GENETIC AND ANTIGENIC CHARACTERIZATION OF FOOT AND MOUTH DISEASE VIRUS STRAINS ISOLATED IN 2011 AND 2015 IN NGAMILAND, BOTSWANA

LATOYA SEOKE

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

2016

ABSTRACT

Foot-and-mouth disease (FMD) is a highly contagious disease of cloven hooved animals that continues to occur in Ngamiland District of Botswana although stringent disease control measures have been put in place. This may be due to irrelevance of currently used vaccine strains. In the present study, genetic and antigenic characteristics of SAT2 viruses isolated from outbreaks which occurred in 2011 and 2015 in Ngamiland districtwere examinedin order to determine any mutational changes of the FMD viruses circulating in that area. The antigenic relationships between the outbreak strains and SAT2 vaccine strains currently used were also determined. Tissue samples collected were subjected to sequencing of the VP1 gene, phylogenetic analysis and vaccine matching with the two SAT2 vaccines strains currently in use in the same region; SAT251 and SAT2035. There was almost 100% amino acid sequence similarity within both outbreaks while minimal mutations (90% sequence similarity) occurred between the outbreaks. Phylogenetic analysis revealed that both outbreaks were caused by genetically similar viruses that belong to SAT2 topotype III. The newer vaccine strain, SAT2035 clustered with the field virus isolates on the phylogenetic tree indicating that it also belongs to the same topotype. However, the older vaccine strain, SAT251, was shown to belong to topotype II. Amino acid variation analysis revealed that mutations occurred post the 2011 outbreak but did not impact on the antigenicity of the field isolates although majority of variability occurred at known FMDV antigenic sites. This is confirmed by $r_1$values obtained against both vaccine strains. The findings are evidence that the vaccines provide satisfactory immunity and are still relevant to confer protection against circulating field strains in the area because minimal mutationsoccurred intra- and inter-outbreaks. Recurrence of the disease is probably due to low vaccination coverage and this should be improved.

DECLARATION

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

_____ _____

LaToya Seoke Date

(Candidate: Master of Science in One Health Molecular Biology)

The declaration is hereby confirmed by;

_____ _____

Prof. Christopher J. Kasanga Date

(Supervisor)

_____ _____

Dr. Joseph Hyera Date

(Supervisor)

COPYRIGHT

ACKNOWLEDGEMENTS

My sincere gratitude goes out to Botswana Vaccine Institute (BVI), my employer, for funding as well as giving me full time off to pursue my studies. This work was conducted at the OIE sub-Saharan Africa Regional Reference Laboratory for FMD (OIE-SSARRL-FMD). I extend my gratitude to the OIE-SSARRL-FMD staff for providing me with technical support.

The bioinformatics analysis was carried out at the Veterinary and Agrochemical Research Centre (CODA-CERVA), Groeseleberg, Brussels, Belgium. I am greatly indebted to staff of CODA-CERVA, in particular Dr Kris De Clercqand Dr Haegmann for the great assistance I received while doing this analysis.

I thank the Director of Veterinary Services, Ministry of Agriculture in Botswana for providing appropriate samples during all outbreaks (2011 – 2015) to the OIE laboratory at BVI and for granting permission to use the samples in these studies.

I am grateful to my supervisors Prof. Christopher J. Kasanga of Sokoine University of Agriculture and Dr. Joseph Hyera of BVI for their willingness to supervise my research work and providing their support during the course of this work.

Appreciation goes to my fellow students for their continued cooperation during our period of study. I am also thankful to my family and friends for their moral support throughout my studies.

DEDICATION

To my mother, Constance Seoke, who prayed for my success without ceasing, and to my dear friends who were there for me every step of the way, I dedicate this work.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

µl	microliter
aa	amino acids
BOT	Botswana
bp	base pairs
BVI	Botswana Vaccine Institute
cDNA	Complementary Deoxyribonucleic Acid
CFT	Complement Fixation Test
CPE	Cytopathic effect
cre	Cis-acting replication element
DIVA	Differentiating Infected from Vaccinated Animals
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
EU	European Union
EuFMD	European Commission for the control of foot-and-mouth disease
FAO	Food and Agriculture Organisation
FMD	Foot-and-mouth disease
FMDV	Foot-and-mouth disease virus
HPV	hypervariable
IRES	Internal Ribosome Entry Site
kb	kilobases
LITS	Livestock Identification and Traceability System
LK-MEM	Lamb Kidney Minimum Essential Medium
LPBE	Liquid-phase blocking ELISA
mAb	monoclonal antibodies

MEGA	Molecular Evolutionary Genetics Analysis
ML	Maximum likelihood
mPCR	multiplex PCR
NASBA	Nucleic acid sequence based amplification
NCBI	National Centre for Biotechnology Information
NJ	Neighbour Joining
nm	nanometre
NSP	Non-structural protein
OIE	World Organisation for Animal Health
OIE-SSARRL-FMD	OIE sub-Saharan African Regional Reference Laboratory for Foot-and-mouth disease
ORF	Open Reading Frame
PCP	Progressive Control Pathway
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
RGD	Arginyl-glycyl-asparatic acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RT-qPCR	Reverse Transcription real time Polymerase Chain Reaction
SAT	Southern African Territories
SPCE	Solid Phase Competitive ELISA
UTR	Untranslated region
VI	Virus Isolation test
VNT	Virus Neutralization Test
VPg	Viral Protein linked genome

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Foot-and-mouth disease (FMD) is a highly contagious disease that affects domestic and wild cloven hoofed animals. FMD is caused by a single stranded, positive sense RNA virus named FMDvirus (FMDV), which is a member of the genus *Aphthovirus* belonging to the family *Picornaviridae*. Globally, seven serotypes are recognised; Southern African Territories (SAT) 1, SAT2, SAT3, A, O, C and Asia 1,of whichSAT1, SAT2 and SAT3 serotypes are endemic in Botswana (Vosloo *et al*., 2006). The capsid protein of FMDV, VP1, is responsible for cell attachment and entry, antigenicity and serotype specificity (Jamal and Belsham, 2013). Antigenic variation between FMDV strains is reflected genetically in the VP1 region (Longjam and Tayo, 2011). This makes VP1 an important gene to study for the determination of the genetic variation of FMDV.

In September 2011 and March 2015,FMD outbreaks occurred in cattle in Itoto and Maxebo crushes, respectively. Both crushes are located in the Kareng extension area in the Ngamiland District of Botswana. Laboratory analysis of FMD-suspected samples identified the responsible FMDV serotype for the outbreaks to be SAT2(OIE-SSARRL-FMD Reports, 2011,2015). These outbreaks and many others have been occurring in the northern region of Botswana for many years even though mass vaccination campaigns are carried out annually in this region (Derah and Mokopasetso, 2005). Botswana is a major exporter of beef to the European Union (EU) market but FMD threatens this trade which is vital for the growth of the economy.

In Botswana,a trivalent vaccine containing SAT1, 2 and 3 strains, produced by Botswana Vaccine Institute (BVI) is used to immunize cattle two to three times a year. There are two types of SAT2 vaccines produced; SAT251 and SAT2035. SAT251 is the older vaccine

strain that has been used since the 1980s while the production of vaccine SAT2035 began in 2010.

The purpose of this study wasto determine the genetic and antigeniccharacteristics of FMD viruses recovered fromthe two outbreaks using complete VP1 coding sequences of samples collected. The results obtained from this study could improve the knowledge of molecular epidemiology of FMDV in Botswana, determine what transpires in the field and provide information required for the development of appropriate vaccine strains and rationale vaccination programme for the control of FMD in the region.

1.2 Problem Statement and Justification of the Study

Outbreaks of FMD continue to occur in some parts of Botswana even though control and preventive measures have been put in place. Serotype SAT2viruses have repeatedly been the cause of outbreaks in the Ngamiland District in Botswana. These outbreaks lead to low production of milk, lean meat and calves' mortality thus impacting negatively on the economy of rural communities whose livelihood depend solely on livestock productivity. The recurrence of the outbreaks may be due to a number of factors including inadequate maintenance of cordon fences leading to contact of cattle with buffalos,low vaccination coverageof cattle and/or genetic evolution of the virus resulting in irrelevance of currently used vaccine strains.

Botswana is divided into FMD vaccination and non-vaccination zones by use of cordon fences. In the vaccination zones, a trivalent vaccine containing SAT1, 2 and 3 strains, produced by Botswana Vaccine Institute (BVI) is used to immunize cattle two to three times a year, yet FMD cases are still being detected. This led to the assumption that, perhaps, mutation of the virus circulating in the field might have occurred thus resulting

inevolution of mutants which could be genetically and/or antigenically diverse from the vaccine strains being used in the production of the hitherto SAT2 vaccines. This assumption therefore needed scientific investigation and hence the objectives of this study. By determining the genetic variation of field strains, appropriate vaccine strains can be produced to assist in the control of FMD in Botswana and elsewhere in Southern Africa. Vosloo *et al.* (2006) and Longjam and Tayo (2011) recommend that circulating strains should be investigated on a regular basis to determine if available vaccines give appropriate immunity because genetic variation of serotypes is vital for determination of vaccine strain to be used in specific geographical regions.

1.3 Research Questions

i. What are the genotype(s) and/or topotype(s) of the sequenced viruses from the 2011 and 2015 outbreaks?
ii. What are the types of mutation(s) and/or antigenic variation(s) that may have occurred over time?
iii. What is the relationship coefficient (r_1 value) between field virus isolates used in this study and vaccine strains currently in use in Ngamiland District of Botswana?

1.4Research Objectives

1.4.1 Overall objective

To determine the genetic and antigenic characteristics of the FMDV serotype SAT2 in outbreaks that occurred in 2011 and 2015 in Kareng area in the Ngamiland District in Botswana focusing on the development of appropriate vaccines.

1.4.2 Specific objectives

i. To determine genotype(s) and/or topotype(s) of the sequenced viruses from the 2011 and 2015 outbreaks.

ii. To examine the type of mutation and/or antigenic variation that may have occurred over time.

iii. To determine the relationship coefficient (r_1 value) between field virusisolates used in this study and vaccine strains currently in use in Kareng, Ngamiland District of Botswana.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Introduction

Foot-and-mouth disease (FMD) was first observed by Hieronymus Fracastorius in 1514 when animals were discovered to have vesicles on their oral cavity and feet as well as redness in the mucous membrane of the mouth but was described years later in 1546. The disease was only confirmed to be a viral disease in the 1800s when Loeffler and Frosch demonstrated it to be a virus, and this marked the beginning of the virology era (Grubman and Baxt, 2004).

FMD is a highly contagious disease which affects cloven hoofed domestic animals such as cattle, sheep, goats and pigs as well as about 70 wildlife species, including the African buffalo and impala(Jamal and Belsham, 2013). The disease in livestock (cattle, sheep, goats and pigs) is characterized clinically by eruption of vesicular lesions in the mouth and feet and hence the name – foot-and-mouth disease. After rupture of the vesicles, there is profuse salivation and lameness(Grubman and Baxt, 2004). The disease is present in more than 100 countries most of which are developing but it has been eradicated in countries that are labelled as developed (Jamal and Belsham, 2013). The FMD virus is classified into seven distinct serotypes globally; O, A, C, Asia 1, Southern African Territories (SAT)1, SAT2 and SAT3 and within each serotype there are subtypes. These serotypes are confined to specific geographical locations, an exception being serotype O and more recently, SAT2(Valdazo-González *et al.*, 2012).

2.2 Etiology

2.2.1 Classification

The disease is caused by the foot-and-mouth disease virus (FMDV) which is a single stranded, positive sense RNA virus belonging to the *Apthovirus* genus of the

Picornaviridae family (Jamal and Belsham, 2013). It is a prototype of this genus, three more viral members have since been added to the genus in recent years; Bovine rhinitis A virus, Bovine rhinitis B virus and Equine Rhinitis A virus (Knowles *et al.,* 2008; ViralZone, 2014)

2.2.2 Structure of the virus

2.2.2.1 Physical structure

The virus is made up of a naked (non-enveloped) icosahedral capsid of 25-30nm diameter which encloses the RNA genome. Four structural proteins, VP1, VP2, VP3 and VP4, interact together to form this viral capsid. These proteins combine to form a protomer of which 60 copies create the complete protein coat. VP1-3 are exposed on the surface of the virion but VP4 is buried within the capsid and has a myristyl group covalently linked to its N-terminus (Belsham *et al.,* 1991). The three surface proteins are responsible for the antigenicity of the virus (Jamal and Belsham, 2013). Of all the picornaviruses, FMDV has the highest buoyant density due to the existence of a channel at the five-fold axis which allows small molecules such as Caesium Chloride to enter (Jackson *et al.,*2003).

2.2.2.2 Genomic structure

The FMDV genome is an 8.4kb single stranded positive sense RNA. It is divided into three sections; 5'untranslated region (UTR), a single open reading frame (ORF) and 3'UTR (Jamal and Belsham, 2013). The organization of the FMDV genome is shown in fig. 1. A small non-structural protein (NSP) of about 24 bases, named VPg (viral protein linked genome), is linked to the 5'end of the genome. This protein is the primer for genome replication (Longjam *et al.,* 2011a).

Following this protein is the 5'UTR which consists of an S fragment, poly C tract, pseudoknot structures, a *cis*-acting replication element (*cre*) and the internal ribosome entry site (IRES), in that order. S fragment is involved in the stability of the genome

(Bunch *et al*., 1994). Poly C tract is of variable length and evidence has shown that the length is linked to virulence of the virus (Longjam *et al*.,2011a). The *cre* element is used as a template for the uridylylation of the VPg and is therefore involved in the initiation of the genomic replication (Mason *et al.,*2002). The IRES is responsible for cap-independent initiation of viral protein synthesis. The roles of the other elements are unknown albeit functional (Grubman and Baxt, 2004).

Downstream the 5'UTR is a single ORF which is about 7000 residues long. The region codes for a single polyprotein which is then cleaved into four structural proteinsand eight different NSPs(Longjam *et al.,* 2011a). It is initially cleaved into three precursors, namely, P1-2A, P2 and P3. P1-2A is then cleaved into the capsid proteins VP1, VP2, VP3 and VP4 as well as 2A. P2 and P3 precursors are translated into non-structural proteins responsible for protein synthesis and RNA replication. P2 encodes three viral NSPs; 2A, 2B, and 2C, and the P3 region encodes NSP 3A, three copies of VPg, 3C protease, and 3D pol which is the viral RNA dependent RNA polymerase (Jamal and Belsham, 2013).

Lastly, the 3'UTR follows the ORF termination codon. It is involved in the replication of the RNA and consists of a stem-loop structure and a poly A tract which plays a role in the translation process (Grubman and Baxt, 2004; Jamal and Belsham, 2013).

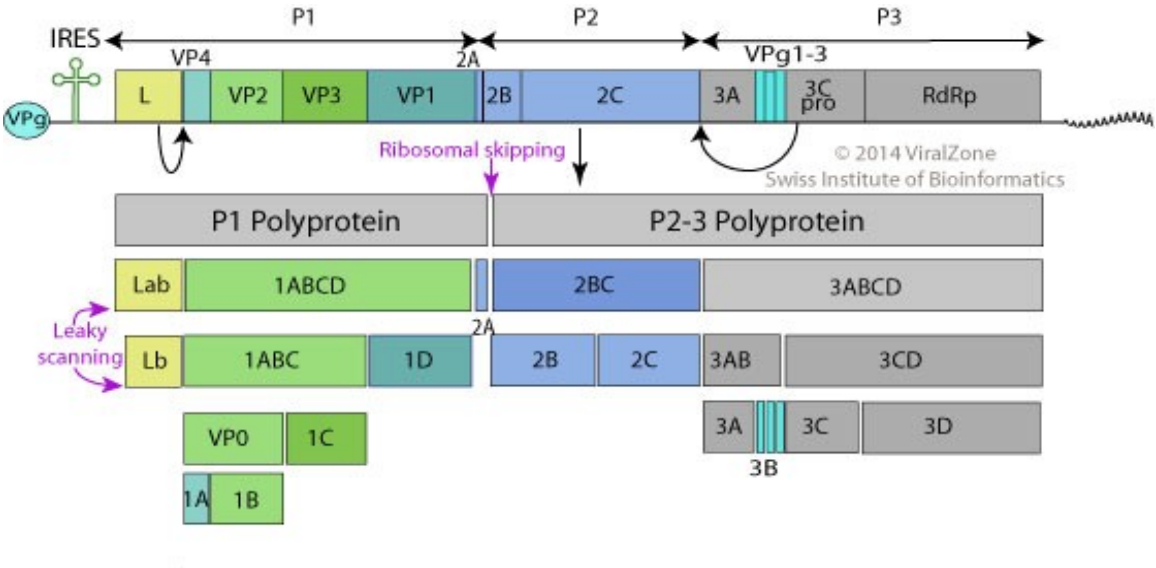

Figure 1: The organization of foot-and-mouth disease virus genome. P1 encodes the structural proteins while P2 and P3 regions encode non-structural proteins. Source: ViralZone, (2014).

2.3 Antigenicity

2.3.1 Antigenic and genetic variation

Antigenic variation is a process by which an infectious organism alters its surface proteins in order to evade the host immune response and is associated with mutation leading to amino acid replacement (Longjam and Tayo, 2011). The RNA-dependent RNA polymerase of RNA viruses lacks the proof reading mechanism and as thus results in error prone replication of the genome. This gives rise to development of genetic and antigenic variants (Domingo *et al.,*1990, 1992). The high mutation rate of FMDV, which have been demonstrated to be in the range of $10^3 – 10^5$per nucleotide per site per genome replication, is one of the causes of antigenic variation (Domingo and Holland, 1988; Drake and Holland, 1999). The quasispecies nature of the virus as well as genetic recombination and continuous circulation of FMDV in the field are other causes that have been identified (Longjam and Tayo, 2011).

Genetic variation may lead to antigenic variants and this may occur in the field due to immunogenic pressure on the virus (Domingo *et al.,*2003) or persistent infection of the animal (Woodbury, 1995). Sobrino *et al.* (1983) and Bolwell *et al.* (1989) demonstrated that antigenic variation may also occur during propagation of the virus in cell cultures in the laboratory.

2.3.2 Antigenic structure

According to Beck*et al*.(1983) studies have shown two variable regions among FMDV type A, O and C strains at positions 40-60 and 130-160. Highly variable sequences were observed even with the same subtypes in the second variable region more than in variable region one (amino acid changes did not alter their functional groups). The second region meets the requirements for an antigenic determinant because, among other things, it differentiates between serotypes (Pfaff *et al.,*1982). All serotypes share a highly variable region of VP1 coding sequence comprising residues 135 to 155 of this protein and this is one of the major antigenic sites of FMDV. It is because of this variability that there is low cross immunity among the serotypes (Cheung *et al.,*1983).

2.3.3 Antigenic sites

In a study conducted by Maree *et al*.(2011) the hypervariable, surface-exposed structural loops observed for SAT1 and SAT2 viruses were; 'βB–βC and βE–βF loops of 1B, the βB–βC and βE–βF loops of 1C, and the N-terminal, βB–βC, βG–βH, βH–βI loops and C-terminal of 1D. No significant variation within the βB–βC loop of 1C for SAT2 viruses was seen but there were additional variations in the βD–βE and βF–βG loops of 1D'. However, Maree *et al*. (2011) recommend that antigenic sites be confirmed by monoclonal antibodies (mAb) and sequencing of virus escape mutants. They further noted that

epitopes identified from mAb escape mutants for other serotypes endorsed the immune relevance of these structural loops.

According to Maree *et al.* (2011)site 2 of serotype O and site 3 of A10are contained in the βB–βC, βE–βF and βH–βI loops of 1B while βB–βC corresponds to D2 of serotype C. The βB–βC loop of 1C compares to site 3 of A10 while βB–βC, βG–βH, βH–βI loops and C-terminal of 1D match epitopes identified for serotypes A, C and O.Antigenic site A which is found within the loop connecting G and H beta sheets is the immunodominant region of the virus (Acharya *et al.,* 1989). RGD (Arginyl-glycyl-asparatic acid)motif receptor binding sequence is also located within the G-H loop (Longjam and Tayo, 2011).

2.4 Epidemiology

2.4.1 Geographical distribution of FMD serotypes

2.4.1.1 Worldwide distribution

FMD is endemic in Africa, Asia, the Middle East and South America. The endemic areas have been grouped into seven pools based on circulation of similar strains of FMDV (Brito *et al.,* 2015) as shown in Fig. 2. An eighth pool was present until the 1980s in Western Europe, but has been eradicated through preventive vaccination and zoo-sanitary measures (Valarcher *et al.,* 2008). Pools 1-3 are located in Asia while pools 4-6 are in Africa and pool 7 is in South America. The rest of the world is free of the disease(Bastos *et al.,* 2003).

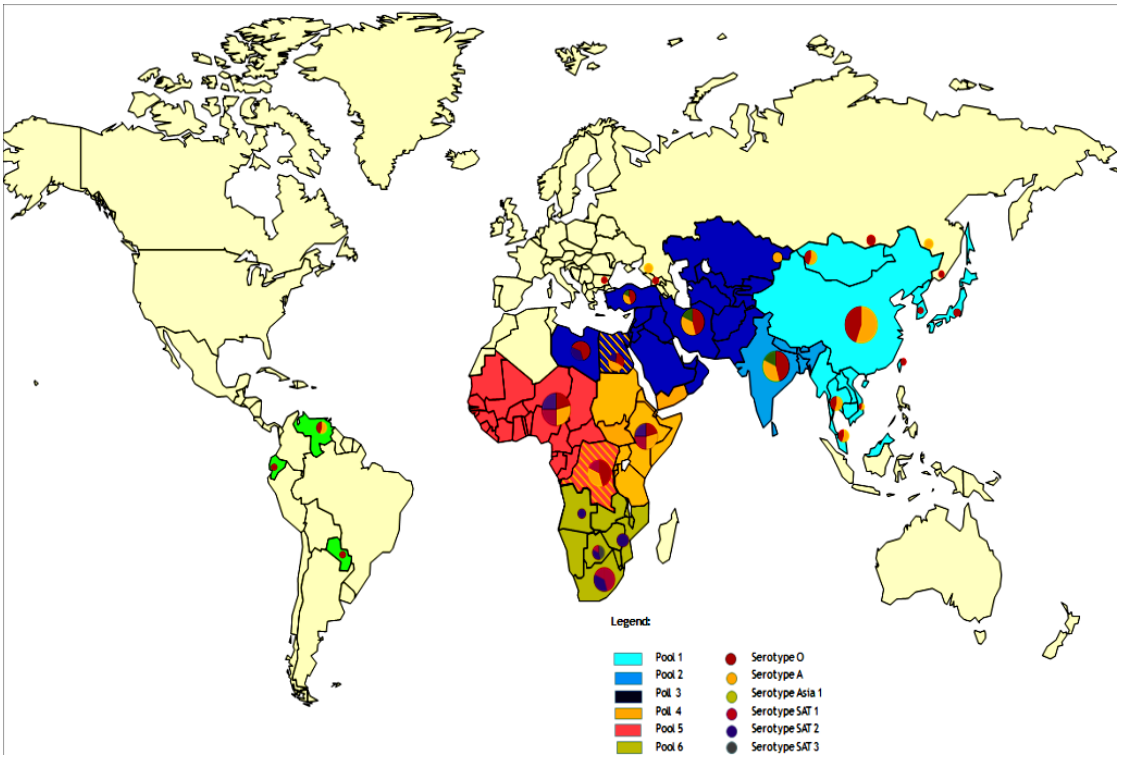

Figure 2: World distribution of FMD virus pools 2011-2015.

Source: Foot and Mouth Disease Situation;OIE Monthly report (February-2016).

The first FMDV serotypes discovered were O and A, named after the places they were discovered in, which are Oise in France for type O and Allemagne in Germany for type A. Serotype C was also initially detected in Germany (Longjam *et al.,* 2011a). After 30 years, novel serotypes were isolated in a South African outbreak and were named Southern African Territories (SAT) 1, SAT2 and SAT3. Asia 1 was first detected in Pakistan. Serotypes O, A, C and Asia 1 are believed to have originated from Asia, spread to America in the 18th century but were eradicated in North America in the 20th century. Earlier than that the serotypes spread to Africa and have since been endemic there (Longjam *et al.,* 2011a).

Serotypes SAT1-3 evolved in sub-Saharan Africa in association with African Buffalo (*Syncerus caffer*) which is a natural carrier of the virus (Thomson, 1994). Serotypes O, A and C have the widest distribution and have been isolated in America, Africa, Asia and Europe FMD outbreaks. Since 2005 no outbreak has been linked to serotype C igniting the assumption that it may no longer exist in the field. Asia 1 has been confined to Asia except when it caused outbreaks in Greece in 1984 and again in 2000 (Thomson, 1994).

2.4.1.2 Distribution in sub-Saharan Africa

FMD is endemic in most parts of sub-Saharan Africa with six of the serotypes occurring in this region. A number of factors complicate the epidemiology of the disease there; the endemic nature of the disease results in samples not being submitted and therefore current strains not documented (Vosloo *et al.,* 2004), uncontrolled movements of domestic and wild animals and the presence of large numbers of persistently infected African Buffaloes and other wild animals (Tekleghiorghis*et al*., 2014a). It is important to know about FMDV populations circulating in not only domestic but wild animals also, as this information is required when implementing vaccination strategies (Vosloo *et al.,*

2004).Due to underreporting of FMD outbreaks in sub-Saharan Africa there are major gaps in knowledge of currently circulating strains (Brito *et al.,* 2015).

In this region, the virus circulates between wildlife and domestic animals as well as among domestic animals without the aid of wildanimals (Vosloo and Thomson, 2004). Majority of the disease-free areas are in Southern Africa, where FMD control strategies such as cordon fencing and vaccination are implemented (Vosloo*et al.,* 2006). Some countries lack animal movement control measuresand only a few practice preventative vaccination required for international trade of animals and animal products (Brito *et al.,* 2015), and this exacerbates the situation.

FMDV serotypeSAT1-3viruses are mainly located in Southern Africa while O is widely distributed in East and West Africa (Knowles and Samuel, 2003).They are normally restricted to Southern Africa but some incursions of SAT1 have been reported in Greece while both SAT1 and SAT2 have occurred in the Middle East (Knowles and Samuel, 2003; Grubman and Baxt, 2004; Rweyemamu *et al.,* 2008; Valdazo-González *et al.,* 2012). Except for Zambia in which serotype O is present, only SAT viruses occur in Southern Africa. Lesotho, Swaziland and Madagascar are free of FMD viruses (OIE, 2012).

It is practically impossible to eradicate FMD in sub-Saharan Africa unlike in North America and Western Europe because of the ever-present threat of the reservoir host, the African buffalo which transmits the disease to domestic animals (Thomson, 1995).

East Africa is plagued by serotypes A, O, SAT1 and SAT2, O being the most common followed by A. In this region only six countries practice preventative immunization (Brito *et al.,* 2015). Serotype C was last detected in 2004 in Kenya (Sangula *et al*., 2011). In

West and Central Africa, the same serotypes as East Africa are endemic there. In 2007, however, SAT3 outbreak was reported in Cameroon. Of all the countries in that region only Guinea and Guinea Bissau have not reported any occurrences of the disease during their surveillance exercises (Brito *et al*., 2015). In all of Africa FMD free areas are found in parts of Botswana, Namibia and South Africa (Tekleghiorghis *et al.*, 2014a).

2.4.2 Transmission

Susceptible animals are infected through direct or indirect contact with infected animals or other objects exposed to live virus. Studies have shown that the most common route of infection of susceptible animals is by direct contact, either by mechanical transfer or by aerosol infection (Alexandersen *et al.*, 2003). Oral transmission is also possible especially when the animal has damaged skin in and around the mouth as well as on pre-existing abrasions on animals (Alexandersen*et al.*, 2003).

Some cases of airborne transmission as far as 300km from source of infection have been described (Sorensen *et al.,* 2000, 2001). Parenteral inoculation has also been implicated in some cases where it was believed that contaminated instruments and medicinal products had been the source of infection. For example, a report exists where an FMD outbreak was linked to the use of vaccines containing live virus (Beck and Strohmaier, 1987). Mechanical dissemination of the disease is contributed to by non-susceptible animals, agricultural tools, vehicles and contaminated animal products (Donaldson *et al.,* 1987).

The most common mode of transmission for cattle is through inhalation of aerosolized virus (Alexandersen *et al.,* 2003). For pigs, this is the least efficient route of transmission, they are more likely to get infected by eating contaminated food (Alexandersen and Donaldson, 2002). The FMD outbreak in South Africa in 2000 and the 2001 United Kingdom (UK) epidemic are evidence of transmission by ingestion of contaminated feed

(Knowles *et al*., 2001). Pigs can be infected by FMDV if placed in premises previously housing infected animals and like cattle, they are at risk of infection due to direct contact with infected animals (Grubman and Baxt, 2004).

2.4.3 Carrier animals

Some animals may develop into a carrier state following acute phase of the disease or after successful vaccination (Alexandersen *et al*., 2002). They are labelled as carrier animals because live virus can be isolated from their pharynx up to a few years after onset of infection (Longjam *et al.*, 2011a). Vosloo *et al*.(1996) have demonstrated that carrier animals infected with SAT serotype virus can cause outbreaks in susceptible animals.Infected cattle, sheep and goats are potential carriers but pigs are cleared of infection within 4 weeks and can therefore, not become carrier animals (Alexandersen *et al.*, 2003). Carrier state of domestic cattle has been recorded as long as 3.5 years (Alexandersen *et al.,* 2002), 9 months for sheep and African buffalos are considered lifelong carriers (De Clercq, 2003).

2.5 Clinical Signs and Pathology

In natural infection, the main route of virus entry is the respiratory tract. The initial virus multiplication usually takes place in the pharynx epithelium, producing primary vesicles (Burrows *et al*., 1981). The clinical outcome of the disease depends on the species of the susceptible animal and on the infectious dose of the virus strain (Longjam *et al*., 2011a). Within 48 hours, fever and viremia are observed in cattle and pigs.The virus then spreads to other organs and causes vesicles in the mouth and feet (Salt, 1993). Incubation periods are in the range of 2 – 14 days depending on the infectious dose and route of infection (Grubman and Baxt, 2004).A week after infection emergence of a strong humoral

response is observed. This coincides with the gradual lessening acute phase of disease (Salt, 1993).

Morbidity and mortality is dependent on the species and age of the animal. Compared to young animals, mortality in the adult population is very low. The clinical signs are more pronounced in cattle and pigs unlike in sheep and goats making it difficult to detect infection in the latter (Knowles *et al*., 2001). The earliest signs of FMD infection are fever, weight loss, reduced milk production, cessation of rumination and excessive salivation (Thomson,1994). The infection of squamous epithelium and constant irritation of infected organs causes development of vesicular lesions in the mouth and feet, which are the main characteristics of FMD. The lesions result in salivation, nasal discharge, difficulty in walking and reluctance to stand (Thomson, 1994).

2.6 Diagnosis

The success of controland eradication FMD involves the ability to accurately and timely diagnose the disease in endemic areas and in backing of stamping out policies in FMD free regions (Rémond *et al.,* 2002). Clinically, FMD cannot be distinguished from other vesicular diseases and therefore diagnosis in the laboratory is vital (OIE, 2012). Due to the highly infectious nature of the virus testing is to be carried out in laboratories that comply with OIE requirements for Containment Group 4 pathogens (OIE, 2012).

2.6.1 Detection of FMD virus antigen or genome

Complement Fixation Test (CFT) was first used in 1929 to type FMDV antiserum, first from guinea pig and later from cattle (Longjam *et al.,* 2011a), it has since been used to differentiate FMDV strains. The test is rapidhowever, it requires a high viral load and can be affected by pro- and anti-complement factors of the sample (Ferris and Dawson, 1988).

Most laboratories have replaced CFT with Enzyme-Linked Immunosorbent Assay (ELISA) as it is a faster method and does not have issues of pro- and anti-complementary activities (OIE,2012). Initially it was used as a serological test but later developed and applied to detection, typing and strain differentiation of FMDV as well (Longjam *et al.*, 2011a). Sandwich ELISA is the preferred test for detection and identification of FMDV (Ferris and Dawson, 1988).

Samples that give out negative results have to be confirmed by virus isolation which is time consuming as it may take up to 4 days to obtain confirmatory results. Reverse Transcription Polymerase Chain Reaction (RT-PCR) was developed as a more reliable and rapid assay system (Longjam*et al.*, 2011a),it is therefore the gold standard for detection of nuclei acids (OIE, 2012). Multiplex PCR (mPCR) is another variation of PCR that is cost effective and increases probability of detection of FMD RNA (Elnifro *et al*., 2000) because it amplifies more than one target sequence using multiple primers (Ferńandez *et al.,* 2008). Longjam *et al*.(2011b) showed that mPCR is more sensitive than sandwich ELISA and virus isolation.

Real time RT-PCR (RT-qPCR) is an even more rapid, sensitive, precise test that has improved quantification and eliminates the need for post PCR processing thus reducing the risk of cross contamination (Longjam *et al.*, 2011a). Shaw *et al.*(2004) compared automated RT-qPCR, virus isolation and antigen ELISA and foundRT-qPCR to be more sensitive to detecting FMDV in epithelial samples.

While RT-qPCR amplifies the FMDV RNA-dependent RNA polymerase 3D(pol) gene for about 2h 30minutes, Reverse-Transcription Loop-Mediated IsothermalAmplification (RT-

LAMP) amplifies the same gene for an hour and the detection of FMDV was determined to be higher (Kasanga *et al*., 2014).

2.6.2 Detection of FMD structural and non-structural proteins

FMD serological tests are divided into those that detect viral structural antibodies and those that detect viral non-structural antibodies (OIE, 2012). Detection of viral antibodies indicate vaccination status or previous infection (De Clercq, 2003).

Virus Neutralization Test (VNT), Solid Phase Competitive ELISA (SPCE) and Liquid Phase Blocking ELISA (LPBE) test for presence of structural proteins. These tests are serotype specific and detect both vaccination status and infection (OIE, 2012). LPBE determines the titers of antibodies in FMD vaccinated animals(Longjam *et al*., 2011a)and can therefore be used for ascertaining the efficacy of the vaccine used.OIE(2012) recommends screening of samples by LPBE or SPCE and confirming results by VNT to minimize the chances of reporting false negatives. Although VNT is the gold standard for detection of FMDV it takes 2-3 days to give out results, requires biosecurity laboratories due to the use of live virus and there are possibilities of bacterial contamination of cell cultures used (De Clercq, 2003).

Non-structural proteins (NSP) tests detect presence of viral non-structural antibodies (De Clercq, 2003). Presence of these antibodies indicate a current or previous infection (OIE, 2012). Although NSPs are produced during vaccine production they are eliminated during manufacturing of highly purified FMD vaccines known as DIVA (Differentiating Infected from Vaccinated Animals) vaccines and therefore cannot be detected after vaccination (De Clercq, 2003).

2.6.3 Advanced diagnostic techniques

Recentlythere have been developments in the field of FMDV diagnostics. Pen side diagnostic methods have been developed which allow for on-site diagnosis and thus eliminating the problems associated with sample transportation.An example of such a method is the rapid chromatographic strip test (Longjam *et al.*, 2011a). Phage display based diagnosis is being carried out in some laboratories to study bovine antibody response at the molecular level (Longjam *et al.*, 2011a).

In the nucleic acid based diagnostic front, nucleic acid sequence-based amplification (NASBA) assay for detection of FMDV has been developed. Compared to other laboratory methods, NASBA detection methods were found to be more sensitive and rapid (Lau *et al.,* 2008). Gajendragad *et al.*(2001) developed an immunobiosensor for the diagnosis and typing of FMDV. Another recently developed test is a microarray based technique produced by Baxi *et al.*(2006) which consists of an FMD DNA chip that was found to detect 23 different FMDV strains which represented all the serotypes.

2.7 Control

FMD has a negative impact on food security and economies of developing countries because it results in high mortality of calves, reduced milk production, decreased fertility, loss of draught power and reduced/prohibited access to markets. (OIE and FAO, 2012) and as thus, control of the disease is vital.

The 178 member states of OIE have been classified into three groups; those that are not free of FMD (102 members), those that have FMD free status (66 members) and 10 that have official free zones (OIE, 2015). The OIE and FAO teamed up to formulate the Global FMD control strategy aiming at fighting FMD worldwide through the use of defined tools and procedures. Examples of these are the FMD-Progressive Control Pathway (PCP),

PVSpathway, field surveillance and improvement of diagnostic laboratory capabilities, just to name a few (OIE and FAO, 2012).

The control of FMD is complicated by a number of factors; FMDV replicates at very rapid rate, it affects a wide range of domestic and wild cloven hoofed animals (Thomson and Bastos, 2002). FMDV also has a high mutation rate which causes the virus to evolve into intratypic subtypes that may not cross protect effectively (Brehm *et al*., 2008).

Control of FMD requires strict zoo- sanitary measures and/or vaccination. The United Kingdom was the first to apply the slaughter policy after an FMD outbreak in the 19th century. Because this application was a financial burden to the country, other European countries opted for quarantine methods, as this was before mass preventive vaccination was introduced (Paton *et al.,* 2009). In the event of an outbreak in FMD free countries, measures such as import controls of animals and animal products, early detection and slaughtering of animals, tracking of undisclosed sources of infection, movement control and surveillance are implemented to reestablish freedom status (Paton *et al*., 2009).

Countries in Southern Africa, unlike those in the rest of the African continent, have implemented FMD control strategies to ensure access of their animals and animal products to international markets. These strategies include control of movement of animals by fencing, restriction of contaminated materials, surveillance to allow for early detection and application of countermeasures during early stages of an outbreak (Vosloo *et al.,* 2002). The fencing separates a country into different zones; FMD free without vaccination, FMD free with vaccination, buffer and FMD zones according to OIE requirements (OIE, 2012). It also prevents contact between domestic animals and wildlife. Cattle located in buffalo

area are vaccinated biannually with a trivalent vaccine containing all three SAT type strains (Vosloo *et al.*, 2002).

2.8 FMD Situation in Botswana

2.8.1 History of FMD in Botswana

Walker(1934) confirmed the first FMD outbreak in Botswana, the causative virus was a variant of FMDV type O. He attributed it to the longstanding drought that had plagued the country at the time. The outbreak occurred as a result of wild animals coming in contact with cattle at watering points thus resulting in the spread of disease to livestock (Baipoledi *et al*., 2004). Eight outbreaks occurred that were linked to contact between cattle and wild animals in the period between 1948 and 1970.These were caused by either SAT1 or SAT3 viruses. This led to the government of Botswana to introduce fencing system to segregate livestock and wild animals (Baipoledi *et al.,* 2004). In 1977 the first FMD outbreak associated with SAT2 serotype occurred; the outbreak was caused by a combination of SAT1 and SAT2viruses (Botswana Ministry of Agriculture, 1977). The first outbreak that was caused solely by SAT2 was detected in 1980 (Botswana Ministry of Agriculture, 1980).

2.8.2 Control strategies

Botswana, like the rest of Southern Africa, has a large population of African buffalo, the main reservoir hosts of SAT serotypes of FMDV. They are largely confined to northern Botswana in the Okavango Delta, the Chobe and Nata river basin. Cattle that are found in these locations are vaccinated biannually with SAT trivalent vaccine (SAT1, SAT2andSAT3) because their close proximity to buffalo puts them at high risk of infection (Baipoledi *et al.,* 2004; Hyera *et al*., 2006; Mapitse, 2008).

FMD control in Botswana has proven successful allowing the country access to the lucrative international livestock markets like the European Union market. The cornerstone of the prevention and control policies of animal diseases in Botswana is the Diseases of Animals Act of 1977; the Act entails movement control, regulation of imports and exports, and control and prevention of animal diseases (Derah and Mokopasetso, 2005).

The country is divided into disease control zones using fences which prevent contact of domestic animals with wildlife (Derah and Mokopasetso, 2005). Before 1963 control of movement of animals was reinforced by aphthisation. In 1965 vaccination using bivalent vaccine was initiated (Falconer, 1972). In 1969 SAT3 serotype was added in the vaccine creating a trivalent vaccine. Vaccination was and is still being carried out twice a year and performed three times for cattle at high risk areas due to being in the vicinity of buffalo (Derah and Mokopasetso, 2005).

Apart from vaccination and fencing, other control measures have been put in place. Movement of animals and animal products between control zones is regulated by use of movement permits. The Livestock Identification and Traceability System (LITS) was adopted in Botswana in 1999 for cattle as a way of keeping track of all the cattle in the country. General disease surveillance is also carried out by veterinarians in the country (Derah and Mokopasetso, 2005). Public awareness of FMD is another important aspect of disease control measure instituted in Botswana(Mapitse, 2008).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

The study was conducted in Maxebo and Itoto crushes located in Ngamiland District which lies in the northwestern part of Botswana between 19 degrees 30' south, and 23 degrees 30' east. It covers a total area of 129 930 km^2. The districtshares borderson the northern and western sides with Namibia. Domestically it borders the Central, Ghanzi and Chobe Districts on the South east, South west and Eastern sides respectively.

3.2 Study Design

This was a retrospective study with a purposive sampling method whereby sampling was carried out from two crushes that had been experiencing FMD outbreak at the time. The crushes are located at Itoto and Maxebo (Fig. 3). Epithelial tissue samples from the oral cavities of the animals were collected from both crushes. The samples were collected from Itoto crush in September 2011 and from Maxebo crush in March 2015.

Figure 3: Map of Botswana showing the two study areas. The overlapping location bubbles, labelled represent Itoto and Maxebo crushes respectively.

3.3 Sample Collection and Handling

3.3.1 Sampling

Twelve epithelial tissue samples were collected in total from cattle that showed clinical signs and symptoms suggestive of FMD. Eight samples were collected from Maxebo and the remaining four samples were collected from Itoto crush. About $3cm^2$ of the specimens were collected from unruptured or newly ruptured lesions in the oral cavities of the infected cattle using a tong after the animals were restrained. The samples were placed in cryovials which were appropriately labelled according to a naming system used by the Veterinary department of the Ministry of Agriculture in Botswana. These labels are regarded as customer identification by the OIE laboratory which the lab states in all correspondences with the customer. Once labelled the samples werestored in liquid Nitrogen.

3.3.2 Sample identification in the laboratory

Thesamples were transported by air and upon arrival in the laboratory, they were removed from the nitrogen tank and stored at -80ºC until laboratory testing commenced. In the laboratory, the samples were labelled according to a system specified by OIE standards (OIE, 2012). The first three letters signify the sample's country of origin i.e. BOT for Botswana, followed by a number representing the number of the particular sample in the batch of samples and finally the last two digits of the year in which the sampling was carried out. In this study, the 2011 samples were labelled BOT/17/11 to BOT/20/11 while the 2015 samples were labelled BOT/03/15 to BOT/10/15.

3.4 Laboratory Tests

The laboratory tests were performed starting November 2015 at the OIE Regional Reference Laboratory of Sub-Saharan Africa for FMD (OIE-RRLSSA-FMD) housed and managed by the Botswana Vaccine Institute (BVI).

3.4.1 Sample treatment and virus isolation

The samples were ground using a pestle and mortar in 3ml of lamb kidney Minimum Essential Media (LK-MEM) and 5% chloroform to degrade the tissue and allow for extraction of viral particles. Supernatants were extracted by centrifugation for 15 minutes at 2400 rpm. The supernatants were used to isolate the virus on lamb kidney primary cell cultures. Once cytopathic effect (CPE) had been observed, the cultures were stopped and frozen at -20^0C to rupture the cells and thereafter centrifuged at 2000 rpm to separate the cell debris from the virus (Appendix 1 and2).

3.4.2 RNA extraction

Total RNA was extracted from the isolated virus particles using the ZR Viral RNA kit™ (Zymo Research Corporation, California, USA) according to manufacturer's instruction (Appendix 3). Positive and negative controls were added to the sample pool. Firstly, they were all lysed using the lysis buffer and subjected to two washes with an ethanol based wash buffer. Finally, RNAse free water was used to elute the extracted RNA.

3.4.3 Reverse Transcription Polymerase Chain Reaction

The master mix was prepared as outlined on the Biolab One Step Reverse Transcription Polymerase Chain Reaction **(**RT PCR) manufacturer's protocol.Table 1 shows the PCR reaction mix per sample. FMD serotype specific SAT2 primers were used for RT PCR on the RNA samples. The forward primer SAT2-1P-1223F and the reverse primer FMD-208R, which amplify the complete VP1 region of the FMD SAT2 genome, were used. Nuclease free water was used as the negative control and BOT/17/09sample was used as the positive control. A volume of 24µl of master mix was mixed with 1µl of RNA on a 96-well reaction plate and the PCR run in the 7500 real time PCR machine (Applied Biosystems, California, USA). The cycling conditions are shown in Table 2.

Table 1: PCR reaction mix per sample

Reagent	Volume (µl) per reaction
OneTaq one step reaction mix (2x)	12.5
OneTaq One Step enzyme mix (25x)	1
Forward primer (µm)	1
Reverse primer (10µm)	1
RNA sample	1
Nuclease free water	8.5
Total	**25**

Source: Biolab One Step RT PCR protocol, 2014

Table 2: RT-PCR Cycling conditions

Cycle steps	Temperature	Time	Number of cycles
Reverse transcription	48	30 min	1
Initial denaturation	94	1 min	1
Denaturation	94	15 secs	40
Annealing	62	30 secs	40
Extension	68	1 min	40
Final extension	68	30 seconds	1
Hold	4	∞	1

Source: Biolab One Step RT PCR protocol, 2014

3.4.4 Agarose gel electrophoresis

A volume of 25µl of PCR products was mixed with 10µl loading dye and loaded on to respective wells on a 2% agarose gel (Appendix 4). The O'GeneRuler 1 kb DNA Ladder (Thermo Scientific, Massachusetts, USA) was added to the run. Electrophoresis was run for 50 minutes at voltage of 100V. The bands were visualised under UV light. Bands of 1145bp size were excised from the gel for further analysis.

3.4.5 cDNA recovery from agarose gel

The desired DNA bands excised from the gel werepurified using Zymoclean™ Gel DNA recovery Kit (Zymo Research Corporation, California, USA). The reagents of the kit

dissolved the gel, denatured proteins bound to the DNA, and removed primers and dNTPs thus allowing for the DNA to be captured on the column matrix. The purified DNA was eluded in DNAse free water. The detailed procedure for elusion is outlined in Appendix 5.

3.4.6 Sequencing

Sequencing was carried out using a BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems, California, USA). The reaction mix per sample is shown on Table 3. Since sequencing uses a dye-termination method, two separate reactions were run, one with forward primer SAT2-1D-209F and another with reverse primer NK72. The cycling conditions are outlined on Table 4.

Table 3: Sequencing reaction mix per sample

Reagent	Volume (µl) per reaction
Terminator ready mix (2.5x)	4
Sequencing buffer (5x)	2
Forward/reverse primer	1
DNA sample	2
Nuclease free water	11
Total	**20**

Source: Big Dye Terminator cycle sequencing kit, 2014

Table 4: Cycling conditions for sequencing

Cycle steps	Temperature	Time	Number of cycles
Initial denaturation	96	1 min	1
Denaturation	9	10 secs	35
Annealing	50	30 secs	35
Extension	60	4 min	35
Hold	4	10 min	1

Source: Big Dye Terminator cycle sequencing kit

3.4.7 Genetic analysis

The sequences produced were analyzed in the 3130xl Genetic Analyzer (Applied Biosystems, California, USA). Sample sequence (2µl) was added to 8µl of nuclease free water in a reaction plate and run in the analyzer. Chromatograms were read and stored for data analysis.

3.4.8 Vaccine matching

Crossed virus neutralization test (VNT) was used to determine the relationship between the two vaccine strains (SAT2035 and SAT251) and the field virus isolates used in this study. Serum neutralisation antibody titres of test samples were determined by mixing respective dilutions of the control sera with a known dose of virus/antigen. The detailed procedure is shown in Appendix 6.

3.5 Data Analysis

3.5.1 Phylogenetic analysis

The chromatograms created during sequencing were visualised and cleaned using Chromas version 2.4.4 (Technelysium PTY Ltd, Australia). The cleaned sequences were transferred to BioEdit software version 7.2.5, both forward and reverse sequences of each sample were assembled into contigs resulting in overlaps. Two representative consensus sequences were chosen from each outbreak. The representative consensus nucleotide sequences (BOT/04/15; BOT/10/15; BOT/17/11; BOT/19/11) trimmed to 648 nucleotides and aligned along with FMDV SAT2 reference sequences from the National Centre for Biotechnology Information(NCBI) database (Appendix 7) on the same software. The aligned consensus sequence file was exported to theMolecular Evolutionary Genetics Analysis(MEGA) software version 7.0.14(Kumar*et al.,* 2015). There, an unrooted tree was constructed according to sequence similarities between nucleotide sequences of the VP1 gene (Knowles and Samuel, 2003)using the neighbour-joining (NJ) method.

3.5.2 Amino acid variation

Nucleotide sequences of both field virus isolates and SAT2 NCBI reference viruses were translated into their respective amino acid sequences using the Expasy translating software (Gasteiger *et al.,* 2003) and aligned using BioEdit software version 7.2.5.

3.5.3 Vaccine matching

Microsoft Excel 2015 was used to analyse the vaccine matching results. The relationship coefficient ('r_1" value) was used to measure the antigenic similarity between the field virus isolates and the vaccine strain and was calculated according to Paton *et al.,* (2005) using the following formula:

$$r_1 = \frac{\text{titre of reference serum against field virus}}{\text{titre of reference serum against vaccine virus}}$$

The mean values and respective standard deviations of the r_1 values were calculated according to standard statistical procedures and the difference between the means were compared by the student t-test (Swinscow, 1980). For r_1 values less than 0.3 it means that the vaccine strain is unlikely to protect against challenge with field virus isolates. $r_1$values greater than 0.3 show that the vaccine strain and the field isolate are antigenically related and immunization with the vaccine will confer protection against the field virus (Paton *et al*., 2005).

CHAPTER FOUR

4.0 RESULTS

4.1 Detection of FMDV by RT-PCR

A total of 12 bovine epithelial samples, eight samples from Itoto crush and four samples from Maxebo crush, were tested for FMDV by RT-PCR. All samples (100%) tested positive for the presence of SAT2 VP1 coding region. The amplicons observed on the agarose gel were approximately 1000bp in size, including the positive controls. No bands were observed on the negative control column thereby eliminating any cross-contamination issues. Sample BOT/18/11 produced a faint DNA band and was thus excluded from further testing.

4.2 Molecular Analysis

4.2.1 DNA sequence characteristics

The consensus sequences were trimmed down to 648 nucleotides which corresponds to the VP1/2A coding region of the virus. The sequences of the field virus isolates were subjected to a pairwise comparison between each other and against the two vaccine strains. When comparing sequences of the 2015 outbreak isolates i.e. BOT/03/15 to BOT/10/15, to each other nucleotide sequence similarity was calculated to be 99.8%. The only difference observed was at nucleotide position 249 with isolates BOT/03/15, BOT/05/15, BOT/06/15 and BOT/07/15 having Adenine (A) while the rest had a Guanine (G). This change was at the third codon and did not result in a variation of the translated amino acid.

A similar occurrence was noted for the 2011 outbreak isolates (BOT/17/11, BOT/19/11 and BOT/20/11); nucleotide differences were observed at positions 401 and 559 whereby

at position 401, BOT/20/11 had Adenine (A) while the rest had Guanine (G) and at position 559, BOT/19/11 had Cytosine (C) while the rest had Thymine (T). These differences, however, did not result in variations of deduced amino acids within samples from the same outbreak.

Comparison of the VP1/2A coding region of samples and the vaccine strains showed much higher nucleotide sequence variations. When SAT251 vaccine strain was aligned against the 2011 outbreak strains a 75.5% (468/648) nucleotide similarity was observed. A similar amount of differences was seen when the same vaccine strain was aligned to the 2015 outbreak strains. There was a difference in 160 nucleotides which equaled 75.79% nucleotide similarity. When comparing the SAT2035 vaccine strain to the 2011 outbreak strains, 55 nucleotides differed (92.3% similarity) and 51 nucleotides differed (91.6% similarity) with 2015 outbreak strains.

4.2.2 Topotypes

A phylogenetic tree was constructed using the Neighbor Joining (NJ) algorithm (Fig. 4) to determine the topotypes of the outbreak strains. The representative FMDV outbreak samples, BOT/17/11 and BOT/19/11 representing the 2011 outbreak and BOT/04/15 and BOT/10/15 representing 2015 outbreak, in this study were determined to belong toSAT2 topotype III as evidenced by the clustering with known topotype III NCBI database sequences. SAT2035 vaccine strain also clusteredwith topotype III strains but SAT251 vaccine strain was determined to belong to topotype II.

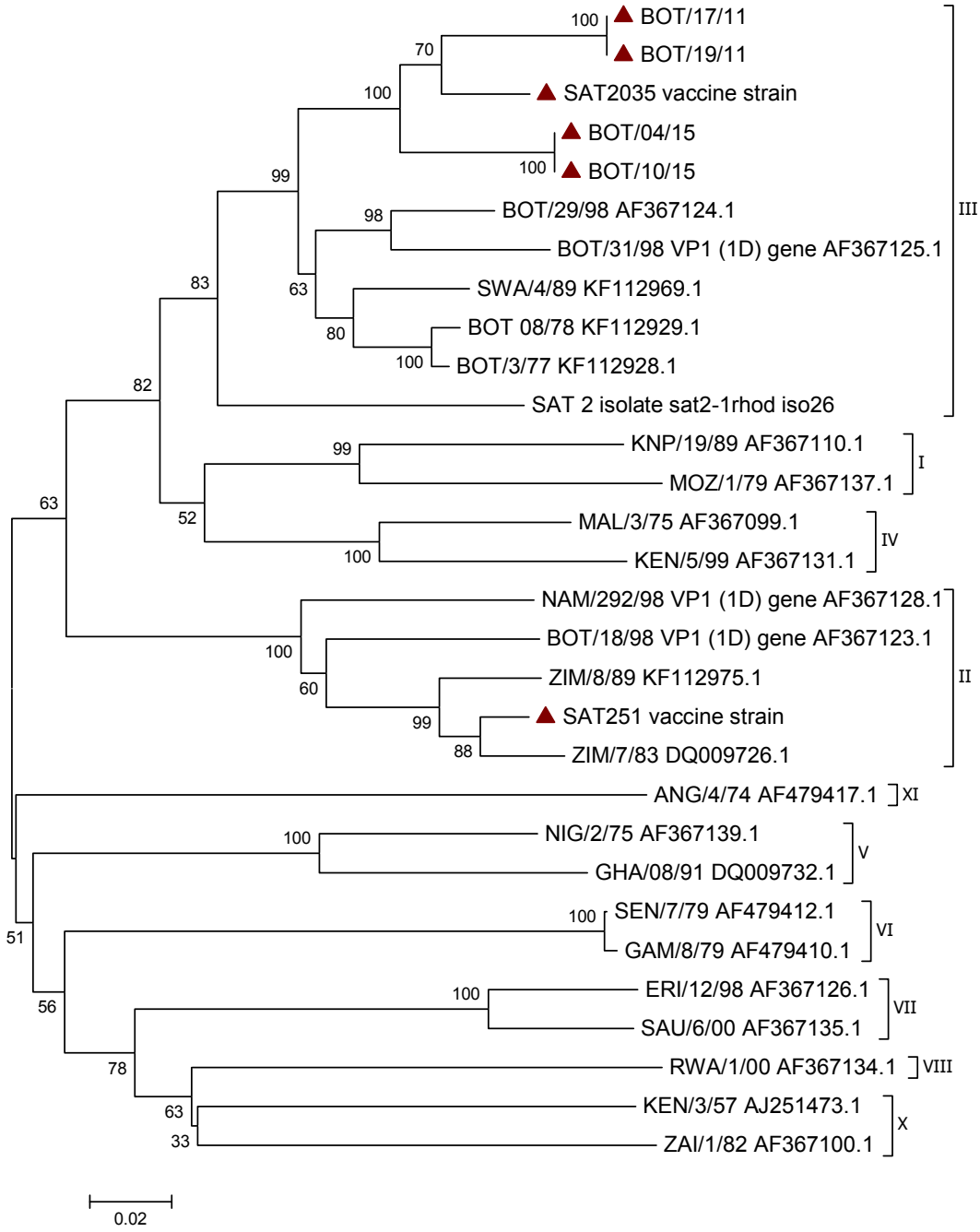

Figure 4: **Phylogenetic tree reconstructed by NJ method depicting the relationship of field virus isolates to relevant reference sequences in the NCBI database. The bootstrap values of each cluster are shown at the respective positions of the tree topology. The field virus isolates and vaccine strains used in this study are shown by the triangles.**

4.3 Amino Acid Variation

4.3.1 Variations within and between outbreaks

The trimmed 648 nucleotide sequences of the FMDVVP1/2A gene of field virus isolates were translated into amino acids sequences which were 216 amino acids long and aligned together(Fig. 5). There was 100% sequence similarity among both the 2011 and 2015 outbreak samples and therefore, it was concluded that no mutation occurred within either outbreak.

Pairwise comparison between the 2011 and 2015 outbreak strains highlighted 21 amino acid differences. Seventeen of these amino acid variations occurred within hypervariable regions and 14 resulted in amino acid side-chain group changes (non-synonymous), thereby changing the properties of the residues at those positions(Fig. 5).

4.3.2 Variations between field virus isolates and vaccine strains

Within the amino acid alignment of the VP1/2A of the field virus isolates shown in Fig. 5, hypervariable (HPV) regions were identified. These regions were at amino acid positions 83 – 88 (βE - BF loop), 98 – 100, 135 (βG-βH loop), 140 – 142 (βG-βH loop), 152, 158 – 160 (βG-βH loop) and 198 – 201 (C- terminal).

The RGD (Arginyl-glycyl-asparatic acid) cell attachment site (located at residues position 144 – 146) within the G-H loop was highly conserved in all the field virus isolates and vaccine strains used in this study, including the isoleucine (I) residue at the -1 position. However, the +1-position residue was varied, the 2011 isolates had methionine (M) while for the remaining isolates an arginine(R) residue was observed at the same position.

4.3.2.1 Field virus isolates versusSAT251 vaccine strain

Comparison of VP1 amino acid sequences of 2011 isolates to SAT251 vaccine strain revealed 35 variable positions. About 11.6% (25 out of 216aa) of the sequences showed

variations that resulted in the alteration of the amino acids and introduction of residues with differing physicochemical properties. This is compared to the 5.1% (11 out of 216aa) observed for 2015 isolates but with a total of 31 variable residue positions. Of these non-synonymous positions, 17 where located at known antigenic sites; positions 45, 48, 50, 57, 69, 86, 88, 100, 101, 135, 139, 141, 156, 158, 160, 174 and 207.

4.3.2.2 Field virus isolates versus SAT2035 vaccine strain

Thirteen amino acid variations were observed when comparing 2011 isolates against the SAT2035vaccine strain. Approximately 5% (10 out of 216aa) of the amino acid positions contained residues that had different physicochemical properties. For the 2015 field virus isolates 4.2% (9 out of 216aa) positions had non-synonymous amino acid alterations. Of these non-synonymous positions, 12 where located at known antigenic sites; positions 45, 58, 86, 100, 111, 135, 140, 141, 152, 156, 163 and 201.

4.3.3. SAT251vaccine strain versus SAT2035 vaccine strain

Thirty-seven (37) amino acid variations occurred between the two vaccine strains. The newer vaccine strain (SAT2035) differed by 17.1% amino acids from the older SAT251 vaccine strain.

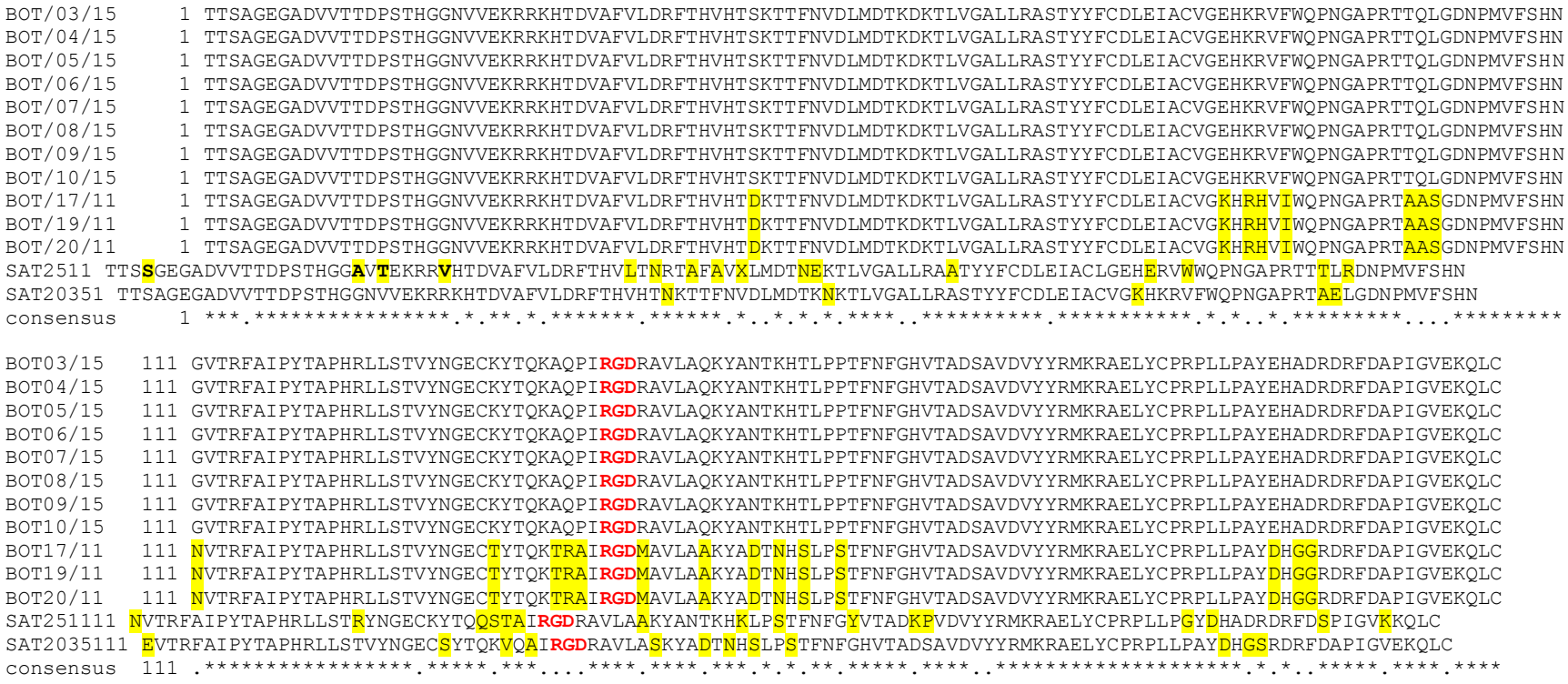

Figure 5: Sequence alignment illustrating amino acid variations between field virus isolates and the vaccine strains. The residues shown in red correspond to the cell attachment site, RGD. Residues highlighted yellow represent amino acid variations observed for each sequence. Asterisks '*' refer to alignments that are 100% identical. Dots '.' refer to locations with different amino acids from the query sequence.

4.4 Vaccine Matching

The antigenic relationship between field virus isolates and vaccine strains currently in use in Ngamiland was determined and the results are presented in Tables 5 and 6 respectively for vaccine strain SAT2035 and SAT251. Positive results were obtained for vaccine matching tests against SAT2035 vaccine strain, relationship coefficient values (r_1 values) were in the range of 0.51 and 0.87. Similar results were obtained when testing against SAT251 vaccine strain, 2011 outbreak strains gave out values between 0.61 and 0.85. The mean r_1 values of the two vaccine strains were statistically similar (SE diff = 0.0601; $t_{(22)}$ = 0.233; $p>0.05$; Tables 5 and 6). According to the OIE manual for Terrestrial animals (2012) these figures are evidence that the vaccines provide satisfactory immunity to the field virus isolates representing FMDV strains currently circulating in the field.

Table 5: Summary of r_1 values between field isolates and SAT2035 vaccine strain
D21 refers to serum obtained 21 days after vaccination. D35 refers to serum obtained after booster vaccination.

Sample ID	D21 titre	D35 titre	r_1 value
SAT2035	2.7	3.6	1.0
BOT/03/15	2.18	2.63	0.76
BOT/04/15	2.18	2.7	0.77
BOT/05/15	1.20	2.7	0.64
BOT/06/15	1.20	2.4	0.61
BOT/07/15	1.05	2.7	0.71
BOT/08/15	2.48	3.0	0.87
BOT/09/15	2.55	2.78	0.85
BOT/10/15	1.95	2.85	0.85
BOT/17/11	0.75	2.65	0.66
BOT/18/11	0.53	2.7	0.51
BOT/19/11	0.45	2.85	0.52
BOT/20/11	1.05	3.0	0.64
Mean r_1 value			0.699
Standard deviation			0.12

Table 6: **Summary of r_1 values between field isolates and SAT251 vaccine strain**

Sample ID	D21 titre	D35 titre	r_1 value
SAT251	2.33	3.53	1.0
BOT/03/15	1.58	2.85	0.85
BOT/04/15	1.43	2.80	0.71
BOT/05/15	1.65	3.15	0.77
BOT/06/15	1.50	3.08	0.78
BOT/07/15	1.20	3.45	0.8
BOT/08/15	1.73	3.00	0.81
BOT/09/15	2.03	2.78	0.82
BOT/10/15	1.65	3.00	0.79
BOT/17/11	0.45	2.10	0.44
BOT/18/11	0.45	1.65	0.36
BOT/19/11	1.05	2.63	0.54
BOT/20/11	0.53	2.70	0.55
Mean r_1 value			0.685
Standard deviation			0.17

D21 refers to serum obtained 21 days after vaccination. D35 refers to serum obtained after booster vaccination.

CHAPTER FIVE

5.0 DISCUSSION

This was a study to investigate intra- and inter-outbreak characteristics of FMDVSAT2that occurred in Ngamiland, Botswana in 2011 and 2015. VP1/2A coding region sequences and their deduced amino acid data were used to determine the genetic and antigenic relationships between the two FMD outbreaks as well as vaccine strains currently used for the commercial production of FMD vaccines against serotype SAT2. The vaccine strains were analysed for protection against the circulating viruses to determine their relevance in the control of FMD in Ngamiland District of Botswana. The findings of this study are showing that the vaccine strains are genetically and antigenically related to the circulating field virus strains and that minimal mutation occurred post the 2011 FMD outbreak.

The field virus isolates were initially passaged once on lamb kidney cell culture monolayers, this exposed the viruses to chances of adaptation to the cell culturewhich could resultin antigenic variationobserved (Sobrino *et al.,*1983; Bolwell *et al.,*1989).However, upon translation of the DNA sequences, all the field virus isolates showed 100% amino acid sequence similarity respectively for the 2011 and 2015 outbreaks. Consequently, it was concluded that no adaptation occurred and that the DNA sequences obtained are a true reflection of what is seemingly occurring in the field.

Pairwise comparison of the VP1/2A coding region of all the field virus isolates revealed a nucleotide sequence similarity of 96.8% indicating that the outbreaks were most likely caused by the same field virus strain. The mutations that had occurred between the 2011 and 2015 outbreaks translated into 14 amino acid variations (6.5%) which occurred at positions that correspond to hypervariable regions (HPV) identified for SAT2 (Maree *et*

al., 2011). The discrete HPV regions observed were shown in a previous study to be responsible for the antigenicity of the virus (Maree *et al*., 2011). The majority of variations occurred in the G-H loop and this is to be expected as it is the most variable region of the 1D coding region (Cheung *et al*., 1983). However the RGD motif was highly conserved in all sequences, including the isoleucine found at the -1 position, this data was identical to that demonstrated by Sahle *et al*.(2007).

This value (6.5%) shows minimal variation between the outbreaks thereby revealing their relatedness. This is consistent with the phylogenetic tree constructed in this study (Fig.4). The field virus strains were shown to belong to the genotype C which was identified by Bastos *et al*.(2003), this genotype is also located in northern Botswana. Comparison of the amino acid variation between both FMD outbreak isolates and the SAT2035 vaccine strain was calculated to be in the range of 4.2% and 4.6% indicating that the strains are closely related. This is confirmed by the neighbor joining (Fig. 4) and maximum likelihood(Appendix 8) trees created as they are observed to have clustered in a strongly supported clade.

Bastos and Sangare (2001) grouped viruses into distinct virus lineages if they differed by more than 20% at the nucleotide sequence level. FMDV SAT251 vaccine strain differed by 24.5% with 2011 field virus isolates and by 24.2% with 2015 field virus isolates and this confirms their theory. The difference in nucleotide sequences between the field virus isolates observed here (0.3%) is rather too small and suggests that the field virus isolates from the 2011 and 2015 FMD outbreaks are possibly from the same lineage. Figure 4 shows the phylogenetic treeof the field virus isolates and the SAT2 vaccine strains. The tree shows that the SAT251 vaccine strain isphylogenetically lessrelated to the field virus isolates and also clusters into a different topotype (topotype II). This vaccine strain

(SAT251) clustered with NCBI database samples that belong to genotype E (Bastos and Sangare, 2001).

FMDV serotype SAT2 is divided into 14 topotypes and Botswana is plagued by three SAT2 topotypes (I, II and III) (Di Nardo *et al*., 2011). All the sequences (field virus isolates and NCBI database samples) observed in this study clustered according to geographical location, illustrating genetically and geographically distinct genotypes and topotypes, confirming the topotype concept proposed by Samuel and Knowles (2001). The analysis was carried out using different models to ascertain the correctness of the data produced. Maximum likelihood (ML) and Neighbor joining (NJ) methods both generated similar tree topologies (ML tree shown in Appendix 8). Phylogenetic analysis of the four representative field virus isolates revealed that they all belonged to topotype III as they clustered, in a single clade with a strong bootstrap value, with isolates known to belong to this topotype (Vosloo *et al*., 1995).This finding supports the outbreak reports submitted to the OIE after they occurred (OIE-WAHID, 2011, 2015) as well as reports published over time, including the OIE monthly report of December 2011 and OIE-FAO-FMD Ref Lab Network reports for 2012 and 2015.

The relationship coefficient (r_1) values (ratio of heterologous to homologous titers) represents the antigenic relationship between vaccine and field virus strains. Values that are close to 1 (highest value possible) are a demonstration that the virus strains could be antigenically similar. Values between 0.4 and 1.0 indicate that the vaccine strain used can confer protection while values below 0.3 suggest that a new vaccine is required (Rweyemamu, 1984; Samuel *et al*., 1990; Paton *et al*., 2005).

Variability of results is a significant challenge of vaccine matching tests (Mittal *et al.,* 2005). One study compared all the different methods used for vaccine matching and Liquid Phase Blocking ELISA (LPBE) was shown to be less variable and more accurate (Tekleghiorghis *et al*., 2014b). In the current study, the vaccine matching of field virus isolates against the two SAT2 vaccine strains was done by the crossed- Virus Neutralization Test (VNT).When vaccine matching of the SAT251 vaccine strain was done against the 2015 field virus isolates, the minimum r_1 value observed was 0.71 while when tested against the 2011 virus isolates the minimum r_1 value was 0.36; a value which is approximately 0.4. When the vaccine matching was done against vaccine strain SAT2035 on the other hand, the minimum r_1 value for the 2015 virus isolates was 0.61 in contrast to a minimum r_1 value of 0.51 observed for the 2011 virus isolates. Both vaccine strains conferred adequate protection (r_1 values 0.4 – 0.9against SAT251; $r_1$values 0.5 – 0.9 against SAT2035) even though the two vaccine strains fell into different topotypes, suggesting that antigenicity is not wholly dependent on virus topotype.

In general, both vaccine strain r_1 values observed for the 2011 field virus isolates are lower than those of 2015 field virus isolates (Tables 5 and 6). This observation may be explained by the variations of deduced amino acid sequences and specific epitopes presented on the virus capsid. Pairwise comparison between amino acid sequences of vaccine strain SAT2035 and the 2015 field virus isolates showed a 4.2% difference and 4.6% against the 2011 isolates. The extra 0.4% difference was observed at the +1 position downstream the important cell attachment site (RGD motif) – the highly conserved R was substituted for an M in the 2011 virus isolates. This could possibly explain the significant difference in the r_1 values between the two sets of samples and this explanation could be extended to challenges with SAT251 vaccine strain as well. With regards to the SAT251 vaccine strain there was a 11.6% difference between the 2011 field virus isolates while a difference of

only 5.1% was observed between this vaccine strain and the 2015 field virus isolates. The vaccine matching values ranged between 0.36 – 0.55 and 0.71 – 0.85 for the 2011 and 2015 field virus isolates respectively.

Botswana is one of the few countries in sub-Saharan Africa that has made considerable success in the control of FMD (Vosloo *et al*., 2002; Scoones *et al.,* 2010). The country's 2010 statistics state that itexports much of its beef to the European Union (59%) and almost 40% is exported to South Africa (Van Engelen *et al.,* 2013). The successful control of FMD is due to a number of control measures that have been cogently stipulated by the OIE. In Botswana, these measures have included *inter alia* routine vaccination of cattle in designated zones with a trivalent vaccine containing SAT1, 2 and 3 strains, erection and maintenance of cordon fences to separate domestic animals from contact with wild animals (more importantly the African buffalo), movement control and efficient disease surveillance (Derah and Mokopasetso, 2005).

Despite the measures put in place, it has not been possible to completely eradicate the disease in Botswana and outbreaks still continued to occur sporadically in the country (Baipoledi *et al.,* 2004). Unlike FMDV types A, O and C, the SAT types of the virus are well established in wild animals (Condy*et al.,* 1969; Hedger*et al*., 1973), and in Southern Africa the African buffalo (*Syncerus caffer*) is known to be the main reservoir host of SAT1, SAT2 and SAT3 types of FMDV (Condy*et al*., 1985; Bengis *et al*., 1986). The main habitats of buffaloes in Botswana are the Okavango delta, the Chobe and Nata river basins (Baipoledi *et al*., 2004). The area covered in this study is neighboring the Okavango delta and some of the outbreaks that occurred in 2015 were associated with cattle having been in contact with buffaloes. It is known that post infection with FMDV, cattle can become persistently infected with the virus and are able to transmit it to other cattle by

contact (Malirat*et al.,* 1994). It is thought that the 2011 FMD outbreak was probably triggered by persistently infected cattle. According to the disease status reports submitted by Ministry of Agriculture of Botswana to the OIE, it was suspected that the outbreak was caused by interaction of cattle in Itoto crush with those from neighboring Kaepe crush which experienced an outbreak of the disease a few months earlier(OIE-WAHID, 2011). Sometimes these cattle come in contact during grazing and at watering points when boreholes break down thus increasing the risk of the transmission of the disease.

After both outbreaks, mass vaccination campaigns were initiated in which vaccination coverage of 98% and 87% were achieved respectively in 2011 and 2015 and these actions prevented the further spreading of the disease(OIE-WAHID, 2011, 2015).

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

Outbreaks that occurred in Ngamiland District of Botswana in 2011 and 2015 were associated with topotype III of FMDV SAT2. This study has conclusively showed that some degree of genetic variation (mutation) occurred for the virus strains that caused the 2015 outbreak. Comparing the amino acid sequences between the two outbreaks, a variation of 6.5% was observed. The variation did, however, not change remarkably the antigenicity of the field virus strains. Antigenic variation of the field viruses as determined by the relationship coefficients (r_1 values) against the two SAT2 vaccine virus strains currently used for the production of FMDV SAT2 vaccines at BVI gave r_1 values greater than 0.3 implying that the use of these vaccines is capable of protecting challenge by circulating SAT2 field viruses.

On the basis of the results of the genetic and antigenic characterization of the SAT2 virus strains provided by the current study, it would therefore appear that the repeated occurrence of SAT2 FMD outbreaks in the Ngamiland District is not an issue of genetic and antigenic variability of the field viruses. Rather than being linked to vaccination failure the problem is probably that of low routine vaccination coverage. Therefore, competent veterinary authorities need to ensure that herd vaccination coverages in all high-risk areas (e.g. Ngamiland District) be maintained to at least 80% to prevent outbreaks of the disease.

6.2 Recommendations

i. Genetic diversity in this study was investigated by partial genome sequencing of VP1. It is recommended that in further studies of genetic characterization of FMD viruses whole genome sequencing be used in order to get a better appreciation of the genetic diversity of field virus isolates and to augment knowledge on molecular epidemiology of FMD viruses circulating in Botswana and elsewhere in Southern Africa.

ii. Further studies of the relationship coefficient between the field virus strains and vaccine strains should be carried out using Liquid Phase Blocking Elisa (LPBE) which has been shown to be less variable and more accurate as compared to crossed virus neutralization test (VNT) which was used in this study.

REFERENCES

Acharya, R., Fry, E., Stuart, D., Fox, G., Rowlands, D. and Brown, F. (1989). The three-dimensional structure of foot-and-mouth disease virus at 2.9 Å resolution. *Nature* 337:709-716.

Alexandersen, S. and Donaldson, A. I. (2002). Further studies to quantify the dose of natural aerosols of foot-and-mouth disease virus for pigs. *Epidemiology and Infection* 128: 313–323.

Alexandersen, S., Zhang, Z. and Donaldson, A. I. **(**2002). Aspects of the persistence of foot-and-mouth disease virus in animals—the carrier problem. *Microbes and Infection*4:1099–1110.

Alexandersen, S., Zhang, Z., Donaldson, A. I. and Garland, A. J. M. (2003). The pathogenesis and diagnosis of foot-and-mouth disease. *Journal of Comparative Pathology*129(1): 1–36.

Baipoledi E.K., Matlho, G., Letshwenyo, M., Chimbombi, M., Adom, E.K., Raborokgwe M. V. and Hyera, J.M.K. (2004). Re-emergence of foot-and-mouth disease in Botswana. *The Veterinary Journal*168: 93-99.

Bastos, A. D. S. and Sangare, O. (2001). Geographic distribution of SAT2 type foot-and-mouth disease virus genotypes in Africa. In: *Proceedings of the Southern African Society for Veterinary Epidemiology and Preventive Medicine.*10 - 11 May, Onderstepoort, South Africa.pp 20-26.

Bastos, A. D. S., Haydon, D. T., Sangaré, O., Boshoff, C. I., Edrich, J. L. and Thomson, G. R. (2003). The implications of virus diversity within the SAT2 serotype for control of foot-and-mouth disease in sub-Saharan Africa. *Journal of General Virology*84(6): 1595–1606.

Baxi, M. K., Baxi, S., Clavijo, A., Burton, K. M. and D. Deregt, D. (2006). Microarray-based detection and typing of foot-and-mouth disease virus.*Veterinary Journal* 172(3): 473–481.

Beck, E., Feil, G. and Strohmaier, K. (1983). The molecular basis of the antigenic variation of foot-and-mouth disease virus. *EMBO Journal*2(4): 555–559.

Beck, E. andStrohmaier, K. (1987). Subtyping of European foot-and-mouth disease virus strains by nucleotide sequence determination. *Journal of Virology*61(5): 1621–1629.

Belsham, G. J., Abrams, C. C., King, A. M., Roosien, J. and Vlak, J. M**. (**1991). Myristoylation of foot-and-mouth disease virus capsid protein precursors is independent of other viral proteins and occurs in both mammalian and insect cells. *Journal of General Virology*72:747–751.

Bengis, R.G., Thomson, G.R., Hedger, R.S., De Vos, V. and Pini, A. (1986).Foot-and-mouth disease and the African buffalo (*Syncerus caffer*): 1. Carriers as source of infection for cattle. *Onderstepoort Journal of Veterinary Research* 53(2): 69 – 73.

Bolwell, C., Brown, A. L., Barnett, P. V., Campbell, R. O., Clarke, B. E., Parry, N. R., Ouldridge, E. J., Brown, F. and Rowlands, D. J. (1989). Host cell selection of antigenic variants of foot-and-mouth disease virus. *Journal of General Virology*70: 45-57.

Botswana Ministry of Agriculture (1977). Annual report of the Department of Animal Health and Production, Ministry of Agriculture, Government printer, Gaborone, Botswana.

Botswana Ministry of Agriculture (1980). Annual report of the Department of Animal Health and Production, Ministry of Agriculture, Government printer, Gaborone, Botswana.

Brehm, K. E., Kumar, N., Thulke, H. H. and Haas, B. (2008). High potency vaccines induce protection against heterologous challenge with foot-and-mouth disease virus. *Vaccine* 26: 1681–1687.

Brito, B. P., Rodriguez, L. L., Hammond, J. M., Pinto, J. and Perez, A. M. (2015). Review of the global distribution of foot-and-mouth disease virus from 2007 to 2014. *Transboundary and Emerging Diseases*pp 1–17. http://doi.org/10.1111/tbed.12373site visited on 15/02/2015.

Bunch, T., Rieder, E. and Mason, P. (1994). Sequence of the S fragment of foot-and-mouth disease virus type A.*VirusGenes*8(2): 173–175.

Burrows, R. Mann, J. A. and Garland, A. J. M. (1981). The pathogenesis of natural and simulated natural foot-and-mouth disease infection in cattle.*Journal of Comparative Pathology* 91(4): 599–609.

Cheung, A., DeLamarter, J., Weiss, S. and Küpper, H. (1983). Comparison of the major antigenic determinants of different serotypes of foot-and-mouth disease virus. *Journal of Virology*48: 451-459.

Condy, J.B., Hermina, K.A.J. and Hedger, R.S. (1969).Foot-and-mouth disease in wildlife in Rhodesia and other African territories. *Journal of Comparative Pathology*79: 27 – 31.

Condy, J. B., Hedger, R. S., Hamblin, C. and Barnett, I. T. R. (1985). The duration of the foot-and-mouth disease virus carrier state in African buffalo (i) in the individual animal and (ii) in a free-living herd. *Comparative Immunology, Microbiology And Infectious Diseases* 8(3): 259-265.

De Clercq, K. (2003). Overview on FMD diagnostic techniques. In:*Foot-and-Mouth Disease: Control Strategies*. (Edited by Dodet, B. and Vicari, M.). EditionsScientifiques et Médicales, Elsevier, SAS, Amsterdam. pp. 345 - 351.

Derah, N. and Mokopasetso, M. (2005). The control of foot-and-mouth disease in Botswana and Zimbabwe. *Tropicultura* 23: 3-7.

Di Nardo, A., Knowles, N. J. and Paton, D. J. (2011). Combining livestock trade patterns with phylogenetics to help understand the spread of foot and mouth disease in sub-Saharan Africa, the Middle East and Southeast Asia.*Revue Scientifique et Technique-OIE* 30(1): 63.

Domingo, E. and Holland, J. J. (1988). High error rates, population equilibrium and evolution of RNA replication systems. In: *RNA genetics, vol. III. Variability of RNA Genomes.*(Edited by Domingo, E., Holland,J. J. and Ahlquist P.)CRC Press, Boca Raton, Florida. pp. 3–36.

Domingo, E., Mateu, M. G., Martinez, M. A., Dopazo, J., Moya, A. and Sobrino, F. (1990). Genetic variability and antigenic diversity of foot-and-mouth disease virus. In:*Applied Virology Research, vol. 2, Virus Variation and Epidemiology*.(Edited by Kurstak, E., Marusyk, R. G., Murphy, S. A.andVan Regenmortel, M. H. V.) Plenum, New York.pp. 233–266.

Domingo, E., Escarmís, C., Martínez, M. A., Martínez Salas, E. and Mateu, M. G. (1992) Foot-and-mouth disease virus populations are quasispecies.*Current Topics in Microbiology and Immunology* 176: 33-47.

Domingo, E., Escarmis, C., Baranowski, E., Ruiz-Jarabo, M. C., Carrillo, E., Nunez, J. I. and Sobrino, F. (2003). Evolution of foot-and-mouth disease virus. *Virus Resesearch* 91:47–63.

Donaldson, A. I., Gibson, C. F., Oliver, R., Hamblin, C. and R. Kitching, R. P. (1987). Infection of cattle by airborne foot-and-mouth disease virus: minimal doses with O1 and SAT2 strains.*Research in Veterinary Science* 43(3): 339–346.

Drake, J. W. and Holland, J. J. (1999). Mutation rates among RNAviruses. *Proceedings of the National Academy of Science of the United States of America*96(24):13910–13913.

Elnifro, E. M., Ashshi, A. M., Cooper, R. J.and Klapper, P. E. (2000). Multiplex PCR: optimization and application in diagnostic virology. *Clinical Microbiology Reviews* 13(4): 559-570.

Falconer J. (1972). The Epizootiology and Control of foot-and-mouth disease in Botswana. *Veterinary Record* 91:354 – 359.

Fernández, J., Agüero, M., Romero, L., Sánchez, C., Belák, S., Arias, M. and Sánchez-Vizcaíno, J. M. (2008). Rapid and differential diagnosis of foot-and-mouth disease, swine vesicular disease, and vesicular stomatitis by a new multiplex RT-PCR assay. *Journal of Virological Methods* 147(2): 301-311.

Ferris, N. P. and Dawson, M. (1988). Routine application of enzyme-linked immunosorbent assay in comparison with complement fixation for the diagnosis of foot-and-mouth and swine vesicular diseases. *Veterinary microbiology*16(3): 201-209.

Gajendragad, M. R., Kamath, K. N. Y., Anil, P. Y., Prabhudas, K. and Natarajan, C. (2001). Development and standardization of a piezo electric immunobiosensor for foot-and-mouth disease virus typing.*VeterinaryMicrobiology* 78(4): 319–330.

Gasteiger, E., Gattiker, A., Hoogland, C., Ivanyi, I., Appel, R.D. and Bairoch, A. (2003). ExPASy: The proteomics server for in-depth protein knowledge and analysis. *Nucleic Acids Research* 31:3784-3788.

Grubman, M. and Baxt, B. (2004). Foot-and-mouth disease. *Clinical Microbiology Reviews*17(2): 465–493.

Hedger, R.S., Forman, A.J. and Woodford, M.H. (1973). Foot-and-mouth disease in East African buffalo. *Bulletin Epizootic Diseases of Africa*21: 45–50.

Hyera, J.K.M., Letshwenyo, M., Monyame, K.B., Thobokwe, G., Pilane, A.R., Mapitse N. and Baipoledi, E.K. (2006). A serological survey for antibodies to Foot and mouth disease virus in indigenous Tswana goats and sheep in Kasane, Maun and Shakawe Districts in North West Botswana. *Onderstepoort Journal of Veterinary Research*73:143-147.

Jackson, T., King, A. M. Q., Stuart, D. I. and Fry, E. (2003). Structure and receptor binding.*Virus Research* 91(1): 33–46.

Jamal, S. M. and Belsham, G. J. (2013). Foot-and-mouth disease: Past, present and future. *Veterinary Research* 44:116 - 129.

Kasanga, C.J., Yamazaki, W., Mioulet, V., King, D.P., Mulumba, M., Ranga, E., Deve, J., Mundia, C., Chikungwa, P., Joao, L., Wambura, P. N.,Rweyemamu, M. M.(2014). Rapid, sensitive and effective diagnostic tools for foot-and-mouth disease virus in Africa, *Onderstepoort Journal of Veterinary Research* 81(2):1 - 5.

Knowles, N. J., Samuel, A. R., Davies, P. R., Kitching, R. P. and Donaldson, A. I. (2001). Outbreak of foot-and-mouth disease virus serotype O in the UK caused by a pandemic strain. *Veterinary Record* 148: 258–259.

Knowles, N. J. and Samuel, A. R. (2003). Molecular epidemiology of foot-and-mouth disease virus. *Virus Research*91(1): 65–80.

Knowles, N. J., Hovi, T., Hyypiä, T., King, A. M., Lindberg, A. M., Minor, P. D., Pallansch, M. A., Palmenberg, A. C., Skern, T., Stanway, G., Yamashita, T. and Roland Zell, R.(2008). Taxonomy of Picornaviridae: current situation and future proposals. *International Committee on Taxonomy of Viruses, Picornavirus Study Group, EUROPIC*. June 2008. [http://www.picornastudygroup.com/posters/europic_2006.pdf] site visited on 14/02/2015.

Kumar, S., Stecher, G. and Tamura, K. (2015)MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets.*Molecular Biology and Evolution* 33(7): 1870-1874.

Lau, L.T., Reid, S. M., King D. P., Lau, A. M., Shaw, A. E., Ferris, N. P. and Yu, A. C. H. (2008). Detection of foot-and-mouth disease virus by nucleic acid sequence-based amplification (NASBA).*Veterinary Microbiology*126:101–110.

Longjam, N. and Tayo, T. (2011). Antigenic variation of foot-and-mouth disease virus - an overview. *Veterinary World*4(10): 475–479.

Longjam, N., Deb, R., Sarmah, K., Tayo, T., Awachat, V. B. and Saxena, V. K. (2011a). A Brief Review on Diagnosis of foot-and-mouth Disease of Livestock: Conventional to Molecular Tools. *Veterinary Medicine International*, *2011*, article 905768. [http://doi.org/10.4061/2011/905768]site visited on 10/01/2015.

Longjam, N., Sarmah, A. K., Deb, R., Mathapati, B., Saxena,V. K. and Tayo, T. (2011b). Detection of foot and mouth diseasevirus serotype O by sandwich ELISA, viral isolation and multiplex PCR. *Online Journal of Veterinary Research*15(1): 76–92.

Malirat, V., De Mello, P. A., Tiraboschi, B., Beck, E., Gomes, I. and Bergmann, I. E. (1994). Genetic variation of foot-and-mouth disease virus during persistent infection in cattle. *Virus Research* 34(1): 31-48.

Mapitse, N. (2008).Botswana's foot-and-mouth disease and beef trade policy. Transboundary animal disease and market access: future options for the beef industry in Southern Africa, Working Paper 3, Brighton: Institute of Development Studies.Brighton. [http://www. steps-centre. org/PDFs/VetScience_Working% 20Paper% 203. pdf] site visited on 10/01/2015.

Maree, F. F., Blignaut, B., Esterhuysen, J. J., de Beer, T. A. P., Theron, J., O'Neill, H. G.and Rieder, E. (2011). Predicting antigenic sites on the foot-and-mouth disease virus capsid of the South African Territories types using virus neutralization data. *Journal of General Virology*92(10): 2297–2309.

Mason, P. W., Bezborodova, S. V. and Henry, T. M. (2002). Identification and characterization of a cis-acting replication element (cre) adjacent to the internal ribosome entry site of foot-and-mouth disease virus.*Journal of Virology* 76(19): 9686–9694.

Mittal, M., Tosh, C., Hemadri, D., Sanyal, A. and Bandyopadhyay, S. K. (2005). Phylogeny, genome evolution, and antigenic variability among endemic foot-and-mouth disease virus type A isolates from India. *Archives of Virology* 150(5): 911-928.

OIE (2012). *Manual of diagnostic tests and vaccines for terrestrial animals*. Chapter 2.1.5: Foot-and-mouth disease. 7th Edition. Paris.1404pp.

OIE (2015). List of FMD free Members. Available online: [http://www.oie.int/animalhealth-in-the-world/official-disease-status/fmd/list-of-fmdfree-members]site visited on 15/01/2016.

OIE and FAO (2012). The global foot-and-mouth disease control strategy: Strengthening animal health sysyems through improved control of major diseases.[http://doi.org/978-92-5-107273-8]site visited 10/08/2015.

OIE-SSARRL-FMD reports (2011). *Customer test report*. Botswana Vaccine Institute Printer, Gaborone, Botswana. 2pp.

OIE-SSARRL-FMD reports (2015). *Customer test report*. Botswana Vaccine Institute Printer, Gaborone, Botswana. 2pp.

OIE-WAHID (2011).Foot-and-mouth disease Botswana.[http://www.oie.int/wahis_2/public/wahid.php/Reviewreport/Review?page_refer=MapFullEventReport&reportid=11037]site visited on08/05/2015.

OIE-WAHID (2015). Foot-and-mouth disease Botswana.[http://www.oie.int/wahis_2/public/wahid.php/Reviewreport/Review?page_refer=MapFullEventReport&reportid=17327]site visited on08/05/2015.

Paton, D.J., Valkarcher, J.F., Bergmann, I., Matlho, G., Zakharav, V.M., Palma, E.L. and Thomson, G.R. (2005). Selection of foot-and-mouth disease vaccine strains – a review. *Revue Scientifique Technique, Office International des Épizooties* 24:981-993.

Paton, D. J., Sumption, K. J. and Charleston, B. (2009). Options for control of foot-and-mouth disease: knowledge, capability and policy. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*364(1530): 2657–2667.

Pfaff,E., Mussgay,M., Bohm,H.O., Schulz,G.E. and Schaller,H. (1982).*EMBO Journal* 1:869-874.

Rémond, M., Kaiser, C. and Lebreton, F. (2002). Diagnosis and screening of foot-and-mouth disease.*ComparativeImmunology, Microbiology and Infectious Diseases* 25(5-6): 309–320.

Rweyemamu, M. M. (1984). Antigenic variation in foot-and-mouth disease: studies based on the virus neutralization reaction. *Journal of Biological Standardization* 13(3): 323-337.

Rweyemamu, M., Roeder, P., Mackay, D., Sumption, K., Brownlie, J., Leforban, Y. and Saraiva, V. (2008). Epidemiological patterns of foot-and-mouth disease worldwide. *Transboundary and Emerging Diseases*55(1): 57–72.

Sahle, M., Dwarka, R. M., Venter, E. H. and Vosloo, W. (2007). Study of the genetic heterogeneity of SAT2 foot-and-mouth disease virus in sub-Saharan Africa with specific focus on East Africa. *Onderstepoort Journal of Veterinary Research*74(4): 289–299.

Salt, J. S. (1993). The carrier state in foot and mouth disease-an immunological review.*British Veterinary Journal* 149(3): 207–223.

Samuel, A. R., Ouldridge, E. J., Arrowsmith, A. E. M., Kitching, R. P. and Knowles, N. J. (1990). Antigenic analysis of serotype O foot-and-mouth disease virus isolates from the Middle East, 1981 to 1988. *Vaccine* 8(4): 390-396.

Samuel, A. R. and Knowles, N. J. (2001). Foot-and-mouth disease type O viruses exhibit genetically and geographically distinct evolutionary lineages (topotypes). *Journal of General Virology* 82(3): 609-621.

Sangula, A. K., Siegismund, H. R., Belsham, G. J., Balinda, S. N., Masembe, C. and Muwanika, V. B. (2011). Low diversity of foot-and-mouth disease serotype C virus in Kenya: evidence for probable vaccine strain re-introductions in the field. *Epidemiology and Infection* 139: 189–196.

Shaw, A. E., Reid, S. M., King, D. P., Hutchings, G. H. and Ferris, N. P. (2004). Enhanced laboratory diagnosis of foot-and-mouth disease by real-time polymerase chain reaction. *Revue Scientifique Technique (Office International des Épizooties)*23(3): 1003-1009.

Scoones, I., Bishi, A., Mapitse, N., Moerane, R., Penrith, M. L., Sibanda, R. and Thomson, G. (2010). Foot-and-mouth disease and market access : challenges for the beef industry in Southern Africa. *Pastoralism*1(2): 135–164.

Sobrino, F., Davila, M., Ortin, J. and Domingo, E. (1983). Multiple genetic variants arise in the course of replication of foot-and-mouth disease virus in cell culture. *Virology* 128: 310-318.

Sorensen, J. H., Mackay, D. K., Jensen, C. O. and Donaldson, A. I. (2000). An integrated model topredict the atmospheric spread of foot-and-mouth disease virus. *Epidemiology and Infection* 124: 577–590.

Sorensen, J. H., Jensen, C. O., Mikkelsen, T., Mackay,D. K. and Donaldson, A. I. (2001). Modelling the atmospheric dispersion of foot-and-mouth disease virus for emergency preparedness. *Physics Chemistry Earth* 26: 93–97.

Swinscow, T.D.V. (1980). *Statistics at Square One*. British Medical Association, London. 86pp.

Tekleghiorghis, T., Moormann, R. J. M., Weerdmeester, K. and Dekker, A. (2014a). foot-and-mouth disease transmission in Africa: Implications for control, a review. *Transboundary and Emerging Diseases* 63(2): 136 - 151.

Tekleghiorghis, T., Weerdmeester, K., Hemert-Kluitenberg, F., Moormann, R. J. M. and Dekker, A. (2014b). Comparison of test methodologies for foot-and-mouth disease virus serotype a vaccine matching. *Clinical and Vaccine Immunology*21(5): 674–683.

Thomson, G.R. (1994). Foot-and-mouth disease. In:*Infectious diseases of livestock with special reference to Southern Africa*, (Edited by J.A.W. Coetzer, G.R. Thomson, andR.C. Tustin). Oxford University Press, Cape Town. pp825-852.

Thomson, G. R. (1995). Overview of foot-and-mouth disease in Southern Africa. *Revue Scientifique et Technique* (*International Office of Epizootics*)14(3): 503–520.

Thomson, G. R. and Bastos, A. D. S. (2002). Foot-and-mouth disease. In:*Infectious diseases of livestock with special reference to Southern Africa2nd edition*(Edited by Coetzer, J. A. W.andTustin, R. C.)Oxford University Press, CapeTown. pp. 1324–1365.

Valarcher, J. F., Leforban, Y., Rweyemamu, M., Roeder, P. L., Gerbier, G., Mackay, D. K., Sumption, K. J., Paton, D. J. and Knowles, N. (2008). Incursions of foot-and-mouth disease virus into Europe between 1985 and 2006. *Transboundary and Emerging Diseases* 55: 14–34.

Valdazo-González, B., Knowles, N. J., Hammond, J. and King, D. P. (2012). Genome sequences of SAT2 foot-and-mouth disease viruses from Egypt and Palestinian Autonomous Territories (Gaza Strip). *Journal of Virology*86(16): 8901–8902.

Van Engelen, A., Malope, P., Keyser, J. and Neven, D. (2013). Botswana agrifood value chain project: Beef value chain study. Food and Agriculture Organisation (FAO), Rome and Ministry of Agriculture, Botswana. 199pp.

ViralZone (2014). Apthovirus. [http://viralzone.expasy.org/all_by_species/98.html] site visited on 12/11/2014.

Vosloo, W., Kirkbride, E., Bengis, R. G., Keet, D. F. and Thomson, G. R. (1995). Genome variation in the SAT types of foot-and-mouth disease viruses prevalent in buffalo (Syncerus caffer) in the Kruger National Park and other regions of Southern Africa, 1986-93. *Epidemiology and Infection* 114(0): 203 - 218

Vosloo,W., Bastos, A. D., Kirkbride, E., Esterhuysen,J. J., Janse van Rensburg, D., Bengis, R. G., Keet, D. W. and Thomson, G. R. (1996). Persistent infection of African buffalo (Syncerus caffer) with SATtype foot-and-mouth disease viruses: rate of fixation of mutations, antigenic change and interspecies transmission.*Journal of General Virology* 77(7): 1457–1467.

Vosloo, W., Bastos, A. D. S., Sangare, O., Hargreaves, S. K. and Thomson, G. R. (2002). Review of the status and control of foot and mouth disease in sub-Saharan Africa. *Revue Scientifique et Technique* (*International Office of Epizootics*)21(3): 437–449.

Vosloo, W. and Thomson, G.R. (2004).Natural Habitats in which foot-and-mouth disease viruses are maintained. In: *Foot-and-mouth disease*. (Edited by Domingo, E. and Sobrino, F.) HorizonScientific Press, Norfolk. pp. 383-410.

Vosloo, W., Bastos, A. D. S. and Boshoff, C. I. (2006). Retrospective genetic analysis of SAT1 type foot-and-mouth disease outbreaks in Southern Africa.*Archives of Virology* 151(2): 285-298.

Walker, J. (1934). Some observations of foot-and-mouth disease in the Bechuanaland Protectorate. *Journal of the South African Veterinary Medicine Association*5: 73-91.

Woodbury, E. L. (1995). A review of the possible mechanisms for the persistence of foot-and-mouth disease virus. *Epidemiology and Infection*114: 1-13.

APPENDICES

Appendix 1: Sample treatment procedure

a) The tissues/scrapings were removed from the transport medium and placed into a clean sterile flask using sterile forceps.

b) Minimum Essential Medium for lamb kidney cells (RM-MEM) was added and the flask shaken to rinse the tissue samples. The samples were then transferred into sterile centrifuge tubes. Sterile scissors used to cut the tissue into smaller pieces.

c) Three ml of RM-MEM medium and 5% $CHCl_3$ was added to the sample. A sterile turrax was used to grind the samples, the mixture then centrifuged at 2 500 rpm for 15 minutes at 4^{o} C in a bench-top centrifuge.

d) The supernatant was collected into sterile 5 ml flasks, labeled properly and used to inoculate monolayer cultures for virus isolation.

Appendix 2: Virus isolation procedure

a) Monolayer cultures of lamb kidney (RM) cell lines were prepared in 150 cm^2 culture flasks. These flasks were labelled with the identity of the sample, date of sample inoculation and sample passage (e.g. 1st passage).

b) The culture flasks were checked using a light microscope to confirm that the monolayer has completely adhered to the bottom of the flask.

c) The cell culture medium was eliminated from the flasks and 2 ml of the supernatant inoculated. The flasks were moved or rolled to spread the virus sample all over the monolayer.

d) The flask was incubated in an incubator at 37^oC for 30 minutes. This was followed by addition of 30ml of RM-MEM medium which was kept warm at 37^oC prior to use. Incubation was continued for a maximum of 48 hours at 37^oC or until the infected cells show maximal cytopathic effect (CPE).

e) Flasks were examined microscopically for CPE. When maximal CPE was observed the flask were shaken vigorously and frozen at –20^oC.

f) The flask contents were thawed at 37^oC and transferred into centrifuge tubes. Centrifugation was run at 2000 rpm for 20 minutes at 4^oC in a bench top centrifuge to separate the cell layer from the virus particles.

g) The supernatant was transferred to sterile 50ml tubes while the pellet, which is the cell debris was discarded.

h) For the 2nd passages, 2 ml of the supernatant was used to inoculate a fresh RM monolayer culture and the same procedure as above followed.

Appendix 3: RNA extractionusing ZR extraction kit

a) In a 1.5ml microcentrifuge tube, add three volumes of ZR Viral RNA buffer to each volume of sample (e.g. 300µl lysis buffer to 100µl supernatant).

b) Transfer mixture to a Zymo-Spin IC column in a collection tube. Centrifuge at around 12000 x g for 1-2 minutes. Discard the flow through from the collection tube.

c) Add 300µl RNA Wash Buffer to the column. Centrifuge at About 12000 x g for 30 seconds. Discard the flow through and place the column back into the collection tube. Repeat the wash step.

d) Centrifuge the column in an empty collection tube at 12000 x g for 2 minutes to ensure complete removal of the wash buffer.

e) Place the column into the DNase/RNase free tube and elute using 6-10µl DNase/RNase free water. To maximize elution let it stand at RT for 2 minutes.

f) Centrifuge the columns at 12000 x g for 1 minute to elute RNA. The RNA can be used immediately or stored at -70°C for some time.

Appendix 4: Agarose gel electrophoresis

a) 1.0 g of low melting point or normal or high efficiency separation agarose is dissolved in 50 ml of TAE buffer and the mixture warmed up to about $55^{O}C$ to speed up dissolution process.

b) The molten agarose is allowed to cool without solidifying and 20µl of gel red is added and mixed.

c) The agarose is poured into a gel trough and the comb inserted. It is then allowed to solidify into a gel at room temperature or at $4^{O}C$ for about an hour.

d) The gel trough is carefully placed in the electrophoresis tank and TAE buffer (x1) is poured to immerse the gel.

e) The sample which is prepared by mixing 25µl of PCR product and 10µl of the loading solution is deposited on the wells. The molecular weight marker is also diluted appropriately with the loading solution and loaded into wells.

f) The electrophoresis process is run at 100-120 volts for 15-30 minutes to facilitate DNA band separation according to their sizes.

g) The gel is visualized by UV illumination and if the desired DNA band is present, always below the tracking dye, it is excised and placed into a 0.5 ml eppendorf tube.

Appendix 5: cDNA recovery from gel

a) Using a clean razor blade or scalpel, excise the slice of agarose containing the DNA fragment to be purified. Cut as close to the DNA band as possible. Cut the slice into several smaller pieces and transfer them to the pre-weighed 1.5 ml microcentrifuge tube.

b) Add 3 volumes of ADB buffer to each volume of agarose excised from the gel e.g. for 100 µl (mg) of agarose gel slice add 300 µl of ADB.

c) Incubate at 37-55°C for 5-10minutes until the slice is completely dissolved. NB: for DNA fragments >8kb, following the incubation step add one additional volume (equal to that of the gel slice) of water to the mixture for better DNA recovery.

d) Transfer the melted agarose solution to a Zymo-Spin™ Column in a Collection tube. Centrifuge at ≥10, 000 x g for 30-60 seconds. Discard the flow-through.

e) Add 200µl of Wash Buffer to the column, and centrifuge at ≥10,000 x g for 30 seconds. Discard the flow-through and repeat the wash step.

f) Add ≥6 µl of water or your preferred Elution Buffer directly to the column matrix. Place column into a 1.5ml sterile DNase free tube and centrifuge at ≥10,000 x g for 30-60 seconds to elute the DNA. The ultra-pure DNA is ready to use for sequencing procedures and can be stored at -70°C for later use.

Appendix 6: Vaccine matching

1. Virus Dilution series

a) NB: The pre-determined virus titre (e.g. $10^{5.5}$) is assumed. The half log dilution series for this example would be in the region of $10^{2.5}$ to $10^{4.5}$. This ensures the theoretical 2 log dose ($10^{3.5}$) is midway and therefore in the middle of the plate.

b) Using the above as a guideline, set out as many haemolytic tubes as needed for each virus to be tested including the homologous reference virus and label accordingly.

c) Using pre-determined theoretical virus titres as guideline, aliquot media as follows;

Table 1: Preparation of log dilutions

Log dilutions	Volume of media (ml)
10^{-1} 0.9	
10^{-2}	1.8
$10^{-2.5}$	2.2
10^{-3}	2.2
$10^{-3.5}$	2.2
10^{-4}	2.2
$10^{-4.5}$	2.2

d) Transfer 0.1ml of the stock virus suspension in to the 1st dilution tube. Mix.

e) Remove 0.2ml of this dilution and transfer it into the second dilution tube. Mix.

f) Remove 1.0ml from this tube and transfer it to the third tube. Mix.

g) Continue transferring 1.0ml of the previous dilution to the end of the series.

h) Repeat for all test viruses.

i) Place all haemolytic tubes at +4°C until required.

2. Virus Neutralisation Plate

Figure 1: Virus neutralisation plate layout

----- Half log pre-prepared virus dilutions----

Cells Control											Virus Control
	A										
	B										
	C										
	D										
	E										
	F										
	G										
	H										

---------- Well columns 1-12----------

a) Add 100µl/well of media to column 1 (cell control) and 50µl/well to columns 2-12.

b) Add 50µl/well of vaccine virus reference sera to the top well row (A2-A11) making an initial serum dilution of ½.

c) Using a pipette and appropriate tips, dilute the sera 2-fold (0.3 log) down the plate (rows A-H) and discard excess. Repeat for all test plates.

d) Add 50µl/well of test virus (starting with the weakest dilution) to each pair of well columns. Begin with adding the weakest dilution (10-4.5) to columns 10, 11 and 12. Add the next, less weak, dilution to well columns 8 and 9 and so on.

e) Repeat in similar plates for each virus in the test.

f) Once the dilution tubes are no longer required, surface decontaminate and discard.

g) Stack the plates and cover the top one(s) and leave for an hour at room temperature.

h) NB: in addition to the test virus plates, include a control plate using a virus with a known titre (reference antigen).

3. Virus Titration Plate

Figure 2: Virus titration plate layout

---------- 0.3 log virus dilution----------

Virus Dilution											Cells control
	A										
	B										
	C										
	D										
	E										
	F										
	G										
	H										

---------- Well columns 1-12 ----------

a) Add 50µl/well of media to well columns 2-12. Leave row 1 empty.

b) Add 100µl/well of the chosen virus dilution e.g. 10-3.0 (this is taken from the dilution tube used in the test plate) to each well in row 1 and dilute 2 fold (0.3 log) across the plate from column 2-11 using multichannel pipettes.

c) Discard excess.

d) Repeat in similar plates for each virus in the test.

e) Overlay each well with 50µl/well of media. Leave to incubate at room temperature with the test plates.

f) Once dilution tubes are no longer required, surface decontaminate and discard.

4. Incubation of Plates

After incubation, add 50µl/well of IBRS2 or BHK21 cells at a seeded rate of between 0.7 – 1 x 10^6 per ml to every plate. Seal each plate with the lid/ semi permeable sealer and incubate in a CO_2 incubator for 48-72hrs.

5. Staining of plates

a) After 72-hour incubation, 50µl of citric acid is added to all the wells.

b) Plates are then incubated at room temperature for 30 minutes.

c) Decant the acid and add 50ul of Amido black stain to all wells.

d) Incubate for a further 30 minutes.

e) Decant the stain and wash the plates thoroughly in cold running tap water.

f) Blot the plates dry.

Appendix 7: Summary of NCBI database nucleotide sequences used in creation of phylogenetic trees

Virus name	Country of origin	Year of sampling	Place of origin	Species of origin	Genbank accession No	References
ZIMR20	Zimbabwe	NK	NK	NK	AF038972	Vosloo *et al.,*1996
KEN/3/57	Kenya	1957	Wamba	Bovine	AJ251473	Unpublished
ANG/4/74	Angola	1974	NK	NK	AF479417	Bastos *et al.,* 2003
MAL/3/75	Malawi	1975	NK	NK	AF367099	Bastos *et al.,* 2003
NIG/2/75	Nigeria	1975	NK	NK	AF367139	Bastos *et al.,* 2003
SEN/5/75	Senegal	1975	NK	NK	AF367140	Bastos *et al.,* 2003
BOT/3/77	Botswana	1977	NK	Bovine	KF112928	Hall *et al.,*2013
BOT/8/78	Botswana	1978	NK	Bovine	KF112929	Hall *et al.,*2013
MOZ/1/79	Mozambique	1979	NK	NK	AF367137	Bastos *et al.,* 2003
ZAI/1/82	DRC	1982	Bibatama, Nord Kivu	Bovine	AF367100	Bastos *et al.,* 2003
ZIM/7/83	Zimbabwe	1983	Nyamandhlovu	Bovine	AF540910	van Rensburg and Nel, 2002
SWA/4/89	Namibia	1989	Sigwe	Bovine	KF112969	Hall *et al.,*2013
ZIM/8/89	Zimbabwe	1989	NK	Bovine	KF112975	Bastos *et al.,* 2003
KNP/19/89	South Africa	1989	Kruger NP	Buffalo	AF367110	Bastos *et al.,* 2003
NAM/1/91	Namibia	1991	NK	Bovine	AY254720	Unpublished
GHA/8/91	Ghana	1991	Tamale province	Bovine	AF479416	Bastos *et al.,* 2003
BOT/18/98	Botswana	1998	Nxaraga	Buffalo	AF367123	Bastos *et al.,* 2003
BOT/29/98	Botswana	1998	Vumbura	Buffalo	AF367124	Bastos *et al.,* 2003
BOT/31/98	Botswana	1998	Vumbura	Buffalo	AF367125	Bastos *et al.,* 2003
ERI/12/98	Eritrea	1998	Erythrea	Bovine	AF367126	Bastos *et al.,* 2003
NAM/292/98	Namibia	1998	East Caprivi GR	Buffalo	AF367128	Bastos *et al.,* 2003
KEN/5/99	Kenya	1999	Athi river, Machakos	Bovine	AF367131	Bastos *et al.,* 2003
RWA/1/00	Rwanda	2000	Gishwati district	Buffalo	AF367134	Bastos *et al.,* 2003
SAU/6/00	Saudi Arabia	2000	Al Kahrj, Riyadh	Bovine	AF367135	Bastos *et al.,* 2003

Appendix 8: Maximum likelihood tree showing relationship of field virus isolates to relevant reference sequences from the NCBI database.