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The 3A non-structural-protein coding region of the southern African SAT type isolates differs from that of other foot-and-mouth disease viruses

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ABSTRACT

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The 3A non-structural protein of foot-and-mouth disease viruses is a relatively conserved protein comprising 153 amino acids. Recent studies have demonstrated correlation between mutations in the 3A non-structural-protein-coding region, including a 10-amino acid deletion, and attenuation of the viruses in cattle. Although the 3A coding region of several type A, O and C isolates has previously been described, nucleotide sequence data of the 3A coding region of the South African Types (SAT) 1, 2 and 3 viruses are limited. Therefore, the 3A non-structural-coding region of different SAT serotypes was determined, analysed and compared to that of European, South American and Asian isolates. The 3A regions of the SAT isolates investigated differed markedly from that of types A, O, C and Asia-1, but were similar within the group.

Keywords: Foot-and-mouth disease virus, picornavirus, SAT types, 3A non-structural protein

INTRODUCTION

Foot-and-mouth disease (FMD), a highly contagious viral disease affecting cloven-hoofed animals, is characterized 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 due to severe marketing restrictions and strict quarantine measures set in place to control the disease (Shahan 1962; Mahul & Durand 1999; Perry, Kalpravidh, Coleman, Horst, McDermott, Randolph & Gleeson 1999; Yang, Chu, Chung & Sung 1999; Prempeh, Smith & Muller 2001). Foot-and-mouth disease virus (FMDV) is a positive sense RNA virus within the *Aphthovirus* genus of the family *Picornaviridae*. There are seven recognized 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 usually responsible for outbreaks of the disease in the region (Thomson 1994). In addition to the SAT types, serotypes A, O and C are occasionally associated with outbreaks in Africa, with Asia-1 being the only FMDV serotype not reported from the continent. Owing to the genetic instability of FMDV, 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 results 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, which is

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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, Pisani, 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 1920s and 1930s respectively, in which pigs were predominantly affected. Cattle were almost completely unaffected in these outbreaks, and it was shown experimentally that the virus isolates were readily transmitted from pig to pig, but not from pigs to cattle. More recently, during 1997, four million pigs were slaughtered in order to contain an outbreak of FMD in Taiwan (Shieh 1997; Yang *et al.* 1999). The outbreak was caused by a strictly porcinophilic phenotype of serotype O virus (O_1Taiwan), 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 signs following needle infection of cattle (Dunn & Donaldson 1997).

Genetic characterization 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). The 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 (O_1C-O/E and C_3R-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 those of the O_1Taiwan isolate. A subsequent study on FMD viruses from the Southeast Asia region (Knowles, Davies, Henry, O'Donnel, Pacheco & Mason 2001) detected 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 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 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*) throughout sub-Saharan Africa are persistently infected with FMDV without exhibiting any of the classical signs 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 characterized by the development of clinical signs that vary in severity. In contrast to the persistent infection in buffalo, there is no clear evidence that the virus is maintained in 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 the 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 region of the SAT type viruses have not been investigated. The nucleotide sequences of the 3A non-structural-protein sequence of several African FMDV isolates obtained from different animal species were therefore determined and compared. 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 regions of the SAT isolates differed markedly from that of types A, O, C and Asia-1, but were closely related among the SAT types.

METHODS

Origin of the viruses

FMDV isolates, representative of all seven serotypes and originating from diverse geographical and host sources, were selected (Table 1). The isolates indicated with asterisks in the table were used to generate additional nucleotide sequence data for this study. Where possible, isolations of the virus grown in primary pig kidney cell cultures were used for direct RNA extraction. In all cases where primary isolates were not available, low passage cell culture

TABLE 1 List of FMD viruses used in comparative studies of the 3A non-structural-protein-coding region

Virus	Country	Year	Species of isolation	Passage origin	Reference history
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 $IB\text{-}RS_2^{\#}$	AF335014
KEN/1/76/A*	Kenya	1976	Bovine	BTY_1 $IB\text{-}RS_2$	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	BTY_1 BHK_2 $IB\text{-}RS_1$	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 $IB\text{-}RS_2$	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 $IB\text{-}RS_1$	AF335010
ZAM/18/96/1*	Zambia	1996	Buffalo	N/A	AF335012
Serotype SAT2					
KEN/3/57/2	Kenya	1957	Bovine	N/A	AV0006
KEN/8/99/2*	Kenya	1999	Bovine	BTY_2 $IB\text{-}RS_4$	AF335008
ZAM/10/96/2*	Zambia	1996	Buffalo	BTY_2 $IB\text{-}RS_4$	AF335013
Serotype SAT3					
KNP/10/90/3*	South Africa	1990	Buffalo	PK_1 $IB\text{-}RS_3$	AF335009

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

N/A Not available

BTY Primary bovine thyroid cells

IB-RS Pig kidney cell line (Instituto Biologica Ruin Suino)

\# Number indicates passage level

BHK Baby hamster kidney cells clone 13

PK Primary pig kidney cells

samples were used. The complete passage history of each virus used is summarized in Table 1.

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 synthesis. 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 1999).

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 50 µl volume in the presence of 0.15 mM dNTPs, 30 pmol sense oligonucleotide (P444 = 5'-GGCCGTTGAAATGAAGAGA-3'), 30 pmol 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 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s and extension at 72 °C for 90 s were performed.

PCR product purification and sequencing

The size of amplified fragments was estimated against a DNA molecular weight marker (*Hin*dIII restricted X174 DNA, Promega) on a 1.5 % agarose gel. Bands of the expected size (860 bp) were excised from the gel and purified by means of the Nucleospin Extract 2 in 1 DNA extraction kit (Mac-Herey-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 programme (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 1 000 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 that occur 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 eleven 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 seven 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 greatest 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 worth noting that the presence of a-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. The 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 programme, 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.

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 neighbour-joining methods when analysing the nucleotide sequence data (results not shown). A neighbour-joining tree based on the alignment of 3A nucleotide sequence data is depicted 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 that 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 and 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 bootstrap value of 100%. The other significant groupings were O_1Campos with O_1C-E/O, the virus that was derived by passaging O_1Campos through eggs, and O_1Kaufbeuren and C_3Resende and its egg-derived strain C_3R-E/O.

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 about this protein in the SAT-type viruses is limited and prompted this investigation into the comparative characteristics of the protein among different SAT-type viruses that originated 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), considered 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 those 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 to 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 at 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

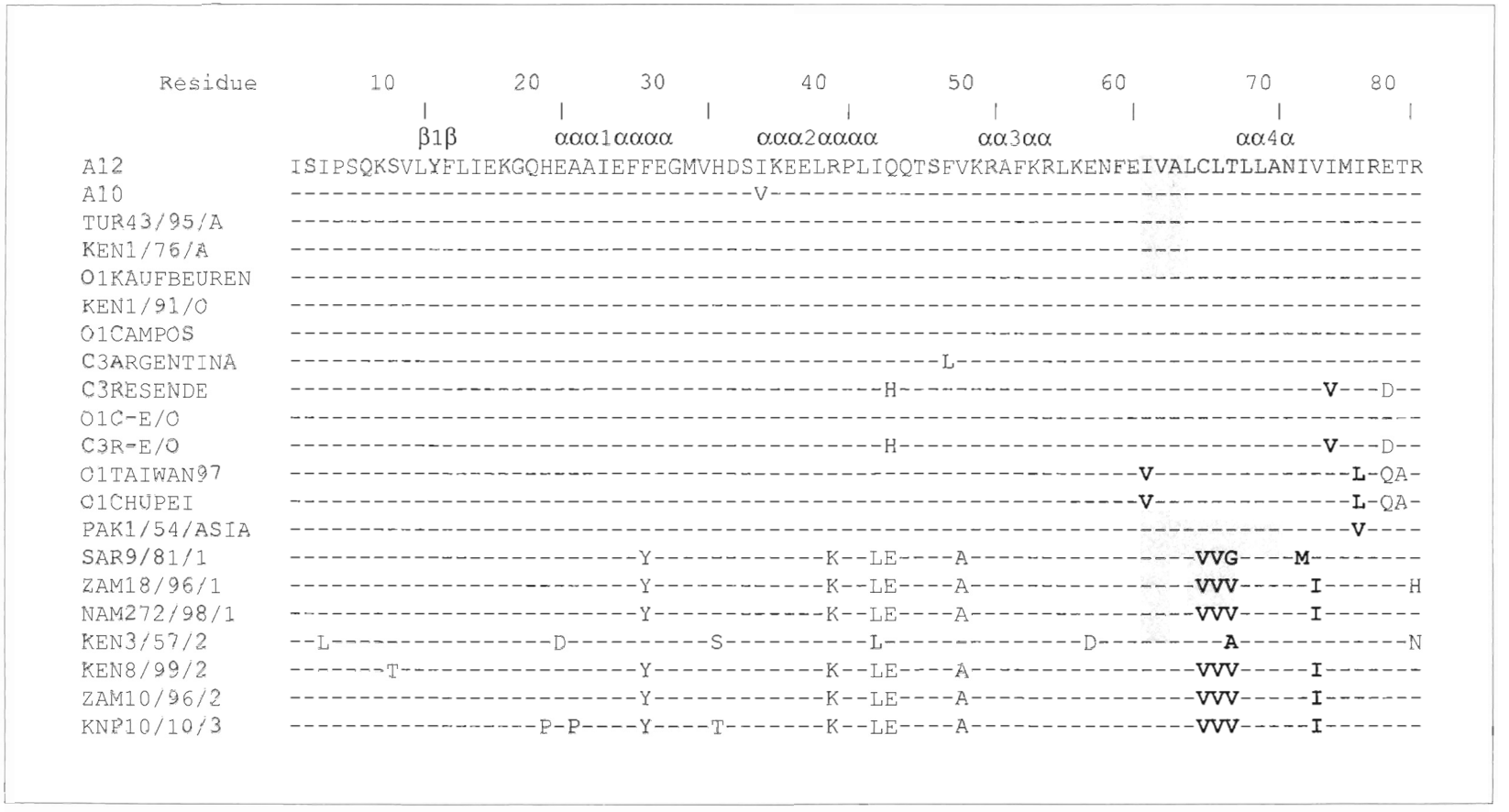

FIG. 1A Multiple alignment of the predicted amino acid sequence (residues 1–80) of 3A non-structural protein of the FMDV isolates used in the study. Secondary structural motifs as predicted with the Garnier, Gibrat and DPM algorithms are indicated with α and β. The hydrophobic domain characteristic of all picornaviruses is shaded in grey. The bold residues within the highlighted area indicate specific amino acid substitutions

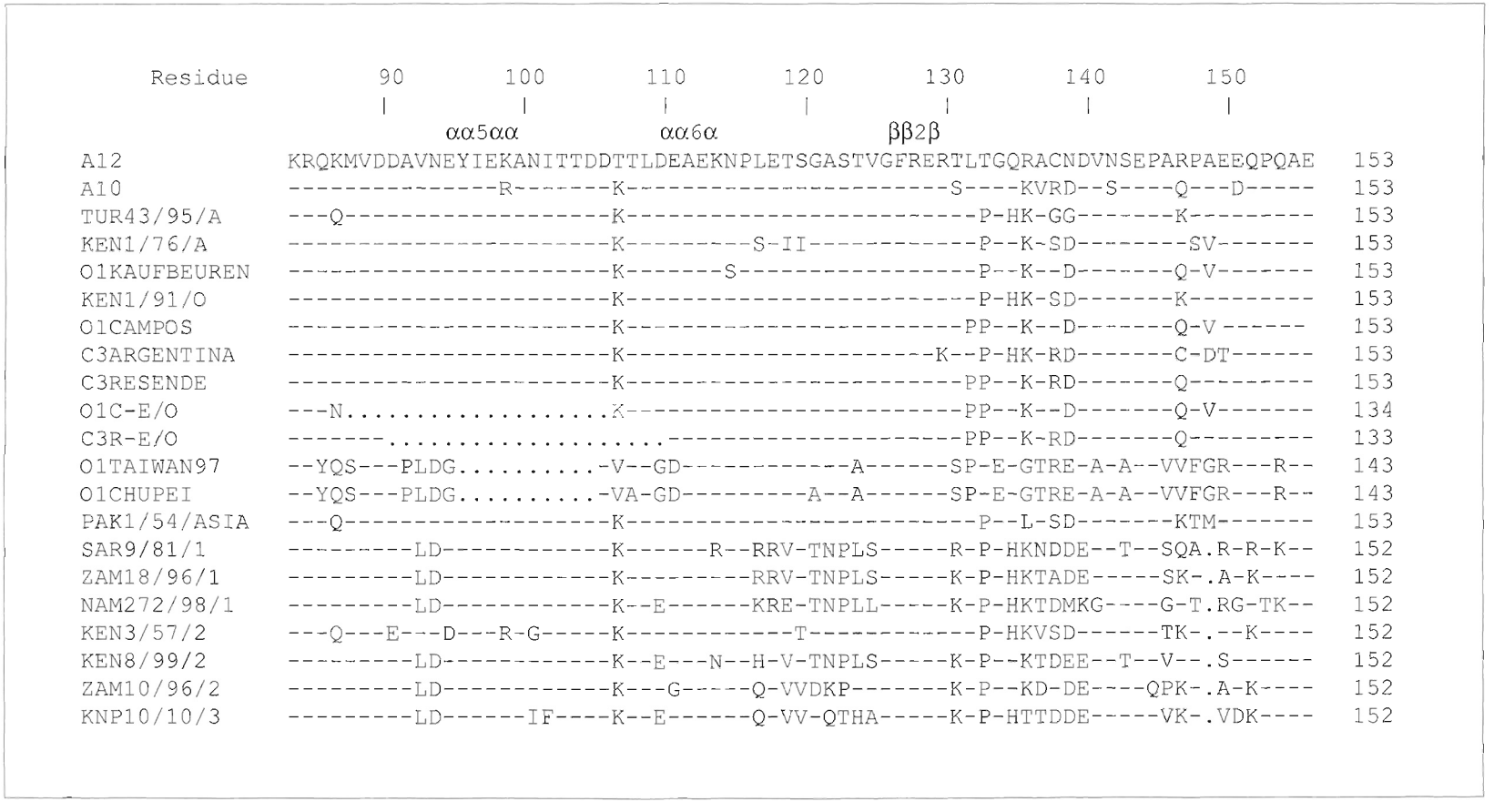

FIG. 1B Multiple alignment of the predicted amino acid sequence (residues 81–153) of 3A non-structural protein of the FMDV isolates investigated in the study. Secondary structural motifs as predicted with the Garnier, Gibrat and DPM algorithms are indicated with α and β. Deletions are highlighted by the shaded boxes

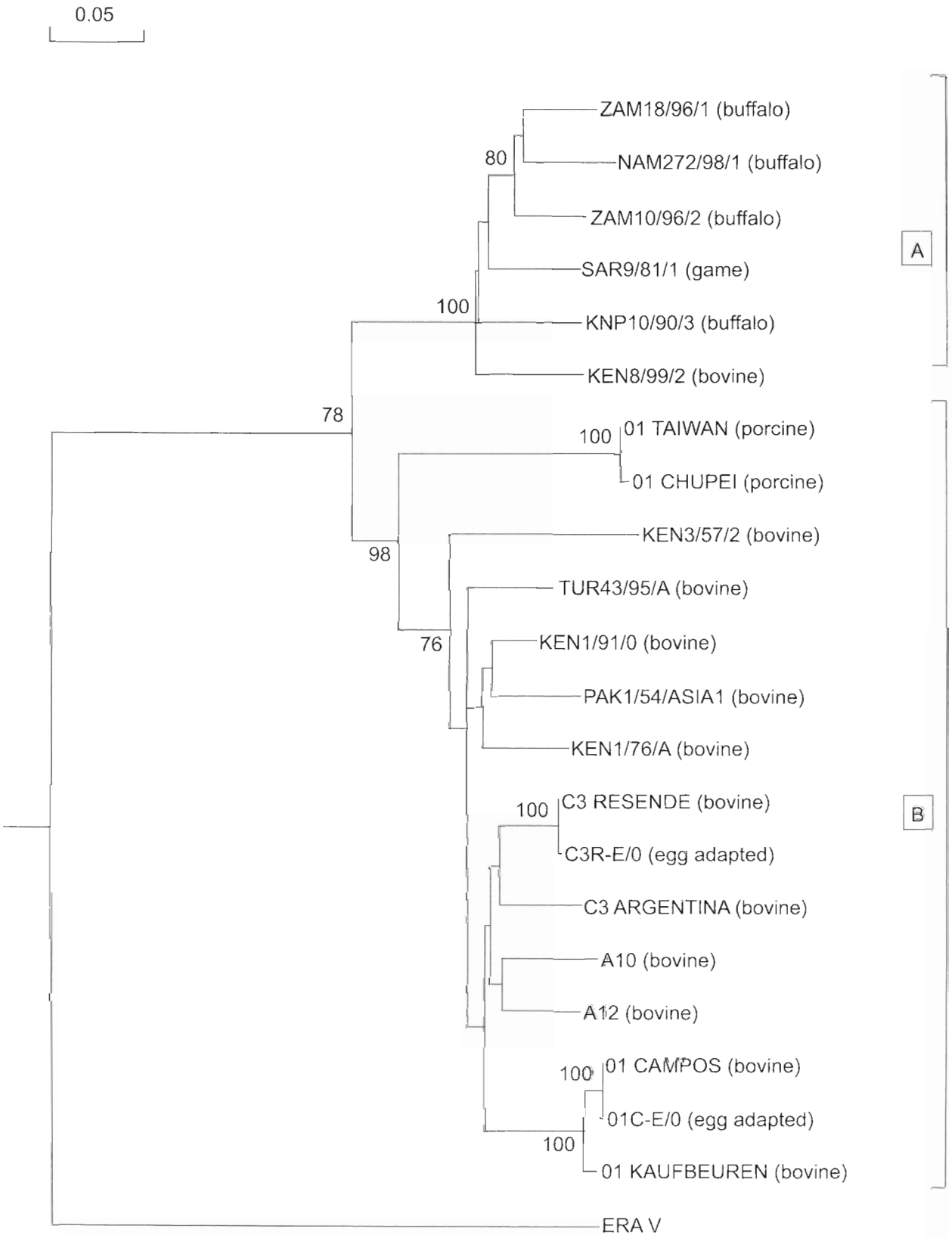

FIG. 2 A neighbour-joining tree based on the nucleotide sequence of the 3A non-structural-protein coding region, depicting the relationship of FMDV isolates originating from diverse geographical and host origins. Equine Rhinitis virus A (ERAV) was selected as appropriate outgroup. A bootstrap trial number of 1000 was applied, and significant branches of > 75 % are indicated. The different FMDV subgroups are indicated in brackets

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-protein 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 parts of Africa need to be investigated to determine whether the 3A genes of SAT isolates from certain regions on the continent are more similar to each other than to those from other regions. This study is the first to investigate the genetic characteristics of the 3A non-structural-protein-coding region of SAT type FMDV 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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