Molecular epidemiology and diagnosis of SAT-type foot-and-mouth disease in southern Africa

By

Armanda Duarte Slager-Bastos

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Armanda Duarte Slager-Bastos

Supervisor Prof. L.H. Nel
Department of Microbiology and Plant Pathology
University of Pretoria

Co-supervisor Dr. G.R. Thomson
ARC-Onderstepoort Veterinary Institute

Degree PhD

Summary

Foot-and-mouth disease (FMD) is an economically devastating picornaviral disease affecting over 40 species of cloven-hoofed animals. The virus occurs as seven immunologically distinct serotypes which are characterized by high levels of intra- and intertypic variation. The three South African Territories (SAT) serotypes 1-3 are endemic to sub-Saharan Africa, a region where the epidemiology of the disease is particularly complex due to the presence of six of the seven serotypes, the role of wildlife in virus maintenance and the apparently higher levels of variation in the endemic serotypes. These factors make it imperative to establish methods suited to elucidating the regional epidemiology. One of the integral parts of this process is the genetic characterization of regionally representative viruses in order to assess the variation in the field and to clarify the role of wildlife. Nucleotide sequence data and methods suited to studying the SAT-types are however limited. A first priority was therefore to establish a PCR-based nucleotide sequencing technique targeting the highly immunogenic and phylogenetically informative 1D genome region encoding the VP1 protein. The screening of multiple serotypes and subtypes prevalent on the African continent confirmed that this method was robust and well-suited to molecular epidemiological studies in the southern Africa region. The method was first applied in the characterization of FMD virus recovered from the reproductive tract of free-living

African buffalo in the Kruger National Park. Nucleotide sequencing assisted in authentication of the results and indicated that carrier status was likely, but it was not possible to unequivocally demonstrate persistent infection of FMDV. In a separate study, the role of impala antelope (*Aepyceros melampus*) in the epidemiology of the disease in South Africa was assessed. Genetic characterization of impala and African buffalo (*Syncerus caffer*) viruses collected over an eleven year period confirmed that inter-species transmission occurred on several occasions and that virus can persist in impala populations for more than 12 months. Inter-species transmission and investigation of the possible mechanisms facilitating virus transmission from persistently infected buffalo focussed on the Kruger National Park in South Africa. In order to ensure regional relevance the study was broadened to incorporate buffalo populations throughout southern Africa. Viruses of the three SAT-types recovered from diverse African buffalo populations were therefore characterized. The results reveal that independently evolving viral lineages occur in distinct geographical regions for each of the SAT-types examined and that the levels of intratypic variation are in the order of 52 - 55 % on nucleotide level across the genome region characterized. Given the strict locality-specific grouping of buffalo viruses the likely usefulness of this database for tracing the origin and course of contemporary and historical SAT-type outbreaks was investigated. Molecular epidemiological studies conclusively show that buffalo are indeed the ultimate source of infection for susceptible cloven-hoofed animals occurring in close proximity, that interspecies transmission occurs between cattle and antelope and that trans-boundary transmission of virus remains a threat to disease security in southern African countries.

SCIENTIFIC PRESENTATION OF RESULTS

Scientific publications emanating directly from this thesis

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.S**, Bertschinger, H.J., Cordel, C., van Vuuren, C. de W.J., Keet, D., Bengis, R.G., Grobler, D.G. & Thomson, G.R. 1999. The possibility of sexual transmission of foot-and-mouth disease from African buffalo to cattle. *Veterinary Record* **145**: 77-79.

3. **Bastos, A.D.S.**, 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.

4. **Bastos, A.D.S**, Haydon, D.T., Forsberg, R., Knowles, N.J., Anderson, E.C., Bengis, R.G., Nel, L.H. & Thomson, G.R., 2001. Genetic heterogeneity of SAT-1 type foot-and-mouth disease viruses in southern Africa. *Archives of Virology (In press)*

Scientific publications incorporating data from this thesis

1. Keet, D.F., Hunter, P., Bengis, R.G., **Bastos, A.** & Thomson, G.R., 1996. The 1992 foot-and-mouth disease epizootic in the Kruger National Park. *Journal of the SA Veteterinary Association* **67**: 83-87

2. Haydon, D.T., **Bastos, A.D.**, Knowles, N.J. & Samuel, A.R., 2001. Evidence for positive selection in foot-and-mouth disease virus capsid genes from field isolates. *Genetics* **157**: 7-15

3. Vosloo, W., **Bastos, A.D.**, Michel, A. & Thomson, G.R. 2001. Tracing movement of African buffalo in southern Africa. *O.I.E. Scientific and Technical Review*, **20** (*In press*)

Publication of results in conference proceedings / as un-refereed papers

1. Hunter, P., **Bastos, A.D**.*, Esterhuysen, J.J. & van Vuuren, C. de W.J., 1996. Appropriate foot-and-mouth disease vaccines for southern Africa. *All Africa Conference on Animal Agriculture, Pretoria, South Africa*, **2.2.7**: 1-4

2. **Bastos, A.D.**, 1997. Detection and characterization of foot-and-mouth disease virus in southern Africa. *African Virology Association Newsletter*. **1**: 7

3. Klemp, E., **Bastos, A.S.**, Vosloo, W., Krige, J. & Thomson, G.R.*, 1998. South Africa: Coping with disease emergencies. *FAO/IAEA/OUA/SADC Subregional workshop on: Emergency preparedness against rinderpest and other trans-boundary animal diseases in southern Africa, Harare, Zimbabwe*: 1-6

3. Thomson, G.R.*, **Bastos, A.S.** & Penrith, M.L., 1998. Proposal for a regional commission for the control of foot-and-mouth disease and other trans-boundary diseases. *FAO/IAEA/OUA/SADC Subregional workshop on: Emergency preparedness against rinderpest and other trans-boundary animal diseases in southern Africa, Harare, Zimbabwe*: 1-9

5. Thomson, G.R., Vosloo, W.* & **Bastos, A.D.S.**, 2000. Foot-and-mouth disease in southern Africa: Re-evaluation of the approach to control. *Proceedings of the 11th symposium on diagnosis and control of transboundary infectious diseases in southern Africa, Utrecht, The Netherlands*: 7-11

6. **Bastos, A.D.S.*** & Sangare, O. 2001. Geographic distribution of SAT-2 type foot-and-mouth disease virus genotypes in Africa. *Proceedings of the southern African Society for Veterinary Epidemiology and Preventive Medicine, 10-11 May, Onderstepoort, South Africa*: 20-26.

Presentation of results at international scientific meetings

1. **Bastos, A.D.*** & Thomson, G.R.. 1995. Genetic variation of SAT-2 isolates of foot-and-mouth disease virus: Implications for phylogenetic reconstruction. *Vth International Congress on the Impact of Viral Diseases on the Developing World, Johannesburg, South Africa* - Talk

2. Hunter, P., **Bastos, A.D.***, Esterhuysen, J.J. & van Vuuren, C. de W.J.. 1996. Appropriate foot-and-mouth disease vaccines for southern Africa. *All Africa Conference on Animal Agriculture, Pretoria, South Africa* - Talk

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4. **Bastos, A.D.S.***, Boshoff, C.I., Esterhuysen, J.J., Anderson, E. & Thomson, G.R. 1998. Topotype variation in SAT1 foot-and-mouth disease viruses in Zimbabwe. *Xth Meeting of the European Study Group on the Molecular Biology of Picornaviruses, Jena, Germany* - Poster

5. **Bastos, A.D.S.***, Esterhuysen, J.J., Forsberg, R., Bengis, R.G., Anderson, E.C. & Thomson, G.R. 1998. VP1 gene variation of SAT 1 type foot-and-mouth disease virus in southern Africa. *Xth Meeting of the European Study Group on the Molecular Biology of Picornaviruses, Jena, Germany* - Poster

6. Vosloo, W.*, **Bastos, A.D.S.** & Knowles, N.J.. 1998. Phylogenetic analysis of historical foot-and-mouth disease virus isolates obtained from southern Africa between 1948 and 1989. *Xth Meeting of the European Study Group on the Molecular Biology of Picornaviruses, Jena, Germany* - Poster

Presentation of results at national scientific meetings

1. **Bastos, A.D.*** & Esterhuysen, J.J.. 1996. Molecular Epidemiology of foot-and-mouth disease virus in southern Africa. *Ninth biennial congress of the South African Society for Microbiology, Pretoria, South Africa* - Talk

2. **Bastos, A.D.*** 1996. Development of a serotype-specific PCR for diagnosis of foot-and-mouth disease virus in southern Africa. *Ninth biennial congress of the South African Society for Microbiology, Pretoria, South Africa* - Poster

3. **Bastos, A.D.S.***, Esterhuysen, J.J., Boshoff, C.I., Nel, L. & Thomson, G.R.. 1998. The use of VP1 gene sequences for studying the epidemiology of foot-and-mouth disease virus in southern Africa. *Congress of the South African Society of Biochemistry and Molecular Biology, Potchefstroom, South Africa* - Talk

4. **Bastos, A.D.S.*** 1998. Improved detection of all seven serotypes of foot-and-mouth disease virus by nested PCR. *Congress of the South African Society of Biochemistry and Molecular Biology, Potchefstroom, South Africa* - Poster

* Indicates presenting author

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aa	amino acid
ANG	Angola
BEC	Becuanaland
BOT	Botswana
BUN	Burundi
BVI	Botswana Vaccine Institute
bp	base pairs
CD	Corridor disease
CPE	cytopathic effect
ERI	Eritrea
FMD	foot-and-mouth disease virus
ICTV	International Committee for the Taxonomy of Viruses
KEN	Kenya
KNP	Kruger National Park
MAL	Malawi
MOZ	Mozambique
NAM	Namibia
NCR	non-coding region
nt	nucleotide
OIE	Office International des Epizooties
OP	oesophageo-pharyngeal
OVI	Onderstepoort Veterinary Institute
PAL	Phalaborwa
PCR	polymerase chain reaction
PFU	plaque forming units
p.i.	post-infection
POT	Potgietersrus
RHO	Rhodesia
RWA	Rwanda
SAR	South African Republic
SAT	South African Territories
SAU	Saudi Arabia
SWA	South West Africa
SWL	Swaziland
TAN	Tanzania
TB	tuberculosis
UGA	Uganda
VP	Virus protein
WRL	World Reference Laboratory
ZAI	Zaire
ZAM	Zambia
ZIM	Zimbabwe

Chapter 1

Literature Review

1.1 Introduction

In the late 1800's numerous researchers described the existence of pathogens that were able to pass through bacteria-proof filters. This included observations by Pasteur on the rabies-causing pathogen in 1887, Ivanovsky's studies of tobacco mosaic disease in 1886 and the ability of the foot-and-mouth disease agent to pass through Berkfeld filters, as documented by Loeffler and Frosch in 1898. These researchers all concurred with the opinion of Pasteur, who believed that microbes were the causative agents of these virulent affections. It was Martinus Beijerinck who described the infectious pathogens as a *contagium vivum fluidum* in 1898. He was the first to propose that these were self-reproducing, sub-cellular entities that did not conform with features characteristic to bacteria in that these microscopically invisible organisms were filterable, inactivated by heat, and could not be cultured *in vitro*. Beijerinck's revolutionary ideas were strongly opposed, but subsequently obtained support from numerous independent studies on novel pathogens and eventually led to the establishment of a new branch of biological science called virology. Nucleic acid made its entrance into virus research in 1934 and the development of the electron microscope in the 1930's permitted the first visualization of these previously invisible pathogens. The study of viruses and ease with which they could be manipulated paved the way for the numerous discoveries in the field of molecular biology. The superficially simple, yet inherently variable and adaptive nature of these entities and their power to shape history ensures that virology will remain a perpetually relevant and dynamic discipline.

1.2 Picornavirus Taxonomy

Virus classification is the area of virology that best reflects the dynamic nature of this field. The International Committee on Taxonomy of Viruses (ICTV) was established in 1966 to address the need for a single, universal taxonomic scheme for viruses. Taxonomic status of known as well as newly discovered viruses is constantly being reviewed by this body (Wildy, 1971; Fenner, 1976; Matthews, 1979, Matthews, 1982; Francki *et al.* 1991; Murphy *et al.* 1995; van Regenmortel *et al.*

2000; Fauquet & Mayo 2001) due to the rapid accumulation of data and discovery of new viruses. In the 6th ICTV report (Murphy *et al*. 1995) 1 order, 50 families and 164 genera were recognized, by 1998 and interim review on virus taxonomy (Mayo & Pringle 1998) identified 2 orders, 54 families and 184 genera. Just 2 years later, in the 7th ICTV report 3 orders, 63 families and 240 genera are recognized (van Regenmortel *et al*. 2000).

1.2.1 Picornavirus genera

Foot-and-mouth disease virus (FMDV) belongs to the picornavirus family, a diverse group of non-enveloped, small RNA viruses which include economically important pathogens of humans and animals. The family name *picornaviridae,* is derived from '*pico*' referring to their small size and '*rna*' referring to their RNA genomes. On the basis of genome size and organization, virus replication strategy and sequence homologies, the family is divided into six genera comprising *cardiovirus, aphthovirus, rhinovirus, hepatovirus, enterovirus* and *parechovirus* (Table 1.1). These genera are further divided into antigenically distinct 'serotypes', a virological category equivalent to the 'species' taxonomic unit. Common properties within a serotype or species include sequence homologies, serological relationships, host range, pathogenicity and geographical distribution. In accordance with the ICTV accepted definition of a species, '*a virus species is defined as a polythetic class of viruses that constitutes a replicating lineage and occupies a particular ecological niche*' (Van Regenmortel 1990). Members of a polythetic class should have one or more properties of the group, but there is no unique group characteristic which excludes or defines membership. Due to the complexity and variability of viruses and the importance of the species hierarchical level it is generally agreed that virus families and genera are best described monothetically (by a few defining characters), whilst species are best defined polythetically (Van Regenmortel 1990). The ICTV does not classify viruses below the level of species.

Most picornaviruses are specific to one or a handful of host species, with the exception of *encephalymyocarditis virus* (EMCV), which has been isolated from over 30 species, and FMDV which is believed to be capable of infecting around 200 mammal species. Viral transmission is usually horizontal and does not involve arthropod vectors, although EMCV has been isolated from mosquito and tick species. Picornavirus genera vary vastly in the number of member serotypes. Some genera are monotypic, eg. *Parechovirus*, whilst others

have over 100 distinct species (summarized in Table 1.1).

TABLE 1.1 Summary of the assigned *picornaviridae* genera and their species composition

Genus	**Total number of species per genus**	**Virus name**	**No of species / serotypes per virus type**
Aphthovirus	8	*foot-and-mouth disease virus*	7
		equine rhinitis A virus (formerly *equine rhinovirus type* 1)	1
Cardiovirus	2	*encephalomyocarditis virus*	1
		Theiler's murine encephalomyelitis virus	1
Enterovirus	98	*bovine enterovirus*	2
		coxsackievirus A	23
		coxsackievirus B	6
		echovirus	30
		human enterovirus	4
		human poliovirus	3
		porcine enterovirus	11
		simian enterovirus	18
		Vilyuisk virus	1
Hepatovirus	2	*hepatitis A virus*	1
		simian hepatitis A virus	1
Parechovirus	1	*human parechovirus type* 1 (formerly *human echovirus type 22*)	1
Rhinovirus	104	*bovine rhinovirus*	3
		human rhinovirus	101
		TOTAL	213

Sources: Murphy *et al.* 1995; Fauquet & Mayo 2001

1.2.2 Picornavirus morphology

Picornavirus virions are icosahedral with no envelope and contain one molecule of infectious, positive sense, single stranded RNA (ssRNA), ranging from 7-8.5 kb in length. A small protein Vpg which is encoded by the 3B genome region is covalently linked to the 5' end of the genome and a poly (A) tract of variable length is located at the 3' terminus. The 25-35 nm capsid is composed of 60 protein subunits or protomers, each consisting of four proteins, VP1-VP4 (Fig. 1.1). VP1-VP3 are exposed on the surface, whilst VP4 is located internally at the pentameric apex of the icosahedron and contains a myristic acid molecule attached to the amino terminal glycine (Chow *et al.* 1987; Minor *et al.* 1995). VP1, VP2 and VP3 share an eight-stranded antiparallel ß-barrel configuration and differ from each other primarily in the loops connecting these beta-barrel segments (Palmenberg 1989). None of the viral proteins are glycosylated and with the exception of *poliovirus*, the virions generally lack lipids.

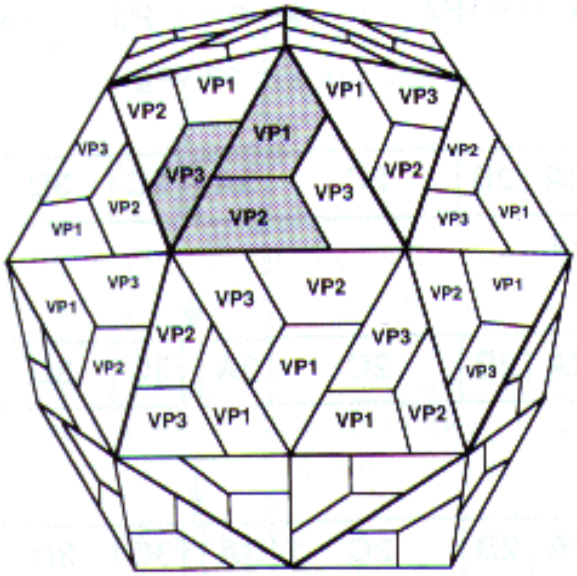

Fig. 1.1 Diagram of the typical picornavirus icosahedral capsid showing the surface exposed VP1, VP3 and VP2 proteins. One of the 60 protomers, each of which contain an internalized VP4 protein is shaded. The pentameric apex about which the VP4 proteins are internally located, and the VP1 proteins are externally clustered, is indicated with a red arrow. Figure from Murphy *et al.* 1995.

1.2.3 Picornavirus genome organization and protein processing

Due to confusion arising from the naming of picornaviral proteins according to the molecular weight of the protein, the so-called L434 convention was adopted at the Europic `84 meeting held at Urbino, Italy to ensure universality of systematic nomenclature of viral proteins (Rueckert & Wimmer 1984). In accordance with the L434 diagram, L represents the leader protein, followed by the P1 region, which encodes four products A-D, which ultimately generate the VP1-4 coat proteins. Similarly, the P2 and P3 regions comprising three (A-C) and four (A-D) products respectively encode the non-structural proteins. FMDV differs from other picornaviruses in that it has 3 non-identical forms of Vpg encoded in the 3B genome region (Fig. 1.2). *Cardiovirus* and *aphthovirus* are the only picornavirus genera that have the L protein or leader proteinase. There are two in-frame translation initiation sites, resulting in two forms of the L protein, termed Lab and Lb in FMDV.

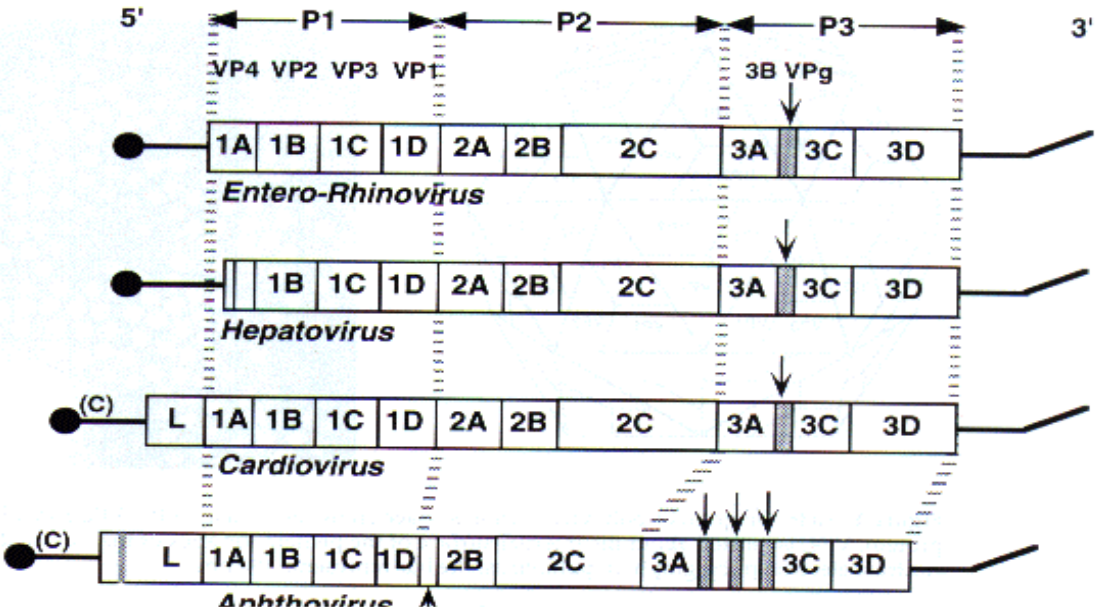

Fig. 1.2 Genome organization of the *picornavirus* genera. Figure from Murphy *et al.* 1995.

1.3 *Foot-and-mouth disease virus*

FMDV is one of two members of the *aphthovirus* genus. The genus name is derived from the Greek word *aphtha* meaning 'vesicles in the mouth' and refers to the historical association of '*aphthovirus*' with foot-and-mouth disease (FMD) alone. Clinically FMD generally manifests as vesicles in the mouth and in the interdigital spaces and coronary bands of the hooves. Mortality in lambs, piglets and calves has been a major feature of some outbreaks. However, in older animals it rarely causes mortality. Virions are unstable below pH 6.5 and above pH 9, and have a buoyant density of 1.43-1.54 g/ cm^3 in CsCl. Seven immunologically distinct serotypes occur, namely types A, O and C, SAT types 1-3 and Asia-1. The classical, or European serotypes A, O and C were identified in the 1920's (Vallée & Carré 1922; Waldmann & Trautwein 1926). Recognition and typing of endemic African strains, the so-called SAT (South African Territories) types only commenced in 1948 (Brooksby 1958) and it was only in 1957 that a seventh serotype, Asia-1, was identified (Brooksby 1957). The different serotypes or FMD virus species differ genetically from each other by more than 50 % across the entire genome (Palmenberg 1989).

1.3.1 FMDV genome organization

FMDV has a single-stranded, positive sense RNA genome which is approximately 8400 bases long, and consists of a 5' non-coding region (NCR), a single large open reading frame and a short 3' NCR. It is polyadenylated on the 3' end and has a small virus encoded protein, Vpg covalently attached to the 5' terminus (Belsham 1993). The 5' NCR of FMDV is about 1300 nucleotides (nt) long, and far exceeds the length of other picornaviruses and typical cellular mRNAs which have 5'NCRs of 740nt and 50-100nt respectively. The first portion of the 5' NCR is termed the S fragment and is approximately 400 nt long. This is followed by the poly C tract, a homopolymeric tract of predominantly cytidyl residues which is 150-250nt long and which only occurs in *cardioviruses* and *aphthoviruses*. The last region of approximately 720 nt contains inverted repeats which are predicted to form pseudo-knots (Clarke *et al.* 1987). The internal ribosome entry site (IRES) which is immediately upstream of the first AUG initiation codon and is approximately 435 nt in length also occurs within this region (Belsham & Brangwyn 1990; Kuhn *et al.* 1990). The major portion of the FMDV genome consists of a single large open reading frame of 6996 nt encoding a polyprotein of 2332 amino acids (type O, Forss *et al.* 1984). Four distinct regions are distinguished for the polyprotein, namely the L, P1, P2 and P3. Another characteristic unique to FMDV is that there are three species of Vpg encoded by protein 3B, termed 3B1, 3B2 and 3B3. All encoded Vpg variants have been shown to be attached to the 5'

terminus of viral RNA (King *et al.* 1980).

1.3.2 Proteolytic processing of FMDV

The first protein processing event in FMDV is the L/P1 cleavage. Aphthovirus L proteinase cleaves co-translationally at its own C terminus and exists in at least two forms, Lab^{pro} and Lb^{pro}, derived from initiation of translation at either of two in-frame AUG codons (Sangar *et al.* 1987). Both forms are able to cleave the L/P1 junction either in *cis* or in *trans* (Medina *et al*. 1993). Site-directed mutagenesis indicates that the L proteinases are thiol proteinases with the active site Cys^{51} being conserved amongst FMDV and *equine rhinitis virus* (ERV) sequences (Piccone *et al.* 1995; Roberts & Belsham 1995). L^{pro} also cleaves host-cell protein eIF-4G in *trans*, a feature it shares with $2A^{pro}$. This is a striking example of convergent evolution as the latter proteinase, although performing the same function, more closely resembles the small serine proteinase sub-class and has a different eIF-4G cleavage site to L^{pro} (Kirchweger *et al.* 1994).

The second cleavage event occurs at the 2A/2B junction, and requires neither the L nor the 3C proteinases. It was shown that a 20 amino acid oligopeptide sequence corresponding to the FMDV 2A region and the N-terminal proline of 2B was able to mediate a co-translational cleavage (Ryan *et al.* 1991; Ryan & Drew 1994) and probably requires the three C-terminal residues (-NPG-) of 2A and the N-terminal residue (proline) of the 2B protein. These residues which are completely conserved are believed to be involved in 2A/2B cleavage do not conform to any known proteinase motifs. *Aphthovirus* polyprotein cleavage occurs between the C terminus of the 2A region and the capsid protein precursor (P1-2A), rather than between P1 and 2A as is the case with most other picornaviruses.

The 3C protease mediates P1 cleavage to produce 1AB (VP0), 1C (VP3) and 1D (VP1). After post-translational processing of the P1-2A, a myristate moeity is attached to the N-terminus of 1AB which appears to be critical for capsid stability (Chow *et al.* 1987). Cleavage of the capsid protein precursor, 1AB, encoding the VP0 protein (VP4 + VP2) only occurs on encapsidation of the virion RNA to form virus particles. This step is often referred to as ‘maturation cleavage’ is it is only at this stage that the four-chain subunits (VP1-VP4) are formed.

1.3.3 FMDV morphology

Amino acid alignment of FMDV with other picornaviruses, indicates that the loop regions connecting the beta-barrels in the VP1 and VP2 genes of foot-and-mouth disease virus are truncated (Palmenberg 1989), particularly in the regions contributing to the pentameric apex of the virion, where poliovirus and rhinovirus have major antigenic sites (Minor 1990). The atomic stucture of serotype O_1 BFS1860 (Acharya *et al.* 1989; Acharya *et al.* 1990) also indicates there is no pit or canyon in FMDV as is observed for other picornavirus genera capsids. There are two disordered regions within the VP1 gene that are exposed on the surface. The first known as the G-H loop extends from residues 133 to 158, and the second from residues 210-213 of serotype O viruses (Acharya *et al.* 1990; Logan *et al.* 1993). The C-terminal residues of VP1 within which this disordered region occurs runs clockwise over the virion surface from VP1, over VP3 to VP1 in the adjacent protomer where it lies close to the G-H loop. These regions correspond to those previously shown to be able to induce neutralising antibodies (Bittle *et al.* 1982; Strohmaier *et al.* 1982). Peptides corresponding to the VP1 gene G-H region of other serotypes inclusive of type A, O, C and SAT-2 strains were also shown to induce neutralizing antibodies to the homologous strain (Clarke *et al.* 1983). Disuphide bridges between the protomers of serotype O in which Cys-130 of VP2 is linked to Cys-134 of VP1 are known to occur in type O viruses, but not in serotype A viruses. Reduction of disulphide bonds favours a more disordered G-H loop structure (Parry *et al.* 1990).

Intact virus particles contain a single copy of genomic RNA, whilst virions lacking RNA are referred to as empty virus particles. The whole virus particle, empty virus particle and pentameric protein subunits have sedimentation coefficients of 146S, 75S and 12S, respectively. The 146S and 75S fraction elicit an immune response, whereas the presence of 12S protein resulting from disassembly of the virion by mild heat or acid treatment, does not induce neutralizing antibodies. 146S determination is therefore a critical part of vaccine production as it provides a measure of the number of intact virus particles which are able to evoke neutralizing antibody and so confer immunity.

1.3.4 FMDV receptor binding

Cell receptors are important determinants of viral host range and cell and tissue tropism. It is known that trypsin treatment abolishes FMDV infectivity (Brown *et al.* 1963) and reduces immunogenicity (Wild *et al.* 1969) and that the trypsin cleavage site is located within the major neutralization antigenic site in the VP1 gene spanning residues 140-160 (Bittle *et al.* 1982, Strohmaier *et al.* 1982). This portion of VP1 contains a highly conserved Arg-Gly-Asp (RGD) site which binds many extracellular ligands to integrin cell surface receptors (reviewed by Ruoslahti & Pierschbacher 1987). Early studies showed that RGD is important for the interaction of FMD virus with cellular receptors (Baxt & Becker 1990) and that SAT, A, O and C viral subtypes use different cellular receptors (Baxt & Barach 1982, Sekiguchi *et al.* 1982). The use of multiple receptors was recently confirmed when it was shown that FMDV is able to ultilize two distinct types of cells surface receptors, namely the heparan sulphate proteoglycans (HSPG; Jackson *et al*. 1996) and the RGD-dependent integrins, $\alpha_v\beta_3$ (Neff *et al.* 1998), $\alpha_v\beta_1$ (Jackson *et al.* 2000a) and $\alpha_v\beta_3$ (Jackson *et al.* 2000b). The $\alpha_v\beta_3$ and HSPG receptors are associated with virulence and loss of virulence following cell culture adaptation, respectively.

1.4 Genetic variation

The observed genetic variation in the FMD viral genome is the result of a two step process. Firstly, the replication of viral RNA is error-prone due to the absence of proofreading in the 3D-encoded RNA-dependent RNA polymerase. Secondly, competitive selection is continuously acting on the genome. Thus those mutants with a selective advantage in the prevailing environment, will be better represented than those with a selective disadvantage.

1.4.1 Mutation

The different genome regions of European serotype viruses accumulate mutations at between 0.9 x 10^{-2} and 7.4 x 10^{-2} substitutions per nucleotide per year (s/n/y) in persistently infected cattle (Gebauer *et al.* 1988). A study of the VP1 protein coding region of SAT-type viruses indicates that mutations become fixed in this portion of the genome at a rate of between 1.54 x 10^{-2} and 1.64 x 10^{-2} s/n/y in persistently infected African buffalo (Vosloo *et al.* 1996). FMD viral genes therefore evolve around 10^{6} times faster than nuclear eukaryotic genes due to increased misincorporation events, with roughly one substitution error occurring per genome replication cycle. This gives rise to a whole population of phylogenetically related FMD variants within a

single infected individual in accordance with the quasispecies concept (Domingo *et al.* 1992; Domingo *et al.* 1995 and references therein).

1.4.2 Selection

One of the major evolutionary mechanisms employed by RNA viruses is the prolific mutant production, detailed above. The immune system of an infected animal, which presumably provides a powerful selective force is another driving force in viral evolution. Evidence that mutations accumulate in the absence of immune selection (Diez *et al*. 1990) seem to accord with the neutral theory of molecular evolution and there has been much debate on whether FMDV evolution is driven by selection or whether the neutral theory applies.

The high mutation rate of FMDV makes this virus an ideal candidate for examining the molecular clock hypothesis, which is intrinsic in the neutral theory of molecular evolution. According to this theory, mutation rates for a given molecule would be equal for all organisms at all times. In contrast, positive Darwinian evolution would result in some sites within a molecule being more likely to change than others due to selective forces. In the latter case, a clocklike behaviour would not be observed. Two independent studies on field and laboratory strains indicate that selective forces are indeed working at sites within the immunogenic region of the VP1 protein (Fares *et al.* 2001; Haydon *et al.* 2001), although this was not supported by all datasets examined. These results indicate that antigenic variants benefit from a selective advantage in their interaction with antiviral antibodies produced by the host immune system.

1.4.3 Recombination

Recombination is another important process driving viral biology and evolution. In RNA viruses, recombination involves the exchange of genetic material between two nonsegmented RNA genomes resulting from polymerase ‘jumping’ during RNA synthesis. Polymerase jumping between different RNA templates is believed to be facilitated by enzyme pausing at sites of strong secondary structure (Wilson *et al.* 1988) and is consistent with the observation of RNA intermediates and the discontinuous nature of both DNA-dependent and RNA-dependent RNA synthesis in experimental systems (Lai 1992). Homologous recombination in RNA viruses (the exchange of two comparable genome regions) was first recognized in the family *picornaviridae*, in *poliovirus* (Hirst 1962) and FMDV (Pringle 1965) with reports in other RNA virus families,

such as coronavirus, following later (Lai *et al.* 1985).

Intratypic recombination occurs more frequently than intertypic recombination and it appears that recombination events in FMDV occur more readily in the 3' half of the genome, than in the capsid genes of FMDV (King *et al.* 1985; King *et al.* 1988). This is probably due to the higher degree of local secondary structure in the C-terminus half of the genome, promoting polymerase pausing (Wilson *et al.* 1988) and to the higher levels of sequence homology between non-structural genes as opposed to structural genes. It was also shown that recombination can involve single or multiple crossover events when two viruses of the same serotype co-infect cell cultures (King *et al.* 1982). Following demonstrations of intertypic recombination *in vitro* (McCahon *et al.* 1985; Giraudo *et al.* 1988), Krebs and Marquardt (1992) identified and characterised the first type O-C intertypic recombinant, from the field.

Recombination is not a frequent occurrence in most RNA viruses and it is unclear why picornaviruses and coronaviruses have such a high frequency of homologous recombination, but this process clearly provides an evolutionary advantage to these viruses. At its most extreme, recombination can give rise to a novel RNA virus following exchange of genetic material between two completely unrelated viruses in a naturally infected host. An example of this is *western equine encephalitis virus*, which appears to be derived from a crossover event between *Sindbis virus* and *eastern equine encephalitis virus* (Hahn *et al.* 1988). Recombination poses a real threat when attenuated vaccines are used, as reversion to virulence following natural infection of a vaccinated individual is likely given the high recombination frequency in FMDV.

1.5 Antigenic variation

One of the consequences of genetic variation through mutation, selection and recombination is that new antigenic variants are constantly being generated. In contrast to *poliovirus* where antigenic variation within a serotype seems to be of minimal epidemiological importance (Minor 1990), given that the existing vaccine strains function effectively despite being over 40 years old, antigenic variation within *aphthovirus* serotypes is extensive and therefore a critical factor in vaccine strain selection (Rweyemamu & Hingley 1984; Mateu *et al.* 1988). Not only is there no cross-protection between the seven FMDV serotypes (Brooksby 1982), but vaccination with one antigenic variant of a serotype does not necessarily protect an animal when challenged with a different virus of the same

serotype (Cartwright *et al.* 1982). Attempts to characterize the extent of the antigenic variation within a FMDV serotype led to the establishment of techniques whereby viral subtypes could be identified (Brooksby 1968). Initially over 60 different subtypes were identified by the World Reference Laboratory (WRL), but it quickly became apparent that there is a continuous spectrum of intratypic antigenic variants, making it difficult to identify specific subtypes (Pereira 1977). Subtype classification was therefore abandoned. Instead, the practical significance of the r-value was recognized (Rweyemamu *et al.* 1977) and is used as a measure of the suitability of a vaccine strain for a given outbreak situation, where r is:

$$r = \frac{\text{Activity of serum against the heterologous virus (field / outbreak strain)}}{\text{Activity of serum against the homologous virus (vaccine strain)}}$$

Thus a suitable vaccine strain is indicated by an r value greater than 0.4, whilst $r < 0.4$ indicates a poor antigenic relationship between the outbreak and vaccine strain. These cut-offs are based on the good correlation between the serum neutralizing antibody titre and protection from challenge in cattle (Rweyemamu *et al.* 1977).

1.5.1 Neutralizing sites of FMDV

Neutralizing monoclonal antibodies (MAb) have been used to map the antigenic sites involved in neutralization of FMDV. Most studies have focussed on the European serotypes (Robertson *et al.* 1984; Xie *et al.* 1987; Thomas *et al.* 1988; Barnett *et al.* 1989; Baxt *et al.* 1989; Kitson *et al.* 1990; Saiz *et al.* 1991; Crowther *et al.* 1993a; Lea *et al.* 1994; Aktas & Samuel 2000), with limited reports on neutralization sites of the Asia-1 serotype (Butchaiah & Morgan 1997; Marquardt *et al.* 2000) and the SAT-type viruses (Crowther *et al.* 1993b). Detailed antigenic profiles of the SAT-1 and SAT-3 serotypes have not been reported.

TABLE 1.2 Summary of immunogenic sites identified in the surface-exposed structural protein genes of different foot-and-mouth disease virus serotypes

Serotype	Antigenic sites; P1 gene	References
A	G-H loop; VP1 C-terminus region; VP1 H-I loop; VP1 B-C loop; VP1 B-C loop; VP2	Robertson *et al.* 1984 Thomas *et al.* 1988 Baxt *et al.* 1989 Saiz *et al.* 1991
O	G-H loop & C-terminus region; VP1 (Site 1) B-C loop & E-F loop; VP2 (Site 2) B-C loop; VP1 (Site 3) VP3 (Site 4) B-C loop; VP2 B-C loop (VP2) & G-H loop (VP1)	Xie *et al.* 1987 Barnett *et al.* 1989 Kitson *et al.* 1990 Crowther *et al.* 1993a Aktas & Samuel 2000
C	G-H loop; VP1	Lea *et al.* 1994
Asia-1	G-H loop; VP1 B-C-loop; VP2 N-terminus; VP2 B-B knb; vp3	Butchaiah & Morgan 1997 Marquardt *et al.* 2000
SAT-2	G-H loop; VP1 (amino acid positions 149, 156 & 158)	Crowther *et al.* 1993b

Most epitopes mapped to loops connecting the ß-strands of the three surface exposed proteins. All studies confirmed that the major antigenic determinants of FMDV are situated in the VP1 gene, of which the G-H loop is the one of the most important neutralizing sites of the virus (Table 1.1). This region, spanning amino acid positions 140-160, protrudes from the virion surface and is highly exposed and mobile. The loop is critical for the process of receptor binding (via the highly conserved RGD motif) as well as being involved in neutralization of viral infectivity. Despite being a target for neutralizing antibodies, the RGD remains intact and experimental modification of amino acid sites in this region are often lethal (Mason *et al.* 1994; Mateu *et al.* 1996; Leipert *et al.* 1997). However, a serotype C escape mutant shown to contain

an RGG instead of the RGD, was viable in BKH-21 cells and antigenically distinct from the parent strain (Ruiz-Jarabo *et al.* 1999), leading to the conclusion that the mutant could enter the cells via an alternative receptor, other than through the RGD-binding integrin receptor, and that single amino acid replacements within the G-H loop, including the receptor recognition site can have profound effects on antigenicity. This finding supports the view that antigenic variation arises through two mechanisms. Firstly, a gradual increase in antigenic distance can be brought about by a steady accumulation of amino acid replacements within site A (G-H loop) of VP1 (antigenic drift), or alternatively, a rapid change can occur following an amino acid substitution at a critical site in the G-H loop (Martínez *et al.* 1991 and references therein).

1.6 Geographical distribution of FMD

FMDV has an essentially global distribution, with the exception of North America, Western Europe and Australia. The European serotypes occur in South America, the Caucasus, Africa, the Middle East and in Asia. Of these, serotype C occurs infrequently, with most outbreaks being due to serotype O (Kitching 1998 & 1999). Asia-1 is limited to the Asian continent and to the Middle East, whilst the SAT-types occur exclusively in sub-Saharan Africa, although rare incursions into other regions have been recorded. This includes the introduction of SAT-2 for the first time into Saudi Arabia and Kuwait in April and May, respectively, in 2000. Similarly, Asia-1 which has a restricted distribution was recorded for the first time in Greece in July 2000 (http://www.oie.int)

Until 2001, western Europe was free of the disease following intensive vaccination campaigns which ceased in 1991. Threats to disease security are however posed by regular outbreaks of the disease in southern and eastern Europe. These include the 1991, 1993, and 1996 outbreaks in Bulgaria, serotype O in Italy, Greece and Turkey in 1993, 1994 &1996 and 1995 & 1996, respectively. The threat posed to Europe by the close proximity and intensive trade in livestock in the Middle East where types O and A are endemic and where Asia-1 and SAT-2 have also been recorded is well-recognized. Of particular concern is the recent introduction of an exotic FMD serotype, Asia-1, into Greece. More recently, the Pan-Asian serotype O virus responsible for outbreaks throughout the Middle East, south-east Asia and South Africa (Knowles *et al.* 2000), was introduced into Great Britain. Prior to this, the UK had been free of the disease for over 30 years, with the last recorded epizootic occurring in 1967/8.

1.6.1 FMD serotype distribution Africa

FMD is endemic in sub-Saharan Africa, but absent from Madagascar (Kitching 1998). Six of the seven serotypes have been recorded on the continent (Brooksby 1972). Types A and O are widespread throughout sub-Saharan Africa, whilst type C occurs rarely (Fig. 1.3). The endemic SAT-types occur predominantly in southern and eastern Africa. SAT-1 and SAT-2 also circulate in West Africa and are the only serotypes to have made incursions into the Middle East. In North Africa serotype O is endemic in Egypt and Libya, but sporadic outbreaks occur in other countries within this region. Type O was introduced into Tunisia from the Middle East in 1989 from where it subsequently spread to Algeria and Morocco. Repeated incursions into Tunisia occurred in the 1990's presumably from Egypt and Libya (Samuel *et al.* 1999). The epidemiological situation is particularly complex in Kenya where numerous outbreaks due to serotypes SAT1, SAT2, O, A and C have been reported following the breakdown of the veterinary control programme. No other country has as wide a range of serotypes in circulation (Kitching 1998).

Due to poor reporting from the African continent, FMD is considered endemic in most African countries with only Morocco (based on serological surveillance), Swaziland, Lesotho, Zimbabwe, Namibia, Botswana and the Republic of South Africa being considered free of the disease by the OIE in 1999 (Kitching 1999). Reports of FMD in Africa from 1996-1999 point to an upward trend in the number of outbreaks (Table 1.3). These figures are unlikely to provide a true reflection of the situation in Africa, due to problematic reporting, but never-the-less represent the only official measure of the impact of this disease on the continent. The upward trend noted for Africa is reflected globally by an increase in outbreaks occurring in countries previously free of the disease. These include the type O outbreaks recorded in 1997 in Taiwan, in 2000 in Japan and in 2001 in the United Kingdom. Prior to this, these countries had been free of the disease for 68 years, 92 years and 32 years, respectively.

1.6.2 Foot-and-mouth disease in southern Africa

Clinical foot-and-mouth disease in cattle in southern Africa was first documented by Le Valliant in 1795 and recorded on numerous occasions in the 19th century by Kruger and Cummings, prior to the arrival of the rinderpest panzootic in southern Africa in 1896 (Bengis *et al.* 1987, Thomson 1994 and references therein). The decimation of cattle and wildlife by the rinderpest epidemic is believed to have led to the temporary disappearance of a large number of ungulate

diseases (Rossiter 1994), including FMD. After an absence of over 30 years, an outbreak was reported in 1931, in Zimbabwe for the first time in the 20th century. Shortly thereafter epizootics occurred in Botwana, South Africa and Zambia, after which FMD outbreaks became a regular occurrence in most southern African countries. The disease continues to threaten the agricultural trade and economic development in this African sub-region.

Six of the seven FMDV serotypes have been recorded in southern Africa. All three SAT-types occur in six of the ten southern African countries, namely South Africa, Zimbabwe, Namibia, Zambia, Botswana and Malawi. SAT-3 has not been recorded in Mozambique or Angola, but has been isolated from African buffalo in Uganda on two separate occasions (Hedger *et al.* 1973; Records of the Onderstepoort Veterinary Institute). The classical or European serotypes A and O (and C on rare occasions) have been identified in the Mozambique and Angola, in northern Namibia, Malawi and more recently in South Africa (Fig. 1.3). The latter serotypes are believed to be exotic to the southern African region, since unlike the SAT-types, antibodies to these classical serotypes do not occur in wildlife (Thomson 1994). Retrospective analysis of type O viruses causing outbreaks in Angola confirms that these outbreaks were due to introduction of the virus from either Europe or South America rather than from an endemic source (Sangare *et al.* 2001) as do studies on serotype A viruses from Malawi and Angola (Knowles *et al.* 1998). The situation in southern Africa contrasts with that in East Africa where type O and A viruses are representative of regional, endemic African genotypes, as indicated by molecular epidemiological studies (Knowles *et al.* 1998; Sangare *et al.* 2001). Furthermore, type O antibodies have been recorded in buffalo in Kenya (Anderson *et al.* 1979) and Uganda (Hedger *et al.* 1973). The latter reports should however be treated with caution as non-specific reactions have previously been recorded against type O in southern African buffalo populations (Condy *et al.* 1969; Records of the Onderstepoort Veterinary Institute).

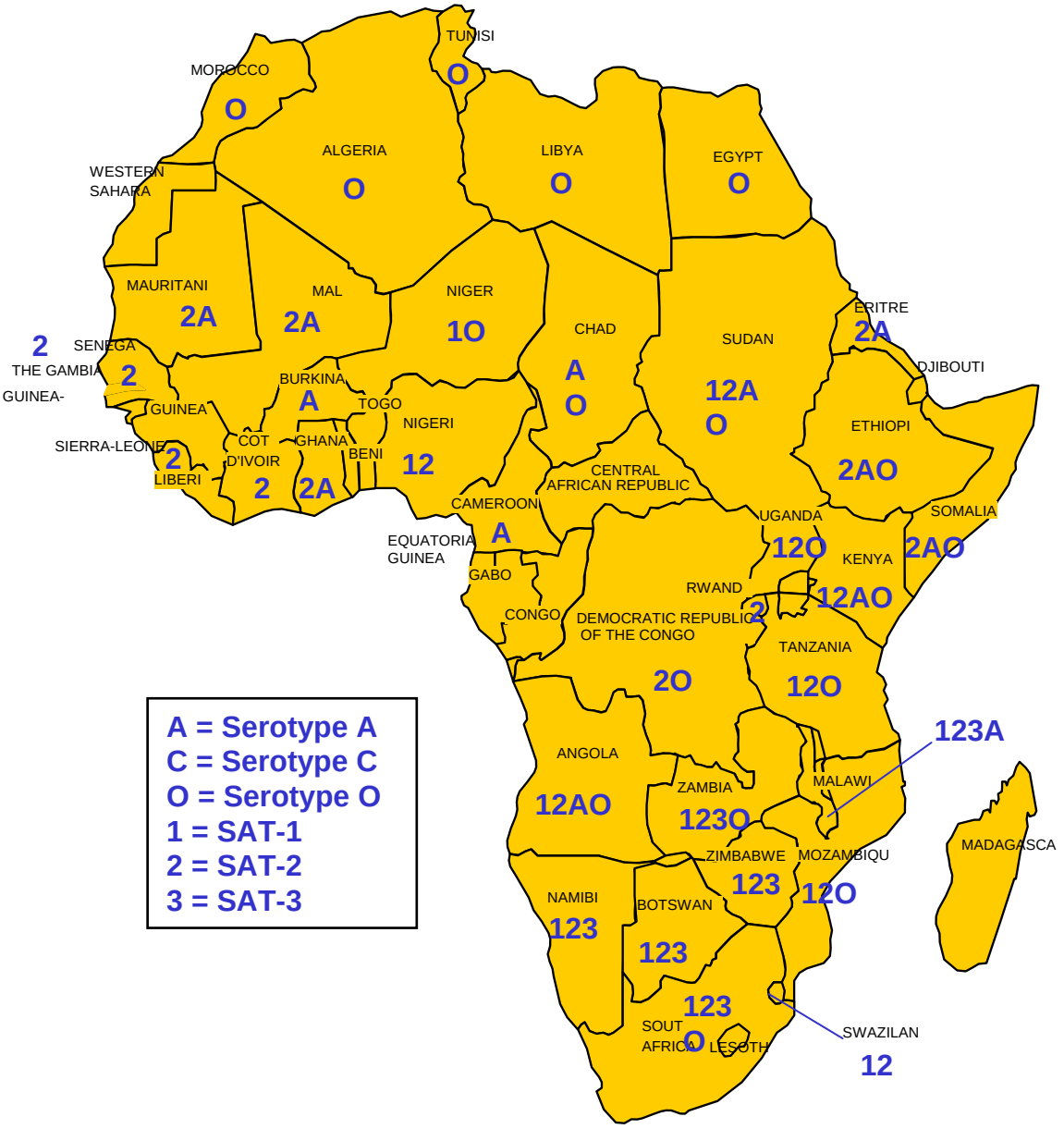

Fig. 1.3 Distribution of FMDV serotypes in Africa

TABLE 1.3 Number of FMD outbreaks reported yearly to the OIE, by African countries (1996-1999)

Country / Territory	1996	1997	1998	1999	Total by country, 1996-1999
Algeria	--	0	0	165	165
Angola	--	--	0	--	0
Benin	20	23	3	59	105
Botswana	--	0	0	0	0
Burkina Faso	56	20	35	90	201
Burundi	--	--	--	(+)	0
Cameroon	2	5	3	10	20
Cape Verde	--	0	0	--	0
Central African Republic	(+)	(+)	--	--	0
Chad	42	4	30	18	94
Comoros	--	0	0	0	0
Congo (Democratic Rep of the)	--	1	(+)	--	1
Côte d'Ivoire	7	7	(+)	1	15
Egypt	--	1	0	0	1
Eritrea	(+)	4	27	3	34
Ethiopia	22	12	75	198	307
Gabon	--	0	0	0	0
Gambia	--	12	(+)	(+)	12
Ghana	58	33	60	22	173
Guinea	--	0	0	9	9
Kenya	3	0	105	139	247
Lesotho	--	0	0	0	0
Libya	--	0	0	0	0
Madagascar	--	0	0	0	0
Malawi	--	0	1	0	1
Mali	8	3	9	18	38
Mauritania	(+)	8	1	4	13
Mauritius	--	0	0	0	0
Morocco	--	0	0	11	11
Mozambique	0	0	0	0	0
Namibia	--	0	0	0	0
Niger	70	84	13	19	186
Nigeria	--	3	--	8	11
Reunion (France)	--	0	0	0	0
Rwanda	--	(+)	(+)	--	0
Sao Tome and Principe	--	--	--	0	0
Senegal	5	28	7	4	44
Seychelles	--	0	0	--	0
South Africa	--	0	1	0	1
Sudan	--	0	0	0	0
Swaziland	--	0	0	0	0
Tanzania	105	55	19	287	466
Togo	--	10	--	--	10
Tunisia	--	0	0	2	2
Uganda	20	1	8	15	44
Zambia	1	1	0	4	6
Zimbabwe	--	1	0	2	3
Total by year	**419**	**316**	**397**	**1088**	**2220**

1996: 14 countries reported FMD outbreaks; 1997: 21 countries reported FMD outbreaks; 1998: 16 countries reported FMD outbreaks; 1999: 22 countries reported FMD outbreaks

1.7 FMD in wildlife in southern Africa

One of the unique aspects of the disease in southern Africa is the role of wildlife in the epidemiology of FMD. Extensive serological surveys in which 3163 sera from 47 species were tested for antibodies to the three SAT-type viruses revealed that 18 artiodactyl species possessed antibodies to one or more serotype (Condy *et al.* 1969; Hedger 1976). In these and in subsequent studies the African buffalo (*Syncerus caffer*) consistently recorded the highest infection rates and antibody titres. Most buffalo populations in southern Africa are infected with the three SAT-type viruses, with the exception of those in the southern most distributional range, at the Hhluhluwe-Umfolozi game reserves (Esterhuysen *et al.* 1985) and Addo Elephant National Park. The high seropositivity rates in buffalo (approximately 80 %) together with the overlap in buffalo distribution and historical outbreaks of the disease (Thomson 1994) points to the role of this species in the epidemiology of the disease.

1.7.1 FMD in buffalo

Buffalo are believed to be the ultimate source of infection for livestock in southern Africa due to their ability to both maintain and transmit the disease. FMDV can persist in an isolated herd of buffalo for up to 24 years, whilst an individual animal can maintain the infection for up to 5 years (Condy *et al.* 1985). Furthermore, buffalo have unequivocally been shown to be the source of infection for cattle under both natural (Dawe *et al.* 1994a) and experimental conditions (Dawe *et al.* 1994b; Vosloo *et al.* 1996). However, transmission from persistently infected buffalo is believed to be an extremely rare event and numerous attempts to demonstrate transmission under experimental conditions have failed (Condy & Hedger 1974; Anderson *et al.* 1979; Hedger & Condy 1985; Bengis *et al.* 1986). Even acutely infected buffalo are not always capable of transmitting SAT viruses to cattle (Gainaru *et al.* 1986). The mechanism facilitating SAT-type virus transmission from persistently infected buffalo to cattle remains obscure, however transmission of virus from buffalo appears to occur readily when:

- There is close contact between the two species
- Buffalo are in an acute stage of infection and shedding large amounts of virus

FMDV is not transmitted vertically, however horizontal transmission occurs, with buffalo calves becoming infected for the first time at around 3-8 months after the waning of maternally-derived immunity (Condy & Hedger 1978). Due to synchronized breeding and calving of buffalo, large

numbers of calves will become infected at around the same time and shed large amounts of virus during the acute stage of infection (Gainaru *et al.* 1986). This results in environmental contamination and provides a potential source of infection for other susceptible cloven-hoofed species.

1.7.2 FMD in impala

In South Africa there is a regular cyclic occurrence of FMD epizootics (approximately every two years) in impala antelope, with sometimes severe signs of clinical infection. This contrasts with other southern African countries where clinical disease is not noted in these antelope and where they are not believed to play a significant role in the epidemiology of the disease (Anderson *et al.* 1993). Although studies have established that individual impala do not become carriers (Hedger *et al.* 1972; Anderson *et al.* 1975), it appears that the disease can persist in impala populations for between 6 and 13 months (Vosloo *et al.* 1992; Keet *et al.* 1996). The extended circulation of FMD virus in this antelope species has only been recorded in recent years in the southern half of the Kruger National Park, an area where impala population density is extremely high. Another unusual feature of the disease in impala in South Africa is that prior to 1982, most outbreaks were caused by SAT-1 virus, yet from 1982 until 1996, all outbreaks in impala were due to SAT-2 type viruses. This trend was recently halted by the recording of a SAT-1 outbreak in impala in 1998 (Records of the Onderstpoort Veterinary Institute).

Impala are particularly susceptible to the aerosol route of infection. In contrast to cattle and sheep which require 25 and 10 cell culture infective doses, respectively, for infection via the respiratory route, impala become infected on exposure to just one cell culture infection dose (Gainaru *et al.* Cited by Thomson 1994). Once infected, these antelopes secrete high levels of FMD virus 1-3 days prior to and 7-14 days after the development of lesions. They have been shown to precipitate infection in buffalo (Vosloo *et al.* 1996) and are also readily infected by buffalo shedding virus (Hedger *et al.* 1972), despite the two species not being in close proximity to each other.

The possibility that these antelope act as intermediaries in disease transmission was recognized in 1962 when clinical cases of FMD in impala were used as an indicator for predicting outbreaks in livestock (Meeser 1962). More recently when the risk posed by African buffalo to cattle in

close proximity to wildlife conservancies was assessed by computer simulated scenario-pathway analyses (Sutmoller *et al.* 2000), it was shown that infected antelope are capable of jumping the outer perimeter fence of a conservancy posed the greatest annual risk of FMD infection to cattle (1:5000). Impala and kudu (*Tragelaphus stresiceros*) antelope are the two species most likely to pose a threat due to their FMD infection rates and to their ability to clear perimeter game fences.

1.7.3 FMD in other antelope species

A number of antelope species have been experimentally infected with SAT-type viruses in order to determine carrier status, routes and amount of virus excreted as well as measuring antibody response, so that experimental and field data can be correlated. Of the species studied only kudu antelope (*Tragelaphus strepsiceros*) were conclusively shown to be persistently infected, with the carrier state of between 106 to 140 days being demonstrated (Hedger *et al.* 1972). Studies on eland (*Taurotragus oryx*), sable (*Hippotragus niger*) and wildebeest (*Connochaetes taurinus*) were either contradictory, or alternatively unable to show virus recovery past 28 days (Hedger *et al.* 1972; Anderson *et al.* 1975; Anderson 1980; Ferris *et al.* 1989).

1.7.4 FMD in *Suidae*

Experimental infection of warthog (*Phacochoerus aethiopicus*) and bush pig *(Potamochoerus porcus*) with SAT-2 type virus resulted in severe clinical signs of infection, antibody response to SAT-2 and transmission to in-contact animals. However no carrier status was demonstrated, nor did interspecies transmission of a SAT-1 from other wildlife species to these species occur. Antibody titres persisted past 300 days in warthog, but were low and of short duration (less than 100 days) in bush pig (Hedger *et al.* 1972). These results together with a lack of serological field data indicate that the bush pig is unlikely to play a role in virus maintenance or disease transmission in southern Africa.

1.7.5 FMD in elephant

The first case of FMD in an Indian elephant (*Elephus maximus*) was reported in 1935 (Ramiah 1935), but no virological data to support the circumstantial and clinical signs was presented until 1976, when natural infection of an Indian elephant with type O virus was detailed (Pyakural *et al.* 1976). The first confirmed report of FMD in African elephants (*Loxodonta africana*), also involved a European serotype (A_7 strain) and affected 15 elephants in a circus in Italy (Piragino

1970). Despite these isolated reports, negative results have consistently been obtained from serological surveys conducted on African elephants and no clinical cases of the disease have ever been reported on the African continent (Howell *et al.* 1973). Furthermore, elephants experimentally infected with SAT-2 type virus failed to react clinically or serologically (Hedger *et al.* 1972). These conflicting reports prompted the experimental infection of 9 African elephants in order to assess their role in the epidemiology of the disease in Africa. The results showed that the pathogenesis of the disease is similar to that observed for other susceptible animals. Elephants developed severe clinical signs of infection, were viraemic for up to 6 days and shed large amounts of virus, however, transmission to other elephants in close contact could not be demonstrated and antibody levels were extremely low (Howell *et al.* 1973). These results together with the lack of field evidence indicates that elephants are unlikely to play an important role as a natural host in the spread of FMD in the enzootic regions of Africa.

1.8 FMD as a zoonosis

Given the concern arising in the public mind following an outbreak of FMD, it is pertinent to mention that FMD is generally not believed to be a zoonosis in man. However a recent review of all available literature reveals that FMD has been recorded on rare occasions, with approximately 40 confirmed cases being reported in humans (Bauer 1997). Clinical signs vary from asymptomatic to severe, with blisters in the mouth and on the hands and feet. However healing is rapid. The virus types most frequently isolated from humans are type O, followed by type C and rarely type A. Antibodies to FMD are of short duration (< 16 weeks) and reach a peak at around 3 weeks after infection (Reviewed by Bauer 1997). Although humans are not readily infected with FMDV, it should be stressed that they can play a role as intermediaries in disease transmission, as demonstrated by the case of a non-infected individual that retained virus in his nasal passages for more than 24 hours before transferring the disease to susceptible cattle (Sellers *et al.* 1971).

1.9 The role of carriers in the epidemiology of FMD

The existence of a carrier state in cattle was first demonstrated by van Bekum and co-workers (1959). Carriers are defined as those animals from which virus can be isolated from the pharyngeal area more than 28 days after infection (Sutmoller *et al.* 1968). Although it is well established that FMD virus persists in buffalo (up to 5 years), cattle (up to 3 years), sheep (up to 9 months) and goats (for between 3-6 months), the mechanisms underlying persistence and the immunological pathways that eventually lead to viral clearance are not well understood. Host variation is however likely to play a role as different breeds of sheep and goats display differential abilities to act as carriers of the FMD virus (McVicar & Sütmoller 1968; Anderson *et al.* 1976). Of the livestock infected by FMD only pigs are believed to be incapable of establishing a persistent infection, although one recent isolated report has indicated that persistence in swine is possible (Merzencio *et al.* 1999). The mechanism of persistence is likely to depend on a number of factors including, viral replication (cytolytic versus lysogenic), target cell, host and strain variability.

1.9.1 Persistence *in vitro*

FMDV is usually cytolytic in cell cultures, but persistent infections have also been established in BHK-21 cells (De la Torre *et al.* 1985), resulting in multiple genetic and phenotypic variations in the persistent viral strain. Upon establishment of a persistent infection *in vitro*, it was shown that cells were resistant to superinfection with FMD viruses of the same or of different serotypes, but not to infection by unrelated viruses such as *encephalomyocarditis virus* (EMCV; De la Torre *et al.* 1985). It was later shown that it is not only the viruses that change during persistent infection, but the cells themselves undergo marked changes in morphology, resistence to infection and in growth characteristics (De la Torre *et al.* 1988; De la Torre *et al.* 1989). This coevolution between the persistently infected BHK-21 cells and the resident virus, was shown to produce viral variants of increased virulence for the parental cells and to have acquired the ability to bind heparin and to infect Chinese hamster ovary (CHO) cells (Escarmis *et al.* 1998). Comparison of the variant and parental viruses revealed amino acid differences involving sites at the five-fold axis of the viral capsid, namely, amino acid position 9 of VP3 (Asp -> Ala) and either amino acid position 108 (His -> Arg) or 110 (Gly -> Arg) of the VP1 gene (Escarmis *et al.* 1998). Drastic antigenic changes can occur *in vitro* in the absence of antibody selection during replication of FMDV in persistently infected cells (Bolwell *et al.* 1989; Borrego *et al.* 1993; Sevilla *et al.* 1996; Mbayed *et al.* 1997). The size of the initial population is a major factor in

determining the repertoire of antigenic variants evolving in viral quasispecies.

1.9.2 Persistence *in vivo*

Approximately 50 % of cattle infected with FMDV become persistently infected, irrespective of their vaccination status, or the serotype of the virus with which they were infected (McVicar & Sütmoller 1969). Persistent infection in cattle may promote the rapid selection of antigenic variants, in addition to being a virus reservoir as demonstrated by the rapid rate of fixation of mutations (0.9×10^{-2} to 7.4×10^{-2} s/n/y) in the VP1 protein of carrier cattle and the accompanying profound antigenic changes (Gebauer *et al.* 1988).

In order for a viral infection to be non-cytopathic, the target cells must be restrictive or non-permissive to the virus. Alternatively, viral variants with reduced cytopathogenicity which replicate at a lower rate must evolve. One or the combination of the afore-mentioned factors would ensure the long-term maintenance of the viral genome in host cells, but not necessarily avoidance of detection and elimination by the hosts' immune system. To achieve this, the virus would need to evade the immune system and / or establish persistence at an immunologically privileged site. The former requirement is readily achieved by the quasispecies nature of FMDV (Domingo *et al.* 1992), where genetic and antigenic variants are generated both in the presence and absence of immune selection (Gebauer *et al.* 1988; Diez *et al.* 1989; Domingo et al. 1993; Domingo *et al.* 1996; Vosloo *et al.* 1996). However, the pharyngeal area which is known to be the most common site of viral persistence is not considered an immunologically privileged site, and there is conflicting evidence regarding the ability of FMDV viral proteins to counteract the host immune defense (Moonen & Schrijver 2000; Haydon *et al.* 2001).

1.9.3 Possible modes of transmission of FMDV from carrier animals

One of the more perplexing problems regarding the role of persistently infected animals, and buffalo in particular, is that the mechanism whereby interspecies virus transmission occurs is unclear. In an attempt to clarify this, numerous studies have investigated possible means of transmission, without success. These include:

- ***Environmental contamination resulting from hunting***

The dissemination of virus through hunting and field dressing of an infected animal was tested

by Condy & Hedger (1974), and shown not to precipitate disease in susceptible animals in close proximity. This possibility is also unlikely as virus disassembly results when the pH in muscle tissue drops following the death of an animal (Bengis 1997).

- ***Stress induction***

Inducing stress in animals was proposed to be one means whereby the shedding of virus by carrier animals could be precipitated (Hedger & Condy 1985). This was tested in cattle through the administration of steroids and dexamethasone (Sutmoller & McVicar 1972; Ilott *et al.* 1997). The former chemical did not lead to increased viral activity in the pharynx, nor to transmission to non-infected in-contact animals (Sutmoller & McVicar 1972) and the latter, a known herpesvirus reactivator had the opposite effect and resulted in a drastic decrease in virus titres in oesophageo-pharyngeal (OP) fluid (Ilott *et al.* 1997).

- ***Mechanical transmission***

The blood feeding African buffalo fly, *Haematobia thirouxin potans* (Bezzi) was assessed for its ability to act as a mechanical vector of FMDV (Thomson *et al.* 1988). The fly failed to transmit FMDV to susceptible cattle after feeding on viraemic animals. The results indicate that this biting fly is an inefficient mechanical transmitter of SAT type viruses. Similarly, investigations into the potential of the ixodid tick, *Rhipicephalus zambeziensis,* the vector for Corridor Disease (CD) to transmit virus after feeding on viraemic animals was unsuccessful, despite virus survival of between 3 and 7 days being demonstrated in the tick (Van Vuuren *et al.* 1993).

1.10 Characterization of field strains of FMDV

Field strains of FMDV were initially characterized by subtyping, but as mentioned in section 1.5, the practical difficulties of discerning subtypes, given the continuous spectrum of antigenic variants, severely impedes the usefulness of this approach. From the 1980's onwards, the characterization of field strains was based on genetic methods and on T_1 oligonucleotide fingerprinting in particular (Domingo *et al.* 1980; Anderson *et al.* 1985; Anderson 1986; Carrillo *et al.* 1990). Briefly this approach involved two-dimensional electrophoretic separation of radioactively labelled RNA digested with T_1 ribonuclease. The resulting T_1 map represents about 5 - 7 % of the RNA genome (Domingo *et al.* 1980; Anderson 1986). Due to gel-to-gel variations, complexity of results and

difficulties in comparing large numbers of field strains with each other, alternative methods were investigated. Of these, the partial nucleotide sequence and analysis of the C-terminus end of the VP1 gene significantly improved the genetic resolution of virue relationships. In 1987 two reports on the application of this approach were published, one on poliovirus (Rico-Hesse *et al.* 1987) and one on FMDV (Beck & Strohmaier 1987), which revolutionized the field of picornaviral molecular epidemiology. The subsequent proliferation in similar studies has provided important epidemiological insights, including evidence of prolonged persistence of outbreak strains in the field (Samuel *et al*. 1999; Freiberg *et al.* 1999), trans-boundary virus transmission (Marquardt & Haas 1999; Samuel *et al.* 1999; Sangare *et al.* 2001) and the presence of virus types specific to different geographical areas (Saiz *et al.* 1993; Stram *et al.* 1995a; Knowles & Samuel 1997; Knowles *et al.* 1998). Outbreaks resulting from improperly inactivated vaccine and those in which there was no vaccine involvement have also been reported (Beck & Strohmaiher 1987; Krebs & Marquardt 1992; Suryanarayana *et al.* 1998). Most of the afore-mentioned studies have addressed the epidemiological situation of types O, A and C. In comparison to the European serotypes, few studies have addressed the continental genetic diversity of the SAT-types. Published studies have primarily focussed on the disease situation in South Africa (Vosloo *et al.* 1992; Vosloo *et al.* 1995; Keet *et al.* 1996) and Zimbabwe (Dawe *et al.* 1994a & 1994b), with only one study addressing genetic variation at a regional level (Knowles 1994). The latter reports were based on partial 1D nucleotide sequences corresponding to the C-terminal 150 to 200 nt of the VP1 protein generated by a direct RNA sequencing approach. The restriction on the length of sequence data generated for the VP1 protein of SAT-type viruses was primarily due to the lack of suitable primers with which to target this region. Difficulties in designing more appropriate primers also arose from the fact that the P1 regions of just two SAT-type viruses have been published (Brown *et al.* 1989; van Rensburg & Nel 1999).

1.11 Objectives of this study

From the review of the literature it is clear that numerous aspects of the molecular epidemiology of FMD in southern Africa remain to be clarified. In particular the mechanism whereby persistently infected buffalo transmit the disease to cattle and the role of impala as possible intermediaries in disease transmission remains obscure. It remains to be established whether buffalo viruses evolve independently in different geographical localities. The role of the African buffalo as the primary source of infection for wildlife and domestic artiodactyl species needs to be investigated further. In this regard, genetic characterization and comparison of field strains from diverse host species may provide insight into the epidemiology of the disease in specific regions. The greatest hurdle in conducting comprehensive molecular epidemiological studies is the lack of suitable PCR-based methods for amplification and characterization of the SAT-type viral genomes. The primary objective is therefore to develop and establish genetic characterization methods and apply these in an investigation of the epidemiology of the disease in southern Africa.

The aims of this study are therefore fourfold:

- To develop a PCR-based method suitable for the detection and genetic characterization of foot-and-mouth disease viruses prevalent in southern Africa

- To apply this new methodology in elucidating factors of regional epidemiological importance, namely:
  - The possibility that sexual transmission of foot-and-mouth disease with particular reference to the role of the African buffalo
  - The role of the impala antelope in disease transmission

- To assess the genetic variability of SAT-type FMD viruses in the buffalo maintenance host populations which occur in southern Africa, by applying the PCR-based methodology

- To use the buffalo virus genetic database as a reference for determining the origin of:
  - Historical outbreak strains in South and southern Africa
  - Recent outbreaks of the disease in livestock

Chapter 2

Development of a PCR-based method for the detection and characterization of foot-and-mouth disease virus in southern Africa

Summary

In order to establish a method suitable for both detection and characterization of SAT-type foot-and-mouth disease virus, published primers were assessed for their ability to amplify variants of these serotypes. Initial screening of field isolates with published primers revealed that SAT-type recognition was low. A primer pair with moderate SAT-type recognition (67%) was however identified and selected for optimization in an effort to improve the detection rate. Modifications to primers improved SAT-type detection (100 %), broadened the recognition range to European (A, O and C) and Asian (Asia-1) serotypes and improved test sensitivity. In addition to being able to confirm the presence of FMDV in a clinical specimen within 6 hours of receipt, the PCR product, which is amenable to nucleotide sequencing, enables genetic characterization of viruses into serotype and genotype within 48 hours. Furthermore, the PCR sequencing approach described here was shown to be superior to conventional direct RNA sequencing and consistently delivered high quality sequences for a variety of heterogenous field isolates.

VP1 gene sequence analysis of isolates from 7 African countries and representative of 5 of the 6 serotypes occurring on the continent, revealed that SAT-types have high levels of intratypic variation. Intratypic variation for the SAT-types ranged from 34 % to 40 % on nucleotide level, and from 24 % to 28 % on amino acid level. The methodology presented here is advocated for studies directed at determining the origin and tracing the course of epizootics in both wild and domestic cloven-hoofed animals in southern Africa.

Parts of the results presented here have been published in the *Onderstepoort Journal of Veterinary Research* 65: 37-47 (1998)

2.1 Introduction

Diagnosis of foot-and-mouth disease (FMD) in developing countries of Asia and Africa is complicated due to the presence of multiple serotypes and the high levels of antigenic variation of the indigenous types (Brooksby 1972). This is particularly true for sub-Saharan Africa where the endemic SAT (South African Territories) types show high levels of intratypic variation (Vosloo *et al.* 1995) and where six of the seven types are known to occur (Odend'hal 1983). European serotypes O, A and C have been recorded in Malawi, Zambia, Angola and in neighbouring Namibia and Mozambique (Brooksby 1972; Odend'hal 1983; Thomson 1994). The close proximity of European types to South Africa necessitates diagnostic methods capable of detecting and characterizing the three European types in addition to the endemic SAT-types.

In 1994, it was estimated that a widespread outbreak of the disease in South Africa would affect an estimated R2 billion-worth of agricultural products annually (Thomson 1994). The last recorded outbreak of FMD in livestock in South African occurred in 1983 (Records of the Directorate of Veterinary Services 1983), with subsequent epizootics being limited to wildlife in the Kruger National Park (Keet *et al.* 1996). The successful control of the disease was achieved by restricting animal (and animal product) movement and by effective vaccination (Hunter 1996; Hunter 1998). This has allowed for international recognition of an infection-free zone by the O.I.E. in May 1996, which has significant implications for South African agricultural export and international trade (Thomson 1996). Early diagnosis is critical in minimizing the potentially detrimental economic effect of an outbreak.

Detection and typing of FMD virus (FMDV) at the Onderstepoort Veterinary Institute, follows similar procedures to those employed by the World Reference Laboratory (Kitching 1992) and is achieved by a combination of methods. These include virus isolation on pig kidney (PK) cells and direct typing of viruses in clinical specimens by a sandwich ELISA (Roeder & Le Blanc Smith 1987). Although these methods are highly sensitive, they each have inherent disadvantages. Isolation of virus on primary tissue cultures usually requires a 16-48 hour incubation period before the cytopathic effect (CPE) of the virus infection becomes apparent. Although CPE confirms the presence of virus, it does not exclusively confirm the presence of FMD virus, as other viruses are able to infect this cell type and display a visually indistinguishable type of CPE. Direct typing by sandwich ELISA remains the fastest (approximately 5.5 hours, J.J. Esterhuysen, personal communication) and most effective method of confirming both the presence and the virus type in a clinical sample (Ferris & Dawson 1988). It does however have limitations when virus is present at low levels as a detection threshold of 2 ng ml^{-1} of

virus has been reported for this method (Ferris & Dawson 1988). In addition, the presence of six serotypes on the African sub-continent requires that the test incorporate all the types relevant to a particular country. The higher the number of serotypes the more expensive the test becomes.

The reported speed and sensitivity of the polymerase chain reaction (PCR; Saiki *et al.* 1988) and its usefulness in the veterinary and FMD diagnostic fields is well recognized (Deacon & Lah 1989; Kitching1992; Bergmann & Malirat 1993; Rodriguez *et al.* 1994) as is the characterization of FMD field strains by direct RNA sequencing of the 1D (VP1) gene (Beck & Strohmaier 1987; Marquardt & Adam 1990).

Advances in molecular epidemiological studies of types A, O, C and Asia-1 in recent years have been facilitated by sequencing VP1-gene amplification products (Saiz *et al.* 1993; Stram *et al.* 1995b; Knowles *et al.* 1998; Marquardt & Haas 1998; Pattnaik *et al.* 1998). By comparison, the molecular epidemiology of SAT-types has relied on direct RNA sequencing of the viral genome due to the lack of suitable primers for these serotypes. Although a variety of primer sequences have been published for detection of multiple FMDV serotypes by PCR, most have been directed at amplification of the conserved, but phylogenetically uninformative 3D gene (Meyer *et al.* 1991; Laor *et al.*1992; Lin *et al.* 1992; Rodriguez *et al.* 1992; Pattnaik *et al.* 1997). Published PCR methods which amplify the more variable structural protein genes have been tested exclusively against European and/or Asia serotypes (Knowles & Samuel 1994; Lochner *et al.* 1995; Tosh *et al.* 1997) or alternatively against a small number of SAT-types (Amarel-Doel *et al.* 1993; Höfner *et al.* 1993; Vangrysperre & de Clercq 1996). Despite the limitations associated with direct RNA sequencing (Knowles & Samuel 1994), analysis of short stretches of sequence, generated by this approach have been instrumental in clarifying some genetic relationships of field and outbreak strains in South Africa and Zimbabwe (Vosloo *et al.* 1992; Dawe *et al.* 1994a; Vosloo *et al.* 1995). Clearly, further advances in epidemiological studies of FMD in southern Africa will benefit greatly from the establishment of a PCR-based nucleotide sequencing approach. The aim of this chapter is therefore to investigate the applicability of PCR methods for diagnosis and characterization of FMD in southern Africa.

2.2 Materials and Methods

2.2.1 Virus strains and cell cultures

FMDV isolates were obtained by preparing 10 % suspensions (W/V) of buffalo (*Syncerus caffer*), impala (*Aepyceros melampus*) and bovine probang and epithelial specimens according to standard procedures. Primary pig kidney (PK) cells were inoculated with these suspensions and propagated further on IBRS2 (Insituto Biologico Rim Suino) or BHK (Baby Hamster Kidney) cells. Additional *aphthovirus* isolates were obtained from the World Reference Laboratory, Pirbright and the Botswana Vaccine Institute, Gaborone. Tissue culture specimens of enteroviruses were provided by C. Chezzi of the National Institute of Virology (NIV), South Africa. Plaque titration of selected strains was performed using IBRS2 cells according to standard procedures.

2.2.2 RNA extraction and cDNA synthesis

RNA was extracted from sucrose gradient purified virus by phenol/chloroform and precipitated in the presence of NaAc (pH 5.2) as previously described (Vosloo 1992). Alternatively, RNA was extracted from cell culture specimens by a modified guanidinium-based nucleic acid extraction method (Boom *et al.* 1990). The RNA viral template was reverse transcribed using AMV-RT (Promega) with the 2A/B junction primer of Beck & Strohmaier (1987). Alternatively, a primer termed 2B (Table 1) which is complimentary to the sequence of primer P32 (Vangrysperre & De Clercq 1996) was used to prime the synthesis. Enterovirus cDNA was prepared by a hexanucleotide method (Meyer *et al.* 1994).

2.2.3 PCR amplification and purification

Genomic amplification of the FMD viral genome was performed with a variety of novel, published and modified primers (Table 2.1). Most of these primers bind to VP1 or neighbouring genes, with the exception of primers #7 and #9 which bind to the RNA polymerase gene. Primers targeting the VP1 gene amplify distinct fragments with the different primer combinations, as outlined in Fig. 2.1. FMDV oligonucleotides VP1a, VP1b and P1 were synthesized by MWG-Biotech GmbH, whilst the W-US, VP1D, VP3U, L-US, #7 and #9 primers were synthesized by the Department of Biochemistry (UCT). The PCR reactions were performed in a 25 µl volume in the presence of 1-2 µl of cDNA template, 0.2 mM dNTP, 0.25 µM of each primer, 1x buffer (DynaZyme) and 0.5 U of *Taq* polymerase (DynaZyme). After an initial denaturation step at 96°C for 1 min, thirty cycles of denaturation at 96°C for 12 s, annealing at pair-specific temperatures (Table 1) for 20 s and extension at 70°C for 40 s were performed. Primers based on those of Rotbart (1990) which target a 154bp fragment in the conserved 5' non-coding region of enterovirus genomes were supplied by C. Chezzi, NIV and used with minor modification to both oligonucleotides and reaction conditions (C. Chezzi, personal communication). Amplification of the expected fragment was confirmed by product size estimation against a DNA molecular weight marker on a 1.5 % agarose gel. Bands of the correct size were excised from the gel and purified by means of the Cleanmix purification system (Talent).

2.2.4 Nucleotide sequencing

Two approaches were followed when determining VP1 gene nucleotide sequence. The first involved a direct RNA sequencing approach where 1-2µg of viral RNA was sequenced in the presence of AMV-RT (Promega), a radionucleotide label and ddNTP's as specified by Vosloo (1992). In the second approach, *in vitro* amplified DNA template generated by PCR, was purified and then sequenced with T7 DNA polymerase (Sequenase Version 2.0, USB) and an [α-^{32}P]dATP radioactive label (Amersham) in the presence of 10% DMSO (Winship 1989).

TABLE 2.1 Summary of the PCR primers used in this study

Name	Sequence 5' 3'	Length	Orientation	Reference/Consensus sequence	Tm	Ta
#7	GTAAAGTGATCTGTAGCTTGG	21mer	antisense	Laor *et al.* 1992	56°C	52°C
#9	TTCGAGAACGGCACGGTCGGA	21mer	sense	Laor *et al.* 1992	64°C	60°C
2B	GACATGTCCTCCTGCATCTG	20mer	antisense	Consensus of A00276, K00554, V01131, A15586, V01130, X00130, X00429, M10975, X00871 / Complimentary to P32 primer (Vangrysperre & De Clercq, 1996)	59°C	55°C
P1	GAAGGGCCCAGGGTTGGACTC	21mer	antisense	Beck & Strohmaier, 1987	65°C	61°C
VP1D	GTCACAAAAGTAATACGTGG	20mer	antisense	Complimentary to W-US primer (Vosloo *et al.*, 1996)	53°C	49°C
P2	CACACAACCAACACCCAGAACAAT	24mer	sense	Höfner *et al.* 1993	61°C	57°C
W-US	CCACGTATTACTTTTGTGAC	20mer	sense	Vosloo *et al.*, 1996	53°C	49°C
VP1Ua	CCACRTATTACTTYTGTGACCT	22mer	sense	Consensus of W-US, M28719 (SAT-3), A06737 (O_1) and M20715 (A_{10})	57°C	53°C
VP1Ub	CCACGTACTACTTYTCTGACCTGGA	25mer	sense	Consensus of W-US, M28719, A06737, M20715, M19760 (C_3) and U01207 (Asia-1)	64°C	60°C
VP3U	GATACTGGTTTGAACTCCAAGTT	23mer	sense	Consensus of M28719, M60118, L29062, L29078, M90381, M90368	53°C	49°C

Sequence, length, orientation and oligonucleotide names of published (#7, #9, P1, P2 & W-US), novel (VP3U) and modified (2B, VP1D and VP1Ua and VP1Ub) are given. Where primer sequences were derived by alignment and identification of a consensus sequence (DAPSA, Harley 1994) Genbank and EMBL accession numbers of the sequences used, are indicated. Melting temperatures (Tm) of each oligonucleotide were calculated according to the following supplier prescribed formula: Tm = [69.3°C + 0.41 (%GC)] - 650/primer length (MWG-Biotech GmbH, Ebersberg, Germany). PCR annealing temperatures (Ta) were determined by applying the following: Ta = Tm - 4°C, with the lowest Ta of a particular primer combination dictating the annealing temperature at which the genomic amplification is performed.

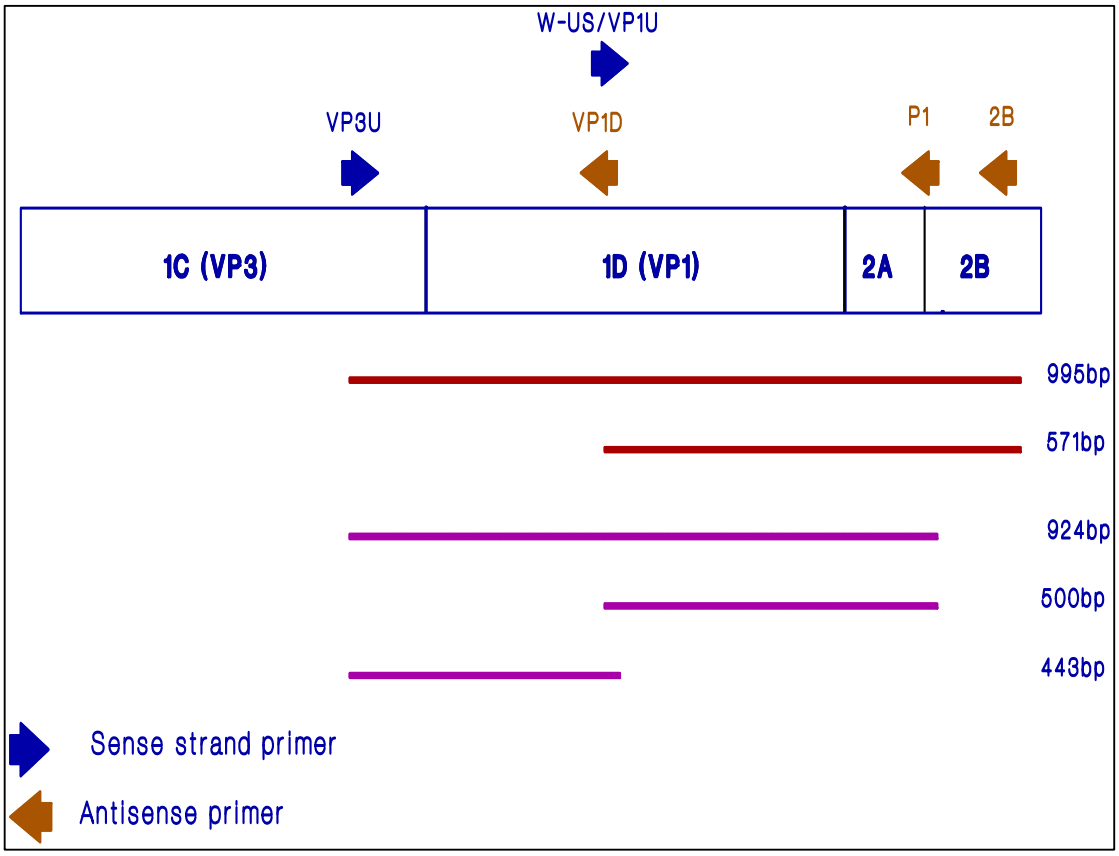

Fig. 2.1: VP1 gene amplification strategy. Arrows indicate primer orientation and binding position with approximate sizes of amplification products obtained with different primer combinations given in base pairs (bp) on the right

2.2.5 Phylogenetic analysis

VP1 gene nucleotide sequences were translated (Harley, 1994) and the deduced amino acid sequences aligned according to the guidelines set by Palmenberg (1989) for picornaviral capsid proteins. Sequences used in the phylogenetic analysis were submitted to Genbank under the accession numbers indicated in Table 2.4. Analyses were conducted on the carboxy-terminal 136 amino acids (aa) of the aligned VP1 gene sequences. Gene trees were constructed using the neighbor-joining method included in the MEGA programme (Kumar *et al.* 1993), with p-distances and pairwise deletions of gaps and missing data being applied. Node reliability was estimated by 1000 bootstrap replications. Published sequences of serotype A (A_{24} Cruzeiro *et al.* 1982) and O (O_1 Kaufbeuren; Forss *et al.* 1984) strains of non-African origin were also included in the analyses.

2.3 Results

2.3.1 Primer-pair recognition of FMDV serotypes

In order to assess the applicability of published primers for FMDV diagnosis in southern Africa, three different primer pairs were initially evaluated. These include the primer pair described by Höfner *et al.* (1993), which targets the P1 structural protein region and the primer pairs of Vosloo *et al.* (1996) and Laor *et al.* (1992) which are designed to amplify the 1D (VP1) and 3D (replicase) genes respectively. Comparison of the three primer pairs revealed that the amplification efficiency was in the order of P1<1D<3D, for the SAT-types tested (results not shown). The difference in amplification efficiency between structural (1D) and non-structural (3D) protein is demonstrated in Figure 2.2. Only two of the seven SAT-2 type field stains from various southern African localities amplified with the 1D primers, whilst all viruses tested with the 3D primers amplified the expected 978 bp band. Although the 3D primers were shown to be suitable for detecting a variety of FMDV types, this conserved, non-structural protein is serotypically non-informative and therefore of no use for genetic differentiation of viruses by nucleotide sequencing.

Five new primers, which amplify a product of under 1000 bp and which specifically target the VP1 gene were subsequently designed, on the basis of consensus sequences of data available in Genbank (Table 2.1). The different primer pair combinations and their expected product sizes are summarized in Fig. 2.1. Each of these primer pairs were tested against one representative of each of the European and Asian serotypes and two of each of the SAT-types in order to establish general FMDV and SAT-type recognition capabilities of different combinations by PCR. Primer pairs 2B+VP3U and P1+W-US scored equally well in terms of SAT-type amplification (Table 2.2), with pair 2B+VP3U having a higher overall recognition for all FMDV types.

The P1+W-US pair was, however, selected for optimization as amplification of all three SAT-types was obtained (2B+VP3U did not amplify the SAT-2 strains) and the level of amplification was generally higher. In addition, the product size of the P1+W-US primer pair is closer to the 200-400 bp amplification efficiency range (Rychlik 1993) and the 500 bp product can potentially be sequenced in its entirety with the external PCR primers alone.

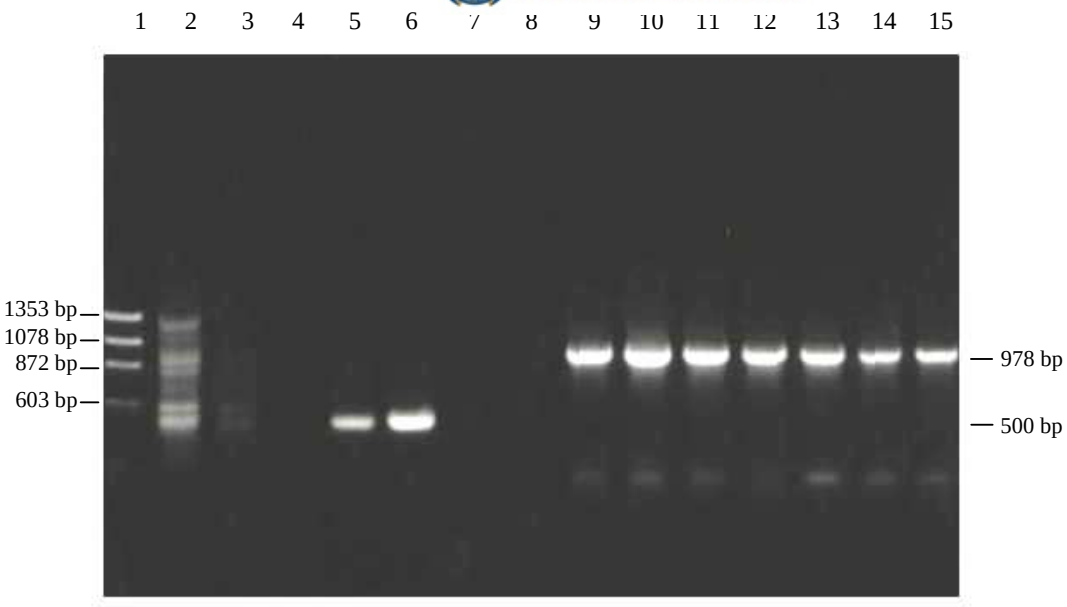

Fig. 2.2: Comparison of amplification results obtained with published VP1 gene-targeting primers (lanes 2-8) and replicase gene-targeting primers (lanes 9-15), with geographically divergent SAT-2 type field strains. cDNA templates used for these reactions were the following:
(2)+(9): ZAM 12/81; (3)+(10): MOZ 4/83; (4)+(11): ZIM 7/83; (5)+(12): KNP 19/89; (6)+(13): KNP 5/92; (7)+(14): NAM 1/92; (8)+(15): KNP 51/93
Size estimation of products was performed against the ∅X 174 (Hae III) DNA molecular weight marker loaded in Lane 1.

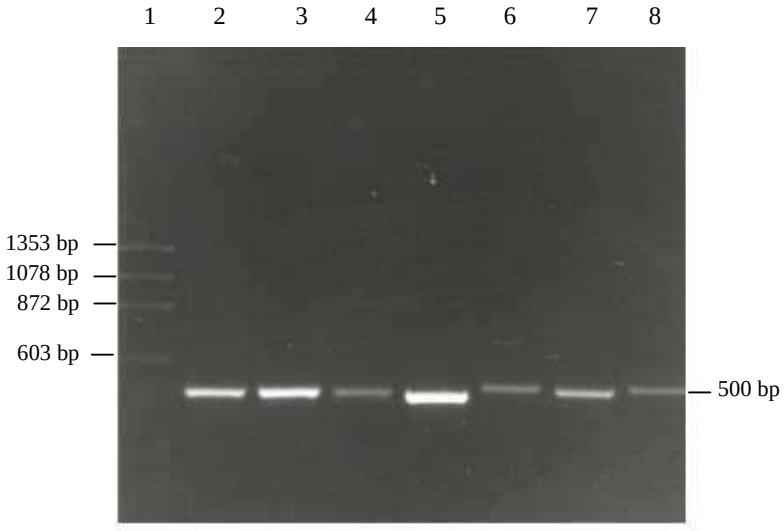

Fig. 2.3: Agarose gel depicting amplification of all seven FMDV serotypes with the VP1Ub and P1 primer set. Lane 1 contains the ∅X 174 (*Hae III*) DNA molecular weight marker (Promega). Lanes 2 through 8 contain PCR products of approximately 500bp, which are amplified in the presence of the following cDNA templates: (2) PAK 1/54 (Asia-1); (3) KEN 37/84 (Type A); (4) KEN 7/78 (Type O); (5) C_1 Noville (Type C); (6) BOT 1/68 (SAT-1); (7) KNP 19/89 (SAT-2); (8) KNP 10/90 (SAT-3)

TABLE 2.2 Summary of PCR results obtained with published and novel primer pairs

Viral Isolate	Serotype	2B+VP3U	P1+VP3U	VP1D+VP3U	2B+W-US	P1+W-US
PAK 1/54	Asia-1	++	x	x	--	x
KEN 37/84	A	--	--	--	--	--
KEN 1/91	O	x	+	--	--	--
C_3 Resende	C	+	x	--	--	x
KNP 196/91	SAT-1	+	x	--	--	++
ZAM 29/96	SAT-1	+	x	--	--	--
ZIM 7/83	SAT-2	x	--	--	--	+
KNP 19/89	SAT-2	--	--	--	--	--
BEC 1/65	SAT-3	+	+	+	--	++
KNP 10/90	SAT-3	++	x	--	--	++
%FMDV		**60%**	**20%**	**10%**	**---**	**40%**
%SAT		66.7%	16.7%	16.7%	---	66.7%

-- = no amplification; + = weak amplification; ++ = good amplification; x = nonspecific amplification
Percentages indicated in bold correspond with the overall FMDV recognition (+ and ++ score as positive, -- and x are considered negative) by PCR, whilst underlined percentages indicate the level of successful amplification of SAT-types.

2.3.2 Primer optimization and testing

It was noted that the 3' terminal nucleotide of the W-US primer corresponds to a third base amino acid position which is known to be highly variable and have a high mutation frequency (Vosloo *et al.* 1996). By comparing the only complete VP1 gene sequence of a SAT-type (Brown *et al.* 1989) with the sequences of European serotypes A, O and C (see Table 2.1), a 22mer primer (termed VP1Ua) based on the consensus sequence of these aligned sequences was identified and synthesized. The primer was designed to end at a 2nd base position of the corresponding amino acid in order to stabilize the terminal end of the oligonucleotide. Although recognition of SAT-types increased to 86 %, the P1+VP1Ua pair was not capable of amplifying all SAT-type field strains tested. This was in all likelihood due to variability in the first base position, one nucleotide upstream of the terminal 3' base. Mismatches in this position are known to affect polymerization, a characteristic exploited for diagnostic purposes in the amplification refractory mutation system (ARMS) PCR (Wenham *et al.* 1991). A second VP1U primer, termed VP1Ub was therefore

synthesized. This primer was based on the consensus sequence of Asia-1, in addition to SAT-3, A, O and C and was extended by 3 nucleotides on the 3' end so that the final oligonucleotide length was 25. Alignment of various representatives of the five serotypes on which the primer sequence was based revealed that the terminal three nucleotides are highly conserved amongst the different FMDV serotypes. In addition, internal stability plots of these primers (results not shown) indicate that VP1Ub and the P1 primer conform to the 3' terminal pentameric requirements of successful PCR and sequencing oligonucleotides (Breslauer *et al.* 1986; Rychlik 1993). Testing of these primers not only revealed significantly improved SAT-type recognition, but also enabled amplification of various subtypes of the European strains in addition to amplification of an Asia-1 isolate. Product sizes varied due to inter- and intratypic differences in VP1 gene amino acid sequence length (Fig. 2.3). The relative amplification efficiencies of the upstream VP1 primers combined with the P1 primer are summarized in Table 2.3. With the exception of KEN 1/91 all 30 isolates tested with the VP1Ub + P1 primer pair amplified the expected band of approximately 500 bp. KEN 1/91 was, however, amplified with the 2B+VP1Ub primer pair, thereby permitting sequencing of the VP1 gene. Testing of the optimized VP1Ub primer in combination with 2B in PCR revealed that this primer pair successfully amplifies all European and Asian types tested but has limited success in amplifying SAT-types (results not shown). These results indicate that of the two potential cDNA and/or antisense PCR primers, P1 (Beck & Strohmaier, 1987) is the more conserved of the two, across all seven serotypes.

2.3.3 Confirmation of specificity

In order to assess the specificity of the VP1 gene primer pair for FMDV alone, cDNA was prepared from genetically and/or symptomatically related *Picornaviridae* such as swine vesicular disease virus (SVDV). The strains tested were representative of the *enterovirus* [coxsackie B2 and - B4; echo 11; polio-1, -2 and -3; bovine enterovirus (BEV); SVDV] and *cardiovirus* (encephalomyocarditis) genera. Integrity of the cDNA was confirmed by amplification with enterovirus-specific primers (Rotbart 1990). Interestingly, all *enterovirus* strains with the exception of BEV isolates amplified the expected 154 bp fragment (results not shown). This is perhaps not surprising in view of the grouping of BEV outside the *enterovirus* and *rhinovirus* cluster on the basis of VP1 gene phylogenetic analysis (Palmenberg 1989). Encephalomyocarditis was negative with the enterovirus primers. All *enterovirus* and *cardiovirus* virus strains tested were negative for PCR with the VP1Ub and P1 primer pair.

TABLE 2.3 Relative recognition of published and modified VP1 gene amplification primers for the endemic SAT-types and for all seven FMDV serotypes

FMDV isolate	Serotype	W-US+P1	VP1Ua+P1	VP1Ub+P1
PAK 1/54	Asia-1	x	++	++
A_5 Allier	A	-	-	++
A_{24} Cruzeiro	A	++	++	++
KEN 1/76	A	-	+	++
KEN 37/84	A	-	+	++
C_1 Noville	C	x	+	++
O_1 BFS	O	-	+	++
KEN 77/78	O	x	x	+
KEN 1/91	O	-	-	-
BOT 1/68	SAT-1	-	++	+
BOT 1/77	SAT-1	-	+	+
MOZ 3/77	SAT-1	-	-	++
SAR 9/81	SAT-1	+	+	++
MAL 1/85	SAT-1	-	+	++
KNP 196/91	SAT-1	++	+	++
KNP 8/95	SAT-1	-	-	++
ZAM 29/96	SAT-1	-	+	++
BOT 3/77	SAT-2	+	++	++
MOZ 4/83	SAT-2	x	+	++
ZIM 7/83	SAT-2	+	++	++
KNP 19/89	SAT-2	-	+	++
SWA 1/89	SAT-2	x	+	++
KNP 51/93	SAT-2	++	+	+
KNP 6/96	SAT-2	+	++	++
BEC 1/65	SAT-3	++	+	+
RHO 3/78	SAT-3	-	-	++
KNP 10/90	SAT-3	++	++	+
KNP 3/94	SAT-3	+	+	++
KNP 25/94	SAT-3	++	++	++
ZAM 4/96	SAT-3	+	+	++
%FMDV recognition		**40%**	**80%**	**97%**
%SAT-type recognition		52%	86%	100%

- = no amplification; + = weak amplification; ++ = good amplification; x = nonspecific amplification
Percentages indicated in bold correspond to the overall FMDV recognition (+ and ++ score as positive, -- and x are considered negative), whilst underlined percentages indicate the level of SAT-type recognition.

2.3.4 PCR sensitivity determinations

Sensitivity was initially determined for the optimized P1+VP1b primed amplification under previously prescribed reaction conditions with one representative of each of the SAT-types. cDNA was synthesized from ten-fold dilutions of infected cell cultures. In addition, ten-fold dilutions of cDNA synthesized from stock virus RNA were also prepared for use as templates in PCR. Comparison of results obtained with the diluted cDNA and the cDNA prepared from diluted cell culture samples allowed assessment of the effect that minimal quantities of virus in the starting material has on genomic amplification. The equivalent of less than one plaque forming unit (PFU) per 25 µl PCR could be detected when the diluted cDNA was used. In contrast, detection levels with the diluted cell culture samples could not match those of the diluted cDNA samples, but nonetheless consistently amplified the 500bp fragment in the presence of 5 or less PFU per PCR. Comparison of amplification capabilities of primer pairs P1+VP1b and P1+W-US revealed that sensitivity was between 10 and 1000 fold lower with the latter primer pair in combination with certain SAT isolates.

2.3.5 Comparison of direct RNA sequencing with PCR sequencing

In order to assess the efficiency of the PCR-based nucleotide sequencing approach, isolates known to be amenable to direct RNA sequencing were selected for sequence analysis. In addition, isolates for which no sequence data could previously be obtained by means of the RNA sequencing method were also included in the comparison. The results obtained using identical viruses but different sequencing methods were notable. Not only did the PCR sequencing method deliver sequences of increased sequence length routinely, but also of superior quality. This is clearly illustrated in Fig. 2.4, where an identical SAT-2 type isolate is used for comparison of the two sequencing approaches. The large number of positions exhibiting secondary structure with the concomitant loss of resolution and readable sequence is striking with the direct RNA sequencing method. For this particular isolate, the RNA sequence method produced in the region of 156 nt of readable sequence with 8 ambiguous sites (5 %), whilst the PCR sequencing method generated 220 nt and 300 nt with sense and antisense primers, respectively. The sequences could be combined to deliver a total sequence length of 440 nt for this strain, with only two ambiguous sites (0.4 %) resulting. This improvement in sequence length and quality of the PCR over RNA approach was obtained for all viruses compared. In addition, the PCR method delivered sequence data for strains which could previously not be characterized because they were not amenable to direct RNA sequencing. These include, among others, the historically important South African cattle outbreak strain, PAL 5/83.

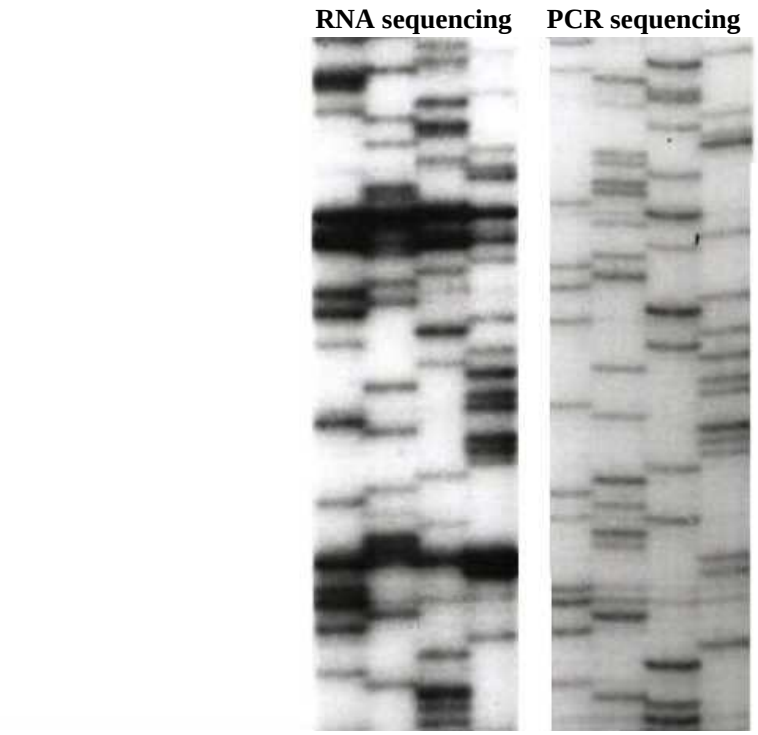

Fig. 2.4: Autoradiogram depicting differences in nucleotide sequencing results obtained with direct RNA sequencing versus PCR sequencing. Sequencing reactions were loaded ACGT, and the antisense primer P1 was used to generate sequences corresponding to the 3' end of the VP1 gene of the SAT 2 type strain (NAM/1/91/2), depicted here.

2.3.6 Phylogenetic relationships and intratypic variation

Between 417 and 507 nucleotides (nt) were obtained for at least one representative of each of the 7 FMDV serotypes, by sequencing the amplification products of the 2B+VP1Ub or P1+VP1Ub primer pairs. The SAT-3 nucleotide sequence of the BEC 1/65 laboratory strain has 97.1 % sequence identity with the published sequence of this virus, over the 450nt compared (Brown *et al* 1989). The Asia-1 deduced amino acid sequence of isolate PAK 1/54 has 100 % identity with the Palmenberg (1989) Asia-1 VP1 sequence. 493nt of PAK 1/54 corresponding to the carboxy-terminal region of VP1, the entire 2A and a partial 2B sequence have been submitted to Genbank under accession number AF024509, but have not been included in the phylogenetic analysis. Likewise, the 417nt sequence corresponding to 1D (3' end) and 2A (5' end) of isolate C_1 Noville has been submitted to Genbank (Accession Number AF024510) and displays >99 % sequence identity with the M90379 Genbank sequence of this strain (Martinez *et al.* 1992).

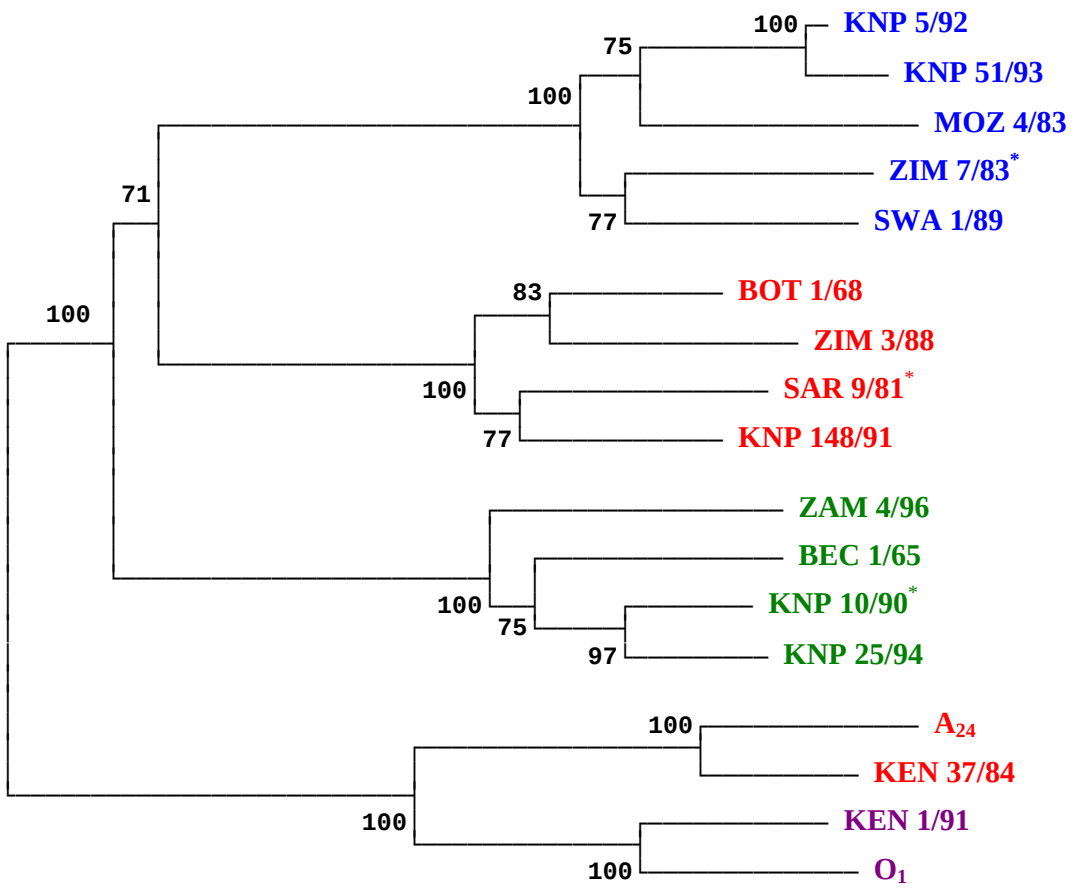

Fig. 2.5: Neighbor-joining tree depicting phylogenetic relationships of southern African FMDV serotypes based on partial amino acid sequences of the VP1 protein. SAT-1 viruses are denoted in red, SAT-2 in blue and SAT-3 in green. Kenyan serotypes O (KEN 1/91) and A (KEN 37/84), indicated in purple and orange respectively, and published sequences of types O and A were included in the analysis. P-distances were used to estimate genetic distance and 1000 bootstrap replications were applied. * Indicates Onderstepoort Veterinary Institute (OVI) vaccine strains

TABLE 2.4 Details of the 15 isolates for which sequencing data was generated for phylogenetic inference purposes

Laboratory name	FMDV Serotype	Country	Animal origin	Year of isolation	Total nucleotide sequence length	Amino acid sequence length used for phylogenetic inference	Genbank accession number
KEN 37/84*	A	Kenya	Bovine	1984	507nt	125aa (375nt)	AF023526
KEN 1/91*	O	Kenya	Bovine	1991	497nt	126aa (378nt)	AF023527
BOT 1/68*	SAT-1	Botswana	Bovine	1968	461nt	132aa (396nt)	AF023524
SAR 9/81*	SAT-1	South Africa	Impala	1981	457nt	132aa (396nt)	AF023514
ZIM 3/88*	SAT-1	Zimbabwe	Buffalo	1988	456nt	132aa (396nt)	AF023515
KNP 148/91	SAT-1	South Africa	Buffalo	1991	456nt	132aa (396nt)	AF023513
MOZ 4/83@	SAT-2	Mozambique	Bovine	1983	436nt	128aa (384nt)	AF023519
ZIM 7/83@	SAT-2	Zimbabwe	Bovine	1983	448nt	128aa (384nt)	AF023523
SWA 1/89*	SAT-2	Namibia	Buffalo	1989	438nt	128aa (384nt)	AF023520
KNP 5/92	SAT-2	South Africa	Impala	1992	436nt	128aa (384nt)	AF023518
KNP 51/93	SAT-2	South Africa	Impala	1993	418nt	128aa (384nt)	AF023516
BEC 1/65*	SAT-3	Botswana	Bovine	1965	450nt	130aa (390nt)	AF023521
KNP 10/90	SAT-3	South Africa	Buffalo	1990	449nt	130aa (390nt)	AF023517
KNP 25/94	SAT-3	South Africa	Buffalo	1994	456nt	130aa (390nt)	AF023522
ZAM 4/96*	SAT-3	Zambia	Buffalo	1996	449nt	130aa (390nt)	AF023525

Total nucleotide (nt) sequence length obtained for each strain and amino acid (aa) sequence length ultimately used for phylogenetic reconstruction are indicated The overlapping VP1 gene amino acid sequences used for gene tree construction have been submitted to Genbank, under the accession numbers indicated above. Virus isolation on specimens obtained from the Kruger National Park (KNP) were performed at the Onderstepoort Veterinary Institute, with viruses obtained from alternative sources being denoted as follows: * World Reference Laboratory, Pirbright; @ Botswana Vaccine Institute

Intratypic amino acid variation levels were highest in the SAT-types, where the total percentage of variable sites (sites not completely conserved across the region sequenced) for SAT-1, -2 and -3 was 25.4 %, 27.5 % and 24.1 % respectively for the isolates used in this analysis (Table 2.4). Kenyan A and O types were compared with non-African isolates of the same type, yet displayed lower levels of intratypic variation. Total amino acid sequence variation was 12 % for each of the A and O type groupings included here. Overall, amino acid variation was 60.1 % for the 13 SAT-types and 34.7 % for the 4 European serotypes used in the phylogenetic analysis.

In the SAT-2 cluster (Fig 2.5), the Namibian (SWA/1/89) and Zimbabwean (ZIM/7/83) isolates form a distinct and separate grouping (77 % bootstrap support) from the southern isolates which originate from Mozambique and South Africa (75 % bootstrap support). As bootstrap proportions ≥70 % generally correspond to a >95 % probability that the corresponding cluster is meaningful (Hillis & Bull 1993), the structuring within the SAT-types is considered to be statistically well supported with distinct northern and southern groupings being observed for all three types.

2.4 Discussion

Initial screening of the two available primer pairs at the OVI revealed a low recognition for SAT-type viruses when structural proteins were targeted. This was due to the high variation in the structural proteins and to poor primer design as both the published W-US primer (Vosloo *et al.* 1996) and L-US (Höfner *et al.* 1993) have 3' terminal nucleotides which correspond to highly variable third base amino acid positions. This emphasizes the importance of critically evaluating published primer sequences prior to synthesis. In this case it necessitated the design of new VP1 gene-targeting primers. Five different primer pairs were subsequently synthesized, but SAT-type recognition with these primers was low. The primer pair initially shown to have a 66.7 % recognition capability for 6 SAT-type strains was later shown to have an even lower detection capability (52 %) for these serotypes when it was tested against 21 field and outbreak strains originating from various sub-Saharan African countries. Initial modification to the primer (VP1Ua) led to a 34 % increase in detection ability, with additional adjustments (VP1Ub) resulting in 100 % recognition for all SAT-types tested. In addition to improving SAT-type recognition, the optimized primer pair (P1+VP1Ub) resulted in a significant improvement in detection of all seven FMDV serotypes. This method was shown to be theoretically capable of detecting <5 PFU and <1 PFU when applied to plaque tirated

tissue culture and diluted cDNA, respectively. This discrepancy in sensitivity could be related to inefficiencies in cDNA synthesis in the presence of limited amounts of viral RNA in the diluted cell culture infected samples. At its least sensitive, the method described here displays a 10-fold increase in sensitivity over that reported for PCR by Rodriguez and co-workers (1994). This in turn translates into a 500-5000 fold increase in sensitivity over conventional indirect ELISA (Rodriguez *et al.* 1994). The wide detection capabilities of the optimized primers were confirmed by testing them against various sub- and topotypes of each of the serotypes. Specificity of the primers for exclusive FMDV genome recognition and amplification was confirmed by the failure to amplify genetically and symptomatically related picornaviruses under optimized reaction conditions.

Sequencing of the amplified fragment of expected size confirmed the specificity of the primers for the VP1 gene of all seven serotypes and consistently produced in excess of 400nt of sequencing data for each of the isolates amplified. This increased sequence length significantly improves phylogenetic resolution of viral strains (Bastos & Thomson 1995), with most clusters being statistically well-supported (Fig. 2.5). European type A and O strains originating from Kenya were clearly and correctly typed by this sequence analysis and reconstruction method. The PCR-based approach also overcomes restrictions on sequence length previously imposed by direct RNA sequencing of the viral genome as well as the need to obtain highly concentrated and purified viral RNA prior to sequencing (Knowles & Samuel 1994).

Nucleotide intratypic variation levels were 34 %, 40.4 % and 36.1 % for SAT-1, -2 and -3 respectively and exceeds the maximum level of variation previously reported for these serotypes (Vosloo *et al*., 1995) by more than 10 %. As the increased sequence length obtained by the PCR approach includes amino acid data on both the G-H loop and C-terminus immunodominant sites and corresponds to approximately 60 % of the total VP1 gene sequence, it is considered more representative and therefore a more accurate estimate of the true VP1 gene intratypic variation for the SAT-types. The lower intratypic variation levels observed here for the European serotypes may in part be attributed to the inclusion of fewer isolates in the analysis than was the case for the SAT-types.

The FMDV detection and characterization approach outlined here has been useful for determining the origin of current field strains and for tracing the course of epizootics in impala in South Africa (Keet *et al.* 1996). In addition to supplying useful information linking current field and outbreak isolates, these primers can potentially be used in retrospective studies on historical outbreak strains as they were shown here to successfully amplify isolates obtained over a 33 year period (1965-1996).

Of the published universal FMDV primers, few fulfil the requirement of both detection and characterization. Primers have been described which detect all seven serotypes, but which are serotypically non-informative because they target non-structural genes (Meyer *et al.* 1991; Laor *et al.* 1992). Alternatively, primers have been described which target the capsid coding region (Höfner *et al.* 1993) and are therefore potentially useful for molecular epidemiological studies, but have limited recognition capabilities for SAT-types. Primers are also available which amplify products of under 350bp from structural coding genes (Amaral-Doel *et al.* 1993). Sequencing of such products results in under 300nt of sequence which is far less than that obtained with the optimized primers presented here. The identification of a primer pair which amplifies all seven serotypes, has a wide recognition range for the many sub- and topotypes occurring within a serotype and whose product is amenable to sequencing, is critical to effective and rapid identification and characterization of field and outbreak strains of FMDV. The primer pair described here is capable of detecting FMDV within 6 hours of receiving a sample and allows for accurate genetic characterization on the basis of nucleotide sequencing within 48 hours of receiving a positive sample. This is a significant improvement on the results obtained by direct RNA sequencing of semi-purified virus which requires 3-4 days for primary virus isolation, adequate virus growth, purification and quantification with a further 2 days needed for sequencing, X-ray exposure and phylogenetic reconstruction. A minimum time lapse of 5-6 days was experienced before results were available. By comparison, direct PCR sequencing consistently delivered superior quality results in a third of the time. In addition, the PCR sequencing approach was capable of generating data for all isolates tested, with only isolations of mixed serotypes presenting problems. This is a significant improvement on the previously used direct RNA sequencing approach.

Although the VP1 gene primers described here amplify all SAT-type viruses tested, they do not meet the requirement of universal FMDV detection, as one of the Kenyan Type O viruses (KEN/1/91) could not be amplified with this primer pair. Given the extensive intratypic variation FMDV serotypes and the limited amount of published full length nucleotide sequence data available for the SAT-types (Brown *et al*. 1989; van Rensburg & Nel 1999), this is not surprising. Clearly, the designing of universal FMDV structural gene primers is problematic and can only be adequately addressed by intensive genetic characterization of SAT and Asia serotype P1 genome regions. Despite being unable to amplify all virus types, these primers significantly improve the sensitivity of FMD recognition for divergent SAT-type field and outbreak strains and clearly fulfil the requirements of SAT-type detection and characterization better than previously described methods.

Chapter 3

Investigating the possibility of sexual transmission of foot-and-mouth disease in African buffalo (*Syncerus caffer*)

Summary

Twenty free-living African buffalo from the Kruger National Park in South Africa were sampled to investigate the possibility of sexual transmission of foot-and-mouth disease virus (FMDV) in this wildlife species. Throat scrapings (probangs), sheath wash and sperm rich samples were collected and examined for the presence of virus and serum was tested for antibodies to the endemic SAT-type viruses.

SAT-3 type virus was isolated from semen and sheath wash specimens of one of the 20 buffalo sampled, and from probang specimens of two additional buffalo. The VP1 gene of these isolates was amplified and sequenced, confirming the SAT-3 type and a close genetic relationship between these viruses. Although the remaining samples were all negative on pig kidney tissue culture isolation, viral RNA could be detected in the sheath wash specimen of an additional buffalo by means of the polymerase chain reaction (PCR).

Circumstantial indicates that the animal from which the positive sheath wash and semen virus isolations was obtained was unlikely to be in an acute stage of infection and presents the possibility of FMD viral persistence in the reproductive system of buffalo bulls. This finding has implications for breeding programmes intending to make use of artificial insemination methods, as sexual transmission between persistently infected and FMD-free buffalo is a distinct possibility. In addition, it supports circumstantial evidence that foot-and-mouth disease may be sexually transmitted between African buffalo and cattle.

Parts of the results presented here have been published in the *Veterinary Record* **145**: 77-79 (1999)

3.1 Introduction

The persistence of FMDV in cloven-hoofed animals was first recognized in 1931, when virus was isolated 246 days post-infection (p.i.) from cattle that had recovered from the disease (Waldmann *et al.* 1931). Subsequent studies confirmed this observation and identified the pharynx as the most frequent site of virus recovery from persistently infected cattle (Van Bekkum *et al.* 1959; Sutmoller & Gragero 1965; Burrows 1966). A carrier animal is defined as one from which FMDV can be isolated from the oesophageal-pharyngeal fluids for longer than 28 days p.i. (Kitching 1992). Although FMD affects up to 70 species of artiodactyls (Woodbury 1995), few of these animals become persistently infected (Table 3.1). Even fewer of these animals are considered to be carriers in the epidemiological sense of the word, namely, that they should not only maintain the virus, but should also be able to transmit it (Thomson 1996).

Table 3.1: Summary of some persistently infected livestock and wildlife species

Species/Animal	Duration of viral persistence	Reference
Goats	2-3 months	Singh 1979 Anderson *et al.* 1976
Sheep	9-12 months	Burrows 1968 McVicar & Sutmoller 1969
Cattle	2.5 to 3.5 years	Hedger 1976 Hargreaves 1994
Wildebeest (*Connochaetes taurinus*)	28 days	Anderson *et al.* 1975
Sable (*Hippotragus niger*)	28 days	Ferris *et al.* 1989
Eland (*Taurotragus oryx*)	32 days	Anderson 1980
Fallow deer (*Dama dama*)	63 days	Forman *et al.* 1974
Kudu (*Tragelaphus strepiceros*)	104-160	Hedger 1972
Water buffalo (*Bubalus bubalis*)	2-24 months	Moussa *et al.* 1979
African buffalo (*Syncerus caffer*)	5 years	Condy *et al.* 1985

Free-living African buffalo are efficient maintenance hosts of the three SAT-types of FMDV in southern Africa (Condy *et al.* 1985). The historical association between the distribution of buffalo and areas in southern Africa prone to outbreaks of FMD, provided circumstantial evidence for the involvement of buffalo in the transmission of the disease to cattle (Condy 1979; Thomson 1995). Attempts at experimental transmission of virus between persistently infected (carrier) buffalo and susceptible cattle have however produced erratic results. Carrier buffalo and cattle were sometimes in direct or close contact for long periods of time without transmission occurring (Condy & Hedger 1974; Anderson *et al.* 1979; Bengis *et al.* 1986; Gainaru *et al.* 1986). Conversely, transmission sometimes occurred after protracted contact (Dawe *et al.* 1994b; Vosloo *et al.* 1996) and in these instances it was shown unequivocally by nucleotide sequencing of the viruses involved, that the buffalo were the source of the infection for cattle. This inconsistency in the ability of carrier buffalo to transmit SAT viruses is difficult to explain, there presently being no indication as to the mechanism involved in FMD transmission by persistently infected ruminants (Thomson 1996).

There is anecdotal evidence from Zimbabwe and South Africa, that inter-species breeding is attempted when male buffalo and domestic cows are held on ranches together (S. Hargreaves & H. Bertschinger, personal communication). Furthermore, the unsuccessful transmission experiments (*vide supra*), involved steers as the indicators of transmission while in those where FMD transmission did occur, male buffalo and female cattle were present together (Hedger & Condy 1985; Dawe *et al.* 1994b; Vosloo *et al.* 1996). Of interest in the latter two experiments is that transmission from buffalo to cattle occurred 5 months post-infection in the Zimbabwe experiment (Dawe *et al.* 1994b), whilst it took approximately 11 months post-infection in the South African experiment (Vosloo *et al.* 1996). One of the notable differences between these two investigations is that the male buffalo in the former experiment were between 2-4 years of age, whilst in the latter they were 15-20 months. Taylor (1985) showed that spermatogenesis commences at 2.5 years of age and that both seminiferous tubule diameter and testes mass increases from August and peaks during and just prior to the breeding season which runs from December to May in most southern African countries (Pienaar, 1969a; Carmichael *et al.* 1977; Brown *et al.* 1991) . It is therefore noteworthy that transmission in both cases occurred in the month of December, at which time sexually mature buffalo were present and at a time which coincides with the start of buffalo breeding season. These factors when taken together have led to the supposition that sexual activity could explain erratic transmission of FMD between buffalo and cattle.

This chapter describes the initial appraisal of the possibility of sexual transmission between buffalo and cattle through the collection of specimens from the uro-genital tracts of buffalo bulls and the examination of these samples for the presence of FMD virus or viral RNA using the polymerase chain reaction (PCR).

3.2 Materials and Methods

3.2.1 Sample collection

Twenty male buffalo were captured in the Shingwedzi and Langtoon Dam areas of the Kruger National Park in May, 1997 for relocation purposes. Serum, oesophageo-pharyngeal (Sutmoller & Gragero 1965), sheath-wash and semen (Bertschinger 1996) specimens were collected. Buffalo were checked for clinical signs of infection and age was estimated on the basis of eruption sequence and wear of the teeth (Pienaar *et al.* 1969a, Grimsdell 1973; Sinclair 1977).

3.2.2 Serology and virus isolation

Serum antibody levels were estimated by a liquid phase blocking ELISA (Hamblin *et al.* 1986; Hamblin *et al.* 1987). Primary pig kidney (PK) cells were inoculated with 10% suspensions prepared from the probangs, semen and sheath-wash samples. Test tubes were incubated at 37°C and examined daily for the development of cytopathic effect (CPE). Three blind passages of probang, sheath wash and semen samples were performed. 19 probang samples and 20 sheath wash and semen samples were ultimately screened. Positive samples were typed using a sandwich ELISA against the three SAT-types (Esterhuysen *et al.* 1985; Esterhuysen 1994).

3.2.3 VP1 gene amplification and sequencing

Detection of FMD viral RNA was attempted on the 10% suspensions prepared for each of the sheath wash and sperm-rich specimens collected, using the methods described in Chapter 2. In addition, the genomic RNA of viruses isolated on primary pig kidney cells was extracted, reverse transcribed and amplified (Bastos 1998a). Characterization was performed by nucleotide sequencing of the amplified product and phylogenetic analysis of the VP1 gene, as previously described in Chapter 2.

3.3 Results

3.3.1 Serological status of buffalo

FMDV infection rates of buffalo with the three endemic SAT-types was confirmed by antibody detection. Sera of 19 of the total of 20 buffalo sampled, were tested and 100% seropositivity to SAT-1 and SAT-3 virus types with a cut-off of log $10^{1.6}$ (Table 3.2) was found. All buffalo sera tested, with the exception of one animal were positive to SAT-2. None of the buffalo showed any signs of clinical infection.

3.3.2 Virus isolation and partial VP1 gene characterization

The results of virus isolation attempts on the specimens collected (shown in Table 3.3) indicates that two buffalo had positive probang specimens. Isolation attempts with uro-genital specimens of these animals, was however negative. Semen and sheath-wash specimens from a different buffalo (Buffalo 3) contained SAT 3 virus but as no probang specimen was collected from this animal, presence of virus at the usual site of persistent infection could not be determined.

Table 3.2: Serum antibody titres (expressed as the reciprocal log value) of KNP buffalo bulls sampled in May 1997

Buffalo No	Age	Date	Locality	SAT-1	SAT-2	SAT-3
1	A	13/5/97	Mashagodzi	2.7	1.8	2.5
2	A	13/5/97	Mashagodzi	2.4	2.2	2.4
3	A	13/5/97	Ndlalophini	1.9	2.0	1.6
4	A	13/5/97	Ndlalophini	2.4	2.0	2.1
5	A	13/5/97	Ndlalophini	2.4	2.3	2.2
6	A	13/5/97	Ndlalophini	2.6	2.3	2.9
7	A	13/5/97	Ndlalophini	2.4	1.6	1.6
8	A	13/5/97	Ndlalophini	2.7	2.1	2.4
9	A	14/5/97	Tsamane	2.1	2.3	1.9
10	A	14/5/97	Tsamane	2.7	1.4	2.3
13	A	14/5/97	Tsamane	2.4	2.5	2.5
14	SA	14/5/97	Tsamane	2.5	2.3	1.8
15	A	14/5/97	North of Dipene	NS	NS	NS
16	A	14/5/97	North of Dipene	2.6	2.9	2.3
1	3y	15/5/97	Langtoon Dam	2.7	2.3	2.7
2	3y	15/5/97	Langtoon Dam	1.8	2.7	1.7
3*	3.5y	15/5/97	Langtoon Dam	3.1	1.8	2.1
4*	3y	15/5/97	Langtoon Dam	3.1	1.9	3.0
5*	3.5y	15/5/97	Langtoon Dam	3.0	3.0	3.1
6	3.5y	15/5/97	Langtoon Dam	3.1	3.0	3.0

NS: no specimen; y: years; A: adult (>4 years); SA: sub-adult (2-4y); * Positive tissue culture isolation

In order to preclude laboratory contamination independent virus isolations were performed. In addition, each of the PCR positive products obtained from sheath wash and sperm specimens and from the PK virus isolations made from these samples was characterized by nucleotide sequencing. Identical sequencing results were obtained for all positive PCR positive uro-genital tract specimens of buffalo 3.

Sheath wash and semen specimens of all buffalo sampled were examined for the presence of viral RNA by VP1 gene amplification. All samples which were positive on tissue culture (TC) isolation were also positive on PCR. In addition, a TC-negative sheath wash specimen of Buffalo 2 (sampling date: 15/5/97) was shown to contain viral RNA (results not shown).

TABLE 3.3: Virus isolation and nucleotide sequencing results of SAT-3 type foot-and-mouth disease virus obtained from buffalo bulls sampled in the Kruger National Park

Buffalo		**Virus isolations & VP1 gene nucleotide sequencing**					
No	Age	Specimen type	Isolate name	Sampling date	Isolation date/s	Sequence length	Genbank accession No
3	3.5 y	Sperm	KNP/1/97/SP	15/5/97	25/9/97 1/10/97	449 nt	AF047481
3	3.5 y	Sheath wash	KNP/1/97/SW	15/5/97	15/9/97 1/10/97	449 nt	AF047482
4	3 y	Probang	KNP/2/97/P	15/5/97	1/10/97	438 nt	AF137399
5	3.5 y	Probang	KNP/3/97/P	15/5/97	1/10/97	438 nt	AF137400

y: years, SP: sperm, SW: sheath wash, P: probang, nt: nucleotides, KNP: Kruger National Park

The virus isolations from sheath-wash and semen specimens of Buffalo 3 were designated KNP/1/97/SW and KNP/1/97/SP respectively and their VP1 gene sequences were shown to be identical to each other, yet unique when compared with sequences of 70 SAT 3 viruses in the Onderstepoort Veterinary Institute (OVI) database. The phylogenetic results (Fig. 3.1) reveal that the sperm and sheath wash strains are most closely related to four other viruses recovered from buffalo probangs.

Two of the related viruses were recovered from buffalo sampled from the same herd and on the same day (KNP/2/97 and KNP/3/97), whilst the remaining two viruses were obtained from the same locality 14 months earlier. All nucleotide and amino acid sequences used in this study have been submitted to Genbank under the accession numbers indicated in Table 3.3.

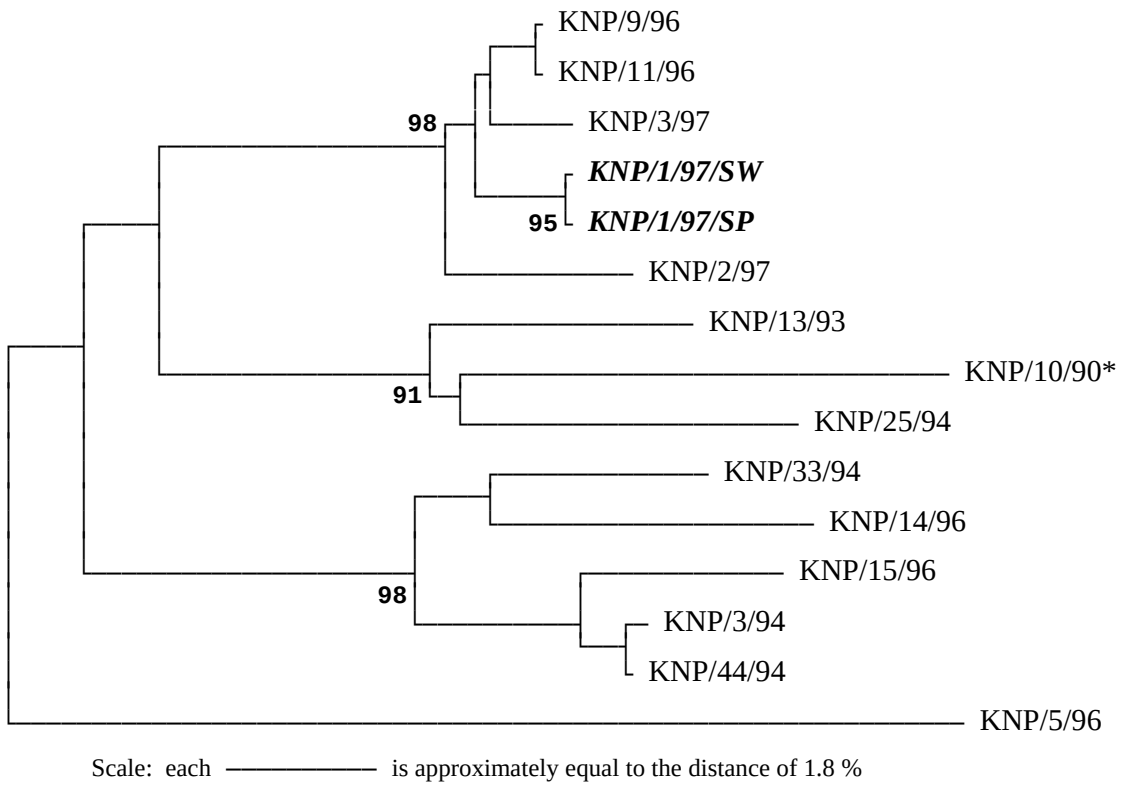

Fig. 3.1: SAT-3 neighbor-joining tree depicting VP1 gene relationships of buffalo isolates (1990-1997) from the Kruger National Park. Identical viruses obtained from buffalo sheath wash and sperm are designated KNP/1/97/SW and KNP/1/97/SP respectively (indicated in bold and italic). Bootstrap values, expressed as a percentage and based on 1000 reiterations, are indicated next to the corresponding clusters. * Denotes Onderstepoort Veterinary Institute (OVI) vaccine strain

3.4 Discussion

This study describes the recovery of FMD virus from the reproductive tract of a 3.5 year old male buffalo in the Kruger National Park. From the twenty buffalo sampled, virus was isolated from semen of one buffalo and from probang samples of two buffalo. Although the isolation rate from semen collected from buffalo was relatively low (5 %) compared to that usually obtained with buffalo probang specimens (Hedger 1976), it most likely reflects sub-optimal sampling and/or isolation, as only two isolations were made from probangs obtained from the same animals (10 %). Recovery of foot-and-mouth disease virus (FMDV) from semen or reproductive tracts of acutely infected cattle (Grunnet 1950; Cottral *et al.* 1968) and buffalo is well documented (Gainaru *et al.* 1986), but the possibility of viral persistence in semen of African buffalo has until now not been reported. There are however reports of virus recovery from clinically normal, domestic bulls in Brazil (Pustiglione-Netto 1971), pointing to the possibility that the virus may persist in the reproductive tract of this known carrier species. No follow-up studies to this work have unequivocally confirmed this.

Genetic characterization of buffalo sheath wash and semen viruses described here, and comparison with other field strains, revealed that the buffalo from which these specimens were obtained was unlikely to have been recently infected as the SAT-3 virus as the sequence differs by 2.9 % on nucleotide level when compared to phylogenetically related viruses (KNP/2/97 and KNP/3/97). Previous studies have shown that buffalo calves which are experimentally or naturally infected initially have identical viral genomes, which become more diverse over time (Bastos unpublished results 1996 & 1998; Vosloo *et al.* 1996). In addition, the age of this animal argues against a recent infection as >95% of buffalo are serologically positive to all SAT types by the age of 2 years (Thomson *et al.* 1992) and this animal was estimated to be 3.5 years of age.

Although it is improbable that the buffalo in this preliminary survey was recently infected, the results are equivocal. It is clinically and serologically difficult to determine when an African buffalo may have been infected, as conventional methods such as virus isolation, serum antibody levels and clinical signs of infection cannot be used as indicators of recent infection in buffalo. Buffalo generally do not show clinical signs of infection and virus can be recovered at regular intervals from carriers which have not been reinfected over a 2 year period. In addition, buffalo can maintain high levels of antibodies, without being reinfected (Condy & Hedger1974). The interpretation of field data is further exacerbated by the phenomenon of ‘original antigenic sin’ (Gainaru *et al.* 1986), where the types against which sera show the highest antibody titres are not necessarily those which most recently caused infection. This is illustrated in the results presented here where buffalo 3 and 4 have higher titres against SAT-1 than against SAT-3, yet a SAT-3 type virus was recovered from both animals.

Disease-free buffalo are an extremely scarce and expensive commodity (Winterbach 1998). Most populations of buffalo in sub-Saharan Africa have high sero-prevalence rates to FMDV, with the exception of two populations in South Africa (Esterhuysen *et al*. 1985). The KNP buffalo, although phenotypically superior and genotypically more diverse than their disease-free counterparts (O'Ryan *et al.* 1998), are undesirable because of their FMD and Corridor disease status. Attempts to boost the phenotypically inferior populations will therefore have to rely on artificial breeding methods because of the risks associated with KNP buffalo (Bertschinger 1996). Cottral and co-workers (1968) showed that artificial insemination of cattle with semen from FMD infected bulls resulted in a 31% FMD infection rate in the heifers. The transmission of FMD via artificial insemination with semen of KNP buffalo is thus a distinct possibility. Attempts to remove or inactivate virus present in cattle semen have previously proved unsuccessful. Virus titres of semen from infected bulls did not decrease during storage at -50°C, over a period of 320 days. In addition, the treatment of washed spermatozoa with acid solution, did not eliminate FMDV (Cottral *et al.* 1968). As no method exists which effectively and reliably eliminates virus, the potential persistence of virus in semen of carrier buffalo, will exclude artificial insemination methods involving KNP buffalo. Alternatives such as embryo transfer, which has been quantitatively shown to have a negligable risk (less than 1 in 100 billion) of FMD transfer in cattle (Sutmoller & Wrathall 1997), should be investigated.

The isolation of virus from semen of African buffalo within the endemic FMD area in South Africa appears not only has implications for buffalo breeding programmes, but also provides the first evidence for the possibility of sexual transmission as a means of transmitting virus from the maintenance host species. This is of epidemiological significance as the mechanism of transmission between buffalo and cattle could not be established, although a number of possibilities have been investigated (Bengis *et al.* 1986; Gainaru *et al.* 1986). It does however shed some light on the observation that transmission of virus from buffalo to cattle, under experimental conditions, occurred only when buffalo and cattle were not of the same sex. It is important to establish whether virus may be recovered from semen in acutely infected buffalo only, or whether virus persists past the stage of acute/recent infection. Future studies should therefore concentrate on extensive sampling of possible sites of persistence in the male and female reproductive systems and should incorporate the sensitivity of PCR detection in clinical specimens (Prato-Murphy *et al.* 1994) in order to characterize viruses by nucleotide sequencing and to clarify the incidence of this preliminary finding in other naturally infected buffalo.

Chapter 4

Natural transmission of foot-and-mouth disease virus between African buffalo (*Syncerus caffer*) and impala (*Aepyceros melampus*) in the Kruger National Park, South Africa

Summary

VP1 gene sequences of SAT-2 type foot-and-mouth disease (FMD) virus isolated from impala and African buffalo in the Kruger National Park (KNP), were used to determine intra- and interspecies relationships of viruses circulating in these wildlife populations. In this way, five distinct lineages of SAT-2 virus were identified in routine sampling of oesophageo-pharyngeal epithelium from buffalo between 1988 and 1996. The different lineages were each found to be associated with a discrete geographic sampling locality. The results presented here clearly show that over the period 1985-1995, four unrelated epizootics occurred in impala in defined localities within the KNP. Evidence for natural transmission of FMD virus between buffalo and impala is presented for the most recent 1995 outbreak, with data linking the 1985 and 1988/9 impala epizootics to viruses associated with specific buffalo herds. Circumstantial evidence indicates that juvenile buffalo infected for the first time are the most likely source of infection for impala and that conditions driving these species to share habitats are likely to facilitate inter-species transmission.

Parts of the results presented here have been published in *Epidemiology and Infection*, **124**: 591-598 (2000)

4.1 Introduction

The Kruger National Park (KNP) is situated in the north eastern corner of South Africa (between 22°25' and 25°32' latitude south and 30°50' and 32°02' longitude east) and is bordered by Zimbabwe and Mozambique, to the north and east respectively. The KNP and surrounding area is the only region in South Africa where foot-and-mouth disease (FMD) occurs, with the remainder of the country being recognized as free of the disease by the O.I.E. (Office International des Epizooties). Since the last recorded outbreak of FMD in livestock in South Africa in 1983, the disease has been restricted to wildlife within the FMD control area (Records of the Directorate of Animal Health). Clinical disease has primarily involved impala (*Aepyceros melampus*), a medium sized antelope, but it is well recognized that infection with SAT-type viruses is endemic to African buffalo (*Syncerus caffer*) herds within the KNP.

Of the eighteen southern African wildlife species found to have antibodies to FMD (Condy *et al.* 1969; Keet *et al.* 1996), only the African buffalo has been shown to be an efficient maintenance host of the virus (Condy *et al.* 1985) and is considered the primary source of infection for domestic and wild ungulates. This is due to its ability to both maintain and transmit the disease (Thomson 1996), with transmission from carrier buffalo to cattle being demonstrated under both experimental and natural conditions (Hedger & Condy 1985; Dawe *et al.* 1994a; Dawe *et al.* 1994b;Vosloo *et al.* 1996). In contrast, evidence of field transmission between buffalo and other cloven-hoofed wildlife species is lacking despite the co-occurrence of buffalo and a variety of antelope in game parks throughout southern Africa and the known susceptibility of some antelope species to FMD virus (FMDV) infection (Hedger *et al.* 1972; Anderson *et al.* 1975).

In South Africa, the diagnosis of clinical FMD in impala has been regularly recorded since 1944 (Rossiter & Albertyn 1947; Meeser 1962; Vosloo *et al.* 1992; Keet *et al.* 1996). Over 90% of these outbreaks have occurred in the months of June to November within the southern region of the KNP. This area, south of the Olifants river (Fig. 1) coincides with the region of the KNP that is most densely populated by both impala and buffalo (Pienaar *et al.* 1966; Pienaar 1974). In addition, the months of June to August are generally rain-free and therefore a period of water scarcity, which leads to animals congregating at permanent water points (Keet *et al.* 1996). This is believed to facilitate disease transmission and is supported by the observation that some outbreaks in impala have spread along water courses (Vosloo *et al.* 1992). In addition to shared drinking water, mutual grazing of at least three grass species by impala and buffalo (Skinner & Smithers 1990) is another factor promoting selection of common environs.

Despite the high incidence of outbreaks in impala (Keet *et al.* 1996) and the active collection and characterization of viruses obtained from buffalo in recent years (Vosloo *et al.* 1995), direct evidence of natural transmission between these species is lacking. VP1 gene nucleotide sequence analysis has, however, been instrumental in determining the apparent unrelatedness of successive epizootics in impala (Vosloo *et al.* 1992; Keet *et al.* 1996). In a study of FMD outbreaks in impala from 1983 to 1989, Vosloo and co-workers (1992) showed that viruses isolated 6 months apart were derived from the same epizootic. A later study focussing on the 1992/3 epizootic in the KNP identified a potential circulation period of 12.8 months (Keet *et al.* 1996). Although these results infer the possibility of viral persistence in impala populations, other studies have shown experimentally, that infected impala develop clinical lesions and excrete virus, but that most do not become carriers (Hedger *et al.* 1972). This was confirmed in a separate study which showed that virus could not be isolated from any tissues of infected impala, 7 or more days after inoculation (Anderson *et al.* 1975). Given the low antibody levels of impala during inter-epizootic periods and their apparent inability to act as long term carriers, it is unlikely that these antelope are capable of maintaining the disease independently and therefore African buffalo are presumed to be the usual source of infection for impala (Keet *et al.* 1996).

In order to determine whether buffalo are indeed the source of infection for impala, a twelve year study period (1985 to 1996) was identified in which clinical FMD was diagnosed on six occasions in impala in the KNP (Fig. 4.1). The availability of impala isolates from these SAT-2 epizootics and buffalo field strains collected within the same time period, together with improved SAT-type genetic characterization methods (Bastos 1998a), prompted an investigation to determine whether inter-species transmission could be demonstrated.

4.2 Material & Methods

4.2.1 Viruses used in this study

Impala viruses used in this study originated from the southern and central region of the Kruger National Park, South Africa (Fig. 4. 1). Impala viruses were isolated on pig kidney cells (PK) from clinical FMD lesions recovered during the epizootics of 1988, 1989, 1992, 1993 and 1995. Tissue culture isolations of an impala virus from the 1985 outbreak was supplied by the World Reference Laboratory, Pirbright. Oesophageo-pharyngeal specimens (probangs) obtained from buffalo during routine surveys in the KNP from 1988 to 1996 (Fig. 4.2), were used as a source of viruses circulating in buffalo. The geographical and species origin of all viruses included in this study are summarized in Table 4.1.

TABLE 4.1: List of SAT-2 viruses of buffalo and impala origin (1985-1996) from the Kruger NP

Strain	Sampling locality	Grid Reference	Species of origin	Sampling date	Genbank Acc. No
KNP 1/85	Gudzani	31°50'E 24°15'S	Impala	21 NOV 1985	AF136986
KNP 7/88	Rietpan	31°58'E-24°54'S	Buffalo	8 JUL 1988	AF137000
KNP 8/88	Rietpan	31°58'E-24°54'S	Buffalo	8 JUL1988	AF137001
KNP 9/88	Shilolweni	31°50'E-24°48'S	Buffalo	27 MAY 1988	AF137002
KNP 10/88	Shilolweni	31°50'E-24°48'S	Buffalo	27 MAY 1988	AF137003
KNP 14/88	Ripape	31°37'E-24°44'S	Buffalo	19 OCT 1988	AF137004
KNP 16/88	Kingfisherspruit	31°26'E-24°27'S	Impala	10 OCT 1988	AF136987
KNP 17/88	Orpen	31°24'E-24°28'S	Impala	7 OCT 1988	AF136988
KNP 19/88	Rabelais Dam	31°30'E-24°27'S	Impala	11 OCT 1988	AF136989
KNP 20/88	Timbavati River	31°28'E-24°26'S	Impala	25 OCT 1988	AF136990
KNP 2/89	Ngotso	31°43'E-24°13'S	Impala	30 APR 1989	AF136991
KNP 19/89	Ripape	31°37'E-24°44'S	Buffalo	25 OCT 1989	AF137005
KNP 25/89	Matjipiri	31°36'E-24°47'S	Buffalo	24 OCT 1989	AF137006
KNP 40/89	Ripape	31°37'E-24°44'S	Buffalo	25 OCT 1989	AF137007
KNP 5/91	Satara	31°47'E-24°24'S	Buffalo	20 JUN 1991	AF137008
KNP 143/91	Reënvoël Dam	31°20'E-23°58'S	Buffalo	13 JUL 1991	AF137009
KNP 147/91	Reënvoël Dam	31°20'E-23°58'S	Buffalo	13 JUL 1991	AF137010
KNP 160/91	Ndziyospruit	31°34'E-23°59'S	Buffalo	18 JUL 1991	AF137011
KNP 183/91	Water Affairs Weir	31°56'E-25°08'S	Buffalo	24 JUL 1991	AF137012
KNP 1/92	Nsemani Dam	31°43'E-24°23'S	Impala	3 AUG 1992	AF136992
KNP 5/92	Timbavati	31°38'E-24°16'S	Impala	3 AUG 1992	AF136993
KNP 8/92	Shibotwana Dam	31°50'E-24°24'S	Impala	3 AUG 1992	AF136994
KNP 32/92	Boyela Vlakteplaas	31°17'E-22°54'S	Buffalo	22 JUN 1992	AF137013
KNP 9/93	Boyelaspruit	31°20'E-23°01'S	Buffalo	17 AUG 1992	AF137014
KNP 16/93	Capricorn	31°26'E-23°29'S	Buffalo	6 AUG 1992	AF137015
KNP 51/93	Tshokwane	31°51'E-24°47'S	Impala	26 AUG 1993	AF136995
KNP 18/95	Monzweni	31°38'E-24°34'S	Buffalo	16 NOV 1995	AF137016
KNP 31/95	Monzweni	31°38'E-24°34'S	Buffalo	16 NOV 1995	AF137017
KNP 43/95	Mbyamiti Mouth	31°46'E-25°18'S	Impala	29 NOV 1995	AF136996
KNP 44/95	Lwakahle Picket	31°41'E-25°23'S	Impala	2 DEC 1995	AF136997
KNP 49/95	Mbyamiti Mouth	31°46'E-25°18'S	Impala	2 DEC 1995	AF136998
KNP 52/95	Lwakahle Picket	31°41'E-25°23'S	Impala	2 DEC 1995	AF136999
KNP 6/96	Mahlanganzwane Dam	32°01'E-25°14'S	Buffalo	17 APR 1996	AF137018
KNP 24/96	Mulalanespruit	31°18'E-23°57'S	Buffalo	29 JUN 1996	AF137019

Fig. 4.1: Geographical origin of impala viruses isolated between 1985 and 1995

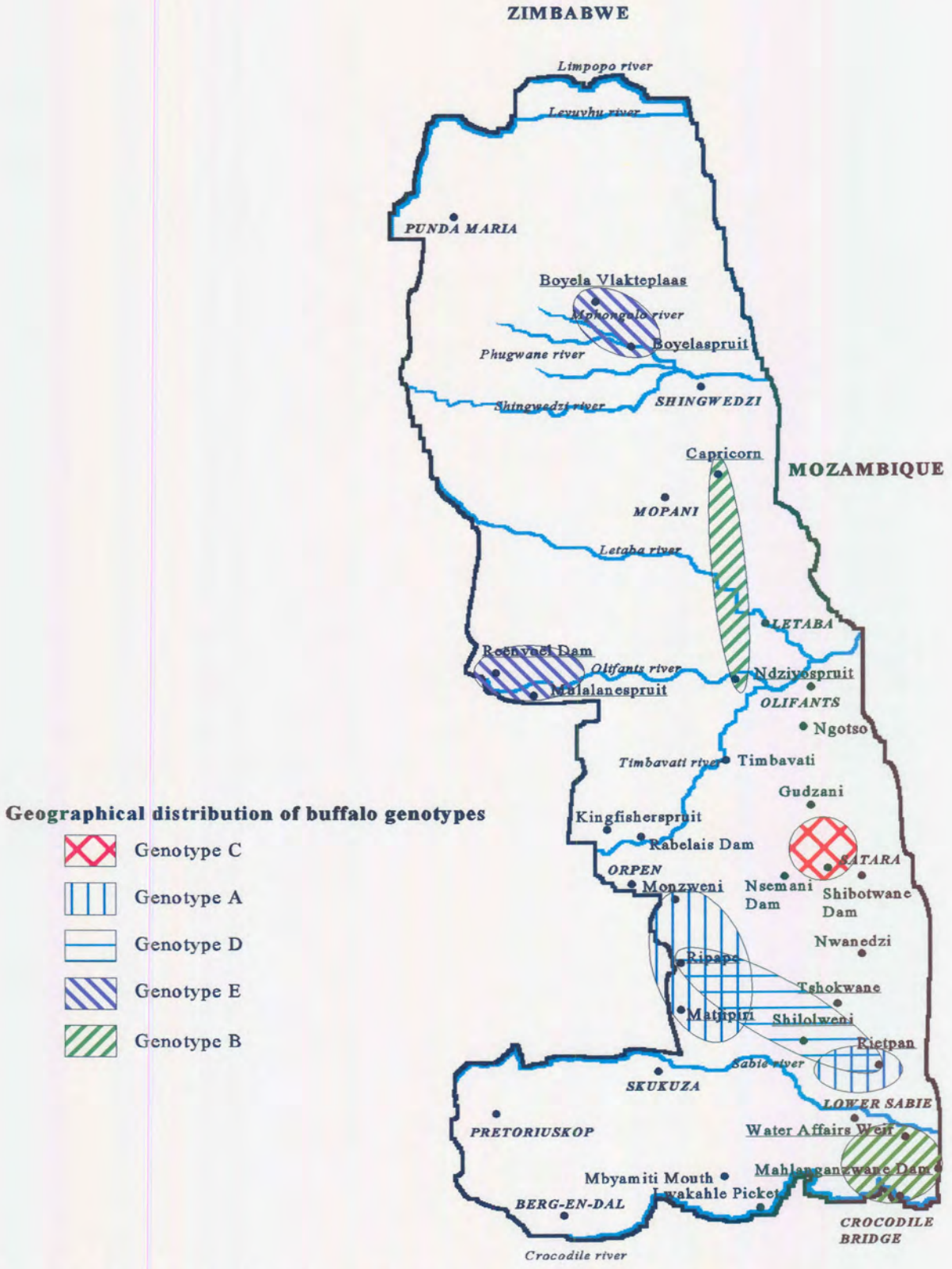

Fig. 4.2: Georgraphical origin and distribution of SAT-2 type buffalo viruses

4.2.2 Genetic characterization

Viral RNA was extracted from clinical specimens and tissue culture isolates of buffalo and impala viruses by a modified guanidinium thyocianate /silica method (Boom *et al.* 1990). A 500 bp fragment corresponding to the C-terminus region of the VP1 gene was amplified, purified and sequenced as described in Chapters 2 and 3. Nucleotide sequences were aligned (Harley 1994) and an homologous 414 nt sequence was identified and selected for further analyses. Pairwise comparisons of amino acid and nucleotide sequences of impala outbreaks strains and field strains of carrier buffalo were determined using the MEGA programme (Kumar *et al.* 1993). Various distance and parsimony methods included in MEGA were also used to construct VP1 gene trees. The cattle outbreak stain, ZIM/7/83 from Zimbabwe (Genbank accession No: AF023523) was selected as an outgroup for the phylogenetic analyses, due to it's distant geographic and genetic relationship to KNP viruses (Bastos 1998a). All nucleotide sequences used in this study have been submitted to Genbank under the accession numbers indicated in Table 4.1.

4.3 Results

4.3.1 Genetic relationships of impala viruses

Four genetically unrelated impala virus lineages were identified by VP1 gene sequence analysis (Fig. 4.3), each from a distinct geographical area indicated in the figure as follows:

(I) Gudzani Area (1985 epizootic)
(II) Orpen Area (1988/9 epizootic)
(III) Satara/Tshokwane Area (1992/3 epizootic)
(IV) Crocodile Bridge Area (1995 epizootic)

Pairwise comparisons of nucleotide and amino acid sequence data reveal that viruses of the same epizootic, sampled within a month of each other are identical or differ by less than 1 %. The 1988/9 and 1992/3 viruses, show marked differences on amino acid level over time. In the 6 months separating the 1989 virus from the 1988 viruses, there was a 1-2 % accumulation in mutations, whilst the 1993 impala virus differed by between 3 and 4 % from the 1992 viruses within a sampling time span of more than 12 months. In the most recent SAT-2 epizootic, the 1995 impala viruses differ from each other by 1 - 4 % on amino acid level, despite being sampled within a five day period. Nucleotide sequence identity (completely conserved sites) for the 14 impala viruses used in this study was 70.5 % across the 414 nt specified in this study. Amino acid sequence identity for the same region was 80.4 %.

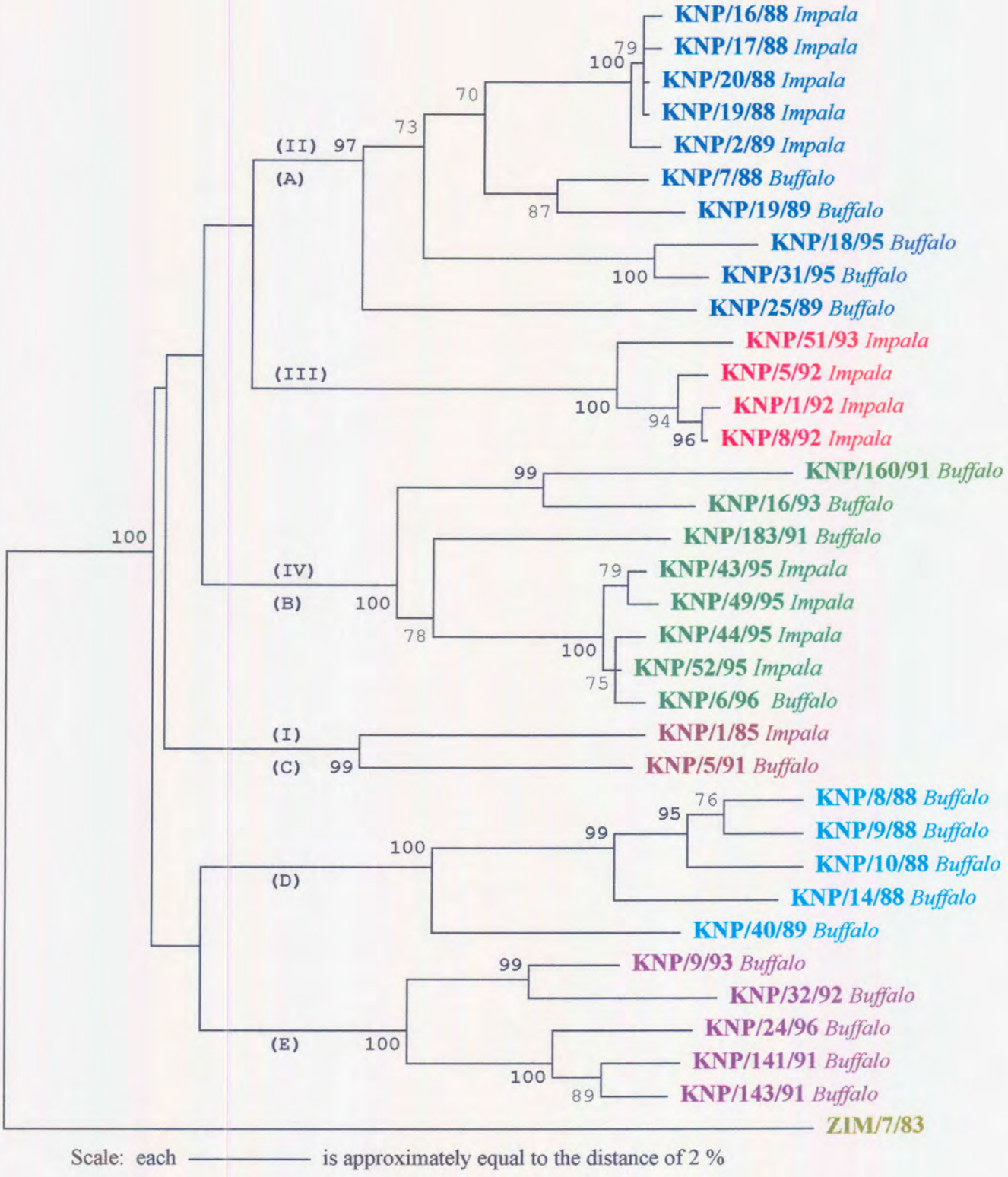

Fig. 4.3: Neighbor-joining tree depicting VP1 gene relationships of buffalo and impala viruses from the Kruger National Park (1985-1996). Bootstrap values indicated as a percentage were determined by 1000 replications.

4.3.2 Genetic relationships of buffalo viruses

VP1 gene trees of SAT-2 type FMDV were used to identify the major buffalo virus lineages within the Kruger National Park. Both distance methods (UPGMA and neighbor-joining) and character-based phylogenetic inference methods produced trees with identical topology (results not shown). The neighbor-joining tree depicted in Fig. 4.3 indicates that there are five distinct buffalo virus lineages (coded A-E) south of the Olifants river (Fig. 4.2), which cluster according to geographical origin in the following manner:

(A) Monzweni (1995), Ripape (1989), Rietpan (1988) and Matjipiri (1989) viruses
(B) Water Affairs Weir (1991) and Mahlanganzwane Dam (1996) viruses
(C) Satara (1991) virus
(D) Rietpan (1988), Ripape (1988 & 1989) & Shilolweni (1988) viruses
(E) Mulalanespruit (1996) virus

The results (Fig. 4.3) also show cases of co-circulation of two different SAT-2 genotypes within a single buffalo herd. This is demonstrated by the grouping of KNP/7/88 and KNP/8/88 with the 'A' and 'D' genotypes respectively, despite having been sampled on the same day and from the same herd. Similarly, the two Ripape viruses KNP/19/89 and KNP/40/89 sampled from the same herd on the 25th of October 1989, fall into two separate clusters. Furthermore, the geographical distribution of buffalo genotypes (Fig. 4.2) reveals that there are shared genotypes across the Olifants river. This is illustrated by the clustering of viruses from the Crocodile Bridge area (KNP/183/91 and KNP/6/96), which is in the southern part of the KNP, with the Capricorn and Ndziyospruit viruses (KNP/160/91 and KNP/16/93) to the north of the Olifants river. In addition, two Reënvoël Dam viruses (KNP/143/91 and KNP/147/91) sampled north of the Olifants river, group with a Mulalanespruit virus (KNP/24/96), sampled south of the river. These form part of a larger cluster (E) containing Boyela viruses (KNP/32/92 and KNP/9/93) sampled in the far north. The five buffalo virus clusters identified in Fig. 4.3 confirm the spacial rather than temporal grouping of FMD viruses, as exemplified by Cluster A (1988-1995) and Cluster B (1991-1996). Overall sequence identity (sites complete conserved across all sequences) for the 20 buffalo viruses used in this study was 59.9 % across the 414 nt regions specified in this study. Amino acid sequence identity for the same region was 74.6 %.

4.3.3 Inter-species relationships of impala and buffalo viruses

The combined buffalo and impala data-set was used to determine inter-species relationships of viruses (Fig. 4.3). Statistically supported phylogenetic clustering for three of the four impala epizootics with specific buffalo lineages, is clearly demonstrated. The

1985 impala strain groups with buffalo virus KNP 5/91 (99% bootstrap support) and the 1988/89 outbreak strains cluster with 1988, 1989 and 1995 buffalo viruses from Rietpan, Ripape and Mondzweni (97% bootstrap support). The 1995 impala viruses from the Crocodile Bridge region are almost identical to a 1996 buffalo virus obtained 4 months later from the same locality (100% bootstrap support). The impala epizootic of 1992/93 did not group with any of the buffalo viruses sampled between 1985 and 1996. Overall nucleotide sequence identity for the 34 Kruger National Park viruses used in this study was 57.2 % across the 414 nt region specified, with amino acid sequence identity being 69.6 %.

4.4 Discussion

The nucleotide sequence data from this study indicate that four unrelated SAT-2 epizootics occurred in impala the KNP from 1985 to 1995. The similarity in nucleotide sequence (>99 %) between impala viruses from the 1995 epizootic and a virus of buffalo origin obtained four months later within the area of the outbreak provided the first evidence for natural transmission between these two wildlife species. Similarly, evidence linking the impala epizootics of 1985 and 1988/89 to distinct buffalo genotypes can be deduced from the phylogenetic analyses of the VP1 genotypes of the viruses involved. One of the impala outbreaks, which occurred in 1992/93 could however not be linked to any buffalo field strains.

The high nucleotide sequence identity (>99%) between the 1995 impala viruses and the 1996 buffalo virus is significant, especially in view of the extent of the genetic variation in the maintenance host species. Amino acid sequence identity of the twenty buffalo field strains used in this study was found to be 74.6 %. Similar intratypic sequence identity determinations for European serotype viruses from diverse geographical origins ranged from 73 % to 83 % (Domingo *et al.* 1990). Clearly the corresponding level of sequence variation (25.4 %) within a buffalo from single game park and country exemplifies the high level of intratypic variation found in the SAT types as it surpasses or almost equals that determined for serotypes A, O and C.

The superimposition of the geographical localities of impala (Fig. 4.1) and buffalo viruses (Fig. 4.2) indicates that shared habitats between these species are likely to be important in transmission, as the origin of impala outbreaks overlap with the geographical range of the buffalo herds to which they can be linked. Although the sampling date of the buffalo viruses did not always

coincide with the year of the impala outbreak to which they were linked, it was shown that buffalo viruses group on the basis of geographic origin, rather than year of isolation, viz. relationships are spatial rather than temporal. Studies of buffalo behaviour have determined that buffalo herds have defined home ranges which they use continuously for several years (Mloszewski 1983; Funston *et al.* 1994). This explains the recovery of related viruses from discrete localities even when separated in time and explains the close genetic relationship between temporally unrelated buffalo viruses from a specific locality. Thus, the clustering of the 1991 buffalo virus with the 1985 impala outbreak strain is not surprising because it is representative of the buffalo herd from which the outbreak strain probably originated. It is known that buffalo home ranges can increase significantly in response to ecological pressures (Funston *et al.* 1994) and may vary from approximately 40 to 1000 square kilometres (Mloszewski 1983; Funston *et al.* 1994), with home range size being dictated by game park boundaries and other buffalo herds. In addition, bulls are known to move between buffalo herds (Mloszewski 1983). These factors would contribute to the findings of this study which demonstrated that some buffalo viruses obtained from the southern and northern regions of the study area are clearly more related than expected.

The occurrence of over 90% of outbreaks of FMD in impala within the time from June to November, is significant in that it coincides with the time at which buffalo calves are likely to become infected for the first time. Strict seasonal breeding of buffalo (Pienaar 1969a) results in synchronized calving, with the majority of births being recorded in January and February in the KNP (Fairall 1968). These buffalo calves, when infected for the first time probably excrete virus in approximately the same quantities and by the same routes as do cattle (Gainaru *et al.* 1986). Studies have shown that this first acute infection is likely to occur at around 3-8 months of age when maternally derived antibodies have waned sufficiently to make them susceptible to infection (Condy & Hedger 1978). On this basis it follows that buffalo calves in the KNP are likely to become infected for the first time from the month of May onwards. It is during this period that they are most likely to be a source of infection for other species within the KNP and therefore notable that most outbreaks in impala have occurred at a slightly later, but overlapping time period.

Factors facilitating inter-species transmission are poorly understood, but as buffalo and impala do not come into direct contact in natural circumstances, it can be assumed that elements driving them to share habitats are important. Shared drinking and grazing localities together with the high population density of both species in the southern part of the KNP are likely to facilitate transmission. It is interesting to note that of the five grazing routes identified by Meeser (1962) to be paths of FMD infection, three overlap with the origin of recent impala epizootics, discussed

here. This lends credence to the possibility that shared grazing may play a role in transmission and is further supported by the observation that impala grazing (as opposed to browsing) peaks at around 85 % in November (Skinner *et al.* 1984), and includes two grass species of preference for the African buffalo, viz. *Panicum maximum* and *Digitaria eriantha* (Pienaar 1969b;Skinner *et al.* 1984).

The role of the impala in the epidemiology of FMD remains unclear, although the historical use of this antelope as an indicator species for predicting both the course of an epizootic and outbreaks in livestock (Meeser 1962), would seem to imply that they may be intermediaries in the transmission of FMD from buffalo to cattle. Conflicting evidence exists in the literature, however, regarding the susceptibility of impala to infection and their ability to transmit the disease. Evidence for transmission of virus from buffalo to impala, where shared drinking and feeding was experimentally enforced, could not be demonstrated (Gainaru *et al.* 1986). Conversely, under experimental conditions where no close contact between the species occurred, it was shown that carrier buffalo inadvertently infected impala (Hedger *et al.* 1972) and acutely infected impala infected carrier buffalo (Vosloo *et al.* 1996). Clearly, a special set of circumstances is required to effect indirect interspecies viral transfer and the role of impala as intermediaries in disease transmission remains to be clarified. Circumstantial evidence does however indicate that infected antelope provided the most likely link between buffalo and cattle in a recent outbreak of FMD in cattle in Zimbabwe. In that instance, genetic characterization of buffalo viruses showed that buffalo were the primary source of infection (Hargreaves *et al.* Unpublished observations).

This study provides the first documented evidence of field transmission between buffalo and impala. As more southern African countries attempt to obtain and retain FMD free zones, it is of fundamental importance to elucidate the role of different wildlife species in the epidemiology of the disease so that appropriate disease control measures are implemented. Clearly, additional factors should be investigated in order to fully understand the epidemiological significance and to permit more accurate risk assessment of this antelope species. These include studies confirming or refuting circumstantial evidence pointing to the role of impala as intermediaries in disease transmission and studies determining the importance of environmental factors in facilitating inter-species transmission of FMD.

Chapter 5

Phylogeographic distribution of SAT-type foot-and-mouth disease viruses in African buffalo populations in southern Africa

Summary

In order to assess the genetic variation and relationships of SAT-type viruses within African buffalo populations in different regions in southern Africa, 30 viruses representative of each the SAT-types were selected for this study. The C-terminus half of the immunogenic VP1 gene was sequenced and relationships were resolved by phylogenetic analysis of the nucleotide data. Independently evolving virus populations were identified for each of the three SAT serotypes endemic to southern Africa, with members of the same genotype being defined as those sharing at least 80 % nucleotide sequence identity across the C-terminus region of the VP1 gene, specified here. Most genotype distributions were shown to correspond with discrete geographic localities in the following manner: Genotype I: South Africa and southern Zimbabwe; Genotype II: Botwana, Namibia and western Zimbabwe; Genotype III: Zambia and Malawi; Genotype IV: northern Zimbabwe.

Intra- and intertypic analyses revealed that regions of hypervariability within the SAT-type viruses correspond with immunogenic sites A (G-H loop) and C (C-terminus region) of the VP1 gene. In addition, SAT-1 and SAT-3 type viruses were shown to accumulate mutations in the H-I and F-H loops, respectively, indicating that structural constraints on VP1 gene evolution probably vary between the different SAT-type viruses. Of importance was the strict conservation of the RGD motif in the G-H loop and the presence of a cysteine residue at the base of the G-H loop in all SAT-type viruses analysed here. In common with serotype O viruses, the longer length of the VP1 protein and high degree of conservation of the cysteine residue upstream of the RGD indicates that conformational epitopes are likely to be important antigenic determinants in the endemic African serotypes.

This study identifies independently evolving virus populations in distinct geographic localities, defines the extent of the genetic heterogeneity among field isolates and provides a powerful reference for determining the origin of outbreaks in livestock and wildlife.

Parts of the results presented here have been accepted for publication in *Archives of Virology*

5.1 Introduction

The epidemiology of foot-and-mouth disease (FMD) in southern Africa is complex due to the circulation of six of the seven known serotypes of FMD virus (FMDV), and the role of wildlife in virus maintenance and disease transmission (Thomson 1996). Due to these epidemiological complexities, emphasis is placed on control rather than eradication of the disease and is achieved by vaccination and restriction on animal movement (Hunter 1998). South African Territories (SAT) type viruses are of particular importance in this region due to the high incidence of these serotypes in FMD outbreaks of livestock and the high infection rates in wildlife (Condy *et al.*. 1969). Of the 350 epizootics reported between 1931 and 1990 in southern Africa, approximately 73 % were caused by SAT-type viruses with European types A, O, C and untyped viruses accounting for the remainder (Thomson 1994).

The African buffalo (*Syncerus caffer*) plays a central role in the epidemiology of the disease due to its ability to maintain and transmit the disease (Thomson 1996). Buffalo populations throughout southern Africa have high infection rates with SAT type viruses (Condy *et al.* 1969; Thomson *et al.* 1992) and the geographical distribution of historical outbreaks of the disease in livestock overlap with the distribution of buffalo populations in southern Africa (Thomson 1994). Furthermore, buffalo are known to be efficient maintenance hosts of SAT-type FMD viruses (Condy *et al.* 1985) and have been shown to be the ultimate source of infection for livestock and wildlife species (Dawe *et al.* 1994a; Bastos *et al.* 2000). Historically, buffalo had a continuous distribution throughout sub-Saharan Africa, but although still widespread, populations are now fragmented due both to natural causes and human intervention. The 1889-1897 rinderpest pandemic which swept through Africa, resulted in an estimated 10 000 fold reduction in buffalo numbers (Sinclair 1977). This may have resulted in some of the southern-most pockets of buffalo becoming free of foot-and-mouth disease (Esterhuysen *et al.* 1985). However, most populations throughout southern Africa retain high infection rates with SAT-type viruses.

The three SAT types (1, 2 and 3) which are endemic to sub-Saharan Africa, differ from each other with regard to geographic distribution, infection rates in wildlife and incidence in FMD outbreaks in livestock. SAT-1 and SAT-2 have extensive distributions throughout sub-Saharan Africa and have made incursions into North Africa and the Middle East (Pereira 1981; www.OIE.org). In contrast, SAT-3 is restricted to five southern African (Brooksby 1972) countries and to Uganda (Hedger *et al.* 1973). SAT-1 has the highest seroprevalence rate in the maintenance host, the African buffalo (Condy *et al.* 1969; Thomson *et al.* 1992). Despite the high infection rate of buffalo with SAT-1 type viruses, this serotype has accounted for only 36 % of the SAT-type

outbreaks of FMD in cattle in southern Africa this century, with most outbreaks (48 %) being caused by SAT-2 type viruses and SAT-3 being responsible for 16 % of outbreaks (Thomson 1994). The reason for this anomaly between SAT-type incidence in cattle outbreaks and seroprevalence rates in the African buffalo maintenance host, is unclear, but may be due to differential abilities of SAT-types to cross species barriers. This possibility is supported by the observation that all clinical cases of FMD in impala antelope in South Africa from 1985 to 1995 were due exclusively to SAT-2 type viruses (Bastos *et al.* 2000), despite the higher recovery rate of SAT-1 type viruses from oesophageo-pharyngeal scrapings of buffalo sampled within the same area and time period (Table 5.1).

The SAT-types are genetically distinct from the European and Asian serotypes, on the basis of RNA hybridization studies (Robson *et al.* 1977) and amino acid sequence comparison of the structural protein genes (Palmenberg 1989). Analysis of complete VP1 gene sequences of European strains has revealed high levels of intratypic variation for the A, O and C serotypes (Domingo *et al.* 1990). Studies based on complete (Brown *et al.* 1989; Van Rensburg & Nel 1999) and partial characterization (Vosloo *et al.* 1995; Bastos 1998a; Bastos *et al.* 2000) of the VP1 gene of SAT-type viruses, although limited, have demonstrated that these serotypes have considerably higher levels of intratypic variation than that documented for other FMDV serotypes. Despite the wide range of variation in the SAT-types it is difficult to identify specific immunological subtypes (Pereira 1977; Esterhuysen 1994).

Genetic characterization of SAT-type viruses of African buffalo has mainly addressed the variation within the Kruger National Park (KNP), South Africa (Vosloo *et al.* 1995; Bastos *et al.* 1999; Bastos *et al.* 2000). Comparisons of SAT-1 viruses from South Africa with viruses from two game parks outside South Africa have however revealed that viruses circulating in buffalo in South Africa appear to be genetically distinct from buffalo viruses of Zambia and Zimbabwe (Vosloo *et al.* 1995; Bastos 1998a). Despite the limited sequence data that is presently available for SAT-type viruses, the genetic characterization of viruses of the maintenance host has been shown to be useful for determining the origin of FMD outbreaks in livestock and wildlife (Dawe *et al.* 1994a; Bastos *et al.* 2000). As SAT type outbreaks of FMD in livestock have been recorded in 8 southern African countries (Thomson 1994), efforts to establish a genetic reference base to assist in determining the origin of outbreaks in southern Africa would require that buffalo viruses of all affected countries be characterized. In this chapter an attempt is made to expand the genetic database of SAT type FMD viruses of the maintenance host species by sequencing representative buffalo viruses from as many southern African countries as possible. This information will be used to establish a regional reference base for determining the geographical origin of FMD outbreaks in livestock and wildlife in southern Africa.

TABLE 5.1 Summary of SAT-type viruses isolated from African buffalo in the Kruger National Park (1986-1996)

Year	No of SAT1 isolates / year	% of SAT1 isolates / year	No of SAT2 isolates / year	% of SAT2 isolates / year	No of SAT3 isolates / year	% of SAT3 isolates / year	Total No of isolations / year
1986	4	40 %	3	30 %	3	30 %	10
1987	3	100 %	0	0 %	0	0 %	3
1988	6	46 %	5	38 %	2	15 %	13
1989	24	73 %	8	24 %	1	3 %	33
1990	3	43 %	2	28 %	2	28 %	7
1991	35	61 %	14	24 %	8	14 %	57
1992	8	89 %	1	11 %	0	0 %	9
1993	5	62 %	2	25 %	1	12 %	8
1994	3	43 %	0	0 %	4	57 %	7
1995	4	67 %	2	33 %	0	0 %	6
1996	4	27 %	1	7 %	10	67 %	15
1986-1996	**99**	**59 %**	**38**	**23 %**	**31**	**18 %**	**168**

5.2 Materials and Methods

5.2.1 Study area

For the purpose of this study, southern African countries are defined as those predominantly occurring south of latitude 10°00'S. This area is inclusive of the following eight countries: South Africa, Namibia, Angola, Botswana, Zimbabwe, Mozambique, Zambia and Malawi.

5.2.2 Viruses used in this study

The viruses used in this study originate from African buffalo occurring within conservancies within 6 of the 8 southern African countries. No buffalo viruses were available for Mozambique and Angola, for historical and logistical reasons. 30 buffalo viruses per SAT serotype, representative of as many southern African countries as possible, were selected for genetic characterization. The geographic origin and strain designation of these viruses is summarized in Table 5.2 (SAT-1 viruses), Table 5.3 (SAT-2 viruses) and Table 5.4 (SAT-3 viruses). FMD viruses were isolated on primary pig kidney (PK) cells inoculated with 10 % suspensions (W/V) of African buffalo (*Syncerus caffer*) oesophageo-pharygeal (probang) samples and propagated further on IBRS2 (Insituto Biologico Rim Suino) cells. Samples and tissue culture isolates that were not available at the Onderstepoort Veterinary Institute and which were obtained from other sources are indicated in Tables 5.2 - 5.4.

3.2.3 RNA extraction, cDNA synthesis and PCR amplification

RNA was extracted from PK isolations or low passage cell culture specimens by a modified guanidinium-based nucleic acid extraction method (Boom *et al.* 1990). The RNA viral template was reverse transcribed using AMV-RT (Promega) in the presence of random hexanucleotides (Boehringer Mannheim) and an antisense primer targeting the 2A/B junction (Beck & Strohmaier 1987). Genomic amplification of the FMD viral genome was performed with primers targeting a 500 bp region corresponding to 1D/2A, as previously described (Bastos 1998a). Reactions were performed in a 50 µl volume in the presence of 0.2 mM dNTP, 0.25 µM of each primer, 1x buffer (DynaZyme) and 1 U of a thermostable DNA polymerase (DynaZyme). After an initial denaturation step at 96°C for 30 s, thirty cycles of denaturation at 96°C for 12 s, annealing at 58°C for 20 s and extension at 70°C for 40 s were performed.

3.3.4 PCR purification and nucleotide sequencing

Amplification of the expected fragment was confirmed by product size estimation against a DNA molecular weight marker (φX174 (*HindIII*); Promega) on a 1.5 % agarose gel. Bands of the correct size were excised from the gel and purified by means of the Cleanmix purification system (Talent). The purified products were sequenced with T7 DNA polymerase (Sequenase Version 2.0, USB) and an [α-^{32}P]dATP radioactive label (Amersham) in the presence of 10% DMSO (Winship 1989). Two or more independent amplification and sequencing reactions were performed per

isolate with each of the sequencing primers.

5.2.5 Genomic region used in this study

Genetic characterization of the carboxy-terminal region of the VP1 gene was performed as described in Chapters 2-4. Amino acid sequence data generated for the three SAT-types were aligned and an homologous region commencing with the conserved amino acid sequence 'WXPNG' in the VP1 protein (Fig. 5.7, Fig. 5.8 and Fig. 5.9) and ending on the C-terminal amino acid was identified for further analyses. This region corresponds to approximately 60 % of the VP1 gene and varies in length between serotypes with SAT-1, SAT-2 and SAT-3 having a sequence length of 132, 128 and 130 respectively. This regions is also inclusive of the two immuno-dominant sites within the VP1 gene, namely the A (G-H loop) and C (C-terminus region) sites.

5.2.6 Analyses of the partial VP1 gene sequencing data

Phylogenetic analyses followed the methods advocated by Martin and co-workers (1995) for the analysis of partial VP1 gene sequences of FMD viruses. Briefly, nucleotide sequences were translated and aligned using the DAPSA programme (Harley 1994) and neighbor-joining trees using the Jukes and Cantor (1969) algorithm were constructed. In order to identify the virus clusters that are well-supported (and repeatedly recovered), 1000 bootstrap replications were performed and a 60 % majority consensus rule was applied.

5.2.7 Amino acid variability and secondary structure prediction

Amino acid sequence data of 30 buffalo viruses of each serotype were aligned and used to plot VP1 gene variability, using the MEGA programme (Kumar *et al.* 1993). Analyses were performed with overlapping windows of 10 amino acids, with windows containing 7 or more variable sites, being considered hypervariable. Secondary structure predictions were performed with the nnpredict programme (Kneller *et al.* 1990), which was available through the BCM search launcher at http://www.cmpharm.ucfs.edu. Tertiary structure could only be inferred by aligning SAT-type reference strains with that of a type O_1BFS (Makoff *et. al.* 1982) whose tertiary structure has been resolved (Acharya *et. al.* 1989).

TABLE 5.2 Species and geographical origin of 30 SAT-1 type viruses from African buffalo populations in southern Africa (1985-1998)

Strain designation	Year of sampling	Country	Game Park / Place of origin	Grid reference
MAL/1/85	1985	Malawi	Kasungu NP	33°30'E-13°00'S
*ZIM/3/88	1988	Zimbabwe	Hwange NP	27°00'E-19°00'S
SWA/2/89	1989	Namibia	Caprivi	23°20'E-17°50'S
*ZIM/HV/3/90	1990	Zimbabwe	Hippo Valley NP	31°37'E-21°07'S
*ZIM/2/90	1990	Zimbabwe	Chirisa SA	28°15'E-18°00'S
*ZIM/Gn/13/91	1991	Zimbabwe	Gonarezhou	32°00'E-21°30'S
KNP/196/91	1991	South Africa	Kruger NP	31°38'E-25°17'S
*ZIM/2/91	1991	Zimbabwe	Bumi Hills	28°22'E-16°49'S
ZAM/2/93	1993	Zambia	Kafue NP	26°00'E-16°30'S
*ZIM/7/93	1993	Zimbabwe	Lone Star	31°54'E-21°07'S
*ZIM/2/94	1994	Zimbabwe	Hwange NP	27°00'E-19°00'S
KNP/8/95	1995	South Africa	Kruger NP	31°38'E-24°34'S
KNP/14/95	1995	South Africa	Kruger NP	31°38'E-24°34'S
KNP/17/95	1995	South Africa	Kruger NP	31°38'E-24°34'S
KNP/41/95	1995	South Africa	Kruger NP	31°38'E-24°34'S
KNP/2/96	1996	South Africa	Kruger NP	31°30'E-24°55'S
KNP/17/96	1996	South Africa	Kruger NP	31°32'E-23°18'S
KNP/22/96	1996	South Africa	Kruger NP	NA
ZAM/18/96	1996	Zambia	Kafue NP	26°25'E-16°17'S
ZAM/29/96	1996	Zambia	Lochinvar NP	27°30'E-15°50'S
*BOT/2/98	1998	Botswana	Nxaraga	23°15'E-19°40'S
*BOT/14/98	1998	Botswana	Nxaraga	23°15'E-19°40'S
*BOT/25/98	1998	Botswana	Vumbura	22°41'E-18°56'S
*BOT/37/98	1998	Botswana	Vumbura	22°41'E-18°56'S
KNP/75/98	1998	South Africa	Kruger NP	31°16'E-22°59'S
NAM/272/98	1998	Namibia	Mamili NP	23°39'E-18°22S
NAM/306/98	1998	Namibia	West Caprivi NP	21°50'E-18°15'S
NAM/307/98	1998	Namibia	West Caprivi NP	21°50'E-18°15'S
NAM/308/98	1998	Namibia	West Caprivi NP	21°50'E-18°15'S
*ZIM/14/98	1998	Zimbabwe	Lubangwa Island	28°20E-16°47'S

SA: Safari Area; NP: National Park; NA: Not available; *Viruses supplied by the World Reference Laboratory, Pirbright, UK

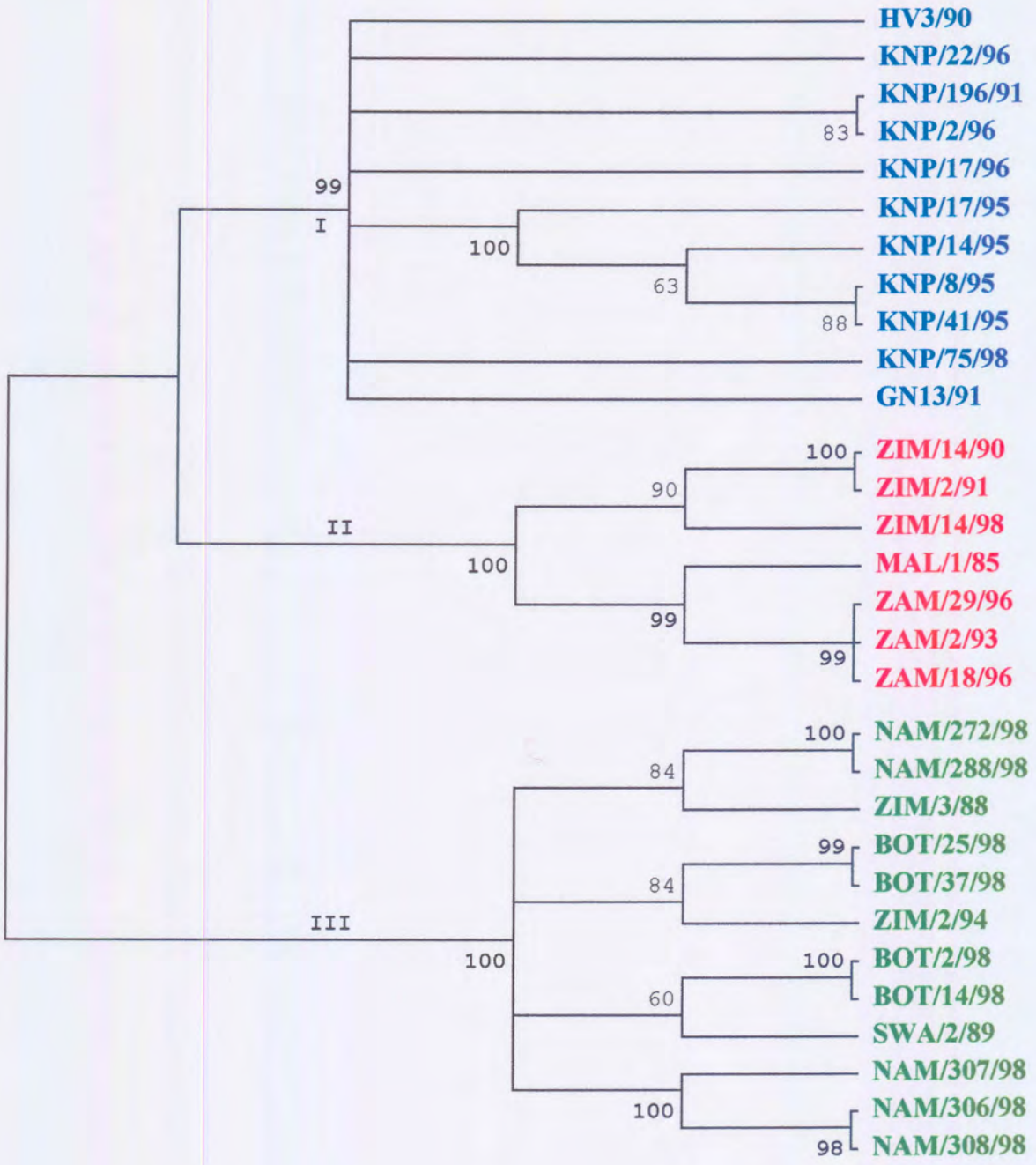

Fig. 5.1: Neighbor-joining tree based on 396 nt of the VP1 gene (amino acid position 87-221) depicting SAT-1 buffalo virus relationships in southern African. Jukes & Cantor algorithm with 1000 bootstrap replications and a 60 % majority consensus rule were applied. Bootstrap values ≥ 95 % confidence level are indicated in bold. Virus clusters (labeled I - III) are colour-coded to coincide with colours used to indicate the geographical distribution of these genotypes in southern Africa (Fig. 5.2).

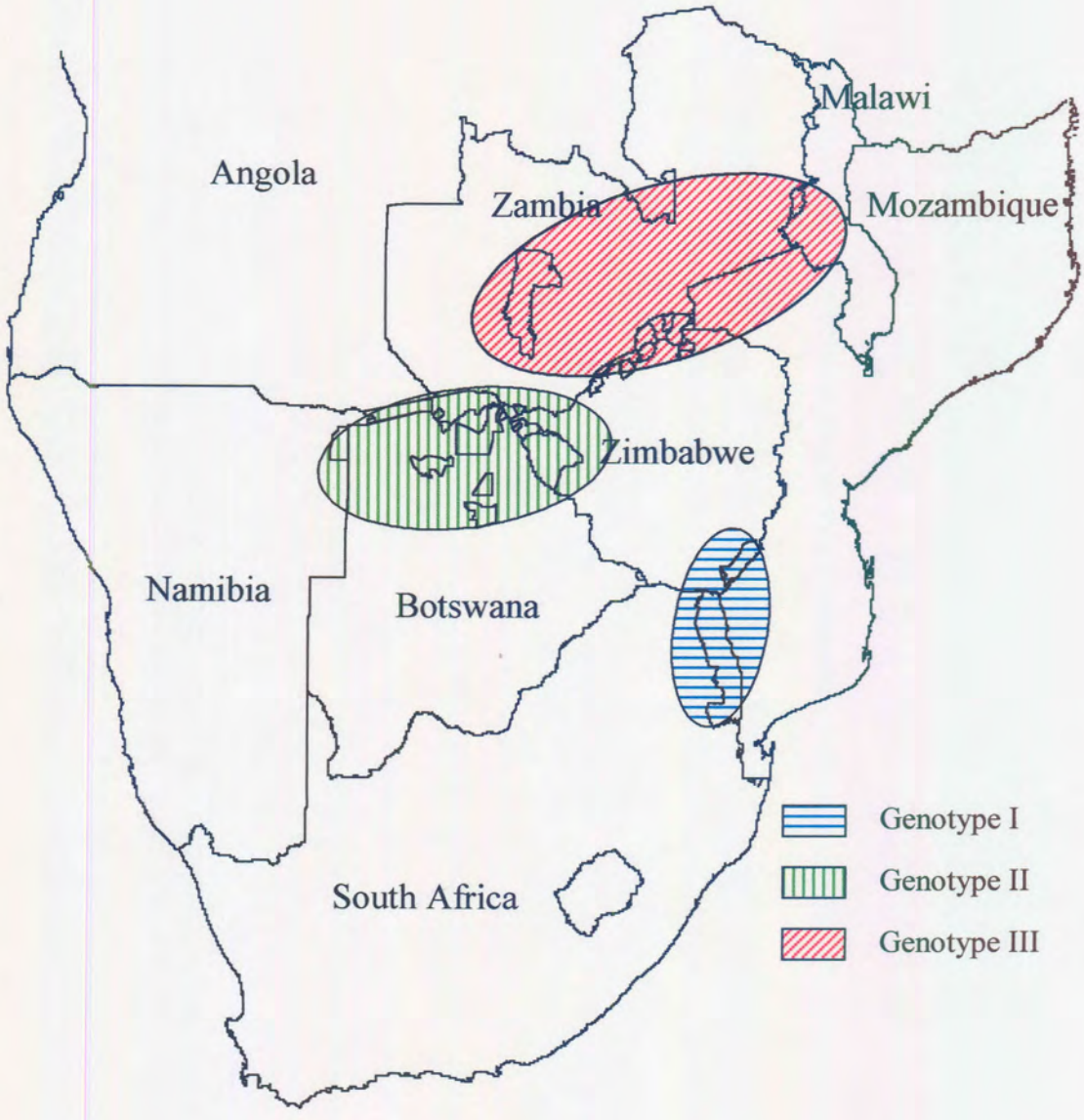

Fig. 5.2: Map of southern African game parks indicating the geographical distribution of SAT-1 buffalo virus genotypes. These regions are colour-coded as follows: blue (genotype I); green (genotype II); red (genotype III)

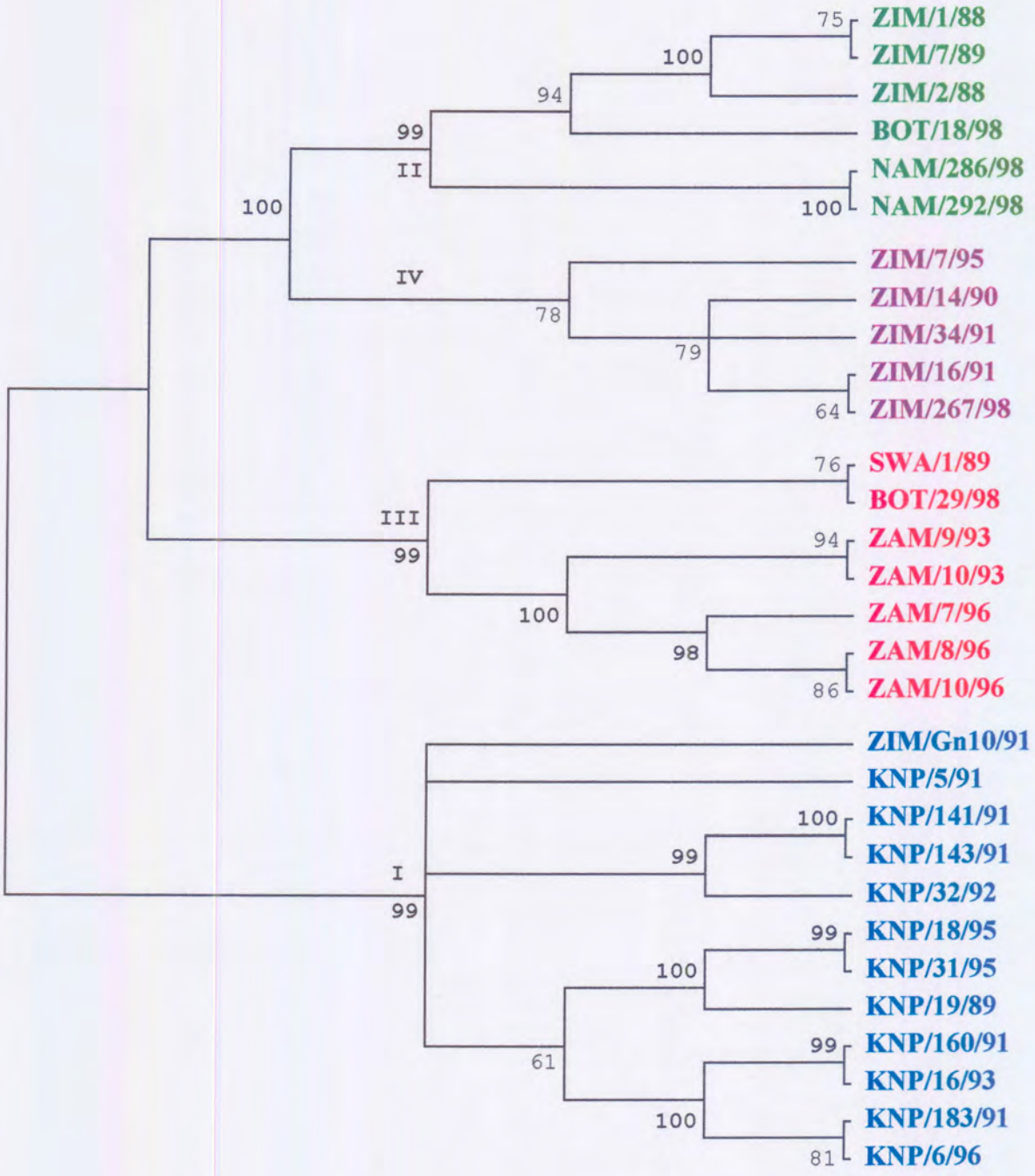

Fig. 5.3: Neighbor-joining tree based on 384 nt of the VP1 gene (amino acid position 89-216) depicting SAT-2 buffalo virus relationships in southern African. Jukes & Cantor algorithm with 1000 bootstrap replications and a 60 % majority consensus rule were applied. Bootstrap values ≥ 95 % confidence level are indicated in bold. Virus clusters (labeled I - IV) are colour-coded to coincide with colours used to indicate the geographical distribution of these genotypes in southern Africa (Fig. 5.4).

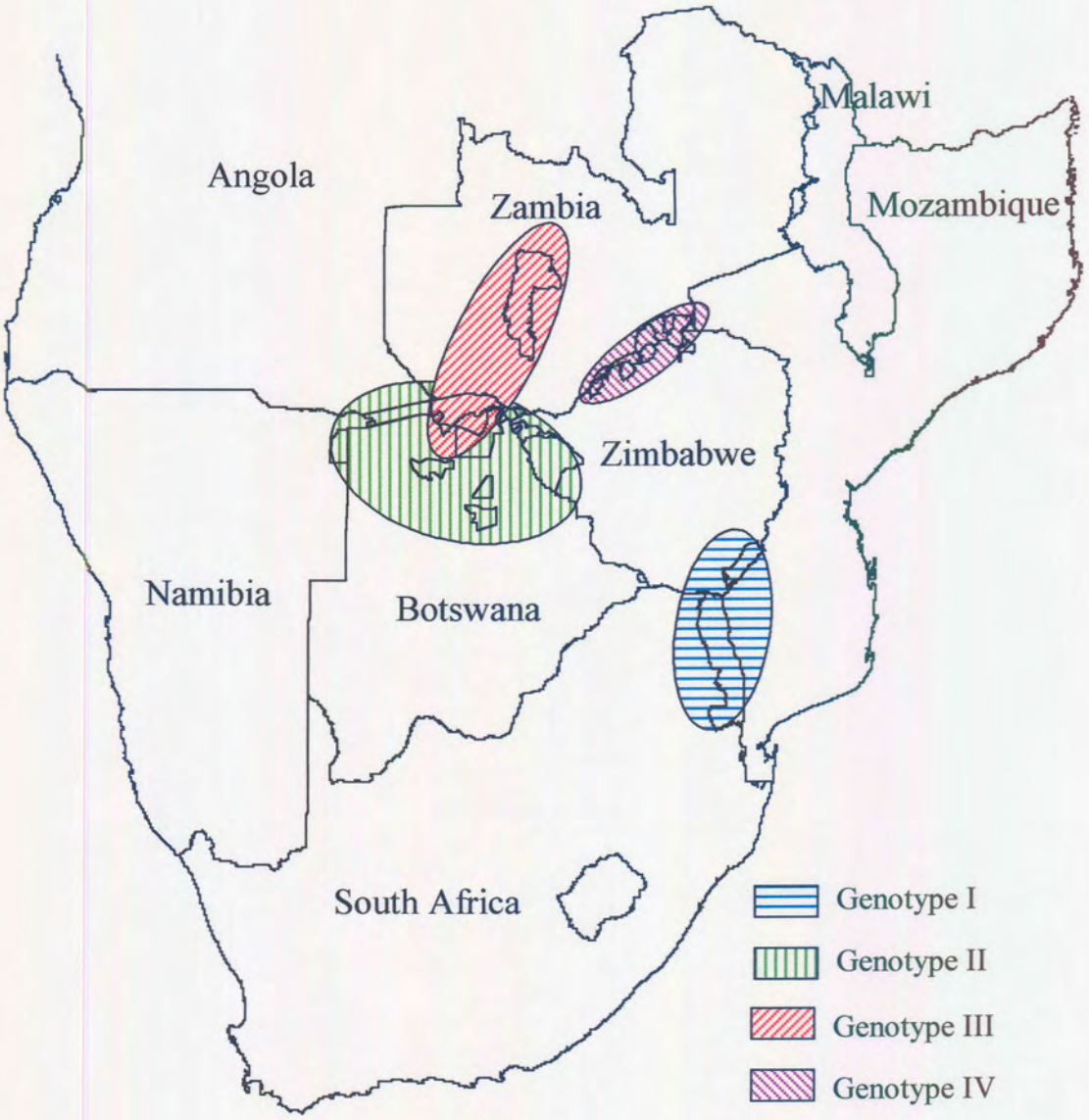

Fig. 5.4: Map of southern African game parks indicating the geographical distribution of SAT-2 buffalo virus genotypes. These regions are colour-coded as follows: blue (genotype I); green (genotype II); red (genotype III); purple (genotype IV)

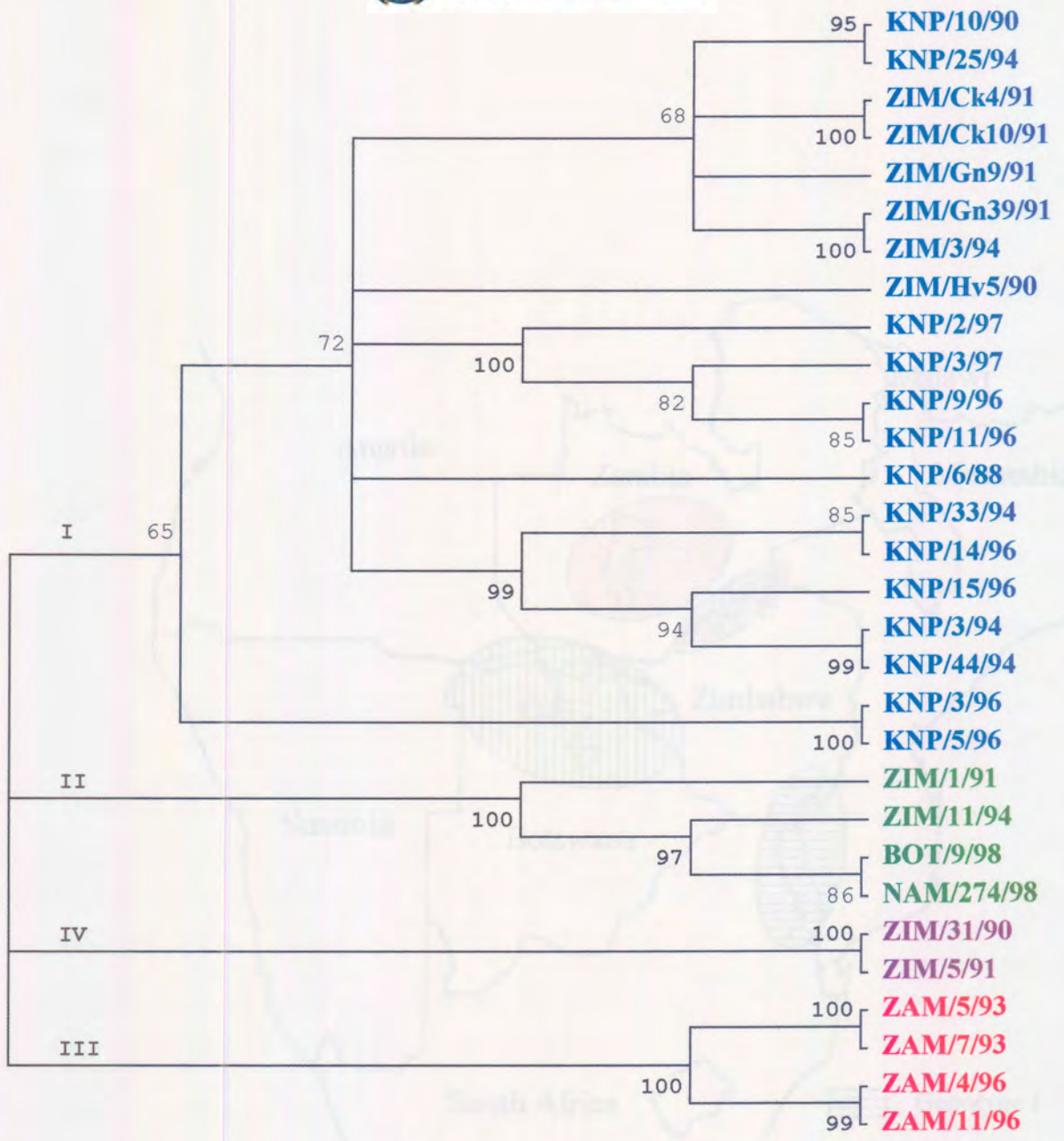

Fig. 5.5: Neighbor-joining tree based on 390 nt of the VP1 gene (amino acid position 88-217) depicting SAT-3 buffalo virus relationships in southern African. Jukes & Cantor algorithm with 1000 bootstrap replications and a 60 % majority consensus rule were applied. Bootstrap values ≥ 95 % confidence level are indicated in bold. Four distinct genotypes (I-IV) are denoted and are colour-coded to correspond to the geographical origin and distribution of SAT-3 viruses, depicted in Fig. 5.6.

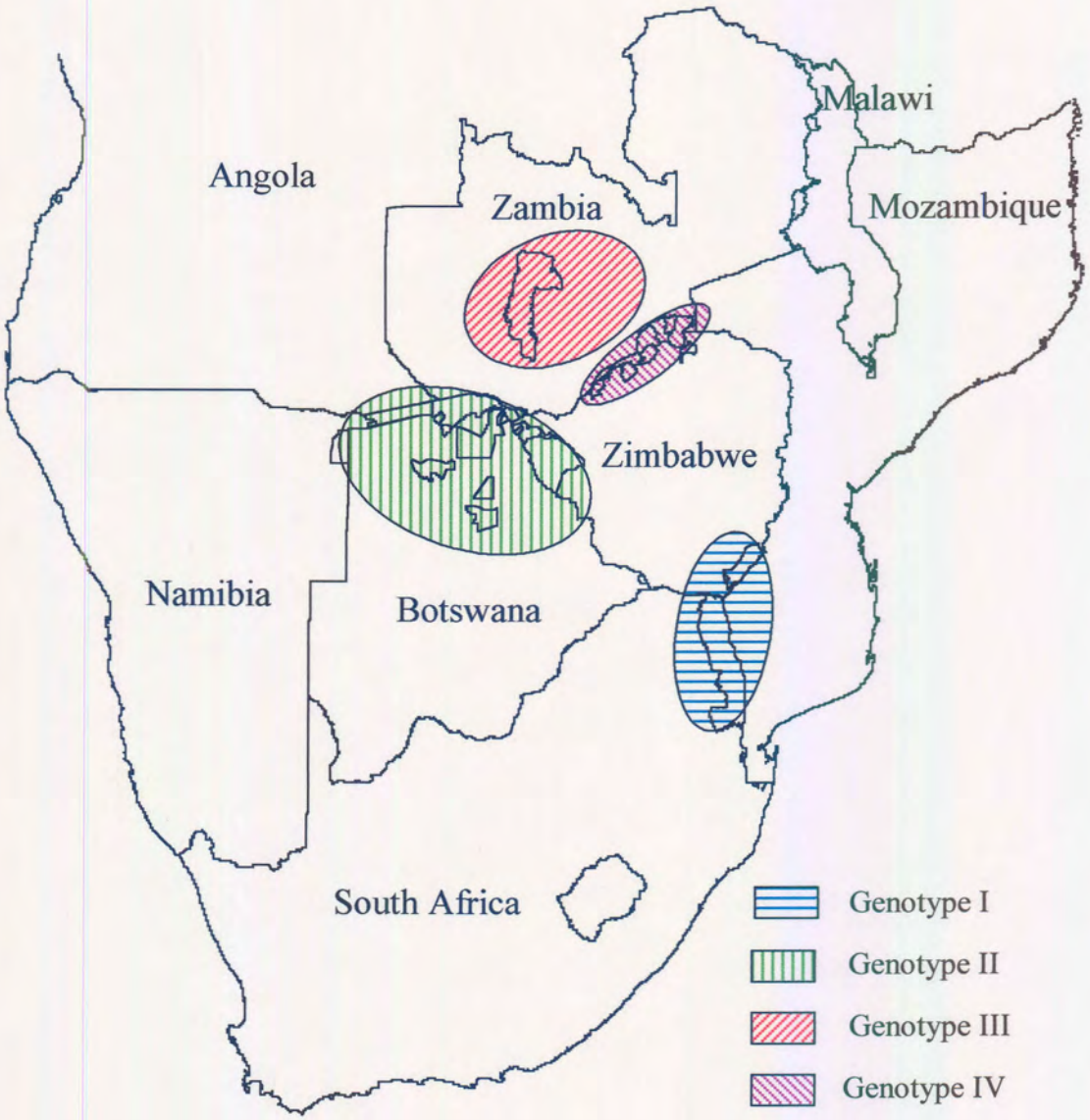

Fig. 5.6: Map of southern African game parks indicating the geographical distribution of SAT-3 buffalo virus genotypes. These regions are colour-coded as follows: blue (genotype I); green (genotype II); red (genotype III); purple (genotype IV)

TABLE 5.3 Species and geographical origin of 30 SAT-2 type viruses from African buffalo populations in southern Africa (1988-1998)

Strain designation	Year of sampling	Country of origin	Game Park / Sampling locality	Grid Reference
*ZIM/1/88	1988	Zimbabwe	Hwange NP	27°E-19°S
*ZIM/2/88	1988	Zimbabwe	Hwange NP	27°E-19°S
*ZIM/4/88	1988	Zimbabwe	Hwange NP	27°E-19°S
KNP/19/89	1988	South Africa	Kruger NP	31°37'E-24°44'S
SWA/1/89	1989	Namibia	Caprivi	23°25'E-14°41'S
*ZIM/7/89	1989	Zimbabwe	Hwange NP	27°E-19°S
*ZIM/14/90	1990	Zimbabwe	Doma SA	30°15'E-16°20'S
KNP/5/91	1991	South Africa	Kruger NP	31°47'E-24°24'S
KNP/143/91	1991	South Africa	Kruger NP	31°20'E-24°00'S
KNP/147/91	1991	South Africa	Kruger NP	31°20'E-24°00'S
KNP/160/91	1991	South Africa	Kruger NP	31°34'E-24°33'S
KNP/183/91	1991	South Africa	Kruger NP	31°56'E-25°08'S
*Gn/10/91	1991	Zimbabwe	Gonarezhou NP	32°00'E-21°30'S
*ZIM/16/91	1991	Zimbabwe	Matusadona NP	28°30'E-16°45'S
*ZIM/34/91	1991	Zimbabwe	Urungwe SA	28°55'E-16°30'S
KNP/32/92	1992	South Africa	Kruger NP	31°17'E-22°54'S
KNP/16/93	1993	South Africa	Kruger NP	31°26'E-23°29'S
ZAM/9/93	1993	Zambia	Kafue NP	26°00'E-16°30'S
ZAM/10/93	1993	Zambia	Kafue NP	26°00'E-16°30'S
*ZIM/8/94	1994	Zimbabwe	Matetsi NP	26°50'E-18°00'S
KNP/18/95	1995	South Africa	Kruger NP	31°38'E-24°34'S
KNP/31/95	1995	South Africa	Kruger NP	31°38'E-24°34'S
*ZIM/7/95	1995	Zimbabwe	Sengwa	28°00'E-17°05'S
KNP/6/96	1996	South Africa	Kruger NP	32°01'E-25°14'S
ZAM/8/96	1996	Zambia	Mulanga	NA
ZAM/10/96	1996	Zambia	Mulanga	NA
*BOT/29/98	1998	Botswana	Vumbura	NA
*BOT/31/98	1998	Botswana	Vumbura	NA
NAM/286/98	1998	Namibia	East Caprivi GR	23°20'E-17°52'S
NAM/292/98	1998	Namibia	East Caprivi GR	23°20'E-17°52'S
ZIM/267/98	1998	Zimbabwe	Chizarira	28°00'E-17°47'S

GR: Game Reserve; NP: National Park; SA: Safari Area; NA: Not available; * Indicates viruses supplied by WRL

TABLE 5.4 Species and geographical origin of 30 SAT-3 viruses from African buffalo populations in southern Africa (1988-1998)

Strain designation	Year of sampling	Country of origin	Game Park / Sampling locality	Grid Reference
KNP/6/88	1988	South Africa	Kruger NP	31°16'E-25°10'S
KNP/10/90	1990	South Africa	Kruger NP	31°20'E-24°00'S
*ZIM HV5/90	1990	Zimbabwe	Hippo Valley NP	31°35'E-21°10'S
*ZIM/31/90	1990	Zimbabwe	Dande SA	30°20'E-15°55'S
*ZIM Ck4/91	1991	Zimbabwe	Chikwarakwara	31°05'E-22°20'S
*ZIM Ck10/91	1991	Zimbabwe	Chikwarakwara	31°05'E-22°20'S
*ZIM Gn9/91	1991	Zimbabwe	Gonarezhou NP	32°00'E-21°30'S
*ZIM Gn39/91	1991	Zimbabwe	Gonarezhou NP	32°00'E-21°30'S
*ZIM/1/91	1991	Zimbabwe	Matusadona NP	28°30'E-16°45'S
*ZIM/5/91	1991	Zimbabwe	Urungwe SA	28°55'E-16°30'S
ZAM/5/93	1993	Zambia	Kafue NP	26°00'E-16°30'S
ZAM/7/93	1993	Zambia	Kafue NP	26°00'E-16°30'S
KNP/3/94	1994	South Africa	Kruger NP	31°55'E-25°15'S
KNP/25/94	1994	South Africa	Kruger NP	31°55'E-25°15'S
KNP/33/94	1994	South Africa	Kruger NP	31°55'E-25°15'S
KNP/44/94	1994	South Africa	Kruger NP	31°55'E-25°15'S
*ZIM/3/94	1994	Zimbabwe	Matetsi	25°50'E-18°06'S
*ZIM/11/94	1994	Zimbabwe	Hwange NP	27°E-19°S
KNP/3/96	1996	South Africa	Kruger NP	31°30'E-24°55'S
KNP/5/96	1996	South Africa	Kruger NP	31°30'E-24°55'S
KNP/9/96	1996	South Africa	Kruger NP	31°23'E-22°52'S
KNP/11/96	1996	South Africa	Kruger NP	31°23'E-22°52'S
KNP/14/96	1996	South Africa	Kruger NP	NA
KNP/15/96	1996	South Africa	Kruger NP	NA
ZAM/4/96	1996	Zambia	Mulanga	NA
ZAM/11/96	1996	Zambia	Kafue NP	26°00'E-16°30'S
KNP/2/97	1997	South Africa	Kruger NP	31°23'E-22°52'S
KNP/3/97	1997	South Africa	Kruger NP	31°23'E-22°52'S
*BOT/9/98	1998	Botswana	Moremi GR?	NA
NAM/274/98	1998	Namibia	Mamili NP	23°39'E-18°22'S

GR: Game Reserve; NP: National Park; SA: Safari Area; NA: Not available; * Indicates viruses supplied by WRL

5.3 Results

5.3.1 Phylogeographic distribution of SAT-1 buffalo viruses

In order to determine the genetic relationships of SAT-1 viruses in southern Africa, an homologous 396 nt region, corresponding to amino acid positions 80-221 of the VP1 protein was identified for phylogenetic analyses. The neighbor-joining method (Saitou & Nei 1987) and Jukes & Cantor (1969) algorithm were used to construct the gene tree as this evolutionary model was shown by Martin and co-workers (1995) to increase the probability of recovering the correct phylogenetic tree when partial VP1 data is used. Alternative distance estimation algorithms produced trees with identical topology as did UPGMA and parsimony methods (results not shown), confirming that the recovered phylogeny is reliable (Kim 1993). In addition, bootstrap support (based on 1000 replications) was consistently above 90 %, irrespective of the type of analysis method or evolutionary model used. Results indicate that there are three independently evolving SAT-1 virus clusters (Fig. 5.1), which correspond to distinct geographical localities (Fig. 5.2) as follows:

Genotype I: South Africa and southern Zimbabwe (99 % bootstrap support)
Genotype II: Botswana, western Zimbabwe and Namibia (100 % bootstrap support)
Genotype III: northern Zimbabwe, Zambia and Malawi (100 % bootstrap support)

As these three viral genotypes correspond to three geographically independent regions, the term 'topotype' is used to reflect this association. This grouping of viruses on the basis of geographical origin has previously been described for serotype O FMDV (Knowles *et al.* 1997) and for numerous unrelated viruses (Deubel *et al.* 1986; Kerschner *et al.* 1986; Monath *et al.* 1986; Calisher *et al.* 1987; Chu *et al.* 1989; Lindsay *et al.* 1993; Yanagihara 1994).

5.3.2 Phylogeographic distribution of SAT-2 buffalo viruses

Four major SAT-2 viral lineages (Fig. 5.3) were similarly obtained when a phylogeny was inferred using partial VP1 sequence data corresponding to amino acid positions 89-216. The geographical distribution of viruses (indicated in Fig. 5.4) comprising each of the four SAT-2 genotypes is as follows:

Genotype I: South Africa and southern Zimbabwe (99 % bootstrap support)
Genotype II: Botswana, Namibia and western Zimbabwe (99 % bootstrap support)
Genotype III: Botswana, Namibia and Zambia (99 % bootstrap support)
Genotype IV: Northern Zimbabwe (78 % bootstrap support)

On the basis of these results, viruses of the same SAT-2 genotype are defined as those sharing at least 80 % sequence identity across the C-terminus region of VP1 specified in this study. In contrast to the genetically and geographically distinct grouping of SAT-1 viruses, co-circulation of 2 distinct genotypes within Botswana and Namibia was observed for the SAT-2 serotype (Fig. 5.4). The map depicting geographic distribution of SAT-2 variants in southern Africa clearly shows that viruses originating from the Okavango Delta (Botswana) and from the eastern tip of the Caprivi strip (Namibia) fall within two sub-clusters of genotypes II and III and have overlapping distributions. In particular, BOT/29/98 and SWA/1/89 are the only viruses originating from Botswana and Namibia that appear to be genetically more closely related to Zambian viruses (Genotype III) than to other viruses originating from Botswana and Namibia (Genotype II).

5.3.3 Phylogeographic distribution of SAT-3 type buffalo viruses

Four viral lineages were identified for SAT-3 type viruses from southern Africa when a phylogeny was inferred using partial VP1 sequence data (Fig. 5.5). These virus clusters were shown to correspond to distinct geographic localities (Fig. 5.6) as follows:

Genotype I: South Africa and southern Zimbabwe (65 % bootstrap support)
Genotype II: Namibia, Botswana and western Zimbabwe (100 % bootstrap support)
Genotype III: Zambia (100 % bootstrap support)
Genotype IV: northern Zimbabwe (100 % bootstrap support)

In contrast to the SAT-1 serotypes, SAT-3 has four genotypes that are phylogeographically distinct from each other. These genotypes therefore concur with the topotype definition. The geographic distribution of the southern genotype (I) is identical to that observed for SAT-1 and SAT-2 as is the distribution of SAT-1 and SAT-3 western genotype (II) viruses. It is noteworthy that genotype III and IV SAT-3 viruses from Zambia and northern Zimbabwe, respectively, form distinct country-specific virus groupings within each of the major genotypes into which they fall.

In 1994, buffalo were translocated from Gonarezhou National Park in southern Zimbabwe to Matetsi National Park in the west. A virus recovered from one of these translocated buffalo, ZIM/3/94 groups with a virus obtained from a Gonarezhou buffalo in 1991 (ZIM/Gn39/91), with 100 % bootstrap support. Clearly, the ZIM/3/94 virus recovered from the Matetsi buffalo reflects its true origins, indicating that VP1 gene characterization may provide a powerful means of identifying the origin of buffalo moved between different genotype localities.

5.3.4 Phylogeographic comparison of the three SAT-type viruses in southern Africa

Sequence identity (percentage of sites that are completely conserved across all the sequences being analysed) within each of the SAT-type buffalo virus genotypes was determined (Table 5.5). Overall variation within the homologous region of the VP1 protein characterized in this study was highest within SAT-3 and lowest in SAT-1 (on amino acid level). In addition, SAT-1 has the lowest number of distinct viral lineages despite having a widespread distribution throughout sub-Saharan Africa. In contrast, SAT-3 which has the most restricted distribution, has the highest level of intratypic variation and occurs as four independently evolving virus clusters. Levels of nucleotide sequence variation by topotype ranged from 32 % to 33 % and from 20 % to 23 % on amino acid level (results not shown).

TABLE 5.5 Comparison of the three SAT-type viruses of the African buffalo (*Syncerus caffer*) maintenance host, in southern Africa

Serotype	No of viruses	No of southern African countries	Nucleotide variation	Amino Acid variation	*No of southern African viral lineages
SAT-1	30	6	52 %	42 %	3
SAT-2	30	5	54 %	43 %	4
SAT-3	30	5	55 %	46 %	4

** Number of lineages obtained with the NJ method with p-distances, 1000 bootstrap replications and a 60% majority consensus rule applied.*

5.3.5 Structurally and immunologically important amino acid sites in SAT-1 viruses

The RGD motif (amino acids 149-151), which is thought to be the cell attachment site of the virus (Geysen *et al.* 1985; Fox *et al.* 1989) and is known to interact with the αv ß3 integrin cellular receptor (Berinstein *et al.* 1995) is completely conserved in the SAT-1 serotype (Fig. 5.7). In addition, leucine at position 152 is also completely conserved in all but one virus (KNP/8/95) as is proline at position 166 (with the exception of one buffalo virus, KNP/17/96). Leu-152 and Pro-166 of SAT-1 type viruses correspond to Leu-148 and Pro-153 of serotype A_{12} which were shown to be important residues in

determining antibody interactions with the highly immunogenic GH-loop (France *et al.* 1994). A conserved cysteine residue at position 135 in the SAT-1 serotype, corresponds to the structurally important Cys-134 in serotype O viruses (Fig.5.10). This cysteine forms a disulphide bond with a cysteine residue in VP2 and is thought to explain why most neutralizing monoclonal antibodies against type O viruses recognize conformational rather than linear epitopes (Acharya *et al.* 1990). The 1D/2A junction site of SAT-1 type viruses is variable, with two distinct forms occurring, viz. 'KQ/MC' in 2/30 buffalo viruses and KQ/LC in the remaining 28 SAT-1 sequences (Fig. 5.7). Of interest is the identification of an additional RGD triplet (position 110-112), upstream of the GH-loop RGD site, in the Namibian virus, designated NAM/307/98.

5.3.6 Structurally and immunologically important amino acid sites in SAT-2 viruses

Amino acid alignment of the 30 SAT-2 buffalo viruses (Fig. 5.8) reveals that the 'RGD' cell-attachment motif (position 144-146) is completely conserved. In contrast to SAT-1, a conserved leucine, adjacent to the RGD motif, does not occur in SAT-2 type viruses. Instead an arginine is observed at position 147 in 28 of the 30 buffalo viruses sequenced. Leu-150 (corresponding to Leu-152 in SAT-1) is however completely conserved as is Pro-162 (which corresponds to Pro-166 in SAT-1). These two sites have been shown to be critical residues in determining antibody interactions with the highly immunogenic G-H loop (France *et al.* 1994). Cys-134 (corresponding to Cys-135 of SAT-1) at the base of the G-H loop (Fig. 5.10) was observed in all SAT-2 viruses sequenced. Of interest is the conservation of the C-terminus 'KQ/LC' at the 1D/2A junction site. In common with SAT-1 type viruses, amino acid positions 110-112, corresponding to positions 109-111 in SAT-2 serotype, were extremely variable, but none of the SAT-2 viruses characterized here had an 'RGD' at this site.

5.3.7 Structurally and immunologically important amino acid sites in SAT-3 viruses

The 'RGD' cell-attachment motif (position 145-147) is completely conserved in the 30 SAT-3 buffalo viruses sequenced (Fig. 5.9). A leucine occurs at position 148 in 20 of these buffalo strains whilst a methionine is present in the remainder. This is in contrast to

the SAT-1 in which a leucine, adjacent to the RGD motif, occurs in all but one strain. In common with the SAT-1 and SAT-2 type viruses, Leu-151 (corresponding to Leu-152 in SAT-1) and Pro-164 (which corresponds to Pro-166 in SAT-1) is completely conserved in the SAT-3 serotype. These two sites are also conserved in serotype A viruses where they are known to be of immunological importance for antibody recognition (France *et al.* 1994). Similarly, Cys-133 (corresponding to Cys-135 of SAT-1) at the base of the G-H loop (Fig. 5.10) occurs in all SAT-3 viruses sequenced. In common with the SAT-1 serotype, the 1D/2A junction site of SAT-3 viruses shows some degree of variability. Although the 'KQ/LC' was the most predominant form (> 80 % of SAT-3 viruses), 'KQ/LS', 'KQ/LY' and 'KQ/TC' forms were also observed. Amino acid positions 108-110 of SAT-3, which correspond to the region in SAT-1 viruses where the additional 'RGD' was observed, were variable. The closest variant on the 'RGD' triplet, was a 'RNG' which was observed in a Zambian isolate (ZAM/11/96; Fig. 5.9).

5.3.8 Distribution of hypervariable regions in the C-terminus half of the VP1 gene

VP1 gene amino acid variability was plotted for all buffalo viruses in order to determine whether mutations were randomly distributed or localized to specific regions of the VP1 gene. Three discrete hypervariable regions, in which 70 % or more of the sites varied (for overlapping windows of 10 amino acids) were identified in this way. In all SAT serotypes, hypervariable regions were located at amino acid positions coinciding with serotype C antigenic sites A (the GH-loop) and C (C-terminus region) of the protein. These hypervariable and immunodominant sites are known to elicit neutralizing antibodies in European serotypes. Additional hypervariable regions were identified for each of the serotypes and are summarized in Table 5.6. Despite the high levels of variability in the primary sequence, secondary structure predictions reveal that the hypervariable regions have highly conserved secondary structures (results not shown). In particular, strict secondary structure conformation is maintained in the form of an alpha helix immediately following the RGD binding motif (positions 152-159; Fig. 5.10).

	99	109	119	129	139	149	159
SWA/2/89	WTPNGSPVMS	EVGDNPVVFS	HQGTTRFALP	YTAPHRVLAT	VYNGDCKYKP	TGTAPRENIR	GDLATLAARI
NAM/272/98	.A.....F.	....	.NN.....I.	....	....	...P..T...	.......E..
NAM/306/98	.......ELT	....	RG........	....	....	...P......	.......K..
NAM/307/98	........LT	....	RGD.......	....	....	...P......	....
NAM/308/98	.......QLT	....	RG........	....	....	.S.P......	.......K..
ZIM/3/88	........LG	....	.NN.....I.	.?........	....	.D.PA.D...	.......E..
ZIM/2/94	.......ELT	....	.NN.......	.......V..	A.........	.SAP......	.......T..
BOT/2/98	........L.	....	.G......F.	......L...	....	......G...	....
BOT/14/98	........L.	....	.G........	....	....	....	....
BOT/25/98	........LE	....	.N........	.......V..	T.........	.S.P....V.	....V.....
BOT/37/98	.......ELT	....	.N........	.......V..	T.........	.S.P....V.	....V.....
ZIM/14/90	.......EL.	....	.KR.....I.	....	T.........	Q.Q...T...	.......Q..
ZIM/2/91	.......EL.	....	.N......I.	....	T.......N.	Q.Q...T...	.......Q..
ZIM/14/98	.....A.EL.	.........A	.NN.......	......C...	T.........	VDQ...T.V.	....V..E..
ZAM/2/93	.....A.EL.	....	.N........	......C...	T...N.....	ATE..PTHV.	....
ZAM/18/96	.....A.EL.	....	.N........	......C...	T.........	VSEE..TH..	....
ZAM/29/96	.....A.EL.	....	.N........	......C...	A.........	VNEV.STH..	....
MAL/1/85	.....A.EL.	....	.N........	......C...	T.........	NSE...T...	....A..E..
HV3/90	.....A.ELN	..C.......	.N........	......C...	T.........	A.....G...	.......H..
ZIM/7/93	.....A.ELD	....	.N........	......C...	T.........	....	.......H..
GN13/91	.....A.ELG	....	.N........	......C...	T.........	A..P......	.......Q..
KNP/196/91	.....A.ELA	....	KG........	......C...	A.........	....	....
KNP/8/95	.....A.EL.	....	AN........	......C...	T.........	.D.P..T...	..F....E..
KNP/14/95	.....A.EL.	....	TN........	......C...	T.........	...P..T...	.......E..
KNP/17/95	.....A.EL.	....	TN........	......C...	P.........	...P..T...	.......E..
KNP/41/95	.....A.EL.	....	AN........	......C...	T.........	.D.P..T...	.......E..
KNP/2/96	.....A.EFL	......C...	KD........	......C...	T.........	....	....
KNP/17/96	.....A.ELT	....	KN........	......C...	S.........	A.DP..G...	....
KNP/22/96	.....A.EFD	....	KNR.A.....	......C...	L.........	AS.T..T...	.......E..
KNP/75/98	.....A.EL.	....	AN........	......C...	S.........	A.....D...	.......N..

	169	179	189	199	209	219	2A
SWA/2/89	ASETHIPTTF	NYGMIYTEAD	VDVYLRMKRA	ELYCPRPVLT	HYDHAGKDRY	KTVLVKPAKQ	MC
NAM/272/98	.N.....	.....E	.....	.....L..	....G.....	Q.A.IR....	L.
NAM/306/98	.....	.....Q.E	.....	.....	....G.....	..A..R....	L.
NAM/307/98	.....	.....Q.E	.....	.....	....S.....	..A..R....	L.
NAM/308/98	.....	.....Q.E	.....	.....	....G.....	..A..R....	L.
ZIM/3/88	.N.....	.....Q.E	.N.....	.....L..	....R.....	..D.T.....	L.
ZIM/2/94	.....	.....Q.E	.....	.....	....G.....	..A.....	L.
BOT/2/98	.G.....	.....Q.E	.....	.....L..	....SN....	RVA.I.....	..
BOT/14/98	.G.....	.....Q.E	.....	.....L..	....SN....	RVA.....	..
BOT/25/98	.....	.....E	.....	.....	....N.....	.AR.....	L.
BOT/37/98	.....	.....	.....	.....	....K.....	Q.A.....	L.
ZIM/14/90	.E.....S.	...R.....E	....V.....	.....	....QN....	.VALT.....	L.
ZIM/2/91	.E.....S.	...R.....E	.TS.V.....	.....	....Q.....	.VALT.....	LY
ZIM/14/98	.A.....	...R.....E	....V.....	.....L..	G...G.....	.VALT.....	L.
ZAM/2/93	.....	...R.....E	....V.....	.....	....Q.....	.VALT.....	L.
ZAM/18/96	....R.....	...R.....E	....V.....	.....	....Q.....	.VALT.....	L.
ZAM/29/96	.....	...R.....E	....V.....	.....	....Q.....	.VALT.....	L.
MAL/1/85	.....	...R.....E	.N..V.....	.....L..	....Q.....	.VALT.....	L.
HV3/90	.....	...R.....E	....V.....	.....	....G.....	..AIT..V..	L.
ZIM/7/93	.....	...R.....E	....V.....	.....	....G.R...	..AIT..V..	L.
GN13/91	.....	...R.....E	....V.....	.....	....G.R...	..AIT..V..	L.
KNP/196/91	.....	...R...DTV	....V.....	.....	....G.....	..AIT..V..	L.
KNP/8/95	.....	...R...H.S	....I.....	.....L..	....G.....	.IAIT..V..	L.
KNP/14/95	.....	...R...Q.S	....I.....	.....L..	....GE....	..AIT..V..	L.
KNP/17/95	.....	...R...K.S	....I.....	.....L..	....G.....	..AIT..V..	L.
KNP/41/95	.....	...R...Q.S	....I.....	.....L..	....G.....	..AIT..V..	L.
KNP/2/96	.....	...RL...GV	....V.....	.....I..	....K.....	..AIT..V..	L.
KNP/17/96	......T...	...R...Q.E	....V.....	.....L..	....G.....	.IAIT.....	L.
KNP/22/96	.....	...RL..Q.E	....V.....	.....L..	.....	..AIT..V..	L.
KNP/75/98	.N.....	...R.....E	....V.....	.....L..	....N.....	..AIT..V..	L.

Fig. 5.7: Amino acid sequence alignment of the C-terminus half of the VP1 gene of 30 SAT-1 type FMD viruses of african buffalofrom southern Africa. The RGD cell-binding site in the GH-loop is underlined (positions 149-151).

	98	108	118	128	138	148	158
KNP/19/89	WQPNGAPRTR	ELGDNPMVFS	NKRVTRFAVP	YTAPHRLLST	VYNGECKYET	PVTAIRGDRA	VLAAKYSNIK
ZIM/1/88	.....T	T.R.....	HNN.....	.....	R.....TQ	QS.....	.....A.TR
ZIM/2/88	.....T	T.R.....	HNN.....	.....	R.....TQ	QS.....	.....A.TR
SWA/1/89	.H.....T	V.....	HNN.....I.	.....A.	.....TQ	RTQ.....E	.....A.TQ
ZIM/7/89	.....T	T.R.....	HNN.....	.....	R.....TQ	QS.....S.	.....A.TR
ZIM/14/90	.....T	Q.R.....	HNS.....L.	.....	R.....N.TQ	RSP.....	.....A.V.
KNP/5/91	.....T	.....	KNG.....	.....	.....G.R.	H.....	.....A.TR
KNP/141/91	.....T	Q.....	RN.....	.....	.....E.K.	R.....	A..S..ASAR
KNP/143/91	.....T	Q.....	HN.....	F.....	.....E.K.	R.....	A..S..ASAR
KNP/160/91	.....T	Q.....	.QG.....	.....	.....DM	.....	.....GKQ
KNP/183/91	.....T	Q.....	.NG.....	.....	.....V.N.	.....	.....GRQ
ZIM/Gn10/91	.....S	.....	HNA.....	.....	.....R.TE	R.....	.....A.TR
ZIM/16/91	.....T	Q.R.....	HNN.....L.	.....	R.....TQ	.SN.....	A.....A.T.
ZIM/34/91	.....?...T	Q.R.....	HNS.....L.	.....	R.....TE	RA.....	.....A.T.
KNP/32/92	.....T	Q.....	RNG.....	.....	.....E.K.	R.....P	..GS..ADGR
KNP/16/93	.....T	Q.....	..G.....	.....	.....D.	....V.....	.....GRQ
ZAM/9/93	.....T	Q.....	HNK.....I.	.....A.	R.....TQ	EAR.....	.....A.V.
ZAM/10/93	.....T	Q.....	HNK.....I.	.....A.	R.....TQ	EAR.....	.....A.V.
KNP/18/95	.....T	.....	SNG.....	.....	.....E.R.	..A.....	.....
KNP/31/95	.....T	V.....	SNG.....	.....	.....E.R.	..A.....	.....T.
ZIM/7/95	.....T	Q.R.....	HNK.....L.	.....	R.....E.AQ	..A.....	.....A.TR
KNP/6/96	.....T	Q.....	.NG.....	.....	.....T.S.	..A.....	.....ERQ
ZAM/7/96	.....T	Q.....	HNK.....I.	.....A.	R.....TQ	EAR.....	.....AGA.
ZAM/8/96	.H.....T	Q.....	HNK.....I.	.....A.	G.....TQ	EAR.....	.....AGA.
ZAM/10/96	.....T	Q.....	HNK.....I.	.....A.	G.....TQ	EAR.....	.....AGA.
BOT/18/98	.....T	T.R.....	HNN.....	.....	R.....TQ	QSA.....	A..QT.A.TR
BOT/29/98	.....T	V.....	HNK.....I.	.....	.....R.TQ	KTQ.....	...Q..A.TR
NAM/286/98	.H...S...T	T.RH.....	HNK.....	.....	R.....TQ	RS.....	.....A.T.
NAM/292/98	.....S...T	?.R.....	HNK.....	.....	R.....TQ	RS.R....P.	.....A.T.
ZIM/267/98	.....T	Q.R.....	HNN.....L.	F.....	R.....TQ	TS.....	.....A.VR

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                 168        178        188        198        208     214 2A
KNP/19/89    HTLPSTFNFG HVTADNSVDV YYRMKRAELY CPRPLLPAYD HASRDRFDAP IGVEKQ  LC
ZIM/1/88     .E........ Y....KP... .......... .......G.. ..A.....S. ......  ..
ZIM/2/88     .E........ Y....KP... .......... .......G.N ..D.....S. ......  ..
SWA/1/89     .A........ .....KV... .......... .........E ..D.....G. ......  ..
ZIM/7/89     .E........ Y....KP... .......... .......G.. ..AK....S. ......  ..
ZIM/14/90    .E........ F....KP... .F....T... .S........ QET..... ......  ..
KNP/5/91     .A........ .....QP... .......... .......... ..N..... ......  ..
KNP/141/91   .......... .....T.... .......... .........E ....Y..... ......  ..
KNP/143/91   .......... .....T.... .......... .........E ..D..... ......  ..
KNP/160/91   .......... Y....KA... .......... .......... ..N..... ......  ..
KNP/183/91   .......... .....AA... .......... .......... .GN..... ......  ..
ZIM/Gn10/91  .A....?... .....KP... .......... .......... ..N.....S. ......  ..
ZIM/16/91    .E........ F....KP..I .......... .......... .G..... ......  ..
ZIM/34/91    .E........ F....AP... .......... .......V.E DGN..... ......  ..
KNP/32/92    ....P..... .....K.... .......... .......... ..N..... ......  ..
KNP/16/93    .......... .....KA... .......... .......... ..N..... ......  ..
ZAM/9/93     .S........ .....AA... .......... .......... .SD.....S. ......  ..
ZAM/10/93    .S........ .....AA... .......... .......... .SD..... ......  ..
KNP/18/95    .......... .....K.... .......... .........N .VD..... ......  ..
KNP/31/95    .......... .....K.... .......... .......... .VD..... ......  ..
ZIM/7/95     .E........ F.....P... .......... .....F.... ..N..... ......  ..
KNP/6/96     .......... .....KA... .......... .........E .GD..... ......  ..
ZAM/7/96     .S........ .....AA... .......... .........E .SD..... ......  ..
ZAM/8/96     .S........ ...?.AV... .......... .........E .SN.....G. ......  ..
ZAM/10/96    .A........ .....AA... .......... .........E .L......G. ......  ..
BOT/18/98    .E........ Y....KP... .......... .......G.. ..N.....S. ......  ..
BOT/29/98    .S........ .....EA... .......... ......?..E .ND...Y.G. ......  ..
NAM/286/98   .E........ Y....KP... .......... .......G.E ..D.....S. ......  ..
NAM/292/98   .E........ Y....KP... .......... .......G.. ..D.....S. ......  ..
ZIM/267/98   .E........ Y..V..P..I .......... ...A...... .QN..... ......  ..
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Fig. 5.8: Amino acid sequence alignment of the C-terminus half of the VP1 gene of 30 SAT-2 type FMD viruses of African buffalo from southern Africa

	97	107	117	127	137	147	157
KNP/10/90	WVPNGCPHTT	RVEDNPVVHA	KGGVTRFALP	YTAPHGVLAT	VYNGNCKYSK	TQHVVPR**RGD**	LAVLAQRVEN
KNP/6/88	.........D	....	.N..A.....	.....R..?.	....	.H..SA....	....S....S
ZIM/Hv5/90	.........D	....	.N..P.....	.....R....	.........T	....PA....	..T..K...T
ZIM/31/90	.........D	....	RNN.......	....	I...S.....	..R.NS....	M.S......S
ZIM/Ck4/91	.........D	.........?	.N..A.....	.....R....	....	....TA....	.....R...T
ZIM/Ck10/91	....?....D	....	.N..A.....	....	..?.......	....TA....	.....R...T
ZIM/Gn9/91	.........D	....	.N........	....	....	....SA....	M........T
ZIM/Gn39/91	.........D	....	.N........	....	...?......	....T.....	M....R...T
ZIM/1/91Mat	.........D	.........S	..S.A.....	....	....	..R.Q.....	..T.S.....
ZIM/5/91	....A....D	....	..N.......	......AV..	T...T.....	..S.K.....	M........G
ZAM/5/93	....?.....	.........YS	.N........	F.......S.	....T.....	..R.S.....	....SE...T
ZAM/7/93	....	......I..S	.N........	F.......S.	....T.....	..R.S.....	....SE...T
KNP/3/94	.........D	....	.N........	.....R....	....	..R.E.....	M...S.....
KNP/25/94	.........D	.........?	.E........	..R.......	....	....T.....	....
KNP/33/94	.........D	....	.N..A.....	.....R....	....	..R.T.....	M...S.....
KNP/44/94	.........D	....	.N........	.....R....	....	..R.E.....	M...S.....
ZIM/3/94	.........D	........Y?	.N..A.....	....	....	....T.....	M....R.A.T
ZIM/11/94	.........D	........YS	..S.A.....	....	........N.	..RTT.....	.T..S.....
KNP/3/96	.........D	....	.N.AA...I.	....	....Y.....	..R.A.....	....SR....
KNP/5/96	....?....D	....	.N.AA...I.	....	....Y.....	..R.A.....	....SR....
KNP/9/96	.........E	......L...	.N..A.....	.....S....	....	....A.....	....Q.....
KNP/11/96	.........E	......L...	.N..A.....	.....S....	....	....A.....	....Q.....
KNP/14/96	.......Y.D	....	.N........	.....R....	....	..R.T.....	M...S.....
KNP/15/96	.........D	....	.N........	.....R....	....	..RIT.....	M...S.....
ZAM/4/96	.........D	.........S	.N........	F.......S.	....T....N	..R.T.....	....ST...T
ZAM/11/96	.........N	.........S	RN........	F.......S.	....T.....	..R.S.....	..A.SA...T
KNP/2/97	.......L.E	........Y.	.N..A.....	.....S....	....	....A.....	..A.Q.....
KNP/3/97	.........E	......L...	.N..A.....	.....S....	....	....A.....	..A.Q.....
BOT/9/98	.........D	.........S	..S.A.....	....	........N.	..R..S....	..A.S.....
NAM/274/98	.........D	.........S	..S.A.....	....	........N.	..R.A.....	....S.....

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                      167        177        187        197        207      215  2A
KNP/10/90   ETTRCRPTTF NFGRLLCDTG DVYYRMKRAE LYCPRALRVR YAHTTDRYKT KLVAPDKQ LC
KNP/6/88    .....L.... .......... TA........ .....P.... .T.......V .....G.. ..
ZIM/Hv5/90  .I...L.... .......... E.......T. .....P...A .T........ .....E.. T.
ZIM/31/90   .QQ..I.... .......ES. ........T. F....P.M.. .T.......V A..S.A.. ..
ZIM/Ck4/91  .I...T.... .....Y..S. ........T. .....P.K.. .T...N.... ........ ..
ZIM/Ck10/91 .I...T.... .....Y..S. ........T. .....P.K.. .T........ ........ ..
ZIM/Gn9/91  .....K.... .......... .......... .....P.... .V..S..... ...S.E.. ..
ZIM/Gn39/91 .......... .......E.. E......... .....P.K.. .T...Y...I ........ ..
ZIM/1/91Mat .....I.... ........K. E......... .....P.... .V........ P..K.E.. ..
ZIM/5/91    .QQ..K.... ........S. .......... .....P.M.. .T.......V A..S.A.. ..
ZAM/5/93    .QE..L.... ........S. .......... .....P.... .T.......V A..K.E.. ..
ZAM/7/93    .QE..L.... ........S. .......... .....P.... .T.......V A..K.E.. ..
KNP/3/94    .....I.... ........S. ........T. .....P.... .V........ ........ ..
KNP/25/94   .....I.... .......... .......... .....P...M .T.......I ........ ..
KNP/33/94   .....I.... ........S. ........T. .....P.... .V........ ........ ..
KNP/44/94   .....I.... ........S. ........T. .....P.... .V........ ........ ..
ZIM/3/94    .......... .......... E......... .....P.K.. .T.......V ........ ..
ZIM/11/94   .AA..I.... ........G. E......... .....P.A.. .T........ T..K.A.. ..
KNP/3/96    .....I.... .Y......S. N......... .....P.M.. .V........ .....E.. .S
KNP/5/96    .....I.... .Y......S. N......... .....P.M.. .V........ .....E.. .S
KNP/9/96    .......... .......... E.......T. .....P.... .I........ ........ ..
KNP/11/96   .......... .......... E.......T. .....P.... .I........ ........ ..
KNP/14/96   .....I.... .......ES. N.......T. .....P.... .V........ ........ .Y
KNP/15/96   .....I.... ........S. ........T. .....P.... .V........ R....... ..
ZAM/4/96    .QE..L.... ........S. .......... .....P.... .T......NL A..K.E.. ..
ZAM/11/96   .QE..L.... ........S. .......... .....P.... .T.......V A..K.E.. ..
KNP/2/97    .......... .......... ........T. .....P.K.. .T........ ........ ..
KNP/3/97    .......... .......... E.......T. .....P.... .I........ .....N.. ..
BOT/9/98    .....I.... ........S. .......... .....P.... .T.VS..... P..R.E.. ..
NAM/274/98  .....I.... .......ES. E......... .....P.... .T.V...... A.IK.E.. ..
```

Fig. 5.9: Amino acid sequence alignment of the C-terminus half of the VP1 gene of 30 SAT-3 type FMD viruses of African buffalo from southern Africa

	βE	αB	βF		βG1		βG2
	....	... .*EEE*....	.*EEE*.	.....*E*	....	.....*H*	*HEEE*
	93	104	109	115	119	125	129
BOT/1/68	WTPN	GSP-VLTEVGDN	PVVFS	RRGTTR	FALP	YTAPHR	VLAT
KEN/3/57	WQPN	GAP-RTTQLGDN	PMVFS	HNNVTR	FAIP	FTAPHR	LLST
BEC/1/65	WVPN	GRP-HTGRVEDN	PVVHS	KGSVVR	FGLP	YTAPHG	VLAT
M20715	WVPN	GAPEAALSNTSN	PTA-N	KAPFTR	LALP	YTAPHR	VLAT
J02185	WVPN	GAPEKALDNTTN	PTAYH	KAPLTR	LALP	YTAPHR	VLAT
AF024510	WVPN	GAPVSALGNTTN	PTAYH	KGPLTR	LALP	YTAPHR	VLAT
	* **	* * *	*	*	*	****	* *

		(G-H loop)		βH
	......	.................*HHHHHHHHH*..	....	.*EEEE*
	135	162	171	176
BOT/1/68	VYNGDC	KYKPTGTAPRENI**RGD**LATLAARIASE	THI--PTTFNY	GMIYT
KEN/3/57	VYNGEC	EYTKTVTA----I**RGD**REVLAQKYSSA	-KHSLPSTFNF	GFVTA
BEC/1/65	VYNGNC	KYSETQRATS--R**RGD**LAVLAQRLENE	TTRCLPRTFNF	GRLLC
M20715	VYNGTS	KYS----A-SGSR**RGD**LGSLATRVA--	TQ--LPASFNY	GAIKA
J02185	VYNGEC	RYS--RNA-VPNL**RGD**LQVLAQKVA-R	T---LPTSFNY	GAIKA
AF024510	AYTGTT	TYT----AS---T**RGD**---LAHLTATH	-ARHLPTSFNF	GAVKA
	* *	* * *** **	* ** **	*

		βI	(C-terminus)	2A
	E.	.*HHHHHHH*..........	...............*HH*.*HH*.*H*	..
		196	219	
BOT/1/68	QA	EVDVYLRMKRAELYCPRP	VLTHYDHNGRDRYKTTLVKPAKQ	LS
KEN/3/57	DK	PVDVYYRMKRAELYCPRA	LLPAYTHAGGDRFDAPIG-VAKQ	LL
BEC/1/65	EE	GDA-YYRMKRAELYCPRP	LRVRYTHTT-DRYKTPLVKPDKQ	MC
M20715	QA	IHELLVRMKRAELYCPRP	LLAMEVSSQ-DRYKQKIIAPAKQ	LL
J02185	TR	VTELLYRMKRAETYCPRP	LLAIHPTEA--RHKQKIVAPVKQ	TL
AF024510	ET	ITELLVRMKRAELYCPRP	ILPIQPTG--DRHKQPLVAPAKQ	LL
		****** ****	* **	

Fig. 5.10: Amino acid sequence alignment of the C-terminus half of the VP1 gene of SAT types 1-3 (BOT/1/68, SAT-1; KEN/3/57, SAT-2 and BEC/1/65; SAT-3) with serotypes A, O and C (A_{10} Holland, J02185, Thomas *et al.* 1988; O_1 BFS, M20715, Makoff *et al.* 1982; C_1Noville, AF024510, Bastos 1998). Amino acid positions correspond to BOT/1/68 (SAT-1). Gaps in sequences represented by '-' and were included for alignment purposes. The '*' symbol below the aligned sequences indicates the positions at which an amino acid is conserved across all serotypes. The 'RGD' motif in the GH-loop of the VP1 gene is indicated in bold, and the upstream cysteine residue which occurs in serotype O and SAT-type viruses are underlined. Secondary structure predictions are indicated in bold and italic above the aligned amino acid sequences and make use of the following codes: H = helix; E = strand; a *dot* denotes those positions where no prediction could be made. Tertiary structure predictions correspond to that determined for O_1BFS (Acharya *et al.* 1989) and are indicated above the secondary structure predictions.

By combining the amino acid alignment data (Fig. 5.7 - Fig. 5.9) with the predicted tertiary structure of type O1BFS (Fig. 5.10), and the results of the amino acid hypervariablility plots, it was possible to identify the virus protein regions where mutations could be accommodated. Not surprisingly, mutations were not randomly distributed. In each of the SAT serotypes, the G-H loop and C-terminus regions were shown to be hypervariable (Table 5.6). Of interest, were differences between serotypes in the distribution of mutations relative to the end of the gene and the position of the RGD. The regions within the C-terminus region of SAT-1 which could accommodate numerous mutations was longer than that of the other two SAT types and was situated right at the end of the VP1 gene. In contrast, the terminal 5-8 amino acids of SAT-2 and SAT-3 were more conserved, with the hypervariable region within the C-terminus end of the gene being located slightly upstream of the 1D/2A junction. Differences in the distribution of mutations within the G-H loop were also notable between serotypes. In SAT-1 viruses, mutations occurred predominantly upstream of the 'RGD', whilst in SAT-3, mutations occurred in two discrete regions, one upstream and one downstream of the 'RGD'. This indicates the amino acid sequences surrounding the 'RGD' in the SAT-3 serotype are critical. In contrast to the other SAT-types, the SAT-2 type had a continuous hypervariable region which was inclusive of the 'RGD'. Very few sites within this 29 amino acid stretch were conserved, indicating that the SAT-2 serotype can accommodate more mutations, than the other SAT-types, within the G-H loop.

Table 5.6 Comparison of partial VP1 gene sequences of the three SAT-types in southern Africa: Location and length of hypervariable regions with respect to the 'RGD' motif and immunogenic sites.

	SAT-1	**SAT-2**	**SAT-3**
Length of VP1	221 aa	216 aa	217 aa
Position of 'RGD'	149-151	144-146	145-147
Amino acid region characterized in this study	90-221 (132 aa)	89-216 (128 aa)	88-217 (130 aa)
Regions of hypervariability			
F-H loop	--	--	102-116 (15 aa)
G-H loop (Site A)	136-152 (17 aa)	133-161 (29 aa)	134-145 (11 aa) 148-157 (10 aa)
H-I loop	172-182 (11 aa)	--	--
C-terminus (Site C)	204-221 (18 aa)	192-208 (17 aa)	199-211 (12 aa)

In addition to accumulating mutations within the G-H loop and C-terminus regions of the protein, SAT-1 and SAT-3 each have an additional hypervariable region corresponding to the H-I loop and F-H loop, respectively. In contrast to the SAT-1 and SAT-3 virus types, SAT-2 appears to have structural constraints limiting most mutations to the G-H loop and C-terminus regions alone.

5.4 Discussion

Four independently evolving virus clusters were identified by genetic characterization of the VP1 gene of SAT-type viruses of buffalo in southern Africa. The virus isolates comprising each of the four clusters (I-IV) were generally found to originate from correspondingly separate geographical localities. On the basis of this strict grouping of viruses according to geographical origin, four southern African genotypes or topotypes are proposed (Table 5.7).

TABLE 5.7 Summary of phylogeographic distribution of SAT-type virus genotypes in southern Africa as determined by partial VP1 gene sequence analysis

Serotype	SAT-1	SAT-2	SAT-3
Genotype I	South Africa southern Zimbabwe	South Africa southern Zimbabwe	South Africa southern Zimbabwe
Genotype II	Botswana Namibia western Zimbabwe	Botswana Namibia western Zimbabwe	Botswana Namibia western Zimbabwe
Genotype III	northern Zimbabwe Malawi Zambia --- ---	--- --- Zambia (Botswana) (Namibia)	--- --- Zambia — —
Genotype IV	---	northern Zimbabwe	northern Zimbabwe

Genotype I viruses of each of the SAT-type viruses have an identical geographical distribution and are genetically and geographically independent of the other genotypes occurring within southern Africa. Genotype II and Genotype III viruses differ between serotypes, although some commonalities are apparent. In particular, Botswana, Nambia and western Zimbabwe feature consistently in Genotype II. Genotype III, although variant between serotypes always includes viruses of Zambian origin. SAT-2 and SAT-3 viruses falling with the northern Zimbabwe genotype (IV) have sufficiently high sequence divergence values to assign them to a genotype. In contrast, although the SAT-1 serotype has a genetically distinct northern Zimbabwe lineage within the genotype III, this virus lineage has not diverged sufficiently to allocate a distinct genotype.

These genetically independent entities generally correspond to geographically discrete regions, in all serotypes but SAT-2, where two distinct genotypes were shown to be circulating simultaneously within Botswana and Namibia. Exclusion of two viruses from the SAT-2 buffalo virus dataset (SWA/1/89 and BOT/29/98), would however result in a similar phylogeographic distribution pattern to that observed for the remaining SAT-types. This indicates, that these viruses are perhaps the exception rather than the rule and may be due to incomplete field data. This is strengthened by the observation of a SAT-3 genotype I virus (ZIM/3/94) in a genotype II locality. In this case information of buffalo translocation from the southern part of Zimbabwe to the western region of the country was made available by authorities, thereby provided an explanation for what would could have been interpreted as a lack of structuring in viruses of the SAT-3 serotype. It is well known that there is extensive animal movement between Zambia and countries converging on the Caprivi strip (Perry & Hedger 1984), which may promote the exchange of viruses from these two topotype areas. The overlap in the geographical origin of genotype II and -III SAT-2 viruses may also be a reflection of past migratory movement of buffalo between the regions, but the trade in cattle between countries converging on the Caprivi and the higher incidence in SAT-2 outbreaks in livestock in the southern African region, provides a more likely explanation.

The strict structuring of viruses according to geographical origins is not supported by similar geographical structuring in populations of the host species (Simonsen *et al.* 1998). A recent study of virus relationships in buffalo populations has however shown that virus genotypes are distinct within different buffalo herds in a single game park (Bastos *et al.* 2000) and that the core buffalo herd unit and home range remain largely unchanged from year to year (Mloszewski 1983). In addition, the once coherent buffalo population ranging over most of sub-Saharan Africa

(Mloszewski 1983) became fragmented by heavy hunting pressure and as a result of the decimation of this species by the rinderpest epidemic as well as the restriction of buffalo movement enforced by fencing. Together, these factors are likely to have resulted in a drastic reduction in field strain variants and in the localization of genotypes to specific areas. The extensively variable yet structured virus population, presently observed is the probable result of a severe genetic bottleneck, coupled with the rapid evolution of SAT-type viruses of buffalo (Vosloo *et al.* 1996), within confined areas.

The genetic characterization of diverse SAT-1 type viruses, has provided insight into important features in the two highly immunogenic sites in the VP1 gene, namely the GH-loop and C-terminus region. Sequence alignment of SAT-type viruses with structurally and genetically well-characterized European serotype viruses (Fig. 5.10) revealed that the GH-loop and C-terminus regions of the SAT serotypes are 5-7 and 1-2 amino acids longer, respectively, than types A and O. This is expected to result in extended protrusions from the surface of the virus particle and may perhaps lead to even greater flexibility in the GH-loop of SAT-1 type viruses. Both the GH-loop and C-terminus region, situated close to each other on the virus surface, are important for the attachment of FMDV to cells via the highly conserved RGD cell binding site (Fox *et al.* 1989). The complete conservation of the RGD binding motif in SAT-type viruses of diverse geographical and species origin was determined in this study. Other immunologically important sites such as leucine at position 152 and 155, and proline at position 166 were highly conserved and probably of similar immunological importance in SAT types as they have been shown to be in the European serotypes (Thomas *et al.* 1988; France *et al.* 1994; Mateu *et al.* 1996).

Intra- and intertypic comparison on partial VP1 gene sequences has provided some insight into the antigenic variation observed for SAT-type viruses. Although many interesting structural implications can be identified by this means, the lack of information on the atomic structure of the SAT-type viruses severely restricts the usefulness of the amino acid data generated here. It can however be predicted that conformation epitopes are likely to be important antigenic determinants for these FMD virus serotypes. This cautions against the use of VP1 gene sequences alone in the development of recombinant vaccines.

Although most southern African countries appear to have a single SAT viral topotype within their borders, these topotypes are usually shared between two or more neighbouring countries, emphasizing the need for a regional approach to disease control if effective management of FMD

is to be achieved. The restriction of FMD infected buffalo populations to game parks in the north-eastern extremes of South African and Namibia, facilitates the control of the disease in these countries. In contrast, control of the disease in Zimbabwe is complicated by the presence of between three to four topotypes per serotype within its borders and the dispersed distribution of FMD-infected buffalo populations in various game parks throughout all but the central region of the country. The presence of multiple topotypes within Zimbabwean borders explains the significantly higher levels of intratypic variation observed for SAT-type viruses from this country. This heterogenous genetic resource is likely to give rise to a wide range of antigenic variants and needs to be addressed for effective control of the disease through vaccination.

Studies based on partial VP1 gene sequences are clearly vital for understanding aspects which are unique to the epidemiology of FMD in southern Africa. In this study, the importance of a regional approach to disease control through vaccination was inferred on the basis of the strict geographical clustering of independently evolving virus populations. The virus characterization approach outlined also provides a means of determining the geographical origin of buffalo infected with SAT-type viruses. This may assist in disease control by acting as a deterrent to illegal movement of diseased buffalo between game parks in southern Africa. The establishment of a SAT buffalo virus database in which regional variants are well-characterized potentially provides a useful means of determining the origin and course of contemporary and historical outbreaks in southern Africa and will be investigated further in Chapter 6.

Chapter 6

Tracing the origin and course of foot-and-mouth epizootics in southern Africa

Summary

Genetic characterization of 54 FMD viruses involved in SAT-type outbreaks in livestock and wildlife were compared to 140 buffalo viruses representative of different southern African buffalo populations in order to determine the origin and course of epizootics in southern Africa. Isolates of historical importance, such as the immunologically distinct regional viruses first identified in 1948, and those from recent outbreaks of the disease were included in this study. The results confirm that African buffalo in close proximity to livestock and wildlife are the most likely source of infection for these cloven-hoofed species. On rare occasions, transboundary movement of animal and animal products have precipitated outbreaks. Further evidence for the role of antelope as intermediaries in disease transmission between buffalo and cattle is presented and the threat to disease security posed by the illegal trade in FMD-infected buffalo is also highlighted.

6.1 Introduction

Following the rinderpest pandemic of 1889-1897, FMDV disappeared from southern Africa until 1931, when a SAT-2 outbreak occurred in cattle in Zimbabwe. In the decades following this outbreak, epizootics were regularly recorded along South Africa's borders with Zimbabwe and Botswana, up until 1960 (reviewed by Thomson 1994). The drop in the number of outbreaks in SADC countries from the 1980's onwards is attributed in part to improved disease control through vaccination and fencing. Vaccination campaigns commenced early in most southern African countries. Initially, aphthisation (the infection of cattle with FMD positive vesicular fluid or ground tissue suspensions) was practiced from the 1930's until the 1960's, after which attenuated vaccines were used, followed by vaccination with inactivated adjuvant vaccines from the 1970's onwards. Recognition of the role of wildlife in disease transmission was also recognized and addressed through the erection of game proof fences. The efficacy of these disease control measures is reflected in the low number of FMD outbreaks relative to other regions in Africa (see Table 1.2, Chapter 1).

Efforts to control the disease were driven by the need to establish disease-free areas in countries where FMD-infected buffalo occur in order to permit trade. A number of SADC countries were successful in their endeavours to obtain international recognition of FMD zoning within their borders by the OIE. These countries include Botswana, South Africa, Namibia and Zimbabwe. The disease-free status which permitted trade in animals and animal products, has recently come under threat due to the increased numbers of outbreaks both within and outside of the FMD-control areas. Prior to 2000, Namibia, South Africa, Botswana and Swaziland had been free of the disease in livestock since 1994, 1983, 1981 and 1980, respectively (Table 6.1). However in 2000, Namibia, South Africa and Swaziland reported FMD outbreaks. South Africa and Namibia both reported outbreaks within their FMD control areas in August 2000. As these outbreaks were effectively dealt with and contained, recognition by the OIE of FMD-free zones in both countries was maintained. However, following two unrelated FMD outbreaks in the FMD-free zones in South Africa, recognition of disease free status was suspended for this country. Botswana and Namibia are currently the only southern African countries to retain recognition by the OIE of their FMD free zones (OIE list of FMD free countries, January 2001; http://www.oie.int).

The economic implications of an outbreak are far-reaching due both to the immediate cost of controlling the outbreak and the loss of revenue resulting from suspension in trade. Following an

outbreak of FMD certain conditions apply before freedom from the disease can be accepted. If widespread vaccination was instituted, a 2-year period without further outbreaks is required from the cessation of vaccination. However, if vaccination to control an outbreak was avoided, or restricted to ring vaccination, the interval to declaration of freedom from the disease is only 3 months. This shorter time period is however dependent on the absence of further outbreaks, the slaughter of all infected animals and those in close contact, and extensive serological surveys demonstrating freedom from antibody-positive animals.

TABLE 6.1 Summary of FMD outbreaks in livestock in southern African countries prior to 2000

Country	**Last recorded outbreak prior to 2000**	**Serotype/s involved**
¥Angola	1978	A, O, C, SAT -1, SAT-2
Botswana	1981	SAT-2
Lesotho	--	--
†Mozambique	1984	SAT-2
*Malawi	1998	O
§ Namibia	1994	SAT-3
§ South Africa	1983	SAT-2
Swaziland	1969	SAT-2
Zambia	1999	SAT-1
Zimbabwe	§1999	SAT-1 & SAT-3

* Based on data provided by the World Reference Laboratory (WRL), Pirbright, UK
† Based on data provided by the Botswana Vaccine Institute (BVI)
¥ Based on data in Thomson 1994
§ Outbreaks occurring within the FMD control areas

It should be noted that following the outbreak of civil war in Angola and Mozambique that reporting of FMD ceased and that the data for these countries is therefore unreliable. It is likely that the serotypes recorded prior to cessation of reporting are still prevalent in these southern African countries.

6.1.1 Outbreaks in South Africa in 2000

Three separate outbreaks occurred in three different provinces in South Africa in 2000. The first in the Northern Province involved a SAT-1 virus, the second in KwaZulu-Natal was caused by an imported type O strain and the third outbreak in Mpumulanga province was due to a SAT-1 virus.

6.1.1.1 SAT-1 in the FMD-control area

In August 2000, 45 serum samples were collected from buffalo and cattle forming part of an experimental project to breed buffalo free of FMD and corridor disease (CD). The experimental farm housing 67 adult buffalo cows, 26 buffalo calves and 25 Jersey cows adjoins the KNP and falls within the FMD-control area. All sera tested positive for SAT-1. Probang and nasal swabs were subsequently collected from 9 buffalo calves and from 29 jersey cows that were acting as foster mothers to the buffalo. SAT-1 type FMDV was isolated from at least four animals, one dairy cow and three buffalo.

6.1.1.2 Type O in Kwazulu-Natal

In September 2000, serotype O FMD was diagnosed in Kwazulu-Natal province for the first time. The virus initially only affected pigs causing 30 % mortalities in these animals. The source of the infection was believed to have been swill obtained from a shipping carrier in Durban harbour, which was fed to pigs. The farm where the index case was identified was placed under quarantine, as was the zone within a 10km radius around this farm. A surveillance zone of 20km radius was declared around the 10km restriction zone. The disease was subsequently spread to cattle, pigs and goats in neighbouring areas. Stamping out procedures were initially carried out, prior to vaccination within a 15km radius with type O_1 Manisa, a 1969 field strain from Turkey, shown to be antigenically related to the outbreak strain. These viruses, although genetically characterized (Sangare *et al.* 2001) are not included in the present study which specifically focussed on the molecular epidemiology of SAT-type viruses.

6.1.1.3 SAT-1 outside the FMD control area, Mpumulanga province

On 23 November SAT-1 type FMD was diagnosed in 8 of the 110 cattle imported from Mpumulanga province in South Africa into Swaziland at an abattoir in Matsapha (26°31'S

- 31°18'E). Subsequent investigations at the feedlot from which these cattle were imported in Middelburg, South Africa, revealed that 30 animals had clinical FMD lesions and were positive for SAT-1 type virus. Quarantine and surveillance zones were identified and emergency vaccination with a trivalent (SAT 1-3) vaccine, supplied by the Onderstepoort Veterinary Institute was carried out.

6.1.2 FMD in Namibia in 2000

In Namibia an outbreak of SAT-1 (typing done by BVI) was reported in August 2000 in the Eastern Caprivi district (25°07' E - 17°48' S) which lies within the foot-and-mouth disease infected zone. Approximately 30,000 cattle around the focus of infection were vaccinated with trivalent (SAT 1-3) vaccine. By 4 October 2000, 138,542 cattle in the area had been vaccinated and an additional 18 000 head of cattle were revaccinated in the Kasika area (http://www.oie.int).

6.1.3 FMD in Zambia in 2000

In Zambia, four FMD outbreaks were reported in three different provinces in 2000. The first was at Mpulungu, Northern Province (31°30'E - 9°24'S) where the infection is estimated to have occurred on 21 February 2000. The affected cattle herds were 10km from the border and the source of infection is believed to have been from a neighbouring country. The serotype involved was not identified. In May 2000 a second outbreak was reported in Chapalonga village, Lundazi district, Eastern Province (11°50'S-33°18'E). In August 2000 SAT-1 outbreaks were reported in two localities in the Western Province, the first was in the Mwandi area, Sesheke (17°32'S - 24°54'E) whilst the second was at Sikuzu crushpen, Sesheke (17°32'S - 24°51'E; http://www.oie.int).

Reports of FMD outbreaks were made by 6 southern African countries at the 14th Conference of the Regional Commission for Africa of the OIE held in Arusha (Tanzania), 23-26 January 2001. In addition to the outbreaks in South Africa, Swaziland, Zambia and Namibia, detailed above, officials from Malawi and Angola reported outbreaks in 2000 (OIE press release, 26 January, 2000, Paris). It is noteworthy that although no official reports of FMD in Angola were made to the OIE in 2000,

the delegate from this country confirmed the presence of the disease there during the year under review confirming the view that there is under reporting to the OIE.

6.1.4 FMD in Zambia in 1999

A SAT-1 virus isolated by the Botswana Vaccine Institute (BVI) from an outbreak reported in August 1999 at Kazungula, Southern Province (25°15'E -17°38'S) was supplied to the Onderstepoort Veterinary Institute. The affected population was a transhumant herd of cattle located on a river island. The affected herd was suspected to have been in contact with young buffaloes from a nearby national game park (http://www.oie.int).

6.1.5 FMD in Zimbabwe 1999

On the 25th of June 1999 samples from an FMD outbreak at Mapanza Estate (20° 55' S - 31° 47' E), Chiredzi, Masvingo Provine were received by OVI where the virus was identified as SAT-3. On 21 July 1999, samples from Mkwasine Ranch (20° 50' S - 32°00' E), also within the Chiredzi district of Masvingo province (http://www.oie.int), were submitted to OVI by Zimbabwean authorities. The virus was identified in the latter outbreak was SAT-1 and therefore unrelated to the outbreak occurring 2 weeks earlier. The source of the infection was believed to be wildlife as the areas adjoining the ranches where the outbreaks occurred were mainly stocked with buffalo (*Syncerus caffer*), impala (*Aepyceros melampus*), kudu (*Tragelaphus strepisiceros*) and eland (*Taurotragus oryx*). Transmission from buffalo to cattle is unlikely as buffalo-proof fences separate wildlife and livestock. It is believed that infected antelope crossed the FMD control fences from wildlife areas to cattle areas and facilitated transmission. The SAT-1 and SAT-3 outbreaks occurred despite the fact that both Mapanza Estate and Mkwasine Ranch fall within the FMD vaccination control zone where vaccination of cattle with trivalent vaccine occurs regularly. No new cases of FMD have been reported from Zimbabwe since 28 July 1999.

6.1.6 FMD in South Africa in 1998

Infection with SAT-3 virus was detected in buffalo in August 1998 in the Potgietersrus district in the Northern Province of South Africa. This area occurs within the zone recognized by the OIE as free from FMD. The 'disease-free' buffalo were purchased from a farm in the Free State Province, which is outside of the FMD control area. Because of the origin of these buffalo, intensive serological surveys in the area surrounding the source farm were conducted in order to ensure that the disease had not spread from the buffalo. Sera from 1295 bovine, 784 ovine and one buffalo were screened, all of which were negative for the three SAT-types. In the interim, the buffalo which had tested positive for FMD were quarantined and later destroyed. SAT-3 type virus was isolated from three of the 7 buffalo purchased.

6.1.7 FMD in Zimbabwe in 1997

In 1997 a SAT-2 outbreak occurred in cattle in the Chiredzi district in southern Zimbabwe. Kudu and impala in adjoining wildlife areas were shown to have antibodies to SAT-2 virus. These results together with the fact that the cattle were separated from buffalo by game proof fences which were of an insufficient height to contain the former antelope species, provides circumstantial evidence for the role of antelope in disease transmission in this case.

In order to preclude future outbreaks of the disease in the southern African region it is essential to establish the origin of recent outbreaks so that appropriate disease control measures can be put in place. In this chapter, both historical and contemporary outbreaks of FMD will be investigated through molecular epidemiological studies. The role of the African buffalo as the ultimate source of infection for both wildlife and cattle will also be addressed by including viruses representative of as many southern African maintenance host populations as possible.

6.2 Materials and Methods

6.2.1 SAT-1 viruses used in this study

A total of 60 buffalo viruses were selected for this study, of which 30 were genetically characterized in Chapter 3. The remaining 30 buffalo viruses were selected to compliment the existing SAT-1 buffalo database (Chapter 3). Twenty-six outbreak viruses recovered from wildlife and cattle, from 1948 to 2000 were selected for genetic characterization. Included in the outbreak virus group is the first SAT-1 virus typed, BEC/1/48, as well as SAT-1 viruses recovered from clinical cases in cattle in Swaziland and South Africa in 2000. Eighty-six viruses were ultimately included in the phylogenetic analysis, the details of which are summarized in Table 5.1.

6.2.2 SAT-2 viruses used in this study

Seventy viruses were used to determine the genetic relationships of SAT-2 type field strains recovered from buffalo, impala and cattle sampled between 1948 and 2000. Due to the extensive characterization of SAT-2 viruses recovered from impala outbreaks in the Kruger National Park (Chapter 3), impala viruses isolated from the 1988-89, 1992-93 and the 1995 epizootics (N=13) were not included here. Instead isolates recovered from recent outbreaks of FMD in Kenya, Eritrea, Saudi Arabia and Zimbabwe were selected for this study and as were outbreaks of historical interest. The latter include RHO/1/48, a historical SAT-2 virus and PAL/5/83, the last SAT-2 type virus causing an outbreak of FMD in cattle in South Africa prior to 2001. The geographical and species origin of previously uncharacterized SAT-2 type viruses are detailed in Table 5.2. They include 20 cattle strains, 4 impala viruses and 8 new buffalo isolates. Details of the remaining 42 buffalo viruses are available in Chapters 3 and 5, Tables 3.2 and 5.3, respectively.

6.2.3 SAT-3 viruses used in this study

In addition to the 30 buffalo viruses previously characterized in Chapter 5, an additional 8 outbreak strains were characterized here. Seven of the viruses are from clinical cases of FMD in cattle and one virus was obtained from a FMD-infected buffalo sourced from a farm outside the FMD control area.

6.2.4 Genetic characterization and analysis

Genomic amplification of the VP1 gene and nucleotide sequencing of the purified product was performed as described in previous chapters. Phylogenetic reconstruction was performed using the neighbor-joining algorithm and Jukes & Cantor correction included in MEGA (Kumar *et al.* 1993), with support for each of the virus clusters being assessed by 1000 bootstrap replications. Gene regions used for the analyses correspond to those previously outlined in Chapter 5, for each of the SAT serotypes.

TABLE 6.2: Summary of previously uncharacterized SAT-1 viruses used in this study

Virus name	Country of origin	Year of sampling	Place of origin	Grid reference	Species of origin
BEC/1/48	Botswana	1948	NA	NA	Cattle
SAR/13/61	South Africa	1961	Potgietersrus	29°01'E-24°11'S	Cattle
BOT/1/68	Botswana	1968	Satau	24°25'E-18°02'S	Cattle
SAR/10/71	South Africa	1971	Sugar Station, Komatipoort	31°00'E-24°00'S	Impala
SAR/11/71	South Africa	1971	Sugar Station, Komatipoort	31°00'E-24°00'S	Impala
SAR/1/73	South Africa	1973	Area 3, Kruger NP	31°16'E-24°15'S	Impala
SAR/5/75	South Africa	1975	Grootdraai, Hectorspruit	31°00'E-25°20'S	Cattle
BOT/1/77	Botswana	1977	Nonaneng	22°11'E-19°40'S	Cattle
BOT/17/77	Botswana	1977	30km SW of Habu	NA	Kudu
BOT/24/77	Botswana	1977	Habu	NA	Cattle
MOZ/3/77	Mozambique	1977	Choque	33°00'E-24°15'S	Cattle
SAR/9/81	South Africa	1981	Pafuri, Kruger NP	Pafuri	Impala
KNP/3/86	South Africa	1986	Tshokwane, Kruger NP	31°51'E-24°47'S	Buffalo
KNP/6/86	South Africa	1986	Tshokwane, Kruger NP	31°51'E-24°47'S	Buffalo
KNP/1/87	South Africa	1987	Mooiplaas, Kruger NP	31°27'E-24°34'S	Buffalo
KNP/4/89	South Africa	1989	Mala Mala, Kruger NP	31°30'E-24°55'S	Buffalo
KNP/8/89	South Africa	1989	Meseldam, Kruger NP	31°13'E-25°07'S	Buffalo
KNP/20/89	South Africa	1989	Kwa Mfamebto, Kruger NP	31°12'E-25°06'S	Buffalo
NAM/2/89	Namibia	1989	W. of Kwando river, Caprivi	23°20'E-17°50'S	Buffalo
ZIM/HV11/90	Zimbabwe	1990	Hippo Valley	31°35'E-21°10'S	Buffalo
ZIM/HV28/90	Zimbabwe	1990	Hippo Valley	31°35'E-21°10'S	Buffalo
ZIM/HV29/90	Zimbabwe	1990	Hippo Valley	31°35'E-21°10'S	Buffalo
ZIM/14/90	Zimbabwe	1990	Bumi Hills	28°22'E-16°49'S	Buffalo
ZIM/16/90	Zimbabwe	1990	Doma SA	30°15'E-16°20'S	Buffalo
ZIM/25/90	Zimbabwe	1990	Chirisa SA	28°15'E-18°00'S	Buffalo
ZIM/26/90	Zimbabwe	1990	Dande SA	30°20'E-15°55'S	Buffalo
ZIM/38/90	Zimbabwe	1990	Chirisa SA	28°15'E-18°00'S	Buffalo
ZIM/47/90	Zimbabwe	1990	Dande SA	30°20'E-15°55'S	Buffalo
KNP/148/91	South Africa	1991	Renosterkoppies, Kruger NP	31°36'E-25°07'S	Buffalo
ZIM/Gn16/91	Zimbabwe	1991	Gonarezhou NP	32°00'E-21°30'S	Buffalo
ZIM/GN34/91	Zimbabwe	1991	Gonarezhou NP	32°00'E-21°30'S	Buffalo
ZIM/1B/91	Zimbabwe	1991	Urungwe SA	28°55'E-16°30'S	Buffalo

Virus name	Country of origin	Year of sampling	Place of origin	Grid reference	Species of origin
ZIM/5/91	Zimbabwe	1991	Larry Cummings GR, Matetsi	25°44'E-10°03'S	Buffalo
ZIM/8/91	Zimbabwe	1991	Urungwe SA	28°55'E-16°30'S	Buffalo
ZIM/6/94	Zimbabwe	1994	Hwange NP	27°00'E-19°00'S	Buffalo
ZIM/3/95	Zimbabwe	1995	Kariba	28°20'E-16°47'S	Buffalo
ZIM/12/95	Zimbabwe	1995	Kariba	28°20'E-16°47'S	Buffalo
UGA/1/97	Uganda	1997	Queen Elizabeth NP	30°00'E-00°00'S	Buffalo
KNP/131/98	South Africa	1998	Phelwane Camp, Kruger NP	31°34'E-24°26'S	Impala
KNP/143/98	South Africa	1998	Kempiana, Kruger NP	NA	Impala
KNP/144/98	South Africa	1998	Kempiana, Kruger NP	NA	Impala
NAM/288/98	Namibia	1998	West Caprivi GR	23°00'E-18°00S	Buffalo
TAN/1/99	Tanzania	1999	Menganyi Range, Matara	NA	Cattle
TAN/2/99	Tanzania	1999	Menganyi Range, Matara	NA	Cattle
ZAM/1/99	Zambia	1999	Kazungula, Southern Province	25°15'E-17°18'S	Cattle
ZIM/5/99	Zimbabwe	1999	Mkwasine Range, Chiredzi	32°00'E-20°50'S	Cattle
ZIM/6/99	Zimbabwe	1999	Mkwasine Range, Chiredzi	32°00'E-20°50'S	Cattle
ZIM/7/99	Zimbabwe	1999	Mkwasine Range, Chiredzi	32°00'E-20°50'S	Cattle
SAR/1/00	South Africa	2000	Phalaborwa District, Northern Province	31°00'E-24°06'S	Buffalo
SAR/2/00	South Africa	2000	Phalaborwa District, Northern Province	31°00'E-24°06'S	Buffalo
SAR/3/00	South Africa	2000	Phalaborwa District, Northern Province	31°00'E-24°06'S	Buffalo
SAR/4/00	South Africa	2000	Phalaborwa District, Northern Province	31°00'E-24°06'S	Cattle
SAR/8/00	South Africa	2000	Phalaborwa Distirct, Northern Province	31°00'E-24°06'S	Buffalo
SAR/32/00	South Africa	2000	Middleburg District, Mpumulanga Province	29°34'E-25°54'S	Cattle
SWL/1/00	Swaziland	2000	Middleburg District, Mpumulanga Province	29°34'E-25°54'S	Cattle
SWL/4/00	Swaziland	2000	Middleburg District, Mpumulanga Province	29°34'E-25°54'S	Cattle
SAR/34/00	South Africa	2000	Middleburg District, Mpumulanga Province	29°34'E-25°54'S	Cattle

GR: Game Reserve; NA: Not available; NP: National Park; SA: Safari Area

TABLE 6.3 Summary of previously uncharacterized SAT-2 viruses used in this study

Virus name	Country of origin	Year of sampling	Place of origin	Grid reference	Species of origin
RHO/1/48	Zambia	1948	NA	NA	Cattle
KEN/3/57	Kenya	1957	Wamba	NA	Cattle
ANG/4/74	Angola	1974	Quibala Raspaguil de Asias da Bola	11°00'E-15°00'S	Cattle
BOT/3/77	Botswana	1977	NA	NA	Cattle
RHO/2/79	Zimbabwe	1979	Chiredzi	31°50'E-21°10'S	Cattle
ZAI/1/82	DRC	1982	Bibatama, Nord Kivu	NA	NA
MOZ/4/83	Mozambique	1983	NA	NA	Cattle
PAL/5/83	South Africa	1983	Phalaborwa	31°00'E-23°58'S	Cattle
SAR/16/83	South Africa	1983	Leeupan	31°48'E-24°50'S	Impala
ZIM/7/83	Zimbabwe	1983	Nyamandhlovu	28°05'E-19°45'S	Cattle
KNP/1/85	South Africa	1985	Gudzane, Kruger NP	31°50'E-24°15'S	Impala
SWA/4/89	Namibia	1989	Sigwe village, East Caprivi	25°01'E-17°44'S	Cattle
BUN/1/91	Burundi	1991	Bujumbura province	NA	Cattle
NAM/1/91	Namibia	1991	East Caprivi	NA	Cattle
NAM/1/92	Namibia	1992	80km E of Rundu, Kavango	NA	Cattle
ZAM/7/96	Zambia	1996	Mulanga	NA	Buffalo
ZIM/1/97	Zimbabwe	1997	Chiredzi	31°48'E-20°50'S	Cattle
ZIM/4/97	Zimbabwe	1997	Chiredzi	31°48'E-20°50'S	Cattle
ZIM/44/97	Zimbabwe	1997	Mukazi Ranch, Chiredzi	31°48'E-20°50'S	Buffalo
ZIM/48/97	Zimbabwe	1997	Mukazi Ranch, Chiredzi	31°48'E-20°50'S	Buffalo
BOT/1/98	Botswana	1998	Nxaraga	23°15'E-19°40'S	Buffalo
BOT/18/98	Botswana	1998	Nxaraga	23°15'E-19°40'S	Buffalo
NAM/304/98	Namibia	1998	Mahango, W Caprivi GR	21°50'E-18°15'S	Buffalo
ERI/12/98	Eritrea	1998	Erythrea	NA	Cattle
KEN/5/99	Kenya	1999	Athi river, Machakos	NA	Cattle
KEN/7/99	Kenya	1999	Kikuya, Kiambu	NA	Cattle
KEN/8/99	Kenya	1999	Jkia, Nairobi	NA	Cattle
KEN/9/99	Kenya	1999	Kaloleni, Kilifi	NA	Cattle
RWA/1/00	Rwanda	2000	Gishwati district	NA	Buffalo
SAU/6/00	Saudi Arabia	2000	Al Kahrj, Riyadh	NA	Cattle
ZIM/1/00	Zimbabwe	2000	Elephant Walk, Tengwe Farm	29°30'E-17°12'S	Buffalo

GR: Game reserve, NA: Not available; NP: National Park; SA: Safari Area

TABLE 6.4 Summary of previously uncharacterized SAT-3 viruses used in this study

Virus name	Country of origin	Year of sampling	Place of origin	Grid reference	Species of origin
BEC/1/65	Botswana	1965	Yaoyaga	NA	Cattle
RHO/3/78	Zimbabwe	1978	Humani Ranch, Fort Victoria	NA	Cattle
NAM/1/94	Namibia	1994	Kasika, Caprivi	25°07'E-17°48'S	Cattle
NAM/5/94	Namibia	1994	Kasika, Caprivi	25°07'E-17°48'S	Cattle
POT/336/98	South Africa	1998	Potgietersrus district	29°03'E-24°10'S	Buffalo
ZIM/1/99	Zimbabwe	1999	Mapanza Estate, Chiredzi	31°47'E-20°55'S	Cattle
ZIM/3/99	Zimbabwe	1999	Mapanza Estate, Chiredzi	31°47'E-20°55'S	Cattle
ZIM/4/99	Zimbabwe	1999	Mapanza Estate, Chiredzi	31°47'E-20°55'S	Cattle

NA: Not available

6.3 Results

Genetic relationships of SAT-type viruses recovered from wildlife and domestic livestock in the southern African region were determined by phylogenetic analysis of nucleotide sequences of the major immunogenic protein of FMDV, the VP1 protein. The interpretation of VP1 data generated for the purpose of molecular epidemiological studies follows the rationale established in previous chapters (Bastos 1998; Bastos *et al.* 2000; Bastos *et al.* 2001) and by others (Saiz *et al.* 1993; O. Marquardt, pers comm 2000; Sangare *et al.* 2001), where the C-terminus half of VP1 (> 380 nt) has been characterized. Briefly, the genetic relationship of any two field strains of FMDV is reflected by their genetic distance and by phylogenetic resolution in the following manner :

- Contemporary isolates of an epizootic differ by 1 % or less
- Isolates from the same epizootic but different years differ by 2- 7 % from each other
- Viruses of the same genotype differ by no more than 14 % from each other
- Viruses from separate evolutionary lineages / topotypes differ by 20 % or more
- Viruses representative of different serotypes differ by > 35 %

6.3.1 Genetic relationships of SAT-1 viruses in southern Africa (1948-2000)

Four distinct evolutionary lineages were recovered by phylogenetic analysis of partial VP1 gene data (Fig. 6.1). The three southern Africa buffalo topotype distributions (I-III) previously identified (Chapter 5) were unaffected by the inclusion of a further 30 buffalo viruses. Inclusion of a buffalo virus from Queen Elizabeth National Park in Uganda (UGA/1/97) revealed that buffalo viruses circulating in this east African region are genetically unrelated to the southern African topotypes. An additional topotype was therefore allocated (topotype IV).

Within topotype I, seven genetically distinct outbreaks occurred in wildlife and cattle between 1971 and 2000. Three of the outbreaks can be directly linked to genotypes associated with specific buffalo herds. These include the link between the 1999 cattle viruses (ZIM 5-7/99) in southern Zimbabwe and buffalo viruses from Hippo Valley and Gonarezhou National Park (99 % bootstrap support), the grouping of impala viruses recovered from the 1998 outbreak in the Orpen area of the Kruger National Park and a buffalo virus, KNP/75/98 (91 % bootstrap support), and the link between cattle viruses from Swaziland and South Africa with a buffalo virus from Lower Sabie, KNP/22/96 (99 % bootstrap support). The viruses recovered from the latter Mpumulanga outbreak are clearly unrelated to the virus involved in clinical FMD in buffalo and cattle, 3 months earlier in the Northern Province. In that case a direct link between cattle (SAR/4/00) and buffalo calves, occurring at a private game farm within the red-line area (100 % bootstrap support), was clearly demonstrated, although the geographical area in the KNP from which this virus originated is not clear. The apparently prolonged circulation of FMDV in impala from 1971 (SAR/10/71 and SAR/11/71) to 1973 (SAR/1/73), is of interest as this aspect of the disease in this antelope species has only recently been recognized by genetic characterization of contemporary viruses (Vosloo *et al.* 1992; Keet *et al.* 1996). The 1971 and 1973 impala viruses, which were sampled 31 months apart differ from each other by 8 % on nucleotide level. Given the rapid rate of accumulation of mutations in the VP1 gene of SAT-type viruses (Vosloo *et al.* 1996) an 8 % difference on nucleotide level is possible for viruses of common origin over this time period. Thus the 1971 and 1973 impala viruses appear to be part of the same epizootic and point to the possibility of a >2.5 year circulation period of FMDV in impala populations, which is longer than that previously identified (Chapter 3) for SAT-2 type virus causing clinical disease in impala (Keet *et al.* 1996; Bastos *et al.* 2000). The possibility of prolonged circulation of a virus strain in the field is also provided by the grouping of MOZ/3/77 and SAR/9/81, where there is 100 % bootstrap support for the grouping of these viruses. The former is of cattle origin, whilst the latter was recovered from impala in the Pafuri area of the Kruger National Park.

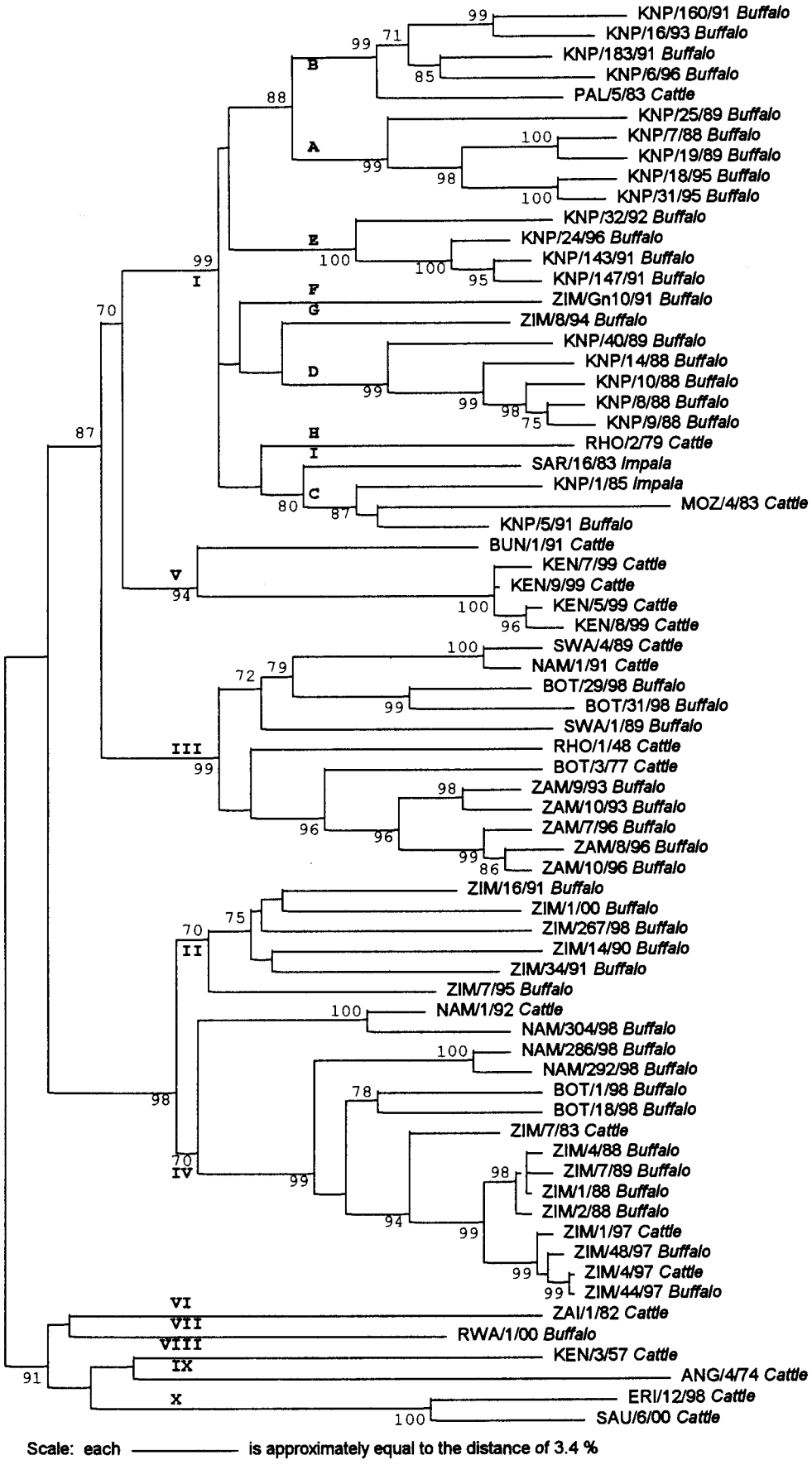

Fig. 6.2 Neighbor-joining tree depicting VP1 gene relationships of SAT-2 type viruses from buffalo, cattle and impala in southern, central and eastern Africa (1948-2000) and in Saudi Arabia (2000)

Five genetically unrelated outbreaks of FMDV are represented within the topotype II buffalo virus cluster, namely the 1948, 1968 and 1977 outbreaks in Botswana, the 1961 outbreak in South Africa and the 1999 outbreak in Zambia. The three 1977 outbreak strains are part of the same epizootic. BOT/1/77 from cattle and BOT/17/77 share more than 99 % nucleotide sequence identity and 100 % bootstrap support, providing additional evidence for inter-species transmission. The BOT/1/68 and SAR/13/61 cannot be directly linked to any contemporary buffalo viruses and the exact origin of these viruses remains unclear, although they do fall within a cluster comprising viruses from Namibia and Botswana (60 % bootstrap support). The direct link between ZAM/1/99 and ZIM/2/94 (94 % bootstrap support) provides evidence for transboundary transmission. Similarly the grouping of a SAR virus within topotype II (Botswana, Namibia and western Zimbabwe region) is also suggestive of virus introduction into South Africa from one of the afore-mentioned topotype II countries. The historical BEC/1/48 strain is unrelated to any of the buffalo virus lineages, but is clearly a topotype II virus (99 % bootstrap support).

In topotype III, all viruses are of buffalo origin with the exception of TAN 1-2/99. Phylogenetic resolution reveals that these Tanzanian viruses are most closely related to Zambian buffalo strains. More accurate determination of the origin was not possible due to the unavailability of SAT virus sequence data from Tanzanian buffalo populations.

6.3.2 Genetic relationships of SAT-2 viruses (1948-2000)

Ten distinct evolutionary lineages (I-X) were identified by phylogenetic analysis of SAT-2 viruses from the Middle East (Saudi Arabia) and from southern, central and eastern Africa (Fig. 6.2). Eight of the lineages correspond to geographically discrete localities, with the remaining two lineages (III and IV) displaying some overlap in distribution. Lineages VI-IX are represented by viruses, from the Democratic Republic of the Congo, Rwanda, Kenya and Angola, respectively. These viruses differ from each other and from all other viruses included in the analysis to such an extent (> 21 % in uncorrected pairwise comparisons), that four independent genetic lineages could be assigned, one for each of the viruses. Angola, represented by ANG/4/74 is the only southern African country which has a distinct evolutionary lineage (IX) within a virus grouping comprising primarily east and central African topotypes (IV-X; 89 % bootstrap support).

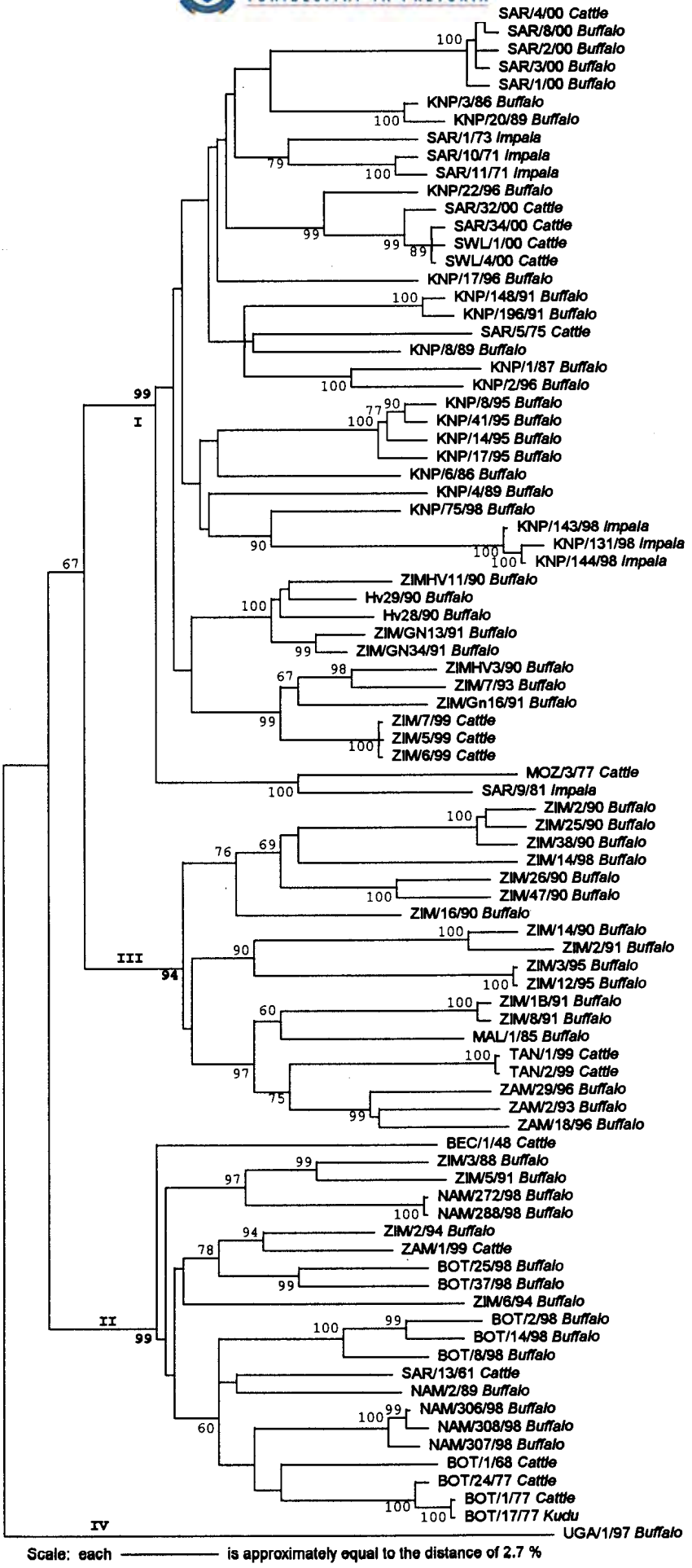

Fig.6.1: Neighbor-joining tree depicting VP1 gene relationships of SAT-1 viruses (1948-2000) recovered from African buffalo (*Syncerus caffer*), impala (*Aepyceros melampus*), kudu (*Tragelaphus strepsiceros*) and cattle

6.3.2.1 VP1 gene relationships of SAT-2 viruses in southern Africa

Within topotype I, nine distinct viral genotypes (A-I) occur, six of which (A-F) were previously identified in Chapter 3 (Bastos *et al.* 2000). Based on genetic distance, the five outbreak strains which fall within this topotype are not part of the same epizootic (differ by > 10 % or more) and cluster within four distinct genotypes (B, C, F and G). PAL/5/83 the last recorded SAT-2 virus causing FMD in cattle in South Africa (prior to the 2001 outbreak in the Northern Province) falls within a group of KNP buffalo viruses (99 % bootstrap support) from Ndziyospruit (KNP160/91), Water Affairs Weir (KNP183/91), Capricorn (KNP/16/93) and Malangangwane Dam (KNP/6/96). These four buffalo viruses were previously shown (Chapter 3) to comprise a single KNP buffalo genotype (B) which occurs both north and south of the Olifants river. RHO/2/79 and SAR/16/83 are genetically distinct from other topotype I viruses, whilst KNP/1/85 and MOZ/4/83 although both representative of genotype C, differ from each other by 14 % on nucleotide level and are therefore not part of the same epizootic.

Within topotype II no outbreak strains are represented, whilst in topotype III, two cattle viruses sampled 2 years apart, SWA/4/89 and NAM/1/91, are clearly part of the same epizootic (differ by less than 2.6 % from each other). Three unrelated outbreaks are presented in topotype IV. ZIM/7/83 and NAM/1/92 can be linked directly to buffalo viruses circulating in Hwange National Park (94 % bootstrap support) and Mahango (100 % bootstrap support), respectively. Viruses recovered from the 1997 outbreak in cattle in the Chiredzi district in southern Zimbabwe were more closely related to buffalo viruses from Hwange National Park (collected in 1988 and 1989) in western Zimbabwe, than to Gonarezhou buffalo viruses in the south. Subsequent field investigations revealed that buffalo from Hwange NP purchased two years prior to outbreak were on a private conservancy in close proximity to where the outbreak occurred. Sampling of these buffalo (ZIM/44/97 and ZIM/48/97) unequivocally showed that they were the source of infection for the affected cattle (99 % bootstrap support).

6.3.2.2 VP1 gene relationships of SAT-2 viruses in eastern Africa

The four Kenyan viruses (KEN/5/99 and KEN/7-9/99) are clearly part of the same epizootic as they differ by 3 % or less from each other. These viruses from Kenya together with an isolate from Burundi comprise Topotype V. Although part of the same topotype (92 %

bootstrap support) the viruses from these two countries represent two distinct genotypes (> 16 % difference). Of interest is the close genetic relationship of topotype V, a virus cluster comprising Kenyan viruses, with southern African topotypes (I-IV; 89 % bootstrap support). Viruses from other east African countries such as Rwanda and Kenya form distinct evolutionary lineages, represented by single viruses, separate from the southern African topotypes. Similarly, a virus from central Africa (ZAI/1/82; topotype VI) is genetically distinct from all other viral lineages. Of note is the link (100 % bootstrap support) between an Eritrean virus (1998) and the SAT-2 strain that was introduced into Saudi Arabia, SAU/6/00 (> 90 % sequence identity) which points to the role of this and perhaps other east African countries in virus introductions into the Middle East.

6.3.3 Genetic relationships of SAT-3 type viruses in southern Africa

Within the SAT-3 serotype, four unrelated FMD epizootics occurred in cattle between 1965 and 1999. Two of the outbreak lineages fall within topotype I and the remaining two within topotype III. In the former topotype, the 1999 outbreak which occurred in cattle in southern Zimbabwe could be directly linked to buffalo viruses circulating in the Gonarezhou National Park (97 % bootstrap support). The historical RHO/3/78 virus is not directly related to any of the buffalo viruses characterized here and is representative of a distinct topotype I genotype. Similarly, within topotype III, BEC/1/65 is unrelated to other viruses within this topotype, as are the 1994 cattle viruses recovered from the outbreak in Namibia. The FMD-infected buffalo (POT/336/98) originating from a farm in the Free State province, is most closely related to KNP buffalo viruses from the Langtoon Dam area in the northern part of the Park (67 % bootstrap support). Seropositivity of these buffalo to both FMD and *Theileria parva lawrencei*, the organism causing corridor disease (CD) in cattle, indicates that their true origin is from a region in South Africa where both diseases are prevalent. The Kruger National Park is the only region in South Africa where buffalo are infected with both FMD and CD. The genetic characterization of the FMD virus presented here confirms that KNP is indeed the origin and further identifies the northern region of the game park as being the most likely origin of these buffalo. This part of the game park is coincidentally an area in which TB infection rates in buffalo are relatively low and the only region from which buffalo translocations to adjoining private game farms in the FMD control area have been permitted in recent years.

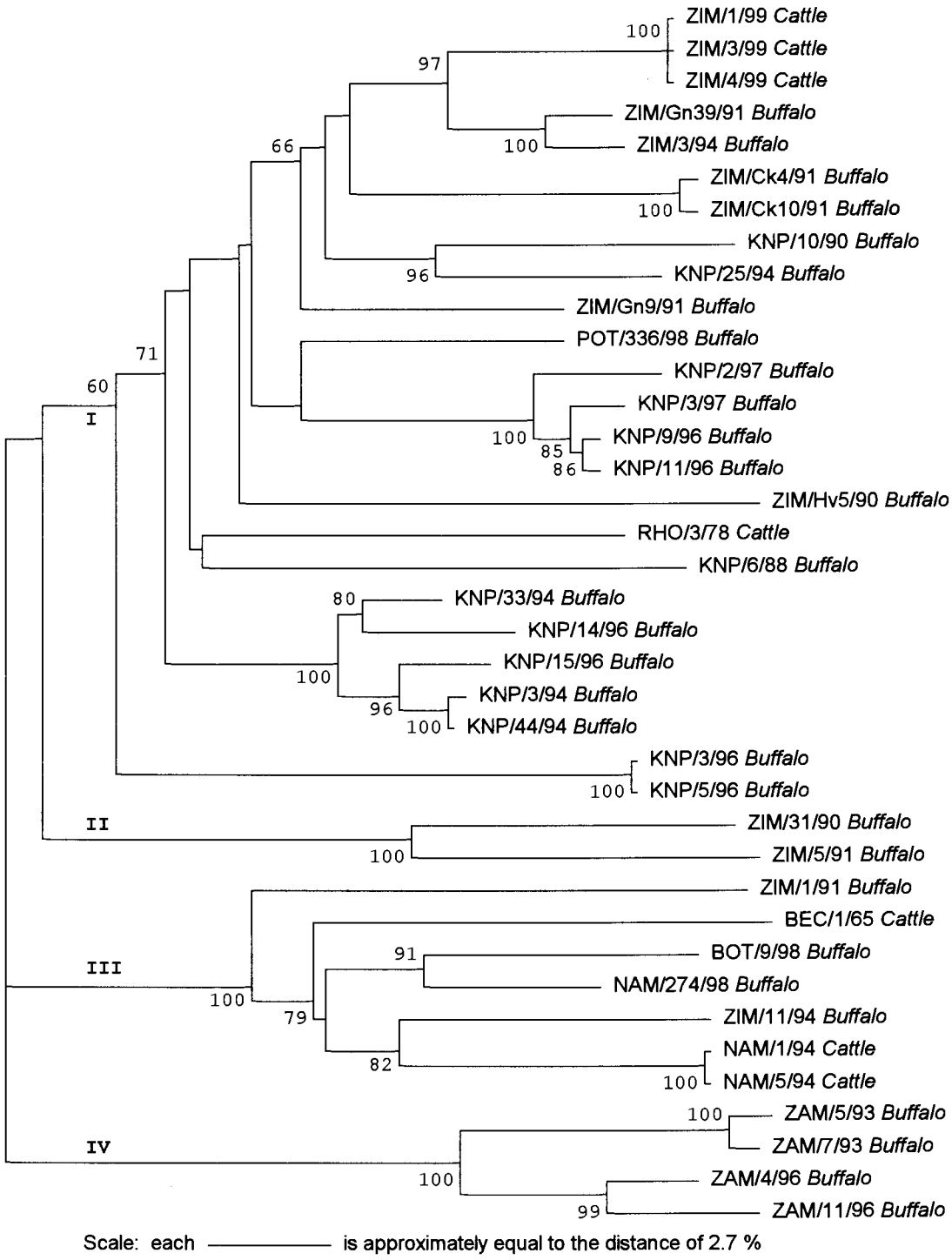

Fig. 6.3 Neighbor-joining tree depicting VP1 gene relationships of SAT-3 type viruses from buffalo and livestock in southern Africa (1965-1999)

6.3.4 Overall levels of genetic variation in the C-terminal half of the VP1 gene

Levels of variation per serotype are expressed as the percentage of sites that are not completely conserved across all viruses used in the analysis. In contrast to results obtained when 30 buffalo viruses were characterized per serotype (Chapter 5), the levels of genetic diversity within the VP1 gene of SAT-type viruses is highest here for SAT-1 and lowest for SAT-3 on both amino acid and nucleotide level (Table 6.5). This is likely due to the higher number of viruses analysed for the former serotype, compared to that analysed for the latter.

TABLE 6.5 Genetic variation in C-terminal VP1 gene sequences of SAT-type viruses of diverse geographical and species origin

Serotype	No of OTUs	Overall nucleotide variation	Overall amino acid variation
SAT-1	87	64 %	57 %
SAT-2	72	63 %	52 %
SAT-3	38	56 %	47 %

6.4 Discussion

By phylogenetic resolution of VP1 gene relationships of SAT-type viruses, it was shown that the geographical origin of outbreak strains generally coincide with the topotype assignment of buffalo viruses from the same localities. This indicates that most outbreaks occur following direct or indirect transmission of virus between animals in close proximity to each other rather than being due to importation of the disease or through the movement of infected animals between countries within the different topotype regions. However, importation of disease has occurred on occasion and is best illustrated by molecular epidemiological studies of SAT-1 type viruses. Prior to the erection of fences between South Africa and neighbouring countries transboundary transmission was implicated in outbreaks along borders. This was confirmed in this study by the finding that the SAT-1 virus causing an outbreak in cattle in the Potgietersrus area in South Africa in 1961 (SAR/13/61) originates from the buffalo topotype II region comprising viruses from Namibia, Botswana and western Zimbabwe. Other evidence of cross-border movement is provided by the link between the cattle outbreak in Mozambique (1977) and the widespread SAT-1 outbreak affecting numerous species of wildlife 1981

(SAR/9/81) in the Kruger National Park (Keet *et al.* 1996). In this case, the affected areas were in close proximity to each other. Records further indicate that movement of wildlife between South Africa and Mozambique was common along the eastern border of the Kruger National Park (Stevenson-Hamilton 1937), prior to the erection of game-proof fences, making it likely that common SAT-1 genotypes occur in these neighbouring countries. Although both cases refer to historical outbreaks, evidence from recent outbreaks suggests that transboundary movement of animals and animal products remains a threat. This is exemplified by the recent importation of SAT-1 virus into Swaziland from South Africa. Prior to this, Swaziland had been free of the disease for 31 years. Phylogenetic results also confirm that the outbreak occurring in Zambia in 1999 (ZAM/1/99) was most likely due to importation of the disease from western Zimbabwe as the outbreak strain groups with a buffalo virus from Hwange National Park (94 % bootstrap support). Of even greater concern was the type O outbreak occurring in KwaZulu-Natal in 2000, which arose after pigs were fed swill obtained from ships in Durban Harbour (Sangare *et al.* 2001). Type O had never been recorded in South Africa prior to this and vaccines against this serotype were not as readily available as those against the endemic SAT-type viruses. These three recent examples stress the need for greater vigilance by veterinary authorities, strict adherence to disease control measures (including the immediate decontamination of waste coming into a country via different means of transportation) and the availability of vaccines and diagnostic methods capable of addressing all FMD virus serotypes.

Molecular epidemiological studies are particularly useful for discerning whether outbreaks occurring in the same year are part of the same epizootic or not. In this study it was shown that the three SAT-2 outbreaks which occurred in 1983 (two in South Africa and one in neighbouring Mozambique) were not part of the same epizootic as the viruses represent three distinct virus genotypes (B,C and I). Similarly, SAT-1 outbreaks occurring in Botswana and Mozambique were shown to be unrelated as were the SAT-1 outbreaks in Tanzania, Zambia and Zimbabwe in 1999 and the two SAT-1 outbreaks in cattle in South Africa in 2000. Genetic characterization of viruses is also useful for identifying viral persistence in the field. In this study, prolonged circulation of viruses was shown to occur in livestock as well as in impala antelope. Two SAT-2 viruses recovered from cattle sampled two years apart, SWA/4/89 and NAM/1/91 were shown to be part of the same epizootic (97.4 % sequence identity) pointing to a circulation period of at least two years in the field. A circulation period of more than 2.5 years was demonstrated for SAT-1 viruses recovered from the 1971 and 1973 impala epizootics. Prolonged circulation of FMDV in impala populations in the KNP has been demonstrated on three

separate occasions and has involved two of the three SAT serotypes circulating in the game park. This is significant as impala and kudu antelope are believed to be intermediaries in disease transmission between buffalo and impala and were historically used as indicator species to determine the rate and spread of the disease from wildlife to livestock (Meeser 1962). Circumstantial evidence indicating that a 1958 SAT-3 outbreak in impala subsequently spread to other species, including cattle, has been presented (Meeser 1962), however unequivocal evidence of a shared outbreak strain between cattle, buffalo and antelope species is presently lacking. The results provided here do however show that viruses recovered from cattle and kudu (SAT-1; Botswana 1977) and from cattle and impala (SAT-1; Mozambique 1977 and South Africa 1981) were part of the same epizootic and that transmission between these species does occur in the field. In Chapter 3, the transmission of FMDV between buffalo and impala was also demonstrated by nucleotide sequence characterization of field strains (Bastos *et al.* 2000). The link between SAT-2 cattle and buffalo strains sampled in 1997 in Zimbabwe and serological evidence of infection of antelope species is perhaps the strongest evidence thus far obtained for the role of antelope as intermediaries in disease transmission.

In summary, this chapter has confirmed the role of African buffalo as the primary source of infection for livestock in southern Africa as most outbreaks could be linked to buffalo in close proximity to where the outbreak occurred. In some cases it was possible to identify herds within specific game parks, from which virus most likely originated, but in others it was only possible to link epizootics to specific genotypes or topotypes. This clearly illustrates that the accuracy with which the origin of an outbreak can be determined is directly dependent on the extent to which the viruses from FMD-infected buffalo populations within a country or region have been genetically characterized. Further genetic studies on buffalo viruses should be encouraged, particularly in Namibia, Botswana, Mozambique, Angola and Malawi, where there is an under-representation or no representation of genetic diversity in the field. This would permit the establishment of a truly representative database for the southern African region and provide a powerful means of determining factors threatening the disease-free status of SADC countries. It would also act as a deterrent to the illegal trade in FMD-infected African buffalo as the geographic origin of buffalo that have been moved illegally was clearly shown by molecular epidemiological studies. The trade in wildlife and 10-fold discrepancy in prices between disease-free and infected buffalo poses a serious threat to disease security and should be addressed through legislation and prosecution, the success of which may be dependent on the availability of accurate and powerful genetic tracking tools.

Chapter 7

Concluding remarks and future prospects

The economic impact of FMD in a country previously free of the disease is significant both in terms of the initial cost of controlling the outbreak and the loss of revenue following the ban on exports. This is exemplified by the 1997 outbreak in Taiwan which cost that country an estimated US$ 2 billion (Yang *et al.* 1999). As an OIE list A disease, FMDV is a major barrier to international export and trade with other regions. Following the outbreaks occurring in livestock in KwaZulu-Natal, Mpumulanga and Northern provinces of South Africa in 2000 and 2001 and the subsequent ban on exports, it is estimated that the outbreak cost the country 2 billion rand in the first five months following the initial type O outbreak. These economic consequences will undoubtedly continue to be felt given the conditions and time constraints precluding international trade following an outbreak (see section 6.1). One of the best defenses a country can have in the event of an outbreak is the rapid diagnosis of the disease and effective containment through the combined efforts of veterinary services and laboratory personnel. Modern molecular epidemiological techniques can assist in pin-pointing the most likely source of infection. This information is critical for assisting field staff in containing the disease in all affected areas, and proved useful in the SAT-1 outbreak initially identified in a feedlot in Middelburg, South Africa in 2000. Nucleotide sequencing showed that the outbreak strain was most closely related to a buffalo virus from Lower Sabie in the Kruger National Park (KNP), which is approximately 200 km east of the index case. Surveillance and sampling was therefore extended to include this region south of the KNP and led to the identification of additional infected animals by veterinary health personnel.

The PCR methodology outlined in this study is not only useful for characterizing outbreak strains, but can also be used to detect virus in a clinical sample, within 6 hours of receipt. The VP1 PCR detailed in Chapter 2, was primarily designed to detect SAT-type viruses in southern Africa. Application of this method to SAT-type viruses from West Africa indicates that the VP1Ub primer does not detect these genetic variants as efficiently as it does those from southern Africa (Sangare & Bastos 2000, unpublished). This is likely due to the occurrence of genetically distinct viruses in different African regions as indicated by nucleotide sequence determination of types A (Knowles *et al.* 1998), O (Sangare *et al.* 2001), SAT-1 (Bastos *et al.* 2001) and SAT-2 (Chapter 5). Furthermore, it was clearly shown that outbreaks sometime result from the transboundary movement of animals or animal products. In light of these factors it is clear that the sole use of the VP1Ub + P1 primers (Chapter 2) for disease diagnosis could potentially lead to false negative results. It is therefore recommended that this PCR be used in combination with primers targeting a more conserved genome region, such as the replicase (3D) gene. Although published primers are available for the 3D genome region, results indicate that they do not amplify all field variants of FMDV occurring on the African continent (Bastos 1998b). For this reason, SAT-type replicase gene sequences were generated and new primers were developed in which the 3D genome region is amplified by a one-step nested PCR approach. This novel 3D PCR test detects all FMDV serotypes and SAT field variants, is more sensitive than the VP1-targeting PCR (Bastos 1998b) and eliminates the possibility of contamination usually associated with the conventional two-step nested PCR. For diagnostic purposes it is therefore imperative that both the 1D (Bastos 1998a) and the 3D PCR (Bastos 1998b) be run simultaneously. If both PCRs are positive, the former product can be purified and used for genetic characterization, whilst the latter provides independent confirmation of a positive result. If however the former is negative, whilst the latter is positive, this would be indicative of inefficient primer binding due to the inherent hypervariablility of the VP1 protein. In such a case, alternative VP1 targeting primers would need to be screened in order to proceed with genetic characterization of the outbreak strain. A double negative result would indicate absence of FMD virus from the sample being analysed.

When comparing PCR with other more conventional FMDV detection methods such as virus isolation and typing, PCR is superior to both methods in terms of sensitivity and speed (when a

light-cycler is being used). Although a PCR-typing method which claims to differentiate all seven serotypes has been published (Callens & de Clercq 1997), the data generated in this thesis indicate that the reported type-specific primers will not accurately detect nor differentiate between all SAT-type variants present in southern Africa. There is therefore no reliable PCR test presently available which simultaneously permits detection and typing of all FMDV serotypes in a clinical sample. Furthermore, the results presented in this thesis indicate that development of a VP1-based serotyping test for use throughout Africa is unlikely as the variation levels within and between genotypes of SAT-type viruses from different regions preclude this. It is however possible to develop accurate regional typing PCRs for SAT-viruses. Preliminary investigations indicate that SAT-1 and SAT-3 viruses from southern Africa are readily distinguished by PCR (Bastos 1996), but differentiation of SAT-2 type viruses is more problematic due to the lack of sufficiently long stretches of type-specific sequences, suitable for designing primers within the VP1-coding region.

It was shown here that genetic characterization of field strains assisted with an investigation into the possibility of sexual transmission of SAT-type viruses from persistently infected buffalo (Chapter 3). The recovery of a genetically unique FMD virus from the reproductive tract of a male buffalo provides the first indication that this may be alternative site of viral persistence, which unlike the oesophageo-pharyngeal area, is immunologically privileged. The results were however equivocal and are likely to remain so until more accurate methods are developed for differentiation between acute and persistently infected animals. The recovery of FMDV from buffalo semen does however indicate that viral transmission by the sexual route is a distinct possibility and has implications for buffalo breeding programmes in which the use of artificial insemination methods is intended.

Nucleotide sequencing of viruses recovered from impala epizootics and their relatedness to FMDV buffalo genotypes (Chapter 4) provided the first evidence of natural transmission between these wildlife species. These results are important not only because they demonstrate the usefulness of detailed genetic characterization of viruses of maintenance host populations, but also because they confirm the possible role of antelope as intermediaries in disease transmission. Further evidence for this was obtained from retrospective studies which showed that cattle and antelope outbreaks were caused by the same virus (Chapter 6), thereby confirming interspecies transmission. Conclusive

evidence, as would be provided by the recovery of an identical virus from the three major role players, buffalo antelope and cattle, in this transmission scenario is however still lacking. The results presented here do at least confirm that interspecies transmission between buffalo and impala and between kudu and cattle has occurred previously. The role of impala and kudu in the epidemiology of the disease should therefore be further investigated in order to allow for the implementation of appropriate disease control measures where these antelope species are implicated as intermediaries in disease transmission.

The cumulative results of this study and those from previous epidemiological studies indicate that the SAT-type FMD virus can be independently maintained within an isolated herd of buffalo for up to 24 years (Condy *et al.* 1985). Under field conditions, virus can be transmitted to cattle (Dawe *et al.* 1994a) and impala (Bastos *et al.* 2000) in close proximity to buffalo. Such transmission would most likely result from the shedding of virus by acutely infected buffalo calves (Gainaru *et al.* 1986). Disease transmission can however also be precipitated on rare occasions by persistently infected buffalo (Hedger & Condy 1985; Dawe *et al.* 1994b; Vosloo *et al.* 1996), possibly by the sexual route (Bastos *et al.* 1999). Circumstantial evidence indicates that interspecies transmission between buffalo and impala results from environmental contamination of shared environs when buffalo calves are simultaneously infected for the first time (Bastos *et al.* 2000). Results from experimental infections indicate that the reverse is also true and that acutely infected impala can act as a source of infection for buffalo in close proximity (Vosloo *et al.* 1996). This scenario presumably applies to any cloven-hoofed species shedding large amounts of virus in close proximity to other susceptible species. Interspecies transmission between livestock and antelope (Chapter 6) was unequivocally demonstrated through the recovery of a common virus in both species. The results of the epidemiological investigations detailed in Chapters 3, 4 and 6, and those from previous studies make it possible to summarize the likely routes of FMDV transmission and the role of different cloven-hoofed species in the southern African sub-region (Fig. 7.1). It should however be stressed that although these routes of transmission are now well-recognized, the mechanisms and conditions facilitating interspecies transmission remain to be clarified.

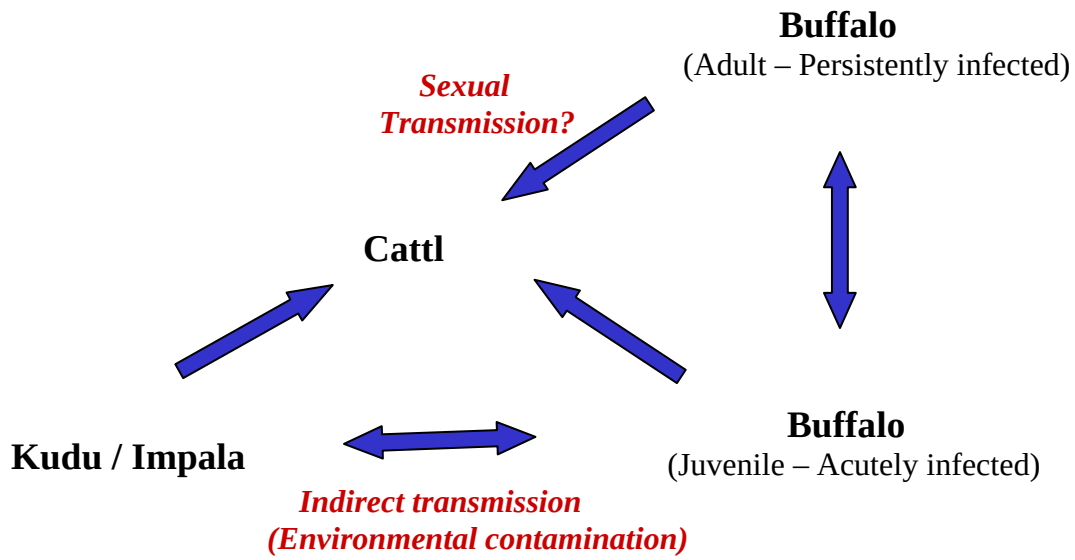

Fig 7.1. Diagrammatic representation of the possible routes of transmission of foot-and-mouth disease virus from African buffalo to other cloven-hoofed species

The identification of locality-specific buffalo virus genotypes (Chapter 5)was shown to be useful for more accurately determining the source of infections in susceptible cloven-hoofed species in close proximity (Chapter 6). The accuracy with which outbreaks could be traced was however dependent on the extent to which viruses from representative buffalo populations had been characterized. In South Africa and Zimbabwe sources of infection were readily established as the viral genotypes within these countries are well studied. In contrast, little or no data was available from Angola, Namibia, Botswana and Mozambique. Clearly the establishment of a regional database requires that more information on the genetic variation of FMD virus isolates within these SADC countries be obtained. This is particularly important in view of the imminent establishment of a 'peace park' incorporating KNP, southern ZIM and Mozambique. The geographical genetic variation of buffalo in Mozambique has not been assessed nor has the impact of buffalo movement from northern and western Zimbabwe into southern Zimbabwe been considered. Confirmation that the introduced topotypes occurring in other regions of Zimbabwe (topotypes II, III or IV) have become established within the southern topotype (I) region to be incorporated within the peace park, would clearly have

implications for disease control though vaccination. It is also likely to have major financial implications as the vaccine would need to contain viruses representative of all circulating topotypes in order to ensure adequate protection. In South Africa, the trivalent vaccine (SAT 1-3) presently in use is primarily inclusive of topotype I virus variants and would therefore not provide adequate protection, following the removal of border fences and likely establishment of new topotype viruses resulting from the peace-park initiative.

FMD, together with vesicular stomatitis, swine vesicular disease and rinderpest are OIE list A diseases and as such pose serious constraints to international trade of live animals and animal products. Many countries in the developing world are embarking on disease control and eradication programmes in order to obtain access to the lucrative foreign markets. For many countries FMD is the only remaining obstacle to free trade and there is therefore always the temptation to delay reporting and eradicate the disease before it halts trade. This delay often results in the disease spreading even further and recent evidence suggests that illegal cattle trade has played a major role in the spread and introduction of FMD (Kitching 1999). In South Africa and Zimbabwe there is an additional threat to disease security posed by the trade in illegal buffalo, as these are the only southern African countries that produce a surplus of buffalo for translocation purposes. The demand for disease-free buffalo for eco-tourism and hunting purposes is high due to their 'big-five' status. Buffalo prices vary according to the disease-status of the animals. In 1998 foot-and-mouth disease (FMD) infected buffalo were valued at between US$500 and US$1000, those infected with Corridor Disease (CD) alone cost between US$4300 and US$7200, whilst disease-free (clean) buffalo fetched upwards of US$16500 (Winterbach 1998). The difference in price paid for diseased versus disease-free buffalo together with limited number of disease-free buffalo and increased demand for animals of this status, are a major driving force in the illegal trade in these animals. The effects of illegal buffalo trade are potentially detrimental and should be curbed by whatever means possible. Extensive genetic characterization of KNP buffalo viruses may act as an effective deterrent as similar molecular epidemiological studies of human viral disease have successfully been used in legal prosecution (Albert *et al.* 1994; Ross *et al.* 1999). To be an affective means of combating illegal movement of buffalo in South Africa, all populations within the KNP would need to be characterized. Although adequate representation of southern KNP populations is available, the

northern KNP buffalo are under-represented in the genetic database. This short-coming and the importance of tracing buffalo is currently being addressed through a project funded by the Red Meat Board of South Africa.

In conclusion, this study has provided numerous epidemiological insights into SAT-type FMD in southern Africa. In particular, the role of the African buffalo and antelope in disease transmission has been addressed and initiation of a regionally representative buffalo virus database has permitted more accurate determination of the source of outbreaks in the southern African region. In addition, methodologies for the detection and characterization of viruses detailed here not only assist with rapid and accurate disease diagnosis, but can potentially be used for the successful legal prosecution of parties transgressing buffalo movement regulations. The results further identify a need for re-evaluating disease control measures, such as the height of perimeter fences intended to be 'game-proof', and for stricter regulation on the importation and incineration of animal products and waste, in order to ensure disease security.

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