MOLECULAR DETECTION AND EVOLUTIONARY CHARACTERISTICS OF RECENTLY RECOVERED SEROTYPES A AND O FOOT-AND-MOUTH DISEASE VIRUSES IN SELECTED AREAS OF TANZANIA

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ABSTRACT

Foot-and-mouth disease (FMD) is a highly contagious disease that infects cloven hoofed animals. FMD is caused by FMD virus (FMDV), a picornavirus with a positive sense single stranded RNA genome of about 8.5 kb in size. FMD is endemic in East African countries including Tanzania. The general objective of this study was to determine the genetic characteristics of recently circulated serotypes O and A FMDV field strains in selected regions of Tanzania in 2019. A total of 36 (four serotype O and 22 serotype A) archived epithelial samples collected from cattle and pig in Kibaha, Morogoro, Sengerema, Butiama and Musoma were used in this study. The laboratory analysis of the samples was performed by reverse transcription polymerase chain reaction (RT-PCR) targeting the 3D and VP1 coding regions, VP1 sequencing and phylogeny. The RT-PCR results revealed that 72% of samples (n= 26) were positive for FMDV genome. Molecular typing of the FMDV genome positive samples was achieved by using serotype specific primers of which serotypes A were 85 % (n= 22) and O were 15 % (n= 4). Phylogenetic reconstructions were determined by neighbour-joining methods. Phylogenetic analysis of VP1 showed genetic diversity among the circulating viruses and their molecular relatedness with previously recorded sequences from East Africa, particularly Kenya and Tanzania. These findings indicate that the 2019 FMDV types A and O responsible for the disease outbreaks in Tanzania were the East Africa 2 (EA-2) and genotype I (GI) which showed a very closely evolutionary relatedness with previous strains collected in Kenya and Tanzania. Further studies are required to design new vaccine strains so as to prevent upcoming outbreaks.

DECLARATION

I, Evance Julieth, hereby declare to the senate of Sokoine University of Agriculture that this dissertation is my own original work done within the period of registration and it has neither been submitted nor being concurrently being submitted to any other institution.

_____ _____

Evance Julieth Date

The above declaration is confirmed

_____ _____

Prof. Christopher J. Kasanga Date

Supervisor

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Prof. Donald P. King Date

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DEDICATION

This work is wholeheartedly dedicated to my Uncle Joel J. Chidabwa and Aunt Siwadaha R. Chidabwa. I would not be the person I am today if it was not for them who have been my inspiration and a hand to lean on when I thought of giving up, who continually provided me with moral, spiritual and financial support.

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LIST OF ABBREVIATIONS AND SYMBOLS

%	Percent sign
^{0}C	Degree Celsius
BBSRC	Biotechnology and Biological Sciences Research Centre
BHK	Baby Hamster Kidney
Ca	Antigen Control
cDNA	Complementary Deoxyribonucleic Acid
CIDLID	Combating Infectious Diseases of Livestock
CVMBS	College of Veterinary Medicine and Biomedical Sciences
DIVA	Differentiation between Infected and Vaccinated Animals
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
DRC	Democratic Republic of Congo
EA	East Africa
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
FMD	Foot-and-Mouth Disease
FMDV	Foot-and-mouth disease virus
g	Relative centrifugal force
IBRS2	Instituto Biologico - Rim Suino- 2
IFPHT	Intermediate Fellowship in Public Health and Tropical Medicine
IRES	Internal Ribosomal Entry Site
Kbp	Kilo basepair
Lpro	Leader Proteinase
MC	Municipal Council
MEGA	Molecular Evolutionary Genetics Analysis
nm	Nanometer
NSP	Non Structural Protein
nt	Nucleotide
OIE	Office International des Epizooties
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
POCT	Point-of-care-test
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SAT	Southern African Territories
SVD	Swine Vesicular Disease

TAE	Tris-acetate EDTA(buffer)
ul	Microliter
UTR	Untranslated Region
v/v	Volume by volume
VES	Vesicular Exanthema Of Swine
VNT	Viral Neutralization Test
VP	Viral Protein
VS	Veterinary Services
VS	Vesicular Stomatitis
WGS	Whole-Genome Sequencing

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Foot-and-mouth disease (FMD) is the most contagious disease that affects cloven-hoofed domesticated livestock (e.g. cattle, sheep, pigs and goats) and wildlife species. The disease is caused by the foot-and-mouth disease virus (FMDV) which is the prototype member of the genus *Aphthovirus*, family *Picornaviridae* and exists in seven genetically distinct serotypes (A, O, C, Asia 1 and Southern African Territories [SAT] 1, SAT 2 and SAT 3) (Freimanis *et al*., 2016). The seven identified serotypes of FMDV cluster into diverse genetic lineages with approximately 30-50% variation in the VP1 region (Knowles and Samuel, 2003).

The RNA genome of FMDV is enclosed in a protein capsid that is formed by sixty copies of different four structural proteins (VP1-4). It encodes for the synthesis of four structural proteins (VP1-4) of which the exposed VP1-3 are on the surface containing the neutralizing epitopes while the VP4 is internal and has eight nonstructural proteins (Mahapatra *et al*., 2017). FMDV genome contains a single long open reading frame (ORF) of about 7000 nucleotides (nt) long followed by a 3’ untranslated region (UTR; about 100 nt) and a poly (A) tail. The RNAs of other picornaviruses have a long 5’UTR but FMDV RNA is featured with a very large 5’ UTR of about 1300 nt (Belsham, 2005).

High genetic and antigenic variations of FMDV are common features of RNA viruses since their RNA polymerase lacks the proof reading ability which results into novel genetic and antigenic variants that emerge within each of the six recently observed

circulating serotypes leading to limitations of cross-protective immunity between some strains, even within the same serotype which brings challenges in developing vaccines that can provide a broad range of both intra and inter-serotypic protection (Longjam and Tayo, 2011b; Bari *et al*., 2015). Currently, broad-range protection is one of the major goals underpinning current research in FMDV vaccine development, making it vitally important to identify those areas of the capsid that are targets for protective immunity (Reeve *et al*., 2016). FMD clinical signs vary between species in which nfected animals are characterized with vesicles on the feet, in and around mouth, and on the mammary gland; these vesicles may also occur to other locations such as vulva, prepuce, or pressure points on the legs and other sites and when they rupture they turn into erosions which bring pain, discomfort, depression, anorexia, excessive salivation, lameness and reluctance to move or rise (Davies, 2002).

Due to existence of multiple serotypes of FMDV in circulation, it is a prerequisite and vital to identify the serotypes causing outbreaks in a particular region so as to select the most appropriate antigens for incorporation in a vaccine preparation. The most important immunogenic site of FMDV is the VP1 surface antigen encoded by the 1D region (Feng *et al*., 2003).

Four serotypes are known to circulate in East Africa and vaccination has been playing a major role in controlling the disease although currently the existing vaccines have been faced with challenges of failing to offer effective protection to the circulating field strains (Bari *et al*., 2014).

Effective vaccines and stringent control measures have enabled FMD eradication in most developed countries, which maintain unvaccinated, seronegative herds in compliance with

strict international trade policies. However, the disease remains enzootic in many regions of the world, posing a serious problem for commercial trade with FMD-free countries (Carrillo *et al*., 2005). Currently, measures for disease control include slaughter of infected and in-contact animals, restriction of animal movement, and vaccination. However, an FMD-free country that has an outbreak might not use vaccination to control the disease because under current OIE policy the use of vaccination delays a return to FMD-free status as compared to only stamping out (Grubman *et al*., 2008).

Both legal and illegal movements of infected animals and their products from one geographical location to another has played an important role in the spread of FMDV (Knowles *et al.,* 2005). FMD outbreaks spread easily from farm to farm, either through movement of animals or animal products, on personnel, transport vehicles, a few to mention (Grubman and Mason, 2002). In Tanzania, the first FMD outbreaks were reported in 1927 and FMDV was isolated for the first time in 1954 (Kasanga *et al*., 2015). In Tanzania mainland, FMD virus serotypes O, A, SAT-1 and SAT-2 have been reported to be endemic and between the years 1954 and 1970, serotypes O and A were regularly reported to cause outbreaks with a fluctuating incidence (Kivaria, 2003).

The disease causes major economic impact due to severe losses of productivity and to the restrictions imposed to the international trade of animals and animal products from FMD aected regions as well as prevention and control by sanitary profilaxis and vaccination of susceptible animals in endemic areas (Cacciabue *et al*.,2017). The disease brings impacts characterized by loses especially in dairy and pig industries as well as high mortality rates in young animals (Kitching *et al*., 2007).

Effective control strategies require knowledge of FMD distribution and epidemiology, the deployment of simple point-of-care test (POCT) platforms for active FMDV detection, monitoring and characterization remains an ongoing research effort (Howson *et al*., 2017). In order to effectively initiate control measures for FMD, the following must be identified: origin of infection, links between outbreaks, extent of genetic variation of the causative viruses, and antigenic relationship of field isolates to the available vaccines (Ayelet *et al*., 2009). In Tanzania, quarantine, restriction of animal movements especially in areas with well-defined farming systems and vaccination are the implemented means which are employed in order to limit the spread and economic impact of the disease (Kivaria, 2003).

Recently, the efforts to control this disease have not been very effective because there is deficiency of knowledge on the actual FMDV serotypes and subtypes (topotypes) which are circulating in the regions hence this knowledge is very vital for successful regional disease control strategies (Kasanga *et al*., 2015). It has also been described that there are several factors contributing to difficulties in controlling the disease such as; its high contagiousness, wide geographical distribution, broad host range, ability to establish carrier status, antigenic diversity leading to poor cross-immunity, and relatively short time of immunity together with poor surveillance and diagnostic facilities as well as insufficient control programs. All these have been stated as major problems in control of this disease in the country (Longjam *et al*., 2011).

Current improvements in sequencing technologies have played a driving role in the application of both partial (viral protein 1(VP1)) and whole-genome sequencing (WGS) to discourse questions relating to FMDV circulation in field locations, and recognition of the origins of FMD outbreaks (Freimanis *et al*., 2016). With the extensive employment of phylogenetic techniques, genetic classifications become more appropriate for serological

classification and subtype classification of FMDV (Zhang *et al*., 2015). In brief, antigenic diversity has been causing a serious effect in vaccine design by failing to confer protection against FMD hence; this study highlights and suggests the use of vaccines strains that match with the circulating field strains.

1.2 Problem Statement and Justification

Globally, most outbreaks of FMD are caused by serotype O followed in frequency by serotype A (Knowles and Samuel, 2003) which is endemic in many developing countries of Africa and Asia. It has been reported that FMD is endemic in East Africa with five circulating serotypes (O, A, SAT1, SAT2 and SAT3) categorized under pool 4 (Paton *et al*., 2009). The main serotype O topotypes found in East Africa are East Africa- 1 (EA-1), EA-2, EA-3 and EA-4 (Lloyd-jones *et al*., 2017) while the circulating genotypes of serotype A are I, II, IV and VII (Bari *et al*., 2014).

In Tanzania, sporadic outbreaks of FMD have been caused by serotype O, A, SAT 1 and SAT 2 (Kasanga *et al*., 2012; Kasanga *et al*., 2015). As RNA virus, it is characterized by the frequent emergence of new variants responsible for recurring disease outbreaks (Bari *et al*., 2015) due to the high feature of genetic variability on the viral protein capsid (Ferretti *et al*., 2018). Due to antigenic diversity, there is no cross protection of vaccine within FMDV serotypes and topotypes hence studying the antigenic heterogeneity characteristics of virus strains becomes necessary to ensure suggestion and selection of appropriate vaccines for control of FMD outbreaks in East African regions.

1.2.1 Research questions

i. What are the chances of detecting FMDV genome following FMDV outbreak?

ii. What are the genotypes of serotypes A and O FMDV recently detected in Tanzania?
iii. What is the genetic diversity of serotypes A and O FMDV with time and space?

1.3 Objectives

1.3.1 General objective

To perform molecular investigation in order to determine the evolutionary characteristics of foot-and-mouth disease virus serotypes A and O of recently circulating in Tanzania.

1.3.2 Specific objectives

i. To assess the presence of FMD virus genome following 2019 FMDV sporadic outbreaks in Tanzania.
ii. To determine the genotype of serotype A and O FMDV samples collected in selected areas of Tanzania.
iii. To determine the genetic diversity of FMDV taking into account of time and space.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Foot – and – Mouth Disease (FMD)

FMD is a highly infectious disease infecting domestic cloven-hoofed animals as well as many wild species. The outbreaks cause the countries to suffer economically due to loses because of low animal productivity as well as posed restrictions to international trade of both animals and animal products (Ali *et al*., 2018). FMD is distributed worldwide and is causing major global animal health problem. The World Organization for Animal Health has documented FMD as one among the identified notifiable disease because it leads to serious economic devastation of the livestock industry (Longjam and Tayo, 2011).

2.2 Foot-and-mouth Disease Virus and Genome organization

2.2.1 Taxonomy and classification of FMDV

The etiology of FMD is a virus (FMDV) which belongs to genus *Aphthovirus*, family *Picornaviridae*. This virus exists as seven genetically distinct serotypes or strains (A, O, C, Asia1, Southern African Territories SAT1, SAT2 and SAT3), distributed on three continents: Asia, Africa and South America (Freimanis *et al*., 2016). Molecular studies have shown that all seven serotypes of FMDV cluster into immunogenically distinct genetic lineages with the difference of about 30-50% in the VP1 region (Knowles and Samuel, 2003). In that purpose, different topotypes defined as geographically clustered viruses form a single genetic lineage generally sharing >85% (O, A, C and Asia 1) or

>80% (SAT1, SAT2 and SAT3) nucleotide identity in the VP1 coding region (Ayelet *et al*., 2009).

2.2.2 FMDV structure and genome organization

FMDV is a non-enveloped virus with a positive sense, single-stranded RNA genome of about 8 300 nucleotides which is surrounded by a protein shell or capsid composed of 60 copies of the capsomers (Figure 1). It is roughly spherical in shape and about 25–30 nm in diameter (Jamal and Belsham, 2013; Ibrahim *et al*., 2014). Its genome contains a single long open reading frame (ORF) of ~7000 nucleotides (nt) and encodes for RNA–dependent RNA polymerase (Carrillo *et al*., 2005; Freimanis *et al*., 2016) as well as a single 260 K polyprotein which is encoded between a major translation initiation site next to the poly (C) tract and the 3'-end of the RNA which undergoes post-translational cleavage into 4 capsid proteins (VP1–4) and 10 non-structural proteins (NSP; leader proteinase (Lpro), 2A, 2B, 2C, 3A, 3B1VPg1, 3B2VPg2, 3B3VPg3, 3Cpro and 3Dpol), bounded by 5′ and 3′ untranslated regions (UTRs) (Carrillo *et al*., 2005). The virus is known to be very fast when multiplying and two non-structural play an important role in virus replication (Chakraborty *et al.*, 2014). Apparently, the genome can be categorized into three main regions: (a) 5; noncoding regulatory region, (b) polyprotein coding region (subdivided into L, P1, P2 and P3) and (c) 3’ noncoding regulatory region (Longjam *et al*., 2011). The 3D protein which is the RNA –dependent RNA polymerase is very important for FMDV genome replication and in that case, the positive sense genome act as a template for synthesizing the anti-sense RNA which will be used for the production of the new infectious genomes (Belsham, 2005).

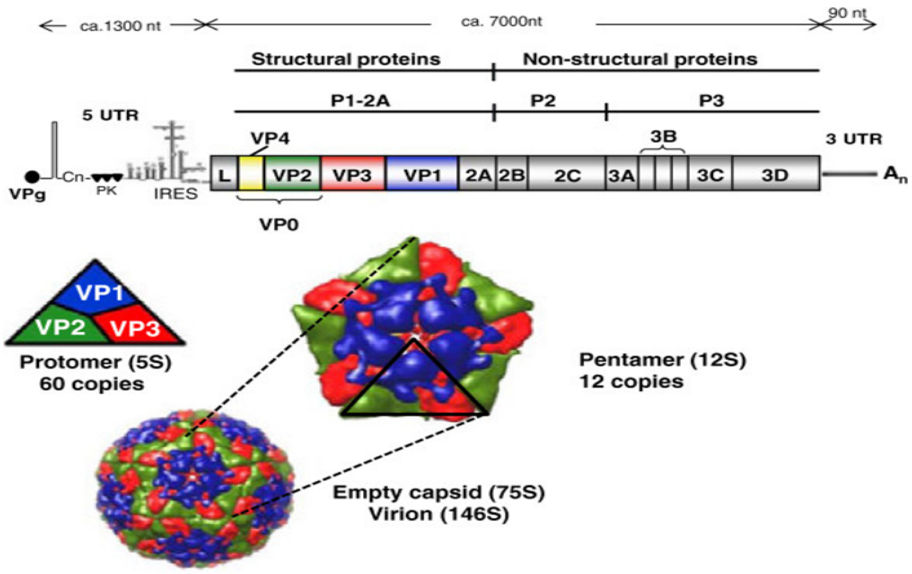

Figure 1: Genome organization of FMDV and the structure of virus. The FMDV genome includes a single large ORF, indicated by the shaded rectangle. (Source: Jamal and Belsham, 2013).

2.3 Antigenic Diversity

Virus antigenicity depends highly on the capsid coating proteins which are VP1-3 (Chakrabotry *et al*., 2014). During virus replication the RNA-dependent RNA polymerase lacks proofreading mechanism as a result newly formed viruses evolve with new features of genetic variation as well as antigenic heterogeneity in their important sites of the VP1-3 which may improve viral fitness (Bari *et al*., 2015). Studies have indicated that the genetic diversity of a virus can occur in a single animal and the rate of change will increase when the virus is transmitted to another animal (Volsoo *et al*., 1996). This leads to serious problems in designing vaccines since there should be an inclusion of multiple independent epitopes in synthetic vaccines so as to decrease the chances of selection of FMDV that are resistant to immune response (Longjam and Tayo, 2011b). Similarly, the strength of cross protection among distinct topotypes of the same serotypes varies significantly, it is very important to understand the circulating strains in a particular region because new variants

keep on evolving hence the need to improve criteria for selecting vaccine strains (Grubman and Mason, 2002).

2.4 Vaccination

It is very important to understand the concept that vaccinating an animal against one serotype of FMDV does not confer protection against other serotypes due to its nature of heterogeneity and this may entirely fail to protect an animal against strains of the same serotype leaving it highly susceptible to infection (Belsham, 2020). In regions where vaccination is implemented as a control measure, it is very crucial to match the vaccine strain with the circulating field isolate (Mahapatra and Parida, 2018). Vaccination of cattle is normally conducted twice from the age when the maternal immunity no longer interferes with the development of active immunity and then every four or six months depending on the exposure risk to infection. Vaccine strains differ between country to country depending on the field circulating strain (Kitching *et al*., 2007).

2.5 Virus – Host Interaction

Most members of the Picornaviridae have the ability to shut off host transcription and cap-dependent translation and they replicate very efficiently in tissue culture which is similar in infected animals as the virus replicates very rapidly at the host's initial site of infection particularly in the respiratory system (Grubman *et al*., 2008).

Similarly, in infected animals, the virus rapidly replicates at the initial site of infection in the respiratory system and spreads to its natural sites of predilection in oral and pedal epithelial regions. In order to achieve this, the virus must evade the immune system by interfering with the host innate immune response which is the first-line of host defense

specified to confine infection in early hours after exposure to infection. Several studies have been conducted and revealed that FMDV infection also has the ability to sabotage the development of the host adaptive immune response (Grubman *et al*., 2008).

2.6 Role of Carriers and Wildlife in FMD

Development of viremia normally occurs when the animals encounter FMDV and can be noticed within few days of exposure where clinical signs commonly last for one to two weeks whereas ersistent infection occurs and last for years in carrier animals, often in buffaloes and other species where the infection progresses in a subclinical condition (Ferretti *et al.*, 2018).

2.6.1 Role of carriers

The occurrence of persistent infection in both domestic and wild animals (cattle and buffalo and to a lesser extent sheep and goats), is a common development to both clinical and subclinical Foot-and-mouth disease and also normally befalls in vaccinated animals that come in a close contact with FMDV, of which the protective levels of circulating antibody restrict the replication of the virus to the oropharynx (Salt, 1993).

2.6.2 Role of wildlife

Wildlife plays a significant role in the circulation of FMDV in Africa where several serotypes may be involved. Studies conducted in West and Central Africa reported the presence of serotype specific antibodies against type C, O and SAT 3 in buffalo samples (Di Nardo *et al.*, 2015). Animals which have shown long-term infections are mentioned to as persistently infected animals or carriers and at 28 days and more after the infection, the virus can be detected from oesophago-pharyngeal scrapings of these animals (Wekesa

et al., 2015). FMD is endemic in most countries of sub-Sahara Africa which involves the circulation of six among the seven FMDV serotypes in the African regions (Vosloo *et al*., 2002). In Africa, African buffaloes (*Syncerus caffer*) play a role in maintenance and transmission of FMD virus thus an important role in the epidemiology of the disease (Bastos *et al*., 2003).

2.7 Epidemiology of FMD

Foot-and-mouth disease is endemic and spread globally in parts of Asia, Africa, the Middle East and South America. The virus exist as seven antigenically distinct serotypes of which serotype O is the most common and found worldwide (Reid *et al*., 2002). These serotypes have shown unequal distribution across the world where, serotypes O and A are broadly distributed which cause outbreaks in many parts of Africa, Southern Asia, the Far East (not type A) and South America, SAT viruses are normally restricted in Sub-Saharan Africa. Countries reported to be free of FMD are; North and Central America, New Zealand, Australia, Greenland, Iceland and Western Europe. These FMDV serotypes cluster into type-specific subtypes when comparing either nucleotide or amino acid sequences (Knowles and Samuel, 2003). Currently, FMDV infection burden is maintained within Asia, Africa and South America that can be further grouped into seven pools (Figure 2) of viral infection; the FMDV pools include pool 1 in East Asia (O, A and Asia 1), pool 2 in Central Asia (O, A and Asia 1), pool 3 in Europe and South Asia (O, A and Asia 1), pool 4 in Southern, Eastern and Horn of Africa (A, O, SAT 1, 2 and 3), pool 5 in Western Africa (O, A, SAT 1 and 2), pool 6 in Southern Africa (SAT 1, 2 and 3) and pool 7 in South America (A,O) (Paton *et al*., 2009).

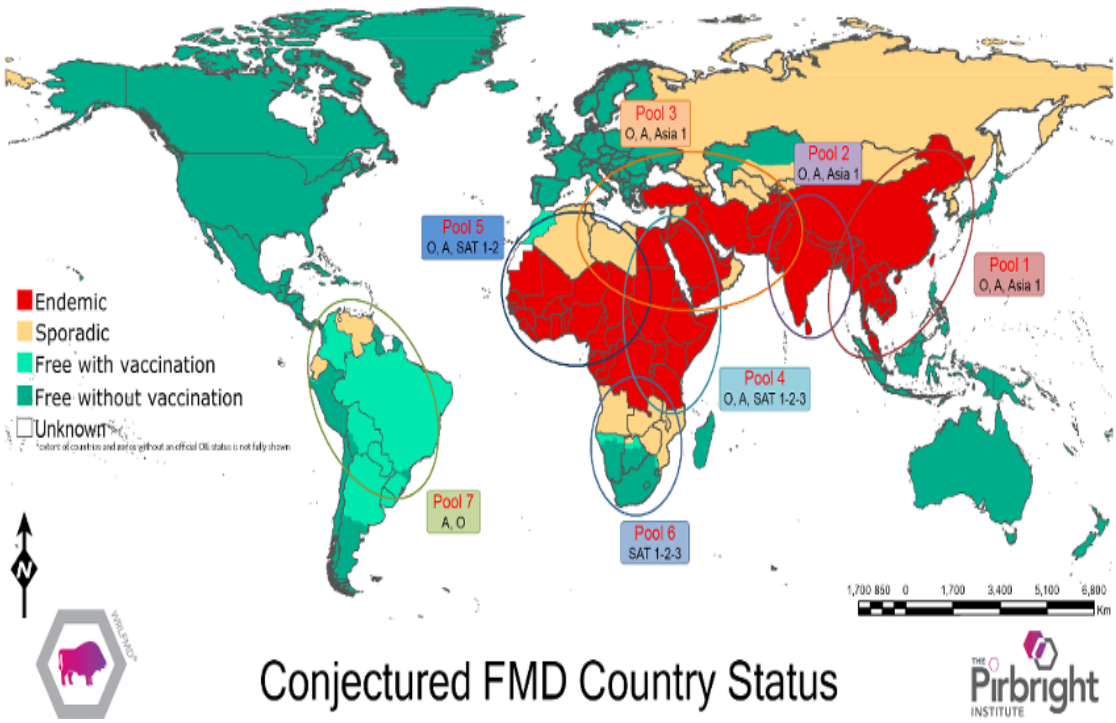

Figure 2: Global distribution of FMDV serotypes (Source: Freimanis *et al.*, 2016)

2.8 Molecular Epidemiology of FMDV in Tanzania

FMD outbreaks occur throughout East Africa with cases being reported and recorded every year in Parts of Tanzania and Uganda where serotype O is the most predominant and serotype A being widely distributed in the regions (Kerfua *et al.*, 2018). The first FMD outbreak in Tanzania was documented in 1927 and the first virus isolation was in 1954 and since then outbreaks have repeatedly occurred in cattle and other susceptible animals (Kasanga *et al.*, 2015).

According to findings, it was reported that the three serotypes (O, SAT1 and SAT2) were detected regularly every year from 2003 to 2010 (Kasanga *et al*., 2012). However, VP1 sequence data have revealed the presence of four serotypes O, A, SAT1 and SAT2 that have been circulating in Tanzania between 1967 and 2009 (Kasanga *et al.*, 2015). Recently, molecular characterization of FMDV in Tanzania has been routine and outbreaks have been documented.

2.9 Clinical Signs and Disease Manifestation

Disease severance varies according to susceptible host species, the serotype and strain of the virus involved as a result farmers may observe depression and exhaustion, rapid loss of condition, moderate or instant drop in milk production either temporarily or permanently, chronic mastitis, reduced growth rate, loss of weight, infertility and poor body condition since the disease is featured by high rise of body temperature, formation of vesicles (blisters) on the mouth, tongue, muzzle, snout, teats, nose, inter digital space of feet and other hairless parts of skin which leads to off-feeding and lameness (Chakraborty *et al.*, 2014).

In animals such as pigs and cattle, viremia and fever starts within 24 to 48 hours of epithelial infection hence viral spread into different organs and tissues (Longjam *et al*., 2011a). Abortions in infected animals may not directly caused by virus replication rather due to elevation of temperature (Kitching, 2002).

2.10 Control of FMD

Regional cooperation with appropriate reporting information on the FMD outbreaks mainly in border areas, timely distribution of information and synchronized adoption of

control measures are needed for effective control of this contagious transboundary disease in line with preparations for detection and responses must be timely for effective containment of the disease (Jamal and Belsham, 2013). Countries in different regions of world adopt FMD control policies depending on the epidemiology of disease. In FMD free countries, slaughter of all infected and susceptible in contact animals, quarantine of infected animals, strict animal and animal product import regulation and animal movement restrictions are practiced (Depa *et al*., 2012).

2.11 Importance of FMD

The importance of FMD is observed on the impacts left after the outbreak has occurred as it causes high mortality rates in young animals compared to the adult ones, restriction to international trade of animals and animal products, poor milk production, permanent damage of animal hooves as well as permanent mastitis leading to short and long term socio-economic impacts such as disruptions of animal feed, veterinary pharmaceutical and tourism associated industries (Kitching *et al*., 2007).

2.12 Diagnosis

Fast and reliable diagnosis of the disease is essential for the implementation of control measures to prevent the spread of the disease. The need is for tests which can give a rapid, accurate result in less than 24 hours (Reid *et al*., 2002).

2.12.1 Clinical diagnosis

The clinical signs, including high temperature, excessive salivation, and formation of vesicles on the oral mucosa, on the nose plus the inter-digital spaces and coronary bands on the feet can be confused with other diseases (e.g. vesicular stomatitis and swine

vesicular disease) and thus laboratory-based diagnosis and determination of the serotype involved in field outbreaks has to be established to permit proper control/ vaccination programs (Jamal and Belsham, 2013).

2.12.2 Laboratory diagnosis

2.12.2.1 Virus neutralization test

The virus neutralization test (VNT) is presently 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). VNT is slower, subject to contamination and requires restrictive biocontainment facilities in contrast to other serological tests which can use inactivated viruses as antigens and various primary cells and cell lines with variable degrees of sensitivities used in the VNTs, are more prone to variability than other serological tests (Jamal and Belsham, 2013).

2.12.2.2 Virus isolation

Virus isolation requires the presence of infectious virus, which depends on sample quality where up to four days may be required to demonstrate the presence of virus, especially when the levels of virus are low (thus it also takes four days to be confident, using this methodology, that no virus is present). Moreover, some FMDVs fail to grow in a specific cell type thus, the absence of apparent growth does not guarantee absence of the virus and therefore samples collected from a suspected case of FMD should be subjected to further investigations, e.g. using another testing system. Additional disadvantages include the problems associated with obtaining and maintaining a regular supply of cells; possible

contamination of cell cultures and the necessity to confirm any apparent virus growth by ELISA (Jamal and Belsham, 2013).

2.12.2.3 Structural and non structural protein (NPS) antibody test

Detection of animals that have been infected with FMDV is of considerable importance for the control of FMD especially in a previously FMD free country or in a country with sporadic outbreaks although both previously infected and vaccinated animals can have neutralizing antibodies in their sera, but it is important for trade purposes to be able to distinguish previously infected animals from those that have been vaccinated against the disease and this is because a high proportion (up to 50%) of animals infected with FMDV can become carrier animals which continue to have infectious virus present within the oropharynx more than 28 days post-infection (Jamal and Belsham, 2013).

2.12.2.4 Reverse transcription-polymerase chain reaction (RT-PCR)

PCR assays are very appropriate for detection and identification of many infectious agents including FMD virus and they use wide range of different samples that is blood tissue, swabs esophageal pharyngeal (OP), scraping feacal sample and milk (King *et al*., 2012). Several RT-PCR techniques have been applied 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. Typing of FMDV by RT-PCR was first demonstrated for the differentiation of the serotypes O, A and C. Designing of serotype specific primers has been achieved for the detection of all seven FMDV serotypes by RT-PCR as they target various regions of the virus genome, including the 5′ UTR, the open reading frame and the 3′ UTR (Jamal and Belsham, 2013).

2.13 Characterization of FMDV below the Level of Serotype (Strains/Subtypes)

Nucleotide sequence analysis is now a decisive method for characterization of FMDV strains. The first study to conduct FMDV phylogenetic analysis using gene encoding capsid protein for VP1 was reported by Dopazo *et al*. (1988). Since then a number of studies have been published on nucleotide sequence analysis for all the seven serotypes of FMDV, some of these have used complete genome sequences for tracing of outbreaks (Jamal and Belsham, 2013). On the course of an outbreak, generation of full FMDV genome sequences is important in the explication of epidemiology data in the field and directly impact measures in controlling the spread of the disease (Cottam *et al*., 2008).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

This study was carried out in Tanzania (Morogoro, Butiama, Kibaha, Sengerema and Musoma) as described in Figure 3. Geographically, Tanzania is among the sub-Saharan countries located in East Africa. The country covers an area of 945 000km^2 whose climate varies from tropical to temperate. It is located between latitude and longitude of -6.369 and 34.8888 respectively. Tanzania borders with Burundi Rwanda and Democratic Republic of Congo (DRC) in the West, Kenya and Uganda in the North, Mozambique, Malawi and Zambia in the south and Indian Ocean in the East.

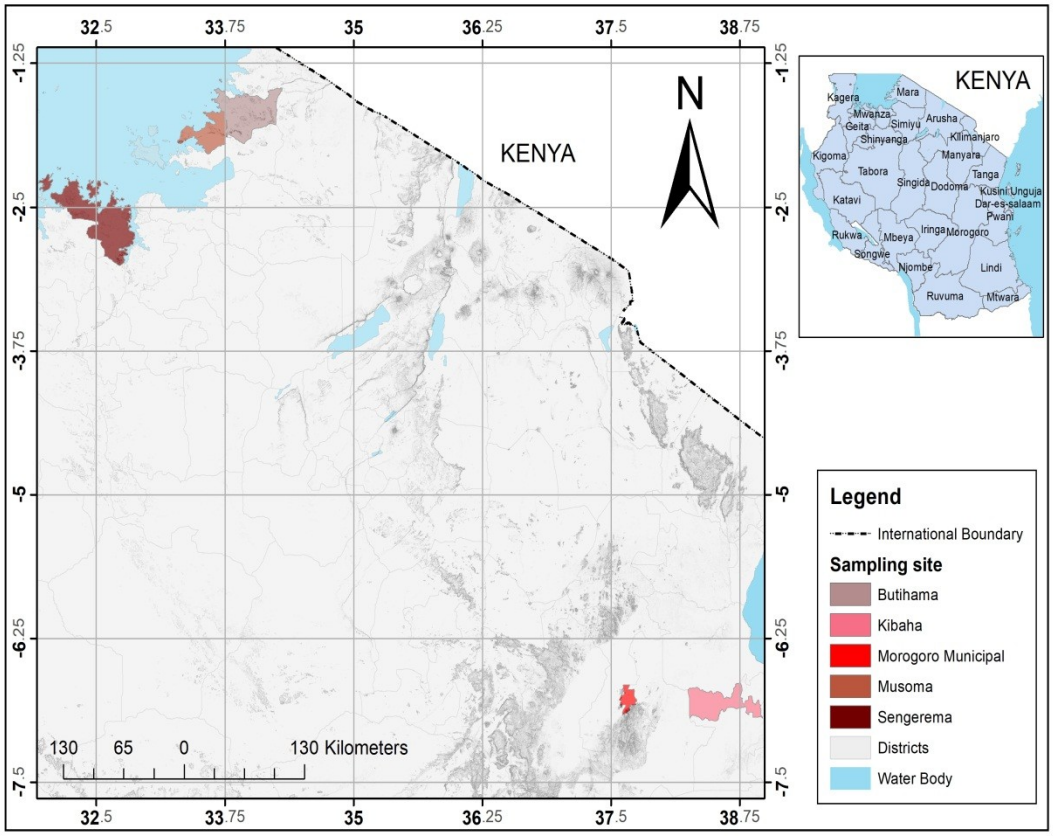

Figure 3: Map of Tanzania illustrating the sampling areas.

3.2 Study Design and Sampling Approach

The design of this research is a cross section study which based on purposive sampling from the five selected areas. Cattle and one pig showing clinical signs of FMD were purposively selected. This study utilized both *in vitro* and *in-silico* experimentation. Laboratory work was conducted at the College of Veterinary Medicine and Biomedical Sciences (CVMBS) in the Molecular Biology and Biotechnology laboratory for viral research and training at the Department of Microbiology, Parasitology and Biotechnology at Sokoine University of Agriculture, Morogoro.

3.3 Sample Size

A total of thirty six (n=36) archived epithelial tissue samples were used and tested during the study. All of these samples originated from oral and foot epithelial tissues. These samples were collected during outbreak cases from February to August 2019 in Tanzania and were stored at Molecular biology and biotechnology Laboratory for FMDV at College of Veterinary Medicine and Biomedical Sciences.

3.4 Sample Description and Preparation

3.4.1 Description of archived samples

A total of thirty - six archived epithelium tissues collected from oral and foot lesions from all suspected FMD infected cattle in different locations between February and August 2019 were used in this study. Morogoro municipality (n = 16), Sengerema (n =9), Butiama (n = 1), Musoma municipality (n = 2) and Kibaha (n = 8). In the field, the collected tissues were immediately placed in a virus transport media composed of equal amount of sterile glycerol (50% v/v) and nuclease free water 50% (v/v).

3.4.2 Preparation of samples

10% epithelial tissue suspension was prepared by grinding 10g of epithelial tissue into 1000µl of Phosphate-buffered saline (PBS) using sterile mortar and pestle. The suspension was used for RNA extraction. The suspension was stored under -80^{0}C freezer but before doing RNA extraction the samples were transferred to a -40^{0}C freezer.

3.5 Laboratory Analysis of Samples

3.5.1 RNA extraction

Total RNA was extracted from 460ul of 10% epithelial tissue suspension using Qiagen RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction as follows: 460µl of epithelial suspension was mixed with 460µl of lysis buffer RLT (containing 1% 2 mercaptoethanol in an Eppendorf tube and vortexed. 460µl 70% ethanol was also added to the same tube and the mixture was vortexed. The maximum loading volume (700µl) was loaded into the RNeasy spin column and centrifuged at 12 000 rpm (7 000-10 000g) for 15 seconds. The flow-through was discarded and the procedure was repeated with the remaining volume using the same collection tube. Volume of 700µl buffer RW1 was added and centrifuged at 12 000 rpm for 15 seconds and the flow-through was discarded. This was followed by washing using 500µl wash buffer RPE and the collection tube with the flow-through was discarded. The washing was repeated with 500µl and centrifuged at 12 000 rpm for 2 minutes to ensure the membrane is dried. Both the flow-through and the collection tube were discarded and the column was transferred to a new collection tube and centrifuged at maximum speed for 1 minute to remove any ethanol traces. Finally, RNA was eluted with 50µl of nuclease free water into a new 1.5ml tube, centrifuged for 60 seconds at 12 000rpm and stored at -40°C until further use.

3.5.2 One step reverse transcription PCR

Extracted RNA was subjected to RT-PCR using Super Qiagen®One Step RT-PCR kit (Hilden, Germany) following manufacturer's instructions and Pan-serotypic primers (gene specific primers) were employed to detect the 5' UTR of FMDV genome. PCR diagnostic assays were deployed to amplify cDNA fragments of the FMDV 5' untranslated region (5' UTR) to confirm the presence of FMDV which was responsible for the outbreak. A

total of 36 epithelial samples from five different locations of outbreak area were purposively selected for confirmatory diagnosis by RT-PCR test. At first, all the 36 samples were screened for FMDV by the universal primer of 5' UTR.

The reaction master mix was prepared in a dedicated PCR clean room (to avoid possible contamination). The reaction mix per one reaction was as follows: nuclease free water 8.0 µl, forward primer 2.5 µl, reverse primer 5.0 µl, dNTPs 1.0 µl, buffer 5 µl and enzyme 1.0 µl. The reaction mix for the thirty six reactions was prepared in the clean room and the final volume per reaction as 22.5 µl plus 2.5 µl of the viral RNA which was added to the RT-PCR tube in a dedicated template room. The tubes containing the reactions mixtures and RNA templates were placed in a thermocycler (GeneAMP® PCR system 9700, Singapore). All the thermocycler conditions (Table 1) were set and Primers specific for targeting the FMDV highly conserved region (5 'UTR) were used (Table 2).

Table 1: RT-PCR conditions for FMDV genome detection

Conditions	Temperature	Time	No. of cycles
Reverse transcription	50^0C	30 min	1
Initial PCR activation	95^0C	15min	1
Denaturation	95^0C	60 sec	
Annealing	55^0C	60 sec	35
Extension	72^0C	120 sec	
Final	72^0C	5min	1
Hold at	4^0C	∞	

Table 2: List of all oligonucleotide primers used for FMDV detection by RT-PCR

Primer	Primer name	Primer sequence (5'-3')	Location	PCR product (bp)
Universal	1F	GCCTGGTCTTTCCAGGTCT	5' UTR	328
	1R	CCAGTCCCCTTCTCAGATC		

3.5.3 Gel electrophoresis

Standard 1.5% (w/v) was prepared by dissolving 1.5 g agarose in 100 ml 1× TAE (Tris base, acetic acid and EDTA) electrophoresis buffer in an Erlenmeyer flask. The mixture was heated in a microwave to allow the agarose to dissolve and form a gel which was allowed to cool to about 20 °C before adding 10µl of EZ-vision® Bluelight DNA dye. The gel was then poured into a horizontal gel tray fitted with appropriate combs. After about 40 minutes of gel polymerisation, the combs were carefully removed and the tray immersed in an electrophoresis tank containing electrophoresis buffer (1× TAE). Then 5µl of DNA products of each individual sample was mixed with 1µl of bromophenol blue dye (Gel loading dye blue 6x) and then loaded into separate lanes (slots) of the submersed agarose gel. The samples were run at 100 volts for 45 min. After the run, the gel was removed and analysed under Utraviolet transmission (TFX 35M, 180W, France). DNA lader (Gene ruler 100 bp plus DNA ladder, Lithuania) was also loaded to gel lanes.

3.5.4 RT-PCR for serotyping

PCR reaction mixture per one reaction was prepared containing; nuclease free water 8 µl, Forward primer 2.5 µl (for serotype A and O), Reverse primer 5 µl (for serotype A and O), dNTPs 1 µl, buffer 5µl and enzyme 1 µl. The reaction mixture for the twenty seven reactions was prepared in the clean room and the final volume per reaction was 22.5 µl plus 2.5 µl from each template RNA. The PCR conditions for FMDV serotyping have been illustrated in table 3. In that aspect, two sets (forward and reverse) primers for both serotype A and O (Table 4) were used for typing a partial region of VP1 and the expected product size was detected on 1.5% gel electrophoresis for serotypes A and O.

Table 3: PCR conditions for serotyping of FMDV

Conditions	Temperature	Time	No. of cycles
Reverse transcription	50^0C	30 min	1
Initial PCR activation	95^0C	15min	1
Denaturation	95^0C	60 sec	
Annealing	60^0C	60 sec	35
Extension	72^0C	120 sec	
Final	72^0C	5min	1
Hold at	4^0C	∞	

Table 4: Oligonucleotide primers used in serotyping

Serotype	Primer name	Primer sequence (5'-3')	Sense
A	FMDV/A/EA/FP	GCCACRACCATCCACGA	+
	FMDV/A/EA/RP	GAAGGGCCCAGGGTTGGACTC	-
O	FMDV/O/EA/FP	CCTCCTTCAAYTACGGTG	+
	FMDV/O/EA/RP	GCCACAATCTTYTGTTTGTG	-

***(+) Represents forward primer **(-) represents reverse primer

3.5.5 Serotype specific RT-PCR for generating VP1

The serotype specific primers were used to anneal within the VP3 coding region (forward primers) and the 2B coding region (reverse primers) to amplify the full length of the FMDV VP1 coding region. This was achieved under specific thermocycler conditions in Table 5. To accommodate the sequence variability that can occur in the target region within respective serotypes, two primer sets for each serotype A and O were used as indicated in Table 6 (Knowles *et al*., 2016).

Table 5: Thermocycler conditions for generating VP1

Steps	Temperature	Time	No. of cycles
Reverse transcription	50^0C	30 min	1
Initial PCR activation	95^0C	15 min	1
Denaturation	95^0C	60 sec	
Annealing	50^0C	60 sec	25
Extension	72^0C	120 sec	
Final	75^0C	5 min	1

Table 6: List of all oligonucleotide primers used for generating VP1

Serotype	Primer name	Primer sequence (5'-3')	Gene	Gene Location	PCR product (bp)	Reference
A	A-1C562F	TACCAAATTACACACGGGAA	VP3	562-581	866	Knowles *et al*.,2016
	A-1C612F	TAGCGCCGGCAAAGACTTTGA		612-632	814	
O	O-1C272F	TBGCRGGNCTYGCCCAGTACTAC	VP 3	272-294	1135	Knowles *et al*.,2016
	O-C244F	GCAGCAAAACACATGTCAAACACCTT		244-269	1165	
A/O	EUR-2B52R	GACATGTCCTCCTGCATCTGGTTGAT	2B	52-77		Knowles *et al*.,2016

3.5.6 Purification of the PCR products

500 µl of capture buffer was added to the 100µl of the PCR products and well mixed. The GFX spin column was then placed in a 2ml collection tubes and the GXF spin tube was set to centrifuge at 13 000rpm for 60 seconds so as to bind DNA. The flow was discarded and the GFX spin column was placed back into the same tube. 0.5mls of Wash buffer Type 1 was added to the GFX column and set to centrifuge at 13 000 rpm for 60 seconds then the GFX column was transferred into a clean 1.5ml micro centrifuge tube. In order to elute DNA, 20µl of Elution buffer Type 6 was added to the center of the GFX membrane and set to centrifuge at 13 000rpm for 60 seconds to complete elution of bound DNA. This process was achieved using the GFX™ PCR DNA and Gel Band purification kit (Buckinghamshire, HP79NA UK).

3.6 Sequencing

3.6.1 Cycle sequencing

A total 90 µl reaction mixture was prepared for the amplification of the whole VP1 region. The reaction mixture consisted of nuclease free water 31.5µl, buffer 18µl, Big Dye 0.5µl,

Primer 27µl and DNA 1µl. The master mix was prepared in laboratory clean room and DNA was added in a PCR laboratory and the thermocycler conditions for cycle sequencing (Table 7) were set in a GeneAmp PCR System 9700 (Applied Biosystems). All amplicons were forward sequenced with the sequencing primers for that serotype and reverse sequenced with internal primer NK72 which is a universal reverse sequencing primer (Table 8).

Table 7: Thermocycler conditions for cycle sequencing

Steps	Temperature	Time	No. of cycles
Initial PCR activation	96^0C	60 seconds	1
Denaturation	96^0C	6 sec	
Annealing	50^0C	3 sec	25
Extension	60^0C	4 min	
Final	4^0C	∞	

Table 8: Primer sets used for Cyclesequencing

Primer	Primer sequence 5'- 3'	sense	Gene	Position	Reference
A-IC612F	TAGCGCCGGCAAAGACTTTGA	+	1C	2834-2854	Knowles *et al*., 2005
O-1C499F	TACGCGTACACCGCGTC	+	1C	2724-2740	
NK 72	GAAGGGCCCAGGGTTGGACTC	-	2A/2B	3558-3578	

3.6.2 Ethanol precipitation

In each of the reaction tube containing the products of sequencing PCR reaction, 5µl of 125 MM EDTA and 60µl of 100% alcohol were added, mixed well by vortexing and incubated in dark for 15 minutes at room temperature to precipitate the extension product. The tubes were centrifuged at 12 000rpm for 30 minutes and all the supernatant was discarded. 60µl of 70% ethanol was added to the pellets and tubes were vortexed again

before set to spin at 12 000rpm for 30 minutes. All the supernatant was removed and the DNA was vacu-dried for 15 minutes in the dark until no ethanol was present.

3.6.3 Sanger sequencing

Following ethanol precipitation, 20µl of Hi-Di Formamide (Applied biosystem, 7Kingsland Grange, UK) was added and the DNA pellet was allowed to resuspend for 15 minutes. The sample was loaded to the plate and the sequencing reactions were run according to the manufacturer's instructions in which purified PCR fragments were sequenced on both strands using primers in Table 8 under Big Dye terminator conditions to obtain the complete VP1 sequences using a Big dye Terminator V 3.1 kit (Applied Biosystems) and run on an automated DNA Sanger sequencer machine (ABI PRISM® 3500).

3.7 Data Analysis

3.7.1 Sequence editing and assembling

The obtained sequences of both forward and reverse strands were visually analyzed using GENIOUS 10.2.3 version computer software (Biomatter Ltd, USA) and assembled into contigs resulting in overlaps. The consensus nucleotide sequences were exported to MEGA 7 software and manually aligned using the same software. Multiple sequence alignments were made using ClustalW (Thompson *et al*., 1994) incorporated in Mega 7.0 (Kumar *et al*., 2016) software and the sequences trimmed to a usable 639 nucleotides covering almost the full VP1 of serotypes A and O.

3.7.2 Serotype identification using the query sequences

Computer-assisted comparisons of the nucleotide sequences was made to find the similarities of nucleotides sequences in the National Centre for Biotechnology Information (www.ncbi.nlm.nih.gov) using default search parameters of the BLASTN search program (Altschul *et al.,* 1990). This considered percentage identity per number of nucleotides as well as geographical locations, topology and the vaccine strains for foot-and-mouth disease.

3.7.3 Phylogenetic analysis

Phylogenetic trees of representatives of one topotype of FMDV type O and one genotype of FMDV type A were constructed using MEGA 7. Sequence alignments were used to construct distance matrices using the Kimura 2-parameter nucleotide substitution model (Kimura, 1980) as implemented in the programme MEGA 7.0 (Kumar *et al*., 2016). Unrooted Neighbour-joining trees were then constructed using MEGA 7.0. The robustness of the tree topology was assessed with 1000 bootstrap replicates as implemented in the program.

CHAPTER FOUR

4.0 RESULTS

4.1 FMDV Genome Detection

The results of this study indicated that twenty six samples (n = 26) representing 72% of the total samples (n=36) were positive for FMDV RNA. Out of sixteen (16) samples collected from Morogoro, 63% (n = 10) were positive for FMDV genome whereas 89% (n = 8) of samples collected from Sengerema were positive for FMDV. It was revealed

further that one (1) sample collected from Butiama was positive for FMDV genome as well. Out of two samples collected from Musoma one (50%) was positive. Eight (8) samples from Kibaha were analysed and 6 (75%) were positive for FMD (Table 9).

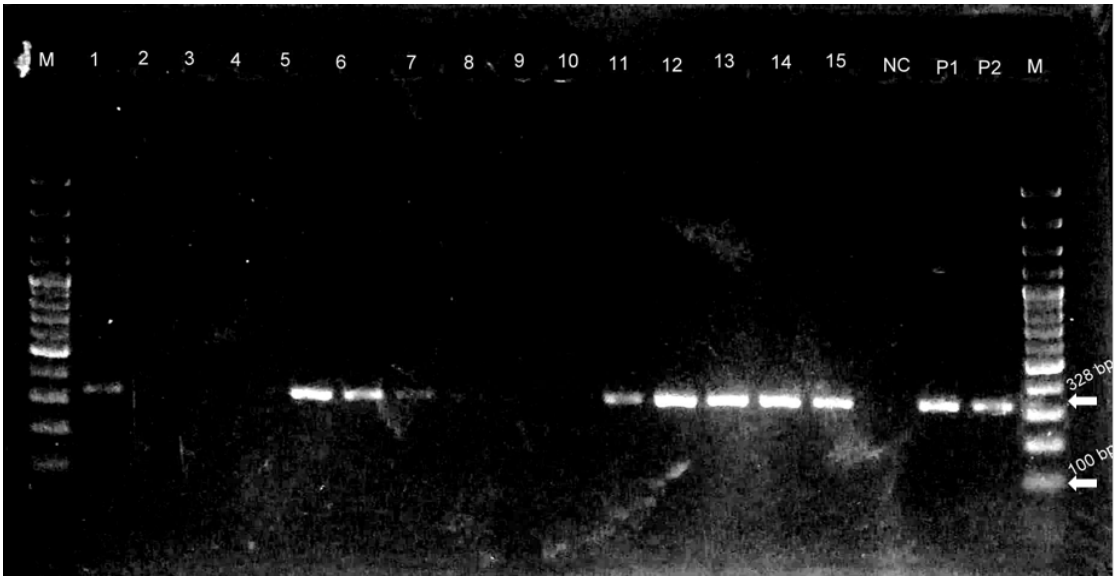

Figure 4: Gel electrophoresis of a 328 bp RT-PCR product. Lane M, DNA size marker (100-bp ladder); lane 1 – 2 were from Musoma, 3-6 were from Morogoro, 7-11 are from Sengerema, 12-14 were from Kibaha and lane 15 was from Butihama. NC was for Negative control, P1 and P2 were for Positive controls

Table 9: Description of RT-PCR results for FMDV genome detection, serotyping and Generation of VP1 region

Sample ID	Location	Source	RT-PCR	Serotyping	VP1 Sequencing	Base size
911	Morogoro	Bovine	-	-	-	-
912	Morogoro	Bovine	-	-	-	-
913	Morogoro	Bovine	-	-	-	-
914	Morogoro	Bovine	-	-	-	-
915	Sengerema	Porcine	+	O	+	328
916	Butiama	Bovine	+	A	-	328
917	Musoma Mc	Bovine	+	A	+	328
918	Musoma Mc	Bovine	-	-	-	-
919	Sengerema	Bovine	+	O	+	328
920	Sengerema	Bovine	+	O	+	328
921	Sengerema	Bovine	-	-	-	-
922	Sengerema	Bovine	+	A	-	328
923	Sengerema	Bovine	+	A	-	328

924	Sengerema	Bovine	+	A	-	328
925	Morogoro	Bovine	+	A	+	328
926	Morogoro	Bovine	+	A	-	328
927	Morogoro	Bovine	-	-	-	-
928	Morogoro	Bovine	+	A	+	328
929	Morogoro	Bovine	+	A	-	328
930	Morogoro	Bovine	+	A	+	328
931	Morogoro	Bovine	+	A	-	328
932	Morogoro	Bovine	+	A	-	328
933	Morogoro	Bovine	-	-	-	-
934	Morogoro	Bovine	+	A	-	328
935	Morogoro	Bovine	+	A	+	328
936	Morogoro	Bovine	+	A	-	328
937	Sengerema	Bovine	+	O	+	328
938	Sengerema	Bovine	+	A	-	328
939	Kibaha	Bovine	+	A	-	328
940	Kibaha	Bovine	-	-	-	-
941	Kibaha	Bovine	+	A	-	328
942	Kibaha	Bovine	+	A	-	328
943	Kibaha	Bovine	-	-	-	-
944	Kibaha	Bovine	+	A	-	328
945	Kibaha	Bovine	+	A	-	328
946	Kibaha	Bovine	+	A	-	328

*+ sign represents Positive **- sign represents Negative

4.2 Sequence Alignment and Identity

A total of 20 (four serotype O and 16 serotype A) positive samples were selected from the 26 FMDV positive typed samples and used for sequencing the VP1 coding region for respective viruses using appropriate primers. In this study, only nine (five out of 16 serotype A and all four serotype O) VP 1 sequences were able to be generated and formed contigs for molecular analysis (Table 9). The alignment of edited VP1 nucleotide sequences for serotype A strains revealed nucleotide identity ranging from 83.12% to 95.46% among the FMDV strains. Similarly, the nucleotide identity among the sequenced VP1 coding regions of serotype O FMD viruses indicated nucleotide identity of 85.83% to 95.31%. There was no significant region within VP1 that revealed consistency in either genetic diversity or similarity among all sequenced viruses.

4.3 Phylogeny

The phylogenetic analysis of 29 complete VP1 sequences of FMDV type A (five generated from this study and 24 from the Genbank) and 30 FMDV type O (four from this study and 26 from the Genbank) showed that the recent FMDV types A (Figure 5) and O (Figure 6) isolates from Tanzania (2019) formed a single genetic lineage which was distinct from the previous reported isolates.

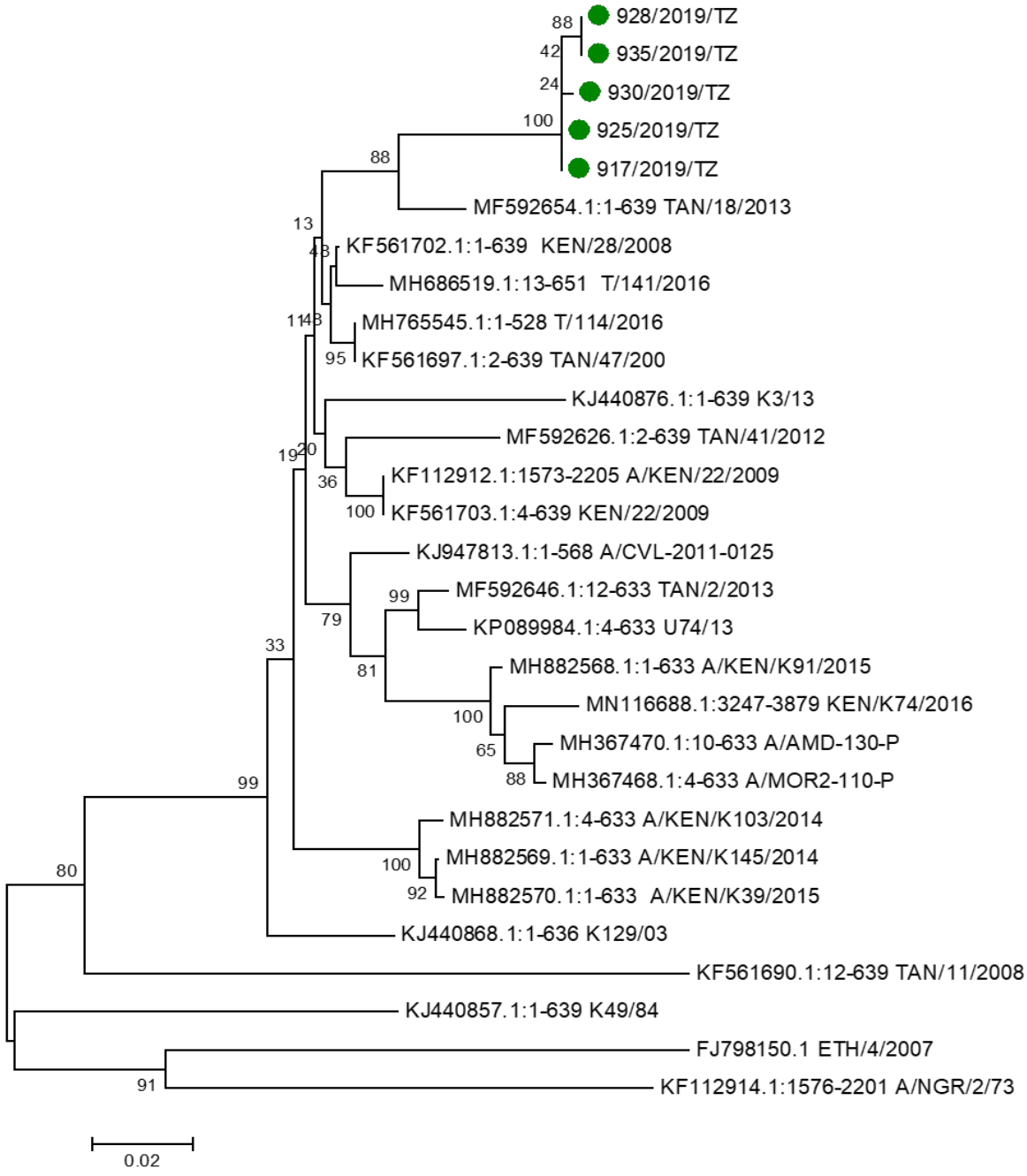

Figure 5: Neighbor-joining tree based on the virus protein VP1 of serotype A coding sequence. The sequences generated for this study are marked with green filled circle symbol.

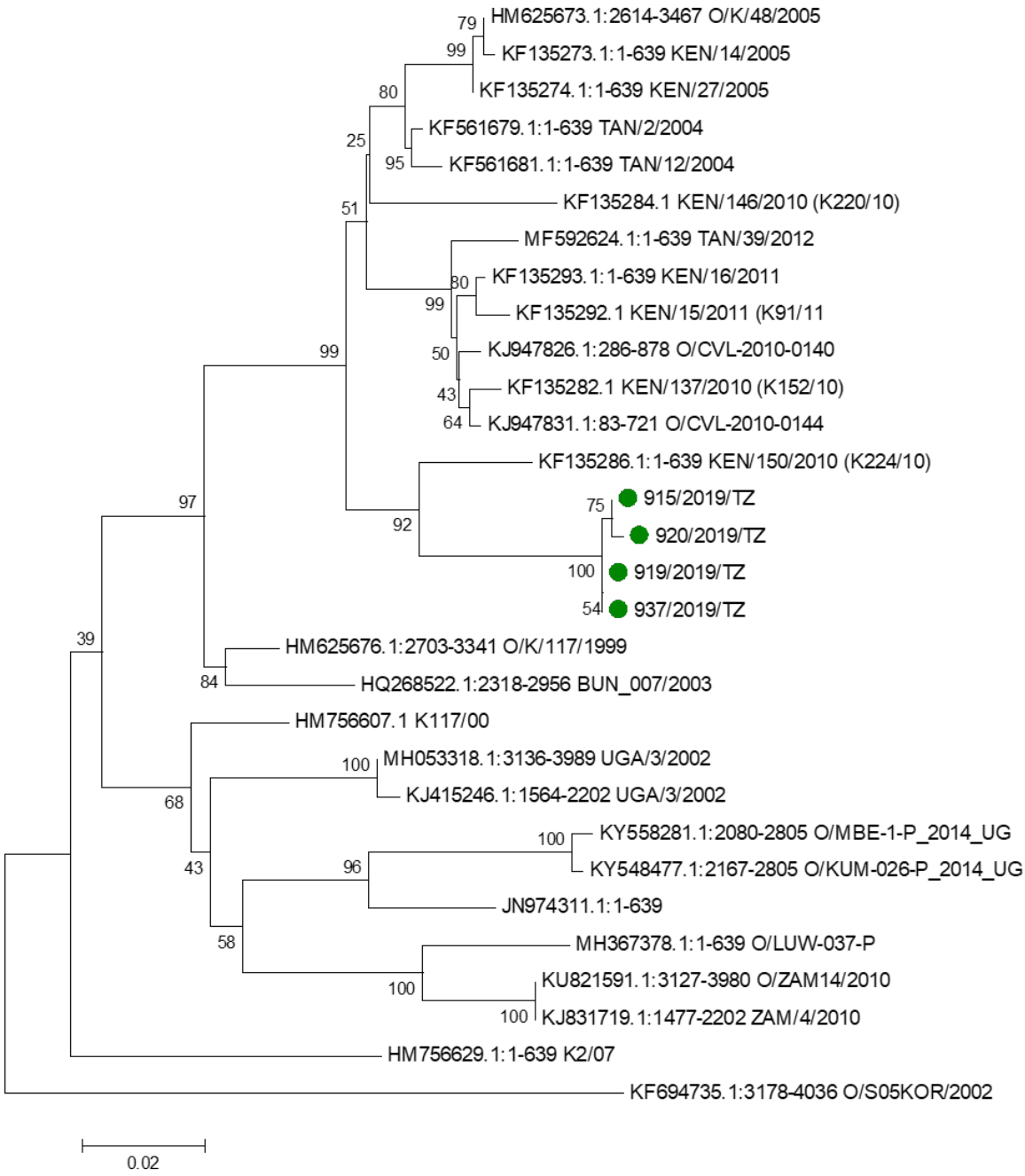

Figure 6: **Neighbor-joining tree based on the virus protein VP1 of serotype O coding sequence. The sequences generated for this study are marked with green filled circle symbol.**

CHAPTER FIVE

5.0 DISCUSSION

This study was based on molecular detection of the FMDV genome and phylogenetic analysis of the VP1 coding region sequence of FMD Viruses that caused outbreaks in Tanzania from February 2019 to August 2019. In view of that, results define molecular evolutionary relationships between recently recovered viruses in Tanzania and previously circulated viruses from Eastern Africa and outside region. The first molecular analysis of FMD virus isolates collected in Tanzania was first conducted in 2013.

RT-PCR was deployed to detect FMD viral RNA in 36 clinical samples and the product band size was 328 base pair (Table 4). The chances of detecting FMDV genome being 72% (n=26) of all the analysed samples from five different geographical locations explains the extent to which the disease has spread in most areas especially the Lake zone and East zone of Tanzania where the cases were reported. The findings showed the disease incidence in Sengerema (89%), Kibaha (75%), Morogoro (63%), Musoma (50%) and 1 sample collected from Butiama tested positive. The spread of the disease in different locations might be due to animal movements. This is consistent with previous conducted studies in Tanzania which indicate that livestock movements are generated as a result of tribal conflicts and cattle rustling, trade, breeding purposes, socio-economic reasons (gifts, debt repayment and dowry) and refugees from neighbouring countries (Kivaria, 2003).

Sequence alignment of the deduced nine (five for serotype A and four for serotype O) complete VP1 sequences indicated that all the virus strains were very similar. The five FMDV type A viruses were compared to 24 VP1 sequences which were derived from the

Genbank. Similarly, four type O viruses were compared to other 26 VP1 sequences from the databases which were previously submitted.

The sequence identity was 95.46% between the 2019 outbreak isolate (TZ 925/2019) and the 2013 outbreak isolate (TAN/18/2013) for FMDV serotype A. For FMDV type O, the high sequence identity of 95.31% was observed between the 2019 outbreak isolate (TZ 915/2019) in Tanzania and 2010 outbreak isolate (KEN/150/2010) from Kenya. These results showing high nucleotide sequence identity among the isolates from two outbreaks indicates that these viruses originate from the same ancestor or outbreaks are from a common source (Knowles and Samuel, 2003).

In this study, the complete VP1 region was amplified by RT-PCR and sequenced on both sides for 16 FMDV type A isolates from Tanzania (Musoma, Sengerema, Butiama, Morogoro and Kibaha) and four FMDV type O isolates from Sengerema only. After sequencing, only nine (four type O and five type A) raw sequences out of 20 were able to be recovered and formed contigs after being assembled (Table 9). The edited VP1 sequences were combined with other 50 (24 for type A and 26 for type O) sequences obtained from the GenBank and phylogenetic analysis of complete VP1 sequences of 29 FMDV type A and 30 FMDV type O showed that the recent FMDV types A isolates from Tanzania (2019) which were collected in this study formed a single genetic lineage which was distinct from the previous isolates as shown in Figure 5.

The FMDV isolates of 2019 have shown to fall under the Africa genotype one (GI) as it was previously described (Kasanga *et al*., 2012) while Genotype III was reported to circulate and cause outbreaks in Tanzania in 2005, 2008 to 2013 (Lloyd-jones *et al*., 2017). This is very important to understand because FMDV serotype A has often caused sporadic outbreaks hence the need for regular monitoring programmes of these circulating

strains especially when selecting a proper vaccine for the disease control in the livestock industry.

Findings depicted that the FMDV type A isolates collected in 2019 were clustered in the same clade with the virus strain that was isolated in 2013 (TAN/18/2013) in Simanjiro region hence suggesting the existence of an epidemiological link between these regions (Morogoro and Musoma) and Simanjiro region. The FMDV isolates from this study also show a close relationship with the virus isolate collected from Kenya in 2008 (KEN/28/2008) in Loitokitok, Rift valley region in Kenya. In that aspect, the results imply that, the history of the ancestral lineage is shared within the East African regions (Kasanga *et al.,* 2015).

The occurrence of FMDV serotype A in different geographical locations in this study, supports previous findings and explains how endemic the disease is since FMDV type A has been reported to cause outbreaks in the Northern, Northern Lakes and Central zones of Tanzania in 1971 (Rweyemamu and Loretu, 1972). Similarly, studies have also reported no outbreaks associated with serotype A since 2003 to 2008 although there was outbreak cases in Morogoro and Dar es salaam in 2009 (Kasanga *et al*., 2012). The reappearance of this serotype is contrary to the previous studies (Sallu *et al*., 2014) and this could have been caused by animal movements inside and outside the country (Sallu *et al*., 2014).

Findings indicate that, serotype A viruses in Figure 5, evolve continuously hence emergence of new variants in the circulation which are genetically distinct. The isolates 928/2019/TZ and 935/2019/TZ have shown some degree of diversion from the rest of the isolates of this study. These two isolates have shown high similarity in their nucleotide sequences. The evidence that serotype A viruses keep on evolving into new variants has been shown by isolates 928/2019/TZ and 935/2019/TZ from this study which have been

collected from Morogoro region. These two isolates are highly similar and have clustered themselves into a sub clade with high bootstrap value support.

On the other hand, recent FMDV type O isolates from Tanzania (2019) formed a single genetic lineage which was separate from the previous isolates as shown in Figure 6. The FMDV isolates from the study formed a clade with Kenyan virus and they all belong to the East African topotype two (EA-2). In Tanzania, EA-2 topotype was reported to circulate in 2008 to 2009, 2012 to 2014 hence monitoring of strains causing outbreaks and carrying out vaccine matching studies are both vital in identification of emerging variants as well as selection of proper vaccine strains to control FMD (Lloyd-jones *et al.*, 2017).

The 2019 isolates are characterized with a close genetic relationship of FMD viruses collected in Kenya in 2010 (KEN/150/2010) high supporting bootstrap value. This evolutionary relatedness suggests possible animal movements from country to country that lead to the transboundary spread of the disease (Kivaria, 2003) within regions of East Africa. In addition to that, the viruses from this study have also shown some evolutionary relatedness by clustering with the Kenyan isolate O/K/117/1999 that was collected in 1999 in Nakuru region. This could allow tracing of virus transmission pathways within disease outbreaks and recommend best ways of disease control. Serotype O is widely spread in the Lake zone of Tanzania causing a number of outbreaks, and this observation agrees with several previous studies that have been conducted (Kasanga *et al*., 2012), all the viruses from this study which were typed as O were collected from Sengerema in Mwanza region.

From Figure 6, two virus strains (915/2019/TZ and 920/2019/TZ) from this study have shown diversity from isolates 919/2019/TZ and 937/2019/TZ by forming a sub-lineage hence possible antigenic and genetic variations are expected to occur. This can be supported by ability of the virus to infect multiple host species as for the virus 915/2019/TZ which was isolated from the porcine oralpharyngeal scrapings. This process

of cross-species transmission is very important for virus evolution and new host adaptation (Geoghegan and Holmes, 2017).

Since the pig industry in Tanzania plays a major epidemiological risk as they breed in huge units and are fed with viral infected dead animals and results lead to the spillover of virus and causing sporadic outbreaks (Kivaria, 2003), more studies should be conducted so as to reduce the disease burden.

Out of the total typed samples (n = 20), four for serotype O and 16 for serotype A, only nine VP1 sequences were able to be generated for phylogenetic analysis. This relatively low rate (45%) of sequence recovery could be a result of several factors such as the low quality of the epithelium tissue sample, long time storage of samples since only archived samples were used, poor transporting but sometimes failure of generating VP1 for sequencing.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

This study has revealed that;

i. Serotype A and O viruses were involved in causing sporadic FMD outbreaks in five geographical locations (Sengerema, Morogoro, Kibaha, Butiama and Musoma) during 2019.
ii. The genetic diversity among serotypes A and O field viruses detected during 2019 outbreaks were East Africa topotype 2 (EA-2) and Genotype I (GI) respectively.
iii. The serotype A viruses detected in Morogoro and Musoma were genetically closely related with FMDV strains detected in Tanzania (Simanjiro) in 2013 and Kenya in 2008, whereas the serotype O viruses were closely related to FMD isolates collected from Kenya in 1999 and 2010. This finding demonstrated the presence of nucleotide deletions and addition among FMDV field strains in different geographic locations in Tanzania.

6.2 Recommendations

i. Consistent monitoring and thorough researches are required to design an efficient FMD vaccines with matching genotypes/topotypes to be kept available in order to confer protection against emerging and reemerging viruses.
ii. The government should impose strict Quarantines in areas where the outbreak has occurred to prevent the spread of the disease to new FMD free areas. This is a challenge since the disease is not of public threat, animal movements in such areas is not controlled.

iii. Molecular characteristics of the FMDV should be identified immediately following FMD outbreaks using Next generation sequence technique to avoid generation of few data that could be caused by primer mismatch or failure to generate VP1 region.

iv. Regular FMD active surveillance as well as molecular survey activities is very important in improving the information on the nature and disclosure of evolutionary features of FMDV topotypes circulating in the country.

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