



# Inherent biophysical stability of foot-and-mouth disease SAT1, SAT2 and SAT3 viruses

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## ABSTRACT

Foot-and-mouth disease (FMD) virus (FMDV) isolates show variation in their ability to withstand an increase in temperature. The FMDV is surprisingly thermolabile, even though this virus is probably subjected to a strong extracellular selective pressure by heat in hot climate regions where FMD is prevalent. The three SAT serotypes, with their particularly low biophysical stability also only yield vaccines of low protective capacity, even with multiple booster vaccinations. The aim of the study was to determine the inherent biophysical stability of field SAT isolates. To characterise the biophysical stability of 20 SAT viruses from Southern Africa, the thermofluor assay was used to monitor capsid dissociation by the release of the RNA genome under a range of temperature, pH and ionic conditions.

The SAT2 and SAT3 viruses had a similar range of thermostability of 48–54 °C. However, the SAT1 viruses had a wider range of thermostability with an 8 °C difference but with many viruses being unstable at 43–46 °C. The thermostable A-serotype A24 control virus had the highest thermostability of 55 °C with some SAT2 and SAT3 viruses of similar thermostability. There was a 10 °C difference between the most unstable SAT virus (SAT1/TAN/2/99) and the highly stable A24 control virus. SAT1 viruses were generally more stable compared to SAT2 and SAT3 viruses at the pH range of 6.7–9.1.

The effect of ionic buffers on capsid stability showed that SAT1 and SAT2 viruses had an increased stability of 2–9 °C and 2–6 °C, respectively, with the addition of 1 M NaCl. This is in contrast to the SAT3 viruses, which did not show improved stabilisation after addition of 1 M or 0.5 M NaCl buffers. Some buffers showed differing results dependent on the virus tested, highlighting the need to test SAT viruses with different solutions to establish the most stabilising option for storage of each virus. This study confirms for the first time that more stable SAT field viruses are present in the southern Africa region. This could facilitate the selection of the most stable circulating field strains, for adaptation to cultured BHK-21 cells or manipulation by reverse genetics and targeted mutation to produce improved vaccine master seed viruses.

## 1. Introduction

Diseases caused by RNA viruses are often difficult to control because of the high mutation rate and the continual emergence of novel genetic and antigenic variants that allow escape from immunity. The degree to which immunity induced by one virus is effective against another is largely dependent on the antigenic differences between them. Foot-and-mouth disease (FMD) virus (FMDV), a member of the genus *Aphthovirus* within the family *Picornaviridae*, is an example of an antigenically variable pathogen with the capacity to evade the immune response

(Martínez et al., 1992; Mateu et al., 1988). FMD remains one of the most economically important diseases of livestock, such as cattle, sheep, pigs and goats (Gibbs, 1981; Suttmoller et al., 2003; Perry and Rich, 2007). Vaccination remains the most effective approach for controlling the seven, clinically indistinguishable serotypes [A, O, C, Asia-1 and Southern African Territories (SAT) 1, 2 and 3] that exist and of the various antigenic subtypes that continuously emerge. Thus, the most effective vaccines need to be of high potency (> 3 PD<sub>50</sub>; where one PD<sub>50</sub> denotes the dose of a vaccine which would protect 50% of vaccinated animals) and closely match the outbreak virus and therefore

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there is a constant need to develop new vaccine strains.

Additionally, the stability of vaccines is of crucial importance for the effective control of FMD in endemic tropical settings of Africa and Asia. In these areas, the logistical process to get the vaccine from the manufacturer to administration to the animal may take several weeks or months and in many remote regions in the absence of a cold-chain. Vaccine stability can be affected by not only temperature but also other factors including pH change, stabilisers or protease activity at any time during manufacturing or administration, shear forces on the antigen or the presence of certain chemicals (e.g. thiomersal). Vaccines with improved biophysically stable antigen (Doel and Baccarini, 1981) and less reliant on a cold-chain are needed and could improve the shelf-life and longevity of immune responses elicited in animals. FMDV, contrary to other picornaviruses, is known to be unstable in mildly acidic pH or at elevated temperatures, especially viruses belonging to the O and SAT2 serotypes. This leads to dissociation of the capsid (146S particle) into 12S subunits and loss of immunogenicity (Curry et al., 1995; Ellard et al., 1999; Doel and Baccarini, 1981). Furthermore, although different FMDVs may show variation in their ability to withstand an increase in temperature (Doel and Baccarini, 1981; Nettleton et al., 1982), all FMDV are surprisingly thermolabile, even though this virus is probably subjected to a strong extracellular selective pressure by heat in hot climatic regions where FMD is prevalent (Mateo et al., 2007). Even worse is the situation for the three SAT serotypes, for which the notoriously low thermostability contributes to vaccines of low protective capacity even when administered multiple times, requiring the producer to compensate for this by increasing the payload (Doel and Chong, 1982; Doel, 2003).

Naturally occurring FMDV adapt to efficient replication in vaccine production cell lines by accompanied changes in viral capsid protein to allow binding to alternative cellular receptors (Jackson et al., 1996; Sa-Carvalho et al., 1997). Mateo et al. (2007) found a reduction in thermostability may occur during adaptation of FMDV to cell culture conditions, perhaps because of the absence, of the selective pressure exerted in the field by occasional heat extremes. The importance of understanding thermostable variants in FMDV populations, should focus on: (i) the number, type and location of mutations needed to confer thermostability and (ii) the effect of those mutations on the biological fitness (Mateo et al., 2007).

Elucidation of the crystal structure of FMDV over 20 years ago (Acharya et al., 1989) enabled assessment of the effects of acid and heat on the virus capsid structure and identification of protein-protein interactions that may correlate with enhanced acid or heat stability (Curry et al., 1995, 1996; Ellard et al., 1999; Mateo et al., 2003). The self-assembly and stability of a multimeric protein capsid, such as FMDV, depends on the occurrence of numerous non-covalent interactions between multiple polypeptide subunits (Liljas, 1986; Rossman and Johnson, 1989; Reguera et al., 2004). The non-enveloped, icosahedral virion of FMDV is composed of 60 copies of four viral structural proteins, VP1–4 assembled into a protomeric subunit (Acharya et al., 1989). Subsequently, five protomers assemble into a pentameric intermediate and finally, 12 pentamers self-assemble through complex protein-protein interactions into a complete capsid (Curry et al., 1996; Lea et al., 1995; Mateo et al., 2003). Even though FMDV, especially the SAT types, exhibit large intra- and inter-serotype genetic variability (Carillo et al., 2005), the multiple and repetitive inter-subunit interactions appear to have evolved under stringent and selective constraints (Acharya et al., 1989; Mateo et al., 2003). Mateo et al. (2007) showed that the presence of thermostable virus variants is not a general feature of FMDV quasispecies. This suggests that no substantial increase in the thermostability of FMDV may readily occur without a negative effect on viral function. Viral capsid inter-subunit interactions are required to be sufficiently robust in order to provide stability to the capsid under environmental denaturing conditions (Curry et al., 1996; Ellard et al., 1999), whilst still permitting intracellular uncoating and release of viral RNA.

A novel approach was developed to accurately measure the inherent biophysical stability (temperature, pH and ionic conditions) of low passage, field viruses of the three SAT serotypes, representative of the geographically linked genotypes or topotypes present in southern African. The establishment of a protocol and the quantification of FMDV stability is vital in the selection of field viruses with stable FMDV 146S properties with potential use as master seed viruses in vaccine production in southern Africa.

## 2. Materials & methods

### 2.1. Sequence analysis

The P1 sequences of 37 SAT 1, 40 SAT2 and 22 SAT3 viruses from southern Africa available from previous studies and retrieved from GenBank were analysed to provide phylogenetic references. From this analysis, field viruses were selected from the virus bank (ARC-OVR) as representatives of the SAT1, 2 and 3 serotypes and their different topotypes.

Dendrograms of each SAT serotype were constructed to determine the relationship that exists between the FMDV SAT isolates from southern Africa. The derived sequences were trimmed using the BioEdit sequence alignment editor (v7.0, Tom Hall, Isis Pharmaceuticals, Inc 1997–2004, CA, USA), and alignments carried out with the ClustalW alignment software (EBI, Cambridge, England) incorporated into the BioEdit software (Thompson et al., 1997).

Mega 5.1 package was used to perform the phylogenetic and distance analyses (Tamura et al., 2011). An unrooted dendrogram was constructed after bootstrapping to 1000 replicates by the minimum evolution (ME) method, which is based on the assumption that the tree with the smallest sum of branch length estimates is most likely to be the true one. A minimum evolution (ME) dendrogram with bootstrap values of more than 70% were generally regarded as providing evidence for a phylogenetic grouping. The graphical output for the 50% majority rule consensus trees was obtained by using the MEGA 5 tree explorer version 5.1 (Tamura et al., 2011).

### 2.2. Cells and viruses

Field viruses (Table 1) were propagated on IB-RS-2 (Instituto Biológico renal suino) cells and Baby hamster kidney (BHK) clone 13 (strain 21; ATCC CCL-10) cells used for purification. IB-RS-2 and BHK-21 cells were maintained and propagated in RPMI medium (Gibco) supplemented with 10% (v/v) foetal calf serum (FCS) and 1% (v/v) antibiotics/antimycotics and Glasgow minimum essential medium (GMEM; Life Technologies) supplemented with L-glutamine, 1% (v/v) antibiotics/antimycotics, 3% (v/v) lactalbumin hydrosylate (LAH), 10% (v/v) tryptose phosphate broth (TPB), 10% (v/v) foetal calf serum (FCS), respectively. Virus stocks were prepared and titrated in BHK-21 cells (Rieder et al., 1993) using virus growth medium (VGM; GMEM with 1% (v/v) FCS, 1% (v/v) HEPES and 1% (v/v) antibiotics).

### 2.3. Construction of SAT1 mutants

The plasmid pSAT1, a previously described genome-length infectious cDNA clone of SAT1/SAR/9/81 (Maree et al., 2011), was used as the genetic backbone in the construction of recombinant cDNA clones harboring capsid stabilizing mutations. The virus recovered from pSAT1 is referred to as vSAT1. Model preparation for molecular dynamics simulation protocol and the estimated degree of stabilization for SAT2/ZIM/7/83 have been described (Kotecha et al., 2015; Scott et al., 2017). In this study, these predicted sites were used to identify corresponding amino acid residues on the 2-fold interface involving VP2 and VP3 regions in SAT1 by aligning the amino acid sequence of SAT1/SAR/9/81 with SAT2/ZIM/7/83. Two mutations were selected: Q2093W and D3197A. Synthetic DNA constructs of the P1 (structural

**Table 1**

SAT serotype FMD viruses used in the study showing the year and species from which they were isolated; the country of origin and the GenBank accession numbers.

Serotype	Virus	Year isolated	Species	Country of origin	GenBank Accession No.
SAT1	KNP/196/91	1991	Buffalo	South Africa	AF283429
	SAR/9/81	1981	Impala	South Africa	DQ009715
	BOT/1/06	2006	Cattle	Botswana	KJ999919
	SAR/33/00	2000	Cattle	South Africa	KJ999908
	MOZ/1/02	2002	Cattle	Mozambique	KJ999928
	TAN/2/99	1999	Cattle	Tanzania	KJ999931
	ZAM/1/06	2006	Cattle	Zambia	KJ999930
SAT2	SAR/3/04	2004	Cattle	South Africa	#
	ZIM/13/01	2001	Cattle	Zimbabwe	JQ639292
	ZIM/7/83	1983	Cattle	Zimbabwe	DQ009726
	ZIM/14/90	1990	Cattle	Zimbabwe	DQ009728
	ZIM/4/97	1997	Cattle	Zimbabwe	JQ639293
	ZIM/8/94	1994	Cattle	Zimbabwe	GU194492
	BOT/18/98	1998	Cattle	Botswana	KJ999940
	MAL/1/08	2008	Cattle	Malawi	KJ999939
	SAR/1/06	2006	Cattle	South Africa	MK415736
SAT3	SAR/14/01	2001	Cattle	South Africa	MK415740
	KNP/14/96	1996	Buffalo	South Africa	MK415741
	KNP/10/90	1990	Buffalo	South Africa	AF286347
	BOT/6/98	1998	Cattle	Botswana	MK415742

# Maree, personal communication.

proteins) genomic region of pSAT1 containing the Q2093 W or D3197 A mutations; flanked with 5' and 3' terminal restriction sites XbaI and SnaBI/BbvCI respectively, and with cell culture adapted residues KRG at position 110–112 of VP1 were designed and synthesized (GenScript USA Inc.). The synthesized constructs were introduced into the pSAT1 genome-length infectious cDNA clone using the IN-Fusion HD Cloning Kit (Clontech, USA), as described previously (Scott, 2017). The recombinant mutated pSAT1 constructs were confirmed by ABI PRISM™ BigDye™ Terminator Cycle sequencing V3.1 (Applied Biosystems, Warrington, United Kingdom). Thereafter, the constructs were linearized with SwaI, *in vitro* transcribed using the MEGAscript™ T7 kit (Ambion) and transfected into BHK-21 cells using Lipofectamine™ 2000 reagent (Life Technologies) according to the manufacturer's instructions. Transfected cells were maintained at 37 °C with a 5% CO<sub>2</sub> influx for 48 h in virus growth medium and frozen at –80 °C (Van Rensburg et al., 2004). Viruses (termed 'passage 0', P0) were subsequently harvested from infected cells by a freeze-thaw cycle and clarified by centrifugation. Foetal goat tongue (ZZ-R CCLV-RIE127) (Brehm et al., 2009) or BHK-21 cells were subsequently used to passage the viruses up to six times or until > 90% CPE was observed. The mutated vSAT1<sup>Q2093W</sup> and vSAT1<sup>D3197A</sup> were confirmed by nucleotide sequencing and used in subsequent experiments.

## 2.4. Virus purification

Infected BHK-21 monolayers were lysed with 0.1% (w/v) Nonidet P40 and 20 mM EDTA (pH 8.0) and clarified by centrifugation at 2000 g for 30 min at 4 °C. Viruses were concentrated using 8% PEG-8000 (Sigma-Aldrich) prior to being purified on 10–50% (w/v) sucrose density gradients (SDG), prepared in TNE buffer (50 mM Tris [pH 7.4], 10 mM EDTA, 150 mM NaCl), as described by Knipe et al. (1997). Following fractionation, peak fractions corresponding to 146S virion particles were calculated using the extinction coefficient E<sub>259</sub> nm = 79.9 (Doel and Mowat, 1985) and pooled for analysis.

## 2.5. Stability thermal release assay

The particle stability thermal release assay (Walter et al., 2012) was performed in 96-well PCR plates using an ABI 7500 PCR machine. All assays were performed using 7.5 µl consisting of 300–500 ng of sucrose density gradient purified virus, and 5 µl of 100 × SYBR green-II dye (Molecular Probes, Invitrogen; diluted 1:100). Additionally, for the

preparation of the 0.8 × PBS reaction, which is for the temperature assay, 37.5 µl of 1 × PBS buffer was added to a final volume of 50 µl. The temperature was ramped from 15 °C to 95 °C in 0.5 °C increments with intervals of 10 s. SYBR green-II fluorescence was read with excitation and emission wavelengths of 490 nm and 516 nm, respectively. The release of RNA and hence the dissociation of capsids was detected by an increase in fluorescence signal and the melting temperature was taken as the minimum of the negative first derivative of the fluorescence curve. The thermostable A-serotype, A24 Cruzeiro virus was used as a positive control.

Ionic buffers were tested at a final concentration of 1 M and 0.5 M NaCl, KCl, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, MgCl<sub>2</sub> and CaCl<sub>2</sub>, 5% glycine, VGM, 30% sucrose. The 50 µl reactions were set up using SYBR green-II dye and virus concentrations as described above, made up to 25 µl with water, with 25 µl of the respective 2 × test buffer added (2 M and 1 M NaCl, KCl, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, MgCl<sub>2</sub> and CaCl<sub>2</sub>, 10% glycine, VGM, 60% sucrose). The 1 × PBS control was made as above using 25 µl of 2 × PBS solution.

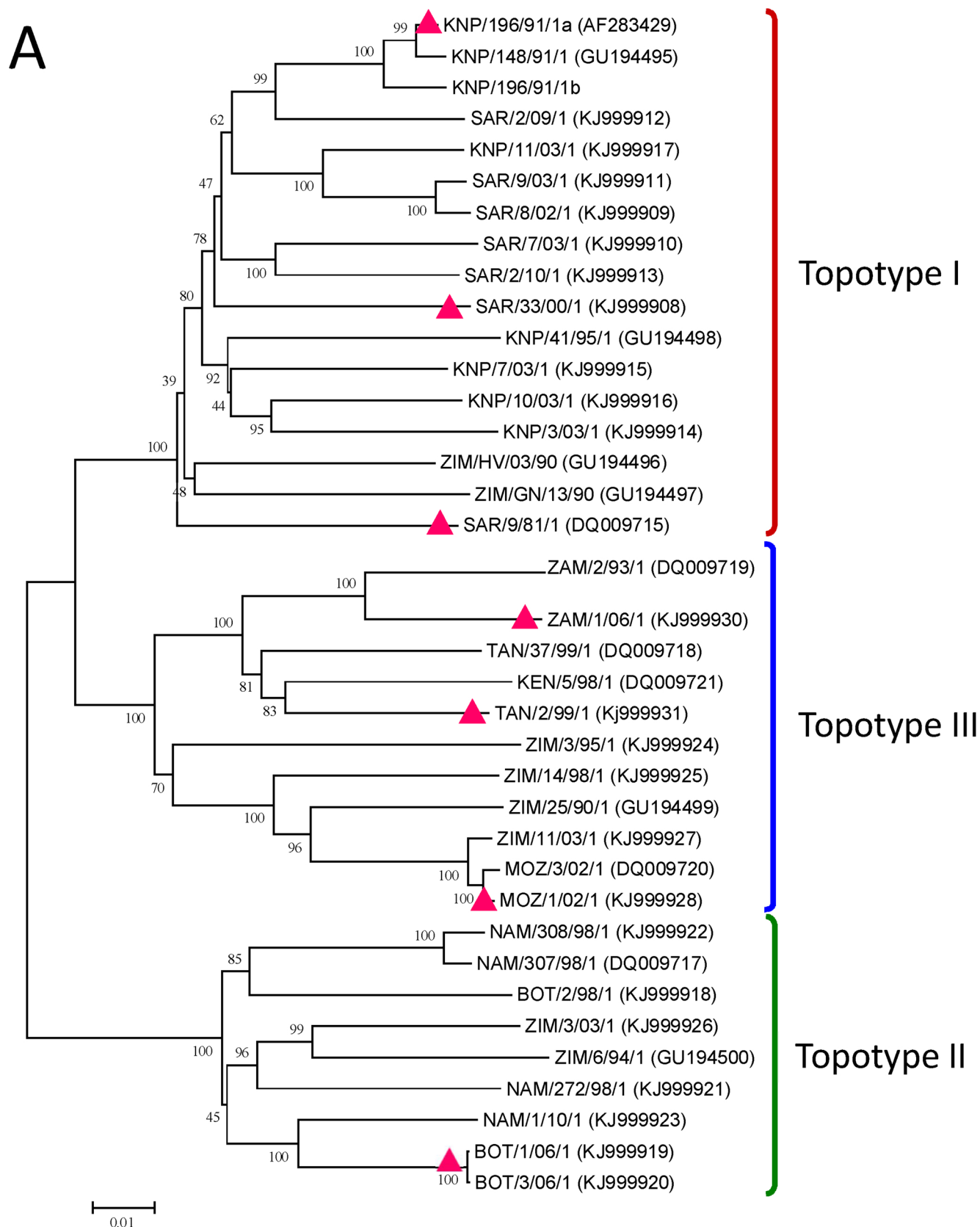
The pH buffers were TNE buffers (Tris pKa = 8.0 at 25 °C) ranging from pH 6.7, 7.2, 7.6, 8.2 and 9.1 (+/-0.02). For pH of 6.1, 2-(N-morpholino) ethanesulfonic acid (MES) buffer (MES pKa = 6.1 at 25 °C) was used. The 50 µl reactions were set up using 7.5 µl purified virus and 5 µl of 100 × SYBR green-II dye as described above, with the 50 µl volume made up with the respective pH test buffer.

The samples were performed in replicate and the mean and standard deviation calculated. The percentage coefficient of variance (%CV) for each virus and treatment was calculated: %CV = (SD/mean) x100. The average %CV was calculated for each virus and treatment. Statistical analyses (one-way ANOVA followed by a Dunnett multiple comparisons test with control) was performed on selected data points to establish significant findings.

## 3. Results

### 3.1. Phylogenetic relationship and variability of the capsid-coding region

Three midpoint rooted Minimum Evolution (ME) trees were compiled using the P1 coding sequences (Fig. 1) of each serotype. Phylogeny was supported by strong bootstrap values and confirmed the major virus lineages, which have evolved separately in each serotype. A cut-off value of 18% nucleotide differences has been calculated and applied to define the toptype clusters in the SAT1 tree. For SAT1 viruses belonging to the southern African geography, at least three



**Fig. 1.** Dendrograms (Minimum Evolution tree) of the P1 sequences of FMDV: (A) SAT1 (topotypes I-III); (B) SAT2 (topotypes I-III); and (C) SAT3 (topotypes I-V) viruses from southern Africa depicted in geographical topotypes. Viruses selected for stability analyses are shown with pink triangles.

B

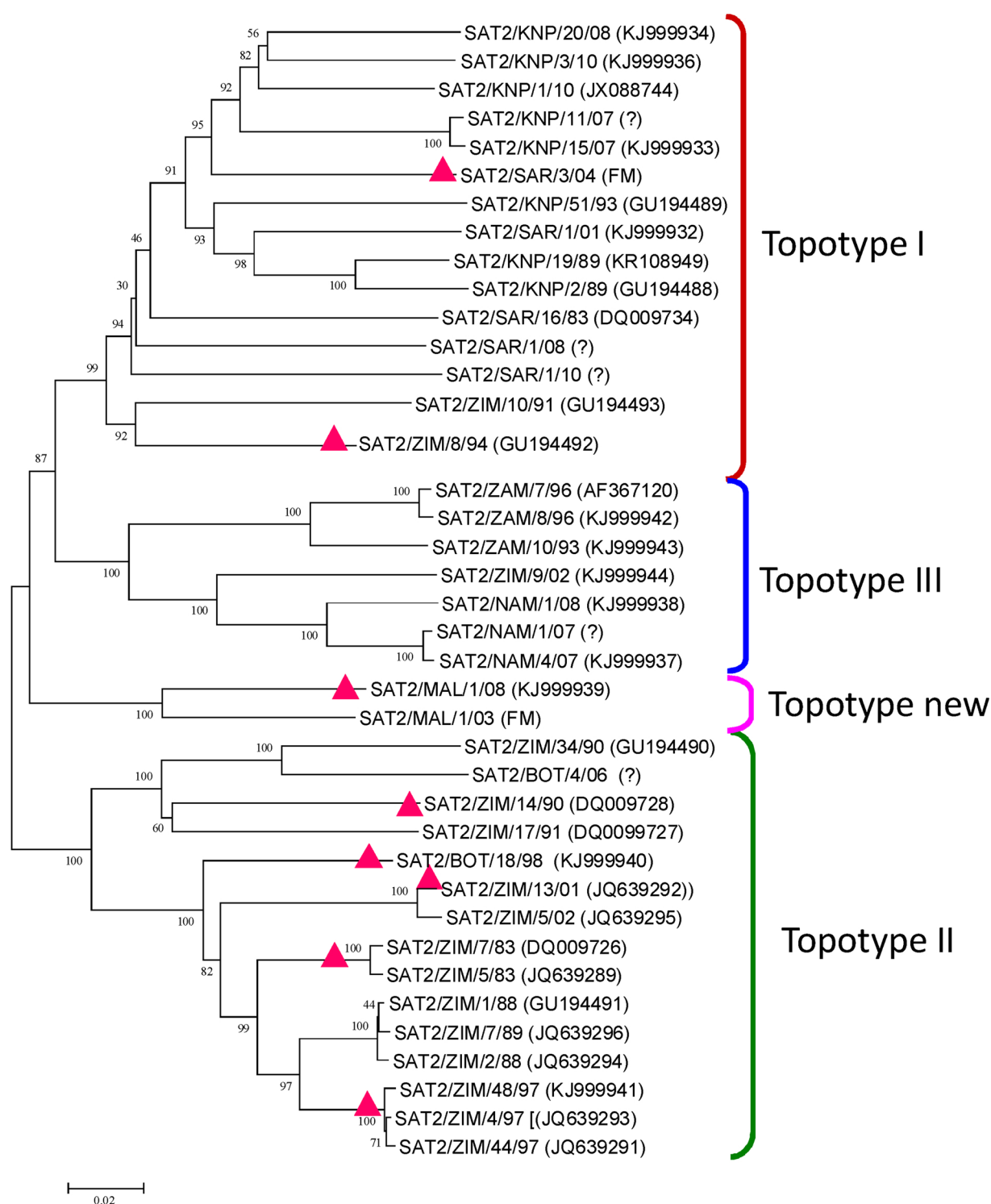


Fig. 1. (continued)

lineages could be resolved, i.e. a southern cluster (I), including viruses from South Africa and Southeast Zimbabwe; a western cluster (II), including viruses from Botswana and Namibia; and a northern cluster (III), including viruses from northern Zimbabwe, Zambia and Mozambique. The lineages corresponded to distinct geographic regions, consistent with the toptype concept (Knowles and Samuel, 2003; Bastos et al., 2001).

Phylogenetic resolution of the P1-coding sequences of 40 SAT2 viruses from southern Africa revealed three toptypes with high bootstrap support (> 86%) and more than 16.4% nucleotide divergence

between them. Topotype I included viruses from South Africa, south-east Zimbabwe and southern Mozambique, whilst viruses from western Zimbabwe and Botswana grouped into toptype II. Viruses that originated from northern parts of Namibia, northern Zimbabwe and Zambia were distinctly different and form part of toptype III. The two Malawian isolates from 2003 and 2008 were distinctly different with more than 16.5% nucleotide differences in pairwise alignments with other SAT2 viruses and warrant placement in a separate toptype, based on P1 phylogeny. Previously the partial VP1 sequences of the SAT2/MAL/1/03 and SAT2/MAL/1/08 viruses grouped with the



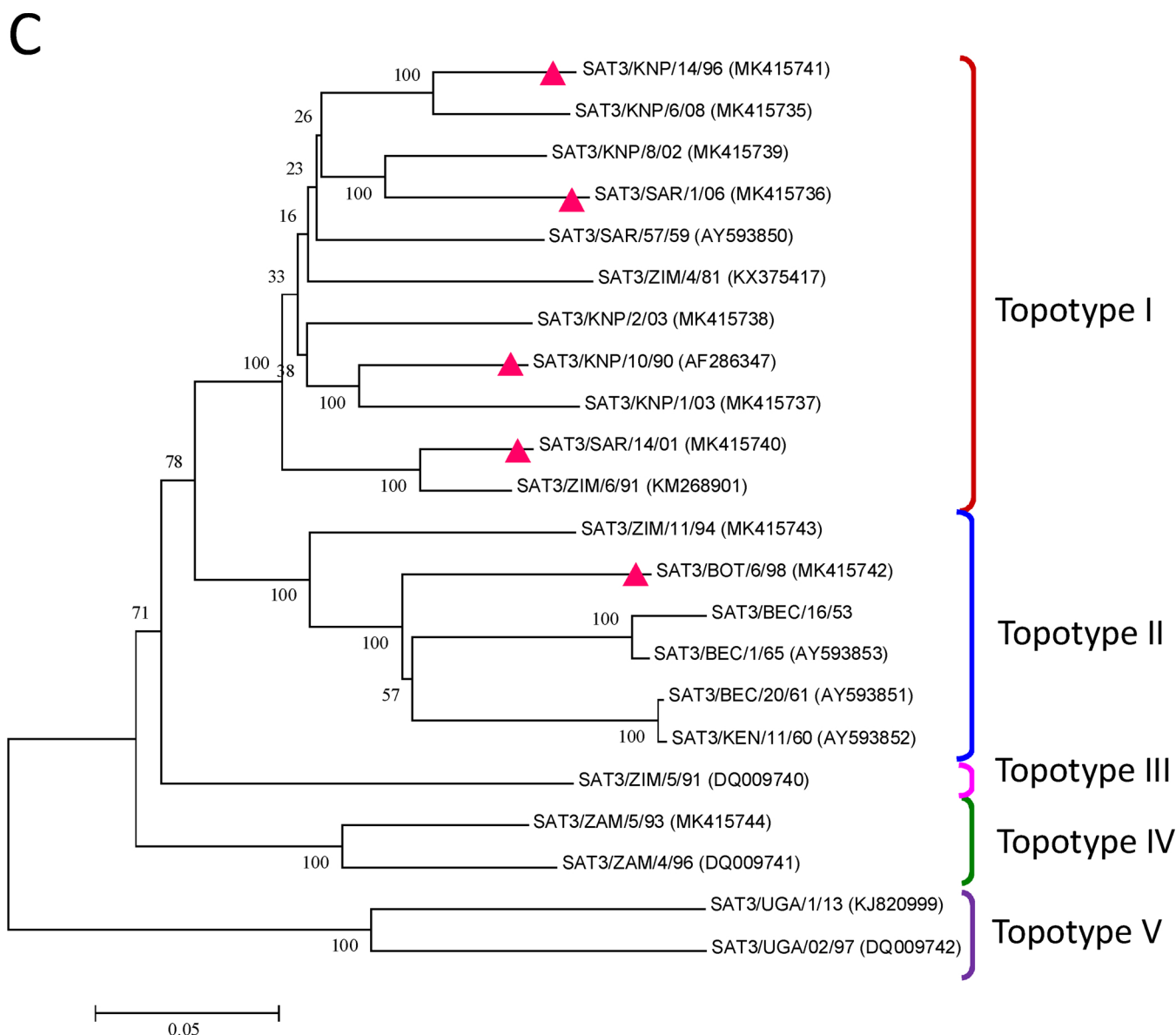


Fig. 1. (continued)

topotype I of SAT2 viruses (Jori and Etter, 2018).

Phylogeny of SAT3 viruses ( $n = 21$ ) revealed four major lineages: (I) viruses from South Africa, and southeast Zimbabwe; (II) includes West Zimbabwe, Botswana and Namibia; (III) includes North-West Zimbabwe and Malawi; and (IV) includes Zambia. Viruses from Uganda (East Africa) clustered in topotype V.

The representatives of the SAT viruses that were selected for thermofluor analysis are summarized in Table 1 and highlighted in the trees depicted in Fig. 1A–C. Also included in the thermofluor analysis was a virus derived from an infectious genome-length clone of SAT1/SAR/9/81 (vSAT1; Maree et al., 2011b). There were no viruses selected from SAT2 topotype III and SAT3 topotypes III–V, as access to these viruses were restricted and/or their purification concentrations were too low for thermofluor analysis.

### 3.2. The effect of biophysical factors on capsid dissociation

Fluorescent thermal shift stability assays (Walter et al., 2012) were performed in triplicate on SDG-purified SAT1, SAT2 and SAT3 viruses. Thermostability was assessed by using only SDG-purified virus in the reaction with  $0.8 \times$  PBS (Fig. 2). The average dissociation temperatures

of the SAT1 viruses ( $n = 7$ ) equating to capsid thermostability ranged from 43 to 51 °C, with a significant difference of 8 ° ( $P < 0.05$ ) between the lowest and highest (Fig. 2). However, only two viruses SAT1/BOT/1/06 and SAT1/ZAM/1/06 had a thermostability of 51 °C, whereas the other SAT1 viruses ( $n = 5$ ) had a much lower range of thermostability between 43–46 °C.

The average dissociation temperatures of the SAT2 viruses ( $n = 8$ ) ranged from 48 to 54 °C, with a significant difference of 6 ° ( $P < 0.05$ ) between the lowest and highest (Fig. 2). SAT2 viral capsids from topotype I ( $n = 2$ ) viruses (SAR/3/04; ZIM/8/94) and a Malawian isolate ( $n = 1$ ) (MAL/1/08) had the highest thermostability and dissociated at 54 °C whilst the topotype II viruses ( $n = 5$ ) ranged from 48 to 52 °C.

Similarly, the average dissociation temperatures of the SAT3 viruses ( $n = 5$ ) ranged from 49 to 53 °C, with the SAT3/SAR/14/01 virus having the highest thermostability (Fig. 2). There were no significant difference ( $P < 0.05$ ) in thermostability between the SAT3 topotypes I and II. Overall, SAT2 and SAT3 viruses had a similar range of thermostability, of 48–54 °C with the more stable capsids dissociating at a temperature similar to that of the notoriously thermostable A24 virus used as control in this study. The SDG-purified A24 virus dissociated at 55 °C in a  $0.8 \times$  PBS buffer. However, the SAT1 viruses had a

significantly lower dissociation temperature with many SAT1 viruses unstable at temperatures between 43–46 °C. There was a 12 °C difference between the most unstable SAT virus (SAT1/TAN/2/99) and the A24 control virus.

The variability [standard deviation, mean and coefficient of variance (%CV)] of the dissociation temperatures of samples within and between runs were determined. The mean %CV of the pH data set was 0.51% (0–5.3 % range) (n = 168) (Sup. Fig. 2A) and the mean %CV of the ionic buffer data set was 0.54% (0–5.8% range) (n = 360) (Sup. Fig. 2B). The virus isolate and/or treatment (pH or ionic buffer) was not responsible for variability within the pH or ionic buffer data set (Sup. Fig. 2). Between run variability was assessed using 6 runs of the A24 and ZIM/7/83 virus 0.8x PBS controls, with mean temperature dissociation values of 55 °C ± 0.25 (0.5 %CV) and 48 °C ± 0.36 (0.8 %CV) respectively.

In an attempt to improve the stability of SAT1 capsids, amino acid residues with potentially stabilizing interactions were introduced at the interfaces between pentamers predicted from structural comparisons and *in silico* calculated binding energies (Kotecha et al., 2015). Of the nine single, two triple and two quadruple mutations introduced into the available SAT1/SAR/9/81 genome-length, infectious clone (vSAT1; Maree et al., 2011), only two mutant viruses could be recovered following transfection of *in vitro* synthesised genome-length viral RNA into BHK-21 cells and successive passaging. The two viable mutant viruses, vSAT1<sup>D3197A</sup> and vSAT1<sup>Q2093W</sup>, were subjected to the thermofluor assay described above. The vSAT1<sup>D3197A</sup> mutant showed a 3 °C improvement in thermostability compared to the vSAT1 wild-type, while vSAT1<sup>Q2093W</sup> displayed the same capsid dissociation as vSAT1.

To investigate the effect of pH on capsid stability, purified viruses

were exposed to MBS and Tris buffers with pH ranging from 6.1 to 9.1 (Fig. 2A). The pH stability of the SDG-purified viruses was measured by the difference in dissociation temperature compared to the 1 × PBS control buffer (pH 7.4) (Fig. 2A). At pH 6.1, SAT1 virus capsids (n = 5/7) were more resilient with average dissociation temperatures showing less difference of 9–14 °C to the control, compared to SAT2 (n = 4/8) and SAT3 (n = 3/5) viruses with significantly higher differences of 12–18 °C and 18–26 °C (P < 0.05), respectively or complete dissociation (Fig. 2A). At pH 6.7–7.6, a similar finding was observed with SAT1 viruses more resilient than the SAT2 and SAT3 viruses. The SAT1/MOZ/1/02 virus was the most resilient of the SAT1 viruses at the pH range 6.7–7.2. In the mild alkaline range of pH (8.2–9.1), SAT1 viruses were more resilient with only a slight decrease (0–3 °C) or increase (+1–4 °C) in the capsid dissociation temperatures compared to the 1 × PBS control. The SAT1 KNP/196/91 virus showed a stabilising effect at pH 9.1 with a 4 °C increase. At pH 8.1, all SAT2 and SAT3 viruses showed a decrease (2–6 °C), and SAT2 topotype II viruses were particularly sensitive to pH 9.1, all showing full dissociation or a 5–12 °C decrease in capsid dissociation temperatures. The vSAT1 mutants did not show any improvement in capsid stability across the pH range compared to the vSAT1 wild-type virus. The SAT1 viruses were the most stable viruses (P < 0.05) over the entire pH range. Overall SAT3 viruses were the most unstable at pH 6.1–7.2 (P < 0.05); and the SAT2 viruses the most unstable (P < 0.05) to mild alkali (pH 8.2–9.1) of the SAT viruses when comparing their decrease in capsid dissociation temperature.

To investigate the effect of ionic buffers on capsid stability, 13 ionic buffers or stabilisers were tested, while keeping the pH constant, in comparison to the 1 × PBS control (Fig. 2B). The following summarizes

**A**

Serotype	topotype	virus isolate	1x PBS	6.1	6.7	7.2	7.6	8.2	9.1
SAT1	I	KNP/196/91	49	-13	-9	-4	-5	-2	4
	I	SAR/33/00	46	ds*	-11	-6	-4	-1	-2
	I	SAR/9/81	47	-14	-8	-5	-2	-1	-2
	I	vSAT1	43	ds*	-8	-3	-1	2	0
	I	vSAT1Q2093W	43	ds*	-8	-6	-2	0	-1
	I	vSAT1D3197A	46	ds*	-8	-6	-3	-1	-2
	II	BOT/1/06	51	-9	-8	-4	-4	-2	-3
	III	ZAM/1/06	52	-14	-9	-5	-4	-2	-3
	III	MOZ/1/02	46	-13	-6	-2	-1	2	-1
	III	TAN/2/99	43	ds*	-7	-6	-3	1	0
SAT2	*	MAL/1/08	56	-17	-8	-7	-5	-3	-8
	I	SAR/3/04	56	-12	-8	-7	-5	-3	-6
	I	ZIM/8/94	56	-18	-12	-9	-6	-3	-5
	II	ZIM/14/90	56	ds*	-13	-12	-9	-4	-12
	II	ZIM/4/97	52	ds*	-13	-10	-7	-4	-9
	II	ZIM/13/01	55	ds*	-12	-9	-7	-4	ds*
	II	BOT/18/98	54	ds*	-11	-8	-5	-2	-12
	II	ZIM/7/83	54	-17	-13	-9	-8	-6	-10
SAT3	I	KNP/10/90	54	ds*	-16	-11	-6	-2	-1
	I	SAR/14/01	58	-26	-14	-11	-7	-3	-4
	I	SAR/1/06	54	-18	-17	-11	-7	-3	-4
	I	KNP/14/96	55	ds*	-20	-15	-11	-6	-7
	II	BOT/6/98	54	-22	-15	-11	-7	-3	-3
A	control	A24 control	58	-20	-15	-8	-8	-6	-9

**Fig. 2.** Fluorescence thermal shift assay (Walter et al., 2012) to determine the dissociation temperatures of SAT viruses. The A24 virus was used as a representative control of an A-serotype virus. Heatmaps are depicted for A and B, showing the average dissociation temperatures of triplicate repeats. The colouring of green is indicative of more stable, whilst red is more unstable. Values depict the difference between the dissociation temperature of the experimental condition (i.e. pH or buffer) to the 1 × PBS controlled condition. A) pH stability tested with a range of pH buffers 6.1–9.1; and B) ionic stability of 13 different buffers. The control represents the dissociation using the standard protocol with a final concentration of 0.8 × PBS where thermostability is assessed. ds\* denotes where viruses were fully dissociated under the experimental condition and no reading was obtained.

## B

Serotype	topotype	virus isolate	NaCl		KCl		MgCl <sub>2</sub>		CaCl <sub>2</sub>		(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>		5% Glycine	VGM	30% sucrose	1xPBS	control
			1M	0.5M	1M	0.5M	1M	0.5M	1M	0.5M	1M	0.5M					
SAT1	I	KNP/196/91	6	1	1	0	-6	-7	-13	-7	-5	-7	-4	0	-3	49	46
	I	SAR/33/00	6	2	1	-1	-8	-2	-7	-3	-5	-6	-9	2	-4	46	44
	I	SAR/9/81	5	2	0	0	-5	-4	-10	-6	-5	-7	-5	2	-3	47	46
	I	vSAT1	4	2	3	0	-11	-9	-11	-3	-7	-8	-2	3	-8	43	nd
	I	vSAT1Q2093W	7	3	1	0	-7	-4	-9	-4	-10	-9	-5	5	-7	43	nd
	I	vSAT1D3197A	5	3	2	1	-3	0	-11	-9	-7	-6	-7	-1	-4	46	nd
	II	BOT/1/06	2	-1	-3	-3	-1	-2	ds*	ds*	0	-3	-6	-1	-2	51	51
	III	ZAM/1/06	5	3	0	-1	-4	-6	-13	-12	-6	-7	-6	0	-5	52	51
	III	MOZ/1/02	4	2	3	0	-13	ds*	-12	-11	-2	-4	-3	-1	-3	46	46
SAT2	I	TAN/2/99	9	3	5	1	-2	-3	-3	1	-4	-7	-6	4	-4	43	43
	*	MAL/1/08	3	1	-2	-3	-15	-10	-11	-1	-5	-8	-15	-5	-6	56	54
	I	SAR/3/04	2	1	0	-2	-16	-11	ds*	ds*	-2	-5	-7	-5	1	56	54
	I	ZIM/8/94	3	1	0	-2	ds*	ds*	ds*	ds*	-3	-6	-11	-6	-1	56	54
	II	ZIM/14/90	6	3	2	-2	-12	-10	ds*	0	0	-4	-14	-8	-7	52	50
	II	ZIM/4/97	3	0	-1	-4	-14	-8	-11	3	-2	-7	-22	-7	-9	56	52
	II	ZIM/13/01	3	1	0	-2	ds*	ds*	ds*	ds*	-5	-8	-10	ds*	-1	55	52
	II	BOT/18/98	4	2	-2	-3	-16	-11	-12	1	-3	-7	-15	-5	-5	54	52
	II	ZIM/7/83	2	-2	-1	-4	ds*	ds*	-9	-6	-11	-15	-1	-3	-7	54	48
SAT3	I	KNP/10/90	-1	-1	0	-3	ds*	ds*	ds*	ds*	-2	-3	-6	1	-8	54	51
	I	SAR/14/01	1	0	1	-1	-10	-9	ds*	-20	-1	-9	-6	-4	-5	58	53
	I	SAR/1/06	0	0	1	-1	ds*	ds*	ds*	ds*	-2	-5	-4	-5	-5	54	50
	I	KNP/14/96	3	0	2	0	ds*	ds*	ds*	ds*	-2	-5	-9	0	-10	55	51
	II	BOT/6/98	2	0	2	0	-11	-10	ds*	-21	0	-5	-6	-6	-9	54	49
A	control	A24 control	2	1	0	-1	1	-1	-3	-4	-2	-3	-6	-3	0	58	55

Fig. 2. (continued)

the trend of the buffers supporting the highest to the lowest stability as assessed by dissociation temperatures. The 1 M and 0.5 M NaCl buffers supported the highest increase in capsid thermostability. The SAT1 and SAT2 viruses had an increased stability of 2–9 °C and 2–6 °C respectively, with the addition of 1 M NaCl. The stability of the SAT1/TAN/2/99, SAT1/KNP/196/91, SAT1/SAR/33/00 viruses and the SAT2/ZIM14/90 virus were greatly improved by 6–9 °C ( $P < 0.05$ ) with addition of 1 M NaCl. The vSAT1<sup>Q2093W</sup> mutant was 3 °C more stable than the vSAT1 wild-type virus at 1 M NaCl. This is in contrast to the SAT3 viruses, which did not show significantly improved stabilisation (0–3 °C) after addition of 1 M and 0.5 M NaCl buffers.

The 1 M and 0.5 M KCl buffers supported little or no improvement (0–2 °C) in capsid stability compared to the control for most of the SAT viruses, with the exception of SAT1/TAN/2/99 and SAT1/MOZ/1/02 viruses, which had a 5 °C and 3 °C improvement respectively with 1 M KCl.

The 1 M and 0.5 M MgCl<sub>2</sub> buffers effected destabilisation of the SAT2, SAT3 and SAT1/MOZ/1/02 viruses by being fully dissociated ( $n = 6/14$ ) whilst other viruses ( $n = 8/14$ ) had a decrease of 8–16 °C in capsid dissociation temperatures. The majority of SAT1 viruses ( $n = 6/7$ ) were less affected with only a 1–8 °C decrease in stability. The stability improved with the mutations to the vSAT1<sup>D3197A</sup> virus by 8–9 °C and to the vSAT1<sup>Q2093W</sup> virus by 4–5 °C compared to the wild-type vSAT1 virus in the presence of 1 M or 0.5 M MgCl<sub>2</sub>.

The 1 M and 0.5 M CaCl<sub>2</sub> buffers effected the greatest decrease in capsid dissociation temperatures with the higher concentration of 1 M having a more drastic destabilising effect of 7–13 °C ( $n = 10/20$ ) or complete dissociation ( $n = 10/20$ ) than 0.5 M. The SAT3 viruses were most destabilised of the SAT viruses by CaCl<sub>2</sub> buffers showing complete capsid dissociation or a 20 °C decrease. The SAT1/TAN/2/99 and A24 viruses were the least affected by 1 M CaCl<sub>2</sub>. At least one SAT1 and four SAT2 viruses were minimally affected by the 0.5 M CaCl<sub>2</sub>. Interestingly, the SAT1 BOT/1/06 virus showed remarkable resilience to the panel of ionic buffers tested with neither stabilizing nor destabilising effects observed, except in CaCl<sub>2</sub>.

The 0.5 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> buffer effected a greater decrease in stability than the 1 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> buffer with the SAT2/ZIM/7/83 virus being least stable.

The addition of 30% sucrose made viruses less stable except for SAT2/SAR/3/04, which was unaffected. The stability improved with the mutation to the vSAT1<sup>D3197A</sup> virus by 4 °C compared to the wild-type vSAT1 virus in the presence of 30% sucrose.

VGM effected a slight increase in stability with SAT1 viruses

(0–4 °C) however; SAT2 and SAT3 viruses were much less stable decreasing by 3–8 °C.

### 3.3. Amino acid variation at the inter-pentamer interfaces

Amino acid residues located at the inter-pentamer interface (or having interaction with the interface) identified in previous studies as having a role in improving FMDV biophysical stability are listed (Table 2) (Twomey et al., 1995; Mateo et al., 2003, 2008; Fowler et al., 2010; Martin-Acebes et al., 2011; Porta et al., 2013; Kotecha et al., 2015; Scott et al., 2017). The residues at these positions were compared to the corresponding residues of the field SAT1, SAT2 and SAT3 virus capsid protein sequences from this study (Table 1) using the A24, A12, A22 viruses as representatives from the A-serotype and the C-S8c1 virus from C-serotype as references from previous studies. This was done to link possible differences observed at amino acid residue positions predicted to be involved in improved stability with changes in metastability (defined as the cumulative effect of stabilizing and destabilizing forces at the pentamer interfaces). The 27 amino acid residues previously identified in stabilization were compared: 6 (VP2: 34; 62; 88; 94; 110; 188) were conserved (according to type of amino acid: charged positive or negative; Polar; Non-polar) amongst the different serotypes; 13 (VP1: 3; VP2: 34; 62; 87; 88; 94; 110; 114; 188; and VP3: 69; 143; 193; 194) were conserved within the SAT serotypes only; and 14 (VP1: 17; VP2: 65; 78; 79; 80; 93; 98; 131; 133; 193; 215; and VP3: 85; 196) were variable amongst the different serotypes. Of the 13 amino acid residues conserved within the SAT serotypes, 6 of these residues are considered to be destabilizing compared to the more stable A-serotype virus residue options.

## 4. Discussion

Previously the focus was on modelling and mutating residues responsible for improving FMDV stability (Kotecha et al., 2015; Scott et al., 2017), however information on the biological range of stability of FMDV within a serotype and between topotypes of different geographical regions is inadequate especially for field SAT viruses. This prompted the determination of the biological variance in capsid stability of SAT viruses. Previous studies were centered on limited isolates of SAT viruses and only those that were available as vaccine master seed viruses with the purpose of improving stability and to derive conclusions about the stability of the SAT serotypes as a whole (Doel and Baccarini, 1981; Kotecha et al., 2016). SAT viruses exhibit different



**Table 2**

Amino acid residues at the interface (VP2 and VP3 proteins) or associated VP1 interaction with the interface of SAT viruses.

Residues	Interaction	amino acid position reference	SAT1	SAT2	SAT3	A24; A12; A22	C-S8c1
A1003S		A12 <sup>#</sup>	S	S/T	S	A	
N1017D	H3140; H3143	C-S8c1 <sup>a</sup>	Q/E	T	T	N	N
F2034L		C-S8c1 <sup>a</sup>	Y	Y	H/Y	H/Y	F
F2062Y	H2087 M/V; H3143V	SAT2 <sup>+</sup>	F	F	F	F/Y	F
A2065H		C-S8c1 <sup>b</sup>	K	K	R/K	Y/H/F	A
L2078S		A <sup>\$</sup>	T	M/L	V	L	M
E2079A		A <sup>\$</sup>	H	Y	H	E	H
K2080R		A <sup>\$</sup>	I/V	I/V	L	K	K
H2087 M/V	F2062Y; H3143V	SAT2 <sup>+</sup>	H	H	H	H	P
T2088A		A <sup>\$</sup>	H/K	K	K	H/K	K
S2093 H/W/Y/F/Q/C*		SAT2 <sup>+</sup> A22*	Q	S	A	H	G
L2094V		SAT2 <sup>+</sup>	L	L	M	L	L
Y2098 F/H	alone or in combination with F2062Y; H2087 V; H3143V	SAT2 <sup>+</sup>	H	Y	H	Y/F	Y
S2110T		A <sup>\$</sup>	S	S/T	S	S	T
T2114N		SAT2 <sup>+</sup>	T	T	T	N	N
E2131K		A12 <sup>#</sup>	K	S	T/S/K	E	D
D2133S		A12 <sup>#</sup>	A/S/D/T	R/K	D	D/T	S
T2188A	D3069E	C-S8c1 <sup>b</sup>	T	T	T	T	T
A2193S		A <sup>\$</sup>	T	Q	N	S	
K2215L		SAT2 <sup>+</sup>	K	K/M	K/M/R	L	L
D3009V		C-S8c1 <sup>a</sup>	A/D/V	A/D	D	D	D
D3069E	T2188A	C-S8c1 <sup>b</sup>	S	–	S	D	D
H3085P		A <sup>\$</sup>	N	V	S	H	H
H3143V	F2062Y; H2087 M/V; Y2098 F/H	SAT2 <sup>+</sup>	H	H	H	V	
D3193H		SAT2 <sup>+</sup>	D	D	D	H	
T3194G		SAT2 <sup>+</sup>	T	T	T	G	
E3196A		A <sup>\$</sup>	Q/E	S	D	A	

+ Kotecha et al., 2015; Scott et al., 2017.

# Twomey et al., 1995.

<sup>a</sup> Martin-Acebes et al., 2011.<sup>b</sup> Mateu et al., 1988.<sup>\$</sup> Fowler et al., 2010.

\* is only applicable for empty capsids (Porta et al., 2013).

phenotypic characteristics such as growth kinetics, cell culture virulence, plaque morphology, cell adaptation, antigenicity, antigen yield and pathogenicity. It is therefore reasonable to expect that there would be a biological range of stability amongst SAT virus populations. Whilst vaccine producers tend to select viruses as vaccine candidates based on good growth and high antigen yield, other important factors such as stability are not always thoroughly assessed before production. This can have negative effects downstream on the efficiency of the vaccine as unstable vaccines may contain dissociated antigen and therefore induce a poor immune response and duration of immunity (Doel and Baccarini, 1981).

To characterise the stability of SAT viruses in southern Africa we used PaSTRy analysis to monitor capsid dissociation by the release of the RNA genome under diverse biophysical conditions (Walter et al., 2012; Kotecha et al., 2015, 2016; Scott et al., 2017). This is considered to date to be the most effective method to assess FMDV capsid stability (Kotecha et al., 2016) as other methodologies such as ELISA-based and comparative infectivity assays are time-consuming, lack suitable antibodies to discriminate 12S vs 146S; and lack the ability to quantify differences. The intrinsic variability of the replicates within and between runs was determined for each data point (virus and treatment) and showed less than 6% CV across the data sets with most of the replicates < 2% CV. Thus, we are confident that the results are reflective of true differences between viruses and treatments and are reliable biological repeats.

A panel of SAT1, SAT2 and SAT3 viruses from different topotypes in southern Africa were selected and their stability studied. There was no representative from the SAT3 topotype III of Southern Africa, as we could not access ZIM/5/81. The capsid thermostability of the SAT1 (n = 7) viruses ranged from 43 to 51 °C, with a significant difference of 8 °C (P < 0.05), whilst the thermostability of the SAT2 (n = 8) viruses ranged from 48 to 54 °C, a significant difference of 6 °C (P < 0.05).

However, only two SAT1 viruses had a high thermostability of 51 °C whereas the two topotype I SAT2 viruses and an Malawian isolate having the highest stability of 54 °C. The vSAT1<sup>D3197A</sup> mutant showed a 3 °C improvement in thermostability compared to the vSAT1 wild-type. Similarly Scott et al. (2017) found the equivalent vSAT2<sup>E3198A</sup> mutant had a 5 °C improvement in thermostability. Overall, SAT3 viruses had a similar range of thermostability compared to SAT2, varying from 49 to 53 °C. Some SAT2 and SAT3 viruses had a similar thermostability to the notoriously thermostable A-serotype A24 control virus of 55 °C. It has been widely accepted that SAT serotype viruses are unstable (Doel and Baccarini, 1981) and this confirms for the first time that more stable SAT2 and SAT3 viruses are naturally present from the southern Africa region. This could facilitate the selection of more stable vaccine master seed viruses or the use of such virus sequences in structural vaccine design. Additionally field viruses that prove to be more unstable but are ideal in other traits important for vaccine viruses (broad antigenicity, growth, antigen yield etc.) could be mutated to include residues responsible for improved stability (Kotecha et al., 2015; Scott et al., 2017). The selection of naturally more stable viruses, which have not been manipulated *in vitro*, could result in the maintenance of genetic stability through laboratory adaptation and upscale processes without the threat of reversion to less stable variants as found previously (Scott et al., 2017).

In a complete alignment of the outer capsid proteins, the SAT1 viruses displayed 18.2% variable amino acid positions, however the variation was not random but located in discrete regions of variability. Similarly, SAT2 and SAT3 viruses showed 15.6% and 13.8% variable residues in the outer capsid proteins, respectively. Mateo et al. (2003) showed that the majority of residues in intersubunit interfaces were critically important for the completion of the virus life cycle. The loss of even one interpentamer contact with a small binding energy may cause a significant change in the stability of an icosahedral virus capsid, like

FMDV, because of at least 60 protomers involved (Zlotnick, 1994). We have shown that mutation of S2093Y improved the capsid thermostability of a SAT2 virus significantly (Kotecha et al., 2015; Scott et al., 2017). SAT1 viruses naturally contained a Gln residue at VP2 position 93, a residue that has been predicted by *in silico* modelling to increase capsid stability. At VP2 position 98, SAT1 and SAT3 viruses, display a His residue previously associated with improved capsid thermostability (Scott et al., 2017). SAT1 viruses were shown in this study to be more stable than SAT2 or SAT3 viruses, possibly due to these amino acid differences. It is unknown at this stage which amino acid residues are critical for stability or what their cumulative effect on metastability would be. We compared the amino acid residues of SAT field viruses in this study with known substitutions that improve stability to determine any correlation to naturally occurring stable SAT viruses. However, it is difficult to predict changes in stability based on an amino acid sequence alone without assessing biologically or generating mutants.

The pH stability of the viruses were compared and SAT1 viruses were found to be more resistant to mild acidic or alkali changes in the pH range of 6.1–9.1, compared to SAT2 and SAT3 viruses. The SAT2 toptotype II viruses were particularly labile at pH 9.1; and the vSAT1 and SAT1/MOZ/1/02 viruses were the most resistant at the pH range 6.7–9.1 compared to other SAT viruses. Overall, the toptotype II SAT2 and SAT3 viruses were the most unstable when comparing the decrease in dissociation temperature over the pH range 6.1–9.1. This highlights the need to be cautious when preparing and storing SAT viruses in certain buffers, which could lead to decreased quantity and quality of 146S particles for vaccine formulation.

In this study the TNE buffer consisting of Tris-HCl was used for testing since it is the same pH buffer used to purify FMD vaccine antigen during production, it however exhibits a large shift in dissociation or pH with a change in temperature equivalent to  $\Delta pK_a/10^\circ\text{C} = -0.31$ . Since the PaSTRy assay relies on  $0.5^\circ\text{C}$  increases in temperature per cycle, a non-linear shift in the pH is expected. It is however important to assess this buffer for its stability across the pH 7–9 range, since it is widely used in production. In the future alternative pH buffers would need to be investigated to draw conclusions on effect of  $\Delta pK_a$  using this temperature assay.

The addition of ionic buffers showed that 1 M NaCl was capable of enhancing stability of the SAT1 and SAT2 viruses by  $2\text{--}9^\circ\text{C}$  confirming the findings by Kotecha et al. (2016). Of particular interest was the significant increase in stability of  $6\text{--}9^\circ\text{C}$  ( $P < 0.05$ ) with addition of 1 M NaCl of the SAT1 viruses TAN/2/99, KNP/196/91, SAR/33/00 and SAT2/ZIM14/90. This is in contrast to the SAT3 viruses, which did not show significant improved stabilisation ( $0\text{--}3^\circ\text{C}$ ) after addition of 1 M and 0.5 M NaCl buffers. This important finding reveals that even within a serotype or toptotype there are differences in inherent stability and the effects afforded by different ionic or pH conditions. Protein-protein interactions, such as those mediating subunit assembly and hence governing capsid stability of the interfaces, are made up of hydrophobic and electrostatic interactions, and hydrogen bonds. In the same way that salts can have either a destabilising effect (chaotropes) or stabilising effect (kosmotropes) on protein structures, they can also modulate protein-protein interactions in various ways depending on the type of salt and its concentration in the buffer environment. Some buffers showed differing results dependent on the virus tested, highlighting the need to test each SAT virus beforehand with different buffers, to establish the most stabilising option before production or storage. Unlike results from a previous study which showed the SAT3/KNP/1/08 virus (unknown passage history) to have increased stability with 30% sucrose (Kotecha et al., 2016), the panel of SAT3 viruses in this study were not stable under this condition.

It must be noted that there is probably a maximum threshold in which a virus can be stabilized through changes to ionic conditions or through mutating residues (Scott et al., 2017) before it is biologically not viable anymore. It was apparent in this study that inherently stable viruses could not be dramatically improved by the addition of

stabilizing ionic buffers and had potentially reached a maximum biological threshold which did not exceed  $60^\circ\text{C}$ .

The thermal shift assay (Walter et al., 2012) is a valuable tool in assessing stability (temperature, pH and salt conditions), for vaccine production systems to assess stability, quality and shelf-life of antigens, effects of storage buffers as also described in Kotecha et al. (2016). Additionally, in Africa, for use in screening viruses as potential vaccine strains and for governments or producers to monitor the quality of the vaccine from production to the field.

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## Appendix A. Supplementary data

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