

Genetic And Phenotypic Characterisation Of Selected African Foot-And-Mouth Disease Virus Isolates

by

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Submitted in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

in the
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University of Pretoria

DECLARATION

I hereby declare that this thesis is my own work. It is submitted in fulfilment of the degree, Doctor of Philosophy, in the University of Pretoria, South Africa. It has not been submitted before for any degree or examination in any other University.

Peninah Nsamba

February 2015

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“Give thanks to the LORD, for He is good and His steadfast love endures forever: Psalm 136”

SUMMARY

Genetic and phenotypic characterisation of selected African foot-and-mouth disease virus isolates

by

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Foot-and-mouth disease (FMD) is a contagious viral disease that affects cloven hoofed animals and can be economically devastating. However, vaccination can reduce the incidence of the disease when used in conjunction with other control methods. Studies described in this thesis address three aspects beneficial to the selection of vaccine seed viruses as well as improving the design of chimeric vaccines customized for use in Africa.

Molecular characterization of the non-structural proteins of African FMD viruses (FMDV) can facilitate the selection of vaccine seed strains with a fitness advantage. In addition, common mutations acquired by South African Territory (SAT) serotypes on the virus surface capsid upon cell culture adaptation may be of value to the design of recombinant constructs for chimeric viruses optimized for vaccine production. Moreover, the antigenicity of a chimeric virus v^{ZIM14}-SAT2 compared to its parental virus, SAT2/ZIM/14/90, in cattle and additionally in vaccine matching assays evaluated its compatibility for future vaccine studies.

Genome comparisons of the non-structural proteins for 79 African FMDV isolates from different serotypes and topotypes, spatially and temporally dispersed, showed that critical functional motifs were conserved and variation occurred away from enzymatic active sites. The southern

African FMDV clustered separately from the other African viruses on phylogenetic analysis. This increased the molecular understanding of FMDV, aiding in the selection of vaccine seed strains.

The BHK-21 cell line (preferred for vaccine production) was used to serially passage eleven SAT1 and four SAT2 FMDV. This resulted in Arg or Lys mutations in the VP1 capsid protein surrounding the 5-fold axis, at positions 84-85 and 111-112 respectively. Arg at position 112 was favoured in the binding of a heparin moiety onto a SAT1 model using molecular docking simulations. This knowledge can be applied to recombinant constructs for the generation of chimeric viruses that adapt rapidly to BHK-21 cell culture for vaccine production.

Vaccines derived from chimeric viruses present a good prospect in the control of FMD. The antigenic nature of an intra-serotype chimeric virus, v^{ZIM14} -SAT2, consisting of the capsid-coding region of SAT2/ZIM/14/90 in a genome-length clone pSAT2, was compared to that of its parental virus. In cattle both chimeric and parental viruses elicited similar neutralizing antibody responses and provided protection against clinical disease following homologous virus challenge. In addition, antigenic matching resulted in comparable cross-reaction results for both chimeric and parental viruses. Therefore, when used as vaccines, the antigenic profiles of chimeric viruses against SAT2 isolates did not differ from parental viruses and could be used as an alternative in vaccine production combined with the advantage of including suitable mutations for cell culture adaptation.

These findings increase our knowledge on the genome structure and replication of the FMDV in BHK-21 cells. The data can be used to facilitate selection of vaccine seed viruses specific to serotype and topotype. Additionally, the information can supplement the design of recombinant constructs for chimeric vaccines for future application. Taken together, these new data broaden our options to control FMD in Africa using tailor made chimeric vaccines.

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Fig. 2.2

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Fig 2.3

Structural representation of FMDV 3C^{pro}. The position of amino acid variation observed in a complete alignment of all African SAT1 (A & B) and SAT2 (C & D) viruses is mapped on the modelled structure. Two

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Fig. 3.1

A minimum evolution tree depicting the gene relationships for the P1-coding regions of SAT1 and SAT2 viruses from southern (Kruger National Park, KNP; Zimbabwe, ZIM; Mozambique, MOZ; and Angola, ANG), western (Nigeria, NIG; Senegal, SEN; and Ghana, GHA) and East Africa (Uganda, UGA; Rwanda, RWA; Kenya, KEN; Tanzania, TAN; Eritrea, ERI; and Sudan, SUD).

(a) The host species from which each virus was isolated is indicated by C (cattle), B (buffalo) or I (impala).

(b) The plaque morphologies of the isolate with the lowest available passage history (PH)

(c) The plaque morphology on BHK-21 cell of cell-culture adapted virus following eight serial passages on BHK-21 cells and two serial passages on CHO-K1 cells. Morphologies are indicated as either (O) for plaques larger than 7 mm in diameter on average, (◐) plaques of 4-6 mm in diameter or, (◑) plaques between 1 and 3 mm in diameter.

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Fig 3.2

Plaque morphologies of the parental and BHK-21 cell culture derived viruses obtained using monolayers of BHK-21 and CHO-K1 cells. Cells infected with the indicated viral strains were incubated with tragacanth overlay for 40 h prior to staining with 1% methylene blue. Plaques for SAT1 and SAT2 wild-type viruses are generally large with opaque edges and eight cytolitic passages on BHK-21 cells were accompanied by smaller to medium plaques and clear edges. The plaque morphology change was associated with the ability to grow on CHO-K1 cells.

Fig. 3.3

Plaque reduction assays for BHK-21 cell-adapted SAT1 (A) and SAT2 (B) viruses following pre-treatment with heparin. Plaques were expressed as a percentage of plaques in the absence of heparin compared to plaques where heparin was present. Each point represents the mean of four repeats. The SAT1/NAM/307/98 virus, with a non-heparin binding phenotype, was included as a control.

Fig. 3.4

A space-filling representation of the SAT1 and SAT2 pentamers. (A) The SAT1 pentamer is based on the protein data bank co-ordinates (2WZR). Amino acid substitutions observed during the adaptation of SAT1 viruses in BHK-21 cells are indicated in yellow. The surface-exposed, positively charged mutations, that occurred more than once in different SAT1 viruses, are highlighted in red. The five copies of VP1 show the positively charged cluster at the five-fold axis. (B) Positively charged mutations are colour-coded based on the frequency of occurrence in different viruses within the SAT1 serotype from orange ($n > 1$) to red ($n > 5$). (C) The SAT2 pentamer is modelled using the SAT1 co-ordinates as a template and the surface-exposed, positively charged mutations are shown in red. In SAT2, a Lys residue appeared twice in VP1 position 1083 in two

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Fig. 3.5

Surface models of wild-type and VP1 positively charged mutant proteins coloured by electrostatic potential. Positively charged surfaces are shown in blue, and negatively charged surfaces are red. The cell culture-adapted mutations at VP1 positions 111 and 112 were mapped onto the SAT1 capsid and the electrostatics surface potential was calculated using APBS module of PyMol (DeLano Scientific LLC).

Fig. 3.6

GRID (Goodford, 1985) was used to find the energetically favourable binding site for HSPG on the SAT1 modelled mutant capsid (A). The GRID calculation was performed for a 20Å radius around the five-fold axis using pyramidal sulfur as probe. The calculations identified the most likely site of interaction in the vicinity of VP1 residue 112 with molecular interaction energy of -8.2 kCal/mole. The interaction energy increased to -10 kCal/mole when the grid was centred at residue 112. (B) Five linked heparin disaccharides were docked using the default parameters of GOLD onto the SAT1 modelled mutant pentamer structures. A 30Å³ region from VP1 residue 112 was defined for docking and the GOLD fitness score function was used to rank the docking poses. The best docking pose is shown. (GOLD score = 127). The equivalent process for the virus produced a less satisfactory docking (GOLD score = 102, docking not shown).

Fig. 4.1

Neutralizing antibody response of Nguni cattle following vaccination detected using VNT assays. Cattle were vaccinated with 12ug of either SAT2/ZIM/14/90 or vSAT2^{ZIM14}-SAT2 BEI-inactivated, SDG-purified antigens mixed with Montanide ISA206 adjuvant. The mean of the Log₁₀ virus neutralizing titres are shown and the error bars represent the standard deviation.

Fig. 4.2 **Clinical picture observed in cattle following challenge with live homologous virus SAT2/ZIM/14/90.** The mean rectal temperature (°C) for cattle vaccinated with the chimeric vaccine (▲), parental vaccine (■) and the controls given a placebo (•) is shown by a line graph on the left axis. The average clinical scores for the cattle vaccinated with the chimeric vaccine (■), parental vaccine (■) and the controls given a placebo (■) are shown by the bar graph on the right axis. The clinical score for each animal was calculated as the sum of the score of the clinical lesions on all four hooves and in the mouth. 104

Supplementary Fig. A **An alignment of 202 amino acids deduced for the Leader protease for 79 African FMDV isolates.** The dots (.) indicate same as top sequence (SAT1/KNP/196/96) and a dash (-) depicts a gap generated during alignment of a particular sequence. Illustrated at the top of the alignment are the critical residues involved in catalysis of L-VP4: C52, H149 and D165 (Guarné et al., 1998), indicated by the downward arrow (↓). Three other residues K200, E94 and E97 associated with catalysis of L-VP4 (Guarné et al., 2000; Guarné et al., 1998) are indicated by the star (★). The position of three deletions (amino acid positions 25-27) in the alignments for the SAT virus isolates is indicated below the alignment with a double underline (==). 170

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Supplementary Fig. D

An alignment of 153 amino acids deduced for the 3A peptide for a 79 African FMDV isolates. The dots (.) indicate identical amino acids to the reference sequence SAT1/KNP/196/96 and a dash (-) depicts a gap generated during the alignment. Illustrated among other things at the bottom of the alignment are the hydrophobic domains (residue positions 1-16: ISIPSQKSVLYFLIEK, 25-30: FYEGMV and 60-74: EIVALVVLLANIII) depicted using a dash-dot bar (.....). A double underline (====) at amino acid position 148 shows the deletion observed in southern SAT virus isolates that grouped cluster I. The eleven residue deletions observed in SAT2/SEN/7/83 are highlighted in the grey horizontal bar.

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Supplementary Fig. E

An alignment of 213 amino acids deduced for the 3C protease for 79 African FMDV isolates. The dots (.) indicate identity with the sequence (SAT1/KNP/196/96). At the bottom of the alignment are horizontal dotted bars (.....) indicate highly conserved areas in the sequence alignment (residue positions 17-48, 68-90, 129-156, 161-164). The horizontal dash-dot bars (.....) depict a hydrophobic domain observed at residue positions 27-44. At the top of the alignment, the residue positions of the active triad (H46, D84 and C163) (Birtley *et al.*, 2005), are shown using the downwards arrow (↓). The residues (154-160, 181-186) that comprise substrate pocket (S1) (Birtley *et al.*, 2005), are indicated by the wavy underline (~~~~~). The three conserved residues Y154, T158 and H181 that donate hydrogen bonds to the P1 substrate (Birtley *et al.*, 2005)(Birtley *et al.*, 2005), are indicated using the kites (◆). Underlined (====) at the top of

the sequence alignment is the conserved motif 95-RVRDI-99 necessary for VPg uridylation (Nayak *et al.*, 2006)(Nayak *et al.*, 2006). Highlighted with grey vertical bars are residues R92 R97 and K101 that contribute towards the uridylation process (Nayak *et al.*, 2006)(Nayak *et al.*, 2006). The following substitutions were observed in the southern SAT virus isolates R92→ S/T, R97→S, I99→L and K101→ G/A. The residue substitutions of 97R→S and 98D→V in this conserved motif for SAT1/NIG/5/81 are underlined.

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**Supplementary
Fig. F**

An alignment of 470 amino acids deduced for the 3D RNA Dependent RNA Polymerase for 79 African FMDV isolates. The dots (.) indicate identical amino acids as the reference sequence SAT1/KNP/196/96. Underlined at the top of the alignment are five highly conserved motifs among the RNA polymerases (Doherty *et al.*, 1999; Ferrer-Orta *et al.*, 2004). KDEIR (positions 164-168), DYSAFD (positions 240-245), PSG (positions 297-299), YGDD (positions 336-339) and FKLK (positions 385-388). The KDEVR motif observed in A/ETH/2/79 and SAT2/KEN/11/60 is highlighted in horizontal grey bars. The block horizontal bar (_____) at the bottom of the alignment indicates a hypervariable region in the sequence alignment between residues 143-154.

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**Supplementary
Fig. I**

A mid-point rooted neighbour joining (NJ) tree showing nucleotide relationships between the sub-Saharan African FMDV (SAT1, SAT2, SAT3, A and O serotypes) for the P3-coding region. The Kimura 2-parameter model in MEGA 5 (Tamura *et al.*, 2011), was used to draw the NJ tree and the scale bar indicates nucleotide substitutions per site. Confidence levels of the tree branches were tested using 1000 bootstrap replications.

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**Supplementary
Fig. II**

A mid-point rooted phylogenetic tree constructed using Minimum Evolution (ME) methods describing nucleotide relationships between the sub-Saharan African FMDV (SAT1, SAT2, SAT3, A and O serotypes) for the P3-coding region. The rate variation among sites was modelled with a

gamma distribution of 0.76.. The robustness of the tree branches was tested using 1000 bootstrap replications. The scale indicates the evolutionary distances used to infer the phylogenetic tree The tree was searched using the Close-Neighbour-Interchange (CNI) algorithm at a search level of 1. Evolutionary analysis was conducted using MEGA 5 (Tamura et al, 2011).

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**Supplementary
Fig. III**

Evolutionary relationships inferred using the Maximum Parsimony methods in MEGA 5, for the sub-Saharan African FMDV (SAT1, SAT2, SAT3, A and O serotypes) for the P3-coding region. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) The tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates).

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**Supplementary
Fig. IV**

Neighbour joining (NJ) tree depicting nucleotide relationships between the sub-Saharan African FMDV (SAT1, SAT2, SAT3, A and O) for the Leader-coding region rooted against the mid-point. The NJ tree was constructed using Kimura 2-parameter model MEGA 5 (Tamura et al., 2011), the scale bar indicates nucleotide substitutions per site. The robustness of the tree topology was assessed using 1000 bootstrap replications.

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**Supplementary
Fig. V**

A mid-point rooted phylogenetic tree constructed using Minimum Evolution (ME) methods describing nucleotide relationships between the sub-Saharan African FMDV (SAT1, SAT2, SAT3, A and O serotypes) for the Leader coding region. The rate variation among sites was modelled with a gamma distribution of 0.795. The robustness of the tree branches was tested using 1000 bootstrap replications. The scale indicates the evolutionary distances used to infer the phylogenetic tree. The tree was searched using the Close-Neighbour-Interchange (CNI) algorithm at a search level of 1. Evolutionary analysis was conducted using MEGA version 5 (Tamura et al, 2011).

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**Supplementary
Fig. VI**

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LIST OF ABBREIATIONS AND SYMBOLS

α	Alpha
β	Beta
$^{\circ}\text{C}$	Degrees Celsius
γ	Gamma
\leq	Greater than or equal to
\geq	Less than or equal to
μl	microliter
%	Percent
\$	United States Dollar
APC	Antigen Presenting Cell
Arg	Arginine
ATP	Adenosine Triple Phosphate
BHK	Baby Hamster Kidney
BD_{50}	50% bovine infectious doses
CD	Cluster of Differentiation
cDNA	copy Deoxyribonucleic acid
CHO-K1	Chinese Hamster Ovary strain K1
CPE	Cytopathic effect
cre	<i>cis</i> -acting replication element
Ct	threshold cycle
DNA	Deoxyribonucleic acid
DRC	Democratic Republic of the Congo
d.p.v	Days Post Vaccination
EAC	East African Community
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FMD	Foot-and-mouth disease
FMDV	Foot-and-mouth disease virus
GAG	Glycosaminoglycan
HS	Heparan Sulphate
HSPG	Heparan Sulphate Proteoglycan
IBRS-2	Instituto Biologico Renal Suino-2
IFN	Interferon
IGAD	Intergovernmental Authority on Development
IL	Interleukin
IRES	Internal ribosomal entry site
Ile	Isoleusine
kb	Kilobase-pairs
Leu	Leusine
LPBE	Liquid phase blocking ELISA
Lys	Lysine
mAbs	monoclonal antibodies
mar	monoclonal antibody resistant virus mutants
MDBK	Madin-Darby Bovine Kidney

ME	Minimum Evolution
Met	Methionine
MHC	major histocompatibility complex
ML	Maximum-Likelihood
MP	Maximum Parsimony
NJ	Neighbour-Joining
NK	Natural Killer
Nt	Nucleotide
OIE	World Organisation for Animal Health (Office International des Épizooties)
OPF	oropharyngeal fluid
ORF	open reading frame
p.c.	Post Challenge
PCR	Polymerase Chain Reaction
PD ₅₀	50% protective dose
PK	pseudo knot
pol	Polymerase
PGP	Protection against Generalized Foot Infection
pro	Protease
RDRP	RNA-dependent RNA polymerase
RGD	arginine-glycine-aspartic acid
RNA	Ribosomal nucleic acid
mRNA	Messenger Ribosomal nucleic acid
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SADC	South African Developmental Community
SAT	South African Territories
Tc-	T-cytotoxic
Th-	T-helper
TNF	Tissue necrotic factor
UTR	Untranslated Region
VN	Virus neutralization
VNT	Virus neutralization test
VLP	Virus-like particle
VP	Virus protein
WRL	World Reference Laboratory

CHAPTER ONE

LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

Foot-and-mouth disease (FMD), a contagious viral disease of cloven hoofed animals, is characterised by vesicles in the mouth and on the hoof. The disease is economically devastating and is among the OIE (World Organisation for Animal Health) listed diseases. The major impact of FMD is not only due to the direct losses in production that is significant among the intensively raised, high-yielding livestock, but also results from trade restrictions on animals and their products due to the contagious nature of the disease (James & Rushton, 2002). The impact of FMD on trade, especially in developed countries where it has been eradicated, is well documented. Over US\$7 billion was spent using a stamping out policy to control an outbreak in the UK in 2001, while in Taiwan 4 million pigs either died or were slaughtered in addition to \$1.6 billion spent on the eradication of the disease (Yang *et al.*, 1999; Leforban & Gerbier, 2002; Sakamoto & Yoshida, 2002). In contrast, the socio-economic losses at farm level in developing countries where the disease is endemic still have to be determined. These include decrease in milk production, mastitis, lower weight gain, infertility, death in young stock and loss of draught animal power for cultivation or transport resulting from lameness (Perry *et al.*, 2002; Perry & Rich, 2007; Forman *et al.*, 2009).

The risk of incursion of FMD from endemic regions into countries free of the disease has increased significantly with globalization, greater mobility of people, opening up of free trade areas, and the expansion of trade. The consequence of such sporadic outbreaks would be immense, considering the occurrence of large susceptible animal populations and intensification of farming practices (Donaldson & Doel, 1992; Sutmoller & Olascoaga, 2002). The devastating FMD pandemic (1999–2001) caused by the pan-Asian type O virus strain, that originated in India, spread rapidly and extensively to include eight countries previously free of the disease (five in western Europe and three in eastern Asia). This pandemic spread was an example of the virulent and contagious nature of the FMD virus (FMDV) (Leforban & Gerbier, 2002; Sakamoto & Yoshida, 2002; Knowles *et al.*, 2005). The virus also occurred in South Africa in 2000 (Sangare *et al.*, 2001). Hence in order to safeguard world animal agriculture and trade, control of FMD, where it is endemic, focuses on the reduction of the foci of infection (Carrillo *et al.*, 1984). Whereas in areas where FMD is eradicated, it is crucial that vigilance and surveillance be maintained to prevent the reoccurrences and to decrease the perceived biorisk threat. Contingency

preparations in case of outbreaks are also essential (Sutmoller *et al.*, 2003; Grubman & Baxt, 2004).

During the past century there has been considerable research on virus structure, biology and vaccines for the FMDV. However, its control is still elusive, and the virus remains a threat to the global livestock and supporting industries (Sobrino *et al.*, 2001; Baxt & Rieder, 2004). Molecular characterisation of the FMDV has complemented epidemiological surveillance by providing for sensitive, rapid identification and characterisation of the virus and establishing the origin of the FMD outbreak and links between outbreaks (Beck & Strohmaier, 1987; Kitching, 1992; Knowles & Samuel, 2003). Whereas the phylogeny of the FMDV is well studied for the viruses in South America and those eradicated in Europe, the converse is true for much of Africa where a limited number of viruses have been studied restraining the implementation of successful control measures (Bastos *et al.*, 2001; Sangare *et al.*, 2001; Bastos *et al.*, 2003a; Bastos *et al.*, 2003b; Sangare *et al.*, 2003; Sahle *et al.*, 2007a; Sahle *et al.*, 2007b; Ayelet *et al.*, 2009; Balinda *et al.*, 2009; Balinda *et al.*, 2010a; Balinda *et al.*, 2010b; Sahle *et al.*, 2010; Sangula *et al.*, 2010a; Sangula *et al.*, 2010b; Maree *et al.*, 2011b; Chitray *et al.*, 2013; Wekesa *et al.*, 2013; Kasanga *et al.*, 2014; Wekesa *et al.*, 2014). Complete characterisation of the FMDV genome offers a holistic understanding of the FMDV structure, evolution and population complexities (Domingo *et al.*, 2002). Again, although many complete genome FMDV sequences are available for viruses in Europe and South America, limited numbers of such sequences have been described for viruses prevalent in Africa (Mason *et al.*, 2003b; Carrillo *et al.*, 2005). Complete characterisation of the African strains of FMDV, including the less studied non-structural proteins involved in virus replication, is important, as it improves our understanding of the replication and pathogenesis of the virus.

Vaccination plays a major role in the control of FMD in addition to quarantine restrictions. While biotechnology is still exploring a safe vaccine to replace the conventional inactivated vaccines, this platform can be used to improve the current cell culture derived vaccines (Kitching, 1992; Parida, 2009; Rodriguez & Gay, 2011). The structure and functional relationships of regions of the virus genome involved in receptor recognition, stability, replication and immunogenicity have been studied alongside the virus biology *in vitro* (Rieder *et al.*, 1994; Mateu, 1995; Wang *et al.*, 2002; Jackson *et al.*, 2003; Grazioli *et al.*, 2006; Maree *et al.*, 2010; Blignaut *et al.*, 2011; Maree

et al., 2011a; Opperman *et al.*, 2012; Maree *et al.*, 2013). Thus predictions for a better design of vaccines can be tested using recombinant biotechnology and genetic engineering techniques. It is hoped that the resultant chimeras will be more immunogenic, adapt faster in cell culture, be more thermostable, availing us with promising options for the control FMD (Parida, 2009; Rodriguez & Gay, 2011). Many biotechnology approaches to improve conventional vaccines have been studied using viruses prevalent in Europe and South America, with yet again, a few viruses from Africa involved (Zibert *et al.*, 1990; Rieder *et al.*, 1994; Sa-Carvalho *et al.*, 1997; van Rensburg *et al.*, 2004; Fowler *et al.*, 2008; Mateo *et al.*, 2008; Maree *et al.*, 2010; Fowler *et al.*, 2011; Maree *et al.*, 2011a; Fowler *et al.*, 2012; Maree *et al.*, 2013). Molecular studies complemented with the biology of FMDV strains prevalent in sub-Saharan Africa, where the disease is endemic, are pertinent if biotechnology is to be harnessed for improved diagnostics and vaccines tailored for control of FMD on the continent.

1.2 EPIDEMIOLOGY OF FMD: A GLOBAL PERSPECTIVE

1.2.1 Global distribution of FMD

FMD was widespread until the late 19th Century, when it was eradicated from the industrialised countries, namely Australia (1872), North and Central America (1929-1954) and more recently by regular vaccination in western Europe (1990) (Leforban & Gerbier, 2002). Eastern Europe and parts of South-east Asia like Japan and Korea suffer sporadic outbreaks of the disease (Leforban & Gerbier, 2002; Park *et al.*, 2013; OIE/FAO Reference Laboratory Reports). In the southern cone of the South American continent, large areas of disease free zones are maintained with or without annual vaccination (Melo *et al.*, 2002). FMD is still endemic in many of the poorer and developing countries of Africa, the Middle East, Asian sub-continent and in the Andean region in northern and central South America (Fig. 1.1), where the disease aggravates the existing farming socio-economic problems.

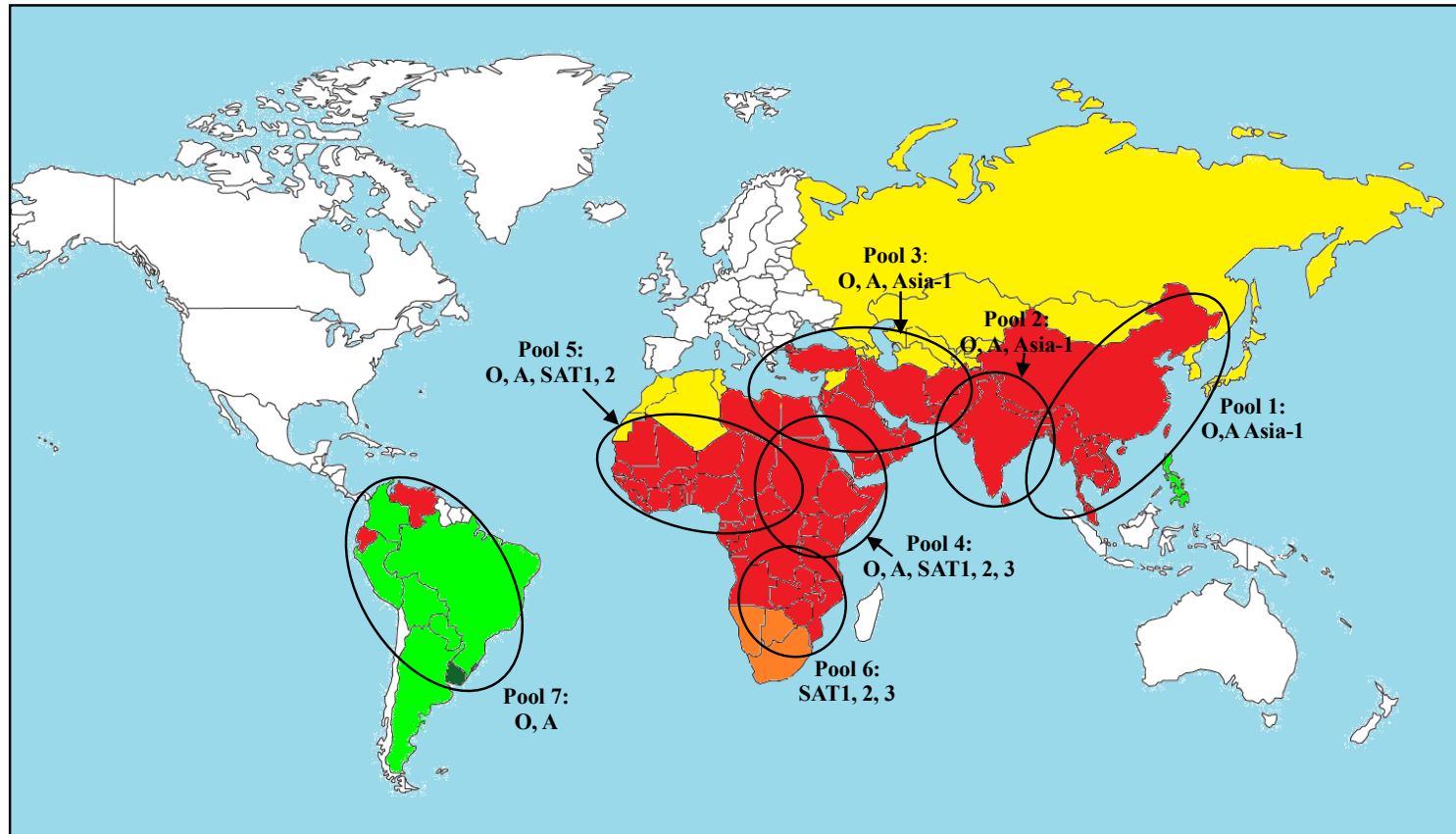


Fig. 1.1 The conjectured global status of FMD in 2011-2012.

Source: [http://www.wrlfmd.org/ref_labs/ref_lab_reports/OIE-FAO FMD Ref Lab Network Report 2011.pdf](http://www.wrlfmd.org/ref_labs/ref_lab_reports/OIE-FAO_FMD_Ref_Lab_Network_Report_2011.pdf); [FAO/EuFMD monthly Report September \(2012\)](#). Outline of world map obtained from <http://www.freeusandworldmaps.com>

1.2.2 Serotypes of FMDV, their distribution and epidemiological patterns

Although FMD is generally referred to as a single disease, the causative agent, FMDV exists as seven distinct serotypes that are distributed globally, *i.e.* A, O, C, Asia-1 and the South African Territories (SAT) types 1, 2 and 3. Although the viruses from different serotypes cause a clinically indistinguishable disease, they differ in their epidemiology and global distribution (Bachrach, 1968; Pereira, 1976; Sobrino *et al.*, 2001; Domingo *et al.*, 2002).

There is no cross-immunity between the different serotypes upon natural infection or vaccination (Pereira, 1981). The seven serotypes of FMD are distributed worldwide; the Asia-1 serotype is restricted to the Asian continent and the SAT serotypes limited to Africa. While serotypes O (the most prevalent serotype in the world) and A, are more widely distributed, occurring in Europe (historically), South America, Asia and Africa. Serotype C was similarly distributed but has most likely disappeared as the last outbreaks have been reported in the Philippines (1991), Brazil (1993), and Kenya (2004) (Melo *et al.*, 2002; Knowles & Samuel, 2003; Rweyemamu *et al.*, 2008; Di Nardo *et al.*, 2011). It has been suggested that improperly inactivated vaccines were the source of re-introduction of the virus into Kenya and that discontinuation of type C vaccines would contribute to its eradication (Sangula *et al.*, 2011). Considering the above and that there are no natural reservoirs known for type C viruses, the existence of circulating type C viruses in the field has been questioned (Paton *et al.*, 2009).

Globally and across multiple serotypes, there are distinct genetic and antigenic strains of FMDV circulating and evolving in defined geographical regions that are hence grouped into seven regional pools. Pools 1 and 2 occur in Asia, while pool 3 occurs across Asia, the Middle East and North Africa. Virus Pool 7 is found in South America while Africa contains 3 different pools roughly spread across East, West and southern Africa respectively (Fig 1.1) (OIE/FAO FMD Reference Laboratory Network Annual Report 2011).

In Africa serotypes A and O are found predominantly north of the equator and do not usually occur in southern Africa (Fig 1.2 a-b) except for sporadic outbreaks in northern Zambia as a result of incursions from southern Tanzania and historically from other continents (Vosloo *et al.*, 2002; OIE/FAO FMD Reference Laboratory Network Report 2011).

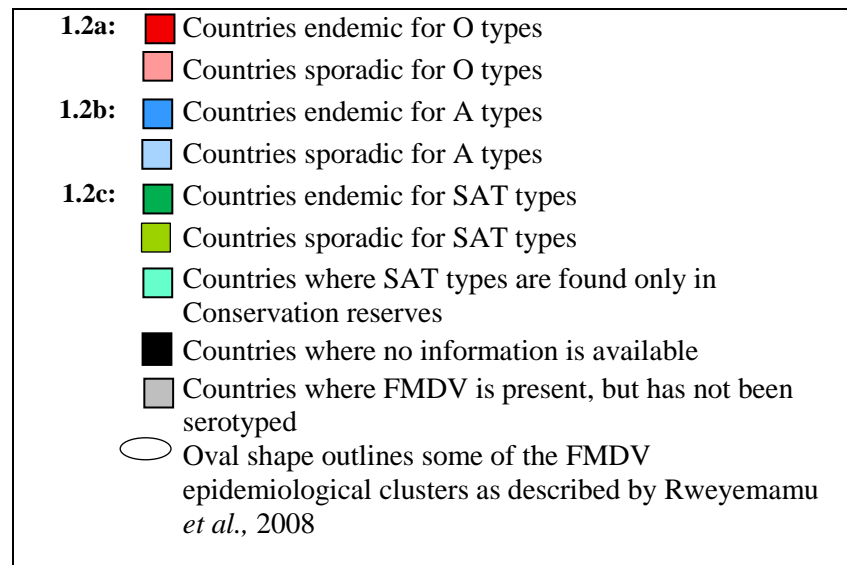
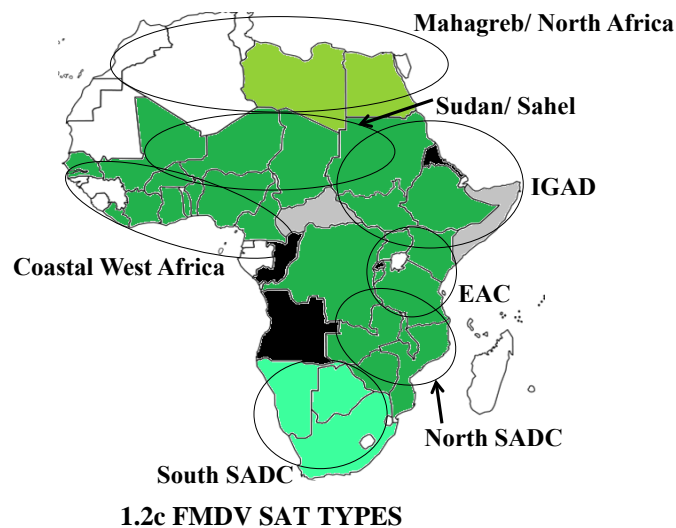
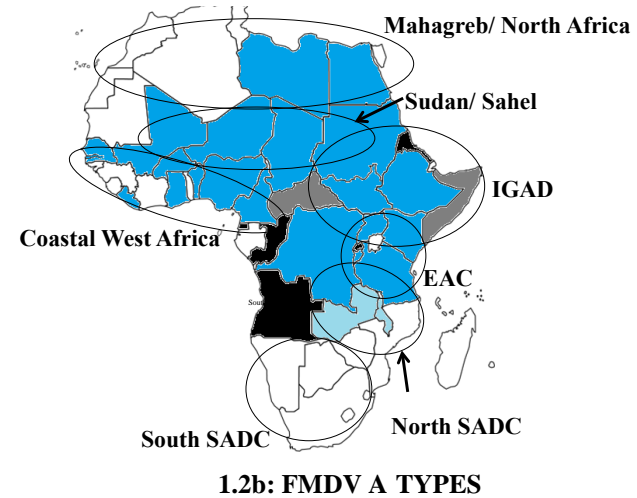
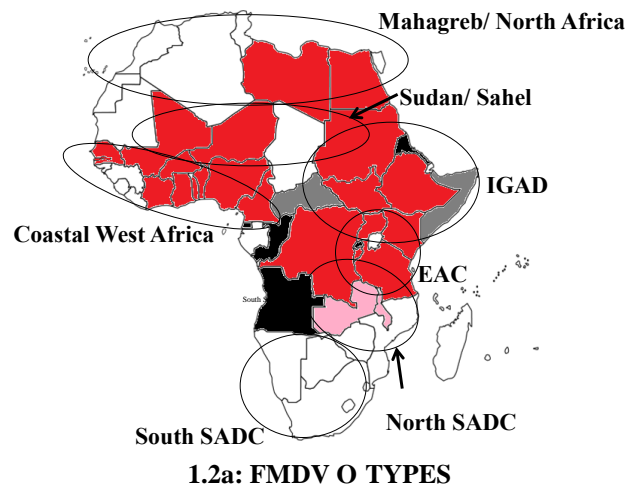


Fig. 1.2 Map showing conjectured the serotype distribution of FMD in Africa 2010-2012.

(Sources: OIE/FAO FMD Reference Laboratory Network Annual Report 2010-2011; FAO/EuFMD Monthly Report September 2012). Source of outline of map of Africa: www.theodora.com/maps

On the other hand the SAT types 1 and 2 are confined to most of sub-Saharan Africa (Fig 1.2c), with SAT2 being responsible for the majority of the FMD outbreaks in southern regions of the continent (Thomson & Bastos, 2004). However, sporadic SAT2 outbreaks occurred in the North African countries of Libya (2003, 2012) and Egypt (2012), and there have historically been incursions of SAT 2 into the Middle East in 1990 and 2000 (Samuel & Knowles, 2001; Vosloo *et al.*, 2002; Knowles & Samuel, 2003; Valarcher *et al.*, 2004; Ahmed *et al.*, 2012). The SAT3 serotype is the least prevalent of all the FMDV serotypes and is maintained in the African buffalo (*Syncerus caffer*) in southern Africa (South Africa, Namibia, Botswana, Zimbabwe Zambia) and Uganda (East Africa) (Bastos *et al.*, 2003a).

1.2.3 Topotype distribution of the various serotypes in Africa

The genetic and antigenic diversity within each of the seven serotypes results in different FMDV strains. These were previously identified antigenically by using serology and were designated as subtypes (reviewed in Kitching *et al.*, 1989). Currently, molecular characterisation of the VP1 coding-region of the virus capsid is used to determine genetic relationships (Beck & Strohmaier, 1987), which groups the FMDV strains into topotypes that refer to limited genetic variation within a defined geographical area. Topotypes generally refer to isolates that have <15% genetic variation for the A, O and C serotypes and < 20% for the SAT serotypes (Samuel & Knowles, 2001; Knowles & Samuel, 2003; Di Nardo *et al.*, 2011).

1.2.3.1 Topotypes for Serotype O

At least five of the eleven topotypes reported globally for serotype O are endemic to the African continent, *i.e.* East Africa (EA-1 to EA-4) and West Africa (WA). These topotypes have the following distribution: EA-3 is circulating most broadly, in three countries in the eastern region; Ethiopia, Sudan, Somalia and two countries in West Africa, Cameroon and Nigeria. Recent sporadic outbreaks of EA-3 have occurred towards the northern region, in Libya and Egypt (2011-2012) and towards the south in Zambia (2010). Topotypes EA-2 and EA-4 are similarly widely distributed in the following countries towards the east: EA-2 is found in four countries, Democratic Republic of the Congo (DRC), Kenya, Uganda and Tanzania, while EA-4 circulates in three countries, Uganda, Ethiopia and Kenya. In contrast, EA-1 has only been reported in Kenya. The WA topotype circulates exclusively in West Africa. Recently there has been sporadic outbreaks of an exotic PanAsia-2 strain belonging to the ME-SE (Middle East-South East Asia)

topotype circulating in in Libya and Egypt (2011-2012), northern Africa (Fig 1.2a) (Knowles & Samuel, 2003; Ayelet *et al.*, 2009; Wekesa *et al.*, 2013; Kasanga *et al.*, 2014; Ludi *et al.*, 2014; FAO/EuFMD Monthly Report September 2012; OIE/FAO Reference Laboratory Reports).

1.2.3.2 Topotypes for Serotype A

Serotype A, although considered to be antigenically the most diverse, has only three topotypes circulating globally and one of them is endemic in sub-Saharan Africa. Within this topotype named Africa, some of the genotypes circulating are as follows: in the eastern part of Africa, Genotype 1 (G-1) both historical and recent viruses have been reported in three countries, DRC, Kenya and Tanzania. Though in the past, Burundi, Ethiopia, Somalia and Uganda have harboured genotype G-I. The genotype G-II is exclusive to Ethiopia while the genotype G-VII is found to the north-east of the continent in three countries, Kenya, Ethiopia and Egypt. Genotype G-IV is most widespread and found spanning the eastern, Central and West Africa regions, in the countries of Cameroon, Eritrea, Mali, Nigeria and Sudan. The G-VI genotype circulates in West Africa in the countries of Cameroon, Cote d' Ivoire, Gambia, Ghana, Mali, and Nigeria. Recently there have been outbreaks of the exotic genotype A/Iran-05 belonging to the Asia topotype in Libya and Egypt (2012) (Fig 1.2b); (Habiela *et al.*, 2010; Di Nardo *et al.*, 2011; Kasanga *et al.*, 2014; Wekesa *et al.*, 2014; OIE/FAO Reference Laboratory Reports).

1.2.3.3 Topotypes for the SAT serotypes

The SAT serotypes are more genetically diverse compared to the A and O serotypes (Bastos *et al.*, 2001; Bastos *et al.*, 2003b). The SAT1 and 2 types have eight and fourteen topotypes documented respectively, while SAT3 a total of six topotypes have been reported (Bastos *et al.*, 2001; Bastos *et al.*, 2003a; Bastos *et al.*, 2003b; Knowles *et al.*, 2010a; Hall *et al.*, 2013). However, with limited sample collection and surveillance in Africa, the geographical distribution described here may not reflect the current endemic status of the SAT topotypes and some may be extinct. Nonetheless the viruses listed hereafter have been circulating on the continent; In southern Africa (Angola, Botswana, Mozambique, Namibia, South Africa, Zambia, Zimbabwe): SAT1 topotypes 1, 2, 3; SAT2 topotypes I, II, III IV and XI; SAT3 topotypes I, II, III and IV. In eastern Africa (DRC, Ethiopia, Eritrea, Kenya, Rwanda, Sudan, Tanzania, Uganda): SAT1, topotypes 3, 4, 5 and 7; SAT2 topotypes IV, VII, (currently causing outbreaks in Egypt and Libya), VIII, IX, X XII, XIII and XIV; SAT3 topotype VI have been documented. Lastly in

western Africa (Cameroon, Gambia, Ghana, Niger, Nigeria, Senegal, Togo) viruses identified include: SAT1 topotypes 7 and 8; SAT2 topotypes V, VI and VII (Fig 1.1d); (Bastos *et al.*, 2001; Bastos *et al.*, 2003a; Bastos *et al.*, 2003b; Di Nardo *et al.*, 2011; Maree *et al.*, 2011b; Ahmed *et al.*, 2012; Kandeil *et al.*, 2012; Hall *et al.*, 2013; Kasanga *et al.*, 2014; OIE/FAO Reference Laboratory Reports; FAO/EuFMD Monthly Report September 2012).

1.2.4 Epidemiological patterns for FMDV in Africa

The four regional virus pools 3, 4, 5 and 6 prevalent in Africa described by FAO/ OIE (discussed previously in section 1.2.2) can be further subdivided into eight epidemiological clusters based on prevalence, topotype distribution, farming systems, animal movements and the impact of wildlife as suggested by Rweyemamu *et al.*, (2008). Namely, the Mahagreb/North Africa cluster, Sudan/Sahel cluster, West Africa/Costal Belt cluster, Intergovernmental Authority on Development (IGAD)/ Horn of Africa cluster, Great Lakes/ East African Community (EAC), Angola cluster and lastly the northern and southern clusters in the South African Developmental Community (SADC) (Table 1.1).

Table 1.1 Epidemiological clusters for sub-Saharan FMDV, geographical distributions and characteristics (adapted from Rweyemamu *et al.*, 2008; Di Nardo *et al.*, 2011).

Epidemiological Cluster		Serotypes	Countries involved	Characteristics
Eastern Africa	The Great Lakes/East African Community (EAC)	O, A, C, SAT1, 2 and 3	Burundi, Kenya, Rwanda, Tanzania, Uganda and eastern part of DRC	<ul style="list-style-type: none"> - Has primary foci of FMDV - Pastoral farming systems with communal grazing and sometimes migration and cross border trade of livestock. - Wildlife like the African buffalo which is a vector of the FMDV, is frequently found at the interface between the pastoralist and conservation areas.
	(IGAD) – Intergovernmental Authority on Development/ Horn of Africa	O, A, C, SAT1 and 2	Djibouti, Eritrea, Ethiopia, Sudan, Somalia and the northern parts of Kenya and Uganda	<ul style="list-style-type: none"> - Has primary foci of infection - It has the highest population of cattle in Africa - Region is involved cross border trade both within Africa and the Middle East.
West Africa	Sudan/Sahel cluster	O, A, SAT1 and 2	Burkina Faso, Chad, Mali, Mauritania, Niger, eastern Senegal, northern Nigeria and western Sudan.	<ul style="list-style-type: none"> - It is a primary endemic focus. - Pastoralism exists with long distance movement of animals due to transhumance or trade.
	Coastal belt	O, A, C, SAT1 and 2	Benin, Cameroon, Cote d' Ivoire, Gabon, Gambia, Ghana, Guinea, Sierra Leon Togo, southern Nigeria, western Senegal, southern Mali and southern Burkina Faso	<ul style="list-style-type: none"> - It is a secondary epidemic focus that gets infection from the Sudan/Sahel cluster.
Southern Africa	Southern SADC cluster	SAT1, 2 and 3	Botswana, Namibia, South Africa,	<ul style="list-style-type: none"> - Virus is only maintained in the wildlife conservational areas. - Game proof fences segregating the Cape/African buffalo (primary foci of infection) from livestock.
	Northern SADC cluster	SAT1, 2 and 3	Malawi, Mozambique, Zambia and Zimbabwe,	<ul style="list-style-type: none"> - Contact with the Cape/African buffalo is the primary source of infection - Prone to secondary epidemics spread from other countries within the group or from southern Tanzania.
	Angola		Angola and western DRC	<ul style="list-style-type: none"> - Not much information available - Possibly, epidemics spread from this cluster to northern Namibia and S. west Zambia
North Africa	North Africa/ Maghreb Cluster	O, A (SAT2 current)	Egypt, Libya	<ul style="list-style-type: none"> - Sporadic cycles of FMD outbreaks occur as a result of incursions from sub-Saharan Africa or the Middle East

In sub-Saharan Africa some sociological factors that predispose spread of FMD include communal grazing, movement of animals in search of pasture, trade, or as part of transhumance (Rweyemamu *et al.*, 2008; Di Nardo *et al.*, 2011; Allepuz *et al.*, 2013; Hall *et al.*, 2013; Hamoonga *et al.*, 2014). As a result there have been incursions of virus into North Africa from the IGAD cluster e.g. Egypt (SAT2, 2012, Type A, 2009, 2006, Type O, 2011-2012), the Middle East in Yemen, (Type O, 1990, 2004, 2006, 2009) and Saudi Arabia (SAT2, 2000) (Knowles & Samuel, 2003; Di Nardo *et al.*, 2011; Valdazo-González *et al.*, 2012). Incursions of type O viruses from the Sudan/ Sahel cluster have also historically occurred into North Africa (1990) in Algeria, Morocco and Tunisia (Aidaros, 2002; Di Nardo *et al.*, 2011). Wildlife like the African buffalo in southern and East Africa is a vector of the FMDV and is frequently found at the interface between the pastoralist and conservation areas (see section 1.6.3 and 1.6.4). However, the role of the African buffalo in maintenance and transmission of the virus to domestic animals has not been studied in detail in the EAC cluster as is the case for the southern SADC cluster (Vosloo *et al.*, 2002; Thomson *et al.*, 2003; Ayebazibwe *et al.*, 2010).

1.2.5 Implications of the genetic and topotype diversity in the FMDV on control using vaccination

The genetic diversity of FMDV, which is the basis for the numerous topotypes prevalent (discussed in section 1.2.3) Genetic variation can sometimes result in antigenic differences between strains of the virus (see section 1.4.4) This has a negative implication on the control of FMD by vaccination. More than one virus topotype of the same serotype may exist within one country and an intraserotype vaccine used in one geographical region may not be effective in another region (Hunter, 1998; Haydon *et al.*, 2001a; Vosloo *et al.*, 2002).

1.2.6 Global perspective on the control of FMD

To safeguard international trade in animals and their products, control and possible eradication of FMD is ideal. This will allow the less developed world to harness their livestock, which is an asset in arid and semi-arid areas, for international trade (Vosloo *et al.*, 2002; Sutmoller *et al.*, 2003; Thomson & Bastos, 2004). Given the contagious nature of FMD it is unlikely that individual effort at farm level or an isolated national intervention would be successful. This is because the disease will be re-introduced from pockets of infection in neighbouring farms, countries or wildlife, therefore a global approach to control FMD is most optimal (Sutmoller *et*

al., 2003; Parida, 2009; Sumption *et al.*, 2012). The OIE and Food and Agriculture Organisation (FAO) have jointly developed the Progressive Control Pathway (PCP) that sets goals for countries that have not yet managed to control the disease to finally attain freedom from FMD. In addition, there are three examples of successful regional co-operation; the Hemispheric Plan for the Eradication of Foot-and-Mouth Disease (PHEFA) together with the Pan American Foot-and-Mouth Disease Centre (PANAFTOSA) in South America; The European Commission for the Control of Foot-and-Mouth Disease (EU-FMD) and the South-east Asia Foot-and-Mouth Disease (SEA-FMD) campaign (Sutmoller *et al.*, 2003; Forman *et al.*, 2009). Similar collaboration's along epidemiological patterns of FMD distribution in sub-Saharan Africa for regional control of FMD are yet to be operational in this region (Rweyemamu *et al.*, 2008). Zoo-sanitary zones created alongside the ecosystems e.g. in South America rather than international boundaries are suggested to give better results, especially when balanced with areas of existing wildlife and traditional pastoralism ecosystems where the virus is maintained (Melo *et al.*, 2002; Rweyemamu & Astudillo, 2002; Vosloo *et al.*, 2002; Rweyemamu *et al.*, 2008). Within each country both public and private input is required to facilitate the manufacture of vaccine, willingness of farmers to reports cases, rapid serotype and strain identification, veterinarians to direct and deliver vaccination, surveillance services, and lastly, zoo-sanitary measures that control movement of animals (Sutmoller *et al.*, 2003). In sub-Saharan Africa much of the above infrastructure for prevention and control of FMD is absent and is aggravated by administratively weak veterinary services (Vosloo *et al.*, 2002; Kandeil *et al.*, 2012; Kasanga *et al.*, 2012; Namatovu *et al.*, 2013). Thus the capacity of much of the continent to implement rapid and accurate control measures for FMD is limited.

1.3 CLASSIFICATION AND PHYSICAL PROPERTIES OF FMDV

1.3.1 Classification

FMDV belongs to the Family *Picornaviridae*, derived from the prefix 'pico' which means small, plus 'RNA'. These are non-enveloped single-stranded, positive-sense RNA viruses with an icosahedral shape, which are important pathogens in animals and human and are classified into twelve genera. The FMDV belongs to the genus *Aphthovirus*, derived from the Greek word 'aphthae' which means vesicles in the mouth, a characteristic symptom of FMD. The genus *Aphthovirus* has two type species, Equine rhinitis A virus and FMDV (Knowles *et al.*, 2012).

1.3.2 Physical properties

The physical features of the FMDV are mostly as a result of the structure of the virus capsid (discussed in 1.4.3). Elucidation of the crystal structure of FMDV 20 years ago (Acharya *et al.*, 1989) enabled assessment of the effects of acid and heat on the virus capsid structure and identification of interactions that may correlate with enhanced acid or heat stability (Curry *et al.*, 1995; Ellard *et al.*, 1999; Mateo *et al.*, 2008; Martín-Acebes *et al.*, 2010; Maree *et al.*, 2013; Porta *et al.*, 2013). Briefly, the FMD virion is 30 nm in diameter with a relatively smooth capsid and a sedimentation coefficient of 146 while empty capsids that have lost its RNA, sediment at 75S. The virion has a buoyancy density of 1.43-1.45g/cm³ (Knowles *et al.*, 2012). In contrast to the enteroviruses, like poliovirus, the FMD virion is unstable at a pH of less than 6.8, where the capsid disintegrates, releasing the RNA (Brown & Cartwright, 1963; Bachrach, 1968; Burroughs *et al.*, 1971; Carrillo *et al.*, 1984; Curry *et al.*, 1995; Curry *et al.*, 1997). Similarly, the virus is labile at pH>10.0 (Bachrach *et al.*, 1957). Therefore common disinfectants applied during outbreaks of FMD include 4% citric acid, 2% Sodium hydroxide, and 2% Sodium bicarbonate (Thomson & Bastos, 2004; OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 2013; OIE Terrestrial Animal Health Code, 2012). Temperatures higher of 56°C-61°C for approximately 30 minutes inactivate the virus (Bachrach *et al.*, 1957; Doel & Baccharini, 1981; Doel & Chong, 1982). Mild heating of FMDV virions leads to irreversible dissociation into pentamers, a characteristic associated with poor vaccine performance that highlights the importance of a cold chain in the preservation of FMD vaccines (Doel & Baccharini, 1981; Ellard *et al.*, 1999; Mateo *et al.*, 2003).

In contrast, the virus can survive in aerosols and micro-droplets in an environment with relative humidity levels above 55% and cool temperatures. The virus can also be protected from degradation in the environment by organic material of approximately neutral or slightly alkaline conditions in fomites like hay, cows hair, dry faeces, winter slurry, and may have implications on the possibility of spread of the virus (Donaldson, 1987; Alexandersen *et al.*, 2003).

1.4 VIRAL RNA GENOME ORGANISATION, FUNCTION AND STRUCTURE, CAPSID STRUCTURE AND ANTIGENIC PROPERTIES

1.4.1 Organisation, structure and function of the RNA genome

The FMDV genome is 8,450 nucleotides (nt) in length (Fig 1.3) and consists of one open reading frame (ORF) of about 6,996 nucleotides (nt) in length, translated into approximately 2,332 amino acids, bordered on either side by two untranslated regions (UTR), the 5' and the 3' UTR's (Forss *et al.*, 1984).

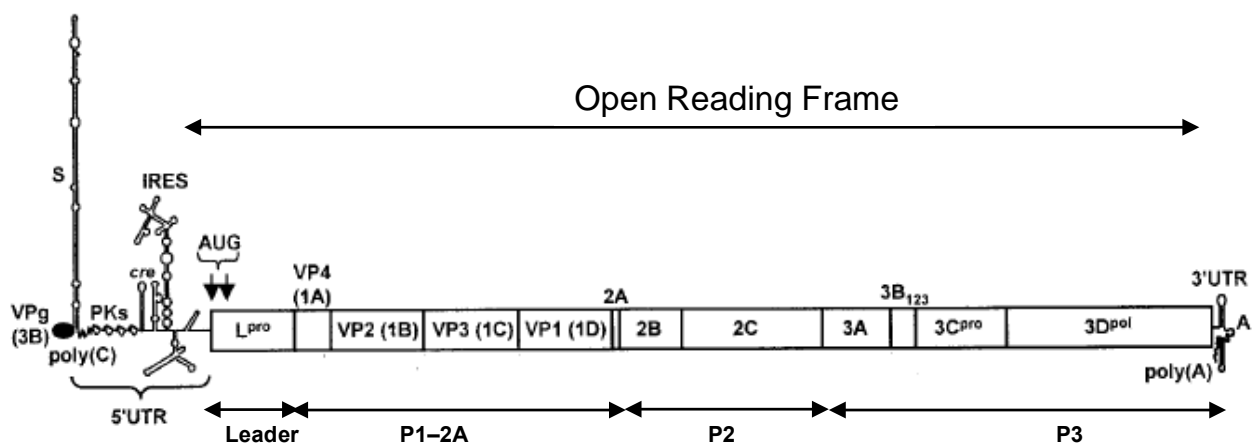


Fig.1.3 Schematic diagram of the FMDV genome showing the position of the genetic elements (adapted from Mason *et al.*, 2003a, with modifications). The 5' and 3' UTR's, Open Reading Frame (ORF) with the four distinct regions, Leader (L^{pro}), P1-2A, P2 and P3 are shown.

1.4.1.1 The 3' and 5' Untranslated Regions (UTR)

The 3' UTR is approximately 90 nts and folds into a stem-loop structure with a genetically encoded polyadenylated tail of varying length and is believed to harbour some *cis*-acting sequences required for initiation of replication (reviewed in Mason *et al.*, 2003a; Grubman & Baxt, 2004). The 5'UTR (Fig. 1.3), approximately 1,200 nt in length, commences as the small virus-encoded protein, VPg, (three non-identical copies, encoded by the 3B protein) covalently attached to the tyrosine residue at the 5' terminus ((Forss & Schaller, 1982); reviewed in (Mason *et al.*, 2003a; Belsham, 2005). The VPg participates in initiation of RNA synthesis as will be discussed shortly (section 1.5.2) and has a role in viral encapsidation (Nayak *et al.*, 2005; Nayak

et al., 2006). The remaining four functional elements include the S-fragment, capable of folding into a stem loop structure whose function is not well understood (Newton *et al.*, 1985; Clarke *et al.*, 1987). The poly-C tract (150 to 250 nt) follows, comprising C (cystine) residues and is associated with virulence (Harris & Brown, 1977; reviewed in Grubman & Baxt, 2004). Distal to the poly-C tract is a region of 720 nts containing inverted repeats which are predicted to form pseudo knots (PKs) whose function is also unknown (Clarke *et al.*, 1987). The PK region may contain large deletions, including a whole PK (Escarmís *et al.*, 1995). Following the PKs is a hairpin loop structure called the *cre* (*cis*-acting replication element), a prerequisite for RNA genome replication (Mason *et al.*, 2002). The *cre* is followed by the internal ribosomal entry site (IRES), the last component of the 5' UTR, and is just proximal to the Leader peptide coding region. The IRES allows for translation initiation in a cap-independent manner and it may play a crucial role in viral pathogenicity (Martinez-Salas *et al.*, 1996; reviewed in Mason *et al.*, 2003a).

1.4.2 The viral structural and non-structural proteins

The ORF encodes proteins that are required for virus genome replication and production of new virus particles and is translated into a polyprotein and cleaved co- and post-translationally by viral proteases into four primary products, Leader, P1-2A, P2 and P3 (Fig 1.3). It also encodes virus proteins that modify the functions of the mammalian cellular translation and transcription mechanisms, thus blocking both protein synthesis and mechanisms of immunity in the host (Vakharia *et al.*, 1987; Rueckert, 1996; reviewed in Belsham, 2005).

1.4.2.1 The leader region

The Leader region (L^{pro}) encodes the N-terminal component of the polyprotein (Robertson *et al.*, 1985; Sangar *et al.*, 1987) and is one of the major virulence factors for the FMDV. This region (Fig. 1.3) contains two functional in frame AUG codons, and is where initiation of translation begins, with the 2nd codon being preferred (Belsham & Brangwyn, 1990; Kühn *et al.*, 1990). The region in between these two functional AUG codons is critical for virulence (Piccone *et al.*, 2010). The L^{pro} (a protease) autolytically cleaves itself from the 5' end of the polyprotein (Strebel & Beck, 1986) following translation, yielding two products, the longer Lab and shorter Lb depending on the AUG codon selected (Beck *et al.*, 1983; Sangar *et al.*, 1987). The protease has an additional role of inhibiting mammalian protein synthesis by modifying the cleavage of the translation initiation factor eIF4G (Devaney *et al.*, 1988; Piccone *et al.*, 1995). The result is

impairment of mammalian mRNA translation including the inhibition of *IFN α/β* -mRNA synthesis, which is necessary for production of interferons' critical for the innate immune system (de Los Santos *et al.*, 2006; Grubman *et al.*, 2008).

1.4.2.2 The structural (capsid) proteins

The P1 region (Fig. 1.3) encodes for the structural or capsid proteins (Sobrinho *et al.*, 2001; Domingo *et al.*, 2002) and consists of the 1A/VP4, 1B/VP2, 1C/VP3, and 1D/VP1-coding regions. Cleavage at the N-terminus by the L^{pro} and at the C-terminus by the 2A peptide (Ryan *et al.*, 1991) releases the P1-2A precursor from the poly protein. The 2A peptide has no protease activity, but rather modifies the activity of the ribosome to promote hydrolysis of the peptidyl(2A)-tRNA^{Gly} ester linkage. This releases the P1-2A precursor in the translational complex in a manner that allows translation of the remainder of the polypeptide (P2-P3) to continue uninhibited (Donnelly *et al.*, 2001). The P1-2A is then processed by the 3C protease (3^{pro}) into VP0, VP3 and VP1. The final cleavage of VP0 to form VP4 and VP2 occurs at a later stage during encapsidation of the virus by an unknown autocatalytic mechanism (Belsham, 2005).

1.4.2.3 The non-structural proteins

The 2BC precursor of the P2 region is processed to 2B and 2C (Fig 1.3) by the 3C^{pro} (Vakharia *et al.*, 1987). The functions of the 2B and 2C proteins are not well understood (Sobrinho *et al.*, 2001), but they have a strong hydrophobic nature (Carrillo *et al.*, 2005) and are involved in many membrane bound activities at sites of FMDV replication in vesicles in the host cytoplasm (Grubman & Baxt, 2004). The 2C has been confirmed to have ATPase activity (Sweeney *et al.*, 2010). The 2B can be located in the endoplasmic reticulum (ER), the site of genome replication. The 2C can also be found to a lesser extent in the ER, however it is mostly located within the Golgi apparatus, in the membrane-associated virus replicating complexes. The 2BC complex blocks the mammalian protein secretory pathway by inhibiting host protein traffic between the ER and the Golgi apparatus (Moffat *et al.*, 2005; Moffat *et al.*, 2007). This interference in the secretory pathway could result in a decrease in the expression of the major histocompatibility complex (MHC) class I molecules on the surface of the infected cells thus hampering the host's cell mediated immune response (Grubman *et al.*, 2008). The 2BC complex in other picornaviruses increases membrane permeability (both the ER and cell membranes) eventually

facilitating release of the virus progeny (Aldabe *et al.*, 1997; van Kuppeveld *et al.*, 1997), while the 2C is implicated in virus encapsidation (Vance *et al.*, 1997).

The P3 region is separated from the P2 and processed by 3C^{pro} (Vakharia *et al.*, 1987) to yield the 3A peptide, three distinct copies of the 3B peptide (VPg), 3C^{pro} and 3D^{pol}, the RNA-dependent RNA polymerase. The 3C^{pro} is the major virus protease processing up to ten of the thirteen cleavage sites in the FMDV polyprotein (Bablanian & Grubman, 1993; reviewed in Belsham, 2005). The 3C^{pro} also induces proteolytic processing of histone H3, which may inhibit the mammalian transcription process (Falk *et al.*, 1990). The 3C^{pro} initiates the cleavage of translation initiation factors eIF4A (part of the cap-binding complex) and eIF4G, making conditions unfavourable for normal mammalian translation (Belsham, 2005). The 3D^{pol} enzyme is necessary in replication (Newman *et al.*, 1979) and requires the uridylylated form of the 3B/VPg peptide to act as a primer (Nayak *et al.*, 2005; Nayak *et al.*, 2006). The function of the 3A protein is unclear in FMDV (Sobrinho *et al.*, 2001; Domingo *et al.*, 2002) and heterogeneity of this protein has previously been associated to differences in pathogenesis and virulence among other genome factors (Giraudou *et al.*, 1990; Knowles *et al.*, 2001; Núñez *et al.*, 2001; Maroudam *et al.*, 2010; Pacheco *et al.*, 2013). The 3A peptide is also hydrophobic in nature (Forss *et al.*, 1984; Carrillo *et al.*, 2005) and is found in association with the 3ABC complex in the perinuclear area of the cells enabling 3C cleavage of histones (Capozzo *et al.*, 2002). In other picornaviruses, the 3AB complex delivers or anchors the 3B/VPg protein to the replication complex (Lama *et al.*, 1994; Towner *et al.*, 1996).

1.4.3 Structure of the FMDV capsid

The *Picornavirus* capsid is non-enveloped, with a pseudo $T=3$ icosahedral architecture. The basic building blocks for the FMDV capsid is the protomer (Fig 1.4a) and consists of the viral structural proteins VP1, VP2, VP3 and VP4. Five protomers subsequently assemble into a pentameric intermediate, and finally, 12 pentamers order themselves structurally via sophisticated protein-protein interactions into a complete capsid (Curry *et al.*, 1995; Ellard *et al.*, 1999; Mateo *et al.*, 2003; Mateo *et al.*, 2008).

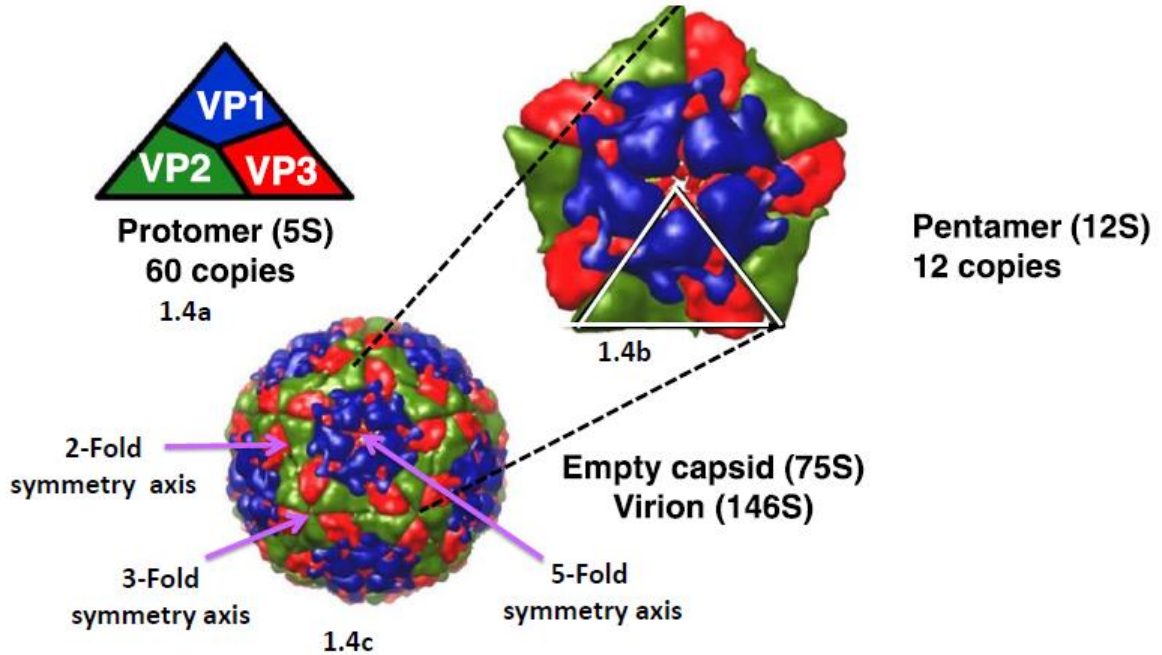


Fig. 1.4 Illustration of a schematic view of the structure of the capsid surface of the **FMDV** (adapted from Jamal & Belsham, 2013 with modifications). **1.4a**: Arrangement of VP1 – VP3 in a protomer (with VP4 hidden); **1.4b**: Arrangement of five protomers (a protomer is outlined) into a pentamer; and **1.4c**: Structure of the capsid. The 5-3-2-fold axis of symmetry for the icosahedral shape is also shown.

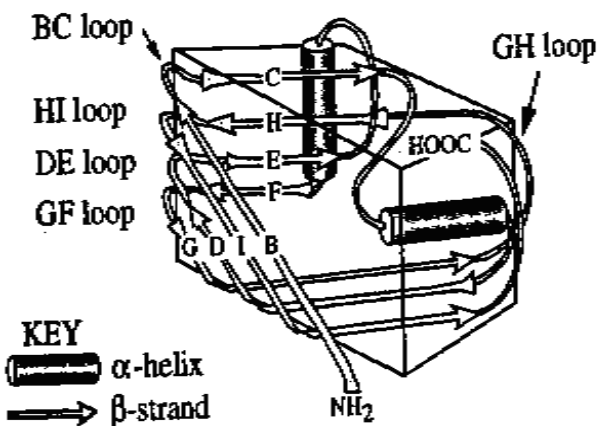


Fig. 1.5. Illustration of a schematic view of the structure of the surface protein (beta barrels) of the **FMDV** (adapted from Rueckert, 1996).

The surface exposed VP1, VP2 and VP3 proteins fold into wedge shape structures, containing an 8-stranded antiparallel β -barrel formation (Fig 1.5), while the VP4 protein is buried within the capsid in close association with the RNA genome. The loops that connect the adjacent β -barrel strands form the outer surface of the virion and are characterised by amino acid residue variation giving FMDV its distinctive morphology and antigenicity. By convention, beta-barrels are labelled B, C, D, etc. to I (Fig 1.5) with the variable loop regions being denoted by the two beta-barrels they connect (Acharya *et al.*, 1989; Acharya *et al.*, 1990; Rueckert, 1996). The VP4 exists initially as an N-terminal extension of VP2 in the precursor VP0. Five protomers assemble into a pentamer (Fig 1.4b) and 12 pentamers into the final capsid structure (Fig 1.4c) consisting of 60 protomers, incorporating a single copy of the RNA genome and one molecule of RNA polymerase. It is then that the final maturation cleavage where VP0 is cleaved into to VP2 and VP4 occurs (Rueckert, 1996).

There are five copies of the VP1 protein located around the icosahedral axis of the 5-fold symmetry while the VP2 and VP3 proteins alternate around the axis of 2- and 3-fold symmetry (Fig 1.4b-c). FMDV differs from the other picornaviruses in that the surface of the capsid is relatively smooth lacking the receptor binding canyon or pit that occur in the entero- or cardioviruses. In contrast the receptor-binding site is located on the protruding, surface-exposed disordered G-H loop of VP1 (Acharya *et al.*, 1989; Acharya *et al.*, 1990; Rueckert, 1996). Another feature, distinguishing the capsid of the FMDV, is the presence of a hydrophobic pore at the 5-fold axis that leaves part of the underlying VP3 exposed and is of unknown biological significance. This pore allows small particles like caesium ions to enter giving FMDV a higher buoyancy among the picornaviruses (Acharya *et al.*, 1989; Acharya *et al.*, 1990; Rueckert, 1996).

The capsid disintegrates into 12 pentameric units at pH lower than 6.8. This occurs when a cluster of histidine residues at the 2-fold axis between VP2 and VP3 are protonated at lower pH weakening the capsid through electrostatic repulsion (Curry *et al.*, 1995; Ellard *et al.*, 1999).

Even though FMDV, especially the SAT types, exhibit large intra- and interserotype genetic variability (Vosloo *et al.*, 1995; van Rensburg & Nel, 1999; Bastos *et al.*, 2001; Bastos *et al.*, 2003b), the multiple and repetitive intersubunit interactions appear to have evolved under stringent and selective constraints (Acharya *et al.*, 1989; Mateu, 1995; Mateu *et al.*, 1996; Ellard

et al., 1999). As a result the viral properties of each serotype are constrained within fundamental structural needs of the virus capsid (Mateu, 1995; Mateu *et al.*, 1996; Samuel & Knowles, 2001). Viral capsid intersubunit interactions are required to be sufficiently robust to provide stability to the capsid against environmental denaturing conditions (Curry *et al.*, 1995; Ellard *et al.*, 1999), whilst still permitting intracellular uncoating and release of viral RNA. Acidification of FMDV in the endosomes disrupts the interactions between VP2 and VP3 at the pentameric interfaces leading to dissociation of the structure into pentamers, thereby releasing the viral RNA (Brown & Cartwright, 1963; Burroughs *et al.*, 1971; Carrillo *et al.*, 1984; Curry *et al.*, 1997).

1.4.4 Antigenic properties

1.4.4.1 Quasispecies nature of FMDV and antigenic variation

Lack of error correction mechanisms of the RNA-dependant RNA polymerase (3D^{pol}) during the replication of RNA viruses leads to high mutation rates (Holland & Domingo, 1998) that occur along the entire genome. Therefore, within any population of virus, all the genome sequences of the strains are not homogeneous, they exist as heterogeneous pools of related genomes called quasispecies (Domingo *et al.*, 1990; Domingo *et al.*, 2003). The resulting average phenotype is virus strains showing fitness in the given environment they are adapted to. This can be the specific cell line or a particular host species (Domingo *et al.*, 2003). Even though mutations occur along the whole FMDV genome, some virus proteins are more prone to positive selection subsequent to amino acid substitution such as capsid proteins, VP1 in the region of the G-H loop followed by the region of 5-fold axis and lastly regions in VP3 (Fares *et al.*, 2001; Haydon *et al.*, 2001b). In contrast, some of the non-structural proteins with enzyme properties like the 3D^{pol} have to maintain structural integrity and similar consensus sequences to ensure proper function (Domingo *et al.*, 2003). Heterogeneity, especially the capsid protein-coding regions can result in resultant phenotype changes such as antigenicity (Haydon *et al.*, 2001b; Domingo *et al.*, 2003).

1.4.4.2 Antigenic sites (epitopes) for the FMDV

For serotype O viruses five antigenically significant sites involved in neutralization (epitopes) have been resolved by sequencing monoclonal antibody resistant (mar) virus mutants and mapping the topography of the mutations on the X-ray crystallographic structure of O₁BFS (Acharya *et al.*, 1989). However, not all five sites may be found in all serotypes (Kitson *et al.*, 1990; Crowther *et al.*, 1993b; Mateu, 1995). Furthermore, monoclonal antibodies (mAbs) have

been used extensively to identify several antigenic sites on the structural proteins of virions belonging to serotypes A (Thomas *et al.*, 1988; Baxt *et al.*, 1989; Bolwell *et al.*, 1989), C (Mateu, 1995) and Asia-1 (Sanyal *et al.*, 2003). Not surprisingly, these antigenic sites are located on structural protrusions on the virus surface, formed mainly by the loops connecting β -barrel structures of the three outer capsid proteins. In contrast, the epitopes for the SAT types have not been studied in detail until recently. In the case of SAT2 serotype viruses, studies involving mar mutants revealed at least three antigenic sites (Crowther *et al.*, 1993a; Grazioli *et al.*, 2006; Opperman *et al.*, 2012). The knowledge of antigenically relevant sites for the SAT1 and SAT2 viruses have been expanded using predictions based on a combination of amino acid variation, cross-neutralization studies together with crystallographic structural data (Maree *et al.*, 2011b). The importance of each of these individual neutralizing antigenic sites in SAT2 viruses is still undefined. The location of the epitopes for serotypes A, O and C are described in Table 1.2, including the locations of the antigenically relevant sites predicted for the SAT types.

Table 1.2. Location of the antigenic sites (both mapped and predicted) on the capsid for different FMDV serotypes.

Axis	Capsid secondary structural element and position of significant nucleotides	FMDV Antigenic Sites				
		O ₁ BFS	A ₁₀	C	SAT2	
3 x	VP2 B-C, 67-79	site 2	site 3	D	+	
2 x	VP2 E-F	site 2				
3 x	VP3 B-B, 56, 58-59	site 4	site 5	D		
3 x	VP3 B-C		site 3			
2 x	VP3 E-F		site 3			
3 x	VP3 H-I		site 3			
5 x	VP1 B-C, 43-45, 48	site 3				
2 x	VP1 G-H, 140-161	site 1a, site 5	site 1	A	+	Major antigenic site
5 x	VP1 H-I		site 4			
3 x	VP1 C _T , 200-213	Site 1b	site 2	C, D	+	Minor antigenic site

(+) Indicates the antigenically relevant sites for SAT serotype

Source: (Baxt *et al.*, 1989; Bolwell *et al.*, 1989; Kitson *et al.*, 1990; Crowther *et al.*, 1993a; Crowther *et al.*, 1993b; Mateu, 1995; Grazioli *et al.*, 2006; Opperman *et al.*, 2012).

1.4.4.2.1 Capsid location of epitopes for A, O and C serotypes

For the A and O viruses three and five sites are described respectively, labelled numerically while for the type C viruses the labelling is alphabetical. Although all the B-epitopes are necessary for adequate immune response, the major immunologically dominant epitope is site 1 or site A, located in the flexible and variable G-H loop of VP1 (residue positions 141-160 mapped on O₁ Kaufbeuren) and is continuous or linear. The β G- β H loop either functions independently (site 5; (Crowther *et al.*, 1993b) or as a discontinuous epitope that encompasses the highly exposed C terminus of VP1, particularly residues 200-213. Neutralizing antigenic site 1 has been mapped to critical residues at positions 144, 148, 154 and 208. The G-H loop also houses the highly conserved RGD sequence motif, used for cell attachment by the integrin receptors. The other epitope sites are discontinuous or conformational and formed by the juxtaposition of amino acid residues from the same protein or different proteins of VP2, VP3 or VP1. Site 2 involves several amino acids in the β B- β C loop of VP2, spanning residues 67-79. Site 3 includes residues 43-45 and 48, inside the β B- β C loop of VP1, while site 4 maps within the β -B “knob” of VP3 with crucial residues at positions 56 and 58-59 (Xie *et al.*, 1987; Barnett *et al.*, 1989; McCahon *et al.*, 1989; Parry *et al.*, 1989; Kitson *et al.*, 1990; Mateu *et al.*, 1990; Mateu, 1995).

1.4.4.2.2 Capsid location of epitopes for the SAT2 serotype

In the case of SAT2 serotype viruses, an antigenic site located in the G-H loop of VP1, downstream of the RGD motif at residues 147, 148, 156, 158 (Crowther *et al.*, 1993a), 154 (Grazioli *et al.*, 2006) and 159 (Opperman *et al.*, 2012), is analogous to site 1 of serotype O₁BFS (Kitson *et al.*, 1990). The other two identified antigenic sites involve residue 210 at the C terminus of the VP1, and residue 154 of VP1 in combination with residue 79 of VP2 (Kitson *et al.*, 1990).

1.5 INFECTIOUS CYCLE OF FMDV

1.5.1 Cell receptor recognition

Virus entry into a susceptible cell is via a cell receptor that is specific, making these molecules of recognition determinants in the pathogenesis of disease (Wimmer, 1994; Baxt & Rieder, 2004). Two major types of receptor molecules are reported for the FMDV; the integrins and the heparan sulfate proteoglycans (HSPGs). During the pathogenesis of disease in livestock the integrin

receptors are utilized, while in cell culture the virus can use either integrins or heparin sulfate receptors (Baranowski *et al.*, 1998; Neff *et al.*, 1998).

1.5.1.1 RGD-dependent mechanism of cell binding

Integrins are the principal receptors used by animal cells during cell migration, adhesion, thrombosis and leukocyte movement (Hynes, 1987, 2002). Integrins are heterodimers containing two trans-membrane glycoprotein subunits (α and β), bound non-covalently to the cell surface. Four members of the integrin family of cellular receptors; $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_6$, $\alpha_v\beta_8$, have been identified for the FMDV entry into cell culture (Berinstein *et al.*, 1995; Jackson *et al.*, 1997; Neff *et al.*, 1998; Jackson *et al.*, 2000; Neff *et al.*, 2000; Jackson *et al.*, 2002; Duque & Baxt, 2003; Jackson *et al.*, 2004). These receptors may be utilized with different efficiencies among the different serotypes *in vitro*, with $\alpha_v\beta_6$ being most preferential however, $\alpha_v\beta_8$ plays a recognizable role in sensitivity of FMD field viruses to cells (Duque & Baxt, 2003; Burman *et al.*, 2006; King *et al.*, 2011). Likewise, a SAT1 virus isolated from impala as well as SAT2 and SAT3 viruses isolated from buffalo were shown to utilize $\alpha_v\beta_6$ preferentially and $\alpha_v\beta_3$ to a lesser extent (Maree *et al.*, 2011a; Maree *et al.*, 2013). The integrin receptors believed to be expressed by some of the common cell lines used in FMDV diagnostics and research are $\alpha_v\beta_8$ for a pig kidney cell line IBRS-2 cells (Istituto Biologico Renal Suino-2), and in primary BTY (Primary Bovine Thyroid) cells it is $\alpha_v\beta_6$ (Burman *et al.*, 2006). Two recent novel continuous cell lines include a foetal goat tongue, ZZ-R 127 and foetal bovine kidney LFBK- $\alpha_v\beta_6$, both expressing the favoured $\alpha_v\beta_6$ integrin receptor (Brehm *et al.*, 2009; LaRocco *et al.*, 2013). The LFBK- $\alpha_v\beta_6$ are highly permissive to all serotypes of FMDV, including the porciphilic viruses that so far have been only detected by using swine cell lines (Brehm *et al.*, 2009; LaRocco *et al.*, 2013). The integrin types expressed on BHK (Baby Hamster Kidney) cell lines are yet to be determined in spite of their universal use in FMD research and vaccine production. The role of the four integrin receptors in the pathogenesis of FMD in the natural host has also not been determined (Baxt & Rieder, 2004).

The highly conserved RGD (arginine-glycine-aspartic acid) sequence motif on the VP1 virus capsid protein mediates the attachment to the integrin receptor molecule (Fox *et al.*, 1989). The motif is located at residue positions 145 to 147 for the O₁ BFS 1860 virus, within the surface

exposed and highly variable region of the G-H loop spanning VP1 residue positions 135-160 (Fox *et al.*, 1989). It has also been suggested that the C-terminus of the VP1, amino acids 203-213 contributes towards receptor binding in BHK cells (Fox *et al.*, 1989). Other residues downstream of the RGD motif at the positions of RGD+1 (leu/arg/met) and RGD+4 (leu/ile) are also significant to the specificity of the integrin receptors (Burman *et al.*, 2006).

The ability of the FMDV to utilize multiple integrin receptors may be an adaptive strategy of the virus and makes the pathogenesis of the FMDV intricate and may influence infection of different tissues and host range (Baranowski *et al.*, 1998; Jackson *et al.*, 2003).

1.5.1.2 RGD-independent mechanisms of cell binding

Upon serial passage of field FMDV isolates in BHK cells, the quasispecies nature of the virus permits selection of isolates with different receptor preference for replication (Neff *et al.*, 1998). The subsequent adaptation of the virus to cell culture may be accompanied by the acquisition of positively-charged residues on the surface exposed capsid protein that enables the virus to use an alternative receptor, the HSPG receptor (Sa-Carvalho *et al.*, 1997). Though these HSPG adapted isolates may gain access to the cells in culture without the use of integrin receptors, they preserve their ability to use integrins. It has recently been shown that the HSPG receptor works in synergy with $\alpha_v\beta_6$ integrin for amplified cell permissiveness (Berryman *et al.*, 2013). Therefore, the virus population exists as a quasispecies “mixture” of HSPG and integrin using individual virus particles (Maree *et al.*, 2011a). They are attenuated in cattle, displaying small, clear plaque phenotypes and with an improved yield in BHK cells (Jackson *et al.*, 1996; Sa-Carvalho *et al.*, 1997; Neff *et al.*, 1998; Baranowski *et al.*, 2000; Maree *et al.*, 2010), a property that is advantageous for vaccine production (Doel, 2003; Maree *et al.*, 2011a). In contrast, field isolates lack the ability to replicate in CHO-K1 (Chinese Hamster Ovary strain K1) cell lines, which do not express integrin receptors used by FMDV for cell entry (Baranowski *et al.*, 1998; Neff *et al.*, 1998; Maree *et al.*, 2010).

HSPG is an example of a glycosaminoglycan (GAG) present ubiquitously on the plasma membranes of many cells as well as in the extracellular matrix, which can bind to many biological substances including growth factors, enzymes, cytokines or even viruses (Byrnes & Griffin, 1998; Jackson *et al.*, 2003). The GAG's are negatively-charged polysaccharides as a

result of N and O sulfation, hence, are able to bind to positively-charged surface exposed regions of virus particles (containing arginine and lysine) using electrostatic interactions (Byrnes & Griffin, 1998).

FMDV isolates serially passaged and adapted to BHK cells have been reported to acquire positively-charged residues on the surface exposed capsid (VP1-3) at the 5-fold or 3-fold axis in positions that vary between serotype or within the same serotype as illustrated in Table 1.3.

Table1.3. A summary of positively charged residues observed to accumulate in the outer capsid proteins, VP1-3, during multiple passage of FMDV in cell culture.

Serotype /Virus isolate	Residue position, positive charge substitution on virus capsid			Secondary Structure	Reference
	VP1	VP2	VP3		
O ₁ Campos		134, Lys	56, Arg	3-fold axis	(Sa-Carvalho <i>et al.</i> , 1997)
O ₁ BFS			56, Arg	3-fold axis	(Fry <i>et al.</i> , 1999)
vac-O/CHA/90	83, Lys 172, Arg			5-fold axis	(Zhao <i>et al.</i> , 2003)
A10 ₆₁		135, Arg	56, Arg	3-fold axis	(Fry <i>et al.</i> , 2005)
A IND 40/2000		131, Lys	85, Arg	3-fold axis	(Anil <i>et al.</i> , 2012)
A/Turkey/2/2006	110, Lys			5-fold axis	(Berryman <i>et al.</i> , 2013)
C ₃ Arg/85	110, Arg 108, Arg			5-fold axis	(Escarmis <i>et al.</i> , 1998)
C-88c1 p100c10	197, Arg		173, Lys 218, Lys		(Baranowski <i>et al.</i> , 1998)
SAT1/SAR/9/81	110, Lys 112, Arg			5-fold axis 5-fold axis	(Maree <i>et al.</i> , 2011a)
SAT1/KNP/196/91	110, Lys 112, Arg	74, Arg		5-fold axis 5-fold axis	(Maree <i>et al.</i> , 2010)
SAT1/ZAM/2/93	111, Lys 112, Arg			5-fold axis 5-fold axis	(Maree <i>et al.</i> , 2010)
SAT1/NAM/304/98			135, Lys 175, Lys	3-fold axis 3-fold axis	(Maree <i>et al.</i> , 2010)
SAT2/KNP/19/89	83, Arg			5-fold axis	(Maree <i>et al.</i> , 2010)
SAT2/ZAM/7/96	85, Lys			5-fold axis	(Maree <i>et al.</i> , 2010)
SAT2/ZAM/14/90		170, Arg	129, Lys	3-fold axis	(Maree <i>et al.</i> , 2010)

Accumulation of positive charges on the virus capsid does not necessarily mean usage of the HSPG receptor. Unidentified pathways of entry for FMDV were reported among viruses that showed high affinity for heparin or replication in cell lines deficient in HSPG like CHO-677 (Baranowski *et al.*, 2000; Zhao *et al.*, 2003; Berryman *et al.*, 2013; Lawrence *et al.*, 2013). The

FMDV can even dispense of the RGD motif after multiple passages in cell culture (Martinez *et al.*, 1997; Lawrence *et al.*, 2013). In addition viruses complexed with antibodies can enter cells that express Fc receptors (Mason *et al.*, 1994).

Identification of the sequences used by FMDV associated with HSPG receptor usage in cell culture can be exploited by reverse genetics technology for use in genetically engineered viruses. These could yield larger quantities antigen within shorter time for use of manufacture of conventional vaccines (Maree *et al.*, 2010). They may be advantageous when compared to the present adaptation techniques involving serial passage that are lengthy and tedious and may result in antigenic drift (Pay *et al.*, 1978; Preston *et al.*, 1982).

There is a notable overlap of the residues involved in antigenicity and the residues involved in the cell receptors used for cell entry. This is observed with the RGD motif located in the variable and immunodominant G-H loop used by integrin receptors and the HSPG binding region located at VP2 position 56 (Verdaguer *et al.*, 1995; Fry *et al.*, 1999), leading to a possibility of the occurrence of coevolution of antigenicity and viral cell receptors (Baranowski *et al.*, 2001).

1.5.2 Viral replication

Viral RNA synthesis occurs in the cytoplasm of susceptible cells within membranous replication complexes that arise from the endoplasmic reticulum and Golgi complex. These complexes contain FMDV non-structural proteins (Grubman & Baxt, 2004). The cell receptors only serve to dock the virus particle onto the surface of a susceptible cell, which is followed by cell entry. The mechanisms of viral uncoating and transfer of the virus genome across the host cell membrane for delivery into the cytoplasm have not been well studied. Virus uptake is by endocytosis that is clathrin-mediated via the integrin receptors (Berryman *et al.*, 2005) and caveola-mediated via the HSPG receptors (O'Donnell *et al.*, 2008). The virus particle is thus delivered to the early acidic endosomes where it disintegrates into 12 pentameric units freeing the viral RNA (Cavanagh *et al.*, 1978; Grubman & Baxt, 2004).

Once in the cytoplasm, translation of the picornavirus genome starts after cleavage of the VPg protein attached to the 5' end of the virus (Ambros *et al.*, 1978). Following down regulation of the host's translation by the L^{pro} as discussed earlier (1.4.2.1), the IRES region of the FMDV

initiates translation of the FMDV genome in a cap-independent manner (1.4.2.1) (Martinez-Salas *et al.*, 1996), resulting in a single polypeptide. The 3'UTR has been suggested to play a role in translation of the FMDV (de Quinto *et al.*, 2002). Two proteases, L^{pro} and 3C^{pro} rapidly process the FMDV polyprotein. In addition the 2A peptide permits the release of the P1-2A peptide from the ribosome while allowing translation of the remainder of the polyprotein to continue downstream. The result is a series of four structural and eight non-structural proteins (Vakharia *et al.*, 1987; Bablanian & Grubman, 1993; Donnelly *et al.*, 2001).

The process of transcription and genome replication is not well studied for FMDV and in many instances analogies have been made from other picornaviruses. For replication of the FMDV genome to start, viral translation ceases by yet unknown mechanisms (Grubman & Baxt, 2004; Belsham, 2005) and the positive-sense genome acts as a template for the synthesis of complementary negative sense strands. The *cre* in the 5' UTR acts as a template for the production of the uridylylated peptide VPg/3B (Nayak *et al.*, 2005; Nayak *et al.*, 2006), which is the primer for the 3D^{pol} to act on during RNA strand synthesis. In the picornaviruses, considering the hydrophobic properties of the 3A peptide, its precursor 3AB is thought to anchor the VPg to the membrane of the virus replicating complex (Lama *et al.*, 1994; Towner *et al.*, 1996). The 3D^{pol} catalyses elongation of the negative strand (still by mechanisms unknown), resulting in a double-stranded molecule called the replicative form (Grubman & Baxt, 2004). A plus strand (infectious genome) is generated from the replicative form after it has been unwound by unknown mechanisms, probably involving the 2C peptide which has both ATPase activity (Sweeney *et al.*, 2010) and helicase motifs (Gorbalenya *et al.*, 1989; Gorbalenya & Koonin, 1989; Klein *et al.*, 2000). A large excess of positive-strand RNA is always produced compared with negative-sense RNA (Belsham, 2005).

The replicative cycle yields a pool of new positive-sense infectious genomes as well as structural and non-structural proteins, which can be assembled into virions (Belsham, 2005). During encapsidation, the cleaved products of the P1 region are assembled to form the protomer structure containing one copy, each of VP0, VP1 and VP3. Five protomers assemble into a pentamer and 12 pentamers into the final capsid structure consisting of 60 protomers, (as discussed previously, 1.4.3), encompassing a single copy of the RNA genome and one molecule of RNA polymerase (Rueckert, 1996). The final step in replication of FMDV is the maturation process. This is where

VP0 is cleaved into VP2 and VP4 by autocatalysis, however the exact mechanism is not clear (Rueckert, 1996). At the end of the replication cycle, a number of intermediate products are identified together with the mature virion that has a sedimentation rate of 146S. These are particles containing RNA with an uncleaved VP0 (provirion) and particles with an uncleaved VP0, lacking RNA (empty capsids) with a sedimentation rate of 75S whose significance is not known (Rueckert, 1996; Grubman & Baxt, 2004).

1.6 FMD TRANSMISSION, PATHOGENESIS AND DISEASE MANIFESTATION

1.6.1 Transmission of virus

The FMDV is transmitted mainly by direct contact between infected and susceptible animals. Infected animals secrete large amounts of virus in all bodily secretions and excretions including aerosols that infect other animals via the respiratory or oral routes (Sutmoller *et al.*, 2003). Infected animals are not infectious until about 12 hours after the onset of clinical symptoms (Charleston *et al.*, 2011) and virus excretion decreases by day 4-5 after development of vesicular lesions that coincide with the onset of antibodies (Alexandersen *et al.*, 2003; Sutmoller *et al.*, 2003). Movement of such infected livestock has been the main source of many of the sporadic epidemics in Europe (1990-2001) and many recurrent FMD outbreaks in Africa (Davies, 2002; Sutmoller & Olascoaga, 2002; Vosloo *et al.*, 2002). FMDV is also spread by contaminated animal products like meat and bones or orally by feeding pigs with untreated swill (Kitching & Alexandersen, 2002; Alexandersen *et al.*, 2003). Additionally, mechanical transmission also occurs through fomites contaminated with animal secretions containing virus e.g. hay or vehicles, farm workers, boots, or bedding (Alexandersen *et al.*, 2003).

1.6.2 Incubation period and pathogenesis

The incubation period is 2-14 days (Kitching & Alexandersen, 2002; Alexandersen *et al.*, 2003). The primary site for virus replication following natural infection is the non-keratinized epithelial cells of the pharyngeal tissues. These include the mucosa-associated lymphoid tissue crypts (MALT), tonsils, the dorsal surface of the soft palate and the roof of the pharynx, just above the soft palate (Zhang & Kitching, 2000; Alexandersen *et al.*, 2001; Arzt *et al.*, 2011). Virus can be detected in the pharynx about 1-3 days before viraemia. From these primary sites, the virus is distributed by viraemia (of about 5 days' duration) via the regional lymph nodes to the secondary

replication sites where stratified and cornified squamous epithelia exist such as the tongue, hooves and teats. There is amplification and spread of virus, which may peak around 2-3 days post-infection (Alexandersen *et al.*, 2001; Oleksiewicz *et al.*, 2001; Alexandersen *et al.*, 2002b; Alexandersen *et al.*, 2003).

1.6.3 Clinical FMD

FMDV causes similar clinical disease in all cloven-hoofed susceptible species that can vary in severity based on the immune status of the host, the virus strain, the infectious dose, to name a few (Clavijo & Kitching, 2003; Baxt & Rieder, 2004). Clinical disease is most obvious and pathogenic in cattle and pigs in highly productive intensive systems. Conversely, in indigenous cattle and porcine breeds of Africa and Asia where the FMDV is endemic, the disease is often less severe (Kitching, 2002a; Kitching & Hughes, 2002; Thomson & Bastos, 2004).

The major clinical signs are acute fever, followed multiple vesicles on the dental pad, tongue, muzzle or snout, the hooves and the teats which rupture within 3 days to leave shallow erosions that heal rapidly. In cattle there is inappetence, copious drooling of saliva due to oral lesions. On the hooves of cattle and pigs, the vesicles are found in the interdigital space, at the bulb of the heel, and along the coronary band. These lesions are preceded by acute inflammatory responses involving reddening, heat and pain. Severe hoof lesions can result in lameness that limits the draught capacity of cattle, or to cause the claws of pigs to be shed (Alexandersen *et al.*, 2003; Thomson & Bastos, 2004). Death is rare in adult animals, but in young animals may result following myocarditis in especially piglets. A sequelae to these lesions is lowered weight gain in all animals, secondary mastitis and infertility in cattle (Thomson & Bastos, 2004). Although the epithelial lesions are usually healed within 1-2 weeks (Alexandersen *et al.*, 2003), there are significant socioeconomic losses at the farm level as a result of the disease (Perry & Rich, 2007) that have been discussed in the General Introduction (section 1.1).

1.6.4 Sub-clinical FMD

Sheep and goats show subclinical disease with oral lesions that are less evident and difficult to detect, thus they are a potential reservoir for infection to other animals (Salt, 1993; Davies, 2002; Kitching & Hughes, 2002; Sutmoller & Olascoaga, 2002). Similarly, the African buffaloes (in southern Africa that are commonly affected with multiple SAT FMDV serotypes, rarely show

clinical disease (Condy *et al.*, 1985; Hedger & Condy, 1985). Likewise these species are a source of FMDV for other wildlife and domestic livestock (Vosloo *et al.*, 2002) as was discussed in the Epidemiological patterns for FMDV in Africa (section 1.2.4).

1.6.5 Persistent FMDV infection

Persistent infection is said to occur when the FMDV is still present in oesophageal-pharyngeal fluids 28 days after acute or subclinical infection (Sutmoller *et al.*, 1968; Salt, 1993). Low levels of FMDV replication still occur in the dorsal soft palate and the dorsum of pharynx (Zhang & Kitching, 2001; Alexandersen *et al.*, 2003). These ruminants can remain persistently infected for a period of 2½ years in cattle (Hedger, 1968), or five months in sheep (Burrows, 1968) and 5 years or longer in African buffalo (Hedger & Condy, 1985). Pigs do not become carriers.

Transmission of FMDV from the persistently infected African buffalo to their calves and occasionally to livestock and impala occurs (Condy, 1979; Condy *et al.*, 1985; Hedger & Condy, 1985; Vosloo *et al.*, 1996; Thomson & Bastos, 2004). In contrast, FMDV transmission from persistently infected livestock is rare and has not been substantially proven except for some incidences in southern Africa (Vosloo *et al.*, 1992; Vosloo *et al.*, 1996; Thomson & Bastos, 2004). Under normal circumstances virus is not shed by the persistently infected livestock, neither is the virus detected in the surrounding environment, however, these animals are still considered a risk to FMD-free countries (Sutmoller *et al.*, 2003).

1.7. IMMUNE RESPONSES

Both innate (inflammation, phagocytosis, cytokines production etc.) and adaptive immune responses (B- and T- cell responses) occur during either natural infection or vaccination with FMDV and are complementary. Natural infection induces protective antibodies for longer duration (up to 4 years) compared to approximately 6 - 12 months observed with inactivated vaccines (Salt, 1993). This is probably due to the development of cell mediated immunity in natural infection, which is less pronounced with vaccination owing to the unstable inactivated antigen or removal of non-structural proteins during the antigen purification process (Salt, 1993; Parida, 2009). Another factor suggested is antigen persisting in the follicular dendritic cells in cases of infection (Juleff *et al.*, 2008).

Core to the host's response to the foreign invading protein are the antigen presenting cells of which dendritic cells are most important, and to a lesser extent, the monocytes and macrophages. The antigen presenting cells endocytose the FMD virion, then migrate to the lymphoid organs and present it for activation of either the B- or T-cell response (Banchereau & Steinman, 1998; McCullough *et al.*, 2004). The fact that the dendritic cells can harbour FMDV for long periods makes them possible reservoirs for the virus. Although replication of the virus occurs in these leucocytes, it is abortive, incomplete and virus progeny is not released (Summerfield *et al.*, 2009; Juleff *et al.*, 2012).

1.7.1. Humoral immune response

The humoral or neutralizing immune response is specific, involving the recognition of antigenic sites or epitopes on the virus particle (Mateu, 1995) by antigenic binding sites or paratopes on the B-lymphocytes (Tizard, 2000; McCullough *et al.*, 2004). For this purposes it is important that the dendritic cells deliver intact FMDV particles to the lymphoid organs. The binding of antigen to antibody interferes with the infectivity of the virus, and later such antibody-antigen complexes can be presented for phagocytosis (McCullough *et al.*, 1992b; McCullough *et al.*, 2004).

The neutralizing antibodies are elicited rapidly upon natural infection or vaccination and correlate with protection subsequent to vaccination (McCullough *et al.*, 1992b). The neutralizing antibodies are suggested to be the most important in resolution of the clinical signs of FMD and in cattle can be induced in the absence the T-lymphocytes helper cells (Salt, 1993; Juleff *et al.*, 2012). Higher neutralizing antibody titres have been observed for convalescent sera compared to post-vaccination sera in the Virus Neutralization (VN) assays (Crowther *et al.*, 1993b; Reeve *et al.*, 2010; Maree *et al.*, 2011b; Mahapatra *et al.*, 2012), indicating possible differences in antibody responses. The nature of the differences in immune responses following infection and vaccination is still not clear. Systemically, IgM antibodies are detected 3-4 days following infection or vaccination, it peaks at 10-14 days, after which it declines and IgG, the major antibody isotype, is detected within 4-7 days. Local antibody responses in the upper respiratory and gastrointestinal tract upon natural infection are initially IgM, followed by IgA and IgG (McCullough *et al.*, 2004; Parida, 2009). In contrast to inactivated FMD vaccines, which elicit

little or no IgA antibodies in mucosal secretions of cattle (Archetti *et al.*, 1995), a higher amount of IgA is secreted in persistently infected animals (Salt, 1993; Parida *et al.*, 2006a).

In cattle IgG1 and IgG2 antibodies appear from five days and onward, and the IgG immune response peaks at 10 to 14 days post-infection (Collen, 1994; Juleff *et al.*, 2009). In pigs, IgG is detected four to seven days post-infection and the maximum levels are reached between 15 to 20 days (Francis & Black, 1983; Doel, 1996; Pacheco *et al.*, 2010). Higher levels of the IgG1 isotype compared to IgG2 have been linked to protection against infection in vaccinated cattle and pigs (Mulcahy *et al.*, 1990; Capozzo *et al.*, 1997; Barnett & Carabin, 2002; Juleff *et al.*, 2009; Capozzo *et al.*, 2011).

1.7.2. Cell-mediated immune response

The T-helper lymphocyte (Th/CD4⁺) and T-cytotoxic lymphocyte (Tc/CD8⁺) responses are considered to be primary in virus infections in vertebrates and recognise antigens processed by the dendritic cells (Salt, 1993; Banchereau & Steinman, 1998). However the specific role of the T-cell response in FMDV infection is yet to be explained (Golde *et al.*, 2011). T-cell epitopes have been identified on residues on the FMDV capsid proteins VP1, VP4 and non-structural proteins, 3A, 3B and 3C mainly in serotypes A, O and C FMDV (Blanco *et al.*, 2000; Blanco *et al.*, 2001; Guzman *et al.*, 2008; Juleff *et al.*, 2009).

The Th/CD4⁺ immune response is elicited much later during infection after the neutralizing antibody (B-cell) response and is important in regulating and maintaining the humoral (T-independent antibody) response (Salt, 1993; Childerstone *et al.*, 1999; Grubman *et al.*, 2008; Golde *et al.*, 2011). Inside early endosomes in the dendritic cells, FMDV antigen is degraded into peptides, a process which alters activity of viral enzymes. These FMDV peptides fuse with the major histocompatibility complex (MHC) Class II molecules in the late endosomes, which are presented on the surface of the dendritic cell. Specific T-cell receptors on Th-lymphocytes recognise the processed antigen and respond by inducing B-lymphocyte proliferation and differentiation into antibody producing cells and later memory cell development (Banchereau & Steinman, 1998; Tizard, 2000; McCullough *et al.*, 2004).

Little is known about the role of the cytotoxic Tc-lymphocytes in protection against FMD. Although Tc/CD8+ immune responses have been reported in infected and vaccinated livestock, cytotoxic Tc-cells have not been detected (Childerstone *et al.*, 1999; Blanco *et al.*, 2001; McCullough *et al.*, 2004; Guzman *et al.*, 2008). Inside the Tc-effector lymphocytes pathogen proteins are degraded into viral peptides by proteosomes. The resultant peptides associate with MHC Class 1 molecules within the endoplasmic reticulum, and are presented on the cell surface, activating the Tc-effector cells. Once the Tc-lymphocytes receive signals from interleukins IL12, IL2 and cytokine IFN- γ , they direct cytotoxicity against cells with endogenous pathogen proteins by apoptosis (Tizard, 2000). It is hypothesized that during the carrier state of FMD in ruminants, the FMDV down regulates expression of MHC Class I molecules (Alexandersen *et al.*, 2002a).

Compared to the high levels of neutralising antibodies in FMDV-infected animals, T-cell responses are weaker (Doel, 1996). However, the duration of such responses may extend for 42-50 days post-infection (Nfon *et al.*, 2008). T-cell responses are serotype cross-reactive and an attractive target for vaccine design (Collen & Doel, 1990; Guzman *et al.*, 2010). Therefore, enhanced T-cell responses, in combination with the B-cell antibody response, could improve long-term protective antibody responses to FMD vaccines (Golde *et al.*, 2011).

1.7.3 The innate immune system

The innate immune system is initiated rapidly against the FMDV before immunity can become effective and is non-specific and of short duration. It includes the inflammatory responses, cytokine stimulation, mononuclear leucocytes like phagocytes, macrophages dendritic cells mast cells and Natural Killer (NK) cells (Tizard, 2000; McCullough *et al.*, 2004; Golde *et al.*, 2011).

1.7.3.1 Cytokines

Cytokines are soluble, cell-to-cell signal molecules involved in cell activation and regulation. Cytokines consist of two families, interleukins and interferons. They are secreted by a variety of cells such as macrophages, Th-lymphocytes, fibroblasts and NK cells. They are triggered by the presence of free antigen, antigen complexed with MHC molecules, or antigen-antibody complexes (Tizard, 2000). Increased cytokine protein levels have been observed following infection and vaccination, and have been noted as modulating the early innate response to limit

the spread of infection (Chinsangaram *et al.*, 2001; Alexandersen *et al.*, 2002a; Zhang *et al.*, 2002; Cox *et al.*, 2003).

Interleukins stimulate and regulate the immune response with an additional advantage of playing a role in the development of specific immune responses towards Th1 and Th2 type immunity (McCullough *et al.*, 2004). Some interleukins observed in FMD infection are IL2, IL1 α (Zhang *et al.*, 2006) and TNF α (Stenfeldt *et al.*, 2012) where it is associated with local inflammatory responses such as increased vascular permeability characteristic for development of vesicular lesions. More specifically, IL-6, IL-8, IL-10 and IL-12 remain at elevated levels following vaccination (Barnard *et al.*, 2005; Cox *et al.*, 2011), and IL-12 has been suggested to be important in induction of monocyte activity and protection (Barnett & Carabin, 2002). Disruption of the secretory pathway (Moffat *et al.*, 2005), restricts secretion the IL-6, IL-8 proinflammatory cytokines in polivirus infection (Dodd *et al.*, 2001) IL-10 production has been associated with FMDV infection in pigs and may be associated with T-cell-independent antibody responses, while suppressing T-cell activation during acute infection (Diaz-San Segundo *et al.*, 2009).

Interferons on the other hand inhibit virus replication by interfering with viral RNA and protein synthesis (Tizard, 2000). Type I interferon, IFN- α , is secreted by the dendritic cells interacting with FMDV immune complexes during acute infection (Reid *et al.*, 2011). However, it is interesting that the L^{pro} of the FMDV blocks the synthesis of IFN- α/β as a survival strategy (de Los Santos *et al.*, 2006) and likewise, the synthesis of IFN- β is reportedly down-regulated in primary tissues of FMDV replication (Stenfeldt *et al.*, 2012). The type II interferon, IFN- γ is released by the NK cells and the Th/CD4⁺ lymphocytes. This type II interferon together with activated Tc /CD8⁺ cells and the activity of NK and macrophages are associated with cell death and inhibition of FMDV in cells (Tizard, 2000; Zhang *et al.*, 2002; Summerfield *et al.*, 2009). It has been reported that during FMDV infection NK cells have a diminished capacity to lyse target cells and secrete IFN- γ (Toka *et al.*, 2009). Recently, a novel type III interferon (IFN- λ) response associated with FMDV infection has been reported (Sheppard *et al.*, 2002; Kotenko *et al.*, 2003) with biological activities similar to type I IFNs. The type III IFN induces antiviral activity in epithelial cells (Ank *et al.*, 2006; Ank *et al.*, 2008; Díaz-San Segundo *et al.*, 2011) and demonstrated antiviral activity of type III IFNs in bovine in the respiratory tract where FMDV

initiates replication (Arzt *et al.*, 2011) and in the skin, another predilection site (Alexandersen *et al.*, 2003; Arzt *et al.*, 2011).

1.7.3.2 Phagocytosis

Phagocytosis by the macrophages is important in eliminating FMDV. This role is enhanced by opsonisation of the FMDV by antibody. This ensures destruction of the virus particles as antibody alone is not capable of destroying the virus (McCullough *et al.*, 1992b; McCullough *et al.*, 2004).

A better understanding of the immune response to FMDV infection should lead to the development of more efficacious vaccines. These should initiate a speedy innate response alongside the T-cell dependent and adaptive B-cell response (Golde *et al.*, 2011). Use of toll-like receptor ligands as adjuvants in the engineered vaccines may improve current control strategies (Parida, 2009).

1.8 CONTROL OF FMD BY VACCINATION

1.8.1 Conventional vaccines

1.8.1.1 Production

Conventional inactivated vaccines (obtained from whole virus preparations) to control FMD have been available since the early 1950s and their protracted use eventually eradicated FMD in Europe by 1984 (Leforban & Gerbier, 2002). Production of inactivated vaccines takes place in high containment facilities where large volumes of virulent whole virus preparations are adapted and propagated on suspension BHK cell lines (Radlett, 1987; Doel, 2003). The virus is inactivated using binary ethyleneimine (BEI) and purified by ultrafiltration or chromatography to remove cellular and media debris and in ideal instances many of the non-structural proteins involved in viral replication, making them compatible with companion tests to differentiate vaccinated from infected animals (Barteling, 2002; Doel, 2003; Parida, 2009). The safety of the antigen is verified, after which it is stored frozen over liquid nitrogen for long periods. The antigen is formulated into a vaccine when needed; the latter having a limited shelf life of about 12-18 months (Doel, 2003). Inactivated antigens are not strongly immunogenic, therefore adjuvants stimulating the immune response are necessary to improve their efficacy. Aluminium

hydroxide/saponin adjuvants (aqueous vaccines) are used in vaccine preparation for ruminants. Nowadays, oil-emulsion adjuvants are preferred because they are more efficacious and compatible in pigs (Sutmoller *et al.*, 2003). The immunity elicited by the inactivated vaccines is humoral in nature with very limited cell-mediated response and is of short duration of about six months (Babiuk *et al.*, 2002; Parida, 2009).

1.8.1.2 Limitations of conventional vaccines

Some disadvantages of conventional vaccines are that their manufacture requires the use of large quantities of live virus in bio-containment facilities, and subsequently, there are risks of FMD outbreaks with virus escaping from production plants. In addition, if the virus has not been inactivated properly, it can cause subsequent outbreaks when formulated into a vaccine. In addition, vaccination also does not fully prevent the development of carrier animals (Beck & Strohmaier, 1987; Sutmoller *et al.*, 2003; Grubman & Baxt, 2004; Rodriguez & Gay, 2011). The viruses used for vaccine antigens require adaptation in cell culture, a procedure that is tedious and lengthy with the risk of antigenic drift and can only be maintained via a cold chain (Pay *et al.*, 1978; Preston *et al.*, 1982; Rogan & Babiuk, 2005; Rodriguez & Gay, 2011). Lastly, the current vaccines stimulate antibodies that are similar to those that occur in infected animals, making it difficult to differentiate vaccinated from infected. This includes those animals that become carriers (Clavijo *et al.*, 2004).

1.8.1.3 Use of conventional vaccines

Vaccination against FMDV can be carried out for purposes of prophylaxis in areas where FMD is endemic or as an emergency measure in FMD-free areas (Forman & Garland, 2002; Sutmoller *et al.*, 2003; Grubman & Baxt, 2004). Serotype-specific vaccines which can be polyvalent are currently employed on a large scale in South America, China and India, while, ironically in many African, south and South-east Asian countries where FMD is endemic, there is limited vaccine use (Parida, 2009). This may be because pastoralists (some nomadic), and other small scale farmers constitute the majority of the farming systems and rear the indigenous breeds of cattle and pigs that sometimes are not adversely affected by FMD. In addition, these farmers are not involved in international trade of animals or animal products; hence there is little incentive for reporting or controlling FMD outbreaks. There is also lack of sustained efficacious vaccine

production in these endemic regions (Kitching, 2002b; Vosloo *et al.*, 2002; Paton *et al.*, 2005; Ayelet *et al.*, 2009; Paton *et al.*, 2009; Balinda *et al.*, 2010a).

1.8.1.4 Vaccine strain matching

The efficiency of a vaccination campaign depends on the relatedness of the vaccine to the outbreak strains (Paton *et al.*, 2009). The genetic and antigenic diversity of FMDV, especially among the sub-Saharan SAT types, limits the control of FMD by vaccination. Thus an intra-serotype vaccine used in one geographical region may not be effective in another region (Hunter, 1998; Haydon *et al.*, 2001a; Maree *et al.*, 2011b). *In vitro* antigenic matching using VN assays or Enzyme-linked immunosorbent assays (ELISA), is used to determine whether a vaccine can offer protection against a variety of field viruses (Rweyemamu *et al.*, 1978; Rweyemamu, 1984; Samuel *et al.*, 1999). Sera from vaccinated animals cross-react with homologous vaccine virus and the field (heterologous) viruses to obtain relationship values (r_1 -values) that give an idea of the serological (antigenic) relatedness between the two viruses (Paton *et al.*, 2009). Values between 0 - 0.19 indicate high antigenic variation, 0.2 – 0.39 indicate significant difference, 0.4 – 1.0 indicate similar antigenic profile and that protection by vaccination is likely (Samuel *et al.*, 1999; Paton *et al.*, 2009). However, data from these *in vitro* cross-reaction studies are limited especially in sub-Saharan Africa where there is a great diversity among FMDV (Balinda *et al.*, 2010a; Muleme *et al.*, 2012) and their interpretation in a field situation is uncertain (Mumford, 2007). Currently there is on-going research into the possibility of utilizing sequence-based prediction methods for vaccine matching which is believed to be rapid and cost effective (Reeve *et al.*, 2010).

1.8.1.5 Assessment of vaccine potency

The effectiveness of the vaccine is judged by its ability to protect primo-vaccinated animals. The 146S particle of the virus is the essential immunogenic component of the vaccine and as it is degraded, vaccine potency reduces (Doel & Baccarini, 1981; Doel & Chong, 1982). Vaccine potency may be assessed directly by the degree of resistance vaccinated animals show to live virus challenge. Two tests are described: the European Pharmacopoeia, 50% protective dose (PD₅₀) test and the South American “Protection against Generalized Foot Infection” (PGF) test (Office International des Épizooties Terrestrial Manual, 2012).

1.8.1.5.1 European Pharmacopoeia, 50% protective dose (PD₅₀) test

The PD₅₀ test is conducted using fifteen test animals divided equally into three groups, and an additional control group consisting of two animals. Vaccine antigen is administered to the test groups in different doses, (1 full dose, 1/4 of a dose and 1/16 of a dose). On the 21st day post-vaccination, the test and control animals are inoculated intradermolingually with live, cattle-adapted, homologous virus at a dose guaranteed to induce FMD in unvaccinated animals (10⁴ 50% Infectious Dose [ID₅₀], delivered in equal volumes at two sites). The cattle are observed daily for eight days for the development of lesions especially on the feet. Protected animals may develop localized lesions on the tongue at the sites of injection, however it should not spread systemically, especially to the feet. Control animals should develop lesions on at least three feet for the test to be valid. The PD₅₀ is defined as the factor by which the concentrate may be diluted such that 50% of vaccinated animals are protected. The PD₅₀ is calculated by a suitable method such as Spearman-Kärber and should be at least 3 for a prophylactic vaccine and a minimum of 6 for an emergency vaccine (Doel, 2003; Parida, 2009; OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 2013). The PD₅₀ test has a high variability and low reproducibility owing to the limited numbers of animals tested (Goris *et al.*, 2007). The animals are costly to maintain especially in high containment, and those not protected succumb to painful lesions causing concern for the welfare of animals (Barnett *et al.*, 2003).

1.8.1.5.2 Protection against Generalized Foot Infection (PGP) test

This test is usually applied in North and South America. Eighteen animals (16 test and two controls) with same characteristics as described in (1.8.1.5.1) are used. The test animals are vaccinated with a single dose whose volume and route are recommended by the manufacturer. After a period of 4 weeks or longer, all animals are challenged with a virulent cattle adapted virus at a dose of 10,000 BID₅₀ (50% bovine infectious doses) delivered intradermolingually at 2 different sites. Seven to eight days later the animals are examined for the clinical development of lesions on the feet. The control animals must develop lesions on at least three feet, while for a prophylactic vaccine at least 12 out of the 16 animals should be protected. However this test does not measure infective doses like the former test, but gives an indication of protection the vaccine provides in a limited cattle population (Periolo *et al.*, 1993; Vianna Filho *et al.*, 1993; Maradei *et al.*, 2008; OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 2013).

1.8.1.6 Emergency and high potency vaccines

In the case of a sporadic outbreak of FMD, in areas free of the disease, vaccination can be considered as an option as opposed to the unpopular mass slaughter/culling methods, with either eventual culling of vaccinated animals or allowing vaccinated animals to live out their productive lives (Scudamore & Harris, 2002; Laddomada, 2003). A vaccine which is closely related to the outbreak strain and able to give reasonable protection within a short time should be used (Barnett *et al.*, 2002). Emergency vaccines contain high levels of antigen (6 PD₅₀) that generates a strong neutralising antibody response within a short time (Pay & Hingley, 1987; Barnett & Carabin, 2002; Doel, 2003). Such high potency vaccines can induce protection against both homologous and heterologous FMDV challenge (Brehm *et al.*, 2008) within at least four days (Cox *et al.*, 1999; Doel, 2003). Brehm *et al.*, (2008), observed high neutralizing antibodies and protection in cattle vaccinated with type A antigens followed by heterologous challenge, likely due to high antigen doses used during immunisation. Such vaccines limit virus replication in the oropharynx, thereby limiting subsequent transmission of the disease to other susceptible animals (Doel *et al.*, 1994; Golde *et al.*, 2005; Parida *et al.*, 2007). Emergency antigen are stored in multinational vaccine banks e.g. the European Union Vaccine Bank and the North America Vaccine Bank, as concentrated inactivated antigen frozen over liquid nitrogen which increases their shelf life and can be formulated into vaccine when needed (Barnett *et al.*, 2002).

1.9 NEW GENERATION FMD VACCINES

The limitations presented by conventional vaccines (discussed in 1.8.1.2), have led to the development of alternative, safe and more efficacious vaccines. These developments have taken advantage of bio-molecular tools and genomics that have increased our knowledge on the immunologically important FMDV antigens and the host defence mechanisms (Rogan & Babiuk, 2005). The new generation vaccines can be more easily manipulated to allow incorporation of field strains, simultaneous expression of multiple antigens or have antigenic markers to facilitate differentiating infected from vaccinated animals when coupled with parallel serological diagnostic tests (Grubman & Mason, 2002; Grubman & Baxt, 2004; Rodriguez & Gay, 2011). They are also envisioned to be thermostable and more immunogenic, eliciting longer lasting protective immunity within a short period of time and easy to administer, avoiding parenteral routes of administration (Grubman & Baxt, 2004; Rodriguez & Gay, 2011).

1.9.1 Marker vaccines

During trade, animals infected with FMDV can be a source of infection for susceptible livestock, therefore there is emphasis on differentiation of potentially infected animals from those vaccinated (Sutmoller & Olascoaga, 2002; Alexandersen *et al.*, 2003). Marker vaccines used for this purpose may lack some antigens or consist of deleted mutants that are expressed in animals infected with the wild-type viruses, thus providing a basis for serological differentiation (van Oirschot *et al.*, 1996; Rogan & Babiuk, 2005). However, the term DIVA (Differentiating Infected from Vaccinated) individuals is preferred over the term negative marker vaccines (van Oirschot, 1999).

1.9.1.1 Types of marker vaccines

1.9.1.1.1 Purified conventional vaccines (negative markers)

Viral replication in infected animals elicits antibodies against both structural and non-structural proteins of FMDV. In contrast, vaccinated animals only produce antibodies to the structural proteins provided the conventional vaccine was well purified to remove all traces of non-structural proteins. The resultant conventional vaccine lacks some antigenic proteins in comparison to the wild type virus and can hence be used as a negative marker vaccine (Doel, 2003; Clavijo *et al.*, 2004). The companion DIVA tests that have been adopted for use together with the conventional vaccines are based on the non-structural proteins of the FMDV and are more sensitive and specific compared to other assays e.g. those that involve IgA antibodies or interferon gamma (IFN- γ) (Parida *et al.*, 2006a; Parida *et al.*, 2006b; Biswal *et al.*, 2008). The non-structural protein assays are advantageous, because they are not sero-specific and there is a considerable degree of conservation among the non-structural proteins (Clavijo *et al.*, 2004). Detection of antibody to the 3ABC polypeptide, using ELISA, is now in use commercially to detect non-structural proteins (Sørensen *et al.*, 2005; Brocchi *et al.*, 2006; Uttenthal *et al.*, 2010). Although the 3A peptide is most immunogenic, inclusion of the 3C peptide increases reliability of the antigens as a negative marker (Mackay, 1998; Mackay *et al.*, 1998a). There is a slight setback when using this assay in that sometimes vaccination can result in subclinical infection where infected animals do not sero-convert (Mackay *et al.*, 1998b).

1.9.1.1.2 Gene-deleted virus antigen (Partial VP1 G–H loop deletion, positive marker)

Fowler *et al.* (2010) reported on the potential use of a naturally selected vaccine virus strain with the region of residues 142–154 of the VP1 G-H loop (including the RGD motif) deleted as a marker vaccine. Cattle vaccinated with this marker vaccine could be distinguished from others using a novel companion $\alpha_v\beta_6$ -integrin ELISA diagnostic assay (Fowler *et al.*, 2010). This marker vaccine (if proved to be viable and immunogenic in other serotypes) could also be applied as a DNA vaccine and is suggested as a possible supplement to the current serological tests that detect non-structural proteins (Fowler *et al.*, 2008; Fowler *et al.*, 2011).

1.9.2 DNA vaccines

A plasmid DNA that contains the gene for the antigen of interest can be delivered into the host cells where transcription and translation occurs thus generating antigen that can elicit an immunological response (Rogan & Babiuk, 2005). Studies have shown that empty capsids (discussed in section 1.5.2) produce comparable immunological response to the virions in animals (Rweyemamu *et al.*, 1979). Recombinant DNA technology has therefore been used to engineer the P1-2A and 3C^{pro} coding region of the FMDV genome. These sections are needed to process and assemble the capsid proteins without the viral RNA and can be referred to as virus-like-particles (VLPs) and they are non-replicative and therefore non-pathogenic (Ansardi *et al.*, 1991; Beard *et al.*, 1999; Rodriguez & Gay, 2011; Porta *et al.*, 2013). This enables the epitopes to be present in a sequential manner similar to parental virus and therefore have similar antigenicity (Beard *et al.*, 1999; Grubman & Mason, 2002). The VLP's can be produced in larger quantities using a number of expression systems like the baculovirus and live viral vectors (discussed in section 1.9.3 and 1.9.4 respectively). DNA vaccines are advantageous because they cannot replicate to cause disease, they are not infectious and therefore do not need high containment facilities and are more stable, being less reliant on the cold chain necessary for conventional vaccines. DNA vaccines also accommodate rapid adjustments to include field isolates and by design are vaccine markers (Gurunathan *et al.*, 2000; Rogan & Babiuk, 2005; Rodriguez & Gay, 2011; Uddowla *et al.*, 2012). Interestingly, FMDV DNA vaccines in sheep not only protected against the challenge virus, but also inhibited localized virus replication, thus preventing the development of the carrier status (Niborski *et al.*, 2006).

1.9.2.1 Enhancing immunogenicity of DNA vaccines

Previous studies on DNA vaccines (consisting of the P1-2A constructs) for FMDV, (Chinsangaram *et al.*, 1998; Cedillo-Barrón *et al.*, 2001) show that eliciting protective mechanisms (humoral and cell-mediated) in farm animals is limited due to the low uptake of DNA by the cells. It is therefore necessary to include cytokines and adjuvants such as plasmid expressing granulocyte macrophage colony stimulating factor (GM-CSF) in the vaccine (Gurunathan *et al.*, 2000; Cedillo-Barrón *et al.*, 2001). Improved systems to deliver DNA include the PLGA Poly (d,l-lactic-co- glycolic acid) micro-particles which encapsulate or absorb plasmid DNA, protecting it against biological degradation by nucleases and delivering the plasmid to the macrophages and dendritic cells as well as electroporation (Walter *et al.*, 2001; Niborski *et al.*, 2006; Wang *et al.*, 2006; Fowler *et al.*, 2012). Recent studies have shown DNA vaccines can fully protect sheep (Niborski *et al.*, 2006), pigs (Li *et al.*, 2008a) and cattle (Fowler *et al.*, 2012) making them a possible strategy of control of FMD.

1.9.3. Protein and peptide vaccines

Peptide vaccines consist of fragments of sequences encoding for antigen epitopes, which are synthesized from amino acids and assembled into molecules (Zhang *et al.*, 2011). Bittle *et al.*, (1982) proved that peptides containing sequences similar to the highly variable G-H loop of VP1, residues 141-160, of FMDV could elicit serotype specific neutralizing antibodies. Similarly, the entire immunogenic VP1 FMDV capsid protein, or fragments thereof, especially the VP1 G-H loop (Strohmaier *et al.*, 1982), expressed in *Escherichia coli*, or *Spodoptera frugiperda* (insect) cells by means of a recombinant baculovirus (Kleid *et al.*, 1981), have induced the production of neutralizing antibodies.

1.9.3.1 Limitations of peptide vaccines

Peptide vaccines have so far met with limited success in some animal species, despite inclusion of Th-cell epitopes (Bittle *et al.*, 1982; DiMarchi *et al.*, 1986; Francis *et al.*, 1987) and in some instances not all animal species were protected in field trials (Taboga *et al.*, 1997). This could be due to the limited number of epitopes presented, since emphasis has been on the VP1 G-H loop (Bittle *et al.*, 1982; Brown, 1992), neglecting multiple discontinuous immunodominant sites in VP2 and VP3, as well as inferior folding of the VP1 FMDV protein in solution limiting the immunogenic sites presented (Domingo *et al.*, 1990; Brown, 1992; Meloen *et al.*, 2001). Escape

mutants were detected in a vaccine trial involving peptide vaccines, probably due to the hypervariability in the VP1 G-H loop together with the presentation of limited epitopes. Such a situation is less common where whole virus vaccine preparations present all epitopes on the virus capsid to recognize the antigen (Taboga *et al.*, 1997). Thus synthetic peptide technologies that include presentation all the discontinuous antigenic sites of the FMDV could accelerate success (Meloan *et al.*, 2001).

1.9.3.2 A commercial peptide vaccine

Despite the above limitations, a synthetic peptide vaccine (UBI peptide) for swine with consensus VP1 amino acid sequences and the inclusion of a novel promiscuous Th1 site to improve immunogenicity, has been reported to offer protection in swine, against the O1 Taiwan FMDV, and is currently on the market (Wang *et al.*, 2002; Rodriguez & Gay, 2011). However, although a similarly generated synthetic peptide vaccine induced peptide specific antibodies in cattle, there was no protection against challenge with O1 Manisa (Rodriguez *et al.*, 2003).

1.9.3.3 Subunit vaccines

Subunit vaccines only contain the essential viral antigens that best elicit an immunological reaction instead of the entire viral particle and are non-replicative (Zhang *et al.*, 2011). In order to have a protein vaccine containing most of the antigenic sites, virus-like particles (VLP's) (section 1.9.2) have been expressed as antigen in insect cell lines using recombinant baculovirus expression systems (Casal, 2001; Kost *et al.*, 2005). Recent advances in the design and production of subunit vaccines include the increased production of the resultant antigen using in silkworm larva expression systems (Li *et al.*, 2008b), in the place of fermenters that are costly (Brun *et al.*, 2011). In addition, rationally designed serotype A VLPs with improved stability as a result of a mutation in the VP2 showed much promise. These mutations created a disulphide bond across inter-pentamer surfaces at the two-fold axis of the capsid surface that enhanced the stability of the empty capsid, thus maintaining its immunogenicity (Porta *et al.*, 2013). The constructs have been further optimized to produce lower amounts of the viral protease 3C^{PRO} used to process the P1 region, lowering the risk of toxicity to mammalian and insect cell expression systems (Porta *et al.*, 2013).

1.9.4 Vector-associated vaccines

This involves the use of chemically attenuated virus to deliver DNA as either VP1 or VLP's (section 1.9.3) into the host cells that stimulate an immune response (Zhang *et al.*, 2011). Several viral vectors have been described including the attenuated pseudorabies, poxviruses (Zheng *et al.*, 2006; Yao *et al.*, 2008) and the recombinant vaccinia virus (Abrams *et al.*, 1995; Berinstein *et al.*, 2000; Porta *et al.*, 2013). It is however, the human defective adenovirus 5-vector system that has to this point been most successful (Grubman & Mason, 2002). The defective human adenovirus type 5 (Ad5) viruses are non-pathogenic in animals and localize in the upper respiratory tract and the gastrointestinal tract, therefore delivering the recombinant VLP's to one of the initial sites for FMDV replication simulating natural infection (Mayr *et al.*, 1999; Grubman & Mason, 2002). In addition, the much desired cell-mediated immune responses is elicited as the vector virus replicates (Sanz-Parra *et al.*, 1999; Russell, 2000). The Ad5 vectored FMD subunit vaccines provide full protection to cattle and swine within 7 days (Moraes *et al.*, 2003; Pacheco *et al.*, 2005) and research into this type of vaccine is currently at an advanced stage after successful field trials (Grubman *et al.*, 2010).

1.9.5 Genetically engineered attenuated strains

Modification of FMDV clones using reverse genetics technology (Zibert *et al.*, 1990) provides a platform to enhance and support production of efficacious inactivated vaccines. Examples of the resultant chimera viruses include the attenuated Leader-less viruses, mutants with altered RGD receptor binding sites, or thermostable isolates (McKenna *et al.*, 1995; Mason *et al.*, 1997; Chinsangaram *et al.*, 1998; Mateo *et al.*, 2008; Maree *et al.*, 2013). More recently, properties of the FMDV such as growth characteristics *in vitro*, antigenicity (both *in vitro* and in animal hosts) were altered by inclusion of restriction enzyme sites that facilitate exchange of capsid sequences like the P1 region (van Rensburg *et al.*, 2004; Maree *et al.*, 2010; Blignaut *et al.*, 2011) or the G-H loop (Rieder *et al.*, 1994; Fowler *et al.*, 2008). Additionally, specific mutations were engineered at the virus receptor binding sites to accommodate growth in particular cell lines (Maree *et al.*, 2010; Maree *et al.*, 2011a), virus stability (Maree *et al.*, 2013) and virus variants with deletions in the VP1 G-H loop, that can be utilized as vaccine markers (Fowler *et al.*, 2010; Fowler *et al.*, 2011). These techniques provide chimeras for custom-made inactivated vaccines to suit prevailing disease outbreak situations, as well as the cell line being used for vaccine manufacture and to enable more robust differentiation assays between vaccinated and infected

animals (Rieder *et al.*, 1994; van Rensburg *et al.*, 2004; Maree *et al.*, 2010; Fowler *et al.*, 2011; Uddowla *et al.*, 2012). These viruses can be attenuated, therefore accidents associated with virus escape from manufacturing plants are less hazardous than when live viruses are used in production of vaccines (Rodriguez & Gay, 2011).

1.10 OBJECTIVES OF THE STUDY

FMD is endemic in most sub-Saharan African countries and the epidemiology of the disease on the continent is complex. It involves the largest diversity of FMD serotypes, has long-term wildlife maintenance hosts and transboundary movement of animals through trade, pastoralism and transhumance. Yet the occurrence of FMDV is poorly documented, and similarly there are scant studies on the biology of the African FMDV. This lack of data poses limitations on the control of FMDV on the continent, and creates foci of infection, which spill over into areas free of the disease, facilitated by local trade patterns. Control of FMDV in Africa is long overdue, and this can only be achieved with comprehensive identification of FMDV as well as knowledge of the biology of the virus. A molecular approach of characterising the FMDV could also enable us to harness the benefits of the improved new generation vaccines. It is against this background that the primary objective of this study focuses on the molecular characterisation of the under reported non-structural proteins for African of FMDV SAT1, 2, 3, A- and O-types, to supplement the available information on the incomplete genome sequences. The resultant full genome sequences can be utilized to study the structure and functional relationships of FMDV proteins that influence phenotypes and viral replication giving us a broader perspective of the biology of the African FMDV.

Secondly, previous studies have identified receptors that some of the southern SAT types use upon adaption to the BHK-21 cell line. These sites have also been engineered into chimeric viruses to address the constraint of poor adaptation of SAT types in cell culture (Maree *et al.*, 2010; Maree *et al.*, 2011a). This study expanded the characterisation (molecular and phenotypic) of BHK-21 cell culture adapted SAT viruses to include that of other field viruses from most of sub-Saharan Africa. This is relevant for the proof of concept, which later can be utilized to

predict and generate chimeric viruses that replicate rapidly in cell culture, from which custom engineered vaccines can be produced.

Thirdly, a genetically engineered chimeric virus obtained from a SAT2 cDNA clone where the capsid-coding sequences were exchanged with that of another SAT2 virus, acquired the antigenic characteristics as well as the cell receptor preferences of the donor virus (van Rensburg *et al.*, 2004; Maree *et al.*, 2010). This chimeric virus was used as seed stock to produce vaccine antigen which was in turn used to vaccinate cattle. The information gap on the immunology response of the chimera vaccines in cattle, which is the target host during vaccination in Africa, is provided by this study. The chimeric vaccine was further characterised by profiling the cross reactivity of serum obtained from chimeric-vaccinated cattle with that of heterologous reference African SAT field viruses.

The objectives of the study therefore cover three aspects:

- To genetically characterise the non-structural proteins of genomes for selected strains of FMDV of type A, O and SAT1, 2 and 3 from various regions in sub-Saharan Africa.
- To adapt SAT1, 2 and 3 viruses to BHK-21 (ATCC) cell lines and characterise the genotypic and phenotypic changes for potential application in reverse genetic techniques for vaccine development.
- To compare the immune response in bovine to a parental and chimera FMDV vaccines.

CHAPTER TWO

DETERMINATION OF COMMON GENETIC VARIANTS WITHIN THE NON-STRUCTURAL PROTEINS OF FOOT-AND-MOUTH DISEASE VIRUSES ISOLATED IN SUB-SAHARAN AFRICA

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2.1. INTRODUCTION

Foot-and-mouth disease (FMD) is widely considered the most economically important disease of livestock, and is a controlled disease in many countries due to its highly contagious nature and associated productivity losses among even-toed ungulates (*Artiodactyla*). The disease is endemic to large parts of the African continent and an impediment to lucrative export markets for animal products (Vosloo *et al.*, 2002). The different serotypes of FMD virus (FMDV) cause a clinically indistinguishable vesicular disease in cloven-hoofed animals and display different geographical distributions and epidemiology (Bastos *et al.*, 2001; Samuel & Knowles, 2001; Bastos *et al.*, 2003a; Bastos *et al.*, 2003b; Knowles & Samuel, 2003; Bronsvort *et al.*, 2004). Of the seven serotypes, the South African Territories (SAT) types 1, 2 and 3 are confined to sub-Saharan Africa, although incursions into the Middle East by SAT1 (1961-65 and 1970) and SAT2 (1990, 2000 and currently in North Africa) viruses have been recorded (Ferris & Donaldson, 1992; Bastos *et al.*, 2001; Valdazo-González *et al.*, 2012; records of the Office International des Épizooties or OIE). In contrast, serotypes A and O occur globally (Samuel & Knowles, 2001) with the exception of southern Africa (Vosloo *et al.*, 2002).

FMDV is a small non-enveloped virus, a member of the *Aphthovirus* genus within the family *Picornaviridae*. The icosahedral capsid consists of 60 copies of four structural proteins, VP1 to 4, arranged in a pseudo T=3 composition. The three surface-exposed proteins, VP1 (1D), VP2 (1B) and VP3 (1C), assemble into a protomeric subunit, with the smaller VP4 (1A) located internally (Acharya *et al.*, 1989; Curry *et al.*, 1995; Sobrino *et al.*, 2001). Despite the high levels of genetic and antigenic variation (Vosloo *et al.*, 1995; Reeve *et al.*, 2010; Maree *et al.*, 2011b), a consequence of the high mutation rate of the virus, the structural arrangement of the capsid is remarkably conserved, indicating plasticity within the three-dimensional structure of the capsid proteins (Acharya *et al.*, 1989; Lea *et al.*, 1994; Curry *et al.*, 1995; Fry *et al.*, 1999). The capsid encloses a *ca.* 8.5 kilobase, positive-sense, single-stranded RNA genome with a single open reading frame and two in-frame translation-initiation codons. Covalently linked to the 5' end of the genome is the small viral protein 3B (or VPg), while the 3' end is poly-adenylated (Carrillo *et al.*, 2005). Upon virus infection, the interactions between VP2 and VP3 at the pentameric interfaces are disrupted by acidification within cellular endosomes, thereby releasing the viral RNA (Knipe *et al.*, 1997; Ellard *et al.*, 1999). The viral genome is rapidly translated into a polyprotein, which is co- and post-translationally cleaved by viral proteinases into several

partially cleaved intermediates and ultimately into 12 mature proteins (Pereira, 1981; Rueckert, 1996).

In addition to the capsid proteins, the ORF of the viral RNA genome encodes eight non-structural proteins, each with its unique function within the replication cycle of FMDV (Belsham, 1993; Belsham, 2005). The non-structural proteins include three proteases, i.e. L^{pro}, 2A and 3C^{pro}, responsible for cleavage of the viral polyprotein and shut-down of the host cap-dependent translational system (Bablanian & Grubman, 1993; Martinez-Salas *et al.*, 1996). Although several of the picornavirus proteins involved in RNA replication (2B, 2C and 3A) have membrane binding properties and disrupt protein trafficking in the cell (Moffat *et al.*, 2005; Moffat *et al.*, 2007), their particular functions during viral replication are still unknown. The 2B protein has been implicated in virus-induced cytopathic effect (CPE) (van Kuppeveld *et al.*, 1997), while the 2C protein has recently been classified as an AAA+ ATPase enzyme that may act as an RNA helicase (Sweeney *et al.*, 2010). The 3D gene encodes the viral RNA-dependent RNA polymerase (RDRP), and together with the 3A co-localizes with ER membrane-associated replication complexes (Lama *et al.*, 1994).

Based on the genetic variability of the VP1-coding region, the FMDV strains that exist among the serotypes, group into topotypes that are geographically specific (Samuel & Knowles, 2001; Knowles & Samuel, 2003). Serotype A has three topotypes, of which the Africa topotype is endemic to sub-Saharan Africa. Of the eleven topotypes defined for serotype O, five are endemic in Africa, the East Africa (EA1 to EA4) and West Africa (WA) topotypes (Di Nardo *et al.*, 2011). On the other hand, the SAT serotypes are more diverse genetically, and nine, fourteen and five topotypes have been defined for SAT1, SAT2 and SAT3 respectively (Bastos *et al.*, 2001; Bastos *et al.*, 2003a; Bastos *et al.*, 2003b; Knowles *et al.*, 2010b).

A few studies have looked at genome comparisons mainly focusing on serotype A, O, C and Asia-1 with a geographic distribution in Euro-Asia and South America (Pereda *et al.*, 2002; Mason *et al.*, 2003b; Carrillo *et al.*, 2005). However, a limited number of complete non-structural protein analyses for viruses belonging to the SAT serotypes have been described (Carrillo *et al.*, 2005). Here we have compared the non-structural proteins and its coding regions for the three SAT serotypes and viruses from serotype A and O found in sub-Saharan Africa from 1974 to

2006. Additionally, the natural variation found within the non-structural protein sequences of 79 viruses to identify structurally and possibly functionally conserved regions were mapped. Variation in the 3C^{pro} and 3D^{pol} was also mapped onto the protein structures to improve understanding of the plasticity of these enzymes. The deduced amino acid sequences of the non-structural proteins of two closely related SAT2 viruses causing an upsurge of outbreaks in North Africa and the Middle East in 2012 were also included (Valdazo-González *et al.*, 2012).

2.2 MATERIALS AND METHODS

2.2.1. Virus isolates

The viruses included in this study were either supplied by the World Reference Laboratory (WRL) for FMD at the Pirbright Institute (United Kingdom) or form part of the virus bank at the Transboundary Animal Diseases Programme, Onderstepoort (South Africa). The SAT1 (n=30), SAT2 (n=26), SAT3 (n=7), serotype A (n=7) and serotype O (n=10) FMDV isolates from 17 countries in sub-Saharan Africa were selected for genomic characterisation. The isolates span a 32-year time period and represent various geographic locations and animal species. The viruses were propagated in IB-RS-2 cells prior to RNA extraction, cDNA synthesis and amplification of the relevant genome regions by PCR (Maree *et al.*, 2011b). A description of the passage histories, host species and representative topotypes can be found in Table 2.1.

2.2.2. RT-PCR, sequencing and analysis

Viral RNA was extracted from infected cell culture supernatant using a modification (Bastos, 1998) of the guanidinium-silica based method described by Boom *et al.*, (1990). To facilitate amplification of the Leader-P1-2A and P2-P3-coding regions, the viral genomic RNA was reverse transcribed with SuperScript IIITM (Life Technologies) using either the oligonucleotide 2B-208R (Bastos *et al.*, 2001) for the Leader-P1-2A coding region or a modified oligo-dT, CCATGGCGGCCGCTTTTTTTTTTTTTTTT, for the P2-P3 coding region. The non-structural coding region and 3'UTR were subsequently amplified by three or four separate PCR reactions using the oligonucleotides detailed in Table 2.2 and Expand Long template Taq DNA polymeraseTM (Roche). The cycling conditions were 95°C for 20 s, 56°C for 20 s, 68°C for 2-3 min (30 cycles). Direct DNA sequencing of amplicons, using the ABI PRISMTM BigDye Terminator Cycle Sequencing Ready Reaction Kit v3.0 (Perkin Elmer Applied Biosystems) and

genome-specific primers (Table I; supplementary data, Appendix1), yielded a consensus sequence representing the most probable nucleotide for each position. For accuracy, at least two sequence reactions (forward and reverse) were generated for each amplicon ensuring that there was more than 50% overlap in the sequencing data. Sequences of the *ca.* 2.8 kb Leader-P1-2A and 4.2 kb P2-P3-coding regions were compiled and edited using BioEdit 5.0.9 software (Hall, 1999). The nucleotide data for eight non-structural protein coding regions (Leader, 2A, 2B, 2C, 3A, 3B, 3C, 3D), were determined in this study, while the nucleotide data for the P1 region have been described (Maree *et al.*, 2011b).

The nucleotide and deduced amino acid sequences were aligned using ClustalX (Thompson *et al.*, 1997). Phylogenetic relationships were inferred using Maximum-Likelihood (ML), Neighbour Joining (NJ), Minimum Evolution (ME), and Maximum Parsimony (MP) methods conducted in MEGA5 (Tamura *et al.*, 2011), with the reliability of the nodes assessed using 1000 bootstrap replications in each method. For the phylogeny using ML method, the jModelTest 2.1.3 (Darriba *et al.*, 2012), was used to predict the most appropriate model of evolution. It was found that the General Time Reversible model with gamma distribution and invariable rates (GIR+I+G) best described the pattern of nucleotide substitution. With the ME and NJ methods, the phylogenetic trees were constructed using the Kimura 2-parameter nucleotide substitution model. The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm at a search level of 1. While the MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm, with search level 1, in which the initial trees were obtained by the random addition of sequences (10 replicates).

MEGA4 software (Tamura *et al.*, 2007) was also used to identify hypervariable amino acid regions in a total alignment of the deduced amino acid sequences, defined as regions with more than 60% variable residues within a window of 10 residues. Entropy plots were drawn from the deduced amino acid alignments using BioEdit 5.0.9 software (Hall, 1999) and were defined as the uncertainty at each amino acid position with high values as an indication of high variation (Schneider & Stephens, 1990). The relative hydrophobicity of the peptides were predicted using the Kyte and Doolittle (1982) method operated in the BioEdit 5.0.9 software (Hall, 1999).

2.2.3. Structural modelling

Homology models of the 3C protease (3C^{pro}) and 3D RNA-polymerase (3D^{pol}) of SAT1 and SAT2 were built using Modeller 9v3 (Sali & Blundell, 1993) with FMDV A10 3C^{pro} (pdb id: 2j92) or type C RNA-polymerase (1UO9) as templates. Alignments were performed with ClustalX. Structures were visualised and the surface-exposed residues identified with PyMol v1.1 (Schrödinger, LLC, New York, NY).

2.2.4. Nucleotide sequence accession numbers

All nucleotide sequences determined in this study have been submitted to GenBank under the accession numbers indicated in Table 2.1.

Table 2.1. Viruses used in this study

	Virus strain	Topo-type ^b	Species ^c	Passage history ^d	Country of origin	GenBank accession number
1	SAT1/KNP/196/91	1	Buffalo	PK1 RS1	South Africa	KF647852
2	SAT1/KNP/148/91	1	Buffalo	PK1 RS5	South Africa	KF647853
3	SAT1/KNP/41/95	1	Buffalo	PK1 RS4	South Africa	KF647856
4	SAT1/SAR/9/81	1	Impala	Epithelium	South Africa	
5	SAT1/ZIM/HV /03/90	1	Buffalo	BTY1 RS3	Zimbabwe	KF647854
6	SAT1/ZIM/GN/13/90	1	Buffalo	BTY1 PK1 RS3	Zimbabwe	KF647855
7	^a SAT1/BOT/1/68	1	N/A	BTY3 BHK5	Botswana	AY593845
8	^a SAT1/RHOD/5/66	1	N/A	BTT1	Rhodesia	AY593846
9	^a SAT1/RV/11/37	1	N/A	IBRS3	Unknown	AY593839
10	SAT1/NAM/307/98	2	Buffalo	PK1 RS4	Namibia	KF647863
11	SAT1/ZIM/6/94	2	Bovine	PK1 RS3	Zimbabwe	KF647864
12	^a SAT1/BEC/1/70	2	N/A	LK2	Botswana	AY593838
13	^a SAT1/SR/2/58	2	N/A	IBRS3	Rhodesia	AY593841
14	^a SAT1/SWA/1/49	2	N/A	IBRS4	Unknown	AY593840
15	^a SAT1/SA/5/61	2	N/A	IBRS3	Unknown	AY593842
16	^a SAT1/SWA/40/61	2	N/A	IBRS3	Unknown	AY593843
17	SAT1/ZIM/25/90	3	Buffalo	BTY2 RS4	Zimbabwe	KF647857
18	SAT1/ZAM/2/93	3	Buffalo	PK1 RS3	Zambia	KF647862
19	SAT1/MOZ/3/02	3	Bovine	PK1 RS5	Mozambique	KF647858
20	SAT1/TAN/1/99	3	Bovine	PK2 RS4	Tanzania	KF647860
21	SAT1/KEN/05/98	3	Bovine	BTY1 RS3	Kenya	KF647861
22	SAT1/UGA/1/97	5	Buffalo	PK1 RS4	Uganda	KF647865
23	SAT1/UGA/3/99	4	Bovine	BTY1 RS4	Uganda	KF647866
24	SAT1/SUD/3/76	7	Bovine	BTY1 RS3	Sudan	KF647868
25	SAT1/NIG/5/81	7	Bovine	BTY2 RS2	Nigeria	KF647867
26	SAT1/NIG/15/75	8	Bovine	BTY1 RS3	Nigeria	KF647869
27	SAT1/NIG/8/76	8	Bovine	BTY1 RS5	Nigeria	KF647871
28	SAT1/NIG/6/76	8	Bovine	BTY1 RS5	Nigeria	KF647870
29	SAT2/KNP/19/89	I	Buffalo	BHK4	South Africa	KJ144902
30	SAT2/KNP/2/89	I	Impala	CFK2 RS2 BHK4	South Africa	KJ144903

Table 2.1 continued

	Virus strain	Topo- type^b	Species^c	Passage history^d	Country of origin	GenBank accession number
31	SAT2/KNP/51/93	I	Impala	PK1 RS6	South Africa	KJ144904
32	SAT2/SAR/16/83	I	Impala	B1 BHK8	South Africa	KJ144905
33	SAT2/ZIM/1/88	I	Buffalo	CFK1 RS4	Zimbabwe	KJ144908
34	^a SAT2/106/67	I	N/A	IBRS3	Unknown	AY593848
35	^a SAT2/ZIM/7/83	II	Bovine	B1 BHK5 B1	Zimbabwe	AF540910
36	SAT2/ZIM/14/90	II	Buffalo	BTY1 RS3	Zimbabwe	KJ144910
37	SAT2/ZIM/17/91	II	Buffalo	BTY2 RS4	Zimbabwe	KJ144911
38	SAT2/ZIM/GN/10/91	II	Buffalo	BTY2 PK1 RS3	Zimbabwe	KJ144907
39	SAT2/ZIM/34/90	II	Buffalo	BTY3 RS4	Zimbabwe	KJ144909
40	SAT2/ZIM/8/94	II	Buffalo	BTY1 RS3	Zimbabwe	KJ144906
41	^a SAT2/RHO/1/48	II	Bovine	BTY2 RS2	Zimbabwe	AY593847
42	SAT2/ZAM/07/96	III	Bovine	BTY1 RS2	Zambia	KJ144912
43	SAT2/KEN/8/99	IV	Bovine	BTY2 RS4	Kenya	KJ144913
44	SAT2/SEN/5/75	V	Bovine	BTY1 RS1 BHK5	Senegal	KJ676543
45	SAT2/GHA/8/91	V	Bovine	BTY1 RS3	Ghana	KJ144917
46	SAT2/SEN/7/83	VI	Bovine	CK1 RS1	Senegal	KJ144916
47	SAT2/ERI/12/98	VII	Bovine	BTY2 PK1 RS5	Eritrea	KJ144921
48	SAT2/SAU/6/00	VII	Bovine	BTY1 RS1	Saudi Arabia	KJ144920
49	SAT2/RWA/2/01	VIII	Bovine	PK1 RS1	Rwanda	KJ144919
50	^a SAT2/KEN/3/57	IX	Bovine	N/A	Kenya	KJ144915
51	^a SAT2/KEN/11/60	IX	N/A	IBRS3	Kenya	AY593849
52	SAT2/ANG/4/74	XI	Bovine	BTY3 RS3	Angola	KJ144914
53	SAT2/UGA/2/02	XII	Bovine	PK1 RS1	Uganda	KJ144918
54	^a SAT2/UGA/MBF/4/02	XII	Buffalo	N/A	Uganda	FJ461346
55	SAT2/EGY/9/12	VII	Bovine	N/A	Egypt	JX014255
56	SAT2/PAT/1/12	VII	Bovine	N/A	Palestinian Autonomous Territories	JX014256
57	SAT3/KNP/10/90	I	Buffalo	PK1 RS1	South Africa	KF647849
58	^a SAT3/SA/57/59	I	N/A	LK1	Unknown	AY593850
59	^a SAT3/BEC/1/65	II	Bovine	LK1	Botswana	AY593853
60	^a SAT3/KEN/11/60	IV	N/A	IBRS4	Kenya	AY593852
61	^a SAT3/BEC/20/61	II	N/A	LK1	Botswana	AY593851
62	SAT3/ZIM/5/91	III	Buffalo	BTY1 RS4	Zimbabwe	KF647851
63	SAT3/ZAM/4/96	IV	Buffalo	BTY1 RS1	Zambia	KF647850

Table 2.1 continued

	Virus strain	Topo- type ^b	Species ^c	Passage history ^d	Country of origin	GenBank accession number
64	A/CIV/4/95	I	Bovine	BTY1	Côte d' Ivoire	KJ144932
65	A/SEN/10/97	I	Bovine	BTY2	Senegal	KJ144933
66	A/SOM/1/78	III	N/A	BTY2	Somalia	KJ144935
67	A/TAN/4/80	III	N/A	BTY2	Tanzania	KJ144937
68	A/ETH/2/79	IV	N/A	BTY5	Ethiopia	KJ144936
69	A/ETH/7/92	IV	N/A	BTY1	Ethiopia	KJ144934
70	A/ERI/3/98	V	N/A	BTY1	Eritrea	KJ144931
71	O/UGA/5/96	II	N/A	IBRS2	Uganda	KJ144924
72	O/UGA/6/76	II	N/A	IBRS2	Uganda	KJ144926
73	O/UGA/17/98	II	N/A	IBRS2	Uganda	KJ144927
74	O/UGA/1/75	II	N/A	IBRS2	Uganda	KJ144925
75	O/UGA/7/03	II	Bovine	PK1	Uganda	KJ144922
76	O/KEN/10/95	II	N/A	IBRS2; IBRS3	Kenya	KJ144928
77	O/SUD/4/80	III	N/A	IBRS2	Sudan	KJ144930
78	O/ETH/3/96	IV	N/A	IBRS2	Ethiopia	KJ144929
79	O/TAN/3/96	VI	N/A	IBRS2	Tanzania	KJ144923

Table 2.1 continued.

^a Genome sequences obtained from GenBank

^b Reference for topotypes : (Bastos *et al.*, 2001; Bastos *et al.*, 2003a; Bastos *et al.*, 2003b; Maree *et al.*, 2011b)

^c The species the virus was isolated from. N/A: Not available

^d The passage history of the virus is indicated as follows: the cell type, followed by the number of times the virus has been passaged in that particular cell type, in numerals (1, 2, 3 etc.).

Cell Types: BHK - baby hamster kidney cells; BOV – bovine; BTT - bovine tongue tissue; BTY- bovine thyroid; BVF - bovine vesicular fluid, CFK - calf foetal kidney; CK - calf kidney; GP - guinea pig vesicular fluid; LK - lamb kidney cells; PK - pig kidney; RS /IBRS – Instituto Biologico Renal Suino-2 Cells.

Table 2.2 A summary of the oligonucleotides that was used for PCR amplification of the L^{PFO}, P2- and P3-coding regions. The orientation, their location on the FMDV genome and the serotype specificity are indicated.

PCR fragment amplified in the different serotypes	Primer oligonucleotide				
	Name	Sequence (5' to 3')	orientation	Location	Serotype specificity
Leader-P1-2A (SATs)	NCR1	TACCAAGCGACACTCGGGATCT (Maree et al., 2011)	Forward	5'UTR	SATs, A & O
Leader-P1 (A & O)	NCR2	GCTTCTATGCCTGAATAGG (Maree et al., 2011)	Forward	5'UTR	SATs
	2B-208R	ACAGCGGCCATGCACGACAG (Bastos et al., 2001)	Reverse	2B	SATs
	2BR-UGA	GCTACAGCGGCCATRCAYGACA	Reverse	2B	SATs East & West Africa
	W-DA	GAAGGGCCCAGGGTTGGACTC (Beck and Strohmaier, 1987)	Reverse	VP1/2B	A & O
2A (A & O)	VP ₁ O	GATTTGTGAAGGTGACACC (Rodriguez et al., 1994)	Forward	VP1	O-type
	A1C-562F	TACCAAATTACACACGGGAA (Reid et al., 2000)	Forward	VP3	A-type
	2B-208R	ACAGCGGCCATGCACGACAG (Bastos et al., 2001)	Reverse	2B	A & O
	2BR-UGA	GCTACAGCGGCCATRCAYGACA	Reverse	2B	A & O
2BC, 3AB ₁₂₃ , 3C (SATs)	SEQ 91	GAGTCCAACCCTGGGCCCTTCTTCTTC	Forward	VP1/2A	All
2BC, 3AB ₁₂₃ (A & O)	SEQ 92	CGCTCYTCAACWTCTCTGGTGTC	Reverse	3D	SATs
	SEQ 126	TCCATRCACACTACAATGTC	Reverse	3C	A & O
3C – 3'UTR (all serotypes)	NC3+	CCKGTGAAGAAGCCTGTGCG	Forward	3B	All
	Poly SAT DT	CCATGGCGGCCGCTTTTTTTTTTTTTTTT	Reverse	Poly A	All

Y=CT; M=AC; K=AG; W=AT; S=CG; K=TG; D=AGT; V=AGC; H=ACT; B=GCT

2.3. RESULTS

2.3.1. Phylogenetic trees of the various non-structural protein coding regions

For the 79 sub-Saharan African FMDV from 5 serotypes (SAT1, 2, 3, A and O) studied, 606, 1416 and 2721 nucleotide positions were aligned for the L^{pro}- P2- and P3-coding regions, respectively. The resultant deduced amino acid alignments, i.e. 202, 472 and 907 positions for the L^{pro}- P2- and P3 peptides respectively, are illustrated in Appendix 2. The L^{pro}-coding region displayed greater nucleotide variability *i.e.* 66.2% as compared to 46.7% and 47.9%, observed for the P2- and P3-coding regions, respectively.

Phylogenetic analyses of the L^{pro}-coding region and the P3-coding regions for the sub-Saharan African FMDV included sequences of two recent SAT2 viruses from the 2012 FMD outbreak in Egypt and the Middle East (Valdazo-González *et al.*, 2012) obtained from GenBank (JX014255 and JX014256). The Maximum-Likelihood, Neighbour Joining, Minimum Evolution, and Maximum Parsimony methods yielded trees with almost similar topologies. Described below are midpoint rooted dendograms based on Maximum-likelihood methods, while the phylogeny depicted by the other methods is illustrated in Appendix 3.

Based on a cut-off value of less than 16% nucleotide difference, three non-serotype specific clusters were observed for the L^{pro}-coding region (Fig. 2.1) and four clusters for the P3-coding regions (Fig. 2.2), supported by strong bootstrap values (over 75%). Based on phylogeny of the L^{pro}- and P3-coding regions, the SAT1 and SAT2 viruses from southern Africa *i.e.* Angola, Botswana, Malawi, Mozambique, Namibia, Zambia, Zimbabwe and South Africa and the SAT3 viruses (from southern and East Africa), as well as two isolates from Kenya (SAT1/KEN/5/98 and SAT2/KEN/8/99) and one from Tanzania (SAT1/TAN/1/99), grouped together (*i.e.* cluster I; Fig. 2.1 and Fig. 2.2). The A and O type viruses that originated from Côte d'Ivoire, Senegal (western Africa), Eritrea, Ethiopia, Kenya, Somalia, Sudan, Tanzania and Uganda (eastern Africa), grouped together with SAT1 and SAT2 viruses from Eritrea, Nigeria, Rwanda, Sudan and Saudi Arabia (*i.e.* cluster II; Fig. 2.1 and 2.2). Three SAT2 viruses, two from Kenya (KEN/3/57 and KEN/11/60), and one from Uganda (UGA/MBF/4/02) were also found in cluster II, based on L^{pro} and P3 phylogeny (Fig. 2.1 and 2.2). It is interesting to note that three SAT2 viruses from Senegal and Ghana in West Africa (SAT2/SEN/5/75, SAT2/SEN/7/83, and

SAT2/GHA/8/91) and a SAT1 virus from Uganda (SAT1/UGA/3/99) formed a strongly supported cluster based on L^{PRO}-sequence phylogeny with 20.4% nucleotide differences within the group (cluster III; Fig. 2.1). Based on the P3-coding region, these West African SAT2 viruses shared a separate cluster III (Fig. 2.2), while three Ugandan isolates grouped together (SAT1/UGA/1/97, SAT1/UGA/3/99 and SAT2/UGA/2/02) (cluster IV; Fig. 2.2). Two virus strains from the recent SAT2 FMD outbreak in Egypt and the Palestinian Autonomous Territories (PAT), (EGY/9/12 and SAT2/PAT/1/12) grouped in cluster II, demonstrating a close genetic relationship with SAT2 viruses from Saudi Arabia and Eritrea (Fig. 2.1 and 2.2).

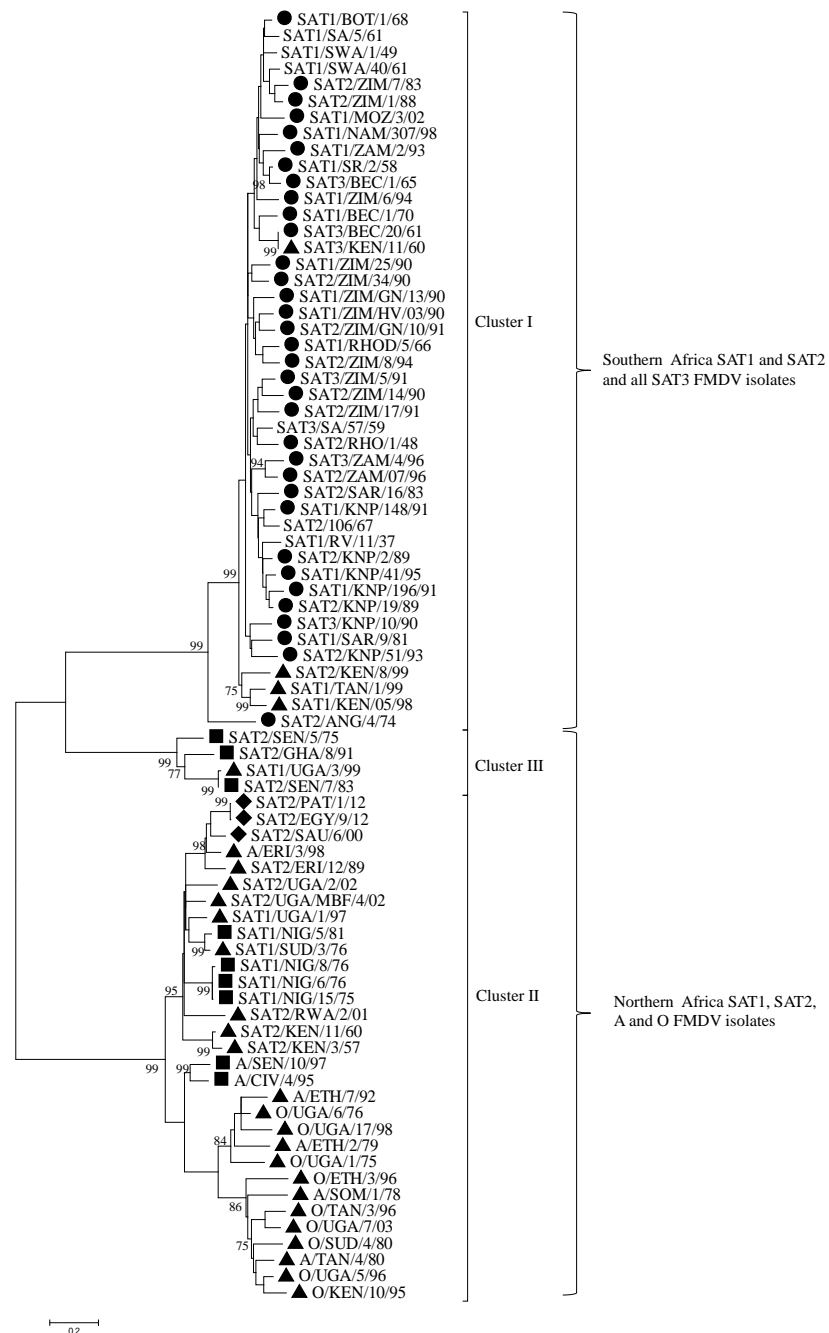


Fig. 2.1 Maximum-Likelihood methods depicting nucleotide relationships between the sub-Saharan African FMDV (SAT1, SAT2, SAT3, A and O) for the Leader-coding region rooted against the mid-point and was constructed using MEGA5 (Tamura *et al.*, 2011). The sub-Saharan southern African viruses are indicated by the closed circles (●) eastern Africa viruses by triangles (▲), western Africa viruses by rectangles (■), while the North African and Middle East viruses by kites (◆). Model assumptions predicted by jModel 2.3 (Darriba *et al.*, 2012) are based on the GIR+I+G (General Time Reversible) model of nucleotide substitution. There was non uniform evolutionary rates among sites modelled using both discrete Gamma distribution(+G) with 4 rate categories and assuming that a certain fraction of sites are evolutionarily invariable (+I). Proportion of invariable sites is 0.2830, gamma shape is 0.795. The scale bar indicates nucleotide substitutions per site. The robustness of the tree topology was assessed using 1000 bootstrap replications.

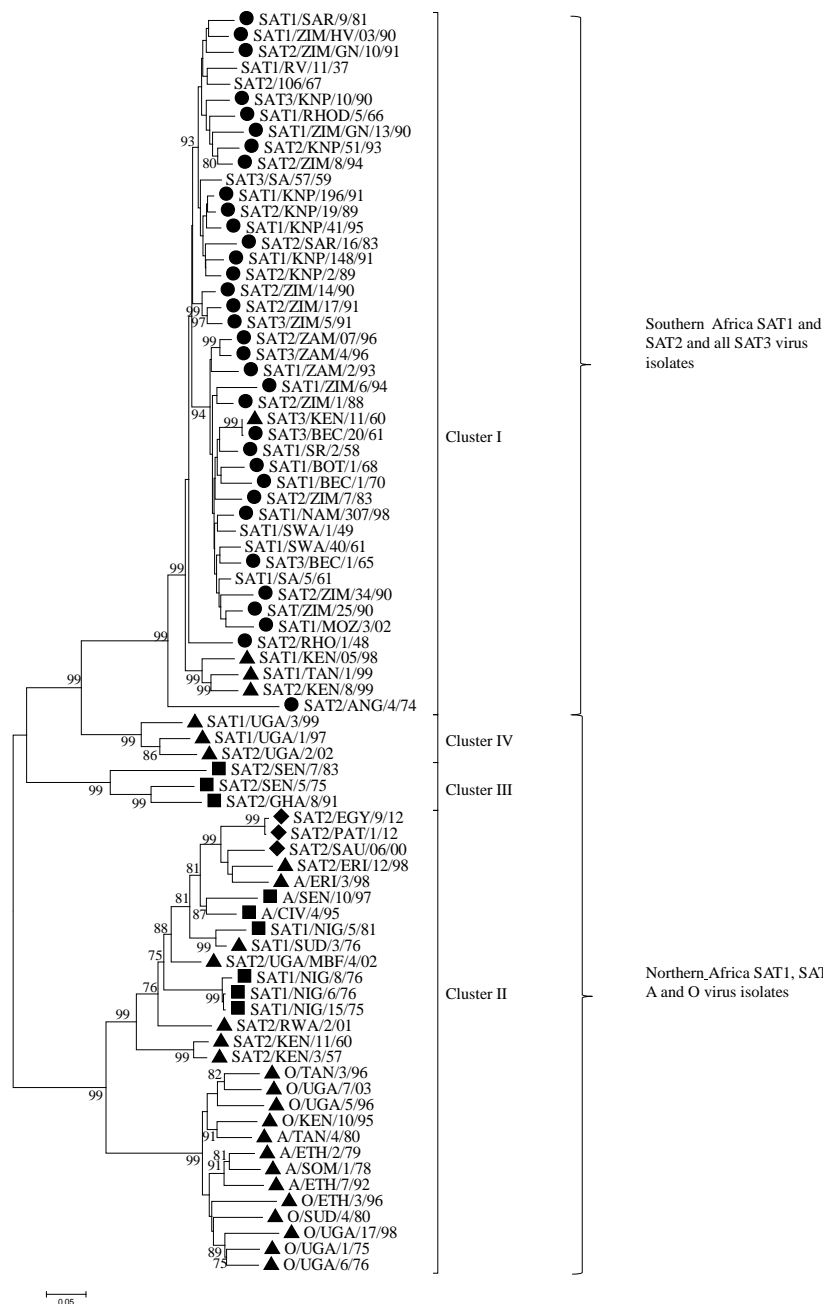


Fig. 2.2 Maximum-Likelihood methods showing nucleotide relationships between the sub-Saharan African FMDV (SAT1, SAT2, SAT3, A and O serotypes) for the P3-coding region. The phylogenetic tree was rooted against the mid-point and was constructed in MEGA5 software (Tamura *et al.*, 2011). The sub-Saharan southern African viruses marked using closed circles (●) eastern Africa viruses with triangles (▲), western Africa viruses with rectangles (■), while the North African and Middle East viruses are marked with kites (◆). The most suitable model for nucleotide pattern substitution was determined by jModel 2.3 (Darriba *et al.*, 2012). It was predicted to be the GIR+I+G (General Time Reversible) model of nucleotide substitution. There was non uniform evolutionary rates among sites modelled using both discrete Gamma distribution(+G) with 4 rate categories and assuming that a certain fraction of sites are evolutionarily invariable (+I). Proportion of invariable sites is 0.428, gamma shape is 0.706. The scale bar indicates nucleotide substitutions per site. Confidence levels of the tree branches were tested using 1000 bootstrap replications.

2.3.2 Amino acid variation in the non-structural proteins of sub-Saharan FMDV

The nucleotide sequences for the non-structural protein-coding regions were translated and the deduced amino acid sequences aligned. A summary of the variability in the non-structural proteins for the African FMDV is summarized in Table 2.3 and discussed subsequently.

Table: 2.3 Variability in aligned nucleotides and the deduced amino acids for the non-structural proteins in the genome for sub-Saharan FMDV.

Region in Genome	No. of nucleotide positions aligned	No. of variant nucleotides	% of variant nucleotides	No. of residue positions aligned	No. of variant residues	% of variant residues
L ^{pro}	606	401	66.2%	202	124	61.4
2A	54	30	55.5%	18	9	50
2B	462	200	43.3%	154	47	30.5
2C	954	455	47.7%	318	110	34.6
3A	459	267	58.2%	153	86	56
3B ₁₂₃	213	120	56.3%	71	38	53.5
3C ^{pro}	639	314	49.1%	213	89	41.7
3D ^{pol}	1410	553	39.2%	470	136	29

2.3.2.1 Leader protease

The L^{pro}, 199-202 residues in length, was the most variable of the non-structural proteins with 61.4% variable amino acid positions in a complete alignment of the 79 sub-Saharan FMDV sequences (Fig. A; supplementary data, Appendix 2). All the SAT sequences and one serotype A virus (ERI/3/98) contained a three amino acid deletion between residues 24 and 28. At least 35.6% of the positions in viruses from southern Africa (Angola, Botswana, Mozambique, Namibia, Tanzania, Zambia, Zimbabwe, and South Africa (*i.e.* cluster I; Fig. 2.1)), were variable. Similarly 38.6% of the positions in the SAT viruses from northern Africa *i.e.* Egypt, Eritrea, Ghana, Nigeria, Senegal, Sudan, Rwanda and Uganda (clusters II and III combined), demonstrated variability. The critical residues used for autocatalysis of the L-VP4, namely C51, H148 and D164, (Guarné *et al.*, 1998), were conserved in all the isolates. According to the numbering in this study, (Fig A; supplementary data, Appendix 2) the catalytic triad is presented as C52, H149 and D165. The residue E77 was conserved in the alignment of the African FMDV

with the exception of SAT2/RWA/2/01 which showed an E77K substitution. The residue H110 in this study was conserved in the 13 SAT isolates from East and western Africa (SAT1: UGA/1/97, NIG/6/76, NIG/8/76, NIG/15/75, NIG/5/81, SUD/3/76; SAT2: SAU/6/00, RWA/2/01, ERI/12/98, UGA/2/02, UGA/MBF/4/02, KEN/3/57, KEN/11/60 (cluster II; Fig. 2.1)). All the SAT viruses from southern Africa (cluster I; Fig. 2.1), except SAT1/KNP/196/91, demonstrated a H110D substitution. For the latter, there was a H110G substitution instead (Fig. A; supplementary data, Appendix 2). Among the non-structural proteins entropy was highest for the L^{pro}, where 29 residue positions had entropy values of 1.0-1.9; especially towards the N-terminus (residues 4-24).

2.3.2.2 Peptides encoded by the P2-coding region

The 2A peptide of 18 amino acids in length (Table 2.4) was the most conserved of the three FMDV encoded proteases with 56% (10 of 18 residues) conserved residues for SAT viruses, irrespective of the geographic location. At least 83% of residues (15 of 18 residues) in a complete alignment of the 2A sequences of African A and O viruses were identical. The C-terminal 13 amino acids of 2A, with the sequence LLKLAGDVESNPG, were highly conserved for all the 79 African viruses. However, a residue substitution, D12N, in the conserved ¹²DVEXNPG¹⁸ motif (Ryan *et al.*, 2004; Carrillo *et al.*, 2005) was observed in two SAT1 viruses from Uganda and Nigeria (SAT1/UGA/1/97 and SAT1/NIG/6/76). The N-terminal residue position 2 of the 2A protein showed most variation with an entropy value of 1.1 (Table 2.4).

Table 2.4 Variation in 2A in a complete alignment of sub-Saharan African FMDV isolates.

2A	Deduced amino acid sequence																	
	Residue position																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
SAT types	L ₅₄	C ₄₇	N ₆₀	F ₅₉	D ₅₇	L ₆₁	L ₆₁	K ₆₀	L ₆₀	A ₆₁	G ₆₁	D ₅₉	V ₆₁	E ₆₁	S ₆₁	N ₆₁	P ₆₁	G ₆₁
	M ₄	A ₆	S ₁	Y ₁	E ₂			M ₁	Q ₁			N ₂						
	T ₃	L ₅		C ₁	A ₂													
		F ₁																
		G ₁																
		S ₁																
O and A- types	<i>L₁₄</i>	<i>L₁₆</i>	<i>N₁₆</i>	<i>F₁₄</i>	<i>D₁₆</i>	<i>L₁₆</i>	<i>L₁₆</i>	<i>K₁₆</i>	<i>L₁₆</i>	<i>A₁₆</i>	<i>G₁₆</i>	<i>D₁₆</i>	<i>V₁₆</i>	<i>E₁₆</i>	<i>S₁₄</i>	<i>N₁₆</i>	<i>P₁₆</i>	<i>G₁₆</i>
	<i>S₁</i>			<i>Y₂</i>											<i>L₁</i>			
	<i>T₁</i>																	<i>P₁</i>

The individual columns represent the deduced amino acid alignment for the 2A for 79 sub-Saharan virus isolates. The consensus sequence for SAT types is given in the 1st row (in normal text) while the consensus for the O and A-types is indicated in the bottom row (in italics). Alternative amino acids occurring in the same position are indicated in the subsequent rows below each consensus. The numerals in the subscripts indicate the number of viruses with that particular amino acid.

The 2B protein (154 amino acids) contained no insertions or deletions and showed 69.5% conserved residues in a complete alignment of all the sub-Saharan FMDV sequences included in this study. Most of the variation was found in two hypervariable domains in the N-terminal half of the protein, i.e. residues 5-29 and 44-53, while the sequence ⁶⁴IKLLSRLSCMAA VAAR(S/A)KDPVLVAIMLADTGLEILDSTFVVKKI¹⁰⁷ was highly conserved among all the sub-Saharan sequences. Another conserved motif, located between residues 115 and 137 (FHVPAPVFSFGAPILLAGLVKVA) contained a hydrophobic domain. Entropy values of 1.0-1.5 were observed at four residue positions (20, 23, 45 and 52) at the N-terminus of the protein.

The FMDV 2C protein is an AAA+ ATPase with RNA binding activity (Sweeney *et al.*, 2010), and was found to be 318 amino acids in length; mostly hydrophilic towards the C-terminus and contained 65.4% invariant residue positions when sequences of isolates in this study were compared. Most variation was focussed in two hypervariable regions, one in the N-terminus (residue 35-101) and one in the C-terminus (258-306) of the protein (Fig. C; supplementary data, Appendix 2). Entropy values of 1.0-1.2 occurred at three residue positions (83, 92, and 291). A highly conserved, hydrophobic motif was present between residues 17-34, i.e. ¹⁷EWLVKLILAIRDWIKAWI³⁴. This amphipathic helix is most probably involved in the

attachment of 2C to the membrane (Echeverri & Dasgupta, 1995). Conserved residues at positions 110-117 (GKSGQGKS), 156-161 (VVVMDD) and 201-207 (VIIATTN) included the characteristic Walker A, Walker B and C motifs of an AAA+ ATPase (Sweeney *et al.*, 2010). However, the C motif for southern Africa SAT viruses displayed a conservative T206S (n=68) substitution while I203V (n=2) and I202L (n=1) substitutions were also observed (Fig. C; supplementary data, Appendix 2). There were residues unique to the SAT1, SAT2 and SAT3 viruses originating from the larger southern African region (Angola, Botswana, Mozambique Namibia, South Africa, Tanzania, Zambia and Zimbabwe). These sequences were located within the N-terminal 100 amino acids of 2C, i.e. residue positions 40-42 (YIS) in the southern African SATs (n=34) as compared to FVT in SATs of the larger northern Africa (Sudan, Ethiopia, Eritrea, Nigeria, Senegal and Ghana (n=11)). Other positions of unique substitutions for the southern SATs are G49R, K53C, D56N, A66S, D71E, Q75E and V92N (Fig. C; supplementary data, Appendix 2).

2.3.2.3 Peptides encoded by the P3-coding region

The 3A protein is 152-153 amino acids in length. Viral genomes of the all southern and the Ugandan SAT serotypes, (clusters I and III; Fig. 2.2) generally encoded a 3A protein one residue shorter compared to the SAT viruses from West Africa and all the African A and O serotypes in cluster II (Fig. 2.2) with a deletion at amino acid position 148 (Fig. D; supplementary data, Appendix 2). An exception is the 3A protein of the SAT2 virus, SEN/7/83, that contained a deletion of 11 amino acids in the C-terminus, between residues 134 and 146. In the total alignment, at least 56% of residue positions were variable, while 34.6% were variable for the SATs from the southern part of Africa (Angola, Botswana, Mozambique Namibia, South Africa, Tanzania, Zambia and Zimbabwe) and 45.7% for the East Africa SAT1, SAT2, A and O viruses (Kenya, Tanzania, Rwanda and Uganda). The N-terminal 41 amino acids were relatively conserved and contained two hydrophobic domains, i.e. ¹ISIPSQKSVLYFLIEK¹⁶ and ²⁵FYEGMV³⁰, while a third hydrophobic domain was located between residues 60 to 74 (⁶⁰EIVALVVLLANIII⁷⁴). The C-terminus was highly variable, with nine residue positions that showed entropy values of 1.0-1.3.

The three copies of 3B varied in length between 23 (3B₁) to 24 (3B₂ and 3B₃) residues demonstrating 53.5% overall variability, whilst each copy varied by 56.5%, 50% and 54.2%,

respectively (Table 2.5). The 3B proteins of the southern African SAT1, SAT2 and SAT3 viruses (n=59) were more similar with 31% variation, while SAT1 and SAT2 viruses (n=9) from Nigeria, Senegal, Ghana, Ethiopia and Eritrea showed 36.6% variability. Nine of the eleven SAT1 and SAT2 viruses in cluster II (Fig. 2.2) from western (Nigeria) and eastern Africa (Ethiopia, Eritrea, Kenya and Uganda) had residues in common with serotype A and O viruses (Table 2.5). The N-terminal motif, GPYXGP (where X is any amino acid), was conserved for all the sub-Saharan viruses

Table 2.5 A complete alignment of the 3B₁₂₃ (VPg) protein for the sub-Saharan African FMDV isolates.

Residue position																								
Deduced amino acid sequence																								
3B ₁	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	
SAT types	G ₅₉	P ₅₉	Y ₅₉	A ₅₇ V ₁ T ₁	G ₅₉	P ₅₉	L ₄₈ M ₈ V ₂ F ₁	E ₅₈ D ₁	R ₅₉	Q ₅₈ H ₁	Q ₄₈ K ₉ R ₁ T ₁	P ₅₉	L ₅₉	K ₅₉	L ₄₉ V ₁₀	K ₅₆ R ₂ Q ₁ V ₁	A ₄₈ T ₁₀ V ₁	K ₅₇ R ₂	L ₅₈ P ₁	P ₅₉	Q ₂₇ K ₂₂ R ₅ L ₂	A ₄₈ Q ₁₁	E ₅₉	
<i>O and A types</i>	<i>G</i> ₁₆	<i>P</i> ₁₆	<i>Y</i> ₁₆	A ₁₀ S ₂ T ₂ V ₂	<i>G</i> ₁₆	<i>P</i> ₁₆	L ₁₂ M ₃ F ₁	E ₁₆	R ₁₆	Q ₁₆	K ₁₅ P ₁₆ Q ₁	L ₁₆	K ₁₅ R ₁	V ₁₆	K ₁₁ R ₅	A ₁₄	K ₁₅ R ₁	L ₁₅ P ₁	P ₁₆	Q ₁₄ L ₂	Q ₁₆	E ₁₆		
3B ₂	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
SAT types	G ₅₉	P ₅₉	Y ₅₉	A ₅₇ V ₂	G ₅₈ R ₁	P ₅₉	L ₄₉ M ₈ T ₁ V ₁	E ₅₉	K ₃₇ R ₂₂	Q ₅₉ K ₁	Q ₅₈	P ₅₉	L ₅₉	K ₅₇ R ₁ E ₁	L ₄₉ V ₉ F ₁	K ₅₅ R ₄	A ₄₅ T ₁₀ V ₂ S ₁ E ₁	K ₄₆ R ₁₄	L ₄₉ A ₅ P ₄ G ₁	P ₅₉	V ₅₉	A ₄₉ V ₁₀	K ₅₉	E ₅₉
<i>O and A types</i>	<i>G</i> ₁₆	<i>P</i> ₁₆	<i>Y</i> ₁₆	A ₁₆	<i>G</i> ₁₆	<i>P</i> ₁₆	M ₁₆ E ₁₆	R ₁₅ K ₁	Q ₁₆	K ₁₂ Q ₄	P ₁₆	L ₁₆	K ₁₆ R ₂	V ₁₆	K ₁₃ R ₂ T ₁	A ₉ V ₇	K ₁₅ R ₁	A ₁₄ T ₁ V ₁	P ₁₆	V ₁₆	V ₁₄ A ₁ T ₁	K ₁₆	E ₁₆	
3B ₃	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	24	24
SAT types	G ₅₉	P ₅₉	Y ₅₉	E ₅₈ D ₁	G ₅₉	P ₅₉	V ₅₈ L ₁	K ₅₉	K ₅₉	P ₅₉	V ₅₆ A ₃	A ₅₉	L ₅₉	K ₅₉	V ₅₇ E ₁ L ₁	K ₅₈ R ₁	A ₅₅ T ₄	K ₅₃ R ₆	A ₄₁ N ₁₀ S ₄ P ₃ T ₁	P ₄₃ M ₉ A ₃ L ₂ V ₂	I ₅₉	V ₅₇ I ₂	T ₅₉	E ₅₉
<i>O and A types</i>	<i>G</i> ₁₆	<i>P</i> ₁₆	<i>Y</i> ₁₆	E ₁₆	<i>G</i> ₁₆	<i>P</i> ₁₆	V ₁₆ L ₁	K ₁₆	K ₁₆	P ₁₆	V ₁₅ A ₁	A ₁₆	L ₁₆	K ₁₆	V ₁₆	K ₁₆	A ₁₆	K ₁₆	N ₁₆	L ₁₃ M ₃	I ₁₆	V ₁₆ I ₂	T ₁₆	E ₁₆

The individual columns represent the deduced amino acid alignment. For each copy of the 3B peptide, the consensus amino acids in the alignment are given in the top row for the SAT types (in normal print) and the complete alignments of A and O 3B sequences (in Italics). Amino acids substitutions that occurred are indicated in the subsequent rows, and the numerals in subscripts indicating the frequency of occurrence of a particular amino acid. The conserved GPYXGP motif (where X is any amino acid) is indicated in bold script.

The 3C^{pro}-coding region translated into 213 amino acids with 41.7% variable positions in an overall alignment (Fig. E; supplementary data, Appendix 2). Forty-two variable residues (19.7%) were observed in the southern African SAT viruses, 35 (16.4%) in the East Africa (Kenya, Uganda, Tanzania and Rwanda) SAT1, SAT2, A and O viruses, 5 variable residues (2.3%) in the West Africa (Ghana and Senegal) SAT2 isolates and only 2 variable residues (<1%) was found

among the four Ugandan SAT1 and SAT2 isolates. The variation was not random, but defined in hyper-variable regions separated by highly conserved residues. The conserved residues point towards the significant contribution of these residues to structural and/or functional constraints. The N-terminal 60 amino acids of 3C^{pro}, especially 17-48 [¹⁷(K/R)PVELILDGK(T/I)VA(L/D)CCATGVFGTAYLVPRHLF⁴⁸], were highly conserved and constituted a hydrophobic domain ²⁷(T/I)VA(L/D)CCATGVFGTAYLV⁴⁴. Other conserved domains included ⁶⁶D(F/Y)RVFEFE(V/D)KVKGQ(D/E)M(L/M)SDAAL(M/R)(V/I)L(H/N)⁹¹, ¹²⁹FSG(D/E)ALTYKD(L/V/I)VVCMDGDTMPGLFAY(R/K)A¹⁵⁶ and ¹⁶¹GYCG¹⁶⁴. Residue substitutions found in these regions were mostly conservative mutations. The active triad of 3C^{pro}, consisting of residues H46, D84 and C163 (Birtley *et al.*, 2005), showed complete conservation except in SAT3/ZIM/5/91 where D84Y substitution was observed. Interestingly, the sequence ¹⁰⁷MKLSKGS¹¹³ in southern African SAT viruses (cluster I; Fig. 2.2) distinguished them from the northern African lineages (cluster II; Fig. 2.2) that had the sequence ¹⁰⁷ARM(R/K)KGT¹¹³. Notably, the four viruses from the Ugandan lineage (cluster IV; Fig. 2.2) displayed ¹⁰⁷VRVAKGN¹¹³ and ¹⁶⁹TKSGSQ¹⁷⁴ potentially specific to this lineage (Fig. E; supplementary data, Appendix 2).

The 3D^{pol} was found to be the most conserved of all the non-structural proteins. The 470 amino acid peptide, the longest of the non-structural proteins, demonstrated 29% variable residues (Fig. F; supplementary data, Appendix 2). The variation was 25% when only the three SAT serotypes (n=67) were considered. The results presented here suggest an average of 15%, 18.5%, 3.2%, 9.8% and 10.2% of variable residues for 3D^{pol} of SAT1, SAT2, SAT3 and serotypes A and O, respectively. The 3D^{pol} variation was not limited to certain areas as seen for 3C^{pro}. Previously five conserved motifs were described for 3D^{pol} of FMDV (Doherty *et al.*, 1999; Ferrer-Orta *et al.*, 2004). The ²⁴⁰DYSAFD²⁴⁵, ²⁹⁷PSG²⁹⁹, ³³⁶YGDD³³⁹ and ³⁸⁵FLKR³⁸⁸ motifs were conserved in all the sub-Saharan FMDV sequences included here (Fig. F; supplementary data, Appendix 2). However, the ¹⁶⁴KDELR¹⁶⁸ motif was present in the A, O and SAT sequences either as KDEIR or KDEVR. Although the sequences analysed here showed some variability in the three hypervariable regions that were previously described for 3D^{pol} (George *et al.*, 2001), it was mostly one or two possible residue substitutions occurring at 14 out of the 23 variable residue positions among the sequences. The 3D^{pol} hypervariable region, between residues 143-154,

showed the highest entropy of 1.15 as a result of 4 possible residues at position 145. All other entropy were less than 0.64.

2.3.3 Structural implications of the non-structural protein variation

Crystallography studies suggest FMDV 3C^{pro} typically consists of two β -barrel domains, each composed by eight anti-parallel β -sheets (Birtley *et al.*, 2005). Upon alignment of the 79 sequences in this study, considerable variation was observed in the amino acid sequences of the β -sheets. However, these were mostly conservative mutations (Fig. 2.3). The substrate (S1) pocket include residues from the β C₂- β D₂ loop (residues 154-160) and the C-terminal end of the β E₂ strand (residues 181-186) and were conserved in this study. The sequence of the S1 pocket, that interact with the P1 amino acid side chain of the substrate, are ¹⁵⁴Y(R/K)A(G/A)TK(V/A)¹⁶⁰ and ¹⁸¹HSAGGN¹⁸⁶. The amino acid variation in the African FMDV 3C^{pro} was almost entirely surface located and directed away from the substrate binding site (Fig. 2.3).

Opposite the active site of the protease, exposed on the surface of the 3C^{pro} was a local region of basic amino acids of which conserved residues R95 and R97 were exposed on the surface (Fig. 2.3E & F). This positively-charged motif was highly conserved for the African A, O and northern African SAT viruses as ⁹⁵RVRDI⁹⁹. The sequence for the southern Africa SAT viruses displayed a characteristic I99L substitution. One SAT1 virus, NIG/5/81, had a characteristic ⁹⁵RVSVI⁹⁹ sequence (Fig. E; supplementary data, Appendix 2). An interesting observation was that the conserved R92 (Nayak *et al.*, 2006), although present in African A, O and northern African SAT viruses, was replaced by either an S or T in all the southern African SAT types. Similarly residue K101 was substituted with a G or A in the southern African SAT viruses (Fig. E; supplementary data, Appendix 2).

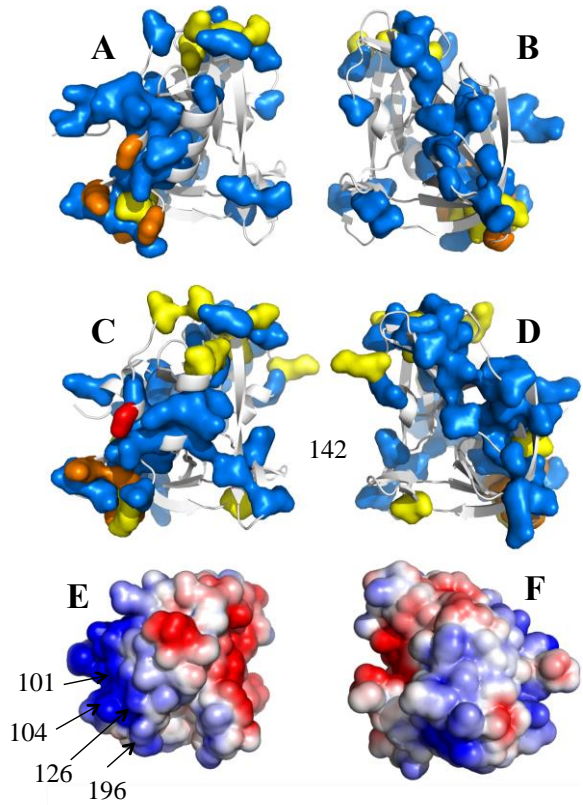


Fig. 2.3 Structural representation of FMDV 3C^{pro}. The position of amino acid variation observed in a complete alignment of all African SAT1 (A & B) and SAT2 (C & D) viruses is mapped on the modelled structure. Two possible residues at a position are indicated in blue, three residues in yellow, four in orange and five in red. The surface electrostatic potential is presented for SAT1/SAR/9/81 3C^{pro} (E & F). The electrostatic potential was coloured with positive charge as blue and negative in red and the scale of colouring was kept constant. The electrostatic potential is conserved in viruses across the five serotypes from Africa

Structural modelling was provided by courtesy of T.A.P. de Beer, European Bioinformatics Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge, CB10 1SD, United Kingdom

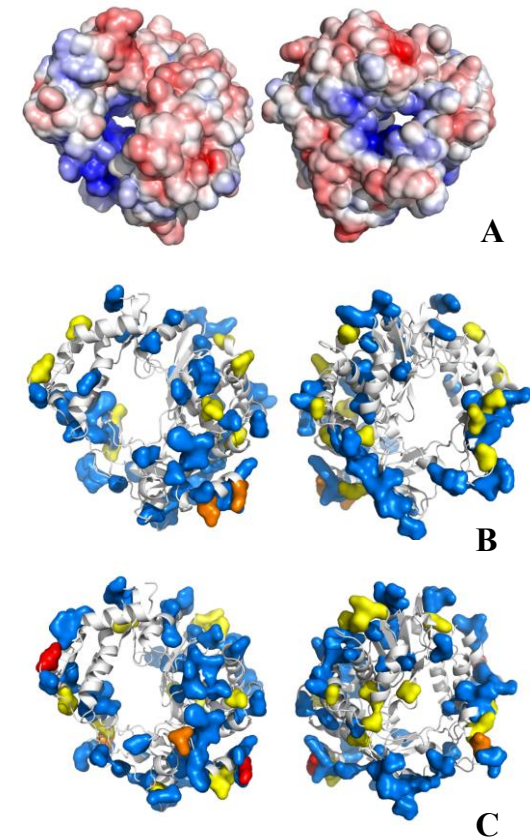


Fig. 2.4 Variation in the 3D^{pol} protein, observed in a complete alignment of African FMDV sequences, has been mapped to the three-dimensional structure of the protein. The electrostatic surface potential is shown with positive charge as blue and negative in red (A). The electrostatic potential is conserved in viruses across the five serotypes from Africa. The variable amino acid positions observed for SAT1 (B) and SAT2 (C) viruses were indicated with two possible residues at a position as blue, three residues as yellow, four as orange and five as red. The orientation of the protein was kept the same

The FMDV 3D^{pol} has a structure similar to that of other RDRP's. Analysis of the variation in relation to the structure indicated that, although conserved, 3D^{pol} was more tolerant to substitutions than previously found (Koonin, 1991; Carrillo *et al.*, 2005). The variation seemed to be limited to the outer edges of the protein, was mostly conservative substitutions and seldom involved more than three possible substitutions in an amino acid position (Fig. 2.4).

2.4. DISCUSSION

The FMDV genome exhibits a quasispecies nature like many RNA viruses resulting in genetic drift due to error prone replication and recombination (Domingo *et al.*, 1992; Eigen, 1996; Holland & Domingo, 1998; Heath *et al.*, 2006). Only limited numbers of non-deleterious mutations can occur in regions of functional conservation in the non-structural proteins like the 2A, 2B, 2C, 3A, 3C^{pro} and 3D^{pol} of FMDV (Tully & Fares, 2009). This preserves both the integrity of structure and function especially of the major enzymes, like the RDRP (Domingo *et al.*, 1990; Domingo *et al.*, 1992). In this study we present the coding regions for the non-structural proteins of 79 FMDV isolates from sub-Saharan Africa (SAT1, 2, 3, A and O types).

The phylogenetic comparisons of nucleotides coding the Leader- and P3-regions, both generated a separate cluster for the southern Africa SAT viruses and the northern Africa SAT, A and O viruses. Three SAT2 viruses from Senegal and Ghana and three isolates from Uganda did not conform to the phylogenetic pattern, forming two separate clusters based on the P3-coding region. However, SAT1/UGA/1/97 and SAT2/UGA/2/02 grouped with A, O and northern Africa SAT viruses in the Leader analysis, implicating intertypic recombination (Jackson *et al.*, 2007) which warrants further investigation.

We observed variation in the deduced amino acid sequence alignments for the eight FMDV non-structural proteins (L^{pro}, 2A, 2B, 2C, 3A, 3B₁₂₃, 3C^{pro}, 3D^{pol}), that ranged from 61.4% for L^{pro}, 56% for 3A, 53.5% for 3B₁₂₃, 50% for 2A, 41.7% for 3C^{pro}, 34.6% for 2C, 30.5% for 2B and 29% for 3D^{pol}. Noteworthy in this study is that the L^{pro} is the most variable among the non-structural proteins in the place of 3A previously reported by Carrillo *et al.*, (2005).

The L^{pro}, which is the first protein to be synthesized, cleaves itself from the rest of the growing polypeptide (Strebel & Beck, 1986; Belsham & Brangwyn, 1990) before cleaving the eukaryotic

translation initiation factor eIF-4G (Piccone *et al.*, 1995; Guarné *et al.*, 1998). Despite the high variability observed (61.4%), residue conservation among the African FMDV is maintained amongst the essential auto-catalytic residues (C52, H149 and D165). Residues involved in substrate specificity were mapped to D50, D164 and D165 for the type O Lb^{pro} (Guarné *et al.*, 2000) and two of the residues (D164 and D165) were conserved for all the isolates investigated. Lb^{pro} from SAT type viruses originating from southern Africa contains a D50G substitution. Amidst the apparent conservation in the non-structural proteins were substitutions causing charge differences in residues of traditional conservation. In the L^{pro}, E77→K, (n=1) occurred in a SAT2 virus; a similar finding has only so far been reported for A/Phillipines/75 (Carrillo *et al.*, 2005). A residue K199 is reported in Guarné *et al.*, (1998) and in this study the concerned residue is found at position 200. Correspondingly, a K200→F substitution was unique to southern Africa viruses while SAT1 and SAT2 viruses from northern Africa showed a K200→Y substitution. The K200 forms weak interactions with E94 and E97 in the crystal structure of a type O virus (Guarné *et al.*, 1998; Guarné *et al.*, 2000). Both residues were replaced with an Asp residue for southern Africa SAT viruses, while an E97K was observed for SAT1/NIG/8/76 and an E97Q for both SAT1/UGA/3/99 and SAT2/SEN/7/83. Whether or not these changes would affect catalysis or the substrate specificity of Lb^{pro}, is unknown.

The 2A protein induces a modification of the cellular translation apparatus resulting in 2A release (Donnelly *et al.*, 2001; Ryan *et al.*, 1991). This is achieved by modifying the activity of the ribosome to promote hydrolysis of the peptidyl (2A)-tRNA^{Gly} ester linkage and the release of the P1-2A precursor in the translational complex (Ryan *et al.*, 1991; Donnelly *et al.*, 2001). We observed only 9 out of the 18 amino acids as invariable in a complete alignment; however the functional domain of ¹²DVEXNPG¹⁸ was conserved indicating structural and functional constraints associated with this domain. 2A is cleaved from the P1 polypeptide by the 3C^{pro} in the later stage of processing and its function as an independent protein is not known.

The small, hydrophobic 2B protein of FMDV associates with the ER and may cause rearrangement of the ER membrane (Moffat *et al.*, 2005; O'Donnell *et al.*, 2011). The hydrophobic motif at residue positions 115-137 is likely to be responsible for positioning 2BC complexes to allow its membrane bound activities at sites of FMDV replication in the ER-derived

vesicles in the host cytoplasm (Grubman & Baxt, 2004; Moffat *et al.*, 2005; Moffat *et al.*, 2007). The hydrophobic character of this domain was highly conserved in the African viruses.

The FMDV 2C protein is an AAA+ ATPase that affects initiation of minus strand RNA synthesis (Sweeney *et al.*, 2010), and localizes with Golgi-derived membrane structures. The three ATPase binding motifs were highly conserved among the African viruses. The interaction of 2C, 3A and a cellular poly (A)-binding protein with the RNA helicase A (RHA) leads to a ribonucleoprotein complex formation at the 5' end of the genome and has been shown to play an important role in FMDV replication (Lawrence & Rieder, 2009). The 2C protein and its precursor, 2BC, induce vesicle formation in the cytoplasm (Moffat *et al.*, 2005). Unique sequences in the 2C peptide for the southern Africa SAT viruses included residues 40-42 (FVT→YIS) and in the Walker C motif, T206→S, I203→V, I202→L which are all conservative substitutions.

The 3A protein is proposed to be the membrane anchor for the picornavirus replication complex (Weber *et al.*, 1996; Xiang *et al.*, 1997). It is associated with viral-induced membrane vesicles and contributes to the cytopathic effect and the inhibition of protein secretion (Doedens & Kirkegaard, 1995; Wessels *et al.*, 2006). The 3A peptide has been associated with virulence in picornaviruses (Giraudou *et al.*, 1990; Heinz & Vance, 1996; Lama *et al.*, 1998; Pacheco *et al.*, 2003), and deletions in this peptide at the C- terminus (133-143), collectively with other genome changes in Asiatic FMDV, correlated to altered host range and slow growth in bovine derived cells but accelerated growth in porcine derived cells (Knowles *et al.*, 2001; Pacheco *et al.*, 2003). Interestingly, a 11 amino acid deletion was observed in the 3A C-terminal region (136-146) of only one virus, SAT2/SEN/7/83, which also did not reach titres of more than 3.0×10^5 pfu/ml after 10 passages in BHK-21 cells (results not shown). The 3A protein is, after the L^{PRO} and VP1, one of the most variable proteins in the FMDV proteome.

Although all three copies of the 3B/VPg protein were present in the African isolates, they were highly variable in the complete alignments. However, the N-terminal motif ¹GPYXGP⁶ was conserved in all the viruses. The VPg protein participates in the initiation of RNA replication and plays a role in the encapsidation of viral RNA (Hogle *et al.*, 1985; Xiang *et al.*, 1997; Barclay *et al.*, 1998). Each of the VPg protein contains a 3Y, which is known to be involved in

phosphodiester linkage to the viral RNA (Forss & Schaller, 1982) and was conserved in the African viruses in this investigation.

The 3C^{pro} is a cysteine protease (Birtley *et al.*, 2005) responsible for catalysing 10 of the 13 proteolytic cleavage events necessary for polyprotein processing (Vakharia *et al.*, 1987; Clarke & Sangar, 1988). In the 3C^{pro}, there were notably two changes to neutral residue substitutions in a conserved motif of a SAT1 virus NIG/5/81, i.e. residues R97→S and D98→V. However, there is conservation of the active triad for the 3C^{pro}, residues H46, D84 and C163 as well as the substrate pocket. The pocket contains a H181 hydrogen bonded with Y154 and T158, which donate hydrogen bonds to the P1 peptide substrate (Birtley *et al.*, 2005), all of which were invariable in the sub-Saharan African viruses. Studies further showed residues 138 to 150 of FMDV 3C^{pro} in a β -ribbon structure that overlies the substrate binding cleft (Sweeney *et al.*, 2007). The C142 at the apex of this loop plays an important role in substrate binding and showed complete conservation in the 3C^{pro} amino acid comparison of the African isolates. Residues located on the surface of the 3C^{pro}, opposite from the catalytic site of the protease, have been shown to be essential for VPg uridylation, which is the first stage in the replication of viral RNA, by binding RNA (Nayak *et al.*, 2005). Although SAT-specific substitutions were observed within this positively charged region, this region was conserved in the 3D structure.

As in other picornaviruses, protein 3D^{pol} is the RDRP responsible for the replication of the RNA genome via negative strand intermediates (Doherty *et al.*, 1999; Ferrer-Orta *et al.*, 2004). The 3D^{pol} was most resistant to variation indicating the importance of conserving the structural and functional integrity of the RDRP. When variation was observed the variable residues were mostly conservative in nature and were pointing away from the active site.

This comparative study of the FMDV non-structural proteins provides an outline into their evolution, sequence variability and common elements among the representative topotypes circulating in the different geographical regions of sub-Saharan Africa. Genetic diversity of the non-structural proteins may be of consequence to control of FMD, as the non-structural proteins are targets for antiviral therapeutics (Curry *et al.*, 2007) or in diagnostic assays for example to differentiate infected from vaccinated animals, or DIVA tests (Mackay, 1998; Clavijo *et al.*, 2004; van Rensburg *et al.*, 2004).

CHAPTER THREE

ADAPTATION OF FOOT-AND-MOUTH DISEASE SAT TYPE VIRUSES TO CELLS IN CULTURE RESULTS IN THE FORMATION OF HEPARIN SULPHATE BINDING SITES AROUND THE FIVE-FOLD PORE OF THE CAPSID

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3.1 INTRODUCTION

Foot-and-mouth disease virus (FMDV) is a small, non-enveloped, icosahedral virus with a polyadenylated, single-stranded, positive-sense RNA genome belonging to the *Aphthovirus* genus of the family *Picornaviridae* (Knowles & Samuel, 2003). The virus capsid comprises 60 copies each of four virus-encoded structural proteins, VP1 to VP4; VP1, VP2 and VP3 form the capsid outer shell, whilst VP4 lines the interior surface (Acharya *et al.*, 1989). FMDV is an important pathogen that causes a highly contagious, vesicular disease affecting cloven-hoofed animals, including cattle, pigs, goats, sheep and buffalo, with severe economic consequences worldwide (Alexandersen & Mowat, 2005).

FMDV naturally infects epithelial cells by adhering to any of four members of the α_v subgroup of the integrin family of cellular receptors, *i.e.* $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_6$ and $\alpha_v\beta_8$ (Berinstein *et al.*, 1995; Jackson *et al.*, 1997; Neff *et al.*, 1998; Jackson *et al.*, 2000; Neff *et al.*, 2000; Jackson *et al.*, 2002; Duque & Baxt, 2003; Jackson *et al.*, 2004). Attachment to these receptors is mediated via a highly conserved Arg-Gly-Asp (RGD) motif (Fox *et al.*, 1989; Baxt & Becker, 1990; Mason *et al.*, 1994; Leippert *et al.*, 1997) located within the structurally disordered β G- β H loop of VP1 (Acharya *et al.*, 1989; Curry *et al.*, 1995; Lea *et al.*, 1995). Following FMDV-receptor interactions, the virus is internalized and the viral genome is released into the cytosol.

While FMDV infection is mediated by the RGD motif, RGD-independent infection can also occur (Baranowski *et al.*, 1998; Zhao *et al.*, 2003; Rieder *et al.*, 2005; Fowler *et al.*, 2010; Maree *et al.*, 2011a). Molecules such as cell-surface glycosaminoglycans (GAGs) have been implicated in FMDV infections in cultured cells and may be involved in RGD-independent infection (Jackson *et al.*, 1996; Sa-Carvalho *et al.*, 1997; Zhao *et al.*, 2003). However, whilst the interactions between FMDV variants and heparin is known at atomic resolution for two strains of virus, the molecular basis of this interaction and mechanism of cell entry to other strains is largely unknown (Fry *et al.*, 1999; Fry *et al.*, 2005).

Control of FMD has been reliant on large-scale vaccinations with whole-virus inactivated vaccines (Bachrach, 1968; Garland, 1999). Available FMD vaccines are chemically inactivated preparations of concentrated, virus infected cell culture supernatants (Office International des Épizooties Terrestrial Manual, 2009) and require that the vaccine strain is adapted and

propagated in cell culture (Amadori *et al.*, 1994; Amadori *et al.*, 1997). This necessitates continuous adaptation of emerging viruses to cell culture whenever a close antigenic relationship to an outbreak virus is required. However, it has been documented that variants of FMDV, virulent to cultured cells, emerge following serial cytolitic infections (Charpentier *et al.*, 1996; Sevilla & Domingo, 1996; Martinez *et al.*, 1997). The culture-adapted, virulent FMDV displayed a shorter replication cycle in BHK-21 cells and an enhanced ability to kill cells compared to the parental field virus (Sevilla & Domingo, 1996). It is thought that FMDV adaptation to cell culture is made possible by the selective pressure of the viral quasi-species, exerted by the cell surface molecules, which may act as virus receptors (Baranowski *et al.*, 1998). However, it has been noted that field SAT viruses are difficult to adapt to BHK-21 cells, thus hampering to enable large-scale propagation of vaccine antigen (Preston *et al.*, 1982; Pay & Hingley, 1987).

Adaptation of FMDV field isolates to enable efficient replication in cultured cells is accompanied by changes in viral properties, including the acquisition of the ability to bind to alternative cellular receptors such as cell-surface GAGs (Jackson *et al.*, 1996; Sa-Carvalho *et al.*, 1997; Zhao *et al.*, 2003). The interactions of a diverse group of ligands, such as growth factors, chemokines, herpes simplex virus (HSV), human immunodeficiency virus, respiratory syncytial virus, alphaviruses, dengue virus, adeno-associated virus and FMDV, to highly sulfated GAGs is typically via a positively charged domain on these proteins (Patel *et al.*, 1993; Fromm *et al.*, 1995; Jackson *et al.*, 1996; Chen *et al.*, 1997; Krusat & Streckert, 1997; Sa-Carvalho *et al.*, 1997; Byrnes & Griffin, 1998; Klimstra *et al.*, 1998; Summerford & Samulski, 1998; Fry *et al.*, 1999; Zhao *et al.*, 2003; Fry *et al.*, 2005). Zhao *et al.* (2003) reported that cell culture adaptation of serotype O FMDV selects for variant viruses with positively charged residues situated at antigenically relevant positions on the VP3 capsid protein. Whilst the genetic alterations associated with the increase of FMDV virulence during cytolitic passages of SAT viruses in BHK-21 cells are largely unknown, it has been noted that amino acid substitutions accumulate in the capsid of SAT viruses during serial cytolitic passages in cell culture (Maree *et al.*, 2010).

In this report we identify amino acid substitutions within the capsid proteins of SAT1 and SAT2 viruses that are consistent with a binding site at a position close to the icosahedral 5-fold axis, for a moiety of roughly the size and charge of a sulphated GAG. Heparan sulfate proteoglycan (HSPG) is an example of such a GAG. The high serial passaged SAT viruses have a specific

affinity for heparin sulphate (HS) thus implying that binding to cell surface HSPG is most likely required for entry in cultured cells.

3.2 MATERIALS AND METHODS

3.2.1 Cells and viruses

Baby hamster kidney (BHK) cells, strain 21, clone 13 (ATCC CCL-10) were maintained in Eagle's basal medium (BME) (GIBCO[®], Invitrogen[™]), supplemented with 10% (v/v) foetal bovine serum (FBS) and antibiotics using methods described by Sobrino *et al.*, (1983) and Rieder *et al.*, (1993) and were used during virus passage and plaque assays. Wild-type Chinese hamster ovary (CHO) cells, strain K1 (ATCC CCL-61) were grown in Ham's F-12 Nutrient medium (GIBCO[®], Invitrogen[™]) supplemented with 10% FBS and 1% (v/v) antibiotics.

Thirty-one (n=31), FMDV isolates belonging to the South African Territories (SAT) serotypes 1 and 2 were used in this study (Fig. 3.1). The viruses were either supplied by the World Reference Laboratory (WRL) for FMD at the Institute for Animal Health, Pirbright (United Kingdom) or are part of the virus bank at Agriculture Research Council (ARC), Onderstepoort Veterinary Institute (South Africa). The viruses originated from both cattle and wildlife (Impala, *Aepyceros melampus* and African Buffalo, *Syncerus caffer*) and were isolated on either primary pig kidney or bovine thyroid cells followed by amplification on Instituto Biologico Renal Suino-2 (IB-RS-2) cell monolayers (Fig. 3.1). The viruses were serially passaged eight times on BHK-21 cells followed by two passages on CHO-K1 cells. The CHO-K1 monolayers were rinsed with acidic MBS, (25mM *N*-morpholino ethanesulfonic acid, pH 5.5, in 145mM NaCl) to remove residual virus that had not been internalized.

3.2.2 Plaque titration

Titration were performed in standard plaque assays by infecting monolayer BHK-21 or CHO-K1 cells in 35 mm cell culture plates (Nunc[™]) with the respective viruses for 1 h, followed by the addition of a 2 ml tragacanth overlay (Grubman *et al.*, 1979; Rieder *et al.*, 1993). Following incubation for 40 h at 37°C, the overlaid infected monolayers were stained with 1% (w/v) methylene blue in 10% ethanol and 10% formaldehyde in phosphate buffered saline, pH 7.4.

Virus titres were calculated and expressed as the logarithm of the plaque forming units per millilitre (PFU/ml).

3.2.3 RNA extraction, cDNA synthesis, PCR amplification and nucleotide sequencing

RNA was extracted from 200 µl infected cell lysates using a guanidium-based nucleic acid extraction method (Bastos, 1998) and used as a template for cDNA synthesis. Viral cDNA was synthesised with SuperScript IIITM (Life Technologies) and oligonucleotide 2B208R (Bastos *et al.*, 2000). The *ca.* 3.0 kb Leader/capsid-coding regions of the viral isolates were obtained by PCR amplification using Expand Long template *Taq* DNA polymerase (Roche) with SAT genome-specific oligonucleotides (van Rensburg *et al.*, 2002; Maree *et al.*, 2010). The consensus nucleotide sequences of the amplicons were determined using a primer-walking approach and the ABI PRISMTM BigDye Terminator Cycle Sequencing Ready Reaction Kit v3.0 (Perkin Elmer Applied Biosystems). Sequences were compiled and edited using Sequencher 4.7 for windows (Gene Codes). The nucleotide and deduced amino acid sequences were aligned using ClustalX (Thompson *et al.*, 1997) and phylogenetic trees constructed using the minimum evolution algorithm within MEGA4 software (Tamura *et al.*, 2007).

3.2.4 Heparin plaque reduction assay

Virus titres were determined using plaque assays on BHK-21 cells (section 3.2.2) and the (PFU/ml) calculated. Virus dilutions (containing $7 \times 10^7 \pm 2 \times 10^7$ PFU/ml) were prepared in 1× PBS (pH 7.4) and added (1:1) to 1× PBS containing heparin (Sigma-Aldrich[®]). Viruses were incubated for 30 min with heparin in 1× PBS at room temperature. Following incubation, 500 µl of virus-heparin mixture was added to sub-confluent cell monolayers and virus was allowed to attach to the cells for 15 min at room temperature. The cell monolayers were then washed with 1× PBS (pH 7.4) and incubated at 37°C for a further 15 min in BME containing 1% FBS (v/v) and 25 mM HEPES to allow virus internalization. Virus that had not been internalized was removed by washing with 1× PBS (pH 6). Following a final wash step, 2 ml of tragacanth overlay was added and the monolayers incubated at 37°C for 48 h. Subsequently the cells were fixed with formaldehyde and stained with 1% methylene blue. Plaques were counted and determined as PFU/ml. In addition, cells were incubated with heparin for 30 min, prior to addition of virus to ascertain if heparin treated cells support plaque formation as well as untreated cells.

3.2.5 Structural modelling and ligand docking

The structure of FMDV SAT1 (2WZR) (Reeve *et al.*, 2010) was used as the starting point for the production of a model of SAT2. This was built manually using COOT (Emsley & Cowtan, 2004) to avoid clashes between symmetry related protomers and was based on a sequence alignment generated using CLUSTALW (Thompson *et al.*, 1997). Only the local geometry was refined in COOT. Pentameric models were generated for each of the SAT1 and SAT2 structures using the non-crystallographic symmetry. In addition, the cell-adaptive mutations were modelled using COOT to make mutant virus structures for both SAT1 and SAT2. Structures were visualised with PyMol v0.98 (DeLano Scientific LLC) and the electrostatic surface potential was calculated using the APBS module of PyMol. GRID (Goodford, 1985) was used to find the energetically favourable binding site of a heparin sulphate moiety. The GRID calculation was performed within a 20Å radius of the 5-fold axis using pyramidal sulphur as a probe. Five linked disaccharides corresponding to the heparin observed bound to FMDV O1BFS (1QQP) (Fry *et al.*, 1999) were then docked onto a SAT1 pentamer (both the wild-type and cell-adapted model) using GOLD 5.1 (Cambridge Crystallographic Data Centre) with default parameters. The best docking ‘poses’ were judged by manual inspection and assessing the GOLD fitness score.

3.2.6 Nucleotide sequence accession numbers

The sequences obtained in this study have been submitted to GenBank and the accession numbers are shown in Fig. 3.1.

3.3 RESULTS

3.3.1 Multiple serial passages of SAT viruses in cultured cells select for variants with increased virulence in BHK-21 cells

Studies performed with the SAT serotypes of FMDV have exhibited altered viral properties after a few passages in BHK-21 cells (Maree *et al.*, 2010). To further investigate this observation we analysed SAT1 and SAT2 viruses that had been serially passaged in BHK-21 cells.

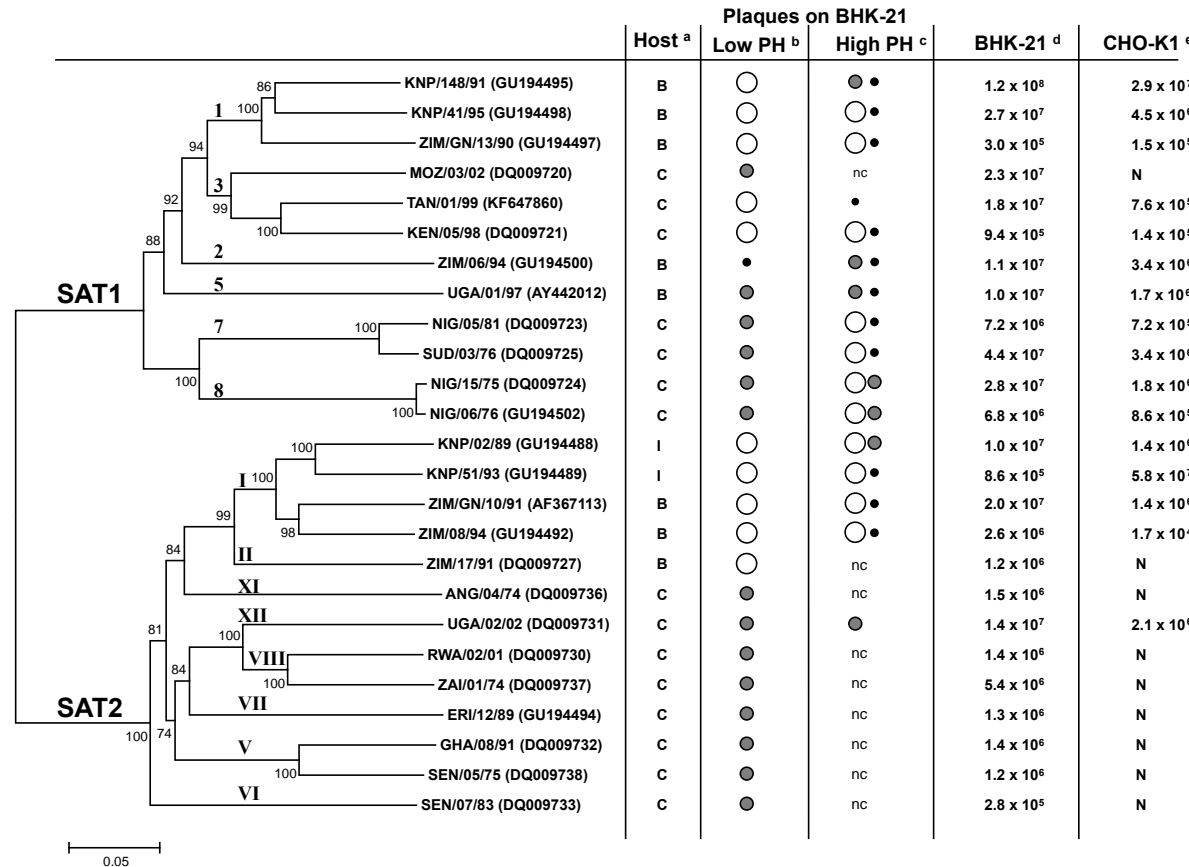


Fig. 3.1 A minimum evolution tree depicting the gene relationships for the P1-coding regions of SAT1 and SAT2 viruses from southern (Kruger National Park, KNP; Zimbabwe, ZIM; Mozambique, MOZ; and Angola, ANG), western (Nigeria, NIG; Senegal, SEN; and Ghana, GHA) and East Africa (Uganda, UGA; Rwanda, RWA; Kenya, KEN; Tanzania, TAN; Eritrea, ERI; and Sudan, SUD).

(a) The host species from which each virus was isolated is indicated by C (cattle), B (buffalo) or I (impala).

(b) The plaque morphologies of the isolate with the lowest available passage history (PH)

(c) The plaque morphology on BHK-21 cell of cell-culture adapted virus following eight serial passages on BHK-21 cells and two serial passages on CHO-K1 cells. Morphologies are indicated as either (O) for plaques larger than 7 mm in diameter on average, (●) plaques of 4-6 mm in diameter or, (●) plaques between 1 and 3 mm in diameter.

(d & e) Virus titres on BHK-21 and CHO-K1 cells following cytotytic passages in cultured cells described in (c). (N) indicates no growth while (nc) indicated no change in plaque morphology.

Recent nucleotide sequence data has facilitated the assignment of FMDV to sero-subtypes or topotypes based on the variability in the capsid-coding region of the RNA genome, which is reflected in their geographic distribution (Samuel & Knowles, 2001). Serial cytopathic passages of SAT1 and SAT2 viruses, belonging to different topotypes and isolated from various host species, resulted in the selection of cell culture-irulent variants. Following eight passages on BHK-21 cells, plaque morphologies changed from medium (4-6 mm) or large (7-8 mm), opaque plaques to the appearance of well defined, medium or small (2-3 mm) plaques (Fig. 3.1 & 3.2). Complete cytopathic effect (CPE) occurred more quickly (data not shown) and the maximum titres observed (Fig. 3.1) were up to 10-fold higher than that of the parental viruses (data not shown). The increased virulence in BHK-21 cells was associated with the ability to infect and replicate in CHO-K1 cells, known to sustain FMDV infection via alternative GAG receptors (Fig. 3.1). The viruses varied in their ability to adapt to BHK-21 cells. A SAT1 virus, ZIM/6/94, acquired the ability to lyse both BHK-21 and CHO-K1 cells, prior to being passaged on BHK-21 cells (after 4 passages on IB-RS-2 cells) (Fig. 3.2). In contrast, other SAT1 and SAT2 viruses (SAT1: KNP/41/95, KEN/5/98, NIG/5/81; SAT2: ZIM/10/91, UGA/2/02, KNP/51/93), infected CHO-K1 cells only after being passaged eight times on BHK-21 cells (Fig. 3.2). Taken together at least 90% of the SAT1 viruses were associated with virulence in BHK-21 and CHO-K1 cells, whilst only 38% of the SAT2 viruses included in this study adopted this phenotype.

To determine how the adaptation process to BHK-21 cells influenced viral growth in these and other cultured cells, we performed plaque assays before and after passaging the viruses on BHK-21 cells. Fig. 3.2 shows the appearance of the small plaque phenotype on BHK-21 cells for SAT1 and SAT2 viruses. In some cases there was a complete transformation to the small plaque phenotype, while in others mixtures of large and small plaques were apparent. There was a direct correlation between the appearance of small plaques on BHK-21 cells and the ability to infect and replicate in CHO-K1 cells.

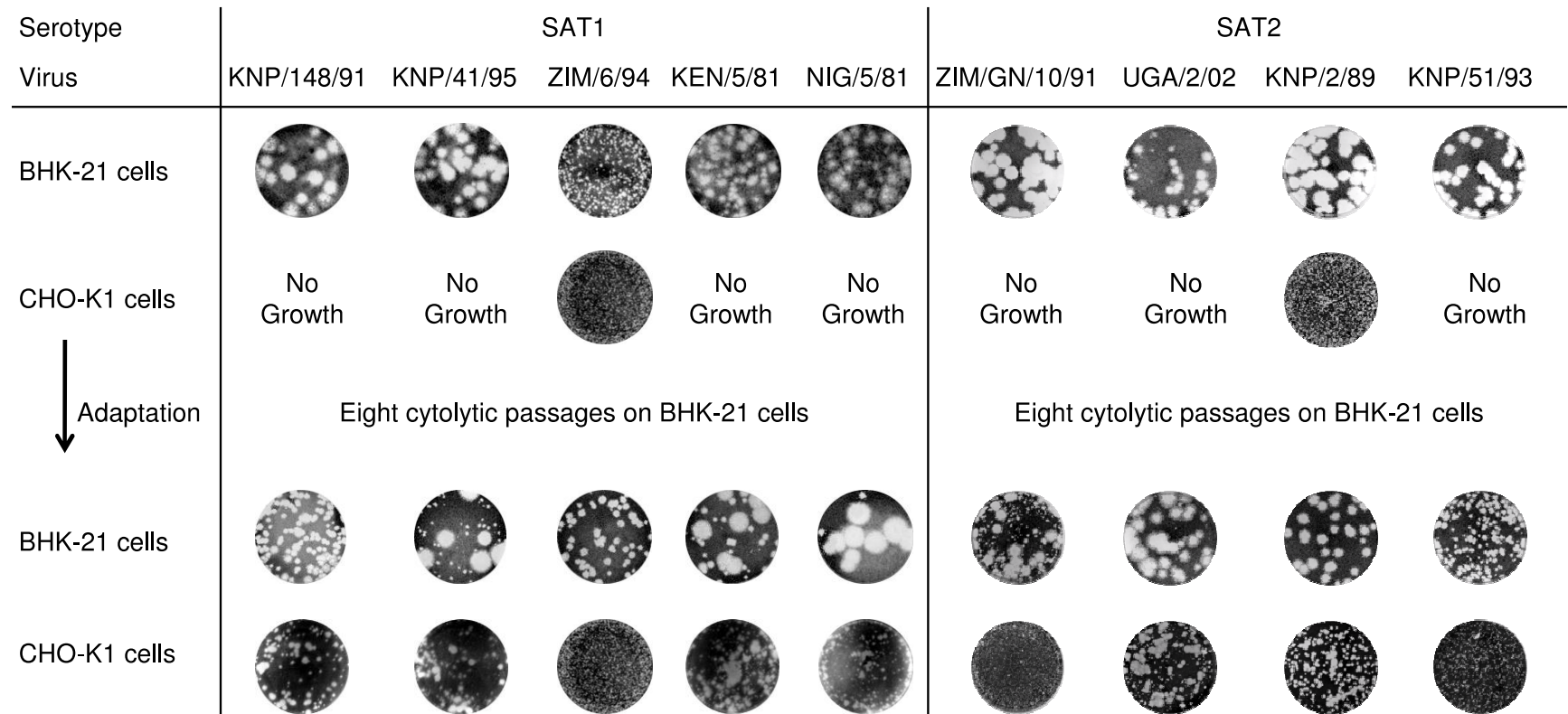
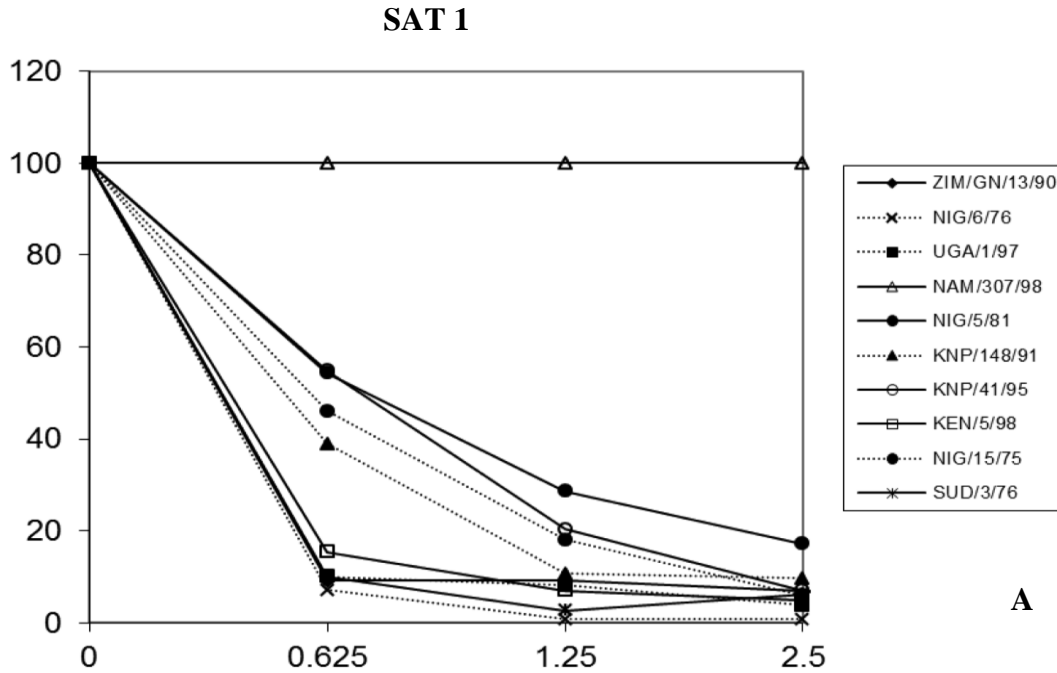


Fig. 3.2 Plaque morphologies of the parental and BHK-21 cell culture derived viruses obtained using monolayers of BHK-21 and CHO-K1 cells. Cells infected with the indicated viral strains were incubated with tragacanth overlay for 40 h prior to staining with 1% methylene blue. Plaques for SAT1 and SAT2 wild-type viruses are generally large with opaque edges and eight cytolitic passages on BHK-21 cells were accompanied by smaller to medium plaques and clear edges. The plaque morphology change was associated with the ability to grow on CHO-K1 cells.

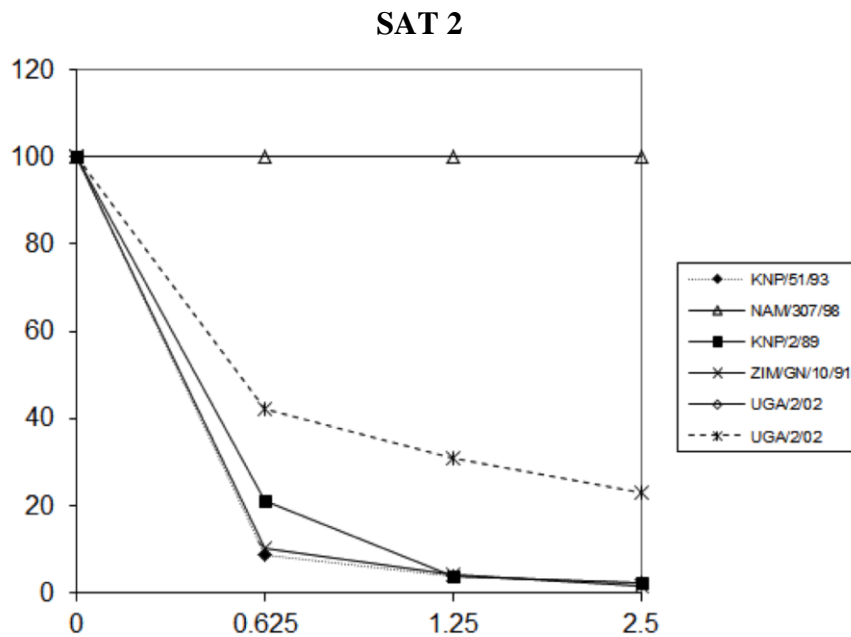
3.3.2 Selection of FMDV variants with increased affinity for heparin

We hypothesized that the changes in the infectivity titres and ability to infect and replicate in CHO-K1 cells were due to more efficient attachment of the adapted virus mutants to the cell surface. These observations suggested that the adapted SAT1 and SAT2 viral mutants might attach to alternative receptors, in particular GAGs. To examine a possible dependence on sulphated proteoglycans during cell entry we used heparin, a soluble form of HS, as a potential inhibitor of virus growth. Following pre-treatment of the viruses with different concentrations of heparin, a rapid decrease in the number of plaques on BHK-21 cells was observed (Fig. 3.3). However, the growth of the control SAT1 virus was not affected by pre-incubation with heparin. The number of plaques was reduced by at least 81% at a concentration of 1.25 mg/ml heparin.

In contrast, treatment with heparin had no effect the number of plaques on BHK-21 cells, irrespective of the heparin concentration, of the control SAT1/NAM/307/98 virus, which naturally does not infect CHO-K1 cells and therefore, does not utilize HSPG as a receptor for infection. The growth of only SAT1 and SAT2 viruses, with the ability to replicate in CHO-K1 cells, was inhibited by an increased concentration of heparin. To validate whether the observed reduction in the number of plaques was a consequence of the interaction between the virus and heparin and not adverse effects of heparin on cell susceptibility, cells were treated with heparin prior to performing plaque assays. Heparin treated and untreated cells supported the same number of plaques.



A



B

Fig. 3.3 Plaque reduction assays for BHK-21 cell-adapted SAT1 (A) and SAT2 (B) viruses following pre-treatment with heparin. Plaques were expressed as a percentage of plaques in the absence of heparin compared to plaques where heparin was present. Each point represents the mean of four repeats. The SAT1/NAM/307/98 virus, with a non-heparin binding phenotype, was included as a control

3.3.3 Analysis of cell culture-adapted SAT virus genomes

Sequence analysis of the SAT1 and SAT2 viruses obtained from eight passages on BHK-21 cells revealed that in general each had acquired three to five amino acid mutations within the outer capsid proteins, VP1, VP2 and VP3 (Table 3.1). The inner VP4 protein remained unchanged in all the viruses. Exceptions include the SAT1 virus, KNP/148/91, which showed 13 amino acid substitutions in the outer capsid proteins, while the SAT2 virus, UGA/2/02 had 7 amino acid substitutions (Table 3.1). SAT1/ZIM/6/94 did not show any amino changes before or after eight passages on BHK-21 cells. A total of 68 amino acid residue substitutions were thus observed between the fourteen SAT1 and SAT2 BHK-21 adapted viruses analysed. The majority (57%, n=39) of these changes occurred in the VP1 protein. The remainder of the residue substitutions occurred in the VP3 (24%, n=16) and VP2 (13%, n=13) proteins. Interestingly, 29% (n=20), of the total amino acid substitutions observed were to positively charged residues located on surface-exposed loops connecting β -strands in VP1 and VP2. The SAT1/SUD/3/76 accumulated three positively charged residues in VP1 (Table 3.1).

The residues in VP2 where positively charged substitutions occurred were Q2074R within the β B- β C loop and E2133K in the β E- β F loop (Table 3.1). The increase in net positive charge in VP1 was most often observed for residues 83, 84 and 85 (33%, n=5) and residues 111 and 112 (53%, n=8). The SAT1/UGA/1/97 virus accumulated positively charged substitutions in a region outside these “hotspots” for mutations. The E1058K change occurred within an existing net positive charge sequence 56-RTEKK-60. The SAT1/KEN/5/98 virus showed a W1087R substitution simultaneously with the E1084K mutation. In the case of SAT1/NIG/5/81 a K appeared at VP1 position 48 (N1048K) simultaneously with the N1111K substitution. In two SAT2 viruses, KNP/2/89 and KNP/51/93, a negatively charged residue located in the β D- β E or β F- β G loops of VP1 mutated to a neutral (D1110G) or weak positive residue (D1083N), simultaneous with the appearance of a positively charged residue in the spatial vicinity (Table 3.1).

None of the mutations in VP3 contributed to a gain in net positive charge, but one resulted in the loss of a negative charge. The mutation of D to N at residue 3009 is spatially close to the cluster of positive charges in the β F- β G loop of VP1. In one instance a negative charge was gained on the VP3 surface and in another a positive charge was lost (Table 3.1).

Table 3.1 Summary of the amino acid substitutions in the outer capsid proteins of SAT1 and SAT2 viruses resulting from cytolytic passages in BHK-21 cells.

<i>Protein</i>	<i>Structure element</i>	<i>SAT1 isolate^a</i>										
		KNP/148/91	KNP/41/95	ZIM/13/90	KEN/5/98	TAN/1/99	UGA/1/97	SUD/3/76	NIG/5/81	NIG/15/75	NIG/6/76	
VP2	β B- β C	-	-	-	D2039A	-	-	-	-	-	-	
		Q2074R*	-	-	-	-	-	-	-	-	-	
		-	-	-	-	-	L2115Q	V2090I	-	-	A2107V	
		D2134E	-	-	-	-	E2133K	-	-	-	-	
		S2196N	-	-	-	-	-	-	-	-		
VP3		D3009N	-	-	-	N3013G	-	A3006G	-	-	-	
		T3043N	-	-	-	M3029V	-	-	-	-	-	
		F3044L	-	-	-	-	-	-	-	-	-	
		N3131H	H3131S	R3129G	-	-	-	-	-	-	-	
		N3135E	-	-	-	-	-	-	-	-	-	
		-	-	-	R3220H	-	-	-	-	-	-	
VP1	β D- β E	-	-	-	-	-	-	C1026R	-	-	-	
		R1049K	-	-	-	-	H1046N	-	N1048K	-	-	
		-	-	-	-	-	-	-	-	-	-	
	β F- β G	-	E1084K	-	E1084K	-	E1058K	-	-	-	A1061V	
		-	-	K1086Q	W1087R	-	-	-	-	-	-	
	β F- β G	-	N1111K	N1111K	-	-	-	-	N111K	N1111K	N1111K	N1111K
		G1112R	-	G1112R	-	E1112K	-	G112R	-	-	-	
		-	-	-	-	-	V1127L	-	-	-	-	
		-	-	-	-	S1141C	-	-	-	-	-	
		V1179E	-	-	-	D1181N	-	-	H1183Y	D1180N	-	
		K1206R	-	-	-	-	-	-	-	-	-	
		K1210R	S1212A	-	-	-	-	-	-	-		

<i>Protein</i>	<i>Structure element</i>	<i>SAT2 isolate^a</i>			
		KNP/2/89	KNP/51/93	ZIM/10/91	UGA/2/02
VP2					L2147F T2158I F2191L
		K2128E			
VP3		C3007W	V3188I		S3120F
		S1050N ,			R1045G ,
VP1	βD-βE	D1083N Q1085R*	E1083K		E1083K
	βF-βG		D1110G	N1110Q	R1189C

Table 3.1 continued

*Amino acid changes to a positive charge in surface exposed loops are shown in bold-italics. The loss of a negative charge is indicated in bold.

^a The amino acid residues have been numbered independently for each protein. For each residue, the first digit indicates the protein (VP1, VP2 or VP3) and the last three digits the amino acid position in either a SAT1 or SAT2 alignment. The P1 polypeptide of SAT1 viruses is 744 amino acids and that of SAT2 viruses 741 amino acids

List of standard amino acid abbreviations used

Amino Acid	3-letter	1-letter	Amino Acid	3-letter	1-letter	Amino Acid	3-letter	1-letter
Alanine	Ala	A	Glycine	Gly	G	Proline	Pro	P
Arginine	Arg	R	Histidine	His	H	Serine	Ser	S
Asparagine	Asn	N	Isoleucine	Ile	I	Threonine	Thr	T
Aspartic acid	Asp	D	Leucine	Leu	L	Tryptophan	Trp	W
Cysteine	Cys	C	Lysine	Lys	K	Tyrosine	Tyr	Y
Glutamic acid	Glu	E	Methionine	Met	M	Valine	Val	V
Glutamine	Gln	Q	Phenylalanine	Phe	F			

Taking the sequence data together, the observed amino acid substitutions were scattered across the outer surface of the three outer capsid proteins. However, the accumulated positively charged residues around the five-fold axis are especially interesting as those alone correlated with the observed phenotypes, i.e. present in heparin binding strains and absent in the non-heparin binding strains.

3.3.4. The molecular basis of HSPG interaction

In order to identify the molecular basis of the heparin inhibition, we looked at the 3D structure of a SAT1 capsid (2WZR) and a modelled structure of the SAT2 capsid, using the PyMol program (DeLano Scientific LLC). Most of the mutations were located on protruding structural elements surrounding the five- and three-fold axis of the virion (Fig. 3.4A & 3.B). It was observed that VP1 positions 111 and 112, located in the β F- β G loop, are highly surface-exposed adjacent to the five-fold pore of the capsid (Fig. 3.4B). Another mutation that occurred more than once in SAT1 viruses was a lysine residue at VP1 position 84 in the β D- β E loop. Five-copies of positively charged residues at both positions formed a tight cluster on the SAT1 capsid around the five-fold axis of symmetry (Fig. 3.4B). For the SAT2 serotype, lysine residues were observed at VP1 position 83 for two viruses and an arginine at VP1 position 84 for KNP/2/89. In the current model of the SAT2 capsid residue 83 is not surface-exposed, but 85 is exposed forming a positively charged cluster around the five-fold axis (Fig. 3.4C). The implication of this is that the positively charged cluster around the five-fold axis would permit HSPG binding at the cell surface

A close inspection of the effect of the mutations on the electrostatic potential of the SAT1 capsid revealed that all the changes to positively charged residues were located in the vicinity of already existing positively charged surface residues. Since the mutations themselves contributed a net gain of positive charge, the result in each case was the formation of an expanded cluster of positive charge on the virion surface, especially focused around the five-fold axis (Fig. 3.5).

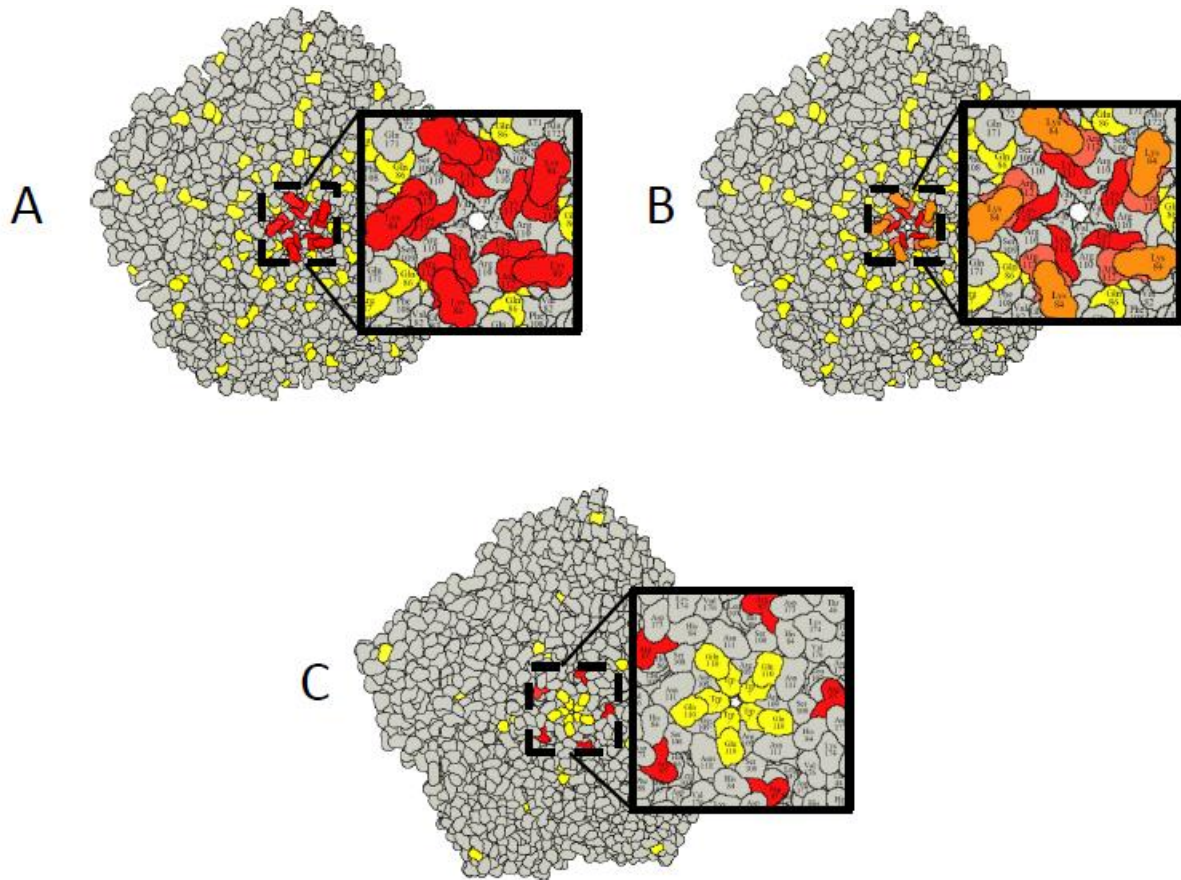


Fig. 3.4 A space-filling representation of the SAT1 and SAT2 pentamers. (A) The SAT1 pentamer is based on the protein data bank co-ordinates (2WZR). Amino acid substitutions observed during the adaptation of SAT1 viruses in BHK-21 cells are indicated in yellow. The surface-exposed, positively charged mutations, that occurred more than once in different SAT1 viruses, are highlighted in red. The five copies of VP1 show the positively charged cluster at the five-fold axis. (B) Positively charged mutations are colour-coded based on the frequency of occurrence in different viruses within the SAT1 serotype from orange ($n > 1$) to red ($n > 5$). (C) The SAT2 pentamer is modelled using the SAT1 co-ordinates as a template and the surface-exposed positively charged mutations are shown in red. In SAT2, a Lys residue appeared twice in VP1 position 1083 in two different viruses, however in the current model 1083 is not surface exposed. Nonetheless 1085R (seen in KNP/2/89) is surface-exposed.

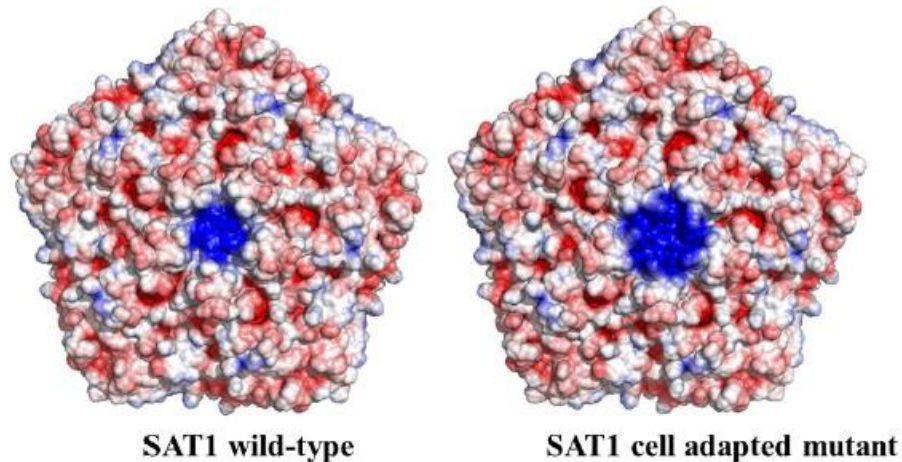


Fig. 3.5 Surface models of wild-type and VP1 positively charged mutant proteins coloured by electrostatic potential. Positively charged surfaces are shown in blue, and negatively charged surfaces are red. The cell culture-adapted mutations at VP1 positions 111 and 112 were mapped onto the SAT1 capsid and the electrostatics surface potential was calculated using APBS module of PyMol (DeLano Scientific LLC).

To understand how the positively charged substitutions at VP1 positions 111 and 112 in SAT1 may exert an effect on the interaction with HSPG we used GRID (Goodford, 1985) to find the most energetically favourable binding site. These calculations identified the most likely residue to interact with heparin as residue 112 of VP1 with molecular interaction energy of -8.2 kcal/mol (Fig. 3.6A & B). The interaction energy increased to -10 kcal/mol when the grid was centred at residue 112 (Fig. 3.6B). Based on this result, a pentamer of heparin disaccharide units [L-iduronic acid (Idu) and D-glucosamine (GlcN)] was docked to both the wild-type capsid and the modelled cell-adapted mutant capsid having a positively charged cluster at the five-fold axis. Fig. 3.6 (A & B) demonstrates that in the vicinity of the 5-fold axis, a heparin oligosaccharide can dock well to the capsid containing the positively charged cluster.

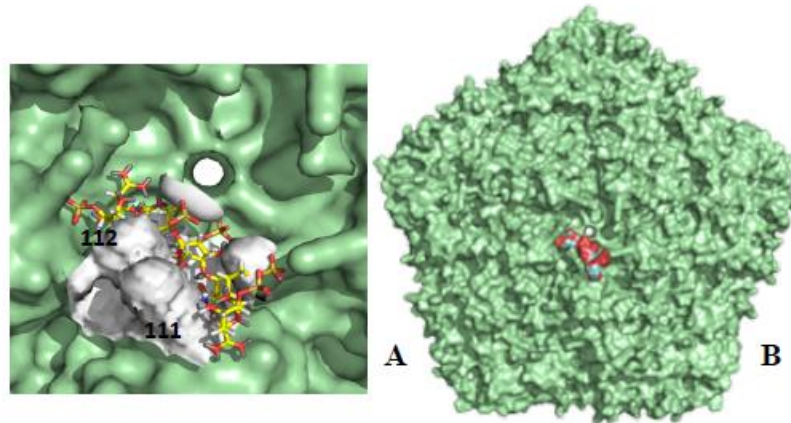


Fig. 3.6 GRID (Goodford, 1985) was used to find the energetically favourable binding site for HSPG on the SAT1 modelled mutant capsid (A). The GRID calculation was performed for a 20Å radius around the five-fold axis using pyramidal sulfur as probe. The calculations identified the most likely site of interaction in the vicinity of VP1 residue 112 with molecular interaction energy of -8.2 kCal/mole. The interaction energy increased to -10 kCal/mole when the grid was centred at residue 112. (B) Five linked heparin disaccharides were docked using the default parameters of GOLD onto the SAT1 modelled mutant pentamer structures. A 30Å³ region from VP1 residue 112 was defined for docking and the GOLD fitness score function was used to rank the docking poses. The best docking pose is shown. (GOLD score = 127). The equivalent process for the wild type virus produced a less satisfactory docking (GOLD score = 102, docking not shown).

3.4 DISCUSSION

HSPG can serve as a cell attachment receptor for FMDV, but only contributes significantly to binding and infection of virus strains adapted to cultured cells. Although infection with low passage SAT1 and SAT2 viruses was predominantly HSPG independent, several cell culture-adapted strains, each with increased clusters of positively charged residues at the five-fold axis, attached to cells through an HSPG-dependent mechanism. Our findings suggest that binding to cell surface HSPG plays little or no role in interaction between natural FMDV isolates and host cells *in vivo*, nonetheless SAT viruses rapidly mutate to utilize HSPG during cell culture adaptation. We demonstrate a molecular interaction of culture-adapted SAT1 and SAT2 viruses with HSPG and identify critical amino acids at VP1 residues 111-112 and 84-85 involved in defining this phenotype.

Growth of FMDV in non-host cells, like cultured hamster cells, is invaluable for the production of vaccines. This necessitates continuous adaptation of new vaccine strains to cell culture whenever a close antigenic relationship to an outbreak virus is required (Amadori *et al.*, 1997). However, cytolitic passages of FMDV in cell culture leads to rapid change in the preference of cell surface molecules for cell entry (Jackson *et al.*, 1996; Sa-Carvalho *et al.*, 1997; Baranowski *et al.*, 1998; Maree *et al.*, 2010). It is thought that adaptation to cell culture is made possible by the selective pressure exerted by the cell surface molecules, which may act as virus receptors for viral quasi-species (Baranowski *et al.*, 1998; Baranowski *et al.*, 2000).

To understand and exploit the dynamics involved in adaptation of viruses belonging to the SAT serotypes to cell culture we showed that small, clear-plaque variants of SAT1 and SAT2 viruses are readily selected upon cytolitic passage in cell culture. An observation from this study that stands out very prominently is the number of mutational patterns that arise during cytolitic passage of SAT viruses, most share the common property of increasing the net positive charge around the five-fold axis of the virion. The mutational patterns around the five-fold axis create local patches of surface area with increased positive charge with the ability to bind a moiety of roughly the same size and charge of a sulfated glycan. Indeed, SAT viruses adopting such a mutation pattern were able to infect CHO-K1 cells while plaque formation in BHK-21 cells was inhibited in the presence of soluble heparin.

An increased number of surface positive charges after cytolitic passage in cell culture has been correlated with the ability to infect CHO-K1 cells and the use of HS receptors and has been observed for a number of diverse virus families. Coxsackievirus A9 (McLeish *et al.*, 2012), Herpesviruses (WuDunn & Spear, 1989; Okazaki *et al.*, 1991), cytomegalovirus (Compton *et al.*, 1993), HIV (Roderiquez *et al.*, 1995; Moulard *et al.*, 2000), pseudorabies virus (Mettenleiter *et al.*, 1990), equine arteritis virus (Asagoe *et al.*, 1997), dengue virus (Chen *et al.*, 1997), porcine reproductive and respiratory syndrome virus (Jusa *et al.*, 1997), classical swine fever virus (Hulst *et al.*, 2000), respiratory syncytial virus (Krusat & Streckert, 1997), Sindbis virus (Klimstra *et al.*, 1998), tick-borne encephalitis (Mandl *et al.*, 2001), vaccinia virus (Chung *et al.*, 1998) and adeno-associated virus (Summerford & Samulski, 1998) have all been reported to select mutants with high affinity for binding to GAGs, especially HS, after repeated passage in cultured cells. Although HS binding sequence motifs have been deduced for known HS binding proteins (Cardin

& Weintraub, 1989; Hileman *et al.*, 1998), the complexity of viral capsids complicates the identification of a primary linear sequence as an HS binding motif. However, X-ray crystallography of HSPG-binding sites in FMDV revealed that residues from both VP1 and VP3 may contribute to positively charged clusters on the viral capsid (Fry *et al.*, 1999; Fry *et al.*, 2005). Our data suggest that positively charged mutations accumulate on SAT1 or SAT2 capsids forming clusters of two to four basic amino acids (lysine or arginine) brought together by protein folding. A pre-requisite of HSPG-interacting regions is that they must be exposed on the virion surface and consist of multiple positively charged amino acids clustered together.

The question arises as to whether the structural requirements for HS binding sites on complex protein structures, like FMDV, are created by chance as a result of random mutations or whether a structurally predisposed binding site exists on the capsid (Fry *et al.*, 1999). Analysis of the capsid region of non-HSPG binding strains revealed that a single basic residue, although rare, may occur in the VP1 β D- β E or β F- β G loops of SAT1 and SAT2 viruses, however on its own it is insufficient to support HSPG-interaction. A mutation of a spatially closely situated residue that may be associated with optimization of cell entry will provide a selective advantage and will be strongly selected for (Baranowski *et al.*, 1998; Baranowski *et al.*, 2000). VP1 positions 111-112 and 84-85 are both unique as they are symmetrically arranged around the five-fold pore and are significantly exposed to the environment. Our data reveals that clustering of positively charged residues at the 111-112 location enables interaction with an HS molecule. This infers that the virus particle has 12 energetically favourable HSPG binding sites, each located at a vertex of the icosahedron.

In confirmation of a role of the HSPG-binding residues at position 111-112, we have recently reported on the construction of two infectious SAT1 genome-length clones, with substitutions of residues 111-112 to positively charged amino acids (Maree *et al.*, 2010; Maree *et al.*, 2011a). This is in agreement to the findings in a type A virus, where a single positively charged substitution at residue 110 of VP1 resulted in the ability to infect CHO-K1 cells (Berryman *et al.*, 2013). Both recombinant SAT1 viruses were capable of infecting CHO-K1 cells, suggesting infection via HSPG-binding. However, an RGD at this position failed to interact with integrin receptors (Storey *et al.*, 2007).

It remains possible that other residues contribute to HSPG binding in SAT1 and SAT2 viruses. Examples from our study include the W1087R substitution of SAT1/KEN/5/98 that appeared simultaneously with the E1084K mutation, and N1048K together with the N1111K substitution in SAT1/NIG/5/81. Both are spatially close to the VP1 β D- β E loop and contribute to the positively charged cluster around the five-fold axis. In several cases the disappearance of negatively charged amino acids or the appearance of residues with a partial positive charge has been observed in association with lysine or arginine residues. Although we found two examples of positively charged substitutions (Q2074R and E2133K) in the shallow depression at the junction of the three major capsid proteins as described for type A and O viruses (Fry *et al.*, 1999; Fry *et al.*, 2005), the role of these substitutions in the interaction of SAT1 capsids to HSPG is still unclear.

Adaptation of FMDV to cultured hamster cells is a common step in the vaccine production process, but time-consuming and often unsuccessful. Knowledge of the HSPG sites for FMDV and the role HSPG plays in assisting cell entry can be applied in the construction of chimeric viruses containing the symmetrical, positively charged clusters that enable interaction with HSPG. This might help avoid the accumulation of random changes that lead to divergence of the vaccine strain from the virus populations circulating in nature.

CHAPTER FOUR

ANTIGENICITY OF AN INTRA-SEROTYPE CHIMERIC FOOT-AND-MOUTH DISEASE VACCINE

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4.1 INTRODUCTION

Foot-and-mouth disease (FMD), of which FMD virus (FMDV) is the causal agent, is a highly infectious vesicular disease of cloven hoofed animals such as cattle, pigs, sheep goats and other artiodactyl species. Although mortality rates are generally low, morbidity is high and outbreaks of FMD results in severe economic losses to the livestock industry, especially in FMD-free regions of the world (Thompson *et al.*, 2002; Alexandersen *et al.*, 2003; Rweyemamu *et al.*, 2008). The disease is widely distributed in the developing world, in particular Africa, Asia and South America (Rweyemamu *et al.*, 2008). In these regions, livestock farming forms the backbone of rural economies that supports approximately 70% of the world's poor. FMD outbreaks particularly affect vulnerable individuals such as women and children since approximately 75% of livestock in Africa are raised under the communal smallholder systems that sustain livelihoods of these groups (Scoones *et al.*, 2010; Ferguson *et al.*, 2013; Miguel *et al.*, 2013).

The epidemiology of FMD in Africa is influenced by two different patterns i.e. a cycle involving wildlife, in particular the African buffalo (*Syncerus caffer*), and an independent cycle maintained within domestic animals (Hedger, 1972; Condy *et al.*, 1985; Bengis *et al.*, 2002; Ayebazibwe *et al.*, 2010). Another unique feature of FMD epidemiology in Africa is the presence of the three South African Territories (SAT) serotypes, i.e. SAT1, SAT2 and SAT3, which are maintained within the African buffalo populations (Vosloo *et al.*, 1996; Bastos *et al.*, 2001; Bastos *et al.*, 2003a; Bastos *et al.*, 2003b; Ayebazibwe *et al.*, 2010). The presence of large numbers of African buffaloes provides a potential source of sporadic infection to domestic livestock and other wildlife species (Dawe *et al.*, 1994; Bastos *et al.*, 2000; Thomson *et al.*, 2003). Although the precise mechanism of transmission of FMD from buffalo to cattle is not well understood, it is facilitated by direct contact between these two species. Once cattle are infected they may maintain SAT infections without the further involvement of buffalo (Dawe *et al.*, 1994; Thomson *et al.*, 2003).

Control of FMD in sub-Saharan Africa revolves around four integrated activities, i.e. vaccination of susceptible animals, separation of infected and susceptible animals, movement restriction and surveillance. Vaccines are based on chemically inactivated whole virus antigen combined with mineral oil adjuvant or aluminium hydroxide/saponin. Despite successful application in the

developed world, the effective administration and optimal induction of protective immunity are hampered by several factors in developing countries. These include poor duration of protective immunity (Doel & Baccarini, 1981), the inability to prevent a sub-clinical persistent infection (Bachrach, 1968; Doel, 2003), biophysical stability of the vaccine antigen (Doel & Baccarini, 1981), the potency of the vaccine (Doel, 2003) and current inactivated vaccines are often unable to control some lineages arising in these regions (Hunter, 1998). The poor duration of immunity has been linked to the temperature liability of viruses belonging to the SAT serotypes (Doel & Baccarini, 1981). One of the foremost factors which influences the potency of vaccine preparations and permits the induction of a protective antibody response is the structural integrity of the intact virion typified by a sedimentation rate of 146S (Doel & Baccarini, 1981). As a consequence countries have to rely on a thrice or quadruple annual vaccination schedule, arguing for the development of structurally improved and custom-made vaccines for use in specific geographic localities (van Rensburg *et al.*, 2002; van Rensburg *et al.*, 2004). However, development of useful cell culture-adapted vaccine strains from field isolates is time consuming and expensive, limiting the availability of custom-made vaccine strains (Doel, 2003).

Improved vaccines, in terms of stability and antigen yield, especially for the SAT viruses are becoming a priority worldwide. One approach is to structurally design vaccines for specific geographic regions (van Rensburg *et al.*, 2002; Blignaut *et al.*, 2011). Viral RNA can be made infectious in the absence of other components of the virion (reverse genetics) making it theoretical possible to genetically engineer new viruses from *in vitro*-generated RNA molecules (Zibert *et al.*, 1990; van Rensburg *et al.*, 2004). The introduction of specific mutations into the cloned genomes of viruses has allowed the manipulation of the biological properties of field and laboratory strains and presents a promising avenue for the design of safe and effective vaccines (Rieder *et al.*, 1994; Piccone *et al.*, 1995; Maree *et al.*, 2011a). Zibert and co-workers (1990) succeeded in the development of this technology for FMDV, thus providing a powerful tool for the development of new methods to control the disease. We have structurally-engineered recombinant SAT viruses, containing desirable antigenic determinants and cell adaptation phenotypes (Storey *et al.*, 2007; Maree *et al.*, 2010; Maree *et al.*, 2013) providing the proof-of-principle to rationally design viruses with the desired biological properties of a good vaccine strain. Several studies have shown that cross-serotype chimeric vaccines successfully induces protective immune responses and protected FMD host species against live virus challenge

(Rieder *et al.*, 1994; Fowler *et al.*, 2008; Blignaut *et al.*, 2011). To this end SAT capsid can be engineered to be thermo-stable whilst encoding the antigens required for vaccines in specific geographic localities.

This paper describes the evaluation of an intra-serotype SAT2 chimeric vaccine in cattle. The feasibility of the chimeric vaccine as a possible alternative to replace conventional inactivated vaccines is assessed. The rationale was to ascertain if the performance of the chimeric vaccine was similar to that of its parental vaccine. We determined the serological profile generated in cattle, using serology and the gold standard virus neutralization (VN) assays as *in vitro* markers of protection. The intra-serotype vaccine was prepared from a chimeric virus containing the external capsid-coding region of a field SAT2 isolate, ZIM/14/90, exchanged in the genetic background of a SAT2 infectious clone (van Rensburg *et al.*, 2004).

4.2. MATERIALS AND METHODS

4.2.1 Cells, viruses and plasmids

The SAT2 virus, SAT2/ZIM/14/90 was obtained from the FMD World Reference Laboratory at the Pirbright Institute, UK and was maintained at the Transboundary Animal Diseases (TAD) of the Agricultural Research Council (ARC) of South Africa. Baby hamster kidney (BHK) cells, strain 21, clone 13 (ATCC CCL-10) were maintained as described previously (Rieder *et al.*, 1994). The SAT2/ZIM/14/90 (GenBank accession number DQ009728), was passaged four times through BHK-21 cells (BHK#4) to produce a vaccine master seed stock. Virus stocks were titrated by plaque assays in BHK-21 cells as described previously (Rieder *et al.*, 1994). The plasmid pSAT2^{ZIM14}-SAT2 containing the outer capsid-coding region of SAT2/ZIM/14/90 in the pSAT2 backbone (Maree *et al.*, 2013) was provided. There were 9.7% amino acid differences in an alignment of the capsid proteins of pSAT2 and SAT2/ZIM/14/90.

The BHK-21 cells were also used for RNA transfection and virus recovery. For serial passages, infected or transfected 35-mm BHK-21 cell monolayers were frozen at 80°C and thawed, and 1/10th of the volume was used to inoculate a fresh BHK-21 monolayer. Following virus adsorption (with periodical rocking for 60 min at 37°C), virus growth medium (VGM; Eagle's basal medium (BME) with 1% FCS, 1% HEPES and antibiotics) was added, and the culture was

incubated for no longer than 48 h at 37°C, after which the infected cells were frozen for subsequent passaging of the viruses.

The IB-RS-2 (Istituto Biologico renal suino) cells were maintained in RPMI medium (Sigma) supplemented with 10% foetal calf serum (FCS, Delta Bioproducts), and were used for virus isolations and as the indicator system in the virus neutralization test (VNT).

4.2.2 Rescue of virus from chimeric genome-length cDNA plasmids

The RNA was synthesized from *SwaI*-linearised plasmid DNA with the MEGAscript™ T7 kit (Ambion). The BHK-21 cell monolayers, in 35-mm cell culture wells (Nunc™), were transfected with 2-3 µg of intact *in vitro*-generated RNA using Lipofectamine2000™ (Invitrogen). The transfection medium was removed after 3-5 h and replaced with VGM followed by incubation at 37°C for up to 48 h with a 5% CO₂ influx. After one freeze-thaw cycle, the transfection supernatants were used for serial passaging on BHK-21 cells. BHK-21 monolayers in 35-mm cell culture wells were infected using 1/10th of clarified infected supernatants and incubated for 48 h at 37°C. Viruses were subsequently harvested from infected cells and passaged as described previously in section 4.2.1. The recombinant viruses derived from the infectious chimeric pSAT2^{ZIM14}-SAT2 clone was designated vSAT2^{ZIM14}-SAT2. Following the recovery of viable virus, the presence of the insert was verified with automated sequencing. The virus that was used for antigen production was passaged four times.

4.2.3 Production of plasmid-derived chimeric FMDV antigen and vaccine formulation

Culture fluids from SAT2/ZIM/14/90 and vSAT2^{ZIM14}-SAT2 infected BHK-21 cells were harvested, inactivated with 5 mM binary ethyleneimine (BEI) for 26 h at 26 °C, clarified by centrifugation, concentrated with 8% PEG (w/v) and resolved on 10-50% (w/v) sucrose density gradients (SDG) by rate zonal centrifugation at 36,000 g for 16 h at 4°C. The gradients were fractionated and analysed spectrophotometrically by measuring the absorbance at 260 nm. Fractions containing 146S virions were calculated using the extinction coefficient $E_{259nm} = 79.9$ (Doel & Mowat, 1985) and pooled for vaccine formulation. The presence of the outer capsid proteins were verified using SDS-PAGE analysis, while the integrity of the chimeric viral RNA was verified by RT-PCR and sequencing of the P1/2A-coding region.

Two separate vaccine formulations, incorporating SAT2/ZIM/14/90 and vSAT2^{ZIM14}-SAT2 inactivated 146S antigens as double oil emulsions with Montanide ISA 206B (Seppic), were prepared. Each vaccine contained 6 µg/ml of the BEI-inactivated, SDG-purified FMDV antigen. The oil adjuvant was subsequently mixed into the aqueous antigen phase (equal volumes) at 30°C for 15 min and stored at 4 °C for 24 h. A placebo vaccine was formulated that contained all the components, but with 1× PBS instead of antigen.

4.2.4 Cattle immunizations and viral challenge

Fourteen Nguni cattle 12-18 months of age were divided randomly into two groups of seven animals and housed separately within the high-containment animal facility at TAD of the ARC-Onderstepoort Veterinary Institute (ARC-OVI). Subsequent to an initial acclimatization period, the cattle were vaccinated with 2 ml of either the SAT2/ZIM/14/90 (group 1) or vSAT2^{ZIM14}-SAT2 (group 2) vaccine. Two control animals were housed in a separate stable and were vaccinated with the placebo vaccine formulation that lacked viral antigen. Blood samples (10 ml clotted and 10 ml heparinised blood) were collected on days 0, 7, 14 and 21 post-vaccination (p.v.).

At 21 days post vaccination (d.p.v.) the cattle from both immunized groups and the two control animals were inoculated intra-dermoligually with 1 ml of 10⁴ TCID₅₀ SAT2/ZIM/14/90 challenge virus (according to the OIE approved dose of 10⁴ ID₅₀) into each of two sites. During each of these procedures cattle were sedated with Rompun (2% Xylazine) at a dosage of 0.67 ml/100 kg. Clotted and heparinised blood was collected on days 0, 2, 4, 7 and 10 days post-challenge (p.c), along with oropharyngeal (OP) fluid and nasal swabs. The animals were examined daily for fever and clinical signs. Body temperatures of >39.5 and <40°C were considered as mild and severe fever, respectively. Generalization of clinical lesions was scored as follows: congestion/small lesion/healing vesicle = 1; moderate vesicle = 2; and severe lesion = 3.

Vaccination, sampling, care and husbandry of the animals during the study, and termination of the cattle at the end of the study were done in accordance to the ARC-OVI intitutional guidelines.

4.2.5 Virus isolation

FMDV in heparinised blood, OP fluid and nasal swabs were detected by the inoculation of IB-RS-2 monolayer cells as described by the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals by Office International des Epizooties (2012). The supernatant was blind passaged at least twice or until cytopathic effect (CPE) was observed. An antigen ELISA was used to confirm the presence of FMDV in cultures showing CPE (Roeder & Le Blanc, 1987; Ferris & Donaldson, 1992).

4.2.6 Viral RNA detection by real-time quantitative RT-PCR

The viral RNA in the heparinised blood, OP fluid and nasal swabs was detected using a two-step real time RT-PCR assay. The guanidinium–silica based method described by Boom *et al.*, (1990) was employed to extract RNA which was stored at -80°C until used. Complementary DNA synthesis from the RNA template was achieved using methods described previously by Bastos (1998) together with a modified oligo-dT (CCATGGCGGCCGCTTTTTTTTTTTTTTTTTT (poly-SAT DT) that annealed to the 3'UTR/polyA tail (see section 2.2.2).

The real time RT-PCR assays were performed using the method described in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals by Office International des Epizooties (2012). For a single 50µl volume reaction, 7µl of cDNA was added to the 43µl of master mix containing the following; 4µl each of 10pmol primer; forward ACTGGGTTTTACAAACCTGTGA and reverse GCGAGTCCTGCCACGGA (Callahan *et al.*, 2002); 2µl of TaqMan[®] probe TCCTTTGCACGCCGTGGGAC (Callahan *et al.*, 2002); 25µl 2x TaqMan universal PCR mix (Applied Biosystems) and 8µl nuclease-free water. Each cDNA sample was tested in duplicate. The PCR amplification was done in a thermal cycler of model sds 7500 (Applied Biosystems) using the following program; 1 cycle of 95°C for 10 min, 50 cycles of 95°C for 15 sec and 60°C for 1 min. The threshold cycle (Ct) values that determined whether a sample was positive or negative were determined by in-house protocols at the TAD laboratory (ARC-OVI). Positive controls have a Ct value <40 and strongly positive controls have a Ct value below 20. The tissue positive control samples were as follows; whole blood samples taken from any of the unvaccinated controls on 23 d.p.v. Oral pharyngeal fluid samples taken from any of the unvaccinated control cattle on 29 d.p.v. Tissue negative controls for both serum and OP fluid

were samples taken from any of the unvaccinated control cattle on 21 d.p.v before the homologous live virus challenge was performed.

4.2.7 Liquid phase blocking ELISA (LPBE)

Anti-SAT2 antibodies in cattle sera from animals vaccinated with SAT2/ZIM/14/90 or vSAT2^{ZIM14}-SAT2 were detected by a SAT2-specific liquid-phase blocking ELISA (LPBE). The LPBE was carried out as described by the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals by Office International des Epizooties (2012). Briefly, the test serum was diluted two-fold in 96-well micro-titre (Nunc) plates, starting with a 1/8 dilution, and mixed with the viral antigen that is homologous to the rabbit antisera that was used to coat the plates and the mixtures were incubated at 37°C for 1 hour. The serum/antigen mixtures were transferred to ELISA plates that were coated with anti-SAT2 rabbit antiserum. The plates were washed with 1× PBS containing 0.05% Tween 20 (PBS-T20) followed by the addition of anti-SAT2 Guinea pig antiserum diluted 1/6000 in 1× PBS containing 0.5% (w/v) casein, to each well for 1 h at 37 °C. The plates were washed with PBS-T20, followed by addition of the substrate solution 3,3',5,5' tetramethylbenzidine (TMB) sodium phosphate/citric acid buffer and H₂O₂. After incubation for 15 min at room temperature, the reactions were stopped with 1 M H₂SO₄ and the optical density (OD) at 492 nm was measured with a Labsystems Multiscan Plus Photometer. The titre was determined from the log₁₀ reciprocal antibody dilution giving 1.0 OD₄₉₂ unit. A cut-off value of log₁₀ 1.7 was considered positive.

4.2.8 Virus neutralization test (VNT)

Neutralizing antibodies against SAT2/ZIM/14/90 in serum samples collected at 0, 7, 14 and 21 d.p.v. from cattle were measured with a VNT, according to the method described in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals by Office International des Epizooties (2012) using IB-RS-2 cells in microtitre plates. Briefly, the test serum was diluted two-fold in 96-well micro-titre (Nunc) plates, starting with a 1/16 dilution, and mixed with a virus suspension containing approximately 100 TCID₅₀ per well. After 1 h of incubation at 37°C, 3 × 10⁵ IB-RS-2 cells were added to each well. Controls included the cell only, a virus titration and positive serum (cells, virus and positive reference serum). After 72 h at 37°C in a humid atmosphere containing 5% CO₂, the plates were analysed microscopically and colorimetrically for CPE and 50% end-point serum titres were calculated according to the method of Kärber (1931). Serum samples

collected at 21 d.p.v. were also used to assess the neutralizing antibody response against a panel of SAT2 reference viruses and field isolates: SAT2/KNP/19/89, SAT2/SAR/3/04, and SAT2/ZIM/7/83. The antibody titres were calculated as log₁₀ of the reciprocal of the final serum dilution that neutralized 100 TCID₅₀ of virus in 50% of the wells (Kärber, 1931).

4.3. RESULTS

To investigate the *in-vitro* dynamics of the neutralizing antibody immune response elicited in cattle following vaccination, standardized VNT and LPBE assays were employed. Both assays complement each other as they correlate directly. (McCullough *et al.*, 1992a; Manual of Diagnostic Tests and Vaccines for Terrestrial Animals by Office International des Epizooties 2012). The ability of the immune response elicited by vaccination to protect the cattle against homologous live virus challenge was determined at 21 d.p.v by intradermolingual challenge with a dose of virus guaranteed to cause lesions in at least three hooves in naïve cattle (McCullough *et al.*, 1992b; Doel, 2003). Protection was established when the FMDV lesions from primary site of virus inoculation on the tongue did not spread to affect all the four hooves (McCullough *et al.*, 1992b; Doel, 2003). On the other hand, evidence of spread of the FMDV from the primary site of inoculation following intradermolingual challenge at 21 d.p.v, in the cattle was detected in either whole blood, tissues of the oral pharynx or nasal secretions using real time RT-PCR and virus isolation methods.

4.3.1 Antibody kinetics of the SAT2/ZIM/14/90 and vSAT2^{ZIM14}-SAT2 vaccines in Nguni cattle

Sera collected on days 0, 7, 14 and 21 post vaccination were tested by LPBE and VNT's to assess antibody and more specifically neutralizing antibody response to vaccination. The kinetics in antibody responses for both assays were similar, therefore the study chose to depict the neutralizing antibodies. Figure 4.1 shows that vaccines produced from the parental SAT2/ZIM/14/90 and chimeric vSAT2^{ZIM14}-SAT2 146S particles produced a similar response and induced detectable levels of anti-SAT2/ZIM/14/90 neutralizing antibodies as early as day 7 post-vaccination. Strong positive neutralizing antibody titres were observed at day 21-post vaccination for animals from both vaccinated groups. No significant differences (P = 0.69, determined by the unpaired T-Test) were observed in the neutralizing antibody titres of animals

that received the vSAT2^{ZIM14}-SAT2 vaccine compared to animal that received the SAT2/ZIM/14/90 vaccine.

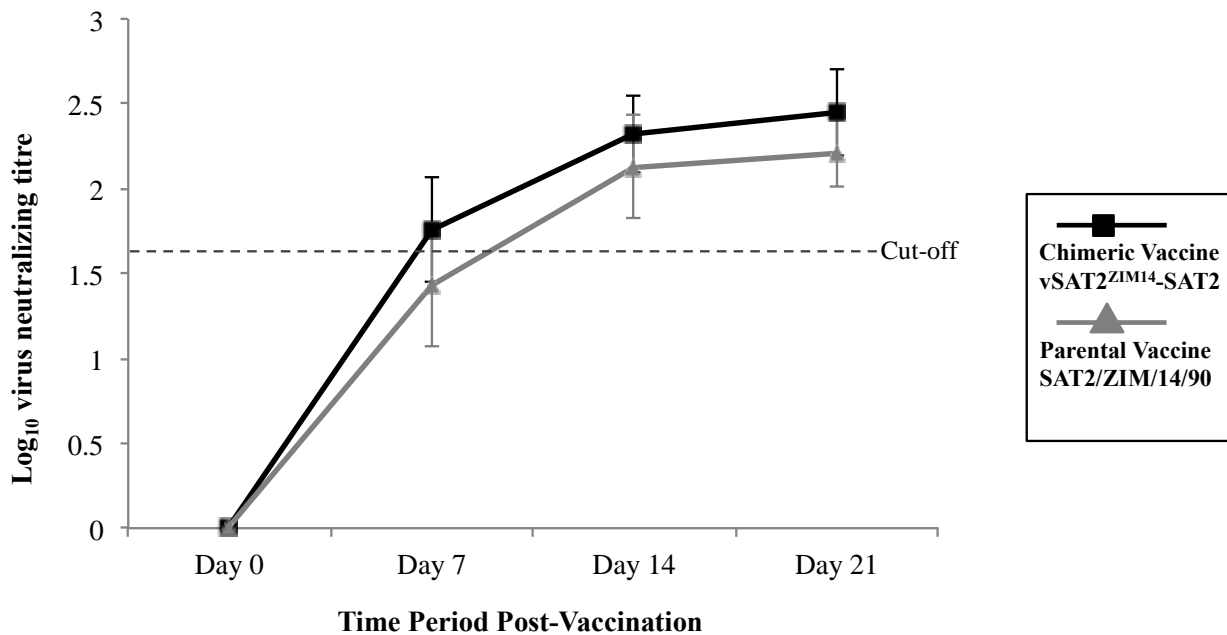


Fig. 4.1 Neutralizing antibody response of Nguni cattle following vaccination detected using VNT assays. Cattle were vaccinated with 12ug of either SAT2/ZIM/14/90 or vSAT2^{ZIM14}-SAT2 BEI-inactivated, SDG-purified antigens mixed with Montanide ISA206 adjuvant. The mean of the log₁₀ virus neutralizing titres are shown and the error bars represent the standard deviation.

4.3.2 Protection of vaccinated cattle against live SAT2/ZIM/14/90 virus challenge

The group of seven cattle, that received the chimeric vSAT2^{ZIM14}-SAT2 vaccine, was fully protected against the intra-dermolingual challenge of SAT2/ZIM/14/90 virus as observed by the absence of generalized lesions on their feet, whilst mild fever was present in three of the animals, 48 hours post-challenge (datum not shown). One animal (16-T25) had severe fever (≥ 40 °C) for two consecutive days and also showed lesions on the tongue other than the site of injection (local spread). However, no systemic spread of FMD was observed in animal 16-T25. This was in contrast to both the un-vaccinated controls, which developed severe pyrexia, and severe lesions that had generalized to all four feet within 48-72 hours post challenge (highest clinical score = 14 at 4-5 days post challenge; Fig. 4.2).

Of the seven cattle that received the SAT2/ZIM/14/90 vaccine, two animals showed generalization with one lesion on one hoof each (clinical scores = 3 at 5 days p.c). The animals had mild (≥ 39.6 < 40 °C) to severe (≥ 40 °C) fever and the tongue lesions were severe, spreading

beyond the sites of inoculation (Fig. 4.2). However the appearance of lesions on the hooves of the two cattle was delayed and mild compared to the un-vaccinated controls (data not shown).

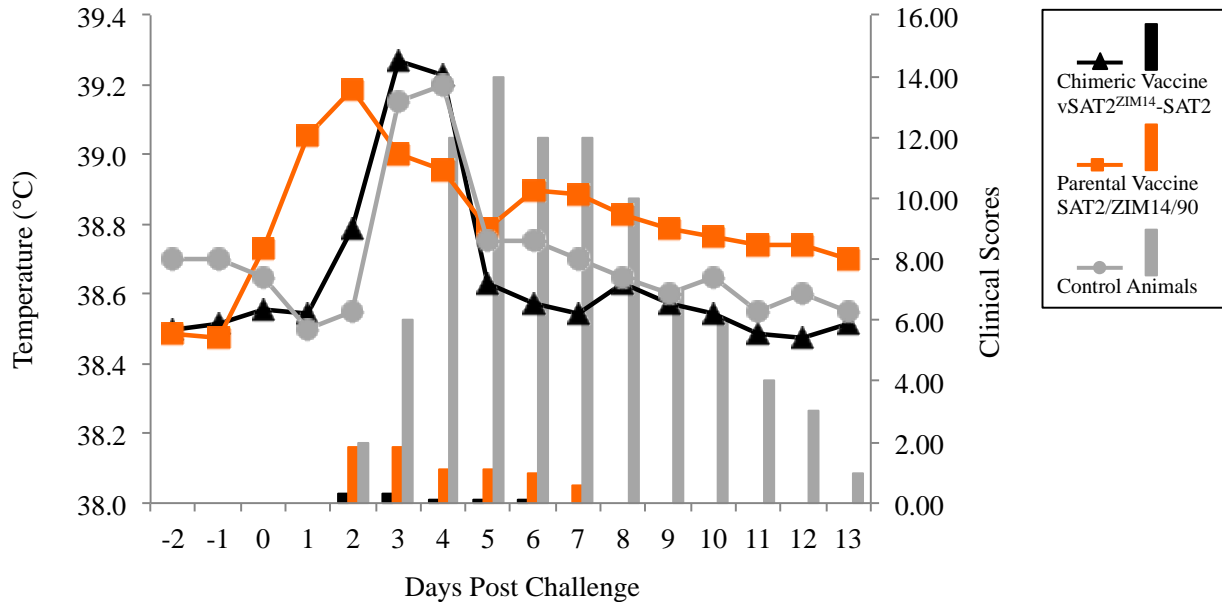


Fig. 4.2 Clinical picture observed in cattle following challenge with live homologous virus SAT2/ZIM/14/90. The mean rectal temperature (°C) for cattle vaccinated with the chimeric vaccine (▲), parental vaccine (■) and the controls given a placebo (•) is shown by a line graph on the left axis. The average clinical scores for the cattle vaccinated with the chimeric vaccine (■), parental vaccine (■) and the controls given a placebo (■) are shown by the bar graph on the right axis. The clinical score for each animal was calculated as the sum of the score of the clinical lesions on all four hooves and in the mouth.

4.3.3 Virus isolation and the presence of viral RNA

FMDV was recovered from OP samples from one parental vaccinated and three chimera vaccinated animals at 48 hours post-challenge. Nasal samples of three animals that received the parental vaccine were positive on virus isolation, but no virus could be isolated from nasal samples of the vSAT2^{ZIM14}-SAT2 vaccinated group. In addition virus was also recovered from oropharyngeal samples from one of the two placebo vaccinated controls. No virus could be isolated from whole blood (Table 4.1).

FMDV RNA was detected by quantitative real time RT-PCR (at a Ct value <40) in oropharyngeal samples taken from the SAT2/ZIM/14/90 vaccinated animals and the two placebo vaccinated controls 2 days post-challenge (Table 4.1). RNA was present in the oropharynx up to 8 days post-challenged but were negative at 11 days post-challenge. FMDV RNA was, however,

detected in nasal samples from one animal in the parental vaccination group (namely 15-169; Table 4.1). Animals that received the chimera vaccine had viral RNA in the oropharynx between 2-7 days post-challenge and in the nasal samples of four of the chimera vaccinated animals were at a Ct value borderline of a positive result (Table 4.1). FMDV RNA was also detected in whole blood (at a Ct value <40) between 2-4 days post-challenge in both vaccinated and placebo groups (Table 4.1). The viral RNA in whole blood decreased as the antibody titres increased. High titres of anti-SAT2 antibodies (>2.0 log₁₀) were observed at 4 days post-challenge which stayed high until the end of the experiment, with the exception of the placebo controls where sero-conversion only took place after 5-8 days post-challenge. No viral RNA could be detected in whole blood after 4 days post-challenge.

Table 4.1 Virus isolation and real time RT-PCR results for whole blood, OP fluid and nasal swab samples collected between 0-11 days post-challenge.

Vaccine	Animal number	Virus Isolation			Real time RT-PCR ¹			Clinical Score ²
		Whole Blood	OP Fluid	Nasal swab	Whole Blood	OP Fluid	Nasal swab	
SAT2/ZIM/14/90	15-T7	-	-	2	2 (36.4)	2 (22.5)	-	3 (2, 3, 5, 6)
	15-T25	-	2	-	2 (35.8)	2 (34.8)	-	0
	15-T29	-	-	2	-	2 (21.2)	-	0
	15-T32	-	-	-	4 (37.5)	2 (34.8)	-	0
	15-TD36	-	-	-	2 (35.7)	2 (29.5)	-	0
	15-169	-	-	2	-	2 (17.3)	2 (36.4)	3 (2, 3)
	15-NT2	-	-	-	4 (37.9)	2 (21.9)	-	0
vSAT2 ^{ZIM14} -SAT2	16-TD16	-	-	-	-	2 (38.7)	<u>2 (40.1)</u>	0
	16-TD20	-	-	-	4 (37.2)	2 (39.7)	-	0
	16-TD25	-	2	-	-	2 (24.3)	-	0
	16-TD31	-	2	-	2 (35.4)	2 (29.8)	<u>2 (40.5)</u>	0
	16-TD37	-	-	-	4 (30.5)	4 (37.9)	<u>2 (40.2)</u>	0
	16-TD41	-	2	-	-	2 (32.8)	<u>2 (40.1)</u>	0
	16-NT1	-	-	-	4 (37.7)	7 (38.2)	-	0
Unvaccinated controls	12-T3	-	-	-	2 (35.5)	2 (35.2)	-	> 10 (4-13)
	12-T13	-	2	-	2 (28.8)	2 (21.5)	-	> 10 (4-11)

¹Numbers indicate the first sample day to be considered positive and the parentheses () indicate the Ct value (Ct values of less than 40.0 are considered positive). Borderline cases are underlined

²Clinical scores were calculated as the total score on all the four hooves. The highest score is indicated and the days post challenge when they were recorded are in the parentheses ().

4.3.4 Cross-reactivity and predicted protection against heterologous field isolates

In order to determine whether a vaccine prepared from SAT2/ZIM/14/90 or vSAT2^{ZIM14}-SAT2 is likely to protect cattle from challenge against heterologous viruses, serum neutralizing antibody titres were used to calculate the degree of predicted cross-protection (Table 4.2). Pooled sera from either SAT2/ZIM/14/90 or vSAT2^{ZIM14}-SAT2 vaccinated groups contained antibodies, which were cross-reactive to the heterologous viruses causing recent outbreaks in the southern African region. The r_1 -values derived from these titres indicate that animals vaccinated with either SAT2/ZIM/14/90 or vSAT2^{ZIM14}-SAT2 would have a close antigenic match against SAT2/SAR/3/04 where $r_1 \geq 0.35$. (r_1 values of 0.4 – 1.0 indicate similar antigenic profile and therefore predict protection by vaccination (Samuel *et al.*, 1999; Paton *et al.*, 2009). Additionally, the SAT2/ZIM/14/90 vaccine and the chimeric vSAT2^{ZIM14}-SAT2 showed relatively similar cross reactivity profiles when measured against SAT2/ZIM/7/83, SAT2/KNP/19/89 and SAT2/SAR/3/04 viruses. There was only a difference of ± 0.46 between the VNT \log_{10} titres of parental and chimeric vaccine sera for each case of cross-reaction with a particular heterologous virus.

Table 4.2 Cross-reactivity and predicted protection against heterologous field isolates.

Virus	Serum					
	SAT2/ZIM/14/90			vSAT2 ^{ZIM14} -SAT2		
	VNT ^a	% Protection ^b	r_1 -value ^c	VNT ^a	% Protection ^b	r_1 -value ^c
SAT2/ZIM/14/90	2.47^d	>94%	1	2.1^d	>94%	1
vSAT2 ^{ZIM14} SAT2	2.97	100%	1	2.39	>94%	1
SAT2/ZIM/7/83	1.82	>85%	0.27	1.49	>79%	0.28
SAT2/KNP/19/89	1.68	>85%	0.2	1.48	>79%	0.27
SAT2/SAR/3/04	1.93	>85%	0.35	1.94	>85%	0.8

^a Neutralizing antibody titres are given as \log_{10} titres.

^b % Predicted protection (Fowler *et al.*, 2010)

^c r_1 values = $\frac{\text{titre of cattle serum against field virus isolate}}{\text{titre of cattle serum against homologous virus isolate}}$

^d homologous neutralizing antibody titre given in bold scrip

4.4. DISCUSSION

In Africa, the diversity of circulating field strains of FMDV makes the selection of sufficiently cross-protective FMD vaccines a challenge. Vaccines therefore need to be custom-made to be effective. One approach to address the problem of antigenic variation in the various epidemiological clusters (see section 1.2.4), would be the development of inter- and intra-serotype chimeric vaccines. The proposed strategy entails the development of chimeric FMDV by substituting antigenic-coding regions such as the external capsid-coding regions in an infectious genome-length cDNA clone of a suitable strain. The production of such recombinant viruses has been achieved for the A and SAT2 serotypes (Rieder *et al.*, 1994; Fowler *et al.*, 2008; Blignaut *et al.*, 2011).

The present study described the evaluation of an intra-serotype chimeric vaccine in cattle. The chimeric vaccine was constructed by exchanging the external capsid coding region (1B-1D/2A) of a SAT2 genome-length clone, pSAT2, with that of the field isolate, SAT2/ZIM/14/90 (Maree *et al.*, 2013).

The serological profile of the resulting chimeric vaccine in cattle in terms of neutralizing antibody response elicited upon vaccination and quantified *in-vivo* using VNT assays was comparable to that of the parental virus. Vaccination of the experimental cattle with either the parental vaccine SAT2/ZIM/14/90 or the chimera induced a humoral neutralizing antibody response recognized at 7 d.p.v and peaking at 21 d.p.v. The mean log₁₀ neutralizing antibody titre in the chimeric vaccine group did not differ significantly from that in the parental vaccine group. This pattern of antibody response is in agreement with the prototype immune response that is widely documented (Alexandersen *et al.*, 2002b; Doel, 2003; Grubman & Baxt, 2004). These data indicate that the neutralizing antibody properties of the parental virus were successfully transferred to the chimeric virus implying that the intra-serotype chimeric virus has vaccine potential.

These virus specific neutralizing antibodies are strongly associated with the ability to protect against challenge virus (McCullough *et al.*, 1992b; Barnett *et al.*, 2003). The antibody titre at which the cattle were considered to be protected from homologous virus challenge *i.e.* sero-

conversion, in this study was interpreted at a neutralizing antibody titre of \log_{10} 1.7 and occurred just after 7 d.p.v. International criteria suggest sero-conversion at titres greater than \log_{10} 1.5 (Van Maanen & Terpstra, 1989). The chimeric vaccine vSAT2^{ZIM14}-SAT2 can be said to have conferred protection against homologous live virus challenge to the cattle because this group did not show any generalized clinical FMD symptoms, albeit having evidence of a viremia and virus excretion in the oropharynx between 2 and 4 days p.c. Similarly, the majority of cattle in the parental vaccine group were also protected against homologous live virus challenge. However there were two cattle in this group that experienced delayed onset of clinical FMD that was of a relatively mild nature. In contrast the unvaccinated controls that were sero-negative on 21 d.p.v., showed rapid onset of severe and generalized clinical signs of FMD with lesions on all four hooves. The failure to correlate strong neutralizing antibody responses with protection from live virus challenge has been reported previously (McCullough *et al.*, 1992a; McCullough *et al.*, 1992b). In our study the two mildly sick cattle that received the parental vaccine had \log_{10} neutralizing titres of 2.15 and 2.02 at 21 d.p.v. This may be as a result of the other elements of the host immune system such cell-mediated immune response, the complement system and phagocytosis (McCullough *et al.*, 1992b) that occur *in-vivo* and were not accounted for in this study.

In view of the results from the VNT's, and homologous virus challenge experiments, it can be suggested that the animals that received the chimeric vaccine were completely protected against clinical disease. Therefore, it can be implied that the chimeric vaccine has successfully imitated the *in-vivo* protection characteristics of the parental vaccine confirming the transfer of properties from the field parental virus strain to the chimera.

An additional desirable attribute investigated was the predicted ability of the chimeric and parental vaccine to protect cattle against heterologous virus challenge for purposes of vaccine matching. This is because a vaccine that can elicit an antibody response that is capable cross-reacting with field virus strains within or between topotypes or genotype is advantageous (Doel, 2003; Mumford, 2007). Similar profiles are observed for the neutralization titres as well as r_1 values for the antisera raised against the chimera virus, vSAT2^{ZIM-14}-SAT2 and that of the parental vaccine SAT2/ZIM/14/90 when cross-matched with heterologous SAT2 viruses. This implies that the chimera maintained its antigenic similarity to parental virus.

In conclusion this study has shown evidence that an intra-serotype chimera vaccine consisting of the capsid of a field strain in a backbone of a genome-length clone, pSAT2 acquired humoral neutralizing immune response *in-vitro* and an immune response *in-vivo* comparable to that of the parental vaccine SAT/ZIM/14/90 in cattle. This supports the argument that better and superior designed chimeric vaccines can be applied to conventional methods to facilitate fast efficacious vaccines for control of FMD.

CHAPTER FIVE

CONCLUDING REMARKS AND FUTURE PROSPECTS

FMD, a highly contagious disease of cloven hoofed animals, is endemic in much of sub-Saharan Africa. It is a threat to food security and restricts profitable trade (both national and international) in animals and their products (James & Rushton, 2002; Vosloo *et al.*, 2002). The potential negative effects of FMD are not limited to developing countries, as endemic foci of the disease can be potential sources of infection for livestock exporting countries where the disease has been eradicated. Costly control measures are needed to keep the virus out of these free areas (Grubman & Baxt, 2004). It is therefore of global interest and concern that FMDV should be controlled and foci of disease reduced (Rodriguez & Gay, 2011; Sumption *et al.*, 2012; Jamal & Belsham, 2013).

Chemically inactivated vaccines have contributed greatly to the reduction in incidence and eradication of FMD in many parts of the world (Ward *et al.*, 2007). However, in southern Africa, control of the disease is hampered by the lack of effective vaccines. The production of effective vaccines necessitate the use of vaccine strains with characteristics such as rapid growth in cell culture, high antigen yields and appropriate immunological specificity (Doel, 2003). Towards addressing these impediments, this investigation concerned the characterisation of the non-structural protein coding regions of prevalent field isolates of FMDV and viruses subsequent to cell culture adaptation as well as evaluating the protective ability and antigenic features of an African FMD engineered virus. In this conclusion, the new information gathered during the investigation will be summarized and suggestions regarding future research made.

This study focused on three different aspects, which when taken together, can be applied to customized vaccine design for the African continent and subsequent control of FMD. In the first part of this investigation, phylogenetic and genetic analyses of the non-structural genomic regions of FMDV serotypes prevalent in sub-Saharan Africa were performed in order to identify conserved functional regions in virus isolates across serotypes and topotypes. These viruses were obtained from wild and domestic species sampled in different countries over 32 years. The results indicated that during naturally occurring variation, the viable genome contains conserved protein domains critical for structure and function. This gives us information on the genetic elements involved in FMDV replication specific to serotype and topotype that can be used in the selection of vaccine seed viruses. Secondly, the genomic and phenotypic characteristics of BHK-21 cell-culture adapted FMDV SAT1 and SAT2 viruses were determined to provide information on the

essential residue substitutions acquired during cell culture growth. These can be applied to chimeric virus constructs improving their design by enhancing fitness for cell culture adaptation necessary for vaccine production. The third aspect of the study involved comparison of the antigenic nature of a SAT2 chimeric virus with that of its parental virus in cattle by live virus challenge post vaccination and testing the cross-reactivity of the chimeric and parental viruses to antisera raised against selected SAT2 reference seed vaccine strains for purposes of vaccine matching. These provided basic information on the biochemical nature of the virus that can be used for future vaccine evaluation studies in cattle should chimeric viruses eventually replace parental viruses.

The non-structural proteins of FMDV are responsible for RNA replication, proteolytic processing of the viral polyprotein precursor, folding and assembly of the structural proteins, and modification of the cellular translation apparatus (Vakharia *et al.*, 1987; Rueckert, 1996; Belsham, 2005). Investigation of the amino acid heterogeneity of the non-structural proteins of seventy-nine FMDV isolates of SAT1, SAT2, SAT3, A and O serotypes revealed between 29-62% amino acid variability. The Leader protease (L^{pro}) and 3A proteins were the most variable whilst the RNA-dependent RNA polymerase (3D^{pol}) was the most conserved. Phylogeny based on the non-structural protein-coding regions showed separate clusters for southern African viruses for both the L^{pro} and 3C protease (3C^{pro}), and sequences unique to this group of viruses, e.g. in the 2C and 3C^{pro} proteins. These groupings were unlike serotype specific groupings based on structural protein-coding regions.

These phylogenetic groupings suggest separate ecological viral pools (described in section 1.2.2); the southern African virus cluster [solely SATs from Mozambique, Namibia, South Africa, Zambia, Zimbabwe, (Pool 6)] and the greater northern Africa virus cluster [SATs, A and O types from Ethiopia, Eritrea, Nigeria, Senegal, Sudan and Somalia, (Pool 5)] (Paton *et al.*, 2009; OIE/FAO FMD Reference Laboratory Network Annual Report, 2011), each with its own distinct virus evolution appropriate to the ecological niche. In contrast, categorizing the FMDV from East Africa (Kenya, Uganda, Tanzania, Rwanda), a region that interfaces between southern and the greater northern Africa, Pools 5 and 6 were ill defined with these viruses being found in either of the Pools. This could be a result of cross border movement of animals (Sangula *et al.*, 2010a; Sangula *et al.*, 2010b; Di Nardo *et al.*, 2011; Maree *et al.*, 2011b; Hall *et al.*, 2013). More

interestingly a unique cluster of SAT FMDV viruses from Uganda is observed, perhaps suggesting an additional ecological system within Pool 4. It still remains to be discovered if there are any FMDV genome markers that increase virus fitness or pathogenicity specific to the ecological niches. This may influence selection of seed virus for vaccine manufacture or universal diagnostic assays established using the 3ABC peptides.

The amino acid substitutions and the nature of the naturally occurring substitutions provided insight into the functional domains and regions of the non-structural proteins that are critical for structure and function. The L^{pro} of southern African SAT type isolates differed from A, O and SAT isolates in northern Africa, particularly in the auto-processing region. However, the critical residues involved in catalysis were conserved i.e. 2A peptide ¹²DVEXNPG¹⁸ that separates the P1-2A region (Ryan *et al.*, 2004); L^{pro}, C52, H149, D165, that cleaves this protease from the rest of the polyprotein (Piccone *et al.*, 1995; Guarné *et al.*, 1998); and 3C^{pro}, H46, D84, C163 which is the active triad (Birtley *et al.*, 2005). The observed conservation is confirmed by the three-dimensional structures of the 3C^{pro} and 3D^{pol} that showed the observed variation does not affect the enzymatic active sites or substrate binding sites. Variation in the 3C^{pro} cleavage sites may be an indication of broad substrate specificity. In general, the trend observed was conservation of all the critical motifs in the non-structural proteins, emphasizing the tendency for continuous selection of viable genomes in the quasispecies, thus maintaining structure and function of viral-specific enzyme activities (Domingo *et al.*, 1990; Bablanian & Grubman, 1993).

Most interesting was an 11 residue deletion occurring at position 136-146 of the 3A peptide sequence of SAT2/SEN/7/83 obtained from cattle. FMDV with deletions in the 3A peptide have been reported to multiply effectively in porcine cell lines and are virulent in pigs, yet such viruses are found to be less virulent in cattle as well as bovine cell lines (Giraudou *et al.*, 1990; Knowles *et al.*, 2001; Pacheco *et al.*, 2003; Maroudam *et al.*, 2010), but this still needs to be confirmed for SAT2/SEN/7/83.

Continuous future full genome sequencing is advised as the FMDV is constantly evolving, leading to changes in circulating viruses (Jamal & Belsham, 2013; Namatovu *et al.*, 2013). The implications of apparent variation in the relatively conserved non-structural proteins may have implications for diagnostic assays such as the 3ABC non-structural protein (NSP) ELISA

currently used for differentiating infected from vaccinated animals (DIVA) (van Rensburg *et al.*, 2002; Clavijo *et al.*, 2004), the proposed negative markers based on the 3B (Uddowla *et al.*, 2012), universal primers based on the 3D^{pol} (Callahan *et al.*, 2002), and proposed antiviral drugs based on the 3C^{pro} (Curry *et al.*, 2007).

Cell culture adaptation is necessary for production of inactivated FMD vaccines (Amadori *et al.*, 1994; Amadori *et al.*, 1997; Doel, 2003). However, this time consuming technique does not yield good results for the SAT viruses (Pay *et al.*, 1978; Preston *et al.*, 1982). This is coupled with the potential of random mutations occurring upon serial virus passage (de la Torre *et al.*, 1985) that may impact negatively on the antigenicity or virus stability (Haydon *et al.*, 2001a; Domingo *et al.*, 2003; Borca *et al.*, 2012). This study described the molecular changes in the surface capsid proteins (VP1-3) and the resultant phenotypes that occurred in SAT viruses upon BHK-21 cell culture adaptation. Fifteen SAT1 and SAT2 viruses, serially passaged in BHK-21 cells, were virulent in CHO-K1 cells and displayed enhanced affinity for heparin, compared to their low passage counterparts. Comparative results with several SAT isolates revealed the fixation of positively charged residues at positions 83-85 of the β D- β E loop and 110-112 in the β F- β G loop of VP1 upon adaptation to cell culture. Molecular docking simulations showed enhanced binding of heparin to a model of the adapted SAT1 virus, with the region around Arginine 112 contributing the most energetically favourable binding site. These results confirm previous studies (Maree *et al.*, 2010; Maree *et al.*, 2011a) and support the importance of these positions during BHK-21 cell culture adaptation. Information on the common heparan sulphate proteoglycan (HSPG) binding sites for the SAT viruses, and the role of HSPG during cell entry can be applied to the construction of chimeric virus subsequently circumventing time-consuming adaptation of SATs to BHK-21 cell lines.

Future experiments should exclude the possibility that the net positive gain in charge by the BHK-21 cell culture adapted viruses can be associated with heparan sulphate (HS) independent binding such as the use of other glycosaminoglycans (GAGs) expressed on the cell surface, or by other unknown mechanisms (Baranowski *et al.*, 1998; Zhao *et al.*, 2003; Berryman *et al.*, 2013). The positive residue mutations reported by this study that seemed to be linked to HSPG binding sites can be introduced individually or collectively into chimeric virus constructs and tested in HS deficient CHO cell lines such as CHO-677 that expresses low levels of only HS and CHO-745

expressing low levels of both HS and chondroitin (Esko *et al.*, 1987; Lidholt *et al.*, 1992). Failure of the recombinant chimeric viruses to replicate in both these cell lines will confirm that the positive charges around the five-fold axis cannot utilize the other GAGs as receptors (Jackson *et al.*, 1996). Furthermore, the likelihood of the use of heparan sulphate independent mechanisms of infection will also have been eliminated, leaving us with the possibility of the BHK-21 cell culture adapted viruses employing the HS receptor for replication in CHO-K1 cells.

Similarly, it should be confirmed if the affinity of the BHK-21 cell culture adapted viruses for heparin arose solely from the gain in net positive charge. The recombinant chimeric viruses with these mutations individually or collectively can be incubated with varying concentrations of heparin salt (as discussed previously in section 3.2.4; Jackson *et al.*, 1996). Decreased plaque formation in BHK-21 or CHO-K1 cells, which have previously supported their replication, will confirm the affinity of the viruses for heparin.

It is also necessary to investigate if the increased growth in BHK-21 cell line gained upon cell culture adaptation is applicable to BHK-21 cell culture suspension systems, the latter being used in large scale antigen production for FMDV vaccines.

Certain positions of positive residue substitution such as in VP2 and VP3 upon cell culture adaptation also determine antigenicity (Verdaguer *et al.*, 1995; Fry *et al.*, 1999). Therefore, additional experiments are needed to determine whether the antigenic traits of the cell culture adapted viruses are unchanged. In most cases the positive substitutions resulted in virus attenuation in the natural hosts of FMD while in some cases they resulted in virulence in suckling mice (Sa-Carvalho *et al.*, 1997; Zhao *et al.*, 2003; Borca *et al.*, 2012). Pathogenicity studies of the cell culture adapted viruses in target species are advised.

A large number of FMDV endemic regions use vaccination as one aspect of disease control and an essential component for the Progressive Control Pathway (PCP) of FMD (Sumption *et al.*, 2012) is the prerequisite for provision of correct vaccine formulations where the vaccine strain protects against the circulating viruses. Chimeric virus backbone constructs (consisting of L-1A, P2-P3), where the antigenic properties on the capsid (1B-1D/2A) are rapidly swapped and replaced with the same region of the outbreak virus, can be used to produce inactivated vaccine

(van Rensburg *et al.*, 2004; Blignaut *et al.*, 2011). Thus the resultant virus acquires the desired antigenic nature while retaining the desired replication properties of the chimeric backbone, which may include rapid replication in cell culture, attenuation or DIVA markers (van Rensburg *et al.*, 2004; Maree *et al.*, 2010; Blignaut *et al.*, 2011; Maree *et al.*, 2011a; Uddowla *et al.*, 2012).

This study compared selected intrinsic qualities of a chimeric virus, v^{ZIM14}SAT2 (Maree *et al.*, 2013), and that of its parental virus, SAT2/ZIM/14/90, in cattle. The parental virus shows characteristics of a good vaccine strain because of its ability to elicit an immune response that cross-reacts broadly against viruses from the southern Africa SAT2 topotype (Maree *et al.* 2011b). It is essential that the performance of a vaccine derived from chimeric virus antigen be at least equivalent to that of its parental virus antigen if it is to replace the parental virus as a seed for vaccine production. The comparisons in this study included the ability of inactivated vaccines produced from the two viruses respectively to elicit a protective immune response in cattle when challenged with a homologous virus. In addition, the capacity of both of the chimeric and parental viruses to cross-react with neutralizing antibodies in post-vaccination cattle sera with a range of inactivated SAT2 reference virus antigens was compared.

The chimeric virus, v^{ZIM14}-SAT2, was obtained by exchanging the capsid-coding region (1B-1D/2A) of a SAT2 genome-length clone, pSAT2, with that of the field isolate, SAT2/ZIM/14/90 (Maree *et al.*, 2013). Our results showed that vaccine formulated from both the inactivated chimera and parental virus antigen then administered to cattle, elicited neutralizing antibodies after 21 days in all the study animals. The immune responses elicited by both vaccines were comparable. The cattle were protected against clinical disease upon challenge with the homologous virus (SAT2/ZIM/14/90). Finally, vaccine-matching using VNTs presented similar antigenic profiles (r_1 -values) for both the chimeric and parental viruses. These results provided evidence that chimeric vaccines containing the external capsid of field isolates can be successfully produced and that they induce protective immune responses in FMDV host species. This approach therefore has the potential to make a valuable contribution to the 16-year framework for the progressive control of FMD in Southern African Development Community (SADC) countries (Lubroth *et al.*, 2007).

However, much research is still needed to develop improved FMD vaccines for use in Africa. Recommendations for the improvement of FMD vaccines include exploring the use of antigen that retain their stability at mild acidic pH or at the environmental temperatures found in the tropics. This will reduce dependency on cold chain delivery systems and increase vaccine shelf life (Mateo *et al.*, 2008; Maree *et al.*, 2013; Porta *et al.*, 2013). It is necessary to prove that each individual clone has retained the property of growing well in cell culture. Although some of these aspects may be addressed through the use of infectious cDNA technology, as utilised in this thesis, alternative approaches should also be explored in future studies. One promising strategy is use of virus-like particles (VLPs) constructed from recombinant structural proteins whereby the antigenic conformation of the FMDV particles is preserved. The VLPs can be delivered by the Ad5 vector (Grubman *et al.*, 2010) and are non-infectious and non-replicating, therefore do not need bio-containment during manufacture (Crisci *et al.*, 2012)

In conclusion, this study compared the genome encoding regions of the non-structural proteins revealing conserved functional motifs and patterns of variation specific to geographical regions for prevalent FMDV serotypes in sub-Saharan Africa. In addition, the genetic characteristics acquired by these viruses upon adaption in BHK-21 cell lines used for vaccine manufacture were described, including the surface oriented Lys/ Arg residue positions on the virus capsid five-fold axis shared among the SAT1 and SAT2 that lead to enhanced binding of a heparin moiety to Arg at position 112 in a SAT1 virus model. These data taken together improve our knowledge on the genome structure, and replicative function of the African FMDV. It is relevant for the selection of seed vaccine strains specific to toptype or geographical region among the serotypes. This knowledge is also key for reverse genetic studies that construct ideal chimeric viruses. These would be permissive to the HS receptors and suitable for inactivated vaccine production systems with shorter high yielding throughputs. Lastly, this study provided evidence that inactivated vaccines formulated from chimeric viruses can elicit protective immunity in cattle under experimental conditions.

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APPENDICES

APPENDIX 1: List of oligonucleotides used for sequencing in study

Table I: List of oligonucleotide primers used for sequencing. The forwards and reverse primers, their location on the FMDV genome and the serotype specificity are provided.

PCR fragment sequenced in the different serotypes	Primer oligonucleotide				
	Name*	Orientation	Sequence (5'-3')	Location	Serotypes sequenced
Leader	NCR1*	Forward	TACCAAGCGACTCGGGATCT	IRES	SAT, A and O
	NCR2*	Forward	GCTTCTATGCCTGAATAGG	IRES	SAT
	SEQ 115 ZIM L-Rev	Reverse	CCAACAAGTCCTGAGAAAGCC	VP4	SAT1/ZIM/14/90
	SEQ 116 HV L-Rev	Reverse	TGTGTGTCCATGGAATTCTGG	VP4	SAT1/ZIM/HV/13/90
	SEQ 117 Nig L-Rev	Reverse	GAGCTGCGTGTCATCGAG'	VP4	SAT1: NIG/6/76, NIG8/76
	O-LINT-F	Forward	CAARCAYYTGCTCCACACCGG	L ^{pro}	O
	O-LINT-R	Reverse	TCRTCRCATCGCGTACCACCC	L ^{pro}	O
	A-LINT-F	Forward	ACCCAGYGAGGTGTGYATGG	L ^{pro}	A
	A-LINT2-F	Forward	CGATGAA YCGTTCTTCGACTGGGTC	L ^{pro}	A
2A	2B-208R*	Reverse	ACAGCGGCCATGCACGACAG	2B	SATA
	2B-R Uga	Reverse	GCTACAGCGGCCATRCAYGACA	2B	SAT (Uganda &Nigeria) O (Uganda, Kenya and Tanzania)
	Seq 109 Uga	Forward	GTGGGCGACAAYCCAGTCGTC	VP1	SAT (Uganda &Nigeria)
	SEQ 113	Forward	CCGCTTGCTATYCCGTTTAC	VP1	SAT2: UGA/2/02, ERI/12/89
	SEQ 178 SEN/10/97	Forward	GAGCTCCTCGTGCGCATGAAG	VP1	A/SEN/10/97
2BC, 3AB ₁₂₃ and partial 3C	SEQ 70	Forward	ACGTCCCGGCCCTGTCTTCAG	2B	SAT, O and A
	SEQ 71	Reverse	TCTTCGGGTGTGGACCGGAAG	2B	SAT, O and A
	SEQ 72	Reverse	GCCGCGGAGGCAAACGACCAC	2C	SAT
	SEQ 73	Reverse	ACATCTTGTTGCAGTCTTTC	2C	SAT
	SEQ 74	Forward	GCCGTTGAAATGAAGAG	2C	SAT
	SEQ 75	Reverse	GCTGTCCTTTCTCGATGAGG	3A	SAT
	SEQ 76 (3A/anti)	Reverse	GCTGTCCCTTCTCAATGAGG	3A	SAT
	SEQ 95	Reverse	GTAGCCGTCGAAGTGGTCAGG	2C	SAT
	SEQ 96	Reverse	AGGCTTTGATCCAGTCGCGGA	2C	SAT

PCR fragment sequenced in the different serotypes	Primer oligonucleotide					
	Name*	Orientation	Sequence (5'-3')	Location	Serotypes sequenced	
2BC, 3AB ₁₂₃ and partial 3C	SEQ 97	Forward	GAGCCTGTCCAATCCGCGGAG	2C	SAT2/ERI/12/98	
	SEQ 159 EA	Reverse	ACATYGCCACYGGGTTGGT	2C	O and A	
	SEQ 160 UGA	Reverse	ACATTGCCACTGGGTTAGT'	2C	O: UGA/17/98,UGA/1/75	
	SEQ 161 EA	Reverse	GGTTGTARCCGTCGAAGTGG	2C	O and A	
	SEQ 162 EA	Reverse	GGTCCTTCAGCTTGYGGTTGT	2C	O and A	
	SEQ 164	Reverse	CACCTCCTGAACGAGTTGGT	2C	A/CIV/4/94	
	SEQ 163	Forward	ACCAACTCGTTCAGGAGGTG	2C	A/CIV/4/94	
	SEQ 77	Reverse	GCGTAGGGTCCTTCAGC	2C/3A	All	
	SEQ 111	Forward	GGACATTACCAAAGCACTTG	3B	SAT2/ERI/12/98	
	SEQ 80	Reverse	GTGGCCATGGCCGGTGTGTCTGTG	3B	SAT1/ZIM/14/90	
	SEQ 79	Reverse	CTCCTGAGAAGATGAGTC	3C	All	
	SEQ 78	Reverse	CCATGACCATCTTTTGCAAG	3C	All	
	SEQ 151 EA 3B Fw	Forward	GATGGTGGATGAWGCAGTGAATG	3A	A/ETH/7/92, A/SOM/1/78, O/UGA/1/75	
	SEQ 126 O & A Rev	Reverse	TCCATRCACACTACAATGTC	3C	O & A	
	3C ^{pro} , partial 3D	NC3+	Forward	CCKGTGAAGAAGCCTGTTCGC	3B	All
		NC3-	Reverse	CGCTCTTCMACATCTCT	3D	SAT and O
SEQ 110 3C Forw		Forward	CAGACAGTGACTTYAGAGTG	3C	SAT	
SEQ118 SAT2 3CF		Forward	GACAGACAGTGACTTCAGAG	3C	SAT	
SEQ 119 SAT2 3CR		Forward	CGTTGTGAAGATGCGAGGCA	3C	SAT	
SEQ120		Forward	GACAGACCGTGATTTACAGG	3C	SAT2/ERI/12/98	
SEQ121		Forward	CGATACCATGCCAGGCCCTCT	3C	SAT2/ERI/12/98	
SEQ122 SEN		Forward	GACGGTGACACCATGCCTG	3C	SAT2/SEN/5/75	
SEQ 165 EA 3C		Reverse	CGCGCTCTTCCACATCTCTGG	3C	O and A	
SEQ 147		Forward	GGTTGATTGTTGATACCAGAGATG	3C	O/UGA/1/75	
SEQ 150		Forward	ACAGGACATGCTCTCAGACGC	3C	A/CIV/495	
SEQ 125		Forward	AARGACATTGTAGTGTGYATGG	3C	O and A	
SEQ 23		Forward	TGCAGATGAAGGCCAC	3C	SAT	

Table I continued

PCR fragment sequenced in the different serotypes	Primer oligonucleotide				
	Name*	Orientation	Sequence (5'-3')	Location	Serotypes sequenced
3D	SEQ 166 3D Rv	Reverse	CGACRATGCGAGTCTTGC	3D	O and A
	SEQ 146 ERI 3D Rev	Reverse	CCTCCTCAAACATGATGTTC	3D	SAT2/ERI/12/98
	SEQ 149 CIV 3D Rev	Reverse	GGTGTCAAGGCTCCATGGCGTC	3D	A/CIV/4/95
	SEQ 90	Reverse	ATGATGTTCATGGCGTCACTGC	3D	SAT
	SEQ 94	Reverse	CATCTGGGTTGCAACCGACCG	3D	SAT
	SEQ 98	Reverse	ATCTGCCAATCATCATTCTG	3D	SAT1/ZIM/25/90
	3D-D	Reverse	CCGCACACGGCGTTACCCA	3D	All
	3D-U	Forward	GCCGGCAAGACTCGCATTGT	3D	All
	SEQ 123 SEN 3D Forw	Forward	GTCAGTCCATTACCGATGTTAC	3D	SAT2/SEN/5/75
	SEQ124 SEN 3D Rev	Reverse	GCCACGGAGATCAACTTCTC	3D	SAT2/SEN/5/75
	SEQ 145 ERI 3D Frw	Forward	GAACATCATGTTTGAGGAGG	3D	SAT2/ERI/12/98
	SEQ 148 EA Type A	Reverse	CGTTGTTYGAGTGCATTTGAGC	3D	A/TAN/4/80, A/SOM/1/78
	SEQ 149	Reverse	GGTGTCAAGGCTCCATGGCGTC	3D	A/CIV/4/95
	SEQ 142	Reverse	CATCGACAATGCGAGTCTTGCC	3D	O/KEN/10/95
	SEQ 143 3D 3' Rev	Reverse	GTCCACGGCGTGCAAAGGAG	3D	SAT ,O
	SEQ 127	Forward	GGCAAGACTCGCATTGTCGATG	3D	O/KEN/10/95
	SEQ 128	Reverse	CGCTGTGCAAGTGTGACGCG	3D	O/ SUD.4/80
	3'UTR	SEQ 93	Forward	CTCCTTTGCACGCCGTGGGAC	3D
#7R		Forward	CCAAGCTACAGATCAC	3D	All

Table I continued

Y=CT, M=AC, K=AG, W=AT, S=CG, K=TG, D=AGT, V=AGC, H=ACT, B=GCT

*The primers were designed by the Onderstepoort Veterinary Institute (with the exception of three primers who's references are indicated).

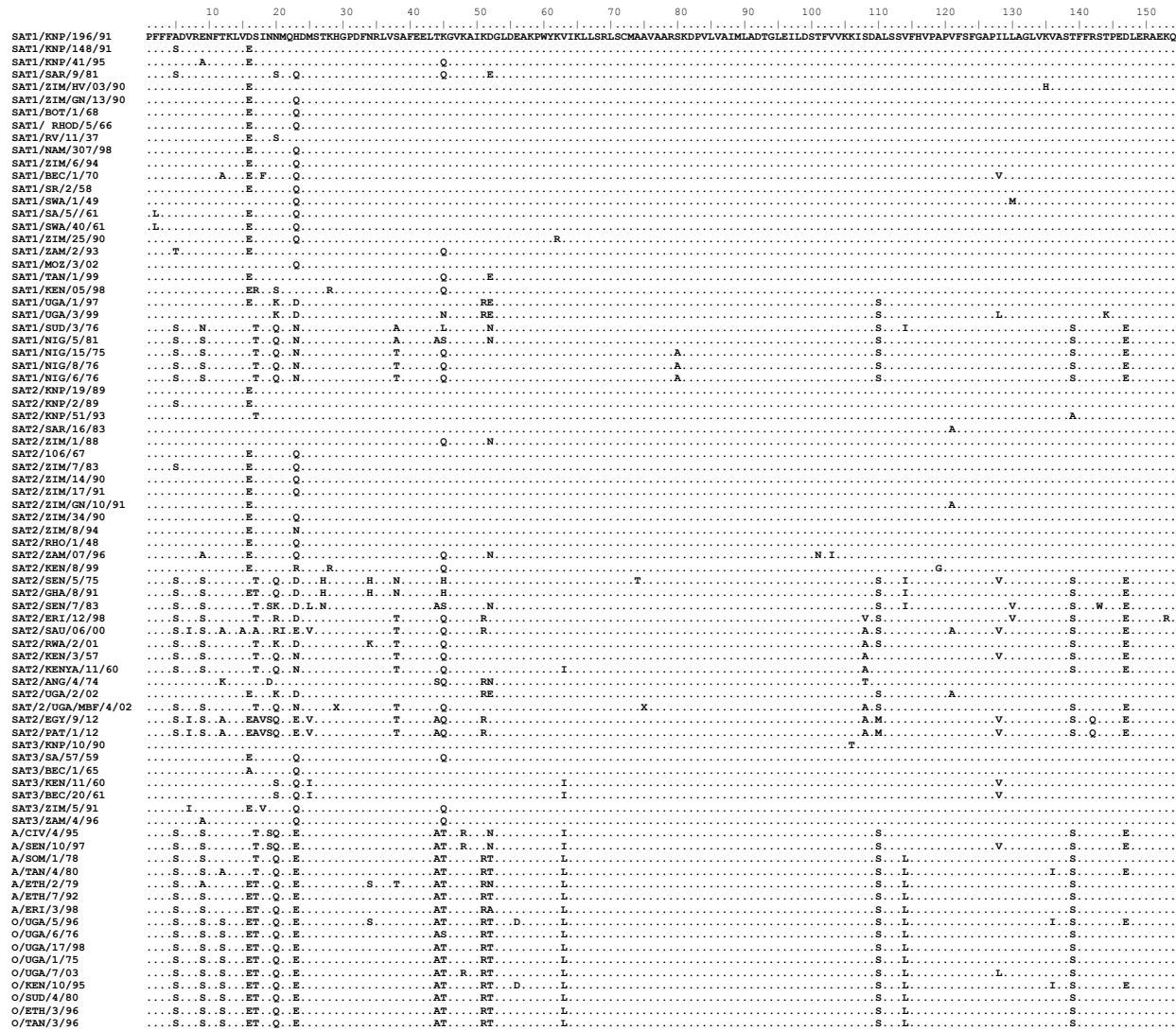
♣(Maree *et al.*, 2011b)

♠(Bastos *et al.*, 2001)

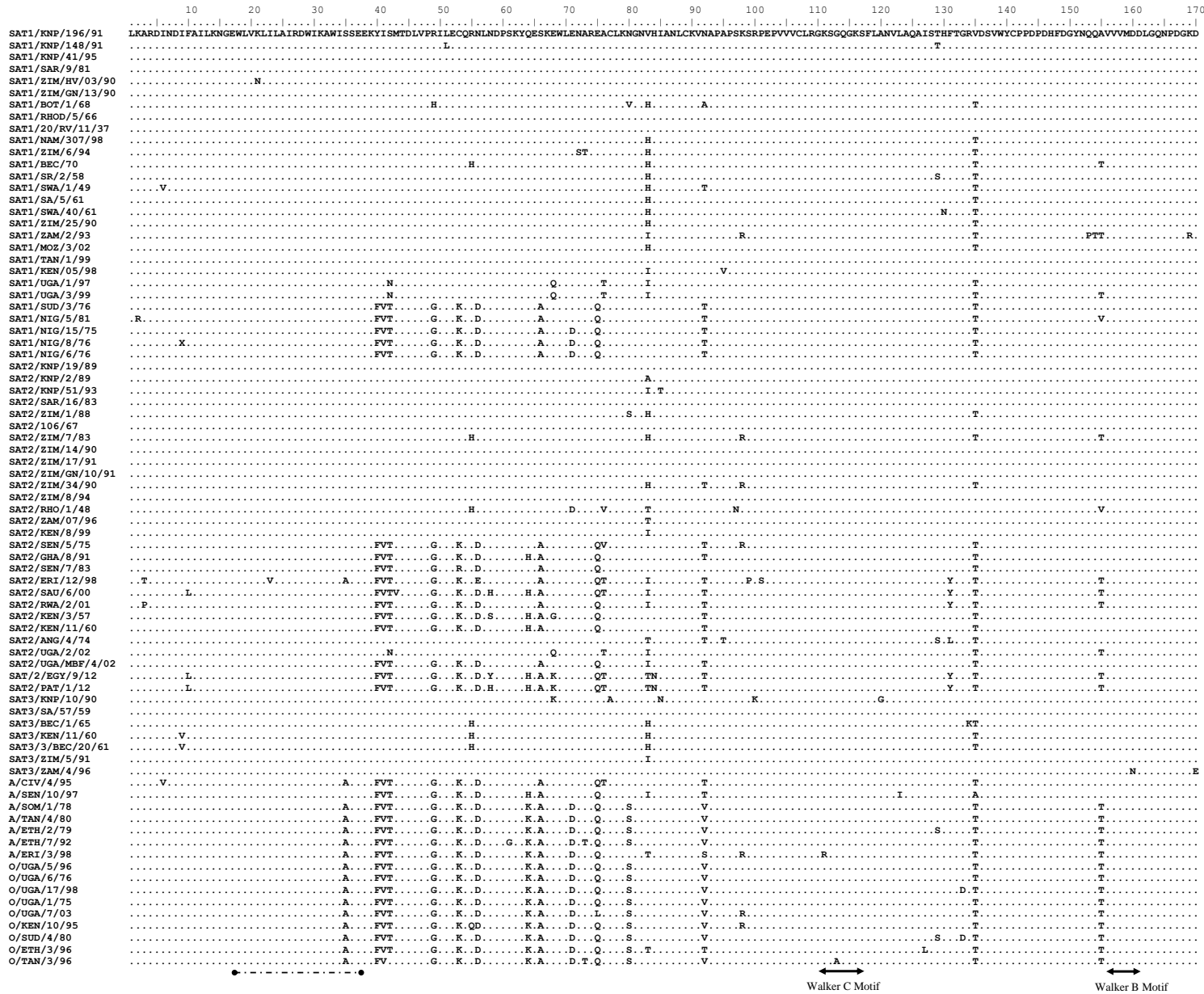
APPENDIX 2: Deduced amino acid sequence alignments for non-structural FMDV proteins

	10	20	30	40	50	60	70	80	90	100	110	120	130	140	150	160	170	180	190	200		
SAT1/KNP/196/91	M	K	T	C	D	S	V	L	F	E	I	H	R	H	L	K	T	K	E	---		
SAT1/KNP/148/91	N	L	F	---	D	---	K	---	T	---	K	---	T	---	K	---	T	---	K	---		
SAT1/KNP/41/95	N	L	F	---	F	---	K	---	T	---	K	---	T	---	K	---	T	---	K	---		
SAT1/SAR/9/91	N	L	F	---	F	---	K	---	T	---	K	---	T	---	K	---	T	---	K	---		
SAT1/ZIM/HV/03/90	W	N	L	---	Q	C	N	D	---	K	---	N	---	G	---	D	---	I	---	V	---	
SAT1/ZIM/GN/13/90	N	L	Y	---	Q	R	S	T	---	K	---	T	---	K	---	T	---	K	---	T	---	
SAT1/BOT/1/68	G	N	L	---	S	---	F	---	Q	F	---	A	---	D	---	D	---	D	---	D	---	
SAT1/RHO/5/66	N	L	I	---	L	---	Q	I	R	A	---	D	---	D	---	D	---	D	---	D	---	
SAT1/SV/11/37	N	L	Q	---	K	Q	A	F	---	A	---	D	---	D	---	D	---	D	---	D	---	
SAT1/NAM/307/98	N	A	L	---	T	---	K	Q	A	F	---	A	---	D	---	D	---	D	---	D	---	
SAT1/ZIM/6/94	G	D	---	Q	F	T	A	---	D	---	D	---	D	---	D	---	D	---	D	---	T	---
SAT1/1/BEC/1/70	N	L	L	---	F	---	F	---	I	---	N	---	V	---	D	---	D	---	D	---	R	---
SAT1/SR/2/58	N	L	F	---	Q	F	---	A	---	D	---	D	---	D	---	D	---	D	---	D	---	
SAT1/SAR/1/49	N	L	F	---	Q	F	---	A	---	D	---	D	---	D	---	D	---	D	---	D	---	
SAT1/SA/5/61	N	L	F	---	Q	F	---	A	---	D	---	D	---	D	---	D	---	D	---	D	---	
SAT1/SWA/40/61	N	S	---	F	---	F	---	G	---	E	---	K	---	T	---	K	---	T	---	K	---	
SAT1/ZIM/25/90	N	L	F	---	Q	F	---	A	---	D	---	D	---	D	---	D	---	D	---	D	---	
SAT1/ZAM/2/93	N	L	---	K	---	F	---	A	---	D	---	D	---	D	---	D	---	D	---	D	---	
SAT1/MOZ/3/02	N	L	---	S	---	Q	F	---	A	---	D	---	D	---	D	---	D	---	D	---	F	---
SAT1/TAN/1/99	A	I	N	---	L	---	F	---	Q	F	---	S	---	T	---	E	---	K	---	T	---	
SAT1/KEN/05/98	N	L	---	F	---	Q	F	---	S	---	T	---	E	---	K	---	T	---	K	---	T	---
SAT1/UGA/1/97	N	---	I	A	---	V	Q	A	I	R	---	K	---	S	---	L	---	F	---	D	---	
SAT1/UGA/3/99	N	---	I	A	---	V	Q	A	I	R	---	K	---	S	---	L	---	F	---	D	---	
SAT1/SUD/3/76	N	---	I	A	---	V	Q	A	I	R	---	K	---	S	---	L	---	F	---	D	---	
SAT1/NIG/5/81	N	---	I	A	---	V	Q	A	I	R	---	K	---	S	---	L	---	F	---	D	---	
SAT1/NIG/15/75	N	---	I	A	---	V	Q	A	I	R	---	K	---	S	---	L	---	F	---	D	---	
SAT1/NIG/8/76	S	---	I	A	---	V	Q	A	I	R	---	K	---	S	---	L	---	F	---	D	---	
SAT1/NIG/6/76	S	---	I	A	---	V	Q	A	I	R	---	K	---	S	---	L	---	F	---	D	---	
SAT2/KNP/19/89	N	---	I	A	---	V	Q	A	I	R	---	K	---	S	---	L	---	F	---	D	---	
SAT2/KNP/2/89	N	---	I	A	---	V	Q	A	I	R	---	K	---	S	---	L	---	F	---	D	---	
SAT2/KNP/51/93	S	---	I	A	---	V	Q	A	I	R	---	K	---	S	---	L	---	F	---	D	---	
SAT2/SAR/16/83	R	---	D	L	---	Y	---	F	---	N	---	F	---	N	---	D	---	D	---	D	---	
SAT2/ZIM/1/88	N	---	I	A	---	V	Q	A	I	R	---	K	---	S	---	L	---	F	---	D	---	
SAT2/1/6/67	N	---	L	---	L	---	S	---	F	---	K	---	T	---	K	---	T	---	K	---	T	---
SAT2/ZIM/7/83	N	---	L	---	I	---	Y	---	F	---	D	---	D	---	D	---	D	---	D	---	D	---
SAT2/ZIM/14/90	N	---	A	---	---	---	Q	---	I	---	N	---	D	---	D	---	D	---	D	---	K	---
SAT2/ZIM/17/91	N	---	L	---	F	---	Q	---	I	---	N	---	D	---	D	---	D	---	D	---	D	---
SAT2/ZIM/GN/10/91	N	---	L	---	F	---	Q	---	F	---	A	---	D	---	D	---	D	---	D	---	D	---
SAT2/ZIM/34/90	N	---	L	---	F	---	Q	---	F	---	A	---	D	---	D	---	D	---	D	---	D	---
SAT2/ZIM/8/94	N	---	L	---	L	---	---	---	Q	---	I	---	N	---	D	---	D	---	D	---	D	---
SAT2/RHO/1/48	N	---	---	---	R	---	Q	---	F	---	A	---	D	---	D	---	D	---	D	---	D	---
SAT2/ZAM/07/96	D	A	---	L	---	A	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
SAT2/KEN/8/99	N	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
SAT2/SAR/5/75	N	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
SAT2/GHA/8/91	D	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
SAT2/SEN/7/83	N	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
SAT2/ERI/12/98	N	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
SAT2/SUD/6/90	N	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
SAT2/RWA/2/01	N	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
SAT2/KEN/3/57	N	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
SAT2/KEN/11/60	N	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
SAT2/MG/4/74	N	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
SAT2/UGA/2/02	N	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
SAT2/UGA/MOZ/41/02	N	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
SAT2/EGY/9/12	N	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
SAT2/PAT/1/12	N	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
SAT3/KNP/10/90	N	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
SAT3/SA/57/59	D	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
SAT3/BEC/1/65	N	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
SAT3/KEN/11/60	N	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
SAT3/BEC/20/61	N	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
SAT3/ZIM/5/91	N	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
SAT3/ZAM/4/96	D	A	---	L	---	A	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
A/CI/14/95	N	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
A/SEN/10/97	N	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
A/SOM/1/78	N	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
A/TAN/4/80	N	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
A/ETH/2/79	N	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
A/ETH/1/92	N	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
A/ERI/3/98	N	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
O/UGA/5/96	N	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
O/UGA/6/76	N	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
O/UGA/17/98	N	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
O/UGA/1/75	N	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
O/UGA/7/03	N	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
O/KEN/10/95	N	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
O/SUD/4/80	D	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
O/ETH/3/96	N	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
O/TAN/3/96	N	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---

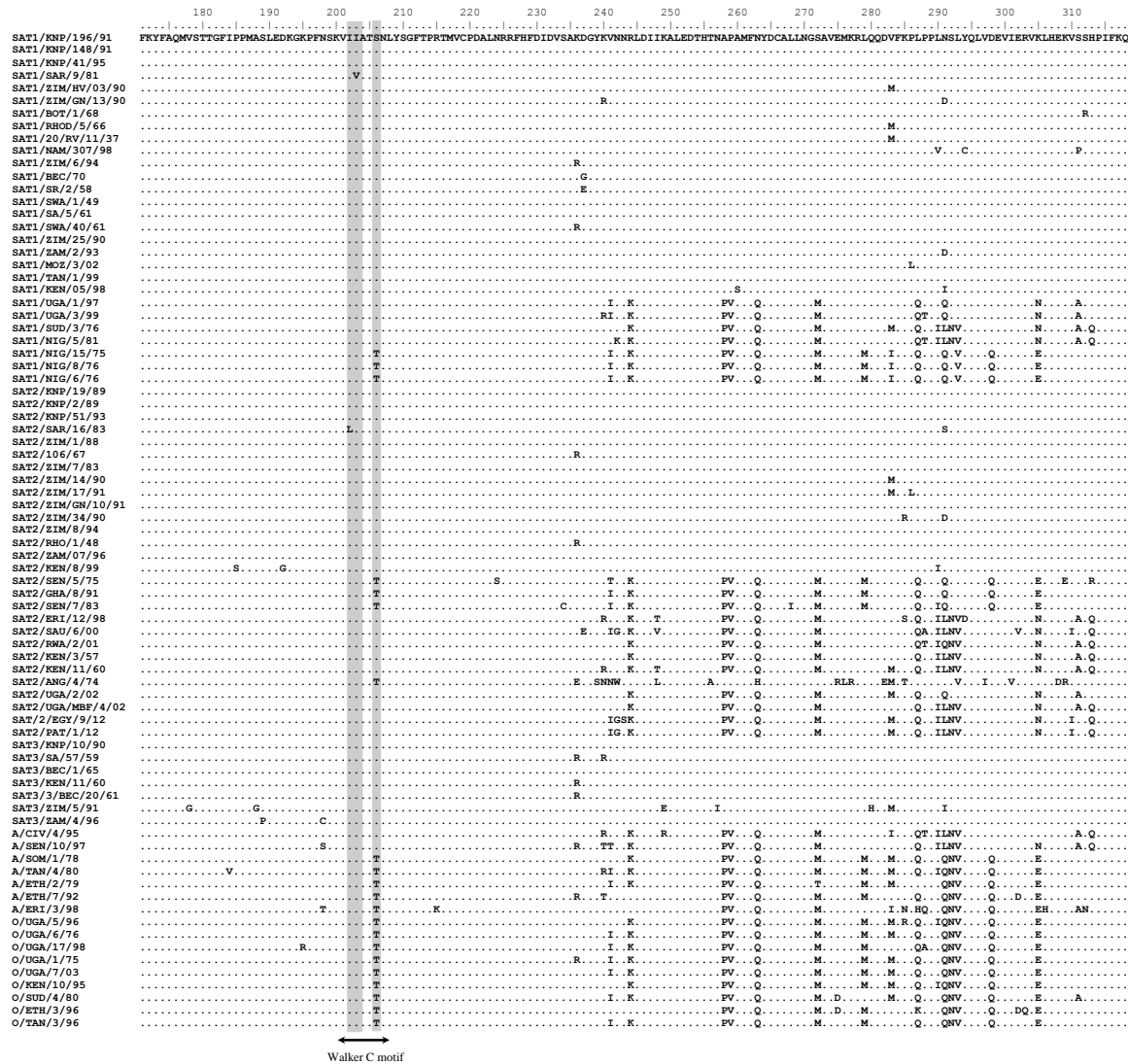
Supplementary Fig. A An alignment of 202 amino acids deduced for the Leader protease for 79 African FMDV isolates. The dots (.) indicate same as top sequence (SAT1/KNP/196/96) and a dash (-) depicts a gap generated during alignment of a particular sequence. Illustrated at the top of the alignment are the critical residues involved in catalysis of L-VP4: C52, H149 and D165 (Guarné et al., 1998), indicated by the downward arrow (↓). Three other residues K200, E94 and E97 associated with catalysis of L-VP4 (Guarné et al., 2000; Guarné et al., 1998) are indicated by the star (★). The position of three deletions (amino acid positions 25-27) in the alignments for the SAT virus isolates is indicated below the alignment with a double underline (==).



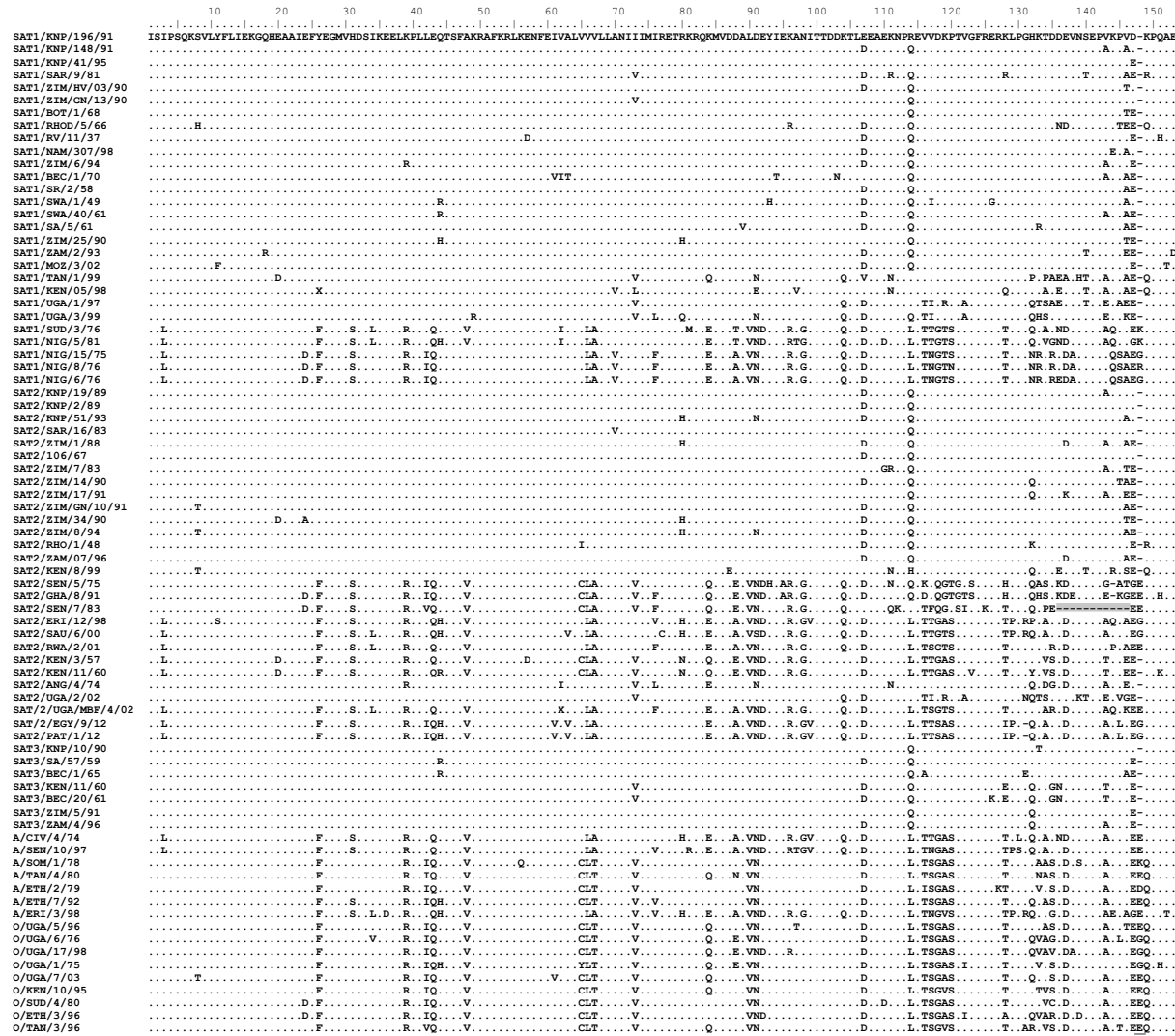
Supplementary Fig. B An alignment of 154 amino acids deduced for the 2B peptide for 79 African FMDV isolates. The dots (.) indicate similarity with the top sequence (SAT1/KNP/196/96). The hypervariable domains (residues positions 5-29 and 44-53) in the alignment are shown by the horizontal block bars (___) at the bottom of the alignment. Highly conserved motifs (residue positions 64-107 and 115-137) are depicted using horizontal dotted bars (.....). The latter conserved motif is a hydrophobic domain.



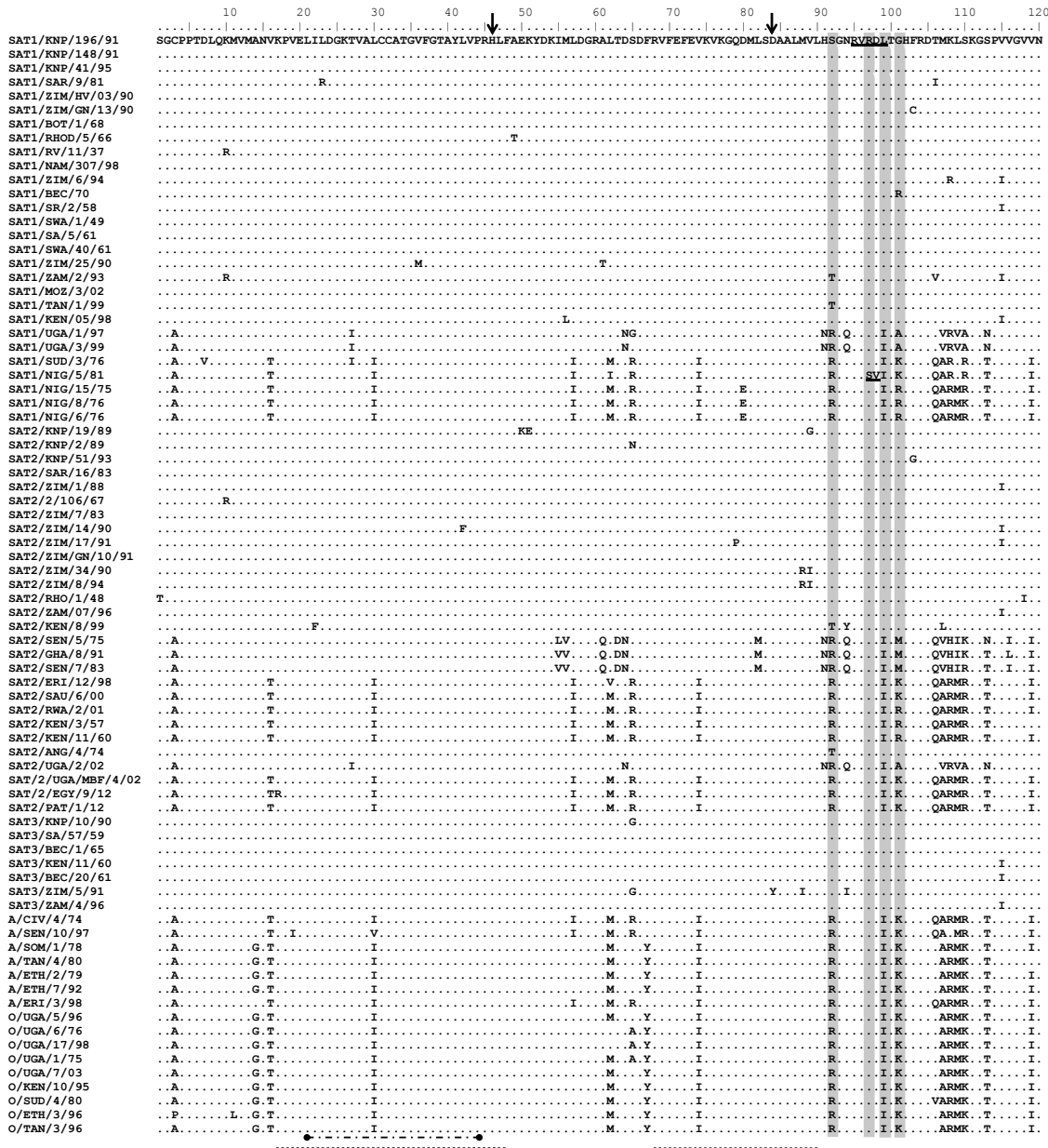
Supplementary Fig C continued



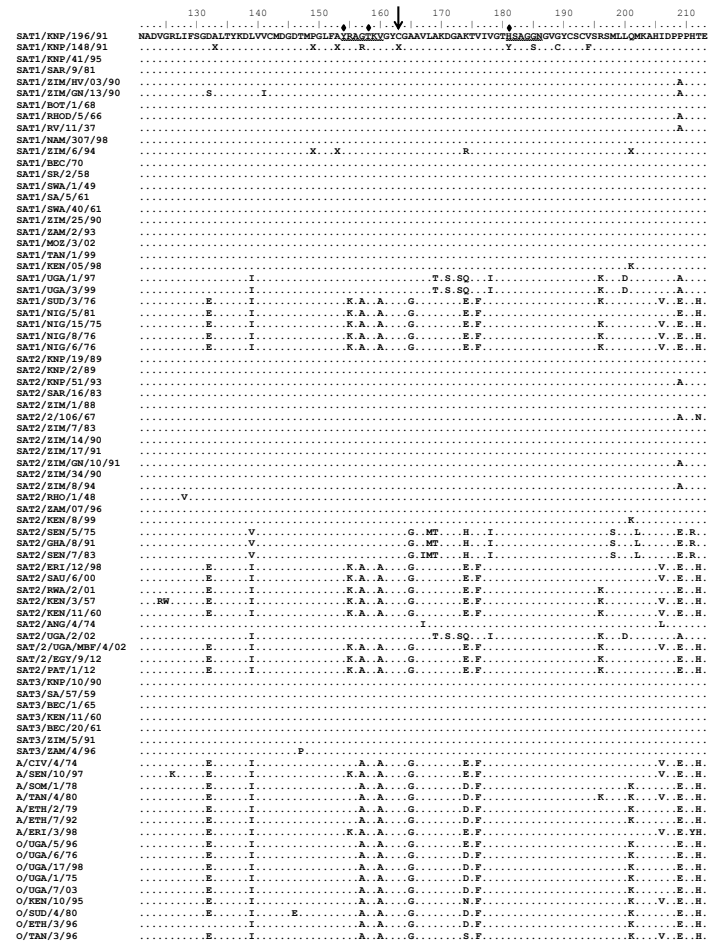
Supplementary Fig. C An alignment of 318 amino acids deduced for the 2C peptide for 79 African FMDV isolates. The dots (.) indicate same as top sequence (SAT1/KNP/196/96). The hydrophobic domain in the alignment (residue positions 17-34) is indicated by horizontal dash-dot bar (_____) at the bottom. The horizontal double arrows (←→) delimit the residue positions in the Walker A motif (110-117), Walker B motif (156-161) and the Walker C motif (201-207) (Sweeney et al., 2010). The vertical grey bars highlight residue substitutions I202→L, I203→V and T206→S observed in the southern SAT virus isolates that occurred in the conserved Walker C motif.



Supplementary Fig. D An alignment of 153 amino acids deduced for the 3A peptide for a 79 African FMDV isolates. The dots (.) indicate identical amino acids to the reference sequence SAT1/KNP/196/96 and a dash (-) depicts a gap generated during the alignment. Illustrated among other things at the bottom of the alignment are the hydrophobic domains (residue positions 1-16: ISIPSQKSVLYFLIEK, 25-30: FYEGMV and 60-74: EIVALVVLLANIII) depicted using a dash-dot bar (-----). A double underline (==) at amino acid position 148 shows the deletion observed in southern SAT virus isolates that grouped cluster I. The eleven residue deletions observed in SAT2/SEN/7/83 are highlighted in the grey horizontal bar.



Supplementary Fig. E continued



Supplementary Fig. E An alignment of 213 amino acids deduced for the 3C protease for 79 African FMDV isolates. The dots (.) indicate identity with the sequence (SAT1/KNP/196/96). At the bottom of the alignment are horizontal dotted bars (.....) indicate highly conserved areas in the sequence alignment (residue positions 17-48, 68-90, 129-156, 161-164). The horizontal dash - dot- bars(____) depict a hydrophobic domain observed at residue positions 27- 44. At the top of the alignment, the residue positions of the active triad (H46, D84 and C163) (Birtley *et al.*, 2005), are shown using the downwards arrow (↓). The residues (154-160, 181-186) that comprise substrate pocket (S1) (Birtley *et al.*, 2005), are indicated by the wavy underline (~~~~~). The three conserved residues Y154, T158 and H181 that donate hydrogen bonds to the P1 substrate (Birtley *et al.*, 2005), are indicated using the kites (◆). Underlined (___) at the top of the sequence alignment is the conserved motif 95-RVRDI-99 necessary for VPg uridylation (Nayak *et al.*, 2006). Highlighted with grey vertical bars are residues R92 R97 and K101 that contribute towards the uridylation process (Nayak *et al.*, 2006). The following substitutions were observed in the southern SAT virus isolates R92→ S/T, R97→S, I99→L and K101→ G/A. The residue substitutions of 97R→S and 98D→V in this conserved motif for SAT1/NIG/5/81 are underlined.


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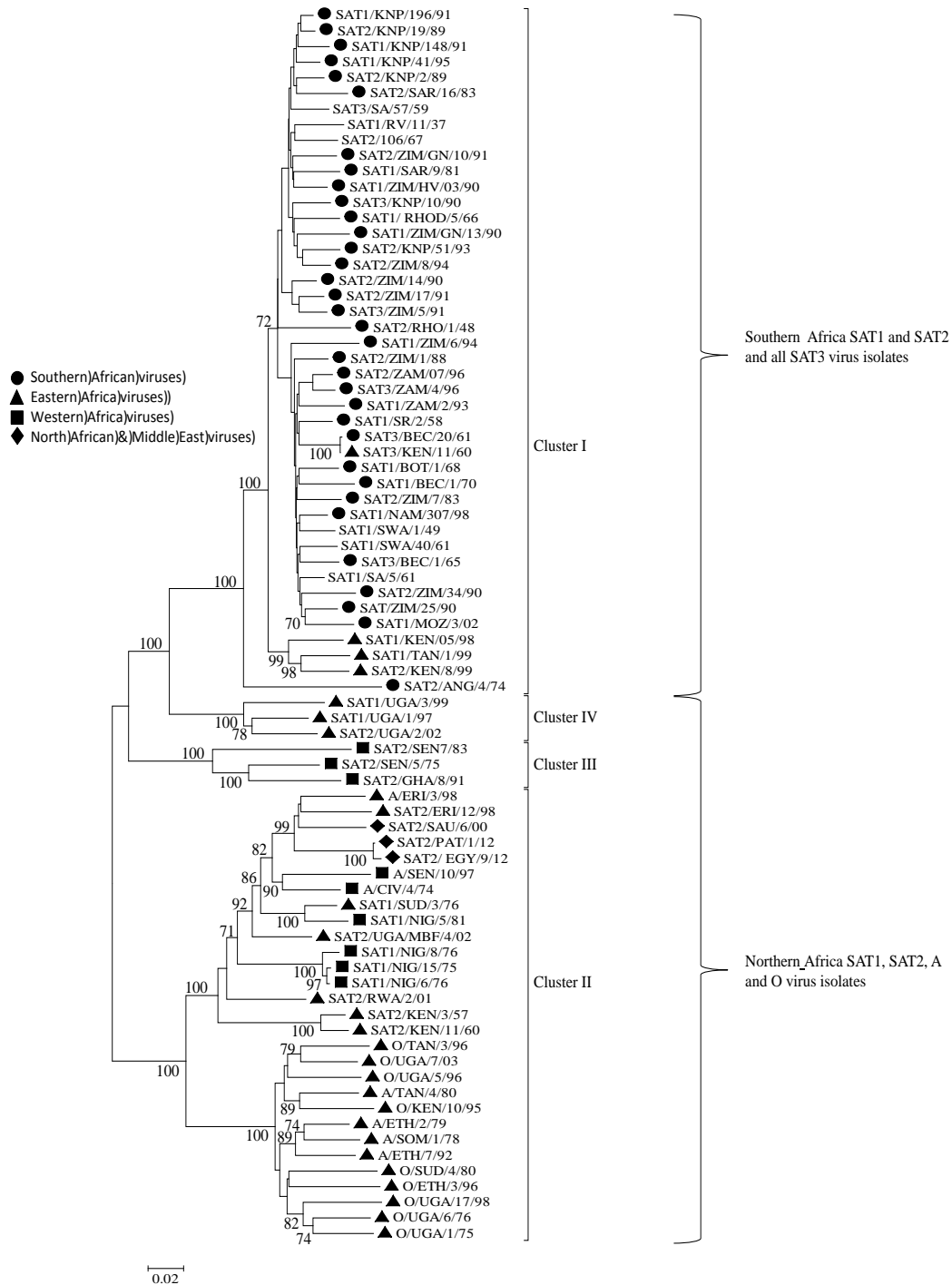
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SAT1/KNP/196/91  GQITTPADKSDKGFVLGQSIIDVTLKRHLDYETGFKPVMASRTLEAILSFARAGTIQEKLISVAGLAVHSGQDEYRRLFEFPQGTFFIIPSYRSLYLKRWNVACGDA
SAT1/KNP/148/91  .....
SAT1/KNP/41/95  .....
SAT1/SAR/9/81  .....X.....G.....P.....
SAT1/ZIM/HV/03/90 .....P.....
SAT1/ZIM/GN/13/90 .....P.....
SAT1/BOT/1/68  .....P.....
SAT1/RHO/5/66  .....P.....
SAT1/RW/11/37  .....H.....
SAT1/NAM/307/98 .....
SAT1/ZIM/6/94  .....
SAT1/BEC/1/70  .....
SAT1/SR/2/58  .....
SAT1/SWA/1/49  .....T.....
SAT1/SA/5/61  .....
SAT1/SWA/40/61 .....G.....
SAT1/ZIM/25/90 .....
SAT1/ZAM/2/93  .....
SAT1/MOZ/3/02  .....
SAT1/TAN/1/99  .....
SAT1/KEN/05/98 .....
SAT1/UGA/1/97 .....G.....P.....T.L.....
SAT1/UGA/3/99 .....G.....P.....T.L.....
SAT1/SUD/3/76  .....FG.....T.....P.....T.L.....
SAT1/NIG/5/81  .....FG.....P.....T.L.....
SAT1/NIG/15/75 .....G.....P.....T.L.....
SAT1/NIG/8/76  .....G.....P.....T.L.....
SAT1/NIG/6/76  .....G.....P.....T.L.....
SAT2/KNP/19/89 .....
SAT2/KNP/2/89 .....
SAT2/KNP/51/93 .....Y.....
SAT2/SAR/16/83 .....G.....
SAT2/ZIM/1/88  .....G.....P.....
SAT2/106/67  .....P.....
SAT2/ZIM/7/83  .....P.....
SAT2/ZIM/14/90 .....
SAT2/ZIM/17/91 .....Y.....
SAT2/ZIM/GN/10/91 .....P.....
SAT2/ZIM/34/90 .....A.....L.....
SAT2/ZIM/8/94  .....S.....P.....
SAT2/RHO/1/48 .....Y.....
SAT2/ZAM/07/96 .....
SAT2/KEN/8/99 .....
SAT2/SEN/5/75 .....FG.....A.L.....
SAT2/GHA/8/91 .....FG.....P.....T.L.....
SAT2/SEN/7/83 .....FG.....P.....N.L.....
SAT2/ERI/12/98 .....M.G.....P.....A.L.....
SAT2/SAU/6/00 .....R.....M.G.....P.....Q.....A.L.....
SAT2/RWA/2/01 .....G.....P.....T.L.....
SAT2/KEN/3/57 .....M.G.....P.....L.....
SAT2/KEN/11/60 .....M.G.....P.....L.....
SAT2/ANG/4/74 .....K.G.....P.....
SAT2/UGA/2/02 .....G.....P.....T.L.....
SAT2/UGA/MBP/4/02 .....FG.....X.P.....X.....LXX.....
SAT2/BET/9/12 .....M.G.....P.....Q.....T.L.....G.....
SAT2/BAT/1/12 .....M.G.....P.....Q.....T.L.....
SAT3/KNP/10/90 .....
SAT3/SA/57/59 .....G.....
SAT3/BEC/1/65 .....S.....
SAT3/KEN/11/60 .....S.....
SAT3/BEC/20/61 .....
SAT3/ZIM/5/91 .....
SAT3/ZAM/4/96 .....
A/CIV/4/74  .....M.FG.....PG.....T.L.....
A/SEN/10/97 .....M.G.....P.....T.L.....
A/SOM/1/78  .....H.....M.G.....P.....L.....
A/TAN/4/80  .....H.....M.G.....P.....L.....
A/ETH/2/79  .....H.....M.G.....P.....L.....
A/ETH/7/92  .....H.A.....M.G.....P.....L.....
A/ERI/3/98  .....M.G.....P.....Q.....T.L.....
O/UGA/5/96  .....A.....M.G.....P.....L.....
O/UGA/6/76  .....H.....M.G.....P.....L.....
O/UGA/17/98 .....P.....V.G.....P.....L.....
O/UGA/1/75  .....HA.....M.G.....P.....L.....
O/UGA/7/03  .....H.....M.G.S.....P.....L.....
O/KEN/10/95 .....H.....M.G.....P.....L.....
O/SUD/4/80  .....H.....M.G.....I.....A.....P.....L.....
O/ETH/3/96  .....H.....M.G.....P.....L.....
O/TAN/3/96  .....H.....M.G.....T.....P.....L.....

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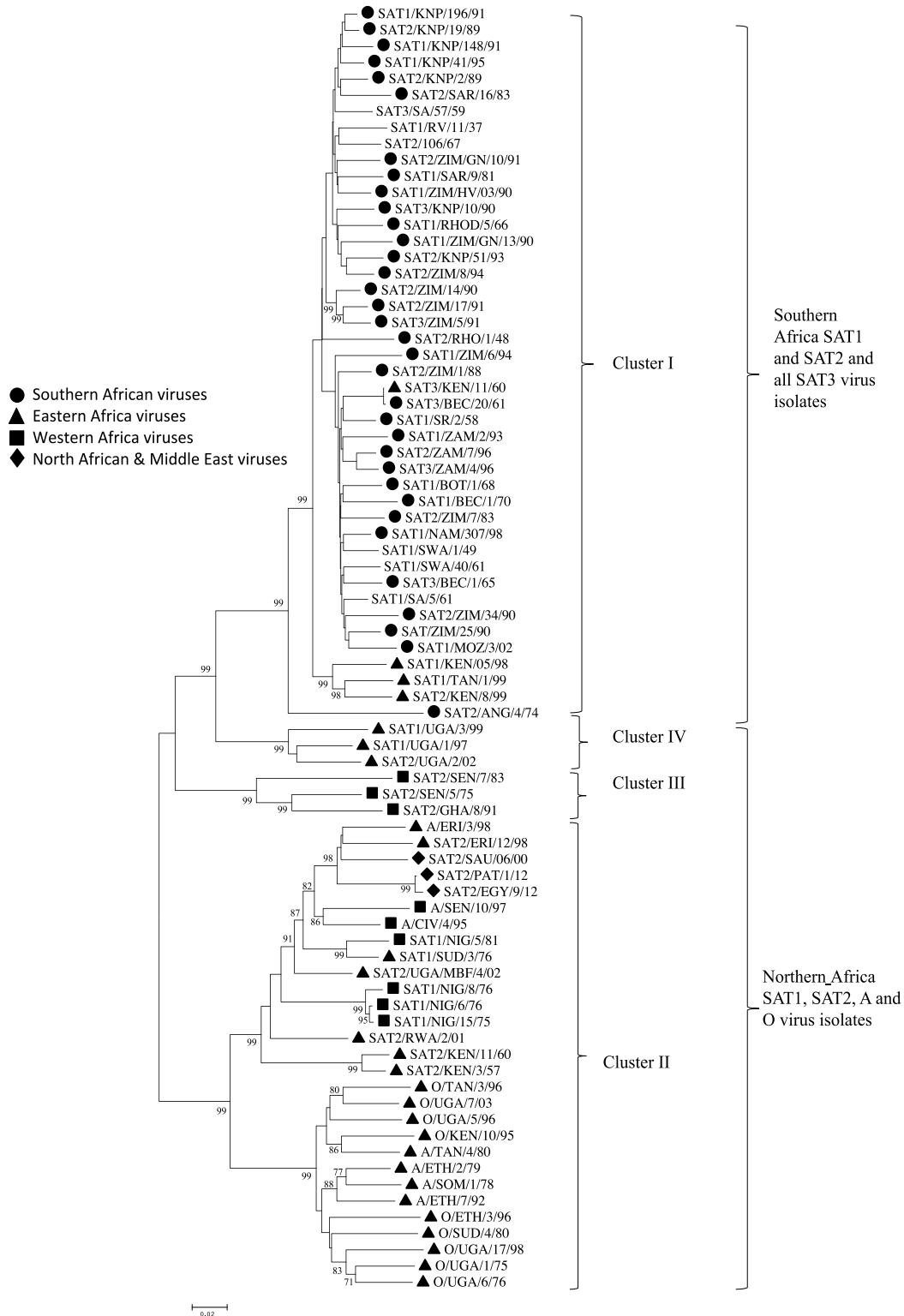
Supplementary Fig. F An alignment of 470 amino acids deduced for the 3D RNA Dependent RNA Polymerase for 79 African FMDV isolates. The dots (.) indicate identical amino acids as the reference sequence SAT1/KNP/196/96. Underlined at the top of the alignment are five highly conserved motifs among the RNA polymerases (Doherty *et al.*, 1999; Ferrer-Orta *et al.*, 2004). KDEIR (positions 164-168), DYSAFD (positions 240-245), PSG (positions 297-299), YGDD (positions 336-339) and FKLR (positions 385-388). The KDEVR motif observed in A/ETH/2/79 and SAT2/KEN/11/60 is highlighted in horizontal grey bars. The block horizontal bar (___) at the bottom of the alignment indicates a hypervariable region in the sequence alignment between residues 143-154.

APPENDIX 3: Supplementary Phylogeny Analyses

(Details describing the four clusters I-IV are given in section 2.3.1)



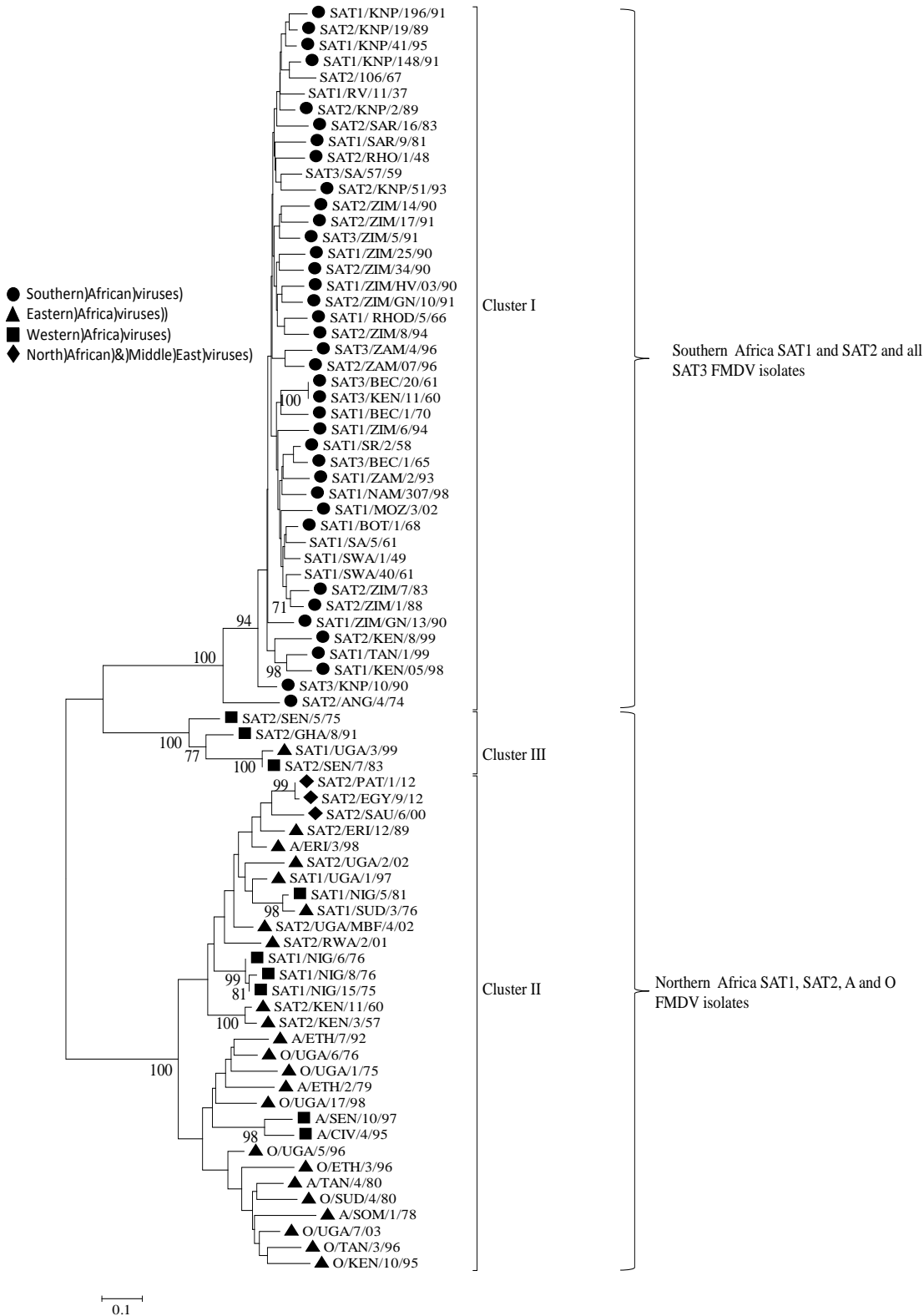
Supplementary Fig. I A mid-point rooted neighbour joining (NJ) tree showing nucleotide relationships between the sub-Saharan African FMDV (SAT1, SAT2, SAT3, A and O serotypes) for the P3-coding region. The Kimura 2-parameter model in MEGA 5 (Tamura et al, 2011) was used to draw the NJ tree and the scale bar indicates nucleotide substitutions per site. Confidence levels of the tree branches were tested using 1000 bootstrap replications.



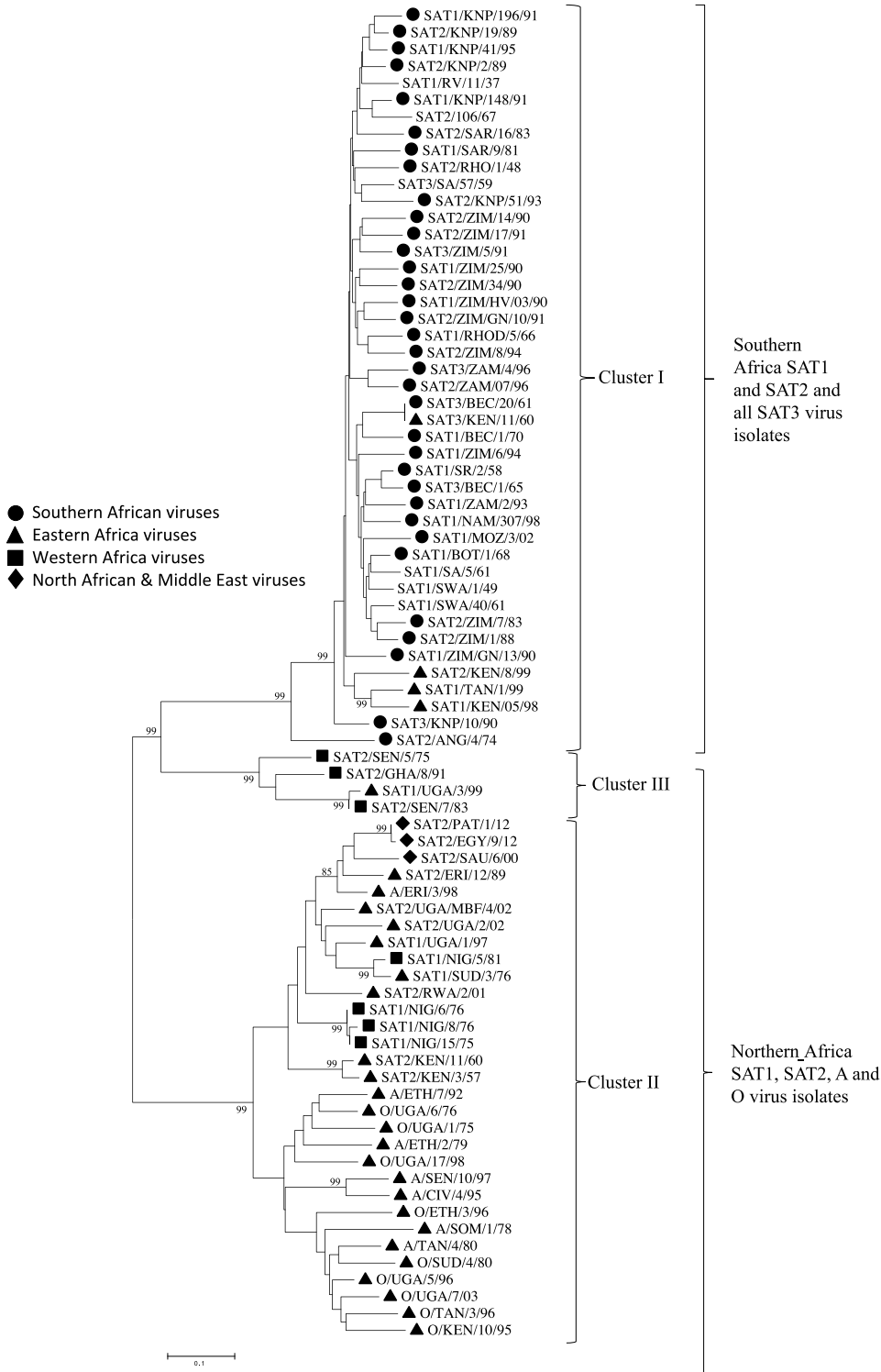
Supplementary Fig. II A mid-point rooted phylogenetic tree constructed using Minimum Evolution (ME) methods describing nucleotide relationships between the sub-Saharan African FMDV (SAT1, SAT2, SAT3, A and O serotypes) for the P3-coding region. The rate variation among sites was modelled with a gamma distribution of 0.76. The robustness of the tree branches was tested using 1000 bootstrap replications. The scale indicates the evolutionary distances used to infer the phylogenetic tree. The tree was searched using the Close-Neighbour-Interchange (CNI) algorithm at a search level of 1. Evolutionary analysis was conducted using MEGA5 (Tamura et al, 2011).



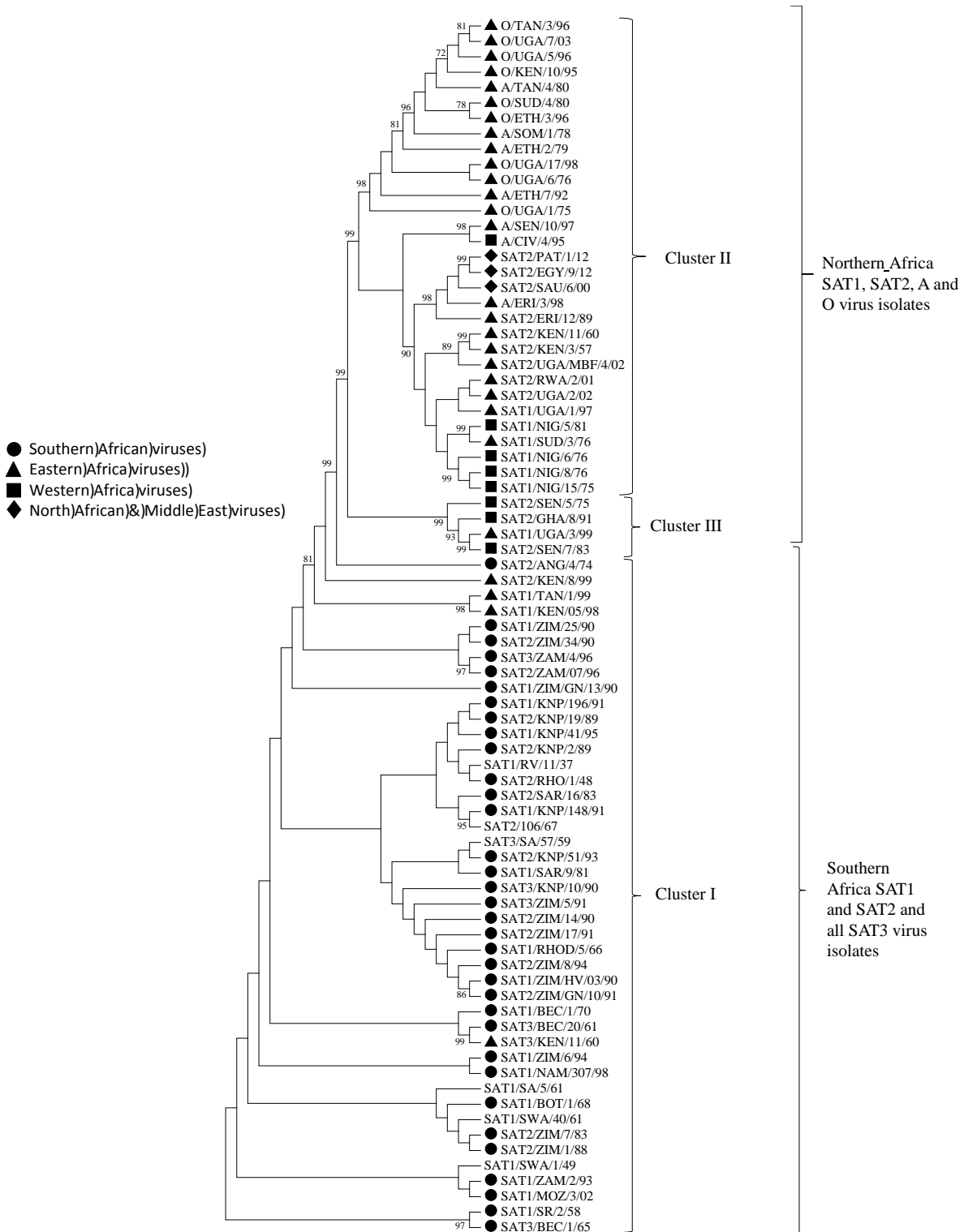
Supplementary Fig. III Evolutionary relationships inferred using the Maximum Parsimony methods in MEGA 5, for the sub-Saharan African FMDV (SAT1, SAT2, SAT3, A and O serotypes) for the P3-coding region. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) The tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates).



Supplementary Fig. IV Neighbour joining (NJ) tree depicting nucleotide relationships between the sub-Saharan African FMDV (SAT1, SAT2, SAT3, A and O) for the Leader-coding region rooted against the mid-point. The NJ tree was constructed using Kimura 2-parameter model MEGA 5 (Tamura et al., 2011), the scale bar indicates nucleotide substitutions per site. The robustness of the tree topology was assessed using 1000 bootstrap replications.



Supplementary Fig. V A mid-point rooted phylogenetic tree constructed using Minimum Evolution (ME) methods describing nucleotide relationships between the sub-Saharan African FMDV (SAT1, SAT2, SAT3, A and O serotypes) for the Leader coding region. The rate variation among sites was modelled with a gamma distribution of 0.795. The robustness of the tree branches was tested using 1000 bootstrap replications. The scale indicates the evolutionary distances used to infer the phylogenetic tree. The tree was searched using the Close-Neighbour-Interchange (CNI) algorithm at a search level of 1. Evolutionary analysis was conducted using MEGA5 (Tamura et al, 2011).



Supplementary Fig. VI Evolutionary relationships inferred using the Maximum Parsimony methods in MEGA 5, for the sub-Saharan African FMDV (SAT1, SAT2, SAT3, A and O serotypes) for the P3-coding region. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) The tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates).

APPENDIX 4: Scientific contributions emanating from this thesis

Conference Proceedings

Posters:

Faculty Day: University of Pretoria, Faculty Veterinary Science (August 2009)

Nsamba P, De beer T, Blignaut B and Maree F

Proteome annotation and phenotype analysis of South African Territories type FMDV.

EU-FMD 2010 (Vienna, September 2010)

P. Nsamba, B. Blignaut and F. Maree

Strategies Towards Effective Vaccination In Sub-saharan Africa: Amino Acid Residues Associated With Alternative Cell Recognition Sties.

Oral Presentations:

South African Society for Microbiology (Durban, September 2009)

1st Southern African Students' Symposium on Vet Tropical Diseases (November 2009)

Nsamba P, De beer T, Blignaut B and Maree F

Proteome annotation and phenotype analysis of South African Territories type FMDV.

TWOWS Fourth General Assembly and International Conference (Beijing June 2010).

P. Nsamba, B. Blignaut, P. Opperman, M.Chitray and F. Maree

Foot-and-mouth Disease In Southern Africa: Improved Control Strategies And Vaccine Matching.

GFRA Session at EUFMD Meeting (September 2010)

Peninah Nsamba

Current situation of FMD in Uganda and comments on her research project at OVI (South Africa).