Improved stability of foot-and-mouth disease virus (FMDV) SAT2 capsid

By

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DECLARATION

I, Katherine Anne Scott hereby declare that this dissertation, except where indicated by a reference, is my own original research and has not been submitted, in part or as a whole, for a degree at any other university.

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The Lord, for His many blessings and for never leaving my side during my challenges.

Dedicated in loving memory of

My dearest father and best friend, Peter James Stewart,

who inspired me throughout my life to attain this PhD degree.

(1946-2009)

My dearly beloved mother Susan Virginia Stewart (nee Abbotts)

her strength, inspiration and love have been present with me every day

(1949-1986)

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LIST OF ABBREVIATIONS

°C	degrees celsius
A	adenine
Ad	adenovirus
ARC	Agricultural Research Council
bp	base pair
BSA	bovine serum albumin
C	cytosine
cDNA	complementary deoxyribose nucleic acid
DNA	deoxyribonucleic acid
dNTP	deoxynucleotriphosphate
ELISA	enzyme-linked immunosorbent assay
FAO	Food and Agriculture Organisation
Fig	figure
FMD	foot-and-mouth disease
FMDV	foot-and-mouth disease virus
G	guanidine
gRNA	genomic ribose nucleic acid
IFN	interferon
IgG	immunoglobulin G
IgM	Immunoglobulin M
IL	interleukin
kDa	kilodalton
LPBE	liquid phase blocking ELISA
M	molar

MAb	monoclonal antibody
MD	molecular dynamics
mg	milligram
$MgCl_2$	magnesium chloride
ml	millilitre
mM	millimolar
mol	mole
MW	molecular weight
N/A	not applicable
NCBI	National Centre for Biotechnology Information
ND	not done
NJ	neighbour-joining
NS	no significance
NSP	non-structural protein
nt	nucleotide
NTR	non translatable region
OD	optical density
OIE	Office International des Epizooties (World organisation for animal health)
P1	polyprotein 1 (leader)
PAGE	polyacrylamide gel electrophoresis
PCP	progressive control pathway
PCR	polymerase chain reaction
PD50	dose/vaccine that protects 50% of animals challenged
RdRp	RNA dependent RNA polymerase
RNA	ribonucleic Acid

RSA	Republic of South Africa
RT	reverse transcription
S	sedimentation rate
SADC	Southern African Development Community
SAT	Southern African Territories
SPCE	solid phase competition ELISA
Sup	supplementary
T	thymine
Taq	taq polymerase
Tm	melting temperature
U	units
UP	University of Pretoria
µg	microgram
µl	microlitre
µM	micro molar
UTR	untranslatable region
UV	ultra-violet
VNT	virus neutralisation assay
VP	virus protein
vRNA	viral ribonucleic acid

THESIS SUMMARY

Improved stability of the foot-and-mouth disease virus (FMDV) SAT2 capsid

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Foot-and-mouth disease virus (FMDV) is notoriously unstable, particularly the O and SAT serotypes. Consequently, vaccines derived from heat-labile SAT viruses have been linked to the induction of poor duration of immunity (Doel and Baccarini, 1981) and hence require more frequent vaccinations to ensure protection. Virus capsids are primed for disassembly, yet capsid integrity is key to generating a protective immune response. FMDV capsids comprise identical pentameric protein subunits held together by tenuous noncovalent interactions and are often unstable. Oxford University devised a computational method to assess the relative stability of protein-protein interfaces and used it to design improved candidate vaccines for two poorly stable, but globally important, serotypes of FMDV i.e. O and SAT2. A restrained molecular dynamics strategy was used to rank mutations predicted to strengthen the pentameric interfaces and we applied the results to produce stabilized SAT2 capsids.

We assessed the stability of 18 recombinant mutant SAT2 viruses for their growth kinetics; antigenicity; plaque morphology; genetic stability and biophysical stability using a novel Pastry thermofluor and inactivation assay, to evaluate potential SAT2 vaccine seed viruses with improved stability. The most stable mutation was the single mutant S93Y in VP2 for temperature and pH stability, whilst other promising single mutants were E198A, L94V, S93H and the triple mutant F62Y-H87M-H143V. Although the S93Y mutant had the greatest stability it exhibited smaller plaques; a reduced growth rate; a change in a monoclonal antibody footprint, and poor genetic stability properties compared to the wild-type virus. However, these factors affecting production can be overcome. Vaccinated guinea pigs generated improved neutralizing-antibody responses to stabilized particles compared to parental viruses and wild-type capsids.

The S93Y and S93H mutants were selected for testing in a cattle vaccine trial. We investigated the efficacy and comparative immunological responses of the two thermostable and a wild-type SAT2 vaccine over 5 months, followed by challenge. We assessed humoral immune responses elicited in cattle in terms of total and neutralising

antibodies and IgG1/2 isotyping; and cell-mediated responses of IFN-ɣ as *in-vitro* markers of protection. Whilst there were significant differences in total and neutralizing antibodies for the vSAT2-93H group compared to other vaccinated groups after the first vaccination, there were no significant differences after the second immunization. Following intra-dermolingual challenge, all vaccinated groups were fully protected as determined by the absence of generalized lesions. These results provide proof that two vaccine doses, consisting of SAT2 antigen combined with ISA206B adjuvant, administered 4-6 weeks apart were able to protect animals up to 5 months post vaccination (pv). Additionally, vSAT2-93Y had significantly higher levels of IFN-γ after challenge and a lower clinical score indicative of better protection compared to other vaccinated groups and suggest the importance of cell mediated responses and antigen stability in protection.

To characterise the stability properties of different wild-type SAT2 viruses we used thermofluor Pastry analysis to monitor capsid dissociation by the release of the RNA genome under a range of temperature, pH and ionic conditions. The dissociation temperatures of 8 SAT2 viruses, belonging to two different topotypes, ranged from 48-54 °C, a difference of 6 degrees. Interestingly, some of these SAT2 viruses had very similar dissociation temperatures to the highly stable A24 control virus. The addition of ionic buffers showed that 1 M NaCl was capable of enhancing stability of the SAT2 viruses by a further 6-8 °C, whereas other solutions showed differing results dependent on the virus tested, highlighting the need to test SAT2 viruses before storage with different solutions to establish the most stabilising option. This confirms, for the first time, that more stable SAT2 viruses are present in the field from the Southern African region. This method could facilitate the selection of the most stable circulating field strains, as well as the comparison of such strains with their recombinant counterparts in vaccine design. The selection of naturally more stable viruses, which have not been manipulated *in-vitro,* could result in genetically stable viruses through laboratory adaptation and upscaled processes.

AIMS OF STUDY

The thermostability of vaccines is of crucial importance in Africa, where the logistical process to get the vaccine from the manufacturer to the animal may take months, and in many remote regions transport and storage is in the absence of a cold-chain. Vaccines with improved stability and less reliance on a cold-chain are needed and could improve the longevity of immune responses elicited in vaccinated animals. In South Africa, cattle in the vaccination zone neighbouring the Kruger National Park have to be vaccinated thrice annually because of declining antibody responses at three months post-vaccination. FMDV is known to be unstable, especially for O and SAT2 serotypes in mildly acidic pH conditions or at elevated temperatures, leading to dissociation of the capsid (146S particle) and loss of immunogenicity. The link between rapidly declining antibody responses and capsid stability have been reported by Doel and Baccarini, 1981. We hypothesized that more stable viruses, especially thermostability, will not only improve the protective immune response in animals but also require less frequent booster vaccinations.

The residues at the capsid inter-pentamer interfaces, and their interactions, are important for the infectivity and stability of the virion and mutations adjacent to these interfaces have an effect on the conformational stability of FMDV. However, experimental studies on the relative importance of residues and molecular interactions in viral capsid assembly, disassembly, and/or stability are still very limited, especially for the SAT serotypes of FMDV. This study investigated the effects of potential residues at the pentameric interfaces that are responsible for increased thermostability and potentially improved stability candidates were tested in small (guinea pigs) and large (cattle) animal vaccination trials to understand the role of stabilised antigens on immune responses. The biological variation in biophysical stability in SAT2 viruses in the southern Africa region was investigated to determine if any naturally occurring viruses have greater capsid thermostability. Naturally occurring stable viruses could be used as prospective candidates in vaccine production and therefore potentially result in increased duration of immune responses.

Our first aim was to investigate the role of different amino acid changes at the interface and their effect on capsid stability using models derived by Oxford University. These changes were introduced by mutating the SAT2 ZIM7/83 infectious genome-length clone (pSAT2) to derive mutated chimeric SAT2 viruses. We quantified the stabilizing

effects of these mutations by using various stability assays. We established the novel thermofluor shift assay that is able to quantify the capsid stability of viruses. The growth kinetics, antigenicity, genetic stability, pH and salt sensitivity were investigated for each of the genetically engineered viruses (Chapters 2 and 3).

The second aim was to further our understanding on the correlation between improved stability and immune responses by performing small animal (Chapter 2) and large animals trials in cattle (Chapter 4) and comparing stabilised and wild-type antigens. This study for the first time for SAT vaccines, determined differences in IgG1 and IgG2 profiles, interferon gamma (IFN-γ) responses and differences in total and neutralising antibodies of stabilised and wild-type antigens over a six month period in cattle (Chapter 4). Animals were intra-dermolingually challenged with live virus to determine levels of protection the antigens have afforded.

In addition, a third aim will be to better understand the inherent thermostability variation of SAT2 viruses in the Southern African region (Chapter 5) by establishing a protocol for screening field isolates as potential vaccine strains and correlating their stability to amino acid residues at the interface of the 146S particles.

CHAPTER 1
LITERATURE REVIEW

1.1 Introduction

Foot-and-mouth disease (FMD) virus (FMDV) is the causative agent of a highly contagious viral disease, which affects cloven-hoofed animals such as cattle, pigs, sheep, goats, and other artiodactyl species (Grubman and Baxt, 2004). FMD ranks as one of the most economically important infectious diseases of animals according to the World Organisation for Animal Health (OIE) (www.oie.int/en/). Typical symptoms are vesicles and ulcers on the tongue and hooves; hence the name of the disease. Not only does this cause much discomfort and secondary infections, but also fever and occasional mortality especially among young animals. Invariable loss of income to the local farmer is a direct consequence of animals becoming immobile, difficulties feeding, substantial loss in milk production of up to 40% and loss of condition with subsequent reduction in meat production. Crop farmers that rely on working cattle for ploughing are also affected due to loss of draught power when an outbreak coincides with important crop activities. Attributable to the highly transmissibility of FMD, it is a notifiable disease and many countries will reject import of animals from FMD positive regions; this is enforced by an international system of export certification (Paton et al., 2009).

FMD affects not only international trade in livestock and animal products, but also results in damaging consequences to the livelihoods of local farmers through loss of productivity, food security and income. In developing regions, livestock farming forms the backbone of rural economies that supports approximately 70% of the world's poor. FMD outbreaks affect particularly vulnerable individuals such as women and children since approximately 75% of livestock in Africa are raised under the communal smallholder, communal-grazing, or pastoral systems that sustain livelihoods of these groups (Scoones et al., 2010; Miguel et al., 2013; Ferguson et al., 2013).

The lack of veterinary infrastructure, human resources, movement controls, and appropriate vaccines render many developing countries particularly exposed to the

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spread of FMD (Doel, 2003, Sutmoller et al., 2003; Perry et al., 2007). Many aspects outlined below have been described in our review article Maree et al. (2014).

Diseases caused by RNA viruses, like FMDV, 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 the host's immune system. FMDV exists as seven serotypes, causing clinical indistinguishable disease, i.e. O, A, C, SAT-1 (southern African territories), SAT-2, SAT-3, and Asia-1. Within each serotype there are many antigenic variants, genetic subtypes, and quasi-species (Knowles and Samuel, 2003; Carillo et al., 2005; Paton et al., 2005; Domingo et al., 2012). The implication of the antigenic variation is that the degree to which immunity induced by one virus is effective against another is largely dependent on the antigenic differences between them. Typically, an FMD vaccine will comprise a separate component for each serotype against which it needs to protect, as a combination vaccine.

Although FMDV causes a clinically indistinguishable disease in cloven-hoofed animals, the seven serotypes display different geographical distributions and epidemiology (Samuel & Knowles, 2001; Bastos et al., 2001; 2003a; 2003b; Knowles & Samuel, 2003; Bronsvoort et al., 2004). Serotypes A and O have an almost worldwide prevalence, whereas there has not been a serotype C outbreak since 2004 (Knowles and Samuel, 2003; Rweyemamu et al., 2008). The three SAT serotypes occur in several regions of Africa and the Middle East, and serotype Asia-1 in Asia and the Middle East.

The seven serotypes also differ in biophysical properties, mainly in their stability. This is relevant as FMDV, apart from being highly contagious, is also quite unstable, and is readily inactivated and dissociated by heat, acidity, shearing, etc. Therefore, all FMD vaccines need to be shipped and stored under strict cold-chain conditions. This is an additional handicap as infrastructure is poor in the certain sub-tropical, tropical and developing regions of the world where FMD is endemic. The virions of serotype A and Asia-1 are relatively more stable than those of other serotypes, and vaccines formulated from these antigens have a workable shelf-life of six months or more. Serotype O vaccines have a very limited biological half-life, typically only a few months. Even worse is the situation for the three SAT serotypes,

for which the notoriously low stability yields vaccines of low protective capacity, even when administered multiple times (Doel and Baccarini 1981; Kotecha et al., 2014).

The epidemiology of FMD in Africa is influenced by two different patterns, i.e. a cycle involving wildlife, in particular the African buffalo (*Syncerus caffer*), and an independent cycle maintained within domestic animals (Hedger, 1972, Hedger et al., 1972, Condy et al., 1985, , Bengis et al., 2002, Thomson et al., 2003). A unique feature of FMD epidemiology in Africa is the presence of the three SAT serotypes (i.e. SAT1, SAT2, and SAT3), which are maintained within the African buffalo populations (Vosloo et al., 1996, Vosloo et al., 2002, Thomson et al., 2003, Ayebazibwe et al., 2010). The presence of large numbers of African buffalo provides a potential source of sporadic infection to domestic livestock and other wildlife species (Dawe et al., 1994, Bastos et al., 2000, Vosloo et al., 2004). Although the precise mechanism of transmission of FMDV from buffalo to cattle is not well understood, it is facilitated by direct contact between these two species. Once cattle are infected, they may maintain SAT infections without the further involvement of buffalo (Thomson, 1994; Thomson et al., 2003; Thomson et al., 2001; Ahmed et al., 2012).

Sub-Saharan Africa is endowed with an abundance of wildlife, which has been preserved within national parks and game reserves (Chardonnet et al., 2002). In communities neighbouring these parks, the livestock/wildlife interface presents unique challenges to livestock disease control (Brückner et al., 2002; Thomson et al., 2003; de Garine-Wichatitsky et al., 2013). In addition, the creation and expansion of transfrontier conservation areas in southern and eastern Africa presents a particular challenge to the management of FMD. In response to this unique epidemiological situation, certain southern (e.g., Botswana, Namibia, and South Africa) and north African (such as Egypt) countries (Ahmed et al., 2012) have invested in regular livestock vaccination programmes to manage the disease and facilitate access to international and regional trade markets in livestock and livestock products.

In southern Africa, the incidence of the disease has increased appreciably over the last decade, and since the eradication of the disease in Africa is unlikely in the near future, more flexible ways of managing FMD are required to obviate clashes between conservation-based and livestock-based initiatives aimed at rural development. Whereas culling is advocated in FMD-free nations, a vaccination

strategy is practiced in endemic countries to combat and contain the disease. Despite systematic use of vaccination, numerous outbreaks of FMD have been recorded, and there is evidence of sustained virus circulation in vaccinated cattle populations in southern and eastern Africa since 2000 (Jori et al., 2009; WAHID) as well as in north Africa where exotic incursions of FMD (serotypes A, SAT2, and O) have caused widespread outbreaks (Knowles et al., 2007; Ahmed et al., 2012). Effective control and prevention of FMD relies largely on the implementation of strategies such as physical separation of wildlife and livestock, repeated vaccination of cattle herds exposed to wildlife, control of animal movements, and careful assessment of the risk of FMDV introduction into disease-free areas (Brückner et al., 2002; Thomson et al., 2003; Jori et al., 2009). The current inactivated vaccines have proven effective in reducing clinical disease in FMD-endemic areas and have been critical to the success of FMD control programs in South America and Europe (Brown, 2003). In Africa, the diversity of circulating field strains of FMDV makes the selection of sufficiently cross-protective FMD vaccines a challenge. Therefore, local and regional programmes in surveillance and diagnostics, to monitor FMDVs circulating in wildlife and livestock populations are a crucial component of vaccine control, by providing vaccine matching data and access to appropriate viral strains that can be used in the development of new vaccines. There is also a need for risk-based surveillance to be able to determine primary endemic areas and factors that influence disease dissemination, to assist the design of targeted, area-wide, or ecosystem-based disease control strategies, as African regions embark on the Food and Agriculture Organization of the United Nations (FAO)-OIE Progressive Control Pathway (PCP) for the Control of FMD (Rweyemamu et al., 2008). The success of any FMD control campaign ultimately depends on the abundant supply of vaccine of the appropriate strain composition and proven potency, adequate vaccine coverage, rapid vaccine development, overall planning and management by a well-resourced veterinary service, and the involvement and cooperation of the livestock farmer (Rweyemamu and Garland, 2006).

Increasing the thermostability of vaccines would increase the time vaccines could be stored, reduce production and quality testing costs, reduce the dependence on a cold chain, increase stability following viral inactivation process, reduce persistent infection (discussed in detail further in the review), and significantly enhance immune

responses. Of particular interest is the potential solution to cold chain-related problems. In most endemic countries, vaccination is during the hotter season of the year and a lag time of 24 - 48 h is typical between the time the vaccine is removed from the major distribution centres and the time of actual vaccination. Increasing the thermostability of vaccines may completely eliminate maintenance of cold chain beyond the distribution centres. In this regard, it is worthwhile noting that the thermostability of the vaccine strain played a critical role in the global eradication of rinderpest (Roeder, 2005). Finally, the duration of immunity following natural infection is much longer compared to that following vaccination (Doel, 2005), and stimulating long-term immunity through immunization is still not achieved for FMD. Although live attenuated vaccines might be an answer, they are not acceptable due to the possibility of reversion to virulence, recombination with field isolates and the inability to differentiate infected and vaccinated animals (Hedge et al., 2009). Current work on thermostable mutants such as A2065H mutant (Mateo et al., 2008) has laid the foundation for a FMD thermostable vaccine, but further research especially with respect to field studies and improved duration of immune responses is warranted (Hedge et al., 2009).

In summary, animal diseases, in particular transboundary animal diseases such as FMD, severely constrain the development of competitive livestock enterprises in developing countries (Perry et al., 2007; Knight-Jones et al., 2013). The aim of this review is to provide a comprehensive summary of the developments in research focussed on FMD stability, the consequences of poor stability and importance of vaccines with improved stability especially to enhance the control of FMD in endemic settings in Africa. This review also highlights different aspects of FMD vaccines, alternative options and gaps in knowledge of vaccinology and vaccine stability. Furthermore, we advocate applied research into vaccination and disease control strategies to enable fit-for-purpose approaches to FMD control in Africa and enhancing stability through reverse genetic approaches.

1.2 Structure of FMD capsid

FMDV belongs to the family *Picornaviridae*, and is the type species of the genus *Aphtovirus*. The virion comprises a single-stranded positive-sense RNA genome, of approximately 8 kb in length, capped at the 5'-end by a virus encoded VPg protein and contained in a non-enveloped, icosahedral capsid of *ca.* 22 - 25 nm in diameter (Acharya et al., 1989). The capsid consists of a symmetrical arrangement of 60 copies of each of four structural viral proteins (VP), i.e. VP1, VP2, VP3 and VP4 (**Fig. 1.1**). Each structural protein interacts to form protomer subunits with a sedimentation coefficient of 5S; five of these protomers come together to form a pentamer of 12S, and 12 pentamers assemble into the complete capsid. This can be a non-infectious empty capsid with a sedimentation coefficient of 70S, or a complete virion of 146S with viral RNA content, which can be infectious (Fry et al., 2005).

Cell entry in infected hosts is followed immediately by translation of the viral RNA, which yields a single polyprotein precursor that has to be processed into fourteen distinct capsid and non-structural proteins for virus replication. The majority of this processing is done by the virus-encoded 3C protease (3Cpro), which cleaves the precursor at ten distinct sites (Curry et al., 2007). The four capsid proteins originate from the post-translationally cleaved P1 region of the polyprotein and are designated 1A/VP4, 1B/VP2, 1C/VP3 and 1D/VP1.

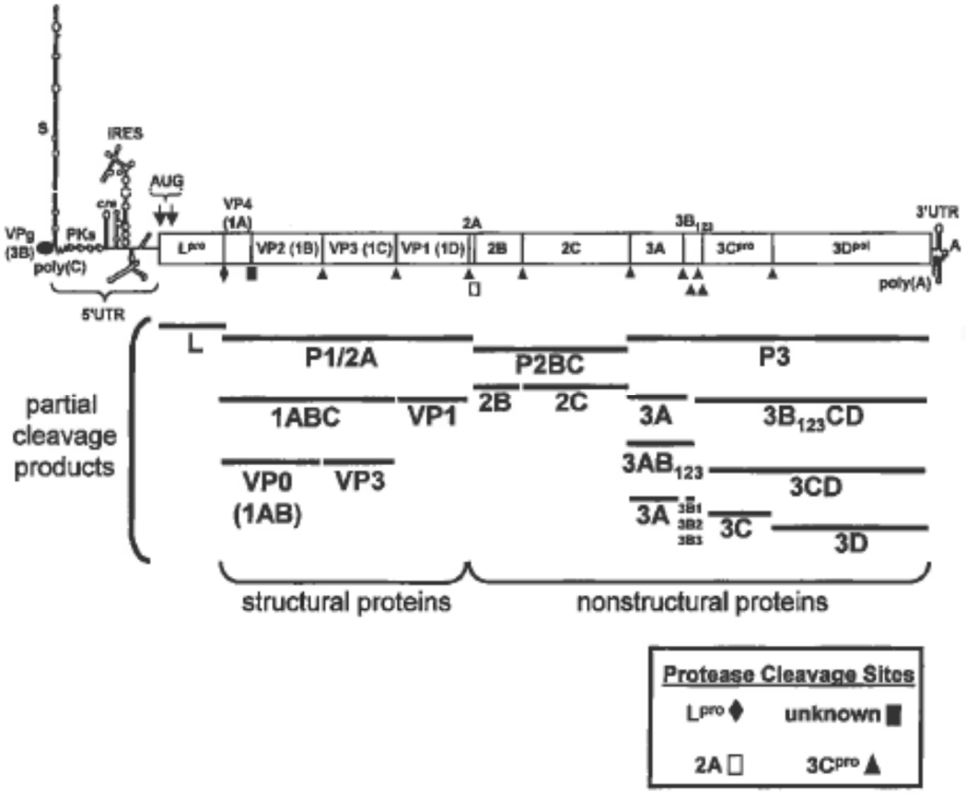

Figure 1.1: Schematic map of the FMDV genome. The ORFs are shown in a boxed area, with the viral proteins named according to the nomenclature of Rueckert and Wimmer (1984). Also shown are the functional elements of the genome and the partial protein cleavage products. The sites of the primary cleavages and the proteases responsible are indicated (Grubman and Baxt, 2004 adapted from Mason 2003).

During assembly the 95 kDa P1 polyprotein is cleaved by the viral 3C protease to yield VP0 (36 kDa), VP1 (32 kDa) and VP3 (27 kDa) which self-assemble to form the capsid. Auto-catalytic cleavage of VP0 into VP2 (28 kDa) and VP4 (8 kDa) occurs during encapsidation of the viral genome to produce the mature virus (Basavappa et al., 1994, Curry et al., 1997). As an icosahedral structure, an FMDV capsid has three natural types of symmetry axis: (1) the 5-fold axis where the protomers meet to assemble into a pentamer, as well as two axes where the pentamer subunits meet, (2) the 2-fold axis of symmetry where neighbouring VP2-VP2 or VP3-VP3 proteins interact, and (3) the 3-fold axis where VP2-VP3 proteins interact (**Fig. 1.2**) (Fry et al., 2005). The capsids are held together by electrostatic interactions, hydrogen bonds and weak hydrophobic interactions between the inter-pentameric subunits (Acharya et al., 1989; Ellard et al., 1999) and release the RNA genome by dissociation into pentamers at pH 7.0 and elevated temperatures. The instability at low pH is essential for FMDV to facilitate uncoating of the viral RNA that is contained inside the capsid

once the virus reaches the acidic environment in the endosomes of infected cells, and it is thus critical for a successful infectious cycle (Carrillo et al., 1985). O'Donnell et al. (2005) showed that FMDV utilizes integrins via the clathrin-mediated endocytosis pathway and/or heparin sulphate-binding via caveola-mediated endocytosis pathway (O' Donnell et al., 2008) to infect cells and viral replication begins due to acidification of endocytic vesicles, causing breakdown of the viral capsid structure and release of the genome by an as-yet-unidentified mechanism.

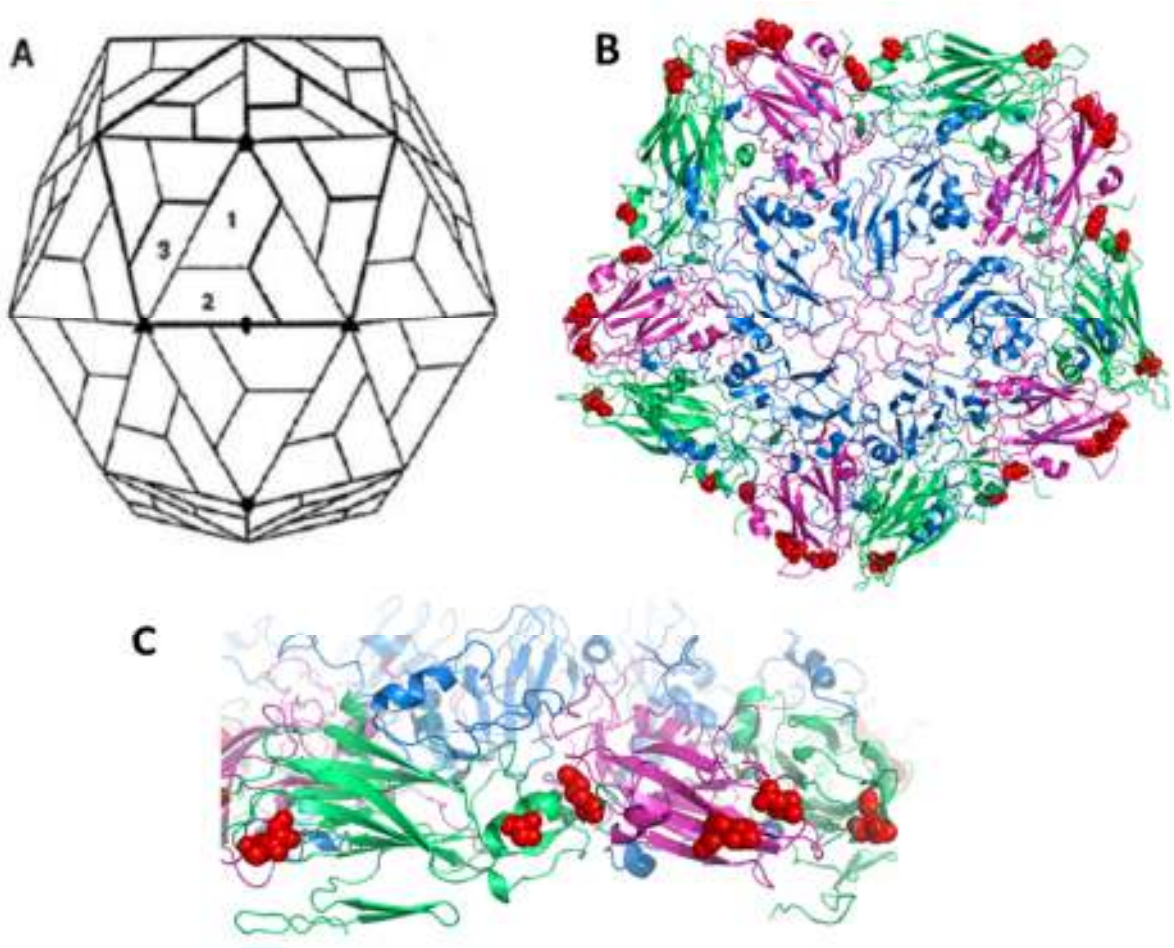

Figure 1.2: The Capsid of FMDV, A: schematic structure of the icosahedral FMDV capsid. VP1 labelled 1, VP2 (2), VP3 (3), VP4 internal of a biological protomer. Five biological protomers and the pentamer subunit form are outlined by thick lines (from Mateo et al., 2003). Twelve pentamers form the capsid. B: The organisation of a pentamer, note the pore located at the top of the fivefold axis. VP1 is in blue, VP2 in green and VP3 in pink. C: Pentameric interface of FMDV SAT2 virus showing amino acid residues (shown in red) that are potentially involved in stability of the 146S particles.

1.3 Stability of FMDV

Conformational stability and dynamics of virus particles play major roles in the infectious cycle (Johnson, 2003; Steven et al., 2005; Mateu, 2013). Mature bacteriophages that inject their genome into the host cell without capsid disassembly are physicochemically very stable (Veesler and Johnson, 2012). Eukaryotic viruses that release their genome through capsid openings, e.g. parvo- and some picornaviruses tend to resist dissociation (Rincón et al., 2014). In contrast, some viruses that must disassemble to release their genome into the cytoplasm e.g. retro- and certain picornaviruses, contain labile capsids. The mechanism of virus entry and uncoating is a function of the route of transmission of the virus. Many viruses have evolved a dynamic, metastable capsid destabilized through controlled conformational rearrangements required for viral nucleic acid uncoating (Veesler and Johnson, 2012). Structure-based analyses have revealed distinct molecular determinants responsible for the wide variations in virus stability (Mateu, 2013). Certain mature virions are surprisingly prone to untimely conformational rearrangements or disintegration in the extracellular environment, leading to their biological inactivation. Investigation of the structural foundations and biological relevance of this low physicochemical stability may provide insights into capsid assembly and genome uncoating. The knowledge acquired could be applied to the design of antivirals and physically robust virus particles for use in medicine, biotechnology, or nanotechnology (Mateu, 2011).

Interfacial residues located at the base of the pore delineating the capsid five-fold axes of the parvovirus minute virus of mice are involved in a heat-induced conformational rearrangement associated with externalisation of the capsid protein N-terminus, and are needed for infectivity (Reguera et al., 2004). Thus, at the subunit interfaces of this virus capsid, only key residues involved in the strongest interactions are critical for assembly and stability, but additional residues fulfil other important biological roles (Reguera et al., 2004). The residues at the FMDV capsid inter-pentamer interfaces, and their interactions, are important for the infectivity and stability of the virion (Mateo et al., 2003; 2008) and mutations adjacent to these interfaces have an effect on the conformational stability of FMDV (Filman et al., 1989; Twomey et al., 1995; Airaksinen et al., 2001; Mateo et al., 2007). However, experimental studies on the relative importance of residues and molecular interactions in viral capsid assembly, disassembly, and/or stability are still very limited.

The FMDV capsid is assembled in a stepwise process: five protomer subunits form a stable pentameric intermediate, and 12 pentamers associate to form the capsid (Rueckert, 1996; See 1.3). Several groups have investigated the stability characteristics of a FMDV capsid, which rapidly dissociates into pentamers above physiological temperatures and below physiological pH (Archarya et al., 1989; Curry et al., 1995). For vaccine use this is unfavourable, as the 12S pentamers are much less immunogenic than the intact capsids (Doel, 1981; Hedge et al., 2009). Twomey et al. (1995) studied natural variants of FMDV A serotype that were less acid-sensitive resulting from amino acid substitutions at amino acid positions 131 and 133 of the VP2 protein. To improve the thermo- and/or the acid stability of an FMDV capsid, several groups have introduced mutations into one or more of the viral structural proteins, and then used such VP mutants to produce FMDV virion capsids, and tested these for their bio-physical properties; termed capsid engineering. Successes varied - to some extent acid sensitivity could be decreased by mutations to histidine residues in positions 140-145 of VP3 (Ellard et al.1999; Martin-Acebes et al., 2011, Liu (patent number CN 101270155)) or by substitution VP1 N17D (Martin- Acebes et al., 2011).

It is important to differentiate between stability against inactivation of infectivity, stability against dissociation into subunits, as well as acid and thermal stability. At moderate temperatures, the inactivation rate of the FMD virion is much higher than its dissociation rate (Mateo et al., 2008). Some FMDV strains with different sensitivity to acid-induced inactivation showed similar sensitivity to thermal inactivation (Maree et al., 2013). Empty capsids are more resistant than virions to acid-induced dissociation (Curry et al., 1995) but less resistant to thermal dissociation (Doel and Baccarini, 1981; Curry et al., 1997). The thermostable VP2 A65H mutant virion (Mateo et al., 2008) is as sensitive to acid-induced dissociation as the thermolabile parent virion (Rincón et al., 2014).

The FMDV capsid thermostability could be improved by mutation of amino acids in regions of the VP2 and VP3 proteins (King et al., 2001; Mateo et al., 2008; Fowler et al. (personal communication) reviewed in Mateu (2011). Fowler et al. (personal communication) found an A-serotype containing a G-H loop deletion of 13 amino acids was more thermostable than the field isolate. Sequencing of the capsid proteins revealed five amino acid substitutions, e.g. VP2 T88A, S110T, A193S and VP3 H85P and E196A. These sites together with VP2 K130E substitution (novel receptor site)

were located close to the inter-pentameric interface. VP2 K130E and S110T were already implicated in thermostability (Twomey et al., 1995; Mateu et al., 2003).

Mateo et al. (2008) applied the substitution to two engineered mutants - VP2 A65H and the combined substitution VP3 D69E/VP2 T188A - and showed it is possible to rationally engineer FMDV with increased stability against thermal dissociation, without disrupting the many biological functions needed for its infectivity. Amino acid side chains located near the capsid inter-subunit interfaces and either predicted or found to be dispensable for infectivity were replaced by others that could establish new disulphide bonds or electrostatic interactions between subunits. The mutants were normally infectious, genetically stable and antigenically indistinguishable from non-mutated FMDV strain, but showed substantially increased stability against irreversible dissociation. The results showed that virion thermostability against dissociation into subunits may not be selectively constrained by functional requirements for infectivity. Thus, the molecular basis and biological consequences of FMDV resistance against inactivation of infectivity, thermal dissociation, or acid-induced dissociation are not the same and must be considered separately (Rincón et al., 2014). However, the precise mechanism by which the mutation increased capsid stability had not been determined. The hypothesis proposed was that the introduced histidine residue exerted its stabilizing effect by partially neutralizing nearby carboxylates, thus reducing interpentamer electrostatic repulsion. To test this hypothesis, Rincón et al. (2014), made a number of acidic-to-neutral substitutions close to the pentamer interfaces and found that two variants displayed no change in stability while four mutants were significantly stabilized compared to the parent, as shown by their slower dissociation rates at 42 ºC. They show that calculations based on the very simple Tanford-Kirkwood theoretical model of electrostatics are able to predict the destabilizing effects of the residues. A simple model was proposed on the structural basis of FMDV thermal dissociation: the instability of the capsid, originating at least in part from intrinsic electrostatic repulsion involving residues VP2 D68, E86, VP3 D134, and D195, will lead to ready dissociation into pentamers even at moderate temperatures. Thus, the energy barrier between the associated and dissociated states is very low. By performing acidic-to-neutral mutations, electrostatic repulsions will be improved. This will decrease the free energy of the assembled (capsid) state, but will not affect the free energy of the transition state where the unfavourable electrostatic interactions have not yet been formed. The net effect will be an increase in the energy

barrier between the two states, leading to a reduced rate of irreversible dissociation at a certain temperature; the capsid will be kinetically stabilized. In summary the low thermal stability of the FMD virion at nonacidic pH is based on the existence of interpentamer electrostatic repulsions involving a few specific carboxylates per capsid protomer (Rincón et al., 2014). There is limited information on how the nucleic acid crosses the endosomal membrane.

Disulphide bonds are used to stabilise many extracellular proteins and also certain virus capsids (Wikoff et al., 2000). Such covalent crosslinks are more robust than the non-covalent interactions that generally hold protein assemblies together. The approach by King et al. (2001) differed from this work in that they attempted to stabilise an A-serotype FMDV capsid by introduction of a covalent rather than a non-covalent bond. By substitution of amino acid 93 of VP2 for a cysteine, they introduced a non-natural disulphide bridge between the two adjoining VP2 proteins at the 2-fold axis of symmetry. This amino acid position 93 in its native structure forms part of an α-helix: encompassing amino acids 88-98 of VP2 (O serotype) (Acharya et al., 1989). This region was studied in relation to the mechanism of uncoating of another picornavirus, the human enterovirus (Wang et al., 2012), however, the mechanism of uncoating differed from that of FMDV. However, this approach was only applicable for stabilising empty capsids (Porta et al., 2013), and not for virion capsids. A Cys residue at position 93 of VP2 resulted in a disulphide bridge across the interpentamer interface causing the capsids to be permanently fixed and virion was no longer able to uncoat upon infection of a host cell (**Fig. 1.3**). Baculovirus expressed wild-type and disulphide bridge-stabilised capsids produced equivalent titres of neutralising antibodies, following a standard immunisation regime over a 34 week period of immunisation (Porta et al., 2013). These results inform the debate on the effect of increased antigen stability on immunogenicity. The rational structure-based approach initiated here could allow the modification of these parameters to match different serotype viruses e.g. SAT and O serotypes (Doel and Baccarini, 1981; Kotecha et al., 2015).

Previous work on Enterovirus (EV)-71 has demonstrated that maintaining the proper positions of the 2-fold helices (the equivalent VP2 H93C mutation in FMDV) is essential for maintaining native antigenicity (Wang et al., 2012), suggesting that the approach demonstrated here may be applicable across a wide range of human and animal picornaviruses, including polioviruses and coxsackieviruses. In spite of all the

efforts, no generally applicable and immunologically effective FMD vaccine, based on engineered FMDV capsids, is available currently.

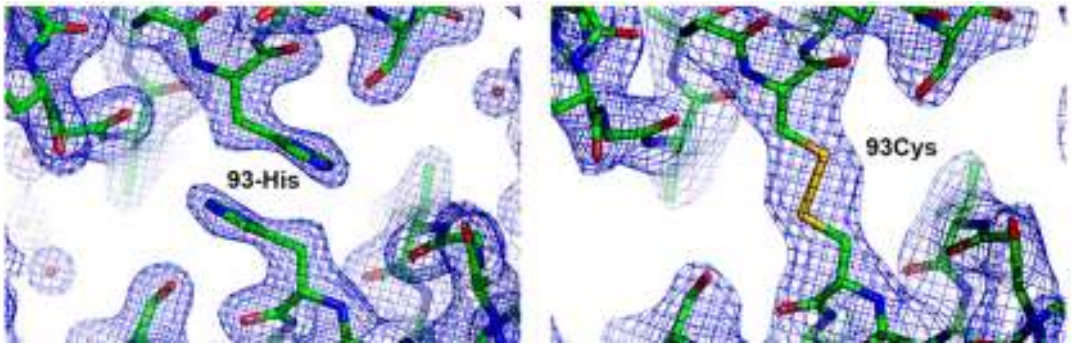

Figure 1.3: Structure analysis of A22-wt and A22-H2093C empty capsids. The electron density map for A22-wt (left panel) presents the expected histidine side chains for VP2 residue 93 whereas the map for A22-H2093C (right panel) shows the disulphide density at the two-fold axis between pentamers. (Taken from Porta et al., 2013).

1.3.1 Thermostability of FMDV quasispecies and biological fitness

Early pioneering studies (Bachrach et al., 1960; Pringle, 1964) described the selection of thermostable variants from a FMDV laboratory population (serotype A). After extensive passaging (76 times) in cell culture these variants were genetically unstable and/or showed a reduced fitness but the genotypic changes involved were not determined. Mateo et al. (2007) attempted detection and selection by heat of thermostable variants from different FMDV populations in order to explore whether FMDV may accept a substantial increase in thermostability without compromising its infectivity. The results obtained with both uncloned and cloned populations of different serotypes, recovered from cytolytic or persistent infections subjected to either very few passages or extensive passaging in cells, indicated that the presence of thermostable virus variants, even in small proportions, were not a general feature of FMDV quasispecies.

Mateo et al. (2007) also considered the possibility that adaptation of FMDV to cell culture could alter the quasispecies equilibrium, leading to the presence of highly thermostable variants. They observed that the inactivation rate constants for laboratory populations were significantly higher than those obtained for natural FMDV populations (i.e. field isolates subjected to limited amplification in cultured cells). This indicates that some reduction in thermostability may occur during adaptation of FMDV to cell culture conditions, perhaps because of the absence, in those conditions, of the selective pressure exerted in the field by occasional heat extremes. A way to reconcile

the presence of thermostable variants in some FMDV populations, may be to contemplate two aspects: (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).

Hedge et al. (2009) speculated that FMDV has evolved to undergo persistency in ruminants by avoiding establishment of thermostable variants. A potential link between acquisition and loss of heparin binding characteristics and persistence of FMDV has been proposed (Sa-Carvalho et al., 1997; Fry et al., 1999). In other words, thermostability might be detrimental to survival, persistence and spread of FMDV in nature (tropism and spread during cold seasons) and therefore this evolutionary adaptation could be blocked by a thermostable vaccine (Hedge et al., 2009).

Most mutations aimed at increasing the stability of FMDV through the rational introduction of inter-subunit salt bridges or disulphide bonds proved lethal (Mateo et al., 2007). While most of the non-lethal mutants showed no increase in stability, only one mutant appeared to lose infectivity at a slower rate, compared with the wildtype control. Although different FMDVs and serotypes may show a somewhat different thermostability (Doel & Baccarini, 1981; Nettleton et al., 1982), all FMDV isolates are 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. Thus, a likely scenario is that the vast majority of single mutations potentially able to increase the stability of the FMDV capsid may have a negative effect on fitness, are strongly selected against, and are heavily under-represented, or not represented, in FMDV quasispecies. On the other hand, compensatory mutations able to counteract the deleterious effect of some mutations in the FMDV capsid, including those located at the inter-pentameric interfaces, have been detected (Mateo et al., 2003). Thus, there is the possibility that particular second-site mutations could compensate for the disadvantage tentatively associated with single mutations that increase thermostability, restoring fitness. However, these variant genomes with combinations of mutations would probably be present at much lower frequencies than single mutants. While some FMDV populations facing a strong selective pressure by heat could easily adapt, many others would face extinction, depending on the stochastic presence or absence of the right combination of mutations. The observations above provide an example for a different, non-deterministic adaptability of different FMDV quasispecies in response to a single selective agent. This suggests that no substantial

increase in the thermostability of FMDV may occur readily without a negative effect on viral function.

Physical stability is one important adaptive property of a virus particle where a compromise between extracellular virion stability and intracellular lability to allow the release of the genome has been inferred (O'Donnell et al., 2005). A clear understanding of the relationship between thermostability and biological fitness is also relevant for the engineering of viral particles of increased thermostability, as a basis for more stable vaccines, where very little is known about whether a virus can accept a substantial increase in thermal stability without compromising its infectivity. A recent study of Rincón et al. (2014) showed that improved thermal stability does not affect infectivity, however more studies are needed. A thermostable poliovirus variant that carried a single mutation in the capsid appeared to be genetically stable (Shiomi et al., 2004) and was selected by Mateo et al. (2003) to explore the possibility of obtaining FMDV of increased stability without compromising viral function. However, the possibility that these variants could have a reduced fitness, because of slower uncoating that would be outcompeted by other, less stable variants could not be excluded. Different studies with small, non-enveloped viruses, including FMDV, have shown that a large majority of mutations in a virus capsid may have a negative effect on infectivity and/or fitness (Filman et al., 1989; Twomey et al., 1995; Airaksinen et al., 2001; Shiomi et al., 2004).

1.4 Control of FMD by vaccination

The existing vaccines against FMD consist of complete, chemically inactivated virions (crude or purified); formulated either with oil or aluminium salt adjuvants (Doel, 2004). The adjuvant used in the vaccine formulation has a huge, undeniable effect on the efficacy and potency of the vaccine and has been reviewed elsewhere (Kitching, 1997; Doel, 2003). Despite successful application in the developed world, the effective administration and optimal induction of protective immunity is hampered by several factors in developing countries. In addition to the vaccine-matching constraints, some viruses adapt with difficulty to cell culture, slowing the introduction of new vaccine strains, reducing vaccine yield, and potentiating the selection of undesirable antigenic changes through prolonged passage (Sa-Carvalho et al., 1997; Zhao, et al., 2003). Vaccination does not induce sterile immunity, and animals may still be able to infect

non-vaccinated animals and may also become persistently infected (Sutmoller and Gaggero, 1965; Sutmoller et al., 1967; Salt, 1993; Mackay et al., 1998). The presence of contaminating non-structural proteins in some vaccine formulations makes it problematic to distinguish between vaccinated and convalescent animals, affecting the ability to export from FMD-controlled regions. In addition, the hot climate in many African regions calls for vaccines with improved stability and which are less reliant on a cold-chain. An extremely critical component of vaccination programmes is the maintenance of a cold chain, not an easy task as most of the endemic countries do not have the infrastructure to achieve it.

During production, the manufacturer has to compensate for temperature instability by increasing the quantity of antigen per vaccine dose, which is expensive and reduces the number of vaccine doses. Based on the findings of Doel and Baccarini (1981) it is believed that unstable vaccines are less immunogenic, due to degradation before and after inoculation. Therefore, FMD vaccines require frequent booster vaccinations in order to be effective. Another limitation associated with stability is the short shelf-life. Lastly, the current vaccines are relatively expensive, especially for small subsistence farmers. Considering that endemic nations are contiguous with other land-locked countries, FMD is a continental or a global problem for international trade, animal movement and maintenance of disease-free zones, requiring a concerted international effort to control and eradicate this disease (Kitching et al., 2007).

Serological assays, such as virus neutralization tests (VNT) and liquid-phase blocking ELISA (LPBE) were designed to detect levels of antibodies against capsid proteins representing a tool for immunological and protective status prediction of vaccinated and susceptible animal populations against FMDV (Barnett et al., 2003; Goris et al., 2008). Although generation of adaptive humoral responses after FMDV infection in cattle were shown to be independent of T-cell collaboration (Juleff et al., 2009; Pega et al., 2013), vaccine-induced antibody responses are dependent on the collaboration of FMDV-specific CD4+ lymphocytes (Carr et al., 2013). However, induction of FMDV-specific cellular immune responses has not usually been considered for the evaluation of vaccine efficacy (Becker, 1994), probably because of the lack of high throughput cell-mediated immunity tests. Cellular responses against FMDV have also been studied by detection of IFN-γ, showing correlation with protection against homologous virus challenge in sheep (Barnett et al., 2004) and

cattle (Oh et al., 2012). In contrast, heterologous anti-FMDV cellular responses in cattle have been reported exclusively in early studies using lymphoproliferation assays (Collen et al., 1998; Collen and Doel, 1990).

Vaccines used in the control of FMD in endemic regions are used mostly for mass prophylactic application. Such vaccines are multivalent to provide protection against multiple serotypes, and should have a potency of at least 3 PD50 per dose (Rweyemamu et al., 2008). Generally, prophylactic vaccines incorporate 146S particles combined with saponin-alhydrogel or oil-adjuvant (Rweyemamu et al., 2008). Oil-adjuvanted vaccines have been used successfully in FMD-eradication campaigns in South America (Dora et al., 1980; Bahneman and Mesquita., 1987; Sutmoller et al., 2003). A study evaluating different adjuvants for SAT vaccines has shown that a double water-in-oil-in-water adjuvant, ISA206, elicited protective antibody responses against SAT2 serotype in cattle (Cloete et al., 2008). Inactivated vaccines induce short-lived immunity, and it is recommended that naïve animals receive two initial vaccinations (a primary and secondary dose) 3 – 4 weeks apart, followed by re-vaccination every 4 – 6 months (Hunter, 1998; Cloete et al., 2008) to prevent spread of disease within populations. However, in the African environment, this may differ for different manufacturers, depending on the potency of the vaccine, and some manufacturers recommend five vaccinations per annum. There is a definite need to assess whether different adjuvants may enhance the duration of immunity against SAT antigens. For these reasons vaccination campaigns should be performed regularly, based on 1) the epidemiological circumstances and risk of disease spread, 2) the value and life expectancy of species, and 3) the economic status of the country. The interval between vaccinations is critical to prevent a “window of susceptibility” and where the continuous or sporadic presence of virus in carrier animals is present.

1.4.1 Control through the Progressive Control Pathway (PCP)

The PCP is the strategy proposed by OIE and FAO to control and ultimately eradicate FMD from endemic countries. Different regions in sub-Saharan Africa are at different developmental stages of control and are thus facing unique challenges and priorities in terms of FMD control. In many African endemic countries, there are various knowledge gaps, such as disease occurrence and mechanisms of virus maintenance

and transmission, and therefore no routine vaccination campaigns are implemented. In other African endemic countries, even where surveillance is conducted to provide knowledge about high-risk populations, often implementation of effective, scheduled vaccination campaigns still does not take place (PCP Stage 2 countries). There are various reasons why governments do not subsidize FMD vaccines, leading to individuals needing to carry the cost and implement their own vaccine schedules. Additionally, individuals need to source vaccines without knowledge of the current circulating strains in their region, leading to a poor vaccine match. This often leads to no or ineffective control in endemic African regions. The development of new vaccines against FMD in endemic countries in Africa should therefore take into account the ecosystem-based synchronization as FMD control strategies employed in these regions (Rweyemamu and Garland, 2006).

1.5 Stability of FMD vaccines

1.5.1 The importance of the cold chain

The stability of vaccines is of crucial importance in Africa, where the logistical process to get the vaccine from the manufacturer to the animal may take months and in many remote regions is in the absence of a cold-chain. Vaccines with improved stability and less reliance on a cold-chain are needed and could improve the longevity of immune responses elicited in animals (Doel and Baccarini et al., 1981). FMD is unstable, especially for O and SAT2 serotypes, in mildly acidic pH conditions or at elevated temperatures, leading to dissociation of the capsid (146S particle) and loss of immunogenicity (Doel and Baccarini et al., 1981; **Fig. 1.4**).

The success of FMDV vaccines is dependent and attributable to the training of staff in the proper storage and transport of vaccines and partly to improvements in the cold chain. However, vaccines are still not being stored and transported properly in many areas. Questions are often raised as to what should be done with stocks of vaccines that have been exposed for varying periods to elevated temperatures. There is no simple and cheap method that can be used in the field to assess whether a vaccine exposed to ambient temperature has retained at least the minimum required potency. In the case of the oral poliomyelitis vaccine (OPV), vaccine vial monitors (VVMs) have been implemented to indicate the level of heat exposure of individual vials. VVM's in combination with knowledge of a vaccine's stability, especially the rate

of decline in potency at a given temperature, can be helpful in determining storage requirements and the decision about the outcome of a vaccine batch exposed for varying periods to elevated temperatures (WHO/GPV/98.07). Vaccine potency can be determined only by costly clinical trials and laboratory assays, the results of which are often delayed for several months. Therefore, only a large number of doses can justify sending a vaccine for retesting.

To ensure the optimal potency of vaccines, storage and handling need careful attention. Adequate electrical power and refrigeration are often lacking in developing countries, where storage, handling and the heat stability of vaccines are consequently matters of great concern. New products have been developed for safe transport and storage, whilst the reliability of vaccine supply has been increased by the introduction of improved management techniques. Extensive training ensures that everyone involved in the cold chain is familiar with all its facets. However, evaluations of medical vaccines in many countries such as India (Sudarshan, 1994) and Tanzania (Simba and Msamanga, 1994) showed that there were still weak points in cold chain performance and that more attention should be paid to it, especially in peripheral facilities.

The importance of maintaining the cold chain has been given little consideration in temperate countries. Although adequate refrigeration is often taken for granted, errors in vaccine handling may occur more commonly than is generally assumed (Bishai, 1992). Substantial drops in vaccine potency caused by unsatisfactory conditions of delivery and storage have been reported (Lerman and Gold, 1991). The most common deficiencies in cold chain performance reported from developed countries are: high temperatures during storage or transport; exposure of adsorbed vaccine to freezing temperatures; refrigerators without thermometers; failure to take and record temperature readings regularly; storage of drugs, drinks, food and pathology specimens with vaccines; and failure to discard unused vaccine after sessions at ambient temperature (WHO/GPV/98.07). A high prevalence of avoidable errors were found where little attention was given to self-monitoring of vaccine storage practices (Bishai, 1992) and considerable weakness in the cold chain during vaccine transportation from manufacturers or from central distribution points to clinics where vaccines were exposed to excessive heat or freezing temperatures when transported (Lugosi and Battersby, 1990).

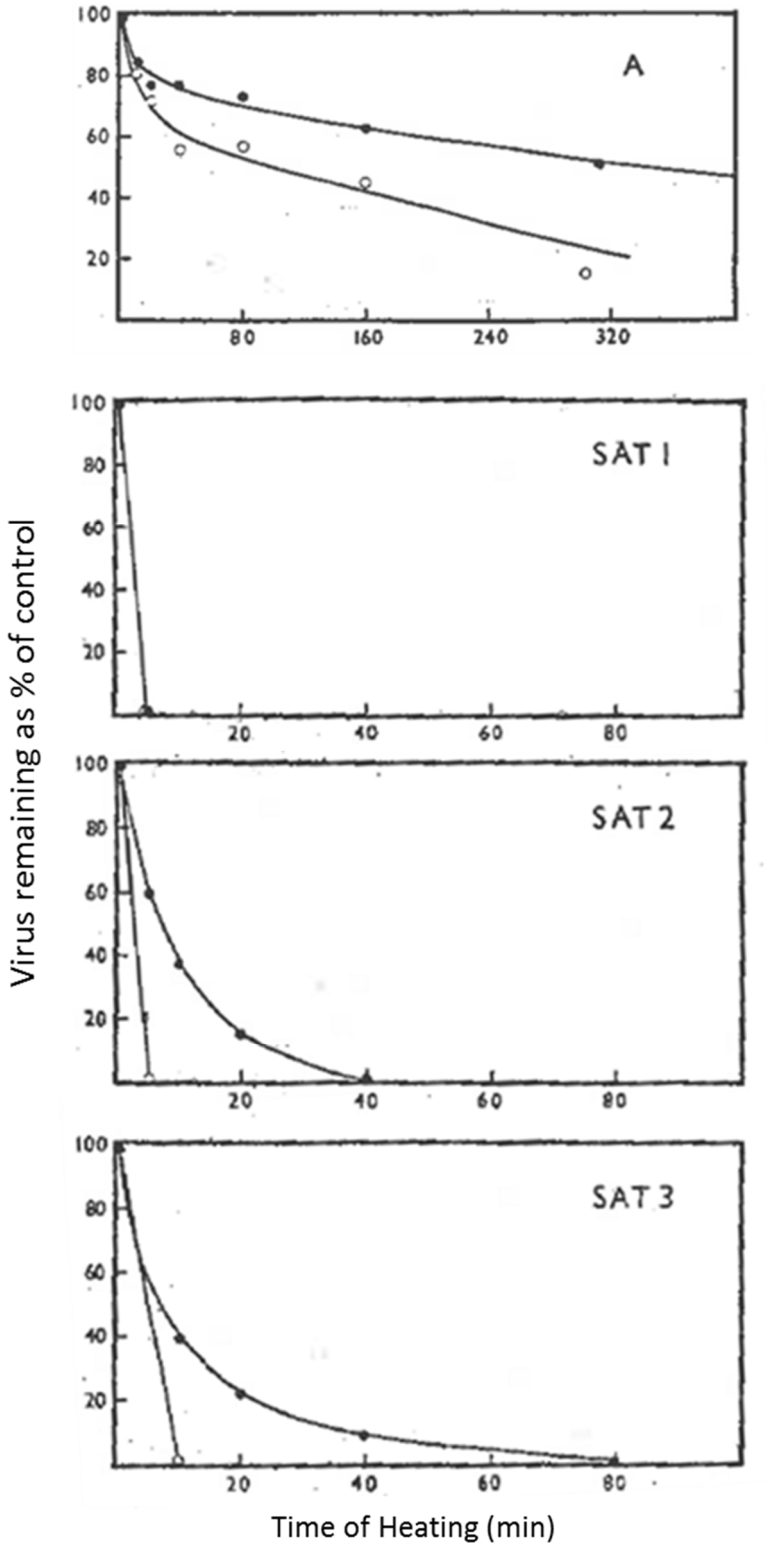

Figure 1.4: Thermal stability of AEI-inactivated preparations of 146S particles of A Cruzeiro (A), BOT1/68 (SAT1), BOT3/77 (SAT2), BEC1/65 (SAT3) annotated with closed circles; unheated control is with open circles. Temperature was 49 °C. (Images taken from Doel and Baccarini et al., 1981).

1.5.2. Vaccine vial monitors and the future of the cold chain

Vaccine vial monitors, which measure exposure to heat, are time- and temperature sensitive labels attached to vials of vaccine at the time of manufacture. Through a gradual colour change they warn officials and storekeepers when a vaccine has been excessively exposed to heat and should not be used (**Fig. 1.5**). They are designed to meet the vaccine's heat stability curve, allowing a margin of safety (WHO/EPI/LHIS/95.01). In the medical field they comply either with WHO requirements for heat stability or with heat stability data provided by the vaccine manufacturer if these are more stringent.

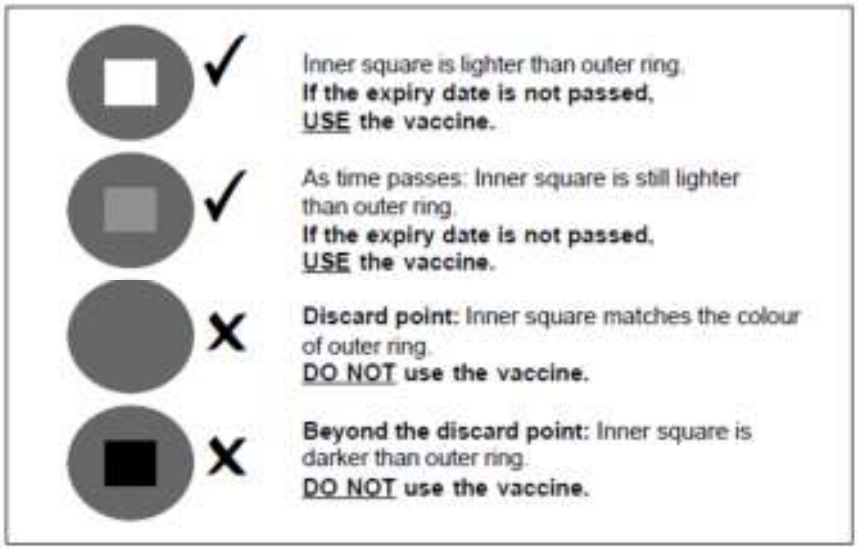

Figure 1.5: Vaccine vial monitor showing four stages of exposure (WHO/GPV/98.07)

When the management and infrastructure of the Expanded Programme on Immunization (EPI) were being established, it was impossible to check whether vaccines retained adequate potency during distribution. More recently, technical adjuncts such as temperature sensors or loggers which have a limited battery life and vaccine vial monitors (VVMs) have evolved where the thermal stresses to which vaccine shipments have been exposed to can be assessed. However, the challenge arises when vaccines leave the manufacturer and are transported to regional stores and in the field where poor regulation and education on the effects of poor cold chain are not understood. The stability of multi-dose vials following opening also has to be considered for FMDV vaccines.

The approach had the merit of simplicity, making the cold chain easy to understand, implement and manage, and presented an uncontroversial concrete

objective to be achieved. Nonetheless, this approach has led to the gradual emergence of a dogmatic view of the cold chain, preventing officials from taking full advantage of the actual heat stabilities of different vaccines (WHO/GPV/98.07).

Vaccines have become more stable, like the Respiratory syncytial virus (RSV) vaccine through structural design (McLellan et al., 2013) and there is a clear prospect of increased or even complete heat stability for other vaccines. In these circumstances the dogmatic approach to the cold chain causes resources to be wasted and places unnecessary restrictions on field operations. The VVM can be seen as a catalyst for much-needed changes in strategies of vaccine distribution via the cold chain. It should eventually allow immunization programmes to exploit the stability of each vaccine to the greatest possible extent, minimize distribution costs, and increase flexibility in the handling of vaccines in the field, thus helping to make operations more effective (WHO/GPV/98.07).

1.5.3. Methods for determining impact of loss of stability

Some authors have sought to determine the validity period of a vaccine by estimating loss of potency during long periods of storage at different temperatures. The accelerated degradation test (ADT) measures samples that are subjected to a range of elevated temperatures at which significant and readily detectable degradation is induced in a relatively short time (WHO/GPV/98.07). The rate at which it occurs is measured and extrapolation is made to the lower temperatures at which vaccines are stored, in accordance with the Arrhenius equation (Tydeman and Kirkwood, 1984). The precision with which the ADT predicts degradation rates differs considerably, depending on the range of temperatures used, the number of samples tested and the design of the test and is further complicated by the different methods used for estimating the potency of vaccines. Potency is expressed in arbitrarily established units or in effective doses providing 50% protection. The results of these tests are often subject to wide biological variation and it is difficult to obtain precise data on vaccine deterioration unless it has been substantial (Perkins, 1976).

With FMDV potency, the most accurate way to assess the degree of protection and cross-protection induced by a vaccine against the field virus is performed using animal challenge studies (Aggarwal et al., 2002; Mattion et al., 2004; Cox et al., 2005). However, routine heterologous challenge studies are rarely performed, as this is time consuming, requires access to expensive bio-containment facilities, and raises serious

animal welfare issues when large numbers of animals need to be vaccinated and challenged (Goris et al., 2007). In South America, a variation of this direct method has been developed, which estimates the likelihood that cattle would be protected against a challenge of 10,000 bovine infective doses after a single vaccination (expected percentage of protection or EPP) (Periolo et al., 1993; Maradei et al., 2008). In South America this method was effective because of extensive testing of the vaccines in many (hundreds) of cattle which were challenged with homologous virus; and the availability of good datasets and sera. However in the African context, this is not a viable approach as many variants exist which require the use of diverse vaccine strains. *In vitro* alternatives to measure cross-reaction of a vaccine is recommended such as the virus neutralization test (VNT) which is used to calculate r_1 values to determine antigenic relationships (Rweyemamu et al., 1978), supporting the need to accurately predict vaccine matching without the involvement of animals. However, interpretation of the test is plagued by limitations, including the uncertainty as to how well the *in vitro* matching data actually correlates to *in vivo* cross-protection, the impact of vaccine potency on protection, availability of reference reagents, and repeatability to overcome the inherent variability of the VNT titres (Rweyemamu et al., 1984).

The degradation rate of a vaccine is determined by the storage temperature: the higher the temperature, the more rapid and extensive is the degradation. There are considerable differences between degradation rates. However, the degradation rate (b) is not the only factor determining the residual potency (Yt) of a vaccine: the time (T) for which a vaccine is stored at a given temperature and the initial potency of the vaccine (Yo) also have an influence. The relationship between the three factors is expressed as follows: $Yt = Yo - bT$. The usefulness of this formula is limited because many of those involved in immunization programmes may not know the initial potency of a vaccine (WHO/GPV/98.07).

FMDV vaccines may be classified as either ‘standard’ or ‘higher’ potency vaccines. Standard potency vaccines are formulated to contain sufficient antigen and appropriate adjuvant to ensure that they meet the minimum potency level required (3 PD50 [50% protective dose]) for the duration of the shelf-life claimed by the manufacturer. This kind of vaccine is usually suitable for use in routine vaccination campaigns. For vaccination in naïve populations to control FMD outbreaks in emergencies, higher potency vaccines > 6 PD50 are recommended for their wider spectrum of immunity as well as their rapid onset of protection (OIE, 2012).

1.5.4 FMDV stability during production and shelf-life

Typically, FMD vaccines are produced in bulk, inactivated, potency assessed and frozen at −80 °C or in liquid nitrogen vapours. When required, the virus concentrates are thawed and formulated. Standard vaccines are formulated to contain a minimum of 3.0 PD50 of antigen per vial (Doel, 2003; Goris et al., 2007). The shelf-life after formulation is generally 1 – 2 years at 2 - 8 °C (OIE, 2012), but once formulated, the vaccine needs to be administered as quickly as possible due to the rapid loss of antigenic payload. The crucial immunogenic component of FMD vaccines is the 146S particle of the virus. Vaccines should never be frozen or stored below the target temperature as this can also cause dissociation. Any degradation of the particle will significantly reduce the potency of the vaccine. Efforts to develop a thermostable vaccine are critical to solve issues of shelf-life and duration of immunity.

Mateo et al. (2008) showed that there is a log reduction of the wild type virus in 20 h at 42 °C, and 30 – 40 days at 4 °C, suggesting that viral structure is lost in less than a day upon exposure to temperatures close to field storage conditions of some endemic countries. If the loss of antigen correlates with dissociation, then antigen derived from the engineered FMDV VP2 mutant A65H would be much more stable than the traditional vaccines derived from wild type strains. The engineered virus had a log reduction in titre when incubated for 60 h at 42 °C, and several months at 4 °C (extrapolated from 20% loss in 2 months). However, the VP2 A65H mutant needs to be evaluated for long-term stability both with and without formulation (Hedge et al., 2009) and if there is any significant difference in immunogenicity.

1.5.5 Consequences of stable vaccines to virus persistence

Regardless of the vaccination status of animals recovered from acute FMD, they will carry the virus persistently except for pigs and can act as reservoirs for other animals. Recent findings suggest that increasing the vaccine antigen payload can reduce sub-clinical infection following virus challenge, leading to fewer persistently infected carrier animals (Cox et al., 2006). Thus, vaccines with greater stability should significantly reduce persistent virus infection during disease outbreaks (Hedge et al., 2009). The role of engineered stability FMD vaccines on virus persistence and secretion needs to be investigated.

1.5.6 Stable vaccines as marker vaccines: inactivation & accidental release

The rarity of the natural occurrence of thermostable variants and inter-serotype recombination being uncommon in the FMDV structural region (Jackson et al., 2007), provides an avenue to develop marker vaccines with stabilised mutations that can be used to detect outbreaks resulting from improper inactivation or accidental release (Hedge et al., 2009).

Formalin and binary ethyleneimine, used for the inactivation of FMDV, are known to affect the integrity of the virus epitopes and may promote dissociation of viral structural proteins (Rweyemamu et al., 1989). The integrity of the capsid is one of the major contributing factors for vaccine potency; therefore, studies have to be performed to confirm that enhanced stability mutants maintain epitope integrity during viral inactivation process (Hedge et al., 2009). FMDV inactivation by non-chemical methods such as induction of virion associated endonuclease activity (Scodeller et al., 1984; Amadori et al., 1987), which degrades the RNA genome but maintains epitope integrity of the virus (Patil et al., 2002), can complement thermostable mutant-based vaccines. In addition, use of new generation molecular adjuvants might enhance the quality of the immune responses (Guy et al., 2007).

1.5.7 Stability vs immunogenicity

Retention of antigenicity (as assessed by immunoblot) or gaining resistance to dissociation may not always translate to immunogenicity, i.e. ability to induce protective immune responses (Hedge et al., 2009). Therefore, potency tests with stability mutants versus wild-type are required to confirm that resistance to dissociation corroborates with improvement in duration and quality of protective immune responses (Hedge et al., 2009).

However, a recent study on the A24/Cruzeiro virus showed that it was a very efficient IFN-γ inducer as a single vaccine strain in vaccinated animals, both when included in the vaccine or as stimulating antigen due to the structural stability of the whole viral particle compared to the less thermostable A/Argentina/2001 or O1/Campos/Brazil/58 viruses (Bucafasco et al., 2015). These results show that IFN-γ production relies on the integrity or stability of 140S particles during the *in vitro* incubation/storage process, and throughout the vaccine´s shelf-life, when used *in vivo* (Bucafasco et al., 2015). This is an important factor to be considered when detecting

FMDV-specific production for this cytokine, and in evaluating and improving immunogenic capacity of FMD vaccines. The use of disrupted capsid particles in the stimulation phase of the IFN-γ production may lead to false negative results, especially when using structurally unstable FMDV strains, such as those from the O or SAT serotypes (Bucafasco et al., 2015). Furthermore, the phenomenon described here may also affect the *in vivo* performance of FMD vaccines reflecting quantitative differences in the antigenicity of 140S and 12S particles (Cartwright et al., 1982). The lower neutralizing antibody titres induced by 12S particles may be obviously related to destruction of neutralizing epitopes from the 140S to 12S particles (Bucafasco et al., 2015). FMDV specific IFN-γ production in vaccinated cattle has been associated with the activation of antigen-specific CD4+ T-cells (Oh et al., 2012) which are responsible for supporting antibody neutralising responses (Carr et al., 2013). Thus, it is also possible to hypothesise that the reduced ability of the 12S particles to trigger amnestic FMDV-specific IFN-γ responses may also contribute to the lower immunogenic capacity in FMD vaccines comprising structurally unstable strains (Bucafasco et al., 2015).

1.5.8 Role of dendritic cells

The primary immune response is initiated by dendritic cells (DC), which have the function of searching for foreign bodies in the surrounding area of an exposed site followed by migration to T-cell regions of the lymph nodes (Steinman et al., 2006). There are two major groups of DCs, conventional and plasmacytoid DCs or natural IFN-producing cells. The bovine plasmacytoid DCs are efficiently killed by live FMDV immune complexes in-vitro (Robinson et al., 2011) and are producers of the type I family of interferon-α and -β, and type III interferon (IFN-λ) in response to FMDV infection (Reid et al., 2011). Initially in the immune response, the host antibodies can destroy DC and block the stimulation of FMDV-specific T-cells (Knight-Jones *et al*., 2016). Whilst only live FMDV is capable of stimulating IFN-α responses and through internalisation triggering apoptosis and viral peptide delivery on MHC class I molecules, inactivated virus does not cause this effect (Langellotti et al., 2015). An increase in IL-10 production by DC and monocytes is capable of directing adaptive immunity towards the development of a stronger humoral response rather than a T-

cell mediated response (Diaz-San Segundo et al., 2009; Robinson et al., 2011). FMDV also inhibits the maturation of *in-vitro* generated DC, subsequently affecting their ability to prime T-cells and inhibiting antigen processing by DC (Diaz-San Segundo et al., 2009; Sei et al., 2016).

Two fundamental problems need to be understood before more effective FMD vaccine control measures can be put in place. These problems are the FMDV ‘‘carrier state’’ and the short duration of immunity after vaccination which contrasts with prolonged immunity after natural infection. Juleff et al. (2008) showed that FMDV locates rapidly to, and is maintained in, the light zone of germinal centres (GCs) following primary infection of naïve cattle. Maintenance of non-replicating FMDV in these sites may represent a source of persisting infectious virus and contributes to the generation of long-lasting antibody responses against neutralising epitopes of the virus (Juleff et al., 2008). The efficient retention within the GCs of intact viral capsids, as opposed to the constituent viral proteins, may be a requirement for sustaining antibody responses relevant for providing protection against challenge. Hence, stabilised vaccines containing stable capsids as antigens may be more efficiently retained within GCs, which could result in sustained antibody immune responses leading to longer duration immunity as apposed to unstable wild-type antigens.

1.6 Design of improved inactivated vaccines and immunity

FMDV is inactivated through binary ethylenimine or BEI inactivation, which converts infectious FMDV into non-infectious FMDV through nicking the RNA genome (Bahnemann, 1990). It is prepared through two substances, 2-bromo-ethylamine (BEA) and NaOH. BEI converts in an alkaline solution to ‘binary’ ethylenimine. The active substance is the ethylenimine ring in all aziridine compounds (Bahnemann, 1990).

Some of the above-mentioned limitations of current vaccines are being addressed by the development of reverse genetic approaches. Additionally, new alternative vaccines that do not require infectious virus as well as efforts to understand the role of innate immunity and cytokines to induce protection and boost the immune response offer tremendous potential for the control of FMD in endemic regions.

Genome-length viral RNA (vRNA) derived from complementary DNA (cDNA) clones of FMDV is infectious when transfected into suitable mammalian cells (Zibert

et al., 1990; Rieder et al., 1994; Almeida et al., 1998; van Rensberg et al., 2004). These cDNA clones are readily amenable to genetic engineering to introduce changes to the virus genome that allows for the replacement of the external capsid-coding region or structural, surface-exposed antigenic loops with the corresponding regions of an emerging virus. The outcome of such a chimeric virus is the transfer of the spectrum of neutralizing epitopes from the aetiological agent to the recombinant virus (Zibert et al., 1990; Rieder et al., 1994; Almieda et al., 1998, van Rensberg and Mason, 2002; van Rensberg et al., 2004) and the ability to antigenically simulate the outbreak virus and induce protective immunity in host animals (Rieder et al., 1994, Maree, 2010; Blignaut et al., 2011). Furthermore, it has been shown that inter-serotype chimeric vaccines with the capsid proteins of SAT1 within a SAT2 background (Blignaut et al., 2011) and O serotype within an A serotype background (Rieder et al., 1994; Fowler et al., 2008) confer protective immunity. However, capsid swapping may transfer other undesirable traits such as capsid instability and poor cell culture adaptation, which are limitations that can be overcome by site-directed mutagenesis of the amino acid(s) associated with improved performance as vaccine candidates (Maree et al., 2010).

1.7 Alternative vaccine strategies: subunit and live viral-vectors

The development of potent new generation vaccines for FMD has been the subject of intense research during the last few decades. In line with other medical and veterinary viral vaccines, research and development on FMD vaccines has focused mostly on subunit and vectored vaccines. Peptides or purified proteins, recombinant DNA, viral vectors and plants expressing FMDV structural proteins, with or without immunopotentiators, have been demonstrated to elicit humoral and cell-mediated immune responses in experimental animals and shown to protect natural hosts to varying degrees in enclosed settings (Balamurugan et al., 2005; Du et al., 2007; Wang et al., 2008; Su et al., 2008; Cubillos et al., 2008; Li et al., 2008; Choudary et al., 2008; Yang et al., 2008; Greenwood et al., 2008). However, while these approaches show promise for use in FMD-free zones, none of them have so far been subjected to large-scale field trials in endemic countries.

Due to the limitations of inactivated vaccines, alternative strategies for vaccine development have focused on the use of VP1-proteins and peptides either isolated from FMDV or produced by recombinant DNA (Bachrach et al., 1975; Kleid et al.,

1981), VP1-derived peptides (Strolmaier et al., 1982) or chemically synthesized VP1 peptides (DiMarchi et al., 1986; Francis et al., 1991; Nargi et al., 1991; Bittle et al., 1992), vectors expressing VP1 fusion proteins (Kit et al., 1991; Kitson et al., 1991; Clarke et al., 1997), inoculation with DNA expressing VP1 epitopes alone (Wong et al., 2000) or with DNA encoding IL-2 (Wong et al., 2002), and transgenic plants or recombinant tobacco mosaic virus expressing VP1 (Mulcahy et al., 1991; Taboga et al.,1997). However, they rarely achieved protection against virus challenge in livestock (Dimarchi et al., 1986; Mulcahy et al., 1990 and 1991), or as a result of a limited subset of epitopes, selected for antigenic variants that escaped from protection (Taboga et al., 1997). The reduced level of protection may be due to a lack of T-cell epitopes (Rodriquez et al., 1994). Whereas, peptides and purified proteins have failed to command an important place in vaccinology due to various reasons, the use of recombinant DNA in large domestic and wild animals may be difficult due to the necessity of injecting large and multiple doses, the period needed to attain optimal responses (compared to the rapidity with which FMD spreads through herds), delivery issues, and the potential for vaccinated animals to be carriers (e.g., suboptimal responses may suppress superinfection with a field virus but may not be able to prevent infection or virus shedding).

Subviral particles or virus-like particles which are assembled as empty particles lack RNA (non-infectious), but are immunogenic, as their antigenic surface is indistinguishable from the complete virus (Grubman et al., 1993). Previous attempts to express the P1, leader, and 3C regions in recombinant baculovirus systems has yielded limited amounts of empty capsid due to toxicity in insect cells (Rooisen et al., 1990) or in *Escherichia coli* it resulted in inefficient capsid assembly (Grubman et al., 1993). Major advances have been made, firstly in developing methods to efficiently express empty capsids in vaccinia virus or baculovirus expression systems by lowering viral protease activity, which is toxic to cells, and secondly by enhancing capsid stability (Porta et al., 2013). Cattle vaccinated with such an A-serotype construct have shown sustained VNT titres and protection from challenge 34 weeks post-vaccination (Porta et al., 2013). This approach has several advantages over conventional vaccines, by reducing production costs, eliminating risk of infectivity, and enhancing stability. However, producing vaccines using insect cells requires highly skilled staff, and scaling up of the manufacturing process is difficult and expensive to optimize, which poses disadvantages for the African market. More studies are needed

to assess whether this strategy would be effective in endemic regions of Africa for SAT serotypes and when applied to the field and to find out whether the production process would be economically viable.

Alternative strategies which incorporate sections of the FMDV genome inserted in a live viral vector have been investigated with: a) bovine rhinotracheitis virus (Kit et al., 1991); b) the G–H loop region has been incorporated in a chimeric poliovirus (Kitson et al., 1991); c) capsid and/or non-structural proteins in vaccinia virus (Berrinstein et al.; 1995; Sanz-Parra et al., 1998); d) fowlpox virus (Zheng et al., 2006) or e) pseudorabies virus (Yao et al., 2007; Li et al., 2008) provided partial protection in pigs and guinea pigs. A chimeric bamboo mosaic virus containing 1D epitopes induced humoral and cell-mediated responses and protection in pigs (Yang et al., 2007). The experimental vaccines developed so far have several disadvantages, such as requirement for multiple doses, low-level antigen expression, and uncertain safety. Additionally, repeated vaccination with a viral-vector vaccine, as is necessary in endemic regions, leads to immunity against the vector and reduced protection. The most successful advances have arisen from incorporating FMDV genes into replication-defective human adenovirus (Ad) vectors (Grubman et al., 2010; Rodriquez et al., 2011). The resulting vaccine has several advantages over conventional vaccines: 1) it can induce protection within seven days and within one day if combined with interferon-α expression, particularly useful for emergency use; 2) it is fully compatible with test systems that allow to differentiate infected from vaccinated animals; 3) incomplete inactivation problems are not a concern; and 4) it has excellent immunogenicity, attributed to its natural adjuvant properties, high transduction efficiency of targeted cells and ability to induce antiviral cytotoxic T-cells (Grubman et al., 2010). However, whether animals vaccinated with Ad-FMD vaccines would be protected from developing a carrier state is unclear, as is the significance of pre-existing immunity in the field. The potential for these in restricting FMD outbreaks or to eradicate them in endemic countries is not clear. Finally, it is of major importance to determine the protective immunity and breadth of antigenic coverage against evolving variants in the field.

To be optimal for use in Africa, new vaccines should be easily delivered to the animal, proven to afford better protection and induce longer lasting immunity than conventional inactivated vaccines, safe and easy to prducein produce and inexpensive. This is especially important for African producers that need to design

vaccines tailored to African continental control, focusing on circulating serotypes and subtypes in endemic regions where eradication is difficult to attain due to the presence of maintenance hosts and continuous wildlife–livestock interfaces.

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CHAPTER 2

Structure-based energetics of protein interfaces guides foot-and-mouth disease virus SAT2 vaccine design – a proof of concept

2.1 Abstract

Virus capsids are primed for disassembly, yet capsid integrity is key to generating a protective immune response. Foot-and-mouth disease virus (FMDV) capsids comprise identical pentameric protein subunits held together by tenuous noncovalent interactions and are often unstable. Chemically inactivated or recombinant empty capsids, which could form the basis of future vaccines, are even less stable than live virus. Oxford University devised a computational method to assess the relative stability of protein-protein interfaces and used it to design improved candidate vaccines for two poorly stable, but globally important, serotypes of FMDV: O and SAT2. A restrained molecular dynamics strategy was used to rank mutations predicted to strengthen the pentameric interfaces and we applied the results to produce stabilized SAT2 capsids. Structural analyses and stability assays confirmed the predictions, and vaccinated animals generated improved neutralizing-antibody responses to stabilized particles compared to parental viruses and wild-type capsids.

2.2 Introduction

FMDV is a small-RNA virus whose icosahedral capsid contains 60 copies each of proteins VP1–VP4. FMDV is responsible for a contagious, economically devastating livestock disease endemic in many developing regions of Asia and Africa; developed countries are also at risk of sporadic but serious outbreaks (for example, in the UK in 2001, in Japan in 2010 and in Egypt in 2012[1–3]). In areas where FMDV is endemic, disease control is predominantly by vaccination. There are seven serotypes of FMDV (A, O, C, Asia, and Southern African Territories (SAT) 1, 2 and 3)[4], and each contains multiple and constantly evolving subtypes. A vaccine against one serotype does not protect against other serotypes or subtypes within a serotype, thus necessitating the continued development of new vaccine strains. The majority of vaccines produced worldwide are serotype O, whereas SAT2 is the most prevalent serotype in sub-Saharan Africa[5].

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FMDV serotypes differ markedly in their capsid stability: A and Asia-1 are relatively stable, whereas O and SAT viruses are more sensitive to heat and low pH. Commercial vaccines are produced by chemical inactivation of virus particles, which renders them even less stable, and above 30 °C they rapidly convert into immunogenically incompetent pentameric subunits[6,7]. Thus, vaccine integrity requires expensive, difficult-to-maintain cold chains, and effective protection requires frequent immunizations[5,8]. Recombinant empty capsids show promise as future vaccines because they overcome many of the disadvantages of preparing vaccines from live virus; however, they are less stable than virus particles[9].

Knowledge of the three-dimensional structure of viral capsids should allow for rational engineering of their properties. Although four different serotypes of FMDV have been analyzed in atomic detail[10–14], few attempts have been made to engineer thermostable capsids[15,16]. The pentameric subassemblies are highly stable, so stabilization of the interpentamer interactions should be sufficient to enhance capsid stability and the effectiveness of vaccine preparations. The introduction of a disulfide bond across the interface between adjacent pentamers has enabled the production of thermostable FMDV empty capsids for an A serotype (A22 Iraq)[17]. However, covalent stabilization may not be compatible with virus viability or with the structure of all viruses[16].

Here, we evaluated mutations designed to increase capsid stability by increasing non-covalent interactions and applied it to the unstable SAT2 serotype. Stability assays confirmed that the method is predictive of increased stability. Furthermore, animals vaccinated with candidate mutant viruses generated improved neutralizing-antibody responses, thus demonstrating potential value for such stabilized vaccine antigens.

2.3 Materials and Methods

2.3.1 Modelling of stabilising mutations

Model preparation for molecular dynamic (MD) simulations; preparation of truncated models for simulation; the design and construction of candidate mutants, MD simulation protocol, and estimation of degree of stabilization ($\Delta\Delta G$) which resulted in

the rational design of a panel of putative stabilizing mutations that might improve hydrophobic and/or electrostatic interactions across the interface (Table S1) were described by Kotecha et al., 2015[18].

2.3.2 Generation of infectious recombinant cDNA

The *ca*. 2.2-kb external capsid-coding region (1B-1D/2A) of the plasmid pSAT2[19], was digested with endonucleases *EcoRI* and *Xma*I and cloned into corresponding sites of the pBluescript® II SK (pBS) (Stratagene) vector to generate the template for site-directed mutagenesis (pBS-P1). Mutations were introduced into pBS-P1 (120 ng) using overlapping inner mutagenesis oligonucleotides (Chapter 3) and the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene) according to manufacturer's instructions. Cycling conditions were performed as follows: 95 °C followed by 18 cycles of 95 °C for 30 sec, 55 °C for 60 sec, 68 °C for 6 min.

The mutated pBS-P1 constructs were confirmed with sequencing (Big Dye Terminator v3.1 cycle sequencing kit, Applied Biosystems). The mutated external capsid-coding region (1B-1D/2A) of plasmid pBS-P1 was digested with endonucleases *SspI* and *Xma*I to recover the mutated capsid-coding region and cloned back into pSAT2. The mutated recombinant pSAT2 constructs were confirmed by nucleotide sequencing.

2.3.3 Preparation of infectious RNA, and transfection

Recombinant, mutated pSAT2 plasmids were linearized with *SwaI* and *in vitro* transcribed using the MEGAscript™ T7 kit (Ambion). The integrity of the RNA was analysed by agarose gel electrophoresis. *In vitro* transcribed RNA (3 µg) was 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_2 influx for 48 h in virus growth medium (VGM; Eagle's basal medium (BME) supplemented with 1% foetal calf serum, 25 mM HEPES) and frozen at -80 °C. 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)[20] or BHK-21 cells were then used to passage the viruses up to six times or until > 90% CPE was observed. The mutated viruses derived from the

genome-length cDNA, were confirmed by nucleotide sequencing and used in subsequent experiments.

2.3.4 Genome amplification and sequencing

Total RNA was extracted from infected cell cultures using the QIAamp Viral RNA Mini Kit (Qiagen), according to the manufacturer's specifications and used as template for cDNA synthesis. Viral cDNA was synthesised with SuperScript III™ (Life Technologies) with oligonucleotide 2B208R (5'GACATGTCCTCC TGCATGTG)[21] and were carried out at 50 °C for 50 min. The Leader/capsid-coding region of the mutated viruses was amplified using Expand Long template Taq DNA polymerase™ (Roche) with genome-specific oligonucleotides (NCR2:5'-GCTTCTATGCCTGAATAGG and WDA: 5'-GAAGGGCCCAGGGTTGGACTC). Direct DNA sequencing of the amplicons or plasmids was performed using the ABI PRISM™ BigDye Terminator Cycle Sequencing Ready Reaction Kit v3.0 (Perkin Elmer Applied Biosystems). The consensus nucleotide sequence of the capsid-coding region was assembled with Sequencher 4.7 DNA sequence analysis software (Gene Codes Corporation, USA) and compared to that of SAT2/ZIM/7/83 (Genbank accession codes: JQ639289 and DQ009726).

2.3.5 Plaque assay

Plaque assays were performed in triplicate by infecting BHK-21 monolayer cells in 35 mm cell culture plates (Nunc™) for 1 h, followed by the addition of 2 ml tragacanth overlay[22]. Following incubation at 37ºC for 48 h the infected monolayers were stained with 1% (w/v) methylene blue in 10% (v/v) ethanol and 10% (v/v) formaldehyde in phosphate buffered saline, pH 7.4. The diameters of 50 parental (wild-type) plaques were measured with a microscope eyepiece micrometer. These were averaged, defined as 100%, and compared against mutant plaque diameters (n=50).

2.3.6 Inactivation and purification of viruses

Infected BHK-21 monolayers were lysed with 0.1% (w/v) Nonidet and 20 mM EDTA (pH 8.0) and clarified by centrifugation at 2000 g for 30 minutes at 4ºC. Virus in the supernatant was inactivated with 5 mM binary ethyleneimine (BEI) for 26 h at 26

°C and 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[23]. Following fractionation, peak fractions corresponding to 146S virion particles were calculated using the extinction coefficient E_{259} nm = 79.9[24] and pooled for analysis. Complete inactivation was verified by titration on BHK-21 cells.

2.3.7 Thermo-stability assay for particles

The particle stability thermal release assay (PaSTRy)[25] was performed in 96-well PCR plates using an Agilent MX3005 PCR machine. The assays were performed using 300-500 ng of purified virus (3-15 µl). A volume of 15 µl of SYBR Green-II dye (Molecular Probes, Invitrogen) was diluted 1:100 in the same buffer as used for virus purification and the volume made up to 150 µl with buffer. The temperature was ramped from 25˚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.

2.3.8 Cattle and guinea pigs vaccination with stabilised FMDV vaccines

Two groups of four 100 to 150 kg Holstein Friesian calves were vaccinated with inactivated: SAT2 Zim7/83-wt or SAT2 Zim7/83-(S93Y) FMDV. Each animal received 15 µg of purified 146S antigen formulated in oil adjuvant ISA206B (SEPPIC) as an intramuscular injection on days 0 and 28 of the study. All eight animals were bled to collect serum on days 0, 28, 35 and 42. Animal experimentation was approved and perfromed by the Pirbright Institute ethical review board under the authority of a Home Office project licence in accordance to the Home Office Guidance on the operation of the Animals (Scientific Procedures) Act 1986 and associated guidelines.

For the guinea pig experiments inactivated SAT2 wild-type and stabilised mutant S93Y were formulated as commercial vaccines using oil-based ISA206B (SEPPIC) adjuvant and stored for 1 month at 4˚C. Aliquots containing 10 µg of 146S antigen at the time of formulation of these vaccines were then used to immunise two

groups of 10 guinea pigs each at MSD in Germany. One group received the wild-type vaccine while the other group received the stabilised vaccine. Serum samples were collected on day 0, followed by 3 and 6 months pv bleeds and virus neutralising antibody titres (VNT) were assessed. The experiments were repeated after the vaccines had been stored for a total of 6 months at 4 ˚C. Serum samples were collected on days 0 and 30 pv and the VNTs performed.

2.3.9 Titration of neutralising antibodies

Sera from guinea pigs and cattle were prepared from blood samples. Sera obtained at weekly intervals from day 0-42 pv were titrated by a standard virus neutralization test (VNT) (OIE, 2012) on porcine kidney IB-RS2 cells using SAT2 wild-type virus. Neutralizing antibody titres, calculated by the Spearmann-Karber method, were expressed as the last dilution of serum that neutralizes 50% of the homologous virus (100TCID50)[22].

2.4 Results

2.4.1 FMDV capsids dissociate into pentameric subassemblies

Inactivated FMDV capsids dissociate into pentameric subunits at pH <7.0, at temperatures >30 °C[1,4,6,15] and after prolonged storage at 4 °C (**Fig. 2.1a,b**). We examined the interface between pentamers for opportunities to stabilize these interpentamer contacts by enhancing noncovalent interactions. From four different serotypes[10,11,13,14,26], we identified 85 amino acids contributing to this interface: 57 from VP2 and 28 from VP3 (**Fig. 2.1c,d**). VP2 shows ~80% sequence identity between different serotypes, and we selected non-conserved interface residues inaccessible to antibodies and thus unlikely to affect antigenicity (**Sup. Fig. 1**) for the introduction of potentially stabilizing interactions (for each residue, a range of mutants was possible). We focused on the region close to the icosahedral two-fold axis because enterovirus uncoating is known to initiate at this point, and disorder of the N terminus of VP2 adjacent to the three-fold axis makes this alternative region less reliable for modelling[27–29]. We chose SAT2/ZIM/7/83 (SAT2) as our target antigen. SAT1 (PDB 2WZR)[14] was used as the model for SAT2 (78% identity in VP2). On the

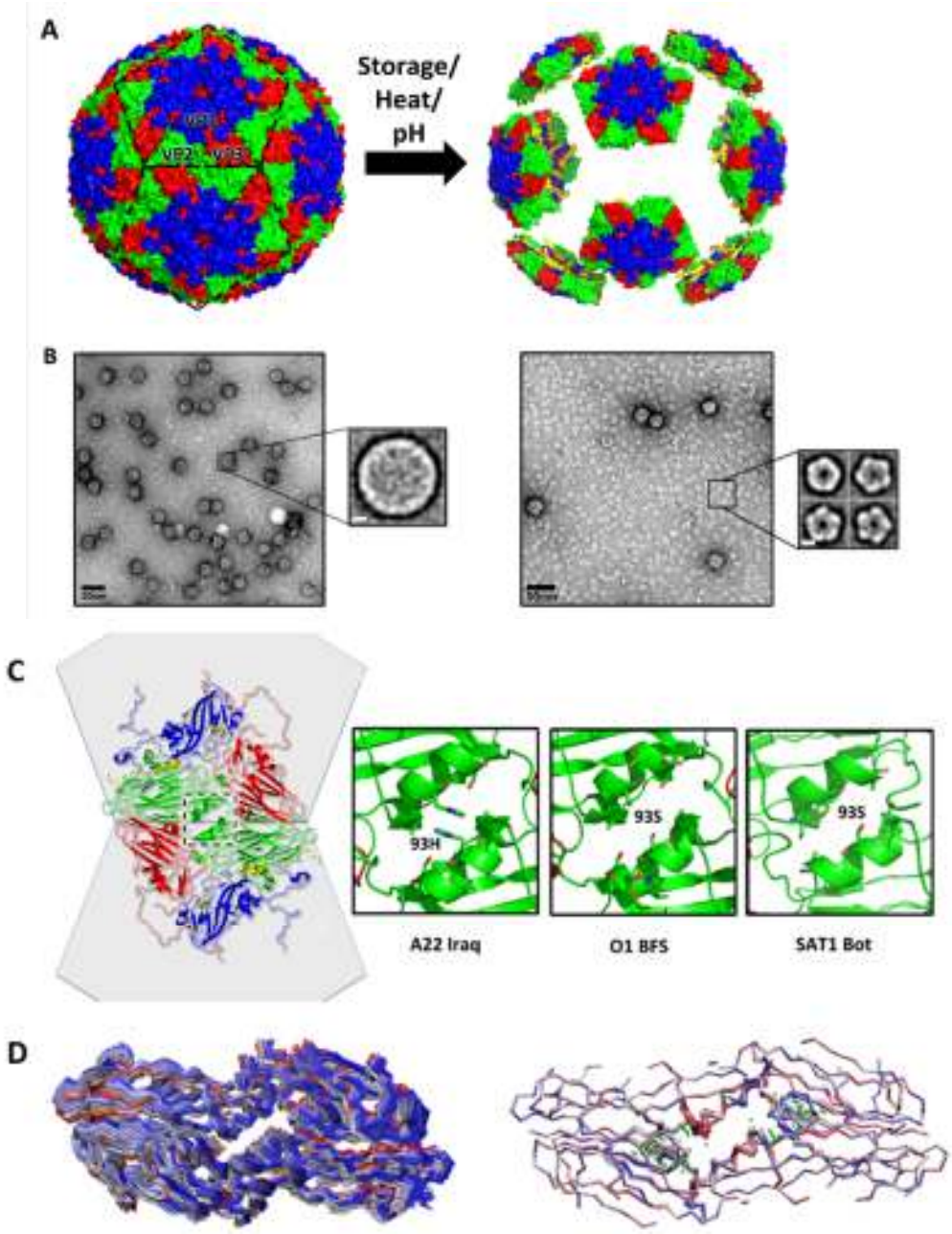

Figure 2.1 Dissociation of FMDV capsids into pentameric assemblies and design of the models used for MD simulations. (**a**) Surface representation of atomic models of the intact FMDV serotype-O capsid (left) and of its dissociation into 12 pentameric assemblies upon storage, heating or lowering of pH (right). Blue,VP1; green,VP2; red,VP3; yellow,VP4. (**b**) Negative-stain EM images of the inactivated O1M wild-type capsids after purification (left, capsids intact) or after 10 d of storage at 4 °C (right, 80% dissociated into pentamers). Scale bars, 50 nm. (**c**) Left, cartoon representation of the atomic structure of the O1M model, showing two icosahedral protomers forming an interpentameric interface. A truncated model was generated by trimming the protomers to include VP2 and VP3 atoms within 13 Å of the interface, as shown in **d**. Right, residues on two-fold symmetry–related helices for O, A and SAT serotypes. (**d**) 1.5-ns MD trajectory. Left, large r.m.s. deviation from the starting structure for an unrestrained model. Right, restrained MD trajectory, with dummy atoms (green) placed at the midpoints of the interprotomeric interface to define the restraints. The beginning of the trajectory is shown in red, the middle in white and the end in blue.

basis of visual inspection of the structures, a panel of potential stabilizing mutants within VP2 were chosen which we then modelled by in silico mutagenesis and localized energy minimization[18].

2.4.2 Putative mutations that confer increased stability

The results of the free-energy calculations are shown (**Table 2.1**). Residue 93 of VP2 is part of an α-helix adjacent to the icosahedral two-fold axis and is largely conserved within but not between serotypes (**Sup. Fig. 1 and Fig 2.1c**). The relatively stable A serotype has a histidine at this position, whose imidazole ring forms a hydrophobic stacking interaction with the corresponding symmetry-related moiety. Simulation results suggested that this virus could be further stabilized by mutation to tyrosine or, more preferably, to phenylalanine. For SAT2, the simulations suggested that stability would be enhanced by mutation of the equivalent serine to histidine (S93H) and would be further improved by mutation to tyrosine, whose side chain was predicted to hydrogen-bond to the backbone of VP2 89. A phenylalanine was predicted to confer even greater stability. Substitution with a bulky tryptophan was less effective, as were residues with non-aromatic hydrophobic side chains (valine, leucine, isoleucine and methionine) (**Table 2.1**). Some VP2 mutations away from the two-fold axis produced stabilization (S97Q, S97I and to a lesser extent V90N), and Y98F was predicted to be most effective.

Table 2.1: Binding free energy of putative mutations that confer increased stability: capsid-stabilising mutants for O1Manisa, SAT2 ZIM7/83 and A22 serotype viruses.

Mutation	O1 Manisa $\Delta\Delta G$ kcal/mol	SAT2 ZIM $\Delta\Delta G$ kcal/mol	A22 Iraq $\Delta\Delta G$ kcal/mol
VP2 93H	−7.7	−5.3	NA
VP2 93Y	−11.8	−12.2	−7.7
VP2 93F	−13.8	−13.2	−10.3
VP2 98F	−10.3	−5.6	–
VP2 97I	−6.8	–	–
VP2 93V	−7.1	–	–
VP2 93L	−1.9	–	–
VP2 93I	−7.2	–	–
VP2 93M	−2.8	–	–
VP2 93W	−9.5	–	–
VP2 97Q	−5.5	–	–
VP2 90N	−3.5	–	–
Negative controls			
VP2 60G	+33.7		
VP2 60L	+24.0		
VP2 57E	+17.7		
VP2 57L	+12.3		

NA, not applicable.

2.4.3 Mutant viruses are infectious and stable

The effects of the amino acid mutations were first tested in recombinant virus. Transcripts were transfected into BHK-21 cells to generate infectious stocks. These stocks were then used to infect ZZ-R127 goat epithelial cells, which are highly susceptible to FMDV infection[20]. Subsequent passages in BHK-21 cells, which are commonly used in the production of FMD vaccines, were possible because the SAT2 virus was cell-culture adapted. All recombinant viruses were sequenced after four passages, and the results revealed no changes or obvious subpopulations. All produced clear, consistent, cytopathic effects (CPE); however, we observed differences between viruses. The patterns of CPE and plaque sizes varied markedly, and in general stabilized viruses yielded substantially smaller plaques than the parental viruses (**Fig. 2**). However, the yields of wild-type and mutant viruses were broadly similar (**Sup. Table 1**).

We determined capsid stability by fluorescence thermal stability assays[25] at pH 7.5. Inactivated SAT2: wild-type and S93W capsids dissociated at a similar temperature (47.0 °C), whereas S93H and S93Y dissociated at 51.0 °C and 53.5 °C, respectively (**Fig. 2.2 and Sup. Table 2**). In conclusion, the mutant viruses were

consistently more stable at elevated temperatures and at low pH, and the results correlated well with the MD simulations. As part of the Wellcome Trust collaboration we purified SAT2 viruses with the aim to crystallise SAT2 at Oxford University. However, this was not possible due to the inability to obtain diffraction-quality crystals and only the structures of inactivated SAT2 S93Y were resolved by cryo-EM. The 8,156 particles of S93Y SAT2 yielded structures of 3.5-Å resolution (Fourier shell correlation of 0.143) (**Fig. 2.3ab, Sup. Fig. 2 & Table 3**).

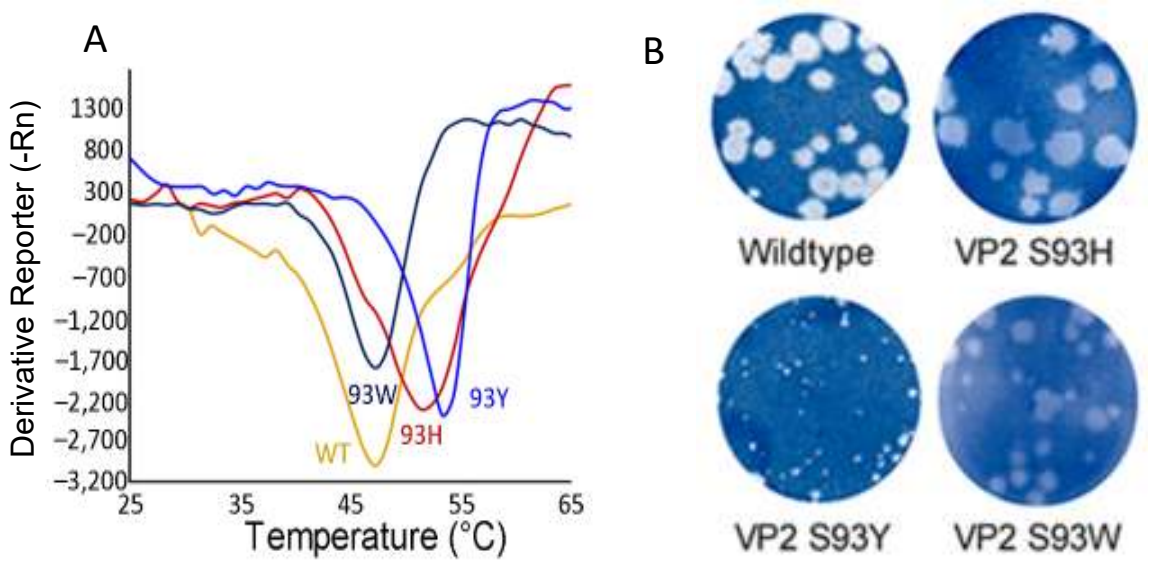

Figure 2.2: Growth characteristics and stability of the engineered capsids. (**a**) Fluorescence assay measuring thermostability of inactivated SAT2 viruses at pH 7.5 (ref. 29). *F*, fluorescence; *T*, temperature. (**b**) Plaques formed in BHK-21 cells by wild-type and mutant SAT2 viruses. The patterns of CPE correlated with plaque size.

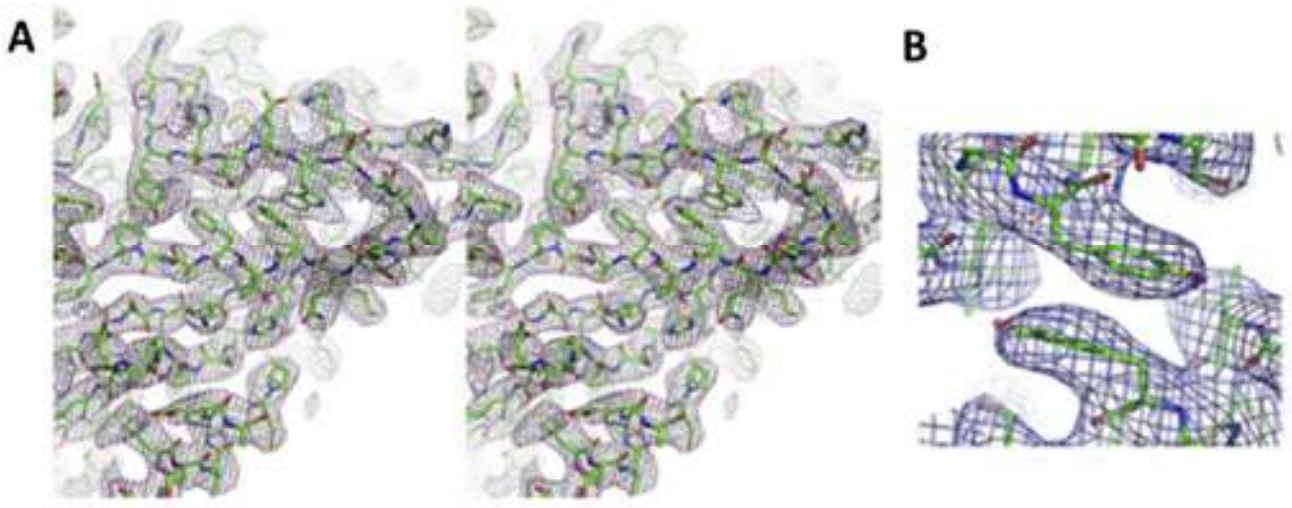

Figure 2.3: Structural analysis of stable engineered capsids. The structures of engineered mutants SAT2 S93Y were determined by X-ray crystallography and cryo-EM. (**a**) Stereo views of the density from cryo-EM reconstructions for SAT2 S93Y at 3.5 Å. The quality of the cryo-EM density maps allowed unambiguous fitting and refinement of the major capsid proteins. (**b**) Cryo-EM maps showing the density for S93Y for SAT2 at 3.5-Å resolution.

2.4.4 Immunogenicity of stabilized mutants

To compare the immunogenicity of wild-type and stabilized viruses, we vaccinated calves with purified inactivated wild-type or mutant (S93Y) virus of the SAT2 serotype. We maintained inactivated antigen at 4 °C and purified it immediately before use. We measured serum virus neutralization antibody titers (VNTs) at different times post vaccination (pv) (**Fig. 2.4a**). In all animals, we observed high VNTs on day 28 at the time of boost immunization (mean antibody titers >2 log10, which is considered protective[30]); titers were substantially increased (>3 log10) by day 42 pv.

Because mutant and wild-type antigens had equivalent immunogenic properties when administered directly after purification, we compared their immunogenicity after storage. For this, we stored inactivated wild-type and mutant S93Y FMDV SAT2 particles at doses of 10 µg each, formulated as described above, for 1 month at 4 °C and used them to vaccinate two groups of ten guinea pigs. We assessed titres using VNT at 3 and 6 months pv (**Fig. 2.4b**). At 3 months pv, we observed substantially higher VNT titres in animals vaccinated with the stabilized SAT2 antigen (>8 log2 considered protective[31]) than in animals vaccinated with the wild-type SAT2 antigen (<3 log2). At 6 months pv, the group mean VNT titres in animals immunized with the stabilized SAT2 antigen remained at >8 log2, whereas

wild type–immunized animals' antibody titers remained below protective levels of <3log2. Thus, the stabilized vaccine performed markedly better than the wild-type vaccine.

In a further iteration, we stored equivalent aliquots of wild-type and stabilized vaccines for 6 months at 4 °C and again used them to immunize two groups of ten guinea pigs. No animal had a VNT titer at day 0, but by day 30 pv, although the mean VNT titer of the group inoculated with wild-type vaccine remained low (~3 log2), the mean titer of the group inoculated with stabilized virus was markedly higher and was consistent with protection, >6 log2 (**Fig. 2.4c**). Thus, only stabilized particles were able to induce VNT titers, a result consistent with protection after 1 month or 6 months of storage at 4 °C.

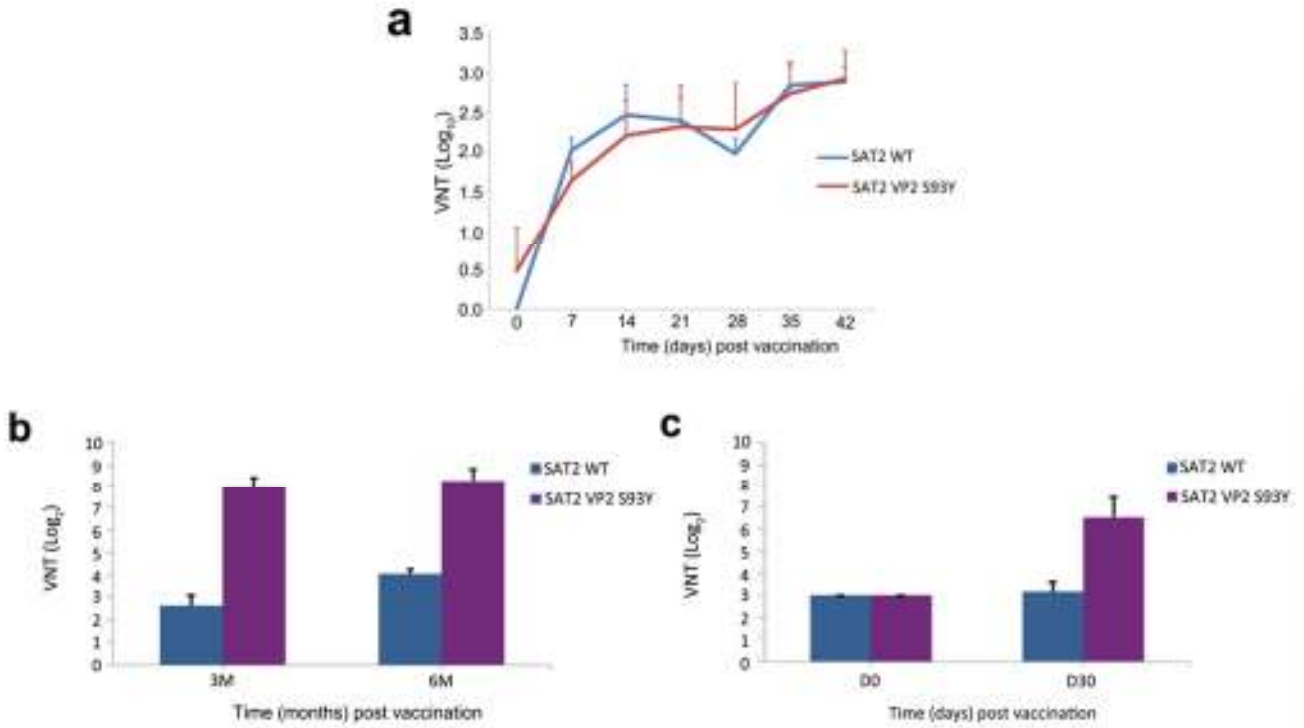

Figure 2.4: Immunogenicity of inactivated wild-type and stabilized viruses. (**a**) Mean virus neutralizing-antibody titer (VNT (log_{10})) of calves vaccinated with purified inactivated virus of the SAT2 serotype. Groups of four calves were vaccinated with either wild-type or mutant S93Y at days 0 and 28; blood samples were assayed at 0, 7, 14, 21, 28, 35 and 42 days pv, error bars, s.d. (**b**) VNT titres of guinea pigs to assess immunogenicity of inactivated particles after 1 month of long-term storage. Before immunization, formulated vaccines were stored for 1 month at 4 °C. Two groups of ten guinea pigs were each immunized with either wild-type or S93Y SAT2 antigen, and VNT titres were assessed at 3 and 6 months pv. Error bars, s.d. (**c**) VNT titres of guinea pigs to assess immunogenicity of inactivated particles after 6 months of long-term storage, showing protective VNT titres for only stabilized antigen. Equivalent aliquots of wild-type and stabilized SAT2 vaccines were stored for 6 months at 4 °C before inoculation of two groups of ten guinea pigs. Error bars, s.d

2.5 Discussion:

FMDV vaccines with higher stability have the potential to be more effective. However, very few natural FMDV variants with increased resistance to heat or pH[32,33] have been reported, possibly because overstabilization hinders the release of the viral genome during cell entry. We therefore investigated the rational introduction of mutations to increase thermostability, earlier attempts at which have had only marginal success[16]. It was previously reported that a stable disulfide bond at the icosahedral two-fold axis between adjacent pentamers in the A22 virus, thus allowing the production of thermostable and pH-stable recombinant empty particles[17]. Because covalent modification was unlikely to be suitable with engineered viruses and may not be generally applicable, we devised a strategy that allows general mutations conferring noncovalent stabilization to be evaluated *in silico*. We exploited highly restrained atomistic MD simulations focused on the appropriate part of the complete virion to efficiently and reliably calculate the changes in binding free energy for interactions between adjacent pentamers within the FMDV capsid, thus allowing screening and ranking of candidate mutations[18]. We found that a single-residue mutation generating hydrophobic stacking of aromatic side chains at the two-fold axis between adjacent pentamers (position 93 of VP2) was effective in stabilizing the particle, results echoing those from studies in enteroviruses that identify the place at which the particle opens up to start uncoating[28,29]. The predicted degree of stabilization correlated with hydrophobicity.

Engineering the stabilization mutations decreased the plaque sizes for both O1M[18] and SAT2 viruses, possibly by delaying viral genome release. Both infectious viruses and recombinant empty capsids carrying these mutations were indeed thermostable and pH stable, and the degree of stabilization mirrored the *in silico* calculations. This noncovalent stabilization of two particularly unstable serotypes produced particles of similar stability to that of covalently cross-linked A22 particles[17]. Formulated vaccines from inactivated wild-type and stabilized SAT2 viruses produced equivalent protective levels of neutralizing antibodies in cattle, thus demonstrating the immunogenicity of the engineered particles. This was also achieved for the O1 Manisa virus of the O serotype in this collaborative study[18]. The stabilized inactivated vaccine antigen was also intact after 96 h at 37 °C[18] for O1 Manisa virus and is expected to be more tolerant

of suboptimal cold-chain performance; goals for the stability of SAT2 have been described by Anderson *et al.*[34]. Furthermore, after storage for 1 or 6 months at 4 °C, stabilized particles produced substantially higher VNT titres in guinea pigs than did wild-type particles, commensurately with an increase in vaccine shelf life.

In conclusion, a new restrained MD protocol to drive structure-based design of stabilized FMDV viruses and empty capsids was reported in this study[18]. Such new effective FMDV vaccines would, in the context of empty capsids, eliminate the considerable risks involved in producing vaccine from infectious virus. This strategy may be relevant to many other picornaviruses that are responsible for causing a wide range of mild–to–life-threatening diseases affecting humans and other animals.

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2.7 Supporting Publication

Supplementary 2.1: The full publication containing the work on the SAT2 virus (ARC-OVI); O-serotype (The Pirbright Institute) and full modelling (Oxford University) was published as:

Kotecha, A., Seago, J., Scott, K., Burman, A., Loureiro, S., Ren, J., Porta, C., Ginn, H.M., Jackson, T., Perez-Martin, E., Siebert, C.A., Paul, G., Huiskonen, J.T., Jones, I.M., Esnouf, R.M., Fry, E.E., Maree, F.F., Charleston, B, Stuart, D.I. (2015). Structure-based energetics of protein interfaces guides foot-and-mouth disease virus vaccine design. Nature Structural and Molecular biology 22(10), 788-7

CHAPTER 3

SAT2 foot-and-mouth disease virus (FMDV) structurally modified for increased thermostability

3.1 Abstract

Foot-and-mouth disease virus (FMDV) is notoriously unstable, particularly the O and SAT serotypes. Consequently, vaccines derived from heat-labile SAT viruses have been linked to the induction of poor duration immunity and hence require repeated vaccinations to ensure protection. *In-silico* calculations predicted residue substitutions that would increase interactions at the inter-pentameric interface supporting increased stability. We assessed the stability of 18 recombinant mutant viruses for their growth kinetics; antigenicity; plaque morphology; genetic stability; temperature, ionic and pH stability using the thermofluor and inactivation assays, in order to evaluate potential SAT2 vaccines candidates with improved stability. The most stable mutation for temperature and pH stability was the single mutant S93Y, whilst other promising single mutants were E198A, L94V, S93H and the triple mutant F62Y-H87M-H143V. Although the S93Y mutant had the greatest stability it exhibited smaller plaques; a reduced growth rate; a change in a monoclonal antibody footprint, and poor genetic stability properties compared to the wild-type virus. However, these factors affecting production can be overcome by reverse-genetic approaches or changes to production. The addition of 1 M NaCl salt further increased the stability of the SAT2 panel of viruses. The S93Y and S93H mutants were selected for future use in stabilising SAT2 vaccines.

3.2 Importance

Foot-and-mouth disease virus (FMDV) causes a highly contagious acute vesicular disease in cloven-hoofed livestock and wildlife. The control of the disease by vaccination is essential especially at livestock-wildlife interfaces. Vaccines produced from unstable serotypes such as SAT2 are often of poor quality and induce a short duration of immunity. We have shown that by mutating residues at the capsid interface

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through predicted modelling we can improve the stability of SAT2. This is an important finding for the potential use of such mutants in improving the stability of SAT2 vaccines in endemic countries that rely heavily on the maintenance of the cold-chain, with potential improvement to the duration of immune responses.

3.3 Introduction

Emerging and re-emerging diseases caused by RNA viruses represent a major threat to both public and animal health, thus affecting food insecurity. Foot-and-mouth disease (FMD) virus (FMDV), an Aphthovirus within the family Picornaviridae, is a highly infectious, antigenically variable pathogen of cloven-hoofed animals. It is often difficult to control because of its high mutation rate leading to the emergence of a novel antigenic variants with the capacity to evade the immune response (Mateu et al., 1988; Martinez et al., 1992; Domingo et al., 1993). The disease, characterised by fever and lesions of the mouth and hoof area, severely affects milk and meat production and draught power when it coincides with the ploughing season. The most effective method of control is for endemic countries to embark on preventative vaccination programmes.

In the developing world FMDV is widely distributed, with unique epidemiological patterns, especially in Africa and Asia (Rweyemamu et al., 2008a; Rweyemamu et al., 2008b). In Africa, FMDV is maintained by the African buffalo (Syncerus caffer), in a cycle involving wildlife, and independently within domestic animals (Hedger et al., 1972, Hedger, 1972, Bengis et al., 2002, Thomson et al., 2003). In Southern Africa, sporadic infection of livestock and wildlife with the three Southern African Territories (SAT) serotypes, i.e. SAT1, SAT2 and SAT3, with multiple topotypes (Hedger et al., 1972, Vosloo et al., 1996, Vosloo et al., 2002, Ayebazibwe et al., 2010, Dawe et al., 1994) readily occur as a result of transmission from buffalo (Bastos et al., 2000, Vosloo & Thomson, 2004, Thomson 1994). Therefore, FMD control in sub-Saharan Africa relies on regular vaccination of susceptible species in high risk areas; fences separating animals at the wildlife-livestock interface; movement restriction and regular surveillance (Jori et al., 2014).

In many FMD endemic countries, the administration of FMD vaccines and the optimal production of protective immunity are poor. This has been linked to factors

including poor duration of immunity (Ahmed et al., 2012), low vaccine potency (Chardonnet et al., 2002), biophysical (temperature and pH) instability of the antigen (Ahmed et al., 2012), and poor cross-protection due to multiple antigenic variants in these regions (Brückner et al., 2002). The poor duration of immunity has been linked to the temperature liability of viruses belonging to the SAT serotypes (Doel and Baccarini, 1981). One of the foremost factors, which influences the potency of vaccine preparations and permits the induction of a protective antibody response is the structural integrity of the intact virion typified by a sedimentation rate of 146S (Doel and Baccarini, 1981). As a consequence, many countries in Africa have to rely on a triple or quadruple annual vaccination schedules.

The self-assembly and stability of a multimeric protein capsid, such as FMDV, depends on the occurrence of numerous non-covalent interactions between viral-encoded polypeptide subunits (Rossmann & Johnson, 1989; Fry et al., 1990; Reguera et al., 2004, 2005). The icosahedral capsid of FMDV is composed of 60 repetitions of four viral structural proteins, VP1-4, which assemble into protomeric subunits and pentameric intermediates. Twelve pentamers self-assemble through complex protein-protein interactions into a complete capsid (Acharya et al., 1989; Curry et al., 1996; Ellard et al., 1999; Mateo et al., 2003; Mateo et al., 2008). Even in the presence of high genetic variability, the viral capsid inter-subunit interactions for the seven serotypes 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). However, the capsid still needs to be sufficiently unstable to permit intracellular uncoating and release of viral RNA during infection. This paradox in capsid metastability appears to be markedly different for the FMDV serotypes. Serotypes A and Asia-1 are relatively stable, whilst O and SAT serotypes are sensitive to heat and pH (Doel & Baccharini, 1981, Maree et al., 2013). Additionally, chemical inactivation of vaccine antigen renders them even less stable and above 30ºC they rapidly convert into immunogenically incompetent pentameric subunits (Doel & Baccarini, 1981, Doel & Chong, 1982, Mateo et al., 2003), requiring frequent immunisation (Doel & Chong, 1982), and expensive, difficult to maintain cold chains for vaccine storage and transport.

Experimental studies on the relative importance of residues and molecular interactions in viral capsid assembly, disassembly, and/or stability are still limited (Reguera et al., 2004). Recent research has compared the inter-pentameric interactions of thermostable serotype A viruses with unstable O and SAT2 viruses in the crystallographic structures of the capsids and used in silico calculations of stability to predict residue substitutions that could increase interactions at the inter-pentameric interfaces (Kotecha et al., 2015). SAT2 and O viruses with improved stability have been developed as a proof-of-concept (Kotecha et al., 2015).

This paper extends the proof-of-concept and describes the evaluation of thermostability of 14 single amino acid substitutions, two triple mutations and two quadruple mutations derived from SAT2/ZIM/7/83 virus, at the inter-pentameric interface of the capsid. We assessed the stability of the recombinant mutant viruses for growth kinetics; temperature and pH inactivation rates; and thermofluor Pastry assay to distinguish capsid dissociation in relation to temperature, pH and ionic strength; antigenicity; plaque morphology and genetic stability in order to evaluate potential SAT2 vaccines candidates with improved stability.

3.4 Materials and Methods

3.4.1 Cells, viruses and plasmids

Baby hamster kidney (BHK) clone 13 cells (strain 21; ATCC CCL-10) were maintained and propagated in Eagle's basal medium (BME; Life Technologies) as described previously (Storey et al., 2007). RNA transfection, virus passage and virus stocks were prepared and titrated in BHK-21 cells (Rieder *et al*., 1993) using virus growth media (VGM; Eagle's basal medium (BME) with 1% (v/v) foetal calf serum (FCS), 1% (v/v) HEPES and antibiotics). The plasmid pSAT2, a previously described genome-length infectious cDNA clone of SAT2/ZIM/7/83 (van Rensburg *et al*., 2004), was used as the genetic backbone in the construction of recombinant cDNA clones harboring capsid stabilizing mutations. The virus recovered from pSAT2 is referred to as vSAT2. BHK-21 #38 suspension cells (Brescia, Italy) were used to upscale the 93Y mutant in vaccine production using virus growth media (VGM; enriched minimum essential medium (MEM) with 1% (v/v) normal bovine serum (NBS).

3.4.2 Identification of stabilising residues

Model preparation for MD simulations, design and construction of candidate mutants; molecular dynamics simulation protocol; estimation of degree of stabilisation for SAT2/ZIM7/83 were described in detail in Kotecha *et al*. (2015). Briefly, a dimer interface was generated from the atomic model of SAT1 (Patent PDB ID: 2WZR) and used as a starting template. A truncated model was then generated which contained all atoms within 13 Å of an interface and simulated for 1.55 ns, with the explicit solvent model using AMBER10 (Case et al., 2005). A set of restraints were introduced by placing the dummy atoms at the interface and all atoms outside the 10 Å radius from them as well as the dummy atoms themselves were heavily restrained (Kotecha et al., 2015). Binding free energy between adjacent protomers was calculated using the Molecular Mechanics Poisson Boltzmann, MM-PBSA method (Kollman et al., 2000). Finally, the difference in binding free energy, $\Delta\Delta G$, between candidate mutant models and the parent wild-type model was calculated to assess the stability of the mutants.

3.4.3 Generation of recombinant SAT2 cDNA mutants

The *ca*. 2.2-kb external capsid-coding region (1B-1D/2A) of the plasmid pSAT2, was digested with endonucleases *EcoRI* and *Xma*I and cloned into corresponding

sites of the pBluescript® II SK (pBS) (Stratagene) vector to generate the template for site-directed mutagenesis (pBS-P1). Mutations were introduced into pBS-P1 (120 ng) using overlapping inner mutagenesis oligonucleotides **(Sup. Table 4)** and the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene) according to manufacturer's instructions. Cycling conditions were performed as follows: 95 °C followed by 18 cycles of 95 °C for 30 sec, 55 °C for 60 sec, 68 °C for 6 min.

The mutated pBS-P1 constructs were confirmed with sequencing (Big Dye Terminator v3.1 cycle sequencing kit, Applied Biosystems). The mutated external capsid-coding region (1B-1D/2A) of plasmid pBS-P1 was digested with endonucleases *SspI* and *Xma*I to recover the mutated capsid-coding region and cloned back into pSAT2. The mutated recombinant pSAT2 constructs were confirmed by nucleotide sequencing.

3.4.4 *In-vitro* RNA synthesis, transfection and virus recovery

Recombinant, mutated pSAT2 plasmids were linearized with *SwaI* and *in vitro* transcribed using the MEGAscript™ T7 kit (Ambion). The integrity of the RNA was analysed by agarose gel electrophoresis. *In vitro* transcribed RNA (3 µg) was 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_2 influx for 48 h in virus growth medium and frozen at -80 °C. 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 viruses derived from the genome-length cDNA were confirmed by nucleotide sequencing and used in subsequent experiments.

3.4.5 Sequence confirmation of mutant recombinant viruses

Total RNA was extracted from infected cell cultures using the QIAamp Viral RNA Mini Kit (Qiagen), according to the manufacturer's specifications and used as template for cDNA synthesis. Viral cDNA was synthesised with SuperScript III™ (Life

Technologies) using the oligonucleotide 2B208R (5'-GACATGTCCTCCTGCATGTG) (Bastos *et al*., 2001) and were carried out at 50 °C for 50 min. The Leader/capsid-coding region of the mutated viruses was amplified using Expand Long template Taq DNA polymerase™ (Roche) with genome-specific oligonucleotides (NCR2: 5'-GCTTCTATGCCTGAATAGG and WDA: 5'-GAAGGGCCCAGGGTTGGACTC). Direct DNA sequencing of the amplicons or plasmids was performed using the ABI PRISM™ BigDye Terminator Cycle Sequencing Ready Reaction Kit v3.0 (Perkin Elmer Applied Biosystems). The consensus nucleotide sequence of the capsid-coding region was assembled with Sequencher 4.7 DNA sequence analysis software (Gene Codes Corporation, USA) and compared to that of SAT2/ZIM/7/83 (Genebank accession codes: JQ639289 and DQ009726).

3.4.6 Plaque, growth kinetic and thermo-inactivation assay

Plaque assays were performed in triplicate by infecting BHK-21 monolayer cells in 35 mm cell culture plates (Nunc™) for 1 h, followed by the addition of 2 ml tragacanth overlay (Rieder et al., 1993). Following incubation at 37ºC for 48 h the infected monolayers were stained with 1% (w/v) methylene blue in 10% (v/v) ethanol and 10% (v/v) formaldehyde in phosphate buffered saline, pH 7.4. The diameters of >50 parental (wild-type) plaques were measured with a microscope eyepiece micrometer. These were averaged, defined as 100%, and compared against >50 mutant plaque diameters.

One-step growth kinetic analyses were performed by infecting BHK-21 cell monolayers with wild-type and mutant viruses 93H, 93Y and 62Y-87M-143V at a m.o.i. of 3-5. After 1 h of adsorption, cells were washed with MBS (MES-buffered saline, 25 mM morpholine-ethanesulfonic acid, 145 mM NaCl, pH 5.5) and then incubated with VGM at 37 °C. At times 0, 1, 4, 6, 8, 12 and 24 hours post-infection (hpi), infected cells were frozen at -80 °C. Virus titres were determined by plaque assays and expressed as plaque forming units per millilitre (pfu/ml) as described above.

Thermo-inactivation was performed on wild-type and mutant viruses in cell culture supernatants diluted in TNE buffer (100 mM Tris pH 7.4, 10 mM EDTA, 150 mM NaCl) essentially as described by Knipe *et al*. (1997). Briefly, 2-5 x 10^5 pfu/ml of infectious particles were incubated in duplicate at temperatures of 42 °C and 49 °C for

0, 15, 30, 45, 60, 120, 180, 240 minutes. Following cooling on ice, the viruses were titrated on BHK-21 cells. Similarly, pH inactivation kinetics was performed in TNE buffer with a pH of 6.0 at a constant temperature (25 °C) for the same time points as above. The samples were subsequently neutralised with 1 M Tris (pH 7.4), 150 mM NaCl and titrated on BHK-21 cells. The respective logarithmic values of the virus titres at the different time points were linearly fitted and the slopes were determined (Mateo & Mateu, 2007, Mateo *et al*., 2007). The percentage of remaining infectious particles was also calculated and plotted along with the exponential decline used to calculate the inactivation rate constant as described by Mateo *et al*. (2003). The percentage of remaining plaque forming units was determined as last titre (4h) divided by the initial titre (0 min) x 100.

3.4.7 Virus purification

Infected BHK-21 monolayers were lysed with 0.1% (w/v) Nonidet and 20 mM EDTA (pH 8.0) and clarified by centrifugation at 2000 g for 30 minutes at 4°C. Virus in the supernatant was inactivated with 5 mM binary ethyleneimine (BEI) for 26 h at 26 °C and 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_{259} nm = 79.9; (Doel & Mowat, 1985) and pooled for analysis. Complete inactivation was verified by titration on BHK-21 cells.

3.4.8 Stability thermal release assay

The particle stability thermal release assay (PaSTRY) (Walter *et al*., 2012) was performed in 96-well PCR plates using an ABI 7500 PCR machine. All assays were performed in triplicate using 300-500 ng of virus, 5 µl of 100X SYBR green-II dye (Molecular Probes, Invitrogen; diluted 1:100), with the volume made up to 50µl with 1X PBS buffer. The temperature was ramped from 15 °C to 95 °C in 0.5 °C increments with intervals of 10 seconds. 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 A-serotype, A24 Cruzeiro virus is very thermostable and was used as a positive control in the thermal shift assays

Ionic buffers were tested at a final concentration of 1 M and 0.5 M NaCl, KCl, $(NH_4)_2SO_4$, $MgCl_2$, $CaCl_2$, 10% normal bovine serum (NBS), 5% glycine, virus growth media (VGM), 30% sucrose, 0.8X (control reaction) and 1X PBS using SYBR green-II dye and virus concentrations as described above. The pH buffers were composed of MBS (MES-buffered saline, pH 6.1, 6.7, 7.2, 7.6, 8.2, 8.5, 9.1).

Alternatively, SDG purified wild-type virus and the 93Y mutant virus (500 ng) were made up to 45 µl with 1X PBS and exposed to 42 °C, 49 °C and 53 °C for 0, 15, 30, 45 and 60 min and then placed on ice. A volume of 5 µl of 100X SYBR green-II dye was added to samples and a stability thermal release assay performed as described above.

3.4.9 Up-scaled production of 93Y mutant in BHK-21 suspension cells

The 93Y mutant virus was serially passaged in suspension BHK-21 #38 cells (Brescia, Italy) up to six passages. During the upscaling process the growth profile, antigen determination and end titres were determined as described. At each passage the P1 region of the 93Y mutant virus was sequenced and the deduced amino acid sequence compared to that of the wild-type.

3.4.10 Sandwich antibody ELISA with SAT2-specific MAbs

Five SAT2-specific MAbs (mouse IgG1 isotype MAbs 1D5 [14 µg/ml], DA10 [8 µg/ml], GE11 [19 µg/ml], GD12 [15 µg/ml], and GG1 [22 µg/ml]) were kindly provided by The Pirbright Institute (Pirbright, Woking, UK). A sandwich ELISA was used to titrate the five SAT2-specific MAbs and to characterize the wild-type ZIM7/83 (Opperman et al., 2014) and nine stabilising mutant viruses (purified or cell supernatant) and repeated three times. Maxisorp™ ELISA plates (Nunc) were coated with an optimal dilution of rabbit SAT2 antiserum (SAT2/ZIM/7/83) in 50 mM carbonate/bicarbonate buffer (pH 9.6) and stored overnight at 4 °C. A serial two-fold dilution (1:5 to 1:40) of the wild-type ZIM7/83 and nine mutant viruses (supernatant of infected cells) in blocking buffer (0.05 M Tris, 0.15 M KCl containing 0.5% [w/v] milk

powder), was applied to the ELISA plates. Viruses were trapped by incubation at 37°C for 1 h after which the plates were washed with 1×PBS containing 0.05% (v/v) Tween-20 (PBS-0.05%T). Two-fold dilutions (1:20 to 1:80) of each of the MAbs, prepared in blocking buffer, was added and the plates incubated at 37°C for 1 h. The ELISA plates were washed with PBS-0.05% Tween and horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG (Sigma-Aldrich), diluted 1:20 000 in blocking buffer, was added. Following incubation at 37°C for 1 h and washing of the plates, the reactions were developed. The binding of the MAbs to the viruses was calculated as follows. The mean absorbance reading at 450 nm (A_{450}) for the binding of each MAb to the viruses were corrected by subtracting the background value. The adjusted A_{450} values for each MAb to the mutant viruses were then expressed as a percentage of the mean A_{450} value obtained against vSAT2.

3.5 Results

3.5.1 Identification of putative mutations that confer increased SAT2 stability

Using the three-dimensional structures of FMDVs [including SAT1 (Reeve *et al*., 2010) and SAT2 (Kotecha *et al*., 2015)], we targeted residues at the interface between the pentamers for stabilisation by enhancing non-covalent interactions (VP2 contributes the majority of the interface residues, followed by VP3). Predicted mutations were based on sequence and structural comparisons with more stable FMDV serotypes and other picornaviruses and *in silico* calculated binding energies but not all mutants could be simulated owing to structural disorder in the vicinity of the three-fold axis (Kotecha *et al*., 2015) **(Table 3.1)**. Mutating residues at the VP2/3 interface **(Fig. 3.1a)**, allowed us to introduce a potentially stabilising non-covalent interaction without disrupting viral antigenicity and infectivity. At the pentamer interface on the two-fold axis there is an alpha-helix. Triple mutants were designed to cap the dipole of this helix (Curry et al., 1995, Ellard et al., 1999) that showed good *in-silico* stability **(Fig. 3.1b-c, Table 3.1)**. The quadruple mutants introduced additional mutations to counter clashes observed in the *in-silico* molecular dynamics simulations with some mutants. Overall, combinations of stabilizing mutations did not produce additive effects in the *in-silico* stability predictions except in the case of the helix-capping mutant 62Y-87M-143V. The infectious clone of the vaccine strain

SAT2/ZIM/7/83 (van Rensberg et al., 2004), was chosen as our target virus, and A24 Cruizero, as a reference strain with known stability.

Table 3.1: SAT2 capsid stabilising mutants located at or near the 2-fold axis, on the α-helix of the inter-pentameric interface. The change in binding free energy is calculated including a negative control, 113G to validate the simulation protocol.

Mutations	***ΔΔG* kcal/mol**
VP2 93H	-5.3
VP2 93Y	-12.2
VP2 93F	-13.2
VP2 93W	-
VP2 94V	-5.2
VP2 98F	-5.6
VP2 113G	-
VP3 193H	-
VP3 198A	-
VP2 62Y, 87M, 143V	-8.3
VP2 62Y, 87M, 98F, 143V	-4.4

To study the effect of individual or combined mutations we constructed recombinant virus mutants in an infectious, genome-length clone of FMDV, pSAT2 **(Fig. 3.2)**. We introduced the following mutations into the VP2-coding region: Y98F, S93H, S93Y, S93W, S93F (Kotecha *et al*., 2015), F62Y, H87M, H87V, H87V, Y98H, S93Q, L94V, T114N, K215L and VP3-coding region: H143V, D193H, T194A, E198A **(Fig. 3.2)**. Two triple mutants consisted of the substitution combinations of F62Y-H87M-H143V and F62Y-H87V-H143V, and quadruple mutants F62Y-H87V-Y98H-H143V and F62Y-H87V-Y98F-H143V (**Fig. 3.2**). The VP2 mutant S113G was selected as a negative control by deliberately disrupting the hydrogen-bond network and was not designed with *in-silico* simulation methods.

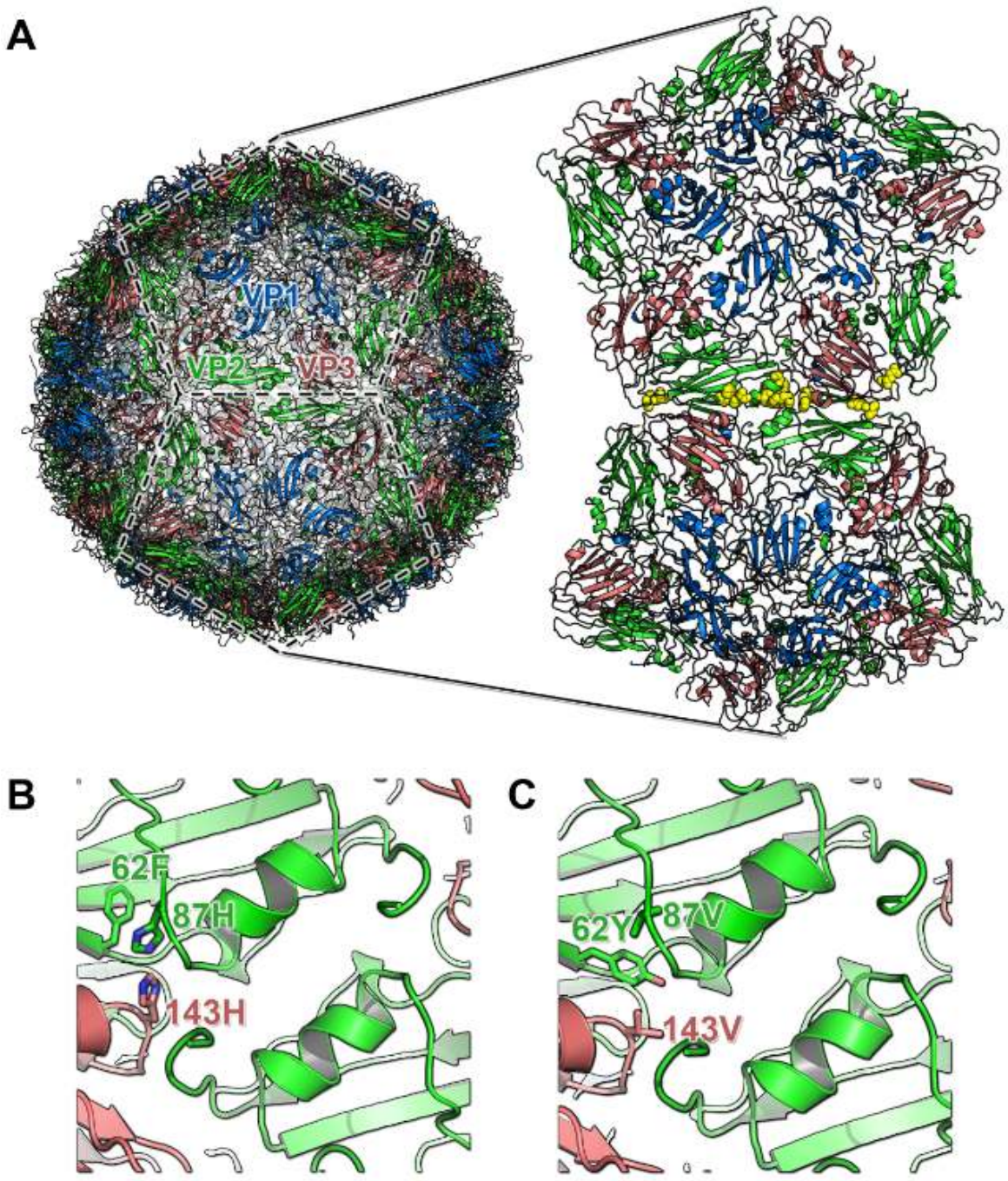


Figure 3.1: Stabilisation of SAT2 inter-pentameric interface and design of the models used for MD simulations. (**A**) Cartoon representation of atomic structure showing portions of two protomers forming an inter-pentameric interface. A truncated model was generated by trimming the protomers to include VP2 and VP3 atoms within a 13Å radius from the interface. (**B** & **C**) Wild-type and substituted residues on 2-fold symmetry related helix for the SAT2 serotype are highlighted.

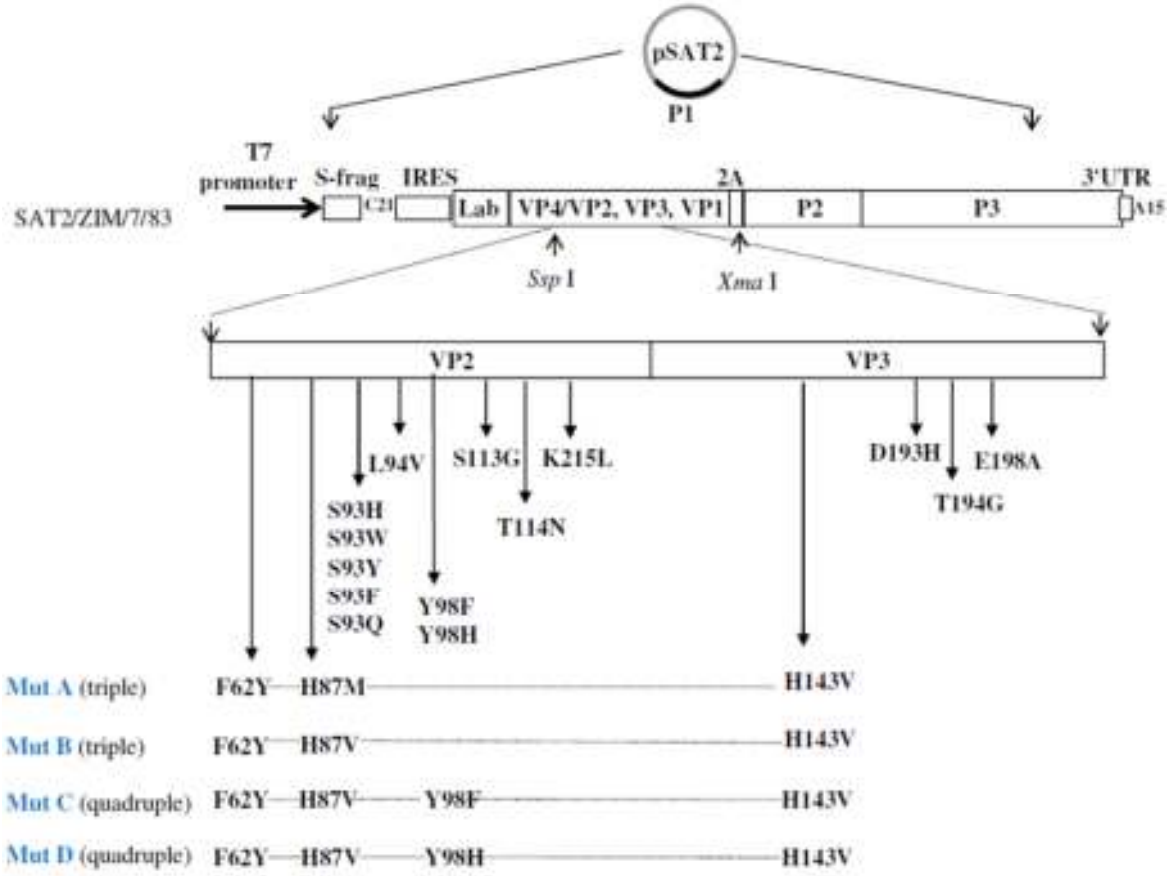


Figure 3.2: Schematic representation of the mutagenesis strategy used to introduce stabilising mutations into SAT2/ZIM/7/83. The predicted mutations as single, triple or quadruple cassettes from the VP2 and VP3 capsid proteins are indicated. Following overlap-extension mutagenesis, the stabilising mutated P1 regions were cloned into the *Ssp* I and *Xma* I sites of pSAT2, a genome-length cDNA clone of SAT2/ZIM/7/83.

3.5.2 Infectivity and growth characteristics of engineered mutants.

The effects of the targeted amino acid mutations on the infectivity of the SAT2 virus in susceptible cells were investigated. Transcripts generated from each construct were transfected into BHK-21 cells to generate infectious FMDV stocks (passage 0 stock (P0)). Lysates from these cells were then used to infect ZZ-R127 goat epithelium cells (P1), which are highly susceptible to FMDV infection (Brehm, *et al.,* 2009). Subsequent passages were performed in BHK-21 cells. Sequencing of each mutant vSAT2 after four consecutive passages (P4) revealed no unintentional changes in any of the mutants and no obvious genetic sub-populations. All mutant vSAT2 viruses produced clear cytopathic effect (CPE). In the later stages of infection, cells infected with the wild-type virus exhibited large areas of cell death and loss of adherence,

correlating to medium to large plaque sizes (3-5 mm medium, 6-8 mm large) whereas cells infected with 93Y and 93W viruses showed numerous microscopic small (<1 mm) foci of dead cells and plaques **(Table 3.2)**. The remaining mutant viruses exhibited a similar pattern of CPE and plaque size to that of the wild-type virus. Viruses could not be recovered from 98F, 98H, 114N, 215L, 194A, 62Y-87V-98F-143V and 62Y-87V-98H-143V mutant constructs. Viruses recovered from 93F, 93Q and 62Y-87V-143V mutant constructs grew to very low titres (5.2-7.2×10^3 pfu/ml) and could not be used for further analyses.

To explore the growth properties of the wild-type, 62Y-87M-143V, 93H and 93Y viruses in more detail, growth kinetics were determined in BHK-21 monolayer cells using a starting multiplicity of infection (m.o.i.) of 5 (**Fig. 3.3**). The growth analysis illustrated that the wild-type, 62Y-87M-143V and 93H viruses were indistinguishable in their growth kinetics. However 93Y showed differences at: 2 hours post infection (h.p.i) with an increased amount of infectious particles, and at 12-24 h.p.i with a statistically significant (P<0.05) ten-fold decrease in growth compared to wild-type viruses (**Fig. 3.3**).

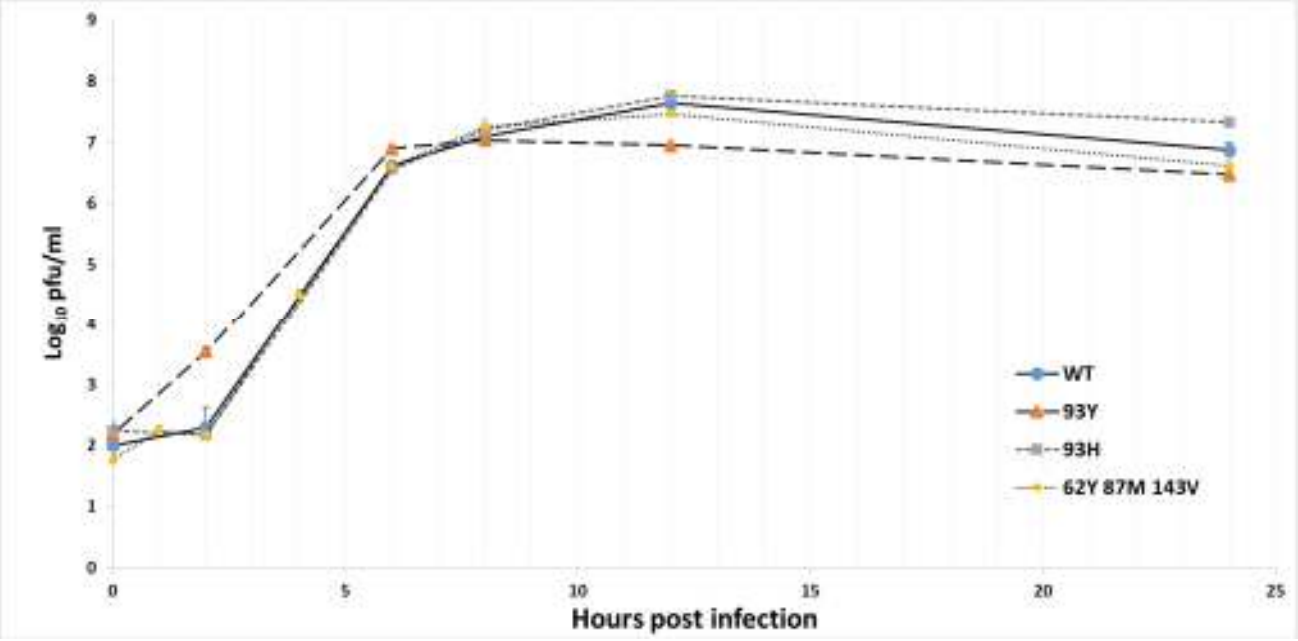


Figure 3.3: One-step growth kinetic study performed in BHK-21 cells. The average (n=4) $\log_{10}$ titres of duplicate wells are shown at different time points (0, 1, 2, 4, 6, 8, 12, 24 hrs post absorption), as indicated on the graph, over a 24 hour period with wild-type, 93H, 93Y and 62Y-87M-143V mutant viruses. The standard deviations of the titres determined from two biological replicates repeated twice (n=4) are indicated on the graph.

Table 3.2: The passage history, plaque morphologies and titres of SAT2 wild-type and stabilising mutant viruses. ND refers to not done as viruses were not viable.

Amino acid change	Passage & Plaque morphology	Titre
93H	BHK Medium/large	1.4×10^7
93Y	BHK ZZR Small	1.87×10^5
93W	BHK Small/medium	1.00×10^7
93Q	BHK Medium/large	7.2×10^3
93F	BHK Medium/large	5.2×10^3
94V	BHK Medium/large	6.2×10^5
98F	not viable	ND
98H	not viable	ND
114N	not viable	ND
113G	BHK Medium/large	4.0×10^5
215L	not viable	ND
193H	BHK Medium/large	2.2×10^7
194A	not viable	ND
198A	BHK Medium/large	4.8×10^5
62Y-87V-143V	BHK Medium/large	6.2×10^3
62Y-87M-143V	BHK Medium/large	8.4×10^7
62Y-87V-98F-143V	not viable	ND
62Y-87V-98H-143V	not viable	ND
wild-type	BHK Medium/large	2.0×10^6

3.5.3 Antigenic profiling of mutant viruses with SAT2-specific MAbs

The viable mutant viruses (193H, 198A, 93H, 93Y, 93W, 94V, 62Y-87M-143V) and 113G (negative control) were characterized based on their sandwich ELISA absorbance readings to SAT2-specific MAbs, The readings were expressed as a ratio in comparison to the wild-type SAT2 virus. The five MAbs reacted as expected to wild-type vSAT2 described in Opperman et al. (2014). All mutants reacted to DA10, GG1, GE11 and 1D5 similarly to the wild-type as shown by similar reactivity profiles (**Fig. 3.4**). However, reactivity of GD12 to mutant 93Y was lowered (37%), with a ratio profile of 1:4 (93Y: wild-type) (**Fig. 3.4**).

Virus / MAb	DA10 :	GG1 :	GE11 :	1D5:	GD12
113G (neg)	0.72	0.71	0.76	0.57	1
193A	0.806	0.78	0.86	0.68	1
198H	0.74	0.7	0.74	0.516	1
93H	0.72	0.64	0.69	0.55	1
93Y	1	0.91	0.99	0.52	0.25
93W	1	0.78	0.96	0.74	0.87
94V	1	0.76	0.85	0.7	0.82
62Y-87M-143V	1	0.83	0.81	0.71	0.86
wild-type	**0.83**	**0.74**	**0.87**	**0.65**	**1**

Figure 3.4: Heatmap of the five SAT2 specific monoclonal antibody (MAb) (DA10, GG1, GE11, 1D5, GD12) reactivity ratios of the mutant and wildtype viruses. Absorbance values, an average of three repeats, are shown as a ratio of each Mab's reactivity to a virus. MOCK-infected cell supernatants were used as background control. This ratio depicts a specific profile for each virus. Green shaded areas represent ratio values closest to 1, indicative of the highest reacting MAb for a given virus. Values of <0.5 (orange to red) are indicative of poor reactivity to that MAb and most disparate from the wildtype profile. The binding pattern ratio of the wildtype virus (0.8: 0.7: 0.9: 0.7: 1) is used as the control to compare mutant virus reactivity against.

3.5.4 Thermo-inactivation assay: thermal and acid stability of the mutants

The differences in the stability of the viruses were elucidated using a thermo-inactivation assay. Duplicates of cell culture supernatants, containing the wild-type and stability-mutated viruses with an approximate titre of 2-10 × 10^5 pfu/ml were either treated at pH 6.0, or heated at 42 °C or 49 °C for different time intervals up to 4 hours following a 1:50 dilution in the appropriate TNE buffer. The number of infectious particles remaining after treatment was determined by plaque titrations on BHK-21 cells. The inactivation of vSAT2 and the mutant viruses at 42 °C or 49 °C followed linear kinetics and the lability of the mutant viruses were reflected by the inactivation rate values calculated from the slope of the graph (**Fig. 3.5**). The lower the value (gradual gradient) the more stable the virus. The inactivation rate constants at 49 °C for wild-type, 198A, 94V, 93Y viruses were 0.022, 0.016, 0.017 and 0.014 min^{-1}, respectively. The percentage of infectious particles remaining after 1 h at 49 °C for mutants 93Y, 198A and 94V was 15, 12 and 10% respectively compared to 5% for the wild-type virus **(Fig. 3.5a-b)**.

The mutant infectious particles that showed increased thermostability at 49 °C were further tested at 42 °C for 4h. The inactivation rate constants at 42 °C for wild-type, 198A and 93Y viruses were 0.007, 0.007 and 0.004 min^{-1}, respectively. Similarly, a slower inactivation rate for the 93Y infectious particles was observed compared to the wild-type and the remaining mutant viruses. The percentage of infectious particles remaining after 1h at 42 °C for mutants 93Y and 198A was 27% and 6.4% respectively compared to 3.3% for the wild-type virus and at 4h for mutants 93Y was 12% compared to 2.5% the wild-type virus **(Fig. 3.5c-d)**.

The inactivation rate values at room temperature at pH 6.0 showed improved pH stability for mutant viruses 93H (0.001 min^{-1}), 93Y (0.002 min^{-1}), 193H (0.002 min^{-1}), 198A (0.002 min^{-1}) compared to the wild-type virus (0.004 min^{-1}), however the negative control mutant 113G (0.017 min^{-1}) which wasn't selected by *in-silico* methods was considerably less pH stable than the wild-type **(Fig. 3.5e-f)**. The percentage of infectious particles remaining after 2h treatment at pH 6.0 for mutant viruses 93H, 193H, 93Y was 77%, 56% and 36% respectively, with 93H having a 28% improvement in pH stability compared to the wild-type **(Fig 3.5e-f)**.

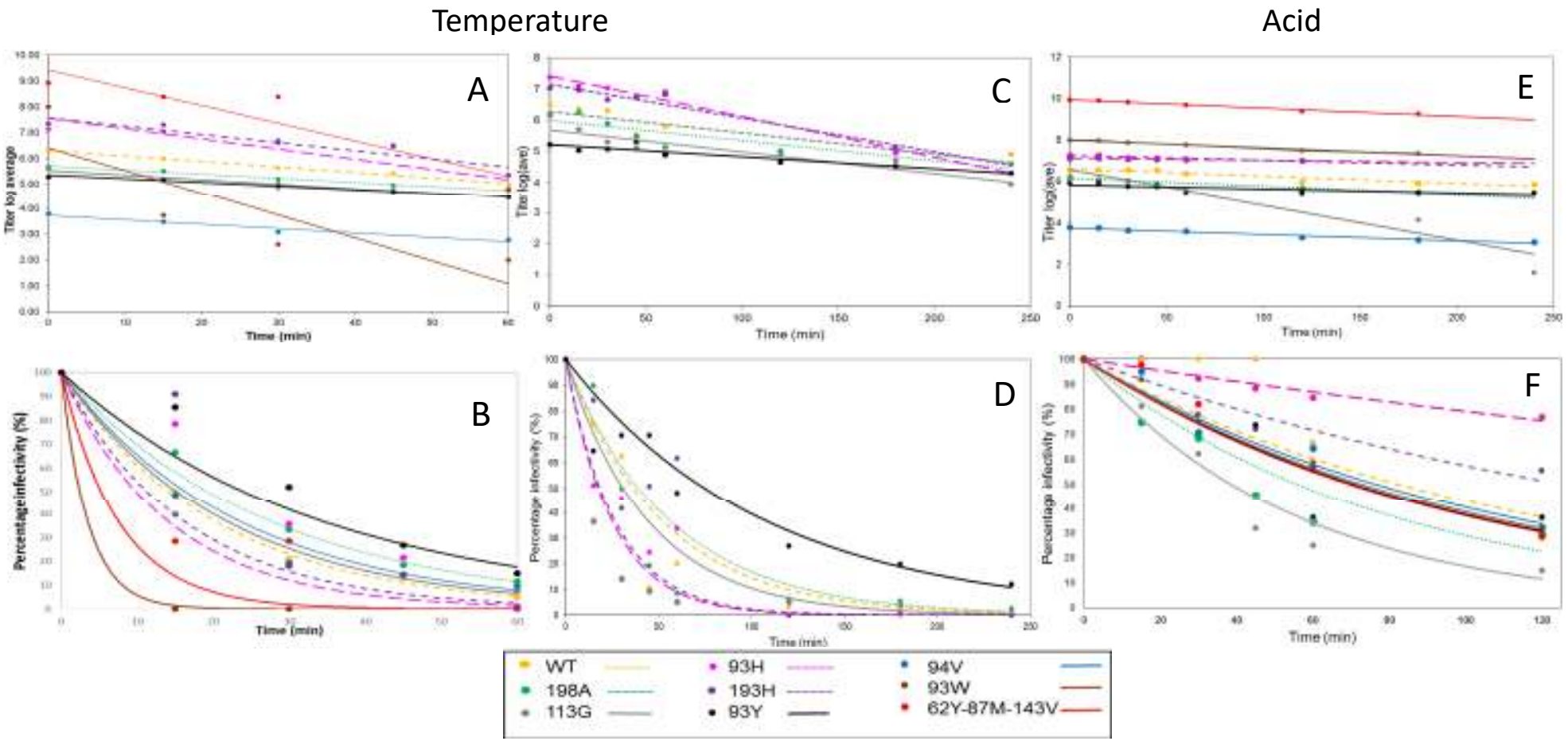


Figure 3.5: Thermal and pH inactivation kinetics of wildtype and mutant SAT2 viruses tested in duplicate. Inactivation of SDG-purified wildtype and mutant SAT2 particles following heat treatment at 49 °C for 1h (A), 42 °C for 4h (C) and with TNE buffer at pH6.0 for 2 h (E). The average $\log_{10}$ virus titres as determined in two different inactivation experiments are shown. The respective logarithmic values of the virus titres at the different time points (0, 15, 30, 45, 60, 90, 120, 180, 240 min p.i) were linearly fitted and the slopes determined. The average virus titres following heat inactivation at 49 °C (B) or 42 °C (D) or pH treatment (F) were used to determine the percentage of residual infectious particles remaining over time.

3.5.5 Thermal shift assay of engineered FMDV mutants

Fluorescent thermal shift assays (Walter *et al*., 2012) were performed on purified wild-type and mutant SAT2 viruses. Wild-type vSAT2 capsids dissociated at 47˚C whereas most mutants were more thermo-stable. The mutant 93Y dissociated at 53.5 °C followed by mutants 198A and 62Y-87M-143V (52 °C), 94V, 93H and 193H (51 °C), 93W (50 °C) **(Fig. 3.6a).** The negative control mutant 113G was less stable with dissociation at 45 °C. The A-serotype A24 control virus had the highest temperature stability of 55.5 °C (**Fig. 3.6a**). The 93Y mutant had a significantly higher capsid stability of 6°C ($P<0.05$), whilst mutants 62Y-87M-143V, 198A, 93H, 94V and 193H had a 4-5 °C increase in capsid stability compared to the wild-type vSAT2 virus. The more stable A24 control capsid dissociation temperature was only 2 °C higher than the 93Y mutant.

Next we investigated whether or not elevated temperatures may affect the conformation of the interface by disrupting the secondary/tertiary protein structure permanently or temporarily. The 93Y mutant and vSAT2 wild-type viruses were heated to 42 °C, 49 °C or 53 °C for different time intervals up to 1 h to allow for varying degrees of dissociation (partial to full) and then cooled on ice to allow re-establishment of conformation before performing a thermal shift stability assay. We found there was no difference in the capsid dissociation temperature of the viruses exposed to different temperatures or time of exposure compared to the normal dissociation temperature (data not shown).

To investigate the effect of ionic buffers on capsid stability, 16 different ionic buffers were examined (**Fig. 3.6b**). The following summarizes the trend of the buffers supporting the highest to the lowest stability as assessed by dissociation temperatures: 1 M and 0.5 M NaCl; 1 M KCl; 1 M $(NH_4)_2SO_4$; 1xPBS; 30% sucrose; VGM; 0.5 M KCl; 0.5 M $(NH_4)_2SO_4$; 5% Glycine; 0.5 M $CaCl_2$; 0.5 M and 1 M $MgCl_2$; 1 M $CaCl_2$; and 10% NBS. The 1 M NaCl solution supported the highest increase in stability (except for 94V) which were significantly different ($P<0.05$) followed by the 0.5 M NaCl solution when compared to the 0.8xPBS control buffer. There was improved stability for all the viruses for: 1 M KCl (except for 93W and 94V), VGM and 1xPBS buffers. The 30% sucrose solution provided improved stability for 93Y, 93H, 198H, 193H and A24. The 1 M and 0.5 M $MgCl_2$ and $CaCl_2$, 10% NBS and 5% glycine

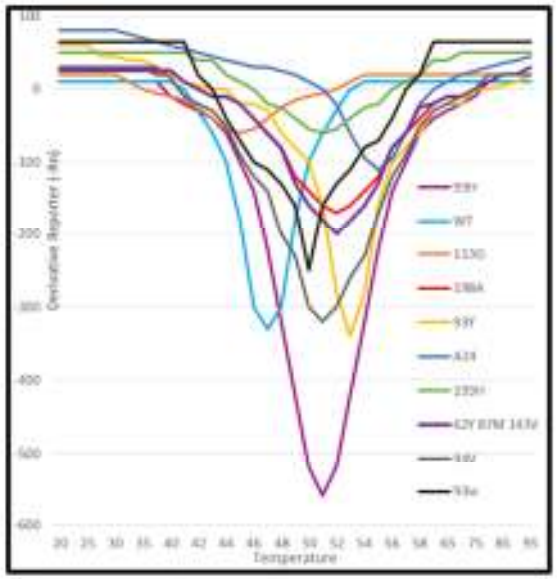
solutions affected the greatest decrease in stability of SAT2 viruses, however, the A24 control virus was not affected. The wildtype virus was destabilised by the 0.5 and 1 M $(NH_4)_2SO_4$ solutions whereas the mutant viruses had improved or equivalent stability.

To investigate the effect of pH on capsid stability, sucrose density gradient SDG purified viruses were exposed to buffers in the pH range 6.1-9.1 (**Fig 3.6c**). Sensitivity to pH was measured by the decrease in dissociation temperature compared to the PBS control (pH 7.4). At pH 6.1 most viruses were fully dissociated except for the wild-type, 93Y and A24 viruses which were more pH resistant. The viruses were most stable at pH 7.6-8.2. The negative control mutant 113G was less thermostable and showed greater sensitivity to pH changes.

3.5.6 Genetic stability of 93Y virus upon cytolytic passage in suspension BHK#38 cells

To evaluate the relationship between virus stability and virus titre following passage in cultured cells, we subjected the 93Y mutant virus to six serial amplifications in suspension BHK-21 #38 (Brescia, Italy) cells and analysed the progeny populations. Initially the 93Y mutant was very slow-growing, but after a few passages it grew faster and had a similar growth profile, CPE/plaque formation and end titre in BHK-21 cells compared to the parental SAT2/ZIM/7/83 virus. A rapid loss of the mutations during virus replication would suggest some biological disadvantage. An in-depth sequence analysis revealed that the originally introduced TAT (Tyr) codon 93 (VP2) had changed to CAT (His), whilst the wild-type codon AGC (Ser) was absent and no other accompanying mutations were observed in the capsid-coding region. This mutation first appeared at passage #3 (33% of the sequencing peaks) and was fully preserved at passage 6. Serial passage of the 93H virus for another 14 cycles did not result in any additional changes, allowing large scale passage of the engineered virus for vaccine production requirements

A



C

virus	control	6.1	6.7	7.2	7.6	8.2	8.5	9.1
Wild-type	47±0.07	37±0	41±0.21	45±0.7	46±0.21	48±0	46±0.14	44±0
93Y	53.5±0.14	36±0	44±0.7	47±0.14	49±0.07	51±0.07	49±0.28	47±0.21
93H	51±0.28	ds*	39±0.28	43±0.21	46±0.28	49±1.4	47±0.21	45±0
93W	50±0	ds*	40±0	45±0.07	49±0.21	51±0	48±0	47±0
94V	51±0	ds*	40±0	45±0.28	48±0	52±0.7	50±0	48±0.35
113G	45±1.9	ds*	34±0.21	36±0.28	41±0	44±0.21	37±0	37±0.28
198A	52±0.21	ds*	41±0	45±0.28	48±0	49±0	47±0	45±0.21
193H	51±0.35	ds*	40±0.21	44±0.21	46±0.35	49±0	44±0	44±0
62Y 87M 143V	52±0.7	ds*	41±0	47±0.07	49±0.14	51±0	48±0.21	ds*
A24	55.5±0.21	38±0.7	43±0.35	50±0.7	50±0.21	52±0.07	50±0.14	49±0.21

B

virus	1M NaCl	0.5M NaCl	1M $(NH_4)_2SO_4$	0.5M $(NH_4)_2SO_4$	1M $MgCl_2$	0.5M $MgCl_2$	1M KCl	0.5M KCl	1M CaCl	0.5M CaCl	10% NBS	5% Glycine	VGM	30% sucrose	1xPBS	control
Wild-type	56±0.14	52±0.21	43±0.7	38±0.7	ds*	ds*	53±0.14	50±0.28	45±0.7	48±0.14	48±2.12	53±0.14	51±0.14	47±0.14	54±0.7	47±0.07
93Y	60±1.4	58±0.07	58±1.4	54±0.2	48±0.07	48±0.2	58±1.4	55±1.4	ds*	52±0.2	ds*	49±1.4	56±1.4	59±1.4	57±1.4	53.5±0.14
93H	60±0.21	57±0.28	56±0.28	52±0.7	48±0.14	49±0.21	56±0.07	54±0.21	45±0.7	50±0.7	49±0.21	42±0.7	50±0.28	57±1.4	56±0.14	53±0.28
93W	60±0.21	57±0.21	56±0.35	52±0.7	ds*	ds*	ds*	53±0.28	ds*	ds*	ds*	46±0.7	53±0.21	53±0.14	56±0.42	50±0
94V	ds*	54±0.42	52±0.14	50±0.21	ds*	ds*	ds*	ds*	ds*	ds*	ds*	42±1.14	52±0.14	50±0.70	55±0.28	51±0
113G	58±0.28	54±0.07	51±0.21	48±0.14	43±0.42	42±0.70	50±0.21	48±0.21	45±0.7	48±0.7	ds*	38±0.28	50±0.35	48±2.12	51±0.21	45±1.9
198A	59±0.21	56±0.14	57±0.21	53±0.7	50±0.28	51±0.7	56±0.14	53±0.14	45±0.07	48±0.07	50±0.21	51±0.21	52±0.14	59±0.14	54±0.07	52±0.21
193H	58±0.28	55±0.14	55±0.14	53±0.7	ds*	ds*	55±0.14	52±0.35	44±0.7	47±0.14	ds*	48±0.28	51±0.14	57±0.7	51±0.14	51±0.35
62Y 87M 143V	60±0.21	57±0.14	52±0.7	49±1.4	47±0.28	48±0.07	57±0.14	56±0.07	ds*	ds*	ds*	53±0.14	55±0.14	54±0.7	56±0.21	52±0.7
A24	60±0.07	59±0.11	56±0.07	55±0.07	59±0.21	57±1.4	58±0.14	57±0.21	55±0.21	54±0.21	53±0.07	52±3.53	55±0.7	58±1.4	58±0.07	55.5±0.21

Figure 3.6: Thermostability measured fluorescence assay (Walter et al., 2012) to determine the dissociation temperatures of wildtype compared to mutant SAT2 viruses. The A24 virus was used as a representative control of an A-serotype stable virus. A) dissociation temperatures of mutants showing improved temperature stability in comparison to controls. Heatmaps are depicted for B-C, showing the average dissociation temperatures of duplicate repeats and error values. The colouring of green is indicative of more stable whilst red is more unstable. B) pH stability tested with a range of pH buffers 6.1-9.1; and C) ionic stability of 15 different buffers and their effect as additional improvement to stability. The control is 0.8x PBS. Ds* denotes where viruses were dissociated under the experimental condition and no reading was obtained.

3.6 Discussion

To date experimental studies on the relative importance of residues and molecular interactions in viral capsid assembly, disassembly, and/or stability are limited. Previously attempts at improving stability have had only marginal success (Mateo et al., 2008) while another study reported a stable disulfide bond at the icosahedral two-fold axis between adjacent pentamers in the A22 virus, which allowed the production of thermo- and pH-stable recombinant empty virus-like particles (Porta et al., 2013). Mateo et al. (2007) observed that thermostable variants are not represented among the quasispecies of FMDV serotypes. Hedge et al. (2009) has therefore speculated that FMDV has evolved to undergo persistency in ruminants by avoiding establishment of thermostable species. We previously investigated the rational introduction of mutations to increase thermostability, by devising a strategy that allows mutations conferring non-covalent stabilization to be evaluated *in silico* (Kotecha et al., 2015). In this study we investigated the effect of a larger panel of amino acid residues, 14 single amino acid substitutions, two triple mutations and two quadruple mutations, at the inter-pentameric interface of the VP2/VP3 capsid proteins of SAT2/ZIM/7/83. The predicted stability mutations were introduced into the full-length SAT2 infectious cDNA clone (pSAT2) and viable mutant viruses were recovered following transfection in BHK-21 cells. We assessed the recombinant mutant virus panel for temperature, pH and ionic stability, antigenicity to SAT2-specific monoclonal antibodies, growth characteristics and genetic stability in suspension cells in order to select candidate mutants for engineering stabilised SAT2 vaccines.

The 93Y and 93W viruses showed much smaller plaque sizes compared to the wild-type virus. This observation was confirmed with a one $\log_{10}$ decrease in the 93Y titre compared to wild-type virus growth over 24 h. It is hypothesised that an increase in stability of 93Y has contributed to delaying viral genome release (uncoating) and slower replication of the virus resulting in a smaller plaque size. O'Donnell et al. (2005) found that the effective release of the viral genome to the cytoplasm is necessary to begin the replication cycle. Some mutant viruses, like the quadruple amino acid mutants, the 93F or 93Q mutants were not viable or grew to $<3\log_{10}$ titres, possibly as a result of over-stabilisation, leading to poor uncoating and replication. This is in contrast to findings of Rincón et al. (2014) in which a different strategy of eliminating

interpentamer repulsions, either by charge neutralization (A2065H) or removal (D2068N, E2086Q, D3134N, or D3195N), were nearly as infectious as the non-mutated virus and yielded similar titres in cultured cells.

The 93Y mutant was upscaled with sequential passage in BHK21 #38 suspension cells and was found to be genetically unstable by acquiring a single nucleotide mutation during passage resulting in 93H. In this study the 93H mutant was shown to be less themostable than 93Y but more thermostable than the wild-type (93S). Genetic instability could be overcome by species-specific codon optimisation in conjunction with the production cell line (Zhou et al., 2011); by the use of stronger base pairing options at the mutation site and choice of cell line that exhibits less reversion (Macadam et al., 2006), compensatory mutations could be introduced near the mutation site (Luongo et al., 2012), adjusting the multiplicitiy of infection (moi), less passaging, improving replication through additional ribosome binding sites or by increasing the fidelity of the viral RNA polymerase (Luongo et al., 2012). Ideally, it would be best to try overcome genetic instability through reverse genetic approaches first.

The antigenic reactivity of the mutants was assessed to ascertain if the amino acid changes had affected the typical binding of five SAT2 MAbs to the epitopes of ZIM/7/83 virus as characterized by Opperman et al. (2014). The reactivity of the 93Y mutant to MAb GD12 was lowered by 37% compared to the wildtype indicative of changes to the GD12 epitope footprint affected by the 93Y mutation in VP2. Although we do not have structural data to confirm this, we hypothesize that increased hydrophobic interactions could possibly cause reduced flexibility or local distortion in surface exposed loops locking the exposed side chains into position not optimally aligned to GD12 complimentary determining regions (CDR's). The exact footprint of GD12 has not yet been mapped but previous evidence (Opperman et al., 2014) suggests that amino acids at the two-fold interface form part of the GD12 footprint. The 93Y mutation is not expected to affect the critical domain for GD12 binding but perhaps to cause imperfect alignment (Colman et al., 1983, Colman, 1997, Lin and Nara, 2007) of the functional binding region of the capsid and GD12 CDRs. However there was no difference in antigenicity of the 93Y and wildtype antigens when

vaccinated in cattle (Chapter 4) and no apparent difference in the structure of the wild-type and 93Y capsids (Kotecha, 2015).

The stability of wildtype and mutant viruses to different temperature, pH and ionic conditions was compared using two methods: a) inactivation assay using live virus particles and b) thermal shift assay (Walter et al., 2012) measuring capsid dissociation using purified particles. There was an increase in thermostability compared to the wild-type virus for mutants 93Y, 94V and 198A using the inactivation assay and for mutants 93Y, 198A, 62Y-87M-143V, 94V, 93H and 193H using the thermal shift assay. The results from the two assays were comparable showing a ten-fold increase in thermostability of mutant 93Y, similar to that of the stable A24 virus.

Additionally, the stability of the mutant viruses to different pH and ionic conditions was evaluated against the wild-type virus. The 93Y, wildtype SAT2 and A24 viruses were more resistant to dissociation at pH 6.1 than most FMDV. It has been shown that FMDV is susceptible to converting rapidly into pentameric subunits at pH 6.0 (Acharya et al., 1989; Curry *et al*., 1996; Ellard *et al*., 1999). The following mutant viruses had improved pH stability compared to the wild-type virus: 93Y and A24 viruses at pH 6.7-9.1; the triple mutant 62Y-87M-143V at pH 7.2-8.5; 93W and 94V at pH 7.6-9.1 compared to the wild-type virus.

The stabilising effect of different ionic buffers are additive to already introduced stability mutations by neutralising destabilising charges on surface exposed amino acids. Most mutant viruses showed improved stability when exposed to: NaCl, $(NH4)_2SO4$, and KCl buffers, PBS, 30% sucrose and VGM solutions. The 1 M NaCl solution supported the highest protective effect on the SAT2 capsid and support the findings by Kotecha et al. (2016). Certain salts like 1 M NaCl are capable of further increasing the stability of these mutant viruses by 6-10 °C. Protein-protein interactions, such as those mediating subunit assembly and hence capsid stability, 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.

Overall, the most beneficial mutation was the single mutant 93Y (temperature and pH stability), while other promising single mutants were 198A, 94V, 93H and triple mutant 62Y-87M-143V. FMDV capsids are held together by weak electrostatic interactions and a series of hydrogen bonds between the pentameric assemblies. We have shown that introducing a single-residue mutation generating hydrophobic stacking of aromatic side chains at the two-fold axis between adjacent pentamers was effective in stabilizing the particle (Kotecha et al. 2015) with little effect on its infectivity. There are a series of histidine residues at the pentameric interfaces and the protonation of a these residues is thought to be responsible for disassembly. Curry et al. (1995) and van Vlijmen et al. (1998) proposed that VP3 His 142 was the trigger for uncoating and Ellard et al. (1999) demonstrated that substitution of this one residue with aspartate renders the capsids stable. The histidine 142 (VP3 143 for SAT2) is facing the positive end of the alpha-helix dipole located at the 2-fold axis. Van Vlijmen et al. (1998) reported that the free energy of dissociation contributed by this histidine is attributable to the polarizing effect of the helix dipole. In addition, there is a second conserved histidine, VP2 87, also at the positive end of the helix dipole which might have a similar effect in destabilising the interface. In enteroviruses such as EV71 (Wang et al., 2012) or polio virus (Basavappa et al., 1994) these histidine residues are not present and there is a tyrosine residue with its OH-group pointing to the positive end of the 2-fold helix. Mutating the histidines in FMDV to valines and introducing an additional mutation, VP2 F62Y of the triple mutant, stabilised the helix dipole at the positive end and showed an increase in binding free energy in simulation as well as a 5˚C increase in thermostability and improved pH stability of the particles compared to the wild-type particles.

The amino acid residues responsible for capsid stabilisation in this study mirrored by *in silico* calculations have the potential to be used in improving the design of SAT2 vaccines by combining reverse genetic approaches that allow a) replacement of external capsid-coding regions or surface exposed antigenic regions to emergency or more suitable field isolates and transfer improved neutralizing epitopes and b) site-directed mutagenesis of amino acids to improve thermostability. Structurally stabilised SAT2 vaccines have a number of advantages in that less strict cold-chain logistics are required especially in Africa where absence of cold chain exists in remote regions and

they have improved duration of immunity due to less degradation (Kotecha et al., 2015). Previously we reported that formulated vaccines from inactivated wild-type and stabilised 93Y SAT2 viruses produced equivalent protective levels of neutralizing antibodies in cattle for 35 days, in addition after storage for 1 or 6 months at 4°C, 93Y particles produced substantially higher neutralising antibodies in guinea pigs than wild-type particles, corresponding with shelf-life improvement (Kotecha et al., 2015). Future work will be to assess the difference in cellular and humoral immune responses, shelf-life and potency between wild-type and stabilised 93Y and 93H antigens in cattle.

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CHAPTER 4

Evaluation of immune responses of stabilised SAT2 antigens of foot-and-mouth disease in cattle

4.1 Abstract

Foot-and-mouth disease (FMD) vaccines with improved stability and less reliant on a cold-chain are needed to improve the longevity of immune responses elicited in animals. This is especially so for serotypes O and SAT2 which are unstable in mildly acidic pH conditions or at elevated temperatures leading to dissociation of the capsid (146S particle) and loss of immunogenicity. Previously, stabilised SAT2 viruses were generated by reverse genetic approaches and assessed *in-vitro* and *in-vivo* in a guinea pig trial. Here we investigated the efficacy and comparative immunological responses of two thermostable and wild-type SAT2 vaccines over 5 months followed by challenge in cattle. We assessed humoral immune responses in terms of total and neutralising antibodies and IgG1/2 isotyping; and cell-mediated responses of IFN-ɣ as *in-vitro* markers of protection. Whilst there were significant differences in total and neutralizing antibodies for the vSAT2-93H group compared to other vaccinated groups after the first vaccination, there were no significant differences after the second immunization. Following intra-dermolingual challenge all vaccinated groups were fully protected as determined by the absence of generalized lesions. These results provide proof that two vaccine doses, consisting of SAT2 antigen combined with ISA206B adjuvant, administered 4-6 weeks apart were able to protect animals up to 5 months pv. Additionally, vSAT2-93Y had significantly higher levels of IFN-γ after challenge and had a lower clinical score indicative of improved protection compared to other vaccinated groups and the importance of cell mediated responses and antigen stability in protection.

4.2 Introduction

Foot-and-mouth disease (FMD) virus (FMDV), an *Aphthovirus* within the family *Picornaviridae*, is highly infectious and one of the most economically important diseases of cloven-hoofed livestock and other artiodactyl species. In the developing

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world FMDV is widely distributed, especially in Africa and Asia [1,2]. In Africa, FMDV is maintained by the African buffalo (*Syncerus caffer*), in a cycle involving wildlife, and independently within domestic animals [3-6]. In Southern Africa, sporadic infection of livestock and wildlife with the three Southern African Territories (SAT) serotypes, i.e. SAT1, SAT2 and SAT3, with multiple topotypes [3, 7-10] readily occur from buffalo transmission [11-13]. Therefore, FMD control in sub-Saharan Africa relies on regular vaccination of cattle in high risk areas; fences separating animals at the wildlife-livestock interface in control zones; movement restriction and constant surveillance.

In some developing countries effective administration of vaccines which is capable of inducing effective immunity is lacking. This has been linked, in part, to factors including poor duration of immunity [14], vaccine potency [15], biophysical (temperature and pH) stability of the antigen [14], and poor cross-protection due to multiple variants in these regions [16]. The poor duration of immunity has been linked to the temperature liability of viruses belonging to the SAT serotypes [14]. One of the foremost factors which influences the potency of vaccine preparations and permits the induction of a protective antibody response is the structural integrity of the intact virion typified by a sedimentation rate of 146S [14]. Due to poor duration of immunity, countries have to rely on a multiple dose yearly vaccination schedule.

Thus, the development of stabilised FMD vaccines with improved immunity are a necessity in Africa. Recent advances in the manipulation of the biological properties of field or laboratory strains [17-19] and structural analyses of the capsid have allowed for the design of thermostable mutations [20, 21] integrated into reverse genetic approaches [22, 23]. Several studies have shown that chimeric vaccines successfully induce protective immune responses and protect FMD host species against live virus challenge [23-25]. Additionally, the SAT capsid can be engineered to encode antigens required for vaccines in specific geographic localities [25] and for improved cell adaptation properties [26, 27].

In many remote areas of Africa, the reliable maintenance of an adequate cold-chain for FMD vaccines from the manufacturer to the field is not possible. Therefore, more stable FMDV vaccines could improve the duration of immune responses in

animals and additionally be less reliant on the cold-chain [14]. FMD is inherently unstable, but especially for O and SAT2 serotypes [14] at raised temperatures or when exposed to mildly acidic pH, which has been linked to loss of immunogenicity due to dissociation of the 146S particle.

The capsids are held together by electrostatic interactions, hydrogen bonds and weak hydrophobic interactions between the inter-pentameric subunits [21, 28]. The inter-pentameric interface residues and their associated interactions are responsible for stabilisation and infectivity of the virion [21, 28]; however, experimental studies on the molecular interactions relating to capsid assembly, disassembly, and stability are still very limited. Furthermore, mutations nearby the FMDV interfaces can affect conformational stability [29–32]. We recently showed that using reverse genetic approaches, we could improve stability by introducing stabilising mutations into a SAT2 infectious clone and recovering stabilised viruses [20]. Residue substitutions were predicted by comparing crystallography structures, sequence data, *in-silico* calculations and modelling of the inter-pentameric interfaces between the thermostable A serotype and the more unstable O and SAT2 serotype viruses [20].

This chapter describes the evaluation of immune responses of two stabilised antigens in comparison to the wild-type SAT2/ZIM/7/83 antigen as vaccines in cattle. We assessed the serological profile generated in cattle, using total and neutralising antibodies, IgG1/2 isotyping and cell-mediated responses of IFN-ɣ as *in-vitro* markers of protection [33, 34]. The two SAT2 stabilised viruses were selected for their potential use as a vaccine strain based on (i) *in vitro* studies that showed improved temperature stability and (ii) initial data showing their ability to elicit an improved immune response with longer duration and shelf-life than wild-type in guinea pigs [20].

4.3 Materials and Methods

4.3.1 Cells, viruses and plasmids

The SAT2 FMDV vaccine strain Zimbabwe (ZIM)/7/83 is a bovine virus originating from an outbreak in western Zimbabwe during 1983 [35] and was maintained at the Transboundary Animal Diseases (TAD) of the Agricultural Research Council (ARC) (South Africa). The plasmid pSAT2, a genome-length infectious cDNA clone of SAT2/ZIM/7/83 [36], was used as the genetic backbone in the construction of recombinant cDNA clones with amino acid substitutions S2093Y and S2093H in VP2

[20]. The cloning, transfection and recovery of viruses were performed as described [20] and were termed vSAT2-wt, vSAT2-93Y and vSAT2-93H.

Baby hamster kidney (BHK) cells, strain 21, clone 13 (ATCC CCL-10) and foetal goat tongue (ZZ-R CCLV-RIE127) cells were maintained as described previously [37]. Mutant viruses were passaged as follows $BHK_2ZZ\text{-}R_2BHK_5$ to produce a vaccine master seed stock. Virus stocks were titrated by plaque assays in BHK-21 cells as described previously [15]. Following the recovery of viable viruses, the presence of the mutation was verified with automated sequencing. The viruses were passaged four times and used for antigen production.

IB-RS-2 (Instituto Biológico renal suino) cells were maintained in RPMI medium (Sigma) supplemented with 10% FCS (Delta Bioproducts), and were used for virus isolations and as the indicator system in the virus neutralization test (VNT).

4.3.2 Production of plasmid-derived chimeric FMDV antigen and vaccine formulation

BHK-21 cell-culture infected fluids from vSAT2-wt, vSAT2-93Y and vSAT2-93H were harvested, inactivated and purified as described before [23]. The inactivated 146S fractions were quantified as described previously [38] and used for vaccine formulation. Three separate vaccines (vSAT2-wt, vSAT2-93Y and vSAT2-93H) containing 6-8 µg each were formulated as double oil emulsions with Montanide ISA 206B (Seppic) as described [23].

4.3.3 Cattle immunizations and viral challenge

Twenty three Nguni cattle 6-9 months of age or 150 kg were divided randomly into 3 groups (n=7) and a control group (n=2). Groups had balanced numbers of females and males. The animal ethics were approved by the ARC-OVI Animal Ethics Committee (25.12), University of Pretoria AEC (V060-15) and Department of Agriculture, Forestry and Fisheries (DAFF, South Africa) Section 20 permit (10/04/2013) (**Supplementary 2.2-2.4**). After an acclimatization period, the cattle were vaccinated intramuscularly on days 0 and 42 with 2 ml of 6-8 µg per dose of (1) vSAT2-wt (wild-type); (2) vSAT2-93Y or (3) vSAT2-93H vaccine. Two control animals were left unvaccinated. Blood samples (clotted and heparinised) were collected every second day from days 0-14, 21, thereafter every second week from 28-160 days post-

vaccination (dpv). The animals were allowed to roam freely in a 0.3 ha camp for 150 days.

At 150 dpv the cattle were brought into the high-containment animal facility at ARC-Onderstepoort Veterinary Institute (ARC-OVI) and each group housed separately. Animals were acclimatized for 12 days. On 162 dpv the three immunized and control groups were inoculated intra-dermolingually at two sites each with 1 ml of 10^4 $TCID_{50}$ SAT2/ZIM/7/83 challenge virus as recommended by the Office International des Epizooties (OIE) [39]. Oropharyngeal (OP) fluid, tonsil swabs (TS) and blood were collected on 0, 2, 4, 7 and 10, 12, 14 days post-challenge (dpc). The animals were examined daily for fever (mild =39.5-40 ºC, severe = >40 ºC) and clinical signs in the mouth and feet (small lesion/healing vesicle = 1, moderate vesicles = 2; severe lesions = 3) and sedated as described [23].

4.3.4 Liquid phase blocking ELISA

Antibody titres in cattle vaccinated with the 3 antigens were detected by a ZIM7/83 SAT2-specific liquid-phase blocking ELISA (LPBE) on days 0, 2, 4, 7, 9, 11, 14, 21, 28 thereafter every 2 weeks until 162 dpv when challenge commenced and sampled 0-14 dpc. The LPBE was essentially carried out as described in the OIE Manual [39]. The optical density (OD^{405nm}) was measured with a Multiskan EX. Samples with serum titres >1/50 (>1.6 log10) were considered positive.

4.3.5 Virus neutralization test (VNT)

Neutralizing antibodies against SAT2/ZIM/7/83 in serum samples collected at 0, 7, 21, 28, 42, 56, 70, 84, 98, 112, 134, 155, 162 dpv from cattle were measured with a VNT, according to the method described in the OIE Manual [39] using IB-RS-2 cells in microtitre plates. The 50% end-point serum titres were calculated according to the method of Kärber [40].

4.3.6 Solid phase competition ELISA (SPCE)

Rabbit anti-serum raised to SAT2 serotype specific FMDV was absorbed onto micro-titre plates and used to capture the FMD type-specific antigen. The SPBE test based on the competition between guinea pig anti-FMDV-SAT2 (GPS) antiserum and antibodies present in the test serum was performed as described in OIE Manual [39].

Positive test sera binding to the antigen that prevents the GPS and conjugate from binding, resulted in a decreased colour reaction.

4.3.7 Non-structural Protein (NSP) ELISA

The Priocheck® NSP FMDV ELISA [41] was used for the detection of antibodies against the 3ABC polypeptide. Samples with percent inhibition of <50% are negative (antibodies against FMDV NS protein are absent) and positive >50% (antibodies are present).

4.3.8 IgG1 and IgG2 isotyping ELISA

The IgGI and IgG2 isotyping ELISA was performed according to Capozzo et al. [33] with slight modification. Maxisorp 96-well plates (Nunc) were directly coated with 100 ng/well solution of sucrose density gradienet (SDG) purified 146S ZIM/7/83 particles in 50 mM carbonate/bicarbonate buffer pH 9.6. Two-fold dilutions of test sera were prepared from 1:50-1:3200. Sheep anti-bovine IgG1 or IgG2 HRP-conjugated antibodies (BD-Serotec) at a dilution of 1:750 and 1:1500 respectively were used. Titers were expressed as the inverse dilution reaching the cut off value (0.2) calculated as mean OD + 2SD achieved by the FMDV-negative Nguni bovine serum samples (n=23).

4.3.9 Whole blood re-stimulation and Bovine Interferon gamma (IFN-γ) ELISA

Whole blood assays [42] were performed using 1.5 ml aliquots of heparinised blood, incubated in 24-well sterile cell culture plates from animals every two weeks from 14-84, 164 and 171 dpv. For each animal, duplicate wells were stimulated with 10 ug/ml Pokeweed mitogen (PWM) as a positive control stimulator of all IFN-γ, 10 ug/ml purified inactivated 146S FMDV ZIM/7/83 or phosphate buffer saline (PBS) as negative control. Plates were incubated for 48 hours with 5% CO_2 and plasma collected. The Bovine Interferon-γ Specific ELISA Assay Kit (MCA5638KZZ, Bio-rad) using two different mouse anti-bovine IFN-γ monoclonal antibodies and recombinant bovine IFN-γ as a standard (0.025-50 ng/ml) was performed.

4.3.10 Virus isolation and plaque assays

FMDV in OP fluid, tonsil swabs and whole blood was detected by the inoculation of IB-RS-2 monolayer cells as described by the OIE Manual [39]. The

supernatant was blind passaged at least twice or until cytopathic effect (CPE) was observed.

Plaque assays were performed in duplicate by infecting IB-RS-2 monolayer cells in 35 mm cell culture plates (Nunc^{TM}) for 1 h, followed by the addition of 2 ml tragacanth overlay [43] and incubated at 37ºC for 48 h. Staining with 1% (w/v) methylene blue in 10% (v/v) ethanol and 10% (v/v) formaldehyde in phosphate buffered saline, pH 7.4 was performed.

4.3.11 Viral RNA detection by real-time quantitative RT-PCR

The viral RNA in OP fluid, tonsil swabs and whole blood was detected using a one-step real-time RT-PCR assay targeting the 3D region [44], tested in duplicate. Total RNA was extracted using QIAamp Viral RNA Mini Kit (Qiagen), according to the manufacturer's specifications and used for cDNA synthesis. Positive test and control samples had a Ct value <30; Ct values 30-40 are designated as weak positive whilst samples Ct values ≥40 negative [44].

4.3.12 Data analysis

Time-course titres obtained by LPBE, SPCE, VNT and IgG-isotype ELISAs were plotted and results between the two experimental groups were compared by ANOVA 2-factor repeated measures followed by Bonferroni multiple comparisons test. Mann–Whitney test was used when data from two groups were compared. The confidence interval was 95%. Statistical analyses were carried out using GraphPad Prism v5.0 (GraphPad Software).

4.4 Results

4.4.1 Characterization of vSAT2-wt, vSAT2-93Y and vSAT2-93H viruses

The construction of the wild-type and thermo-stabilised SAT2$^{ZIM7/83}$ viruses have been described [20]. The vSAT2-wt, vSAT2-93Y and vSAT2-93H viruses were already adapted to BHK-21 cells. Although the vSAT2-93Y virus had a slower growth rate in BHK-21 monolayers, differences in recovery of antigen were negligible (results not shown).

4.4.2 Antibody kinetics of the vSAT2-wt, -93Y and -93H vaccines in Nguni cattle

Sera collected from 0-162 dpv were tested by LPBE, SPCE and VNT's to assess total antibody and neutralizing antibody responses to vaccination (**Fig 4.1**). Total antibody titres from LPBE peaked at 9 dpv for vSAT2-wt and -93H groups and at 14 dpv for the vSAT2-93Y group (**Fig 4.1a**). From 42-162 dpv (after the second vaccination) there was no significant difference ($p<0.05$) in the total antibody titres from LPBE (**Fig 4.1a**) and SPCE (**Fig 4.1b**) between the vaccinated groups. One third of animals from each of the vaccinated groups still had positive LPBE titres ≥1.6 at 162 dpv (**Fig 4.1a**). After challenge, the circulating antibodies were consumed 0-6 dpc, as animals cleared the virus completely both vaccinated and non-vaccinated cattle elicited high levels of total antibodies (**Fig 4.2a,b**). At 21 dpv (**Fig 4.1c**) high positive anti-SAT2/ZIM/7/83 neutralizing antibody titres were observed from vSAT2-wt (2.3-3.2 $\log_{10}$) and vSAT2-93H (2.7-3.2 $\log_{10}$) groups which were statistically significant ($p<0.05$) to the vSAT2-93Y group (**Fig 4.1d**). At 42 dpv VNT titers of vSAT2-wt group were significantly different ($p<0.05$) to both vSAT2-93H and -93Y groups (**Fig 4.1c,d**). At 56-113 dpv (after the second vaccination) there were no significant differences ($p<0.05$) in the neutralizing antibody titres of the vaccinated groups with mean group titres >2.0 $\log_{10}$ (**Fig 4.2c,d**). Vaccinated animals had high levels of neutralising antibodies at 2 dpc (2.74-3.07 $\log_{10}$), whereas non-vaccinated animal levels were low ($0.05\pm0.07\log_{10}$). There was no significant difference ($p<0.05$) between vaccinated groups from 2-9 dpc (**Fig 4.2e**).

4.4.3 IgG iso-typing and interferon gamma (IFN-γ) responses

IgG1 and IgG2 anti-FMDV serum titres were determined at 21, 42, 56 dpv (**Fig 4.1d**) and for 0-9 dpc (**Fig 4.2cd**), with an increase in all vaccinated animals and no statistical difference between vaccinated groups ($p<0.05$) (**Fig 4.1d**). The kinetics of IgG1 and IgG2 titres at 21 and 56 dpv were comparable between the vaccinated groups (**Fig 4.1d**). At 7 dpc IgG1 titres increased, peaking at 9 dpc (**Fig 4.2c**) for vaccinated animals, however there was no statistical difference ($p<0.05$) in IgG1 and IgG2 titres between all groups 0-9 dpc. The ratio of IgG1/IgG2 titres were higher at all the time points.

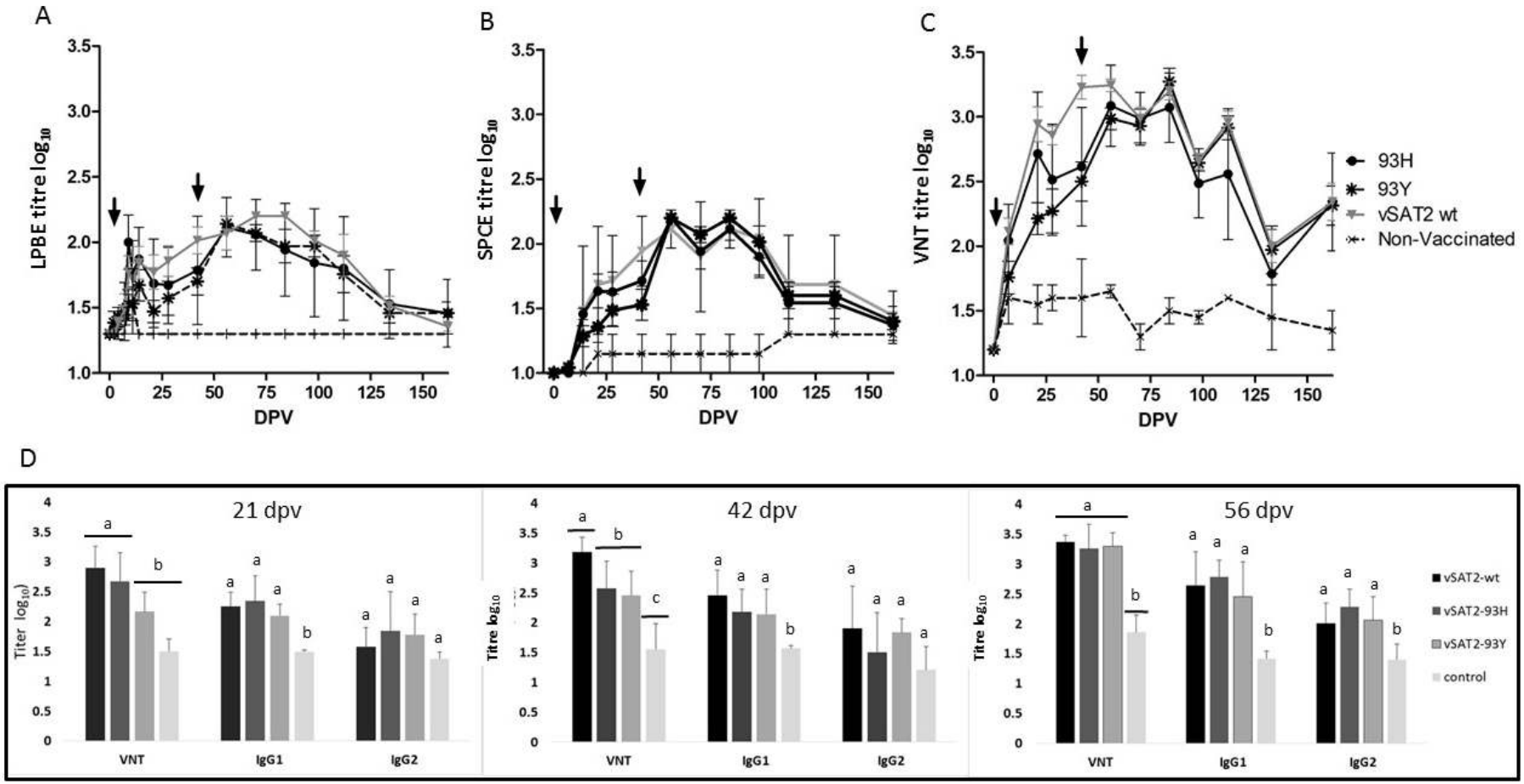

Figure 4.1: Antibody kinetics of vaccination responses to wildtype and stabilised antigens. Mean serum titres (log_{10}) measured by (A) SPCE; (B) LPBE; (C) VNT and (D) kinetics of IgG1, IgG2 and neutralising antibody titres at 21, 42 and 56 dpv of Nguni cattle immunized twice with a 6-8 µg dose of either vSAT2-wt (n=7), vSAT2-93H (n=7) or vSAT2-93Y (n=7) BEI-inactivated, SDG-purified antigens with Montanide ISA206 adjuvant. Arrows indicate the time of first vaccination (0 dpv) or second vaccination (42 dpv). Error bars represent the standard deviation. Animals regarded as negative had log_{10} titres ≤1.6 as shown by the unvaccinated control animals (n=2). a,b or c refers to statistically significant groupings where a is statistically significant to b and c, and b is statistically significant to c.

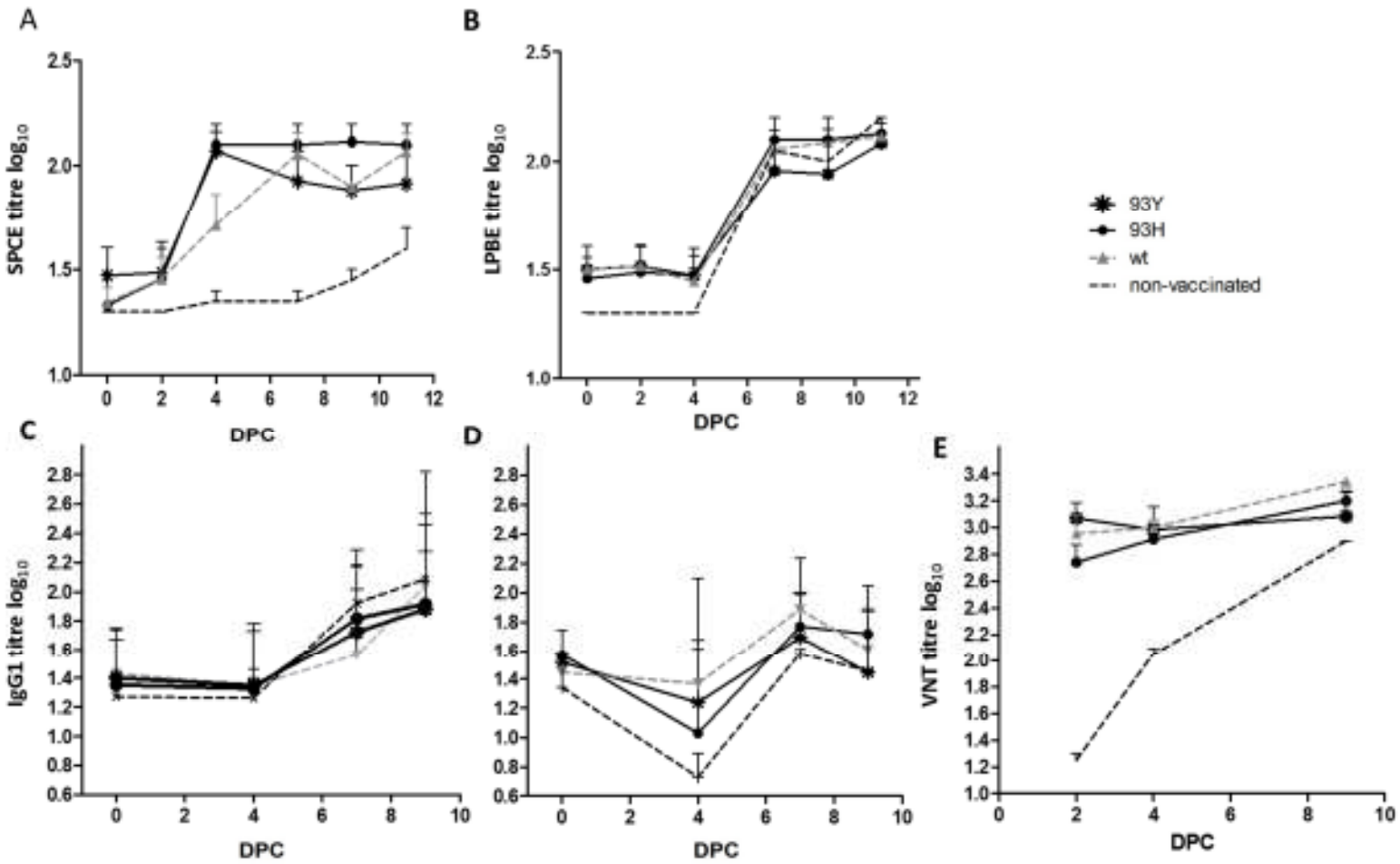

Figure 4.2: Antibody kinetics of challenge responses to wildtype and stabilised antigens. Mean serum titres (log_{10}) of cattle challenged with live SAT2/ZIM/7/83 virus at 0-12 days post challenge (dpc) measured by (A) SPCE; (B) LPBE; (C) IgG1; (D) IgG2 and (E) VNT after being vaccinated twice with either vSAT2-wt (n=7), vSAT2-93H (n=7) or vSAT2-93Y (n=7) antigens and unvaccinated control animals (n=2) at 0-162 dpv. Error bars represent the standard deviation. * Denotation refer to statistically significant groupings.

IFN-γ levels measured from whole blood are extremely variable [45], however validation and implementation of the test was based on previous findings [42]. Blood was incubated with PBS (negative control <1 ng/µl), SAT2-FMDV and PWM (positive control >3 ng/µl). Stimulated blood of both naïve and vaccinated animals were tested with a commercial kit which includes a IFN-γ standard 0.025-50 ng/µl. IFN-γ responses peaked at 28 dpv with vSAT2-93H (8.55 ± 7.89 ng/µl) followed by vSAT2-93Y (4.86 ± 5.17 ng/µl) and vSAT2-wt (2.74 ± 4.55 ng/µl) (**Fig 4.3a**). IFN-γ responses before challenge (84 dpv) showed high systemic IFN-γ levels for vSAT2-wt and vSAT2-93Y. After challenge (2 dpc) IFN-γ levels induced by the vSAT2-93Y vaccine were significantly higher ($p < 0.05$) than the others (**Fig 4.3b**).

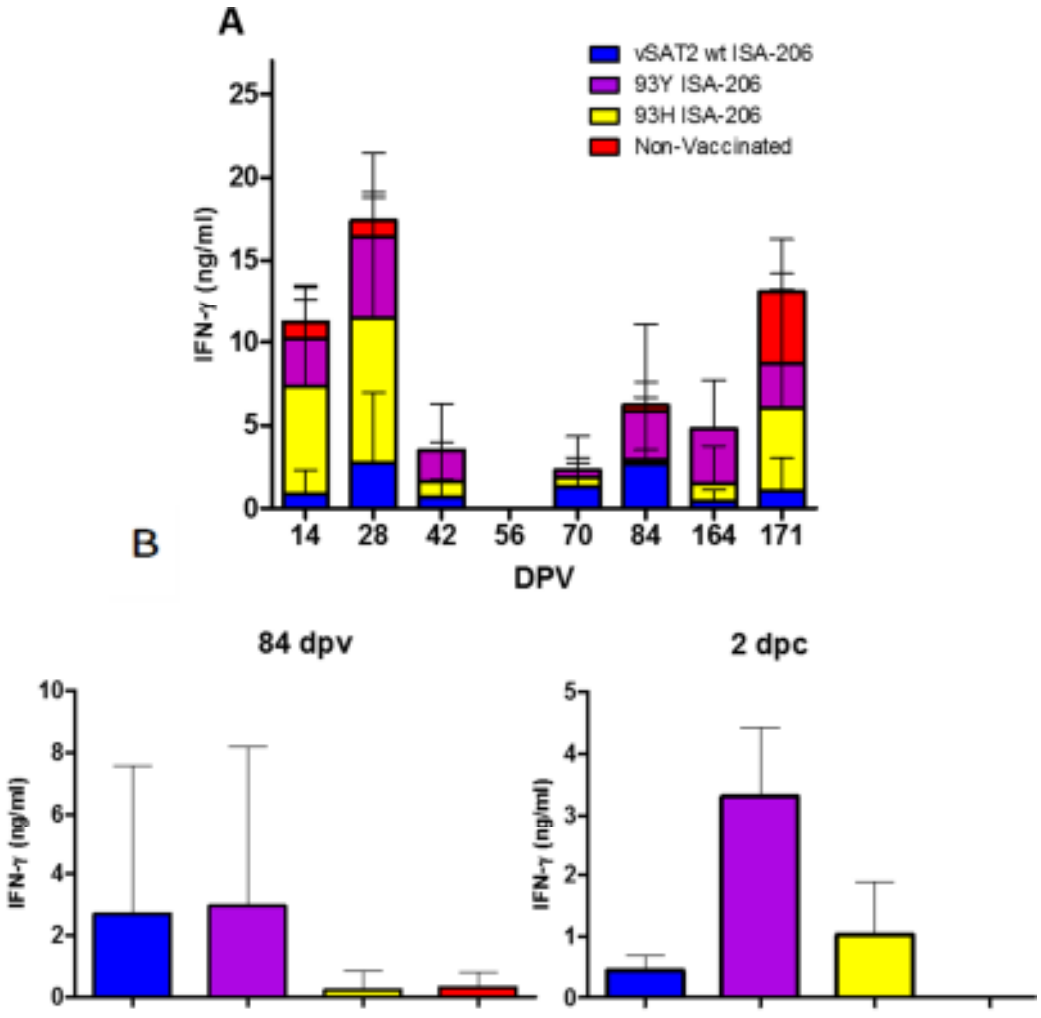

Figure 4.3: Cell mediated IFN-γ responses. A) Mean serum titres of IFN-γ (ng/ml) of Nguni cattle after being vaccinated twice with either vSAT2-wt, vSAT2-93H or vSAT2-93Y antigens 14-84 dpv. Error bars represent the standard deviation. Arrows indicate the time of first vaccination (0 dpv) or second vaccination (42 dpv) and challenge 162-171 dpv (0-9 days post challenge). B) Comparison of IFN-γ (ng/ml) levels at 84 dpv and 2 dpc, showing significantly higher levels ($p<0.05$) of vSAT2-93Y at 2 dpc compared to other antigens.

4.4.4 Protection of vaccinated cattle against live SAT2/ZIM/7/83 virus challenge

The 3 groups (n=7), that received the vSAT2-wt, vSAT2-93Y and vSAT2-93H vaccines, were protected against systemic spread of FMD after the intra-dermolingual challenge of SAT2/ZIM/7/83 virus as observed by the absence of generalised lesions on their hooves, whilst mild fever (39.5-40 ºC) was present in animals vaccinated with vSAT2-wt (n=1) and vSAT2-93H (n=2) 48 hours pc. (**Fig 4.4b**). There was no statistical difference in clinical scores between the vaccinated groups ($p<0.05$). This was in contrast to the unvaccinated control animals (n=2) which developed severe pyrexia and severe lesions that were generalised to all four hooves within 48-72 hours of challenge (**Fig 4.4b**). The vSAT2-93Y group had the shortest period of 2-4 days of subclinical infection compared to 2-7 days with the other vaccinated groups. Additionally, less animals showed subclinical infection in the vSAT2-93Y group (n=3) compared to vSAT2-wt (n=6) and vSAT2-93H (n=5).

4.4.5 Virus isolation and the presence of viral RNA

FMDV was recovered from cell culture and FMDV RNA was detected by real-time RT-PCR (positive sample Ct value <32) in OP and tonsilar swab (TS) (**Fig 4.4a**) samples from 0-11 dpc.

The following results were found for OP samples (**Fig 4.4a, Suppl table 1.5**): vSAT2-wt group (n=7): virus and/or viral RNA was detected 2-7 dpc (n=4) whilst virus or viral RNA (n=1), low viral RNA (n=2) only detected 4 dpc; vSAT2-93Y group (n=7): virus and/or viral RNA detected 2 dpc (n=2) whilst virus or viral RNA (n=1), viral RNA (n=3) or low viral RNA (n=1) detected from 4 dpc. Overall there was low detection of RNA or virus compared to other vaccinated groups and detection was later from only 4 dpc; vSAT2-93H group (n=7): virus and/or viral RNA was detected 2-7 dpc (n=6) whilst viral RNA (n=1) detected 4 dpc; and the unvaccinated group (n=2): virus and viral RNA detected from 2-4 dpc and virus or RNA from 7-9 dpc (n=2) and also developed severe symptoms with lesions on the hooves. No animals from the vaccinated groups that were positive on virus isolation developed any lesions at sites other than site of inoculation. TS samples showed a similar result as OP samples (**Fig 4.4a, Suppl table 1.5**).

Viraemia was assessed 2-7dpc and virus was only isolated in the unvaccinated group (n=2) on 2-4 dpc, with a corresponding high viral RNA load. Viral RNA was detected in blood later at 4 dpc for the vSAT2-wt group compared to the earlier

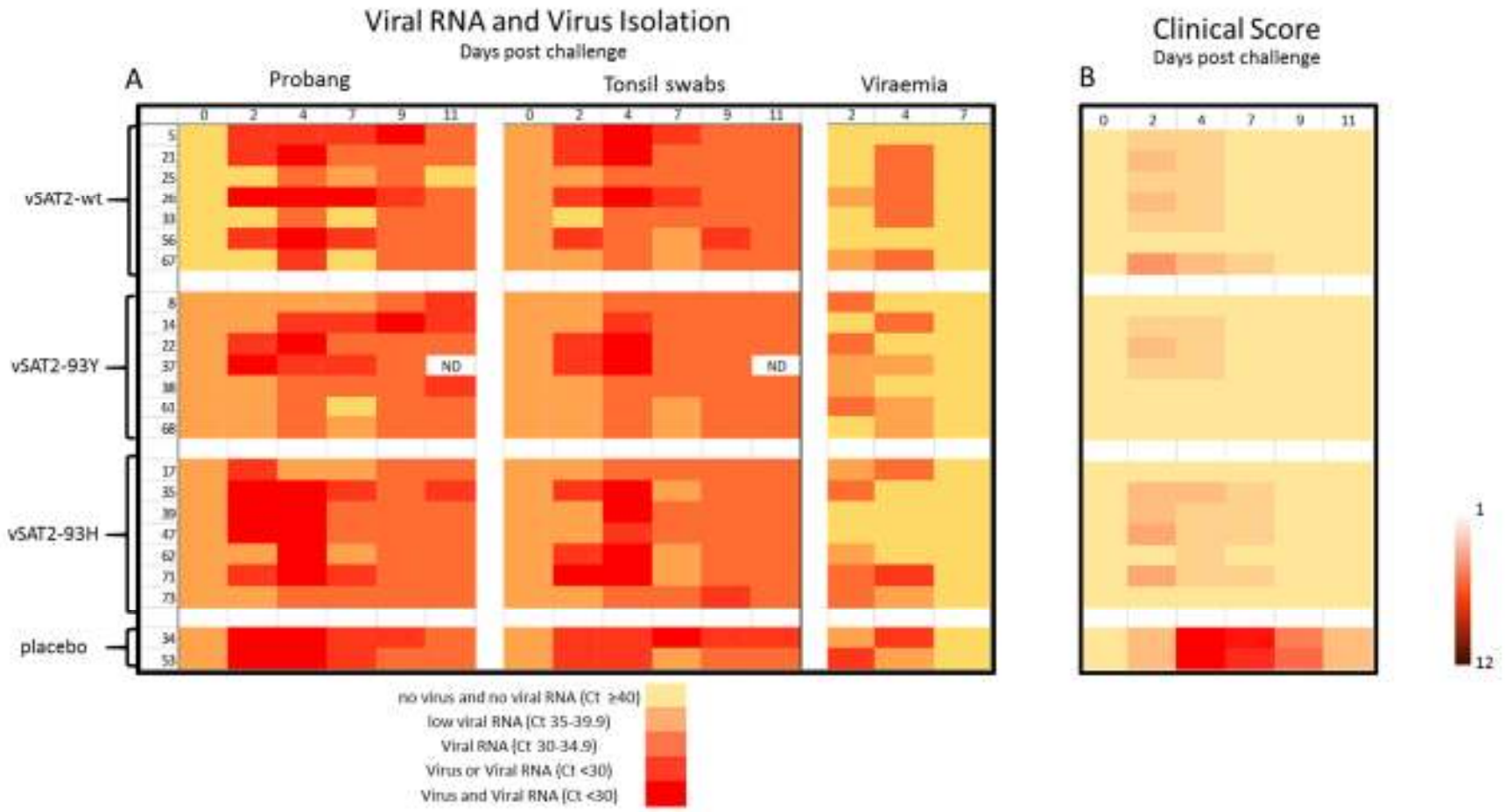

Figure 4.4: Clinical scores and presence of FMDV and viral RNA 0-14 days post challenge. Summary of the (A) presence of viral RNA (determined by real-time RT-PCR) and virus isolation in oropharyngeal fluid (probang), tonsil swab and whole blood samples and (B) clinical scores of animals vaccinated with vSAT2-wt (n=7), vSAT2-93H (n=7) or vSAT2-93Y (n=7) antigens and non-vaccinated controls (n=2) challenged at 162 days with SAT2/ZIM/7/83 virus. The clinical scores were calculated as described in materials and methods, with 0-12 indicating low to high severity.

presence of viral RNA from 2 dpc in the vSAT2-93Y and -93H groups (Fig 4.4a, Suppl table 1.5).

The presence of non-structural protein (NSP) antibodies were positive using the NSP ELISA indicative of presence of live FMDV in challenged animals.

4.5 Discussion

In Africa, the duration of immunity and improved stability of SAT2 vaccines [14] are important considerations for FMD vaccines to be less reliant on a cold-chain in many remote areas in Africa [45]. The control of SAT2 in Africa is dependent on: (i) the selection of cross-protective strains to protect against the high antigenic diversity circulating strains and (ii) stable vaccines with intact 146S particles that will induce a strong and sustainable serological response [46]. Although SAT2 antibody responses were observed following vaccination of cattle in Mnisi, South Africa, the proportion of cattle with significant titres to previous vaccination was low suggesting that the interval between vaccinations was too long to maintain adequate antibody response [46].

In the present study, the efficacy and comparative immunological responses of two thermostable and wild-type SAT2 vaccines were assessed. Viruses generated by reverse genetic approaches with modified residues identified through *in-silico* modeling [20, 47] that improve stability provided the basis for the structural engineering of two improved SAT2 vaccines. The stabilised SAT2 146S antigens were previously formulated as vaccines and elicited improved immune responses with longer duration and shelf-life than wild-type in guinea pigs [20].

Cattle vaccinated with vSAT2-wt and vSAT2-93H produced strong neutralizing antibodies titres (>2.3 $\log_{10}$) at 21 dpv whereas the neutralizing antibody titres to vSAT2-93Y were delayed, however after second vaccination there was no significant difference ($p<0.05$) between the vaccinated groups. By 162 dpv all vaccinated animals still had neutralizing antibodies titres between 1.7-2.4$\log_{10}$, correlative to protection [34, 47].

Total antibody titres peaked earlier at 9 dpv for the vSAT2-wt and -93H groups compared to 14 dpv for -93Y group, however there was no difference in titre after the second vaccination. Even though by 162 dpv total antibody titres were <1.6 for most animals, which is suggestive of poor protection there were however a few animals from each group that still maintained >1.6 titres. However, following intra-dermolingual challenge all vaccinated groups were fully protected as determined by the absence of generalized lesions even though virus excretion in the oropharynx was detected 2-4 dpc. As expected unvaccinated animals developed pyrexia 1 dpc and generalized lesions

within 2 dpc. These results also provide proof that two vaccine doses, consisting of SAT2 antigen combined with ISA206 adjuvant, administered 4-6 weeks apart were able to protect animals up to 5 months pv as advised by the OIE [39]. It has been reported that FMD vaccine immunity is short-lived and cattle in endemic areas require revaccination at regular intervals of 4-6 months to ensure protective levels of antibodies [16, 49]. We have not tested the duration of immunity afforded by the stabilized vaccines in cattle after 6 months, this will be tested in the future.

There were no significant differences ($p<0.05$) between IgG1 and IgG2 titres of the vaccinated groups with IgG1>IgG2 titres across all time points. The role of the IgG1 isotype in protection of cattle from disease has been reported [33] and a predominance of IgG1 over IgG2 antibodies in animals receiving infectious or inactivated virus [50]. T-cell responses were measured by IFN-γ (cytokine) [34] that is mainly produced by activated cells [34] in anamnestic responses. SAT2-specific IFN-γ responses peaked at 28 dpv with vSAT2-93H having the highest concentrations followed by vSAT2-93Y. Moreover, IFN-γ anamnestic T-cell responses in the 93Y group were higher at 84 dpv for vaccinated animals even when total Ab responses had decayed. IFN-γ responses were boosted at 2 dpc, with higher levels for vSAT2-93Y than -93H and -wt vaccinated animals. Previously it was shown that increased structural stability of the whole A24 virus correlated with the ability to induce greater IFN-γ [42]. This data may explain the improved ability of the 93Y vaccine to have a shorter subclinical period or no symptoms at all and the importance of IFN-γ T-cell responses in vaccine-mediated protection [42].

Although we have not exposed the thermostable and wild-type vaccines to any period of shelf-life storage or any break in the cold-chain to truly test the differences in stability and immunogenicity of the two antigens it is not unreasonable to expect from the *in vitro* stability data and guinea pig trial [20] that the two antigens will outperform the wild-type antigen during long term storage. Previously it was shown that ISA 206B oil adjuvant was the most suitable adjuvant for SAT serotypes and able to elicit immune response in cattle for >50 weeks pv, however when stored for 6 months at 4 °C, it failed to protect the cattle against a homologous infection even though the vaccine's pH level and emulsion were still of acceptable quality at the time of vaccination [48]. Improved storage of SAT2 vaccines will be advantageous for the vaccine producer, which currently has to increase the antigen load to make up for the rapid decrease during storage [15].

After the second vaccination (>52 dpv) there were no differences observed between vaccinated groups. Interestingly vSAT2-93Y had significantly higher levels of IFN-γ after challenge compared to other vaccines indicative of the importance of cell mediated responses and a shorter or no subclinical period. We did not measure antibody

avidity in this study, however it is not unrealistic to assume that after the second vaccination cattle developed high avidity antibodies through affinity maturation [25].

In conclusion, we have shown that two thermostable and the wild-type SAT2/ZIM7/83 vaccines are capable of eliciting strong antibody titres and IFN-γ with full protection more than 5 months pv, this is the first step in proof of concept to now advance our knowledge on where problems of SAT2 stability occur from vaccine production to the administration in the field. We intend to follow up this work with thermostable vaccination trials assessing different shelf-life conditions, disturbances in cold-chain maintenance and potency to improve stability and SAT2 control.

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CHAPTER 5

Inherent biophysical stability of foot-and-mouth disease SAT2 viruses

5.1 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. Even worse is the situation for the three SAT serotypes, for which the notoriously low biophysical stability only yield vaccines of low protective capacity, even when administered multiple times. The aim of the study was to determine the inherent biophysical stability of 8 field SAT2 isolates from 2 different topotypes. To characterise the stability of the different SAT2 viruses we used thermofluor analysis to monitor capsid dissociation by the release of RNA genome under a range of temperature, pH and ionic conditions. The dissociation temperatures of the 8 SAT2 viruses ranged from 48-54 °C, a substantial difference of 6 degrees ($P<0.05$), with the southern topotype I viruses having the highest stability of 54 °C whilst the western topotype II viruses ranged from 48-52 °C. Some of these SAT2 viruses had very similar dissociation temperatures to the highly stable A24 control virus at 56 °C. The addition of ionic buffers showed that 1 M NaCl was capable of enhancing stability of the SAT2 viruses by a further 6-8 °C whereas some solutions showed differing results dependent on the virus tested, highlighting the need to test SAT2 viruses before storage to establish the most stabilising option with different solutions. This confirms for the first time that more stable SAT2 viruses are present in the field from the Southern Africa region that could facilitate the selection of the most stable circulating field strains, as well as the comparison of such strains with their recombinant counterparts in vaccine design. The selection of naturally more stable viruses, which have not been manipulated *in-vitro,* could result in genetically stable viruses through laboratory adaptation and upscaling process.

5.2 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), an *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 is one of the most economically important diseases of livestock, such as cattle, sheep, pigs and goats (Gibbs, 1981, Sutmoller et al., 2003, Perry and Rich, 2007). Vaccination remains the most effective approach for controlling the seven, clinical indistinguishable serotypes, (A, O, C, Asia-1 and Southern African Territories (SAT) 1, SAT2 and SAT3) that exist and of the various antigenic subtypes that continuously emerge. Thus the most effective vaccines need to closely match the outbreak virus resulting in 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 months and in many remote regions happens in the absence of a cold-chain. Vaccines with improved biophysical stability of the antigen and less reliant on a cold-chain are needed and could improve the longevity of immune responses elicited in animals (Doel and Bacharini *et al*., 1981). FMD, in constrast to other picornaviruses, is known to be unstable, especially viruses belonging to the O and SAT2 serotypes, in mildly acidic pH conditions or at elevated temperatures, leading to dissociation of the capsid (146S particle) and loss of immunogenicity (Doel and Baccarini *et al*., 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 climate regions where FMD is prevalent. Even worse is the situation for the three SAT serotypes, for which the notoriously low biophysical stability is one reason for the yield vaccines of low protective capacity, even when administered multiple times.

Early pioneering studies (Bachrach *et al*., 1960; Pringle, 1964) described the selection of thermostable variants from a FMDV laboratory population (serotype A) after extensive passaging (76 times) in cell culture. However, these variants were genetically unstable, showed a reduced fitness in cell culture and the genotypic changes associated with increase in thermostability were not determined. Mateo *et al*. (2007) considered the possibility that adaptation of FMDV to cell culture could alter the quasispecies equilibrium, leading to the presence of highly thermostable variants. They observed that the inactivation rate constants for three unheated laboratory populations of FMDV (serotype C) were significantly higher than those obtained for natural FMDV populations (i.e. a field virus subjected to limited amplification in cultured cells). This indicates that some reduction

in thermostability may occur during adaptation of FMDV to cell culture conditions, perhaps because of the absence, in those conditions, of the selective pressure exerted in the field by occasional heat extremes. A way to reconcile the presence of thermostable variants in some FMDV populations, may be to contemplate two aspects: (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., 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 et al., 1986; Rossmann & Johnson, 1989; Reguera et al., 2004). The non-enveloped, icosahedral virion of FMDV is composed of 60 repetitions of four viral structural proteins, VP1-4 that assemble 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; Ellard et al., 1999; Mateo et al., 2003). Even though FMDV, especially the SAT serotypes, exhibit large intra- and inter-serotype genetic variability (Carrillo et al., 2005), the multiple and repetitive intersubunit 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

Based on the above studies, we followed a novel approach to accurately measure the inherent biophysical stability of low passage field viruses of the SAT serotypes in varying temperature, pH and ionic conditions. We selected representative viruses of the three genetically distinct topotypes of SAT2 in southern Africa. This information is vital in the selection of field viruses with more stable capsid properties for vaccine production.

5.3 Materials & Methods

5.3.1 Sequence Analysis

The P1 sequences of SAT2 viruses from southern Africa available from previous studies (ref) and retrieved from GenBank were analysed to provide phylogenetic references. The following sequences were analysed: KNP/1/10 (JX088744); KNP/20/08 (KJ999934); KNP/03/10 (KJ999936); KNP/15/07 (KJ999933); SAR/03/04 (personal communication); KNP/51/93 (GU194489); SAR/01/01 (KJ999932); KNP/19/89 (KR108949); KNP/02/89 (GU194489); SAR/16/83 (DQ009726); ZIM/10/91 (GU194493); ZIM/8/94 (Maree, unpublished); MAL/01/08 (KJ999939); ZAM/07/96 (AF367120); ZAM/08/96 (KJ999942); ZAM/10/93 (KJ999943); ZIM/09/02 (KJ999944); NAM/04/07 (KJ999937); NAM/01/08 (KJ999938); ZIM/14/90 (DQ009728); ZIM/17/91 (DQ009727); ZIM/34/90 (GU194490); BOT/18/98 (KJ999940); ZIM/05/02 (JQ639295); ZIM/07/83 (DQ009726); ZIM/05/83 (JQ639289); ZIM/01/88 (GU194491); ZIM/02/88 (JQ639294); ZIM/7/89 (JQ639296); ZIM/48/97 (KJ000041); ZIM/4/97 (JQ639293); ZIM/44/97 (JQ639291). A dendrogram was constructed to determine the relationship that exists between the FMDV SAT2 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 by the ClustalW alignment software (EBI, Cambridge, England) incorporated into the BioEdit software (Thompson *et al*., 1997).

The Mega 5.1 package was used to perform the phylogenetic and distance analyses (Kumar *et al*., 2003). Unrooted dendrogram was constructed after bootstrapping to 1000 replicates by the neighbour-joining method using the Jukes-Cantor parameter. A neighbour-joining (NJ) 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 (Kumar *et al*., 2003).

5.3.2 Cells and viruses

Field viruses (**Table 5.1**) with low passage history were propagated on IB-RS-2 (Instituto Biologico renal suino) cells and Baby hamster kidney (BHK) clone 13 cells (strain 21; ATCC CCL-10) for purification. IB-RS-2 cells and BHK-21 cells were maintained and propagated in RPMI medium 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 media (VGM; GMEM with 1% (v/v) FCS, 1% (v/v) HEPES and 1% (v/v) antibiotics).

Table 5.1: SAT2 Foot-and-mouth disease viruses used in the study showing the year and species from which it was isolated; the country of origin and the GenBank accession numbers.

Serotype	Virus	Year isolated	Species	Country of origin	GenBank Accession No.
SAT2	SAR/03/04	2004	Cattle	South Africa	F.Maree,unpublished
	ZIM/13/01	2001	Cattle	Zimbabwe	JQ639292
	ZIM/07/83	1983	Cattle	Zimbabwe	DQ009726
	ZIM/14/90	1990	Cattle	Zimbabwe	DQ009728
	ZIM/04/97	1997	Cattle	Zimbabwe	JQ639293
	ZIM/08/94	1994	Cattle	Zimbabwe	JQ639290
	BOT/18/98	1998	Cattle	Botswana	KJ999940
	MAL/01/08	2008	Cattle	Malawi	KJ999939

5.3.3 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 minutes 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 mMTris [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_{259} nm = 79.9; (Doel & Mowat, 1985) and pooled for analysis.

5.3.4 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 300-500ng of virus, 5 µl of 100X SYBR green-II dye (Molecular Probes, Invitrogen; diluted 1:100), with the volume made up to 50 µl with 1X PBS buffer. The temperature was ramped from 15 °C to 95 °C in 0.5 °C increments with intervals of 10 seconds. 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 concentrations of 1 M and 0.5 M NaCl, KCl, $(NH_4)_2SO_4$, $MgCl_2$ and $CaCl_2$, 5% glycine, VGM, 30% sucrose, 0.8X PBS (control reaction) and 1X PBS using SYBR green-II dye and virus concentrations as described above. The pH buffers were composed of MBS (MES-buffered saline, pH 6.1, 6.7, 7.2, 7.6, 8.2, 8.5, 9.1).

5.4 Results:

5.4.1 Phylogenetic tree of P1 region

The P1 coding region of approximately 2kb was obtained from sequences on Genbank which included 33 sub-Saharan African SAT2 FMDV isolates previously sequenced. Midpoint rooted neighbor-joining (NJ) trees were used to determine the phylogenetic relationships of the P1 coding region. Based on the cut-off value of less than 16% nucleotide difference, three clusters were observed, supported by strong bootstrap values. The three clusters represented the (a) southern topotype viruses from South Africa, southern Zimbabwe; (b) northern topotype viruses from Malawi, Zambia, northern Namibia and Zimbabwe; and (c) western topotype viruses from Zimbabwe and Botswana. The following viruses available and representative of the two topotypes I and II were selected for analysis with the thermofluor assay: (a) southern topotype I: MAL/01/08, ZIM/04/97 and SAR/03/04; and (b) western topotype II: ZIM/14/90, BOT/18/98, ZIM/13/01, ZIM/07/83 and ZIM/04/97 (**Fig 5.1**) (Bastos et al., 2003; Knowles and Samuel et., 2003).

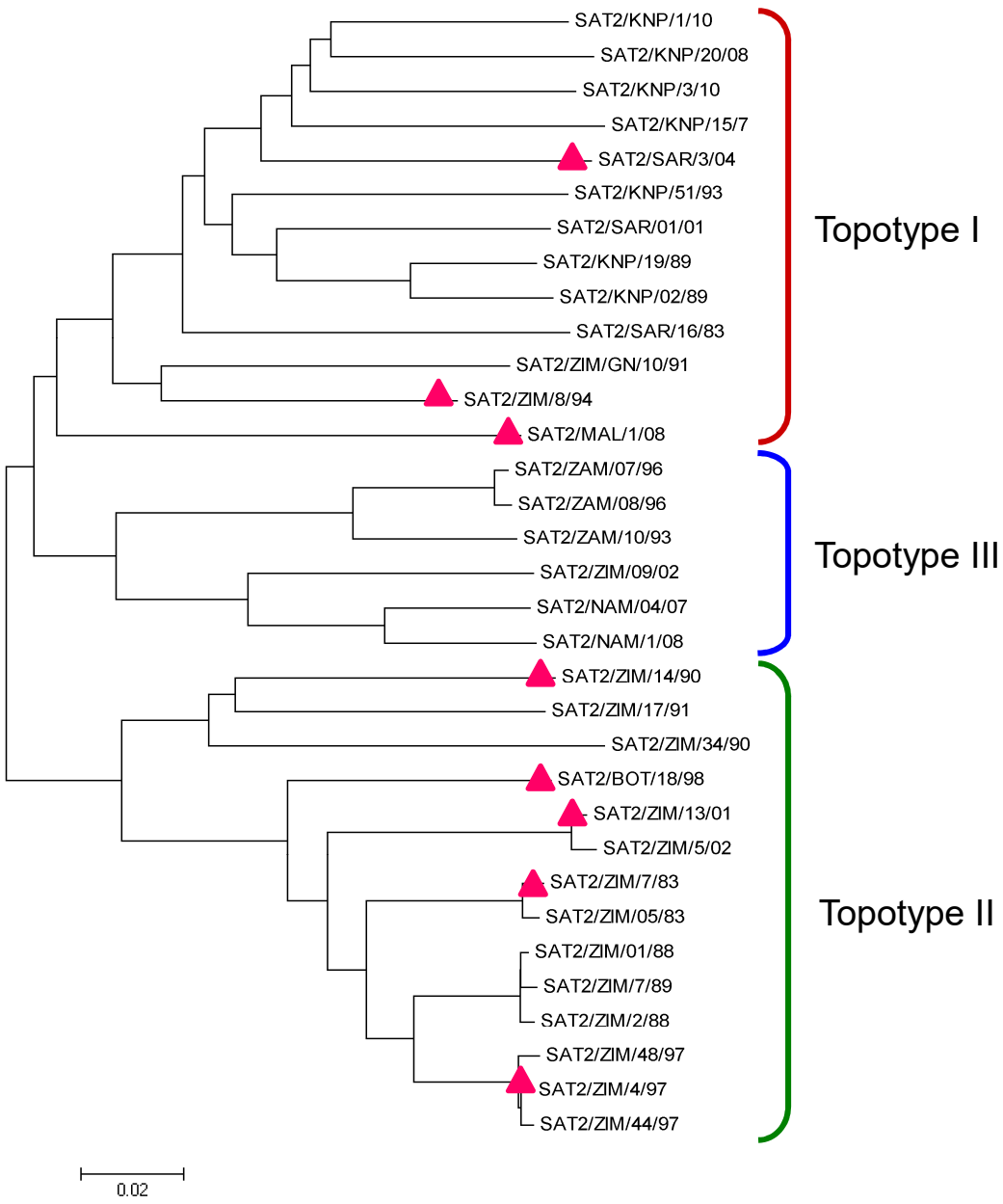

Figure 5.1: A dendrogram (NJ tree) of P1 sequences of FMDV SAT2 viruses from southern Africa depicted as topotypes I-III. Viruses selected for stability analysis from topotypes I and II (Southern and Western) are shown with pink triangles.

5.4.2 Amino acid analysis at 2-fold interface

Amino acid residues located at the interface of the SAT2 viruses identified as being responsible for stability were compared to the ZIM/07/83 virus sequence that was used in a previous study (Scott et al., submitted). This was in an attempt to link any differences observed in stability with amino acid differences at the interface. There were no differences between the amino acid residues of ZIM/07/83 and the SAT2 viruses analyzed (ZIM/13/01, ZIM/14/90, ZIM/04/97, BOT/18/98, MAL/01/08, SAR/03/04 and ZIM/08/94)

(Table 5.2). The following amino acid positions were compared in VP2: 62, 87, 93, 98, 113, 114, and 215; and in VP3: 143, 193, 194 and 198.

Table 5.2: Amino acid residues situated at the interface encompassing VP2 and VP3 proteins of SAT2 viruses.

Viruses	Amino acid positions										
	VP2							VP3			
	62	**87**	**93**	**94**	**98**	**114**	**215**	**143**	**193**	**194**	**198**
ZIM7/83	Phe	His	Ser	Leu	Tyr	Thr	Leu	His	Asn	Thr	Glu
***SAT2 viruses**	Phe	His	Ser	Leu	Tyr	Thr	Leu	His	Asn	Thr	Glu
#Stable amino acids	Tyr	Met Val	Tyr Phe Trp His Gln	Val	Phe His	Asp	Lys	Val	His	Gly	Ala

*denotes ZIM13/01, ZIM14/90, ZIM4/97, BOT18/98, MAL1/08, SAR3/04, ZIM8/94 and KNP 1/10 viruses. # denotes stable amino acids identified by Kotecha et al., 2015 and Scott et al., submitted.

5.4.3 Thermal release assay

Fluorescent thermal shift stability assays (Walter et al., 2012) were performed in triplicate on purified SAT2 viruses. The average dissociation temperatures equating to capsid stability of the 8 SAT2 viruses ranged from 48-54 °C, with a difference of 6 degrees ($P<0.05$) between the lowest and highest **(Fig. 5.2b)**. Viral capsids from topotype I (n=3) viruses had the highest stability and dissociated at 54 °C whilst the topotype II viruses (n=5) ranged from 48-52 °C. The A-serotype A24 control virus had the highest temperature stability of 55 °C.

To investigate the effect of pH on capsid stability, purified viruses were exposed to buffers in a pH range 6.1-9.1 (**Fig. 5.2a**). Sensitivity to pH was measured by the decrease in temperature compared to the PBS control. At pH 6.1 most viruses were fully dissociated except for topotype II virus ZIM/07/83 and topotype I viruses (n=3), which were more pH resistant. At pH 9.1 the topotype II viruses were most labile, ranging from fully dissociated at 44 °C whereas topotype I viruses were more resilient, ranging from 48-51 °C. Between pH 6.7-7.6, topotype I viruses were more resilient especially MAL/01/08 and SAR/03/04

viruses, whereas the ZIM/14/90 virus (topotype II) was most labile. The panel of viruses were most stable at pH 8.2.

To investigate the effect of ionic buffers on capsid stability, 13 ionic buffers were tested (**Fig. 5.2b**). The following summarizes 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 stability respectively compared to the control buffer, with the western topotype viruses (n=5) having highest increase in stability of 6-8 °C and 4-5 °C with 1 M and 0.5 M NaCl buffers respectively. The 1 M and 0.5 M KCl buffer supported improved stability compared to the control with topotype II viruses supporting highest increase of 6-8 °C. The 0.5 M CaCl2 and 30% sucrose had conflicting results where some viruses were fully dissociated or less stable whilst others had improved stability (3-6 °C). The 1 M (NH4)2SO4 buffer improved stability for only two topotype II viruses ZIM/14/90 and ZIM/04/97 whilst the 0.5 M $(NH_4)_2SO_4$ buffer showed decreased stability for all viruses. The VGM, 1 M CaCl, 5% Glycine, 1 M and 0.5 M MgCl2 buffers affected the greatest decrease in stability and are not suitable buffers for storage of the SAT2 viruses however, the A serotype virus A24 was not affected by these buffers.

A

Serotype	topotype	virus isolate	1x PBS	6.1	6.7	7.2	7.6	8.2	9.1
SAT2	I	MAL/1/08	56	39	48	49	51	53	48
	I	SAR/3/04	56	44	48	49	51	53	50
	I	ZIM/8/94	56	38	44	47	50	53	51
	II	ZIM/14/90	56	ds*	43	44	47	52	44
	II	ZIM/4/97	52	ds*	39	42	45	48	43
	II	ZIM/13/01	55	ds*	43	46	47	51	ds*
	II	BOT/18/98	54	ds*	43	46	49	52	42
	II	ZIM/7/83	54	37	41	45	46	48	44
A	control	A24	58	38	43	50	50	52	49

B

Serotype	topotype	virus isolate	NaCl		$(NH_4)_2SO_4$		$MgCl_2$		KCl		CaCl		5% Glycine	VGM	30% sucrose	1xPBS	control
			1M	0.5M	1M	0.5M	1M	0.5M	1M	0.5M	1M	0.5M					
SAT2	I	MAL/1/08	59	57	51	48	41	46	54	53	45	55	41	51	50	56	54
	I	SAR/3/04	58	57	54	51	40	45	56	54	ds*	ds*	49	51	57	56	54
	I	ZIM/8/94	59	57	53	50	ds*	ds*	56	54	ds*	ds*	45	50	55	56	54
	II	ZIM/14/90	58	55	52	48	40	42	54	50	ds*	52	38	44	45	52	50
	II	ZIM/4/97	59	56	54	49	42	48	55	52	47	57	34	49	47	56	51
	II	ZIM/13/01	58	56	50	47	ds*	ds*	55	53	ds*	ds*	45	ds*	54	55	52
	II	BOT/18/98	58	56	51	47	38	43	52	51	42	55	39	49	49	54	52
	II	ZIM/7/83	56	52	43	39	ds*	ds*	53	50	45	48	53	47	51	54	48
A	control	A24	60	59	56	55	59	57	58	57	55	54	52	55	58	58	53

Figure 5.2: Thermostability measured fluorescence assay (Walter et al., 2012) to determine the dissociation temperatures of SAT2 viruses. The A24 virus was used as a representative control of a stable virus. Heatmaps are depicted for A and B, showing the average dissociation temperatures of duplicate repeats and error values. The colouring of green is indicative of more stable whilst red is more unstable. **A)** pH stability tested with a range of pH buffers 6.1-9.1; and **B)** ionic stability of 14 different buffers and their effect as additional improvement to stability. Ds* denotes where viruses were dissociated under the experimental condition and no reading was obtained. The control represents the natural dissociation temperature excluding pH and ionic effects.

5.5 Discussion:

Previously we have focused on modelling and mutating viruses to improve stability (Kotecha et al., 2015, Scott et al., submitted), here we focus on the natural range of stability exhibited by SAT2 viruses. Many studies have focused on representative SAT2 viruses to either improve stability of that particular virus and to derive conclusions about the stability of the serotype as a whole (Doel and Bacharini et al., 1981, Kotecha et al., 2016). Studies based on few isolates have focused on differences in stability between different serotypes. There is very limited information about the range of stability that occurs within a serotype and between topotypes of different geographic regions. In many cases SAT2 viruses have very different growth kinetics, virulence, growth characteristics like plaque morphologies, ability to adapt to different cell lines, antigenicity and antigen yield. Whilst vaccine producers tend to select viruses as vaccine candidates based on good growth, and high antigen yield other important factors such as the stability of the virus are not 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 et al., 1981).

To characterise the stability properties of the different SAT2 viruses we used PaSTRy analysis as described previously (Walter et al., 2012, Kotecha et al., 2015 and 2016) to monitor capsid dissociation by the release of RNA genome under a range of temperature, pH and ionic conditions. This is considered the most effective method to assess FMDV capsid stability (Kotecha et al., 2016). The dissociation temperatures of the 8 SAT2 viruses ranged from 48-54 °C, a substantial difference of 6 degrees ($P<0.05$), with the southern topotype I viruses having the highest stability and dissociated at 54 °C whilst the western topotype II viruses ranged from 48-52 °C. Interestingly, some of these SAT2 viruses had very similar dissociation temperatures to the highly stable A24 control virus that dissociates at 56 °C. It has been widely accepted that SAT serotype viruses are unstable (Doel and Baccarini et al., 1981) and this confirms for the first time that more stable SAT2 viruses are naturally present from the southern Africa region. This could facilitate the selection of the most stable circulating field strains, as well as the comparison of such strains with their recombinant counterparts in vaccine design. Additionally viruses that prove to be more unstable from the field but are ideal in other traits as vaccine candidates could be mutated to include residues responsible for improved stability (Kotecha *et al*., 2015; Scott *et al*., 2016). The selection of naturally more stable viruses,

which have not been manipulated in vitro, could result in genetically stable viruses through laboratory adaptation and upscale processes.

The addition of ionic buffers showed that 1 M NaCl was capable of enhancing stability of the SAT2 viruses by a further 6-8 °C confirming the findings by Kotecha et al. (2016). Some buffers showed differing results dependent on the virus tested, highlighting the need to test SAT2 viruses with different buffers before storage to establish the most stabilising option.

Amino acids previously identified at the interface (Kotecha et al., 2015, Scott et al., 2016) as having a role in stability were analysed and no differences between the SAT2 residues in VP2 and VP3 inter-pentamer interfaces were found. The conservation of the SAT2 interface residues might be an inherent characteristic of SAT2 viruses. The stability differences observed in this study could not be attributed to any known residues responsible for stability.

The thermal shift assay (Walter et al., 2012) was a very 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 it could be used for screening viruses for use a potential vaccine strains and for governments to monitor the quality of the vaccine from being transported to the field and before being administered to animals.

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CONCLUDING REMARKS

FMDV vaccines with higher stability have the potential to be more effective by improving the potency of the vaccine and therefore the duration of antibody response and enhanced exposure to T-helper cells affording better cellular immunity. The vaccine producer would benefit from improved shelf-life and less antigen required to produce vaccines of same potency. However, very few natural FMDV variants with increased resistance to heat or pH (Mateo et al., 2007) have been reported, possibly because overstabilization hinders the release of the viral genome during cell entry. We therefore investigated the rational introduction of mutations to increase thermostability, earlier attempts at which have had only marginal success (Mateo et al., 2008). Porta et al. (2013) previously reported a stable disulfide bond at the icosahedral two-fold axis between adjacent pentamers in the A22 virus, thus allowing the production of thermostable and pH-stable recombinant empty particles. Because covalent modification was unlikely to be suitable with engineered viruses and may not be generally applicable, we devised a strategy that allows general mutations conferring non-covalent stabilization to be evaluated *in silico* by using the 3 Å crystallographic SAT1 virus structure as a structural template and model for the SAT2 virion (collaborative project with Oxford University and the Pirbright Institute). Highly restrained atomistic molecular dynamic (MD) simulations focused on the appropriate part of the complete virion to efficiently and reliably calculate the changes in binding free energy for interactions between adjacent pentamers within the FMDV capsid, thus allowing screening and ranking of candidate mutations *(*Kotecha et al., 2015*)*. We further investigated a larger panel of amino acid residues, involved in inter-pentameric protein interactions, which may contribute to stability of the capsid identified based on *in silico* analysis of 70 residues. This together with the structural data available and knowledge about stability of other serotypes we identified a panel of 14 single amino acid substitutions, two triple mutations and two quadruple mutations, at the inter-pentameric interface of the VP2 and VP3 capsid proteins of SAT2/ZIM/7/83. The predicted stability mutations were introduced into the full-length SAT2 infectious cDNA clone (pSAT2) and viable mutant viruses were recovered following transfection of the plasmid DNA into BHK-21 cells. We assessed the recombinant mutant virus panel for temperature, pH and ionic stability, antigenicity to SAT2-specific monoclonal antibodies, growth characteristics and genetic stability in suspension cells in order to select candidate mutants for engineering stabilised SAT2 vaccines. Single-residue mutations generating hydrophobic stacking of aromatic side chains at the two-fold axis between adjacent pentamers (position 93 of VP2)

were effective in stabilizing the particle (Kotecha *et al.,* 2015, Scott et al., 2016a), results resembling those from enterovirus studies identifying residues where the particle opens up to initiate uncoating (Ren et al., 2013). Engineering the stabilization mutations decreased the plaque sizes of SAT2 viruses, possibly by delaying viral genome release. Infectious viruses carrying these mutations were indeed thermostable and pH stable, and the degree of stabilization mirrored the *in silico* calculations.

Formulated vaccines from inactivated wild-type and stabilized SAT2 146S vaccine antigen produced equivalent protective levels of neutralizing antibodies in cattle, thus demonstrating the immunogenicity of the engineered particles. The stabilized inactivated vaccine antigen is expected to be more tolerant of suboptimal cold-chain performance; goals for the stability of SAT2 have been described by Anderson et al. (1982). Furthermore, after storage for 1 or 6 months at 4 °C, stabilized particles produced substantially higher VNTs in guinea pigs than did wild-type particles, commensurately with an increase in vaccine shelf life. In conclusion, we have devised a new restrained MD protocol to drive structure-based design of stabilized FMD viruses and empty capsids. This strategy may be relevant to many other picornaviruses that are responsible for causing a wide range of mild–to–life-threatening diseases affecting humans and other animals.

It has long been assumed that all field SAT2 viruses are inherently unstable, however previous studies have concentrated on one or more strains like ZIM7/83. We investigated the biophysical stability (a range of temperature, pH and ionic conditions) of eight SAT2 viruses from the southern and western topotypes using the thermofluor assay, which monitors capsid dissociation. The dissociation temperatures of the eight SAT2 viruses ranged from 48-54 °C, a substantial difference of 6 degrees, with the southern topotype I viruses having the highest stability of 54 °C whilst the western topotype II viruses ranged from 48-52 °C. Interestingly, some of these SAT2 viruses had very similar dissociation temperatures to the highly stable A24 control virus at 56 °C. The addition of ionic buffers showed that 1 M NaCl was capable of enhancing stability of the SAT2 viruses by a further 6-8 °C whereas some solutions showed differing results dependent on the virus tested, highlighting the need to test SAT2 viruses before storage with different solutions to establish the most stabilising option. This confirms for the first time that more stable SAT2 viruses are present in the field from the Southern Africa region. This could facilitate the selection of the most stable circulating field strains, as well as the comparison of such strains with their recombinant counterparts in vaccine design. The selection of naturally more stable viruses, which have not be manipulated in-vitro, could result in genetically stable viruses through laboratory adaptation and upscale processes.

The thermal shift assay (Walter *et al*., 2012) was a valuable tool in assessing stability with temperature, conformation, pH and salt conditions. However it can be used in other applications such as vaccine production systems to assess stability, quality and shelf-life of antigens, effects of storage buffers and in screening viruses for use a potential vaccine strains. For governments it can be used to determine the quality of the vaccine once transported to the field before being administered to animals. Additionally in research, it can be used to assess the quality of purified 146S particles.

The amino acid residues responsible for stabilisation in this study mirrored by *in silico* calculations have the potential to be used in improving the design of SAT2 vaccines by combining reverse genetic approaches that allow a) replacement of external capsid-coding regions or surface exposed antigenic regions to emergency or more suitable field isolates to transfer improved neutralizing epitopes and b) site-directed mutagenesis of amino acids to improve thermo-stability. The improved stability of FMDV capsids result in a number of advantages in that it can be transported with less strict requirement on cold-chain logistics, and they provide an improved immune-response due to less degradation of the 146S particle (Kotecha *et al*., 2015, Scott et al., 2016b). The stability of vaccines is of crucial importance in Africa, where the logistical process to get the vaccine from the manufacturer to the animal may take months and in many remote regions is in the absence of a cold-chain. Vaccines with improved stability and less reliance on a cold-chain are needed and could improve the longevity of immune responses elicited in animals due to less degradation of the antigen (Doel and Baccarini, 1981).

We followed up this work with an extensive cattle trial (n=30) in which we studied the efficacy and comparative immunological responses of two thermostable and wild-type ZIM7/83 SAT2 vaccines and challenged with live virus 5.5 months pv. We showed that all vaccinated groups (stabilised or wildtype) were capable of eliciting strong antibody titres and good IFN-γ responses with full protection 5.5 months pv which to our knowledge has not been shown before. This is the first step in the proof of concept to now advance our knowledge on where problems of SAT2 stability occur from vaccine production to the administration in the field. This also proves that optimal protection is afforded by the recommended strategy of vaccinating two vaccines, 4-6 weeks a part.

The most thermostable mutant 93Y derived in this study however had a few limitations. (1) Its growth was slower with a $\log_{10}$ decrease in titre compared to wild-type virus over 24h. Possibly its increase in stability has contributed to a delay in viral genome release (uncoating) and slower replication. In such a study the balance between increasing the stability whilst maintaining efficient viral genome release is an important criteria if such a candidate is to be used in a vaccine production system where optimal

growth and antigen yield is a prerequisite. Howeve,r the benefits of a stable antigen would result in potentially the need for less antigen in the vaccine and could outway the cost of the use a slower growing virus. It is hypothesized that you would need less potent vaccines if the vaccine is stabilised.

(2) 93Y was found to be genetically unstable when upscaled through passage in suspension BHK cells. Since 93Y is more thermostable it is probably undesirable for the fitness of the virus and therefore this amino acid was negatively selected by mutating to 93H. This genetic instability could be overcome by species-specific codon optimisation in conjunction with the production cell line; by choosing amino acids with stronger base pairing options at the mutation site and/or choice of cell line which exhibits less reversion; compensatory mutations introduced near the mutation site; adjusting the multiplicitiy of infection (moi); less passaging; improving replication through additional ribosome binding sites or by increasing the fidelity of the viral RNA polymerase.

(3) The antigenic reactivity of 93Y to the SAT2 specific MAb GD12 was considerably lower compared to the wild-type indicative of changes in the epitope footprint. Although we do not have structural data to confirm this, we hypothesize that increased hydrophobic interactions could possibly cause reduced flexibility or local distortion in surface exposed loops locking the exposed side chains into position not optimally aligned to GD12 complimentary determining regions (CDR's). The exact footprint of GD12 has not yet been mapped but previous evidence suggests that amino acids at the two-fold interface form part of the GD12 footprint (Opperman et al., 2014). The 93Y mutation is not expected to affect the critical domain for GD12 binding but perhaps to cause imperfect alignment of the functional binding region of the capsid and GD12 CDRs. However there was no difference in antigenicity of the 93Y and wildtype antigens when vaccinating cattle and no apparent difference in the structure of the wild-type and 93Y capsids (Kotecha et al, 2015). However in light of these findings it appeared that 93H which is slightly less stable than 93Y, but more stable than the wild-type virus, is not prone to these limitations and also performed best overall in the cattle trial. This might be the best candidate mutant to take forward for improved stability and immunogenicity of SAT2 vaccines.

The thermostable and wild-type vaccines were never exposed to any period of shelf-life storage or any break in the cold-chain to truly test the differences in possible stability of the thermostable versus wild-type vaccines as well as their differences in immunogenicity, which will be addressed in our continuous studies. We were not able to measure antibody avidity in this study and it is possible that after the second vaccination cattle developed high avidity antibodies through affinity maturation. It is also possible that

if we only administered one vaccine dose that differences in stability would be more conclusive.

Whilst in this study efforts were made to design SAT2 viruses with improved thermostability and their immunogenicity evaluated in a cattle trial this only constitutes improvement to the actual virus. Other important factors such as shelf-life; potency; maintenance of the cold-chain from the producer to the field; and correct administration of the vaccine are all critical in assessing stability, efficacy and duration of immunity of SAT serotype vaccines in the field. We intend to follow up this work with vaccination trials using thermostable vaccines assessing different shelf-life conditions, disturbances in cold-chain maintenance and potency to improve stability and SAT2 control. Additionally, more field work is needed to assess and monitor the administration of vaccines in the field in terms of maintenance of the cold-chain in the field (from the first dose in the vial until the last is administered), and that the correct dosage is administered. In addition, it is important to track the immune responses of animals vaccinated in the field over different periods of time post-vaccination.

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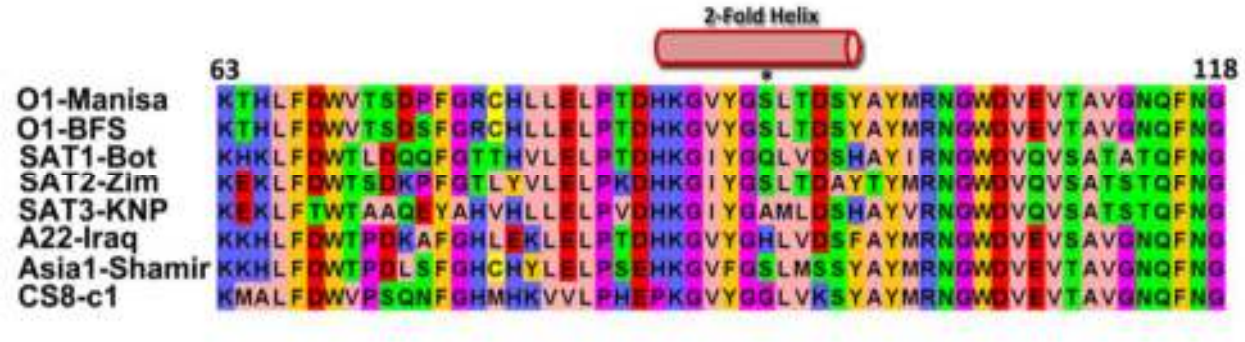
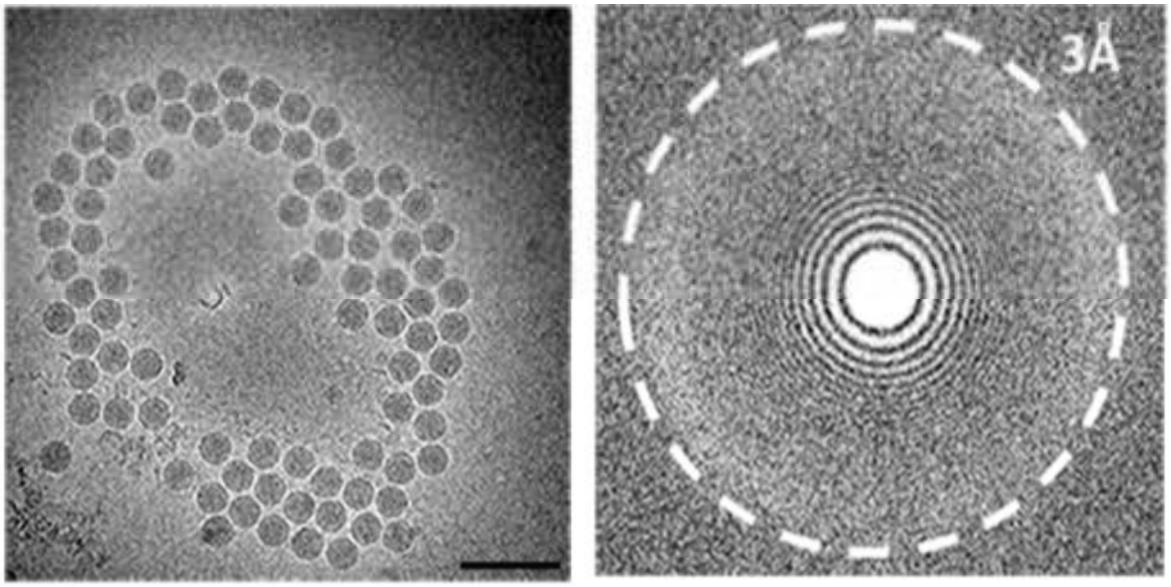
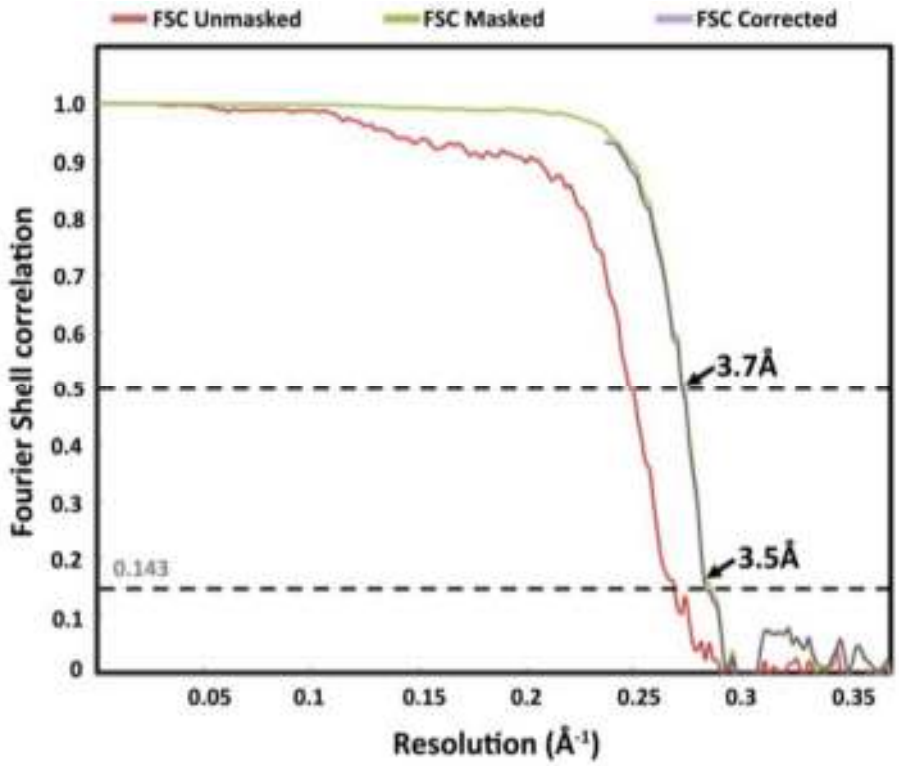
SUPPLEMENTARY 1



Supplementary Figure 1.1: Sequence alignment of a region of VP2 from representatives of seven FMDV serotypes (VP2 dominates the interactions at the interpentamer interface). The position of the α-helix found in all picornaviruses adjacent to the icosahedral 2-fold symmetry axis (residues 87-98) is shown as a red cylinder with position 93 indicated by a star (amino acid sequences are coloured using the Zappo Colour scheme in Jalview, http://www.jalview.org/).





Supplementary Figure 1.2: Cryo-EM analysis of stabilized FMDV particles. Purified inactivated SAT2 S93Y particles were used for data collection by cryoEM. Representative aligned average (motion corrected) image of SAT2 VP2 S93Y particles and the corresponding Fourier transform. The FSC curves of the final 3D reconstruction obtained using gold-standard refinement using RELION, marked with the resolution corresponding to a Fourier shell correlation (FSC) of 0.5 and 0.143. The scale bar indicates 100nm.

Supplementary Table 1: Growth analysis comparison of parental wild-type and thermostable FMDVs. BHK-21 cells were infected with the respective virus and samples analysed at 8 h post-infection by plaque assay to determine viral titres. Similar results were obtained from three individual experiments.

Serotype		Titre (pfu/ml)
SAT2	Wild-type	3.2×10^6
	VP2 S93H	2.4×10^7
	VP2 S93Y	1.7×10^5
	VP2 S93W	2.8×10^6

Supplementary Table 2: Thermostability of inactivated SAT2 viruses measured in triplicates by fluorescence assay at pH 7.5.

		Tm (n=3) °C
SAT2	Wildtype	47.1 ± 0.2
	93H	51.2 ± 0.3
	93Y	53.4 ± 0.3
	93W	47.2 ± 0.1

Supplementary Table 3: CryoEM data collection and refinement statistics.

CryoEM Detector	
Data set	**SAT2 VP2 S93Y**
Particles	8156
Pixel Size (Å)	1.35
Defocus Range (μm)	0.8-2.5
Voltage (kV)	300
Electron Dose (e^{-} $Å^{-2}$)	18
Resolution (Å)	3.5
Map Sharpening B-factor ($Å^2$)	-121.4
Model Refinement	
Fo-Fc Correlation	0.87
Protein atoms	5246
R.m.s.d., bonds (Å)	0.01
R.m.s.d., angles (°)	0.98
Clashscore, all atoms (percentile)	8.75 (78^{th})
Rotamer Outliers (%)	0.0
Ramachandran outliers (%)	2.29
MolProbity score (percentile)	1.91(80^{th})

Supplementary Table 4: Capsid stabilising mutations for SAT2 serotype showing the nucleotide substitutions, amino acid changes, position of mutation, and the sequences of the forward and reverse mutagenesis primer sets.

Mutation name	Nucleotide seq	Amino acid change	Protein	Mutagenesis forward primer (5' to 3')	Mutagenesis reverse primer (3' to 5')
S2093H	AGC → CAT	93H	VP2	ACCACAAGGGGATCTACGGCCATCTGACCGACGCGTATACTTAC	GTAAGTATACGCGTCGGTCAGATGGCCGTAGATCCCCTTGTGGT
S2093Y	AGC → TAT	93Y	VP2	ACCACAAGGGGATCTACGGCTATCTGACCGACGCGTATACTTAC	GTAAGTATACGCGTCGGTCAGATAGCCGTAGATCCCCTTGTGGT
S2093W	AGC → TGG	93W	VP2	AAGGGGATCTACGGCTGGCTGACCGACGCG	TTCCGCGTCGGTCAGACCGCCGTCGATCCC
S2093Q	AGC → CAG	93Q	VP2	CAAGGGGATCTACGGCCAGCTGACCGACGCGTATAC	GTTCCGCGTCGGTCAGGTCGCCGTCGATCCCATATG
S2093F	AGC → TTT	93F	VP2	ACCACAAGGGGATCTACGGCTTTCTGACCGACGCGTATACTTAC	GTAAGTATACGCGTCGGTCAGAAAGCCGTAGATCCCCTTGTGGT
L2094V	CTG → GTC	94V	VP2	GGGATCTACGGCAGCGTCACCGACGCGTATAC	CCCGTCGGTCAGTCGCAGGTCGATCCCATATG
Y2098F	TAT → TTC	98F	VP2	GCCTGACCGACGCGTTCACTTACATGCGCAACGG	CGGACTGGCTGCGCAAGTGAATGTACGCGTTGCC
Y2098H	TAT → CAC	98H	VP2	GCCTGACCGACGCGCACACTTACATGCGCAACGG	CGGACTGGCTGCGCGTGTGAATGTACGCGTTGCC
T2114N	ACG → AAT	114N	VP2	GTCCAGGTTTCCGCCACCAGCAATCAGTTCAACGGCGGG	CCCGCCGTTGAACTGATTGCTGGTGGCGGAAACCTGGAC
S2113G	AGC → GGT	113G	VP2	GTTTCCGCCACCGGTACGCAGTTCAACGGCGGG	CCCGCCGTTGAACTGCGTACCGGTGGCGGAAAC
K2215L	AAG → CTT	215L	VP2	GTCTTCGTTGCTGGCGAACTTCCTGCGAAACAGGGC	GCCCTGTTTCGCAGGAAGTTCGCCAGCAACGAAGAC
D3193H	GAC → CAT	193H	VP3	GCGGTGTTCCAGGTGACTCATACCCATTCGGCCGAAGCC	GGCTTCGGCCGAATGGGTATGAGTCACCTGGAACACCGC
E3194A	ACC → GGT	194A	VP3	GTGTTCCAGGTGACTGACGGTCATTCGGCCGAAGCCGCT	AGCGGCTTCGGCCGAATGACCGTCAGTCACCTGGAACAC
E3198A	GAA → GCT	198A	VP3	GACACCCATTCGGCCGCGGCCGCTGTGGTTGTG	CACAACCACAGCGGCCGCGGCCGAATGGGTGTC
F2062Y	TTC → TAT	62Y	VP2	CAAGCAGAGCGGTTCTATAAGGAAAAGCTTTTTGATTGG	GTTCGTCTCGCCAAGATATTCCTTTTCGAAAAACTAACC
H2087V	CAC → GTC	87V	VP2	GGAATTGCCCAAGGACGTCAAGGGGATCTACGGC	CCTTAACGGGTTCCTGCAGTTCCCCTAGATGCCG
H2087M	CAC→ ATG	87M	VP2	GGAATTGCCCAAGGACATGAAGGGGATCTACGGCAGC	CCTTAACGGGTTCCTGTACTTCCCCTAGATGCCGTCG
H3143V	CAC → GTG	143V	VP3	CCCGAGGACGCAGCCGTGTGCTACCACTCG	GGGCTCCTGCGTCGGCACACGATGGTGAGC

Supplementary Table 5: The presence of FMDV and viral RNA after 0-14 days challenge. (A) represents the presence of viral RNA (determined by real-time RT-PCR) and (B) virus isolation in probang, tonsil swab and whole blood samples for animals vaccinated with vSAT2-wt (n=7), vSAT2-93H (n=7) or vSAT2-93Y (n=7) antigens and non-vaccinated controls (n=2) for 162 days. Thereafter challenged with SAT2/ZIM/7/83 virus.

A

			Days post-challenge															
			Oropharyngeal fluid (Probang)						**Retropharyngeal tonsil**						**Viraemia**		
	Animal	stable	**D0**	**D2**	**D4**	**D7**	**D9**	**D11**	**D0**	**D2**	**D4**	**D7**	**D9**	**D11**	**D2**	**D4**	**D7**
vSAT2-wt	5	5	>40	>40	35.84	31.80	28.82	35.45	>40	>40	25.62	31.92	33.35	31.28	>40	>40	>40
	21	5	>40	>40	30.03	32.53	34.32	31.97	>40	>40	29.19	33.71	32.36	33.01	>40	34.1	>40
	25	5	>40	>40	33.33	37.67	33.33	>40	>40	>40	32.77	34.35	33.23	34.46	>40	34.4	>40
	26	5	>40	18	27.18	29.11	29.41	33.14	>40	>40	29.62	38.05	32.30	33.22	35.1	34.5	>40
	33	5	>40	>40	33.37	>40	32.81	34.06	>40	>40	33.19	34.72	32.44	31.41	>40	33.90	>40
	56	5	>40	>40	25.18	32.48	32.64	34.53	>40	>40	31.27	37.09	29.86	31.94	>40	>40	>40
	67	5	>40	>40	30.38	35.36	32.71	33.36	>40	>40	32.24	>40	31.55	32.05	34.7	33.8	>40
vSAT2-93Y	8	6	>40	>40	35.59	36.99	33.41	34.79	>40	>40	31.95	32.99	32.35	30.44	33.5	>40	>40
	14	6	>40	>40	>40	31.35	29.03	29.17	>40	>40	32.78	32.88	32.05	32.70	>40	33.3	>40
	22	6	>40	>40	25.62	36.34	33.05	33.79	>40	>40	28.65	33.98	32.24	36.32	34.2	>40	>40
	37	6	>40	18	26.95	33.80	32.79	ND	>40	>40	28.90	33.70	33.71	ND	35.1	34.9	>40
	38	6	>40	>40	33.58	36.02	33.76	>40	>40	>40	33.26	33.45	32.47	31.78	34.8	>40	>40
	61	6	>40	>40	34.17	>40	34.78	34.05	>40	>40	34.15	>40	31.12	31.99	34.1	34.8	>40
	68	6	>40	>40	32.78	39.55	34.19	32.40	>40	>40	32.01	36.48	32.36	34.93	>40	34.8	>40
vSAT2-93H	17	15	>40	>40	36.13	36.25	32.91	31.99	>40	>40	31.69	33.41	32.14	32.32	34.9	33.9	>40
	35	15	>40	18	22.74	31.93	31.59	33.82	>40	>40	24.07	>40	33.73	30.72	33.8	>40	>40
	39	15	>40	18	23.16	31.31	33.69	33.58	>40	>40	24.60	34.49	31.88	30.37	>40	>40	>40
	47	15	>40	18	25.04	32.86	33.14	32.01	>40	>40	31.12	34.39	33.15	30.94	>40	>40	>40
	62	15	>40	>40	27.02	36.27	33.42	32.93	>40	>40	21.08	>40	31.46	31.89	34.2	>40	>40
	71	15	>40	14	26.02	33.92	32.82	34.32	>40	16	27.35	35.98	31.37	33.82	33.8	24.4	>40
	73	15	>40	>40	32.02	34.61	33.00	34.17	>40	>40	33.32	34.80	29.51	32.98	33.7	34.6	>40
controls	34	9	>40	18	29.29	24.87	28.32	33.17	>40	>40	33.52	28.66	24.29	29.78	34.15	31.8	>40
	53	9	>40	18	32.47	31.05	30.30	33.96	>40	>40	32.51	>40	32.48	30.33	33.5	34.3	>40

B

			Days post-challenge														
			Oropharyngeal fluid (Probang)						**Retropharyngeal tonsil**						**Viraemia**	
	Animal	stable	**D0**	**D2**	**D4**	**D7**	**D9**	**D11**	**D0**	**D2**	**D4**	**D7**	**D9**	**D11**	**D2**	**D4**
vSAT2-wt	5	5	0	RS1	RS1	RS1	RS1	0	0	RS1	RS1	RS1	0	0	0	0
	21	5	0	RS1	RS1	0	0	0	0	RS1	RS1	0	0	0	0	0
	25	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	26	5	0	RS2	RS1	RS2	0	0	0	RS2	RS1	RS3	0	0	0	0
	33	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	56	5	0	RS2	RS1	RS2	0	0	0	RS1	0	0	0	0	0	0
	67	5	0	0	RS2	0	0	0	0	0	0	0	0	0	0	0
vSAT2-93Y	8	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	14	6	0	0	0	RS1	RS1	0	0	0	RS2	0	0	0	0	0
	22	6	0	RS1	RS1	0	0	0	0	RS1	RS2	0	0	0	0	0
	37	6	0	RS1	0	RS1	0	0	0	RS1	RS1	0	0	0	0	0
	38	6	0	0	0	0	0	RS2	0	0	0	0	0	0	0	0
	61	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	68	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0
vSAT2-93H	17	15	0	RS2	0	0	0	0	0	0	0	0	0	0	0	0
	35	15	0	RS1	RS1	RS1	0	RS2	0	RS1	RS1	0	0	0	0	0
	39	15	0	RS1	RS1	0	0	0	0	0	RS1	0	0	0	0	0
	47	15	0	RS1	RS1	0	0	0	0	0	0	0	0	?	0	0
	62	15	0	0	RS1	0	0	0	0	RS1	RS1	0	0	0	0	0
	71	15	0	0	RS1	RS2	0	0	0	RS1	RS1	0	0	0	0	0
	73	15	0	0	0	0	0	0	0	0	0	0	0	0	0	0
controls	34	9	0	RS1	RS1	0	0	0	0	RS1	RS2	RS1	0	0	0	RS2
	53	9	0	RS1	RS2	ND	0	0	0	RS1	RS2	0	0	0	RS2	0

SUPPLEMENTARY 2

2.1 Published article

ARTICLES

nature structural & molecular biology

Structure-based energetics of protein interfaces guides foot-and-mouth disease virus vaccine design

Abhay Kotecha[1,8], Julian Seago[2,8], Katherine Scott[3], Alison Burman[2], Silvia Loureiro[4], Jingshan Ren[1], Claudine Porta[1,2], Helen M Ginn[1], Terry Jackson[2], Eva Perez-Martin[2], C Alistair Siebert[1], Guntram Paul[5], Juha T Huiskonen[1], Ian M Jones[4], Robert M Esnouf[1], Elizabeth E Fry[1], Francois F Maree[3,6], Bryan Charleston[2] & David I Stuart[1,7]

Virus capsids are primed for disassembly, yet capsid integrity is key to generating a protective immune response. Foot-and-mouth disease virus (FMDV) capsids comprise identical pentameric protein subunits held together by tenuous noncovalent interactions and are often unstable. Chemically inactivated or recombinant empty capsids, which could form the basis of future vaccines, are even less stable than live virus. Here we devised a computational method to assess the relative stability of protein-protein interfaces and used it to design improved candidate vaccines for two poorly stable, but globally important, serotypes of FMDV: O and SAT2. We used a restrained molecular dynamics strategy to rank mutations predicted to strengthen the pentamer interfaces and applied the results to produce stabilized capsids. Structural analyses and stability assays confirmed the predictions, and vaccinated animals generated improved neutralizing-antibody responses to stabilized particles compared to parental viruses and wild-type capsids.

FMDV is a small-RNA virus whose icosahedral capsid contains 60 copies each of proteins VP1–VP4. FMDV is responsible for a contagious, economically devastating livestock disease endemic in many developing regions of Asia, Africa and South America; developed countries are also at risk of sporadic but serious outbreaks (for example, in the UK in 2001, in Japan in 2010 and in Egypt in 2012 (refs. 1–3)). In areas where FMDV is endemic, disease control is carried out predominantly by vaccination. There are seven serotypes of FMDV (A, O, C, Asia, and Southern African Territories (SAT) 1, 2 and 3)[4], and each contains multiple and constantly evolving subserotype strains. A vaccine against one serotype does not protect against other serotypes or subtypes within a serotype, thus necessitating the continued development of new vaccine strains. The majority of vaccines produced worldwide are type O, whereas SAT2 is the most prevalent serotype in sub-Saharan Africa[5].

FMDV serotypes differ markedly in their capsid stability: A and Asia 1 are relatively stable, whereas O and SAT viruses are more sensitive to heat and pH[6]. Commercial vaccines are produced by chemical inactivation of virus particles, which renders them even less stable, and above 30 °C they rapidly convert into immunogenically incompetent pentameric subunits[6,7]. Thus, vaccine integrity requires expensive, difficult-to-maintain cold chains, and effective protection requires frequent immunizations[5,8]. Recombinant empty capsids show promise as future vaccines because they overcome many of the disadvantages of preparing vaccines from live virus; however, they are even less stable than virus particles[9].

Knowledge of the three-dimensional structure of viral capsids should allow for rational engineering of their properties. Although four different serotypes of FMDV have been analyzed in atomic detail[10–14], few attempts have been made to engineer thermostable capsids[15,16]. The pentameric subassemblies are highly stable, so stabilization of the interpentamer interactions should be sufficient to enhance capsid stability and the effectiveness of vaccine preparations. The introduction of a disulfide bond across the interface between adjacent pentamers has enabled the production of thermostable FMDV empty capsids for an A serotype (A22 Iraq)[17]. However, covalent stabilization may not be compatible with virus viability or with the structure of all viruses[16].

Here, we developed a molecular dynamics (MD)-based strategy for the evaluation of mutations designed to increase capsid stability by increased noncovalent interactions and applied it to the less stable serotypes O and SAT2, and the more stable A viruses. Structural analyses and stability assays confirmed that the method is predictive of increased stability. Furthermore, animals vaccinated with candidate mutant viruses generated improved neutralizing-antibody responses, thus demonstrating potential value for such stabilized vaccine antigens.

RESULTS

FMDV capsids dissociate into pentameric subassemblies

Inactivated FMDV capsids dissociate into pentameric subunits at pH <7.0, at temperatures >30 °C (refs. 1,4,6,15) and after prolonged storage at 4 °C (**Fig. 1a,b**). We examined the interface between pentamers for opportunities to stabilize these interpentamer

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2.2 Animal ethics approval – ARC-OVI

APPROVED
AEC 25.12

ARC • LNR

Onderstepoort Veterinary Institute

Animal Ethics

Decision of the Animal Ethics Committee for the use of living vertebrates for research, diagnostic procedures and product development

APPROVAL PERIOD: 2012 / 2013

PROJECT NUMBER:	OV23/11/C211			
PROJECT TITLE:	**Structurally Modified Master Seed Viruses to Enhance Conventional Foot-and-mouth Disease Virus Vaccines.**			
PROJECT LEADER:	Dr F.F. Maree			
DIVISION:	Transboundary Animal Diseases Programme			
CATEGORY:	D			
SPECIES OF ANIMAL:	Bos taurus			
NUMBER OF ANIMALS:	34			
NOT APPROVED:				
APPROVED:	APPROVED			

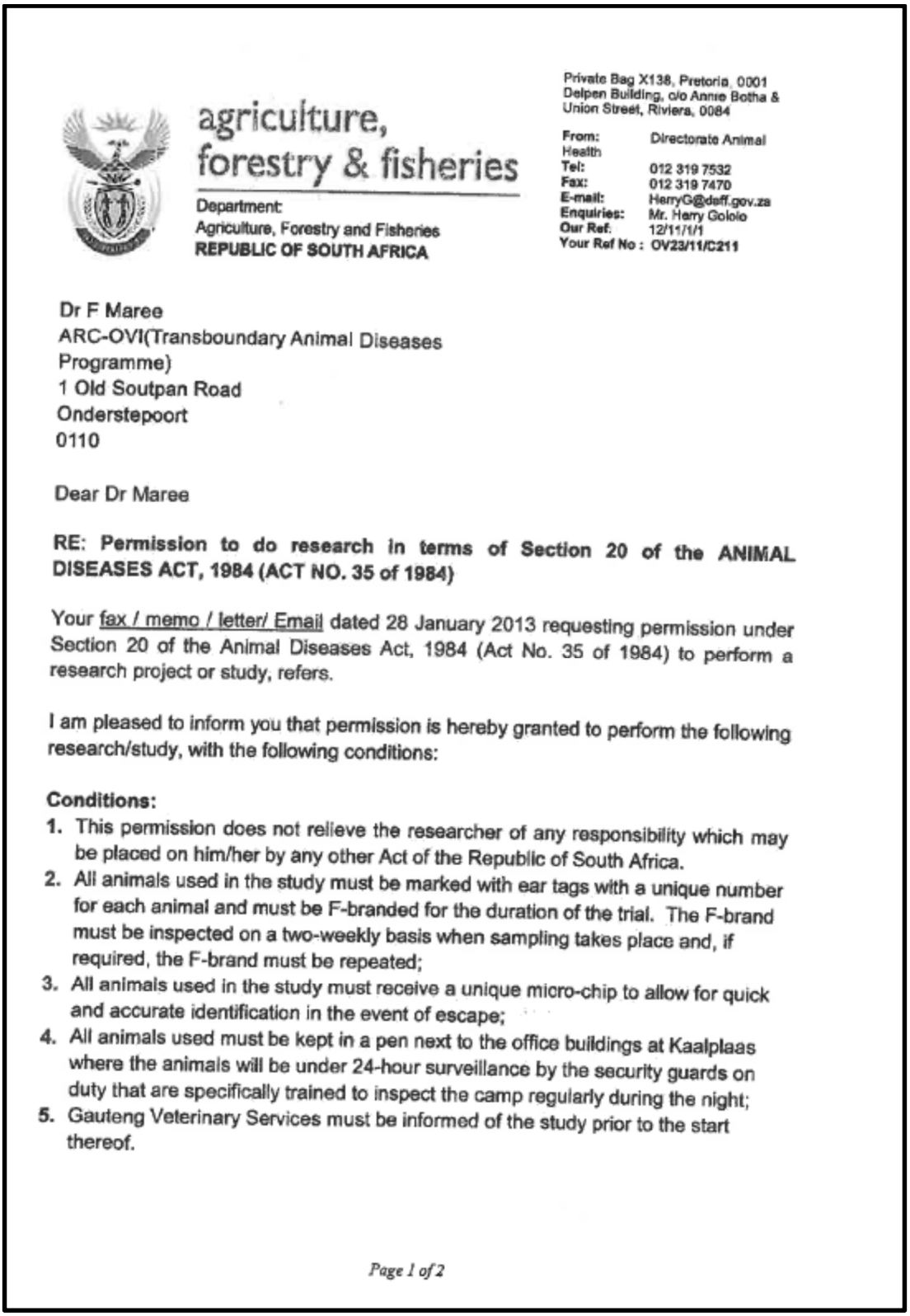
PLEASE NOTE: Should the number or species of animal(s) required, or the experimental procedure(s) change, please submit a revised animal ethics clearance form to the animal ethics committee for approval before commencing with the experiment

SIGNATURE: Dr L. Lopez
CHAIRPERSON ANIMAL ETHICS COMMITTEE

DATE: 27-12-2012

17

Figure 3: Section 20 approval DAFF



agriculture, forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Private Bag X138, Pretoria, 0001
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From: Directorate Animal Health
Tel: 012 319 7532
Fax: 012 319 7470
E-mail: HenryG@daff.gov.za
Enquiries: Mr. Henry Gololo
Our Ref: 12/11/1/1
Your Ref No : OV23/11/C211

Dr F Maree
ARC-OVI(Transboundary Animal Diseases Programme)
1 Old Soutpan Road
Onderstepoort
0110

Dear Dr Maree

RE: Permission to do research in terms of Section 20 of the ANIMAL DISEASES ACT, 1984 (ACT NO. 35 of 1984)

Your fax / memo / letter/ Email dated 28 January 2013 requesting permission under Section 20 of the Animal Diseases Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

I am pleased to inform you that permission is hereby granted to perform the following research/study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him/her by any other Act of the Republic of South Africa.
2. All animals used in the study must be marked with ear tags with a unique number for each animal and must be F-branded for the duration of the trial. The F-brand must be inspected on a two-weekly basis when sampling takes place and, if required, the F-brand must be repeated;
3. All animals used in the study must receive a unique micro-chip to allow for quick and accurate identification in the event of escape;
4. All animals used must be kept in a pen next to the office buildings at Kaalplaas where the animals will be under 24-hour surveillance by the security guards on duty that are specifically trained to inspect the camp regularly during the night;
5. Gauteng Veterinary Services must be informed of the study prior to the start thereof.

Page 1 of 2

Title of research/study: Structurally modified master Seed viruses to enhance conventional Foot-and-Mouth Disease virus vaccines.
Researcher (s): Dr .F. Maree
Institution: ARC-OVI (Transboundary Animal Diseases Programme)
Your Ref./ Project Number: OV23/11/C211
Our ref Number: 12/11/1/1

Kind regards

DIRECTOR: ANIMAL HEALTH

DATE: 2013-06-10

Figure 4: Animal ethics approval – University of Pretoria

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Improved Stability of Foot-and-mouth disease virus(FMDV) SAT2 *capsid***
PROJECT NUMBER	**V060-15**
RESEARCHER/PRINCIPAL INVESTIGATOR	**KA Scott**

STUDENT NUMBER (where applicable)	**UP_04125258**
DISSERTATION/THESIS SUBMITTED FOR	**PhD**

ANIMAL SPECIES	**Bos taurus**	
NUMBER OF ANIMALS	**34**	
Approval period to use animals for research/testing purposes		**February 2016-February 2017**
SUPERVISOR	**Dr FF Maree**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 2 March 2016
CHAIRMAN: UP Animal Ethics Committee	Signature