MOLECULAR INVESTIGATION OF FOOT-AND-MOUTH DISEASE DURING THE 2021 OUTBREAK IN MVOMERO DISTRICT, MOROGORO

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

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ABSTRACT

Foot-and-mouth disease is a highly contagious infection of cloven hoofed animals. The disease is caused by an RNA virus from the genus *Aphthovirus* in the *Picornaviridae* family. Foot and mouth disease virus (FMDV) affect mostly cattle at all stage of age and cause severe economic loss. In Tanzania FMDV has become endemic despite the efforts in its control. Outbreaks are still occurring and cause economic losses due to different reasons including vaccine failure. To avoid this crisis, vaccine matching can be done to ascertain a proper vaccine candidate that can create immunity in cattle against the circulating strains. To accomplish this, updated knowledge of the circulating FMDV strains in the country is required through regular epidemiological surveys and vaccine matching exercise. The aim of this study was to investigate FMDV serotype(s) responsible for the recent outbreak that occurred in Mvomero district, Tanzania. Seventeen (n=17) epithelial tissues were taken from feet and mouth of diseased cattle and transported aseptically to the Laboratory at Sokoine University of Agriculture for analysis. Detection, molecular typing and identification were done using One-step reverse transcription polymerase chain reaction (RT-PCR) followed by Sanger sequencing and phylogenetic analysis to establish the relationship to the existing FMDV sequences in GenBank. The findings indicated the morbidity and detection rates to be 27.5 % and 17.6% respectively. Further analysis revealed that the FMDV strain responsible for the outbreak was Serotype O, genotype EA-2 which clustered in the same clades with the isolates from Uganda (OUGA2009) and Kenya (O/KEN/150/2010) with accession numbers JN974311.1 and KF1352286.1 respectively. It is recommended that vaccines formulated using the characterised genotype need to be administered in cattle from that region. Continuous epidemiological studies and close follow up of the

circulating strains is important so that the proper prophylactic doses can be administered before the outbreak occurs.

DECLARATION

I, Igizeneza Acsa, do hereby declare to the Senate of 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 for a higher degree award in any other institution.

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DEDICATION

This work is dedicated to my late father Muzirikana Jason, may we meet in the resurrection morning. I am dedicating this work to my caring mom Nyirabaganizi Damarce, my brother Masengesho Amouram, Sengiyumva Kandusi and to all the rest of my family members.

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LIST OF ABBREVIATIONS

µl	Microliter
BLAST	Basic Local Alignment Search Tool
Bp	Base pair
cDNA	Complementary Deoxyribonicleic acid
CPE	Cytopathic effect
DNA	Deoxyribonucleic acid
DNTP	Deoxynucleotide triphosphate
EA	East Africa
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme linked immunosorbant assay
EURO SA	Europe–South America
FMD	Foot-and-mouth disease
FMDV	Foot-and-mouth disease virus
FP	Forward Primer
ISA	Indonesia
MESA	Middle East–South Asian
NCBI	National Center for Biotechnology Information
NCEs	Non-coding elements
NSPs	Nonstructural proteins
PBS	Phosphate Buffered Solution
Pmol	Picomole
RNA	Ribonucleic acid
RP	Reverse Primer
RT-LAMP	Reverse-transcription loop-mediated isothermal amplification

RT-PCR	Reverse-transcription polymerase chain reaction
SAT	Southern African Territories
SEA	South-East Asia
UTR	Untranslated region
WA	West Africa

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background information

Foot-and-mouth disease is a highly contagious infectious disease caused by a virus in the genus *Aphthovirus* from the *Picornaviridae* family (Samuel and Knowles, 2001). The virus is known to cause infections in cloven hoofed animals such as cattle, sheep, goats, pigs and buffalo (Paton *et al*., 2018). Foot-and-mouth disease virus (FMDV) affect cattle mostly due to their large respiratory capacity and requirement of low dose of virus to induce the disease (Markey *et al*., 2013). Mortality rate in adult cattle is rare while young calves may die due to acute myocarditis (Markey *et al*., 2013), this poses a threat to the economy due to low milk and beef meat production (Kivaria, 2011).

Foot-and-mouth disease virus has the ability to undergo high antigenic variation in its genome which results in different serotypes, topotypes and strains (Grubman and Baxt, 2004). Hence, seven major serotypes of FMDV are known which are: serotype A, O, C, Asia-1, and Southern African Territories (SAT)-1, SAT-2 and SAT-3 (Knowles and Samuel, 2003). Each serotype causes a different infection and recovering from one serotype cannot give immunity against the remaining serotypes making the control of the virus difficult. This complicate designing FMDV control strategies especially in terms of vaccine development (Grubman and Baxt, 2004; Rodriguez and Grubman, 2009).

Foot-and-mouth disease virus can be transmitted by different means which are; direct contact between animals or contact with infected animal products such as semen, by aerosols (airborne route) and by mechanical carrier such as vehicles, people, and fomites (Paton *et al*., 2018). Following infection, the incubation period of FMDV is normally

between 1 and 14 days depending on the serotype and the inoculated quantity and after that time, the animal can shed the viruses in secretion and excretion (Kitching, 2005). Infected animals present the following clinical symptoms: fever, formation of vesicles, and lesions in foot and mouth (Grubman and Baxt, 2004). Though not considered as a zoonotic disease, FMDV can infect human who deals with infected samples without protection and they can develop fever and vesicles on the skin and mucous membranes (Markey *et al*., 2013).

Foot-and-mouth disease virus has the ability to persist in the pharyngeal region of animals (Markey *et al*., 2013) and as a result, its eradication has become a challenge worldwide (Brito *et al*., 2017). In Tanzania, FMDV has become endemic and different serotypes have been identified in cattle, which are: serotype A, O, SAT-1, SAT-2 and SAT-3 with serotype O being the most prevalent and have been reported in all regions of Tanzania where FMDV has already occurred (Sallu *et al*., 2014). Other serotypes have been found to be distributed according to regions depending on the climate conditions (Kivaria, 2011; Kasanga *et al*., 2015). Molecular detection and characterisation of FMDV is the necessary tool to understand the spread and distribution of FMDV hence helps in looking for the control strategies to eradicate the disease.

1.2 Problem statement and justification of the study

Foot-and-mouth disease has become a worldwide concern and its impact to the economy is alarming. Despite the efforts to control and manage the disease, its eradication has become problematic. FMDV is highly contagious and has high mutation rate that range between 10^{-3} to 10^{-5} per nucleotide for each replication, which enable it to produce different variants which sometimes cannot respond to the vaccine used during control

process hence, in different countries around the world it has become endemic (Grubman and Baxt, 2004; Rweyemamu *et al*., 2008a; Rodriguez and Grubman, 2009).

Tanzania is among the countries where Foot-and-mouth disease (FMD) has become endemic and so far there are five serotypes (A, O, SAT-1, SAT-2 and SAT-3) reported from different regions within the country (Sallu *et al*., 2014; Kasanga *et al*., 2015). Despite using different control measures such as vaccination, FMD outbreaks are still seen in different regions of Tanzania. This has contributed to low animal production which has led to reduction of income to the farmers as well as to the GDP of the country (Kitching, 2005). Foot-and-mouth disease, though caused by one virus, each of the seven serotypes differ from one another and has its own different strains with their own antigens and epidemiological factors that differ from other strains. So, it is quite difficult to predict the causative serotype of a particular outbreak (Kitching *et al*., 2005). In addition, there is no cross-protection between the different serotypes that requires a separate vaccine for each serotype which is currently not available. This triggers the need for the identification of the serotype that caused the outbreak is required for awareness and proper control.

The aim of this study was to detect and molecularly characterize FMDV from tissue samples collected in Mvomero district, Morogoro region in Tanzania during the 2021 outbreak. Information from this study will give awareness about the causative serotypes responsible of that outbreak to the government and other stakeholders so that different control measures put in place designed and implemented.

1.3 Research questions

1. What are the serotypes of FMDV that caused the 2021 outbreak in cattle in Mvomero district?

2. What are the genotypic relatedness of the serotypes detected to the previously reported in Tanzania and other countries?

1.4 Objective of the study

1.4.1 General objective

To establish the genetic characteristics profiles of FMDV serotypes that caused the 2021 outbreak in Mvomero district, Morogoro, Tanzania.

1.4.2 Specific objectives

1. To examine the presence of FMDV genome from tissue samples of cattle in the recent FMD outbreak in Mvomero district and establish circulating serotypes.
2. To determine the phylogenetic relationships of circulating FMDV serotypes in cattle from Mvomero district.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Epidemiology and risk factors of foot-and-mouth disease virus

Foot-and-mouth disease has become enzootic in different developing countries located in Africa, Asia and South America which lack effective control techniques to eradicate the disease (Knowles and Samuel, 2003; Rweyemamu *et al*., 2008b). The seven FMDV serotypes are not equally distributed throughout the endemic areas as described by Figure 1 (Freimanis *et al*., 2016). FMD serotype distribution worldwide is commonly affected by the similarity in ecological ecosystem and mutual exchange of livestock between regions (Brito *et al.*, 2017).

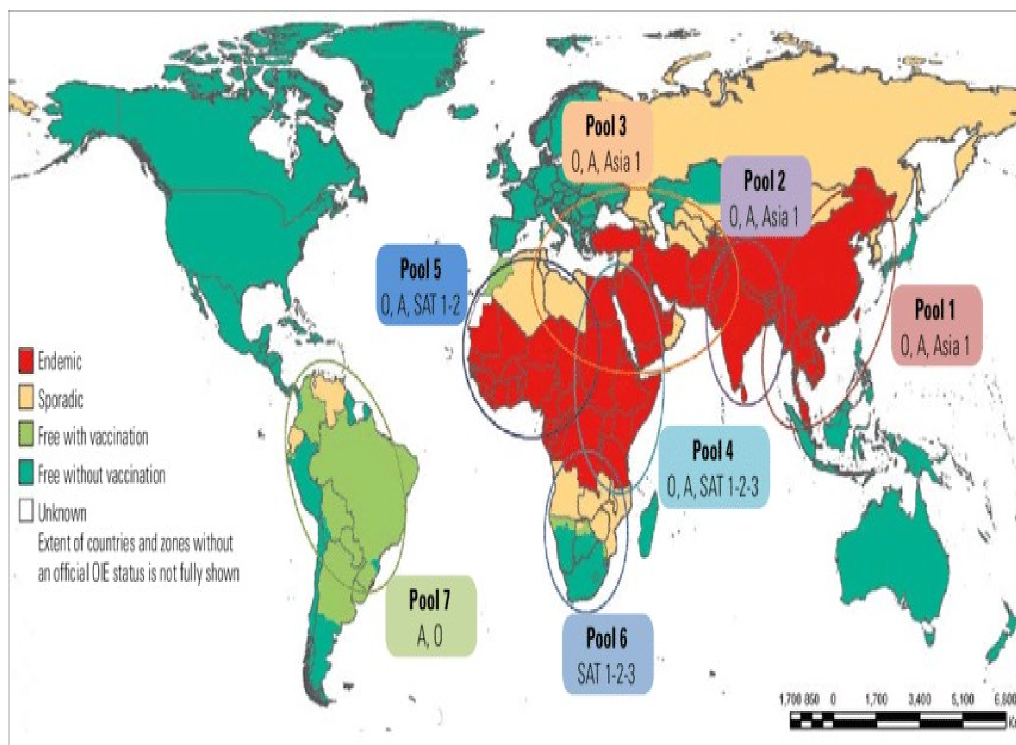

Figure 1: Geographical distribution for Foot-and-mouth disease virus (FMDV) globally by 2016.

Source: (Freimanis *et al*., 2016)

In Africa, there exist six serotypes: A, O, C, SAT-1, SAT-2 and SAT-3, while in Asia four serotypes have been detected O, A, C and Asia-1, and South America has serotypes A, O and C.

In East Africa, according to epidemiological records the most common serotypes are A, O, SAT-1, SAT-2 and SAT-3. Tanzania is among the countries in East Africa in which Foot-and-mouth disease virus has become endemic. All the five serotypes have been detected (A, O, SAT-1, SAT-2 and SAT-3) making it difficult to eradicate the virus (Kivaria, 2011; Sallu *et al*., 2014; Kasanga *et al*., 2015).

Different risk factors have been considered to be involved in the transmission of the virus and occurrence of outbreaks. Contact with contaminated secretion such as milk (breast feeding) and semen by natural breeding among animals has been found to be a risk factor that results in spreading of virus among animals (Paton *et al*., 2018; Udahemuka *et al*., 2020). Trading and movement of infected or carrier livestock, impose high risk of spreading of the virus to the susceptible animals in different regions/countries (Rweyemamu *et al*., 2008b). It has been documented that susceptible animals that have recovered from FMD virus infection and those which have been vaccinated against the virus, if they get exposed to live virus, they can become the carrier of the viruses in their epithelial tissue of the pharynx for a certain period of time (Markey *et al*., 2013). Cattle, sheep and goats can carry the virus for up to three years, nine months and four months respectively (Paton *et al*., 2018). Also, interaction between wildlife animals and livestock cause spread of FMDV. Buffaloes are known to be carrier of SAT serotypes of

FMDV; they have the ability to keep the virus up to five years. Other susceptible wild animals includes elephants, deer, hedgehogs and antelope (Vosloo *et al*., 2002).

Since the virus has the ability to remain in the environment in summer as well as in winter for 3 and 28 day respectively, in case of grazing at the same places with livestock, the virus can be spread in the FMDV free animals (Rodriguez and Grubman, 2009; Paton *et al*., 2018). FMD virus favorable conditions are low temperature, high humidity, and it is sensitive to PH ranging between 6.0 to 9.0 (Markey *et al*., 2013).

2.2 Molecular structure of foot-and-mouth disease virus

Foot-and-mouth disease is caused by a virus in the from genus *Aphthovirus* from the family *Picornaviridae* (Markey *et al*., 2013). FMD virus is an enveloped virus, with symmetric icosahedral capsid of 30nm which is composed by 60 copies of each of the structure proteins being VP1 [1D], VP2 [1B], VP3 [1C], and VP4 [1A] (Grubman and Baxt, 2004). Its genome is a positive sense single stranded RNA and has approximately the length of 8.3 kilo base (kb) that encode a single Open Reading frame (ORF) of about 7 kb (Gao *et al*., 2016).

The ORF is located between a long 5′-untranslated regions (5′-UTR) and is divided into four functional regions which are L that encodes a protease L^{pro}; P1 responsible for a precursor for capsid polypeptide (that generates capsid proteins VP4, VP2, VP3, and VP1); P2 that generates viral proteins: 2A, 2B, and 2C and P3 region encodes viral proteins 3A, 3B, $3C^{pro}$ (a protease) and $3D^{pol}$ (an RNA-dependent RNA polymerase) (Jamal and Belsham, 2013). The ORF is translated into a polyprotein of about 250 kDa, which is then cleaved to produce viral structural and nonstructural proteins (NSPs) (Gao *et al*., 2016). Figure 2 demonstrates FMDV genome structure and its transcription and translation process.

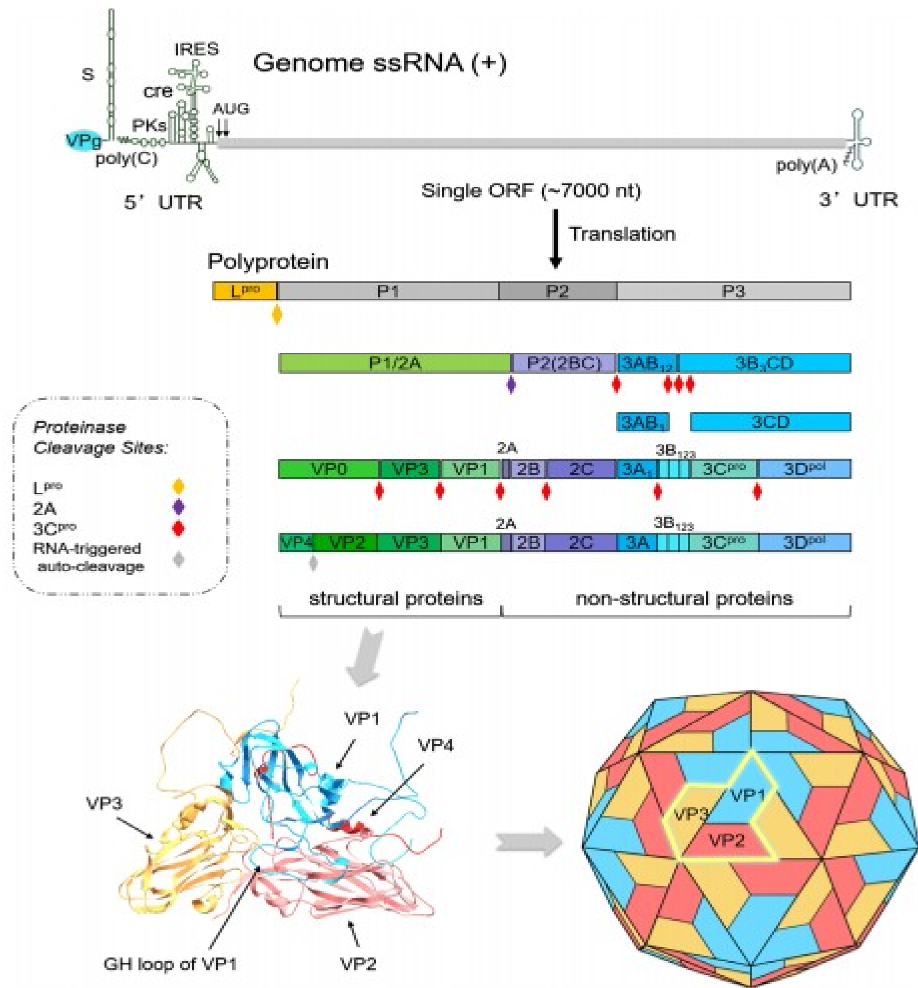

Figure 2: Foot-and-mouth disease viral genome structure and its processing.

Source: (Gao *et al*., 2016).

Foot-and-mouth disease virus genome has different non-coding elements (NCEs) that play an important role in viral evasion of the host cell as summarized in Table 1. FMD virus doesn't have a 5' terminal cap structure hence, it uses the 5′-UTR to initiate replication and translation of the viral genome. The 3' UTR contains a poly (A) tail and a structural sequence of 90 nt that involves in FMD virus virulence and replication (Jamal and Belsham, 2013).

FMDV nonstructural proteins (NSPs) are: L^{pro}, 2A, 2B, 2C, 3A, 3B, $3C^{pro}$and $3D^{pol}$. L^{pro} is ^{required} for viral pathogenesis, it is also involved in repressing the host cell translation machinery by cleaving the host initiation factor. The P2 portion of FMDV genome encodes viral proteins 2A, 2B and 2C. 2B and 2C play an important role in modifying the host membrane where; 2B proteins are viroporins, they increase membrane permeability hence play a role in virus pathogenicity by permitting the release of viral particle, and 2C play a role in membrane reorganization, construction of viral replication complex and induction of cytopathic effects (Jamal and Belsham, 2013). 2A protein has proteolytic activity (Grubman and Baxt, 2004). 3A has an importance role in virulence of FMD virus and the change in host range. 3B (VPg) involves in viral replication, $3C^{pro}$ is responsible for proteolytic cleavage activity of viral proteins and it is known to interrupt transcription and translation machinery of the host cell. $3D^{pol}$ is the virus RNA dependent polymerase involved in virus replication (Grubman and Baxt, 2004).

Table 1: The role played by FMD viral non-coding elements and Nonstructural proteins in viral infection

Viral elements	Region	Role played
Non-coding elements	5′-UTR	-Replication and translation initiation
	3’ UTR	-Viral virulence and replication
	L^{pro}	-Viral pathogenesis
		-Block host translation machinery
	2A	-Assembly and maturation
	2B	-Viral release
Nonstructural proteins	2C	-Reorganization of viral replication complex
		- Apoptosis
		-Membrane rearrangement
	3A	-Host range
		-Viral virulence
	3B	-Viral replication
	$3C^{pro}$	-Proteolytic activity
		-Disruption of host cell transcription and translation
	$3D^{pol}$	-Viral RNA dependent polymerase

Source: (Grubman and Baxt, 2004; Gao *et al.,* 2016).

FMD viral RNA polymerase does not have a proofreading property hence, it is prone to high rate of mutation that range between 10^{-3} to 10^{-5} per nucleotide for each replication process (Grubman and Baxt, 2004) .This contribute to rapid evolution of FMDV and development of new strains. FMD virus already has seven different serotypes which are serotype O, A, C, South African territories (SAT)-1, SAT-2, SAT-3 and Asia-1 and each serotype has different topotypes, genetic lineage and strains (Brito *et al*., 2017).

2.3 Pathogenesis and clinical manifestation of foot-and-mouth disease virus

Foot-and-mouth disease, though caused by one virus, it is considered as seven different diseases since each of the seven serotypes differ from one another and has its own different strains with their own antigens and epidemiologically factors that differ from other strains (Knowles and Samuel, 2003). The severity of the disease depends on the causative serotype, infective dose and route of infection. The seven serotypes shares common virulence factors however they have some differences which makes them differ in the severity (Grubman and Baxt, 2004).

Inhalation is considered to be the main route of FMD infection, however there are other routes of infection through which animal can get exposed to the virus, those are; via insemination, ingestion of infected materials, inoculation or contact with abraded skin (Paton *et al*., 2018). FMD virus replication occurs primary in pharynx then spread via the blood stream to different sites such as epithelia of feet, mouth, muzzle and teats (Rodriguez and Grubman, 2009). Following exposure, incubation period can vary between 1 to 14 days and after five days of clinical signs development (lesions), virus infectivity reduce gradually (Grubman and Baxt, 2004).

Infected cattle are characterized by fever, loss of appetite, and noticeable drop in milk production (Markey *et al*., 2013). Wounds resulted from the rapture of formed vesicular lesions on snout, tongue, teats, may be infected by bacteria and lead to second infection (El-Bayoumy *et al*., 2014).

2.4 Diagnosis and control of foot-and-mouth disease virus

2.4.1 Diagnosis of FMDV

Foot-and-mouth disease viral infection can be mistakenly confused with other infections which are characterized by vesicle formation such as vesicular stomatitis; hence laboratory confirmation is needed. The preferred samples is epithelium tissue collected from an unruptured or recently ruptured vesicle (El-Bayoumy *et al*., 2014).

Different techniques have been adopted to diagnose and differentiate FMDV serotypes. Electro-focusing and SDS-polyacrylamide gel electrophoresis diagnose based on charges and/or size of the genome (Knowles and Samuel, 2003). To understand cytopathic effect produced by FMD virus, culture technique can be done in sensitive cell lines such as primary bovine thyroid or kidney cells, where visible cytopathic effect (CPE) can be seen after 48 hours (Markey *et al*., 2013). To detect the presence of viral antigens and/or antibody against the virus, Enzyme linked immunosorbent assay (ELISA) test can be performed (Grubman and Baxt, 2004; Habiela *et al*., 2010).

Currently the mostly used technique which is sensitive and specific is reverse transcription polymerase chain reaction (RT-PCR), which can be followed by sequencing to know the serotypes and trace back the origin of the virus (Reid *et al*., 2000; Jamal and Belsham, 2013; Knowles *et al*., 2016). Also, reverse-transcription loop-mediated isothermal amplification (RT-LAMP) assay which is very sensitive is can be used for the detection of foot-and-mouth disease virus especially in field practice (Farooq *et al*., 2015; Bath *et al*., 2020).

2.4.2 Control of FMDV

Most of the countries which managed to control FMD, have been using stamping out methods, quarantine of suspected animals, slaughtering of infected animals, control of animal movement within the country as well as Transboundary vaccination of herds and continuous surveillance of the animals (Rweyemamu *et al*., 2008b).

Though all control approaches are useful in different ways, vaccination is the best effective measure that can be used. Different vaccines have been developed for FMDV control which can be monovalent, bivalent, or multivalent depends on the constituent. Types of vaccines that have been developed for FMDV falls into five broad categories: Inactivated vaccines, Live attenuated vaccines, DNA vaccines, Recombinant protein and peptide vaccines, Viral vector vaccines (Kamel *et al*., 2019). Ring vaccination have been adopted in East Africa which involve the use of multivalent vaccines that target more than one serotype (Muleme *et al*., 2012; Hammond *et al*., 2021).

However, outbreak can occur despite vaccination practice because of the presence of different subtypes in each serotype, causes the problem in developing FMDV vaccines since antigenic variation in each variant need to be considered (Rodriguez and Grubman, 2009; Muleme *et al*., 2012; Maree *et al*., 2014). FMDV vaccines failure can also be due to short shelf life of used product in production hence, requirement of booster dose is needed which cannot be affordable by every farmer (Rodriguez and Grubman, 2009). Also another challenge in controlling FMDV is that, exposure and recovery from a particular serotype, does not provide immunity against the other remaining serotypes (Rweyemamu *et al*., 2008b).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study area

The study was conducted in Mvomero ward located in Mvomero district in Morogoro region, Tanzania. The district faced an outbreak of FMD in May, 2021 based on information of the outbreak obtained from the district veterinary officer. Samples were collected in the sites indicated in the Map below (Figure 3).

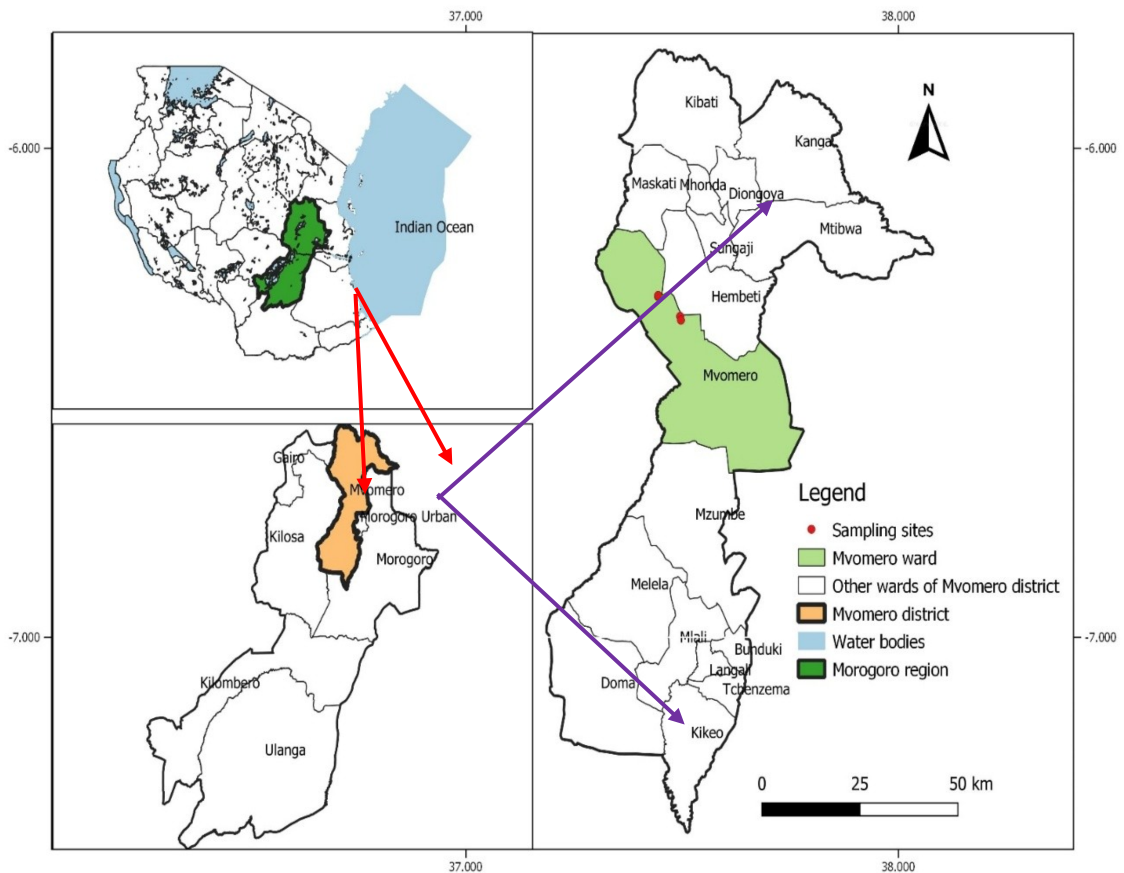

Figure 3: Map showing the study areas and sampling sites in Mvomero district, Morogoro region, Tanzania (created using qGIS 3.4).

3.2 Research design

The study was purposive, cross-sectional design, where cattle having symptoms suggestive of FMD were targeted for sampling. In collaboration with the cattle owner and the veterinarians, tissues samples were collected from feet and mouth lesions of diseased cattle, stored into viral transport media and transported to the laboratory at Sokoine University of Agriculture (SUA). The samples were used for viral RNA extraction, cDNA synthesis, RT-PCR, sequencing, and phylogenetic analysis.

3.3 Sample collection and handling

Epithelia tissues were collected from two farms with a total of 167 cattle where, farm A had 79 and farm B had 88 cattle totalling 167 cattle. Seventeen (17) epithelia tissues were collected from 17 cattle suspected to have FMD based on clinical symptoms. Samples were taken from lesions in feet and/or mouth and placed in containers containing viral transport media (50% of glycerol) and carried in a cool box to Sokoine University of Agriculture (SUA) where they were kept at -80ºC until further use.

3.4 Laboratory analysis

3.4.1 Sample processing and RNA extraction

Tissue samples were ground using pestle and mortal, while mixing with 1000 µl PBS (Phosphate Buffered Solution) and the homogenised mixture was centrifuged for RNA extraction. RNA extraction of the supernatant was done using RNeasy Minikit for total RNA (Qiagen Company, GmbH, Germany) by following the manufacturer's instructions. Briefly, 500µl of sample was mixed with 500µl volume of lysis buffer (RLT) containing 1% of Beta mercaptoethanol; 500µl of 70% ethanol was added to the tubes then mixed by vortexing. The mixture was added to the RNeasy spin column and centrifuged for 15sec at 12 000 rpm. Followed by the washing steps which was done using RW1 buffer (700 µl)

and RPE buffer (500 µl) respectively. To remove any remaining trace of ethanol, the column was dried by centrifuging at maximum speed for 2 minutes and elusion was done using nuclease free water (20 µl) followed by centrifuging at maximum speed for one minute. The extracted RNA was stored at -80ºC until use.

3.4.2 Screening and serotyping of FMDV

One-Step Reverse Transcription Polymerase Chain Reaction kit (Qiagen Company, GmbH, Germany) was used. Primers targeting highly conserved region 5'UTR (Table 2) were used for detecting the presence of FMDV. For serotyping purpose, positive samples were subjected to specific primers for serotype A, O, SAT-1 and SAT-2 (Table 2) targeting a gene fragment in VP1 region. Those serotypes were chosen based on the epidemiological history, that showed that Tanzania is mostly affected by those four serotypes (Kasanga *et al*., 2012; Sallu *et al*., 2014).

Table 2: List of primers used for screening and serotyping of FMDV

FMDV serotype	Primer ID	Sequence	Amplicon size (bp)	Location	References
Screening	1FP	GCCTGGTCTTTCCAGGTCT	328	5'UTR	(El-Bayoumy *et al*., 2014)
	1RP	CCAGTCCCCTTCTCAGATC			
O	Forward	CCTCCTTCAAYTACGGTG	160	Fragment of VP1 region	(Bachanek-Bankowska *et al*., 2016)
	Reverse	GCCACAATCTTYTGTTTGTG			
A	Forward	GCCACRACCATCCACGA	160		
	Reverse	GAAGGGCCCAGGGTTGGACTC			
SAT-1	Forward	CTYGACCGGTTCACYCTG	160		
	Reverse	CCGAGAAGTAGTACGTRGC			
SAT-2	Forward	CRATCCGCGGTGAYCG	160		
	Reverse	CGCTTCATYCTGTAGTARACGTC			

Legend: FP= Forward Primer, **RP**= Reverse primer, **R** =A or G, **Y** = C or T and **M** =A or C.

To conduct amplification process, RT-PCR master mix was prepared that contained seven constituents making a total of 25µl reaction mixture as shown in Table 3 (Knowles *et al*., 2016).

Table 3: RT-PCR master mix used and RNA quantity

S/N	Components	Quantity(µl)
1	RNA free water	8
2	5X Buffer	5
3	Reverse primer (4 pmol/µl)	5
4	Forward primer (4 pmol/µl)	2.5
5	Enzyme	1
6	dNTPs	1
7	Extracted RNA	2.5
	Total volume	25

One step RT-PCR conditions used were as follow: cDNA synthesis was done at 50ºC for 30 minutes; denaturation of reverse transcriptase and activation of polymerase was done at 95ºC for 15 minutes; followed by 35 subsequent cycles of initiation step for 1 minute at 95ºC, annealing at 55ºC for 2 minutes, and elongation at 72ºC for 5 minutes, termination was done at 72ºC for 5 minutes, followed by cooling until 4ºC. The amplification was performed using Gene Amp® PCR System 9700, (Applied Biosystem, USA).

3.4.3 Gel electrophoresis and visualization of RT-PCR product

For PCR products visualization, 1.5% agarose gel was prepared by mixing agarose powder (Carlsbad, USA) and 1x TBI Buffer (Hedwin, USA) that was stained with safe view stain (abm inc, Canada). Gel wells were loaded with 5µl of PCR products mixed with 1µl of 1X Tritrack DNA loading dye (vilinius). After subjecting it to 120 volts for 45 minutes, the gel was visualized under Ultraviolet fluorescent light.

3.4.4 RT-PCR and sequencing of the VP1 region

RT-PCR of the VP1 region was performed as in section 3.4.2 with the same PCR conditions and master mix but with different primers. Primers targeting serotype O for VP1 region were used. Two forward primers; **O-1C244F** (5'-GCA GCA AAA CAC ATG TCA AAC ACC TT-3') and **O-1C272F** (5'-TBG CRG GNC TYG CCC AGT ACT AC-3') were used. The reverse primer used was **EUR-2B52R** (5'-GAC ATG TCC TCC TGC ATC TGG TTG AT-3') (Knowles *et al*., 2016).

Positive RT-PCR products was purified using GFX™ PCR DNA and Gel Band purification kit (GE Healthcare UK Limited) according to the manufacturer instructions. Briefly, 500µl of capture buffer type 3 was added in the column followed by the PCR product (25 µl) then centrifuged at 13 000rpm for one minute. After discarding the flow through, 500 µl of wash buffer type 1 was added followed by centrifugation at 13 000rpm for one minute. Elusion was done using 20 µl of buffer type 6 followed by centrifugation at 13 000rpm for one minute.

Partial sequencing was done using Sanger sequencing platform. Prior to sequencing, cycle sequencing was done for the purified PCR products using Big Dye™ terminator V3.1 cycle sequencing kit (Thermo scientific Baltius UAB, Lithuania). Five primers were used that helped to generate VP1 fragments.

The three reverse primers used were:

NK72R (GAAGGGCCCAGGGTTGGACTC);

O-ID487fR (TGATGGCACCGTAGTTGAA);

O-IC605cF (TGGCTAGCGCCGAGGACTTTGAG) and two forward primers used were: **O-IC499F (**TACGCGTACACCGCGTC);

O-IC283F (GCCCAGTACTACACACAGTACAG).

The master mix for cycle sequencing contained 3.5 µl RNase-free water, 2 µl of 5x Buffer, 0.5 µl Big dye terminator, 3µl (1.6 pmol) sequencing primer and 1µl DNA making a total of 10µl. The conditions used at this stage were: denaturation at 96 ºC for 1 minutes, 96ºC for 10 seconds, annealing done at 50ºC for 5 secs, and elongation at 60ºC for 4 minutes 25 cycles were made (Knowles *et al*., 2016).

After cycle sequencing, Ethanol precipitation was done by adding cold absolute Ethanol in 1:4 EDTA, followed by sample and mixed by vortexing. The tubes containing the mixture were left in dark for 15 minutes and centrifuged for 30 minutes at 13 000 rpm (Hettich Zentrifugen, Germany). After carefully removing the supernatant, 70% of cold Ethanol was added and centrifuged at 13 000 rpm. The supernatant was removed carefully and the DNA Pellet was dried at 45ºC for one hour and stored at -20ºC.

Prior to Sanger sequencing, the DNA pellet was dissolved using 20µl of Hi-Di™ formamide (Applied Biosystem, USA). The product was loaded in bar coded plates and placed in the 3500 Genetic Analyzer (A&B Applied Biosystem, HITACHI, USA) and run according to the instructions.

3.5 Data analysis

To create consensus sequence form raw data created after Sanger sequencing process, the software SeqManPro within the DNA STAR LASER GENE V.9 was used by targeting the protein sequence without the stop codon. The obtained consensus was blasted using Basic Local Alignment Search Tool (BLAST) on National Center for Biotechnology Information (NCBI) to check for the similarity and construct phylogenetic tree using Mega X software.

CHAPTER FOUR

4.0 RESULTS

Cattle were suspected to have FMDV by looking at the clinical signs and the one with fresh lesions in the mouth and/or foot were targeted for sample collection (Figure 4). From both farms (farm A with 79 and farm B with 88 cattle), 46 cattle were reported to have experienced the symptoms of FMD, with 19 and 27 sick cattle from farm A and B respectively. From seven samples taken in farm A, only one (1) sample was detected to be positive and from farm B in ten samples two (2) were positives totalling three (3) positives from seventeen (17) samples. By referring to the total from both farms (A and B), the morbidity and detection rate were 27.5 % and 17.6% respectively.

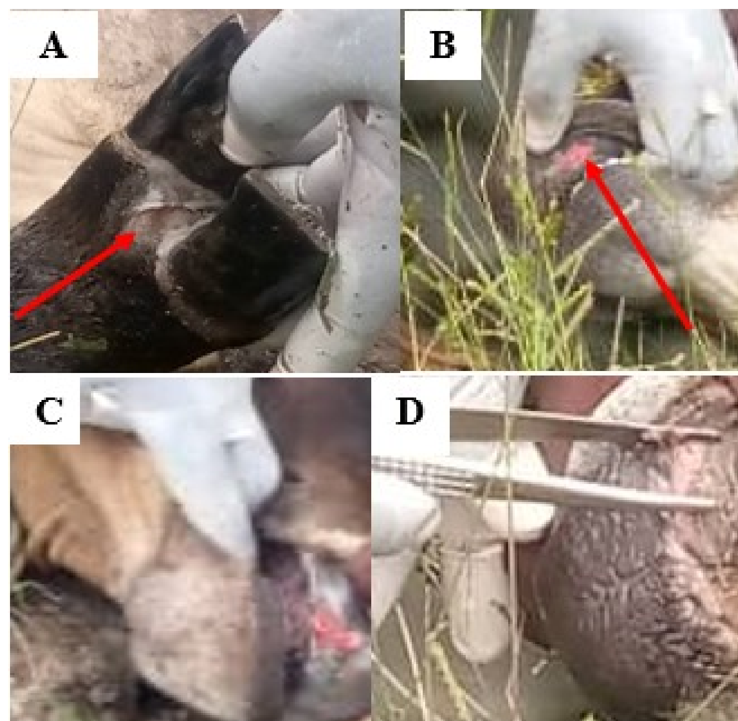

Figure 4: FMD lesions (pointed by arrows) in the infected cattle, in the foot (A) and in mouth (B, C and D).

The RT-PCR results for detecting the presence of FMDV (pan serotyping)) using primers targeting 5’UTR showed three positives, the expected band size is 328 bp as shown in Figure 5.

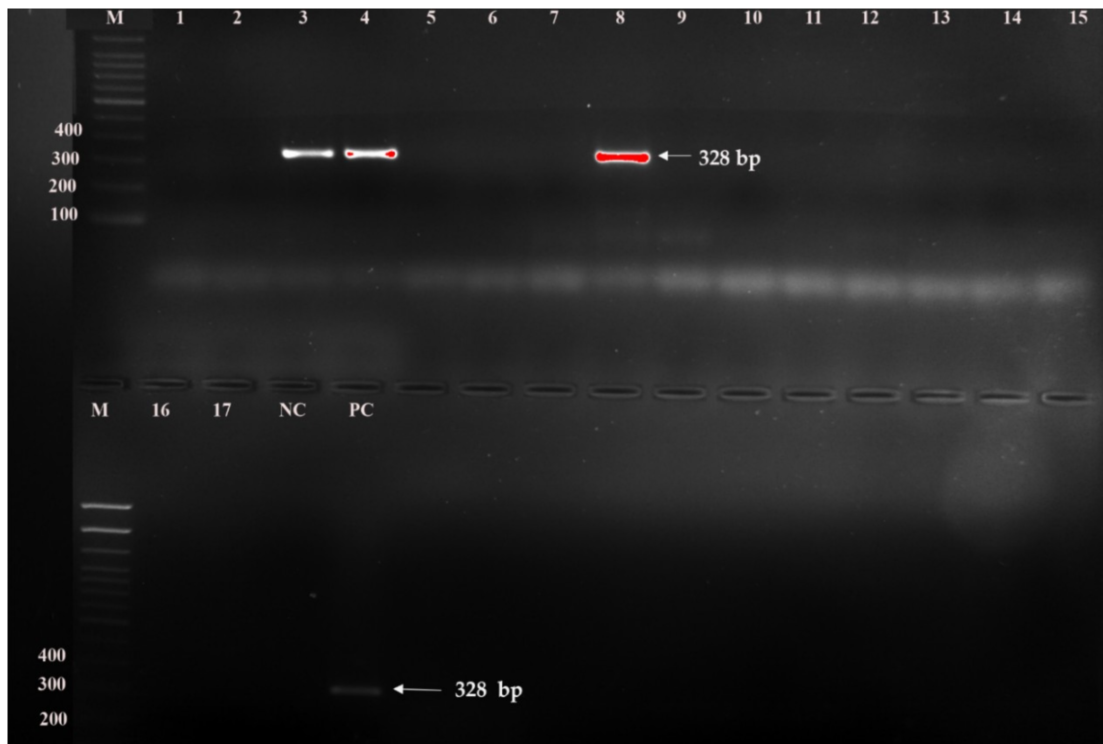

Figure 5: Gel electrophoresis of the RT-PCR products for the 17 samples, obtained after Pan serotyping. M stands for Marker or gel ladder that separates from 100 base pair (bp), NC is negative control, PC is positive control. Numeric from 1-17 are numbers assigned to tissue samples.

Obtained positive samples were further analysed to serotyping level using specific sets of primers (Table 2) for Serotype A, O, SAT-1 and SAT-2 and were all found to be of serotype O, with the band size falling between 160 bp of the ladder as shown in Figure 6.

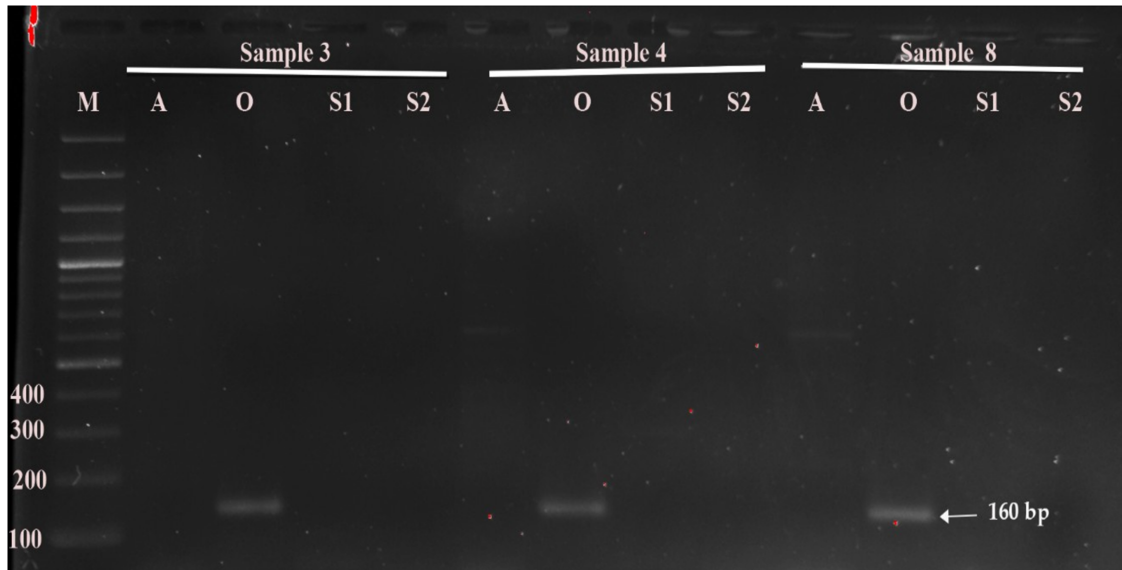

Figure 6: Typing of the positive samples. M stands for Marker, A is serotype A, O is serotype O, S1 is SAT-1, S2 is SAT-2.

After doing sequencing of VP1 region, Sample no 3 failed to give sequence, so the analysis continued with sample no 4 and 8 that succeeded to give clear sequences which were compared to other serotype O sequences retrieved from NCBI (Table 4). The size of the consensus was 651, both sequences were similar with a single nucleotide difference at the 586 base pair with 99.85% identity and query cover of 100%. The resulted sequences from two samples (sample 4 and 8) were falling in topotypes EA-2 as shown in the phylogenetic tree (Figure 7).

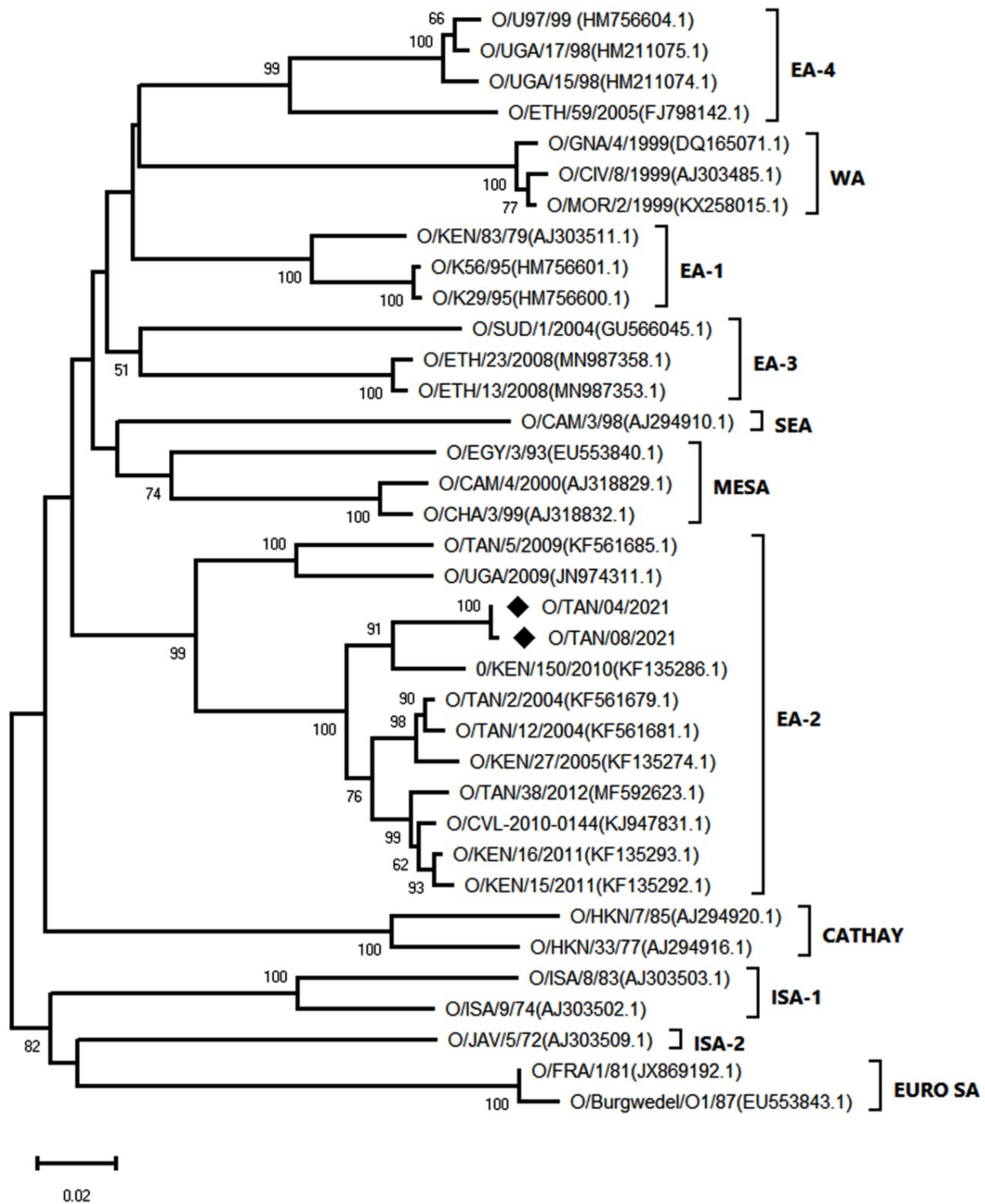

Figure 7: Midpoint-rooted Neighbor-Joining tree showing the relationship between the sequenced samples (marked ♦) and other serotype O topotypes (EA-1, EA-2, EA-3, EA-4, SEA, ME-SA, ISA-1, ISA-2, EURO SA and CATHAY collected in Tanzania and other countries. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The bootstrap value above 50 was shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site (Kumar *et al.,* 2018).

Table 4: List of sequences used in comparisons to the studied FMDV VP1region.

No	Virus ref no	Accession number	Topotypes	Year	Country
1	O/CIV/8/99	AJ303485.1	WA	1999	Cote d'Ivoire
2	O/MOR/2/99	KX258015.1	WA	1999	Morocco
3	O/GNA/4/99	DQ165071.1	WA	1999	Ghana
4	O/CAM/4/2000	AJ318829.1	MESA	2000	Cameroun
5	O/CAM/3/98	AJ294910.1	SEA	1998	Cameroun
6	O/CHA/3/99	AJ318832.1	MESA	1999	Chad
7	O/EGY/3/93	EU553840.1	MESA	1993	Egypt
8	ISA/8/83	AJ303503.1	ISA-1	1983	Indonesia
9	ISA/9/74	AJ303502.1	ISA-1	1974	Indonesia
10	O/JAV/5/72	AJ303509.1	ISA-2	1972	Indonesia
11	O/FRA/1/81	JX869192.1	EURO SA	1981	France
12	O1/Burgwedel/87 VP1	EU553843.1	EURO SA	1987	Germany
13	O/HKN/7/85	AJ294920.1	CATHAY	1985	Hong Kong
14	O/HKN/33/77	AJ294916.1	CATHAY	1977	Hong Kong
15	K56/95	HM756601.1	EA-1	1995	Kenya
16	KEN/83/79	AJ303511.1	EA-1	1979	Kenya
17	K29/95	HM756600.1	EA-1	1995	Kenya
18	ETH/23/2008	MN987358.1	EA-3	2008	Ethiopia
19	ETH/13/2008	MN987353.1	EA-3	2008	Ethiopia
20	SUD/1/2004	GU566045.1	EA-3	2004	Sudan
21	UGA/17/98	HM211075.1	EA-4	1998	Uganda
22	ETH/59/2005	FJ798142.1	EA-4	2005	Ethiopia
23	U97/99	HM756604.1	EA-4	1999	Uganda
24	UGA/15/98	HM211074.1	EA-4	1998	Uganda
25	0/KEN/150/2010	KF135286.1	EA-2	2010	Kenya
26	O/CVL-2010-0144	KJ947831.1	EA-2	2010	Tanzania
27	O/TAN/2/2004	KF561679.1	EA-2	2004	Tanzania
28	O/TAN/12/2004	KF561681.1	EA-2	2004	Tanzania
29	O/KEN/16/2011	KF135293.1	EA-2	2011	Kenya
30	O/KEN/27/2005	KF135274.1	EA-2	2005	Kenya
31	O/TAN/38/2012	MF592623.1	EA-2	2012	Tanzania
32	O/KEN/15/2011	KF135292.1	EA-2	2011	Kenya
33	TAN/5/2009	KF561685.1	EA-2	2009	Tanzania
34	OUGA2009	JN974311.1	EA-2	2009	Uganda

CHAPTER FIVE

5.0 DISCUSSION

The current study was conducted to investigate the FMDV serotype(s) that caused the recent outbreak of FMD in Mvomero district, Morogoro, Tanzania. The detection rate of FMDV in this study using RT-PCR was 17.6%. This is lower detection rate compared to the findings obtained by Loth *et al*. (2011) and Diab *et al*. (2019) that showed FMDV detection rates of 54% and 86. 27% respectively from the active outbreaks that occurred in Bangladesh and Egypt.

The small detection rate from the current investigation could be ascribed to the results of small viral load in the samples which was not enough to be detected by the RT-PCR technique. The decline in viral titter is because samples were taken 3 days following the outbreak of which at that time the lesions could have started to heal.

Genotyping and VP1 sequencing of the detected virus showed that serotype O, genotypes EA-2 of FMDV was responsible for the outbreak. This is not surprising because most of investigated outbreaks in Tanzania were found to be caused by serotype O, genotypes EA-2 (Kasanga *et al*., 2015). By pairwise comparison the two sequenced VP1 region of the detected viruses in this study demonstrated 99.85% nucleotide sequence identity with a single nucleotide difference with FMDV detected from farm A and farm B respectively. This is not surprising because they were collected from the same outbreak that might have shared the common source of infection and transmission dynamics.

The serotype O virus detected in this study was genetically closely related to the strains isolated from Kenya (O/KEN/150/2010) and Uganda (OUGA2009) with 95.93% and

87.79% nucleotide identity respectively and clustered in the EA-2 genotype. One of the cause of FMDV spread among cattle is livestock exchange by selling and buying that contributes to virus transmission between animals. By considering the years of isolation of the closely related strains 2009 from Uganda and 2010 from Kenya, and the period of isolation of the current strains (2021) from Tanzania, this might be associated with the trans boundary exchange of cattle between Kenya, Uganda and Tanzania. The study done by Kerfua *et al*. (2018) has demonstrated the circulation of FMDV serotypes O in cattle between the border of Uganda and Tanzania. There is a possibility of trans-boundary transmission of the serotypes between Tanzania and the neighbouring countries that had happened in the past years which should have contributed to spread of the serotypes within East Africa.

As shown on the phylogenetic tree (Figure 7), there is quite a difference between the characterised genotype and other serotypes O genotype EA-2 that have been detected in Tanzania. The closely related isolate from Tanzania to the isolates from this investigation was having the similarity of 94.37% nucleotide identity (KF561679.1) reported in 2004. Hence, the possibility of antigenic variation in FMDV serotypes (Grubman and Baxt, 2004) cannot be overlooked in the current study. The differences in the nucleotides of the isolated genotype EA-2 virus in this study (2021) to the already identified in Tanzania (2004) could be ascribed to genetic mutation that happened in the past within the highly variable part of FMDV VP1 region.

Serotype O has been detected in East Africa in Kenya and Uganda, different topotypes have been identified according to the phylogenetic investigation being, EA-1, EA-2, EA-3 and EA-4 (Balinda *et al*., 2010; Wekesa *et al*., 2015). As it has been documented that cattle that have suffered from FMDV can keep the virus within their pharynx for three

year (Paton *et al*., 2018), this can be another possible source of transmission within and between countries.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

Foot-and-mouth disease virus detection rate by using RT-PCR following FMDV outbreak was 17.6% which was relatively low as compared to detection rates from other outbreaks in different geographic locations (Bangladesh and Egypt).

The current study showed that FMDV serotype O genotype EA-2 was responsible for the outbreak that happened in Mvomero district, Morogoro in May, 2021. From this piece of research work, it is revealed that the isolated virus can be used for vaccine matching that will provide the vital information that are needed in developing a vaccine that could be used to control FMDV in the region.

6.2 Recommendations

i) Passive and active surveillance should complement the molecular epidemiological studies following FMDV outbreaks should be conducted for identification of the strain/topotypes/serotype responsible;

ii) Using the relevant scientific information generated from the current and previous studies, the Ministry of Livestock and Fisheries responsible for FMDV control in Tanzania can adhere to the FMD control pathway and come up with the vaccines against the main serotype circulating in the country including the genotype EA-2 of serotype O FMDV recovered from Mvomero district in Morogoro region;

iii) Continuous communication, awareness, and molecular surveillance of FMDV is needed to provide necessary information required for effective control of FMD in Tanzania;

iv) Imposing of quarantine whenever there is an outbreak in some locality as one of the control measure of this diseases;

v) Routine vaccination of animals using the selected existing vaccines candidate in the market especially those with the best-approved cocktail of FMDV serotype/topotypes derived after the vaccine matching exercise and should be done regularly.

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