Appendix

Table A.1.1 Summery of oligonucleotides used in the sequence determination of the P1 fragment of FMDV KNP/10/90/3.

Oligonucleotide	*Sequence*	*Purpose*
NCR1 (22 mer)	5'-TACCAAGCGACACTCGGGATCT-3'	PCR amplification Nucleotide sequencing
WDA (21 mer)	5'-GAAGGGCCCAGGGTTGGACTC-3'	cDNA synthesis, PCR amplification Nucleotide sequencing
VP4U (20 mer)	5'-TCTGGCAAYACTGGTATAGCAT-3'	Nucleotide sequencing
VP4D (20 mer)	5'-CTACCAGTATTGCCAGATTG-3'	Nucleotide sequencing
SEQ1 (18 mer)	5'-CGTCGATGAGCCACTCTT-3'	Nucleotide sequencing
SEQ2 (17 mer)	5'-CATCAAAGGCACTGAAC-3'	Nucleotide sequencing
SEQ3 (18 mer)	5'-ACAACACGACACGGTACC-3'	Nucleotide sequencing
SEQ4 (19 mer)	5'-TTGTGCGAAGCGTGGTTGT-3'	Nucleotide sequencing
SEQ5 (18mer)	5'-CACCAGCACGCAGTTCAA-3'	Nucleotide sequencing
SEQ6 (17 mer)	5'-GTTCGTGTTGCCAAGG-3'	Nucleotide sequencing
SEQ8 (18 mer)	5'-ACGTTSGTCGGNGCKATG-3'	Nucleotide sequencing
SEQ9 (20 mer)	5'-GCKTAACCGTAGGTGAYKCC-3'	Nucleotide sequencing
SEQ10 (17 mer)	5'-GACCCBAAGACCGCAGA-3'	Nucleotide sequencing
SEQ11 (18 mer)	5'-GGGAYACAGGAYTGAACT-3'	Nucleotide sequencing
SEQ12 (15 mer)	5'-CGTAGATMCCCTTGT-3'	Nucleotide sequencing
SEQ13 (17 mer)	5'-GCGACGTCCAACAKGTT-3'	Nucleotide sequencing
SEQ14 (19 mer)	5'-CAACGGTGAGTGCAAKKAC-3'	Nucleotide sequencing

Table A.1.1 (Continued) Summery of oligonucleotides used in the sequence determination of the P1 fragment of FMDV KNP/10/90/3.

Oligonucleotide	*Sequence*	*Purpose*
SEQ15 (19 mer)	5'-GTMMTTGCACTCACCGTTG-3'	Nucleotide sequencing
SAT3LH (20 mer)	5'-CCCTCACCTGCGCTGGTTGT-3'	Nucleotide sequencing
FOR (17 mer)	5'-GTAAAACGACGGCCAGT-3'	Nucleotide sequencing
REV (17 mer)	5'-GTTTTCCCAGTCACGAC-3'	Nucleotide sequencing

A.2 List of Figures and Tables

A.3 Abbreviations

µg	Micro-gram
µl	Micro-litre
^{32}P	Radio-active Phosphorus
A	Adenine
ATP	Adenosine 5'-triphosphate
C	Cytosine
Cdna	Complementary Deoxyribonucleic Acid.
Cfu	Colony forming units
Ci	Curie
CPE	Cytopathic effect
dATP	Deoxyadenosine triphospate
dCTP	Deoxycytidine triphosphate
ddNTP	Dideoxy-ribonucleotide 5'-triphosphate
dGTP	Deoxyguanosine triphosphate
dH_2O	Distilled Water
DMSO	Dimethylsulfoxide
DTT	Ditioltrytol
dTTP	Deoxyribosylthymine triphosphate
EDTA	Ethelene diaminetetraacetate
FMD	Foot-and-mouth disease.
FMDV	Foot-and-mouth disease virus.
G	Guanine
HCl	Hydrochloric Acid
IPTG	Isopropyl-β-D-thiogalactoside
Kbp	Kilo base pairs
M	Molar
mg	Milligrams
$MgCl_2$	Magnesium chloride
ml	Millilitre.
mm	Millimetre
mM	Millimolar

NCBI	National Centre for Biotechnology Information.
ng	Nanno-grams
°C	Degrees Celsius.
OD*	Optical Density (* the applicable wavelength is indicated in subscript)
P1	First polyprotein precursor coding region of foot-and mouth disease virus consisting of the structural protein coding region and the 2A non-structural-protein-coding region of the genome.
Par	Section
PBS	Phosphate Buffer Saline
PCR	Polymerase chain reaction
pmol	Pico-moles
RNA	Ribonucleic Acid.
RPM	Revolutions per Minute
SAT	Southern African Territories.
T	Thymine
TAE	Tris/Acetic acid/EDTA buffer
Tris	Tris(hydroxymethyl)aminomethane
U	Units
V	Volts
v/v	Volume to volume ratio
W	Watt
X-Gal	5-bromo-4chloro-3indol-β-galactoside

A.4 Buffers and Reagents

Versene-Trypsin

2M NaCl

0.1M KCl

01.M $NaHCO_3$

0.1M Glucose

0.01M EDTA

5g/L Trypsin

0.035% Neommycin

0.015% Penicilin

0.032% Streptomycin

Phosphate Buffer Saline (PBS)

Solution A:

0.1M NaCl

0.03mM KCl

0.1mM $Na_2HPO_4.2H_2O$

0.01Mm KH_2PO_4

Solution B:

0.01mM CaCl2

0.01mM $MgCl_2.6H_20$

Autoclave separately and ad 800ml Solution A to 200ml Solution B.

1M Tris/HCl Buffer (pH 8.0 @ 25oC)

121.1g Tris

800ml dH_2O

Adjust pH to 8.0 using HCl

Adjust final volume to 1L using dH_2O

0.1M Tris/HCl Buffer (pH 7.6 @ 25oC)

100ml 1M Tris/HCl buffer (pH 8.0 @ 25°C)

800ml DH2O

Adjust pH to 7.6 using HCl

10% Sodium Dodecyl Sulphate solution

100g SDS

900ml dH_2O

Adjust pH to 7.2 using HCl

0.1M Tris/HCl saturated Phenol

Melt the crystalline phenol at 68oC. Add 8-hydroxyquinolene to a final concentration of 0.1%. Extract consecutively with 1.0M Tris/HCl (pH 8.0), 0.1M Thris/HCl (pH 8.0) and β-mercaptoethanol until the pH of the aqueous phase in 7.6.

5 x Reverse Transcriptase Baffer

250mM Tris/HCl (pH 8.3 @ 25°C)

250m MKCl

50mM $MgCl_2$

2.5mM Spermidine

50mM DTT

Deoxynucleotide-triphosphate mixture (2.5 mM)

2.5mM ATP

2.5mM TTP

2.5mM GTP

2.5mM CTP

10 x T4 Ligase Buffer

300mM Tris/HCl (pH 7.8 @ 25°C)

100mM MgCl2

100mM DTT

10mM ATP

10 x ExpandTM Long Template PCR Reaction Buffer

20mM Tris/HCl (pH 7.5 @ 25°C)

100mM KCl

1.0mM DTT

0.1mM EDTA

0.5% Tween 20 (v/v)

0.5% Nonidet P40 (v/v)

50% Glycerol (v/v)

1 x TAE Buffer

242g Tris

5.7g Glacial acetic acid

100ml 0.5M EDTA (pH 8.0 @ 25°C)

900ml dH_2O

Dilute 1:50 in dH_2O

LB Liquid medium

10g Tryptone

5g Yeast extract

10g NaCl

1L dH_2O

SOC Liquid medium

20g Tryptone

5g Yeast extract

0.5g NaCl

10ml 250mM KCl (pH 7.0 @ 25°C)

965ml dH_2O

Add after sterilisation:

20ml 1M Glucose (Filter sterilised)

5ml 2M MgCl2 (Filter sterilised)

SOB-agar culture plates

20g Tryptone

5g Yeast extract

0.5g NaCl

10ml 250mM KCl (pH 7.0 @ 25°C)

15g Agar

985ml dH_2O

Add after sterilisation:

5ml 2M MgCl2 (Filter sterilised)

2% X-Gal solution

0.1g X-Gal

5ml N,N-dimethylformamide

100mM IPTG

0.119g IPTG

5ml sterile dH2O

1% Sodium Dodecyl Sulphate / 0.1M Sodium Hydroxide Solution

1ml 10% Sodium Dodecyl Sulphate

200µl 10M NaOH

8.8ml dH2O

SET Buffer

50mM Tris/HCl (pH 8.0 @ 25°C)

50mM EDTA

20% Sucrose

Poly-ethylene Glycol / Sodium Chloride Solution

27g Poly-ethylene Glycoll 6000

66ml 5M NaCl

34ml dH2O

Formamide dey mix

90% deionised Formamide (v/v)

10mM Tris/HCl (pH 7.4)

5mM EDTA

0.1% Xylene cyanol (w/v)

0.1% Bromophenol blue (w/v)

1 x TBE Buffer

0.1M Tris/HCl (pH 8.0)

0.1M Boric acid

0.2mM EDTA

Dilute 1:10 using dH_2O

Biotools DNA plymerase reaction buffer.

75mM Tris/HCl (pH 9.0)

2mM $MgCl_2$

50mM KCl

20mM $(NH_4)_2SO_4$

List of conferences and symposiums at which the work was presented.

1999 International Union of Microbiological Societies Sydney, Australia
XIth International congress of Virology
Poster Presentation: H.G. van Rensburg, L.H. Nel & L.E. Heath.
Toward the construction of a recombinant vaccine against Foot and Mouth Disease.

1999 Conference of Workers in Animal Diseases Chicago, USA
80th Annual meeting of the Conference of workers in animal diseases
Poster Presentation: H.G. van Rensburg, L.E. Heath, L.H. Nel & P.Mason
Aspects in the development of recombinant vaccines against FMDV.

1999 Agricultural Research Council Pretoria, RSA
Biennual report of the Exotic Disease Division, Onderstepoort Veterinary institute.
Oral Presentation: H. G van Rensburg & L.E. Heath.
Development of recombinant vaccines against foot-and-mouth disease.

2000 South African Society for Microbiology Grahamstown, RSA
XIth Biennial congress of the Soth African society for microbiology
Oral Presentation: L. E. Heath, H.G. van Rensburg, W. Vosloo & L.H. Nel.
Genetic characterisation of the structural-protein-coding region of several Southern African Type foot-and-mouth disease virus.

2000 South African Society for Microbiology Grahamstown, RSA
XIth Biennial congress of the Soth African society for microbiology
Poster Presentation: L. E. Heath, W. Vosloo & L.H. Nel.
The Possible role of the foot-and-mouth disease virus 3A non-structural-protein-coding region in determining host range specificity and virulence.

Publication to be submitted to the Onderstepoort Journal of Veterinary Research

Analyses of the 3A non-structural-protein coding region of foot-and-mouth disease virus isolates from southern and eastern Africa

L.E. HEATH[1], H.G. VAN RENSBURG[1], W. VOSLOO[2*] AND L.H. NEL[1].

ABSTRACT

L.E. HEATH[1], H.G. VAN RENSBURG[1], W. VOSLOO[2*] AND L.H. NEL[1]. Analyses of the 3A non-structural-protein coding region of foot-and-mouth disease virus isolates from southern and eastern Africa.[1]

The 3A non-structural protein of foot and mouth disease viruses is a highly conserved protein of 153 amino acids. Recently correlation between the presence of a 10 nucleotide deletion as well as other mutations within the 3A non-structural-protein coding region and attenuation of FMDV in cattle was demonstrated (Beard & Mason, 2000). Although the 3A coding region of several type A, O and C isolates have previously been described, nucleotide sequence data of the 3A coding region of the South African Types (SAT) 1, 2 and 3 viruses, is limited. Therefore, the 3A non-structural-coding region of different SAT serotypes were determined, analysed and compared to that of European, South American and Asian isolates. Our results indicated that the 3A region of the SAT isolates investigated differed markedly from that of types A, O, C and Asia-1, but were closely related within the group.

Key Words: Picornavirus, Foot-and-mouth disease virus, 3A non-structural protein, SAT types.

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INTRODUCTION

Foot-and-mouth disease (FMD), a highly contagious viral disease affecting cloven-hoofed animals, is characterised by a predominantly non-lethal infection culminating in temporary oral and pedal vesicles. Infections commonly result in a significant reduction in the production of meat or dairy products. Affected producers suffer substantial losses as a result of severe marketing restrictions and strict quarantine measures set in place to control the disease (Shahan 1962). Foot-and mouth disease virus (FMDV) is a positive sense RNA virus within the *Aphthovirus* genus of the family *Picornaviridae*. There are seven recognised FMDV serotypes *viz*. A, O, C, Asia-1 and South African Territories (SAT) types 1, 2 and 3. The SAT types are three serotypes unique to sub-Saharan Africa and are predominantly responsible for outbreaks of the disease in the region (Thomson 1994), although incidence of SAT 1 and SAT 2 has been reported in the Middle East. In addition to these, serotypes A, O and C are also occasionally found to be associated with outbreaks in Africa with Asia-1 being the only exotic FMDV type to the continent. Due to the genetic instability of the FMD virus, new viruses with altered antigenic and phenotypic properties frequently emerge (Rueckert 1990) making control of the disease by vaccination complex.

The FMDV genome contains an open reading frame encoding a single polypeptide. A cascade of proteolytic processing events carried out by three viral proteases result in 12 mature gene products. In addition to four structural proteins, the genome encodes several non-structural proteins that are involved in different stages of the viral replication cycle. One of these is the 153 amino acid 3A protein that is known to be involved in viral replication and has been shown to mediate the relocation of the viral RNA replication machinery to the cellular membrane (Xiang, Cuconati, Hope, Kirkegaard & Wimmer 1998). Furthermore, 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, Giddings & Kirkegaard 1997). The 3AB polypeptide, the precursor of the 3A and 3B viral proteins, stimulates the *in vitro* synthesis of poly(U) viral RNA, directed by the RNA-dependant RNA polymerase (Lama, Sanz & Carrasco 1998). Changes in 3A have also been associated with altered host range specificity in

the hepatoviruses (Lemon, Murphy, Shields, Ping, Feinstone, Cromeans & Jansen 1991; Morace, Oisani, Beneduce, Divizia & Pana 1993; Graff, Normann, Feinstone & Flehming 1994), rhinoviruses (Heinz & Vance, 1996) and enteroviruses (Lama *et al.* 1998).

Historical accounts of FMD outbreaks have indicated that the FMDV isolates involved in some outbreaks demonstrated a marked species restriction. For example, Brooksby (1950) cited reports of epidemics in Germany and Britain during the 1920's and 1930's respectively, in which pigs were predominantly affected. Cattle were almost completely unaffected 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 virus (O_1Taiwan), demonstrated an extensive porcinophilic phenotype with no infection observed 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 & Donaldson 1997).

Genetic characterisation of the 1997 O_1Taiwan outbreak virus revealed distinct differences in the 3A sequence when compared to that of representative European and South American isolates. The most significant of these was a 10 amino acid deletion corresponding to residues 93 to 102 of the other isolates (Beard & Mason 2000). These authors were able to demonstrate a direct correlation between the presence of this deletion within the 3A protein and attenuation of 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 passage in chicken embryos (Giraudo, Beck, Strebel, Augè de Mello, La Torre, Scodeller & Bergmann 1990). These attenuated viruses exhibited similar porcinophilic phenotypes to that observed for the O_1Taiwan isolate. A subsequent study on FMD viruses from the Southeast Asia region (Knowles, Davies, Henry, O'Donnel, Pacheco & Mason 2001), found a similar deletion in the earliest isolates from the region. In contrast to previous results (Beard & Mason 2000), these viruses did not display similar attenuation on bovine keratinocytes, suggesting that the deletion (residues 93 –102) in

the 3A region alone can not 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 FMDV susceptible 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 most African buffalo (*Syncerus caffer*) across sub-Saharan Africa are persistently infected with FMDV without exhibiting any of the classical symptoms of the disease (Condy, Hedger, Hamblin & Barnett 1985). However, occasional outbreaks of FMD in impala (*Aepycerus 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 in 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 circumstantial evidence and more recently on nucleotide sequence data, that buffalo are the source of infection in impala (Bastos, Boshoff, Keet, Bengis & Thomson 2000).

Although the 3A-coding region of several European and Asian isolates has previously been described, the sequence characteristics of the 3A of the SAT type viruses have not been investigated. Therefore, the nucleotide sequences of the 3A non-structural-protein sequence of several African FMD virus isolates from different species were determined and comparatively analysed. We compared this region of the genome of different SAT serotypes with that of types A, O, C and Asia-1 isolates. Our results indicated that the 3A region of the SAT isolates differed markedly from that of types A, O, C and Asia-1, although closely related within the SAT types.

METHODS

Origin of the viruses

FMDV isolates, representative of all seven serotypes originating from diverse geographical and host origins, were selected (Table 1). Isolates indicated with asterisks were used to generate additional nucleotide sequence data for this study. Where possible, primary pig kidney cell

isolations of the viruses were used for 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 used is summarised in Table 1.

INSERT TABLE 1 HERE

RNA extraction and cDNA synthesis

RNA was extracted from cell culture specimens by a modified guanidium-based nucleic acid extraction method (Boom, Sol, Salimans, Jansen, Wertheim-van Dillen & van der Noorda 1990). The viral RNA was reverse-transcribed using 10 U of AMV-RT (Promega). In addition to random hexanucleotides, an antisense oligonucleotide (P445 = 5'-ACCATCTTTTGCAAGTC-3') targeting a region within the 3B region was used to initiate cDNA construction. Oligonucleotides used in this study were designed from the partial nucleotide sequence of the SAT 2 isolate, ZIM/7/83 (H.G. van Rensburg, unpublished data).

PCR amplification

Amplification of the FMD viral genome was performed using primers targeting an 860bp region, which includes the entire 3A-coding region. The primers were designed to anneal to conserved areas within the 2C and 3B coding regions of the FMDV genome respectively. The reaction conditions were optimised using a method described by Cobb & Clarkson (1994). Reactions were performed in a 50µl volume in the presence of 0.15mM dNTP's, 30pmol sense oligonucleotide (P444 = 5'-GGCCGTTGAAATGAAGAGA-3'), 30pmol antisense oligonucleotide (P445), 1 x Biotools DNA polymerase reaction buffer and 2 U of thermostable DNA polymerase (Biotools). After an initial denaturation step at 94°C for 2 minutes, 30 cycles of denaturation at 94°C for 30 seconds, annealing 53°C for 30 seconds and extension at 72°C for 90 seconds were performed.

PCR product purification and sequencing

The size of amplified fragments were estimated against a DNA molecular weight marker (*HindIII* restricted X174 DNA, Promega) on a 1.5% agarose gel. Bands of the expected size (860bp) were excised from the gel and purified by means of the Nucleospin Extract 2 in 1 DNA extraction kit (MacHerey-Nagel). The purified products were sequenced using the ABI PRISM™ BigDye™ Terminator Cycle sequencing ready reaction kit (Perkin-Elmer Applied Biosystems). To ensure the validity of the data, both the sense and antisense strands of the amplified fragments were independently sequenced using the P444 and P445 oligonucleotides.

Computer-assisted analyses of the nucleotide sequences

In addition to isolates sequenced in this study, the published 3A nucleotide sequences of several isolates were obtained from GenBank and included in the analysis (Table 1). All nucleotide sequences determined in this study have been submitted to GenBank under the accession numbers indicated in Table 1. Nucleotide sequences, as well as predicted amino acid sequences, were aligned using the DAPSA program (Harley 1998). The multiple nucleotide 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, Copyright©1994-1999). Bootstrap supported trees were constructed using a random seed generator of 111 and 1000 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 was used to determine the hydrophobic regions within the predicted proteins.

RESULTS

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 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 (Fig. 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, Fig. 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 (Fig. 2).

The amino acid sequence was found to be highly conserved within the N-terminus region of the protein. In Fig. 1, only secondary structural motifs that could be predicted with the most certainty are indicated. 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-helices 1 and 2 (Fig. 1a). Secondary structure predictions in the C-terminus region of the protein were more complicated and only three regions could be indicated with confidence (Fig. 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 (Fig. 1b).

A distinct and conserved hydrophobic domain is situated between residues 61 and 77 (Fig. 1b) 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 (Fig. 1b). Although the identity of residues 65 to 67 as well as residue 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, it may be assumed that the variation in amino acid sequence would not necessarily affect the functionality of the domain.

INSERT FIG. 1 HERE

Phylogenetic relationships of FMDV based on the 3A protein

Genetic relationships of the FMD 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 Fig. 2.

All SAT type viruses, with the exception of KEN/3/57/2, were found in a single distinct phylogenetic cluster (group A, Fig. 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 type Asia-1 were contained (group B, Fig. 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 (O_1Taiwan and O_1Chupei) with a high bootstrap value 100%. The other significant groupings were that of O_1Campos together with O1C-E/O, the virus that was derived by passaging O_1Campos through eggs and O_1Kaufbeuren and C_3Resende and its egg-derived strain C3R-E/O.

INSERT FIG. 2 HERE

DISCUSSION

The 3A protein of the FMD virus has recently been shown to play an important role in host range specificity and virulence (Beard & Mason 2000; Knowles *et al.* 2001). Information regarding this protein of the SAT-type viruses is limited and prompted an investigation into the comparative characteristics of the protein among different SAT-type viruses, originating from different host species in southern and eastern Africa. The 3A proteins of the SAT type viruses were found to be 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. Results obtained by Knowles and co-workers (2001) indicated that amino acid mutations surrounding the described deletion in some East Asian isolates might play a role in the observed species restriction. The functional implication of the deletion in the SAT-type 3A protein was however not investigated in this study.

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 types A and O were identical to that of the European isolates.

Phylogenetic analysis based on the nucleotide 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 (Vosloo, Kirkbride, Bengis, Keet & Thomson 1995; Bastos 1998). FMDV, being a single-stranded RNA virus with no proof-reading ability during replication (Holland, Spindler, Horodyski, Grabau, Nichol & van de Pol 1982) has a high

rate of mutation. 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, Davila, Ortin & Domingo 1983; McCahon 1986; Sobrino, Palma, Beck, Davila, De la Torre, Negro, Villaneuva, Ortin & Domingo 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. KEN/3/57/2 groups with the SAT 2 serotype when VP1 gene sequences are compared (Bastos 1998), 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 supposition 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 did not conform to the SAT-specific pattern but rather to that of the European serotypes. However, 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. No evidence was found to suggest that 3A variation might be implicated in the host range specificity or virulence of the SAT type viruses.

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REFERENCES

1. BASTOS, A.D.S. 1998. Detection and characterization of foot-and-mouth disease virus in sub-Saharan Africa. *Onderstepoort Journal of Veterinary Research,* 65: 37-47.

2. BASTOS, A.D., BOSHOFF, C.I., KEET, D.F., BENGIS, R.G. & THOMSON, G.R. 2000. Natural transmission of foot-and-mouth disease virus between African buffalo (*Syncerus caffer*) and impala (*Aepyceros melampus*) in the Kruger National Park, South Africa. *Epidemiology and Infection*, 124: 591-598.

3. BEARD, C.W. & MASON, P. W. 2000. Genetic determinants of altered virulence of Taiwanese foot-and-mouth disease virus. *Journal of Virology*, 74: 987-991.

4. BOOM, R., SOL, C.J., SALIMANS, M.M.M., JANSEN, C.L., WERTHEIM-VAN DILLEN, P.M.E. & VAN DER NOORDA, J. 1990. Rapid and simple method for purification of nucleotide acids. *Journal of Clinical Microbiology*, 28: 495-503.

5. BROOKSBY, J.B. 1950. Strains of the virus of foot-and-mouth disease showing natural adaption to swine. *Journal of Hygiene*, 47: 184-195.

6. COBB, B.D. & CLARKSON, J.M. 1994. A simple procedure for optimising the polymerase reaction (PCR) using modified Taguchi methods. *Nucleic Acid Research*, 22: 3801-3805.

7. CONDY, J.B., HEDGER, R.B., HAMBLIN, C. & BARNETT, I.T. 1985. The duration of the foot-and-mouth disease virus carrier state in African buffalo (i) in the individual animal and (ii) in a free-living herd. *Comparative Immunology, Microbiology and Infectious Diseases*, 8: 259-65.

8. DELAEGE, G. 1999. *Anthoprot release 4*. Institut de Biologie et Chemie des Protéines. Lyon.

9. DOEDENS, J.R., GIDDINGS, T.H. & KIRKEGAARD, K. 1997. Inhibition of endoplasmic reticulum-to-Golgi traffic by poliovirus protein 3A: genetic and ultrastructural analysis. *EMBO Journal*, 14: 9054-9064.

10. DUNN, C.S. & DONALDSON, A.I. 1997. Natural adaptation to pigs of a Taiwanese isolate of foot-and-mouth disease virus. *Veterinary Record*, 141: 174-175.

11. GIRAUDO, A.T., BECK, E., STREBEL, K., AUGÈ DE MELLO, P., LA TORRE, J.L., SCODELLER, E.A. & BERGMANN, I.E. 1990. Identification of a nucleotide deletion in parts of ploypeptide 3A in two independent attenuated Aphthovirus strains. *Virology*, 177: 780-783.

12. GRAFF, J., NORMANN, A., FEINSTONE, S.M. & FLEHMING, B. 1994. Nucleotide sequence of wild-type hepatitis A virus GBM in comparison with two cell culture-adapted variants. *Journal of Virology*, 68: 548-54.

13. HARLEY, E.H. 1998. *DAPSA – a program for DNA and Protein sequence analysis, Version 4.31.* Cape Town: Department of Chemical Pathology, University of Cape Town.

14. HEINZ, B.A. & VANCE, L.M. 1996. Sequence determinants of 3A-mediated resistance to enviroxime in rhinoviruses and enteroviruses. *Journal of Virology*, 70: 4854-7.

15. HOLLAND, J.J., SPINDLER, K., HORODYSKI, F., GRABAU, E., NICHOL, S. & VAN DE POL, S. 1982. Rapid evolution of RNA genomes. *Science*; 215: 1577-85.

16. KNOWLES, N.J., DAVIES, P.R., HENRY, T., O'DONNELL, V., PACHECO, J.M. & MASON, P.W. 2001. Emergence in Asia of foot-and-mouth disease viruses with altered host-range: characterization of alterations in the 3A protein. *Journal of Virology*, 75: 1551-6.

17. LAMA, J., SANZ, M. A. & CARRASCO L. 1998. Genetic analysis of poliovirus protein 3A: characterization of a non-cytopathic mutant virus defective in killing Vero cells. *Journal of General Virology,* 79: 1911-21.

18. LEMON, S.M., MURPHY, P.C., SHIELDS, P.A., PING, L.H., FEINSTONE, S.M., CROMEANS, T. & JANSEN, R.W. 1991. Antigenic and genetic variation in cytopathic hepatitis A virus variants arising during persistent infection: evidence for genetic recombination. *Journal of Virology*, 65: 2056-65.

19. MCCAHON, D. 1986. The genetics of foot and mouth disease virus. *Rev. sci. tech. Off int,* 5: 279-97.

20. MORACE, G., PISANI, G., BENEDUCE, F., DIVIZIA, M. & PANA, A.. 1993. Mutations in the 3A genomic region of two cytopathic strains of hepatitis A virus isolated in Italy. *Virus Research*, 28: 187-94.

21. RUECKERT, R.R. 1990. Picornaviridae and their replication in *Virology 2nd edition*, edited by D.M. Knipe & B.N. Fields, New York. Raven press: 507-548.

22. SHAHAN, M.S. 1962. The virus of foot and mouth disease. *Annals of the New York Academy of Sciences,* 101: 444-454.

23. SOBRINO, F., DAVILA, M., ORTIN, J. & DOMINGO, E. 1983. Multiple genetic variants arise in the course of replication of foot and mouth disease virus in cell culture. *Virology,* 128: 310-8.

24. SOBRINO, F., PALMA, E.L., BECK, E., DAVILA, M., DE LA TORRE, J.C., NEGRO, P., VILLANEUVA, N., ORTIN, J. & DOMINGO, E. 1986. Fixation of mutations in the viral genome during an outbreak of foot-and-mouth disease: heterogeneity and rate variations. *Gene*, 50: 149-59.

25. THOMSON G.R. 1994. Foot-and-Mouth Disease, in *Infectious Diseases of Livestock with special reference to Southern Africa*, edited by J.A.W. Coetzer, G.R. Thomson & R.C. Tustin, Oxford: Oxford University Press: 826-852.

26. VOSLOO W., KIRKBRIDE, E., BENGIS, R.G., KEET, D.F. & THOMSON, G.R. 1995. Genome variation in the SAT types of foot-and-mouth disease viruses prevalent in buffalo (*Syncerus caffer*) in the Kruger National Park and other regions of southern Africa, 1986-93. *Epidemiology and Infection*, 114: 203-218.

27. XIANG, W., CUCONATI, D., HOPE, D., KIRKEGAARD, K. & WIMMER E. 1998. Complete protein linkage map of poliovirus P3 proteins. Interaction of polymerase $3D^{pol}$ with VPg and with genetic variants of 3AB. *Journal of Virology*, 72: 6732-6741.

Fig 1.a.

Multiple alignment of the predicted amino acid sequence (residues 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 shaded in grey. The bold residues within the highlighted area indicate specific amino acid substitutions.

Fig. 1.b

Multiple alignment of the predicted amino acid sequence (residues 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 highlighted by the shaded boxes.

Fig. 2.

A neighbour-joining tree based on the nucleotide sequence of the 3A non-structural-protein coding region, depicting the relationship of FMDV isolates originating from diverse geographical and host origins. Equine Rhinitis virus A (ERAV) was selected as appropriate outgroup. A bootstrap trial number of 1000 was applied, and significant branches of >75% are indicated. The different FMDV subgroups are indicated in brackets. Bovine isolates are indicated by §, porcine isolates by #, African buffalo isolates * and impala isolates by ⊗

Table 1. 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_1C-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_3R-E/O			Egg Passage	N/A	Girauda *et al.* 1990

Serotype SAT1					
SAR/9/81/1*	South Africa	1981	Impala	B_1 BHK_4 B_1 BHK_1	AF335011
NAM/272/98/1*	Namibia	1998	Buffalo	PK_2 IB-RS_1	AF335010
ZAM/18/96/1*	Zambia	1996	Buffalo	Not Available	AF335012
Serotype SAT2					
KEN/3/57/2	Kenya	1957	Bovine	N/A	AV0006
KEN/8/99/2*	Kenya	1999	Bovine	BTY_2 IB-RS4	AF335008
ZAM/10/96/2*	Zambia	1996	Buffalo	BTY_2 IB-RS4	AF335013
Serotype SAT3					
KNP/10/90/3*	South Africa	1990	Buffalo	PK1 IB-RS3	AF335009

* Indicates the viruses of which the nucleotide sequence of the 3A coding region was determined in this study.

N/A Not Available