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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.

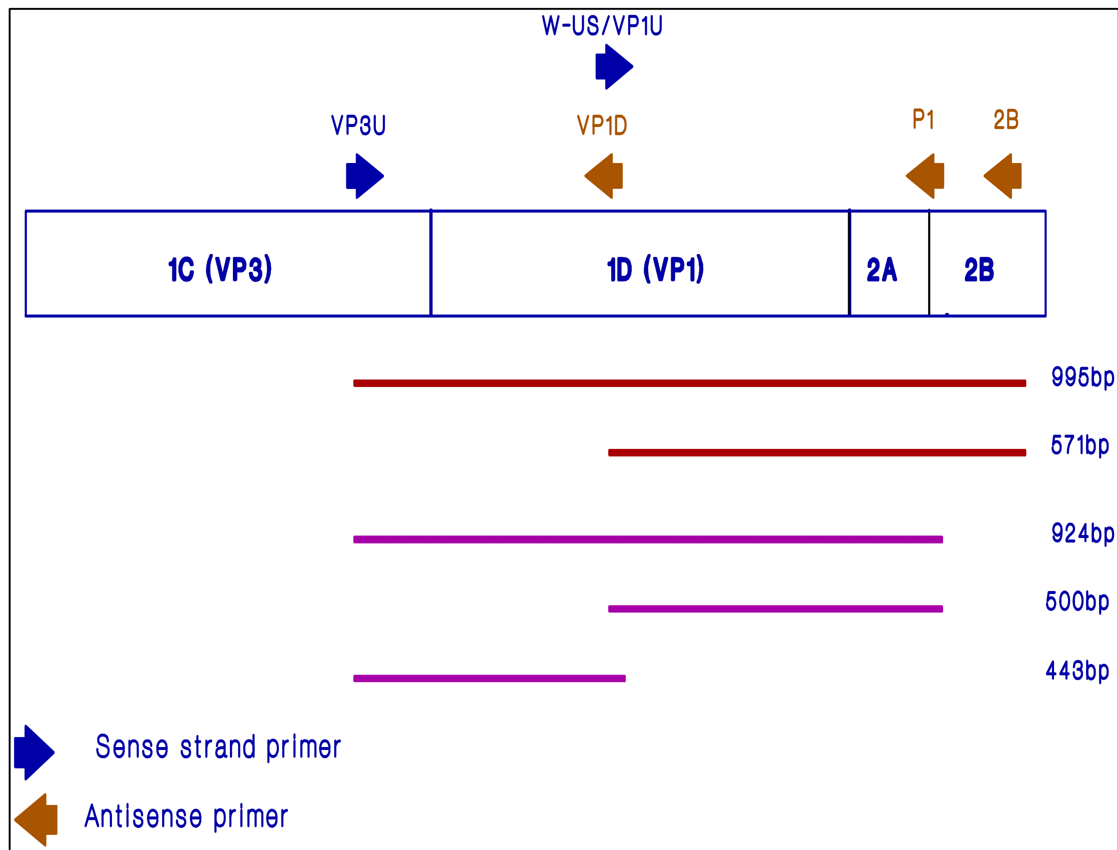

Fig. 2.1: VP1 gene amplification strategy. Arrows indicate primer orientation and binding position with approximate sizes of amplification products obtained with different primer combinations given in base pairs (bp) on the right

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.

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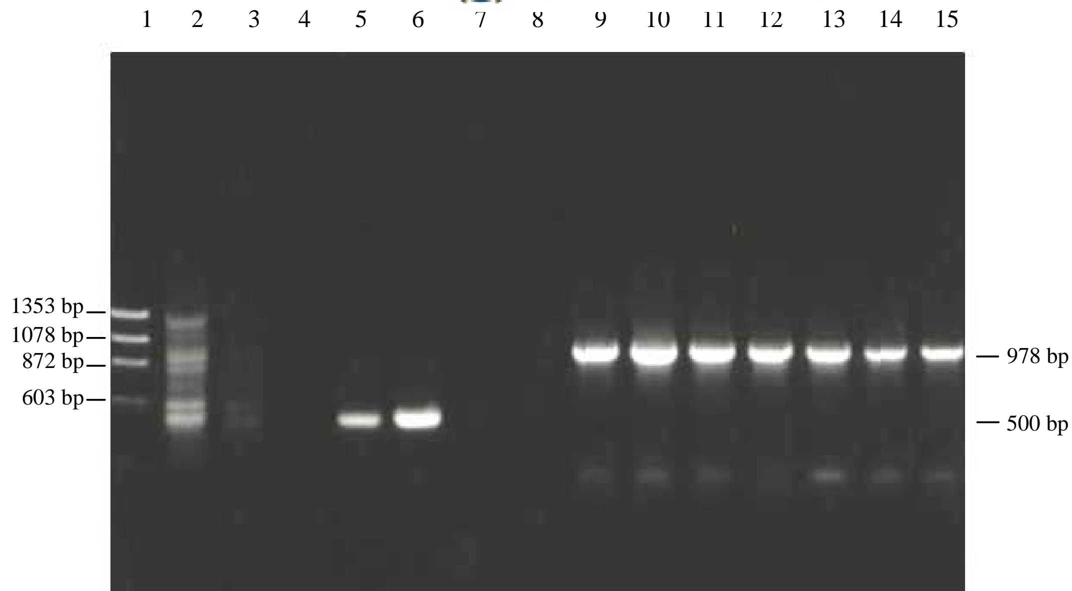


Fig. 2.2: Comparison of amplification results obtained with published VP1 gene-targeting primers (lanes 2-8) and replicase gene-targeting primers (lanes 9-15), with geographically divergent SAT-2 type field strains. cDNA templates used for these reactions were the following:
(2)+(9): ZAM 12/81; (3)+(10): 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.

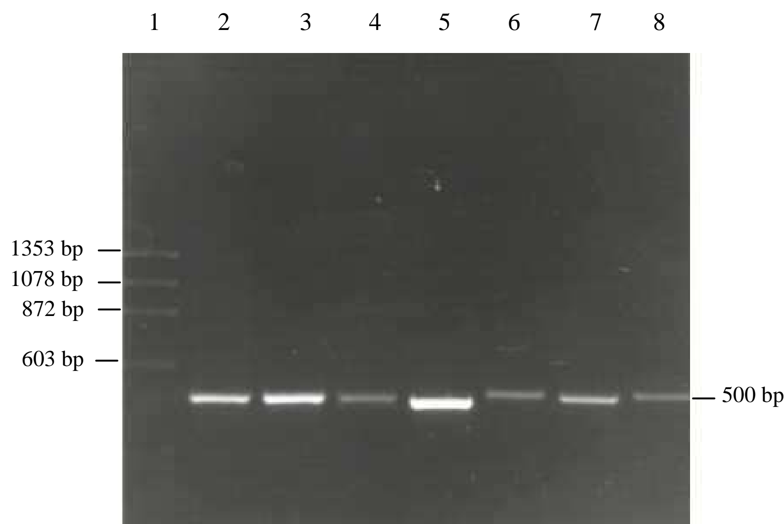


Fig. 2.3: Agarose gel depicting amplification of all seven FMDV serotypes with the VP1Ub and P1 primer set. Lane 1 contains the ∅X 174 (*Hae III*) DNA molecular weight marker (Promega). Lanes 2 through 8 contain PCR products of approximately 500bp, which are amplified in the presence of the following 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		<u>66.7%</u>	<u>16.7%</u>	<u>16.7%</u>	---	<u>66.7%</u>

-- = 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		<u>52%</u>	<u>86%</u>	<u>100%</u>

- = 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.

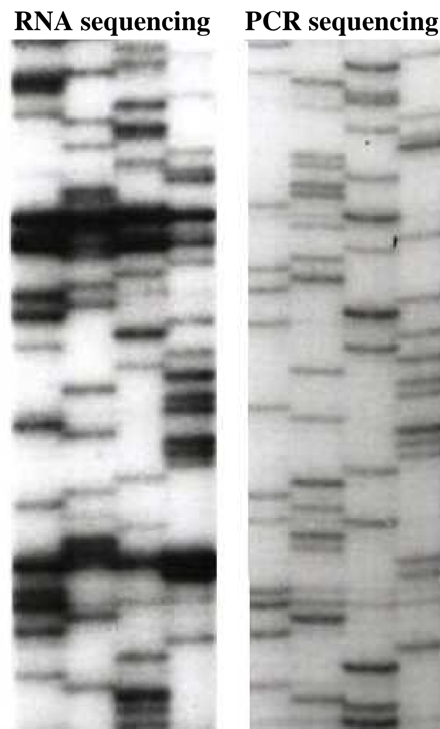
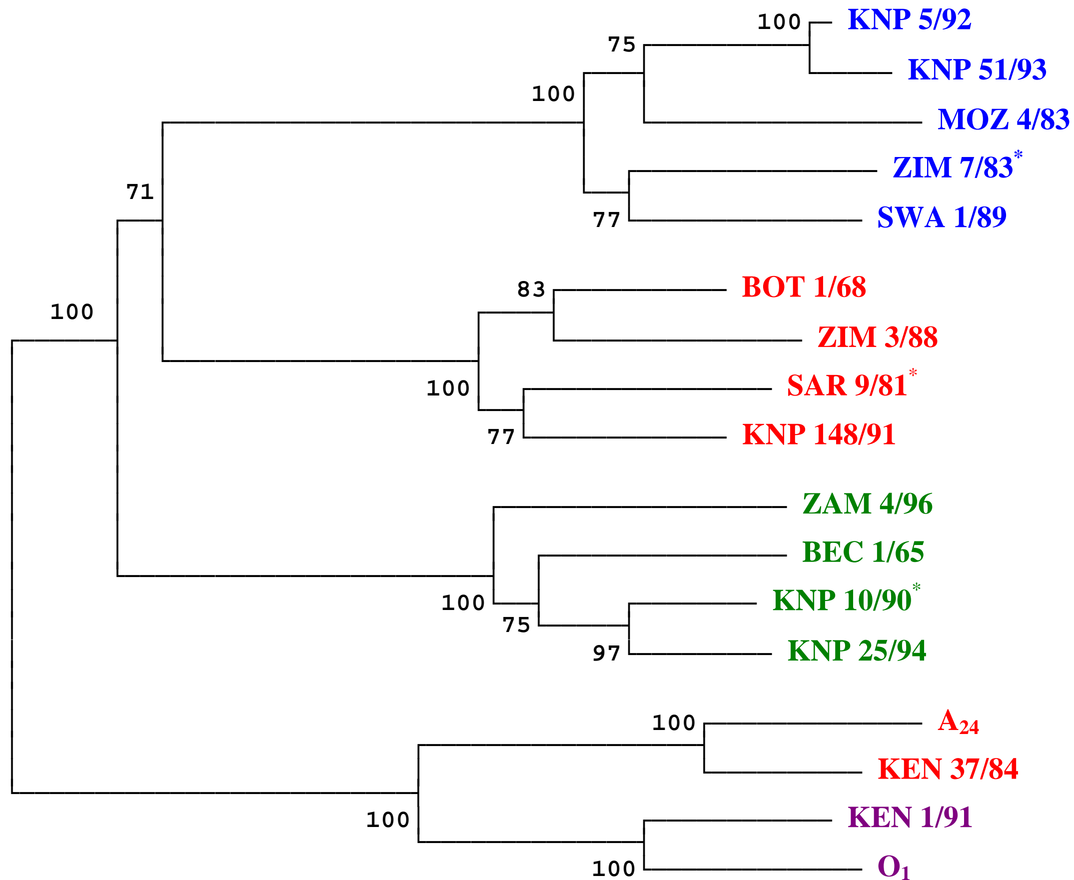


Fig. 2.4: Autoradiogram depicting differences in nucleotide sequencing results obtained with direct RNA sequencing versus PCR sequencing. Sequencing reactions were loaded ACGT, and the antisense primer P1 was used to generate sequences corresponding to the 3’ end of the VP1 gene of the SAT 2 type strain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



Scale: ———————— is equal to a distance of approximately 4.9 %

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.

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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.

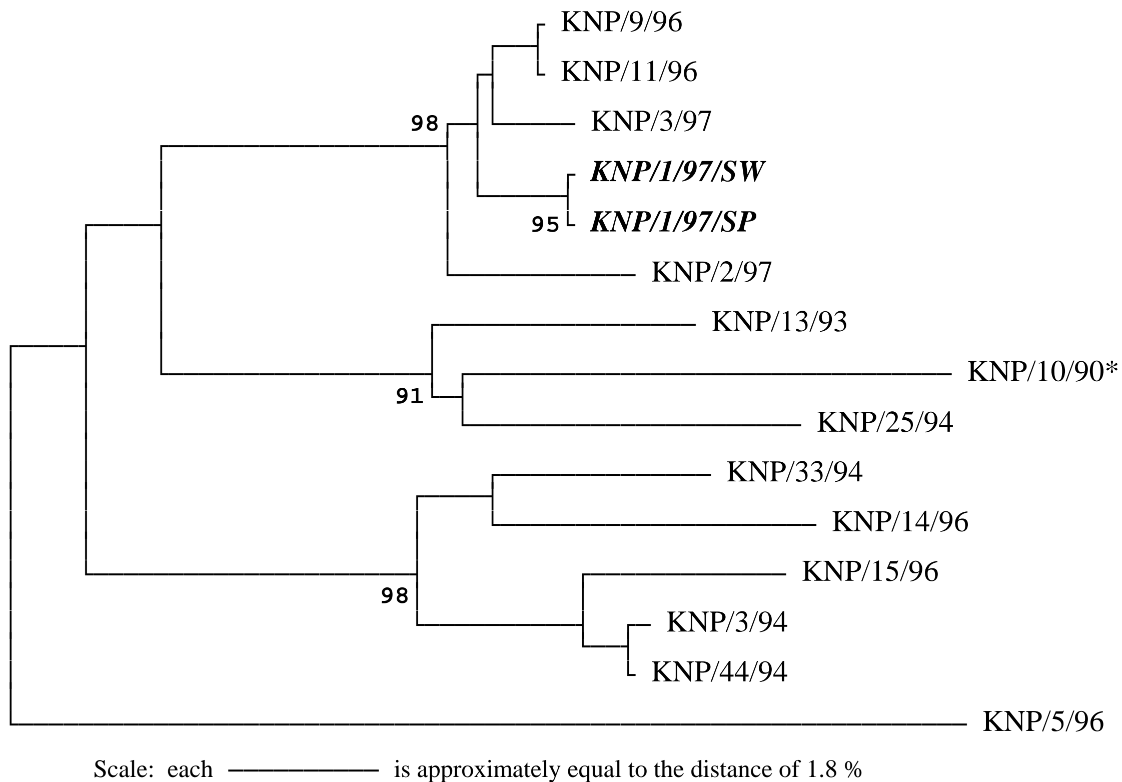

Fig. 3.1: SAT-3 neighbor-joining tree depicting VP1 gene relationships of buffalo isolates (1990-1997) from the Kruger National Park. Identical viruses obtained from buffalo sheath wash and sperm are designated KNP/1/97/SW and KNP/1/97/SP respectively (indicated in bold and italic). Bootstrap values, 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.