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LIST OF ABBREVIATIONS

α	alpha
aa	amino acid
Ad	adenovirus
$Al(OH)_3$	aluminium hydroxide
AMV	avian myeloblastosis virus
ARC	Agriculutural Research Council
ATCC	American Type Culture Collection
β	beta
BaMV	bamboo mosaic virus
BEA	bromoethylamine hydrobromide
BEI	binary ethylenimine
BHK	baby hamster kidney
BME	Eagle's basal medium
C	carboxy
°C	degrees Celsius
ca.	approximately
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CHO	Chinese hamster ovary
CMI	cell-mediated immunity
CPE	cytopathic effect
CpG	cytosine-phosphate-guanine
cre	*cis*-acting replication element
CsCl	caesium chloride
CTL	cytotoxic T lymphocyte
Da	Dalton
DIVA	differentiating infected from vaccinated animals
DMSO	dimethyl sulfoxide
dN	non-synonomous
DNA	deoxyribonucleic acid
d.p.v.	days post-vaccination
dS	synonomous
EDTA	ethylenediaminetetra-acetic acid
EE	early endosomes

e.g.	*exempli gratia* (for example)
eIF	eukaryotic initiation factor
ELISA	enzyme-linked immunosorbant assay
EMCV	encephalomyocarditis virus
EmPCR	emulsion-based clonal amplification
ER	endoplasmic reticulum
et al.	*et alia* (and others)
FAO	Food and Agriculture Organisation of the United Nations
FCS	fetal calf serum
Fig.	figure
FMD	foot-and-mouth
FMDV	foot-and-mouth disease virus
γ	gamma
g	gram
GAG	glycosaminoglycans
GlcN	D-glucosamine
GM-CSF	granulocyte-macrophage colony-stimulating factor
GS	Genome Sequencer
GuSCN	guanidinium thyocianate
h	hour
HBcAg	hepatitis virus core antigen gene
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	human immunodeficiency virus
H_2O_2	hydrogen peroxide
HRP	horseradish peroxidase
HS	heparan sulfate
H_2SO_2	hyposulfurous acid
HSPG	heparan sulfate proteoglycans
IB-RS-2	Instituto Biologico Renal Suino-2 cells
ICAM	intercellular adhesion molecule
ID_{50}	median infective dose
Idu	L-iduronic
i.e.	*id est* (that is)
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IRES	internal ribosome entry site

kb	kilobase pair
kg	kilogram
KNP	Kruger National Park
M	molar
mAb	monoclonal antibody
MALT	mucosa-associated lymphoid tissue
MBS	MES-buffered saline
MEGA	Molecular Evolutionary Genetics Analysis
MES	[*N*-morpholino]ethane-sulfonic acid
MHC	major histocompatibility complex
min	minute
ml	millilitre
mm	millimetre
m.o.i.	multiplicity of infection
mM	millimolar
N	amino
n	number
NCBI	National Center for Biotechnology Information
NCR	non-coding region
ND	not determined
ng	nanogram
NGS	next-generation sequencing
nm	nanometer
no.	number
NS	non-structural
nt	nucleotide
OD	optical density
OIE	Office des Epizooties
OP	oesophageal-pharyngeal
ORF	open reading frame
OVI	Onderstepoort Veterinary Institute
PABP	poly(A) binding protein
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCBP	poly(C)-binding protein
PCR	polymerase chain reaction
PD_{50}	50% protective dose

PDB	Brookhaven Protein database
PEG	polyethylene glycol
PEO	polyethylene oxide
pfu	plaque forming units
PK	pig kidney
pKa	acid dissociation constant
PKs	pseudoknots
PLG	poly(D,L-lactide-*co*-glycolide) microparticles
poly(A) tract	polyadenylate tract
poly(C) tract	polycytidylate tract
PTB	polypyrimidine tract-binding protein
RGD	arginine-glycine-aspartic acid
RHA	RNA helicase A
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute-1640 medium
RT-PCR	reverse transcriptase-polymerase chain reaction
S	Svedberg unit
SAT	South African Territories
SD	standard deviation
SDS	sodium dodecyl sulphate
SNP	single-nucleotide polymorphism
SPCE	solid-phase competition ELISA
TCID	tissue culture infective dose
TMB	3,3'.5,5' tetramethylbenzidine
TNF	tumour necrosis factor-alpha
TPB	tryptose phosphate broth
Tris	Tris-hydroxymethyl-aminomethane
U	Unit
µg	microgram
µM	micromolar
UMP	uridine monophosphate
UTR	untranslated region
v.	version
v/v	volume per volume
VGM	virus growth medium
VNT	virus neutralisation test
VP_g	viral genome-linked protein

VSV-G	vesicular stomatitis virus
w/v	weight per volume
WRL	World Reference Laboratory

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CHAPTER ONE

LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

Foot-and-mouth disease (FMD), of which foot-and-mouth disease virus (FMDV) is the causative agent, is a highly contagious, acute infection of cloven-hoofed animals and is a compulsory notifiable disease (Grubman and Baxt, 2004). Susceptible species include domestic animals such as cattle, sheep, goats and pigs, as well as more than 30 species of wild ruminants. Although FMD is characterised by low mortality rates (less than 5%) (Thomson, 1994; Beard and Mason, 2000), the disease has a major economic impact on the livestock industry (Perry and Rich, 2007). FMD leads to disruption of trade in animals and animal products due to losses in milk production, delays in animals reaching maturity for marketing, abortions and death in young animals (Gibbs, 1981; Sutmoller *et al*., 2003). The devastating effects of a large outbreak of FMD have been witnessed by the extensive spread of the Pan-Asia strain in 2000 from Asia to South Africa and in 2001 from the United Kingdom to four other European countries (Sangare *et al*., 2001; Samuel and Knowles, 2001, 2003). The total cost of the FMD epidemic was estimated at 10 billion pounds in the United Kingdom alone (Samuel and Knowles, 2001).

FMD spreads rapidly to susceptible animal populations and transmission of the disease may either be direct or indirect. The most common route of infection is through direct contact between infected animals, which excrete the virus, and susceptible animals. The virus is excreted through the breath, excretions and from the skin of infected animals (Alexandersen and Mowat, 2005). The disease can be transmitted indirectly through contact with virus-contaminated animal products (*e.g*. meat, milk, semen, embryos and aerosols), while humans and vehicles have been implicated in the mechanical transmission of the disease (Hyslop, 1970; Sellers, 1971). The natural route of FMDV infection is via the respiratory tract. Virus entry and multiplication take place in the pharyngeal area, whereafter the virus spreads rapidly in the lymphatic system and blood to infect many tissues and organs. The earliest clinical signs of disease include fever, dullness, inappetence, anorexia, lameness and excessive salivation. These clinical signs develop after an incubation period of two to five days and also coincide with the development of vesicles and erosions in the mucosa of the mouth, as well as on the skin of the interdigital space and coronary bands (Burrows *et al*., 1981; Thomson, 1994; Arzt *et al*., 2011a; Charleston and Rodriguez, 2011; Golde *et al*., 2011).

Rapid and accurate diagnosis of FMD is a prerequisite for effective control of the disease (Paton *et al*., 2005; Rweyemamu *et al*., 2008). Diagnosis is based on a combination of clinical, epidemiological and laboratory observations. Vesicle fluid, epithelial tissue and serum samples are used for laboratory tests (Thomson, 1994). The enzyme-linked immunosorbant assay (ELISA) is a sensitive and rapid method for detection of the virus or anti-virus antibodies and is used in many diagnostic laboratories (Abu Elzein *et al*., 1979; Van Maanen and Terpstra, 1990; De Clercq *et al*., 2008a). Other diagnostic techniques include virus neutralisation tests (VNT) (Rweyemamu, 1984), virus isolation in pig kidney cells and bovine thyroid cells (Ferris *et al*., 2006), and molecular techniques such as the reverse transcriptase-polymerase chain reaction (RT-PCR) and real-time RT-PCR for rapid detection of FMDV nucleic acid (Bastos, 1998; Reid *et al*., 2003; Ferris *et al*., 2006; Mohapatra *et al*., 2007; Shaw *et al*., 2007; Reid *et al*., 2009). Diagnostic tests have also been described that allow for detection of the different FMDV serotypes (Ferris *et al*., 2005; Nordengrahn *et al*., 2008; Ferris *et al*., 2009). Not only do these diagnostic tests allow for a rapid turn-around time, but may also be used in an outbreak situation in the field.

In this review of the literature, aspects relating to FMDV epidemiology, structure and its infectious cycle will be discussed. Also included are discussions of FMD pathogenesis and immune repsonses elicited against FMDV following infection or vaccination. This will be followed by a discussion regarding the control of FMD by vaccination, and bioinformatic approaches to vaccine matching and antigenic cartography will also be highlighted. Finally, this section will be concluded with a brief description of the aims of this investigation.

1.2 FMD: A GLOBAL PERSPECTIVE

FMD is a disease with global distribution and has affected most countries in the world (Lubroth *et al*., 2007). Australia, New Zealand, Central and North America have been traditionally free of FMD. Systematic vaccination and a stamping-out policy have been used up to 1991 to control FMD in Europe and Scandinavia. The disease has entered South America from European breeding stock, but today several South American countries are FMD-free with vaccination. The disease, however, often spreads from Eastern Europe, North Africa, Asia and the Middle East to the rest of the world. Despite attempts to control the disease, FMDV infection is maintained within three continental epidemiological clusters in

Africa, Asia and South America that can be further subdivided into seven major virus pools (Fig. 1.1). Pools 1-3 are found in Asia, pools 4-6 in Africa and pool 7 in South America. Multiple serotypes co-circulate and distinct patterns of virus evolution occur within each of the defined pools. Six of the seven serotypes have been recorded in Africa (O, A, C, SAT1, SAT2 and SAT3), while in the Middle East and Asia only four serotypes (O, A, C and Asia-1) are generally present (Di Nardo *et al.*, 2011). Asia has been the main source of outbreaks affecting the Middle East and Europe (Valarcher *et al*., 2008). In contrast, outbreaks in Africa have been limited to the continent with only occasional incursions into the Middle East (FMD Reference Laboratory Network Reports).

Serotype A viruses have been associated with outbreaks caused by the European type A vaccines used in the 1980s (Beck and Strohmaier, 1987). Type A viruses are considered the most antigenically diverse of the European serotypes (types O, A and C) (Davie, 1964; Domingo *et al*., 1990; Knowles and Samuel, 2003; Mohapatra *et al*., 2011) and are prevalent in North and East Africa, the Middle East, Asia, as well as South America where closely related type A viruses have been responsible for outbreaks in Argentina, Uruguay, Venezuela and Brazil (Knowles and Samuel, 2003; FMD Reference Laboratory Network Report, 2010; Madin *et al*., 2011). The type A Iran05 virus appeared in Iran in 2003 and spread to Saudi Arabia in 2005 and then to Pakistan, Turkey and Jordan in 2006 (FMD Reference Laboratory Network Report, 2006). In 2007, outbreaks of Iran05 were reported in Jordan and Turkey and in 2008 in Bahrain. In 2010, type A Iran05 caused outbreaks in Afghanistan, Iran, Pakistan and Turkey (FMD Reference Laboratory Network Report, 2010). The continued circulation of type A viruses in this region is cause for concern, as it presents a significant threat to Europe and the former Soviet Union. Outbreaks of type A in Tanzania were the furthest south that this serotype has been observed (FMD Reference Laboratory Network Report, 2009).

FMD type O viruses have historically been associated with outbreaks caused by type O vaccines used in the 1970s and 1980s (Beck and Strohmaier, 1987). Although type O has a wide geographical distribution and remains the most prevalent serotype (Grubman and Baxt, 2004; FMD Reference Laboratory Network Reports), it is not prevalent in southern Africa. The type O Pan-Asia strain, which caused FMD outbreaks in many parts of the world, was first isolated in India in 1990 (Knowles *et al.*, 2001; Grubman and Baxt, 2004). From there it spread to Saudi Arabia in 1994 (Samuel *et al*., 1997), Turkey in 1996 and was isolated in Taiwan in 1999 (Huang *et al*., 2001). In 2000, this Pan-Asia strain spread to the Republic of

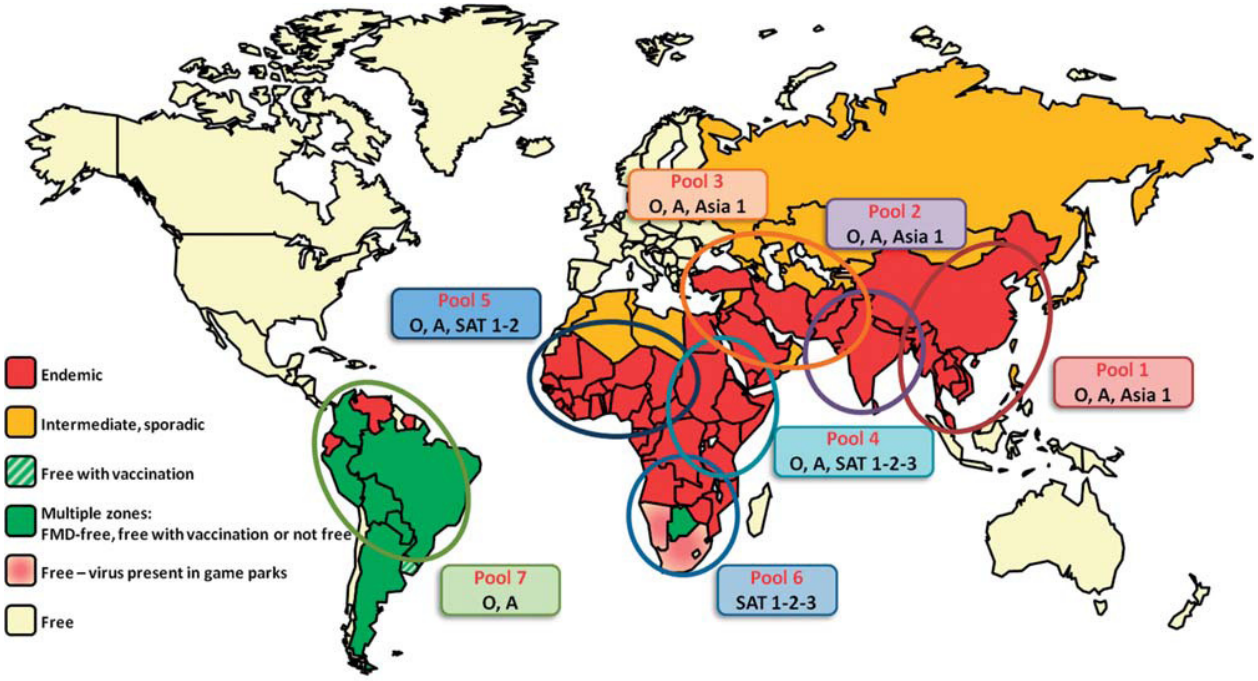

Fig. 1.1 Global estimation of the prevalence of foot-and-mouth disease in 2010 (taken from Di Nardo *et al*., 2011).

Korea, Japan, Russia and Mongolia, and led to the first isolation of a type O virus in South Africa (Sangare *et al*., 2001). In 2001, the Pan-Asia strain was isolated in Britain (Samuel and Knowles, 2001) from where it spread to Northern Ireland, the Republic of Ireland, France and the Netherlands within a month. In 2002, this virus strain was still causing FMD outbreaks in Mongolia (Knowles and Samuel, 2003). A highly transmissible Pan-Asia strain, *i.e*. Middle East-South Asia (ME-SA)/PanAsia-2, has spread from India to the east into Malaysia (2003-2005), west into Pakistan and Iran (2005-2006), and to Turkey and Jordan in 2006 (FMD Reference Laboratory Network Report, 2006). During 2007, this virus spread to Lebanon, Israel, Palestine and Egypt (FMD Reference Laboratory Network Report, 2009). The ME-SA/PanAsia-2 strain has since spread as far as North Africa in 2009, Afghanistan, Iran, Pakistan and Turkey in 2010, and Bulgaria in 2011 (FMD Reference Laboratory Network Report, 2009; WRLFMD Quarterly Report, 2011). ME-SA/PanAsia-2 is currently the most prevalent strain in the Middle East, South East Asia and the Far East (Valarcher *et al*., 2004; Lee *et al*., 2011).

In South America, outbreaks of type O have been reported in FMD-endemic countries, *i.e.* Ecuador, Bolivia and Venezuela, as well as in FMD-free countries such as Argentina and Brazil (FMD Reference Laboratory Network Report, 2006, 2007, 2010; Malirat *et al*., 2007). However, in 2009, type O_1 was circulating only in Ecuador and Venezuela (FMD Reference Laboratory Network Report, 2009). Several outbreaks of type O have occurred in South East Asia and the Far East (Le *et al*., 2010; Madin *et al*, 2011; WRLFMD Quarterly Report, 2011). In 2006, the type O Cathay virus, which is a pig-adapted strain, spread to Malaysia and Thailand following initial reports in the Philippines, Vietnam and China (FMD Reference Laboratory Network Report, 2006). In 2007, an outbreak of FMD caused by type O_1 BFS was reported in the United Kingdom. This outbreak, which originated from the Pirbright site, was quickly contained and trade restrictions were lifted within three months (FMD Reference Laboratory Network Report, 2007). In 2010, type O viruses were causing outbreaks across Asia in countries such as Bhutan, Japan, Kazakhstan, Mongolia, China, South Korea, the Russian Federation, Taiwan, as well as the United Arab Emirates (FMD Reference Laboratory Network Report, 2010).

Type C FMDV occurs infrequent and the last outbreak in Europe was in Italy in 1989. In the last decade, outbreaks of type C have been reported in Brazil, the Philippines and Kenya (Knowles and Samuel, 2003; Di Nardo *et al*., 2011). It has been suggested that improperly

inactivated vaccines were the source of re-introduction of the virus into Kenya and that discontinuation of type C vaccines would contribute to its eradication (Sangula *et al*., 2011). Considering the above and that there are no natural reservoirs known for type C viruses, the existence of circulating type C viruses in the field has been questioned (Paton *et al*., 2009).

Asia-1 is not a very genetically and antigenically diverse FMDV serotype and is mainly restricted to the Indian subcontinent (Doel, 2003; Kumar *et al*., 2004; Mohapatra *et al*., 2004). In an epizootic of Asia-1, it spread across the east from Iran and Turkey to Greece in 2000 and an Asia-1 type virus has also been isolated in Saudi Arabia in 1994 (Knowles and Samuel, 2003). In 2005, one of the main epidemiological events was the spread of Asia-1 in Asia (FMD Reference Laboratory Network Report, 2005). In addition to incursions into the Middle East, periodic incursions into former Soviet Union countries, North Korea, Vietnam and China have also been reported (FMD Reference Laboratory Network Report, 2006, 2007; Paton *et al*., 2009). Recently, Asia-1 outbreaks were reported in Bahrain and Iran in 2011, and these outbreaks have been linked to viruses originating from Pakistan (WRLFMD Quarterly Report, 2011).

Although the South African Territories (SAT) type viruses are considered to occur mostly in sub-Saharan Africa (Vosloo *et al*., 2001, 2002a), outbreaks have been recorded in the Middle East (1962-1965 and 1969-1970), as well as in Greece (1962) (Pereira, 1981; Ferris and Donaldson, 1992; Knowles and Samuel, 2003). Furthermore, the SAT2 type viruses have also spread to the Middle East in 1990 and again in 2000 (Bastos *et al*., 2003b). In southern Africa, African buffalo (*Syncerus caffer*) are the maintenance hosts of FMDV and provide a potential source of infection for domestic livestock and wildlife (Dawe *et al.*, 1994; Vosloo *et al*., 2007). Moreover, persistently infected buffalo not only maintain co-infection of different virus serotypes, but also facilitate genetic and antigenic evolution of the virus (Condy *et al.*, 1985; Esterhuysen, 1994; Vosloo *et al*., 1996, 2006). The SAT2 type viruses are responsible for most FMD outbreaks in southern Africa (Condy *et al*., 1969; Vosloo *et al*., 2002a), followed by SAT1 type viruses, whereas SAT3 type viruses are the least associated with outbreaks in domestic animals (Knowles and Samuel, 2003). During the last decade, several SAT type outbreaks have occurred in Namibia, Botswana and South Africa (Records of the OIE). SAT1 outbreaks occurred in Mozambique during 2001-2002, Zimbabwe in 2003, Zambia during 2004-2006/2008, Botswana in 2006 and Namibia in 2010. SAT2 outbreaks were reported from Zimbabwe during 2000-2003/2009-2010, Zambia during 2005-2009,

Botswana during 2002/2006-2010, Namibia during 2007-2009, Mozambique in 2010 and Malawi during 2008-2009 (Records of the OIE). With specific reference to South Africa, many outbreaks of all three SAT serotypes have occurred since the turn of the century, *e.g.* SAT1 outbreaks were reported during 2000/2002-2003/2009-2011, SAT2 outbreaks during 2001/2003-2005/2009/2011-2012 and a SAT3 outbreak in 2006 (FMD Reference Laboratory Network Reports; Records of the OIE and ARC).

Based on the above epidemiological data, FMD appears to have remained mostly confined to FMD-endemic countries and only a few outbreaks have been reported from FMD-free countries. Moreover, the majority of outbreaks were reported from Asia and Africa. Recent data from the field suggest that antigens available in international Vaccine Banks may not be adequate in providing protection against outbreak virus strains. For example, vaccine strains used locally in Asia are not available from European Vaccine Banks (FMD Reference Laboratory Network Report, 2009), and the Asia-1 strain responsible for an outbreak in Pakistan in 2009 is different to the Asia-1 Shamir antigen that is held in European Vaccine Banks (Parida, 2009; FMD Reference Laboratory Network Report, 2009). Moreover, the type A Iran05^{ARD-07} strain is unique to Turkey and is less related to the A_{22} Iraq vaccine strain, which is widely used in the Middle East (FMD Reference Laboratory Network Report, 2008). In addition, in contrast to the Pan-Asia virus that caused the 2001 outbreaks, the ME-SA/PanAsia-2 strain (2007) is antigenically dissimilar to the vaccine strain O_1 Manisa. Also, a SAT2 outbreak in Botswana in 2008 was probably due to the fact that the field virus was not related to the vaccine strains (FMD Reference Laboratory Network Report, 2009). Therefore, continuous efforts by regional FMD laboratories to characterise viruses remain essential in order to evaluate which viruses should be used for vaccine manufacturing. This aspect will be addressed in more detail in subsequent sections.

1.3 CLASSIFICATION AND PHYSICAL PROPERTIES OF FMDV

1.3.1 Classification

FMDV belongs to the *Picornaviridae* family, which consists of numerous widely-studied viruses, including important human and animal pathogens. This family contains nine genera, namely *Entero-*, *Rhino-*, *Cardio-*, *Aphtho-*, *Hepato-*, *Pareho-*, *Erbo-*, *Kobu-* and *Theschoviruses* (King, 2000). FMDV, together with equine rhinitis A virus (Li *et al.*, 1996),

is classified under the genus *Aphthovirus* and is characterised by high genetic and antigenic variation (Rueckert, 1996). Seven FMDV serotypes (A, O, C, Asia-1, SAT1, 2 and 3) have been identified based on their ability to induce cross-protection in animals (Pereira, 1981). FMDV has played a historic role in the field of virology. Although the first description of FMD was provided by the Italian Hieronymus Fracastorius in 1546 (Sobrino *et al*., 2001), it was only in 1898 that Loeffler and Frosch identified FMDV as the first filterable infectious agent to cause an animal disease (Brown, 2003; Enquist, 2009). Today, FMDV is a compulsory notifiable disease of animals according to the Office International des Epizooties (OIE).

1.3.2 Physical properties

FMDV is highly labile at pH ≤6; a characteristic that is also shared by *Rhinoviruses* (King, 2000). In contrast, *Entero*- and *Cardioviruses* are stable at pH 3. Unlike other picornaviruses, the capsid of FMDV has a hydrophobic pore at the icosahedral 5-fold axis that allows penetration of molecules such as caesium ions, thereby resulting in FMDV particles having a high buoyant density in CsCl gradients (1.41-1.45 g/ml) (Acharya *et al*., 1989; Thomson, 1994). FMD virions are 25 nm in diameter, spherical with icosahedral symmetry (Bachrach, 1968; Putnak and Phillips, 1981), and consist of 70% protein, 30% RNA and a limited amount of lipid (Bachrach *et al*., 1964). The virus particle has a sedimentation constant of 146S in sucrose density gradients (Rueckert, 1996). The latter is a characteristic widely used in vaccine production to determine the intact virion content present in cell harvests (Doel, 2003; Blignaut *et al*., 2011).

1.4 VIRAL RNA GENOME, CAPSID AND ANTIGENIC PROPERTIES

1.4.1 Structure of the RNA genome

The positive-sense single-stranded RNA genome of FMDV is *ca*. 8 500 nucleotides in length (molecular weight of 2.5×10^6 Da) and is enclosed within a protein capsid (Forss *et al*., 1984; Belsham, 2005). The viral genome consists of a single open reading frame (ORF) flanked by two untranslated regions (UTRs), both displaying complex secondary structure (Sobrino *et al*., 2001). The organisation of the FMDV genome is similar to those of other picornaviruses and the nomenclature of the viral proteins was established by Rueckert and Wimmer (1984).

A schematic representation of the FMDV RNA genome and protein coding regions is presented in Figure 1.2.

FMDV has a long 5' UTR of more than 1 300 bases in length (Forss *et al*., 1984). Covalently linked to the 5' UTR of the genome is the virus-encoded protein VP_g (viral genome-linked protein) (Grubman, 1980). The protein is encoded by the 3B coding region, which is located in the P3 region of the ORF. FMDV is unique in containing redundant copies of 3B, never fewer than three copies in tandem. The VP_g protein may be involved in the initiation of RNA synthesis and encapsidation of viral RNA (Sanger *et al*., 1977; Forss and Schaller, 1982), and its function may furthermore be related to pathogenesis and host range determination of the virus (Mason *et al*., 2003a). The 5' UTR also contains the small (S) fragment and polycytidylate tract (poly(C) tract), which is followed by four tandem repeats of pseudoknots (PKs), a *cis*-acting replication element (*cre*) and the internal ribosome entry site (IRES) (Mason *et al*., 2003a). The S fragment has a sequence that is capable of forming a stem-loop structure, but it does not encode proteins (Newton *et al*., 1985). The FMDV genome is divided into the S fragment and the large (L) fragment by the poly(C) tract (Sanger, 1979). The poly(C) tract is unique to *Aphtho-* and *Cardioviruses* and its length varies among different isolates (Brown *et al*., 1974). Although this region has been implicated to play a role in virulence, viruses containing a poly(C) tract of only two residues have been reported to be virulent in mice (Rieder *et al*., 1993). The poly(C) tract is predicted to be associated with a cellular poly(C)-binding protein (PCBP), as in the case of poliovirus, which may function in genome circularisation (Mason *et al*., 2003a). Three to four pseudoknot motifs are located downstream of the poly(C) tract, but their function is not yet known (Clarke *et al*., 1987a). In contrast to other picornaviruses, the *cre* is situated in the 5' UTR of FMDV and not within the protein coding region. It consists of a highly conserved hairpin-loop structure that contains the consensus AAACA motif and may play a role in viral replication (Mason *et al*., 2002; Tiley *et al*., 2003). The picornavirus IRES is highly structured and directs cap-independent translation of the viral RNA (Mason *et al*., 2003a; Beales *et al*., 2003).

The viral ORF consists of the Leader proteinase (L^{pro}), a structural protein coding region (P1) and two non-structural protein coding regions (P2 and P3) (Fig. 1.2). The L^{pro} coding region of FMDV (Sanger *et al*., 1987) contains two in-frame AUG codons and result in the synthesis of two proteins; Lab and Lb, respectively. The Lb protein is the major protease synthesised *in vivo* (Coa *et al*., 1995). Although it is an important determinant of viral virulence and

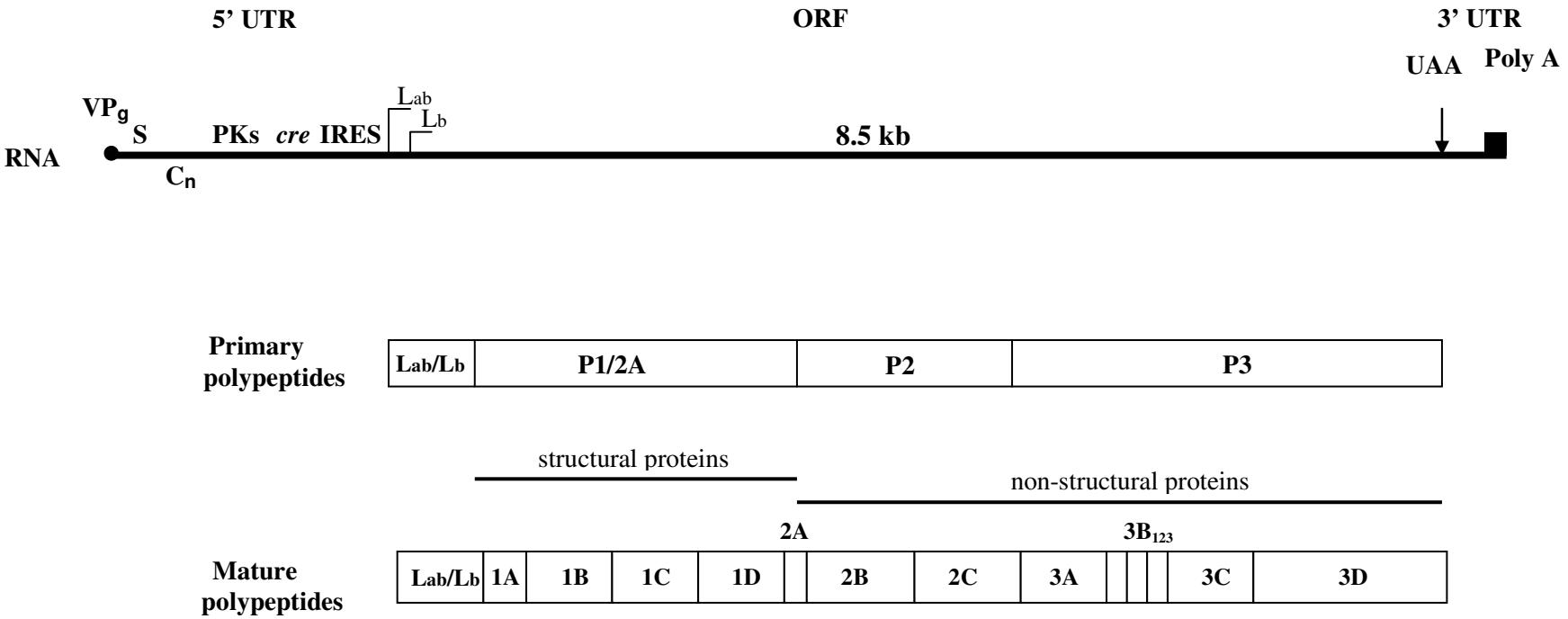

Fig. 1.2 Schematic diagram of the FMDV RNA genome, the untranslated regions (UTR) and open reading frame (ORF), as well as primary and mature polypeptides. The structural elements of the 5' UTR are indicated on the RNA genome, which is indicated by a thick line.

essential for pathogenesis (Mason *et al*., 1997; Chinsangaram *et al*., 1998; de Los Santos *et al*., 2006; Piccone *et al*., 2010), functional studies, using genetically engineered viruses, have indicated that the L^{pro} is not essential for virus replication (Piccone *et al*., 1995). The L^{pro} is a protease unique to the *Aphthoviruses* (Strebel and Beck, 1986). Four other picornaviruses also encode for L^{pro} proteins that are structurally different and do not have protease activity (Stanway *et al*., 2002). Sixty copies of each of the structural proteins 1A, 1B, 1C and 1D form the capsid of FMDV (Belsham, 1993; Jackson *et al*., 2003). These mature viral proteins are the products of several proteolytic processing events of the P1 polyprotein precursor.

The non-structural proteins encoded by the P2 and P3 coding regions of FMDV are involved in RNA replication, and in the folding and assembly of the structural proteins (Porter, 1993). The P2 polyprotein precursor is proteolytically processed into 2A, 2B and 2C. The 18-amino-acid peptide 2A remains attached to the P1 polyprotein precursor following primary cleavage (Donnelly *et al*., 2001; Mason *et al*., 2003a) and appears to be an autoproteinase. Protein 2B has been implicated in virus-induced cytopathic effects (CPE), and has been shown to enhance membrane permeability and block protein secretory pathways (Doedens and Kirkegaard, 1995). The 2C protein is involved in viral RNA synthesis since a mutation that confers resistance to guanidine, an inhibitor of viral RNA replication, is located close to its C terminus (Saunders *et al*., 1985). The 2C protein contains ATPase domains and a helicase domain, and assembly of 2C into hexameric rings appears to be required for its ATPase and non-specific RNA binding activities (Sweeney *et al*., 2010). Recently, RNA helicase A (RHA), a cellular helicase, was shown to play an essential role in replication of the FMDV through ribonucleoprotein complex formation at the 5' end of the genome and by interactions with 2C, 3A and PABP, a cellular poly(A)-binding protein (Lawrence and Rieder, 2009). RHA is thought to assist in the unwinding of double-stranded RNA intermediates during replication of the virus genome either by itself or in combination with 2C. Moreover, transient expression of 2B and 2C or of the 2BC precursor impedes trafficking of proteins between the endoplasmic reticulum (ER) and Golgi apparatus, which may have implications for development of persistent infections (Moffat *et al*., 2005, 2007).

The P3 polyprotein precursor is proteolytically processed to yield the non-structural proteins 3A, 3B, 3C and 3D. The 3A protein is involved in pathogenesis of FMDV, as deletions in the C terminus of 3A led to a type O outbreak strain being attenuated in cattle, but causing a devastating disease in pigs (Beard and Mason, 2000; Pacheco *et al*., 2003). The 3A protein

has also been implicated in adaptation of the virus to guinea pigs through a single amino acid change, lending further support to it playing a role in determining the host range and virulence of the virus (Núñez *et al*., 2001; Mason *et al*., 2003a). In addition, the 3A protein is believed to be a membrane anchor for the replication complex (Weber *et al*., 1996). Similar to other picornaviruses, the FMDV 3A protein is associated to viral-induced membrane vesicles, and contributes to CPE and to the inhibition of protein secretion (Doedens and Kirkegaard, 1995; Wessels *et al*., 2006). As mentioned previously, the 3B coding region encodes the VP_g protein and the level of FMDV infectivity has been reported to correlate with the number of copies of 3B present on the RNA genome (Falk *et al*., 1992; Pacheco *et al*., 2003).

The 3C protein is a protease ($3C^{pro}$) and is responsible for 10 of the 13 proteolytic cleavages in the FMDV polyprotein (Ryan *et al*., 1989), except for cleavage of L^{pro} from P1, the 2A cleavage between P1 and P2 and the maturation cleavage of 1AB to 1A and 1B. Structural studies revealed that the FMDV $3C^{pro}$, like that of other picornaviruses, is a cysteine protease and has an overall fold similar to serine proteases (Birtley *et al*., 2005). The β-ribbon surface loop folds over the peptide binding cleft of $3C^{pro}$ and contributes to substrate recognition via direct contact with peptide sequences (Sweeney *et al*., 2007). Opposite the active site and exposed on the surface of $3C^{pro}$ is a region of basic amino acids that is involved in RNA binding during replication (Nayak *et al*., 2006). The proteolytic processing of histone H3 is also induced by $3C^{pro}$, thereby resulting in the inhibition of transcription in infected host cells (Falk *et al*., 1990). The $3C^{pro}$ has also been implicated as being responsible for inducing a loss of γ-tubulin from the microtubule organising centre in cells, thus resulting in altered cellular morphology (Armer *et al*., 2008). However, the mechanism through which $3C^{pro}$ exerts its effect on γ-tubulin remains unclear since the γ-tubulin is not targeted for destruction.

The 3D protein is a RNA-dependant RNA polymerase ($3D^{pol}$) (Newman *et al*., 1979) and has been implicated in specific interaction with the viral RNA genome during the replication process (Ferrer-Orta *et al*., 2009; Bentham *et al*., 2012). The $3D^{pol}$ catalyses the covalent linkage of uridine monophosphate (UMP) to a tyrosine on the small protein VP_g and the uridylylated VP_g then serves as a protein primer for the initiation of RNA synthesis (Nayak *et al*., 2005). Downstream of the 3D coding region is the 3' UTR. In addition to the polyadenylate tract (poly(A) tract), the 3' UTR includes *ca.* 100 nucleotides of heterogeneous sequence and its deletion blocks infectivity of the viral RNA (Sáiz *et al*., 2001). The 3' UTR

is involved in viral replication through interactions with the 5' UTR and stimulation of IRES activity (Belsham, 2005).

1.4.2 Structure of the FMDV capsid

The structure of type O (Acharya *et al*., 1989; Logan *et al*., 1993; Lea *et al*., 1995), type C (Lea *et al*., 1994), type A (Curry *et al*., 1996; Fry *et al*., 2005) and SAT1 (Fry *et al*., unpublished; Protein Data Bank ID: 2wzr, r2wsrf) virions have been elucidated by X-ray crystallography. FMDV capsids have the classic structural organisation of picornaviruses (Fig. 1.3d). The non-enveloped capsid consists of 60 asymmetrical units (protomers), the majority of which contain one molecule of each of the structural proteins (1A, 1B, 1C and 1D) (Fig. 1.3b). A few protomers within each capsid are immature and consist of 1AB (the precursor of proteins 1A and 1B). Viral particles consist of 12 pentamers, with each pentamer consisting of five protomers (Fig. 1.3c), and the single-stranded RNA genome (Vasquez *et al*., 1979). Upon encapsidation of the RNA, 1AB is autocatalytically cleaved into proteins 1A and 1B (Curry *et al*., 1996).

Structural proteins 1B, 1C and 1D have an eight-stranded anti-parallel β-barrel core structure composed of two four-stranded β-sheets of which the loops joining these strands, as well as the C termini of these three proteins, are exposed on the surface of the capsid (Acharaya *et al*., 1989; Logan *et al*., 1993) (Fig. 1.3a). These proteins have a similar arrangement to other picornaviruses, where 1D proteins are located around the icosahedral 5-fold axes and 1B and 1C alternate around the 2- and 3-fold axes. Protein 1A is located internally (Acharya *et al*., 1989), which corresponds with the observation that it displays the least genetic variation (Carillo *et al*., 2005). The N-terminal portion of 1A is myristylated, which has been reported to be essential for capsid assembly and stability (Chow *et al*., 1987). Despite similarities in the spatial arrangement of proteins 1B, 1C and 1D with other picornaviruses, FMDV has some unique structural characteristics. The capsid surface is relatively smooth, except for the βG-βH (G-H) loop in protein 1D (Fry *et al*., 1999). The crystal structure of the O_1BFS virus (Acharya *et al*., 1989; Logan *et al*., 1993) revealed that the G-H loop is mobile and surface-exposed. This protruding G-H loop spans about 20 amino acid residues in the region of residues 140-160 and the length varies for viruses from different serotypes (Maree *et al*., 2011b). At the apex of the G-H loop is the highly conserved Arg-Gly-Asp (RGD) triplet, which is the main cellular receptor recognition site (Fox *et al*., 1989; Baxt and Becker, 1990) and forms part of the major neutralisation site (Mateu *et al*., 1995).

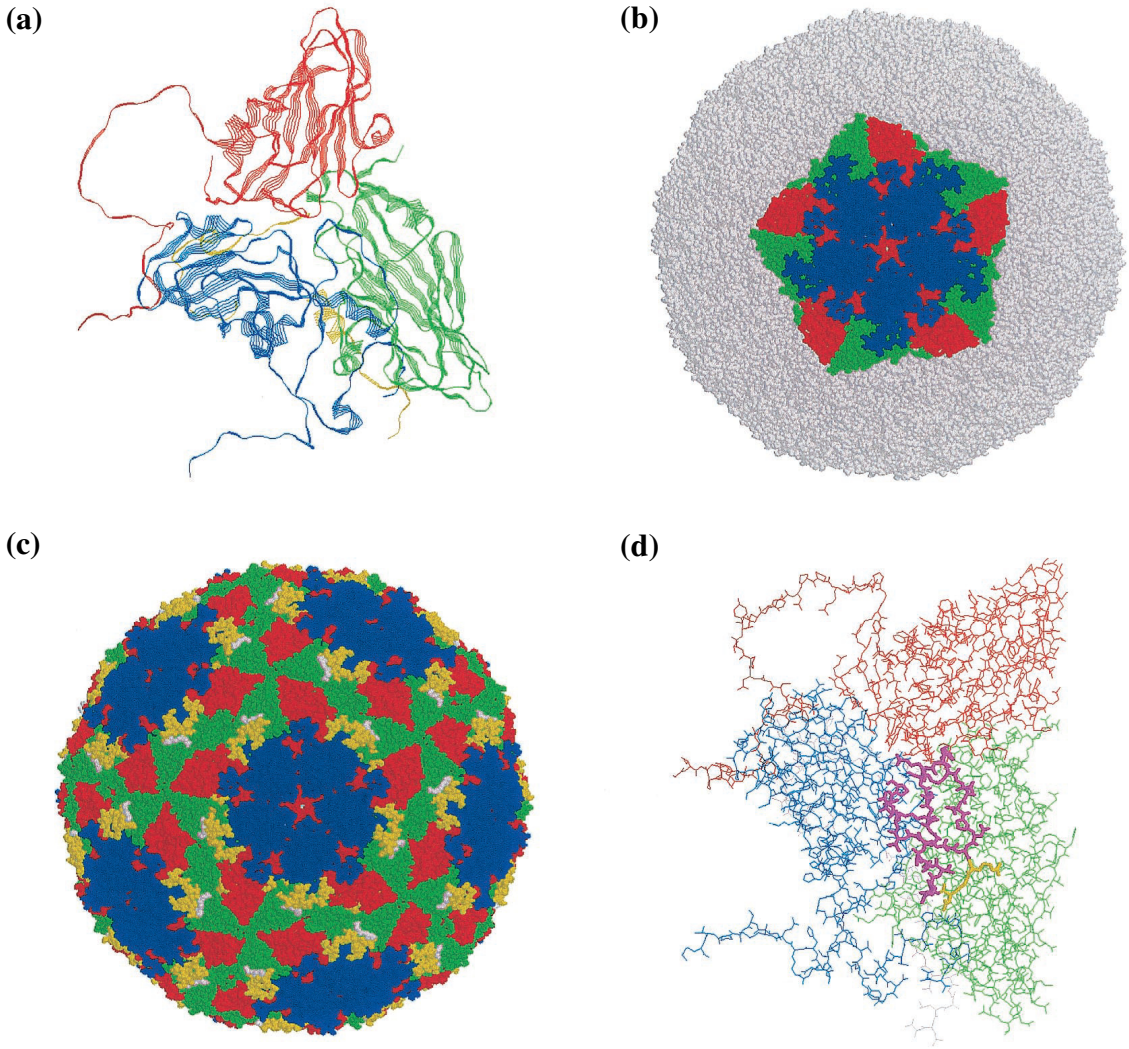

Fig. 1.3 The structure of the FMDV external capsid proteins, the subunits and viral capsid. Structure of the mature type O_1BFS FMD virion based on X-ray crystallographic data determined by Acharya *et al*. (1989) and Logan *et al*. (1993). (a) Structure of the viral protomer indicating the β-barrel-and-loop organisation of the proteins. (b) Arrangement of the five protomers into a pentamer positioned on the virion (looking at the 5-fold symmetry axis). (c) Structure of the FMDV capsid, consisting of 60 protomers, highlighting the G-H loop (yellow) and the RGD sequence (white). (d) The three external capsid proteins assemble into a protomer, consisting of 1B (green), 1C (red) and 1D (blue) exposed on the virus surface and 1A which is internal (indicated in yellow in panel a). The positions of the G-H loop (purple) and the RGD sequence (yellow) are indicated. Nomenclature is adapted from Grubman and Baxt (2004).

Unlike most other picornaviruses, FMDV is susceptible to low pH-induced capsid disassembly, a characteristic that has been used in the taxonomic classification of this virus (Knipe *et al*. 1997; King, 2000) and is also considered to be of great importance to its pathogenicity (Mateo *et al.*, 2007). The acid sensitivity varies between different FMDV serotypes, as well as subtypes. Studies on type A viruses, for example, indicated that type A_{22} is *ca*. 0.5 pH unit less stable under low pH conditions compared to types A_{10} and A_{24} (Curry *et al*., 1995). Notably, the pKa of FMDV dissociation corresponds to approximately that of the amino acid histidine (His). Consequently, a His-rich region at the pentamer interface (1B/1C) has been suggested as the reason for capsid disassembly (Ellard *et al*., 1999). A His-α-helix dipole interaction has been identified at the pentamer interface close to the icosahedral 2-fold symmetry axis (Curry *et al*., 1995). This interaction involves residues 89 to 98 of 1B, which is associated with the side chain of His-142 from 1C. Protonation of residues H-142 and H-145 in 1C at low pH is proposed to cause repulsive electrostatic forces across the pentamer interface, thereby resulting in the capsid opening up (Curry *et al*., 1995; Twomey *et al*., 1995; van Vlijmen *et al*., 1998). Moreover, acidification of FMDV within cellular endosomes can also disrupt interactions between 1B and 1C at the pentameric interfaces, thus leading to dissociation of the structure into pentamers and thereby release of the viral RNA (Burroughs *et al*., 1971; Carrillo *et al.*, 1984; Curry *et al*., 1997). The low pH-induced instability of FMDV not only leads to differences in the mechanism of its uncoating upon infection of cells as compared to other picornaviruses, but potentially also plays a role in the targeting of the virus to specific tissues and organs in susceptible hosts (Grubman and Baxt, 2004).

It has been reported that the stability of the FMD virion is influenced by multimeric electrostatic and hydrophobic interactions between the protein subunits and that disruption of these interactions causes dissociation of the capsid (Acharya *et al.*, 1989; Lea *et al.*, 1994, 1995; Mateo *et al*., 2003). It can therefore be expected that these interactions should be sufficiently robust in order to provide stability to the capsid under environmental conditions (Curry *et al*., 1995; Ellard *et al*., 1999), while still permitting intracellular uncoating and release of viral RNA. The availability of crystal structures for FMD viruses has facilitated more detailed studies aimed at identifying protein-protein interactions that relate to virion stability and also to assess the effects of environmental factors, such as temperature and pH, on capsid stability. Subsequently, amino acid residues involved in protein-protein interactions at the pentameric interfaces and their intolerance to variation within and between

serotypes have been demonstrated (Curry *et al.*, 1995; Twomey *et al.*, 1995; Ellard *et al.*, 1999; Mateu, 2011). At least 42 amino acid side chains of each protomer were shown to be involved in non-covalent interactions between the pentamers in the virus capsid (Mateo *et al.*, 2003). Recently, Borca *et al.* (2012) have suggested that residue H-56 in 1C contributes to thermal stability through interactions with residues in 1B and 1D, whereas an A118V mutation in 1C was shown to enhance acid sensitivity of type C viruses (Martín-Acebes *et al.*, 2010).

1.4.3 Antigenic properties

The antigenicity of the virus is determined mainly by linear and discontinuous epitopes formed by amino acid residues exposed on one or more of the capsid surface proteins. These antigenic sites form the main areas for antibody neutralisation (Mateu *et al.*, 1995) and are serotype-specific. Initial studies on type A_{12} FMDV indicated that the 1D protein induces sufficient neutralising antibodies to protect animals (pigs and cattle) from homologous virus challenge (Bachrach *et al.*, 1975; Kleid *et al.*, 1981). Indeed, the major antigenic site of FMDV is the G-H loop located in the 1D protein (Strohmaier *et al.*, 1982; Baxt *et al.*, 1989), as was deduced from the immunogenicity of peptides consisting of amino acid residues 140-160 in the 1D protein (Mateu *et al.*, 1995). Using bovine sera of convalescent or vaccinated animals, Francis *et al.* (1990) reported that 50% of the neutralising antibodies were directed to this site. This region has subsequently become known as site 1 for serotypes O_1 and A or site A for serotype C. Notably, results from animal trials with chimeric FMD vaccines with substitutions in the G-H loop indicated that the G-H loop may not be essential to induce a neutralising antibody response and protection in cattle (Fowler *et al.*, 2008, 2010).

The conformation of the G-H loop of type O viruses differs markedly from other serotypes due to the formation of a disulphide bond between the base of the G-H loop (Cys-134) and protein 1B (Cys-130). The loop may have a more ordered structure and the disulphide bond may contribute to the loop being in the less intrinsically stable ‘up’ conformation (Logan *et al.*, 1993). This correlates with the finding that type O-specific antibodies have a propensity for binding to conformational epitopes (Parry *et al.*, 1990), in contrast to type A, type C and SAT2 viruses, which recognise immobilised peptides representing the G-H loop (Mateu *et al.*, 1987; Bolwell *et al.*, 1989; Opperman *et al.*, 2012).

Many of the antigenic sites for FMDV are involved in more than one structural element. Although site 1 (or site A) is often referred to as the immunodominant site, other neutralisation and non-neutralisation sites also exist, of which some are important in the host immune response to FMDV. A total of five non-overlapping sites have been identified for serotype O_1 and several sites have been described for type C and A viruses (Kitson *et al.*, 1990; Crowther *et al.*, 1993a). Similar to other picornaviruses, most of the antigenic sites are formed by residues from different parts of a protein or from different proteins at the particle surface. For type O_1, site 1 involves the G-H loop and C terminus of the 1D protein (residues 144-149 and 206-207). Site 2 involves residues from the B-C loop and E-F loop of the 1B protein (residues 70-77/78 and 131-134), and has been suggested to be as important as site 1 in 1D in eliciting immune responses in infected hosts (Barnett *et al.*, 1998; Mahapatra *et al.*, 2008, 2012). Site 3 is located on the B-C loop of the 1D protein (residues 43-48) and site 4 is located in the 1C protein (residues 56-58). Site 5 is a second distinct site in the 1D protein (residue 154). For type A viruses, multiple antigenic sites have been determined in the outer capsid proteins (1B, 1C and 1D), as well as in the 1A protein (Thomas *et al.*, 1988; Baxt *et al.*, 1989; Bolwell *et al.*, 1989; Mahapatra *et al.*, 2011). These sites for the type A viruses agree with sites 1, 2, 4 and 5 of type O_1. Type C has similar sites to type O_1, but these are denoted alphabetically. Site C is located on the C terminus of the 1D protein and is continuous and independent from the G-H loop. Site D is discontinuous and comprises 1B (residues 72, 74 and 79), 1C (residue 58) and the C terminus of 1D (residue 193) (Mateu *et al.*, 1990; Lea *et al.*, 1994).

In contrast to the above European serotypes, not much is known about the nature of the antigenic sites of the SAT type viruses. Studies using monoclonal antibodies against the SAT2 virus RHO/1/48 led to the identification of epitopes that were all associated with the G-H loop region of 1D (Crowther *et al.*, 1993b). Similarly, for the SAT2 virus ZIM/7/83, residue 159 in 1D has recently been proposed as an epitope (Opperman *et al.*, 2012). In addition, Grazioli *et al.* (2006) identified antigenic sites for both SAT1 and SAT2 viruses. For the SAT1 virus BOT/1/68, four sites were determined that comprise of 1B (residue 72), 1C (residues 71, 76 or 135), 1D (residue 111) and the G-H loop (residue 179 or 181). Two independent linear sites were identified in 1D for the SAT2 virus ZIM/5/81. These were located in the G-H loop (residue 154), which may be associated with residue 79 in 1B, and in the C terminus (residue 210).

1.5 INFECTIOUS CYCLE OF FMDV

1.5.1 Cell recognition

The FMDV infection cycle is initiated by the interaction of the virus with receptors exposed on the cell surface. *Apthoviruses*, unlike *Enteroviruses*, do not need a specific cell surface receptor for cell entry. Competition-binding studies with the seven FMDV serotypes have indicated that although these viruses share a common receptor on cultured cell surfaces, some serotype viruses bind to other high-copy-number cell surface proteins (Baxt and Bachrach, 1980). These results were the first to indicate that *Apthoviruses* utilise multiple cellular receptors for infection.

1.5.1.1 RGD-dependant mechanism of cell binding

Structural and functional studies have indicated that the RGD motif is important in the interaction of FMDV with integrin cellular receptors on the cell surface (Fig. 1.4a). Integrin subunits are transmembrane glycoproteins that are composed of a large N-terminal extracellular domain, a smaller transmembrane region and a short cytoplasmic tail (Jackson *et al*., 2004) (Fig. 1.4b). Ligand binding is regulated by conformational changes in the integrin ectodomain in a process called integrin activation (Luo *et al*., 2004). Binding of FMDV to the integrin receptor is facilitated by conversion of the integrin to an extended or open conformation and the RGD-binding pocket becomes exposed (Frelinger *et al*., 1990; Blystone *et al*., 1997).

Despite being located in a highly variable region among virus isolates, the RGD is highly conserved (Robertson *et al*., 1983; Pfaff *et al*., 1988). The RGD sequence is located on the apex of the long, flexible G-H loop of the 1D protein (Acharya *et al*., 1989; Logan *et al*., 1993). Synthetic peptides, containing the RGD sequence, have been used to inhibit binding of FMDV to cells, thereby confirming the importance of the RGD motif in cell binding (Fox *et al*., 1989; Baxt and Becker, 1990). Reverse-genetic studies, using viruses in which the RGD sequence has been mutated or deleted, furthermore implicated this sequence as being important for binding of FMDV to cells (Mason *et al*., 1994; Leipert *et al*., 1997; Storey *et al*., 2007). In addition to the RGD motif, the regions flanking this triplet are also important as changes in these residues may influence binding of the virus to RGD-dependant cellular receptors (Baxt and Becker, 1990; Jackson *et al*., 2000, 2003; Burman *et al*., 2006).

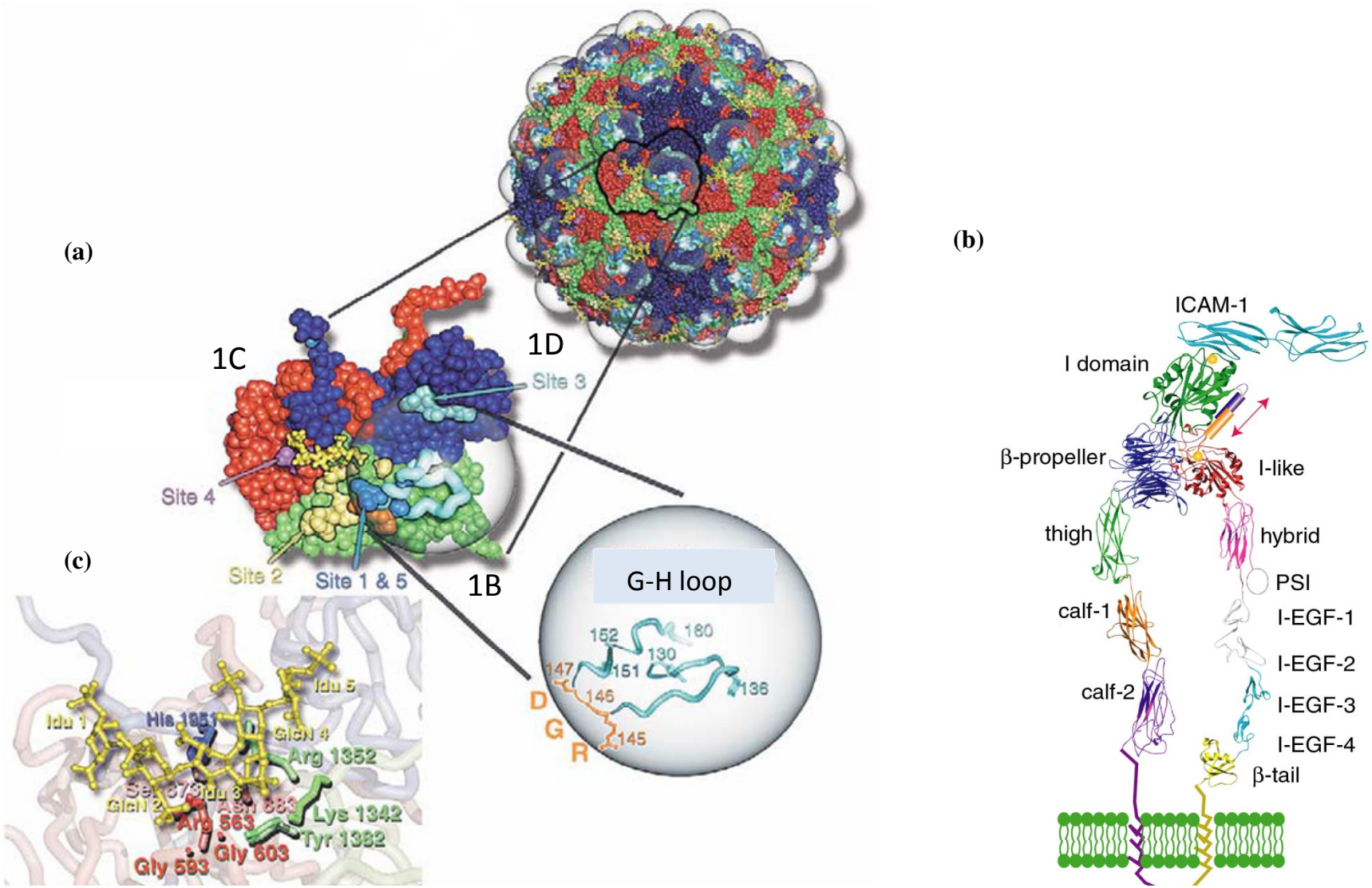

Fig. 1.4 FMDV internalisation by the RGD-dependant and -independant mechanism of cell binding. (a) Atoms on the structure of the reduced type O virus (Logan *et al*., 1993) are depicted as solid spheres: 1D is blue, 1B is green and 1C is red (1A is not visible). The G-H loop on 1D (residues 130-160) is shown in cyan with the Arg-Gly-Asp (RGD) residues in orange. Antigenic residues are colour-coded according to their classification into sites (Crowther *et al*., 1993a, 1993b; Kitson *et al*., 1990): site 1 and 5 (mid-blue), site 2 (pale yellow), site 3 (light blue), site 4 (magenta). The potential positioning of the G-H loop is modelled by a transparent sphere. The residue numbers are shown for the conserved residues for the RGD integrin binding motif (orange). (b) Representation of the integrin receptor in an extended or open conformation when the RGD-binding pocket becomes exposed. (c) The heparin motif which has been visualised in complex with the virus (Fry *et al*., 1999) is drawn in yellow ball-and-stick. The heparan-binding site is enlarged to show the protein side chains which act as ligands for the five sugar residues IDU1 to IDU5. The protein backbone is shown in the background colour coded as above and the side-chains interacting with the heparan are in ball-and-stick and coloured as the protein backbone; His 195 of 1D, Lys 134, Arg 135 and Tyr 138 of 1B and Arg 56, Gly 59, Gly 60, Ser 87 and Asn 88 of 1C. Adapted from Jackson *et al*. (2003) and http://www.google.co.za/imgres?q=Integrin+receptors.

The integrin $\alpha_v\beta_3$ was the first cellular receptor implicated in the RGD-dependant binding of FMDV to cells (Berinstein *et al*., 1995). Most laboratory strains of FMDV utilise this cellular receptor for cell entry and it is also used in bovine hosts (Neff *et al*., 1998). A second integrin, $\alpha_v\beta_6$, also functions as a cellular receptor for FMDV and is exposed on the surface of epithelial cells, which are the first target cells in infected cattle (Jackson *et al*., 2000; Monoghan *et al*., 2005). Subsequently, integrin $\alpha_v\beta_1$ has been implicated in binding of FMDV to cultured cells (Jackson *et al*., 2002) and $\alpha_v\beta_8$ has also been identified as a fourth integrin receptor used by FMDV (Jackson *et al*., 2004). Integrins $\alpha_v\beta_6$ and $\alpha_v\beta_8$ have been identified as important receptors for successful isolation of FMDV from clinical specimens (King *et al*., 2011). Despite its ability to utilise different integrins, it has been reported that the integrin-binding G-H loop of FMDV has a higher affinity for $\alpha_v\beta_6$ than for $\alpha_v\beta_3$ and $\alpha_v\beta_8$ (Boettiger *et al*., 2001; Burman *et al*., 2006). Likewise, a SAT1 virus isolated from impala, as well as SAT2 and SAT3 viruses isolated from buffalo were shown to utilise $\alpha_v\beta_6$ preferentially and $\alpha_v\beta_3$ to a lesser extent (Maree *et al*., 2011a).

1.5.1.2 RGD-independant mechanisms of cell binding

In addition to integrins, FMDV has also been reported to utilise various other cell surface molecules as receptors *in vitro*. Propagation of FMDV in cell culture has led to the selection of mutant viruses that are characterised by several phenotypic alterations such as a small-plaque phenotype (Maree *et al*., 2010), improved replication (Baranowski *et al*., 1998), altered antigenic variants with enhanced resistance to neutralising monoclonal antibodies (Martínez *et al*., 1997), broadened cell tropism (Escarmís *et al*., 1996, 1998), the ability to bind to heparan sulphate (Baranowski *et al*., 1998; Jackson *et al*., 1996, 2000), and are attenuated in cattle and pigs (Sa-Carvalho *et al*., 1997; Zhao *et al*., 2003; Borca *et al*., 2012).

Despite the presence of the RGD motif, viruses that have been propagated in cell culture often use RGD-independant mechanisms of cell recognition. Such virus variants have a high affinity for the cell surface receptor heparan sulphate (HS) (Jackson *et al*., 1996; Fry *et al*., 2005). Glycosaminoglycans (GAGs), such as HS proteoglycans (HSPGs), are negatively charged, randomly sulphated polymers of disaccharide repeats of L-iduronic (Idu) and D-glucosamine (GlcN) (Fig. 1.4c). HSPGs are expressed on the surface of most cell types. Binding of FMDV to HS involves positively charged amino acids on the capsid surface, which vary for different virus strains (Jackson *et al*., 2003). The mechanism of binding is

similar to that of other viruses, *i.e.* through electrostatic interactions between negatively charged N- and O-sulfated groups of the HSPGs and positively charged residues on the virion (Gromm *et al*., 1995; Byrnes and Griffen, 2000). For type A and O viruses, the presence of positively charged Arg residues at position 134 of 1B and 56 of 1C has been associated with binding to HS (Jackson *et al.*, 1996; Sa-Carvalho *et al.*, 1997; Fry *et al*., 1999, 2005; Cottam *et al*., 2008). In contrast, for SAT1 and SAT2 viruses, acquisition of positively charged amino acid residues at positions 83-85 and 110-112 in 1D surrounding the 5-fold axis of the virion was observed (Maree *et al*., 2010).

The HS-binding site for FMDV may neither be continuous in sequence nor be composed of a single peptide chain, but possibly distant surface-exposed structural motifs may play a role (Fry *et al*., 2005). Notably, despite small molecular changes in cell-adapted viruses a range of phenotypic alterations may be introduced. The ability of a single amino acid to change the receptor-binding characteristics of FMDV is probably due to the enhanced number of protein binding sites on the virion. The shallow depression at the junction of capsid proteins 1B-1D within the protomer, which is associated with HS binding, is distinct in its location from the integrin binding site (Fry *et al*., 2005). The RGD motif is unaffected by HS binding (Fry *et al*., 1999) and the two receptor recognition sites appear to function independantly of each other. Cell-adapted viruses can use either class of receptor. It should be noted that although HS-adapted viruses retain the ability to use intergrins as cellular receptors, in some cases FMDV can dispense with the RGD integrin-binding site (Martínez *et al*., 1997; Neff *et al*., 1998; Baranowski *et al*., 2000; Rieder *et al*., 2005).

FMDV can also enter cells by an antibody-dependant pathway using Fc receptors (Mason *et al*., 1993, 1994). A single chain FMDV-specific antibody fused to intercellular adhesion molecule-1 (ICAM-1) was expressed in cells lacking FMDV receptors and was found to function as an artificial cell receptor (Rieder *et al*., 1996). These results indicated that integrin and HS receptors are not essential for FMDV cell-binding. Indeed, FMDV may bind to cells via integrin- and HS-independant pathways, suggesting the existence of additional uncharacterised receptors, and thus of multiple mechanisms whereby FMDV can adapt to growth in cell culture (Baranowski *et al*., 2000; Zhao *et al*., 2003). However, integrins are the primary receptors required for infection of host species by FMDV (Neff *et al*., 1998; Monaghan *et al*., 2005; Rieder *et al*., 2005).

1.5.2 Protein synthesis and processing of the FMDV polyprotein

The mechanism of FMDV cell entry is dependant on the receptor to which the virus binds. Early internalisation events, following binding to integrins, include the use of a clathrin-dependant mechanism to enter the cell, moving from early endosomes (EE) into recycling endosomes (Berryman *et al*., 2005; O'Donnell *et al*., 2005). In Baby Hamster Kidney cells (BHK-21) and Instituto Biologico Renal Suino-2 (IB-RS-2) cells, FMDV endocytosis depends on the presence of cholesterol in the plasma membrane which is required for the formation of clathrin-coated vesicles (Martín-Acebes *et al*., 2007, 2009). In contrast, viruses that utilise HS enter cells via a caveola-mediated endocytic pathway (O'Donnell *et al*., 2005, 2008). Irrespective of the pathway used for virus internalisation, viral uncoating is believed to occur within the internalised endocytic vesicles. Acidification of the vesicles leads to the release of 1A and unfolding of the hydrophobic regions of capsid proteins 1B-1D. Fusion of the lipid bilayer with hydrophobic regions of the exposed capsid proteins leads to the formation of a pore through which the viral RNA can be transferred to the cytoplasm (Rueckert, 1996). In the cytoplasm, the VP_g protein, which is linked to the 5' UTR of the viral RNA, is released and polyprotein synthesis is initiated in a cap-independant manner at the internal ribosome entry site (IRES) (Kühn *et al.*, 1990). Translation is initiated at two in-frame AUG codons, 84 nucleotides apart, in the large (L) fragment of the viral genome (Beck *et al*., 1983; Sanger *et al*., 1987). The eukaryotic initiation factor (eIF)-4B (Meyer *et al*., 1995), as well as the cellular polypyrimidine tract-binding protein (PTB) (Niepmann *et al*., 1997) have been implicated in the translation initiation at the IRES structure. The RNA strand directs synthesis of the polyprotein, which undergoes a series of proteolytic cleavages to yield individual proteins as synthesis progresses (Rueckert, 1996; Belsham, 2005).

The L^{pro} is the first protein to be synthesised and cleaves itself from the rest of the growing polypeptide (Strebel and Beck, 1986). The L^{pro} cleaves eIF-4G, thereby resulting in the inhibition of cap-dependant mRNA translation of the host cell (Devaney *et al.*, 1988; Mayer *et al*., 2008). With the progression of polyprotein synthesis, the polyprotein is cleaved into several structural and non-structural proteins (Ryan *et al.*, 1989), mainly by $3C^{pro}$ that functions *in trans* (Vakharia *et al.*, 1987; Clarke and Sanger, 1988). The FMDV $3C^{pro}$ cleavage sites show heterogeneity among different isolates and cleavage occurs at multiple dipeptides, unlike the poliovirus $3C^{pro}$, which only cleaves at Gln-Gly dipeptides (Robertson *et al*., 1985; Palmenberg, 1990). As the recognition of these $3C^{pro}$ cleavage sites is dependant

on their position within the polyprotein, the three-dimensional structure of these processing regions should be conserved (van Rensburg *et al*., 2002). The $3C^{pro}$ also cleaves histone H3, removing 20 N-terminal amino acids (Falk *et al*., 1990). Neither poliovirus nor EMCV infection results in this cleavage; therefore FMDV may have developed different mechanisms to inhibit host cell transcription. The excision of P1-2A from 2B is catalysed *in cis* by 2A (Clarke and Sanger, 1988).

Viral RNA replication entails the synthesis of complementary negative-sense RNA, which serves as template for the synthesis of many positive-sense copies. Many of the progeny positive-sense RNA strands are initially recruited as viral mRNA, but later, as the synthesis of positive-sense RNA predominates, almost 50% of these molecules are packaged into virions (Rueckert, 1996; Nayak *et al*., 2005). Virion assembly involves the formation of capsid protomers and association of five such protomers results in the formation of a pentamer, which is followed by assembly into either empty capsids or provirions, which contain packaged positive-sense VP_g-RNA (Rueckert, 1996). Autolytic cleavage of protein 1AB present in the provirion completes the assembly process and is required for the generation of infectious virus particles (Palmenberg, 1990). The mechanism of this maturation cleavage is not known. Virion assembly and RNA encapsidation leads to the formation of about 10^4-10^5 viral particles per cell, of which 0.1-1% are infectious. The virions are released from the host cells by disintegration of the cells as a consequence of the infection (Belsham, 1993; Rueckert, 1996).

1.6 PATHOGENESIS

Infection of susceptible livestock species with FMDV results in an acute, febrile illness and is characterised by the rapid onset of clinical signs within two to five days of exposure. Clinical signs of disease include fever, malaise and the development of vesicles on the coronary bands of the feet, in the mouth and on the tongue and teats (Burrows *et al*., 1981; Thomson, 1994). Viraemia is detectable in the same time frame and resolves very quickly, as do the clinical signs, by which time an infected individual may have secreted large amounts of virus into the environment and infected many other susceptible animals (Arzt *et al*., 2011b). The interaction between the virus and the host occurs at many levels, namely molecular, cellular and in tissues. Understanding the host-virus interaction is key to both understanding

transmission between animals and the rational design of intervention strategies. It has been shown previously that the success of disease control strategies depend on the timing of the onset of infectiousness relative to the onset of detectable clinical signs (Charleston *et al.*, 2011).

Following transmission of FMDV to susceptible cattle, three stages of FMD pathogenesis have been identified (Arzt *et al.*, 2011b), namely pre-viraemia (infection and replication at primary site/s), viraemia (active viral replication, generalisation and vesiculation at secondary infection sites) and post-viraemia/convalescence (clearance of clinical disease, shedding and possible long-term persistent infection).

Pre-viraemia is recognised as the period when the animal is first infected with virus until virus is first detected within the intravascular compartment (*i.e.* blood) (Arzt *et al.*, 2011b). During this period, FMDV can be detected in the nasal mucosae and nasopharynx of cattle. Prior to detection of virus in blood or formation of vesicles, virus can be isolated from oesophageal-pharyngeal (OP) fluid (Sellers *et al.*, 1968; Burrows *et al.*, 1971). Primary virus replication could be detected two to six hours following intranasal administration of FMDV, depending on the dose and strain used (McVicar and Sutmoller, 1976). In addition to the pharynx of cattle, high titres of virus have been detected in the dorsal soft palate and retropharyngeal lymph node, while virus was also recovered from the lungs, tonsils and nasal mucosae. Recent studies showed that virus infection was initiated within six hours of exposure to FMDV at the crypt regions of the follicle-associated epithelia at mucosa-associated lymphoid tissue (MALT) regions of the nasopharynx (Arzt *et al.*, 2010; Pacheco *et al.*, 2010). Shortly thereafter, at 12 hours post-infection, FMDV was detected in the pulmonary alveolar septa. The FMDV infection was in epithelial cells of the pharyngeal and pulmonary sites, which express mostly $\alpha_v\beta_6$ integrin receptors (Monaghan *et al.*, 2005). During the pre-viraemia period in cattle no virus could be detected in the lymph nodes and, as viraemia approached, viral load decreased in the pharynx and increased in the lungs (Arzt *et al.*, 2010; Pacheco *et al.*, 2010).

During viraemia, FMDV is detected within the intravascular compartment. This is indicative of active viral replication and is associated with the clinical phase of FMD (Arzt *et al.*, 2011b). Viraemia results in widespread distribution of FMDV to tissues and organs from one to two days following contact virus exposure in cattle (Arzt *et al.*, 2010). This phase is

characterised by vesiculation and erosion of epithelial cells of the mouth, feet, teats, prepuce and pillars of the rumen. Virus is also detected in the myocardium, palatine tonsils, lungs, spleen, liver, lymph nodes and vesicle-free epithelial sites such as the skin (Zhang and Alexandersen, 2004; Arzt *et al*., 2010, 2011b). However, viral proteins have not been detected in the spleen or liver and therefore FMDV replication in these organs has yet to be confirmed (Juleff *et al*., 2008). During the viraemic phase of the disease, all excretions and secretions may contain virus (Alexandersen *et al*., 2003) and is a potential source of infection for other susceptible animals.

The relationship between transmission and clinical signs was characterised in cattle by investigating individual transmission events occurring at specific time points (Charleston *et al*., 2011). The results indicated that the incubation period was four to five days. However, the infectious period in cattle is short (1.7 days) and does not start until clinical signs appear (after 0.5 days). Successful virus transmissions occurred one day after high levels of viraemia was detected. The onset of clinical signs followed and live virus was found in nasal fluid (Charleston *et al*., 2011). Notably, the conditions optimal for transmission exists only briefly in cattle.

In small ruminants such as sheep and goats, the onset of viraemia is 24 to 30 hours post intranasal inoculation and could last up to five days (Hughes *et al*., 2002). Within 12 to 48 hours, clinical signs such as fever and vesicles are visible. In sheep and goats, FMDV infection is often asymptomatic and oral lesions are not observed frequently. In contrast, clinical signs on the feet may be more frequently observed (Arzt *et al*., 2011b). The viraemic phase in pigs starts after 24 to 48 hours post contact exposure and high quantities of viral RNA are present in lingual and pedal epithelial lesions and blood (Murphy *et al*., 2010). In buffalo, the viraemic period follows one to three days after experimental infection and primary infection occurs in the pharynx and dorsal soft palate similarly as in cattle (Thomson and Bastos, 2004).

The period following viraemia, starting with the first negative blood tests, characterises post-viraemia and includes clearance of clinical signs, short-term and persistent infections, as well as other chronic long-term manifestations such as heat-intolerance (Arzt *et al*., 2011b). In most cattle, the virus is cleared within 14 days post-infection (Zhang and Alexandersen, 2004). However, during the convalescent period, FMDV may be detected in lesions at high

titres. Following clearance of virus from lesions, it may persist in other tissues for extended periods. In the absence of sterilising immunity to FMDV (*i.e.* total protection against infection), *ca.* 50% of ruminants may develop chronic asymptomatic infection and is referred to as persistence or the carrier state (Alexandersen *et al.*, 2003; Kitching, 2005). The exact mechanisms for viral persistence in hosts are still not clear (Zhang *et al.*, 2009). However, it has been proposed that the persistence of FMDV in the oropharynx, in the presence of neutralising antibody, may be due to antigenic diversification through the selection of variants from the existing quasispecies and is species- and virus strain-dependant (Vosloo *et al.*, 1996; Sutmoller *et al.*, 2003; Pacheco *et al.*, 2012).

A recent study showed that viral RNA and capsid antigen could be detected in germinal centres within the mandibular lymph nodes of cattle between 29 and 38 days post-infection, irrespective of the carrier state (Juleff *et al.*, 2008). However, it is not clear whether the virus in these germinal centres contributes to virus replication in other cells in the pharynx, nor if the virus present in the germinal centres of the lymph nodes are replicating. The importance of the pharyngeal tissues in the carrier state has recently been elucidated in a study by Pacheco *et al.* (2012). Structural and non-structural proteins were detected in these tissues, indicating replication of virus during persistence. Interestingly, a lower incidence of carrier cattle has been detected among vaccinated than in non-vaccinated animals (Sutmoller *et al.*, 2003). Characterisation of the viruses isolated from carrier animals indicated that these viruses displayed altered phenotypes. Not only were they temperature-sensitive, but they also produced different plaques sizes on cell monolayers compared to the corresponding parental viruses (Moonen and Schrijver, 2000).

Studies in other species have noted persistent infections in sheep up to nine months following experimental infection and to a lesser extent in goats (Thomson *et al.*, 2003; Parida, 2009). In contrast, FMDV infection in pigs is cleared within three to four weeks post-infection and the carrier state has not been demonstrated in pigs (Alexandersen *et al.*, 2003; Parida *et al.*, 2007). Buffalo recover from FMDV infection within three weeks (Thomson *et al.*, 2003), but become persistently infected (Vosloo *et al.*, 1996). This is a very important factor in FMD epidemiology since buffalo could be a source of infection for other species, *e.g.* cattle, as was evidenced from outbreaks of FMD in Zimbabwe and South Africa (Dawe *et al.*, 1994; Vosloo *et al.*, 2002b).

Current FMD vaccines do not prevent primary infection and persistence. Improved understanding of FMD pathogenesis will assist with targeted design of new generation vaccines and antiviral agents by preventing or impairing viral infection, generalisation, clinical disease and persistence. The latter is of particular importance as the threat of carrier animals necessitates most countries to adopt mass slaughter instead of a vaccination-to-live policy (Ward *et al*., 2009). This application of stamping out may be abrogated by a better understanding of the various stages of pathogenesis with specific reference to mitigation of viral persistence in hosts.

1.7 IMMUNE RESPONSES

1.7.1 Humoral immune response

Due to the economic importance of FMD, comprehensive studies on disease immunity and development of vaccines were initiated at the start of the 20th century. Following immunisation or infection, the immune response elicited against FMDV consists of the production of specific neutralising antibodies to the structural proteins of the virus (Pay and Hingley, 1987; Salt, 1993). Induction of high levels of neutralising antibodies is believed to be the principle method of protection for FMDV; therefore initial immunological studies have been directed mainly at the humoral immune response. However, anti-viral antibody responses may be T-cell independant or T-cell dependant subject to the need for T-cell assistance in antibody production.

The nature of the differences in immune responses following infection and vaccination is still not clear. Higher neutralising antibody titres have been observed for convalescent sera compared to post-vaccination sera in the VNT (Crowther *et al*., 1993a; Reeve *et al*., 2010; Maree *et al*., 2011b; Mahapatra *et al*., 2012), indicating possible differences in antibody responses. The first neutralising antibodies elicited against FMDV are IgM antibodies and appear three to five days following infection or vaccination, and reaches a peak between 10 to 14 days post-infection (Doel, 1996; Golde *et al*., 2008; Juleff *et al*., 2009). The initial IgM response is followed by IgA antibodies, which are detected in upper respiratory secretions early during infection (Salt, 1993; Pacheco *et al*., 2010). In contrast to inactivated FMD vaccines, which elicit little or no IgA antibodies in mucosal secretions of cattle (Archetti *et*

al., 1995), a higher amount of IgA is secreted in persistently infected animals (Salt *et al*., 1996; Parida *et al*., 2006).

In cattle, IgG1 and IgG2 antibodies appear from five days and onward, and the IgG immune response peaks at 10 to 14 days post-infection (Collen, 1994; Juleff *et al*., 2009). In pigs, IgG is detected four to seven days post-infection and the maximum levels are reached between 15 to 20 days (Francis and Black, 1983; Doel, 1996; Pacheco *et al*., 2010). Higher levels of the IgG1 isotype compared to IgG2 have been linked to protection against infection in vaccinated cattle and pigs (Mulcahy *et al*., 1990; Capozzo *et al*., 1997, 2011; Barnett *et al*., 2002; Juleff *et al*., 2009). In comparison, higher IgG2 antibody titres characterised a second round of DNA vaccine administration and is indicative of the Th1 immune response profile associated with genetic-derived vaccines (Capozzo *et al*., 2011).

The rapid humoral-specific response elicited upon FMDV infection or vaccination results in protection against re-infection with homologous virus strains (Pay and Hingley, 1987; McCullough *et al*., 1992; Salt, 1993). Conversely, Brehm *et al*. (2008) observed high neutralising antibodies and protection in cattle vaccinated with type A antigens followed by heterologous challenge. These results are most likely due to high antigen doses used during immunisation. Immunodominant neutralising epitopes have been identified for several FMDV serotypes, with the G-H loop in the 1D protein being of particular importance in the specificity of the response (Rowlands *et al*., 1983; Grubman and Baxt, 2004). When sufficient immunity is induced to FMDV, the development of clinical signs of disease is prevented (Doel, 1996; Paton *et al*., 2009). However, contrary to disease, FMDV infection may not be prevented as circulating antibodies do not prevent primary infection, *i.e.* at the site of indradermal inoculation or in the pharynx (McVicar and Sutmoller, 1976).

Convalescent ruminants may be protected against re-infection by a long-term immune response as opposed to a shorter vaccine-induced immunity (Alexandersen *et al*., 2003). This long-term protective antibody response following infection may be due to antigen retention on follicular dendritic cells within the germinal centres of mandibular lymph nodes (Juleff *et al*., 2008). Virus neutralisation in the host is achieved by phagocytosis of opsonised virus by macrophages (McCullough *et al*., 1992; Rigden *et al*., 2002). Dendritic cells are important for inducing and regulating immune responses (Golde *et al*., 2011), whereby the germinal centres play an important role in the humoral immune response in terms of B-cells during the

early stage of infection. FMDV binds to the light zone of the germinal centre cells and a virus-specific IgG response is induced. These results were confirmed by dendritic cell internalisation of FMDV, where an IgG immune response was induced following antigen presentation to lymphocytes (Harwood *et al*., 2008). Interestingly, dendritic cell internalisation was more efficient for HS-binding vaccine virus compared to non-HS-binding virus, indicating a possible advantage for antigen production as efficient interaction with dendritic cells may optimise vaccine efficiency. Furthermore, following the generation of secondary follicles, long-term protective antibody responses may be induced through efficient antibody class switching, as well as the development of memory B-cells (Juleff *et al*., 2008).

1.7.2 Cellular immune response

In recent years the contribution of cellular and innate (cytokines) immune responses, as well as their role in induction of protective B-cell responses to FMDV in the host has become evident. However, the contribution of the T-cell-mediated response to immunity and protection is still not fully understood. Despite several advances in investigating the nature of these interactions, different immune responses are observed *in vitro* and *in vivo*, depending on the host species or virus strain used, as well as whether the virus is live or inactivated (Golde *et al*., 2011; Reid *et al*., 2011). T-cell responses have, however, been observed with infection and vaccination. In the case of FMD vaccines, protection in hosts is induced prior to detection of FMDV-specific antibodies or at antibody levels that are not normally considered to be protective (Barnett *et al*., 2002; Mason *et al*., 2003b; Grubman, 2005). This suggests that cellular and innate immune responses may be involved in early protective immunity.

Specific T-cell anti-viral responses ($CD4^+$ and $CD8^+$) have been observed in cattle and pigs during FMDV infection and following vaccination (Collen and Doel, 1990; Sanz-Parra *et al.*, 1999a). FMDV infection results in a rapid reduction of major histocompatibility complex (MHC) class I molecules, which are expressed on the surface of susceptible cells. This mediates escape of the virus from the host anti-viral response since viral proteins are ineffectively presented to cytotoxic T-cells by infected cells (Sanz-Parra *et al*., 1998). Recently, Guzman *et al.* (2008) reported a cytotoxic T lymphocyte (CTL) response to FMDV and inactivated antigen. $CD8^+$ T-cells kill virus-infected cells following recognition of viral peptides bound to MHC class I molecules. In addition to antigen presentation to the MHC class I pathway, $CD4^+$ T-cells are activated by the MHC class II pathway following

vaccination and infection (Li *et al*., 2008a; Golde *et al*., 2011). Antigen presenting cells, in combination with antigen, activates $CD4^+$ class II MHC-restricted T-cells to produce a Th1 (interferon (IFN)-γ) response and Th2 (interleukin (IL)-4, IL-5 and IL-13) response (Golde *et al*., 2008). Early induction of T-helper ($CD4^+$) cells not only induces B-cell activation and antibody production, but also contributes to maintaining the appropriate microenvironment for a synergistic immune response (Collen, 1994; Sáiz *et al*., 2002; Eblé *et al*., 2006). However, recently, Juleff *et al*. (2009) determined that the complete depletion of $CD4^+$ T-cells by treatment of cattle with monoclonal antibody did not effectively control primary infection and that $CD4^+$ cells were not necessary for IgM induction or isotype class switching.

Increased cytokine protein levels have been observed following infection and vaccination, and have been noted as modulating the early innate response to limit the spread of infection (Chinsangaram *et al*., 2001; Alexandersen *et al*., 2002; Zhang *et al*., 2002; Cox *et al*., 2003). An additional advantage of cytokines is their role in development of specific immune responses towards Th1 and Th2 type immunity. Specifically, IL-6, IL-8, IL-10 and IL-12 remain at elevated levels following vaccination (Barnard *et al*., 2005; Cox *et al*., 2011), and IL-12 has been suggested to be important in induction of monocyte activity and protection (Barnett *et al*., 2002). IL-10 production has been associated with FMDV infection in pigs and may be associated with T-cell-independant antibody responses, while supressing T-cell activation during acute infection (Diaz-San Segundo *et al*., 2010). IFN-γ is induced by immunisation and appears to be related to antigen payload and is indicative of a lack of sub-clinical infection (Zhang *et al*., 2002; Barnett *et al*., 2004; Parida *et al*., 2005; Eblé *et al*., 2006). FMDV replication and spread is affected by IFN-γ production through natural killer cell and macrophage activation (Moraes *et al*., 2007; Summerfield *et al*., 2009), and has been associated with protection following vaccination (Parida *et al*., 2006). Type 1 IFN (IFN-α or IFN-β) has been linked with early vaccine-induced protection prior to the onset of adaptive immune responses (Grubman, 2005). These IFNs are also produced in dendritic cells during acute stages of infection in cattle, a process which required immune-complexes with live virus (Reid *et al*., 2011). In FMDV-infected pigs, monocytes differentiate into dendritic cells and antigen is processed through active antigen presentation. However, suppression of IFN-α leads to viral spread and increased pathogenesis during acute FMDV infection in pigs (Nfon *et al*., 2008). Dash *et al*. (2010) used porcine mucosal epithelial cell cultures to analyse FMDV interactions with mucosal epithelial cells. In addition to the cytokines IFN-α, IFN-β and RANTES mRNAs, IL-8 protein and granulocyte-macrophage colony-stimulating factor

(GM-CSF) were observed in infected epithelial cells, suggesting an important role in modulating the immune response against FMDV infection in pigs. Although cytokine production and cell proliferation has been observed in γδ T-cells (gamma delta T-cells) in vaccinated pigs, these cells do not appear to play a major role during FMDV infection in cattle (Juleff *et al*., 2009).

Compared to the high levels of neutralising antibodies in FMDV-infected animals, T-cell responses are weaker (Doel, 1996). However, the duration of such responses may extend for 42-50 days post-infection (Nfon *et al*., 2008). Both T-cells specific for structural and non-structural proteins assist B-cells in IgM to IgG class switching (Foster *et al*., 1998; Blanco *et al*., 2001; Paton *et al*., 2009). In addition, since T-cell responses are serotype cross-reactive, this is an attractive target for vaccine design (Collen and Doel, 1990; Guzman *et al*., 2010). Therefore, enhanced T-cell responses, in combination with the antibody response, could improve long-term protective antibody responses to FMD vaccines.

1.8 CONTROL OF FMD BY VACCINATION

Countries free of FMD have restrictions on the movement of animals and animal products originating from countries where FMD occurs. In the event of an outbreak of FMD in these countries, a quarantine area is immediately declared and enforced. A stamping-out policy during which infected or susceptible in-contact animals are slaughtered is favoured above vaccination as it ensures a rapid return to FMD-free status (Barteling and Vreeswijk, 1991; Grubman and Baxt, 2004). Nevertheless, conventional type-specific inactivated vaccines can be used prophylactically or as an emergency control measure.

In countries where FMD is endemic, the disease is controlled by zoo-sanitary measures, such as control of animal movement and importation of animals and animal products from affected areas, and by prophylactic vaccination (Leforban, 1999; Kitching *et al*., 2007; Rweyemamu *et al*., 2008; Paton *et al*., 2009). Annually, *ca.* two billion animals are vaccinated, with large-scale vaccination campaigns mostly in China, South America, India and the Middle East. However, not all countries are able to afford extensive vaccination campaigns or have the manpower or infrastructure to sustain such an endeavour. In addition, high herd immunity is necessary to prevent re-introduction of virus to FMD-free host species. The situation in

Africa is furthermore complicated by infrequent reporting of outbreaks and the uncertainty regarding vaccine efficacy (Paton *et al*., 2009). In southern Africa, FMD control is hampered by the large genetic (Maree *et al*., 2011b) and antigenic (Esterhuysen, 1994; Reeve *et al*., 2010; Maree *et al*., 2011b) variability, as well as regional differences in the distribution and prevalence of serotypes within the SAT types, whereby different lineages establish themselves in different geographical regions (Vosloo *et al*., 1995; Bastos *et al*., 2001) described as topotypes (Samuel and Knowles, 2001). The wildlife/domestic animal interface remains a potential source of FMD infection, as evidenced by SAT2 outbreaks in South Africa in January and April 2012 (FMD Reference Laboratory Network Report, 2012). Therefore, successful disease control necessitates separation of buffalo and livestock by fences, movement restrictions and vaccination (Hunter, 1996; Vosloo *et al*., 2002b).

1.8.1 Conventional vaccines

Vallée and co-workers discovered in the 1920s that FMDV could be inactivated by formaldehyde, but retained its antigenicity. Due to concerns regarding residual infectivity and loss of immunising ability, the use of aluminium hydroxide ($Al(OH)_3$) gel as adjuvant was subsequently investigated by Schmidt in the 1930s. However, the virus and $Al(OH)_3$ gel mix caused disease in cattle. When Waldmann (1937) first absorbed the virus to the $Al(OH)_3$ gel, followed by inactivation with formaldehyde, the formulation contained no live virus and had conserved immunopotency. $Al(OH)_3$ was found to be toxic for cells, complicating measurement of inactivation kinetics by *in vitro* methods. However, ultracentrifugation in caesium chloride separated the virus from the gel, thereby allowing titration tests to be performed successfully (Barteling and Woortmeijer, 1984). In the early 1950s, Frenkel developed a procedure whereby large-scale *in vitro* production of FMDV was possible in bovine tongue epithelium cells. The cell cultures were incubated for 20-24 hours at 37°C, clarified and filtered, absorbed onto $Al(OH)_3$ gel and inactivated with formaldehyde. An increase in the formaldehyde concentration (0.04%) in vaccines led to proper inactivation of the virus and improved safety (Barteling and Vreeswijk, 1991). This inactivated vaccine was introduced into vaccination programs in Europe during 1953 (Barteling and Vreeswijk, 1991; Brown, 1991).

1.8.1.1 Production of FMDV in cell lines

FMD vaccine production entails viral amplification to high quantities in cell culture in containment facilities. Cell culture-methods have been developed in the 1950s using primary cells such as calf kidney cells. BHK-21 cells became available in the 1960s and are considered a better host cell system for propagation of FMDV (Mowat and Chapman, 1962). Currently, FMD vaccines are still prepared using BHK cells. BHK monolayer cells used for large-scale virus production are generally considered to be better for virus propagation than BHK suspension cells (Panina, 1985). Despite this, the fact that BHK cells grow in suspension is very useful in vaccine production (Amadori *et al*., 1997). BHK suspension cultures can easily be scaled up to larger volumes, the process is less labour-intensive and it is a closed production system, thus making it less prone to contamination (Capstick *et al*., 1965; Barteling and Vreeswijk, 1991; Doel, 2003). Vaccines obtained from such virus harvests are potent immunogens and have been used successfully worldwide for FMD control and eradication.

1.8.1.2 Inactivation of FMDV

Conventional FMD vaccines are based on chemically inactivated viruses (Barteling and Vreeswijk, 1991). Virus inactivation and subsequent safety tests, which are necessary to determine complete inactivation, are two vital steps in the production process. The importance of this has been exemplified by FMD outbreaks in Europe, which have been associated with vaccines improperly inactivated using formaldehyde (Beck and Strohmaier, 1987). Due to these problems, aziridines, such as acetylthyleneimine, have been investigated as alternative inactivation agents (Brown *et al*., 1963). However, they were found to be very toxic and were soon replaced with bromoethylamine hydrobromide (BEA), which is less hazardous and is transformed into active binary ethylenimine (BEI) at pH >8 (Bahneman, 1975). Inactivation of FMDV is achieved through alkylation of the viral RNA genome and subsequent disruption of RNA-protein interactions, without adversely affecting the antigenic properties of the 146S particles (Brown, 2002). The toxicity and non-infectivity of the vaccine is assessed with an *in vivo* test, as prescribed by the European Pharmacopoeia (2006), whereby cattle are inoculated with the vaccine and observed for a minimum of seven days for evidence of toxicity or clinical signs of FMD. Another measure of safety testing is based on inactivation kinetics and the virus titre determined should reach the minimal safety level of $-5\log_{10}$ plaque forming units for a 100-litre batch (Barteling and Vreeswijk, 1991).

1.8.1.3 Purification and concentration of the FMDV antigen

The purity and concentration of the antigen is of utmost importance in the production of efficacious vaccines. Polyethylene glycol (PEG), as well as polyethylene oxide (PEO) is used successfully to precipitate FMDV on a large scale. PEO removes virus harvest proteins and concentrates the virus. Similarly, a single precipitation step with PEG removes unwanted compounds from vaccine preparations (Wagner *et al.*, 1970). The precipitated antigen is finally collected by centrifugation or filtration. Alternatively, industrial-scale chromatography or ultrafiltration systems can also be used to purify the antigen (Barteling and Vreeswijk, 1991).

1.8.1.4 Formulation of the antigen

The serotype-specific immunity induced by FMDV infection necessitates careful selection of a vaccine strain (Paton *et al.*, 2005, 2009). Antigenic variation in viruses may lead to a vaccine that does not provide full protection, as it might not be of the same serotypic subtype as the field strain. Therefore, formulation of the vaccine depends on the virus strain needed and animal species to be vaccinated. The appropriate antigen is removed from liquid nitrogen and diluted with buffers before blending it with an adjuvant, which serves as an immune stimulant. Three such adjuvants are used for FMD vaccines, namely $Al(OH)_3$ gel, saponin and oil emulsions (Barteling and Vreeswijk, 1991). In addition, the antigen payload is influenced by the potency level required for the vaccine, which may vary between 1 to 10 µg or more of 146S antigen. The relationship between the 146S antigen and vaccine potency is not a linear function and therefore complicates the formulation of the final product (Rweyemamu and Ouldridge, 1982; Barnett *et al.*, 2003).

Aqueous vaccines consist of $Al(OH)_3$ gel, supplemented with saponin, and are administered subcutaneously. These vaccines induce high levels of neutralising antibody, protecting animals for up to nine months (Frenkel *et al.*, 1982). The $Al(OH)_3$ vaccines are widely used for cattle, but are less effective in pigs. Therefore, a formulation has been developed for pigs where the antigen is emulsified in mineral oil such as Montanide ISA 206 (Iyer *et al.*, 2001; Rigden *et al.*, 2003). The advantage of oil adjuvant vaccines is longer-lasting protection and consequently, a possible reduction in the frequency of vaccination (McKercher and Graves, 1977; Barnett and Carabin, 2002; Parida *et al.*, 2007; Cloete *et al.*, 2008). To establish basic immunity, inactivated FMD vaccines should be administered with a primary dose and a

secondary dose two to eight weeks later. The immune response to inactivated vaccines is relatively short-lived and necessitates booster immunisation every four to twelve months (Hunter, 1998; Lubroth *et al*., 2007; Paton *et al*., 2009).

1.8.1.5 Assessment of vaccine potency

The virus neutralisation test (VNT) is used for the indirect assessment of vaccine potency of the final product (Rweyemamu, 1984). Potency testing consists of the vaccination of three groups of five cattle, with a different dose of vaccine for each group. Two control animals are also included. Three weeks post-vaccination, the animals are challenged with 10 000 ID_{50} of a homologous virulent strain by the intradermolingual route, followed by daily inspection for a period of eight days or more to observe signs of disease and to collect samples for laboratory tests. The 50% protective dose (PD_{50}) is calculated by a method such as that described by Kärber (1931). The vaccine potency is expressed as the number at which 50% of the cattle used for the challenge experiments were protected, and the minimum potency required by international standards is 3 PD_{50} (Brown, 1991; Doel, 2003; European Pharmacopoeia, 2006; De Clercq *et al*., 2008b; OIE Manual of Standards, 2009). Several groups have investigated the current potency tests relating PD_{50} to protection (Sutmoller, 1986; Vianna Filho *et al*., 1993; Goris *et al*., 2008a). As a means to decrease the variability in the results of these tests, suggestions have been made such as increasing the number of animals used in the tests (Goris *et al*., 2007) or adapting potency trial regimes to test two groups of six animals immunised with either a one-third or a one-sixth dose of the vaccine (Reeve *et al*., 2011).

1.8.2 Emergency and high potency vaccines

FMD-free countries, with a non-vaccination policy, have moved towards strategic reserves of FMD vaccines that could be used if necessary in the event of an outbreak. Such reserves or vaccine banks are the North American Vaccine Bank, the European Union Vaccine Bank and the International Vaccine Bank (Barnett *et al*., 2001; Doel, 2003; Lombard and Füssel, 2007). The formulated vaccines are replaced every 12 to 18 months or stored as concentrated antigen over liquid nitrogen that can be rapidly formulated into a vaccine in the event of an outbreak. Emergency vaccines contain antigen doses of ≥6 PD_{50} to ensure both rapid protective immunity and wider antigenic coverage within FMDV serotypes (Salt *et al*., 1998; Barnett *et al*., 2002; Cox *et al*., 2003, 2005, 2006, 2007; Rigden *et al*., 2003; Parida *et al*., 2008). Such

vaccines limit virus replication in the oropharynx, thereby limiting subsequent transmission of the disease to other susceptible animals (Doel *et al*., 1994; Salt *et al*., 1998; Barnett *et al*., 2004; Golde *et al*., 2005; Parida *et al*., 2007). The duration of the immune response induced by emergency vaccines is 6 months for sheep and pigs (Cox *et al*., 2003). Ideally, FMD vaccine antigens should match the outbreak strain closely, but existing vaccine strains may not be closely related to field strains. However, high potency vaccines could be used when no closely related vaccine antigen is available. In a study conducted by Brehm *et al*. (2008), animals vaccinated with 6 PD_{50} were protected against heterologous challenge. Although animals with low VNT titres were protected, the probability of protection decreased with decreasing vaccine doses.

1.9 NEW GENERATION FMD VACCINES

In the past, outbreaks of FMD have been associated with the use of improperly inactivated virus in conventional vaccines. The introduction of recombinant DNA technology has led to a new approach in the development of FMD vaccines based on the reconstruction of viruses and their immunogens. Increased understanding of the FMDV structure at the molecular level has led to the identification of regions on the virus particle that elicits a protective immune response. Alternatives to current inactivated FMD vaccines, which target these antigenic determinants in the absence of the entire infectious virus particle, have been investigated. These alternative approaches are expected to result in the elimination of the production of large quantities of infectious virus and the risks involved in the production of improperly inactivated vaccines. Such alternative vaccines would, however, have to be innocuous, as good or better than inactivated virus vaccines, have competitive prices and be attractive to the market (Brown, 1992; Zhang *et al*., 2011).

1.9.1 Protein and peptide vaccines

Cloned gene products can be formulated into subunit vaccines, where the coding regions of critical antigenic determinants are isolated, cloned and expressed in an appropriate host system. The 1D protein of the type A_{12} FMDV has been expressed as a fusion protein in *Escherichia coli* and the purified protein induced a protective immune response in cattle and pigs upon challenge with the homologous virus (Kleid *et al*., 1981). However, the level of immunogenicity obtained by 1D alone was much lower than that obtained for virus particles

(Brown, 1992). This may have been due to inadequate folding of the protein in solution that limits presentation of the expressed antigenic sites to the host immune system. In addition, these results may be suggestive of other antigenic regions on other parts of the capsid coding region being required for immunity.

Several peptide vaccines using segments of the 1D protein have been developed based on information regarding the antigenic regions in the viral proteins (Strohmaier *et al*., 1982; Meloen *et al*., 1995). Advantages of such vaccines would be a product that is chemically defined, stable and antigenic variation can be detected on a chemical basis (Brown, 1992, 2003). Chemical peptide synthesis has thus been used to synthesise peptides corresponding to the highly variable G-H loop (amino acid residues 138-160) in 1D (Pfaff *et al*., 1982), or in combination with the C terminus (residues 200-213). These synthetic peptide vaccines induced neutralising antibodies in mice and guinea pigs (Bachrach, 1985), and provided partial protection against homologous FMDV challenge in cattle and pigs (DiMarchi *et al*., 1986). However, the immunogenicity was much lower than that elicited by conventional vaccines. Presenting the peptide on hepatitis B core-like particles did provide promising results, since a smaller amount of peptide was necessary for protection (Clarke *et al*., 1987b).

Results obtained from the above studies indicated that the immunogenicity obtained upon vaccination of animals with peptide vaccines was lower than with conventional inactivated vaccines and the protection in natural hosts, such as cattle and pigs, was less than in guinea pigs. The reduced level of protection with peptide vaccines may be due to a lack of T-cell epitopes on these peptides, which is not efficiently recognised by MHC molecules of the host species (Rodriquez *et al*., 1994). In addition, escape mutants were possibly present that may have led to a lack of protection of cattle, suggesting rapid generation and selection of FMDV antigenic variants *in vivo* (Tobago *et al*., 1997). This could be due to the quasispecies nature of FMDV (Domingo *et al*., 1990) whereby antigenic variants arise that could cause disease in vaccinated animals (Krebs *et al*., 1993). Moreover, the epitopes on 1D, or parts thereof, are not the only neutralising epitopes on the virion and are not recognised equally well in different host species (Mateu *et al*., 1995). To be effective, an epitope must assume the same conformation as that on the intact viral particle. As seen from the above, a single epitope may not be sufficiently immunogenic to stimulate protective immune responses to FMDV. In addition, peptides mimic the linear conformation of epitopes, whereas many FMDV epitopes are conformational (Mateu, 1995).

Rather than using peptides, greater emphasis is being placed on the development of recombinant FMDV epitope vaccines. Early reports have indicated that two distinctive B-cell epitopes in the 1D capsid protein elicit protective neutralising antibody responses in cattle (Bittle *et al.*, 1982), and a T-cell epitope was subsequently shown to aid the B-cell epitopes to induce FMDV-neutralising antibodies (Collen *et al.*, 1991). Various reports have since highlighted the construction and production of recombinant proteins consisting of multiple copies of B-cell epitopes and T-cell epitopes of 1D (Cubillos *et al.*, 2008; Fang *et al.*, 2012), or the G-H loop domain of 1D and an optimised artificial promiscuous T helper site for broad immunogenicity (Wang *et al.*, 2002). The results generally showed that the recombinant epitope-based vaccines induced the production of neutralising antibodies and could either prevent the development of significant clinical signs in immunised pigs or protect immunised pigs from challenge with the homologous virus. Attempts have been made to strengthen the immunogenicity of these epitopes by generating fusion proteins of the B-cell and T-cell epitopes with porcine IFN-α (Du *et al.*, 2008) or host-self immunoglobulin molecules (IgG) (Shao *et al.*, 2011). With regards to the latter, a multiple-epitope vaccine that consisted of three copies each of residues 141 to 160 and 200 to 213 of 1D of type O fused with the pig IgG heavy-chain constant region was constructed. The recombinant epitope vaccine induced immune responses for at least 6 months in immunised pigs and protected the pigs against homologous virus challenge (Shao *et al*., 2011). In addition to the 1D capsid protein, T-cell epitopes have also been identified on other FMDV structural and non-structural proteins (Blanco *et al*., 2001; Garcia-Briones *et al*., 2004; Moraes *et al*., 2011). Consequently, Wang *et al.* (2007) reported that type Asia-1 recombinant epitope vaccines, consisting of amino acids 133 to 163 of 1D and amino acids 1 to 33 of 1B, both induced lymphoproliferation in guinea pigs. Although only the 1D fragment was capable of inducing neutralising antibodies, a higher neutralising antibody response was nevertheless obtained when guinea pigs were immunised with a mixture of the 1B and 1D epitope vaccines.

Antigen production in transgenic plants has received some attention as an alternative method of expressing recombinant proteins. The 1D protein of type O_1 Campos FMDV has been expressed successfully in plants such as alfalfa (Wigdorowitz *et al*., 1999b) and *Nicotiana benthamiana* (Wigdorowitz *et al*., 1999a). In an attempt to improve heterologous protein levels expressed in plants, as well as easier selection of transgenic plants presenting the highest levels of protein expression, Dus Santos *et al.* (2002) expressed the major antigenic epitope (G-H loop in 1D) fused to a reporter gene. Both transgenic plant-derived vaccines

(comprising the G-H loop region and complete 1D) were highly immunogenic in mice, which were completely protected upon challenge with the homologous virulent virus. Subsequent to these reports, the production of transgenic alfalfa plants expressing the P1 polyprotein and 3C protease was reported (Dus Santos *et al.*, 2005). Although processing of the viral polyprotein into empty capsids could not be demonstrated, the expressed products were immunogenic in mice and provided complete protection against challenge with virulent virus. Expression of the P1-2A and 3C protease coding regions in transgenic tomato plants (Pan *et al.*, 2008) and the P1 coding region in transgenic rice (Wang *et al.*, 2012) has also been reported. Although the expressed antigens were immunogenic, they afforded limited protection upon virus challenge in guinea pigs and mice, respectively. In an alternative approach, the region spanning the G-H loop in 1D was inserted into the hepatitis virus core antigen gene (HBcAg) and the recombinant protein was expressed in *Nicotiana tabacum* (Huang *et al.*, 2005). Not only did mice immunised with a soluble crude extract of transgenic tobacco leaves produce antibody responses to both HBcAg and FMDV 1D, but the mice were also protected against virulent virus challenge. Since large quantities of antigen are necessary to elicit immune responses, a major disadvantage of this approach, which is of great relevance to vaccine production, is the low expression levels obtained in the transgenic plants. Moreover, few of the studies reporting on transgenic plant-derived vaccines tested its efficacy in host species such as cattle and pigs and therefore their true potential is not yet known.

The ability to assemble subviral particles (Clarke and Sanger, 1988) has significance in vaccine production, as these empty capsids lack the viral RNA and are indistinguishable from the complete virus by monoclonal antibody detection (Ryan *et al.*, 1989). The empty virus capsid particles are therefore non-infectious, but retain most of the immunogenic and antigenic properties of viral particles (Rowlands *et al.*, 1975; Grubman *et al.*, 1993). Construction of cDNA cassettes containing the capsid precursor (P1), together with the Leader and 3C coding regions of type O_1 Kaufbeuren and type A_{10} viruses, followed by expression in a recombinant baculovirus system yielded limited amounts of empty viral capsids; possibly because the Leader protease is toxic to the insect cells (Rooisien *et al.*, 1990). Similarly, expression of the P1, partial P2 and 3C coding regions of the type A_{12} virus in *E. coli* resulted in efficient synthesis and processing of the structural protein precursor. Although empty capsids were formed, the capsid assembly was found to be inefficient (Grubman *et al.*, 1993). Expression of the P1 region of type A_{12} virus by a baculovirus recombinant protected pigs from clinical disease following challenge with a homologous

virus, whereas the empty capsid extracts from *E. coli* failed to protect the animals (Grubman *et al*., 1993). This was probably due to the toxic effect of the 3C protease for the cells and the fact that no myristoylation of the 1A protein occured in *E. coli*, which is required for capsid assembly and stability (Lewis *et al*., 1991).

Recently, the synthesis of empty viral capsids by means of the baculovirus expression system has been revisited. Based on reports indicating that expression levels in silkworms are 50-1000 times higher compared to that in insect cells, Li *et al.* (2008c) made use of a silkworm-baculovirus expression system to express the P1-2A and 3C coding regions of a type Asia-1 strain. A single vaccination with the hemolymph from infected silkworms induced protection in 80% of cattle when challenged with virulent homologous virus. Notably, the potency of the vaccine was 6.34 PD_{50} per dose, which is higher than the 3 PD_{50} per dose recommended by the OIE (OIE Manual of Standards, 2009). The suitability of pseudotype baculoviruses, which display the glycoprotein of vesicular stomatitis virus (VSV-G) on the envelope in order to extend its host range and make use of mammalian promoters to drive expression in different mammalian cells, have also been investigated (Cao *et al.*, 2011). Two recombinant pseudotype baculoviruses expressing the P1-2A and 3C coding regions, with or without a T-cell epitope fragment, were constructed and expressed in BHK-21 cells. The T-cell epitope fragment comprised of two universal T-cell epitopes and several conserved T-cell epitopes present on the FMDV structural and non-structural proteins. Intramuscular inoculation of mice with the respective recombinant baculoviruses induced production of FMDV-specific neutralising antibodies and IFN-γ. Although the recombinant pseudotype baculovirus with T-cell epitopes had better immunogenecity than the recombinant without T-cell epitopes, an inactivated vaccine produced the highest titer of neutralising antibodies and thus indicated that the amount of capsid protein expressed by the pseudotype baculoviruses did not reach that present in the inactivated vaccine (Cao *et al.*, 2011). Although vaccines derived from recombinant particles may have advantages such as safety, the ability to differentiate between vaccinated and infected animals and engineering of cDNA constructs containing conformational epitopes of different viruses, this technology has not been applied in the field since large quantities of properly processed viral proteins have not yet been produced.

1.9.2 Genetically engineered attenuated strains

When the development of attenuated vaccines for FMDV was first attempted, some of the challenges faced were the possibility of outbreaks caused by the virus reverting back to its virulent state (Coa *et al*., 1991; Mason *et al*., 1997; Grubman and Baxt, 2004) and the possibility that attenuated strains might be virulent in a different host (Sagedahl *et al*., 1987). Since classical attenuated strains have the potential for variation and adaptation, new attenuated virus vaccines have been designed by genetically engineering type A_{12} virus genome-length cDNA clones (Chinsangaram *et al*., 1998). Modification of these clones resulted in recombinant viruses of which the RGD sequence (McKenna *et al*., 1995) or Leader coding region (Mason *et al*., 1997) was deleted. Protection was observed in cattle vaccinated with RGD-deleted viruses, without development of clinical symptoms, upon challenge with homologous virus (McKenna *et al*., 1995). In contrast, partial protection of cattle was observed upon challenge with homologous recombinant viruses lacking the Leader coding region. Two of the three animals did not develop lesions, but showed signs of FMDV infection post-challenge (Mason *et al*., 1997). More recently, it was reported that humoral and cellular immune responses were elicited in pigs inoculated with self-replicating RNA transcripts derived from an infectious clone with a deletion in the 3' non-coding region of the genome (Rodríguez Pulido *et al*., 2009). This deletion contributed to attenuation of the type O virus and showed promise as an alternative FMD vaccine through RNA-based immunisation. Despite promising results, the wide host range of FMDV, the possibility of the disease spreading to non-vaccinated livestock, the high potential for variation and difficulties in determining whether the animal is vaccinated or infected, are all complications that may have led to this approach not receiving more attention. However, identification of new virulence determinants in the FMDV genome, together with detailed knowledge of the virus-host interaction and mechanisms of pathogenesis, may address some of these concerns and contribute to making informed decisions on where to introduce mutations or deletions in order to attenuate the virus.

1.9.3 DNA vaccines

Vaccination with plasmid DNA elicits humoral and cellular immune responses, and allows for modulation of the induced response by co-expression of FMDV immunogens and cytokines (Lai and Bennet, 1998; Zhang *et al*., 2011). Immune responses to DNA vaccines can also be enhanced through addition of synthetic substances such as oligodeoxynucleotides containing

unmethylated CpG dinucleotides as an additional adjuvant in combination with Montanide ISA 206 (Ren *et al*., 2011). DNA vaccines consisting of FMDV attenuated genome-length cDNA clones with deletions in the Leader or RGD regions have been reported to provide protection in pigs (Ward *et al*., 1997; Beard *et al*., 1999). Vaccination of mice or pigs with DNA expressing empty viral capsids has also been reported to induce a neutralising antibody response (Chinsangaram *et al*., 1998; Cedillo-Barrón *et al*., 2001). DNA vaccines have been constructed to contain the G-H loop and C terminus of the 1D protein, as well as host-self IgG. These DNA plasmids elicited immune responses in both mice and pigs, and protected pigs against challenge with FMDV by T-cell proliferation and induction of neutralising antibodies (Wong *et al*., 2000). The immune responses elicited by DNA vaccines have been shown to be enhanced with the co-administration of the cytokine IL-2 (Wong *et al*., 2002; Zhang *et al*., 2008). Co-inoculation of a 1D DNA vaccine with constructs expressing either IL-6 or Tumour necrosis factor-alpha (TNF-α) was also shown to significantly enhance the humoral and cellular immune responses in immunised mice (Su *et al*., 2008). Studies aimed at further improving the immune responses induced by FMD DNA vaccines have focused on DNA vaccines encoding B-cell and T-cell epitopes. Results from these studies have indicated that these vaccines conferred protection in mice (Borrego *et al*., 2006), but did not protect against FMDV challenge in pigs (Ganges *et al*., 2011). However, the induction of humoral and cellular immune responses was nevertheless confirmed in both cases. These approaches have been refined by generating DNA vaccines encoding FMDV B-cell and T-cell epitopes fused to the VSV glycoprotein (Capozzo *et al*., 2011) or the single-chain variable fragment of a 1F12 mouse monoclonal antibody that recognises Class II pig leukocyte antigens (Borrego *et al*., 2011). It was shown that the chimeric VSV-G DNA vaccine induced neutralising antibodies in calves and was able to neutralise FMDV infectivity *in vitro*, whereas the second approach provided full protection in two of four DNA-immunised pigs upon viral challenge.

In addition to the use of adjuvants highlighted above, it has been reported that a prime-boost strategy can also lead to a significant improvement in DNA vaccine efficacy. The strategy entailed priming pigs with a DNA vaccine that comprised of the type O_1 Kaufbeuren P1-2A and 3C coding regions together with the 3D polymerase for added T-cell stimulation, followed by a boost with chemically inactivated FMDV antigen and recombinant 3D protein (Li *et al*., 2008b). This immunisation strategy not only induced very high titre FMDV-specific antibody and enhanced cellular immune responses in the vaccinates, but the immunised pigs were also fully protected from heterologous virus challenge. Interestingly, it

was also demonstrated by VNT and ELISA tests that the immunised pigs generated antibodies that were cross-protective against types A, C and Asia-1, albeit that the mechanism responsible for the observed cross-reactivity is unclear (Li *et al.*, 2008b). Using the identical DNA vaccine construct as above, Niborski *et al.* (2006) reported that the DNA vaccine did not induce any detectable immunity against FMDV in sheep. However, coating of poly(D,L-lactide-*co*-glycolide) microparticles (PLG) with the plasmid DNA or dilution of the plasmid DNA in lipofectine resulted in the triggering of divergent immune responses in vaccinated sheep. In contrast to the DNA vaccine/PLG formulation, which stimulated a T-cell response and elicited neutralising antibody titres, the DNA vaccine/lipofectine formulation generated higher antibody titres but no significant T-cell response. In addition to pigs and sheep, a DNA vaccination regime that included a protein boost and electroporation was recently reported to protect cattle against FMD (Fowler *et al.*, 2012). A DNA vaccine containing the P1 coding region together with the non-structural proteins 2A, 3C and 3D of type O_1 Kaufbeuren was used in combination with an adjuvant plasmid expressing bovine GM-CSF. DNA vaccinations were administered intramuscularly with the use of electroporation and the vaccinated cattle received a protein boost of 146S FMDV antigen and non-structural protein 3D. This vaccination regime provided clinical protection in 75% of the cattle following FMDV challenge. Despite promising results from the research on DNA vaccines, it has not replaced traditional inactivated FMD vaccines because of concerns about safety, difficulty of administration and it being usually poorly efficient in target host species.

1.9.4 Vector-associated vaccines

Alternative vaccines have been investigated whereby sections of the FMDV coding regions have been included as part of a live viral vector. For example, a hybrid virus containing the genetic material coding for the amino acid region 138-160 in the 1D protein of FMDV was incorporated onto the genome of the bovine rhinotracheitis virus. The FMDV G-H loop region in the hybrid virus elicited protective levels of neutralising antibodies in calves (Kit *et al.*, 1991). In addition, a chimeric poliovirus containing 1D epitopes induced neutralising antibody responses in guinea pigs (Kitson *et al.*, 1991). Expression of capsid proteins, non-structural proteins or both in vaccinia virus (Sanz-Parra *et al.*, 1998; Berinstein *et al.*, 2000), fowlpox virus (Zheng *et al.*, 2006) or pseudorabies virus (Li *et al.*, 2008a; Yao *et al.*, 2008) provided partial protection following vaccination of pigs and guinea pigs. However, recently, chimeric bamboo mosaic virus (BaMV) virions that contain 1D epitopes not only induced

humoral and cell-mediated immune responses but also provided full protection against FMDV in pigs (Yang *et al.*, 2007). Disadvantages associated with these types of vaccines are that multiple doses may be required and expression levels of the appropriate proteins are typically low.

The most promising platform for vector-associated vaccines relies on the use of adenovirus vectors (Grubman *et al.*, 2010; Rodriguez and Gay, 2011). Replication-defective adenovirus vectors are able to infect cells of several animal species, including cattle and pigs (Prevec *et al.*, 1989), and effective immunisation of animals has been achieved with such vectors expressing proteins from other pathogens (Grubman and Mason, 2002). This strategy avoids the problem of possible introduction of mutations in the viral capsid during passage in cells for conventional vaccine production. Since adenovirus serotype 5 infects the upper respiratory tract, the recombinant adenovirus allows for the expression and assembly of FMDV empty capsids in the respiratory tract, which is the region of initial infection by FMDV (Paton and Taylor, 2011). An additional advantage of direct immunisation with a recombinant adenovirus vector is that the FMDV capsids are expressed and assembled in the animal, thus potentially inducing both humoral and cellular immunity (Rodriguez and Grubman, 2009). Replication-defective human adenovirus serotype 5 (Ad5) vectors containing FMDV type A capsids and either the wild-type or inactive $3C^{pro}$ have been engineered (Mayr *et al.*, 1999, 2001; Moraes *et al.*, 2002). Inoculation of pigs with the recombinant vector containing the wild-type $3C^{pro}$ led to the production of a neutralising antibody response upon challenge with homologous virus, but not when a recombinant vector containing the inactive $3C^{pro}$ was used. When a replication-competent Ad5 vector, containing the capsid coding region of type C_1 Oberbayern was used to inoculate cattle and pigs, no protection was observed (Sanz-Parra *et al.*, 1999a, 1999b). This emphasises the necessity of proper processing of the P1 protein precursor by the $3C^{pro}$ for the correct presentation of antigenic sites on the virus particle.

An alternative use for the Ad5 vector has also been investigated whereby rapid protection is stimulated before the start of vaccine-induced adaptive immune responses (Grubman *et al.*, 2005). Since type I alpha/beta-interferon (IFN-α/β) is expressed and secreted in virus-infected cells and FMDV is highly sensitive to IFN-α/β (Chinsangaram *et al.*, 1999), the usefulness of type I alpha/beta-interferon as an anti-viral agent has been investigated. IFN-

α/β that are continuously expressed by a recombinant, replication-defective Ad5 is not rapidly cleared from the body, as under normal circumstances. Pigs inoculated with such a replication-defective Ad5 expressing IFN-α were protected 24 hours post-challenge (Chinsangaram *et al*., 2003) and this protection lasted up to five days (Moraes *et al*., 2003). In comparison, conventional inactivated vaccines take a week to induce a protective immune response. Although cattle inoculated with a replication-defective Ad5 vector expressing IFN-α were only partially protected upon challenge with FMDV type A_{24}, one animal developed no clinical signs of disease (Wu *et al*., 2003). In a subsequent study, Pacheco *et al*. (2005) reported that a single dose of 5×10^9 p.f.u./animal of an Ad5-vectored FMD subunit vaccine was sufficient to induce protective immune responses in cattle. In addition to the fast immune response elicited by these IFN-expressing Ad5 vectors, the vaccine may be used immediately during a FMD outbreak to facilitate rapid onset of immunity in susceptible animals or as prophylactic anti-viral treatment (Grubman *et al*., 2010). Since this method should provide protection against all FMDV serotypes, it would thus not have to be compared with the outbreak strain and thereby reduce the time needed before the vaccine is released and distributed. In addition, when applied by FMD-free countries to control an outbreak, the large-scale slaughtering of animals could be prevented. Despite the great promise shown by recombinant Ad5-FMD vaccines, a number of limitations remain that are currently being addressed. These include the addition in the vector of FMDV non-structural protein coding regions as a means to enhance empty capsid assembly (Pena *et al*., 2008), as well as the use of synthetic adjuvants, alternative routes of delivery, tissue targeting and Ad5-FMD vaccines with enhanced capsid stability (Rodriguez and Grubman, 2009).

1.9.5 Marker vaccines

Despite the availability of effective conventional FMD vaccines, they cannot be used in FMD-free countries as it may interfere with disease surveillance based on serological testing and may result in the loss of a country's disease-free status and hence, compromise international trade (Paton *et al*., 2009). The ability to selectively delete genes from a pathogen has allowed the development of marker vaccines that, combined with suitable diagnostic assays, allow differentiating infected from vaccinated animals (DIVA) by differentiation of antibody responses induced by the vaccine (no antibodies generated to deleted genes) from those induced during infection with the wild-type virus (Grubman, 2005; Root-Bernstein, 2005; Vannier *et al*., 2007). Such DIVA vaccines and their companion

diagnostic tests are available or in development for FMD. For example, the commercial vaccine Cedivac-FMD does not induce antibodies to the FMDV non-structural proteins in vaccinated animals. This therefore provides the basis whereby vaccinated and infected animals can be differentiated from each other (Espinoza *et al*., 2004), and the Ceditest FMDV®-NS ELISA can be used for this purpose (Gilles Chénard *et al*., 2008). However, assays relying on the detection of antibodies to non-structural proteins have associated problems since vaccine preparations may contain traces of non-structural proteins. In such instances, the assay may therefore detect a response to a poorly purified vaccine rather than infection in a vaccinated animal. In an alternative approach, chimeric FMDV containing 1D G-H loop substitutions were evaluated as potential marker vaccines and it was shown that the chimeric FMD vaccines protected cattle against challenge (Fowler *et al*., 2008). Moreover, these findings also highlighted the importance of epitopes outside the G-H loop for protective immunity in host species. Subsequently, the marker vaccine potential of a naturally selected FMD vaccine virus lacking a large proportion of the 1D G-H loop, including the RGD motif (Fowler *et al*., 2010), was investigated. Not only were cattle vaccinated with this marker vaccine fully protected following virus challenge, but the marker potential of the vaccine was confirmed with an indirect ELISA (Fowler *et al*., 2011). This assay is based on the principle that vaccinated-uninfected animals would lack antibodies directed at the G-H loop, whereas vaccinated-infected animals would have circulating G-H loop antibodies.

1.10 VACCINE MATCHING

Despite tremendous research efforts aimed at improving FMD vaccines, as highlighted in the sections above, FMD viruses are highly variable and it is thus difficult to provide adequate protection by vaccination as immunity will only be conferred against related field strains (Mattion *et al*., 2004). Consequently, several vaccine antigens may be required to ensure protection following vaccination (Doel, 2003). Improved methods for predicting viral cross-protection could assist with evaluations regarding the appropriateness of current vaccines (vaccine match) and the extent of coverage afforded by new vaccine strains. In response to these needs, various approaches have recently been reported, including antigenic cartography using multidimensional scaling and prediction of cross-reactivity by applying linear mixed-effect models in combination with structural data.

1.10.1 Classical techniques

Sequence analysis and phylogenetic comparisons provide important initial information about the serotype and topotype of a particular FMD outbreak isolate. Molecular epidemiological studies of the SAT type viruses have focused almost exclusively on the 1D coding region to determine the genetic relatedness of isolates (Vosloo *et al*., 1992, 1995, 1996; Bastos *et al*., 2000, 2001, 2003a, 2003b; Samuel and Knowles, 2001). However, with the advent of modern sequencing techniques the complete P1 sequence is readily obtained. Using all three the capsid protein coding regions the confidence or bootstrap level is increased compared to 1D analysis alone (Carillo *et al*., 2005). During the last decade, FMD-endemic countries have begun to sequence the P1 region of virus isolates causing disease outbreaks and several publications have highlighted the value of this approach in producing more comprehensive sequence analyses compared to 1D only (Mohapatra *et al*., 2004; Jangra *et al*., 2005; Mingqiu *et al*., 2008; Maree *et al*., 2011b).

Phylogenetic analysis has proven useful in broadly classifying FMDV isolates into major groupings or genotypes and in tracing the origin of an outbreak. However, it does not provide sufficient resolution regarding the antigenic properties of the virus to know whether it is antigenically closely related to the vaccine antigen. Antigenic differences between strains determine whether the immunity induced by one strain is effective against another (Doel, 2003). To accurately determine the potency of a vaccine, dose reduction trials are conducted in animals (De Clercq *et al*., 2008a). However, this is not always feasible due to the extensive resources required to conduct such trials. Such studies are expensive, require ethical approval and are time-consuming (preparation of vaccines and experimental trial). In addition, statistical analysis has revealed that several repetitions of a potency trial may give varying results (Goris *et al*., 2007). In comparison, serological tests allow for immediate and extensive screening of field isolates compared to reference strains representative of viruses used as vaccine antigens (De Clercq *et al*., 2008a; Goris *et al*., 2008b). The VNT is widely accepted as the preferred serological test for evaluation of cross-reactivity of field isolates with reference sera representative of existing vaccines (Barnett *et al*., 2003; Mattion *et al*., 2004; Robiolo *et al*., 2010; Maradei *et al*., 2011). Despite its usefulness, factors that limit the quantitative analyses of VNT results are reliability of the assays, irregularities in the data such as higher heterologous than homologous titres, as well as variation between individual sera raised from the same virus inoculum or antigen batch (Smith *et al*., 2004; Hanlon *et al*., 2005).

1.10.2 Antigenic cartography using multi-dimensional scaling

Antigenic cartography is a theory and computational method that allows for quantitative analyses and visualisation of data determined by serological assays (Smith *et al*., 2004; de Jong *et al*., 2007). Antigenic distances are measured by the positioning of each virus by multiple sera, and the sera are positioned by the distances to the viruses only and expressed as antigenic units. The distance between an antigen and antiserum in the map directly corresponds to the serological test measurement determined in the VNT (Smith *et al*., 2004; Horton *et al*., 2010). Antigenic maps to characterise the antigenicity of pathogens have been applied for influenza A (H1N1), A (H3N2) and B influenza viruses (de Jong *et al*., 2007; Russell *et al*., 2008; Garten *et al*., 2009; Barr *et al*., 2010). For influenza A (H3N2) virus the strains grouped in clusters that were in chronological order. It was shown that a single amino acid substitution resulted in change in antigenicity, thus moving the virus into a different genetic cluster (Smith *et al*., 2004). Sequence information, together with neutralisation titre data, has also been applied in combination to predict antigenic distances between lyssaviruses (Horton *et al*., 2010). As antigenic cartography could be applied to other viruses for which serological data is available, it may prove a valuable approach to test predictions of the antigenic effects of amino acid substitutions for FMDV and to evaluate vaccine efficacy for FMD control. The combination of antigenic maps and epidemiological data could furthermore be used to determine to which extent field isolates may escape host immunity by determining antigenic distances between vaccine and circulating strains, as has been done in the influenza field (Smith *et al.*, 2006).

1.10.3 Linear mixed-effects models

To guide vaccine strain selection, continued cross-reactivity assessment of sera from FMD vaccine strains and field isolates is required (Paton *et al*., 2005). Towards this end, an *in silico* tool was recently developed that not only complements but could also potentially replace conventional vaccine strain selection methods. This computational tool, based on a linear mixed-effects model (Yates, 1934), combines sequence information, virus neutralising titres and structural data to relate cross-reactivity to sequence variation, and to identify and quantify the significance of predicted epitopes (Reeve *et al*., 2010). Interestingly, significant fixed effects observed were the reference and field viruses and their interaction (virus neutralisation titres), whereas serotype or sera prepared by vaccination or infection were not significant. These findings suggest that convalescent antisera are acceptable as test reagents,

albeit that there is some controversy regarding this approach since the OIE Manual of Standards (2009) suggests the use of antisera from vaccinated animals.

By making use of the above *in silico* tool, residues were identified that affected r_1-values (*i.e.* led to loss of cross-reactivity). For 1D of SAT1, these were associated with the G-H and H-I loops (residues 132-174) and in 1C the H-I loop (residues 191-202), and for the 1D of SAT2 the C terminus (residues 200-224) and H-I loop (residue 178), and in 1B the B-C loop (residues 70-82) (Reeve *et al*., 2010). Notably, these residues are exposed on the surface of the virion and are located in highly variable regions that correspond with previously identified epitopes. Cross-reactivity (r_1-value) prediction for new vaccine candidates using sequence data (predictive models) identified 98% matches, compared to 87% determined by serology for SAT1 and 77%, compared to 66% for SAT2 (Reeve *et al*., 2010). Predictions and serological measurements were less accurate for SAT2 compared to SAT1, possibly due to greater variation in the serology data of SAT2. The two most significant predictors of loss of cross-reactivity were residue 138 on the E-F loop of 1C and residue 198 on the H-I loop of 1B. The expected loss of cross-reactivity in these instances was 25% and 16%, respectively (Reeve *et al*., 2010). The value of this computational approach lies in its applicability across all FMDV serotypes and it may thus be used to identify viruses with a broad cross-protection that can subsequently be incorporated into FMD vaccines.

1.11 VACCINATION IN SOUTH AFRICA: AIMS OF THIS INVESTIGATION

The efficacy of FMD control strategies is heavily dependent on accurate and detailed knowledge of the epidemiology of the disease. The seven FMDV serotypes cause a clinically indistinguishable disease in infected animals, but they are serologically distinct from each other. FMD in southern Africa differs significantly from the disease in other parts of the world, such as South America, the Middle East and Asia, as the SAT serotypes are genetically and antigenically distinct and the epidemiology uniquely revolves around wildlife. In South Africa, FMD is endemic in the Kruger National Park (KNP), where it is maintained through persistent infections of African buffalo (*Syncerus caffer*) (Thomson *et al.*, 2003; Vosloo *et al.*, 2002b). In communities within the proximity of the KNP and adjacent game farms, the wildlife/livestock interface presents unique challenges to FMD control and constitutes a continual threat to the livestock industry in the rest of the country. For this reason, South Africa invests heavily in fences to separate wildlife and livestock, as well as regular

vaccination programmes in an effort to effectively manage FMD. Cattle in the control zone surrounding the KNP are vaccinated twice annually (Brückner *et al*., 2002; Thomson *et al.*, 2003) with a chemically inactivated trivalent (SAT1, 2 and 3) vaccine (Hunter, 1996; Cloete *et al*., 2008). Despite these efforts, outbreaks of FMD have occurred frequently between 2009 and 2012 in the control zone. Therefore, improved vaccines in terms of protection against emerging FMDV, as well as continuous vaccine matching are essential for controlling the disease and maintaining the FMD-free status of South Africa.

An important aspect of an efficient vaccination strategy is the provision of adequate antigenic coverage in the region where control measures is to be applied (Paton *et al*., 2009). This is not a trivial matter, since distinct genetic and antigenic variants within a serotype contribute to intratypic variation in different geographical regions and may therefore render available vaccines inadequate. Despite the fact that the current FMD vaccine contains strains that are representative of all three SAT serotypes, outbreaks of FMD still do occur. This necessitates that new vaccine strains are identified. However, adaptation of new vaccine strains in production cells (BHK-21) is time-consuming and often hampered by low yields of stable antigen, rendering it unsuitable for large-scale production. In comparison, genetic manipulation through a reverse genetic approach can be used to make potential vaccine candidates with the necessary antigenic match to current field or outbreak viruses. As such, the capsid coding region of a field virus cloned into a stable genome-length cDNA clone allows for recovery of infectious recombinant viruses that are antigenically similar to the circulating field viruses or outbreak virus. The potential therefore exists to generate more effective new generation chemically inactivated FMD vaccines. Consequently, the main aim of this study was to evaluate if chimeric vaccines containing the external capsid of field isolates can be successfully produced and induce protective immune responses in FMD host species. The specific objectives of this study were:

1. To predict antigenic sites of SAT1 and SAT2 type viruses based solely on the use of capsid sequences and virus neutralisation titre data.

2. To determine and compare the population diversity of chimeric and host-adapted SAT1 viruses using next-generation sequencing.

3. To evaluate a custom-engineered chimeric FMD vaccine by a dose-dependent potency trial in pigs.

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CHAPTER TWO

PREDICTING ANTIGENIC SITES ON THE FOOT-AND-MOUTH DISEASE VIRUS CAPSID OF THE SOUTH AFRICAN TERRITORIES TYPES USING VIRUS NEUTRALISATION DATA

2.1 INTRODUCTION

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

The South African Territories (SAT) types 1, 2 and 3 are confined to sub-Saharan Africa, although incursions into the Middle East by SAT1 (1961-1965 and 1970) and SAT2 (1990 and 2000) viruses have been recorded (Ferris and Donaldson, 1992; Bastos *et al*., 2001; Records of the OIE). The SAT3 serotype has a restricted distribution and essentially occurs only in southern Africa (Bastos *et al*., 2003b), Asia-1 is restricted to Asia and the Middle East (Ansell *et al*., 1994), while serotypes A and O occur globally (Samuel and Knowles, 2001). The distribution of FMD samples received from Africa from 2000 to 2010 revealed that the majority of outbreaks (41%) were caused by serotype SAT2 viruses. The prevalence of SAT1 and type O viruses was similar with 19 and 23%, respectively. Outbreaks caused by type A viruses were identified for 15% of the reported cases. In contrast, SAT3 viruses caused only two outbreaks during this period in the Democratic Republic of Congo and South Africa, respectively, whereas type C was only detected in Kenya in 2004 (Annual OIE/FAO FMD Reference Laboratory Network Reports; Records of the OIE). Eradication of the disease from the African continent is unlikely due to the presence of large numbers of the free-living maintenance host, the African buffalo (*Syncerus caffer*). These animals provide a potential source of infection for domestic livestock and wildlife (Dawe *et al.*, 1994; Bastos *et al.*, 2000; Vosloo and Thomson, 2004) and pose a constant threat to susceptible livestock in the rest of the world (Vosloo and Thomson, 2004).

Persistently infected buffalo are the ideal hosts to maintain co-infection of different virus serotypes and to facilitate antigenic and molecular evolution of the virus (Condy *et al*., 1985;

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

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

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

2.2 MATERIALS AND METHODS

2.2.1 Cells and viruses

Instituto Biologico Renal Suino-2 cells (IB-RS-2) were propagated on T-265 flasks at 37ºC in RPMI medium (Sigma-Aldrich) supplemented with 5% fetal calf serum (FCS; Invitrogen) and 1 × antibiotics (Invitrogen). Upon reaching 80% confluence, cell suspensions were prepared at 3×10^5 cells/ml in RPMI medium containing 5% FCS.

The viruses included in this study were either supplied by the World Reference Laboratory (WRL) for FMD at the Institute for Animal Health, Pirbright (United Kingdom), or form part of the virus bank at the Transboundary Animal Diseases Programme, Onderstepoort (South Africa). The 20 SAT1, 23 SAT2 and 5 SAT3 FMDV isolates from 19 countries in Africa, selected for genetic characterisation, represented a broad geographical distribution. These isolates, together with their GenBank accession numbers, a description of the passage histories and representative topotypes are indicated in Table 2.1.

2.2.2 Viral RNA extraction, cDNA synthesis and cloning

Viruses were propagated in IB-RS-2 cells and the viral RNA was extracted from cell culture samples using a guanidinium thyocianate (GuSCN)/silica method (Boom *et al*., 1990). The viral RNA was reverse-transcribed in a reaction mixture containing 5 µl RNA, 0.23 µM of the FMDV-specific antisense oligonucleotide 2B208R (Table 2.2), 0.42 U RNasin (Promega), 1 × AMV-RT buffer, 0.34 mM dNTPs, 2% (v/v) DMSO and 4.55 µM of a random hexanucleotide mixture (Roche Diagnostics). The first strand cDNA was synthesised at 42°C for 2 h by addition of 0.6 U AMV-Reverse Transcriptase (Promega). The Leader-P1-2A-coding region of the virus isolates were subsequently amplified using the Expand Long Template PCR system (Roche Diagnostics), together with oligonucleotides NCR1 or NCR2 and WDA (Table 2.2), as described previously (van Rensburg and Nel, 1999). The cycling profile consisted of initial denaturation at 94ºC for 2 min, followed by 30 cycles of denaturation at 95ºC for 30 s, oligonucleotide annealing at 55ºC for 30 s and elongation at 68ºC for 4 min. The amplicons, *ca*. 3 kb in length, were purified from agarose gels with the Nucleospin® Extract kit (Macherey-Nagel) and cloned into the pGEM®-T Easy vector (Promega). Following transformation of competent *E. coli* DH5α cells (Invitrogen), plasmid DNA was extracted from recombinant clones with the QIAprep® Spin Miniprep Kit (Qiagen). These procedures were performed in accordance with the recommendations of the respective manufacturers' instructions.

Table 2.1 FMDV isolates used in this study

Serotype	Virus strain	Topotype	Passage history	Country of origin	GenBank Accession No.
SAT1	KNP/196/91[a]	1	PK_1RS_1	South Africa	AF283429
	KNP/148/91	1	PK_1 RS_5	South Africa	GU194495
	ZIM/HV/3/90	1	BTY_1 RS_3	Zimbabwe	GU194496
	ZIM/GN/13/91	1	BTY_1 PK_1 RS_3	Zimbabwe	GU194497
	KNP/41/95	1	PK_1 RS_4	South Africa	GU194498
	SAR/9/81[a]	1	Epithelium	South Africa	DQ009715
	NAM/307/98	2	PK_1 RS_4	Namibia	AY770519
	ZIM/6/94	2	PK_1 RS_3	Zimbabwe	GU194500
	TAN/37/99	3	BTY_1 RS_4	Tanzania	DQ009718
	ZAM/2/93	3	PK_1 RS_3	Zambia	DQ009719
	ZIM/25/90	3	BTY_2 RS_4	Zimbabwe	GU194499
	MOZ/3/02	3	PK_1 RS_5	Mozambique	DQ009720
	KEN/5/98	3	BTY_1 RS_3	Kenya	DQ009721
	UGA/3/99	4	BTY_1 RS_4	Uganda	DQ009722
	UGA/1/97	5	PK_1 RS_4	Uganda	AY043300
	NIG/5/81[a]	7	BTY_2 RS_2	Nigeria	DQ009723
	SUD/3/76	7	BTY_1 RS_3	Sudan	DQ009725
	NIG/15/75	8	BTY_1 RS_3	Nigeria	DQ009724
	NIG/8/76	8	BTY_1 RS_5	Nigeria	GU194503
	NIG/6/76	8	BTY_1 RS_5	Nigeria	GU194502
SAT2	KNP/19/89[a]	I	BHK_4	South Africa	DQ009735
	KNP/2/89	I	CFK_2 RS_2 BHK_4	South Africa	GU194488
	KNP/51/93	I	PK_1 RS_6	South Africa	GU194489
	ZIM/1/88	I	CFK_1 RS_4	Zimbabwe	GU194491
	SAR/16/83	I	B_1 BHK_8	South Africa	DQ009734
	ZIM/14/90	II	BTY_1 RS_3	Zimbabwe	DQ009728
	ZIM/17/91	II	BTY_2 RS_4	Zimbabwe	DQ009727
	ZIM/GN/10/91	II	BTY_2 PK_1 RS_3	Zimbabwe	GU194493
	RHO/1/48	II	BTY_2 RS_2	Zambia	AJ251475
	ZIM/7/83[a]	II	B_1 BHK_5 B_1	Zimbabwe	AF540910
	ZIM/34/90	II	BTY_3 RS_4	Zimbabwe	GU194490
	ZIM/8/94	II	BTY_1 RS_3	Zimbabwe	GU194492
	KEN/8/99	IV	BTY_2 RS_4	Kenya	AY254730
	KEN/3/57	IX	N/A[b]	Kenya	AJ251473
	GHA/8/91	V	BTY_1 RS_3	Ghana	DQ009732
	SEN/5/75	V	BTY_1 RS_1 BHK_5	Liberia	DQ009738
	SEN/7/83	VI	CK_1 RS_1	Senegal	DQ009733
	SAU/6/00	VII	BTY_1 RS_1	Saudi Arabia	AY297948
	ERI/12/89[a]	VII	BTY_2 PK_1 RS_5	Eritrea	GU194494
	RWA/2/01[a]	VIII	PK_1 RS_1	Rwanda	DQ009730
	ANG/4/74	XI	BTY_3 RS_3	Angola	DQ009736
	UGA/2/02	XII	PK_1 RS_1	Uganda	DQ009731
	ZAI/1/74	XII	BTY_2 RS_4	Zaire	DQ009737
SAT3	KNP/10/90	1	PK_1RS_1	South Africa	AF286347
	BEC/1/65	2	BHK_5B_1	Botswana	M28719
	ZIM/5/91	3	BTY_1RS_4	Zimbabwe	AY168799.1
	ZAM/4/96	4	BTY_1RS_1	Zambia	AF023525.1
	UGA/2/97	6	PK_1RS_2	Uganda	AY192556

[a] Reference viruses for which homologous antisera was used in serological tests

[b] Not available

Table 2.2 Oligonucleotides used for cDNA synthesis, PCR amplification and nucleotide sequence determination

Oligonucleotide	Nucleotide sequence[a]	Reference	Purpose
2B208R	5'-ACAGCGGCCATGCACGACAG-3'	N. Knowles, unpublished	cDNA synthesis (2B)
NCR1	5'-TACCAAGCGACACTCGGGATCT-3'	van Rensburg & Nel (1999)	PCR amplification, nucleotide sequencing (5' UTR)
NCR2	5'-GCTTCTATGCCTGAATAGG-3'	This study	PCR amplification, nucleotide sequencing (5' UTR)
WDA	5'-GAAGGGCCCAGGGTTGGACTC-3'	Beck & Strohmaier (1987)	PCR amplification, nucleotide sequencing (2A)
LINT	5'-GWTACGTCGATGARCC-3'	This study	Nucleotide sequencing (Leader)
FOR	5'-GTAAAACGACGGCCAGT-3'	Messing (1983)	Nucleotide sequencing (pUC/M13)
REV	5'-GTTTTCCCAGTCACGAC-3'	Messing (1983)	Nucleotide sequencing (pUC/M13)
SEQ1	5'-CGTCGATGAGCCACTCTT-3'	This study	Nucleotide sequencing (Leader)
SEQ2	5'-CATCAAAGGCACTGAAC-3'	This study	Nucleotide sequencing (Leader)
SEQ3	5'-ACAACACGACACGGTACC-3'	This study	Nucleotide sequencing (1B)
SEQ4	5'-TTGTGCGAAGCGTGGTTGT-3'	This study	Nucleotide sequencing (1D)
SEQ5	5'-CACCAGCACGCAGTTCAA-3'	This study	Nucleotide sequencing (1B)
SEQ6	5'-GTTCGTGTTCGCCAAGG-3'	This study	Nucleotide sequencing (1D)
SEQ7	5'-GGTAGCAGTGGGCYGC-3'	This study	Nucleotide sequencing (1C)
SEQ8	5'-ACGTTSGTCGGNGCKATG-3'	This study	Nucleotide sequencing (1B)
SEQ9	5'-GCKTAACCGTAGGTGAYKCC-3'	This study	Nucleotide sequencing (1B)
SEQ10	5'-GACCCBAAGACCGCAGA-3'	This study	Nucleotide sequencing (1C)
SEQ11	5'-GGGAYACAGGAYTGAACT-3'	This study	Nucleotide sequencing (1C)
SEQ12	5'-CGTAGATMCCCTTGT-3'	This study	Nucleotide sequencing (1B)
SEQ13	5'-GCGACGTCCAACAKGTT-3'	This study	Nucleotide sequencing (1B)
SEQ14	5'-CAACGGTGAGTGCAAKKAC-3'	This study	Nucleotide sequencing (1D)
SEQ15	5'-GTMMTTGCACTCACCGTTG-3'	This study	Nucleotide sequencing (1D)
SEQ 16	5'-GTGGAACAAGCAGAGAGGT-3'	This study	Nucleotide sequencing (1B)
SEQ 17	5'-GTGTAGTACTTGGGCAG-3'	This study	Nucleotide sequencing (1C)
SEQ 18	5'-CAACTGCAACGTCCTTCTC-3'	This study	Nucleotide sequencing (1C)
Linternal	5'-GWTACGTCGATGARCC-3'	This study	Nucleotide sequencing (Leader/1A)

Table 2.2 Continued

Oligonucleotide	Nucleotide sequence[a]	Reference	Purpose
VP4internal	5'-GGTGGKTCMAATGAGG-3'	This study	Nucleotide sequencing (1A/1B)
VP2internal	5'-GAACTSCCCAMAGACCAC-3'	This study	Nucleotide sequencing (1B/1C)
SAU1	5'-CACTCTMTACCCACACCAG-3'	This study	Nucleotide sequencing (1C)
SAU2	5'-GTGTGTKGAWGGGTCSGTGG-3'	This study	Nucleotide sequencing (1D)
SAU3	5'-CTGTCTACAAYGGTGAGTG-3'	This study	Nucleotide sequencing (1D)
SAU4	5'-GAGGACTTTTACCCATGGAC-3'	This study	Nucleotide sequencing (1A/1B)
SAU5	5'-CACTCACCKTTGTAGAYKGT-3'	This study	Nucleotide sequencing (1D)
SAU6	5'-GCTGGGTCGCGAGGCAGTGC-3'	This study	Nucleotide sequencing (Leader)
SAU7	5'-GTGTGGACGTGTGTACTGCGG-3'	This study	Nucleotide sequencing (1C)
SAU8	5'-CCACGACCTCCACCACGC-3'	This study	Nucleotide sequencing (1B)
SAU9	5'-GACCCCGTGCGACAGACCAC-3'	This study	Nucleotide sequencing (1D)
SEQ29	5'-GATGTCCTGGTGTTTGTTC-3'	This study	Nucleotide sequencing (Leader)
SEQ30	5'-ACACAGGTCCTACTCAC-3'	This study	Nucleotide sequencing (1C)

[a] Abbreviations representing ambiguities are Y (C/T), M (A/C), K (A/G), B (C/G/T), W (A/T), S (C/G) and K (T/G)

2.2.3 Nucleotide sequence determination and analyses

The nucleotide sequence of cloned insert DNA or PCR amplicons was determined using an ABI PRISM™ Big Dye™ Terminator Cycle Sequencing Ready Reaction kit v3.0 (Applied Biosystems). Oligonucleotides used in nucleotide sequencing procedures are described in Table 2.2. After cycle sequencing, the extension products were purified and resolved on an ABI PRISM™ 3100 Genetic Analyzer (Applied Biosystems). The nucleotide sequence of overlapping areas of the P1-coding region of 20 SAT1, 23 SAT2 and 5 SAT3 viruses were determined using BioEdit 5.0.9 software (Hall, 1999). The obtained sequences were aligned using ClustalX (Thompson *et al.*, 1997) and then assembled to obtain the full-length nucleotide sequence from which the amino acid sequence of the viral proteins (1A-1D) was deduced. The complete P1-coding region was selected for phylogenetic analysis. Phylogenetic trees were constructed in MEGA 4 software (Tamura *et al.*, 2007) using minimum-evolution (ME) algorithms. Bootstrap values were applied and values of ≥65% were considered as significant. Amino acid sequences were aligned using ClustalX and the predicted secondary structure was determined using NNpredict software (www.cmpharm.ucsf.edu). Amino acid hypervariable plots were drawn following analysis with MEGA 1.0 software using either 5 or 10 overlapping frames. Hypervariable regions were defined as ≥4 amino acid changes in a window of 5, or ≥7 amino acid changes in a window of 10. Entropy plots were drawn from the deduced amino acid alignments using the BioEdit 5.0.9 software. Francois Maree, Thabiso Lekoana, Pamela Opperman and Wilna Vosloo are gratefully acknowledged for contributions towards sequencing of the P1-coding regions.

2.2.4 Animal sera and virus neutralisation test

The antigenic diversity of the field isolates was determined using cross-neutralisation assays in microtitre plates on IB-RS-2 cells, carried out as described in the OIE Manual of Standards (2009). Cattle sera prepared by two consecutive vaccinations (vaccinated at day 0, boosted at day 28 and bled at day 38) or convalescent sera obtained from cattle 21 days post-infection with reference viruses (SAT1: SAR/9/81, KNP/196/91 and NIG/5/81; SAT2: ZIM/7/83, KNP/19/89/2, ERI/12/89 and RWA/2/01) were used. For cattle immunisations, oil emulsion vaccines were prepared using binary ethylenimine (BEI)-inactivated sucrose density gradient-purified antigens for strains SAT1/SAR/9/81, SAT1/KNP/196/91, SAT2/ZIM/7/83 and SAT2/KNP/19/89/2, respectively. Each 2-ml dose, containing 4 µg of 146S antigen and

Montanide ISA 206 (Seppic) as the oil adjuvant, were mixed 50:50 with the aqueous antigen phase to produce a water-in-oil-in-water emulsion. Convalescent sera were prepared by inoculating cattle intradermolingually with 10^4 $TCID_{50}$ of the SAT1/NIG/5/81, SAT2/ERI/12/89 and SAT2/RWA/2/01 viruses, respectively.

The end-point titre of the serum against homologous and heterologous viruses was calculated as the reciprocal of the last dilution of serum to neutralise 100 $TCID_{50}$ in 50% of the wells (Rweyemamu *et al*., 1978). All neutralisation titre determinations were repeated at least twice, each time using sera from two animals. One-way antigenic relationships (r_1-value) of the field isolates and vaccine viruses relative to the reference strains were calculated as the ratio between the heterologous and homologous serum titres and were interpreted as proposed by Samuel *et al*. (1990). Briefly, r_1-values between 0-0.19 indicated highly significant antigenic variation from the reference strains; values of 0.20-0.39 showed antigenic relatedness where some protection may be provided by a potent vaccine based on the reference strain; and values of 0.40-1.0 demonstrated that the reference and field strains are sufficiently antigenically similar for the reference strain to provide good protection. The means of the r_1-values were calculated (Tables 2.4 and 2.5) and plotted against total amino acid changes between the field strains and reference viruses. Jan Esterhuysen is thankfully acknowledged for performing the serological assays.

2.2.5 Structural modelling

To build homology models of the capsid proteins 1A, 1B, 1C and 1D of a SAT1 and SAT2 virus, *i.e*. SAT1/SAR/9/81 and SAT2/ZIM/7/83, respectively, a closely related structural template was required. Thus, the coordinates and sequence from the X-ray structure of FMDV O_1BFS (1FOD) was obtained from the Brookhaven Protein database (PDB) and used as the template for model building (Logan *et al*., 1993). The sequences were aligned with ClustalX and modelling scripts were generated by the structural module of FunGIMS. Models for each of the SAT1 and SAT2 capsid proteins were generated using the satisfaction of spatial restraints-method as found in Modeller 9v3 (Sali and Blundell, 1993). After building, the models were refined in Modeller 9v3 and the model parameters were validated using PROCHECK (Laskowkski *et al*., 1993). Structures were visualised and the surface-exposed residues identified with PyMol v1.1rc2pre (DeLano Scientific LLC). Tjaart de Beer and Francois Maree are thanked for providing the structural models and figures.

2.2.6 Relating r_1-value variability to amino acid variance and prediction of antigenically significant sites

Amino acid variance of field isolates compared to reference strains for SAT1 and SAT2 were determined in BioEdit 5.0.9 software and plotted against r_1-values derived from titres determined in serological tests. Regions with high amino acid variation and low and high r_1-values were investigated to determine the amino acids located at those positions. In addition, low sequence variation with low and high antigenic relatedness was compared. Residues that were identified as putative epitopes coincided with hypervariable regions and surface exposed residues on the viral capsid.

2.3 RESULTS

2.3.1 Phylogenetic relationships and topotype diversity in sub-Saharan Africa

The minimum evolution phylogeny, based on P1 nucleotide sequences of representative SAT viruses that caused outbreaks across the African continent over the last 28 years, is indicated in Fig. 2.1. The SAT viruses clustered according to serotype with high bootstrap support. The distribution of the SAT3 serotype is limited to southern Africa. The five SAT3 isolates represented five of the six previously described topotypes. Three major virus lineages, which have evolved separately, exist within serotypes SAT1 and SAT2. The lineages clustered according to their geographic location (southern, western and eastern Africa) and are in agreement with the FMD topotype concept described for the European and SAT serotypes (Samuel and Knowles, 2001a, 2001b; Bastos *et al*., 2001, 2003a, 2003b; Knowles and Samuel, 2003). Exceptions, however, were observed for SAT1 and SAT2, where isolates from East Africa, *i.e.* SAT2/KEN/8/99, SAT1/KEN/5/98 and SAT1/TAN/37/99, clustered in the southern topotypes (Fig. 2.1), possibly due to historical movement of buffalo and livestock between the two regions (Bastos *et al*., 2001; Sangare *et al*., 2003). Similarly, an isolate from Sudan, SAT1/SUD/3/76, demonstrated high sequence similarity, based on the complete P1-coding region, with West African isolates, *e.g.* SAT1/NIG/5/81, indicating historical spread of the disease between East and West Africa. Using a 16% nucleotide difference cut-off value (Bastos *et al.*, 2003b), seven of eight previously described distinct lineages could be identified within the SAT1 serotype, while nine of the fourteen SAT2 topotypes could be resolved using the P1 phylogeny (Fig. 2.1). Within the southern African region, Zimbabwe shared different SAT1 (1, 2 and 3) and SAT2 topotypes (I, II and III) with

neighbouring countries. This correlates well with phylogeny based on the 1D sequence only (data not shown). P1 phylogeny did not provide significantly more resolution for epidemiology purposes, although stronger bootstrap values were observed. An exception was the Angolan SAT2 isolate, ANG/4/74, which grouped separately from other southern African SAT2 isolates according to 1D phylogeny and was previously assigned to a different topotype (Bastos *et al*., 2003b; Sangare *et al*., 2004). However, using complete capsid sequence data (Fig. 2.1), this isolate was more closely related to the other southern Africa isolates with good bootstrap support.

The intratypic nucleotide variation of the P1 region for SAT1, 2 and 3 was calculated to be 47.3% (n = 20), 48.9% (n = 23) and 39.5% (n = 5), respectively, and found to be considerably higher than the intratypic variation reported previously for types A, O and C (<18%) (van Rensburg and Nel, 1999; Knowles and Samuel, 2003). The nucleotide and amino acid variation in a complete alignment of the SAT P1-coding region is summarised in Table 2.3. The complete P1 region of the SAT1 viruses was 2232 nucleotides (nt) in length, encoding 744 amino acids (aa), with the exception of the SAT1 isolates NIG/15/75, NIG/6/76 and NIG/8/76 (topotype 8) that consisted of 2229 nt. The corresponding region of the SAT2 viruses was 2220 nt in length, with the exception of a West African isolate, *i.e*. SAT2/SEN/7/83 (topotype VI). The P1-coding region of this isolate is 2217 nt in length and has a 6-nt deletion in the 1C-coding region and a 3-nt insertion in 1D. The five SAT3 isolates revealed the most variation in sequence length of the P1 region, ranging from 2214 to 2223 nt and encoding a polypeptide of between 738 and 741 aa in length. The maximum number of nucleotide differences observed intratypically in any pairwise alignment of the P1 regions of isolates within SAT1, SAT2 and SAT3 serotypes was 581 (26.1%), 558 (25.1%) and 617 (27.74%), respectively. The average proportion of nucleotide differences was estimated to be 53.6%, 55.7% and 57% over synonymous sites, and 9.4%, 9.2% and 11.2% over non-synonymous sites for the three serotypes, respectively. Therefore, the data suggests an overall ratio of synonymous: non-synonymous changes to be *ca.* 6:1 for SAT 1, 2 and 3.

2.3.2 Analysis of antigenic properties of SAT1 and SAT2 viruses

One-way antigenic relationships (r_1-values) were used to compare SAT1 and SAT2 viruses from various topotypes in sub-Saharan Africa to select reference strains within each serotype. Since SAT3 viruses has the most restricted distribution and are the least frequently recovered

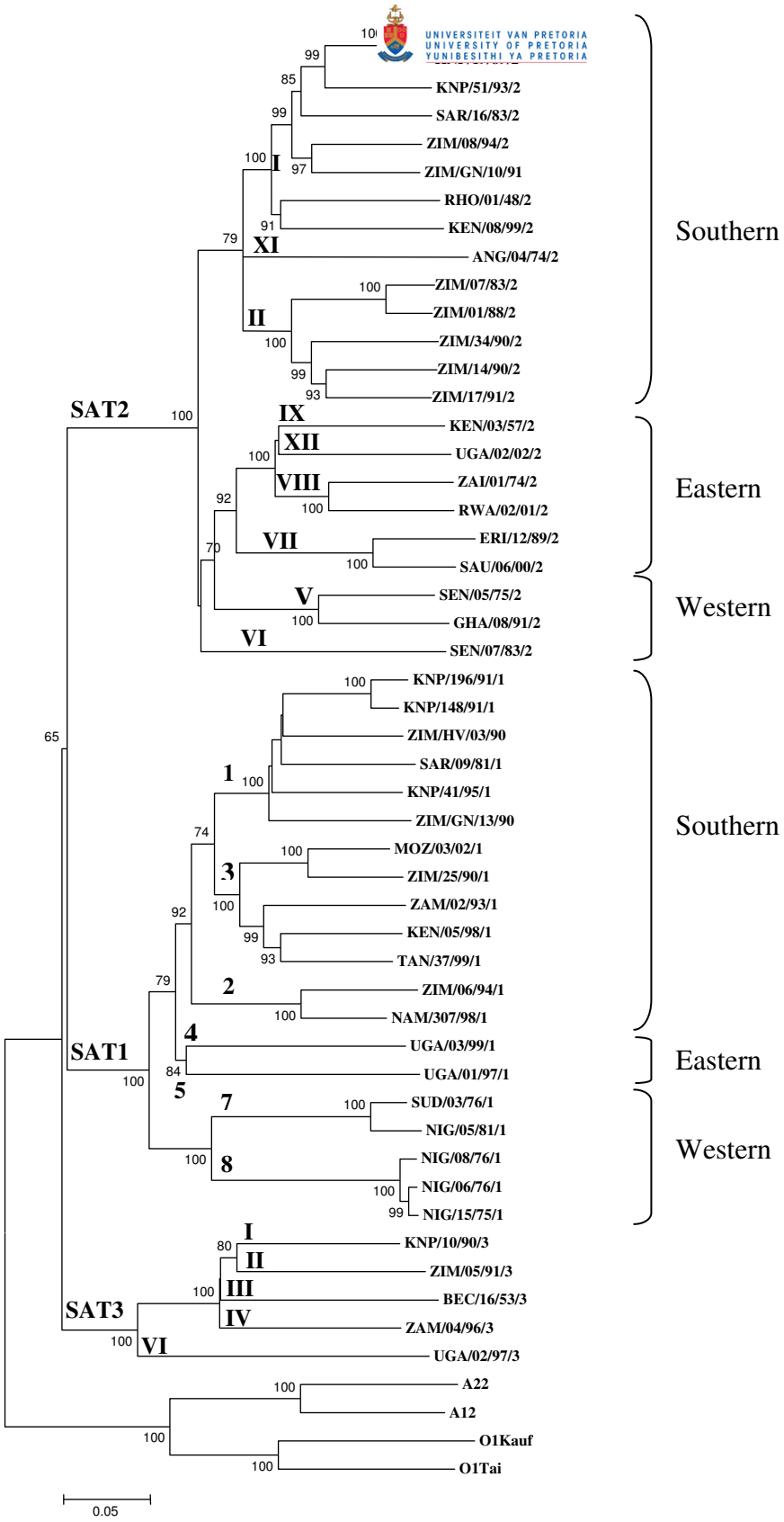


Fig. 2.1 Minimum evolution tree depicting the gene relationships for the P1-coding regions, respectively, of SAT1, 2 and 3 viruses from southern (Kruger National Park, KNP; South Africa, SAR; Zimbabwe, ZIM; Rhodesia, RHO; Mozambique, MOZ; Namibia, NAM; Zambia, ZAM; and Angola, ANG), western (Nigeria, NIG; Senekal, SEN; and Ghana, GHA) and eastern Africa (Uganda, UGA; Rwanda, RWA; Zaire, ZAI; Kenya, KEN; Tanzania, TAN; Eritrea, ERI; Saudi Arabia, SAU; and Sudan, SUD). The major topotypes for SAT1 (labelled 1 - 8), SAT2 (labelled I to XII) and SAT3 (labelled I - VI) viruses are indicated. No representative isolates of SAT1 topotype 6 and of SAT2 topotypes III, IV, X, XIII and XIV were included. Bootstrap support ≥65 is based on a 1000 replicates and is indicated next to the relevant node.

Table 2.3 Variation within the nucleotide and amino acid sequences of the P1 polyprotein in a complete alignment of the SAT1, 2 and 3 viruses

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

[a] The number of nucleotides and amino acids were based on ClustalX alignments of the complete P1-coding region of the SAT serotypes.

[b] The number of variant nucleotides or amino acids for each genomic region or capsid protein relative to the total number of positions was used to estimate the percentage (%) variability.

from buffalo (Bastos *et al.*, 2003b), these isolates were not included in this study. The virus isolates and the average r_1-values are summarised in Tables 2.4 and 2.5. Sera from cattle vaccinated twice with the two SAT1 viruses, SAR/9/81 and KNP/196/91, both belonging to topotype 1, demonstrated r_1-values that were higher with isolates belonging to the same topotype compared with isolates from other topotypes. Among the 20 SAT1 isolates analysed against the vaccine strains, 15% (n = 3) and 15% (n = 3) showed r_1-values ≥0.4 with the SAR/9/81 and KNP/196/91 viruses, respectively, all belonging to topotype 1 (Table 2.4). Furthermore, 55% (n = 11) and 70% (n = 14) of the isolates had r_1-values in the range of 0.2 to 0.39, indicating that a high potency vaccine may still afford protection in animals. The remaining isolates reacted poorly to antisera directed against these vaccine strains, indicating that the vaccines will most probably not protect animals in the field against these strains. Convalescent sera to a West African SAT1 virus, *i.e.* SAT1/NIG/5/81, showed reasonable cross-protection (r_1-values of 0.2-0.39) against 75% (n = 15) of the SAT1 viruses (Table 2.4). Only one isolate had an r_1-value of ≥0.4, *i.e.* SUD/3/76, which belongs to the same topotype as the Nigerian isolate. The difference in cross-reaction with the reference sera might be indicative of differences in shared epitopes of the isolate in question to the reference virus.

The SAT2 reference antisera used in the study were from the SAT2 vaccine strains ZIM/7/83 and KNP/19/89 that belong to SAT2 topotypes I and II, respectively, and two convalescent antisera, that of ERI/12/89 and RWA/2/01 from topotypes VII and VIII, respectively (Table 2.5). The antigenic variation for the SAT2 isolates was more pronounced compared to SAT1 isolates, with r_1-values below 0.2 even when vaccine and field strains from the same topotype were compared. With the exception of the reaction of the ZIM/7/83 isolate to the KNP/19/89 sera, none of the other SAT2 isolates showed r_1-values in the range of 0.4-1.0 against any of the four reference strains. Although none of the SAT2 isolates had r_1-values of 0.2-0.39 against ZIM/7/83 antisera, at least 16.6% (n = 4) of the isolates fell into this range using KNP/19/89 antisera. Furthermore, 28% and 25% (n = 6 and n = 5) of isolates had r_1-values of 0.2-0.39 when tested against ERI/12/89 and RWA/2/01 antisera, respectively. From the data in Table 2.5 the convalescent sera (ERI/12/89 and RWA/2/01) appear to be more cross-reactive than the sera derived from the immunised animals (ZIM/7/83 and KNP19/89).

Table 2.4 The average r_1-values and number of variable amino acids in the capsid proteins of SAT1 isolates as measured against reference strains (SAR/9/81, KNP/196/91 and NIG/5/81)

SAT1 isolates[a]	Topotype[b]	SAR/09/81[c]		KNP/196/91[c]		NIG/05/81[d]	
		Variable amino acids	r_1-value	Variable amino acids	r_1-value	Variable amino acids	r_1-value
KNP/196/91[e]	**1**	49	0.44	**0**	**1.00**	109	0.21
SAR/9/81[e]	**1**	**0**	**1.00**	49	0.44	105	0.34
ZIM/GN/13/91	1	44	0.50	38	0.55	107	0.23
ZIM/HV/3/90	1	42	0.40	31	0.48	100	0.20
KNP/148/91	1	46	0.37	17	0.36	106	0.34
KNP/41/95	1	54	0.25	50	0.36	118	0.23
NAM/307/98	2	73	0.21	67	0.28	109	0.22
ZIM/6/94	2	75	0.23	77	0.33	113	0.17
MOZ/3/02	3	63	0.24	59	0.37	107	0.32
ZIM/25/90	3	68	0.27	62	0.10	109	-
TAN/1/99	3	-	0.13	-	0.28	-	0.25
TAN/37/99	3	55	0.25	55	0.36	105	0.27
ZAM/2/93	3	63	0.16	60	0.14	106	0.10
KEN/5/98	3	56	0.23	58	0.34	101	0.23
UGA/3/99	4	73	0.26	80	0.25	100	0.32
UGA/1/97	5	91	0.24	102	0.27	114	0.29
SUD/3/76	7	103	0.28	107	0.25	17	0.60
NIG/5/81[e]	**7**	105	0.19	109	0.28	**0**	**1.00**
NIG/15/75	8	105	0.17	108	0.26	65	0.21
NIG/6/76	8	104	0.10	106	0.12	63	0.17
NIG/8/76	8	103	0.14	106	0.36	63	0.18

[a] The passage histories and country of origin of the study viruses, as well as GenBank accession numbers of the P1 sequences are indicated in Table 2.1.

[b] The topotype designations is based on 1D phylogeny proposed by Bastos *et al*. (2001); Sangare *et al.* (2003); Vosloo *et al*. (2006) and Sahle *et al.* (2007a).

[c] The reference SAT1 test sera used in the VNTs. The sera were prepared by two consecutive vaccinations on days 0 and 28 with reference SAT1 viruses and subsequently bled on day 38.

[d] The convalescent test sera were prepared by inoculating cattle intradermolingually with 10^4 $TCID_{50}$ of SAT1/NIG/5/81 virus and collecting blood 21 days post-infection.

[e] The homologous viruses used as reference strains. The bold values indicate the homologous reaction in VNTs or alignment with homologous sequence.

-, VNTs were not done.

Table 2.5 The average r_1-values and number of variable amino acids in the capsid proteins of SAT2 isolates as measured against reference strains (ZIM/7/83/2, KNP/19/89/2, ERI/12/89/2 and RWA/2/01)

SAT2 isolates[a]	Topotype[b]	ZIM/07/83[c]		KNP/19/89[c]		ERI/12/89[d]		RWA/2/01[d]	
		Variable amino acids	r_1-value	Variable amino acids	r_1-value	Variable amino acids	r_1-value	Variable amino acids	r_1-value
KNP/19/89[e]	**I**	59	0.14	**0**	**1.00**	86	0.25	90	0.10
KNP/2/98	I	54	0.09	16	0.11	81	0.12	84	0.19
KNP/51/93	I	56	0.07	33	0.09	81	0.12	83	0.18
SAR/16/83	I	60	0.03	39	0.03	83	0.05	85	0.10
ZIM/8/94	I	52	0.08	39	0.12	83	-	79	-
ZIM/GN/10/91	I	49	0.11	42	0.11	81	0.09	80	0.09
ZIM/7/83[e]	**II**	**0**	**1.00**	59	0.41	96	0.20	90	0.19
ZIM/14/90	II	49	0.05	57	0.09	91	0.14	85	0.10
ZIM/17/91	II	51	0.13	56	0.05	90	0.14	88	0.24
ZIM/1/88	II	19	0.18	51	0.13	87	0.16	86	0.13
ZIM/34/90	II	61	0.14	67	0.28	88	-	91	-
RHO/1/48	II	60	0.17	58	0.12	86	0.21	87	0.27
KEN/8/99	IV	68	0.05	53	0.07	86	0.04	86	0.06
GHA/8/91	V	89	0.09	80	0.09	74	0.18	62	0.21
LBR/1/74	V	-	0.03	-	0.05	-	0.12	-	0.16
SEN/5/75	V	100	0.02	90	0.05	82	0.03	71	0.06
SEN/7/83	VI	96	0.10	84	0.20	106	0.25	98	0.35
SAU/6/00	VII	97	0.06	86	0.06	38	0.26	73	-
ERI/12/89[e]	**VII**	96	0.14	86	0.31	**0**	**1.00**	70	0.32
RWA/2/01[e]	**VIII**	90	0.13	90	0.09	70	0.23	**0**	**1.00**
KEN/03/57	IX	93	-	94	-	87	-	56	-
ANG/4/74	XI	85	0.09	88	0.10	91	0.04	80	0.05
UGA/02/02	XII	93	0.02	92	0.03	84	0.04	57	0.08
ZAI/01/74	XII	92	0.02	86	0.05	66	0.12	33	0.17

[a] The passage histories and country of origin of the SAT2 viruses, as well as the GenBank accession numbers of the P1 sequences are indicated in Table 2.1.

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

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

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

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

-, VNTs were not done.

2.3.3 Comparison of amino acid variation within the SAT1 and SAT2 serotypes

The study was extended to investigate potential regions involved in the antigenic variability of SAT1 and SAT2 viruses based on the identification of hypervariable regions and positions of high entropy in the capsid-coding regions for SAT viruses. Hypervariable regions, in which 70% or more of the residue positions varied (using overlapping windows of 10 aa), were identified in the outer capsid proteins, *i.e.* 1B, 1C and 1D (Appendix A to this thesis). Within hypervariable regions, entropy was used to measure the uncertainty at each amino acid position within the SAT alignment (Schneider and Stephens, 1990). Interestingly, differences were observed between hypervariable sites for SAT1 and 2 isolates obtained from eastern, western and southern Africa. The hydrophilicity and deduced surface exposure were also considered for each residue and highly variable regions correlated with regions of significant hydrophilicity (Appendix A to this thesis). Comparison of the P1 amino acid sequences of SAT1 and 2 with type A and O sequences, revealed that many of the SAT1 and 2 hypervariable regions corresponded or were located in close proximity to previously identified immuno-dominant sites on types O and A (Table 2.6) (Xie *et al.*, 1987; Thomas *et al.*, 1988; Baxt *et al.*, 1989; Bolwell *et al.*, 1989; McCahon *et al.*, 1989; Kitson *et al.*, 1990; Saiz *et al.*, 1991; Crowther *et al.*, 1993a). Some hypervariable regions were located within flexible structural loops or downstream of protease cleavage sites.

As expected, the internally located 1A protein was the most conserved of the structural proteins with only 27% variable positions on amino acid level (only 6% identified as parsimony-informative) between SAT1 and SAT2 (Table 2.3). Alignment of the amino acid sequences of the 1A polypeptide revealed no hypervariable regions, but common substitutions that are unique to each serotype were observed. Two amino acids in the 1A protein that are potentially specific for SAT2 and for SAT1 viruses are an Iso or Val at position 76 and Phe at position 80 for SAT2 viruses, while SAT1 viruses present with Phe and Val at the corresponding positions (Appendix A to this thesis).

The 1B protein, 219 aa in length, contained 24% and 29% variable amino acid positions for SAT1 and 2, respectively (57% in a complete alignment) (Table 2.3). Within the 1B capsid protein, four hypervariable sites were identified within each serotype (Table 2.6), *i.e.* βA-βB loop (aa positions 31-45), βB-βC loop (aa 64-82), βC-βD loop (aa 93-101) and βE-βF loop (aa 130-134/141). Only the βB-βC and βE-βF loops have significant surface exposure in the

complete virion, while the N terminus of 1B (βA-βB loop) is located on the inner surface of the capsid in close proximity with the N terminus of 1D.

The 1C protein varied from 221 to 222 aa in length for SAT1 and SAT2 isolates, respectively. The exception was a SAT2 isolate from West Africa, SEN/7/83, which was only 220 aa in length. For 1C, the overall variable amino acids positions was 65% (37% variation within SAT1 and 30% within SAT2) (Table 2.3). The genetic heterogeneity of 1C was confined to four hypervariable regions (Table 2.6), *i.e.* N terminus (aa 30-45), βB-βC loop (aa 63-77), βE-βF loop (aa 125-142) and βG-βH loop (aa 165-183/172). The heterogeneity was more pronounced in SAT1 viruses with some unique amino acids confined to different topotypes. The latter three of these hypervariable regions (consisting of the βB-βC, βE-βF and βG-βH loops) correlated with hydrophilic surface-exposed regions, indicating its possible contribution to antigenic determinants (Appendix A to this thesis).

The 1D protein, the most variable of the outer capsid proteins, varied in length from 213 aa for SAT2 to 219 aa for SAT1 with 71% overall variable amino acid positions (48% within SAT1 and 50% within SAT2) (Table 2.3). The SAT2 virus, SEN/7/83, was the exception with 214 aa in length. The inserted amino acid of SEN/7/83 is a Trp between residues 28 and 29 within a conserved positively charged motif (KRRXH for SAT2 or RRXH for SAT1) located in a region similar to a previously identified T-cell epitope of 1D for serotypes C and O (Pérez Filgueira *et al.*, 2000; Guzman *et al.*, 2010). At least seven discrete hypervariable regions were identified in 1D of SAT1 and SAT2 viruses (Table 2.6). These regions corresponded with the N terminus (residues 9-40 in SAT viruses), βB-βC loop (residues 43-62/71 for SAT1 and 2), βE-βF loop (residues 80-103), βF-βG loop (residues 110-122), βG-βH loop (residues 136-167), βH-βI loop (residues 176-187) and the C terminus (residues 199-220/192-212 for SAT1 and 2). With the exception of the N terminus and βF-βG loop, the hypervariable regions coincided with hydrophilic surface-exposed regions.

Amino acid residues situated within regions of hypervariability in the outer capsid proteins, which exhibit high entropy and hydrophilicity, and on structurally exposed loops were regarded as having the potential for involvement in antibody recognition sites. These regions are therefore possibly involved in the antigenicity of the virion (Table 2.6 and Appendix A to this thesis).

Table 2.6 A summary of the hypervariable regions observed in an alignment of the amino acid sequences of SAT1 and SAT2 outer capsid proteins

The hypervariable regions were considered in relation with structurally exposed loops and with known antigenic sites in serotype A and O sequences. *Residues within the starred regions have significant surface exposure (Fig. 2.3) and entropy that could contribute to the changes in antigenicity observed.

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

[a] The hypervariable regions have been derived from the alignment presented in Appendix A to this thesis.

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

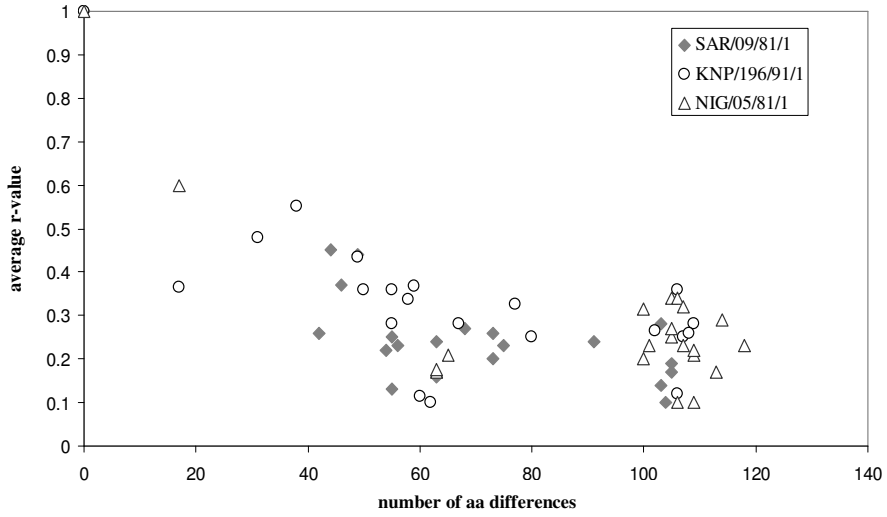
2.3.4 Amino acid changes and antigenic types within the SAT serotypes

To obtain information regarding the relationship between genetic distance and mean r_1-values for SAT1 and SAT2 viruses, the mean r_1-values obtained for each field isolate against the reference sera were plotted against the total amino acid changes between the field strain and reference virus (Fig. 2.2). For SAT1 viruses, a close genetic relationship with the reference strain, as observed by considerable amino acid identity, did agree with sufficient serological cross-reaction. However, for the SAT2 viruses the observed r_1-values were low, even for viruses with considerable identity with the reference strain. The availability of the deduced amino acid sequences for the P1 regions of the VNT analysed viruses allows for the direct comparison of amino acid residue changes between any two isolates. In addition, residues located in hypervariable regions that are surface-exposed can be identified and counted between any two sequences. The changes specifically corresponding to surface-exposed residues are expected to correlate directly with antigenic variability as it may abrogate antibody binding and neutralisation *in vitro*. This can be explained using two examples, one for SAT1 and SAT2, respectively.

In a pairwise alignment of the P1 polypeptide of the SAT1/SAR/9/81 virus and five additional topotype 1 isolates (KNP/196/91, KNP/148/91, KNP/41/95, ZIM/GN/13/91, ZIM/HV/3/90), 17 to 55 variable amino acids were observed (Table 2.4). The r_1-values for these viruses varied from 0.22 to 0.45, indicating poor but some cross-reaction to good cross-reaction (Table 2.4 and Fig. 2.2a). Since the outer-capsid protein surface-exposed residues are directly involved in antigenicity (Logan *et al*., 1993; Lea *et al*., 1994), clusters of variable surface-exposed amino acids may constitute antigenic regions on the virion. Using this approach, the putative residues directly involved in antigenicity could be narrowed down compared to amino acid alignment alone. Indeed, residue positions with high entropy and surface exposure were located in the βB-βC (aa 71-74) and βE-βF (aa 133-134) loops and aa 196 of 1B, the βB-βC (aa 64-68) and βE-βF (aa 134-136) loops of 1C, and the 1D βB-βC loop (aa 46-49), residues 142-148 and 157 in the βG-βH loop, 177-179 in the βH-βI loop and 208 and 214 in the C terminus.

Within the SAT2 serotype, similar observations were made. Comparison of the P1 polypeptide of six SAT2 isolates that belong to topotype 1 (KNP/19/89, KNP/02/89, KNP/51/93, SAR/16/83, ZIM/08/94 and ZIM/GN/10/91) revealed 16 to 42 variable residue

(a)



(b)

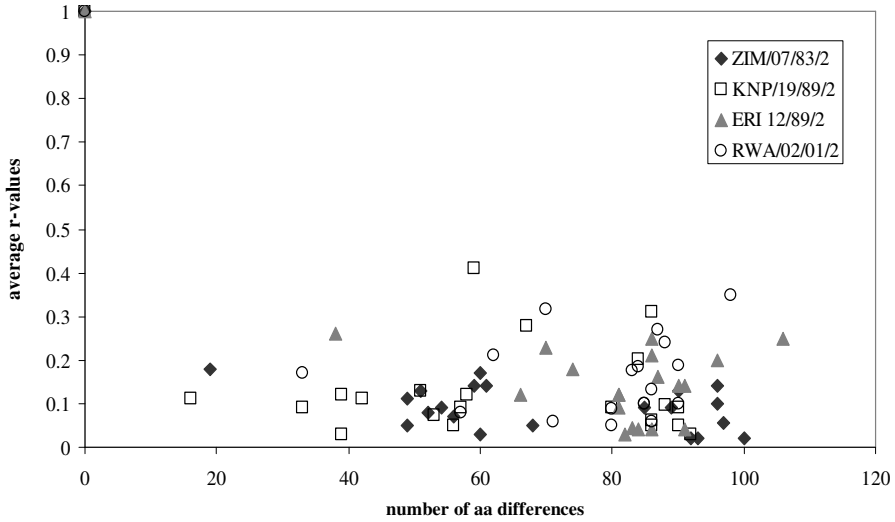


Fig. 2.2 The average r_1-values obtained for SAT1 (a) and SAT2 (b) viruses plotted against the number of amino acid differences determined in pairwise comparisons with the reference strains. The reference viruses used for the SAT1 serotype was SAR/9/81 (grey diamonds), KNP/196/91 (open circles) and NIG/5/81 (open triangles), and for the SAT2 serotype was KNP/19/89 (open squares), ZIM/7/83 (black diamonds), ERI/12/89 (grey triangles) and RWA/02/01 (open circles).

positions (Table 2.4). The r_1-values against SAT2/KNP/19/89 sera varied from 0.04 to 0.39 where the genetically closely related KNP/2/89 with 16/744 variable residues compared to KNP/19/89, had a maximum r_1-value of only 0.17 against KNP/19/89 sera (Table 2.5 and Fig. 2.2b). These 16 variable residues are likely involved in abolishing the neutralisation by SAT2/KNP/19/89 antisera. Using structural data, surface-exposed residues with high entropy could be identified in the βE-βF (aa 130-134) loop of 1B, the βE-βF (aa 129-134) loop of 1C, and βB-βC (aa 63-68), βE-βF (aa 83-85), βF-βG (aa 110-112), βG-βH (aa 136-140 and 156-161), βH-βI (aa 172-176) and C terminus (aa 200-202) of 1D.

Taken together, the inability of anti-SAT1/SAR/9/81 or anti-SAT2/KNP/19/89 antiserum from vaccinated animals to neutralise genetically related viruses may be the result of amino acid variation in the above-mentioned surface-exposed β-loops.

2.3.5 Mapping putative antigenic sites on the virus capsid

The SAT1 and SAT2 capsid structures were modelled using O_1BFS (Logan *et al.*, 1993) as template. The model was based on the optimal alignment of the SAT1 virus, SAR/9/81, or the SAT2 virus, ZIM/7/83, P1 sequences to the corresponding sequence of O_1BFS. The homology modelled structure was calculated by the satisfaction of spatial restraints as described by empirical databases. Using this method and mapping the observed variable loops (Table 2.6) to the structures, several putative antigenic sites on the virus capsid were identified (Fig. 2.3). In particular, differences between serotypes were observed, as well as differences within topotypes in a given serotype.

Taking all the topotype information into consideration, the variable residues (high entropy) within surface-exposed loops were regarded as immune relevant and were mapped to the SAT1 and SAT2 pentamer structures, respectively (Fig. 2.3 and Table 2.6). For both serotypes the variable regions outside the 1D βG-βH loop were concentrated around the 5-fold and 3-fold axis of the virion and the C-terminal of 1D. These regions correlated strongly with previously identified neutralising epitopes of serotypes A and O (Table 2.6). The role of these residues as antigenic determinants can therefore not be excluded. Furthermore, it can be hypothesised that some of the putative epitope regions are probably discontinuous. The close proximity of 1B residues 71-74, 133-134 and 196 or 1B residues 71-74, 196 and 1C residues 64-68 around the 3-fold axis of the virion or 1D residues 46-49, 84-86 and 177-179 around the 5-fold axis of SAT1 viruses are examples (Fig. 2.3).

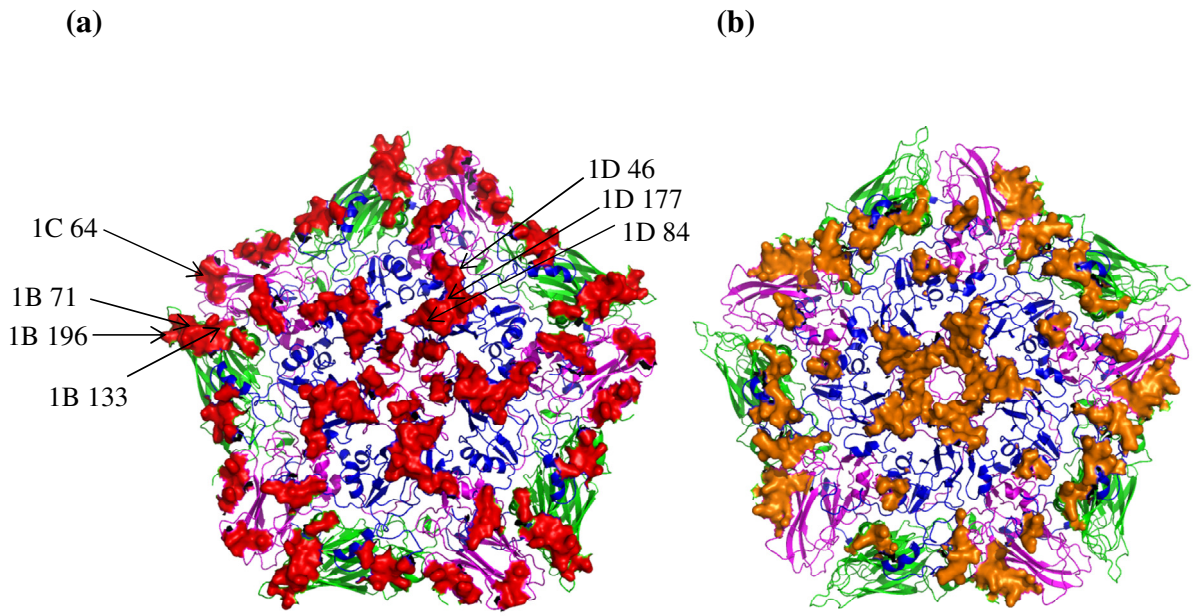


Fig. 2.3 Three-dimensional structure of a SAT1 (a) and SAT2 (b) pentamer, modelled using the O1BFS co-ordinates (1FOD; Logan *et al*., 1993), as template. The protein subunits are colour coded: 1D (blue), 1B (green) and 1C (cyan). The position of surface-exposed residues with high entropy is indicated in red (A) or orange (B) on the SAT1 or SAT2 pentamers, respectively. Putative discontinuous epitopes are indicated for SAT1 (A) as the capsid protein followed by the first residue number. The alignment used for the extraction of data is available in Appendix A of this thesis.

2.4 DISCUSSION

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

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

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

less than 16% aa variation). In the past, the combination of SAT1/SAR/9/81 and SAT1/KNP/196/91 in a tetravalent vaccine, containing also a SAT2 and a SAT3 strain, were able to protect against SAT1 outbreaks in southern Africa (Hunter, 1998).

The SAT2 reference strains ZIM/7/83 and KNP/19/89, on the other hand, did not have good antigenic relationships with most SAT2 isolates, even within the same topotype. ZIM/7/83 antisera cross-reacted weakly to the SAT2 viruses in this study and were not able to neutralise the genetically closely related ZIM/1/88 (19 aa differences in a pairwise comparison). From the data set, it seems unlikely that SAT2/ZIM/7/83 alone will provide protection against the large genetic and antigenic diversity among the SAT2 viruses. Antigenic relationships against KNP/19/89 was better with at least 16% of the isolates having an r_1-value >0.2, while ERI/12/98 and RWA/2/01 antisera were able to neutralise at least 20% of the viruses *in vitro*. Similar to the SAT1 viruses, better antigenic relationships to ERI/12/98 and RWA/2/01 were obtained for SAT2 viruses belonging to the same topotypes. The results of this study suggest that extensive antigenic variation occurs for SAT2 viruses across Africa and has serious implications for vaccine strain selection. A previous serological comparison of SAT2 viruses from Kenya suggested extensive antigenic variation in this country alone (Ndiritu *et al.*, 1983). The poor antigenic coverage of existing vaccine strains against field strains call for urgent development of multiple region-specific or topotype-specific vaccine strains. Additionally, there is a need for a SAT2 vaccine strain that will provide protection against a wide range of antigenic types in the field.

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

In this study, a different approach was followed by combining amino acid variation and calculated r_1-values from *in vitro* cross-protection titres in VNTs, together with structural data, to predict areas on the surface of the capsid as antigenically relevant. This modelling

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

Although the putative antigenic sites need to be confirmed using mAbs and sequencing of virus escape mutants, the methodology employed offers a potentially quick, easy and cheap alternative to the identification of antigenic relevant sites on field FMD strains. This approach may furthermore be used for any FMDV serotype and potentially for any similar virus where cross-reactivity, sequencing and structural studies have been performed. The information obtained may thus be used not only to identify putative epitopes, but also to predict the best vaccine match for new isolates and to estimate the efficacy of new candidate seed strains.

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CHAPTER THREE

PYROSEQUENCING ANALYSES REVEALS COMPARABLE POPULATION DIVERSITY OF CHIMERA AND HOST-ADAPTED FOOT-AND-MOUTH DISEASE VIRUSES

3.1 INTRODUCTION

Foot-and-mouth disease (FMD), of which FMD virus (FMDV) is the aetiological agent, is a contagious, acute infection of cloven-hoofed animals, including cattle, pigs, goats, sheep and several wildlife species. Despite the restricted global distribution of FMD, it remains a compulsory notifiable disease of the OIE. FMD not only poses a constant threat world-wide as was evidenced by its extensive spread in South Korea and Japan in 2010, but it also has a major economic impact on the livestock industry as has been illustrated by recent FMD outbreaks in South Africa from 2000 to 2012 (Records of the OIE). Rapid and accurate diagnosis of FMD is a prerequisite for effective control of the disease and is based on a combination of clinical, epidemiological and laboratory observations.

FMDV belongs to the *Picornaviridae* family, which consists of numerous widely-studied RNA viruses, including important human and animal pathogens. FMDV, together with equine rhinitis A virus (Li *et al*., 1996; King, 2000), is classified under the genus *Aphthovirus* and is characterised by high genetic and antigenic variation (Rueckert, 1996). The positive-sense single-stranded RNA genome of FMDV is *ca*. 8 500 nucleotides in length. During viral RNA replication high mutation rates occur (0.2-1 mutation per plus or minus strand copied) (Drake and Holland, 1999). A consequence of the high mutation rate is a continuous generation of genetic variants that are observed in infected hosts or cell culture. Thus, diversity within the FMDV population is generated as new genetic and phenotypic variants arise during the process of genome replication (Domingo *et al*., 2003). Many of the progeny positive-sense RNA strands are initially recruited as viral mRNA, of which almost 50% of these molecules are packaged into virions (Rueckert, 1996). Virion assembly involves the formation of capsid protomers and association of five such protomers results in the formation of a pentamer, which is followed by assembly into either empty capsids or provirions (Rueckert, 1996). Autolytic cleavage of the 1AB polypeptide present in the provirion is required for the generation of virus particles (about 10^4-10^5 viral particles per cell), of which 0.1-1% are infectious (Palmenberg, 1990; Belsham, 1993; Rueckert, 1996).

High variability in the RNA genome population gives rise to distinct but related viruses, referred to as the quasispecies of FMDV (Domingo *et al*., 2003). *In vivo* and *in vitro* the quasispecies exist due to mutation, recombination and selection. Sanger sequencing using conventional methods allows for sequence determination of the virus at a most-represented

basis of the entire population present. The consensus nucleotide sequence is an average determination of many different sequences and it is possible that such a genome does not exist in a viral population. Since the quasispecies of RNA viruses consists of viruses where the genome is statistically defined but not determined on an individual level (Domingo *et al*., 2002), it therefore does not discriminate between variants in the population that may be present at a lower frequency. Consequently, the true sequence diversity in the viral population is not always accounted for. This makes next-generation sequencing (NGS) (Ronaghi, 2001) unique in its ability to decipher the genetic identity of any genomic region of several numbers and types of viruses. Several studies have applied next-generation sequencing to detect sequence variation in human viruses such as HIV (Knapp *et al*., 2011), hepatitis virus (Lindström *et al*., 2004; Solmone *et al*., 2009; Wang *et al*., 2010), cytomegalovirus (Görzer *et al*., 2010) and rotavirus (Jere *et al*., 2011). This sequencing technology has not yet been applied extensively in the veterinary field (Leifer *et al*., 2010; Wright *et al*., 2011).

The application of next-generation sequencing to study viral population diversity in picornaviruses is currently limited (Silva *et al*., 2008; Wright *et al*., 2011). Host infection with FMDV results in fixation of variants different to viruses that have been cultured *in vitro* in cells. In addition, a chimeric virus that has its origin as a plasmid DNA clone is potentially less variable due to the narrow representation of the population, which resembles passages of small viral populations or repeated bottleneck effects (such as plaque-to-plaque transfers) (Escarmís *et al*., 2008). The 454 Life Sciences platform allows for pyrosequencing of individual DNA molecules using the Genome Sequencer (GS) FLX. Next-generation sequencing technology provides rapid and simple methods to characterise and investigate complex virus populations through in-depth sequence data analyses where genetic identity is represented in large numbers.

In this study the population diversity of two dissimilar FMD viruses, *i.e*. a chimera derived from a cloned population characterised by cell adaptation to BHK-21 cells and an animal-adapted virus isolated from pigs, was characterised. Next-generation sequencing was applied to investigate the variation present in the capsid sequences and to assess key viral properties. Direct PCR sequencing was used as reference in the analysis and polymorphisms in the P1 region were determined. Pyrosequencing detected considerable heterogeneity in the diversity of the two FMD populations. An improved understanding of the FMDV population diversity

may impact positively on aspects relating to disease control such as the genetic and antigenic characteristics of circulating field strains, as well as the choice of appropriate vaccine antigens and diagnostic reagents that could be applied.

3.2 MATERIALS AND METHODS

3.2.1 Cell lines

Baby hamster kidney cells-21 clone 13 (BHK-21, ATCC CCL-10) were maintained in Eagle's basal medium (BME; Invitrogen) supplemented with 10% (v/v) tryptose phosphate broth (TPB; Sigma-Aldrich), 1 mM L-glutamine (Invitrogen), antibiotics and 10% (v/v) fetal calf serum (FCS; Delta Bioproducts). Both sources of pig cells, primary pig kidney cells 337 (PK) and Instituto Biologico Renal Suino-2 cells (IB-RS-2), were maintained in RPMI medium (Sigma-Aldrich) supplemented with 10% FCS and antibiotics. Chinese hamster ovary cells (CHO strain K1, ATCC CCL-61) were grown in Ham's F-12 medium supplemented with 10% FCS and antibiotics.

3.2.2 Viruses and plasmids

The SAT1/KNP/196/91 strain (designated KNP/196/91) was isolated at the Transboundary Animal Diseases Programme (ARC-OVI) from a buffalo sample received from the Kruger National Park, South Africa. The full spectrum of the virus population was recovered by passage once in PK (KNP_{PK1}) and four times in IB-RS-2 (KNP_{PK1RS4}) cells. For vaccine production purposes, KNP_{PK1RS4} was adapted to the FMDV host-species of choice, cattle (B = bovine), and subsequently propagated in BHK-21 cells. This virus, $KNP_{PK1RS4B1BHK4}$, was used to infect pigs (P = pig) and the recovered host-adapted viruses $KNP_{PK1RS4B1BHK4P2}$ and $KNP_{PK1RS4B1BHK4P3}$ are henceforth referred to as KNP_{P2} and KNP_{P3}, respectively.

The external capsid-coding region (1B-1D/2A) of plasmid pSAT2, a genome-length infectious cDNA clone of SAT2/ZIM/7/83 (van Rensburg *et al*., 2004), was replaced with that of KNP/196/91 to yield plasmid pKNP/SAT2 (constructed by H.G. van Rensburg). In constructing the recombinant clone, pSAT was digested with endonucleases *Ssp*I and *Xma*I to excise the *ca*. 2-kb external capsid-coding region from the pSAT2 clone to facilitate cloning of the corresponding KNP/196/91 amplicon containing the same restriction endonuclease sites.

3.2.3 Transcription of viral RNA, transfection and amplification of chimera viruses

Plasmid DNA for use as template in *in vitro* transcription reactions was obtained by plasmid extraction using the QIAprep® Spin Miniprep kit (Qiagen). For RNA synthesis, plasmid pKNP/SAT2 was linearised with *Swa*I and *in vitro* transcribed using the MEGAscript™ T7 kit (Ambion). *In vitro*-transcribed RNA was transfected into cells using Lipofectamine 2000™ reagent (Invitrogen). BHK-21, CHO-K1 and IB-RS-2 cell monolayers in 35-mm wells were maintained at 37°C for 48 h in virus growth medium (VGM: the appropriate medium supplemented with 1% HEPES and 1% FCS) and passaged as described previously (van Rensburg *et al*., 2004). The transfected cell cultures were harvested and aliquots of the supernatant were stored at -80°C. Ten percent of the thawed supernatant was used to infect freshly prepared cells and observed for cytopathic effect (CPE) up to 48 h post-infection. The same procedure was followed for serial passage of the viruses in the respective cells, and the viruses were designated vKNP/SAT2$_{RS3}$, vKNP/SAT2$_{BHK5}$ and vKNP/SAT2$_{CHO4}$.

3.2.4 Viral RNA extraction, cDNA synthesis and DNA sequencing

The FMDV were characterised by RT-PCR of the capsid-coding region (P1), followed by nucleotide sequencing of the amplicon. The viral RNA was extracted with TRIzol® reagent (Life Technologies) according to the specifications of the manufacturer and used as template for cDNA synthesis. The viral RNA was reverse-transcribed using AMV-Reverse Transcriptase (Promega) and the antisense oligonucleotide 2B208R, which is situated in the 2B region. The Leader-P1-2A-coding region was amplified using Expand Long Template polymerase (Roche), as described previously (Chapter 2, Section 2.2.2). The PCR-amplified products were purified from the agarose gel with the Nucleospin® Extract kit (Macherey-Nagel) and the nucleotide sequence was determined with an ABI PRISM™ Big Dye™ Terminator Cycle Sequencing Ready Reaction kit v3.0 (Applied Biosystems). SAT-specific oligonucleotides (Chapter 2, Table 2.2) were used to determine the sequence of the full-length P1 and to obtain good overlaps. After cycle sequencing, the extension products were purified and resolved on an ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems). The sequence data was analysed using BioEdit 5.0.9 software (Hall, 1999) and Sequencher 5.0 DNA sequencing software (Gene Codes Corporation). All nucleotide possibilities were taken in consideration, which resulted in polymorphisms in the deduced amino acid sequences. The

nucleotide sequences were submitted to the NCBI GenBank under the accession numbers JQ692595 for KNP_{P3} and JQ692596 for $vKNP/SAT2_{BHK5}$.

3.2.5 Plaque assays

Plaque titration assays were performed on BHK-21, IB-RS-2 and CHO-K1 cells. VGM was used to prepare log_{10} dilutions of each virus. The medium was aspirated from the cell monolayers and 250 µl of each virus dilution added to the cells. Following incubation for 1 h at 37ºC, 2 ml of tragacanth overlay was added to each well and incubated for 48 h (Rieder *et al*., 1993). The cell monolayers were then stained with 1% (w/v) methylene blue in 10% ethanol and 10% formaldehyde, prepared with PBS (pH 7.4). All plaque assays were performed in duplicate.

3.2.6 Pyrosequencing

Amplicons for pyrosequencing were generated by using cDNA from $vKNP/SAT2_{BHK5}$ and KNP_{P3} as template. Amplicon sequencing fusion oligonucleotides were designed to anneal to the P1 region of $vKNP/SAT2_{BHK5}$ and KNP_{P3} with overlaps, and rendering products of 200 to 300 bp (Appendix B to this thesis). In addition, the fusion oligonucleotides for the respective viruses included a 19-mer adapter sequence at the 5'-end ("A" and "B") to match the components for forward and reverse reactions, and a 4-bp tag sequence allowing for the recognition of the two viruses upon sequence analysis. The tag sequences were "ACGT" and "CATG" for KNP_{P3} and $vKNP/SAT2_{BHK5}$, respectively. The 3' 20-29 nucleotides of the fusion oligonucleotides were complimentary to the FMDV genomes. PCR was performed using the Expand Long Template system (Roche Diagnostics) with added *Pfu* DNA polymerase (Promega). PCR reactions consisted of 30 cycles of denaturation at 95°C for 20 s, annealing at 56°C for 20 s and extension at 68°C for 3 min, with a final extension step of 68°C for 7 min. The PCR products were purified and then quantified using the Picogreen DNA Quantification kit (Invitrogen) on a fluorometer (BMG Labtech), as well as a DNA Pico chip on a 2100 Bioanalyzer (Agilent Technologies). Large volume emulsion-based clonal amplification (EmPCR) was performed at predetermined DNA copies per bead and the sequencing run was performed using the GS FLX instrument (Roche, 454 Life Sciences, Branford, CT, USA) at Inqaba Biotechnical Industries, Pretoria, South Africa.

3.2.7 Pyrosequencing data analysis

Low-quality reads were removed and the remaining sequences were built into a multi-sequence assembly using the Sanger-obtained sequences as references. The assemblies were performed using CLC Genomic Workbench (CLC bio). Single-nucleotide polymorphisms (SNPs) detected between pyrosequencing data reads and the reference sequences were exported to Excel® (Microsoft Windows) where the frequency of each SNP was calculated. Antoinette van Schalkwyk is thankfully acknowledged for assisting with data analysis.

3.3 RESULTS

3.3.1 Generation and characterisation of inter-serotypic chimera progeny

Several FMDV variants representing populations of the KNP/196/91 virus were recovered by propagation in PK, IB-RS-2 and BHK-21 cell cultures, as well as FMDV host-species such as cattle and pigs (Fig. 3.1). In order to obtain infectious vKNP/SAT2 chimeric virus, BHK-21, IB-RS-2 and CHO-K1 cells were transfected with *in vitro*-synthesised RNA transcripts and the results obtained following repeated passaging in cells are shown in Table 3.1. Initially, 80% CPE was observed for BHK-21 cells transfected with RNA derived from the chimeric pKNP/SAT2 clone, compared to 20% CPE at the second passage (after 48 h) in IB-RS-2 cells. Recovery of viable virus was less effective in the CHO-K1 cells where 5% CPE was only observed after four passages and 48 h. In agreement with the aforementioned recovery results, virus titres were the highest on BHK-21 and IB-RS-2 cells, followed by 10-fold lower titres on CHO-K1 cells, which have a restricted receptor phenotype (Jackson *et al*., 2003).

Phenotypic properties of the virus populations recovered from an infectious clone and from pigs were compared to other KNP/196/91 viruses, and are described in Table 3.2. The parental KNP/196/91 virus (KNP_{PK1}) was unable to replicate in CHO-K1 cells. However, during four subsequent passages in IB-RS-2 cells, KNP_{PK1RS4} acquired the necessary adaptations to produce plaques on CHO-K1 cells, a characteristic that has been linked to the ability to interact with heparan sulfate proteoglycan (HSPG) receptors during cell entry (Jackson *et al.*, 1996; Fry *et al.*, 1999; Zhoa *et al.*, 2003). Interestingly, the original viruses (KNP_{PK1} and KNP_{PK1RS4}) and pig-adapted viruses (KNP_{P2} and KNP_{P3}) produced larger sized, turbid plaques on BHK-21 and IB-RS-2 cells. In contrast, the vaccine seed stock ($KNP_{PK1RS4B1BHK5}$) and chimera viruses produced clear plaques on the above-mentioned cells in addition to displaying similar infectious titres and plaque morphology (Tables 3.1 and 3.2).

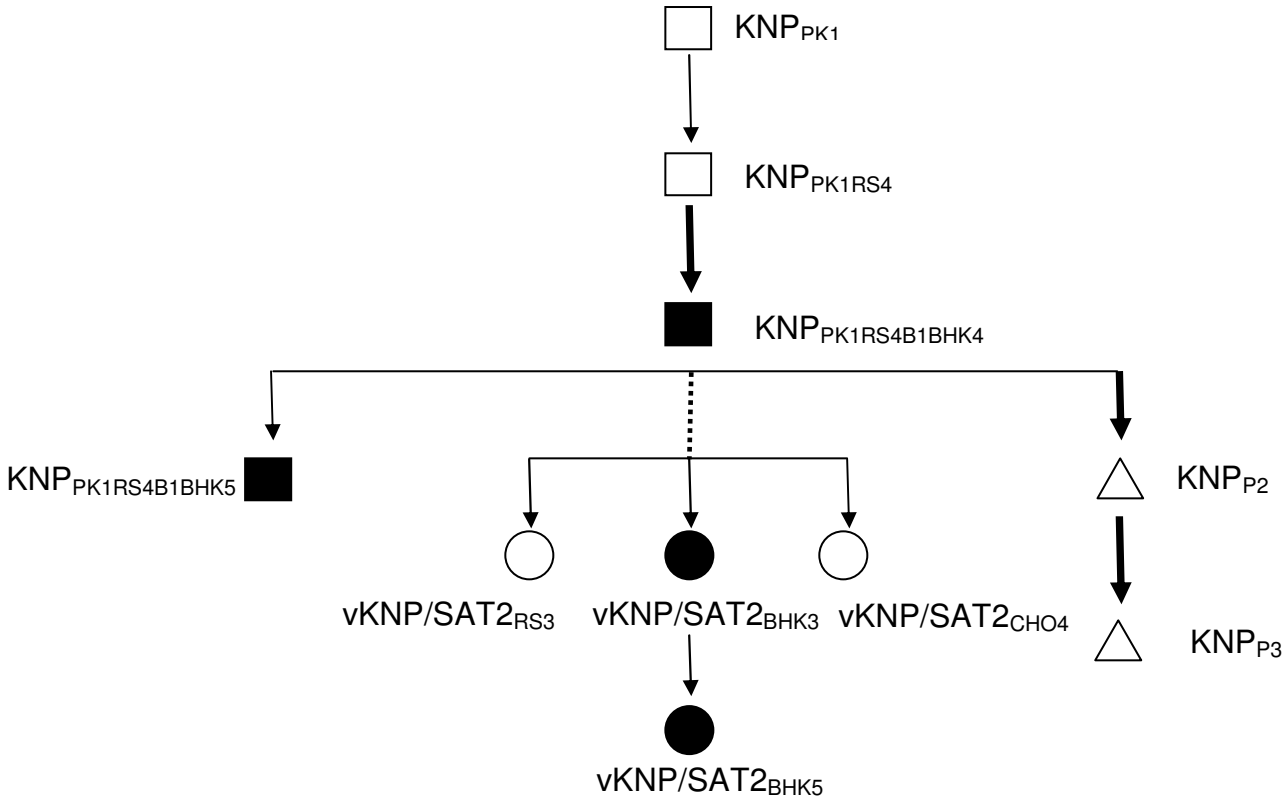

Fig. 3.1 Schematic representation of the origin of SAT1 type FMDV variants analysed in the present study. Uncloned populations are depicted by squares (derivatives of the parental virus) and triangles (pig-adapted virus). Recombinant viruses recovered from infectious cDNA clones are depicted by circles. Filled squares and circles represent FMDV adapted to BHK-21 cells. Thin arrows describe passage in cell lines (PK, IB-RS-2, BHK-21 and CHO-K1), large arrows signify adaptation to FMD host species (cattle and pigs) and the dashed line indicates transfection of *in vitro*-synthesised RNA into cells.

Table 3.1 Recovery and infectious titres of FMDV in host and non-host cells

FMDV	Passages	Percentage CPE and infectious titre[a]		
		BHK-21[b]	IB-RS-2[c]	CHO-K1[c]
vKNP/SAT2	1	80%	-	-
	2	100%	20%	-
	3	100%	100%	-
	4	ND	ND	5%
vKNP/SAT2	5	1.4×10^8	2.4×10^8	2.6×10^7
KNP/196/91	5	2.8×10^8	2.5×10^9	4.0×10^7

[a] Titres were determined in cells and expressed as p.f.u./ml.
[b] Percentage CPE measured at 24 h.
[c] Percentage CPE measured at 48 h.
-, not observed.
ND, not determined.

Table 3.2 Phenotypic properties of KNP/196/91 virus variants

FMDV	Description	Passages[a]	Plaque size[b] and morphology in cells			Virulence in animals[c]
			BHK-21	CHO-K1	IB-RS-2	
KNP_{PK1}	Isolation of field isolate from buffalo and passage in primary PK cells	1	L[†], turbid	NA	S/M, turbid	ND
KNP_{PK1RS4}	Adaptation to IB-RS-2 cells for use in the VNT	5	S/L, turbid	Mi/S, clear	S/M, turbid	ND
$KNP_{PK1RS4B1BHK4}$	Adaptation to cattle and multiple passages in BHK-21 cells	10	M/L, clear	Mi/S, clear	Mi/M, clear	Yes/10^{6} [d]
$KNP_{PK1RS4B1BHK5}$	Vaccine seed stock	11	S/L, clear	Mi/S, clear	Mi/M, clear	ND
KNP_{P2}	Pig adaptation	12	S/L, turbid	Mi, clear	Mi/M, turbid	Yes/10^{9}
KNP_{P3}	Challenge virus	13	S/L, turbid	Mi, clear	Mi/M, turbid	Yes
$vKNP/SAT2_{BHK3}$	Infectious clone recovered in BHK-21 cells	3	Mi/L, clear	Mi, clear	Mi/S, clear	ND
$vKNP/SAT2_{BHK5}$	Chimera vaccine seed stock	5	Mi/M, clear	Mi, clear	Mi/S, clear	ND
$vKNP/SAT2_{CHO4}$	Infectious clone recovered in CHO-K1 cells	4	Mi/S, clear	Mi, clear	Mi, clear	ND

All variant FMDV compared are derived from the parental KNP_{PK1} as depicted in Fig. 1 and as described in Materials and Methods.

[a] Total number of passages in cells and animals. Plaques were generated by infection of BHK-21, IB-RS-2 and CHO-K1 cells with the indicated virus strains, followed by the addition of tragacanth overlay and incubation at 37ºC for 24 h (IB-RS-2) or 44 h (BHK-21 and CHO-K1), prior to staining. Viruses that yielded plaques under the conditions of the assay were considered to have Mi, micro (<1 mm); S, small (1-2 mm); M, medium (3-4 mm); L, large (5-10 mm) plaque phenotypes.

[b] The diameter of the large plaques varied depending on the origin of the virus: PK and BHK-21, 7 mm; IB-RS-2, 8 mm; pig (P), 10 mm. NA, not applicable.

[c] Animals were inoculated 10^{4} $TCID_{50}$/ml FMDV and examined daily for lesions.

[d] Titres were determined in PK cells and expressed as $TCID_{50}$/ml.

ND, not determined.

3.3.2 Comparative analysis of outer capsid consensus sequences of SAT1 variants

The capsid-coding (P1) sequences for each of the nine FMDV described in Table 3.2 were determined by Sanger sequencing and compared for variation that may implicate differences in the biological properties of the viruses. Genetic heterogeneity for the outer capsids of KNP/196/91 variants is described in Table 3.3. Sequence differences for 1D of the KNP/196/91 viruses were 3.8% and 3.2% nucleotide and amino acid variation, respectively. Five amino acid changes were observed specific to vKNP/SAT2$_{BHK5}$ adapted to BHK-21 cells and the pig-adapted KNP$_{P3}$ isolate, of which two were located in 1B. The S18R amino acid difference for the vKNP/SAT2$_{BHK5}$ corresponded to a mutation that was introduced into the recombinant clone during the construction of pKNP/SAT2 and a mixed population (E108Q/E) was observed for KNP$_{P3.}$ Three areas of variability were observed in 1D. (1) G112R was present in the chimera populations (both for viruses recovered in BHK-21 and CHO-K1 cells), whereas G112R/G was observed for the KNP$_{P3}$ population. (2) The vKNP/SAT2$_{BHK5}$ chimera existed as a mixed population of R149S/C/R residues within the RGD of the G-H loop. (3) The residue substitution (D202Y) was unique to KNP$_{P3}$ and of possible antigenic importance, as it is associated with site 2 in type A in the C terminus of 1D. These mutations in the 1B and 1D capsid proteins did not relate to changes in the secondary structure of the SAT1 variant; however, residue-specific differences in hydrophilic profiles were observed (results not shown).

Further to the above differences between the chimera and pig-adapted viruses, other areas of variability within the amino acid sequences of the KNP/196/91 populations were observed across the outer capsid that may play a role in the functioning of the viral capsid. Fourteen mutations occurred between the low-passage viruses in cells from pig origin (KNP$_{PK1}$ and KNP$_{PK1RS4}$) and high-passage viruses that have been used to infect cattle, followed by additional rounds in cell culture (BHK-21) and adaptation to pigs. The mutations observed in 1B were Q74R and S196N for the cell- and pig-adapted viruses. In 1D, the residue change Y18H was observed, which is located in the T-cell epitope determined for O$_1$BFS and within a region of hypervariability for SAT1 viruses. Two identical mutations occurred in the C terminus of 1D (K206R and K210R) for the high-passage variants. Three residues were unique to KNP$_{PK1RS4}$ (residue changes E134D and Q170H in 1B and E135K/D/N in 1C), of which E135K possibly contributed to the acquired ability to infect CHO-K1 cells (Table 3.2). For the chimera population recovered in the CHO-K1 cells, a unique amino acid change

Table 3.3 Amino acid substitutions in the outer capsid consensus sequences of FMDV variants

FMDV	**1B**							**1C**		**1D**							
Polyprotein position[a]	**103**	**159**	**193**	**219**	**239**	**255**	**281**	**313**	**439**	**543**	**574**	**637**	**674**	**704**	**727**	**731**	**735**
Protein position	**18**	**74**	**108**	**134**	**154**	**170**	**196**	**9**	**135**	**18**	**49**	**112**	**149**	**179**	**202**	**206**	**210**
KNP_{PK1}[b]	S	Q	E	E	T	Q	S	D	E	Y	R	G	R	V	D	K	K
KNP_{PK1RS4}				D		H/Q			K/D/N/E								
$KNP_{PK1RS4B1BHK4}$		R					N	V		H	K	R		E		R	R
$KNP_{PK1RS4B1BHK5}$		R					N	V		H	K	R		E		R	R
KNP_{P2}	R	R					N	V		H	K			E		R	R
KNP_{P3}	R/S	R	Q/E				N	V		H	K	R/G		E	Y	R	R
$vKNP/SAT2_{BHK3}$	R	R					N	V		H	K	R		E		R	R
$vKNP/SAT2_{BHK5}$	R	R					N	V		H	K	R	S/C/R	E		R	R
$vKNP/SAT2_{CHO4}$	R	R			N		N	V		H	K	R		E		R	R
Epitope[c]		Site 2 (types O&A)							Site 3 (type A)	T-cell	Site 3 (type O)		Site 1 (type O)	Site 1 (type A12)	Site 2 (type A10)		
Charge[d]	P to +	P to +	- to P	None	None	P to +	None	- to H	- to +/-/P	P to +	None	H to +	+ to P	H to -	- to P	None	None

[a] Specific residue number in the SAT1 type FMDV capsid sequence.
[b] The amino acid sequence of the KNP_{PK1} isolate is assigned as the parental consensus sequence.
[c] Epitopes determined by monoclonal antibodies (taken from Mateo, 1995).
[d] H, hydrophobic; +, positive; -, negative; P, polar.

(T154N) in the 1B protein was observed. Nine mutations occurred after infection of cattle with KNP/196/91 virus and passage in BHK-21 cells for vaccine production purposes. These mutations were maintained during successive passages in cells with the addition of four more mutations in the KNP_{P3}, $vKNP/SAT2_{BHK5}$ and $vKNP/SAT2_{CHO4}$ populations (Table 3.3).

3.3.3 Pyrosequencing data analyses

Genetic differences detected by Sanger sequencing were further explored by pyrosequencing, as this method provides clonal sequences and individual variants can be characterised. Accuracy of this analysis was ensured by using PCR enzyme with high fidelity. A total of 3181 reads (average read length of 237 bp) and 2625 reads (average read length of 259 bp) were obtained for the $vKNP/SAT2_{BHK5}$ and KNP_{P3} amplicon libraries, respectively. The individual libraries were assembled to the corresponding reference sequence obtained by conventional Sanger sequencing. The assembled reads had an average of 200- to 300-fold coverage at each nucleotide position (Table 3.4). The greatest representation of nucleotides at any position was 694 and 496 for $vKNP/SAT2_{BHK5}$ and KNP_{P3}, respectively. Coverage was variable across the amplified region with $vKNP/SAT2_{BHK5}$ displaying the most coverage ($\leq$29.39%) for 101-400 repeats per nucleotide. Similarly, for KNP_{P3}, the distribution of repeat reads was highest with 101-300 at $\leq$44.8%.

Sequences containing indels (-) or base-uncertainties (N) and no other positions of variability were excluded from subsequent analyses. A total of 809 indels occurred over 281 nucleotides (frequency of 0.36) in $vKNP/SAT2_{BHK5}$ and 622 indels over 235 nucleotides (frequency of 0.27) for KNP_{P3}. Variable nucleotides and amino acids for the complete P1 region were determined using CLC Genomic Workbench (CLC bio) and BioEdit (Hall, 1999). The sequences were split according to two criteria. The data sets were grouped according to the frequency of a nucleotide at a specific position and the total number of positions per group was determined. In addition, the data was subdivided to represent the individual capsid-coding regions and the variation for each of these was described.

Table 3.4 Coverage (distribution of number of repeats/reads per nucleotide position in population)

FMDV	Coverage for reads[a]						
	<100	101-200	201-300	301-400	401-500	501-600	601-700
vKNP/SAT2$_{BHK5}$	17 (0.76)	597 (26.75)	656 (29.39)	461 (20.65)	152 (6.89)	149 (6.68)	200 (8.96)
KNP$_{P3}$	211 (9.45)	711 (31.86)	1000 (44.8)	127 (5.69)	183 (8.2)	-	-

[a] Number of reads. The value in brackets is the percentage coverage per nucleotide position (2232 nucleotides correspond to 100%).

3.3.4 Population diversity of chimera and pig-adapted viruses

The representation of nucleotides at any position on the genome allows for in-depth sequence analyses of the genetically related vKNP/SAT2$_{BHK5}$ and KNP$_{P3}$ viral populations. Heterogeneity was observed in frequency and diversity of the variants detected. Nucleotide substitutions occurred at 681 and 621 positions of the P1-coding region of vKNP/SAT2$_{BHK5}$ and KNP$_{P3}$, respectively. Of these, 253 substitutions occurred at the same position of the capsid-coding region between the two viruses and 190 of the substitutions resulted in identical nucleotide replacement.

Positions of variability with one or two representative nucleotides for vKNP/SAT2$_{BHK5}$ were 355 and 93, respectively (Table 3.5). Similarly for the KNP$_{P3}$ virus, the frequency of one to two substitutions was high, varying between 390 and 95. In comparison, high frequency of substitutions (>3) was less for both viruses (Table 3.5). The number of positions with low frequency substitutions was high for both vKNP/SAT2$_{BHK5}$ and KNP$_{P3}$ (frequency of between 0.1-1% were 488 and 425, respectively). In contrast, KNP$_{P3}$ had 1.5-times more substitutions with a frequency between 1-10% compared to vKNP/SAT2$_{BHK5}$ (79 and 51), yet vKNP/SAT2$_{BHK5}$ had one position with a frequency higher than 10%. Generally, the frequency of substitutions in the capsid sequence was less for KNP$_{P3}$ compared to vKNP/SAT2$_{BHK5}$. The most variable nucleotide frequency observed at a site for the pyrosequencing data was determined. The number of nucleotide possibilities compared to the reference sequences at a site was described as 2, 3 or 4. This was similar for vKNP/SAT2$_{BHK5}$ and KNP$_{P3}$ where 2 and 3 nucleotides were observed in *ca.* 21% and 1 to 2% of the population, respectively. In comparison, 4 nucleotides were observed at 2-fold more positions (2 and 1%) for vKNP/SAT2$_{BHK5}$ and KNP$_{P3}$, respectively.

The distribution of nucleotide variation for all four capsid proteins of KNP_{P3} was similar (Table 3.6). However, the 1A-coding region of $vKNP/SAT2_{BHK5}$ displayed 36.9% variable nucleotides. In contrast, the least number of variable nucleotides was observed for the 1A-coding region (20.0%) of KNP_{P3}, and 24.1%, 21.9% and 22.7% variable nucleotides was observed for the outer capsid-coding regions 1B, 1C and 1D, respectively. Similarly to the sequence disparity observed for 1A of $vKNP/SAT2_{BHK5}$, the variation in 1B was partly due to the first 37 nucleotides that are derived from SAT2/ZIM/7/83 (incorporated during cloning) of which 15 nucleotides contribute to variation. The 1C-coding region was the least variable with 16.9% variable nucleotides. In comparison, the 1D-coding region was the most variable (26.5%). Transitions were detected at 424 and 418 positions for $vKNP/SAT2_{BHK5}$ and KNP_{P3}, respectively. Transversions were detected at 123 and 101 positions for $vKNP/SAT2_{BHK5}$ and KNP_{P3}, respectively. The ratio of non-synonomous and synonomous substitutions (dN/dS) was 0.78 and 0.67 for $vKNP/SAT2_{BHK5}$ and KNP_{P3}, respectively. The distribution of amino acid variation was comparable for the outer capsids of $vKNP/SAT2_{BHK5}$ and the KNP_{P3} virus (Table 3.6), except for 1A of the chimera that was the most variable capsid protein (50.6%) and for KNP_{P3} the least variable (9.4%).

Amino acid sequences determined by standard Sanger sequencing and pyrosequencing were also compared. Four mutations were observed in the consensus sequences for the outer capsid proteins of $vKNP/SAT2_{BHK5}$ and KNP_{P3} viruses (Table 3.7). In 1B, a S18R change was observed by Sanger sequencing that remained S18 in the KNP_{P3} population as confirmed by pyrosequencing and occurred as polymorphic populations in $vKNP/SAT2_{BHK5}$ where R18 and Q18 appeared as the majority (99.8%) and minority (0.2%). Furthermore, in the 1B protein, E108 was observed by Sanger sequencing and pyrosequencing for $vKNP/SAT2_{BHK5}$. Whereas Q108 was observed for KNP_{P3} by Sanger sequencing, E108 (98.96%), as well as G108 (1.04%) was detected by pyrosequencing. In 1D, G112R was observed where the G112 was detected by pyrosequencing in KNP_{P3} and in $vKNP/SAT2_{BHK5}$ R112 was observed as the majority (99.75%) of the population with S112 and G112 as the minority (0.25 and 0.06%, respectively). In comparison to the Y202 and D202 in the variable C terminus of 1D for the KNP_{P3} and $vKNP/SAT2_{BHK5}$, respectively, both virus populations contained a D202 as the majority determined by pyrosequencing. N202, G202 or S202 was observed for $vKNP/SAT2_{BHK5}$ (<1%), whereas only G202 was observed for KNP_{P3} (0.8%).

Table 3.5 Comparison of nucleotide and amino acid data for pyrosequencing of FMDV from cells and pigs

Genetic composition	FMDV	Presence and frequency of substitutions at position[a]															
		1	2	3	4	5	6	7	8	9	10	11	13	17	19	23	50
Nucleotide	vKNP/SAT2$_{BHK5}$	355	93	29	17	9	6	1	1	-	1	1	2	-	2	2	1
	KNP$_{P3}$	390	95	25	8	1	-	-	1	3	-	-	-	1	-	-	-
Amino acid[b]	vKNP/SAT2$_{BHK5}$	ND	24	4	-	-	-	-	-	-	-	-	-	-	-	-	-
	KNP$_{P3}$	ND	31	2	-	-	-	-	-	-	-	-	-	-	-	-	-

[a] For each site the highest number of nucleotide or amino acids are indicated.
[b] The number of variable amino acids per position are indicated as the possibility of 2 or 3 variable amino acids.
-, none detected.
ND, not determined.

Table 3.6 Genetic heterogeneity observed by pyrosequencing of the cell- and pig-adapted viruses

	Capsid region	Total nucleotides	Variable nucleotides		Total amino acids	Variable amino acids	
FMDV			**vKNP/SAT2$_{BHK5}$**	**KNP$_{P3}$**		**vKNP/SAT2$_{BHK5}$**	**KNP$_{P3}$**
	1A	255	94[a] (36.9)	51 (20.0)	85	43[b] (50.6)	8 (9.4)
	1B	657	137 (20.9)	158 (24.1)	219	64 (29.2)	85 (38.8)
	1C	663	112 (16.9)	145 (21.9)	221	58 (26.2)	64 (29)
	1D	657	174 (26.5)	149 (22.7)	219	76 (34.7)	72 (32.9)
	P1	2232	517 (23.2)	503 (22.5)	744	241 (32.4)	229 (30.8)

[a] Total number of variable nucleotides (percentage variability).
[b] Total number of variable amino acids (percentage variability).

Table 3.7 Polymorphic sites in the capsid proteins for KNP_{P3} and $vKNP/SAT2_{BHK5}$

		KNP_{P3}							$vKNP/SAT2_{BHK5}$											
Protein	**Codon[a]**	**Ref [b]**	**Ref AA[c]**	**NGS AA[d]**	**AA1[e]**	**F1[f]**	**AA2**	**F2**	**Ref AA**	**NGS AA**	**AA1**	**F1**	**AA2**	**F2**	**AA3**	**F3**	**AA4**	**F4**	**AA5**	**F5**	**AA6**	**F6**
1A	4	X	Q	Q					H	H												
	8	X	A	A					V	X	V	99.03	G	0.33	A	0.33	I	0.32	T	0.11	S	0.11
	14	Q	Q	X	Q	99.00	R	1.00	Q	Q												
	51	S	S	S					S	X	S	99.69	R	0.16	G	0.16						
	76	X	F	X	F	99.58	L	0.42	I	X	I	99.71	T	0.15	V	0.15	A	0.02				
	80	X	V	V					F	X	F	99.29	S	0.71								
1B	103/18	X	S	S					R	X	R	99.80	Q	0.20								
	159/74	R	R	X	R	99.11	Q	0.89	R	R												
	193/108	X	Q	X	E	98.96	G	1.04	E	E												
	219/134	E	E	X	E	99.52	G	0.48	E	X	E	99.66	D	0.35								
	239/154	T	T	T					T	T												
	255/170	Q	Q	Q					Q	Q												
	281/196	N	N	N					N	N												
1C	313/9	V	V	X	V	99.45	I	0.55	V	V												
	439/55	E	E	E					E	X	E	99.37	G	0.63								
1D	543/18	H	H	H					H	H												
	574/49	K	K	K					K	K												
	637/112	X	G	G					R	X	R	99.75	S	0.25	G	0.06						
	674/149	R	R	R					R	X	R	99.56	H	0.22								
	704/179	E	E	E					E	X	E	99.71	D	0.15								
	727/202	X	Y	X	D	99.20	G	0.80	D	X	D	98.81	N	0.39	G	0.79	S	0.32				
	731/206	R	R	X	R	99.20	G	0.80	R	R												
	735/210	R	R	X	R	99.20	G	0.80	R	R												

[a] Polyprotein and protein position.
[b] IUPAC for the references of the viruses KNP_{P3} and $vKNP/SAT2_{BHK5}$. X refers to changes in amino acids.
[c] Reference amino acid sequence determined by standard Sanger sequencing.
[d] IUPAC for the NGS data.
[e] Amino acid differences described as AA1 whereby the numbers indicate all possible residues.
[f] Frequency at which the amino acid was detected.

3.4 DISCUSSION

Properties of FMDV in cell culture and *in vivo* are influenced by high mutation rates of this RNA virus and quasispecies dynamics where related but non-identical viral genomes exist within a population. Two FMDV of the same origin, but with different properties, *viz.* a chimera derived from a cloned population characterised by adaptation to BHK-21 cells and an animal-adapted virus isolated from pigs were compared in genetic and phenotypic composition. Although much work has been done on clonal populations, these were all from plaque-purified viruses (Escarmĩs *et al*., 1996, 2002). Passage of such cloned or small viral populations leads to bottleneck effects. In contrast, large population passages increases viral fitness (Domingo *et al*., 2003). Here, a chimera representing a conceivably small population and pig-adapted FMDV were compared and the diversity in the populations estimated for the viral capsids. In addition, the P1 region for the FMDV was compared for sequence variation that may implicate differences in cell-binding abilities or changes to the antigenic component of the virus.

Prior to the acquisition of positively charged amino acid residues R74 (1B), R112, R206 and R210 (1D) following multiple passage in cells and animals, the KNP_{PK1RS4} virus produced plaques in CHO-K1 cells. A single residue change (E135K) results in the acquisition of positive charges on the capsid to facilitate interaction with HSPG receptors as has been observed for the SAT1/NAM/307/98 virus (Maree *et al*., 2010). When viruses are replicating in cells in a constant environment, increased capacity to induce CPE and progeny will be selected (Domingo *et al*., 2003). Recovery of viable FMDV from genome-length cDNA clones were within two passages in BHK-21 and IB-RS-2 cells. In comparison, the recovery rate of vKNP/SAT2 in CHO-K1 cells was low. Comparable varying levels of virulence displayed by FMDV in different cell lines have been reported (Baranowski *et al*., 1998). The T154N change in 1B is not likely to affect the cell-binding capacity of the virus, which suggests that other virulence determinants for FMDV may be involved.

The diversity within the consensus sequence of the KNP/196/91 viruses was compared. Within this population, mutants with substitutions relative to a dominant genome sequence occur as is expected within the variation in quasispecies viruses (Domino *et al*., 1996). Introduction of amino acid changes on the capsid during FMDV infection has been well documented in different cell lines (Sobrino *et al*., 1983; de la Torre *et al*., 1988; Diez *et al*.,

1989; Sevilla *et al*., 1996; Carillo *et al*., 2007; Maree *et al*., 2011a) and animal hosts (Gebauer *et al*., 1988; Tami *et al*., 2003). The importance of a single amino acid change in viral properties was illustrated where the 3A protein has been implicated in determining the host range through adaptation of the virus to guinea pigs (Núñez *et al*., 2001). The sequences of the chimera viruses that were derived from the genome-length cDNA clone were all similar, but different compared to the earlier passage viruses (prior to adaptation to bovine and BHK-21 cells). Genetic heterogeneity for the KNP/196/91 viruses was comparable to a study of cell- and pig-adapted FMDV by Zhao *et al*. (2003). Sanger sequencing identified 14 positions of variable amino acids for the outer capsid proteins. Most variation between low-passage and high-passage viruses were observed in 1B and 1D, similar to observations for *in vitro* studies by Carillo and co-workers (2007). Acquisition of positively charged amino acid residues in 1D (positions 112, 206 and 210) has been associated with cell adaptation for KNP/196/91 (Maree *et al*., 2010). In addition to recognition of cellular receptors, amino acid residue substitutions were associated with regions of hypervariability and coincided with areas identified as antigenic determinants (Maree *et al*., 2011b).

Two KNP/196/91 isolates were exposed to the host's immune response (pig-adapted viruses). The genetic similarity between the two pig isolates (KNP_{P2} and KNP_{P3}) is in agreement with previous characterisation studies of FMDV in pigs (Carillo *et al*., 1990, 1998, 2007), where the consensus sequence represents the majority of the progeny population. The KNP_{P2} virus displayed G112 as observed for the original two isolates (KNP_{PK1} and KNP_{PK1RS4}), whereas all the other viruses contained a R112. For the KNP_{P3} virus, there was a mixed population of G and R at this position. This genetic heterogeneity may be associated with viruses at different cell adaption levels as observed previously (Botha, 2002; Maree *et al*., 2010). Among chimera viruses that contain polymorphisms, R149S/C were observed within the RGD triplet and similar mutations were also detected in the RGD for type C (Martínez *et al*., 1997). Contrary to other observations (Gebauer *et al*., 1988; Tami *et al*., 2003), there were no additional mutations in the G-H loop. In addition to the involvement of the RGD in cell binding, it is also critical in interaction with neutralising antibodies (Verdaguer *et al*., 1995). Overlapping regions on the FMDV capsid may be involved in cell binding and serve as antigenic sites, and is therefore co-functional in determination of host range and antigenicity (Baranowski *et al*., 2001). The pig-adapted virus (KNP_{P3}) displayed additional changes; E108E/Q in 1B and D202Y in 1D. In contrast, no amino acid changes were observed in 1D in a study where type O virus was subjected to serial passage in pigs and most changes were in

2C and 3D (Carillo *et al*., 2007). Such virus variants with dissimilar host range and virulence can arise *in vivo* as has been described by others (Beard and Mason, 2000; Núñez *et al*., 2001). *In vivo* there is great antigenic diversity (Mateu *et al*., 1988) as replication in an animal host increases the variation in the FMDV quasispecies since the viruses are subjected to positive selection, negative selection and random drift within the animal (Domingo *et al*., 2002). In addition, fitness loss can occur in host species as was introduced during serial passage of type O virus in pigs (Carillo *et al*., 2007). However, in this study the KNP/196/91 population retained diversity and virulence in animals.

Using Sanger sequencing, a mutation may appear to be fixed within the FMDV population although there may still be extensive genetic variability. The variation in functionally important regions of the viral genome was further explored by determining the quasispecies population of the chimera and pig-adapted viruses by pyrosequencing as it has the advantage of being a high-throughput method to sensitively detect and characterise variants. Multiple repeats of the sequence at any position of the capsid were obtained by pyrosequencing on the GS FLX platform and indicated variability in nucleotides and amino acids for both populations. In the host species, inter-host competition will select for more virulent forms and in cells viral genomes with increased fitness will result in the generation of plaques (Manrubia *et al*., 2005). Next-generation sequencing techniques were applied to detect SNPs across the capsid-coding regions at many different positions (>300) for both KNP/196/91 populations. In contrast, more than one substitution occurred at fewer positions (<100) for vKNP/SAT2$_{BHK5}$ and KNP$_{P3}$. The level of heterogeneity for the outer capsid proteins was comparable for both viruses and in most cases the amino acid that was present in the Sanger sequence was also detected by pyrosequencing. However, for vKNP/SAT2$_{BHK5}$ and KNP$_{P3}$ unique amino acid residues were detected within the population that were different compared to the sequences determined by conventional methods. The G112 in 1D of the majority of the pig-adapted population explains the similar turbid plaque morphology as the low-passage viruses and confirms the contribution of this region to cell-binding properties of SAT1 viruses (Maree *et al*., 2010, 2011a). It is uncertain how much of the population observed by pyrosequencing are viable viruses as this was not determined. It has been established that fitter viruses will out-compete viral genomes and virions with defects in replication and stability (Domingo *et al*., 2003).

The results obtained in this study indicated that pyrosequencing may be used for better understanding of population diversity of RNA viruses. Taken together, most variation observed for the outer capsid proteins was due to *in vitro* or *in vivo* adaptation of FMDV. Residue changes involved in altering cell-binding capacity and plaque morphology were observed at positions 135 of 1C and 112 of 1D. KNP/196/91 viruses with an altered plaque morphology phenotype retained infectivity and virulence in animals. Despite different environments (cell culture/animal host species) that contributed to intra-population variation, pyrosequencing detected considerable heterogeneity within the clone-derived chimera and pig-adapted virus populations. The variance in viral sequences for both the chimera and host-adapted populations of FMDV was unexpected as the origin from a cDNA clone and adaptation to host species could potentially limit variation. The results of this study support the notion of using genome-length cDNA clones to generate chimeric vaccines with improved biological properties (Rieder *et al*., 1994; van Rensburg *et al*., 2004; Fowler *et al*., 2008; Blignaut *et al*., 2011). Chimera viruses with comparable genetic heterogeneity to viruses subjected to *in vivo* and *in vitro* environments may be used as vaccine antigens with advantageous cell-binding abilities or enhanced antigenic determinants for improved FMD control.

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CHAPTER FOUR

CUSTOM-ENGINEERED CHIMERIC FOOT-AND-MOUTH DISEASE VACCINE ELICITS PROTECTIVE IMMUNE RESPONSES IN PIGS

4.1 INTRODUCTION

Most of Europe, North America and some countries in South America have eradicated foot-and-mouth disease (FMD) through the administration of inactivated FMD vaccines since the 1960s (Ward *et al*., 2007). Despite international regulations limiting routine vaccination in many parts of the world, it remains an important strategy for disease control in countries where FMD is endemic. In sub-Saharan Africa, control of FMD is complicated by the African buffalo that serves as a reservoir of the FMD virus (FMDV) and contributes to the spread of FMD to other wildlife species and livestock. Moreover, genetic and antigenic characterisation of FMDV field isolates has revealed that these viruses evolve rapidly in different geographical areas (Esterhuysen, 1994; Vosloo *et al*., 1995; Bastos *et al*., 2001, 2003). This has led to the available vaccines being unable to adequately cover the extent of antigenic variation within the South African Territories (SAT) types of FMDV (Reeve *et al*., 2010). Thus, for vaccination to be effective in sub-Saharan Africa, it requires the incorporation of vaccine strains representative of viruses circulating in that geographical area and that such strains should be available for specific regions. However, adaptation of wild-type SAT viruses in cell culture to produce high yields of stable antigen is an intricate and time-consuming process that is often associated with a low success rate.

Infectious cDNA clone technology (Zibert *et al*., 1990; Rieder *et al*., 1993, 1994) for FMDV SAT viruses has provided a valuable tool for genetic manipulation and biological characterisation of field and laboratory strains (van Rensburg *et al*., 2004; Storey *et al*., 2007; Maree *et al*., 2010). Chimeras, containing the external capsid-coding region (1B-1D/2A) of another FMDV, retain the replication machinery of the backbone as the P1, 2A and 3C proteins are required for FMD viral capsid assembly (Beard *et al*., 1999), whilst acquiring the antigenic properties of the parental virus, including its ability to bind to cellular receptors that allow host-cell internalisation (Maree *et al*., 2010). This technology may therefore be utilised towards the production of custom-made vaccines specific to geographical regions or in cases of sudden FMDV outbreaks. For such a chimera-derived vaccine to be a viable alternative to currently used vaccines, it should have high titre replication in cell culture, stability of the virus particle and antigen, recovery of high 146S antigen mass following chemical inactivation, appropriate immunological specificity and the ability to elicit a protective immune response in animals (Rweyemamu, 1978; Doel, 2003).

In this study, the feasibility of a chimeric vaccine was assessed by determining its immunogenicity and protective ability following immunisation of pigs. The vaccine was prepared from a chimeric virus containing the external capsid-coding region of a rapidly replicating, cell-adapted SAT1 vaccine strain, KNP/196/91, that was exchanged into the genetic background of a SAT2 infectious clone. The recovered chimeric virus exhibited comparable plaque morphologies, infection kinetics, virion stability and antigenic profiles to the KNP/196/91 parental virus. In a full potency test of the chimeric vaccine in pigs, good humoral immune responses were elicited and the majority of the animals were protected against homologous FMDV challenge and passed the 50% protective dose (PD_{50}) requirement of the Office International des Epizooties (OIE) for emergency vaccine use. These results suggest that custom-engineered chimeric FMD vaccines can be produced and applied in a fashion similar to the current inactivated vaccines.

4.2 MATERIALS AND METHODS

4.2.1 Cell lines, viruses and plasmids

Baby hamster kidney-21 cells clone 13 (BHK-21; ATCC CCL-10), Instituto Biologico Renal Suino-2 cells (IB-RS-2) and Chinese hamster ovary cells strain K1 (CHO-K1; ATCC CCL-61) were propagated as described previously (Storey *et al*., 2007). Primary pig kidney cells (PK) were maintained in RPMI medium (Sigma-Aldrich) supplemented with 10% fetal calf serum (FCS; Delta Bioproducts). The SAT1 virus KNP/196/91 (passage history: PK_1RS_4), originally isolated from a buffalo, was passaged in cattle and BHK-21 cells (passage history: $PK_1RS_4B_1BHK_4$), as described previously (Chapter 3, Section 3.2.2). Homologous challenge virus was prepared by three passages of KNP/196/91 in pigs (passage history: $PK_1RS_4B_1BHK_4P_3$) to adapt the virus to the host-species. The construction of plasmid pKNP/SAT2 has been described previously (Chapter 3, Section 3.2.2). In essence, the external capsid-coding region (1B-1D/2A) of plasmid pSAT2, a genome-length infectious cDNA clone of SAT2/ZIM/7/83 (van Rensburg *et al*., 2004), was replaced with that of KNP/196/91 (passage history: $PK_1RS_4B_1BHK_4$) to yield plasmid pKNP/SAT2.

4.2.2 RNA synthesis, transfection and virus recovery

For RNA synthesis, plasmids pKNP/SAT2 and pSAT2 were linearised with *Swa*I and *Not*I, respectively, and *in vitro* transcribed using the MEGAscript™ T7 kit (Ambion). The *in vitro*-

transcribed RNA was transfected into cells using Lipofectamine 2000 reagent (Invitrogen) and viruses were recovered and characterised according to the procedures described previously (Chapter 3, Sections 3.2.3 and 3.2.4). The viruses derived from the genome-length cDNA, designated vKNP/SAT2 and vSAT2, were confirmed by nucleotide sequencing and used in all subsequent experiments. Viral amplification was performed in BHK-21 cell monolayers for chimeric (vKNP/SAT2), parental (KNP/196/91) and vSAT2 viruses. Viruses were harvested at 90% cytopathic effect (CPE) at passage five.

4.2.3 Plaque and growth kinetics assays

BHK-21 cell monolayers were infected with KNP/196/91, vKNP/SAT2 and vSAT2 at a multiplicity of infection (m.o.i.) of 2 pfu/cell. After 1 h of adsorption, cells were washed with MBS (MES-buffered saline, pH 5.5) and then incubated at 37°C with virus growth medium (VGM; Eagle's basal medium supplemented with 1% HEPES and 1% FCS). At several time points post-infection, cells and culture medium were frozen at -80°C. Virus titres were determined by plaque assays as described previously (Chapter 3, Section 3.2.5).

4.2.4 Virus purification and stability

The vKNP/SAT2 and KNP/196/91 viruses were concentrated using 8% PEG-8000 (Sigma-Aldrich) and purified on sucrose density gradients (10-50%), 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 (extinction coefficient $E_{259nm}(1\%)$ = 79.9; Doel and Mowat, 1985) were pooled, the amount of antigen (µg) calculated from the peak area, analysed by SDS-PAGE and titrated on BHK-21 cells. The stability of the purified virion particles at different temperatures was determined by incubating the virion particles, diluted 1:50 in 2 × TNE buffer (100 mM Tris [pH 7.4], 10 mM EDTA, 150 mM NaCl), at 25, 37, 45 and 55°C for 30 min, and at 4 and 25°C over a period of four weeks. Following cooling on ice, the viruses were titrated on BHK-21 cells. The stability of the purified virion particles at different pH (pH 6.5 to 9.0) and NaCl concentrations (50 mM to 1.5 M) was determined by diluting the virion particles 1:50 in TNE buffer and incubation at room temperature for 30 min. After pH neutralisation with buffer (1 M Tris [pH 7.4], 150 mM NaCl), the viruses were titrated on BHK-21 cells.

4.2.5 Antigen preparation and vaccine formulation

The vKNP/SAT2 and KNP/196/91 viruses harvested from infected BHK-21 cell monolayers were inactivated with 5 mM binary ethyleneimine (BEI) for 26 h at 26°C, concentrated and purified as described above. Two separate vaccine formulations, incorporating vKNP/SAT2 and KNP/196/91 inactivated 146S antigens as double oil emulsions with Montanide ISA 206B (Seppic), were prepared. The antigen was diluted in Tris/KCl buffer (0.1 M Tris, 0.3 M KCl, pH 7.5) to the required concentration. The oil adjuvant was subsequently mixed into the aqueous antigen phase (50:50) at 30°C for 15 min and stored at 4°C for 24 h. This was followed by a second brief mixing cycle for 5 min. A placebo vaccine was formulated that contained all the components, except antigen.

4.2.6 Immunisation of guinea pigs

The above formulated vaccines were initially tested in guinea pigs to determine the immunogenicity of the respective antigens. Six groups of 20 female guinea pigs (400-800 g each) were immunised intramuscularly with vaccines containing 0.3, 0.6 and 1.2 µg of the inactivated vKNP/SAT2 or KNP/196/91 antigen. Control animals received the placebo vaccine formulation. Ten animals from each group were bled alternately on 0, 7, 14, 21 and 28 days post-vaccination (d.p.v.). Animals were anaesthetised intramuscularly with a combination of Xylazine and Ketamine.

4.2.7 Vaccination and viral challenge of pigs

Thirty-four FMD-seronegative pigs (3-4 months of age and weighing 25-30 kg) were divided randomly into six groups of five animals each, and one control group of four animals. Each group was housed in a separate room in the high-containment animal facility of the Onderstepoort Veterinary Institute (ARC-OVI). Subsequent to an initial acclimatisation period, the pigs were vaccinated by the intramuscular route immediately caudal to the ear with 2 ml (full dose), 0.5 ml (quarter dose) and 0.125 ml (one-sixteenth dose) of 3 µg/ml of either the vKNP/SAT2 or KNP/196/91 vaccines. Control animals were vaccinated with a placebo vaccine formulation that lacked viral antigen. Serum samples were collected at 0, 7, 14, 21 and 28 d.p.v. for serological assays. At 28 d.p.v. the pigs were inoculated intra-epidermally in the coronary band of the left hind heel bulb with 10^4 $TCID_{50}$ KNP/196/91 challenge virus. During each of these procedures the pigs were sedated using Azaperone. The animals were examined daily for fever and clinical signs. Body temperatures of ≥39.6

and ≥40°C were considered as mild and severe fever, respectively. Upon observation of generalisation of clinical lesions to other sites (*e.g.* to the snout and other legs), pigs were removed from the experiment and euthanised. At day 10 post-infection the remainder of the animals were euthanised.

4.2.8 Antibody detection in guinea pigs

A SAT1/KNP/196/91-specific sandwich ELISA was performed on guinea pig sera to measure the amount of anti-KNP/196/91 antibodies present in each sample. The KNP/196/91 virus was added to a 96-well microtitre plate (Nunc™ Maxisorp) coated with rabbit anti-KNP/196/91 antiserum. After incubation overnight at 4ºC the plates were washed with 1 × PBS containing 0.05% (v/v) Tween-80 (PBS-T80). Of each sample, 100 µl was added to normal bovine serum followed by diluting 1:1000 in 1 × PBS containing 0.5% (w/v) casein (PBS-casein). The dilution was added to the plates in triplicate and diluted 1:1 in PBS-casein. The plates were then incubated for 1 h at 37°C and washed with PBS-T80. Horseradish peroxidase (HRP)-conjugated rabbit anti-guinea pig IgG (Sigma-Aldrich), diluted 1:100 in PBS-casein, was added and the plates incubated for 1 h at 37°C. The plates were washed with PBS-T80, followed by addition of the substrate solution consisting of 1 ml of 3,3'.5,5' tetramethylbenzidine (TMB), sodium phosphate/citric acid buffer and 30 ml of H_2O_2. After incubating for 15 min at room temperature, the reactions were stopped with 1 M H_2SO_4 and the optical density (OD) at 492 nm was measured with a Labsystems Multiscan Plus Photometer. The titre was determined from the $\log_{10}$ reciprocal antibody dilution giving 1.0 OD_{492} unit. Sera were considered positive when the titre was equal or greater than twice the value of the negative control sera.

4.2.9 Antibody detection in pigs

Antibodies in pig sera against KNP/196/91 were detected by a SAT1/KNP/196/91-specific solid-phase competition ELISA (SPCE). The SPCE was essentially carried out as described above. Briefly, the KNP/196/91 virus was trapped by rabbit anti-KNP/196/91 antiserum. After incubation overnight at 4ºC the plates were washed with PBS-T80. Of each sample, 100 µl of a 1:20 dilution in PBS-casein was added in triplicate and diluted 1:1 in 50 µl of PBS-casein across the plate. Guinea pig anti-KNP/196/91 antiserum diluted 1:6000 in PBS-casein was added to the wells and then incubated and washed. The addition of anti-species conjugate and the subsequent detection steps were as described before. Antibody titres were

determined at the dilution where 50% inhibition was observed between the pig sera and the guinea pig anti-KNP/196/91 antisera. A cut-off value of $\log_{10}$ 1.7 was considered positive.

4.2.10 Virus neutralisation assay

Neutralising antibodies against SAT1/KNP/196/91 in serum samples collected at 28 d.p.v. from pigs were measured with the virus neutralisation test (VNT), according to the method described in the OIE Manual of Standards (2009) using IB-RS-2 cells in microtitre plates. The antibody titres were calculated as the $\log_{10}$ of the reciprocal of the final serum dilution that neutralised 100 $TCID_{50}$ of virus in 50% of the wells.

4.2.11 Data analysis

Antibody titres were presented on a $\log_{10}$ scale as means and standard deviations (SD). Repeated measures ANOVA analyses were performed to estimate the effects of vaccination strain and dose on ELISA antibody titres over time. VNT titres were compared using the Student's *t*-test with Bonferroni adjustment for multiple comparisons. Statistical analyses were performed using commercially available software (SPSS v.17.0 for Windows, SPSS Inc., Chicago, IL, USA) and results interpreted at the 5% level of significance.

4.3 RESULTS

4.3.1 Parental viral properties are retained in the recovered vKNP/SAT2

The FMDV SAT1 isolate KNP/196/91 has been proven to be an efficient vaccine strain displaying the required qualities for a good vaccine candidate (see Introduction). Hence, KNP/196/91 virus that displayed broad antigenic coverage (Reeve *et al*., 2010) was utilised to investigate the prospect of engineering chimeric vaccines to provide protection against FMD for specific southern African regions affected by circulating SAT1 viruses.

Plaque morphologies for the vKNP/SAT2, KNP/196/91 and vSAT2 viruses were compared on BHK-21, IB-RS-2 and CHO-K1 cell lines (Fig. 4.1a). As shown in Fig. 4.1a, the vKNP/SAT2 and KNP/196/91 viruses formed micro (<1 mm) to medium (3-5 mm) or small (1-2 mm) to large (6-7 mm) plaques on BHK-21 cells, respectively. On IB-RS-2 cells, the plaques were micro to small and large for the vKNP/SAT2 and KNP/196/91 viruses,

respectively. The vKNP/SAT2, KNP/196/91 and vSAT2 viruses each formed clear plaques on CHO-K1 cells, which is characteristic of viruses that use heparan sulfate (HS) as receptors. The comparable ability to infect cultured cells originating from different species is indicative that the vKNP/SAT2 capsid retained the characteristics of the KNP/196/91 virus. The deduced amino acid sequences of the external capsid-coding region for the vKNP/SAT2 and KNP/196/91 viruses were identical following transfection and recovery in BHK-21 cells (Appendix C to this thesis).

In growth curves performed in BHK-21 cells (Fig. 4.1b), vKNP/SAT2 displayed similar replicative ability in BHK-21 cells to KNP/196/91 up to 6 h post-infection. At 12 h post-infection, KNP/196/91 and vKNP/SAT2 had peak infectivity titres of 8.9×10^8 and 8.2×10^8 pfu/ml, respectively. For vaccine purposes the optimal harvest time for both vKNP/SAT2 and KNP/196/91 is 12 h post-infection. This compares favourably to the time balance for stable particle survival resulting from the processes of virus assembly, rate of inactivation and the effect of proteolytic enzymes released during virus-infected cultures (Doel and Collen, 1983). The rate of production of infectious vSAT2 particles was lower than for the SAT1 viruses from 4 to 12 h.

To determine the antigenic profiles of vKNP/SAT2 and KNP/196/91, a virus neutralisation test was performed against a panel of SAT1 and SAT2 pig and cattle antisera (Fig. 4.2). The data showed similar neutralisation values for the wild-type (passage history: PK_1RS_4) and cell culture-adapted (passage history: B_1BHK_9) isolates, indicating that the amino acid variation with adaptation to BHK-21 cells did not significantly influence the major antigenic determinants. As expected, SAT2 antisera showed no cross-neutralisation with either of the SAT1 isolates (Fig. 4.2).

4.3.2 Antigen yield is determined by the biophysical properties of FMDV

Biophysical stability of the infectious virus or antigen has been correlated with the protective nature of FMD vaccines (Doel and Baccarini, 1981). With this in mind, vKNP/SAT2 and KNP/196/91 were compared in terms of their biophysical properties after treatment at different temperatures, pH and salt concentrations. Following virus exposure to temperatures of 25, 37, 45 and 55°C (Fig. 4.3a), viable virus particles were detected at decreased concentrations for vKNP/SAT2, similar to those of KNP/196/91. After 30 min at 55°C, the

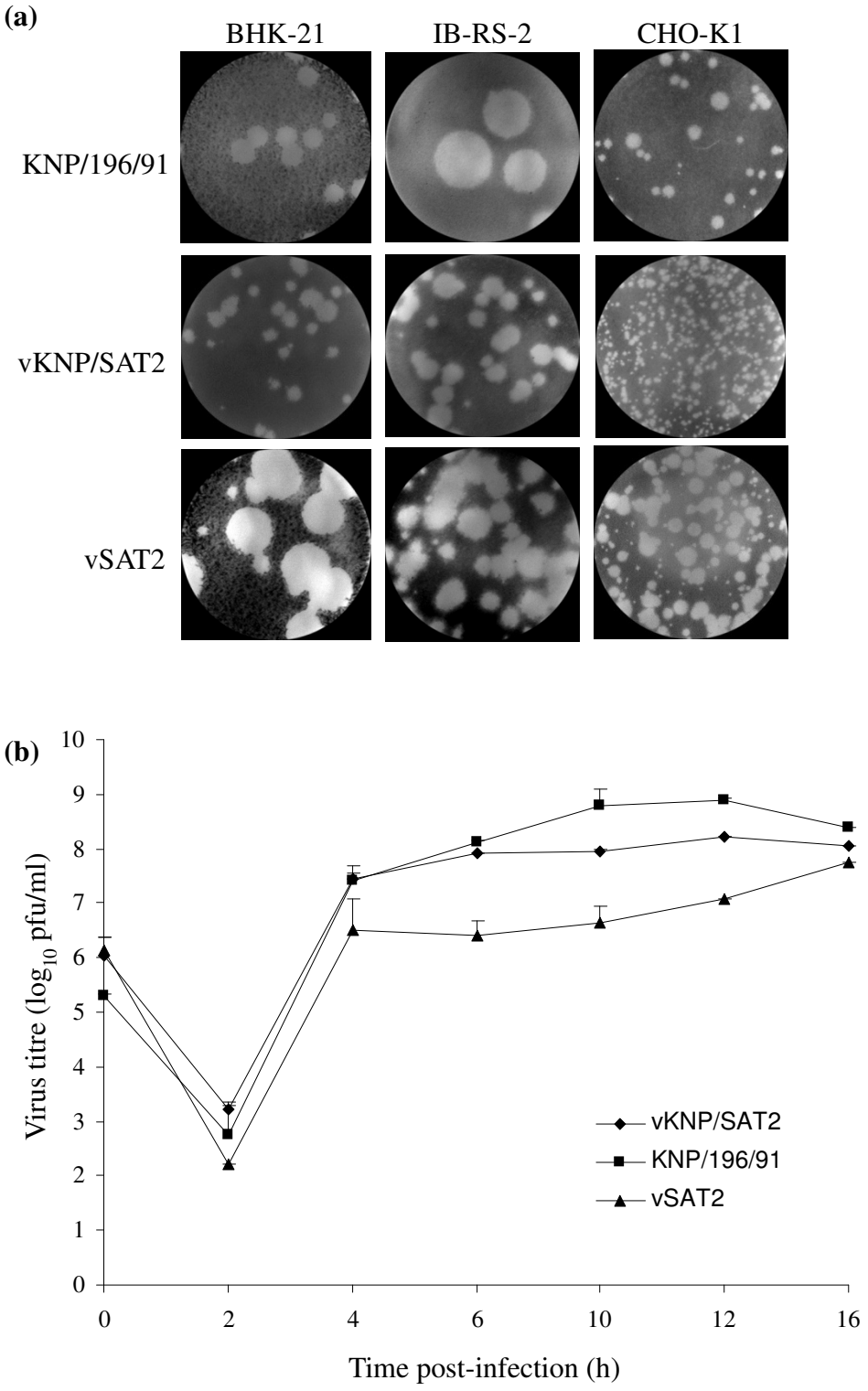

Fig. 4.1 (a) FMDV replication in BHK-21, IB-RS-2 and CHO-K1 cell cultures expressing diverse cellular receptors utilised by FMDV for cell entry. Morphology of plaques for the KNP/196/91 parental virus, vKNP/SAT2 chimera and vSAT2 were determined by plaque assay at 48 h post-infection. (b) Growth curves for the KNP/196/91 parental virus, vKNP/SAT2 chimera and vSAT2. BHK-21 cells were infected at a m.o.i. of 2 pfu/cell for 1 h and subsequently washed with MES-buffered saline (pH 5.5). Samples were frozen at selected time intervals and titrated on BHK-21 cells. Each data point represents the mean (± SD) of at least two wells.

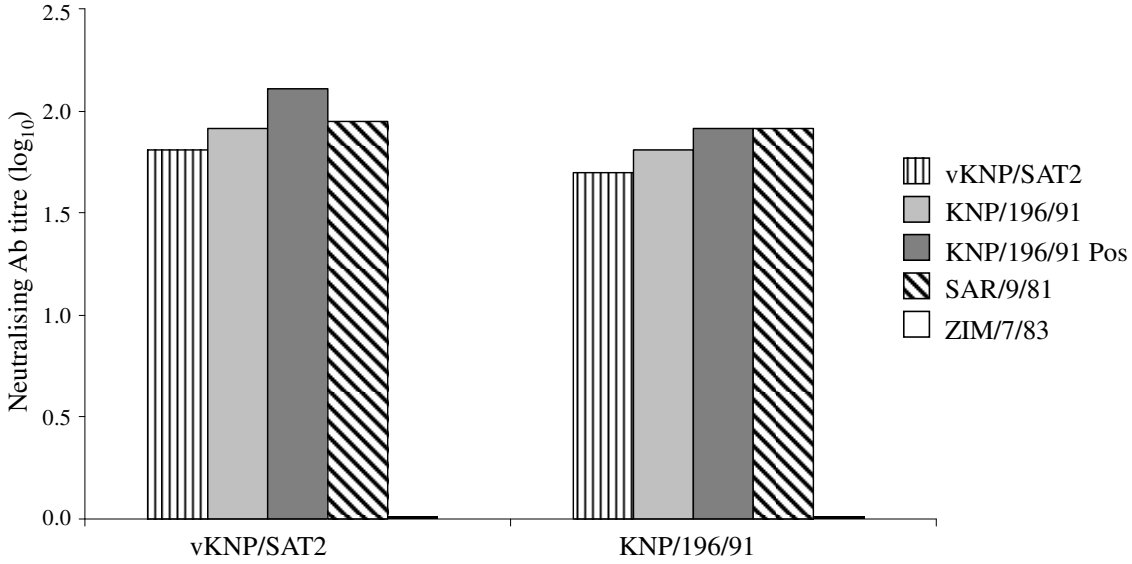

Fig. 4.2 Antigenic profiles of the FMD viruses vKNP/SAT2 and KNP/196/91, which have not been cell culture-adapted in BHK-21 cells, tested against FMDV SAT1 antisera (vKNP/SAT2 chimera, KNP/196/91 parental and SAR/9/81) and SAT2 antisera (ZIM/7/83) in a VNT. Pig antisera vKNP/SAT2 and KNP/196/91 were from the vaccinated animals in this potency trial, whereas all other antisera originated from cattle. A positive-control serum of high titre (KNP/196/91 Pos) was included. Serum titres of $\geq$1.7 were regarded as positive.

infectious particles of vKNP/SAT2 and KNP/196/91 had been completely inactivated. After extended incubation at 4°C, both viruses were present up to 28 days (Fig. 4.3b) and the infectious titres observed for vKNP/SAT2 and KNP/196/91 were 2.6×10^3 and 5.7×10^3 pfu/ml, respectively, under these conditions. Moreover, no significant variation in thermal stability was observed between the two viruses when they were incubated at 25°C for 14 days (Fig. 4.3b). However, no infectious particles were detected at 28 days under these conditions.

The stability of the viruses was tested at pH values ranging from 6.5 to 9.0 (Fig. 4.3c). Treatment of vKNP/SAT2 and KNP/196/91 at pH 6.5 yielded a slight decrease in the titre of infectious particles after 30 min. The titre of infectious particles for both viruses was similar following treatment with buffers at alkaline pH for 30 min (Fig. 4.3c). The capsid stability of vKNP/SAT2 was comparable to that of KNP/196/91 at various NaCl concentrations ranging from 50 mM to 1.5 M (Fig. 4.3d). Taken together, these results indicated no differences in the biophysical properties of the parental and chimeric viruses.

4.3.3 Guinea pig antibody titres in relation to vaccine dose

Two separate formulations incorporating inactivated 146S antigens of vKNP/SAT2 and KNP/196/91 were used to assess the antibody response to immunisation in guinea pigs (Fig. 4.4). Following animal immunisation, serum samples were collected at weekly intervals and tested by using a sandwich ELISA specific for KNP/196/91. Sero-conversion occurred at *ca.* 7 days post-vaccination (d.p.v.) and serological responses varied with time ($P < 0.001$), dose ($P < 0.001$), and vaccine ($P = 0.001$; Fig. 4.4). High antibody titres were obtained for guinea pigs vaccinated with all three of the vKNP/SAT2 vaccine doses and titres peaked between 21 and 28 d.p.v. (Fig. 4.4a). In comparison, the antibody responses elicited by the KNP/196/91 vaccine were similar to those of the same dose of the vKNP/SAT2 vaccine and peaked at 28 d.p.v. (see Fig. 4.4b).

4.3.4 FMDV-specific antibody responses induced by vKNP/SAT2 and KNP/196/91 vaccines in pigs

To investigate vKNP/SAT2 as a potential chimeric vaccine, a 6 µg dose was taken as the full dose in a decreasing dose potency trial in pigs. Two separate double oil emulsions incorporating inactivated 146S antigens of vKNP/SAT2 and KNP/196/91 were prepared and used for vaccination in a full potency trial (European Pharmacopoeia, 2006; OIE Manual of

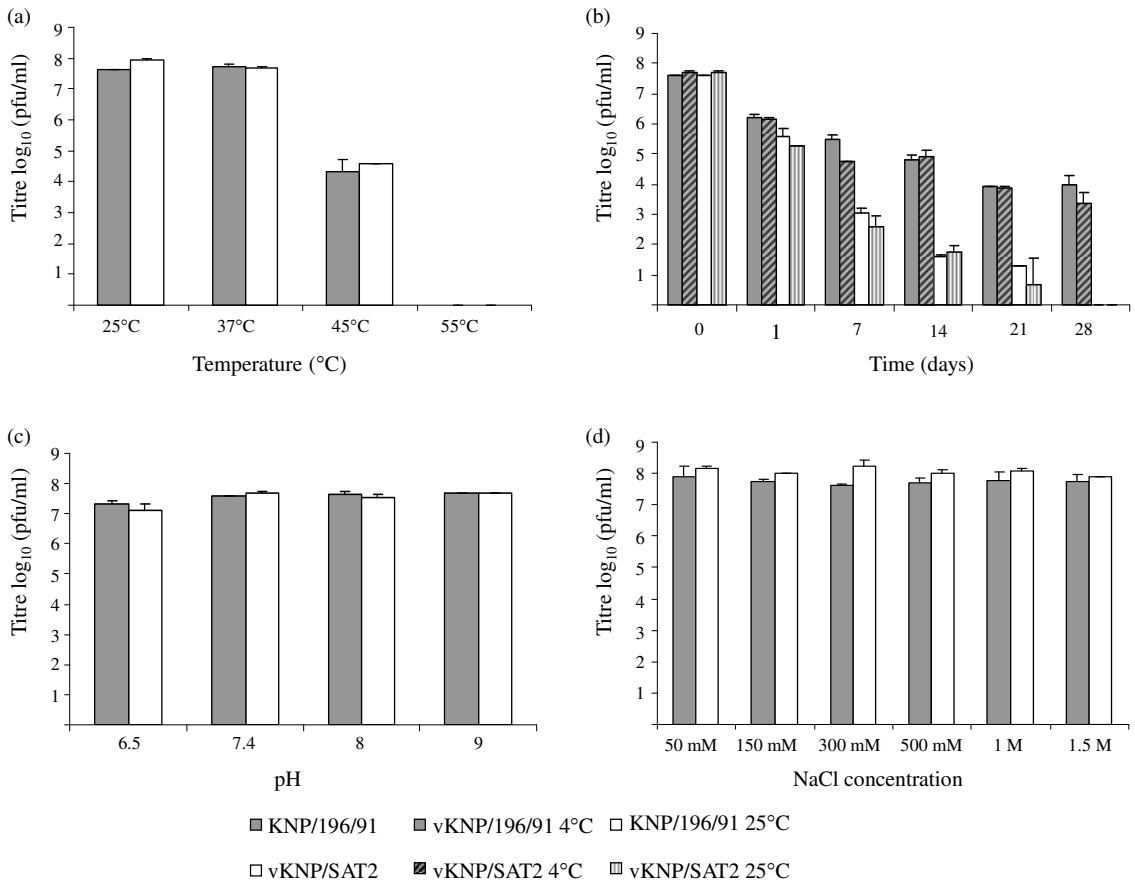

Fig. 4.3 Biophysical stability of 146S virion particles of KNP/196/91 and the recovered vKNP/SAT2 chimera at different temperatures (a, b), pH (c) and NaCl concentrations (d). Purified virion particles were tested for temperature stability by incubation at the indicated temperatures for 30 min (a), and at 4°C and 25°C over a period of four 4 weeks (b). All samples were titrated on BHK-21 cells.

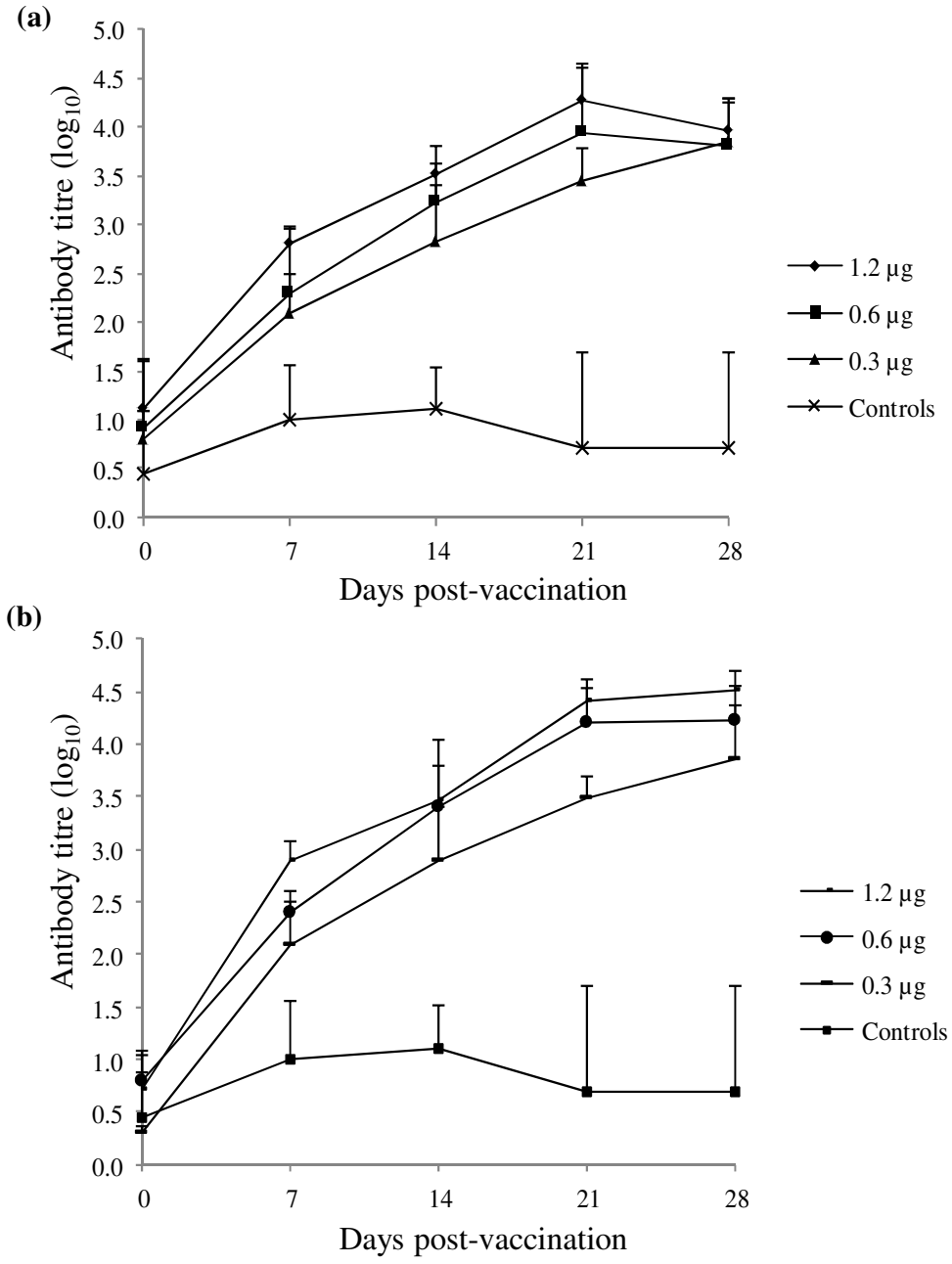

Fig. 4.4 Antibody responses elicited in guinea pigs for varying antigen payloads (0.3, 0.6 and 1.2 µg) of the vKNP/SAT2 (a) and KNP/196/91 (b) vaccines. Serum samples were tested by using a sandwich ELISA specific for KNP/196/91 and the mean titres per group of 10 animals for each vaccine dose were determined at weekly intervals. Bars indicate the mean (± SD) of each group.

Standards, 2009). The antibody response elicited in pigs varied with time ($P < 0.001$), dose (full, 6.0 µg; quarter, 1.5 µg; one-sixteenth, 0.375 µg; $P = 0.003$), and vaccine ($P = 0.011$) when monitored at weekly intervals using a KNP/196/91-specific SPCE (Fig. 4.5). The full and quarter doses of the vKNP/SAT2 vaccine (Fig. 4.5a, b) elicited an antibody response comparable to the equivalent doses of the KNP/196/91 vaccine (Fig. 4.5d, e) up to 21 d.p.v., whereafter the titres for vKNP/SAT2 remained similar until the day of challenge. Most animals vaccinated with the vKNP/SAT2 one-sixteenth dose (Fig. 4.5c) were borderline positive at the time of challenge. However, the results from the SPCE indicated that the KNP/196/91 vaccine elicited positive antibody responses for all vaccine doses (Fig. 4.5f).

4.3.5 Comparison of neutralisation titres and protection

Serum neutralising-antibody responses were measured by a VNT at the day of challenge for the vaccinated and control animals (Table 4.1). All of the pigs were negative for FMDV-specific neutralising antibody at the onset of the study (data not shown). Positive neutralising antibody titres were induced for the full doses of both vaccines. For the quarter dose of the vKNP/SAT2 and KNP/196/91 vaccines, three and four animals, respectively, were positive for neutralisation of the KNP/196/91 virus. Whilst four pigs vaccinated with the KNP/196/91 one-sixteenth dose had positive neutralising antibody titres, the entire vKNP/SAT2 one-sixteenth group had no detectable neutralising virus titre. Statistical analysis indicated that a significant difference in neutralising antibody response was induced by the chimeric and parental vaccines for the one-sixteenth dose ($P = 0.02$), but not for the full and quarter doses. Comparisons between the immune responses for the three doses of the respective vaccines indicated no difference for KNP/196/91, suggesting that the one-sixteenth dose for the parental vaccine already gives a protective response and appears to be as efficient as the full dose under these conditions. For vKNP/SAT2, the neutralising antibody response conferred by the full and quarter dose was not different. The response against the one-sixteenth dose was significantly lower compared to the full ($P = <0.02$) and the quarter dose ($P = 0.03$). Similar antibody response profiles at day 28 were observed using both the SPCE (Fig. 4.5) and VNT (Table 4.1) for animals from all the groups and vaccinated with both antigens.

The mean neutralising-antibody titres for the animals protected by the vKNP/SAT2 full and quarter dose were 1.98 (SD = 0.09) and 1.97 (SD = 0.19) log titre, respectively, and ranged between 1.7 and 2.1 for individual pigs. Despite the fact that a mean 1.8 (SD = 0.06) log titre

for the two unprotected animals in the vKNP/SAT2 full-dose group was similar to titres observed for protected animals and that it is considered positive for SAT types in the VNT, these animals developed FMD lesions. The three animals of the vKNP/SAT2 one-sixteenth dose that were protected had a lower mean titre of 1.09 (SD = 0.25) compared with the unprotected animals in the quarter dose, which had mean antibody levels of 1.49 (SD = 0.06) log titre. Following challenge, 60% of the animals receiving the chimeric vaccine were fully protected against disease (Table 4.1). The onset of FMD lesions in animals with clinical disease was delayed and restricted in distribution compared with the control animals, indicative of partial protection in these animals. For most of the vaccinated pigs increased body temperatures were observed for one day only, whereafter temperatures decreased (data not shown). In comparison, the KNP/196/91 vaccine elicited mean titres ranging from 2.21 (SD = 0.39) for the full dose, 1.9 (SD = 0.27) for the quarter dose and 1.81 (SD = 0.34) for the one-sixteenth dose. Notwithstanding the absence of measureable positive neutralising-antibodies titres for two animals that received the KNP/196/91 vaccine, all animals were protected against challenge. The PD_{50} for the vKNP/SAT2 and parental vaccine was >6.4 and >39.4, respectively, as calculated by the Kärber method (Kärber, 1931). The vaccine potency is expressed as the number at which 50% of the animals used for the challenge experiments were protected.

4.4 DISCUSSION

FMD vaccine candidates should be closely related to field strains (Doel, 1996) and induce an immune response with a broad immunological cross-reactivity (Esterhuysen *et al.*, 1988) for appropriate protection against subtypes (Pay, 1983). This is especially relevant in sub-Saharan Africa where the high degree of antigenic variability in FMDV and the presence of several serotypes, including several subtypes, have important implications for vaccine strain selection. In the present study, by engineering a chimeric virus, a possible alternative to the conventional inactivated FMD vaccine production of the SAT type viruses was investigated for the development of custom-engineered inactivated FMD vaccines.

A cross-serotype chimeric virus, vKNP/SAT2, replicated stably in both FMD host- and non-host-species-derived cell lines and the ability to produce plaques was similar for vKNP/SAT2 and KNP/196/91. Although BHK-21 cells are derived from a non-host species, the integrin

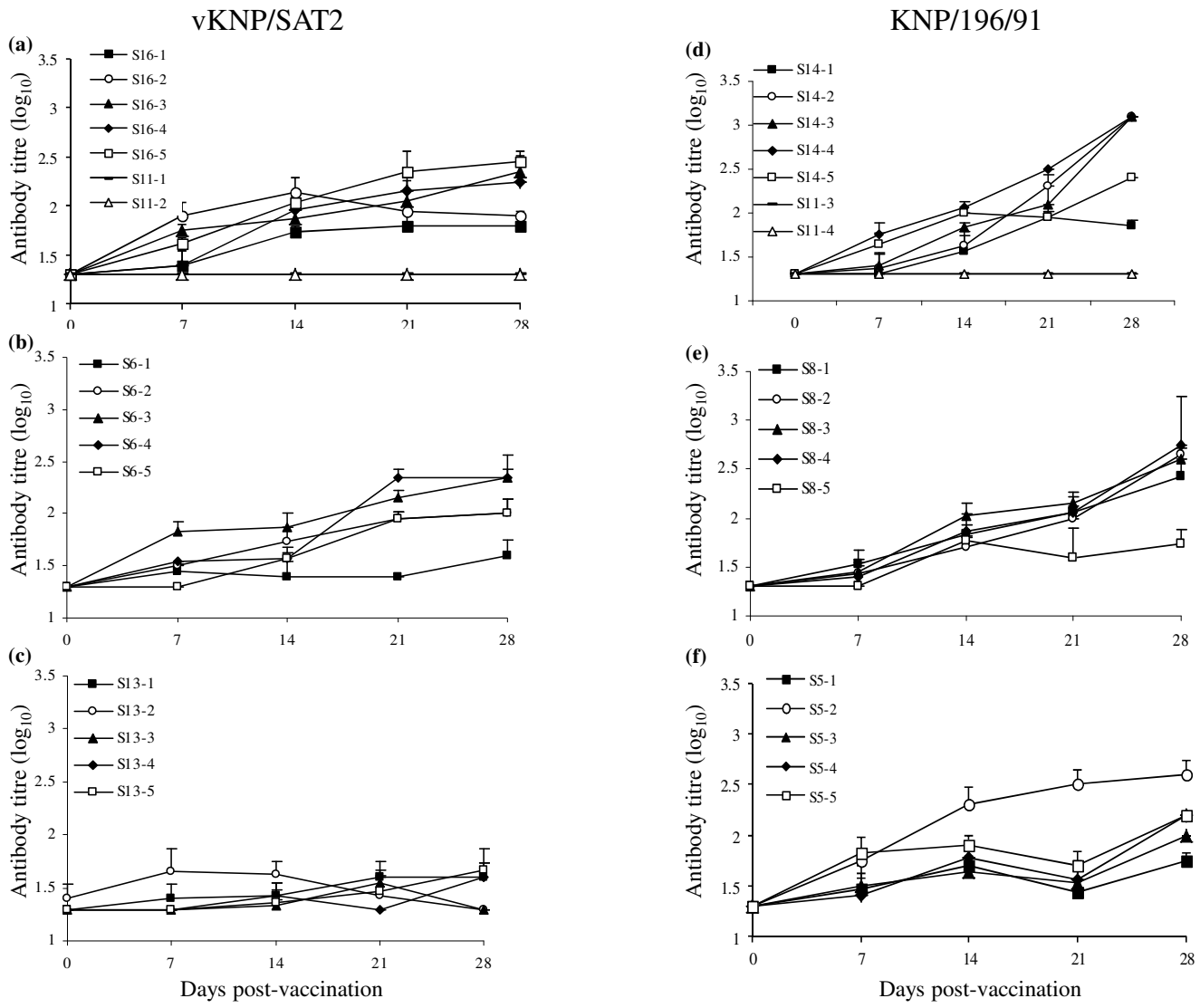

Fig. 4.5 Antibody responses elicited in pigs by full (6.0 µg), quarter (1.5 µg) and one-sixteenth (0.375 µg) doses of the vKNP/SAT2 (a-c) and KNP/196/91 (d-f) vaccines, respectively. Two separate double oil emulsions incorporating inactivated 146S antigens of vKNP/SAT2 and KNP/196/91 were prepared and used for vaccination. Serum samples collected were tested by using a KNP/196/91-specific SPCE and mean titres per group of five animals for each vaccine dose were determined at weekly intervals. Means were determined from at least two repeats. Animals of the control group (S11 animals) were inoculated with a placebo formulation without antigen.

Table 4.1 Neutralising antibody responses and post-challenge clinical data for pigs vaccinated with vKNP/SAT2 and KNP/196/91

Vaccines were administered as a single inoculation at day 0. Neutralising antibody titres were determined on day 28 post-vaccination and are the mean values of three replicates of the VNT. Titres of $\geq \log_{10}$ 1.7 were regarded as positive. The maximum temperature observed for each animal after challenge is shown. Temperatures of ≥39.6°C were regarded as positive for fever. The degree of disease post-challenge was defined as: none, no lesions on any feet; mild, lesions on only one foot; severe, lesions on two to three feet. Clinical signs of FMD were regarded as lesions on any foot, other than the limb used for challenge, and elevated body temperatures of ≥39.6°C. Total protection was considered as complete absence of lesions.

Vaccine	Animal	Neutralising antibody titre	Post-challenge clinical data	
			Temperature (°C)	Degree of disease
vKNP/SAT2				
6 µg	16-1	1.7	39.9	Mild
	16-2	1.8	39.7	Mild
	16-3	1.9	39.2	None
	16-4	2.0	39.8	None
	16-5	2.0	39.2	None
1.5 µg	6-1	1.4	39.8	Mild
	6-2	1.5	39.6	Mild
	6-3	2.1	39.3	None
	6-4	2.1	39.3	None
	6-5	1.7	39.2	None
0.375 µg	13-1	0.8	40.4	None
	13-2	0.4	40.6	Severe
	13-3	0	41.0	Mild
	13-4	1.2	39.8	None
	13-5	1.2	39.9	None
KNP/196/91				
6 µg	14-1	1.7	39.2	None
	14-2	2.5	39.9	None
	14-3	2.5	39.3	None
	14-4	2.5	39.0	None
	14-5	1.9	39.6	None
1.5 µg	8-1	2.0	39.3	None
	8-2	2.2	39.0	None
	8-3	1.9	39.1	None
	8-4	1.8	39.1	None
	8-5	1.5	39.4	None
0.375 µg	5-1	1.3	39.9	None
	5-2	2.2	39.5	None
	5-3	1.7	39.6	None
	5-4	1.9	39.4	None
	5-5	1.8	39.4	None
Controls				
Placebo[a]	11-1	0	39.9	Severe
	11-2	0	40.1	Severe
	11-3	0	41.0	Severe
	11-4	0	40.2	Severe

[a] Control animals received a placebo formulation containing Tris/KCl buffer.

cellular receptors expressed by these cells while growing in monolayers are recognised by FMDV in a RGD (arginine-glycine-aspartic acid)-dependant manner (Fox *et al*., 1989; Baxt and Becker, 1990; Mason *et al*., 1994; DiCara *et al*., 2008 and references therein). The porcine cell line IB-RS-2 was utilised for virus isolation and the VNT, and expresses mainly $\alpha_v\beta_8$ on the cell surface (Burman *et al*., 2006). In contrast, CHO-K1 cells lack the integrin cellular receptors used by FMDV for cell entry and express HSPG, which is used as an alternative receptor by cell culture-adapted FMDV (reviewed in Jackson *et al*., 2003). Notably, the presence of the external capsid proteins of a SAT1 virus in the genetic background of a SAT2 virus did not alter the biological properties of the chimera markedly, suggesting that it is a method that leads to the design of good vaccine candidates. In fact, vKNP/SAT2 retained the rapid infection kinetics of KNP/196/91 in BHK-21 cells that is imperative for production of a high yield of inactivated 146S particles to be used as FMD vaccines. Both vKNP/SAT2 and KNP/196/91 displayed the cell culture-adaptation phenotype (Maree *et al*., 2010), possibly because of interactions with HS receptors (Jackson *et al*., 1996; Sa-Carvalho *et al*., 1997; Fry *et al*., 1999; Jackson *et al*., 2003). Such alteration in FMD viruses' ability to utilise HS is important as it is associated with more rapid replication in BHK-21 cells, a change in cell tropism, a more effective cell killing capacity and could contribute to improved production of FMD vaccines in suspension cultures (Amadori *et al*., 1994; Sevilla *et al*., 1996; Baranowski *et al*., 1998).

The potential of vKNP/SAT2 as a vaccine strain producing high yields of stable antigen was emphasised by its comparable biophysical properties to those of KNP/196/91. Virion stability is of importance during the vaccine manufacturing process as the maintenance of intact 146S particles is relevant to the amount of immunogenicity induced by antigens and to vaccine efficacy (Doel and Baccarini, 1981; Doel, 2003). In the present study, a comparable decrease in infectivity was observed for the respective viruses at room temperature and above. The similar capsid stability of vKNP/SAT2 and KNP/196/91 observed at various NaCl concentrations is applicable to the range of ionic strengths that might exist during the purification of 146S particles in the vaccine production process. Unlike most other picornaviruses, FMDV is susceptible to low pH-induced capsid disassembly, a characteristic of great importance to its pathogenicity (King, 2000). Both the KNP/196/91 virus and its derivative were stable throughout a range of acidic to basic pH treatments. In addition to the above-mentioned qualities necessary for successful vaccine manufacturing, KNP/196/91 also displays good r_1-values, which are an indication of the probability to protect vaccinated

animals against circulating variant viruses in the field (Esterhuysen *et al*., 1994; Reeve *et al*., 2010). Moreover, comparable neutralisation profiles were obtained for the respective viruses tested against other SAT1 and 2 antisera, confirming that vKNP/SAT2 retained the antigenic properties of KNP/196/91.

The advantages of using oil formulations for FMD vaccines have been well established and Montanide ISA 206B has proven to be an efficient adjuvant (Barnett *et al*., 1996) for the SAT types. Long-lasting immune responses were observed for cattle following immunisation with vaccine containing KNP/196/91 antigen formulated in Montanide ISA 206B oil-based adjuvant (Hunter, 1996; Cloete *et al*., 2008). The vKNP/SAT2 and KNP/196/91 vaccines elicited good humoral immune responses in guinea pigs and pigs. The majority of the pigs vaccinated with the vKNP/SAT2 were protected against live virus challenge. In addition, the onset of disease was delayed for most of the vKNP/SAT2-vaccinated pigs when compared with the control animals, and the clinical signs were less severe. This is indeed promising as antigen doses of 6 µg correlate with emergency FMD vaccines (PD_{50} ≥6) that induce rapid protective immune responses (Barnett and Carabin, 2002).

Similar neutralising-antibody titres were elicited in pigs vaccinated with the vKNP/SAT2 and KNP/196/91 vaccines as were observed for SAT type vaccines that conferred protection *in vivo* by Barnett *et al.* (2003). However, for some of the animals it is difficult to find a correlation between protection and neutralising-antibody response. Protection was observed for three animals of the vKNP/SAT2 one-sixteenth group, which showed no detectable titre in the VNT. Although higher antigen doses elicited more neutralising antibodies for most animals vaccinated with vKNP/SAT2 and KNP/196/91, even at low antigen concentrations protective immunity was induced. This phenomenon may be explained by additional neutralising mechanisms that exist in the host, such as complement-enhanced neutralisation. Another explanation might be the presence of a cell-mediated immunity (CMI) component contributing to the extent of protection, which could have a different response depending on the difference in non-capsid proteins in the virion. CMI parameters were not measured in this study. Previous studies have indicated that even though antibodies were non-neutralising *in vitro* and/or at low concentrations, these were in fact protective *in vivo* (Anderson *et al*., 1971; McCullough *et al*., 1992; Filho *et al*., 1993; Brehm *et al*., 2008).

The antigen payload, the integrity of the FMDV particle and the conformation of the viral epitopes are important factors to consider for FMD vaccine efficacy. The reason for the lesser extent of protection in pigs conferred by the vKNP/SAT2 chimeric vaccine is not clear. This matter could be addressed by characterisation with well-defined monoclonal antibodies directed against SAT1 FMDV or re-vaccination of animals to determine the stability of the formulated antigen. To refine the construction of SAT type chimeras, the external capsid-encoding region (1B-1D/2A) can be further manipulated to make such viruses more specific in their ability to infect and replicate in the cells most commonly used for vaccine production. It would also be possible to design SAT type FMD vaccines where regions of the genome are engineered to optimise epitope representation. An additional benefit of using this recombinant DNA technology is that reverse genetics allows for modifications that could be incorporated to support serological differentiation of infected from vaccinated animals for surveillance of FMD in sub-Saharan Africa. The potential now exists to generate more effective new generation chemically inactivated FMD vaccines for this highly infectious and economically important disease, which are custom-engineered and specifically produced for geographic areas.

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CHAPTER FIVE

CONCLUDING REMARKS

Chemically inactivated vaccines used for control of foot-and-mouth disease (FMD) have contributed greatly to its eradication and control in many parts of the world (Ward *et al.*, 2007). However, in southern Africa, control of FMD is hampered by the extensive variability of the South African Territories (SAT) type viruses, which exist as distinct genetic and antigenic variants in different geographical regions (Vosloo *et al.*, 1995; Bastos *et al.*, 2001, 2003). To be efficacious, vaccine candidates should be closely related to field strains and induce a response with a broad immunological cross-reactivity for appropriate protection against subtypes (Paton *et al.*, 2005; Paton and Taylor, 2011). Moreover, production of effective vaccines necessitates the use of good vaccine strains with characteristics such as maximal infectivity and rapid growth in tissue culture, high antigen yields and appropriate immunological specificity (Doel, 2003). Towards addressing these impediments, this investigation concerned the evaluation of a chimera-derived FMD vaccine in relation to a current SAT type vaccine. In this conclusion, the new information that has evolved during this investigation will be summarised and suggestions regarding future research will be made.

The FMDV outer capsid proteins (1B, 1C and 1D) contribute to antigenic variants that exist within each of the seven serotypes. Consequently, in the first part of this investigation, phylogenetic, genetic and antigenic analyses of SAT types prevalent in sub-Saharan Africa were performed in order to identify possible epitopes that could be prone to antigenic variation in the SAT type viruses. The results indicated that the high levels of genetic diversity in the capsid (P1)-coding region within the SAT types are reflected in the antigenic properties of these viruses and therefore have implications for the selection of vaccine strains that would provide the best vaccine match against emerging viruses. Interestingly, although SAT1 and SAT2 viruses displayed similar genetic variation within each serotype (32% variable amino acids), antigenic disparity, as measured by r_1-values, was less pronounced for SAT1 viruses compared with SAT2 viruses, thus emphasising the high antigenic variation within the SAT2 serotype. Furthermore, amino acid variation and the r_1-values were combined with crystallographic structural data to predict areas on the surface of the FMD virion as antigenically relevant. Although the putative antigenic sites identified during the course of these analyses need to be validated either through a reverse-genetics approach or through comparison with monoclonal antibody (MAb) escape mutants, the putative antigenic sites were nevertheless consistent with antigenic sites determined previously for type A, O and C viruses.

Since the identification of antigenic sites on individual FMDV isolates is time-consuming, data are therefore not available for all serotypes, much less for all isolates. Indeed, very little is known about the important epitopes for SAT1 and SAT2 viruses (Crowther *et al.*, 1993; Grazioloi *et al.*, 2006; Opperman *et al.*, 2012). This paucity of information impacts negatively on the design of vaccines with either a broad or targeted antigenic coverage and to predict the efficacy of a particular vaccine strain against circulating viruses in the field. The availability of sequence data and related virus neutralisation test (VNT) titres could, however, provide an opportunity to relate cross-reactivity to sequence variation. Such a relationship may facilitate the selection of suitable candidate vaccine seed strains, as well as rapid identification of vaccine matches without the need for additional serological work for existing vaccines. Recently, Reeve *et al.* (2010) reported a mathematical model to predict a match to vaccine strains. In the study reported in this thesis, focus was placed on the potential escape from protection by field strains through comparisons to the current SAT1 and SAT2 reference strains. Despite the use of different approaches and methodologies, these respective studies nevertheless support each other's observations. Not only was similar antigenic-relevant sites for FMDV field strains predicted, but both the respective approaches may be used towards predicting the most suitable vaccine match for new isolates. Based on the analyses performed and described in this thesis, the SAT1 virus, KNP/196/91, was specifically chosen for further use in this investigation since it is currently used as a vaccine strain that is capable of producing high yields of antigen and has an r_1-value of above 0.4 against topotype 1 field viruses, indicating a good level of cross-protection (Samuel *et al.*, 1990; Esterhuysen, 1994).

A potentially powerful approach in the control of FMD in sub-Saharan Africa relies on the use of chimeric FMDV of which the antigenic properties can be readily manipulated. However, as highlighted above, FMD vaccine application is complicated by the extensive variability of the SAT type viruses, which exist as distinct genetic and antigenic variants in different geographical regions. Thus, if a chimeric FMD vaccine is to be efficacious it would need to reflect a heterogeneous mixture of genetic variants as is encountered in the infected host animal. Consequently, in the second part of this investigation, the population diversity of a chimeric FMDV derived from a cloned cDNA was examined and compared to that of the parent virus. For this purpose, a cross-serotype chimeric virus, vKNP/SAT2, was engineered by replacing the external capsid-coding region (1B-1D/2A) of a genome-length infectious cDNA clone of a SAT2 vaccine strain, ZIM/7/83, with that of SAT1/KNP/196/91. The

sequence heterogeneity of several different FMDV, which were obtained following growth either in cell culture or within pigs, was then examined using Sanger consensus sequencing and pyrosequencing. Sanger sequencing identified 14 positions of amino acid variability for the outer capsid of the SAT1 variants of which the majority of variation was in 1B and 1D. Residue changes that may be involved in altering cell-binding capacity and plaque morphology were observed at positions 135 of 1C and 112 of 1D. Pyrosequencing detected considerable genetic diversity in the two FMDV populations, which was comparable for the outer capsid proteins. The results obtained in this part of the investigation suggested that chimeras derived from cDNA clones display similar genetic heterogeneity compared to viruses that have been subjected to *in vivo* and *in vitro* environments.

Although the above study provided potentially interesting data relating to cell culture adaptation and host animal adaptation, caution is required in interpreting the results. It should be noted that various sources may contribute to the observed sequence heterogeneity. For example, when virus is recovered from a cDNA clone there may be heterogeneity introduced in a variety of different ways. These could include errors introduced by the RNA polymerase used to synthesise RNA transcripts *in vitro* and it can be expected that further heterogeneity will be introduced as the viral RNA is replicated within cells. Since the error rate of the viral RNA polymerase is such that it is likely that every genome made will contain a mutation, then the large number of molecules produced within each cell in a single cycle of infection will rapidly generate a huge "quasispecies" of RNA. Moreover, heterogeneity could also be introduced during RT-PCR of the capsid-coding regions prior to sequencing. Errors introduced during the reverse transcription step or during initial PCR rounds will be amplified and thus be observed as mutations. Indeed, many of the low frequency mutations observed could have been caused by this process. These factors therefore mean that careful analysis of the events involved in generating sequence diversity has to be performed before definitive conclusions can be drawn regarding the importance of the observed mutations in processes such as cell and host adaptation.

In the final part of this investigation, the genetically engineered chimeric virus vKNP/SAT2 was characterised with regards to properties important in FMD vaccinology. The vKNP/SAT2 virus exhibited comparable infection kinetics, virion stability and antigenic profiles to the KNP/196/91 parental virus, thus indicating that the functions provided by the capsid can be readily exchanged between serotypes. High titres of stable chimeric virus were

obtained, which are properties of importance in vaccine manufacturing (Doel, 2003). Chemically inactivated vaccines, formulated as double-oil-in-water emulsions, were subsequently produced from intact 146S virion particles of both the chimeric and parental viruses. Inoculation of guinea pigs with the respective vaccines induced similar antibody responses. In order to show compliance to commercial vaccine requirements, the vaccines were also evaluated in a full potency test. Pigs vaccinated with the chimeric vaccine produced neutralising antibodies and showed protection against homologous FMDV challenge, albeit not to the same extent as for the vaccine prepared from the parental virus. However, the SAT1 chimera vaccine passed the PD_{50} ≥6 requirement of the Office International des Epizooties (OIE) for emergency vaccine use.

The results of the above study provide support that chimeric vaccines containing the external capsid of field isolates can be successfully produced and that they induce protective immune responses in FMDV host species. This approach therefore has the potential to make a valuable contribution to the 16-year framework for the progressive control of FMD in Southern African Development Community (SADC) countries (Lubroth *et al*., 2007). However, much research is still needed to develop improved FMD vaccines for use in Africa. Recommendations for the improvement of FMD vaccines include the development of vaccines with broad spectrum antigenic coverage (Hunter, 1996, 1998; Mattion *et al*., 2004; Cloete *et al*., 2008; Paton *et al*., 2005, 2009), marker vaccines enabling differentiation between infected and vaccinated animals (DIVA vaccines) (Grubman, 2005; Fowler *et al*., 2011) and vaccines that provide earlier onset of protection, as well as long-lasting immunity, preferably longer than 12 months (McCullough *et al*., 1992; Parida, 2009; Grubman *et al*., 2010). Although some of these aspects may be addressed through the use of infectious cDNA technology, as utilised in this thesis, alternative approaches should also be explored in future studies. A promising approach is based on the use of empty FMDV capsids that are expressed by means of recombinant replication-defective human adenovirus type 5 (Ad5) vectors (Grubman *et al*., 2010; Golde *et al*., 2011; Mateu, 2011). In addition, future research could also focus on improving the stability of the viral capsid since the thermal stability of complete 146S particles, amongst other, has been linked to vaccine efficacy (Doel and Baccarini, 1981; Doel and Chong, 1982). Such improved thermal stability may also lessen the dependence on a faultless cold chain. In this regard, structural analysis of SAT type virus capsids and subsequent mutational studies have enabled the introduction of targeted

mutations into the capsid proteins, resulting in capsids that are stable at temperatures of 42-55°C (Maree *et al*., unpublished results). The potential therefore exists to generate thermostable FMD vaccines that could improve the efficiency of immunisations by preventing temperature damage to vaccine antigens (especially in the absence of the cold chain), reducing vaccine wastage and decreasing logistical requirements, as well as the costs of vaccine transportation and storage (Kristensen *et al*., 2011).

In conclusion, vaccines are an important component of strategies aimed at effective control and regional eradication of FMD. Chimera-derived vaccines, as described in this investigation, are useful since they can be custom-engineered to specific geographical localities through cloning of the external capsid-coding region of relevant field isolates. However, it is unlikely that any single vaccine approach will be sufficient in controlling FMD, since different stages during control and eradication will require a combination of different vaccine strategies (Rodriguez and Grubman, 2009). FMD enzootic regions may require highly efficacious vaccines that can induce broadly protective and long-term immune responses in order to decrease virus transmission and incidence of clinical disease, whereas eradication might require vaccines that will allow differentiating infected from vaccinated animals (DIVA). Moreover, emergency responses to outbreaks will require fast acting high potency vaccines with long-term stability of the formulated ready-to-use product. Efforts aimed at developing more efficacious FMD vaccines should furthermore be supported through the implementation of other control measures. These should include restriction of animal movement and maintenance of disease barriers (*i.e.* fences), as well as effective disease surveillance and diagnostic tests. All of these may contribute to improving livestock health, thereby securing agricultural income and ensuring trade opportunities with lucrative markets.

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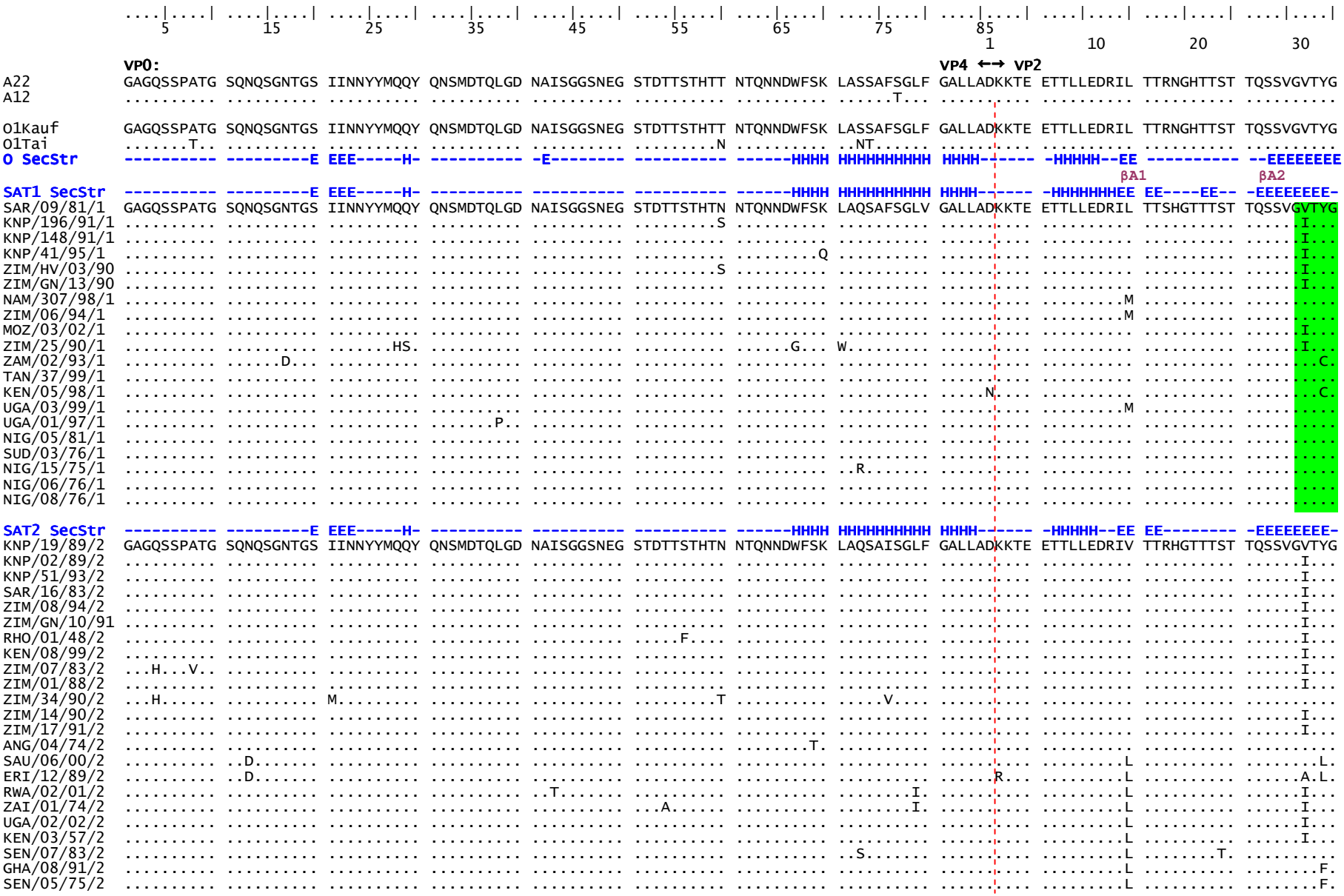
APPENDICES

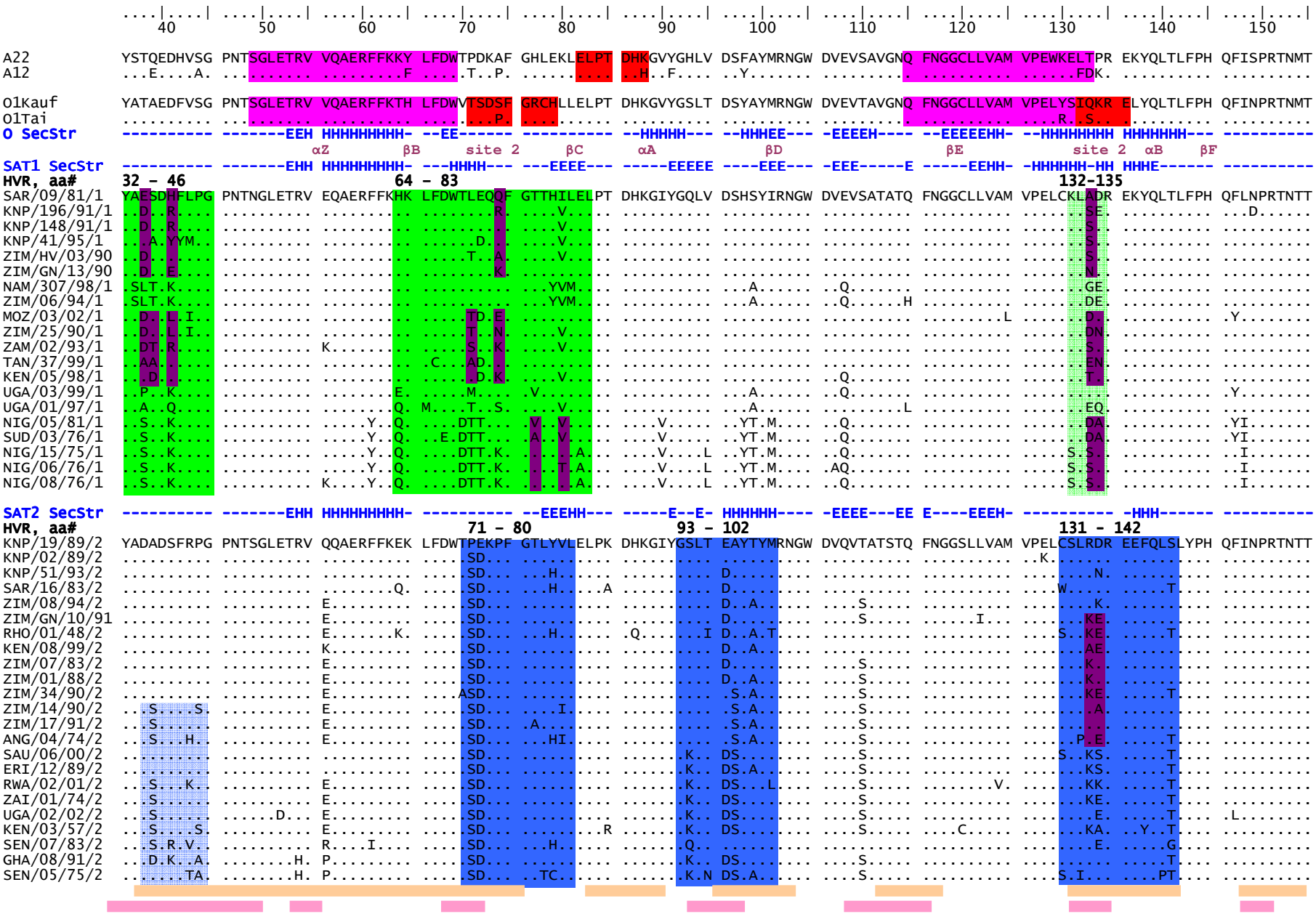
A: Alignment of SAT1 and SAT2 deduced capsid protein sequences.

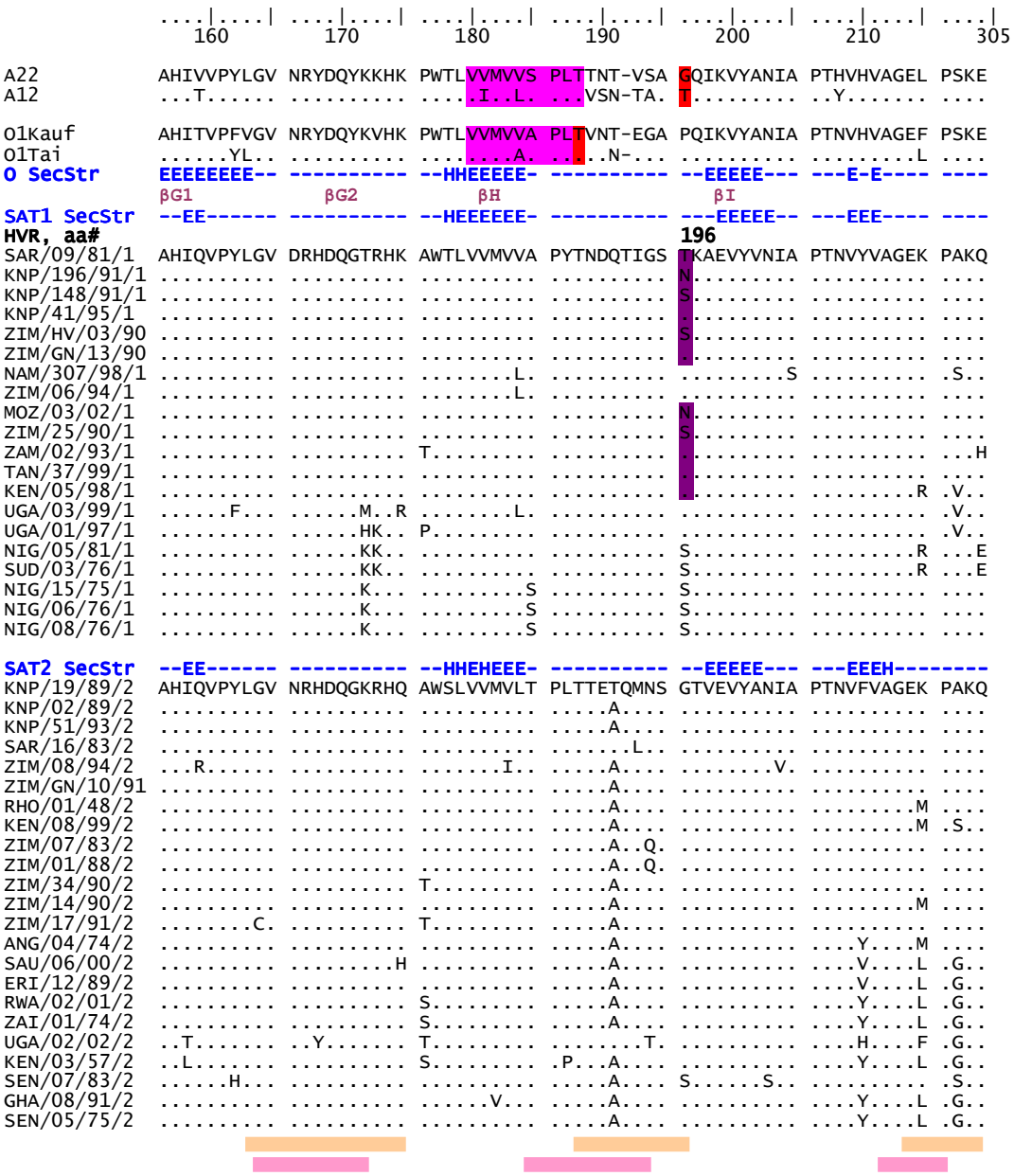
An alignment of the SAT1 and SAT2 deduced capsid protein sequences used to predict antigenic sites on the foot-and-mouth disease virus capsid of the South African Territories (SAT) types (Chapter 2). Variable regions on the capsid proteins of SAT1 and SAT2 isolates were combined with structural data and serological relatedness to identify possible epitopes that could be prone to antigenic variation in the SAT viruses. The study is based on amino acid and secondary structure alignments of SAT1 and SAT2 viruses, followed by the identification of hypervariable regions and amino acid positions of high entropy in the capsid-coding regions for SAT viruses. Hypervariable regions, in which 70% or more of the residue positions varied (using overlapping windows of 10 amino acids), were identified in the outer capsid proteins, *i.e.* 1B, 1C and 1D (VP2, VP3 and VP1). This data was mapped in juxtaposition of predicted hydrophilic regions and antigenic regions using Antheprot software. The hydrophilicity and deduced surface exposure were also considered for each residue and highly variable regions correlated with regions of significant hydrophilicity. Amino acid residues, situated within regions of hypervariability in the outer capsid proteins, which exhibit high entropy and hydrophilicity, and on structurally exposed loops were regarded as having the potential for involvement in antibody recognition and antigenicity of the virion. Taken all the topotype information into consideration, the variable residues (high entropy) within surface-exposed loops were regarded as immune relevant and were mapped to the SAT1 and SAT2 pentamer structures, respectively.

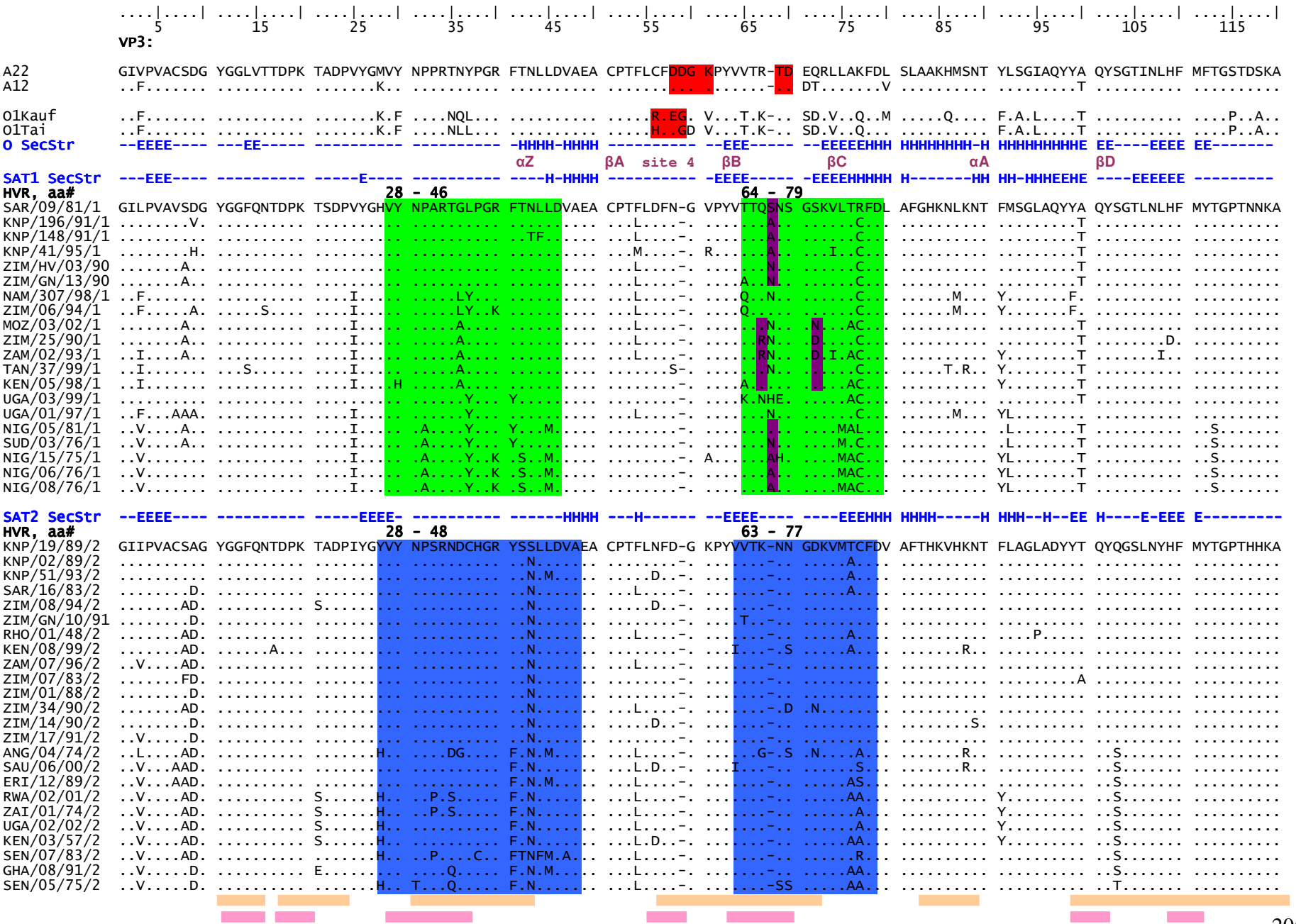
Legend:

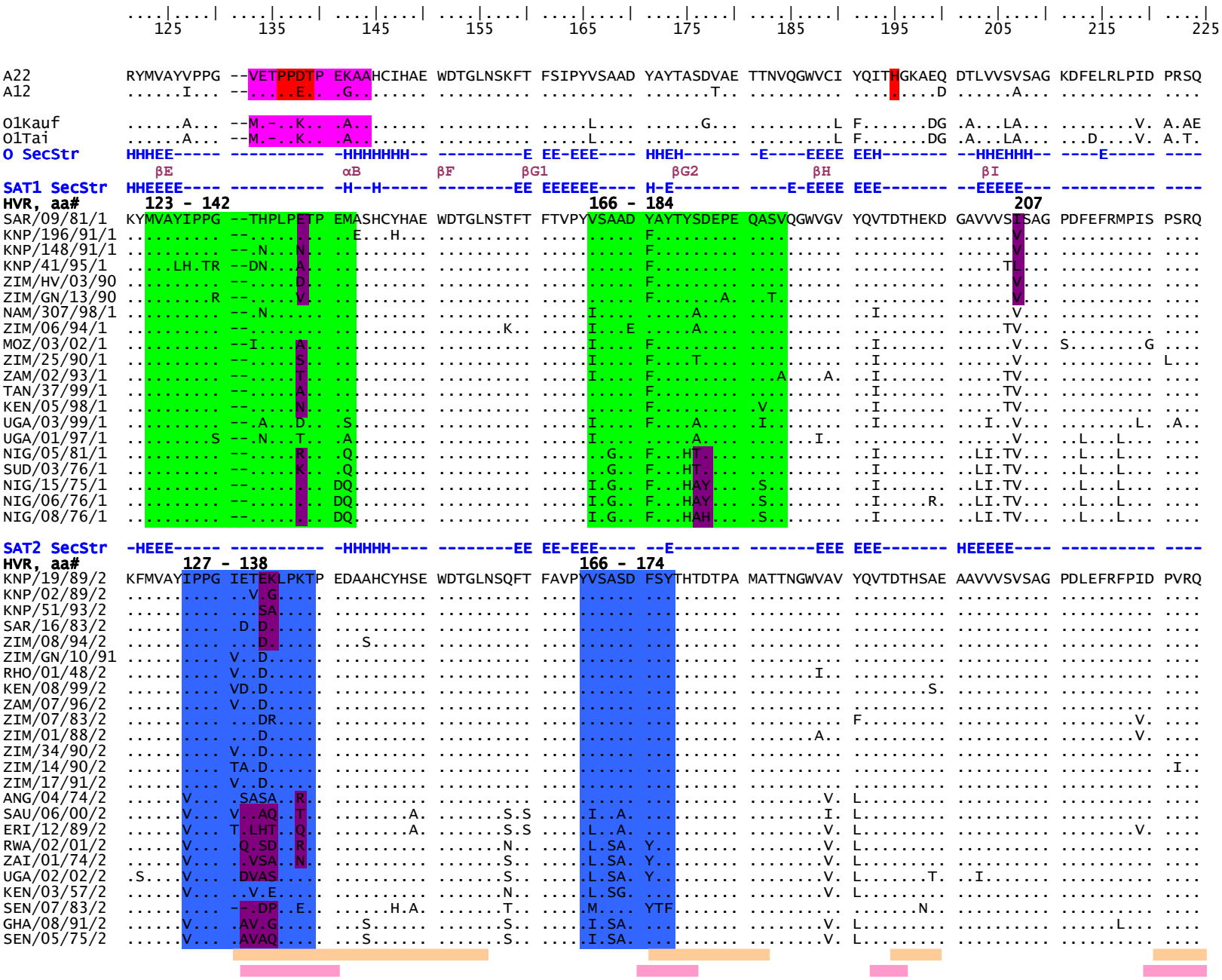
	Hypervariable regions in the alignment of SAT1 P1 amino acid sequences defined as five or more variable positions in a window of 10 residues.
	Hypervariable regions in the alignment of SAT2 P1 amino acid sequences defined as five or more variable positions in a window of 10 residues.
	Residue position with entropy of more than 1 as calculated using BioEdit software (Hall, 1999).
	Neutralising epitopes identified using monoclonal antibodies and virus escape mutants (Thomas *et al.*, 1988; Baxt *et al.*, 1989; Bolwell *et al.*, 1989; Kitson *et al.*, 1990; Crowther *et al.*, 1993).
	T-cell dependent epitopes (Pérez Filgueira *et al.*, 2000).
	The conserved integrin binding RGD motif on the G-H loop of 1D.
	The average hydrophilicity in SAT1 or SAT2 sequences as determined with the Antheprot software.
	The average antigenicity in the alignment as determined with the Antheprot software.
-----	VP4-VP2 auto-cleavage site
HHH	Alpha helices
EEE	Beta sheet structures
HVR	Hypervariable regions

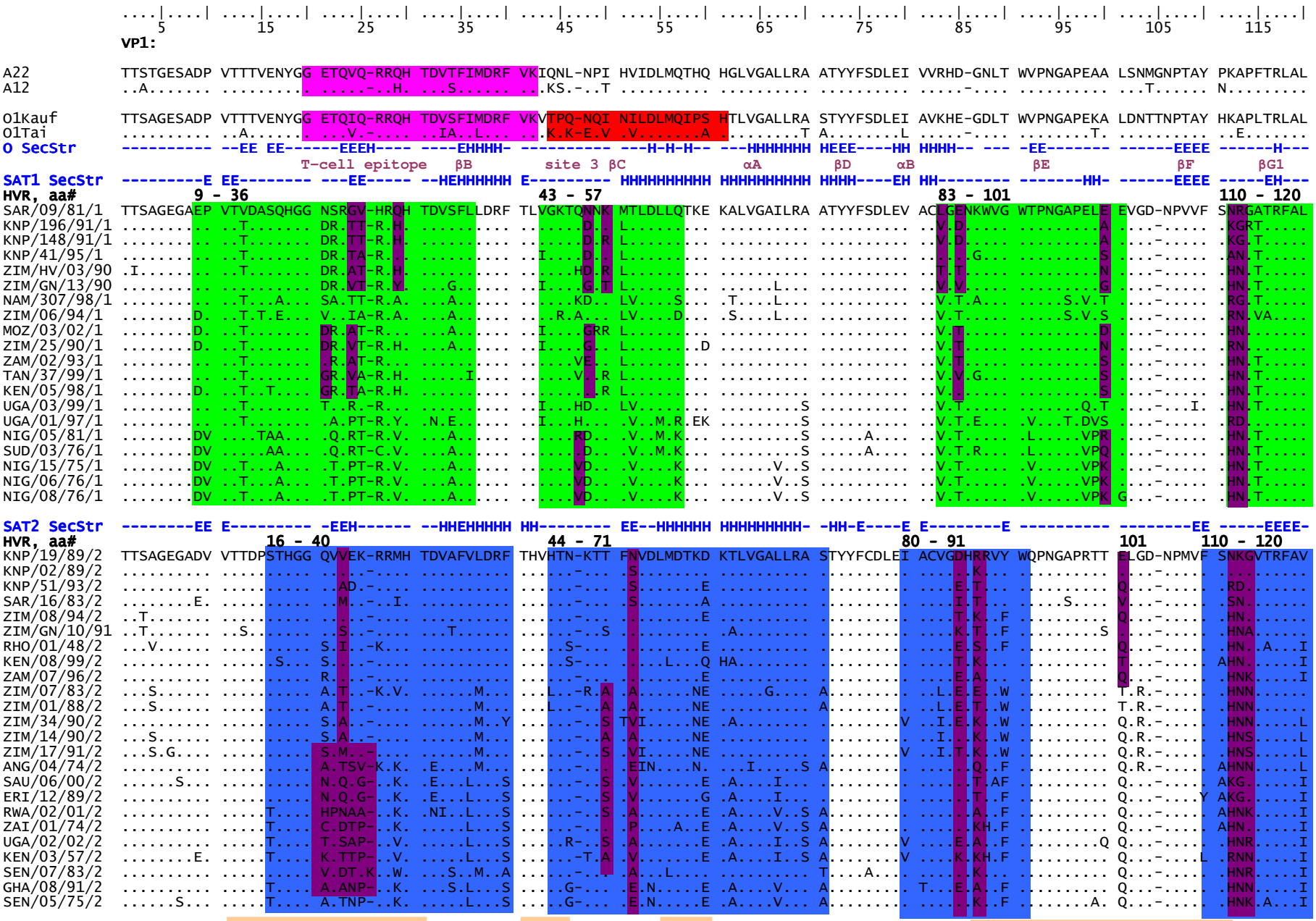


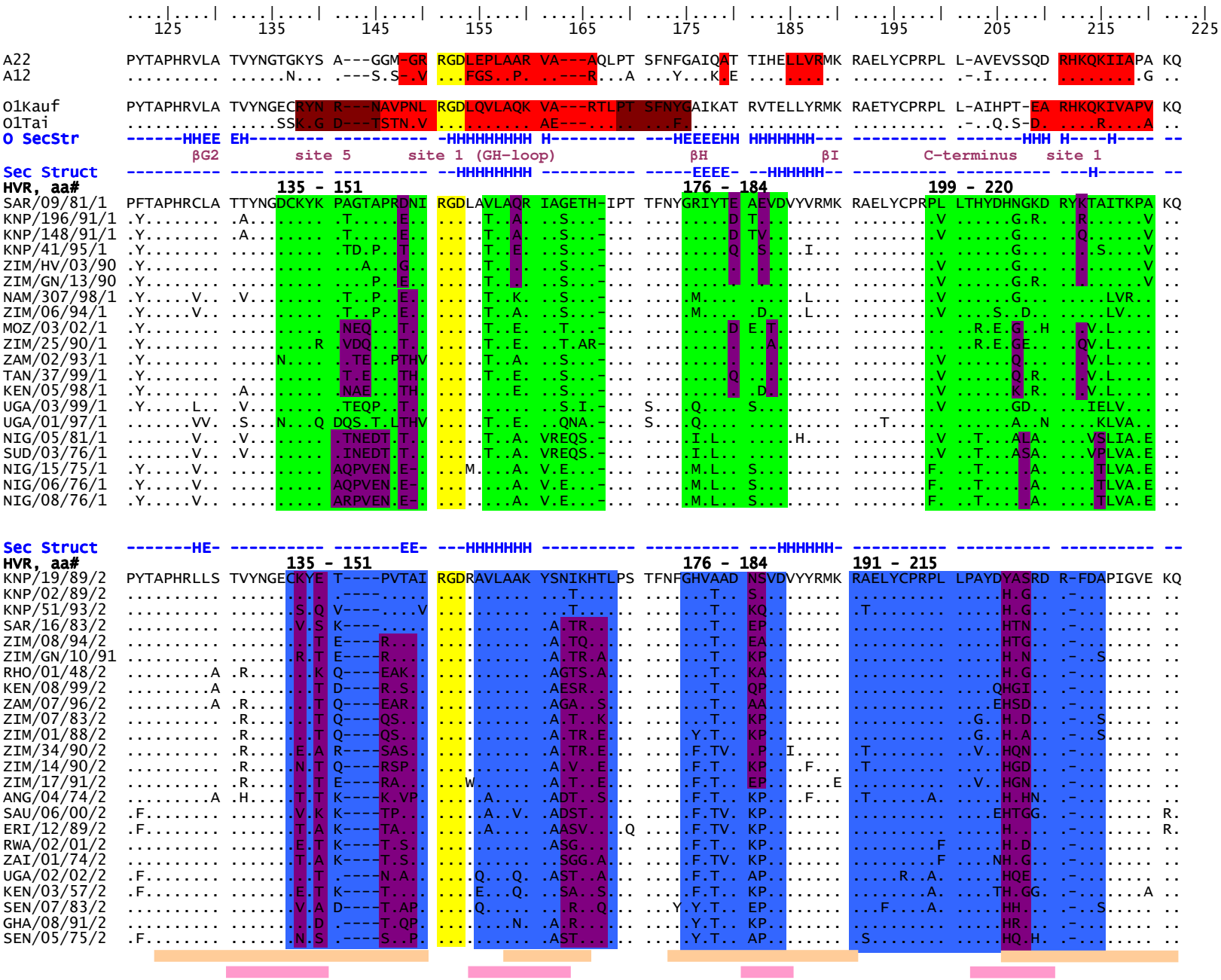












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B: Pyrosequencing oligonucleotide sequences for KNP_{P3} (set 1-9) and $vKNP/SAT2_{BHK5}$ (set 10-18).

Amplicon sequencing fusion oligonucleotides were designed to anneal to the P1 region of KNP_{P3} and $vKNP/SAT2_{BHK5}$ with overlaps, and rendering products of 200 to 300 bp (Chapter 3).

	Oligonucleotide A sequence (5' - 3')	**Oligonucleotide B sequence (5' - 3')**
Set 1	GCCTCCCTCGCGCCATCAGACGTGGGTCTGTTCCTCCGTGGAG	GCCTTGCCAGCCCGCTCAGACGTGAACTTTGTGTGGTCGAGGTGG
Set 2	GCCTCCCTCGCGCCATCAGACGTGTTGGTGCGTTGCTTGCCGAC	GCCTTGCCAGCCCGCTCAGACGTGAATGTATGAGTGGGAGTCAACAAG
Set 3	GCCTCCCTCGCGCCATCAGACGTCTGCCCACAGACCACAAAGG	GCCTTGCCAGCCCGCTCAGACGTGCTTTGTTCGAGCCAATCGTC
Set 4	GCCTCCCTCGCGCCATCAGACGTGCCACGACCAAGGAACACGC	GCCTTGCCAGCCCGCTCAGACGTGGGTGGTCACGTACGGAACTC
Set 5	GCCTCCCTCGCGCCATCAGACGTCCTGGGAGGTTCACAAACCTC	GCCTTGCCAGCCCGCTCAGACGTGGTTGAGTTCAGGCCTGTGTCC
Set 6	GCCTCCCTCGCGCCATCAGACGTGGCTAAGTACATGGTGGCCTAC	GCCTTGCCAGCCCGCTCAGACGTGGAAGCATCAGTCGTGACAGG
Set 7	GCCTCCCTCGCGCCATCAGACGTCCGACTTCGAGTTCAGGATGC	GCCTTGCCAGCCCGCTCAGACGTCTTCCGCAAGTTCTGGAGCTCC
Set 8	GCCTCCCTCGCGCCATCAGACGTCGCAGCCACGTACTACTTCTC	GCCTTGCCAGCCCGCTCAGACGTCACGTCGACCTCAGTGTCTGTG
Set 9	GCCTCCCTCGCGCCATCAGACGTCACTGCCCTACACCGCTCC	GCCTTGCCAGCCCGCTCAGACGTCCACCAGCTTTGTGAAGTTCTCCC
Set 10	GCCTCCCTCGCGCCATCAGCATGGGGTCTGTTCCTCCGTGGAG	GCCTTGCCAGCCCGCTCAGCATGGAACTTTGTGTGGTCGAGGTGG
Set 11	GCCTCCCTCGCGCCATCAGCATGGCCCAGTCAGCGATCTCGG	GCCTTGCCAGCCCGCTCAGCATGGAATGTATGAGTGGGAGTCAACAAG
Set 12	GCCTCCCTCGCGCCATCAGCATGCTGCCCACAGACCACAAAGG	GCCTTGCCAGCCCGCTCAGCATGGCTTTGTTCGAGCCAATCGTC
Set 13	GCCTCCCTCGCGCCATCAGCATGGCCACGACCAAGGAACACGC	GCCTTGCCAGCCCGCTCAGCATGGGGTGGTCACGTACGGAACTC
Set 14	GCCTCCCTCGCGCCATCAGCATGCCTGGGAGGTTCACAAACCTC	GCCTTGCCAGCCCGCTCAGCATGGGTTGAGTTCAGGCCTGTGTCC
Set 15	GCCTCCCTCGCGCCATCAGCATGGGCTAAGTACATGGTGGCCTAC	GCCTTGCCAGCCCGCTCAGCATGGGAAGCATCAGTCGTGACAGG
Set 16	GCCTCCCTCGCGCCATCAGCATGCCGACTTCGAGTTCAGGATGC	GCCTTGCCAGCCCGCTCAGCATGCTTCCGCAAGTTCTGGAGCTCC
Set 17	GCCTCCCTCGCGCCATCAGCATGCGCAGCCACGTACTACTTCTC	GCCTTGCCAGCCCGCTCAGCATGCACGTCGACCTCAGTGTCTGTG
Set 18	GCCTCCCTCGCGCCATCAGCATGCACTGCCCTACACCGCTCC	GCCTTGCCAGCCCGCTCAGCATGCCACCAGCTTTGTGAAGTTCTCCC

KNP_{P3} oligonucleotides:

Adapter A sequence ---------- KNP_{P3} TAG (4 bases) ---------- genome-specific FWD seq
GCCTCCCTCGCGCCATCAG---- ACGT ----------genome-specific FWD seq

Adapter B sequence ---------- KNP_{P3} TAG (4 bases) ---------- genome-specific REV seq
GCCTTGCCAGCCCGCTCAG---- ACGT ---------- genome-specific REV seq

vKNP/$SAT2_{BHK5}$ oligonucleotides:

Adapter A sequence ---------- vKNP/SAT_{BHK5}TAG (4 bases) ---- genome-specific FWD seq
GCCTCCCTCGCGCCATCAG---- CATG ---------- genome-specific FWD seq

Adapter B sequence ---------- vKNP/SAT_{BHK5}TAG (4 bases) ---- genome-specific REV seq
GCCTTGCCAGCCCGCTCAG---- CATG ----------genome-specific REV seq

Legend:
The fusion oligonucleotides for the respective viruses included a 19-mer adapter sequence at the 5'-end ("A" and "B") to match the components for forward and reverse reactions, and a 4-bp tag sequence allowing for the recognition of the two viruses upon sequence analysis. The tag sequences were "ACGT" and "CATG" for KNP_{P3} and vKNP/$SAT2_{BHK5}$, respectively. The 3' 20-29 nucleotides of the fusion oligonucleotides were complimentary to the FMDV genomes.

C: Alignment of the deduced amino acid sequence of the external capsid-coding region of the vKNP/SAT2 chimeric virus with that of the parental KNP/196/91 virus (Chapter 4).

```
                  ↓1A
KNP/196/91        GAGQSSPATGSQNQSGNTGSIINNYYMQQYQNSMDTQLGDNAISGGSNEG
vKNP/SAT2         GAGHSSPVTGSQNQSGNTGSIINNYYMQQYQNSMDTQLGDNAISGGSNEG
                                                      ↓1B
KNP/196/91        STDTTSTHTNNTQNNDWFSKLAQSAFSGLVGALLADKKTEETTLLEDRIL
vKNP/SAT2         STDTTSTHTNNTQNNDWFSKLAQSAISGLFGALLADKKTEETTLLEDRIL

KNP/196/91        TTSHGTTTSTTQSSVGITYGYADSDRFLPGPNTNGLETRVEQAERFFKHK
vKNP/SAT2         TTRHGTTTSTTQSSVGITYGYADSDRFLPGPNTNGLETRVEQAERFFKHK

KNP/196/91        LFDWTLEQRFGTTHVLELPTDHKGIYGQLVDSHSYIRNGWDVEVSATATQ
vKNP/SAT2         LFDWTLEQRFGTTHVLELPTDHKGIYGQLVDSHSYIRNGWDVEVSATATQ

KNP/196/91        FNGGCLLVAMVPELCKLSEREKYQLTLFPHQFLNPRTNTTAHIQVPYLGV
vKNP/SAT2         FNGGCLLVAMVPELCKLSEREKYQLTLFPHQFLNPRTNTTAHIQVPYLGV

KNP/196/91        DRHDQGTRHKAWTLVVMVVAPYTNDQTIGSNKAEVYVNIAPTNVYVAGEK
vKNP/SAT2         DRHDQGTRHKAWTLVVMVVAPYTNDQTIGSNKAEVYVNIAPTNVYVAGEK
                     ↓1C
KNP/196/91        PAKQGILPVAVSVGYGGFQNTDPKTSDPVYGHVYNPARTGLPGRFTNLLD
vKNP/SAT2         PAKQGILPVAVSVGYGGFQNTDPKTSDPVYGHVYNPARTGLPGRFTNLLD

KNP/196/91        VAEACPTLLDFNGVPYVTTQANSGSKVLTCFDLAFGHKNLKNTFMSGLAQ
vKNP/SAT2         VAEACPTLLDFNGVPYVTTQANSGSKVLTCFDLAFGHKNLKNTFMSGLAQ

KNP/196/91        YYTQYSGTLNLHFMYTGPTNNKAKYMVAYIPPGTHPLPETPEMASHCYHA
vKNP/SAT2         YYTQYSGTLNLHFMYTGPTNNKAKYMVAYIPPGTHPLPETPEMASHCYHA

KNP/196/91        EWDTGLNSTFTFTVPYVSAADFAYTYSDEPEQASVQGWVGVYQVTDTHEK
vKNP/SAT2         EWDTGLNSTFTFTVPYVSAADFAYTYSDEPEQASVQGWVGVYQVTDTHEK
                                                  ↓1D
KNP/196/91        DGAVVVSVSAGPDFEFRMPISPSRQTTSAGEGAEPVTTDASQHGGDRRTT
vKNP/SAT2         DGAVVVSVSAGPDFEFRMPISPSRQTTSAGEGAEPVTTDASQHGGDRRTT

KNP/196/91        RRHHTDVSFLLDRFTLVGKTQDNKLTLDLLQTKEKALVGAILRAATYYFS
vKNP/SAT2         RRHHTDVSFLLDRFTLVGKTQDNKLTLDLLQTKEKALVGAILRAATYYFS

KNP/196/91        DLEVACVGDNKWVGWTPNGAPELAEVGDNPVVFSKGRTTRFALPYTAPHR
vKNP/SAT2         DLEVACVGDNKWVGWTPNGAPELAEVGDNPVVFSKGRTTRFALPYTAPHR

KNP/196/91        CLATAYNGDCKYKPTGTAPRENIRGDLATLAARIASETHIPTTFNYGRIY
vKNP/SAT2         CLATAYNGDCKYKPTGTAPRENIRGDLATLAARIASETHIPTTFNYGRIY

KNP/196/91        TDTEVDVYVRMKRAELYCPRPVLTHYDHGGRDRYRTAITKPVKQLC
vKNP/SAT2         TDTEVDVYVRMKRAELYCPRPVLTHYDHGGRDRYRTAITKPVKQLC
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Legend:
The protease cleavage sites are indicated by arrows. The cloning region is indicated in grey. The amino acid sequence change obtained in the 1B protein is indicated in green and was introduced during cloning. The amino acid sequence differences between KNP/196/91 and ZIM/7/83 are indicated in yellow.