Predicting antigenic sites on the foot-and-mouth disease virus capsid of the South African Territories types using virus neutralization data

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Foot-and-mouth disease virus (FMDV) outer capsid proteins 1B, 1C and 1D contribute to the virus serotype distribution and antigenic variants that exist within each of the seven serotypes. This study presents phylogenetic, genetic and antigenic analyses of South African Territories (SAT) serotypes prevalent in sub-Saharan Africa. Here, we show that the high levels of genetic diversity in the P1-coding region within the SAT serotypes are reflected in the antigenic properties of these viruses and therefore have implications for the selection of vaccine strains that would provide the best vaccine match against emerging viruses. Interestingly, although SAT1 and SAT2 viruses displayed similar genetic variation within each serotype (32 % variable amino acids), antigenic disparity, as measured by r_1-values, was less pronounced for SAT1 viruses compared with SAT2 viruses within our dataset, emphasizing the high antigenic variation within the SAT2 serotype. Furthermore, we combined amino acid variation and the r_1-values with crystallographic structural data and were able to predict areas on the surface of the FMD virion as antigenically relevant. These sites were mostly consistent with antigenic sites previously determined for types A, O and C using mAbs and escape mutant studies. Our methodology offers a quick alternative to determine antigenic relevant sites for FMDV field strains.

INTRODUCTION

Foot-and-mouth disease (FMD) is widely considered an economically important disease of livestock, and is a compulsory OIE notifiable disease as it remains a global threat to national and international trade in livestock and livestock products. In 2010, outbreaks have occurred in South America, Asia, the Middle East and Africa (FMD Reference Laboratory Network Report, 2010). FMD is of particular importance in Africa where the disease is endemic and six of the seven immunologically distinct serotypes occur (Thomson, 1994; Vosloo *et al.*, 2002). Although foot-and-mouth disease virus (FMDV) causes a clinically indistinguishable vesicular disease in cloven-hoofed animals, the seven serotypes display different geographical distributions and epidemiology (Samuel & Knowles, 2001; Bastos *et al.*, 2001, 2003a, b; Knowles & Samuel, 2003; Bronsvoort *et al.*, 2004).

The South African Territories (SAT) types 1, 2 and 3 are confined to sub-Saharan Africa, although incursions into the Middle East by SAT1 (1961–1965 and 1970) and SAT2 (1990 and 2000) viruses have been recorded (Ferris & Donaldson, 1992; Bastos *et al.*, 2001; Records of the OIE). The SAT3 serotype has a restricted distribution and essentially occurs only in southern Africa (Bastos *et al.*,

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2003b), Asia-1 is restricted to Asia and the Middle-East (Ansell *et al.*, 1994), while serotypes A and O occur globally (Samuel & Knowles, 2001). The distribution of FMD samples received from Africa from 2000 to 2010 revealed that the majority of outbreaks (41 %) were caused by serotype SAT2 viruses. The prevalence of SAT1 and type O viruses was similar with 19 and 23 %, respectively. Outbreaks caused by type A viruses were identified for 15 % of the reported cases. In contrast, SAT3 viruses caused only two outbreaks during this period in the Democratic Republic of Congo and South Africa, respectively, whereas type C was only detected in Kenya in 2004 (Annual OIE/FAO FMD Reference Laboratory Network Reports; Records of the OIE). Eradication of the disease from the African continent is unlikely due to the presence of large numbers of the free-living maintenance host, the African buffalo (*Syncerus caffer*). These animals provide a potential source of infection for domestic livestock and wildlife (Dawe *et al.*, 1994; Bastos *et al.*, 2000; Vosloo & Thomson, 2004) and pose a constant threat to susceptible livestock (Vosloo & Thomson, 2004) in the rest of the world.

Persistently infected buffalo are the ideal hosts to maintain co-infection of different virus serotypes and to facilitate antigenic and molecular evolution of the virus (Condy *et al.*, 1985; Esterhuysen, 1994; Vosloo *et al.*, 1996, 2006). Genetic variation results from changes to the viral genome as a consequence of the high mutation rate of the virus. Therefore, the genetic diversity will most likely be reflected in antigenic differences. The outer capsid proteins are directly involved in antigenicity since 30–50 % of their residues are exposed on the virion surface, many of which constitute neutralizing epitopes (Acharya *et al.*, 1989; Logan *et al.*, 1993; Lea *et al.*, 1995; Mateu, 1995; Usherwood & Nash 1995). Although several antigenic sites have been identified for A and O serotypes (Xie, *et al.*, 1987; Thomas *et al.*, 1988; Baxt *et al.*, 1989; Bolwell *et al.*, 1989; McCahon *et al.*, 1989; Kitson *et al.*, 1990; Saiz *et al.*, 1991; Crowther *et al.*, 1993a), a dearth of knowledge exists for the epitopes of the SAT types. Identification of those residues that comprise the antigenic determinants of the SAT viruses will allow the identification of those changes in outbreak strains that may cause escape from protection afforded by the vaccine. Once such epitopes for the SAT types have been identified, it may be possible to predict the protection afforded by a vaccine against a specific outbreak virus.

The SAT types display appreciably greater intratypic genomic and antigenic variation than the 'Euro-Asian' types (Vosloo *et al.*, 1992, 1995, 1996; Esterhuysen, 1994; Bastos *et al.*, 2001, 2003a, b). Even within a serotype, distinct genetic and antigenic variants exist in different geographical regions. This has implications for the control of the disease by vaccination, since it may render available vaccines less effective (Hunter, 1998). Consequently, the ability to predict vaccine efficacy would be a valuable tool in an effective control strategy as control of FMD in Africa is essentially via strategic vaccination and restriction of animal movements.

Traditionally, the *in vitro* virus neutralization test (VNT) and statistically calculated r_1-values are used to determine antigenic relationships between a vaccine strain and an outbreak virus (Rweyemamu *et al.*, 1978). Meanwhile molecular epidemiological techniques have offered the possibility of more detailed analyses of FMD epidemiology (Vosloo *et al.*, 1992; Samuel & Knowles, 2001; Bastos *et al.*, 2001, 2003a, b; Knowles & Samuel, 2003). We have investigated the genetic variation of the complete capsid-coding region of representative FMDV strains found in sub-Saharan Africa during a 28 year period from 1974 to 2002. Variable regions on the capsid proteins of SAT1 and SAT2 isolates were then combined with structural data and serological relatedness to identify possible epitopes that could be prone to antigenic variation in the SAT viruses.

RESULTS

Phylogenetic relationships and topotype diversity in sub-Saharan Africa

The minimum evolution phylogeny, based on P1 nucleotide sequences of representative SAT viruses that caused outbreaks across the African continent over the past 28 years, is indicated in Fig. 1. The SAT viruses clustered according to serotype with high bootstrap support. The distribution of SAT3 serotype is limited to southern Africa. The five SAT3 isolates represented five of the six previously described topotypes. Three major virus lineages, which have evolved separately, exist within serotypes SAT1 and SAT2. The lineages clustered according to their geographical location (southern, western and eastern Africa) and are in agreement with the FMD topotype concept described for the European and SAT serotypes (Samuel & Knowles, 2001; Bastos *et al.*, 2001, 2003a, b; Knowles & Samuel, 2003). Exceptions, however, were observed for SAT1 and SAT2, where isolates from East Africa, i.e. SAT2/KEN/08/99, SAT1/KEN/05/98 and SAT1/TAN/37/99, clustered in the southern topotypes (Fig. 1), possibly due to historical movement of buffalo and livestock between the two regions (Bastos *et al.*, 2001; Sangare *et al.*, 2003). Similarly, an isolate from Sudan, SAT1/SUD/3/76, demonstrated high sequence similarity, based on the complete P1-coding region, with West African isolates, e.g. SAT1/NIG/05/81, indicating historical spread of the disease between East and West Africa. Using a 16 % nt difference cut-off value (Bastos *et al.*, 2003b), seven of eight previously described distinct lineages could be identified within the SAT1 serotype, while nine of the fourteen SAT2 topotypes could be resolved using the P1 phylogeny (Fig. 1). Within the southern African region, Zimbabwe shared different SAT1 (1, 2 and 3) and SAT2 topotypes (I, II and III) with neighbouring countries. This correlates well with phylogeny based on the 1D sequence only (data not shown). P1 phylogeny did not provide significantly more resolution for

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epidemiology purposes, although stronger bootstrap values were observed. An exception was the Angolan SAT2 isolate, ANG/4/74, which grouped separately from other Southern African SAT2 isolates according to 1D phylogeny and was previously assigned to a different topotype (Bastos *et al.*, 2003b; Sangaré *et al.*, 2004). However, using complete capsid sequence data (Fig. 1), this isolate was more closely related to the other South African isolates with good bootstrap support.

The intratypic nucleotide variation of the P1 region for SAT1, 2 and 3 was calculated to be 47.3 (n=20), 48.9 (n=23) and 39.5 % (n=5), respectively, and found to be considerably higher than the intratypic variation reported previously for types A, O and C (<18 %) (van Rensburg & Nel, 1999; Knowles & Samuel, 2003). The nucleotide and amino acid variation in a complete alignment of the SAT P1-coding region is summarized in Table 1. The complete P1 region of the SAT1 viruses was 2232 nt in length, encoding 744 aa, with the exception of the SAT1 isolates NIG/15/75, NIG/06/76 and NIG/08/76 (topotype 8) that consisted of 2229 nt. The corresponding region of the SAT2 viruses was 2220 nt in length, with the exception of a West African isolate, i.e. SAT2/SEN/07/83 (topotype VI). The P1-coding region of this isolate is 2217 nt in length and has a 6 nt deletion in the 1C-coding region and a 3 nt insertion in 1D. The five SAT3 isolates revealed the most variation in sequence length of the P1 region, ranging from 2214 to 2223 nt and encoding a polypeptide of between 738 and 741 aa in length. The maximum number of nucleotide differences observed intratypically in any pairwise alignment of the P1 regions of isolates within SAT1, SAT2 and SAT3 serotypes was 581 (26.1 %), 558 (25.1 %) and 617 (27.74 %), respectively. The average proportion of nucleotide differences was estimated to be 53.6, 55.7 and 57 % over synonymous sites, and 9.4, 9.2 and 11.2 % over non-synonymous sites for the three serotypes, respectively. Therefore, our data suggests an overall ratio of synonymous : non-synonymous changes to be approximately 6 : 1 for SAT 1, 2 and 3.

Analysis of antigenic properties of SAT1 and SAT2 viruses

One-way antigenic relationships (r_1-values) were used to compare SAT1 and SAT2 viruses from various topotypes in sub-Saharan Africa to select reference strains within each serotype. Since SAT3 viruses has the most restricted distribution and are the least frequently recovered from buffalo (Bastos *et al.*, 2003b) these isolates were not included in this study. The virus isolates and the mean r_1-values are summarized in Tables 2 and 3. Sera from cattle vaccinated twice with the two SAT1 viruses, SAR/09/81 and KNP/196/91, both belonging to topotype 1, demonstrated r_1-values that were higher with isolates belonging to the same topotype compared with isolates from other topotypes. Among the 20 SAT1 isolates analysed against the vaccine strains, 15 (n=3) and 15 % (n=3) showed

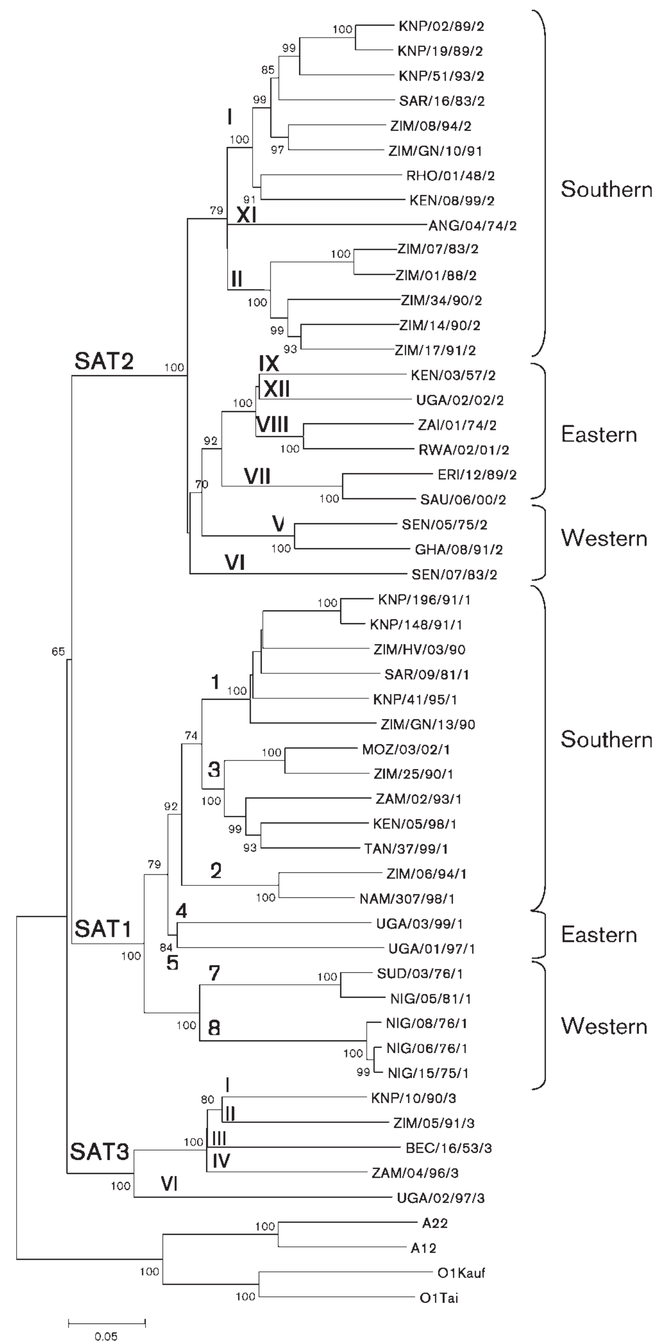

Fig. 1. Minimum evolution tree depicting the gene relationships for the P1-coding regions, respectively, of SAT1, 2 and 3 viruses from South (Kruger National Park, KNP; South Africa, SAR; Zimbabwe, ZIM; Rhodesia, RHO; Mozambique, MOZ; Namibia, NAM; Zambia, ZAM; and Angola, ANG), West (Nigeria, NIG; Senekal, SEN; and Ghana, GHA) and East Africa (Uganda, UGA; Rwanda, RWA; Zaire, ZAI; Kenya, KEN; Tanzania, TAN; Eritrea, ERI; Saudi Arabia, SAU and Sudan, SUD). The major topotypes for SAT1 (labelled 1–8), SAT2 (labelled I–XII) and SAT3 (I–VI) viruses are indicated. No representative isolates of SAT1 topotype 6 and of SAT2 topotypes III, IV, X, XIII and XIV were included. Bootstrap support ⩾65 is based on a 1000 replicates and is indicated next to the relevant node.

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Table 1. Variation within the nucleotide and amino acid sequences of the P1 polyprotein in a complete alignment of the SAT1, 2 and 3 viruses

Serotype	Genome region	No. nucleotide positions aligned*	No. variant nucleotide				Variant nucleotide (%)†				No. amino acid positions aligned*	No. variant amino acids				Variant amino acids (%)†			
			All	SAT1	SAT2	SAT3	All	SAT1	SAT2	SAT3		All	SAT1	SAT2	SAT3	All	SAT1	SAT2	SAT3
	1A	258	120	100	106	72	46.5	38.8	41.1	27.9	84	24	11	15	2	28.5	13.1	17.9	2.4
	1B	654	391	285	315	230	59.8	43.6	48.2	35.2	219	125	54	64	48	57.1	24.7	29.2	21.9
	1C	675	413	304	308	231	61.2	45.0	45.6	34.0	224	146	82	68	52	65.5	37.1	30.6	23.4
	1D	677	498	379	379	341	73.6	56.0	56.0	50.4	224	158	105	107	91	70.5	48.0	50.2	42.7
	All capsid proteins	2232	1422				62.8				744	429				57			

*The number of nucleotides and amino acids were based on CLUSTAL_X alignments of the complete P1-coding region of the SAT serotypes.
†The number of variant nucleotides or amino acids for each genomic region or capsid protein relative to the total number of positions was used to estimate the percentage (%) variability.

r_1-values $\geqslant 0.4$ with the SAR/09/81 and KNP/196/91 viruses, respectively, all belonging to topotype 1 (Table 2). Furthermore, 55 (n=11) and 70 % (n=14) of the isolates had r_1-values in the range from 0.2 to 0.39, indicating that a high potency vaccine may still afford protection in animals. The remaining isolates reacted poorly to antisera directed against these vaccine strains, indicating that the vaccines will most probably not protect animals in the field against these strains. Convalescent sera to a West African SAT1 virus, i.e. SAT1/NIG/05/81, showed reasonable cross-protection (r_1-values of 0.2–0.39) against 75 % (n=15) of the SAT1 viruses (Table 2). Only one isolate had an r_1-value of $\geqslant 0.4$, i.e. SUD/3/76, which belongs to the same topotype as the Nigerian isolate. The difference in cross-reaction with the reference sera might be indicative of differences in shared epitopes of the isolate in question to the reference virus.

The SAT2 reference antisera used in the study were from the SAT2 vaccine strains ZIM/07/83 and KNP/19/89 that belong to SAT2 topotypes I and II, respectively, and two convalescent antisera, that of ERI/12/89 and RWA/02/01 from topotypes VII and VIII, respectively (Table 3). The antigenic variation for the SAT2 isolates was more pronounced compared with SAT1 isolates, with r_1-values below 0.2 even when vaccine and field strains from the same topotype were compared. With the exception of the reaction of the ZIM/07/83 isolate to the KNP/19/89 sera, none of the other SAT2 isolates showed r_1-values in the range of 0.4–1.0 against any of the four reference strains. Although none of the SAT2 isolates had r_1-values of 0.2–0.39 against ZIM/7/83 antisera, at least 16.6 % (n=4) of the isolates fell into this range using KNP/19/89 antisera. Furthermore, 28 and 25 % (n=6 and n=5) of isolates had r_1-values of 0.2–0.39 when tested against ERI/12/89 and RWA/02/01 antisera, respectively. From the data in Table 3 the convalescent sera (ERI/12/89 and RWA/02/01) appear to be more cross-reactive than the sera derived from the immunized animals (ZIM/07/83 and KNP19/89). However, the best consensus estimate model described by Reeve *et al.* (2010) did not show a difference for sera prepared by vaccination or collected from infected animals.

Comparison of amino acid variation within the SAT1 and 2 serotypes

We extended our study to investigate potential regions involved in the antigenic variability of SAT1 and SAT2 viruses based on the identification of hypervariable regions and positions of high entropy in the capsid-coding regions for SAT viruses. Hypervariable regions, in which 60 % or more of the residue positions varied (using overlapping windows of 10 aa), were identified in the outer capsid proteins, i.e. 1B, 1C and 1D (see Supplementary material, available in JGV Online). Within hypervariable regions, entropy was used to measure the uncertainty at each amino acid position within the SAT alignment (Schneider & Stephens, 1990). Interestingly, differences were observed

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Table 2. The mean r_1-values and number of variable amino acids in the capsid proteins of SAT1 isolates as measured against reference strains (SAR/09/81, KNP/196/91 and NIG/05/81)

−, VNTs were not done.

SAT1 isolates*	Topotype†	SAR/09/81‡		KNP/196/91‡		NIG/05/81§	
		Variable amino acids	r_1-value	Variable amino acids	r_1-value	Variable amino acids	r_1-value
KNP/196/91‖	**1**	49	0.44	**0**	**1.00**	109	0.21
SAR/09/81‖	**1**	**0**	**1.00**	49	0.44	105	0.34
ZIM/GN/13/91	1	44	0.50	38	0.55	107	0.23
ZIM/HV/03/90	1	42	0.40	31	0.48	100	0.20
KNP/148/91	1	46	0.37	17	0.36	106	0.34
KNP/41/95	1	54	0.25	50	0.36	118	0.23
NAM/307/98	2	73	0.21	67	0.28	109	0.22
ZIM/06/94	2	75	0.23	77	0.33	113	0.17
MOZ/03/02	3	63	0.24	59	0.37	107	0.32
ZIM/25/90	3	68	0.27	62	0.10	109	−
TAN/01/99	3	−	0.13	−	0.28	−	0.25
TAN/37/99	3	55	0.25	55	0.36	105	0.27
ZAM/02/93	3	63	0.16	60	0.14	106	0.10
KEN/05/98	3	56	0.23	58	0.34	101	0.23
UGA/03/99	4	73	0.26	80	0.25	100	0.32
UGA/01/97	5	91	0.24	102	0.27	114	0.29
SUD/03/76	7	103	0.28	107	0.25	17	0.60
NIG/05/81‖	**7**	105	0.19	109	0.28	**0**	**1.00**
NIG/15/75	8	105	0.17	108	0.26	65	0.21
NIG/06/76	8	104	0.10	106	0.12	63	0.17
NIG/08/76	8	103	0.14	106	0.36	63	0.18

*The passage histories and country of origin of the study viruses as well as GenBank accession numbers of the P1 sequences have been described in Reeve *et al.* (2010).
†The topotype designations is based on 1D phylogeny proposed by Bastos *et al.* (2001); Sangare *et al.* (2003); Vosloo *et al.* (2006); Sahle *et al.* (2007a).
‡The reference SAT1 test sera used in the VNTs. The sera were prepared by two consecutive vaccinations on days 0 and 28 with reference SAT1 viruses and subsequently bled on day 38.
§The convalescent test sera were prepared by inoculating cattle intradermolingually with 10^4 $TCID_{50}$ of SAT1/NIG/5/81 virus and collecting blood 21 days post-infection.
‖The homologous viruses used as reference strains. The bold values indicate the homologous reaction in VNTs or alignment with homologous sequence.

between hypervariable sites for SAT1 and 2 isolates obtained from eastern, western and southern Africa. The hydrophilicity and deduced surface exposure were also considered for each residue and highly variable regions correlated with regions of significant hydrophilicity (see Supplementary material). Comparison of the P1 amino acid sequences of SAT1 and 2 with type A and O sequences, revealed that many of the SAT1 and 2 hypervariable regions corresponded or were located in close proximity to previously identified immuno-dominant sites on types O and A (Table 4; Xie *et al.*, 1987; Thomas *et al.*, 1988; Baxt *et al.*, 1989; Bolwell *et al.*, 1989; McCahon *et al.*, 1989; Kitson *et al.*, 1990; Saiz *et al.*, 1991; Crowther *et al.*, 1993a). Some hypervariable regions were located within flexible structural loops or downstream of protease cleavage sites.

As expected, the internally located 1A protein was the most conserved of the structural proteins with only 27 % variable positions on amino acid level (only 6 % identified as parsimony-informative) between SAT1 and SAT2 (Table 1). Alignment of the amino acid sequences of the 1A polypeptide revealed no hypervariable regions, but common substitutions that are unique to each serotype were observed. Two amino acids in the 1A protein that are potentially specific for SAT2 and for SAT1 viruses are an Iso or Val at position 76 and Phe at position 80 for SAT2 viruses, while SAT1 viruses present with Phe and Val at the corresponding positions (see Supplementary material).

The 1B protein, 219 aa in length contained 24 and 29 % variable amino acid positions for SAT1 and 2, respectively (57 % in a complete alignment) (Table 1). Within the 1B capsid protein four hypervariable sites were identified within each serotype (Table 4), i.e. βA–βB loop (aa positions 31–45), βB–βC loop (aa 64–82), βC–βD loop (aa 93–101) and βE–βF loop (aa 130–134/141). Only the

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Table 3. The mean r_1-values and number of variable amino acids in the capsid proteins of SAT2 isolates as measured against reference strains (ZIM/07/83/2, KNP/19/89/2, ERI/12/89/2 and RWA/02/01/2)

−, VNTs were not done.

SAT2 isolates*	Topotype†	ZIM/07/83‡		KNP/19/89‡		ERI/12/89§		RWA/02/01§	
		Variable amino acids	r_1-value	Variable amino acids	r_1-value	Variable amino acids	r_1-value	Variable amino acids	r_1-value
KNP/19/89‖	**I**	59	0.14	**0**	**1.00**	86	0.25	90	0.10
KNP/02/98	I	54	0.09	16	0.11	81	0.12	84	0.19
KNP/51/93	I	56	0.07	33	0.09	81	0.12	83	0.18
SAR/16/83	I	60	0.03	39	0.03	83	0.05	85	0.10
ZIM/08/94	I	52	0.08	39	0.12	83	−	79	−
ZIM/GN/10/91	I	49	0.11	42	0.11	81	0.09	80	0.09
ZIM/07/83‖	**II**	**0**	**1.00**	59	0.41	96	0.20	90	0.19
ZIM/14/90	II	49	0.05	57	0.09	91	0.14	85	0.10
ZIM/17/91	II	51	0.13	56	0.05	90	0.14	88	0.24
ZIM/01/88	II	19	0.18	51	0.13	87	0.16	86	0.13
ZIM/34/90	II	61	0.14	67	0.28	88	−	91	−
RHO/01/48	II	60	0.17	58	0.12	86	0.21	87	0.27
KEN/08/99	IV	68	0.05	53	0.07	86	0.04	86	0.06
GHA/08/91	V	89	0.09	80	0.09	74	0.18	62	0.21
LBR/01/74	V	−	0.03	−	0.05	−	0.12	−	0.16
SEN/05/75	V	100	0.02	90	0.05	82	0.03	71	0.06
SEN/07/83	VI	96	0.10	84	0.20	106	0.25	98	0.35
SAU/06/00	VII	97	0.06	86	0.06	38	0.26	73	−
ERI/12/89‖	**VII**	96	0.14	86	0.31	**0**	**1.00**	70	0.32
RWA/02/01‖	**VIII**	90	0.13	90	0.09	70	0.23	**0**	**1.00**
KEN/03/57	IX	93	−	94	−	87	−	56	−
ANG/04/74	XI	85	0.09	88	0.10	91	0.04	80	0.05
UGA/02/02	XII	93	0.02	92	0.03	84	0.04	57	0.08
ZAI/01/74	XII	92	0.02	86	0.05	66	0.12	33	0.17

*The passage histories and country of origin of the SAT2 viruses and the GenBank accession numbers of the P1 sequences have been described in Reeve *et al.* (2010).

†The SAT2 topotype designations is based on 1D phylogeny proposed by Bastos *et al.* (2003b); Sangaré *et al.* (2004); Sahle *et al.* (2007b).

‡The reference SAT2 test sera used in the VNTs. The sera were prepared by two consecutive vaccinations on days 0 and 28 with reference SAT2 viruses and subsequently bled on day 38.

§The convalescent test sera were prepared by inoculating cattle intradermolingually with 10^4 $TCID_{50}$ of SAT2/ERI/12/89 and SAT2/RWA/02/01 viruses, respectively, and collecting blood 21 days post-infection.

‖The homologous viruses used as SAT2 reference strains. The bold values indicate the homologous reaction in VNTs or alignment with homologous sequence.

βB–βC and βE–βF loops have significant surface exposure in the complete virion, while the N terminus of 1B (βA–βB loop) is located on the inner surface of the capsid in close proximity with the N terminus of 1D.

The 1C protein varied from 221 to 222 aa in length for SAT1 and SAT2 isolates, respectively. The exception was a SAT2 isolate from West Africa, SEN/7/83, which was only 220 aa in length. For 1C, the overall variable amino acid positions was 65 % (37 % variation within SAT1 and 30 % within SAT2) (Table 1). The genetic heterogeneity of 1C was confined to four hypervariable regions (Table 4), i.e. N terminus (aa 30–45), βB–βC loop (aa 63–77), βE–βF loop (aa 125–142) and βG–βH loop (aa 165–183/172). The heterogeneity was more pronounced in SAT1 viruses with some unique amino acids confined to different topotypes. The latter three of these hypervariable regions (consisting of the βB–βC, βE–βF and βG–βH loops) correlated with hydrophilic surface-exposed regions, indicating its possible contribution to antigenic determinants (see Supplementary material).

The 1D protein, the most variable of the outer capsid proteins, varied in length from 213 aa for SAT2 to 219 aa for SAT1 with 71 % overall variable amino acid positions (48 % within SAT1 and 50 % within SAT2) (Table 1). The SAT2 virus, SEN/07/83, was the exception with 214 aa in length. The inserted amino acid of SEN/07/83 is a Trp between residues 28 and 29 within a conserved positively

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Table 4. A summary of the hypervariable regions observed in an alignment of the amino acid sequences of SAT1 and two outer capsid proteins

The hypervariable regions were considered in relation with structurally exposed loops and with known antigenic sites in serotype A and O sequences. *Residues within the starred regions have significant surface exposure (Fig. 2) and entropy that could contribute to the changes in antigenicity observed.

	Amino acid hypervariable region†														
	Capsid secondary structure elements														
Capsid	1B	1B	1B	1B	1C	1C	1C	1C	1D	1D	1D	1D	1D	1D	1D
β-Sheets	A–B	B–C	D–D	E–F; H–I	A–B	B–C	E–F	G–H; H–I	N_T	B–C	E–F	F–G	G–H	H–I	C_T
Axis		3 ×		2 ×		3 ×	2 ×			5 ×			2 ×	5 ×	3 ×
SAT1	31–44	62–82*	97–101	130–134*	30–45	59–78*; 83–91	124–141*	165–183	9–35	43–57*	93–102	113–123	135–151; 156–169	176–187	201–222
SAT2	37–43	69–79*	91–100	129–140*	27–47	62–76*	125–137*	165–173	16–42	45–72*	80–92; 100–104	108–118	136–145; 151–166	172–183	193–217
	FMDV antigenic sites†														
Type O		Site 2 70–77/78		Site 2 131–134; T188I		Site 4 56–58			T-cell	Site 3 43–48			Site 1a, 5 144–149, 154		Site 1b 206–207
Type A		Site 3 82–88; 196				Site 5 58–61; 69–70	Site 3 136–139; 195						Site 1 142–157	Site 4 169; 175–178	Site 2 200–212

*The hypervariable regions have been derived from the alignment in the Supplementary material.

†The antigenic sites are a summary of that described in Thomas *et al.* (1988); Baxt *et al.* (1989); Bolwell *et al.* (1989); Saiz *et al.* (1991); Kitson *et al.* (1990); Crowther *et al.* (1993a); Guzman *et al.* (2010).

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charged motif (KRRXH for SAT2 or RRXH for SAT1) located in a region similar to a previously identified T-cell epitope of 1D for serotype C and O (Pérez Filgueira *et al.*, 2000; Guzman *et al.*, 2010). At least seven discrete hypervariable regions were identified in 1D of SAT1 and SAT2 viruses (Table 4). These regions corresponded with the N terminus (residues 9–40 in SAT viruses), βB–βC loop (residues 43–62/71 for SAT1 and 2), βE–βF loop (residues 80–103), βF–βG loop (residues 110–122), βG–βH loop (residues 136–167), βH–βI loop (residues 176–187) and the C terminus (residues 199–220/192–212 for SAT1 and 2). With the exception of the N terminus and βF–βG loop, the hypervariable regions coincided with hydrophilic surface-exposed regions.

Amino acid residues situated within regions of hypervariability in the outer capsid proteins, which exhibit high entropy and hydrophilicity, and on structurally exposed loops were regarded as having the potential for involvement in antibody recognition sites. These regions are therefore possibly involved in the antigenicity of the virion (Table 4 and Supplementary material).

Amino acid changes and antigenic types within the SAT serotypes

To obtain information regarding the relationship between genetic distance and mean r_1-values for SAT1 and SAT2 viruses, the mean r_1-values obtained for each field isolate against the reference sera were plotted against the total amino acid changes between the field strain and reference virus (Supplementary Fig. S1, available in JGV Online). For SAT1 viruses, a close genetic relationship with the reference strain, as observed by considerable amino acid identity, did agree with sufficient serological cross-reaction. However, for the SAT2 viruses the observed r_1-values were low, even for viruses with considerable identity with the reference strain. The availability of the deduced amino acid sequences for the P1 regions of the VNT analysed viruses allows for the direct comparison of amino acid residue changes between any two isolates. In addition, residues located in hypervariable regions that are surface-exposed can be identified and counted between any two sequences. The changes specifically corresponding to surface-exposed residues are expected to correlate directly with antigenic variability as it may abrogate antibody binding and neutralization *in vitro*. This can be explained using two examples, one for SAT1 and SAT2, respectively.

In a pairwise alignment of the P1 polypeptide of the SAT1/SAR/9/81 virus and five additional topotype 1 isolates (KNP/196/91, KNP148/91, KNP/41/95, ZIM/GN/13/91 and ZIM/HV/03/90), 17 to 55 variable amino acids were observed (Table 2). The r_1-values for these viruses varied from 0.22 to 0.45, indicating poor but some cross-reaction to good cross-reaction (Table 2 and Supplementary Fig. S1). Since the outer-capsid protein surface-exposed residues are directly involved in antigenicity (Logan *et al.*, 1993; Lea *et al.*, 1995), clusters of variable surface-exposed amino acids may constitute antigenic regions on the virion. Using this approach the putative residues directly involved in antigenicity could be narrowed down compared to amino acid alignment alone. Indeed, residue positions with high entropy and surface exposure were located in the βB–βC (aa 71–74) and βE–βF (aa 133–134) loops and aa 196 of 1B, the βB–βC (aa 64–68) and βE–βF (aa 134–136) loops of 1C, and the 1D βB–βC loop (aa 46–49), residues 142–148 and 157 in βG–βH loop, 177–179 in the βH–βI loop and 208 and 214 in the C terminus.

Within the SAT2 serotype similar observations were made. Comparison of the P1 polypeptide of six SAT2 isolates that belong to topotype 1 (KNP/19/89, KNP/02/89, KNP/51/93, SAR/16/83, ZIM/08/94 and ZIM/GN/10/91) revealed 16 to 42 variable residue positions (Table 3). The r_1-values against SAT2/KNP/19/89 sera varied from 0.04 to 0.39 where the genetically closely related KNP/02/89 with 16/744 variable residues compared to KNP/19/89, had a maximum r_1-value of only 0.17 against KNP/19/89 sera (Table 3 and Supplementary Fig. S1b). These 16 variable residues are likely involved in abolishing the neutralization by SAT2/KNP/19/89 antisera. Using structural data, surface-exposed residues with high entropy could be identified in the βE–βF (aa 130–134) loop of 1B, the βE–βF (aa 129–134) loop of 1C, and βB–βC (aa 63–68), βE–βF (aa 83–85), βF–βG (aa 110–112), βG–βH (aa 136–140 and 156–161), βH–βI (aa 172–176) and C terminus (aa 200–202) of 1D.

Taken together the inability of anti-SAT1/SAR/09/81 or anti-SAT2/KNP/19/89 antiserum from vaccinated animals to neutralize genetically related viruses may be the result of amino acid variation in the above-mentioned surface-exposed β-loops.

Mapping putative antigenic sites on the virus capsid

We modelled the SAT1 and SAT2 capsid structures using O_1BFS (Logan *et al.*, 1993) as template. The model was based on the optimal alignment of the SAT1 virus, SAR/09/81, or the SAT2 virus, ZIM/07/83, P1 sequences to the corresponding sequence of O_1BFS. The homology modelled structure was calculated by the satisfaction of spatial restraints as described by empirical databases. Using this method and mapping the observed variable loops (Table 4) to the structures, we identified several putative antigenic sites on the virus capsid (Fig. 2a, b). In particular, we observed differences between serotypes, but also within topotypes in a given serotype.

Taking all the topotype information into consideration, the variable residues (high entropy) within surface-exposed loops were regarded as immune relevant and were mapped to the SAT1 and SAT2 pentamer structures, respectively (Fig. 2a, b and Table 4). For both serotypes the variable regions outside the 1D βG–βH loop were concentrated around the fivefold and threefold axis of the virion and the C-terminal of 1D. These regions correlated strongly with

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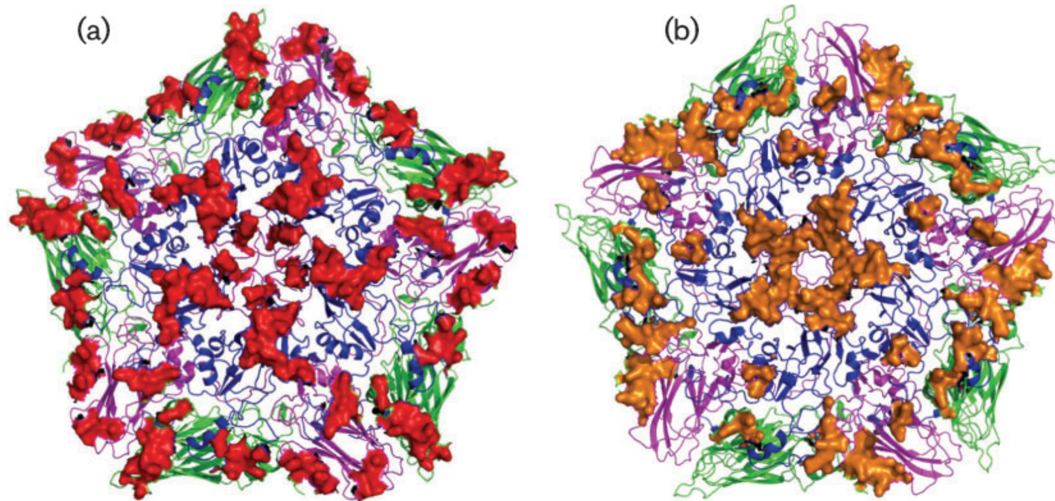

Fig. 2. Three-dimensional structure of (a) SAT1 and (b) SAT2 pentamer, modelled using the O_1BFS co-ordinates (1FOD; Logan *et al.*, 1993), as template. The protein subunits are colour coded: VP1 (blue), VP2 (green) and VP3 (cyan). The position of surface-exposed residues with high entropy is indicated in red (a) or orange (b) on the SAT1 or SAT2 pentamers, respectively. The alignment used for the extraction of data are available as in Supplementary material.

previously identified neutralizing epitopes of types A and O (Table 4). The role of these residues as antigenic determinants can therefore not be excluded. Furthermore, it can be hypothesized that some of the putative epitope regions are probably discontinuous. The close proximity of 1B residues 71–74, 133–134 and 196 or 1B residues 71–74, 196 and 1C residues 64–68 around the threefold axis of the virion or 1D residues 46–49, 84–86 and 177–179 around the fivefold axis of SAT1 viruses are examples (Fig. 2a, b).

DISCUSSION

This study reports on a genetic and phylogenetic analysis of the entire capsid-coding region and its deduced amino acid sequence of the three SAT serotypes of FMDV. We have also consolidated amino acid variation and r_1-values with structural data in order to predict areas on the surface of the FMD virion that are antigenically significant.

Based on the complete P1-coding region the phylogeny of 48 SAT isolates with diverse geographical distribution in Africa revealed the same evolutionary lineages within a serotype as previously described for 1D phylogeny (Bastos *et al.*, 2001, 2003a, b). Phylogenetic analysis of the complete P1- or 1B- and 1C-coding regions of the SAT isolates indicated similar tree topologies compared to 1D phylogeny (data not shown). Phylogeny, based only on the internally located 1A genomic region, revealed similar structuring than that described previously for the Leader and 3C protease-coding regions (van Rensburg *et al.*, 2002), although different from the outer capsid-coding regions. Darwinian selective pressure for the evolution of the non-structural proteins and the internally located 1A, exist to preserve structure and functionality and escape from immune response is less important (van Rensburg *et al.*, 2002).

High levels of genetic diversity in the P1-coding region within the SAT serotypes are reflected antigenically and therefore have implications for the control of the disease through vaccination. Antigenic analysis of field isolates in relation to vaccine strains, based on VNTs, play a significant role in evaluating the suitability of existing vaccine strains (Jangra *et al.*, 2005; Paton *et al.*, 2005; Brehm *et al.*, 2008), despite significant variation having been reported with VNTs (Rweyemamu *et al.*, 1978, 1984). It is generally accepted that r_1-values higher than 0.4 demonstrates a good cross-protection of the vaccine against the field isolate (Samuel *et al.*, 1990). Although SAT1 and SAT2 viruses displayed a similar number of variable amino acids (32 %) in a complete alignment, antigenic variation within SAT1 was less pronounced (73 % of r_1-values >0.2) than for SAT2 viruses (17 % of r_1-values >0.2) within the dataset. The implication for control by vaccination is that a high potency or bi-valent SAT1 vaccine will most likely be effective across topotypes. Indeed, it has been shown for serotype A viruses that a high potency vaccine provides protection against heterologous challenge despite low r_1-values (Brehm *et al.*, 2008). Better antigenic relationships were obtained for the SAT1 viruses belonging to the same topotype of the reference viruses (with less than 16 % aa variation). In the past, the combination of SAT1/SAR/9/81 and SAT1/KNP/196/91 in a tetravalent vaccine, containing also a SAT2 and a SAT3 strain, were able to protect against SAT1 outbreaks in Southern Africa (Hunter, 1998).

The SAT2 reference strains ZIM/7/83 and KNP/19/89, on the other hand, did not have good antigenic relationships

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with most SAT2 isolates, even within the same topotype. ZIM/7/83 antisera cross-reacted weakly to the SAT2 viruses in this study and were not able to neutralize the genetically closely related ZIM/1/88 (19 aa differences in a pairwise comparison). From our dataset, it seems unlikely that SAT2/ZIM/7/83 alone will provide protection against the large genetic and antigenic diversity among the SAT2 viruses. Antigenic relationships against KNP/19/89 was better with at least 16 % of the isolates having an r_1-value >0.2, while ERI/12/98 and RWA/02/01 antisera were able to neutralize at least 20 % of the viruses *in vitro*. Similar to the SAT1 viruses, better antigenic relationships to ERI/12/98 and RWA/02/01 were obtained for SAT2 viruses belonging to the same topotypes. Our study suggests that extensive antigenic variation occurs for SAT2 viruses across Africa and has serious implications for vaccine strain selection. A previous serological comparison of SAT2 viruses from Kenya suggested extensive antigenic variation in this country alone (Ndiritu *et al.*, 1983). The poor antigenic coverage of existing vaccine strains against field strains call for urgent development of multiple region-specific or topotype-specific vaccine strains. Additionally, there is a need for a SAT2 vaccine strain that will provide protection against a wide range of antigenic types in the field.

mAbs against FMDV were used in the past to identify antigenic variants and to resolve epitopes that play a role in the neutralization of the virus. The majority of these mAbs were against Euro-Asian serotypes A, O and C (Xie *et al.*, 1987; Thomas *et al.*, 1988; Baxt *et al.*, 1989; Bolwell *et al.*, 1989; McCahon *et al.*, 1989; Kitson *et al.*, 1990; Saiz *et al.*, 1991; Crowther *et al.*, 1993a). Only one report described the mapping of epitopes to a SAT2 virus (RHO/1/48) isolated in 1948 (Crowther *et al.*, 1993b), with residues 147–149 and 156 in the flexible and surface-exposed G–H loop of 1D proven to be important in *in vitro* neutralization by a mAb.

We followed a different approach by combining amino acid variation and calculated r_1-values from *in vitro* cross-protection titres in VNTs, together with structural data, to predict areas on the surface of the capsid as antigenically relevant. This modelling approach has identified putative antigenic regions that correlated with cross-neutralization *in vitro* for the SAT1 and SAT2 serotypes, and may contain neutralizing epitopes for each serotype. These antigenic sites were consistent within, but not between serotypes, and were found to match some of the mAbs identified antigenic sites in other serotypes. These are the hypervariable, surface-exposed structural loops observed for SAT1 or SAT2 viruses, i.e. βB–βC and βE–βF loops of 1B, the βB–βC and βE–βF loops of 1C, and the N-terminal, βB–βC, βG–βH, βH–βI loops and C-terminal of 1D. The antigenic sites for SAT2 viruses differed by having no significant variation within the βB–βC loop of 1C, but with additional variation in the βD–βE and βF–βG loops of 1D. Epitopes identified from mAb escape mutants for other serotypes confirms the immune relevance of these structural loops. The βB–βC, βE–βF and βH–βI loops of 1B contains site 2 of serotype O (Kitson *et al.*, 1990; Crowther *et al.*, 1993a), site 3 on A10 (Thomas *et al.*, 1988) and βB–βC also correlates with subsite D2 on C (Mateu *et al.*, 1995). The βB–βC loop of 1C is in agreement with site 3 on A10 (Thomas *et al.*, 1988), while βB–βC, βG–βH, βH–βI loops and C-terminal of 1D all agree with epitopes identified for serotypes A, O and C (Kitson *et al.*, 1990; Crowther *et al.*, 1993a; Thomas *et al.*, 1988; Mateu *et al.*, 1995).

Although the putative antigenic sites need to be confirmed using mAbs and sequencing of virus escape mutants, the methodology employed in this study offers a potentially quick, easy and cheap alternative to the identification of antigenic relevant sites on field FMD strains.

METHODS

Virus isolates, RT-PCR, sequencing and analysis. The viruses included in this study were either supplied by the World Reference Laboratory (WRL) for FMD at the Institute for Animal Health, Pirbright (UK) or form part of the virus bank at the Transboundary Animal Diseases Programme, Onderstepoort (South Africa). The 20 SAT1 and 23 SAT2 FMDV isolates from 17 countries in Africa, selected for genetic characterization, represented a broad geographical distribution. The viruses were propagated in IB-RS-2 cells prior to RNA extraction, cDNA synthesis and PCR amplification. A description of the passage histories, host species and representative topotypes can be found in Reeve *et al.* (2010); Bastos *et al.* (2001, 2003a, b) and Sangare *et al.* (2003). The GenBank accession numbers are as provided in Reeve *et al.* (2010). The SAT3 viruses included in the P1 phylogenetic analysis were SAT3/BEC/01/65 (GenBank accession no. M28719), SAT3/KNP/10/90 (GenBank accession no. AF286347), SAT3/UGA/02/97 (GenBank accession no. AY192556), SAT3/ZAM/04/96 (GenBank accession no. AF023525.1) and SAT3/ZIM/05/91 (GenBank accession no. AY168799.1).

The Leader-P1-2A-coding regions of the isolates were obtained via RT-PCR of viral genomic RNA as described previously (Bastos, 1998; van Rensburg *et al.* 2002). Direct DNA sequencing of amplicons yielded a consensus sequence representing the most probable nucleotide for each position. Sequences of the approximately 2.2 kb P1-coding region were compiled and edited using the BioEdit 5.0.9 software (Hall, 1999). The nucleotide and deduced amino acid sequences were aligned using CLUSTAL_X (Thompson *et al.*, 1997) and phylogenetic trees constructed using the minimum evolution algorithm within MEGA4 software (Tamura *et al.*, 2007). Entropy plots were drawn from the deduced amino acid alignments using the BioEdit 5.0.9 software (Hall, 1999).

Animal sera and VNT. The antigenic diversity of the field isolates was determined using cross-neutralization assays in microtitre plates on IB-RS-2 cells, carried out as described by the OIE (2009). Cattle sera prepared by two consecutive vaccinations (vaccinated at day 0, boosted at day 28 and bled at day 38) or convalescent sera obtained from cattle 21 days post-infection with reference viruses (SAT1: SAR/09/81, KNP/196/91 and NIG/05/81; SAT2: ZIM/7/83, KNP/19/89/2, ERI/12/89 and RWA/02/01) were used. For cattle immunizations oil emulsion vaccines were prepared using binary ethylenimine-inactivated sucrose density gradient purified antigens for strains SAT1/SAR/09/81, SAT1/KNP/196/91, SAT2/ZIM/07/83 and SAT2/KNP/19/89/2, respectively. Each 2 ml dose, containing 4 μg 146S antigen and Montanide ISA 206 (Seppic) as the oil adjuvant, were mixed 50 : 50 with the aqueous antigen phase to produce a water-in-oil-in-water emulsion. Convalescent sera were prepared by inoculating cattle

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intradermolingually with 10^4 $TCID_{50}$ of the SAT1/NIG/5/81, ERI/12/89 and RWA/02/01 viruses, respectively.

The end-point titre of the serum against homologous and heterologous viruses was calculated as the reciprocal of the last dilution of serum to neutralize 100 $TCID_{50}$ in 50 % of the wells (Rweyemamu *et al.*, 1978). All neutralization titre determinations were repeated at least twice, each time using sera from two animals. One-way antigenic relationships (r_1-value) of the field isolates and vaccine viruses relative to the reference strains were calculated as the ratio between the heterologous and homologous serum titres and were interpreted as proposed by Samuel *et al.* (1990). Briefly, r_1-values between 0 and 0.19 indicated highly significant antigenic variation from the reference strains; values of 0.20–0.39 showed antigenic relatedness where some protection may be provided by a potent vaccine based on the reference strain; and values of 0.40–1.0 demonstrated that the reference and field strains are sufficiently antigenically similar for the reference strain to provide good protection. The means of the r_1-values were calculated (Tables 1 and 2) and plotted against total amino acid changes between the field strains and reference viruses.

Structural modelling. Homology models of the capsid proteins of SAT1 and SAT2 were built using Modeller 9v3 (Sali & Blundell, 1993) with O_1BFS coordinates (1FOD) as template (Logan *et al.*, 1993), alignments were performed with CLUSTAL_X and modelling scripts were generated with the structural module of FunGIMS. Structures were visualized and the surface-exposed residues identified with PyMol v1.1rc2pre (DeLano Scientific LLC).

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