MOLECULAR CHARACTERIZATION OF FOOT-AND-MOUTH DISEASE VIRUS RECENTLY RECOVERED IN ZAMBEZI REGION, NAMIBIA

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A DISSERTATION SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN ONE HEALTH MOLECULAR BIOLOGY OF SOKOINE UNIVERSITY OF AGRICULTURE.

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ABSTRACT

Foot-and-mouth disease (FMD) is a severe highly contagious viral disease of cloven-hoofed animals that has significant economic impacts. FMD causes significant economic loss in Namibia in which the molecular epidemiology of FMD virus (FMDV) responsible for the outbreaks has not been consistently studied. The general objective of this study was to determine molecular characteristics of FMD viruses of 2019 outbreak in Zambezi region, Namibia. A total of 11 epithelial tissue samples collected from cattle showing FMD clinical signs were used in this study. The RNA extraction and detection of FMDV genome was done by One-step Reverse Transcription Polymerase Chain Reaction (RT-PCR) of 5' untranslated region (5'UTR) using universal primers. Typing and sequencing was performed using serotype-specific oligonucleotide primers. Phylogenetic analysis was performed using distance matrix neighbour joining method employing the Kimura-2-parameter option. The findings indicated that all 8 sequenced viruses revealed 100% nucleotide identity among themselves. Phylogenetic analysis of 36 sequences including 8 sequences of this study, 7 from publication and other sequences from GenBank revealed that the 2019 isolates clustered together with historic Namibian isolates and those from neighbouring countries. In-depth typing and phylogeny proved that SAT3 viruses of topotype II were responsible for 2019 outbreak. Further analysis revealed that the 2019 isolates closely related to isolates from Botswana, some isolates from Zimbabwe and Kenya but were distantly related to isolates from Zambia and South Africa and no genetic linkage with isolate from Uganda. These findings indicate that topotype II SAT 3 FMD viruses were involved in the 2019 FMD outbreak in Zambezi region, Namibia. Further in-depth studies are required to elucidate transmission dynamics and factors associated with

the outbreaks so that appropriate FMD control measure (s) in Namibia can be recommended.

DECLARATION

I, Simaneka Saara Lukas, 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 concurrently being submitted to any other institution.

Simaneka Saara Lukas Date

(MSc. Student)

This declaration is confirmed by:

Prof. Christopher. J. Kasanga Date

(Supervisor)

Prof. Mark M. Rweyemamu Date

(Supervisor)

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DEDICATION

I dedicate this work to my paternal grandmother Hileni Nepolo and maternal grandmother Helena Kauluma. God Bless You.

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LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

%	Percentage
°C	Degree Celsius
µL	Microliter
AU-IBAR	African Union Inter-African Bureau of Animal Resources
bp	Base pair
cDNA	Complementary Deoxyribonucleic Acid
CVL	Central Veterinary Laboratory
DIVA	Differentiation between Infected and Vaccinated Animals
DNA	Deoxyribonucleic acid
dNTP	Dioxy-nucleosidetriphosphate
EDTA	Ethylenediaminetetraacetic Acid
FAO	Food and Agriculture Organisation
FMD	Foot-and-mouth disease
FMDV	Foot-and-mouth disease virus
g	Relative centrifuge force or gram
GDP	Gross Domestic Product
IFPHTM	Intermediate Fellowship in Public Health and Tropical Medicine
Km^2	Square kilometre
LPBE	Liquid-Phase Blocking Enzyme Linked Immunosorbent assay
m/v	mass by volume
MEGA	Molecular Evolutionary Genetics Analysis
MgCL2	Magnesium Chloride
mL	Millilitre
m-PCR	Multiplex-Polymerase Chain Reaction
NCBI	National Centre for Biotechnology Information
NGS	Next Generation Sequencing
nm	Nanometer
nt	Nnucleotide
OIE	Office International des Epizooties
ORF	Open Reading Frame
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
rpm	revolutions per minute
RT-LAMP	Reverse transcription loop-mediated isothermal amplification
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SACIDS	Southern African Centre for Infectious Diseases Surveillance
SADC	Southern African Development Community
SAT	South African Territories
S-ELISA	Sandwitch-Enzyme Linked Immunosorbent Assay

SSA	Sub-Saharan Africa
TAE	Tris-acetate EDTA
UTR	Untranslated region
VI	Virus Isolation
VP	Viral Protein

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Foot- and- mouth disease (FMD) is one of the most important livestock diseases globally. It is a viral disease affecting domestic and wild cloven hoofed animals (Lycett *et al*., 2019). The disease is of major economic importance since it is associated with high potential of production losses, increased disease control measures as well as constraints to international trade of livestock and livestock products.

Seven immunologically distinct serotypes exist namely, Serotype O, A, C, Asia 1, SAT 1, SAT 2 and SAT 3 (Kasanga *et al.,* 2015), all of which are known to be highly transmissible and with devastating impacts when introduced into FMD free countries or zones (Lyons *et al*., 2014). To implement an effective FMD control programme, it is essential to understand the complex epidemiology of the disease, which is affected by different viral, host and environmental factors. FMD is highly contagious and the virus has high antigenic diversity that makes the disease difficult to control. The genetic diversity of FMD virus (FMDV) is a consequence of high mutation rate due to error-prone RNA polymerase lacking proofreading activity (Tekleghiorghis *et al*., 2014). Timely and accurate diagnosis of FMD is essential in controlling the disease. Many serological and genomic based assays have been developed for FMD diagnosis.

Sub-Saharan Africa (SSA) is an enormous geographical area with a huge diversity of culture, cattle rearing practices and wildlife including Cape buffalo (*Syncerus caffer*), an important reservoir of SAT serotypes. The role of wildlife in epidemiology of the disease

makes the control very difficult. Therefore, rapid identification of FMDV serotypes especially during outbreaks is very important in order to use appropriate emergency vaccine and determine genetic and geographical origin of infection (El-khabaz and Al-hosary, 2017).

1.2 Problem Statement and Justification of Study

FMD is the animal disease of economic importance in terms of world trade; it also affects food security and livelihood especially in developing countries where the disease is endemic. The disease causes direct production losses in adult animals, significant mortality in young stock as well as economic losses ascribed by limited access to lucrative international markets of livestock and their products.

Despite obligatory vaccination of cattle against Foot-and-mouth disease in Sub-Saharan Africa including Namibia, still there are reports of FMD outbreaks with variable spatio-temporal characteristics. This situation calls for improving knowledge of FMD virus, its epidemiological field patterns, and its risk magnitude posed at livestock-wildlife interfaces for enhancing FMD mitigation strategies.

In Namibia, the serotypes SAT1, SAT2 and SAT3 are responsible for FMD outbreaks (FAO, 2018). In order to achieve a better control of FMD, it is crucial to monitor variants of the prevalent serotypes of FMDV in the field in order to understand their genetic and antigenic characteristics to ensure that appropriate vaccines are used to combat the circulating virus strains.

1.3 Research Questions

The key research questions for this study were:

(i) What are the circulating FMDV serotypes in Zambezi region?

(ii) What are the top types and/or genotypes of FMDV recovered in Zambezi region during 2019?

(iii) What are the evolutionary features of FMDV strains in Namibia and neighbouring countries?

i.

1.4 Objectives

1.4.1 General objective

To determine the molecular characteristics of FMDV strains involved in confirmed FMD outbreak (s) in Zambezi region, Namibia. The obtained information will help on the development of appropriate FMD control method in the region.

1.4.2 Specific objectives

1. To examine circulating FMD virus serotypes in Zambezi region;
2. To determine genotypes of FMD virus involved for FMD outbreak(s) in Zambezi region;
3. To determine the evolutionary relationships of the circulating strains within Namibia and neighbouring countries.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Foot-and-Mouth Disease

Foot-and-mouth disease (FMD) is a highly contagious, transboundary viral disease principally affecting cloven hoofed animals (EL-Bayoumy *et al*., 2014). The disease is ranked first in the World Organisation for Animal Health (OIE) list of notifiable diseases due to its ability for rapid and considerable spread within and between countries (Fowler *et al.*, 2014). Clinically, the disease cannot be differentiated from other vesicular diseases, such as swine vesicular disease, vesicular stomatitis and vesicular exanthema (OIE, 2017). Therefore, laboratory diagnosis of any FMD suspected case is a matter of urgency. Clinically, the disease is characterised by fever, loss of appetite, salivation, vesicular eruptions in the mouth, on the feet and teats and sudden death of young stock (Sulayeman *et al*., 2018).

2.2 Aetiology and Genome Organisation of Foot-and-mouth Disease Virus

The disease is caused by FMD virus (FMDV), the species of the genus *Aphthovirus* in the family *Picornaviridae* (Belsham, 2005). The virus is non-enveloped, with a single stranded, positive sense RNA genome, about 8.5kb in length. The genome is enclosed in a

nearly spherical protein capsid of about 28nm, which consist of 60 copies of each of the four different structural polypeptides e.i VP1 (1D), VP2 (1B), VP3 (1C) and VP4 (1A) as depicted in figure 1. The VP4 is entirely internal in the virus whereas VP1, VP2 and VP3 are surface exposed and contribute to antigenic properties of the virus (Jamal *et al.,* 2011). The genomic RNA encodes a single long open reading frame (ORF) of about 7kb with two initiation sites. The ORF is flanked by a long 5' untranslated region (5'UTR) and a short 3'UTR and ends with a genetically encoded poly-(A) tail (Gao *et al*., 2016). Primary processing of FMDV ORF bring about three large intermediate polyproteins (L/P1, P2 and P3). Protease cleavage by FMDV proteins L, 3C and 2A produces smaller sub-products and 12 final mature proteins (LA, 1A, 1B, 1C, 1D, 2A, 2B, 2C, 3A, 3B, 3C and 3D), (Carrillo, 2012). Seven (7) serotypes are currently circulating globally. These are serotypes O and A found around the world, the Southern Africa Territories (SAT) 1,2 and 3 serotypes found predominantly in sub-Saharan Africa and Asia 1 found in Asia (Lycett *et al*., 2019). The last outbreak due to serotype C FMDV was in Ethiopia during the 2005 outbreak (Rweyemamu *et al.,* 2008) but it had a widespread distribution formerly.

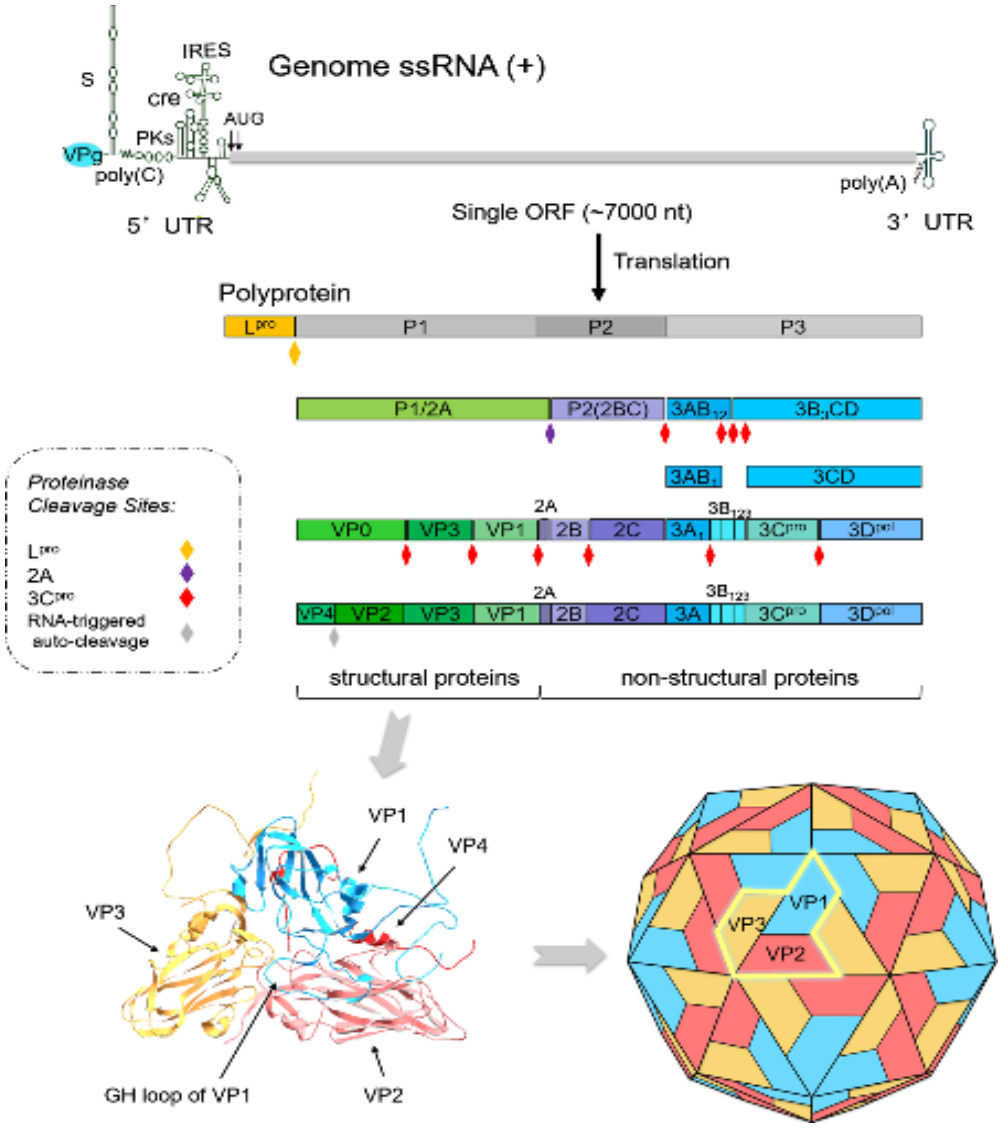

Figure 1: Schematic diagram of FMDV genome, processing of viral polypeptides and conformation of structural proteins. Source: Gao *et al*. (2016)

2.3 Clinical Signs of FMD

Clinical signs range from mild to severe, and death may occur, especially in young animals. In some species the infection may be subclinical, e.g. African buffalo (*Syncerus caffer*) (OIE, 2017). Typical cases of FMD are characterised by blisters or vesicular eruptions on the mouth, muzzle, tongue, snout, on the feet and teats and between the hooves (Chakraborty *et al*., 2014) which results into off-feeding and lameness. These clinical signs are followed by pyrexia, anorexia, shivering, reduction in milk production for 2–3 days, then smacking of the lips, grinding of the teeth, drooling, stamping or kicking of

the feet; caused by vesicles (aphthae) on buccal and nasal mucous membranes and/or between the claws and coronary band. After 24 hours, vesicles rupture leaving erosions (OIE, 2013).

2.4 Epidemiology of FMD

FMD is one of extensively distributed diseases worldwide. The disease is endemic in sub-Saharan Africa where new virus strains are likely to emerge. The epidemiology of FMD is complex, and it is affected by different viral, host, and environmental factors (El-Khabaz and Al-Hosary, 2017). The seven FMDV serotypes differ in distribution across the world. The SAT serotypes are mainly found in Africa while Asia 1 is confined to Asia. Serotypes A and O have the widest distribution, occurring in Africa, Asia and South America. Each serotype behaves differently and one vaccine does not confer immunity to all serotypes.

The global FMDV population can be roughly split into seven regional pools. Pool 1 covers south-east Asia with spill over into eastern Asia. Pool 2 represents southern Asia. Pool 3 covers west Eurasia and Middle East. In these three pool serotype O, A and Asia 1 are circulating serotypes. Pools 4, 5 and 6 cover eastern, western and southern Africa, respectively. In pool 4 serotype O, A and SAT 1, SAT 2 and SAT 3 are circulating. In pool 5 serotype O, A, SAT 1 and SAT 2 and in pool 6, only the SAT serotypes are circulating, with serotype O and A in northern Zambia, a spill over from pool 4. Pool 7 covers South America and has only type A and type O circulating (FAO, 2018).

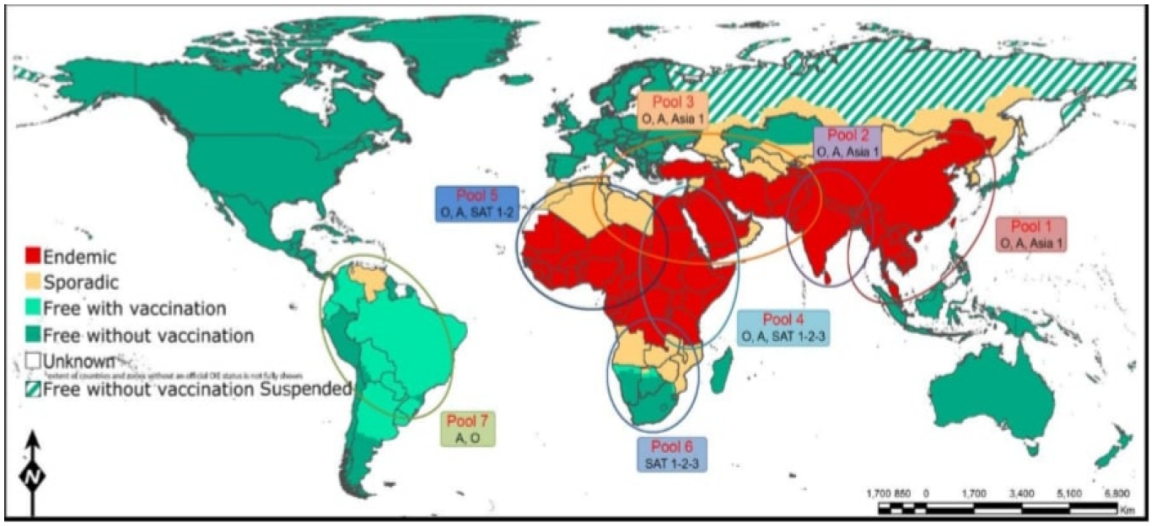

Figure 2: Mapping of seven regional pools with estimated FMD outbreak status during 2017. Source: Wubshet *et al.* (2019)

According to Tekleghiorghis *et al.* (2014), two cycles of FMD that influence livelihood exist in sub-Saharan Africa, one in which the virus circulates between wildlife hosts and domestic animals and one in which the virus spread among domestic animals without involvement of wildlife. In Africa, African buffaloes (*Syncerus caffer*) play a role in maintenance and transmission of FMD virus thus a crucial role in the epidemiology of the disease (Bastos *et al*., 2003). Elsewhere in the world, cattle are usually the main reservoir for FMD viruses, although in some instances the viruses involved appear to be specifically adapted to pigs (OIE, 2017).

FMD is a highly transmissible disease and a limited number of infective particles can initiate infection. Transmission of FMDV occurs mostly by direct contact or aerosol droplets although indirect transmission is also possible. Contaminated animal products, non-susceptible animals, agricultural tools, people, vehicles, and airborne transmission can contribute to the mechanical dissemination of FMDV (Longjam *et al*., 2011; Lyons *et al*., 2014).

Of the domesticated species, cattle, pigs, sheep, goats and water buffalo (*Bubalus bubalis*) are susceptible to FMD. Many species of cloven-hoofed wildlife may become infected, and the virus has occasionally been recovered from other species as well. Amongst the Camelidae, Bactrian camels and new world Camelids have been shown to be susceptible (Larska *et al.*, 2009). The disease affects both adult animals and young stock. The morbidity rate can reach 100% while mortality rate in adults is very low (Depa *et al*., 2012) but, may reach 50% in young stock due to myocarditis.

2.5 Molecular Epidemiology of FMD Virus

Molecular epidemiology and evolution of FMDV has been studied using the sequences coding for VP1 (627–657 nt, depending on serotype), the most variable capsid structural protein containing relevant antigenic domains. This region reliably classifies the viruses into serotypes, topotypes and strains for most practical epidemiological applications (Lycett *et al*., 2019). Nucleotide sequences of field strains of FMDV contribute to the understanding of the distribution and evolution or viral lineages that circulate in different regions across the globe (Knowles *et al*., 2016).

Seven non-cross protective FMDV serotypes exist around the world, namely O, A, C, Asia 1, SAT 1, SAT 2 and SAT 3 with an ability to infect a wide range of domesticated and wildlife species of cloven hooved animals (Fowler *et al*., 2014). In Africa, six topotypes have been identified for serotype O, two for serotype A, three for C and 9, 14 and 5 topotypes for SAT 1, SAT 2 and SAT 3, respectively (Tekleghiorghis *et al*., 2014). Africa has been divided into three FMDV pools: East Africa (pool 4) with serotypes O, A, SAT 1, SAT 2 and SAT 3; West Africa (pool 5) with serotypes O, A, SAT 1 and SAT 2; and

southern Africa (pool 6) with serotypes SAT 1, SAT 2 and SAT 3 (FAO, 2018). These pools only relate to countries currently infected with FMDV. For SAT 1, eight topotypes were identified in sub-Saharan Africa, most have localised geographic distribution. Isolates from Zambia, Malawi, Tanzania, Kenya and northern Zimbabwe clustered in topotype III, demonstrating that related viruses were found in Southern and East Africa and describing a link between the two regions. SAT 2 demonstrates the most genetic diversity with a total of 14 topotypes. Topotypes VI and V occurred in West Africa. Topotype IV occurred in countries from East and southern Africa. For SAT 3, 6 genotypes exist. Zimbabwe had the most diversity with three topotypes. Topotype I (South Africa, southern Zimbabwe), II (Namibia, Botswana and western Zimbabwe) and III (Malawi and northern Zimbabwe). Topotype V and VI are found in East Africa (Vosloo *et al*., 2004).

2.6 Diagnosis of FMD

Accurate and rapid diagnosis of FMD is very crucial in controlling the disease. Preliminary diagnosis of FMD in suspected cases is based on clinical signs and confirmation by laboratory methods. Numerous serological and genome based assays have been developed for FMD diagnosis (Subramaniam *et al*., 2012). Epithelial samples, vesicular fluid, oropharyngeal fluid, throat swab, blood samples, semen samples and serum samples can be used for diagnosis.

Diagnosis of FMD can be done using different techniques such as Virus Isolation (VI), Sandwich-ELISA (S-ELISA), Liquid-Phase Blocking ELISA (LPBE), Multiplex-PCR (m-PCR) and real time-PCR. Nucleotide sequencing for serotyping, microarray as well as recombinant antigen-based detection, biosensor, phage display, and nucleic-acid-based diagnostic are used for rapid and specific detection of FMDV (Longjam *et al*., 2011).

Various pen side tests such as lateral flow, RT-LAMP, Immunostrip tests are also developed for detection of the virus under field conditions. Detection of antibodies against non-structural proteins using indirect ELISA (DIVA) can be used for differentiation between infected and vaccinated animals (Mahmoud *et al.*, 2019).

2.7 Economic Importance of FMD

Livestock enterprises and animal production contribute significantly to the world economy by providing household source of income, food security, draft power for crop cultivation, high quality animal proteins and vitamins, manure, raw materials (hides and skins) and generate a livelihood for thousands of people in the world (Baluka *et al*., 2014). In many African countries, the livestock sector contributes 30% agricultural GDP and approximately 60% of the value of edible livestock products is generated by cattle (AU-IBAR, 2010). However, infectious animal diseases generate a range of devastating economic impacts and undermine the livestock sector capacity, consequently lessening household incomes. Animal diseases cause losses of up to 30% of the annual livestock output in low and middle income countries (Tambi *et al*., 2006).

Foot-and- mouth disease is the most important livestock disease in the world in terms of economic impact (James and Rushton, 2002) due to its potential to cause production losses, and those related to the reaction of veterinary services to the presence of the disease and to animals trade restrictions both locally and internationally. At farm level, FMD epidemic can significantly increase costs for affected farmers through disease-induced mortality and loss of production animals, higher input costs (medicine and feed) and control measures such as culling of affected animals (Rich and Niemi, 2017). Production losses due to FMD

also include, reduced milk production, suppressed growth rate, loss of traction power and abortion in cows.

Concomitant with farm level losses are the control costs carried out by the state veterinary services such as vaccination, outbreak control, culling of animals and compensation in addition to significant amount spent by the private sector. Countries free of FMD or with FMD free zones have on-going costs due to effort to prevent disease introduction including veterinary fences repair, vaccination and import control (Knight-Jones and Rushton, 2013). Countries infected with FMD cannot trade live animals with FMD free countries. Trade of livestock products is also restricted. As a consequence, lack of access to lucrative market have devastating impacts on the economy and can restrict development of commercial farming.

2.8 Prevention and Control of FMD

Different national, regional and international control measures can be undertaken against foot-and-mouth disease. The measures taken depend on whether the country is free from the disease, is subject to sporadic outbreaks or is endemic of the disease. Routine vaccination against FMD is used in many countries or zones recognised as free from foot – and-mouth disease. However, a number of disease-free countries have never vaccinated their livestock but have preferred the use of strict movement controls and culling of infected and contact animals when outbreaks have occurred (OIE, 2017).

The control of FMD in endemic settings in Africa is mainly through repeated vaccination of domestic animal populations (usually only cattle) with multivalent FMD vaccines to reduce incidence of clinical FMD where the cattle population is at risk of infection from

sympatric or nearby wildlife populations. Nevertheless, vaccination alone is not sufficient to contain the disease and is coupled with physical separation of infected or high-risk populations of wildlife and/or domestic livestock from uninfected populations enabled through fencing systems, restrictions of animals movement and their products between localities of different FMD-status or FMD-risk, commonly executed through permit systems operated by the official veterinary service of the country concerned (Tidashe, 2018). However, several factors such as social customs, religious festivals and trade of animals in live markets can complicate control of animal movement (Jamal and Belsham, 2013).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

The study was conducted in Sigwe village in the Kabbe North-constituency (E 25° 03, 418’ S, 17° 73, 418’) of the Zambezi region, Namibia. Zambezi region is 14 785 km^2 in size, located in the Caprivi Strip; North-East part of Namibia. In Northwest, it borders the Cuado Cubago province of Angola, in the North it borders the western province of Zambia, in the south it borders the North-west district of Botswana and in the East it borders Zimbabwe. Laboratory analysis of clinical samples was conducted at the College of Veterinary Medicine and Biomedical Sciences, Sokoine University of Agriculture, Morogoro, Tanzania.

3.2 Study Design

This is a cross-sectional based on 2019 FMD outbreak. Zambezi region, Kabbe constituency was purposively selected since it is where recent FMD outbreak occurred. Cattle showing clinical signs of FMD were purposively selected.

3.3 Study Animals, Sample Identification and Transportation

A total of 11 epithelial tissues samples (from the 2019 outbreak) were used in this study. Epithelial tissues from un-ruptured or recently ruptured vesicles from the tongue, buccal mucosa or hooves were collected from 11 cattle showing clinical signs of FMD in two kraals that were few meters apart. Among those 11 samples, 7 samples (64%) were

collected from kraal A and the remaining 4 (36%) were from kraal B as shown in Table 1 below.

Table 1: Sample identification

S/N	Animal ID	Source/kraal	Sampling date	FMD vaccination date before outbreak
1	SSL227349	B	All sampled on 9 August 2019	February 2019
2	SSL328042	A		February 2019
3	SSL239042	A		February 2019
4	SSL267704	B		February 2019
5	SSL062015	A		February 2019
6	SSL328136	A		February 2019
7	SSL355501	A		February 2019
8	SSL328110	A		February 2019
9	SSL328041	A		February 2019
10	SSL270505	B		February 2019
11	SSL125699	B		February 2019

The samples were transported from the field to Central Veterinary laboratory (CVL) in Namibia by Air Namibia under cold chain and stored at-80°C. From CVL, clinical samples were triple packaged. The primary receptacle was leak-proof cryovials containing one sample each. All cryovials were packaged into the secondary receptacle (Biopack) which is also leak proof and with absorbent materials. The biopack was package into the third receptacle (styroafoam box) which was the outer shipping packaging. The outer package was properly labelled and the samples were shipped to Sokoine University of Agriculture, Morogoro, Tanzania. The collected samples were transported from Namibia by air with import permit number: 0005 914 to Tanzania. These samples fall under category of infectious substances affecting animals with UN Number 2900. Upon arrival, samples were stored at -80°C until use. The consignee was informed on the date of samples arrival by the consignor.

3.4 Study Methodology

3.4.1 Sample preparation

A total of eleven (n=11) epithelial tissue samples were used in this study. 10% epithelial tissue suspension was prepared by grinding 1g of epithelial tissue into 100 µl of Phosphate-buffered saline (PBS) at the pH of 7.2 using sterile mortar and pestle. The suspension was used for RNA extraction. Excess suspensions from different samples were stored in cryo-vials at -80°C for future use.

3.4.2 RNA extraction

Total RNA was extracted from 460 µl 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. 460 µl 70% ethanol was added to the same tube and the mixture was vortexed. The maximum loading volume (700 µl) was loaded into the RNeasy spin column and centrifuged at 12 000 rpm (7000-10 000 g) for 15 seconds. The flow-through was discarded and the procedure was repeated with the remaining volume using the same collection tube. Volume of 700 µl buffer RW1 was added, centrifuged at 12 000 rpm for 15 seconds and the flow-through was discarded.

Similarly, washing was done using 500 µl wash buffer RPE and the collection tube with the flow-through was discarded. The washing was repeated with 500 µl and centrifuged at 12 000 rpm for 2 minutes to dry the membrane. 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 traces of ethanol. Lastly, RNA

was eluted with 50 µl of nuclease free water into a new 1.5ml tube, centrifuged for 60 seconds at 12 000 rpm and stored at -40°C until further use.

3.4.3 One-Step Reverse Transcription Polymerase Chain Reaction (RT-PCR)

One-step reverse transcriptase polymerase chain reaction (RT-PCR) was carried out for the extracted RNA for detection of the presence of FMDV genome using primers that target the highly conserved RNA sequences within the 5'-untranslated region (5'UTR). This assay was combined with reverse transcription. Therefore, the composition of the 25 µl of master mix for one-step RT-PCR consisted of the following: 8 µl nuclease free water, 1µl dNTPs, 5µl reaction buffer (containing 12.5Mm $MgCl_2$), 2.5µl forward primer (4 pm/µl), 5 µl reverse primer (4 pm/µl) and 1 µl of One-Step RT-PCR enzyme. Preparation of the master mix was carried in the laboratory clean room inside the PCR Workstation. The RT-PCR was carried out in GeneAmp PCR System 9700 (Applied Bio systems) in the PCR room. Thermal profile is shown in table 2 while primers used shown in table 3 below.

In this assay, negative and positive controls were included in each run. However, the controls used were not certified controls. The positive control was a known FMD positive sample from Tanzania while the negative control was nuclease free water.

Table 2: Temperature profile for RT-PCR

Step	Temperature	Time	Cycle
reverse transcription	50°C	30 min	1
inactivation of reverse transcriptase	95°C	15 min	1
Denaturation	95°C	1 min	35
Annealing	55°C	1 min	
Extension	72°C	2 min	
Final extension	72°	5 min	1
Holding	4°C	∞	

Table 3: Oligonucleotide primers used for RT-PCR

Primer	Serotype	Sense	Sequence (5'-3')	Gene	Size (bp)
1F	Universal	Forward	GCCTGGTCTTTCCAGGTC	5'UTR	328

1R	Reverse	TCCAGTCCCCTTCTCAGATC

Source: Reid *et al.* (2000)

3.4.4 Detection of PCR products by Agarose Gel Electrophoresis

PCR products were analysed by agarose gel electrophoresis. cDNA fragments of 328bp were determined by 1.5% gel electrophoresis. For this purpose, 1.5g (1.5% m/v) of agarose gel in 100 ml of 1X TAE (Tris base, acetic acid and EDTA) buffer was prepared. The gel was allowed to cool to about 37°- 40°C and 10 µl of EZ-vision® Bluelight DNA dye was added to the molten gel. The gel was then casted into an electrophoresis apparatus on the leveled surface and the combs were inserted and allowed to solidify at room temperature for 60 minutes. The electrophoresis tank was filled with 1X TAE buffer to the maximum level. The electrophoresis apparatus was placed in the chamber so that the gel is submerged in the buffer and the combs were removed. Samples were prepared on para film by mixing 5 µl of PCR product with 1 µl of the loading dye. The samples were loaded in parallel with DNA ladder. Amplified products were run under 100 volts for 1 hour. Fragment sizes were determined by comparing with 100bp ladder (GeneRuler 100bp DNA ladder plus) that confirm the correct size of the product. The gel was then viewed under UV Transilluminator and photographed.

3.4.5 Serotype identification

To determine the serotypes, all samples (n=11) were subjected to another PCR assay. Primers that target the VP1 coding region for five serotypes (SAT 1, SAT 2, SAT 3, serotype O and serotype A) were used as shown in table 4 below. The reaction mix

consisted of; 8 µl nuclease free water, 1 µl dNTPs, 5µl reaction buffer, 2.5 µl forward primer, 5 µl reverse primer and 1 µl enzyme was used.

Table 4: Oligonucleotide primers used in serotyping

Serotype	Primer name	Primer sequence (5'-3')	Sense
SAT1	FMDV/SAT1/EA/FP	CTYGACCGGTTCACYCTG	+
	FMDV/SAT1/EA/RP	CCGAGAAGTAGTACGTRGC	-
SAT2	FMDV/SAT2/EAiV/FP	CRATCCGCGGTGAYCG	+
	FMDV/SAT2/EAiV/RP	CGCTTCATYCTGTAGTARACGTC	-
SAT3	SAT3-1D208F	GCYACGTAYTACTTYTGTGACCT	+
	SAT3-1D520R	ACARTCKHCCGAAGTTGAA	-
A	FMDV/A/EA/FP	GCCACRACCATCCACGA	+
	FMDV/A/EA/RP	GAAGGGCCCAGGGTTGGACTC	-
O	FMDV/O/EA/FP	CCTCCTTCAAYTACGGTG	+
	FMDV/O/EA/RP	GCCACAATCTTYTGTTTGTG	-

Source: Bachanek-Bankowska *et al*. (2016) *(+) Represents forward primer

****(-) represents reverse primer**

3.4.6 Amplification of VP1 Coding region

Amplification of the VP1 coding region was done using serotype specific primers shown in table 5 below. Two different forward primers were used for SAT 3 and SAT 1 serotypes and only one reverse primer was used for each serotype for all samples (n=11) in this assay. 1.5% agarose gel electrophoresis was done to determine the product size. The thermal profile used for the amplification of VP1 sequence was as follows: 50°C for 30 minutes, 95°C for 15 minutes, 35 cycles of 95°C for 60 seconds, 50°C for 60 seconds, 72°C for 2 minutes followed by final extension at 72°C for 5 minutes. The PCR reactions were carried out in GeneAmp PCR System 9700 (Applied Biosystem) in the PCR room.

Table 5: Oligonucleotide primers used for VP1 amplification

Serotype	Sense	Primer name	Sequence (5'- 3')	Gene	Product size (bp)
SAT 1	Forward	SAT1-IC559F	GTGTATCAGATCACAGACACAC A	VP3	1,043
	Forward	SAT1U-OS	GTGTACCAGATCACTGACAC	VP3	1,043
SAT 3	Forward	SAT3-1C559F	CTGTACCAAATYACAGACAC	VP3	1,034
	Forward	SAT3-P1-1222F	AATCTGCATTTCATGTACAC	VP3	1,277
SAT1 & SAT3	Reverse	SAT–2B208R	ACAGCGGCCATGCACGACAG	2B	-

Source*:* Knowles *et al.* (2016)

3.4.7 DNA purification and concentration determination

Purification of DNA was carried out using GFX™ PCR DNA and gel band Purification kit (GE Healthcare Life Sciences, UK) following manufacturer's instruction to remove unincorporated primers and free nucleotides before sequencing. Briefly, 5 volume of capture buffer was added to 1 volume of the PCR sample and mixed. The GFX spin column was placed in a collection tube and centrifuged for 60 seconds at 13 000 rpm to bind DNA. The flow-through was discarded and the spin column was placed back into the same collection. Washing was done by adding 0.5ml wash buffer type 1 to the GFX column and centrifuged for 60 seconds. The GFX column was placed in a clean 1.5ml microcentrifuge tube and DNA was eluted by adding 20µl elution buffer 6 to the centre of the GFX membrane. The column was left to stand for 1 minute and centrifuged for 1 minute at 13 000 rpm. Detection of purified DNA was done by agarose gel electrophoresis as described above. The concentration of purified DNA was determined by adding 2µl of DNA on NanoDrop™ spectrophotometer (Biochrome, Cambridge, England).

3.4.8 Cycle sequencing, ethanol precipitation and sanger sequencing

Amplified cDNA products were sequenced using sequencing primers shown in Table 6. The cycle sequencing reaction was set up using different sequencing primers both for SAT 1 and SAT 3 serotypes using BigDye® Terminator v3.1 cycle sequencing kit (Thermofisher, Vilnius, Lithunia) following instructions by the manufacturer. All amplicons were forward sequenced with the sequencing primers for that serotype and reverse sequenced with universal reverse primer NK72.

Table 6: Oligonucleotide primers used for sequencing

Serotype	Primer name	Primer sequence	Direction	Gene
All	NK72	GAAGGGCCCAGGGTTGGACTC	Reverse	2A/2B
SAT1	SAT1-ID394R	GGYTTGTACTTRCARTCACCGTTGTA	Reverse	VP1
	SAT1-P1-1228F	AACCTGCACTTCATGTACAC	Forward	VP3
	SAT1-1D200F	TGCGYGCIGCCACGTACTAYTTCTC	Forward	VP1
	SAT-1D209F	CCACATACTACTTTTGTGACCTGGA	Forward	VP1
SAT3	SAT3-1D208F	GCYACGTAYTACTTYTGTGACCT	Forward	VP1
	SAT3-1D520R	ACARTCKHCCGAAGTTGAA	Reverse	VP1
	SAT-1D209F	CCACATACTACTTTTGTGACCTGGA	Forward	VP1

Source: Knowles *et al.* (2016)

The master mix consisting of 3.5 µl nuclease free water, 2.0 µl sequencing buffer, 0.5 µl BigDye Terminator v3.1, 3µl specific sequencing primer and 1.0 µl target cDNA template was used. The master mix was prepared in laboratory clean room and cDNA was added in a PCR laboratory. The PCR reactions were carried out in individual 0.2 ml thin walled

tubes at the following thermo-profile; 96°C for 1 minute and 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes followed by holding at 10°C in a GeneAmp PCR System 9700 (Applied Biosystems).

Following cycle-sequencing, desalinating and concentrating of DNA was done by ethanol precipitation. Briefly, 5 µl of fresh 125 Mm EDTA at pH 8.0 and 60 µl of 100% ethanol were added to each tube containing products of the PCR reaction. The tubes were vortexed and incubated in dark for 15 minutes at room temperature to precipitate the extension product. The tubes were then spinned at 13 000 rpm for 30 minutes and the supernatants were discarded leaving the pellets. The pellets were washed with 60 µl 70% ethanol, centrifuged at 13 000 rpm for 30 minutes and the supernatants were discarded. The pellets were vacuum dried in a vacuum drier for 1 hour. The pellets were re-suspended in 20 µl Hi-Di formamide and incubated in dark for 15 minutes. The samples were then loaded into the 96-well plate and placed into the sequencer. Amplified DNA was then directly sequenced on both strands using automated ABI PRISM®3500 DNA sequencer machine (Applied Biosystems) to obtain completed VP1 sequence.

3.4.9 Sequence editing, alignment and phylogenetic tree reconstruction

The chromatograms obtained for each individual reaction using forward and reverse primer were assembled into contigs using Geneious computer software version 10.2.3. The consensus nucleotide sequences were then trimmed manually to 417 nucleotides long. A total of 8 SAT 3 serotype VP1 nucleotide sequences from this study were used for online blast search to retrieve highly similar sequences from GenBank using National Centre for Biotechnology Information (NCBI) nucleotide BLAST. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The tree was 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 (Kimura, 1980) and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 256 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al*., 2016). Topotypes and genotypes were determined using previously identified strains (Knowles and Samuel, 2003).

CHAPTER FOUR

4.0 RESULTS

4.1 Detection of FMDV Genome

All eleven (n=11) epithelial tissue samples were subjected to one-step reverse transcription polymerase chain reaction (RT-PCR) assay using universal primers targeting the 5'UTR. Positive and negative controls were also included for reliability and reproducibility of results. All samples tested positive for FMDV genome with detection rate of 100%. Results are shown in Figure 3 below.

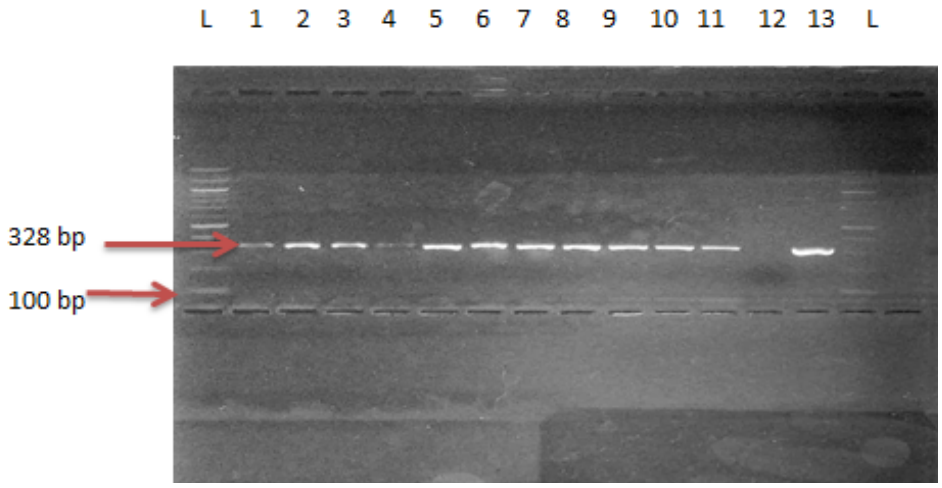

Figure 3: Gel electrophoresis of amplified RT-PCR products using 5'UTR universal primers. L represents 100bp DNA ladder. Wells 1-11 were FMDV samples of 2019 outbreak in Zambezi. Wells 12 and 13 were negative and positive controls respectively.

4.2 Serotype Identification

All samples were serotyped by molecular based technique using serotype specific primers targeting the highly variable VP1 region of FMDV genome for serotypes A, O, SAT 1, SAT

2 and SAT 3. Following gel electrophoresis, the PCR products for SAT 1 and SAT 3 serotypes were detected (Table 7).

Table 7: Serotype identification

Sample #	Serotype				
	SAT1	SAT2	SAT3	A	O
1	+	-	+	-	-
2	+	-	+	-	-
3	+	-	+	-	-
4	-	-	-	-	-
5	+	-	+	-	-
6	+	-	+	-	-
7	+	-	+	-	-
8	+	-	+	-	-
9	+	-	-	-	-
10	-	-	-	-	-
11	+	-	+	-	-

4.3 VP1 Amplification

All eleven samples confirmed positive for the presence of FMDV genome as described above. After serotyping, the PCR products were subjected to amplification of VP1 region using two sets of forward primers and one reverse primer both for SAT 1 and SAT 3 serotypes (as described in 3.4.6 above). Out of eleven samples, 73% (n=8) were amplified, while three (27%) samples were not amplified by any primer for either of the serotype (Table 8). A total of forty four (44) reactions were run, out of which, twenty two (50%) were by SAT 1 primers while the remaining half (50%) were amplified by SAT 3 primers. Most of the samples were amplified by primers for SAT 3 while most of SAT 1 reactions gave negative results (no amplification). VP1 amplification results both for SAT 1 and SAT 3 are shown on Table 8 below. SAT 1 samples gave false positive results with SAT 1 specific primers. However, sequencing of these samples revealed that they belonged to SAT 3 serotype.

Table 8: Amplification of VP1 coding region

Sample no.	SAT1 primers		SAT3 primers	
	SAT1-IC559F	SAT1U-OS	SAT3-IC559F	SAT3-P1-1222F
1	-	-	-	-
2	+	-	+	+
3	-	-	-	+
4	-	-	-	-
5	-	+	+	+
6	-	+	+	-
7	-	+	+	-
8	-	+	+	+
9	-	+	+-	+
10	-	-	-	-
11	-	-	+	-

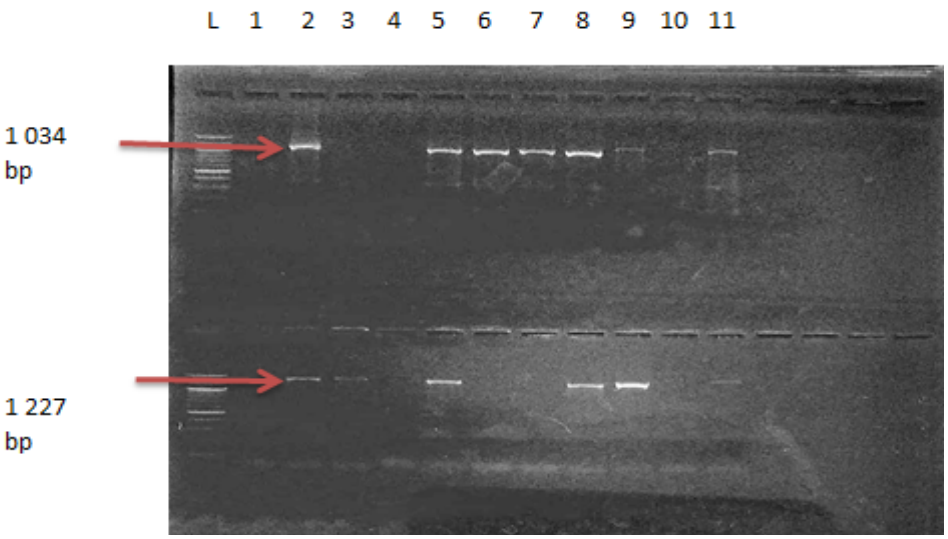

Figure 4: Gel electrophoresis of PCR products of VP1 gene of FMDV SAT3 amplified using forward primers SAT3-1C559F and SAT3-P1-122F with SAT-2B208R as reverse primer. L is 100bp DNA ladder. Wells 1-11 represent FMDV samples.

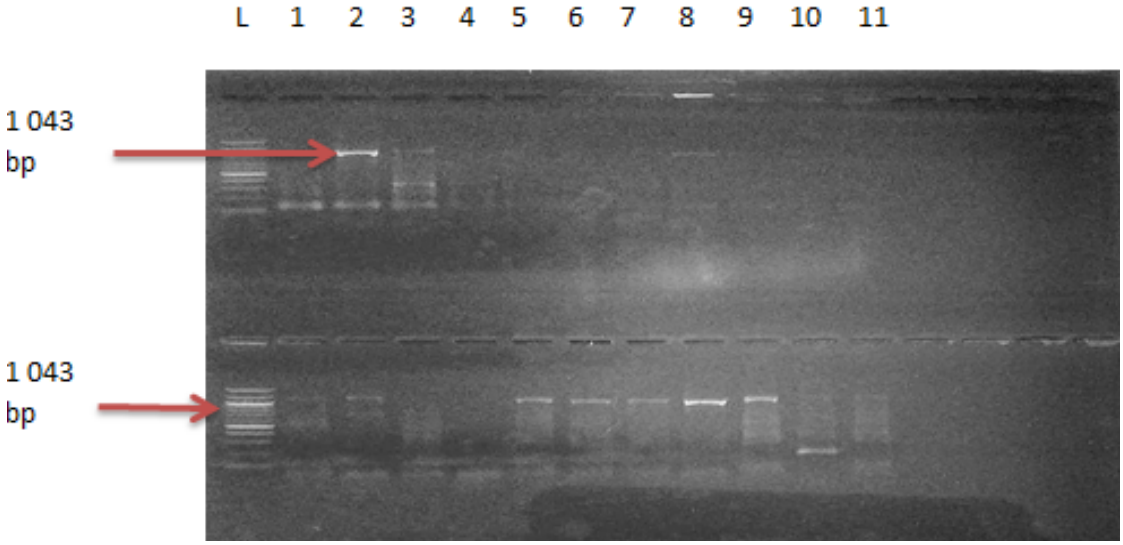

Figure 5: Gel electrophoresis of PCR products of VP1 gene of FMDV SAT1 amplified using forward primers SAT1-1C559F and SAT1U-OS with SAT-2B208R as reverse primer. L is 100bp DNA ladder. Wells 1-11 represent FMDV samples

4.4 Concentration of Purified cDNA

Using a spectrophotometer, with complementary findings from electrophoresis, it was found that all samples analysed are of good quality with purity measurement A260/280 ratio 1.836 and above (Table 9).

Table 9: Concentration of purified cDNA

Sample no.	Conc. cDNA (ng/µl)	A260/280	A260/230
02.SAT1-IC559	83.5	1.856	0.815
05-SAT1-IU-OS	39.0	1.837	0.681
06-SAT1-IU-OS	43.0	1.870	1.117
07-SAT1-IU-OS	26.0	1.898	0.233
08-SAT1-IU-OS	35.0	1.842	0.424
09-SAT1-IU-OS	67.0	1.836	0.899
02-SAT3-559	92.0	1.859	1.243
05-SAT3-559	53.5	1.945	0.301
06-SAT-559	59.5	1.919	0.730
07-SAT3-559	46.0	1.917	0.211
08-SAT3-559	60.0	1.846	0.359
09-SAT3-559	26.5	1.906	0.654
11-SAT3-559	56.0	1.898	0.926
02-SAT3-1222	23.5	1.911	0.560
03-SAT3-1222	13.5	1.949	0.274
05-SAT3-1222	25.0	1.838	0.370
08-SAT3-1222	35.0	1.842	0.295
09-SAT3-1222	49.5	1.868	0.798

4.5 Sequencing and Phylogenetic Analysis

Each sample gave two PCR products on the basis of two different forward primers used for amplification of VP1 region. Sequence analysis revealed the presence of FMDV serotype SAT 3 only. Genetic relationships of SAT 3 viruses were determined by phylogenetic analysis of partial VP1 gene sequence data by comparing sequences from this study with other Namibian and African isolates especially those circulating in SADC and East African region. A total of 21 highly similar SAT 3 VP1 sequences were retrieved from GenBank and other 7 were taken from previous publication as shown in Table 10 below.

Table 10: Details of the foot-and-mouth disease viruses used in this study

Isolate	Year isolated	Host species	Country	Accession number	Reference
2/NAM/2019	2019	Bovine	Namibia	N/A	This study
3/NAM/2019	2019	Bovine	Namibia	N/A	This study

5/NAM/2019	2019	Bovine	Namibia	N/A	This study
5/NAM/2019	2019	Bovine	Namibia	N/A	This study
7/NAM/2019	2019	Bovine	Namibia	N/A	This study
8/NAM/2019	2019	Bovine	Namibia	N/A	This study
9/NAM/2019	2019	Bovine	Namibia	N/A	This study
11/NAM/2019	2019	Bovine	Namibia	N/A	This study
NAM/5/94	1994	Bovine	Namibia	AY168806	GenBank
NAM/1/94	1994	Bovine	Namibia	AY168805	GenBank
Sat3-3kenya iso 22	1960	N/A	Kenya	AY593852	GenBank
Sat3-3bech iso 29	1961	N/A	Botswana	AY593851	GenBank
BOT-BUFF/13/70	1970	Buffalo	Botswana	MHO53339	GenBank
ZIM/2/1984	1984	Bovine	Zimbabwe	MHO53351	GenBank
ZIM 2/1984	1984	N/A	Zimbabwe	HQ268511	GenBank
Sat3-4bech iso 23	1965	N/A	Botswana	AY5938853	GenBank
M28719	N/A	N/A	N/A	M28719	GenBank
NAM/294/98	1998	Buffalo	Namibia	AY258052	GenBank
RHO/7/74	1974	Bovine	Zimbabwe	MHO53344	GenBank
KNP/33/94	1994	Buffalo	South Africa	AY168803	GenBank
KNP/14/96	1996	Buffalo	South Africa	AY168813	GenBank
ZAM/11/96	1996	Buffalo	Zambia	AY168815	GenBank
ZAM/P2/96	1996	Buffalo	Zambia	MHO53342	GenBank
KNP/6/88	1988	Buffalo	South Africa	AY168791	GenBank
KNP/02/03	2003	Buffalo	South Africa	MK415738	GenBank
SAT3/KNP/14/96	1996	Buffalo	South Africa	MK415741	GenBank
RHO/3/78	1978	Bovine	Zimbabwe	AY168790	GenBank
RHO/11/77	1977	Bovine	Zimbabwe	MHO53348	GenBank
RHO/16/76	1976	Bovine	Zimbabwe	MHO53346	GenBank
ZIM/3/94	1994	Buffalo	Zimbabwe	AY168807	Bastos *et al*., 2003
ZIM/11/94	1994	Buffalo	Zimbabwe	AY168808	Bastos *et al*., 2003
BOT/6/98	1998	Buffalo	Botswana	AY258050	Bastos *et al*., 2003

ZIM/1/99	1999	Bovine	Zimbabwe	AY168819	Bastos *et al*., 2003	
ZAM/1/93	1993	Buffalo	Zambia	AY258049	Bastos *et al*., 2003	
BOT/9/98	1998	Buffalo	Botswana	AY168816	Bastos *et al*., 2003	
SAT3/UGA BUFF/27/70	1970	Buffalo	Uganda	AJ303480	Bastos *et al*., 2003	

*N/A: Not Available

The evolutionary relationships of 36 nucleotide sequences of FMDV SAT 3 isolates including 8 sequences of the present study is depicted below (Fig. 6). The optimal tree with the sum of branch length = 1.95754798 is shown (Fig. 7). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The optimal tree with the sum of branch length = 1.95754798 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985).

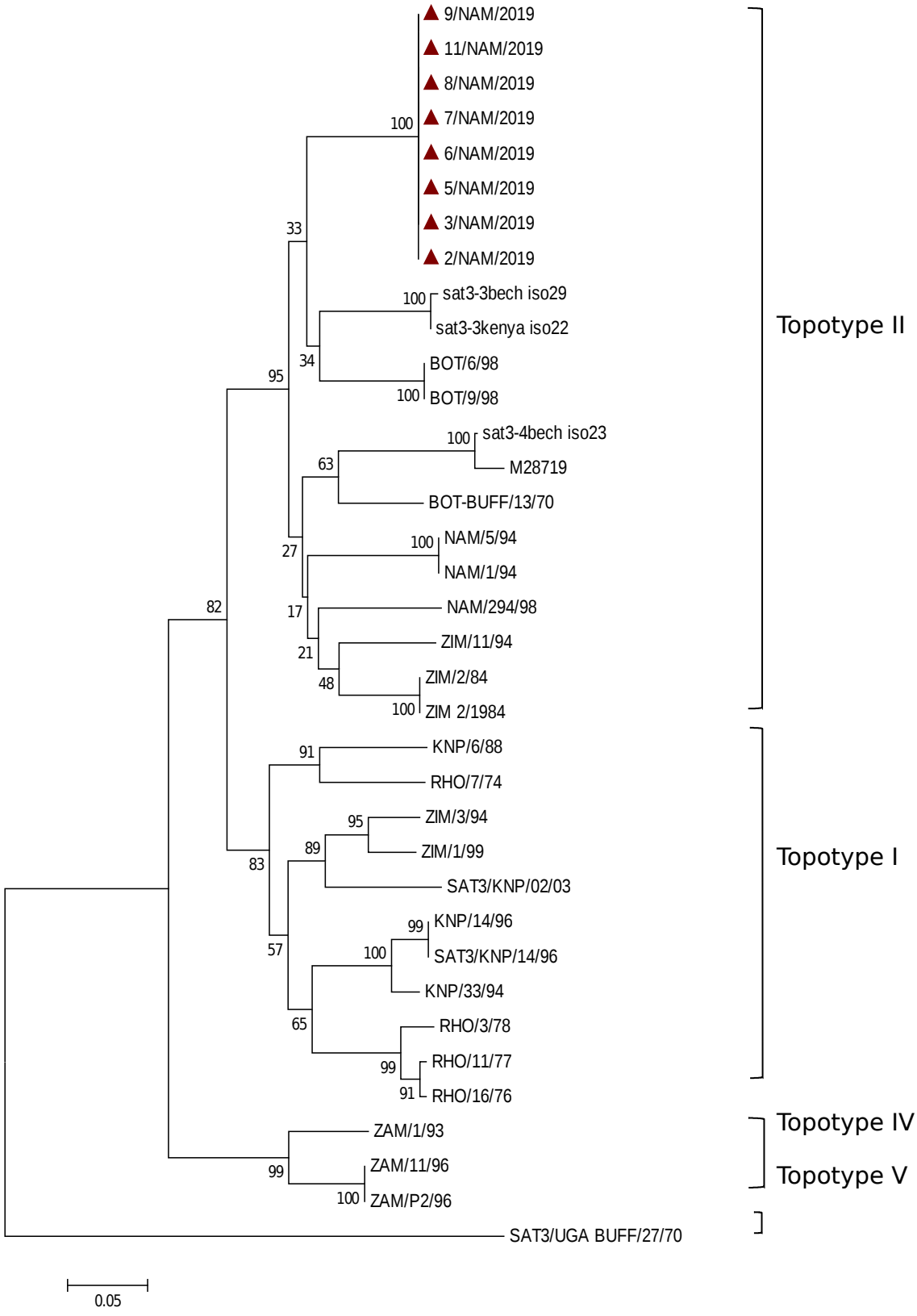

Figure 6: Neighbor-Joining tree showing the relationship between selected SAT 3 FMDVs based on VP1 gene sequences

The phylogenetic analysis of 417 nucleotide sequence of VP1 gene of FMDV SAT 3 serotype 2019 Namibian isolates showed that the strains were clustered with other strains within SADC and East Africa and indicated significant grouping which showed genetic variation of virus isolates. The tree consisted of three main clusters. The first cluster consisted of virus strains mainly from SADC region and one strain from East Africa while the second cluster consisted of viruses circulating in the SADC region only.

Cluster one is further divided into two clades of which isolates from this study formed one clade. Isolates from this study are closely related to two Namibia historic isolates (NAM/5/94 and NAM/1/94) of which they shared 86.81% and 86.75% nucleotide identity respectively. The VP1 sequences of FMDV SAT 3 from this study were also compared to other African isolates available in GenBank database. Isolates from this study shared 86.75% nucleotide identity with sat3-3kenya iso22 from Kenya, 86.33% with sat3-3bench iso29 and 86.16% identity with isolate BOT-BUFF/13/70 from Botswana. Furthermore, SAT 3 virus isolates from this study shared only 85.44% with Namibian isolate NAM/294/98 and were distantly related to isolate RHO/11/77 from Zimbabwe and KNP/33/94 from South Africa with 22.41% and 20.48% nucleotide diversity respectively. The isolates were also compared to other strains including those retrieved from publication as depicted in Figure 6 above.

CHAPTER FIVE

5.0 DISCUSSION

Foot-and- mouth disease is considered as one of the most contagious and acute disease in domestic ruminants and pigs. The disease imposes a massive threat to livestock industry and consequently induces negative economic impacts. Therefore, identification of the correct FMDV causing a particular outbreak plays a crucial role in taking measures to control and eradicate the disease (Ranaweera *et al*., 2019).

In this study, all eleven (100%) epithelial tissue samples tested by RT-PCR for the presence of FMDV genetic materials were found positive. This is justified by Urge and Gizaw, (2018) that epithelial tissues are preferred samples for FMDV detection since it is in epithelial tissue where virus multiplication starts from the pharynx epithelium and spread to oral mucosa to form vesicles. Other studies have also demonstrated that FMDV can as well be harvested from probang samples comprising oropharyngeal scraping and fluid (Dhikusooka *et al*., 2015).

In this study, sequencing revealed that most samples belonged to SAT 3 serotype. However, during serotyping, SAT1 positive samples were detected using SAT1 specific primers (FMDV/SAT1/EA/FP/ FMDV/SAT1/EA/R). This indicated that, serotyping alone might not be enough and can misguide in diagnosis of FMDV infection since cross-reactivity of primers between serotypes may occur. The serotype of 2 samples was not determined using serotype-specific primers. This could be ascribed to low concentration of viral RNA in the samples. Further studies using highly sensitive molecular techniques such as Next Generation Sequencing (NGS) are highly recommended to unravel the serotype, genotype

and genetic diversity of the undetermined FMD virus samples. Previous study by Ali *et al*. (2018) disclosed that mutation at primer binding sites affect detection by RT-PCR making the specificity of primers questionable. FMDV is continuously evolving virus and all assay developed for serotype identification are designed in the most variable region (VP1) of its genome. Therefore, the introduction of new lineages or the finding of new strain in this region with changes within primer attachment sites limits detection capability of these assays.

Sequencing of VP1 coding region of FMDV of the present study confirmed that SAT 3 serotype was responsible for the 2019 outbreak in Zambezi region. This is in agreement with findings by studies that reported that SAT serotypes are prevalent and dominant causing FMD outbreaks in southern Africa including Namibia (Knowles and Samuel, 2003). However, SAT 3 only accounts for 16% of FMD outbreaks in cattle (Bastos *et al*., 2001).

Phylogenetic analysis of the VP1 coding region of FMDV has been broadly used to determine evolutionary dynamics and epidemiological relationships of genetic lineages (Samuel and Knowles, 2001), thus it is used to identify FMDV isolates. These techniques have helped to define genetic relationships between FMDV isolates and geographical distribution of lineages and genotypes. They have also helped establish genetically and geographically linked topotypes to trace the source of outbreaks. FMDV topotypes are defined as clustered viruses that form a single genetic lineage based on nucleotide differences of up to 15% (O, A,C and Asia 1) or 20% nucleotide differences for SAT viruses (Knowles and Samuel, 2003).

The phylogenetic analysis of FMDV VP1 sequences of this study revealed that viruses that caused 2019 outbreak in cattle in Zambezi region in Namibia are clustered together with Namibia historic isolates and isolates from neighbouring Botswana and Zimbabwe. Since the study area is bordered by these countries, this probably indicate trans-boundary transmission, but further molecular epidemiological studies across countries and detailed genetic analysis are needed to have more insight of genetic and geographical linkage of these viruses.

Furthermore, phylogenetic analysis of SAT 3 sequences disclosed that 2019 isolates are clustered with historic sequences from African buffaloes, indicating possible interspecies transmission at wildlife-livestock interface. This also suggests the existence of natural reservoir for FMDV in Zambezi region. This is in agreement with studies from previous studies that show a role of African buffaloes in maintenance and transmission of SAT serotypes to domestic animals in SSA (Knowles and Samuel, 2003). However, close genetic relationship of 2019 isolates to Namibia historic isolates recovered in cattle communicate a possibility of passing the virus from long-term carrier animals to susceptible hosts. In addition, viruses recovered in this study were related to historic Namibia isolates recovered in African buffaloes over 20 years ago. There is high population of free loaming African buffaloes in Kabbe constituency because of its geographical location and presence of conservancies (Kasika Conservancy, Nakabolelwa Conservancy, Impalila Conservancy and Kabulabula conservancy) in this constituency.

In agreement with a study by Bastos *et al*. (2003), the phylogenetic analysis revealed presence of distinct lineages, of which three corresponding to southern Africa and one unique to East Africa. Isolates from this study fall under topotype II, and clustered together with isolates from Botswana and Zimbabwe. Therefore, viruses that caused the 2019

outbreak in Zambezi region and viruses from Botswana (BOT/9/98, BOT/6/98), Namibia (NAM/1/94, NAM/5/94) and Zimbabwe (ZIM 2/1984, ZIM/2/84) are genetically related. However, viruses from other parts of Zimbabwe, South Africa and Zambia were genetically distinct from the 2019 Namibia isolates. Based on genetic relatedness of 2019 Namibia isolates to viruses in neighbouring countries, there is a possibility that the virus has originated from neighbouring countries.

The study animals were vaccinated against FMD virus in February 2019 using a trivalent (SAT 1, SAT 2 and SAT 3) vaccine (Yule, J. personal communication, 2019). This is approximately six months prior to the outbreak. Parida and Mahapatra, (2018) pointed out that, FMD vaccines only provide a short-lived protection (4-6 months) and there is also incomplete protection between some subtypes of the same serotype which affect the application of vaccine in the field. Furthermore, new variant viruses are emerging periodically and antigenic mismatch makes the existing vaccines inefficient. The antigenicity of FMDV change due to mutations occurring in its genome which sometimes allows evasion of the immunity provided by the vaccines resulting into emergency of immunologically distinct variants.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

This study has revealed that the FMD outbreak of 2019 on cattle in Zambezi region, Namibia was due to SAT 3 viruses of topotype II in a wide host range including both domestic and wildlife species that are roaming freely across borders between neighbouring countries, communal grazing areas and watering points. Diagnosis of FMDV infection requires highly sensitive molecular techniques such as Next Generation Sequencing technique because sequencing alone might misguide in the diagnosis of FMD. Furthermore, the presence of multiple serotypes and genotypes/topotypes of FMDV as well complex epidemiology of FMD complicates the control of the disease through vaccination.

6.2 Recommendations

i. Further molecular epidemiological studies need to be undertaken that include sampling both domestic animals and wildlife to have a deeper understanding on the genetic diversity, evolutionary dynamics, epidemiological relationships of genetic lineages and geographical distribution of genotypes of foot- and-mouth disease virus in sub-Saharan Africa.
ii. Further studies also need to be undertaken in the field of diagnosis, vaccines and epidemiology to accelerate progress for FMD control at national, regional and international levels.
iii. In any case of FMD outbreak, vaccine matching should be done before vaccination to ensure that the vaccines used could provide protection against field isolates and

vaccination (s) should be carried out at regular interval using vaccines prepared from circulating strains since matching vaccines with circulating viruses is crucial for successful implementation of vaccination based FMD control in Namibia and neighboring countries.

iv. Namibian government should consider setting up FMDV research laboratory that could routinely evaluate vaccine strains and characterize the field isolates using the highly sensitive molecular biology tools and/or platforms.

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