2012-0359	663	660	99.5	04
	T1/TAN-CVL-2012-0361	663	660	99.5	0.4
	T1/TAN-CVL-2012-0360	663	659	99.4	0.6
SAT1/TAN-CVL-2012-0355	T1/TAN-CVL-2012-0354	663	662	99.8	0.1
	T1/TAN-CVL-2012-0359	663	661	99.7	0.3
	T1/TAN-CVL-2012-0361	663	661	99.7	0.3
	T1/TAN-CVL-2012-0360	663	660	99.5	0.4
SAT1/TAN-CVL-2012-0359	T1/TAN-CVL-2012-0361	663	663	100.0	0.0
	T1/TAN-CVL-2012-0355	663	662	99.8	0.1
	T1/TAN-CVL-2012-0355	663	661	99.7	0.3
	T1/TAN-CVL-2012-0355	663	660	99.5	0.4
SAT1/TAN-CVL-2012-0360	T1/TAN-CVL-2012-0359	663	662	99.8	0.1
	T1/TAN-CVL-2012-0355	663	662	99.8	0.1
	T1/TAN-CVL-2012-0355	663	660	99.5	0.4
	T1/TAN-CVL-2012-0355	663	659	99.4	0.6
SAT1/TAN-CVL-2012-0361	T1/TAN-CVL-2012-0359	663	663	100.0	0.0
	T1/TAN-CVL-2012-0355	663	662	99.8	0.1
	T1/TAN-CVL-2012-0355	663	661	99.7	0.3
	T1/TAN-CVL-2012-0355	663	660	99.5	0.4

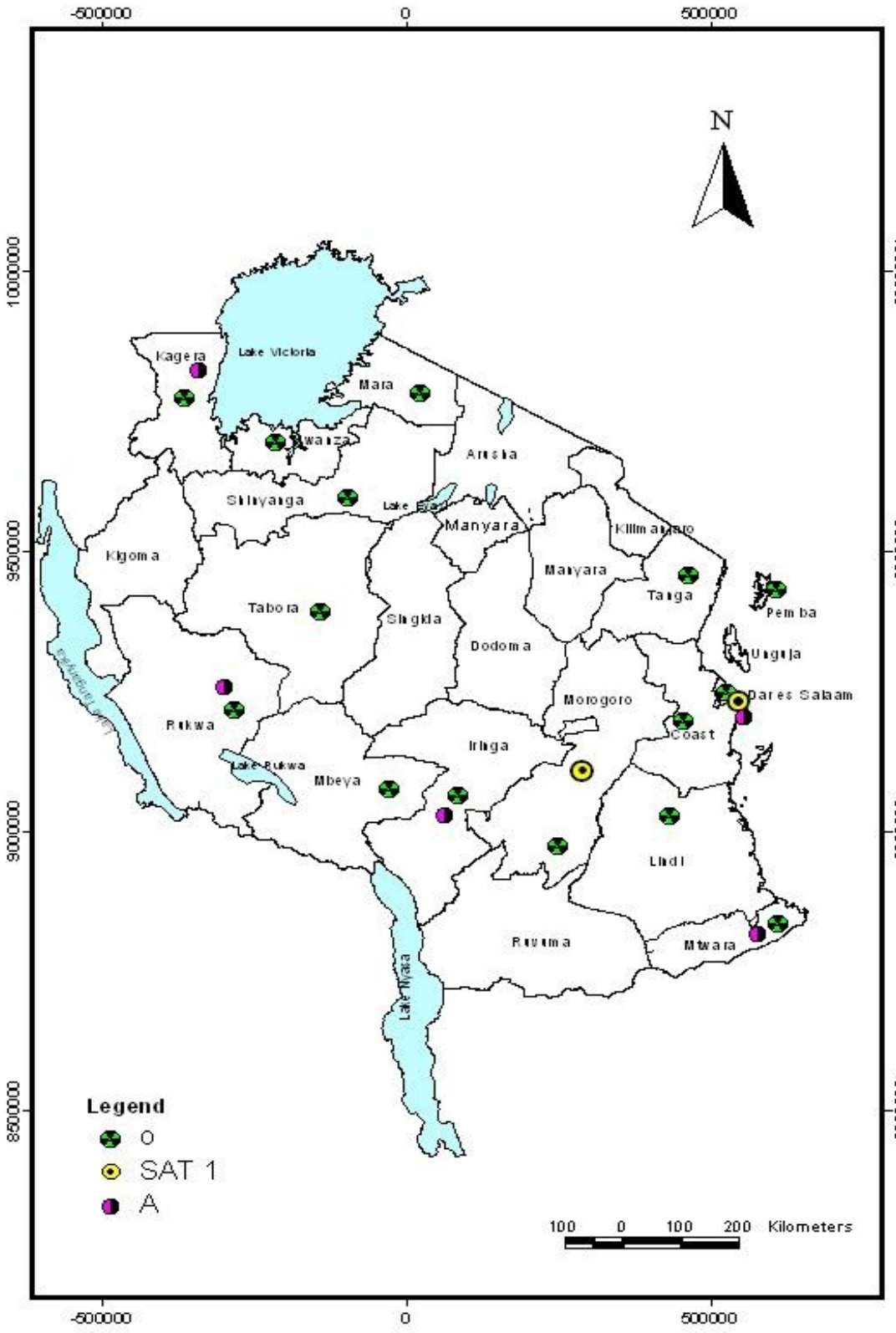

Figure 10: Distribution of serotypes in Tanzania (2008-2013). Source: Current study

Table 8: Summary of FMD outbreaks over six years from 2008 to 2013

Region	Serotype A	Serotype O	SAT1	Year
Mtwara	√	√		2011
Lindi		√		2013
Morogoro		√	√	2008,2012
Dar Es Salaam	√	√	√	2009,2010,2012
Mwanza		√		2010
Kagera	√	√		2010, 2010,201
Iringa	√	√		2009, 2013
Mbeya		√		2012
Rukwa	√	√		2008, 2010
Kigoma	√			2008, 2012
Mwanza		√		2010
Shinyanga		√		2010
Tanga		√		2008
Coast		√		2010
Tabora	√	√		2009,2010,2011
Zanzibar		√		2010

CHAPTER FIVE

5.0 DISCUSSION

5.1 Determination of Sensitivity and specificity

Foot-and-mouth disease (FMD) is endemic in Tanzania, with outbreaks occurring almost each year in different ecological zones of the country. In recent years, the spread of FMDV in Tanzania is at higher rate and it is advisable by OIE that the control measures and confirmation of the presence of FMDV should be based on the laboratory findings. And also, there is a strong political desire to control this contagious disease and other animal diseases as part of National Strategy for Growth and Reduction of Poverty (MKUKUTA).

Experimental evaluation for detection and confirmation the presence of FMDV is very much based on how is the sensitivity and its specificity of the assay. To date, various assays have been used to diagnose and confirm FMDV serotypes prevailing and causing outbreaks in the field. Molecular diagnostic tests play an important role in monitoring the spread of these viruses and controlling outbreaks in susceptible livestock (Jamal and Belsham *et al*., 2013).

In the mentioned study the performance of automated rRT-PCR had been compared with Virus Isolation (VI) and antigen-detection ELISA using samples submitted. The results as per (Shaw *et al.,* 2004), showed that all VI-positive samples and those samples positive by VI and ELISA combined were also positive by rRT-PCR. Although methods based on virus isolation or the demonstration of FMD viral antigen or nucleic acid in samples of tissue or fluid or culture products is sufficient for a positive diagnosis, in general, the ELISA (Ferris and Donaldson, 1992) using type-specific serological reagents is the

preferred procedure for the detection of FMD viral antigen and identification of viral serotype in the early stages of research.

As in this study to two techniques were compared for their sensitivity and specificity. It is proven in other studies, that TaqMan assay has been shown to be very robust and as effective for primary detection of FMDV as virus isolation in conjunction with antigen ELISA (Reid *et al.,* 2003). The rRT-PCR has been shown to be a useful tool for the diagnosis of FMD as it fast, sensitive and reliable diagnosis (Jamal and Belsham, 2013). In this study, for rRT-PCR sensitivity was 86.66% and specificity was 70.0%. The sensitivity results of 86.6%, mean that the chance to detect FMDV was 86.6% (Fig. 6). This test has good chance of capturing all possible positive conditions and can miss very few due to the degree of sensitivity. Also, the specificity of 70.0% entail that the degree of detecting the true negative is moderate. With regards to this study, the Ag-ELISA test, gave a positive result of only about 70-80.0% in epithelial suspensions that contain the virus due even though it has sensitivity high enough to detect a viral antigen in samples of saliva and/or nasal discharge (Mohapatra *et al.,* 2007). Although the current Ag- ELISA is able to distinguish between the seven serotypes of the FMDV antigen and is a useful method, it has low sensitivity (Mohapatra *et al*., 2007; Reid *et al.,* 1998) and may have low adaptability to types O and A because of their wide range of antigenicity (OIE, 2008; 2009; 2013).

For Ag-ELISA the sensitivity of 70.0% and specificity of 70.0% is of moderate range however propagation of the virus in tissue culture would increase the degree of sensitivity. This will allow further and subsequent testing by Ag-ELISA for detecting the virus and ascertain the serotype (Jamal and Belsham, 2013). The lower concentration of the virus in the epithelial tissue could be caused by several factors such as poor preservation of

samples due to poor cold chain facilities. Also, could be due to improper timing of sample collection, and poor quality/amount collected from the clinical animal. Most of the samples collected when the entire visible clinical lesions have healed affect the quality of the samples submitted. This assay is more rapid, but has lower analytical sensitivity and is inappropriate for use with certain sample types (Ferris and Dawson, 1988).

Although only a limited number of samples (30 vesicular epithelial) were available for this study, it was possible to demonstrate the sensitivity and specificity of the two important assays that can be used for rapid laboratory detection of the FMDV genome from the field. The rRT-PCR had 86.6% sensitivity, 70.0% specificity and Ag-ELISA had 70.0% for both specificity and sensitivity. Therefore, for rapid and accuracy the rRT-PCR is the assay of choice for detection of FMDV due to its higher sensitivity.

5.2 Molecular screening, epidemiology of FMDV in endemic settings of Tanzania

5.2.1 Molecular screening of FMDV

The present study provides a partial picture of spread and distribution of FMDV isolates, focusing on the circulating FMDV in Tanzania, thus filling a gap of knowledge of country's molecular screening of this economically important virus. The molecular survey of FMDV is a very important step in controlling the spread of the disease and also the production of specific vaccine which will cater for a specific virus strain at given time and space.

Results from molecular survey indicated that 49.8% of samples (n = 181) were positive for FMDV genome by rRT-PCR with Ct values <32. The samples with Ct value <20 were 65 (39.9%), Ct value between 21-25 were 51 (28.1%), Ct of 25-32 were 39.9%, Ct value ≤ 32 to <40 were 80 (44.9%) and negative samples with no Ct value were 100 (27.7%). This

molecular screening of FMDV was focused on screening of clinical cases collected from the field to ascertain the status of FMDV circulating in Tanzania from 2008-2013. The overall result was very much affected by poor storage conditions on the samples that causing FMDV loss. However, viral RNA tracing was performed by rRT-PCR and it showed that, this method can be used as a rapid and initial screening test in outbreaks (Fosgate *et al* 2008). Also, despite high genetic mutation in FMDV genome and the circulating FMDV serotypes in different hosts, detection of virus genetic materials in clinical samples is most important. This molecular screening revealed the spread and reappearance of FMDV within the country that could possibly be caused by the movement of animals across the border or within the country by nomadic livestock keepers and by high density of animals in other areas of the country (Fig.11). This study provides an overview of the molecular screening of FMDV in Tanzania that is giving a pre requisite knowledge on the distribution of FMDV.

5.2.2 Molecular Epidemiology of FMDV serotype O, A and SAT1

Knowledge on the epidemiology of a FMD and the phylogeography of FMDV is essential for formulation of the most effective control strategies. This determines the possible source of outbreaks and is achieved by undertaking VP1sequencing to provide an important element of epidemiological investigation (Samuel and Knowles, 2001).

The intensive description of the molecular epidemiology of FMDV in sub-Sahara and East Africa has not been studied as extensively in as some other regions in the world. It is also difficult to control the diseases as the capacity of FMDV to cross large geographical extensions could be assisted by the movement of infected/carrier animals, sharing of grazing land and watering points with wildlife animals (Fig. 11 and Appendix iv). The epidemiology of FMDV in Tanzania is complex and is not clearly understood to date. This is due to several factors such as free movement of livestock/animals, the presence of wild

ungulate animals that are disease carriers and the lack of good policy to control the disease from spreading.

Since it was detected in 1954, FMD has been widespread and endemic in many regions of Tanzania (Appendix v). The endemicity of FMD in Tanzania has been described in the past by (Kasanga *et al*., 2012, 2014; Swai *et al*., 2009, Kivaria, 2009; Rweyemamu *et al.,* 2008b) showing that FMDV serotypes O, A, SAT1 and SAT2 are prevailing in most of the regions (Fig.1 and Fig. 11) and (Appendix v). In Tanzania, the 2010/2011 statistics indicate that, there are about 25.8 million cattle, 15.2 million goats and 6.4 million sheep distributed all over the regions (Appendix iv).

As in this study the genetic characterization and phylogenetic analysis of thirty-nine (39) field isolates of serotype O, twelve (12) isolates of A and five (5) of SAT1 obtained during the last six years of FMD epizootic in Tanzania. These results showed a small genetic diversity within FMDV but revealed independent evolution of the isolates e.g. O/TAN-CVL-2010-0021. Also, phylogenetic analysis showed that all emergency/sporadic viruses from the southern highlands circulating between the years 2008 and 2012 clustered as part of a clade, supported by 99.0% bootstrap value. The clustering of profile B showed considerable genetic relationships between viruses in Kenya (Fig.8) (O/KEN/137/2010, O/KEN/151/2010, O/KEN/15/2010 and O/KEN/162011) which shares a common boundary.

The isolate number O/TAN-CVL-2013-0452, caused an outbreak in the Southern region of Tanzania in 2013 had an average nucleotide difference of 3.3% (Appendix ii) with other viruses in clade B. In terms of phylogenetic interpretations, FMDV that differ by 2–5% from each other are generally believed to originate from the same epizootic (Samuel *et al.,* 1997). In general, distances <5.0% are considered to indicate very closely related

strains (Samuel *et al.,* 1997). Differences in the genetic sequences of viruses from the same serotype do not necessarily reflect differences in antigenicity (Esterhuysen, 1994).

According to Mateu *et al*. (1995, 1996) very limited genetic variations in the immuno dominant region can alter the antigenic specificity of FMDV isolates. This is consistent with previous studies which have indicated that serotype O is highly prevalent in Tanzania (Kasanga *et al*., 2012; Kasanga *et al*., 2014a) and (appendix v). And although these samples were collected from different ecological set-ups in different regions, they were very closely related (Appendix i and iii). Other findings indicated that, the dissemination of the same virus-lineage of GI-Africa serotype A in Tanzania such as cluster A (Fig. 8) consisting of isolates from different parts of Tanzania (A/TAN-CVL-2011-0160 and A/TAN-CVL-2011-0146 from Dar Es Salaam, A/TAN-CVL-2011-0125-Mtwara, A/TAN-CVL-2011-0289 and 0293-Kigoma) were supported with bootstrap of 98 to 99.0% (Fig. 9) and (Tab. 6) shows that these isolates were genetically the same with nucleotide identity between 98.5 to 100.0% and nucleotide difference of 0.00 to 3.2% (Tab.6).

The phylogenetic analysis showed that all emerging FMD serotype A viruses in Tanzania circulating between the years 2008 to 2013 clustered as part of a clade, as supported by 73 to 99.0% bootstrap values (Fig 9). The clustering pattern showed considerable genetic relationships of viruses with common boundaries (Tab. 7) isolates from Kenya (A/KEN/28/2009 and A/KEN/28/2008) clustered together with Tanzanian isolates (A/TAN/-CVL-2009-42 and A/TAN/-CVL-2009-52b) (Fig. 9). At bootstrap 96.0% the two major clusters were separated within small groups showing genetic separation with four isolates (A/TAN/9/2009-Njombe, A/TAN/11/2008-Coast, A/TAN/12/2008-Iringa and A/TAN/-CVL-2008-0155-Rukwa) with bootstrap of 99 to 100.0%. These isolates, except for isolate A/TAN/11/2008, were isolated from the Coastal region at the animal

holding ground ready to be marketed and originated from Southern Highlands referred cluster C (A/TAN/9/2009, A/TAN/11/2008, A/TAN/12/2008 from Iringa and A/TAN-CVL-2008-0155 from Rukwa). This can be evidenced by the outbreak in Dar Es Salaam in 2009 which was caused by isolate A/TAN/-CVL-2009-042 and in the Southern Highland by isolate A/TAN/45/2009 caused the outbreaks within the same month of 2009. Also, these isolates are most closely related (96.0%) to viruses of 2012/13 outbreaks from North-West of Tanzania, which shows that the same virus is circulating within those areas.

Currently, the epidemiology of FMDV is very complex as the spread of disease is rampant and little efforts are applied to stop the spread of FMD. Additionally, studies have demonstrated the presence of viral topotypes in both wildlife and domestic animals, information that should be heeded when planning FMD vaccination strategies (Vosloo *et al*., 1992; Vosloo *et al*., 1995; Bastos, 1998; Bastos *et al*., 2001; Bastos *et al*., 2003a; Bastos *et al*., 2003b; Sangare *et al*., 2003; Sangare *et al.,* 2004). As with other RNA viruses, the FMDV continually evolves and mutates and the analysis of its genome has become a primary means of classification (Carrillo *et al.,* 2007; Simmonds, 2006). The complete nucleotide sequence of the VP1-coding region was determined for 39 type O viruses, 12 A and five SAT1 obtained from epithelium samples collected in 16 of the endemic geographical regions of Tanzania (Fig. 5; (Appendix iii). The aim was to establish the epidemiological relationship between the viruses causing the epizootic in Tanzania and the viruses circulating in the region. The viruses from this study were also compared to the viruses responsible for the previous emergencies or sporadic cases in the Great Lakes countries, including those from the East African Community, as well as with other relevant strains from the continent, and with representative exogenous viruses. These phylogenetic relationships were used to indicate whether FMD viruses were introduced on a regular basis, or alternatively were maintained for long periods of time within Tanzania.

As shown in (Figs.7, 8, 9) these phylogenetic trees based on the VP1 coding region revealed that the strains used in this study were clustered into one group with other exotic viruses from neighbouring countries (Rwanda, Democratic Republic of Congo, Zambia, Kenya, Burundi and Uganda. The livestock movement is the one factor for the virus dissemination which can be generated by market pressures, cattle rustling, and social economic reasons and in search of water and pastures (Appendix iv and Fig. 12).

These data from this study do not provide information regarding the origin of viruses and a significant role in the genetic evolutionary dynamics of FMDV. But the results can be used for the future planning and control strategies for FMD, as the knowledge of disease epidemiology is a paramount and most important factor. The phylogenetic statistical analysis clearly showed that groups of viruses share common geographical characteristics, and epidemic histories. For examples; the four isolates originated from Southern Highlands (Fig. 9) clustered at clade C (A/TAN/9/2009, A/TAN/11/2008, A/TAN/12/2008 all three were from Iringa and A/TAN-CVL-2008-0155 were from Rukwa). The lack of the sequences of 1970s or 1980s for Tanzanian topotypes that are available in the public database may be one of the reasons for small genetic distances from the root observed in EA-2 topotype (Fig. 8).

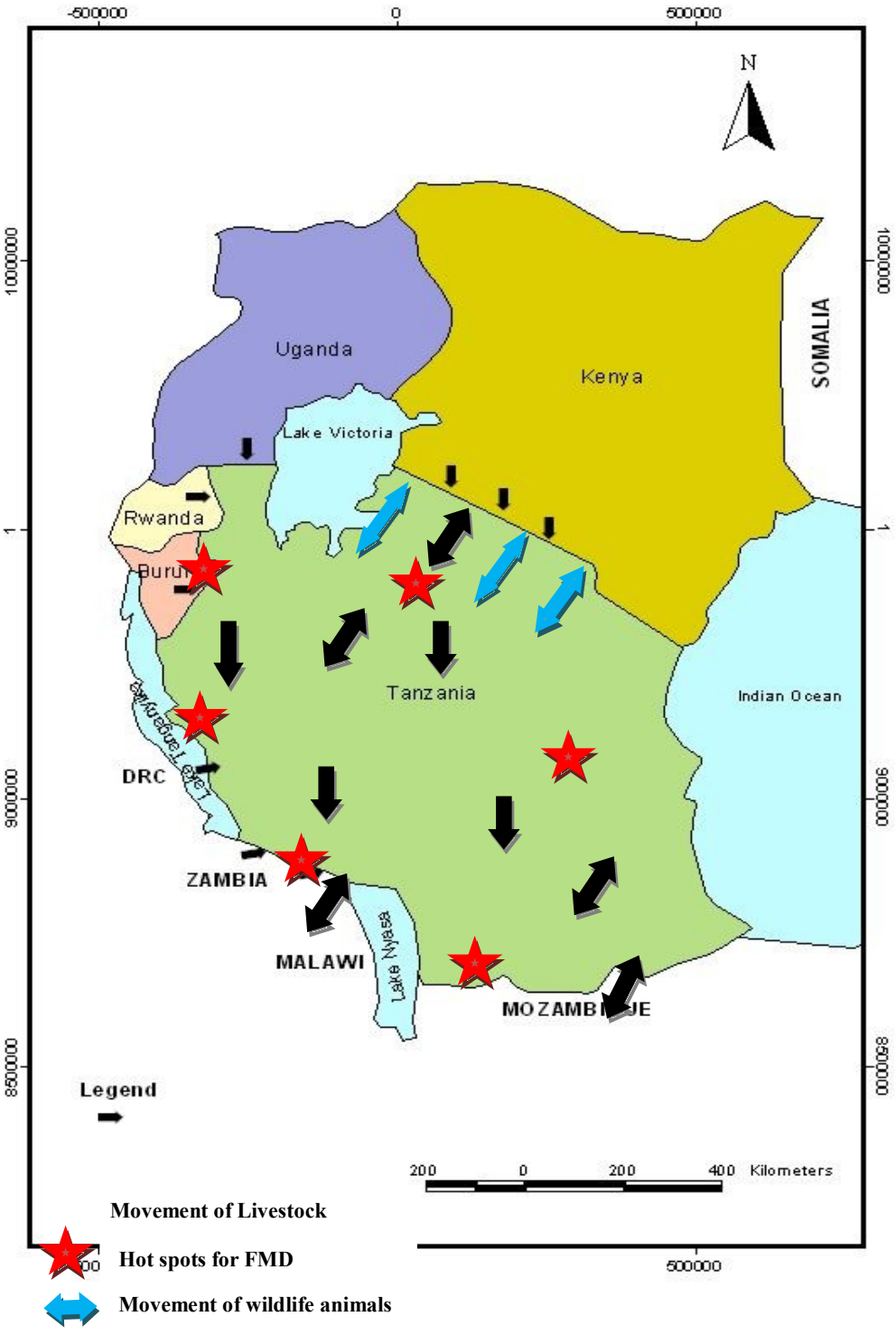

Figure 11: Map of Great Lakes region showing the movement of livestock and wildlife animals towards Tanzania. Source: MoL&FD (2012).

The epidemiology of FMDV in Tanzania is complicated over the last 16 years by the occurrence of novel subtypes (Topotypes) within the existing serotypes (A, O, SAT1 and SAT2). The findings of this study, together with those reported by Kasanga *et al*. (2014a) suggested that the serotype O is the most prevalent and widely distributed serotype of FMDV in Tanzania followed by serotype A (Fig. 11 and Appendix ii). The relationship between viruses circulating in this region is described by the results obtained from phylogenetic analyses using neighbour-joining (Tamura *et al.,* 2011). The results assisted in knowing where these genotypes are found and whether are a contributing factor for the risk of FMD for the disease to occurrence.

Indeed, for appropriate disease control measures in these endemic settings, it is important to monitor the genotype that is causing the outbreaks in the field. The results would provide science-based support for the design of vaccination control program/policy that would ensure the most appropriate vaccine candidate strain to be used. Furthermore, information obtained from this study will help to indicate the possible sources of outbreaks, the factors for the transmission of FMD and predictions on the causes of the outbreaks. Gathering and analyzing information on disease dynamics in sub-Saharan Africa has been pointed out as one of the priorities in FMD control in the region (Kivaria and Kapaga, 2002; Rweyemamu *et al.,* 2008a).

The government of Tanzania is planning to establish FMD-free zones that will be sources of exporting livestock and its products. The results from this study should help delimiting the most suitable regions and identifying the areas that require special attention. This task, however, will need strong political commitment and technical input, considering the heterogeneity observed on the spatial and temporal distribution of the FMDV.

In FMD-endemic countries like Tanzania, the implementation of an effective vaccination program requires intensive molecular epidemiological surveillance of currently circulating

field isolates and continuous monitoring to ensure that the vaccine strains are protective against field viruses. The importance of the movement of infected animals as a mechanism responsible for the spread of FMDV among countries in the Eastern and Southern African region has been highlighted previously by Rweyemamu *et al.* (2008b). These movements occur within countries or across borders from Uganda (20.0%), Kenya (25.0%), DRC (5.0%) and Rwanda (15.0%) towards Zambia (5.0%) and Malawi (2.0%) and are driven by a higher demand and market price (Fig.11).

The epidemiological surveys of FMD, molecular characterization of FMDV genetic diversity will provide insights into the relationships between different field isolates. Regular monitoring of field outbreak strains by VP1 sequence analysis is essential to arrive at overall epidemiological scenario and for tracking the virus movement. Knowledge on prevalent lineage/topotype is essential for implementation of any control programme through vaccine strategy (Jangra *et al.,* 2005).

A rapid evolution of these antigenic sites in FMDV in nature hinders synthetic vaccines from providing efficient protection (Mateu, 1995; Martinez *et al*., 1997). As a phenotypic variability of viral RNA is strongly influenced by high mutation rates and quasispecies dynamics (Sobrino *et al.,* 2001). Despite significant advances in our understanding of viral pathogenesis and the development of new antiviral strategies in the last two decades, FMDV remains a major threat to animal husbandry in Tanzania.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

As part of the Tanzanian national poverty alleviation strategies, there is an intense pressure to control animal diseases especially the Transboundary Animal Diseases (TADs). Since FMD is one of TADs, its control requires improving the current knowledge on the disease dynamics and factors associated with FMD occurrence for appropriate implementation of rational control measures. Despite considerable information on the virus, disease and vaccines being available FMD remains a major threat to the livestock industry in Africa and world-wide. New sublineages of FMDV continue to evolve to produce novel strains which sometimes break through vaccine induced immunity and can result in major epidemics. FMD is endemic in most of African countries including Tanzania. The current situation is alarming considering that it is an economic driver of poverty, although it varies in different agro-ecological and livestock production systems.

Two assays rRT-PCR and Ag-ELISA were tested for sensitivity and specificity. The results revealed that rRT-PCR is more sensitive (86.6%) than Ag-ELISA (70%), although both have the same specificity of 70%. This showed that molecular-based assay has more potential over the conventional Ag-ELISA as it is a “gold” standard test, as recommended by OIE, for other laboratory test beside virus isolation as proposed by OIE/FAO World Reference Laboratory for Foot-and-Mouth Disease (WRL for FMD) for some resource compromised laboratory. The molecular genotyping assays (rRT-PCR, LAMP, LFD) are rapid with high sensitivity, are cost effective and portray high throughput such that they can be used in the field and for export validation.

The phylogenetic relationships revealed that FMD viruses were introduced into Tanzania on a regular basis, or alternatively were maintained/generated within for long periods of

time or in neighboring countries. This study revealed rapid and countrywide spread of FMD, it was noted that the overlapping serotypes distribution in the country and its neighbours provides the basis for this endemicity within this region. The study showed that FMDV serotypes O and A are most prominent in Tanzania and there is a gradual increase in the amount of genetic diversity of current serotype O field strains. Highest numbers of reported outbreaks caused by serotype O were recorded during 2008–2013 and had been the most widely distributed serotype in the country.

The genetic diversity observed within VP1 nucleotide sequences was 95%–100%. It was observed that type O strains from Republic of Kenya, DRC, Zambia compared from the GenBank shared the highest nucleotide identity (98.3%) to Tanzanian strains. This finding suggests that a single group of type O viruses could be spreading throughout Great Lakes countries while type A viruses are likely to represent the phylogeographic strains common in Tanzania. With this current study, it seems that this serotype's reappearance is contrary to the previous reports. The spread and reappearance of serotype A could possibly be caused by the movement of animals across the border or within the country by nomadic livestock keepers. When comparing serotype A using VP1 sequences with other strains from previous studies it was found that some are 100% similar to each other, suggesting that the same viruses are circulating within the country.

In the two previous studies by Kasanga *et al*. (2012; 2014) it was indicated that spatiotemporal distribution of FMDV serotype A is in southern and central parts of the country. But, the findings of the current study show that the occurrence and distribution of FMDV during 2008-2013 changed as serotype A was found in Nzega, Mtwara, Tabora, Kibondo (Kigoma), Dar es Salaam and Sumbawanga (Rukwa) and that FMDV is heading towards Mozambique.

The results indicated that FMDV endemicity is still prevailing and extending to the Great Lakes zone, inspite of the fact that this study was conducted in Tanzania only. The phylogenetic analysis results revealed that the currently existing serotypes (A, O and SAT1) are circulating or generated within the countries of this region. For example, in this study phylogenetic analysis for serotype O revealed a sharing of topotype EA-2 within the Great Lakes zone. To stress on this issue of virus maintenance, in Uganda serotype SAT3 was isolated in 1973, since then, in year 2015 it was isolated in long horn Ankole cattle, showing that, the virus is still generated and maintained within the endemic settings without going to cattle. Therefore, due to high phylogenetic links found in both serotypes which are very much associated with human activities within the region it is advisable to combine all the efforts regionally to control the disease by the use of a vaccine that contains a cocktail of candidates' antigens of FMDV strains and controlling cattle movements crossing their borders.

Results presented in this study show that molecular epidemiological studies can help in understanding the FMD dynamics even with limited data since active and passive, when applied routinely, could be used to set up FMD risk-based surveillance. Also, this study, has improved the understanding of FMDV circulating strains and risk factors that provide opportunities for exploring livestock vaccination strategies such as performing vaccine-matching before recommending a vaccine for FMD control in specific areas. This knowledge of the FMDV diversity and circulation pattern of FMDV strains in different hosts across the region is also growing, allowing vaccine strains to be selected that can be tailored to local settings, increasing their livelihood of being more effective.

Due to gradual increases of the amount of diversity within Tanzanian strains (e.g. serotype O) it is advisable for the future study to focus on molecular evolution of FMDV with the

main focus on understanding the rates of nucleotide substitution for VP1 gene sequences using Bayesian MCMC approach implemented in the BEAST program. Also detailed phylogenetic analyses should be done for field samples from the selected hot spots in order to trace the origin of FMD outbreaks that will give us the possibility of more detailed analyses of outbreak strains.

In this aspect it is proposed that, further antigenic characterization, together with full genome genetic analyses, should be undertaken to confirm whether the Tanzanian strains are undergoing antigenic diversification or there is evolution of individual isolates. Although only a limited number of samples were available for the sequence analysis, this study provides an overview of genetic characterization and epidemiological situation of FMDV circulating in the field within the six years (2008-2013).

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APPENDICES

Appendix 1: Topotype distribution of FMDV serotypes O, A, C, and SAT types 1-3 in Africa, Table adapted from (Vosloo et al., 2001)

SAT1	I	South Africa, Southern Zimbabwe, Mozambique	Vosloo *et al*.,1995
	II	Botswana, Namibia, Western Zimbabwe	
	III	Zambia, Malawi, Tanzania, Kenya, Northern Zimbabwe	Bastos *et al.,* 2001
		Uganda	Reid *et al*., 2001
	IV	Uganda	
	V	Uganda	Sahle 2003
	VI	Nigeria, Sudan	
	VII	Nigeria, Niger	Sangare *et al.,* 2003
	VIII	South Africa, Mozambique, Southern Zimbabwe	
		Namibia, Botswana, northern and western Zimbabwe	
SAT2	I	Botswana, Zambia, Zimbabwe	
	II	Burundi, Malawi, Kenya, Tanzania, Ethiopia	Bastos *et al*., 2003b
		Nigeria, Senegal, Liberia, Ghana, Mali, Ivory Coast	
	III	Gambia, Senegal	Vosloo *et al*., 1995
	IV	Eritrea	
	V	Rwanda	
		Kenya, Uganda	
	VI	Democratic Republic of the Congo, Uganda	Sangare *et al.,* 2003
	VII	Angola	
	VIII	Uganda	Sahle 2003
	IX	Sudan	
	X	Ethiopia	Sangare *et al.,* 2004
	XI		
	XII	South Africa, southern Zimbabwe	
	XIII	Namibia, Botswana, western Zimbabwe	
	XVI	Malawi, Northern Zimbabwe	
SAT3		Zambia	
	I	Uganda	
	II	Uganda	Vosloo *et al.,* 1995
	III		
	IV	Ethiopia, Eritrea, Kenya, Somalia, Sudan, Tunisia, Egypt	Bastos *et al.,* 2003a
	V	Algeria, Ivory Coast, Guinea, Morocco, Niger, Ghana, Burkina Faso, Tunisia, Sudan	
O	VI	Uganda, Kenya, Sudan	Reid *et al*., 2001

		Uganda	
	I	Uganda	
		Tanzania, Uganda	
	II	South Africa	Samuel and Knowles 2001
		Angola	
	III		
	IV	Mauritania, Mali, Ivory Coast, Ghana, Niger, Nigeria, Cameroon, Chad, Senegal, Gambia, Sudan	
	V	Angola, Algeria, Morocco, Libya, Tunisia, Malawi	Sangare *et al.,* 2002
	VI	Tanzania, Burundi, Kenya, Somalia, Malawi	
	VII	Ethiopia	Sahle 2003
	VIII	Sudan, Eritrea	
		Uganda, Kenya, Ethiopia	Sangare *et al.,* 2001
A	I	Kenya	
		Ethiopia, Kenya	
		Angola	
	II		
	III		Samuel and Knowles 2003
	VI		
	V		
	VI		Knowles *et al*., 1998
C	I		
	II		
	II		Reid *et al*., 2001
			Knowles and Samuel 2003

Appendix 2: Closely related FMDV to Tanzanian type O isolates

Name of the virus (WRLFMD Ref. No.)	Reference virus	No. Nucleotide composition	No. of Nucleotide Match	% Identity	% difference
TAN-CVL-2010-0004	O/TAN-CVL-2010-0019	639	637	99.6	0.3
	O/COD/1/2010	639	636	99.5	0.4
	O/COD/1/2010	639	636	99.5	0.4
	O/TAN-CVL-2010-0109	639	636	99.5	0.4
TAN-CVL-2010-0008	O/TAN-CVL-2010-0009	639	639	100.0	0.0
	O/TAN-CVL-2010-0142	639	639	100.0	0.0
	O/TAN-CVL-2010-0144	639	639	100.0	0.0
	O/TAN-CVL-2011-0039	639	639	100.0	0.0
TAN-CVL-2010-0009	O/TAN-CVL-2010-0009	639	639	100.0	0.0
	O/TAN-CVL-2010-0142	639	639	100.0	0.0
	O/TAN-CVL-2010-0144	639	639	100.0	0.0
	O/TAN-CVL-2010-0039	639	639	100.0	0.0
TAN-CVL-2010-0011	O/TAN-CVL-2010-0140	639	639	100.0	0.0
	O/TAN-CVL-2010-0008	639	635	99.3	0.6
	O/TAN-CVL-2010-0009	639	635	99.3	0.6
	O/TAN-CVL-2010-0037	639	635	99.3	0.6
TAN-CVL-2010-0013	O/TAN-CVL-2010-0014	639	639	100.0	0.0

	O/TAN-CVL-2010-0139	639	639	100.0	0.0
	O/TAN-CVL-2010-0015	639	633	99.0	0.9
	O/TAN-CVL-2011-0006	639	633	99.0	0.9
TAN-CVL-2010-0014	O/TAN-CVL-2010-0009	639	639	100.0	0.0
	O/TAN-CVL-2010-0142	639	639	100.0	0.0
	O/TAN-CVL-2010-0144	639	633	99.0	0.9
	O/TAN-CVL-2011-0006	639	633	99.0	0.9
TAN-CVL-2010-0015	O/TAN-CVL-2010-0009	639	635	99.3	0.6
	O/TAN-CVL-2010-0142	639	633	99.0	0.9
	O/TAN-CVL-2010-0144	639	633	99.0	0.9
	O/TAN-CVL-2010-0139	639	633	99.0	0.9
TAN-CVL-2010-0019	O/COD/1/2010	639	638	99.8	0.1
	O/COD/2/2010	639	638	99.8	0.1
	O/TAN-CVL-2010-0109	639	638	99.8	0.1
	O/ZAM/P3/2010	639	638	99.8	0.1
TAN-CVL-2010-0037	O/TAN-CVL-2010-0046	639	639	100.0	0.0
	O/TAN-CVL-2010-0008	639	635	99.3	0.6
	O/TAN-CVL-2010-0009	639	635	99.3	0.6
	O/TAN-CVL-2010-0011	639	635	99.3	0.6
TAN-CVL-2010-0047	O/TAN-CVL-2010-0036 O/TAN-CVL-	639	639	100.0	0.0

	2010-0031	638	638	100.0	0.0
	O/TAN-CVL-2010-0143	639	632	98.9	1.1
	O/TAN-CVL-2010-0008	639	631	98.7	1.2
	O/TAN-CVL-2010-0009	639	631	98.7	1.2
TAN-CVL-2010-0071	O/KEN/150/2010	564	562	99.6	0.3
	O/TAN/2/2004	564	541	95.9	4.0
	O/TAN/3/2004	564	541	95.9	4.0
TAN-CVL-2010-0086	O/TAN-CVL-2010-0009	639	634	99.2	0.7
	O/TAN-CVL-2010-0008	639	634	99.2	0.7
	O/TAN-CVL-2010-0011	639	634	99.2	0.7
	O/TAN-CVL-2010-0037	639	634	99.2	0.7
TAN-CVL-2010-0021	O/TAN-CVL-2010-0008	637	637	100.0	0.0
	O/TAN-CVL-2010-0009	637	637	100.0	0.0
	O/TAN-CVL-2010-0039	637	637	100.0	0.0
	O/TAN-CVL-2010-0142	637	637	100.0	0.0
	O/TAN-CVL-2010-0144	637	637	100.0	0.0
TAN-CVL-2009-0040	O/TAN/44/2009	639	639	100.0	0.0
	O/TAN/5/2009	639	639	100.0	0.0
	O/UGA/18/2004	639	621	97.1	2.8
	O/UGA/1/2004	639	621	97.8	3.1

TAN-CVL-2011-0106	O/TAN-CVL-2010-0019	639	639	100.0	0.0
	O/COD/1/2010	639	638	99.8	0.1
	O/COD/2/2010	639	638	99.8	0.1
	O/TAN-CVL-2010-0108	639	638	99.8	0.1
	O/TAN-CVL-2010-0109	639	638	99.8	0.1
TAN-CVL-2010-0109	O/TAN-CVL-2010-0019	636	638	99.8	0.1
	O/COD/1/2010	639	637	99.6	0.3
	O/COD/2/2010	639	637	99.6	0.3
	0/ZAM/P3/2010	639	637	99.6	0.3
TAN-CVL-2010-0139	O/TAN-CVL-2010-0013	639	639	100.0	0.0
	O/TAN-CVL-2010-0014	639	639	100.0	0.0
	O/TAN-CVL-2010-0015	639	633	99.0	0.9
	O/TAN-CVL-2011-0006	639	633	99.0	0.9
TAN-CVL-2011-0031	O/TAN-CVL-2010-0036	638	638	100.0	0.0
	O/TAN-CVL-2010-0047	638	638	100.0	0.0
	O/TAN-CVL-2010-0143	638	631	98.9	1.1
	O/TAN-CVL-2010-0008	638	630	98.7	1.2
	O/TAN-CVL-2010-0009	638	630	98.7	1.2
TAN-CVL-2010-0143	O/TAN-CVL-2010-0008	639	636	99.5	0.4
	O/TAN-CVL-2010-0009	639	636	99.5	0.4
	O/TAN-CVL-2010-0011	639	636	99.5	0.4

	O/TAN-CVL-2010-0021	639	634	99.5	0.4
	O/TAN-CVL-2010-0037	639	636	99.5	0.4
TAN-CVL-2010-0039	O/TAN-CVL-2010-0008	639	639	100.0	0.0
	O/TAN-CVL-2010-0009	639	639	100.0	0.0
	O/TAN-CVL-2010-0142	639	639	100.0	0.0
	O/TAN-CVL-2010-0144	639	639	100.0	0.0
	O/TAN-CVL-2010-0039	639	639	100.0	0.0
TAN-CVL-2010-0017	O/COD/1/2010	447	446	99.7	0.2
	O/TAN-CVL-2010-0019	447	446	99.7	0.2
	O/TAN-CVL-2010-0108	447	446	99.7	0.2
	O/TAN-CVL-2011-0106	447	446	99.7	0.2
	O/ZAM/P3/2010	447	445	99.5	0.4
TAN-CVL-2010-0140	O/TAN-CVL-2010-0011	639	639	100.0	0.0
	O/TAN-CVL-2010-0008	639	635	99.3	0.6
	O/TAN-CVL-2010-0009	639	635	99.3	0.6
	O/TAN-CVL-2010-0037	639	635	99.3	0.6
TAN-CVL-2010-0142	O/TAN-CVL-2010-0009	639	639	100.0	0.0
	O/TAN-CVL-2010-0008	639	639	100.0	0.0
	O/TAN-CVL-2010-0144	639	639	100.0	0.0
	O/TAN-CVL-2011-0039	639	639	100.0	0.0

TAN-CVL-2011-0006	O/TAN-CVL-2010-0015	639	635	99.3	0.6
	O/TAN-CVL-2010-0013	639	633	99.0	0.9
	O/TAN-CVL-2010-0014	639	633	99.0	0.9
	O/TAN-CVL-2010-0139	639	633	99.0	0.9
TAN-CVL-2011-0046	O/TAN-CVL-2010-0037	639	639	100.0	0.0
	O/TAN-CVL-2010-0008	639	635	99.3	0.6
	O/TAN-CVL-2010-0009	639	635	99.3	0.6
	O/TAN-CVL-2010-0011	639	635	99.3	0.6
TAN-CVL-2010-0144	O/TAN-CVL-2010-0009	639	639	100.0	0.0
	O/TAN-CVL-2010-0008	639	639	100.0	0.0
	O/TAN-CVL-2010-0144	639	639	100.0	0.0
	O/TAN-CVL-2011-0039	639	639	100.0	0.0
TAN-CVL-2010-0108	O/TAN-CVL-2010-0019	639	638	99.8	0.1
	O/TAN-CVL-2010-0106	639	638	99.8	0.1
	O/COD/1/2010	639	637	99.6	0.3
	O/COD/2/2010	639	637	99.6	0.3
	O/TAN-CVL-2010-0109	639	637	99.6	0.3
TAN-CVL-2010-0039	O/TAN-CVL-2010-0008	639	639	100.0	0.0
	O/TAN-CVL-2010-0009	639	639	100.0	0.0
	O/TAN-CVL-2010-0142	639	639	100.0	0.0
	O/TAN-CVL-2010-0144	639	639	100.0	0.0

	O/TAN-CVL-2011-0039	639	639	100.0	0.0
TAN-CVL-2010-0034	O/TAN-CVL-2010-0037	639	636	99.5	0.4
	O/TAN-CVL-2010-0046	639	636	99.5	0.4
	O/TAN-CVL-2010-0143	639	635	99.3	0.6
	O/TAN-CVL-2010-0008	639	634	99.2	0.7
	0/TAN-CVL-2010-0009	639	634	99.2	0.7
TAN-CVL-2010-0003	O/TAN-CVL-2010-0013	639	639	100.0	0.0
	O/TAN-CVL-2010-0014	639	639	100.0	0.0
	O/TAN-CVL-2010-0139	639	639	100.0	0.9
	O/TAN-CVL-2010-0015	639	633	96.0	0.9
	O/TAN-CVL-2010-0006	639	633	99.0	0.9
TAN-CVL-2013-0362	O/TAN-CVL-2010-0366	639	639	100.0	0.0
	O/TAN-CVL-2010-0364	639	638	99.8	0.1
	O/TAN-CVL-2010-0321	639	632	98.9	1.1
	O/TAN-CVL-2010-0318	639	631	98.7	1.2
TAN-CVL-2013-0318	O/TAN-CVL-2012-0321	639	637	99.6	0.3
	O/TAN-CVL-2010-0019	639	627	98.1	1.8
	O/COD/1/2010	639	626	97.9	2.0
	O/COD/2/2010	639	626	97.9	2.0
TAN-CVL-2012-0321	O/TAN-CVL-2010-0009	639	637	99.6	0.3

	O/TAN-CVL-2010-0008	639	629	98.4	1.5
	O/COD/1/2010	639	628	98.2	1.7
	O/COD/2/2010	639	628	98.2	1.7
TAN-CVL-2013-0366	O/TAN-CVL-2013-0362 O/TAN-CVL-	639	639	100.0	0.0
	2013-0364	639	638	99.8	0.1
	O/TAN-CVL-2012-0321	639	632	98.9	1.1
	O/TAN-CVL-2012-0318	639	631	98.7	1.2
TAN-CVL-2013-0364	O/TAN-CVL-2013-0362	639	638	99.8	0.1
	O/TAN-CVL-2012-0366	639	638	99.8	0.1
	O/TAN-CVL-2012-0321	639	633	99.0	0.9
	O/TAN-CVL-2012-0318	639	632	98.9	1.1
TAN-CVL-2013-0452	O/TAN-CVL-2010-0143	639	619	96.8	3.1
	O/TAN-CVL-2010-0037	639	618	96.7	3.2
	O/TAN-CVL-2010-0046	639	618	96.7	3.2
	O/TAN-CVL-2010-0034	639	617	96.5	3.4
TAN-CVL-2013-0376	O/TAN-CVL-2013-0378	639	638	99.8	0.1
	O/TAN-CVL-2010-0004	639	624	97.6	2.3
	O/TAN-CVL-2010-0019	639	624	97.6	2.3
	O/TAN-CVL-2011-0106	639	624	97.6	2.3

TAN-CVL-2013-0378	O/TAN-CVL-2013-0376	639	638	99.8	0.1
	O/TAN-CVL-2010-0004	639	625	97.8	2.1
	O/TAN-CVL-2010-0019	639	625	97.8	2.1
	O/TAN-CVL-2010-0106	639	625	97.8	2.1
TAN-CVL-2010-0116	O/TAN-CVL-2010-0143	639	635	99.3	0.6
	O/TAN-CVL-2010-0008	639	634	99.2	0.7
	O/TAN-CVL-2010-0009	639	634	99.2	0.7
	O/TAN-CVL-2010-0011	639	634	99.2	0.7
	O/TAN-CVL-2010-0021	637	632	99.2	0.7
TAN-CVL-2010-0036	O/TAN-CVL-2010-0047	639	639	100.0	0.0
	O/TAN-CVL-2011-0031	638	638	100.0	0.0
	O/TAN-CVL-2010-0143	639	632	98.9	1.1
	O/TAN-CVL-2010-0008	639	631	98.7	1.2
	0/TAN-CVL-2010-0009	639	631	98.7	1.2

Appendix 3: Sources of FMD virus used in this study

Serotype	Topotype/ Lineage	WRLFMD Ref. No.	Geographic origin	species	Collection date	Accession	References
O	EA-2	O/TAN-CVL-039	Misungwi, Mwanza Region, Tanzania	Cattle	13/11/2010	KJ947823	This study
O	EA-2	O/TAN-CVL-0144	Mabuki, Mwanza Region, Tanzania	Cattle	16/12/2010	KJ947831	This study
O	EA-2	O/TAN-CVL-0142	Mabuki, Mwanza Region, Tanzania	Cattle	16/12/2010	KJ947824	This study
O	EA-2	O/TAN-CVL-008	Mabuki, Mwanza Region, Tanzania	Cattle	16/12/2010	KJ947810	This study
O	EA-2	O/TAN-CVL-009	Mabuki, Mwanza Region, Tanzania	Cattle	16/12/2010	KJ947807	This study
O	EA-2	O/TAN-CVL-086	Kahama, Shinyanga Region, Tanzania	Cattle	18/09/2010	KJ947832	This study
O	EA-2	O/TAN-CVL-011	Musoma Rural, Mara Region, Tanzania	Cattle	22/11/2010	KJ947804	This study
O	EA-2	O/TAN-CVL-140	Musoma Rural, Mara Region, Tanzania	Cattle	23/11/2010	KJ947808	This study
O	EA-2	O/TAN-CVL-037	Municipal, Tabora Region, Tanzania	Cattle	21/09/2010	KJ947806	This study
O	EA-2	O/TAN-CVL-046	Nzega, Tabora Region, Tanzania	Cattle	02/09/2010	KJ947827	This study
O	EA-2	O/TAN-CVL-015	Serengeti, Mara Region, Tanzania	Cattle	15/11/2011	KJ947828	This study

			Rorya, Mara Region, Tanzania				
O	EA-2	O/TAN-CVL-006	Musoma Rural, Mara Region, Tanzania	Cattle	02/03/2010	KJ947830	This study
O	EA-2	O/TAN-CVL-013	Musoma Rural ,Mara Tanzania	Cattle	22/11/2010	KJ947829	This study
O	EA-2	O/TAN-CVL-014	Serengeti, Mara Region, Tanzania	Cattle	22/11/2010	KJ947833	This study
O	EA-2	O/TAN-CVL-139	Iringa Region, Tanzania	Cattle	16/11/2010	KJ947825	This study
O	EA-2	O/TAN-CVL-040	Mbeya Region, Tanzania	Cattle	09/11/2009	KJ947805	This study
O	EA-2	O/TAN-CVL-318	Mbeya Region, Tanzania	Cattle	31/05/2012	KJ947834	This study
O	EA-2	O/TAN-CVL-321	Ilala, Dar Es Salaam Region, Tanzania	Cattle	31/05/2012	KJ947835	This study
O	EA-2	O/TAN-CVL-019	Ngara, Kagera Region, Tanzania	Cattle	16/10/2010	KJ947811	This study
O	EA-2	O/TAN-CVL-109	Wasela, Tanga Region, Tanzania	Cattle	28/08/2010	KJ947836	This study
O	EA-2	O/TAN-CVL-004	Masasi, Mtwara Region, Tanzania	Cattle	02/12/2010	KJ947826	This study
O	EA-2	O/TAN-CVL-039	Kisarawe, Coastal Region, Tanzania	Cattle	21/09/2011		This study
O	EA-2	O/TAN-CVL-003	Tabora Municipal, Tanzania	Cattle	12/08/2008		This study
O	EA-2	O/TAN-CVL-108	Mafinga, Iringa Region, Tanzania	Cattle	20/07/2010		This study
			Mafinga, Iringa Region, Tanzania				

O	EA-2	O/TAN-CVL-031	Muleba, Kagera Region, Tanzania	Cattle	15/12/2011	This study
O	EA-2	O/TAN-CVL-036	Muleba, Kagera Region, Tanzania	Cattle	21/09/2011	This study
O	EA-2	O/TAN-CVL-362	Unguja , Zanzibar	Cattle	25/04/2013	This study
O	EA-2	O/TAN-CVL-364	Kilwa, Lindi Region, Tanzania	Cattle	25/04/2013	This study
O	EA-2	O/TAN-CVL-366	Ngerengere, Morogoro Region, Tanzania	Cattle	25/04/2013	This study
O	EA-2	O/TAN-CVL-376	Sumbawanga, Rukwa	Cattle	04/04/2013	This study
O	EA-2	O/TAN-CVL-378	Tabora Municipal, Tabora region, Tanzania	Cattle	04/04/2013	This study
O	EA-2	O/TAN-CVL-0116	Ilala Dar Es Salaam Region, Tanzania	Cattle	23/10/2010	This study
O	EA-2	O/TAN-CVL-452	Tabora Municipal, Tabora Region, Tanzania	Cattle	13/12/2013	This study
O	EA-2	O/TAN-CVL-071	Madale, Ilala Municipal, Dar Es Salaam Region, Tanzania	Cattle	12/07/2010	This study
O	EA-2	O/TAN-CVL-017	Kyela, Mbeya Region, Tanzania Bagamoyo, Coastal Region, Tanzania	Cattle	16/11/2010	This study
O	EA-2	O/TAN-CVL-034	Morogoro Region, Tanzania Kibaha, Pwani Region, Tanzania	Cattle	21/09/2010	This study
O	EA-2	O/TAN-CVL-358	Chuya, Mbeya Region, Tanzania Morogoro Region, Tanzania Makete, Iringa Region, Tanzania	Cattle	31/08/2012	This study

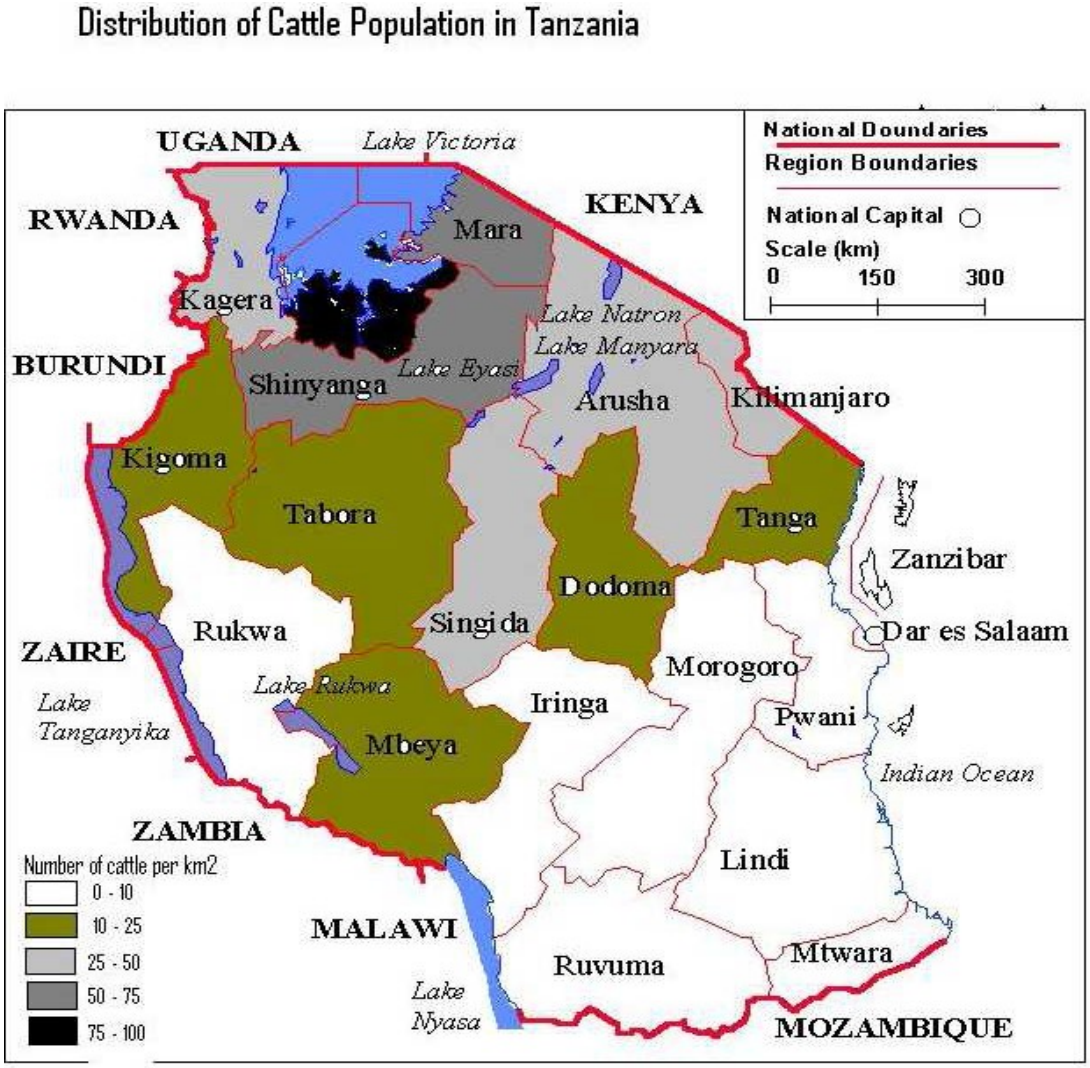
			Iringa Region, Tanzania				
O	EA-2	O/TAN-CVL-047	Songea, Ruvuma Region, Tanzania	Cattle	02/09/2010		This study
			Nkasi, Rukwa Region, Tanzania				
O	EA-2	O/TAN-CVL-109	Iringa region, Tanzania	Cattle	19/12/2011		This study
			Tanzania				
O	EA-2	O/TAN/09/1998	Tanzania	Cattle	22/10/1998	KF561677	Kasanga et al., (2014a)
O	EA-2	O/TAN/01/2004	Tanzania	Cattle	15/11/2004	KF561678	Kasanga et al., (2014a)
O	EA-2	O/TAN/16/2008	Tanzania	Cattle	25/09/2008	KF561684	Kasanga et al., (2014a)
O	EA-2	O/TAN/02/2004	Arusha Region, Tanzania	Cattle	15/11/2004	KF561679	Kasanga et al., (2014a)
			Iringa Region, Tanzania				
O	EA-2	O/TAN/12/2004	Iringa Region, Tanzania	Cattle	2004	KF561681	Kasanga et al., (2014a)
			Morogoro Region, Tanzania				
O	EA-2	O/TAN/05/2009	Njombe, Iringa Region, Tanzania	Cattle	01/05/2009	KF561685	Kasanga et al., (2014a)
O	EA-2	O/TAN/44/2009	Kibaha, Coastal Region, Tanzania	Cattle	27/10/2009	KF561686	Kasanga et al., (2014)
O	EA-2	O/TAN/01/1985	Mpwapwa, Dodoma Region, Tanzania	Cattle	11/07/1985	KF561676	Kasanga et al., (2014a)
O	EA-2	O/TAN/14/2004	Iringa Rural, Iringa Region, Tanzania	Cattle	01/09/2014	KF561682	Kasanga et al., (2014a)
O	EA-2	O/TAN/17/2004	Bagamoyo, Coastal Region,	Cattle	01/08/2004	KF561683	Kasanga et al.,

			Tanzania				(2014a)
O	EA-2	O/TAN/03/2004	Sumbawanga, Rukwa Region, Tanzania	Cattle	15/11/2004	KF561680	Kasanga et al., (2014a)
O	EA-2	O/TAN/38/2012	Ilala Municipal, Dar Es Salaam, Tanzania	Cattle	04/04/2012		WRLFMD Report 2012
O	EA-2	O/TAN/39/2012	Ilala Municipal, Dar Es Salaam, Tanzania	Cattle	04/04/2012		WRLFMD Report 2012
A	AFRICA/G-1	A/TAN/02/1968	Ilala Municipal, Dar Es Salaam, Tanzania	Cattle	01/01/1968	KF561687	Kasanga et al., (2014a)
A	AFRICA/G-1	A/TAN/03/1968	Kasulu, Kigoma Region, Tanzania	Cattle	01/01/1967	KF561688	Kasanga et al., (2014a)
A	AFRICA/G-1	A/TAN/04/1980	Tabora Municipal, Tabora Region, Tanzania	Not known	17/10/1980	KF561689	Kasanga et al., (2014a)
A	AFRICA/G-1	A/TAN/11/2008	Masasi, Mtwara Region, Tanzania	Cattle	01/08/2008	KF561690	Kasanga et al., (2014a)
A	AFRICA/G-1	A/TAN/12/2008	Ilala Municipal, Dar Es Salaam,Tanzania	Cattle	01/08/2008	KF561691	Kasanga et al., (2014a)
A	AFRICA/G-1	A/TAN/04/2009	Ilala Municipal, Dar Es Salaam,Tanzania	Cattle	01/05/2009	KF561692	Kasanga et al., (2014a)
A	AFRICA/G-1	A/TAN/09/2009	Kasulu, Kigoma Region, Tanzania	Cattle	13/06/2009	KF561693	Kasanga et al., (2014a)
A	AFRICA/G-1	A/TAN/11/2009	Kasulu, Kigoma Region, Tanzania	Cattle	21/06/2009	KF561694	Kasanga et al., (2014a)
A	AFRICA/G-1	A/TAN/42/2009	Misenyi, Kagera Region, Tanzania	Cattle	07/09/2009	KF561695	Kasanga et al., (2014a)
A	AFRICA/G-1	A/TAN/45/2009	Misenyi, Kagera Region, Tanzania	Cattle	06/11/2009	KF561696	Kasanga et al., (2014a)

A	AFRICA/G-1	A/TAN/47/2009	Kingolowila , Morogoro Region,	Cattle	21/11/2009	KF561697	Kasanga et al., (2014a)
A	AFRICA/G-1	A/TAN-CVL-155	Kingolowila, Morogoro Region	Cattle	02/07/2008	KJ947815	This study
A	AFRICA/G-1	A/TAN-CVL-042	Kimara, Ilala, Dar Es Salaam, Tanzania	Cattle	01/05/2009	KJ947814	This study
A	AFRICA/G-1	A/TAN-CVL-052		Cattle	09/07/2009	KJ947812	This study
A	AFRICA/G-1	A/TAN-CVL-052b	Kimara, Ilala, Dar Es Salaam, Tanzania	Cattle	09/07/2009	KJ947822	This study
A	AFRICA/G-1	A/TAN-CVL-032	Kimara, Ilala, Dar Es Salaam, Tanzania	Cattle	21/09/2011	KJ947819	This study
A	AFRICA/G-1	A/TAN-CVL-066		Cattle	21/09/2011	KJ947813	This study
A	AFRICA/G-1	A/TAN-CVL-125		Cattle	21/09/2011	KJ947821	This study
A	AFRICA/G-1	A/TAN-CVL-146		Cattle	19/02/2011		This study
A	AFRICA/G-1	A/TAN-CVL-160		Cattle	19/02/2011	KJ947837	This study
A	AFRICA/G-1	A/TAN-CVL-289		Cattle	13/07/2011		This study
A	AFRICA/G-1	A/TAN-CVL-293		Cattle	13/07/2011		This study
A	AFRICA/G-1	A/TAN-CVL-370		Cattle	04/04/2013		This study

A	AFRICA/G-1	A/TAN-CVL-374	Cattle	04/04/2013	This study
SAT1	I (NWZ)	SAT1/TAN-CVL-354	Cattle	31/08/2013	This study
SAT1	I (NWZ)	SAT1/TAN-CVL-355	Cattle	31/08/2012	This study
SAT1	I (NWZ)	SAT1/TAN-CVL-359	Cattle	02/10/2012	This study
SAT1	I (NWZ)	SAT1/TAN-CVL-360	Cattle	02/102012	This study
SAT1	I (NWZ)	SAT1/TAN-CVL-361	Cattle	02/10/2012	This study

Appendix 4: **Cattle population in Tanzania, map adapted from MLDF (2012)**



Appendix 5: FMD outbreaks that occurred in Tanzania from 1954-2013, adapted from MLFD (Ministry of Livestock and Fisheries Development).

No.	Year	SAT1	SAT2	SAT3	O	A
1.	1954		√		√	
2.	1955					√
3.	1958	√	√		√	√
4.	1960		√		√	√
5.	1961				√	√
6.	1962		√		√	√
7.	1963	√			√	√
8.	1964		√		√	√
9.	1967					√
10.	1968		√		√	√
11.	1969		√		√	
12.	1970		√			√
13.	1971	√			√	√
14.	1972	√	√			
15.	1975		√			
16.	1976				√	√
17.	1977	√				
18.	1980	√			√	√
19.	1984				√	
20.	1985				√	
21.	1986		√			
22.	1992	√	√			
23.	1996	√	√			
24.	1998	√	√		√	
25.	1999	√	√			
26.	2000	√	√		√	
27.	2004		√		√	
28.	2008				√	√
28	2009		√			
30.	2010	√			√	
31	2011		√			√
32.	2012	√	√		√	
33.	2013				√	√

Notes: SAT1, SAT2, and possibly SAT 3, types are endemic in the African buffalo population

Appendix 6: RNA Extraction, Adapted from Qiagen Mini Kit

310µl of buffer AVE (RNase free water that contains 0.04% sodium azide) was added to a tube containing 310µg lyophilized RNA carrier to obtain a solution of 1µg/µl. The carrier RNA was thoroughly dissolved and the amount to be added to AVL buffer was calculated using the following formulas,

n x 0.56ml = y ml or yml x 10 µl/ml = z µl whereas, n=number of samples to be processed simultaneously, y= calculated volume of Buffer, z= volume of carrier RNA-Buffer AVE to add to buffer AVL. Buffer AW1 was prepared according to the number of preparations, for 50 samples with an AW1 concentrate 25 ml of ethanol was added. And for AW2 the 50 samples required about 30 ml. of ethanol to make a final volume of 43ml. The protocol used is for purification of viral RNA from 140 µl plasma, serum, urine, cell-culture media, or cell-free body fluids using a micro centrifuge. 560 µl of prepared Buffer AVL containing carrier RNA was put into a 1.5 ml micro centrifuge tube.140 µl plasma, serum, urine, cell-culture supernatant, or cell-free body fluid was added and pulse-vortexed for 15 seconds.

Then the mixture was incubated at room temperature of (15–25°C) for 10 min and briefly centrifuged to remove drops from the inside of the lid. A 560 µl of ethanol (96–100%) was added to the sample, and mixed by pulse-vortexing for 15 seconds and briefly centrifuged to remove drops from inside the lid. And carefully, about 630 µl of the solution from above step was applied to the QIAamp Mini column (in a 2 ml collection tube) without wetting the rim. The cap was closed and centrifuged at 6000 x g (8000 rpm) for 1 minute (Eppendorf 5415D). The QIAamp Mini column was placed into a clean 2 ml collection tube, and the tube containing filtrate was discarded. The above step was repeated twice. 500 µl of Buffer AW1 was added and centrifuged at 6000 x g (8000 rpm) for 1 min. The QIAamp Mini column was placed in a clean 2 ml collection tube and discarded the tube

containing the filtrate. Then, 500 µl of Buffer AW2 was added and centrifuged at full speed (20,000 x g; 14,000 rpm) for 3 min. The QIAamp Mini columns were placed in a clean 2 ml collection and centrifuged at full speed for 1 minute. The old collection tubes containing the filtrate were discarded and the QIAamp Mini columns placed in a clean 1.5 ml micro centrifuge. 60 µl of Buffer AVE equilibrated to room temperature was added incubated at room temperature, then centrifuged at 6,000 x g(8,000 rpm) for 1 minute. The pure nucleic acid (RNA) stored at -81°C for future molecular work.

Appendix 7: Geographic origin of FMD type O, A and SAT1 Viruses and Nucleotide Sequences Examined in this study

Isolate	ccession number	Date of collection	Place of Origin	Reference
O/TAN-CVL-2010-0039	KJ947825	13/11/2010	Mabuki, Mwanza	This study
O/TAN-CVL-2010-0144	KJ947831	16/12/2010	Mabuki, Mwanza	This study
O/TAN-CVL-2010-0142	KJ947824	16/12/2010	Mabuki, Mwanza	This study
O/TAN-CVL-2010-0008	KJ947810	16/12/2010	Mabuki, Mwanza	This study
O/TAN-CVL-2010-0009	KJ947807	16/12/2010	Mabuki, Mwanza	This study
O/TAN-CVL-2010-0086	KJ947832	18/09/2010	Kahama, Shinyanga	This study
O/TAN-CVL-2010-0011	KJ947804	22/11/2010	Musoma, Mara	This study
O/TAN-CVL-2010-0140	KJ947808	23/11/2010	Serengeti, Mara	This study
O/TAN-CVL-2010-0037	KJ947809	21/09/2010	Ngara, Kagera	This study
O/TAN-CVL-2010-0046	KJ947827	02/09/2010	Nzega, Tabora	This study
O/TAN-CVL-2010-0015	KJ947828	15/11/2010	Serengeti, Mara	This study
O/TAN-CVL-2010-0006	KJ947830	02/03/2010	Rorya, Mara	This study
O/TAN-CVL-2010-0013	KJ947829	22/11/2010	Musoma, Mara	This study
O/TAN-CVL-2010-0014	KJ947833	22/11/2010	Musoma, Mara	This study
O/TAN-CVL-2010-0139	KJ947825	16/11/2010	Musoma, Mara	This study
O/TAN-CVL-2009-0040	KJ947823	09/11/2009	Iringa Region	This study
O/TAN-CVL-2010-0318	KJ947834	31/05/2012	Mbeya Region	This study
O/TAN-CVL-2010-0321	KJ947823	31/05/2012	Mbeya Region	This study
O/TAN-CVL-2010-0019	KJ947811	16/10/2010	Sumbawanga	This study
O/TAN-CVL-2010-0109	KJ947825	28/08/2010	Dar Es Salaam	This study
O/TAN-CVL-2010-0004	KJ947825	02/12/2010	Ngara, Kagera	This study
O/TAN-CVL-2008-0003	-	08/12/2008	Wasela, Tanga	This study
O/TAN-CVL-2010-0047	-	09/02/2009	Tabora Region	This study
O/TAN-CVL-2010-0108	-	20/07/2010	Masasi, Mtwara	This study
O/TAN-CVL-2010-0034	-	01/09/2010	Tabora, Region	This study
O/TAN-CVL-2010-0036	-	21/09/2010	Tabora, Region	This study
O/TAN-CVL-2010-0116	-	23/09/2010	Zanzibar	This study
O/TAN-CVL-2010-0017	-	16/11/2010	Rukwa Region	This study
O/TAN-CVL-2010-0071	-	07/12/2010	Morogoro Region	This study
O/TAN-CVL-2010-0143	-	16/12/2010	Musoma, Mara	This study

				This study
O/TAN-CVL-2010-0021	-	16/12/2010	Mabuki, Mwanza	This study
O/TAN-CVL-2010-0031	-	15/12/2011	Kisarawe, Coast	This study
O/TAN-CVL-2010-0106	-	01/09/2011	Muleba, Kagera	This study
O/TAN-CVL-2010-0376	-	04/04/2013	Muleba, Kagera	This study
O/TAN-CVL-2010-0378	-	04/04/2013	Muleba, Kagera	This study
O/TAN-CVL-2010-0362	-	25/04/2013	Mafinga, Iringa	This study
O/TAN-CVL-2010-0364	-	25/04/2013	Mafinga, Iringa	This study
O/TAN-CVL-2010-0366	-	25/04/2013	Mafinga, Iringa	This study
O/TAN-CVL-2010-0452	-	13/12/2013	Kilwa, Lindi	This study
O/TAN/016/2008	KF561684	25/09/2008	Morogoro	;anga et al., 2014
O/TAN/002/2004	KF561679	15/11/2004	Kibaha, Pwani	;anga et al., 2014
O/TAN/012/2004	KF561681	15/11/2004	Chunya, Mbeya	;anga et al., 2014
O/TAN/005/2009	KF561685	01/05/2009	Morogoro	;anga et al., 2014
O/TAN/044/2009	KF561686	27/10/2009	Makete, Iringa	;anga et al., 2014
O/TAN/003/2004	KF561680	15/11/2004	Iringa Region	;anga et al., 2014
SAT1/TAN-CVL-2012-0354	KJ947841	31/08/2012	Morogoro, Region	This study
SAT1/TAN-CVL-2012-0355	KJ947840	31/08/2012	Morogoro, Region	This study
SAT1/TAN-CVL-2012-0359	KJ947838	02/10/2012	Dar Es Salaam	This study
SAT1/TAN-CVL-2012-0360	KJ947839	02/10/2012	Dar Es Salaam	This study
SAT1/TAN-CVL-2012-0361	KJ947837	02/10/2012	Dar Es Salaam	This study
A/TAN-CVL-2012-0052	KJ947812	09/07/2009	Dar Es Salaam	This study
A/TAN-CVL-2012-0125	KJ947813	21/11/2011	Masasi, Mtwara	This study
A/TAN-CVL-2012-0042	KJ947814	01/05/2009	Dar Es Salaam	This study
A/TAN-CVL-2012-0155	KJ947815	07/02/2008	Rukwa Region	This study
A/TAN-CVL-2012-0374	KJ947817	04/04/2013	Muleba, Kagera	This study
A/TAN-CVL-2012-0370	KJ947818	04/04/2013	Muleba, Kagera	This study
A/TAN-CVL-2012-0066	KJ947819	21/112011	Tabora, Region	This study
A/TAN-CVL-2012-0293	KJ947820	13/07/2011	Kibondo, Kigoma	This study
A/TAN-CVL-2012-0160	KJ947821	12/09/2011	Dar Es Salaam	This study
A/TAN-CVL-2012-052b	KJ947822	09/07/2009	Dar Es Salaam	This study
A/TAN-CVL-2012-0146	-	19/02/2011	Dar Es Salaam	This study
A/TAN-CVL-2012-0289	-	13/07/2011	Kibondo, Kigoma	This study

Appendix 8: Shows the results obtained from rRT-PCR and Ag-ELISA

Sample number	Date collected	Place	Specie Species	Ag ELISA	qRT-PCR (Ct value)
Negative control	30/03/13	-	-	Negative	No Ct value
Positive control	16/12/10	Kenya	-	Positive	15.9
O/TAN-CVL-2010-08	16/12/10	Mwanza	Bovine	Positive	25.9
O/TAN-CVL-2010-09	16/12/10	Mwanza	Bovine	Positive	26.1
O/TAN-CVL-2010-11	22/11/10	Mara	Bovine	Positive	26.8
O/TAN-CVL-2010-13	22/11/10	Mara	Bovine	Negative	40.3
O/TAN-CVL-2010-14	22/11/10	Mara	Bovine	Positive	18.9
O/TAN-CVL-2010-15	15/11/10	Mara	Bovine	Negative	No Ct
O/TAN-CVL-2010-19	16/10/10	Rukwa	Bovine	Positive	20.45
O/TAN-CVL-2010-37	21/09/10	Kagera	Bovine	Negative	No Ct
O/TAN-CVL-2010-39	13/11/10	Mwanza	Bovine	Negative	28.5
O/TAN-CVL-2010-46	02/09/10	Tabora	Bovine	Positive	17.1
O/TAN-CVL-2010-086	18/09/10	Shinynga	Bovine	Positive	19.3
O/TAN-CVL-2010-109	28/08/10	Dar-Es-S	Bovine	Positive	24.8
O/TAN-CVL-2010-139	16/11/10	Mara	Bovine	Positive	15.2

O/TAN-CVL-2010-140	23/11/10	Mara	Bovine	Positive	16.7
O/TAN-CVL-2010-142	16/12/10	Mwanza	Bovine	Positive	21.4
O/TAN-CVL-2010-144	16/12/10	Mwanza	Bovine	Positive	20.3
O/TAN-CVL-2010-318	31/05/10	Mbeya	Bovine	Positive	14.3
O/TAN-CVL-2010-321	31/05/10	Mbeya	Bovine	Positive	21.5
O/TAN-CVL-2010-06	02/03/10	Mwanza	Bovine	Positive	22.9
O/TAN-CVL-2010-04	02/12/10	Kagera	Bovine	Positive	28.2
O/TAN-CVL-2010-048	2/9/2010	Tabora	Bovine	Positive	26.8
O/TAN-CVL-2010-066	24/9/2011	Tabora	Bovine	Negative	20.9
O/TAN-CVL-2010-021	16/12/2010	Mwanza	Bovine	Positive	25.1
O/TAN-CVL-2011-007	3/03/2011	Rorya	Bovine	positive	13.9
O/TAN-CVL-2013-450	30/12/2013	Lindi	Bovine	Positive	16.3
O/TAN-CVL-2013-452	30/12/2013	Lindi	Bovine	Positive	26.5
O/TAN-CVL-2010-106	19/12/2011	Kagera	Bovine	Positive	21.6
O/TAN-CVL-2012-122	21/09/2010	ZNZ	Bovine	Negative	No Ct
O/TAN-CVL-2010-002	03/03/2010	Mwanza	Bovine	Negative	No Ct
O/TAN-CVL-2010-0452	15/12/2013	Lindi	Bovine	Positive	13.4