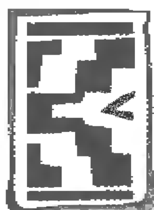

Detection and characterization of foot-and-mouth disease virus in sub-Saharan Africa

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ABSTRACT

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Genomic amplification of the VP1 gene of SAT-type foot-and-mouth disease virus (FMDV) was performed with published and novel oligonucleotide primers. The primer pair with the highest SAT-type recognition (67 %) was identified and selected for optimization. Modifications to primers significantly 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 h of receipt, the PCR product, which is amenable to nucleotide sequencing, enables genetic characterization of viruses into serotype and topotype within 48 h. VP1 gene sequence analysis of isolates from seven African countries and representative of five of the six serotypes occurring on the continent, revealed that SAT-types have the highest levels of intratypic variation. Intratypic variation for the SAT-types ranged from 34–40,4 % on nucleotide level, and from 24,1–27,5 % on amino acid level. In addition, the methodology presented here was shown to be useful for determining the origin and tracing the course of epizootics in both wild and domestic cloven-hoofed animals.

Keywords: Intratypic variation, nucleotide sequencing, phylogenetic analysis, polymerase chain reaction (PCR), SAT-types, VP1 gene, subtypes, topotypes

INTRODUCTION

Foot-and-mouth disease (FMD) is a highly contagious, economically devastating disease of cloven-hoofed animals. Seven serotypes occur, three of which are endemic to sub-Saharan Africa, namely SAT (South African Territory) type-1, -2 and -3. Traditionally, European serotypes O, A and C which were historically exotic to southern Africa have been identified in a number of northern African countries and in Namibia, Angola, Malawi and Zambia (Brooksby 1972; Thomson 1994).

Although disease eradication has been achieved in North America and Western Europe, the probability of achieving this in sub-Saharan Africa is remote due to the role that wildlife plays in the epidemiology of the disease and the greater complexity of antigenic types (Brooksby 1972). African buffalo (*Syncerus caffer*) are efficient maintenance hosts of the virus and a potential source of infection for domestic livestock and wildlife (Thomson 1994). The situation is further exacerbated by the presence of six of the seven serotypes and high levels of intratypic variation in the indigenous virus types (Vosloo, Kirkbride, Bengis, Keet & Thomson 1995), making prevention and control problematic. At present, the disease is controlled in South Africa by the restriction of animal movement, usually involving fences, and by vaccination (Hunter 1996).

The applicability of current vaccine strains is assessed by antigenic comparison of these strains with field strains circulating in the Kruger National Park (KNP) and neighbouring countries. The last recorded outbreak

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of FMD in livestock in South Africa occurred in 1983 (Directorate of Veterinary Services 1983), with subsequent outbreaks being limited to wildlife in the KNP. The effective control of FMD in South Africa has allowed for recognition internationally of an infection-free zone. It is estimated that a widespread outbreak of the disease would affect an estimated R2 billion-worth of agricultural products annually (Thomson 1994). Early diagnosis and virus characterization is thus critical to minimizing the potentially detrimental economic effect of an outbreak. Presently this is achieved by virus isolation on tissue culture and/or serological techniques. The reported speed and sensitivity of virus detection by means of the polymerase chain reaction (PCR) prompted an investigation into the applicability and usefulness of the technique in the African context. Although a number of primer sequences have been published for detection of foot-and-mouth disease virus (FMDV) by PCR, these primers do not readily amplify the SAT types (A. Bastos, unpublished results 1996). Given the extensive intratypic variation reported for all FMDV virus types, and the SAT-types in particular (Vosloo *et al*. 1995), it is clear that universal FMD detection can only be substantiated by testing a wide range of divergent viral subtypes or topotypes.

In order to address the problem of low SAT-type recognition, the suitability of published and novel primer sequences for detecting the FMDV serotypes which occur in sub-Saharan Africa was evaluated. The VP1 gene was targeted for this study as amplification of this portion of the genome not only allows for confirmation of FMDV in a sample, but also facilitates viral characterization by means of nucleotide sequencing analysis (Beck & Strohmaier 1987). Emphasis was thus placed on SAT-type recognition when selecting a primer pair for possible modification and optimization, with the aim of improving the detection range and sensitivity of South and sub-Saharan African viruses by PCR. In addition, primer specificity for FMDV, the recognition range of primers and sensitivity levels were investigated in order to critically assess the diagnostic potential of viral detection by PCR in sub-Saharan Africa.

MATERIALS AND METHODS

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.

RNA extraction and cDNA synthesis

RNA was extracted from cell culture specimens by a modified guanidinium-based nucleic acid extraction method (Boom, Sol, Salimans, Jansen, Wertheim-Van Dillen & Van der Noordaa 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, Pacciarini, Hilyard, Ferrari, Vakharia, Donini, Brocchi & Molitor 1994).

PCR amplification

Genomic amplification of the FMD viral genome was performed with a variety of novel, published and modified primers (Table 1). These primers bind to VP1 or neighbouring genes and amplify distinct fragments with the different primer combinations, as outlined in Fig. 1. FMDV oligonucleotides VP1a, VP1b and P1 were synthesized by MWG-Biotech GmbH, whilst the W-US, VP1D and VP3U primers were synthesized by the Department of Biochemistry, University of Cape Town. The reactions were performed in a 25 $\mu\ell$ volume in the presence of 1–2 $\mu\ell$ 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, 30 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 154 bp 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 1997).

PCR purification and nucleotide sequencing

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). 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 independent amplification and sequencing reactions were performed per isolate.

TABLE 1 Summary of the PCR primers used in this study

Name	Sequence 5' 3'	Length	Orientation	Reference/consensus sequence	Tm	Ta
2B	GA CAT GTC CTC CTG CAT CTG	20mer	Antisense	Consensus of A00276, K00554, V01131, A15586, V01130, X00130, X00429, M10975, X00871/complimentary to P32 primer (Vangrysperre & De Clerq 1996)	59 °C	55 °C
P1	GAA GGG CCC AGG GTT GGA CTC	21mer	Antisense	Beck & Strohmaier 1987	65 °C	61 °C
VP1D	GTC ACA AAA GTA ATA CGT GG	20mer	Antisense	Complimentary to W-US primer (Vosloo *et al.* 1996)	53 °C	49 °C
W-US	CC ACG TAT TAC TTT TGT GAC	20mer	Sense	Vosloo *et al.* 1996	53 °C	49 °C
VP1Ua	CC ACR TAT TAC TTY TGT GAC CT	22mer	Sense	Consensus of W-US, M28719 (SAT-3), A06737 (O_1) and M20715 (A_{10})	57 °C	53 °C
VP1Ub	CC ACG TAC TAC TTY TCT GAC CTG GA	25mer	Sense	Consensus of W-US, M28719, A06737, M20715, M19760 (C_3) and U01207 (Asia-1)	64 °C	60 °C
VP3U	GAT ACT GGT TTG AAC TCC AAG TT	23mer	Sense	Consensus of M28719, M60118, L29062, L29078, M90381, M90368	53 °C	49 °C

Sequence, length, orientation and oligonucleotide names of published (P1 and 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

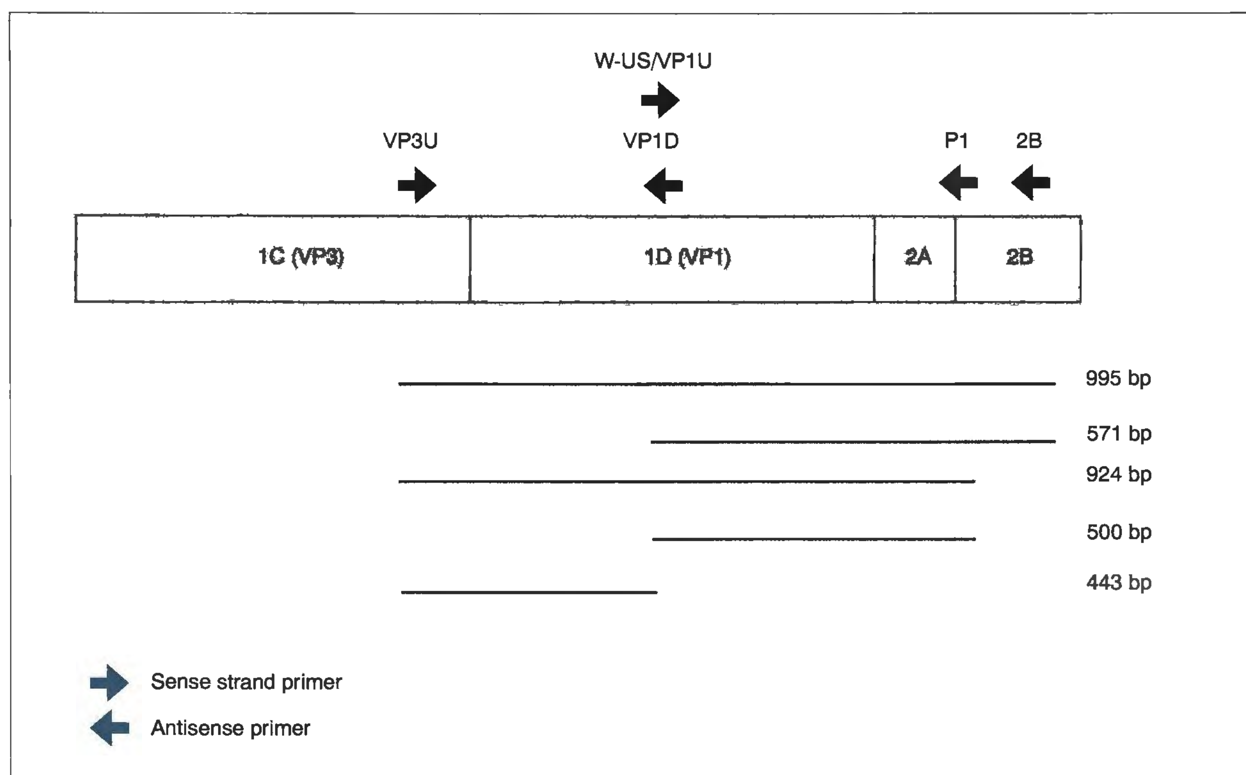

FIG. 1 VP1 gene amplification strategy Arrows indicate primer orientation and binding position with approximate sizes of amplification products obtained with different primer combinations given in base pairs (bp) on the right

Phylogenetic analysis

VP1 gene nucleotide sequences were translated and the deduced amino acid sequences aligned according to the guidelines set by Palmenberg (1989) for picornaviral capsid proteins. These sequences have been deposited in Genbank under the accession numbers indicated in Table 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, Tamura & Nei 1993), with p-distances and pairwise deletions of gaps and missing data being applied. Node reliability was estimated by 1 000 bootstrap replications. Published sequences of serotype A (A_{24} Cruzeiro; Makoff, Paynter, Rowlands & Boothroyd 1982) and O (O_1 Kaufbeuren; Forss, Strebel, Beck & Schaller 1984) strains of non-African origin were also included in the analyses.

RESULTS

Primer-pair recognition of FMDV serotypes

Five different 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 primer pairs by PCR. Primer pairs 2B+VP3U and P1+W-US scored equally well in terms of SAT-type amplification (Table 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.

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, Bastos, Kirkbride, Esterhuysen, Janse van Rensburg, Bengis, Keet, & Thomson 1996). By comparing the only complete VP1 gene sequence of a SAT-type (Brown, Campbell & Clarke 1989) with the sequences of European serotypes A, O and C (cf. Table 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 second base position of the corresponding amino acid in

TABLE 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

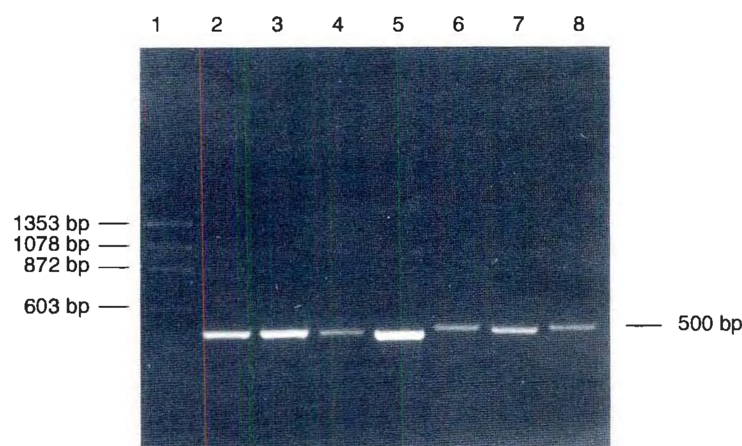

FIG. 2 Agarose gel depicting apmplification of all seven FMDV serotypes with the VP1Ub and P1 primer set

Lane 1 contains the øX 174 *(Hae III)* DNA molecular weight market (Promega)

Lane 2 through 8 contain PCR products of approximately 500 bp, which are amplified in the presence of the following cDNA termplates:

(2) PAK 1/54 (Asia-1)
(3) KEN 37/84 (Serotype A)
(4) KEN 77/78 (Serotype O)
(5) C_1 Noville (Serotype C)
(6) BOT 1/68 (SAT-1)
(7) KNP 19/89 (SAT-2)
(8) KNP 10/90 (SAT-3)

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 nucleotide position one residue in from the terminal 3' nucleotide. Mismatches in this position are known to affect polymerization, a characteristic exploited for diagnostic purposes in the amplification refractory mutation system (ARMS) PCR (Wenham, Newton & Price 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 three 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. 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). The relative amplification efficiencies of the upstream VP1 primers combined with the P1 primer are summarized in Table 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

TABLE 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

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.

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 enterovirus band (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.

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

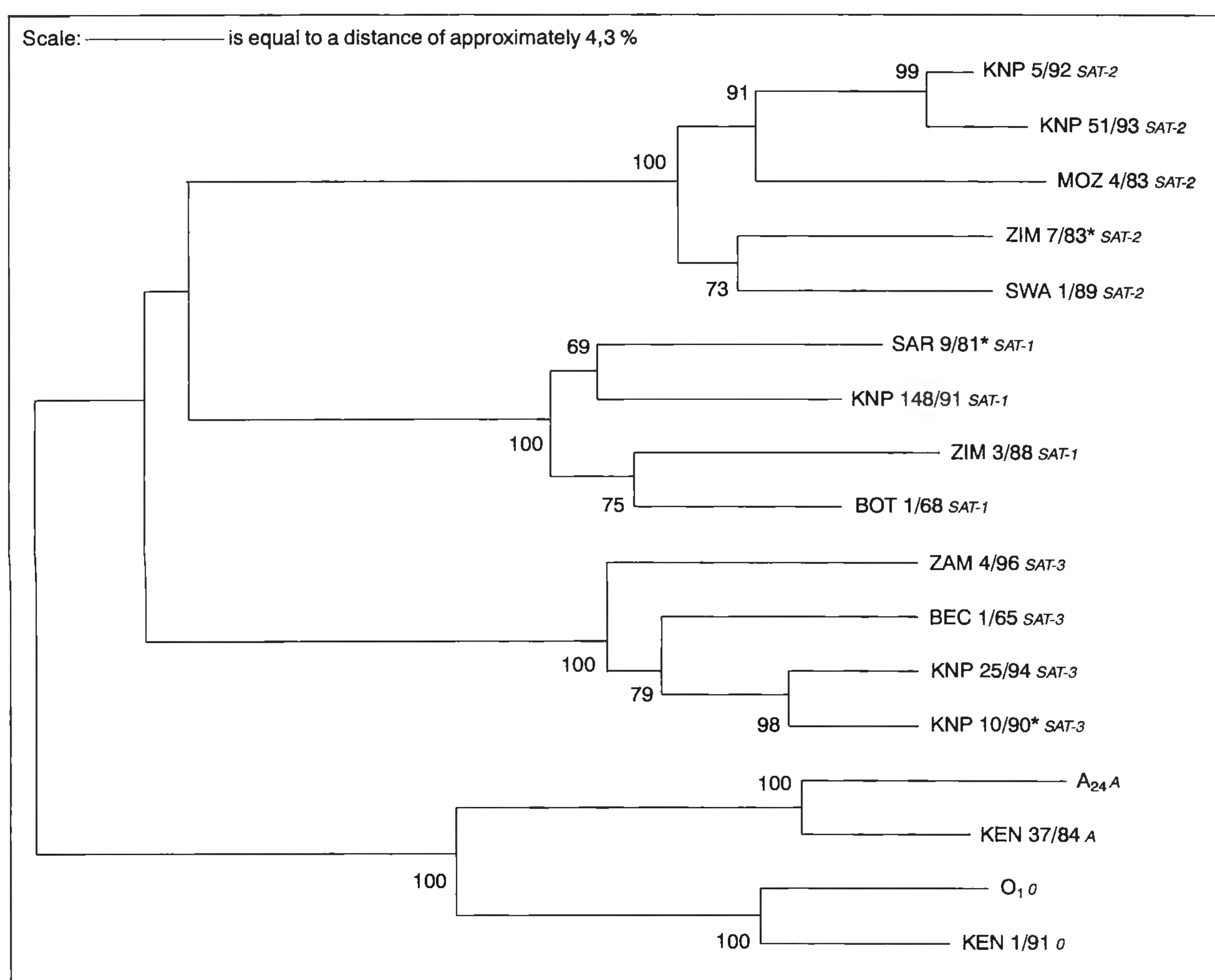

FIG. 3 Neighbour-joining tree depicting the phylogenetic relationshipts of five sub-Saharan African FMDV serotypes bases on BP1 gene amino acid sequences

Bootstrap proportions >65 (based on 1 000 reiterations) are indicated on the branches with FMDV serotype being indicated in italic to the right of the isolate name

* Indicates Onderstepoort Institute for Exotic Diseases vaccine strains

quantities of virus in the starting material has on genomic amplification. The equivalent of less than one PFU per 25 $\mu\ell$ 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 500 bp 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 1 000 fold lower with the latter primer pair in combination with certain SAT isolates.

Phylogenetic relationships and intratypic variation

Between 417 and 507 nucleotides (nt) were determined for at least one representative of the seven FMDV serotypes by sequencing the amplification products of the 2B+VP1Ub or P1+VP1Ub primer pairs. The SAT-3 nucleotide sequence of the OIED BEC 1/65 isolate has 97,1 % sequence identity with the published sequence of this strain, over the 450 nt 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 gene sequence. 493 nt 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 417 nt 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, Dopazo, Hernandez, Mateu, Sobrino, Domingo & Knowles 1992).

TABLE 4 Details on 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 phylo-genetic inference	Genbank accession number
KEN 37/84*	A	Kenya	Bovine	1984	507 nt	125 aa (375 nt)	AF023526
KEN 1/91*	O	Kenya	Bovine	1991	497 nt	126 aa (378 nt)	AF023527
BOT 1/68*	SAT-1	Botswana	Bovine	1968	461 nt	132 aa (396 nt)	AF023524
SAR 9/81*	SAT-1	South Africa	Game	1981	457 nt	132 aa (396 nt)	AF023514
ZIM 3/88*	SAT-1	Zimbabwe	Buffalo	1988	456 nt	132 aa (396 nt)	AF023515
KNP 148/91	SAT-1	South Africa	Buffalo	1991	456 nt	132 aa (396 nt)	AF023513
MOZ 4/83@	SAT-2	Mozambique	Bovine	1983	436 nt	128 aa (384 nt)	AF023519
ZIM 7/83@	SAT-2	Zimbabwe	Bovine	1983	448 nt	128 aa (384 nt)	AF023523
SWA 1/89*	SAT-2	Namibia	Buffalo	1989	438 nt	128 aa (384 nt)	AF023520
KNP 5/92	SAT-2	South Africa	Impala	1992	436 nt	128 aa (384 nt)	AF023518
KNP 51/93	SAT-2	South Africa	Impala	1993	418 nt	128 aa (384 nt)	AF023516
BEC 1/65*	SAT-3	Botswana	Bovine	1965	450 nt	130 aa (390 nt)	AF023521
KNP 10/90	SAT-3	South Africa	Buffalo	1990	449 nt	130 aa (390 nt)	AF023517
KNP 25/94	SAT-3	South Africa	Buffalo	1994	456 nt	130 aa (390 nt)	AF023522
ZAM 4/96*	SAT-3	Zambia	Buffalo	1996	449 nt	130 aa (390 nt)	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 Institute for Exotic Diseases, with viruses obtained from alternative sources being denoted as follows: *World Reference Laboratory, Pirbright; @ Botswana Vaccine Institute, Gaborone

Intratypic amino acid variation levels were highest in the SAT-types with SAT-1, -2 and -3 displaying differences of 25,4 %, 27,5 % and 24,1 %, respectively, for the isolates used in this analysis (Table 4). Kenyan A and O types were compared with non-African isolates of the same type, yet displayed lower levels of intratypic variation. Amino acid sequence differences were 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 four European serotypes used in the phylogenetic analysis.

In the SAT-2 cluster, the Namibian and northern Zimbabwe isolates form a distinct and separate grouping from the southern isolates which originate from Mozambique and South Africa. 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.

DISCUSSION

Initial screening of five different primer pairs, all of which target the VP1 gene revealed that recognition for the SAT-types was low. The primer pair initially shown to have a 66,7 % recognition capability for six 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 optimization led to a 34 % increase in detection ability and with additional modifications 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 and was shown to be theoretically capable of detecting < 5 PFU per PCR on dilution of plaque titrated TC samples and < 1 PFU/PCR when diluted cDNA was used as a template. This discrepancy in sensitivity can be related to inefficiencies in cDNA synthesis in the presence of limited amounts of viral RNA in the diluted cell culture infected samples. The universal 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 for all seven serotypes and consistently produced in excess of 400 nt of sequencing data for each of the isolates amplified. This increased sequence length significantly improves phylogenetic resolution of viral strains as exemplified by the high node support obtained from 1 000 bootstrap replications and overcomes restrictions on sequence length previously imposed by direct RNA sequencing of the viral genome. 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, Hunter, Bengis, Bastos & Thomson 1996). Two SAT-2 impala strains KNP 5/92 and KNP 51/93 although isolated 1 year apart (1992 and 1993) were shown to be part of the same epizootic, yet unrelated to a previous epizootic in impala in the KNP which occurred in 1988/89. 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 fulfill 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, Brown, House, House & Molitor 1991; Laor, Torgersen, Yadin & Becker 1992). Alternatively, primers have been described which target the capsid coding region (Hofner, Carpenter & Donaldson 1993) and are therefore potentially useful for molecular epidemiological studies, but have limited recognition capabilities for SAT-types (A. Bastos, unpublished results 1996). Primers are also available which amplify products of under 350 bp from structural coding genes (Amaral-Doel, Owen, Ferris, Kitching & Doel 1993). Sequencing of such products results in under 300 nt of sequence which is insufficient sequence length to accurately infer a FMDV VP1 gene phylogeny (A. Bastos, unpublished findings 1995) and are therefore generally not suitable for accurate viral characterization. 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 h of receiving a sample and allows for accurate genetic characterization on the basis of nucleotide sequencing within 48 h of receiving a positive sample. These primers clearly fulfil the requirements of FMDV detection and characterization better than others described previously and are therefore recommended for molecular epidemiological clarification of FMDV in sub-Saharan Africa and elsewhere.

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