Chapter 3:
Analysis of the 3A
non-structural-protein-coding region of
foot-and-mouth disease virus isolates
from Africa

3.1 Introduction

The mature 3A protein of FMDV has been shown to be highly conserved among European isolates (Knowles *et al.*, in press). This 153 amino acid protein is kwon to be involved in viral replication and has been shown to mediate the relocation of the RNA replication machinery to the cellular membrane (Xiang *et al.*, 1998). The 3AB polypeptide, the precursor of the 3A and 3B viral proteins, stimulates the *in virto* synthesis of poly(U) RNA, directed by the 3Dpol viral polymerase (Lama *et al.*, 1994). FMDV is unique among the picornaviruses in that it encodes three copies of genome-bound 3B (VPg) protein, which serves as a primer for genome replication (Rueckert, 1996). The association of the 3A protein with the cellular membrane is known to prevent the surface expression and excretion of cellular proteins thereby contributing to the death of infected cells (Doedens *et al.*, 1997). Changes in 3A have been associated with altered host range in the hepatoviruses (Lemon *et al.*, 1991; Morace *et al.*, 1993; Graff *et al.*, 1994), rhinoviruses (Heinz & Vance, 1996), and enteroviruses (Lama *et al.*, 1994).

Historical accounts of FMD outbreaks commonly showed a marked species restriction of FMD virus isolates involved. For example, Brooksby (1950) cited reports of epidemics in Germany during the 1920's and Britain during the 1930's in which pigs were predominantly affected. Cattle were almost completely excluded in these outbreaks and it was experimentally shown that the virus isolates were readily transmitted from pig-to-pig, but not from pig-to-cattle. More recently, during 1997, four million pigs were slaughtered in order to contain an outbreak of FMD in Taiwan. The outbreak caused by serotype O viruses (O1 Taiwan), demonstrated an extensive porcinophilic phenotype while being completely absent in the bovine population. Results of several *in vitro* and *in vivo* studies showed that the viruses isolated during the outbreak were unable to infect bovine thyroid cells or to cause typical disease symptoms following needle infection of cattle (Dunn *et al.*, 1997).

Genetic characterization of the causative isolate revealed distinct differences in the 3A sequence when compared to that of representative European and South American isolates. The most significant of these is a 10 amino acid deletion corresponding to codons 93 through 102 of the European and South American isolates (Beard and Mason, 2000). These

authors were able to demonstrate a direct correlation between the presence of this deletion within the 3A protein and attenuation of the FMDV in cattle. Similar deletions were previously found within the 3A genomic region of two viruses (O1C-O/E and C3R-O/E) attenuated through repeated passages in chicken embryos. These attenuated viruses exhibited similar porcinophilic phenotypes to that observed for the O1 Taiwan isolate (Giraudo *et al.*, 1990). In all three cases the deletion was tolerated without affecting the pathogenicity of the virus in swine, while severely restricting growth in other systems. These results strongly suggest the involvement of the 3A protein in virulence and possibly host range specificity (Beard and Mason, 2000). A subsequent study on FMD viruses from the Southeast Asia region (Knowles *et al.*, 2001) indicated the presence of this deletion in the oldest isolates from the region. In contrast to previous results (Beard and Mason, 2000), these viruses did not display similar attenuation on bovine keratinocytes, suggesting that this deletion (residues 93 –102) in the 3A region alone cannot be attributed to the observed growth restriction (Knowles *et al.*, 2001). Additional mutations in the 3A genome observed for O_1Taiwan might however be responsible for the species specificity.

The occurrence of a vast diversity of wildlife and domestic livestock across the African continent presents researchers with a unique opportunity to study the epidemiology of FMDV. It is generally accepted that African buffalo (*Syncerus caffer*) occurring across sub-Saharan Africa are persistently infected with FMDV without exhibiting any of the classical symptoms of the disease (Thomson, 1994). However, occasional outbreaks of FMD in impala (*Aepyceros melampus*), an abundant species of antelope found in southern Africa, are characterised by the development of clinical symptoms ranging in severity. In contrast with the persistent infection of buffalo, there is no clear evidence that the virus is maintained within these antelope through interepidemic periods (Thomson, 1994). It is generally accepted, based on nucleotide sequence data, that buffalo are the source of infection of other susceptible species (Bastos *et al.*, 2000).

Although the 3A coding region of several European isolates have previously been described, the sequence characteristics of the 3A of the SAT type viruses have not been investigated. In light of this, the nucleotide sequences of the 3A non-structural-coding sequence of several African FMD viruses isolated form different host species were

determined and comparatively analysed. We compared this region of the genome among different SAT serotypes and with that of European, South American and Asian isolates.

3.2 Materials and Methods*

3.2.1 Origin of the viruses
FMDV isolates representative of all seven serotypes, originating from diverse geographical and host origins were selected for this study. Isolates indicated with asterisks were used to generate additional nucleotide sequence data for this study. Where possible, primary isolations of the viruses were used in direct RNA extraction. In all cases where primary isolations were not available, low passage cell culture samples were used. The complete passage history of each virus is summarised in Table 3.2.1.

3.2.2 RNA extraction from foot-and-mouth disease virus
RNA was extracted from cell culture specimens by a modified guanidium-based nucleic acid extraction method (Boom *et al.*, 1990). A tissue culture sample, previously stored at −70°C, was slowly thawed on ice and 100µl transferred to an RNase-free 1.5ml micro-test tube. A volume of 1ml L6 lysis buffer was added to the sample together with 40µl particulate silica The sample was briefly vortexed and incubated at room temperature for 5 minutes after which it was thoroughly mixed by inversion and centrifuged at maximum revolutions for 15 seconds using a Hettich bench top centrifuge. The supernatant was discarded and the resulting pellet resuspended in 900µl L2 buffer before again being concentrated by centrifugation. The supernatant was again discarded and the pellet washed by resuspension in 900µl 70% ethanol. The sample was centrifuged at 16000xg, the supernatant discarded and 900µl acetone added to the pellet. The silica was concentrated by centrifugation, the bulk of the supernatant removed using a micropipet and residual acetone evaporated by incubating the tube at 56°C for 20 minutes. The RNA was eluted from the silica by adding 30µl prewarmed (56°C) 1 x TE buffer, containing 5U of RNase Inhibitor (Roche Molecular Biochemicals) to the pellet and incubating it at 56°C for a further 2 minutes. The aqueous phase containing the RNA was carefully transferred to a clean micro-test tube and stored at −70°C.

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* The recipes for the preparation of all buffers and reagents referred to in the texts are summarised in appendix A.4.

Table.3.1.1. (Continued) List of FMD viruses used in comparative studies of the 3A non-structural-protein-coding region.

Virus	Country	Year of Isolation	Species of Origin	Passage History	Reference
Serotype A					
A_{10} **Argentina/61**	Argentina	1961	Bovine	N/A	X00429
A_{12} **119/Kent/32**	United Kingdom	1932	Bovine	N/A	M10975
TUR/43/98/A*	Turkey	1998	Bovine	BTY_1 $IB-RS_2$	AF335014
KEN/1/76/A*	Kenya	1976	Bovine	BTY_1 $IB-RS_2$	AF335007
Serotype O					
O_1 **Kaufbeuren**	Germany	1965	Bovine	N/A	X00871
O_1 **Campos**	Brazil	1958	Bovine	N/A	Girauda et al. 1990
O_1**C-O/E**			Egg Passage	N/A	Girauda et al. 1990
O_1 **Tau-Yuan**	Taiwan	1997	Porcine	N/A	AF154271
O_1 **Chu-Pei**	Taiwan	1997	Porcine	N/A	AF026168
KEN/1/91/O*	Kenya	1991	Bovine	BHK_2 BTY_1 $IB-RS_1$	AF335006
Serotype Asia1					
PAK/1/54/Asia1*	Pakistan	1954	Bovine	Not Available	AF335015
Serotype C					
C_3 **Argentina/85**	Argentina	1985	Bovine	N/A	AJ007347
C_3 **Resende/55***	Brazil	1955	Bovine	BTY_2 $IB-RS_2$	Giraudo et al. 1990; AY026896
C_3**R-E/O**			Egg Passage	N/A	Girauda et al. 1990

* Indicates the viruses of which the 3A region was successfully sequenced.
N/A Not Available

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KEN/1/76/A*	Kenya	1976	Bovine	BTY_1 IB-RS$_2$	AF335007
Serotype O					
O_1 **Kaufbeuren**	Germany	1965	Bovine	N/A	X00871
O_1 **Campos**	Brazil	1958	Bovine	N/A	Girauda et al. 1990
O_1**C-O/E**			Egg Passage	N/A	Girauda et al. 1990
O_1 **Tau-Yuan**	Taiwan	1997	Porcine	N/A	AF154271
O_1 **Chu-Pei**	Taiwan	1997	Porcine	N/A	AF026168
KEN/1/91/O*	Kenya	1991	Bovine	BHK_2 BTY_1 IB-RS$_1$	AF335006
Serotype Asia1					
PAK/1/54/Asia1*	Pakistan	1954	Bovine	N/A	AF335015
Serotype C					
C_3 **Argentina/85**	Argentina	1985	Bovine	N/A	AJ007347
C_3 **Resende/55***	Brazil	1955	Bovine	BTY_2 IB-RS$_2$	Giraudo et al. 1990; AY026896
C_3**R-E/O**			Egg Passage	N/A	Girauda et al. 1990

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3.2.3 Preparation of cDNA

The oligonucleotide used to initiate the reverse transcription was designed from the partial nucleotide sequence of the SAT 2 isolate, ZIM/7/83 (H. G. van Rensburg, unpublished data). It was designed to anneal to an area approximately 100 nucleotides downstream of the 3A non-structural-protein-coding region (Figure. 3.2.1). The primer was obtained commercially from Gibco BRL and designated P445 (5'-ACCATCTTTTGCAAGTC-3'). The cDNA synthesis reaction was performed as described in Section 2.2.7 with the only alteration being the substitution of the WDA primer with the P445 oligonucleotide. After completion of the reaction, the cDNA was stored at –20°C.

3.2.4 Amplification of the 3A non-structural-protein-coding region

The 3A non-structural-protein-coding region of the FMDV genome was amplified by PCR using cDNA prepared as described above. The first ®oligonucleotide used during the amplification was designed to anneal to an area within the 2C non-structural-protein-coding region of the genome (Figure. 3.2.1). This primer was obtained commercially from Gibco BRL and designated P444 (5'-GGCCGTTGAAATGAAGAGA-3'). The P445 oligonucleotide was used as the second primer in the amplification.
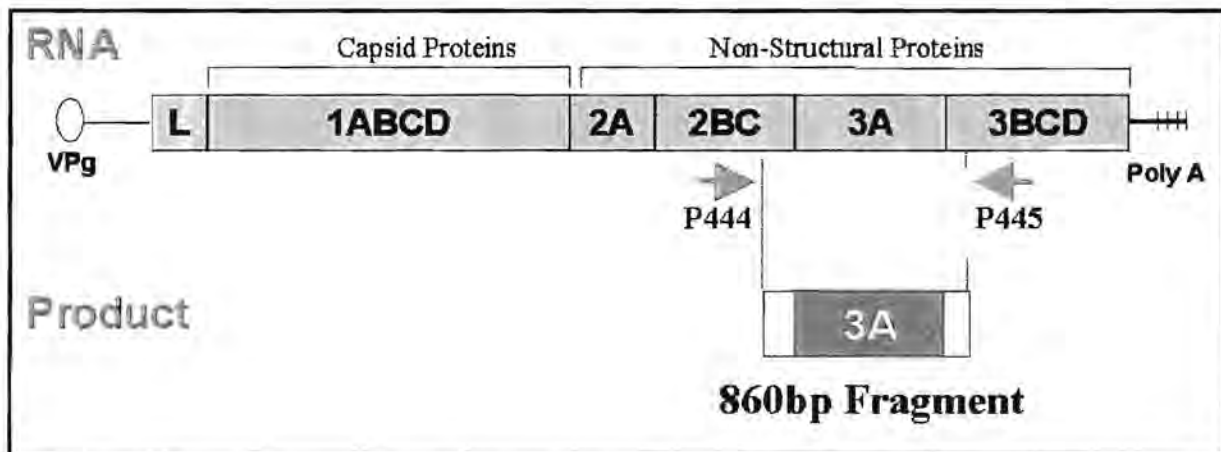

Figure 3.2.1 *A graphical representation of the polymerase chain reaction strategy used to amplify the 3A non-structural-protein-coding region of FMDV. The primers used in the reaction are represented by the red arrows (→).*

Reaction conditions were optimised using a method described by Cobb *et al.* (1985). This involved setting up a series of reactions in which the effect of a chosen number of variables on the outcome of the reaction was tested. Three variables, namely deoxyribonucleotide concentration, primer concentration and magnesium chloride concentration were chosen, limiting the number of reactions needed to test all permutations of these variables to seven. A summary of the reactions set up in order to optimise the reaction conditions is presented in Table 3.2.2. The PCR was performed using an OmniGene Hybaid machine. The sample was initially denatured at 94°C for 2 minutes followed by 30 repetitions of a cycle starting off with denaturation at 95°C for 30 seconds followed by a 30 seconds annealing period at 53°C and ending with polymerisation for 90 seconds at 72°C.

After completion of the thermocycling the amplified fragment was separated from the unincorporated primers on a 1.5% agarose gel by standard electrophoreses techniques (Sambrook, 1987). The gel was prepared by dissolving 1.5g of Multi-Purpose Agarose (Roche Molecular Biochemicals) in 100ml 1 x TAE buffer. After allowing sufficient time for the gel-mix to cool to approximately 60°C, 2µl Ethidium bromide (10µg/ml) was added, the gel cast and left to set at room temperature. A 10% volume of DNA loading buffer (40% sucrose solution coloured with bromo-phenol-blue) was added to the PCR reaction mix and 50µl of the mixture loaded onto the gel. A commercial DNA marker (*HindIII* restricted phage φX174 genomic DNA) was loaded as a reference. The DNA was electrophoresed at 100V for approximately 30 minutes after which the fragment was visualised by exposing the gel to short wavelength ultra-violet light using a bench top transilluminator. Recovery of the amplified fragment (0.8kbp) from the agarose gel was done as previously described in Section 2.2.9. The purified DNA was stored at -20°C until needed.

3.2.5 Sequence determination of the amplified 3A non-structural-protein-coding region
The ABI PRISM™ BigDye™ Terminator Cycle Sequencing ready reaction kit (Perkin-Elmer Applied Biosystems) was used to determine the nucleotide sequence of the amplified fragments. The reaction was performed as described in Section 2.2.14. Sequencing reaction mix was set up as prescribed by the manufacturer and consisted of 2µl Terminator Ready Reaction Mix, between 90 to 150ng double-stranded DNA template and

20pmols of the appropriate oligonucleotide. The final volume of each reaction was adjusted to 20µl with the appropriate volume of deionised water

To ensure the validity of the data, both the negative and positive sense strands of the amplified fragment were independently sequenced using the P444 and P445 primers respectively. The data obtained was verified by manual inspection of the electropherograms and analysed as discussed in Section 3.2.6.

Table 3.2.2. Composition of reactions used to optimise the polymerase chain reaction conditions used during the amplification of 3A non-structural-protein-coding region.

Reaction number		1	2	3	4	5	6	7
Template		2µl	2µl	2µl	2µl	2µl	2µl	2µl
Reaction buffer	X10	2.5µl	2.5µl	2.5µl	2.5µl	2.5µl	2.5µl	2.5µl
*$MgCl_2$	25mM	0µl	0µl	0µl	0.5µl	0.5µl	0.5µl	1µl
*dNTP's	2.5mM	1,5µl	3µl	6µl	3µl	6µl	1.5µl	6µl
*P444 primer	10pmol/µl	1.5µl	3µl	6µl	1.5µl	3µl	6µl	1.5µl
*P445 primer	10pmol/µl	1.5µl	3µl	6µl	1.5µl	3µl	6µl	1.5µl
Enzyme	1U/µl	1µl	1µl	1µl	1µl	1µl	1µl	1µl
dH_2O		15µl	10.5µl	1.5µl	13µl	7µl	5.5µl	9.5µl
Total volume		25µl	25µl	25µl	25µl	25µl	25µl	25µl

* Indicates variables tested

3.2.6 Computer assisted analysis of the nucleotide sequences

In addition to isolates sequenced in this study, the 3A non-structural-protein-coding region sequence of several isolates obtained from GenBank and included in the analysis (National Centre for Biotechnology Information). All nucleotide sequences determined in this study have been submitted to GenBank under the acession numbers indicated in Table 3.2.1.

Nucleotide sequences, as well as predicted amino acid sequences, were aligned using the DAPSA program (Harley, 1998). These multiple sequence alignments were subsequently used to determine the phylogenetic relationships of the isolates. Phylogenetic analyses for the full data set (N=21) were carried out using DNAMAN version 4.13 (Lynnon Biosoft). Bootstrap supported trees were constructed using a random seed generator of 111 and 1000

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bootstrap trials. The 3A coding region of equine rhinitis A virus (GenBank Acc. No. X96870) was selected as outgroup. Secondary structural protein predictions were carried out using the Garnier, Gibrat and DPM algorithms contained in Antheprot version 4.9 (Delaege, 1999), while DNAMAN version 4.13 (Lynnon Biosoft) was used to determine the hydrophobic regions within the predicted proteins.

3.3 Results

3.3.1 Amplification of the 3A non-structural-protein-coding region

Initial attempts to amplify the 3A non-structural-protein-coding region of the A_{10} FMD virus yielded four distinct DNA products ranging in size from approximately 420 to 860bp (results not shown). The size of the largest fragment ($\approx$860bp) corresponded to the size of the product expected to contain the 3A coding region as predicted by computer assisted analysis of the FMDV genome. In order to verify whether this product indeed contained the 3A coding region, it was purified from the agarose gel and sequenced. The results indicated that the fragment did contain the 3A coding region.

After verifying that the 3A non-structural-protein-coding region of the A_{10} FMDV was being amplified, amplification of the corresponding region of several other isolates was attempted. In addition to the non-specific amplification, the concentrations of the products varied significantly depending on the isolate used. It was therefore necessary to optimise the PCR reaction conditions in order to minimise amplification of non-target regions and to maximise the yield of the specific target region. KNP/10/90/3 was chosen as a representative isolate and subsequently used in all experiments designed to optimise the reaction conditions.

PCR conditions were optimised by employing a strategy described by Cobb *et al.* (1985). Results of the optimisation experiments indicated the optimal reaction mixture to consist of 5μl 10x Biotools DNA polymerase reaction buffer, 3μl dNTP's (2.5mM), 3μl of the P445 oligonucleotide (10pmol/μl), 3μl of the P445 oligonucleotide (10pmol/μl), 2μl polymerase enzyme [(1U/μl) Biotools SA] and 30μl dH$_2$O. Amplification of the target region using the optimal conditions yielded a single fragment of approximately 860bp at a concentration 4

times higher than that observed during initial experiments. Similar results were obtained for several other isolates tested under these conditions.

3.3.2 Genetic heterogeneity of the 3A non-structural protein

To determine the genetic heterogeneity of the 3A non-structural-protein-coding region of African isolates of FMDV, representatives of the six serotypes occurring on the continent were selected and compared to isolates originating from Europe, South America and Asia (Table 3.3.1). Comparative analysis of these sequences demonstrated that none of the 11 isolates of which the nucleotide sequences were determined in this study, contained any insertions (Figure 3.3.1). However, a single amino acid deletion was observed in all the SAT type 3A proteins, located 7 amino acids from the carboxyl terminus of the protein when compared to the corresponding region of types A, O, C and Asia-1.

The nucleotide sequence identity calculated for all isolates amounted to 47.4%, while the amino acid identity was calculated as 50.3%. Nucleotide sequence identity within the 3A coding region of the SAT types (group A, Figure 3.3.2) was calculated to be 73.6%, which was markedly higher than the 63% calculated for group B, containing types A, O, C and Asia-1 (Figure 3.3.2). The amino acid sequence was found to be highly conserved within the N-terminus region of the protein.

In Figure 3.3.1, only secondary structural motifs that could be predicted with the most certainty are indicated. It is evident that these motifs were conserved among all the different FMDV serotypes in the N-terminus region of the 3A protein. Limited amino acid substitutions could be found for the SAT types in α-helix 1 and 2 (Figure 3.3.1a). Secondary structure predictions in the C-terminus region of the protein were more complicated and only three regions could be indicated with confidence (Figure 3.3.1b). It is worthy to note that the presence of α-helix 6 in the two isolates from Southeast Asia, O_1Taiwan and O_1Chupei, is not well supported (results not shown). The most variable regions of the 3A protein for all the isolates were found to be located between residues 113 and 151 (Figure 3.3.1b).

A distinct and conserved hydrophobic domain is situated between residues 61 and 77 (Figure 3.3.1) of the FMDV 3A protein (Beard & Mason, 2000) and is common to all

picornaviruses (Xiang *et al.*, 1998). The hydrophobic domain was found to be highly conserved in all isolates belonging to types A, O, C and Asia-1 (Figure 3.3.1b). Although the identity of residues 65 to 67 as well as 73, differed for the SAT type viruses, the hydrophobicity of the region remained conserved. These differences involved the change of CLT (residues 65-67) in types A, O, C and Asia-1 to VVV in the SAT types, with the exception of SAR/9/81/1 (VVG) and KEN/3/57/2 (CLA). Residue 73 changed from V found in A, O, C, Asia-1 and KEN/3/57 to I in the SAT type isolates. According to the prediction made using the DNAMAN program, the hydrophobicity of all the 3A proteins remained constant. Therefore, the variation in amino acid sequence should not affect the functionality of the domain.

3.3.3 Phylogenetic relationship of FMDV based on the 3A protein

Genetic relationships of the FMDV viruses were determined by phylogenetic analysis of the 3A gene sequence data. Consistent and comparable results were obtained with the use of parsimony, maximum likelihood and neighbor-joining methods when analysing the nucleotide sequence data (results not shown). A neighbor-joining tree based on the alignment of 3A nucleotide sequence data is shown in Figure 3.3.2.

All SAT type viruses, with the exception of KEN/3/57/2, were found in a single distinct phylogenetic cluster (group A, Figure 3.3.2) separate from the other types and supported by a bootstrap value of 100. In contrast, isolates of serotypes A and O which originated from Africa, as well as the SAT 2 isolate KEN/3/57/2, formed part of a second phylogenetic cluster in which types A, O, C from Europe and South America and Asia-1 were also contained (group B, Figure 3.3.2). This cluster was similarly supported by a significant bootstrap value of 98. However, sub-grouping within these clusters was limited. The only well-supported sub-groupings in cluster B involved the grouping of the Southeast Asian isolates (O₁Taiwan and O₁Chupei) with a high bootstrap value 100. The other significant groupings were that of O₁Campos together with O1C-E/O, the virus that was derived by passaging O₁Campos through eggs and O₁Kaufbeuren and C₃Resende and its egg-derived strain C3R-E/O.

Phylogenetic analysis using the predicted amino acid sequences of the isolates yielded similar results as discussed above (results not shown). Isolates were again found in two major phylogenetic clusters consistent with the pattern generated using the nucleotide

sequence data. Although the phylogenetic arrangement of isolates belonging to subgroup B were somewhat different from that represented in Figure 3.3.2, bootstrap values were generally very low and did not statistically support the pattern generated using the amino acid sequence data.

3.4 Discussion

Previous results indicated that the 3A protein might play a role in host range specificity (Beard and Mason, 2000), and in this study, 3A genes of FMDV isolated from different species in Africa were investigated for obvious amino acid differences. The 3A proteins of the SAT type viruses were conserved in length, although a single amino acid deletion corresponding to residue 145 of types A, O, C and Asia-1 was observed for all the SAT type isolates investigated. As this deletion was found in virus isolates obtained from impala, cattle and buffalo, it is not thought to play a role in host range specificity. Knowles *et al.* (in press) also found that for the East-Asian isolates mutations other than deletions seem to play a role in host range specificity. The possible effect of this deletion on the functional role of the 3A protein was not investigated in this study.

Furthermore, the hydrophobic domain within the FMDV 3A protein (Beard & Mason, 2000), thought to mediate the association of the 3A protein with cellular membranes (Xiang *et al.*, 1998), was found to be conserved in all isolates investigated. Despite the amino acid changes observed in the SAT isolates within the domain when compared to the O, A, C and Asia-1 isolates, the predicted hydrophobicity was consistent. Therefore, the variation in amino acid sequence should not affect the functionality of the domain. The amino acid sequences of the hydrophobic domain of African isolates belonging to type A and O were identical to that of the European isolates.

Phylogenetic analysis based on the nucleotide and amino acid sequence data of the 3A coding region confirmed that the SAT type viruses differ significantly from types A, O, C and Asia-1 isolates and group within a single distinct phylogenetic group. This is in contrast with phylogenetic studies based on the VP1 (containing the major antigenic determinant) coding sequences where viruses group strictly according to serotype, as would be expected (Bastos, 1998). FMDV, being a single-stranded RNA virus has a high

rate of mutation as the virus has no proof-reading ability during replication (Holland *et al.*, 1982). It is generally accepted that more variation can be tolerated in the structural proteins than in the functional, non-structural proteins, although the rate of mutations across the whole genome would be similar. Selection would be on the functional level (Sobrino *et al.*, 1983; McCahon 1986; Sobrino *et al.*, 1986). Therefore, although the VP1 gene sequences vary by up to 34-40% between SAT serotypes (Bastos, 1998), variation of only 26.4% was found between the limited number of 3A gene sequences investigated in this study.

Notably a single SAT isolate, KEN/3/57/2, grouped consistently within the European lineage using both nucleotide and amino acid sequence data of 3A. KEN/3/57/2 groups with the SAT 2 serotype when VP1 gene sequences are compared, but groups with types A, O and C isolates when comparing 3A non-structural-proteins sequence data, suggesting that this isolate may have been the product of a recombination event. This is further supported by the multiple alignment of the amino acid sequence of 3A, which showed that the hydrophobic domain of KEN/3/57/2 more closely resembled that of the European serotypes than other SAT isolates. More isolates from all over Africa need to be investigated to determine whether the 3A genes of SAT isolates from certain regions on the continent are more similar than from other regions.

This study is the first to investigate the genetic characteristics of the 3A non-structural-protein-coding region of SAT type FMD virus isolates. The results of this exploratory study will prove useful in identifying significant variations in the 3A coding region of prevalent isolates in sub-Saharan Africa.
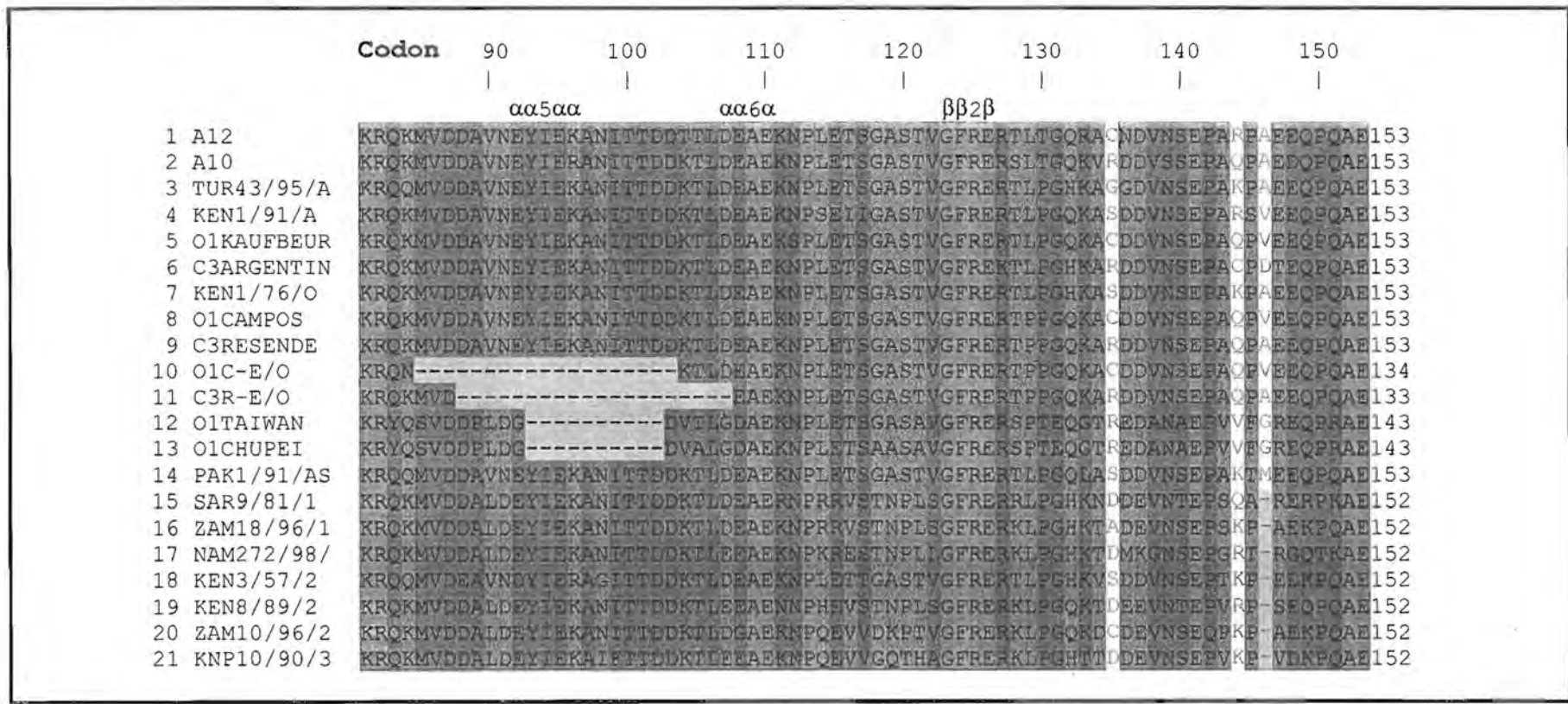
Figure 3.3.1.b Multiple alignment of the predicted amino acid sequence (codons 81-153) of 3A non-structural protein of several FMDV isolates. Secondary structural motifs as predicted with the Garnier, Gibrat and DPM algorithms, are indicated with α and β. Deletions are shown as — . Amino acids identity: 100%, >75%, >50%, <50%]

Codon 10   20   30   40   50   60   70   80

β1β   ααα1αααα   ααα2αααα   αα3αα   αα4α

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 1 A12          ISIPSQKSVLYFLIEKGQHEAAIEFFEGMVHDSIKEELRPLIQQTSFVKRAFKRLKENFEIVALCLTLLANIVIMIRETR  80
 2 A10          ISIPSQKSVLYFLIEKGQHEAAIEFFEGMVHDSVKEELRPLIQQTSFVKRAFKRLKENFEIVALCLTLLANIVIMIRETR  80
 3 TUR43/95/A   ISIPSQKSVLYFLIEKGQHEAAIEFFEGMVHDSIKEELRPLIQQTSFVKRAFKRLKENFEIVALCLTLLANIVIMIRETR  80
 4 KEN1/91/A    ISIPSQKSVLYFLIEKGQHEAAIEFFEGMVHDSIKEELRPLIQQTSFVKRAFKRLKENFEIVALCLTLLANIVIMIRETR  80
 5 O1KAUFBEUR   ISIPSQKSVLYFLIEKGQHEAAIEFFEGMVHDSIKEELRPLIQQTSFVKRAFKRLKENFEIVALCLTLLANIVIMIRETR  80
 6 C3ARGENTIN   ISIPSQKSVLYFLIEKGQHEAAIEFFEGMVHDSIKEELRPLIQQTSIVKRAFKRLKENFEIVALCLTLLANIVIMIRETR  80
 7 KEN1/76/O    ISIPSQKSVLYFLIEKGQHEAAIEFFEGMVHDSIKEELRPLIQQTSFVKRAFKRLKENFEIVALCLTLLANIVIMIRETR  80
 8 O1CAMPOS     ISIPSQKSVLYFLIEKGQHEAAIEFFEGMVHDSIKEELRPLIQQTSFVKRAFKRLKENFEIVALCLTLLANIVIMIRETR  80
 9 C3RESENDE    ISIPSQKSVLYFLIEKGQHEAAIEFFEGMVHDSIKEELRPLIQHTSFVKRAFKRLKENFEIVALCLTLLANIVVMIRDTR  80
10 O1C-E/O      ISIPSQKSVLYFLIEKGQHEAAIEFFEGMVHDSIKEELRPLIQQTSFVKRAFKRLKENFEIVALCLTLLANIVIMIRETR  80
11 C3R-E/O      ISIPSQKSVLYFLIEKGQHIAAIEFFEGMVHDSIKEELRPLIQHTSFVKRAFKRLKINFEIVALCLTLLANIVVMIRETR  80
12 O1TAIWAN     ISIPSQKSVLYFLIEKGQHEAAIEFFEGMVHDSIKEELRPLIQQTSFVKRAFKRLKENPEVVALCLTLLANIVIMLRQAB  80
13 O1CHUPEI     ISIPSQKSVLYFLIEKGQHEAAIEFFEGMVHDSIKEELRPLIQQTSFVKRAFKRLKENFEVVALCLTLLANIVIMLRQAB  80
14 PAK1/91/AS   ISIPSQKSVLYFLIEKGQHEAAIEFFEGMVHDSIKEELRPLIQQTSFVKRAFKRLKENFEIVALCLTLLANIVIMVRETR  80
15 SAR9/81/1    ISIPSQKSVLYFLIEKGQHEAAIEFYEGMVHDSIKEELKPLLEQTSFAKRAFKRLKENFEIVALVVGLLANMVIMIRETR  80
16 ZAM18/96/1   ISIPSQKSVLYFLIEKGQHEAAIEFYEGMVHDSIKEELKPLLEQTSFAKRAFKRLKENFEIVALVVVLLANIIIMIRETR  80
17 NAM272/98/   ISIPSQKSVLYFLIEKGQHEAAIEFYEGMVHDSIKEELKPLLEQTSFAKRAFKRLKENFEIVALVVVLLANIIIMIRERF  80
18 KEN3/57/2    ISLPSQKSVLYFLIEKGQHDEAIEFFEGMVHDSIKEELRPLIQQTSFVKRAFKRLRENFEIVALCLALLANIVIMIRETR  80
19 KEN8/89/2    ISIPSQKTVLYFLIEKGQHEAAIEFYEGMVHDSIKEELKPLLEQTSFAKRAFKRLKENFEIVALVVVLLANIIIMIRETR  80
20 ZAM10/96/2   ISIPSQKSVLYFLIEKGQHEAAIEFYEGMVHDSIKEELKPLLEQTSFAKRAFKRLKENFEIVALVVVLLANIIIMIRETR  80
21 KNP10/90/3   ISIPSQKSVLYFLIEKGQPEPAIEFYEGMVTDSIKEELKPLLEQTSFAKRAFKRLKENFEIVALVVVLLANIIIMIRETR  80
```
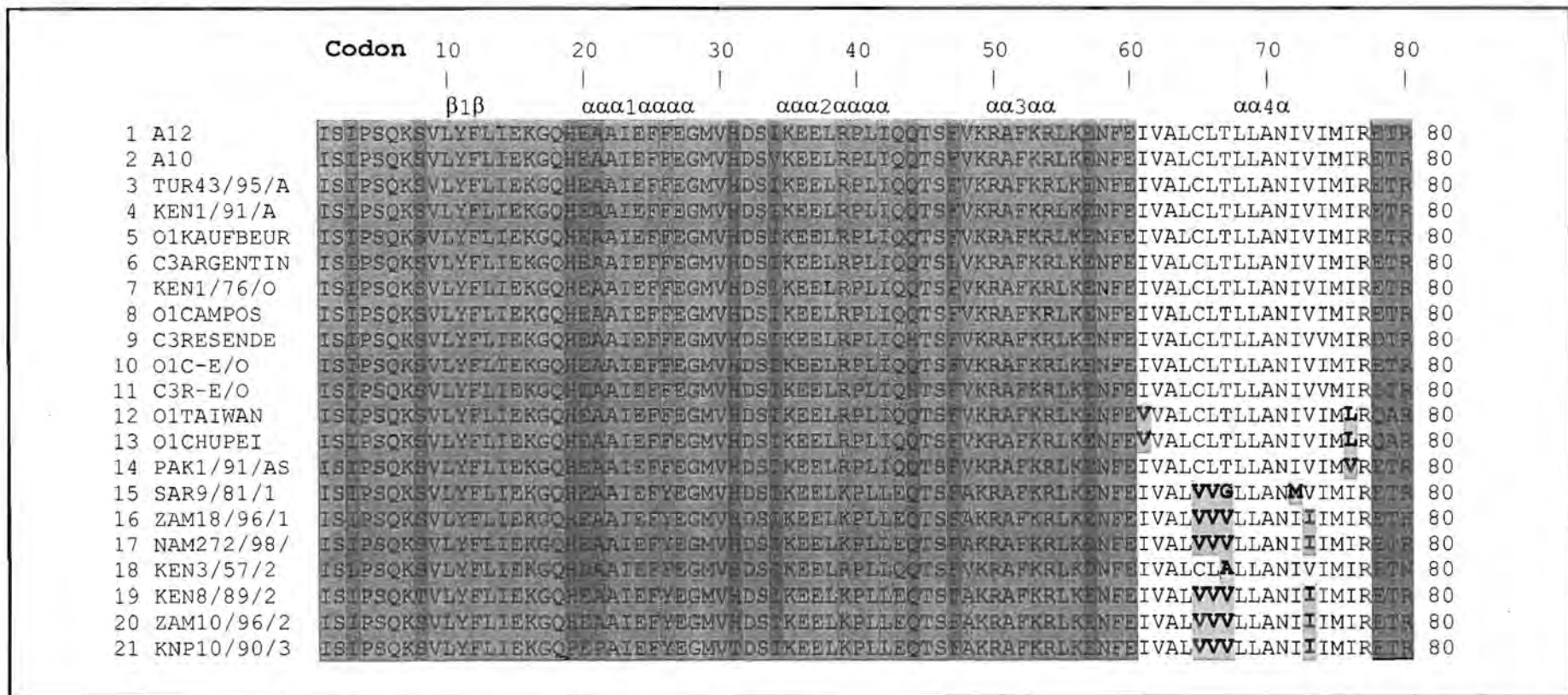
Figure 3.3.1.a. Multiple alignment of the predicted amino acid sequence(codons 1- 80) of 3A non-structural protein of several FMDV isolates. Secondary structural motifs as predicted with the Garnier, Gibrat and DPM algorithms, are indicated with α and β [The hydrophobic domain characteristic of all picornaviruses is highlighted in yellow. The green shaded boxes within the highlighted area ( ) indicate specific points of amino acid substitutions. Amino acids identity: 100%, >75%, 50%, <50%]
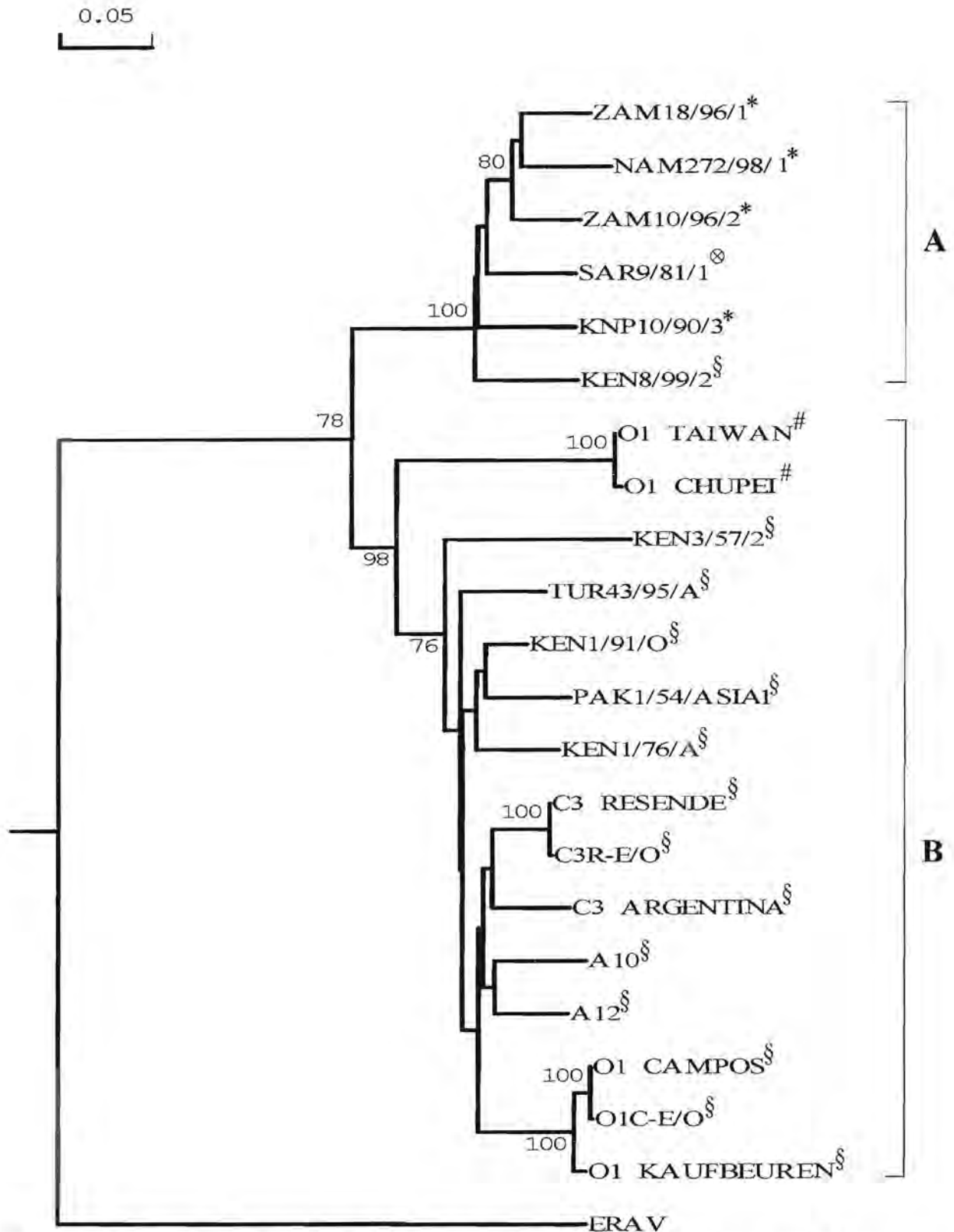
0.05

*Figure 3.3.2. A neighbour-joining tree based on the nucleotide sequence of the 3A non-structural-protein coding region, depicting the relationship of FMDV isolated originating from diverse geographical and host origin. Equine Rhinitis virus A (ERAV) was selected as appropriate outgroup. A trial number of 1000 was applied. The different FMDV subgroups are indicated by the brackets. Bovine isolates are indicated by a superscripted §, Porcine isolates by a superscripted #, African Buffalo isolates by a superscripted * and Impala isolates by a superscripted ⊗.*

A.1 Experimental strategies and Oligonucleotide sequences
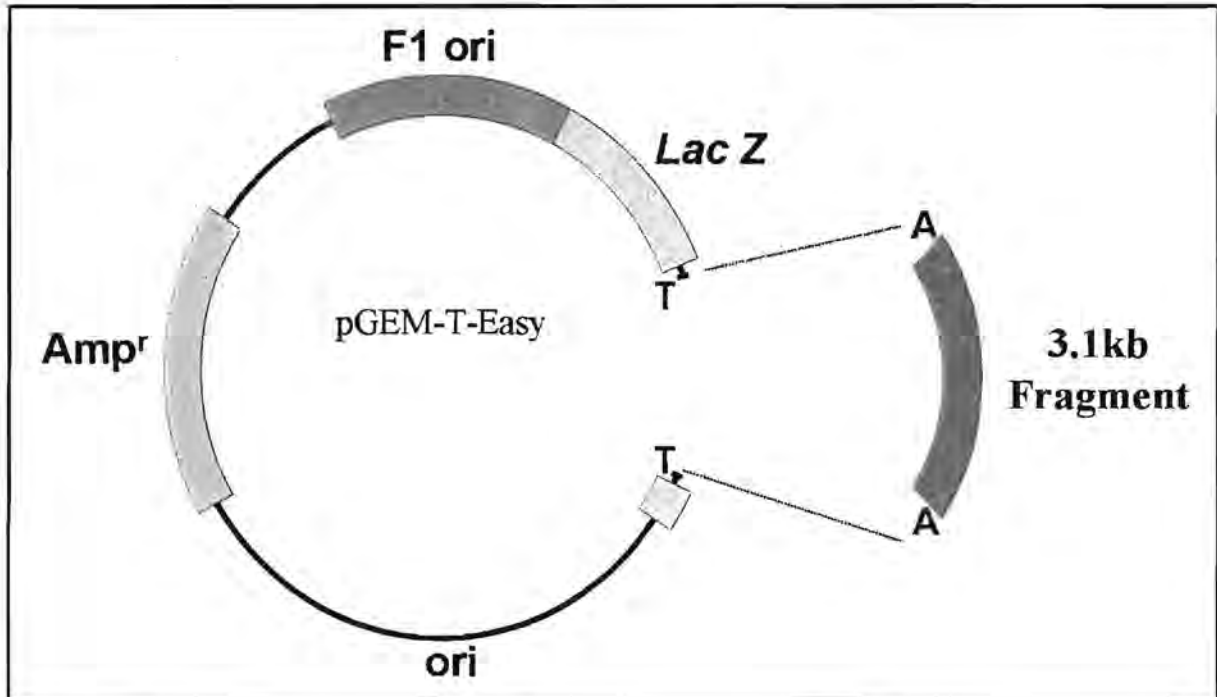
Figure A.1.1. A graphical representation of the cloning strategy used to construct pGEM.SAT3P1. The amplified fragment (dark blue) was ligated into a liniarised pGEM-T-Easy vector by employing a T/Acloning strategy. Insertion of the foreign fragment leads to the inactivation of the Lac Z gene (turquoise). This inactivation, in conjunction with resistance to ampicillin conferred by the Amp^r gene (   ), is used for the selection of possible recombinant clones.
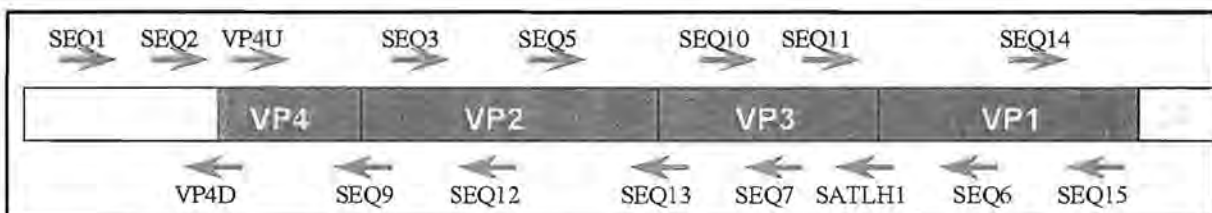
Figure A.1.2. A graphical representation of the relative positions of the oligonucleotides used in the nucleotide sequence determination of the P1 precursor. The structural-protein-coding region is represented in blue. The position and orientation the oligonucleotides are represented by the red arrows.

Chapter 4:
General Discussion

4.1 Significance of the sequence variation in the P1 region of FMDV

Due to the economical impact of the disease, FMD is of particular importance to countries dependent on animal production and related agriculture. Trade embargoes are instituted not only to regions where FMD is endemic, but also where outbreaks occur in non-endemic areas. Countries that are free of FMDV maintain strict import restrictions to prevent the introduction of the disease. Continued global surveillance of the disease is therefore crucial.

A case in point is the recent outbreak of an exotic strain of FMD in KwaZulu-Natal, a province of South Africa free of FMDV since 1958. The outbreak occurred after infected swill taken from an Asian ship was fed to pigs at a local farm and lead to the introduction of a new serotype into South Africa. The causative isolate was shown to be a type O virus originating from the Far East. Within hours of the first reported case the surrounding area was quarantined and a general restriction placed on the movement of any cloven hoofed animals to and from the affected area. In order to contain the outbreak all susceptible animals on the affected farm, as well as the surrounding farms, were destroyed and a major vaccination program launched in the region. The international community responded by banning the import of all meat and meat products from the whole KwaZulu-Natal province until the outbreak cleared. Several countries banned imports from the whole of South Africa until several months after the outbreak was brought under control. Farmers in the region suffered significant losses in profits during this period.

The involvement of wildlife in virus maintenance and disease transmission in sub-Saharan Africa makes the eradication of disease in the region highly unlikely. For this reason more emphasis is placed on the control of the disease in Africa, rather than outright eradication. Control of FMD in southern Africa is predominantly achieved through the implementation of vaccination programs in areas where FMDV is endemic (Hunter, 1998). Although vaccination proved to be an effective strategy to control the disease in sub-Saharan Africa, current vaccines frequently fail to adequately address the extent of antigenic variation within the SAT types of FMDV (Domingo et al., 1990). One effective approach to address this problem could be to develop recombinant vaccines. Such a strategy is discussed in detail in Section 2.4, but briefly involves the development of recombinant FMD viruses by

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substituting the antigenic determinants of a full-length infectious clone. Under ideal conditions this approach would be modular, allowing the rapid development of effective recombinant vaccines to be used in specific geographical locations. During the course of this study, an investigation into the approach of infectious recombinant virus vaccines was started in a parallel PhD study within the program (H.G. van Rensburg)

The major consideration in developing recombinant viruses is the effect of genetic variation within the genome on the post-translational processing events. Of specific consequence is the ability of the 3C protein to effectively process the cleavage sites within the P1 precursor. We have shown that the amino acids situated at the catalytic centre of two of the three cleavage sites within the P1 precursor, are conserved across six seroptypes (With H.G. van Rensburg, PhD thesis; This study provided the SAT 3 sequence data towards the analysis). An amino acid substitution found at the remaining cleavage site in all of the SAT type viruses, was of such a nature as to suggest that it would not affect functionality of the site (Section 2.4). This conclusion is supported by reports showing that the 3C protease is able to cleave the P1 protein of heterologous European serotypes (Clarke & Sanger, 1988) and suggests that the specificity of the viral protease resides in structural features at the cleavage sites rather than the precise identity of the amino acids involved (Brown *et al.*, 1989).

An alternative approach to the substitution of the entire P1 coding region would be to exchange only parts of this region (van Rensburg & Nel, 1999). The VP1 gene contains the major immunodominant sites and has been shown to be antigenic in its purified form (Laporte *et al.*, 1973; Sobrino *et al.*, 1989). The advantage of this alternative approach would be the exclusion of foreign proteolytic sites limiting the effect of genetic incompatibility. However, protection after vaccination with different regions of the capsid protein VP1, produced as recombinant proteins (Giavedoni *et al.*, 1991) or reproduced in synthetic peptides (Bittle *et al.*, 1982), has been lower than that achieved with classical vaccines composed of inactivated virus particles (Corchero & Villaverde, 1996). This is probably due to the improper exposure of the neutralising epitopes (Blanco *et al.*, 2000). The disruption of conformational neutralising epitopes due to partial substitution of the P1 polyprotein may limit the efficiency of such an approach in a similar manner.

4.2 Significance of sequence variation in the 3A non-structural-protein-coding region of FMDV.

Phylogenetic reconstruction based on the nucleotide and amino acid sequence data of the 3A protein showed that the SAT type viruses differ significantly from the European serotypes. This is consistent with results of phylogenetic studies based on the VP1 structural-protein-coding sequences of FMDV (Bastos, 1998). The level of genetic variation within the 3A coding region of this subgroup was found to be markedly lower than the level of variation within the same region of the European serotype. This may be due to the fact that geographical distribution of the SAT types is restricted to sub-Saharan Africa whilst the European isolates are more globally distributed.

Although the nucleotide sequences of the 3A protein of SAT type viruses differ from that of the European serotypes, the proteins appear to be similar in structure. This would be expected, since the 3A protein plays a crucial role during infection and virus replication and is thus under significant pressure to remain functionally conserved (Beard and Mason, 2000). This is especially evident when one compares functional domains within the protein. The hydrophobicity of the domain responsible for mediating the association of the 3A protein with cellular membranes (Xiang et al., 1998), were found to be conserved between the two subgroups despite the fact that the SAT type viruses contained several amino acid substitutions within this domain when compared to that of the European serotypes.

Historically, the VP1 gene has been used to determine the phylogenetic relationship of FMDV virus isolates. Characterisation of naturally occurring variants by phylogenetic analyses of the VP1 gene facilitates the rapid determination of the serotype and geographical origin of the isolates (Bastos et al., 2000). It has further revealed that the SAT type viruses evolve independently in different geographical areas (Vosloo *et al.*, 1995). In contrast to the phylogenetic studies based on the VP1 gene, no distinction could be made between SAT isolates belonging to different serotypes or originating from different geographical localities, based on the 3A sequence data. This can again be contributed to the fact that mutations occur more readily within the structural protein-coding regions of the genome and that the level of genetic changes fixed within the 3A coding region is restricted by the functionality of the resulting protein. The high level of homology between

the 3A proteins of diverse isolates and reduced number of informative sites thus limits the depth of the phylogenetic information attainable from these sequences.

One of the major questions of this study was whether the 3A protein of the SAT types is conserved or variable in length and features. Previous reports have shown that the 3A protein plays a role in determining the host range specificity of FMD virus isolates circulating in the Far East (Beard and Mason, 2000, Núñez et al., 2001), but nothing had been known about the involvement of the 3A protein in determining the host range of the SAT viruses. We have shown that the 3A proteins of the SAT type viruses are conserved in length and structure. We found no evidence to suggest that 3A variation may be implicated in the host range specificity of the SAT type viruses. Although isolates were carefully selected to represent diverse viruses, the limited number of isolates in this study leaves room for more expanded research in this regard.

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Appendix