CHAPTER 1

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. Susceptible species include domestic animals such as cattle, sheep, goats and pigs, as well as more than 30 species of wild ruminants, including the African buffalo, deer, mountain gazelle and giraffe. Although FMD is characterized by low mortality rates (less than 5%) (Thomson, 1994; Beard and Mason, 2000), the disease has a major economic impact on the livestock industry. FMD leads to disruption of trade in animals and other animal products due to loss of milk production, delay in animals reaching maturity for marketing, abortions and death in young animals (Gibbs, 1981). The devastating effects of a large outbreak of FMD have been seen recently in Europe, where 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 (up to 10^{12} infectious units per animal) is excreted through the breath, excretions and from the skin of infected animals. The disease can be transmitted indirectly through contact with viral-contaminated animal products (*e.g.* meat, milk, semen, embryos and aerosols), whilst 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 eight days and are followed by 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).

Rapid and accurate diagnosis of FMD is a prerequisite for effective control of the disease. 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 and is used in many diagnostic laboratories (Abu Elzein *et al.*, 1979). Other diagnostic techniques include virus neutralization tests (VNT) (Rweyemamu, 1984), virus isolation in cell lines such as pig kidney and bovine thyroid cells, and molecular techniques such as the reverse transcriptase-polymerase chain reaction (RT-PCR) for rapid detection of FMDV nucleic acid (Bastos, 1998).

1.2 RECENT OUTBREAKS OF FMD

FMD is a disease with global distribution and has affected most countries in the world. 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 the disease 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 and the Middle East to the rest of Europe. Despite attempts to control the disease, FMD persists in most African countries, South America, the Middle East and central and south-east Asia (Thomson *et al.*, 2003).

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). During the last decade, many type O outbreaks have been reported in India (Knowles *et al.*, 2001). The Pan-Asia type O strain, which recently caused FMD outbreaks in many parts of the world, was first isolated in India in 1990. 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 Korea, Japan, Russia and Mongolia, and in September of 2000 led to the first isolation of a type O virus in South Africa (Sangare *et al.*, 2001). In February of 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 August of 2002, this virus strain was still causing FMD outbreaks in Mongolia (Knowles and Samuel, 2003).

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) and are prevalent in South America, where closely related type A viruses have been responsible for outbreaks in Argentina, Uruguay and Brazil in 2001 (Knowles and Samuel, 2003). Type C FMDV has a limited distribution due to the fact that there are no natural reservoirs for this specific virus type. This serotype occurs infrequent, with the last outbreak in Italy in 1989 and the last reports being from Africa and Asia in 1996 (Knowles and Samuel, 2003). Therefore, there is a possibility that type C FMDV may be eradicated. Asia-1 is not a very diverse FMDV serotype. In a recent epizootic of Asia-1, it spread across the east from Iran and Turkey in 1999 to Greece in 2000. An Asia-1 type virus has also been isolated in Saudi Arabia in 1994 (Knowles and Samuel, 2003).

Although the South African Teritories (SAT) type viruses are considered to occur mostly in sub-Saharan Africa, outbreaks have been recorded in the Middle East (1962-1965 and 1969-1970), as well as in Greece (1962). Furthermore, the SAT2 type viruses have also spread to the Middle East in 1990 and again in 2000 (Bastos *et al.*, 2003). In southern Africa, buffalo are the maintenance hosts of FMDV and a source of infection for other cloven-hoofed animals (Thomson *et al.*, 2003). Whereas SAT2 type viruses are responsible for most FMD outbreaks in southern Africa (Vosloo *et al.*, 2002), followed by SAT1 type viruses, the SAT3 type viruses are least associated with outbreaks in domestic animals (Thomson, 1994; Knowles and Samuel, 2003).

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 characterized 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, as it was only the second virus and first animal disease to be discovered. The first description of FMD was provided by the Italian Hieronymus Fracastorius and dates back to 1546 (Sobrino *et al.*, 2001). FMDV was also the first filterable infectious agent to be identified as causing an animal disease by Loeffler and Frosch (1898), and indicated the existence of infectious agents smaller than bacteria (Brown, 2003). Today, FMDV ranks first in the A-list of infectious diseases of animals according to the Office International des Epizooties (OIE Manual of Standards).

1.3.2 Physical properties

FMDV is highly labile at pH≤6; a characteristic which is also shared by *Rhinoviruses* (King, 2000). By contrast, *Entero-* and *Cardioviruses* are stable at pH 3. Unlike other Picornaviruses, the capsid of FMDV has a highly hydrophobic hole at the icosahedral five-fold axis, which 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 28 to 30 nm in diameter, spherical with icosahedral symmetry (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 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).

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 x 10^6 Da) and is enclosed within a protein capsid. 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). Figure 1.1 indicates a schematic representation of the FMDV RNA genome and protein-coding regions.

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 a virus-encoded protein VPg (viral genome-linked protein) (Fig. 1.1). 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 3 copies in tandem. The VPg protein may be involved in initiation of RNA synthesis and encapsidation of viral RNA (Sanger *et al.*, 1977; Forss and Schaller, 1982), and its function may furhermore be related to pathogenesis and host range determination of the virus (Mason *et al.*, 2003). 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.*, 2003) (Fig. 1.1). 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 circularization (Mason *et al.*, 2003). Three to four pseudoknot motifs are located downstream of the poly(C) tract, but their function is not yet known (Clarke *et al.*, 1987a). By 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

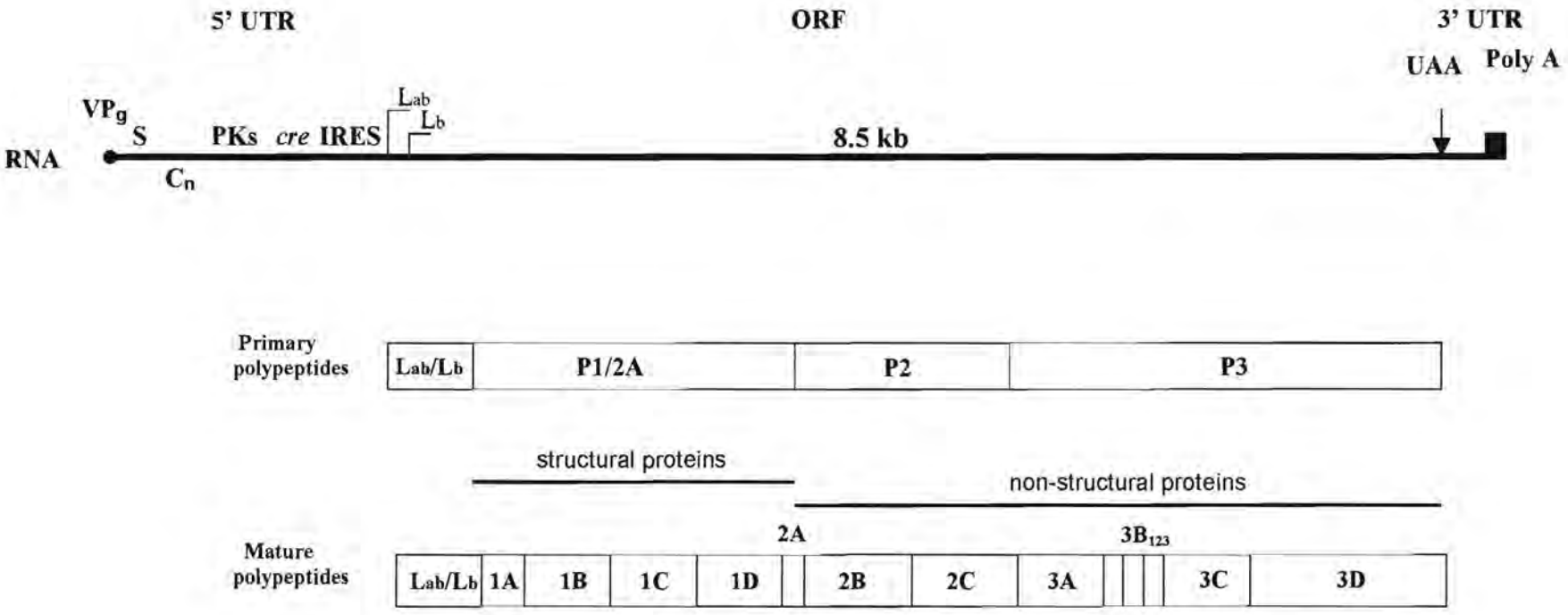

Fig. 1.1. Schematic diagram of the FMDV RNA genome, the untranslated regions (UTR) and open reading frame (ORF), as well as primary and mature polypeptides. The structural elements of the 5' UTR are indicated on the RNA genome, which is indicated by a thick line.

replication (Mason *et al.*, 2002). The Picornavirus IRES is highly structured and directs cap-independent translation of the viral RNA (Mason *et al.*, 2003).

The viral ORF consists of the Leader proteinase (L^{pro}), structural protein-coding region (P1) and non-structural protein-coding regions (P2 and P3) (Fig. 1.1). The L^{pro} protein-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 synthesized *in vivo* (Coa *et al.*, 1995). Although it is an important determinant of viral virulence and essential for pathogenesis (Mason *et al.*, 1997; Chinsangaram *et al.*, 1998), functional studies, using genetically engineered viruses, have indicated that the L^{pro} is not essential for virus replication (Piccone *et al.*, 1995). The L protein is a protease unique to the *Aphthoviruses* (Strebel and Beck, 1986). Four other picornaviruses also encode for L proteins which 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 (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. The P2 polyprotein precursor is proteolytically processed into 2A, 2B and 2C (Fig. 1.1). The 18-amino-acid peptide 2A remains attached to the P1 polyprotein precursor following primary cleavage (Donnelly *et al.*, 2001; Mason *et al.*, 2003) 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 contains a helicase domain (Porter, 1993) and the protein is involved in viral RNA synthesis, as mutations that confer resistance to guanidine, an inhibitor of viral RNA replication, are located in this region (Saunders *et al.*, 1985). The 2C protein and its precursor 2BC are associated and induce cell membrane vesicle proliferation (Bienz *et al.*, 1990).

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 for 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). In addition, the 3A protein is believed to be a membrane anchor for the replication complex (Weber *et al.*, 1996) and is associated to viral-induced membrane vesicles and contributes to CPE and to the inhibition of protein secretion (Doedens and Kirkegaard, 1995). As mentioned previously, the 3B-coding region encodes the VPg 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). The 3C protein is a thiol-protease ($3C^{pro}$) and is responsible for most of the 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. 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 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. Downstream of the 3D region is the 3' UTR and the polyadenylate tract (poly(A) tract). The poly(A) tract is involved in viral replication (Sáiz *et al.*, 2001).

1.4.2 Structure of the FMDV capsid

The structure of type O (Acharya *et al.*, 1989), type C (Lea *et al.*, 1994) and type A (Curry *et al.*, 1996) virions have been elucidated by X-ray crystallography. FMDV capsids have the classic structural organization of Picornaviruses (Fig. 1.2d). 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. 1.2b). 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. 1.2c), and the single-stranded RNA genome. 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) (Fig. 1.2a). These proteins have a similar arrangement to other Picornaviruses, where 1D proteins are located around the icosahedral five-fold axes and 1B and 1C alternate around the two- and three-fold axes. Protein 1A is located internally and its N-terminal portion 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, and lacks the peaks and depressions of other Picornaviruses (Fry *et al.*, 1990). This protruding G-H loop spans about 20 amino acid residues in the region of residues 140-160 and contains the highly conserved Arg-Gly-Asp (RGD) triplet, which is the main cellular receptor recognition site (Baranowski *et al.*, 2001) and forms part of the major neutralization site (Mateu *et al.*, 1995).

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 and is of great importance to its pathogenicity (King, 2000). This acid sensitivity varies between different FMDV serotypes, as well as subtypes. A His-rich region at the pentamer interface (1B/1C) has been suggested as the reason for capsid disassembly. The His-142 in the 1C protein is conserved in FMDV and protonation of these residues at a low pH may cause repulsive electrostatic forces across the pentamer interface, thereby resulting in the capsid opening up (Acharaya *et al.*, 1989; Curry *et al.*, 1995).

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 neutralization (Mateu *et al.*, 1995) and are serotype-specific. Initial studies on type A_{12} FMDV indicated that the 1D protein induces sufficient neutralizing antibodies to protect animals (swine and cattle) from homologous virus challenge

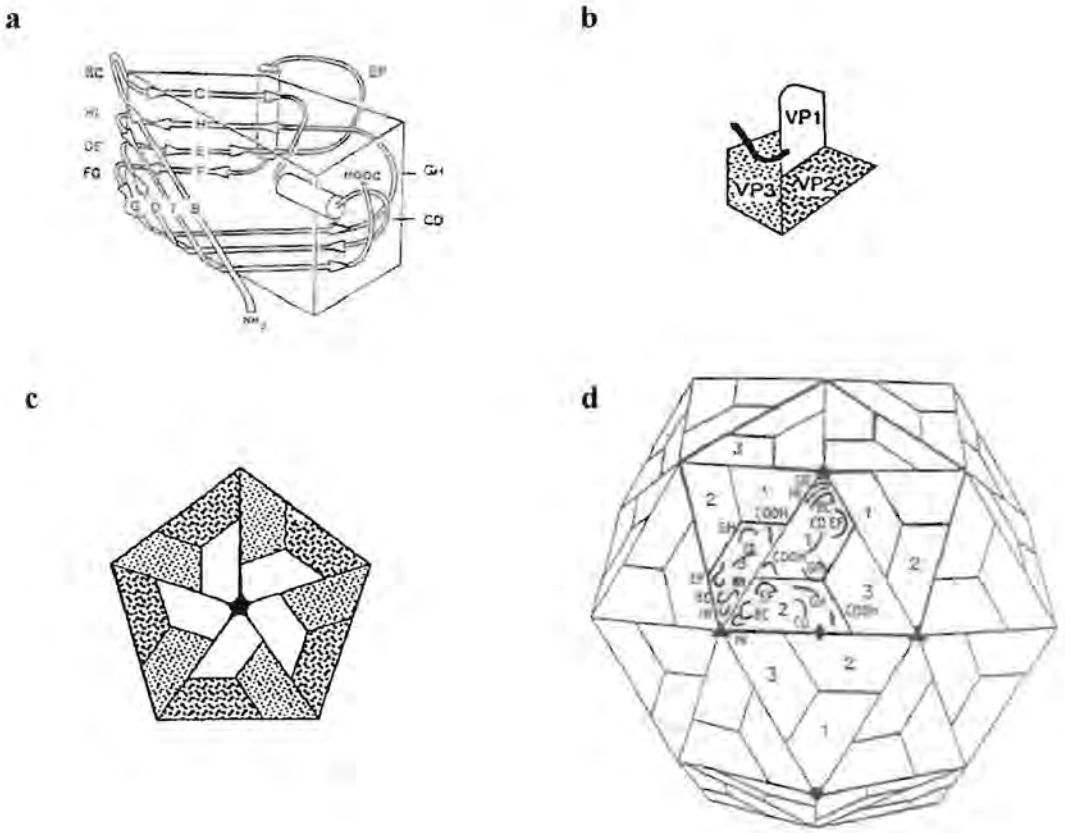

Fig. 1.2. Schematic view of the structure of the FMDV external capsid proteins, the subunits and viral capsid. (a) Schematic tertiary structure of 1B, 1C and 1D; β-chains (arrows); α-chains (cylinders); connecting loops (identified by the β-chains joined). The upper side of the trapezoid is exposed on the virion surface. (b) The three external capsid proteins assemble into a protomer, consisting of 1B (VP2), 1C (VP3) and 1D (VP1) exposed on the virus surface and 1A (VP4) which is internal (C-terminus of 1D is indicated by a black ribbon). (c) Arrangement of the five protomers into a pentamer (the 5-fold symmetry axis is indicated, pentagon). (d) Structure of the FMDV capsid, consisting of 60 protomers. The icosahedral symmetry axis are labelled (2-fold axis, oval; 3-fold axis, triangle; 5-fold axis, pentagon). Nomenclature is adapted from Mateu, 1995 and Sobrino *et al.*, 2001.

(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 deduced from the immunogenicity of peptides consisting of amino acid residues around 140-160 in the 1D protein (Mateu *et al.*, 1995). This region has subsequently become known as site 1 for serotypes O_1 and A or site A for serotype C. The antigenic sites identified for type O, type A and type C FMD viruses are indicated in Table 1.1.

The conformation of the G-H loop of type O viruses differs markedly from other serotypes due to the formation of a disulfide 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 and type C viruses, which recognize immobilized peptides representing the G-H loop (Mateu *et al.*, 1987; Bolwell *et al.*, 1989).

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 neutralization and non-neutralization 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. Site 2 involves residues from the B-C loop and E-F loop of the 1B protein. Site 3 is located on the B-C loop of the 1D protein and site 4 is located in the 1C protein. Site 5 is a second distinct site in the 1D protein. For type A viruses, the antigenic sites are similar to that of type O_1 (Table 1.1; Fig. 1.2d). Type C also 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)

Table 1.1. Secondary structure elements of FMDV involved in antigenic sites of type O, type A and type C. The closest axis to the capsid structural element is indicated (Taken from Mateu, 1995)

Axis	Capsid secondary structural element	FMDV antigenic sites					
		Type O	Type A_{10}	Type A_{12}	Type A_{22}	Type A_5	Type C
5 x	1D B-C	3					
	1D H-I		4	1			
2 x	1D G-H	1, 5	1	2	1		A
	1B E-F	2					
3 x	1B B-C	2	3			1	D
	1C B-B	4	5				D
	1C B-C		3				
	1C H-I		3				
	1D C_T	1	2	3, 4			C, D
	1C E-F		3				
	1C G-H			4			

(Mateu *et al.*, 1990; Lea *et al.*, 1994). By contrast, 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).

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* utilize multiple cellular receptors for infection. After cell binding, when FMDV is present in the low pH endosomal compartment, the virus particle disassembles and viral replication proceeds (Rieder and Wimmer, 2002).

1.5.1.1 RGD-dependant mechanism of cell binding

Structural and functional studies over the last decade (Mateu *et al.*, 1995) have indicated that the RGD motif is important in the interaction of FMDV with the cell surface. Despite being located in a highly variable region between virus isolates, the RGD is highly conserved (Robertson *et al.*, 1983; Pfaff *et al.*, 1988). The RGD is located on the apex of the long, flexible G-H loop of the 1D protein (Acharya *et al.*, 1989). 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 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). Although viral particles could be obtained from *in vitro*-transcribed RNA upon transfection into cultured cells, these particles were non-infectious as a result of not being able to bind to cells.

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 utilize 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). Integrin $\alpha_v\beta_1$ is implicated in cell binding of FMDV to cultured cells (Jackson *et al.*, 2002). In addition to the RGD motif, the regions flanking this triplet are also important for cell binding, as changes in these residues may influence binding of the virus to RGD-dependant cellular receptors (Jackson *et al.*, 2000).

1.5.1.2 RGD-independant mechanism of cell binding

In addition to integrins, FMDV has been reported to utilize 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 characterized by phenotypic alterations such as improved replication (Baranowski *et al.*, 1998), enhanced resistance to neutralizing monoclonal antibodies, broadened cell tropism (Escarmís *et al.*, 1996), the ability to bind to heparan (Jackson *et al.*, 2000) and are attenuated in cattle (Sa-Carvalho *et al.*, 1997).

Viruses propagated in tissue culture often use RGD-independent mechanisms of cell recognition. Despite the presence of the RGD motif, alternative receptors on the cell surface such as heparan sulfate glycosaminoglycan residues are utilized (Jackson *et al.*, 1996; Sa-Carvalho *et al.*, 1997). Binding to heparan involves positive amino acids on the capsid surface, which vary for different virus strains (Jackson *et al.*, 2003). Viruses that utilize heparan sulfate are, however, still capable of using intergrins as cellular receptors (Baranowski *et al.*, 2000). The virus 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 heparan sulfate receptors are not essential for FMDV cell-binding. In addition to the above, FMDV may bind to cells via integrin- and heparan sulfate-independent pathways, suggesting the existence of additional

uncharacterized receptors, and thus of multiple mechanisms whereby FMDV can adapt to growth in cell culture (Baranowski *et al.*, 2000; Zhao *et al.*, 2003).

1.5.2 Protein synthesis and processing of the FMDV polyprotein

Viral replication occurs in the cytoplasm of infected cells in association with cell membranes (Bachrach, 1977). Viral uncoating is believed to proceed via receptor-mediated endocytosis. Virus-receptor complexes form on the plasma membrane, cluster at clathrin-coated pits and are subsequently internalized by invagination and internalization to form clathrin-coated vesicles (Levy *et al.*, 1994). Acidification of these resulting clathrin-coated vesicles then 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 protein leads to the formation of a pore through which the viral RNA can be transferred to the cytoplasm (Rueckert, 1996). In the cytoplasm, the VPg protein, which is linked to the 5' UTR of the viral RNA, is released and polyprotein synthesis is initiated in a cap-independent 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).

The leader protease (L^{pro}) is the first protein to be synthesized 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-dependent mRNA translation of the host cell (Devaney *et al.*, 1988). With the progression of polyprotein synthesis, the polyprotein is cleaved into several structural and non-structural proteins (Ryan *et al.*, 1989), mainly by $3C^{pro}$ which functions *in trans* (Vakharia *et al.*, 1987; Clarke and Sanger, 1988). The FMDV $3C^{pro}$ cleavage sites show heterogenicity 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-

dimentional 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 catalyzed 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). Virion assembly involves the formation of capsid protomers and association of five such protomers result in the formation of a pentamer, which is followed by assembly into either empty capsids or provirions, which contain packaged positive-sense VPg-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 IMMUNE RESPONSE

Due to the economic importance of FMD, comprehensive studies on disease immunity and development of vaccines have been initiated at the start of the 20th century. Following immunization or infection, the immune response elicited against Picornaviruses consists of the production of specific neutralizing antibodies to the structural proteins of the virus (Salt, 1993). Induction of high levels of neutralizing antibodies is believed to be the principle method of protection; therefore immunological studies have been directed mainly at the humoral immune response. The first neutralizing antibodies elicited against FMDV are IgM antibodies and appear 3 to 5 days following infection or vaccination and reaches a peak between 5 to 10 days post-

infection (Doel, 1996). IgG antibodies appear from 4 days and onward. In cattle, the IgG immune response has a peak at 10 to 14 days post-infection (Collen, 1994). In pigs, IgG is detected 4 to 7 days post-infection and the maximum levels are reached between 15 to 20 days (Francis and Black, 1983; Doel, 1996). This rapid humoral-specific response elicited upon infection or vaccination with FMDV, results in protection against re-infection with homologous virus strains (McCullough *et al.*, 1992; Salt, 1993). B-cell epitopes on the viral capsid induce neutralizing antibodies. These immunodominant neutralizing 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). When sufficient immunity is induced to FMDV, the development of clinical signs of disease is prevented (Doel, 1996).

The host animal T-cell immune response to FMDV infection has only been investigated recently, with studies performed mainly on inactivated virus vaccines and synthetic peptides. Compared to the high levels of neutralizing antibodies in FMDV-infected animals, T-cell responses have been lower and the duration of T-cell responses is unknown (Doel, 1996). Specific T-cell responses have, however, been observed with infection and vaccination, where T-helper cells ($CD4^+$) not only contribute to protective immunity with B-cell activation and antibody production, but also contribute to maintaining the appropriate microenvironment for a synergistic immune response (Collen, 1994; Sáiz *et al.*, 2002). FMDV infection results in a rapid reduction of major histocompatibility complex (MHC) class I molecules, which are expressed on the surface of susceptible cells, thus mediating escape of the virus from the host antiviral response, since viral proteins are ineffectively presented to cytotoxic T-cells by infected cells (Sanz-Parra *et al.*, 1998). It has been proposed that viral clearance *in vivo* could also be facilitated by phagocytosis of virus-antibody complexes (McCullough *et al.*, 1992).

In the absence of sterilizing immunity to FMDV (*i.e.* total protection against infection), viral replication may prevail at the epithelial surface and hosts could become carrier animals. Where animals are asymptomatic and persistently infected, virus is isolated from the oesophagus for four weeks or longer (Salt, 1993). This carrier state is species- and strain-dependant and has not been demonstrated in pigs. Domestic animals can be a source of infection for up to 2.5 years in cattle and six to nine months in sheep and goats (Thomson *et al.*, 2003). In southern Africa, this

is a very important factor in FMD epidemiology. African buffalo become persistently infected (Vosloo *et al.*, 1996) and could be a source of infection for other species, *e.g.* cattle, as was evidenced from an outbreak of FMD in South Africa during 2001 (Vosloo *et al.*, 2002).

1.7 CONTROL OF FMD

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, the first step is to confirm clinical diagnosis by laboratory tests. A quarantine area should immediately be declared and enforced, whereafter the extent of the spread of the disease should be established. A stamping-out policy is then followed during which infected or susceptible in-contact animals are slaughtered (Barteling and Vreeswijk, 1991). In countries where FMD is endemic, the disease is controlled by prophylactic vaccination and zoo-sanitary measures, such as control of animal movement and importation of animals and animal products from affected areas. In vaccinated animals, a consistent humoral response and solid, although short-lived lymphoproliferative response is produced (Sáiz *et al.*, 2002). Should an outbreak occur, it is controlled by vaccination and stamping-out of infected or in-contact animals is applied only at the final stage of an eradication campaign.

1.7.1 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 immunizing ability, the use of aluminium hydroxide ($Al(OH)_3$) gel as adjuvant was subsequently investigated by Schmidt in the 1930s. However, this virus and $Al(OH)_3$ gel mix caused disease in cattle. When Waldmann 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). An increase in the formaldehyde concentration (0.04%) in

vaccines, led to proper inactivation of the virus and improved safety (Barteling and Vreeswijk, 1991). 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 h at 37°C, clarified and filtered, absorbed onto $Al(OH)_3$ gel and inactivated with formaldehyde. This inactivated vaccine was introduced into vaccination programs in Europe during 1953 (Barteling and Vreeswijk, 1991; Brown, 1991).

1.7.1.1 Production of FMDV in cell lines

FMD vaccine production entails viral amplification to high quantities in tissue culture in containment facilities. Tissue culture-methods have been developed in the mid-1960s using primary cells such as calf kidney cells. Baby hamster kidney cells (BHK-21, clone 13) became available soon afterwards and is 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. 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). Vaccines obtained from such virus harvests are potent immunogens and have been used successfully worldwide for FMD control and eradication.

1.7.1.2 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 ethyleneimine (BEI) at pH>8 (Bahneman, 1975). The toxicity

and non-infectivity of the vaccine is assessed with an *in vivo* test, as prescribed by the European Pharmacopoeia (OIE Manual of Standards), 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).

1.7.1.3 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) are 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).

1.7.1.4 Formulation of the antigen

The serotype-specific immunity induced by FMDV infection necessitates careful selection of a vaccine strain. 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 of 146S. The relationship between the 146S and vaccine potency is not a linear function and therefore complicates the formulation of the final product (Rweyemamu and Ouldridge, 1982).

Aqueous vaccines consist of $Al(OH)_3$ gel, supplemented with saponin, and are administered subcutaneously. These vaccines induce high levels of neutralizing 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, an incomplete Freund adjuvant formulation has been used for pigs where the antigen is emulsified in mineral oil. When cattle were injected intramuscularly using such vaccines, severe adverse reactions have been observed. Reduced dose volumes and subcutaneous injection gave more satisfactory results. The advantage of oil adjuvant vaccines is longer-lasting protection and consequently a possible reduction in the frequency of vaccination (McKercher and Graves, 1977). 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 immunization every four to six months (Thomson, 1994).

The virus neutralization test (VNT) is used for the assessment of vaccine potency of the final product and in determining the relationship between field isolates and vaccine strains. 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 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).

1.7.2 Emergency 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 banks are the North American Vaccine Bank, the European Union Vaccine Bank and the International Vaccine Bank. 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 a higher antigen dose (≥6 protective doses [PD_{50}]) than conventional vaccines to ensure both rapid protective immunity and wider antigenic coverage within FMDV serotypes (Salt *et al.*, 1998; Cox *et al.*, 2003). Such vaccines

limit virus replication in the oropharynx, thereby limiting subsequent transmission of the disease to other susceptible animals (Salt *et al.*, 1998).

1.8 ALTERNATIVES TO CONVENTIONAL 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 lead to a new approach in the development of FMD vaccines based on the reconstruction of viruses and its immunogens. Increased understanding of the FMD virus structure at the molecular level has led to the identification of regions on the virus particle that elicit 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).

1.8.1 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} FMD virus 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). 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 synthesize 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 carboxy terminus (residues 200-213). These synthetic peptide vaccines induced neutralizing antibodies in mice and guinea pigs (Bachrach, 1985) and provided partial protection against homologous FMDV challenge in cattle and swine (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 swine, 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 recognized by MHC molecules of the host species (Rodriquez *et al.*, 1994). In addition, escape mutants were possibly present which may have led to less than 40% protection of cattle, suggesting rapid generation and selection of FMDV antigenic variants *in vivo* (Tobago *et al.*, 1997). To be effective, an epitope must assume the same conformation as the intact viral particle. As seen from the above, a single epitope may not be sufficiently immunogenic. In addition, peptides mimic the linear conformation of epitopes, whereas many FMDV epitopes are conformational (Mateu, 1995).

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 foreign protein levels expressed in plants, as well as easier selection of transgenic plants presenting the highest levels of protein expression, Dus Santos and co-workers (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. However, a disadvantage of this system, which is of great relevance to vaccine production, is the low expression levels obtained.

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 insufficient (Grubman *et al.*, 1993). Expression of the P1 region of type A_{12} virus by a baculovirus recombinant protected swine 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). Although vaccines derived from empty virus 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, as large quantities of properly processed viral proteins have not been produced.

1.8.2 Genetically engineered attenuated strains

When the development of attenuated vaccines for FMDV were 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) 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 (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). By contrast, partial protection of cattle was observed upon challenge with homologous recombinant viruses lacking the Leader-coding region. Two out of the three animals did not develop lesions, but showed signs of FMDV infection post-challenge (Mason *et al.*, 1997). Despite initial 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, were complications which may have lead to this approach being discontinued.

1.8.3 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). 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 with DNA expressing empty viral capsids has also been reported to induce a neutralizing antibody response (Chinsangaram *et al.*, 1998). DNA vaccines have been constructed to contain the G-H loop and C-terminus of the 1D protein, as well as host-self immunoglobulin molecules (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 neutralizing antibodies (Wong *et al.*, 2000). This immune response was enhanced with the co-administration of the cytokine interleukin-2 that enhances cellular immunity (Wong *et al.*, 2002). Despite all the research on DNA vaccines, it has not replaced traditional inactivated FMD vaccines, due to concerns about safety and difficulty of administration.

1.8.4 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 neutralizing antibodies in calves (Kit *et al.*, 1991).

Replication-defective adenovirus vectors are able to infect cells of several animal species, including cattle and pigs (Prevec *et al.*, 1989) and effective immunization of animals has been achieved with such vectors expressing proteins from other pathogens (Grubman and Mason, 2002). 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. Replication-defective human adenovirus serotype 5 (Ad5) vectors containing the FMDV type A_{12} capsid and either the wild-type or inactive $3C^{pro}$ have been engineered (Mayr *et al.*, 1999; 2001). Inoculation of pigs with the recombinant vector containing the wild-type $3C^{pro}$ led to the production of a neutralizing antibody response upon challenge with homologous virus (Mayr *et al.*, 1999; 2001), 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 emphasizes 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 is currently being investigated. 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 is 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 h 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 addition to the fast immune response elicited by these interferon-expressing Ad5 vectors, the vaccine may be used immediately during a FMD outbreak. Since this method should provide protection against all FMDV serotypes, it would thus not have to be compared with the outbreak strain. In addition, when applied by FMD-free countries to control an outbreak, the large-scale slaughtering of animals could be prevented.

1.9 VACCINATION IN SAUDI ARABIA: AIMS AND SCOPE OF THIS INVESTIGATION

Saudi Arabia imports *ca.* 6.5 million animals annually. Outbreaks of FMD occur often among livestock such as cattle, sheep and goats (Hafez *et al.*, 1994). As mentioned previously, FMD is of great economic importance, resulting in a reduction in growth and fertility of animals, as well as mortality in young animals (Thomson, 1994). Consequently, FMD leads to the disruption of farming practices through loss of income and breeding stock in this region. On a national level, it determines the availability and prices of livestock and other related products, whilst export markets are lost due to trade embargoes (Doel, 2003). FMD is also associated with a reduction of up to 25% in milk yield in infected animals (Hafez *et al.*, 1993). This is a very important consideration in Saudi Arabia which has a well-established dairy industry.

Uncontrollable movement of animals between countries in the Middle East, especially the herds and flocks of nomadic people, complicates the control of FMD. In the large dairy herds of Saudi Arabia, sub-clinical FMDV infection has been found on a number of occasions to spread amongst the cattle before clinical signs of disease were seen. Another contributing factor to the difficulty in controlling the disease is that some owners are unwilling to report the disease (Kitching, 1998; Hutber *et al.*, 1999).

Vaccines used in Saudi Arabia contain different serotypes of FMDV and are used in four vaccinations annually. Using a mathematical model of the decay of protective antibodies, it has

been calculated that 95% of animals are protected with an antibody half-life of 43 days, indicating that it is unlikely that FMD outbreaks can be prevented (Woolhouse *et al.*, 1996). Indeed, a severe outbreak of FMD occurred in Saudi Arabia in 2000 (Knowles and Samuel, 2003). Genetic analysis revealed that the SAT2 virus responsible for an outbreak in Eritea during 1998 and the Saudi Arabian SAT2 outbreak strain, SAU/6/00, are members of the same lineage. Therefore, the virus may have been introduced from north-eastern Africa to the Middle East (Bastos *et al.*, 2003). As the Saudi Arabian SAT2 outbreak strain is genetically different from other SAT2 vaccine strains, it necessitated the production of a vaccine containing the SAU/6/00 antigen. The vaccine is being produced at the Exotic Diseases Division, Onderstepoort Veterinary Institute, but batch analysis of the vaccine has indicated low and variable yields in the production of antigen.

Despite recombinant DNA techniques having been used to produce different kinds of vaccines for FMD, it is still only the inactivated virus vaccine that is effective in the field. Therefore, to investigate an alternative means towards production of the inactivated Saudi Arabian SAT2 vaccine, reverse genetics techniques will be used in this study to engineer a chimeric FMDV for potential use in inactivated vaccine production. This will entail replacing the external capsid-coding region of a previously constructed SAT2 genome-length cDNA clone (Van Rensburg *et al.*, 2004) with that of the Saudi Arabian SAT2/SAU/6/00 outbreak strain. The SAT2 genome-length cDNA clone has been constructed from the ZIM/7/83 virus, which is a stable vaccine strain and produces high concentrations of antigen (Esterhuysen *et al.*, 1988). It is therefore hypothesized that the newly constructed SAT2 chimeric virus will have improved growth properties and result in a constant production of high concentrations antigen. Such reverse genetics approaches have proven useful for studies on genetically engineered European type viruses (Rieder *et al.*, 1994), the investigation of attenuated viruses (Almeida *et al.*, 1998) and genetically engineered viruses (Baranowski *et al.*, 2001; Van Rensburg and Mason, 2002; Van Rensburg *et al.*, 2004).

The aims of this study were therefore:

(i) to molecularly characterize the external capsid-coding region of the Saudi Arabian outbreak strain SAU/6/00;

(ii) to engineer a chimeric FMD virus by cloning the external capsid-coding region from SAU/6/00, encoding the determinants for antigenicity, into the SAT2 genome-length cDNA clone lacking the corresponding region; and

(iii) to characterize the newly engineered vSAU6/SAT2 chimera in terms of properties important in inactivated vaccine production.

CHAPTER 2

MOLECULAR CHARACTERIZATION AND ANALYSIS OF THE CAPSID-CODING REGION FROM THE SAUDI ARABIAN SAT2 FOOT-AND-MOUTH DISEASE VIRUS VACCINE STRAIN SAU/6/00

2.1 INTRODUCTION

Foot-and-mouth disease (FMD) is endemic in most of the middle-Eastern countries, with serotype O being the most prevalent. Sporadic outbreaks are caused by serotypes A and Asia-1, whereas outbreaks caused by serotype C occur infrequently (Woodbury *et al.*, 1994). Although the SAT type viruses usually occur in sub-Saharan Africa, outbreaks of SAT1 have been recorded in the Middle East. Recently, in 2000, a severe outbreak of SAT2 was confirmed in dairy herds in Saudi Arabia (Knowles and Samuel, 2003). Genetic analysis has indicated that a SAT2 virus (ERI/12/98), responsible for an outbreak of FMD in Eritrea during 1998, and the Saudi Arabian outbreak strain, SAU/6/00, isolated in 2000, are members of the same lineage. Therefore, the virus may have been introduced to the Middle East from the north-eastern region of Africa (Bastos *et al.*, 2003). Such a scenario is not unlikely, as Saudi Arabia annually imports more than 6 million animals, mainly cattle, goats and sheep, from Africa, Asia and Australasia. The majority of the animals originate from countries in Africa and Asia where FMD is enzootic (Kitching, 1998).

The imported livestock may be carrier animals that act as a source of FMDV infection or they may be sub-clinically infected animals that excrete virus (Hafez *et al.*, 1994). In a study performed over 11 years, 47% of dairy herds in Saudi Arabia were found to be sub-clinically infected with FMDV (Hutber *et al.*, 1999). Since FMD is a highly contagious disease, the increased levels of virus secretion by infected animals and the close contact between infected and susceptible animals cause herds to become infected and/or re-infected; a situation that is made worse by the high animal densities found at feedlots (Hutber and Kitching, 2000). Furthermore, the uncontrollable movement of animals between countries in the Middle East, especially the

herds and flocks of nomadic people, as well as the unwillingness of some owners to report the disease, make it difficult to effectively control FMD (Hafez *et al.*, 1993).

The control of FMD in Saudi Arabia has, however, been improved by vaccination (Hutber *et al.*, 1998; Sutmoller *et al.*, 2003). Although data suggests that vaccines give 81-98% protection for 2.5 months in cattle and despite vaccination every 4 to 6 months, outbreaks of FMD persist in dairy herds (Woolhouse *et al.*, 1996; Hutber *et al.*, 1998). This is of particular concern, as Saudi Arabia has a well-established dairy industry with dairy herds consisting of up to 5 000 cattle, which may give 12 000 litres of milk per lactation (Kitching, 1998). It has also been reported that the critical interval between protection and vaccination is impractical, especially for a heterologous challenge (Woolhouse *et al.*, 1996). In addition, the diverse origins of FMD viruses make it difficult to determine which strains to include in the vaccine. Although the recommended vaccines contain serotypes A, O, C and Asia-1 (Kitching, 1998), the heptavalent vaccine that has been used to protect herds is not providing full protection.

Currently, the Exotic Diseases Division, Onderstepoort Veterinary Institute, produces a formalin- and binary ethyleneimine (BEI)-inactivated vaccine that contains the SAT2/SAU/6/00 antigen. However, antigen production has shown great variability between batches (Fig. 2.1). In addition, the SAU/6/00 antigen is harvested at 42 h post-infection, which is much later than the standard time of 26 h used for harvesting SAT type vaccine strains (Mr N. Ismail-Cassim, pers. comm.). For vaccine preparation, a constant production of high concentrations antigen is ideal. To address these problems, an alternative approach to improve the growth properties and obtain a higher yield of antigen within a shorter time will be explored through the engineering and characterization of a chimeric FMD virus. Towards this objective, the capsid (P1)-coding region of SAU/6/00 was obtained by RT-PCR, cloned into the pGEM®-T Easy vector by means of T/A cloning and the nucleotide sequence determined. The sequence data was subsequently analyzed with regards to intratypic variation, hypervariable regions and 3C protease cleavage sites in order to assess its potential impact on the construction of the desired chimeric SAT2 virus.

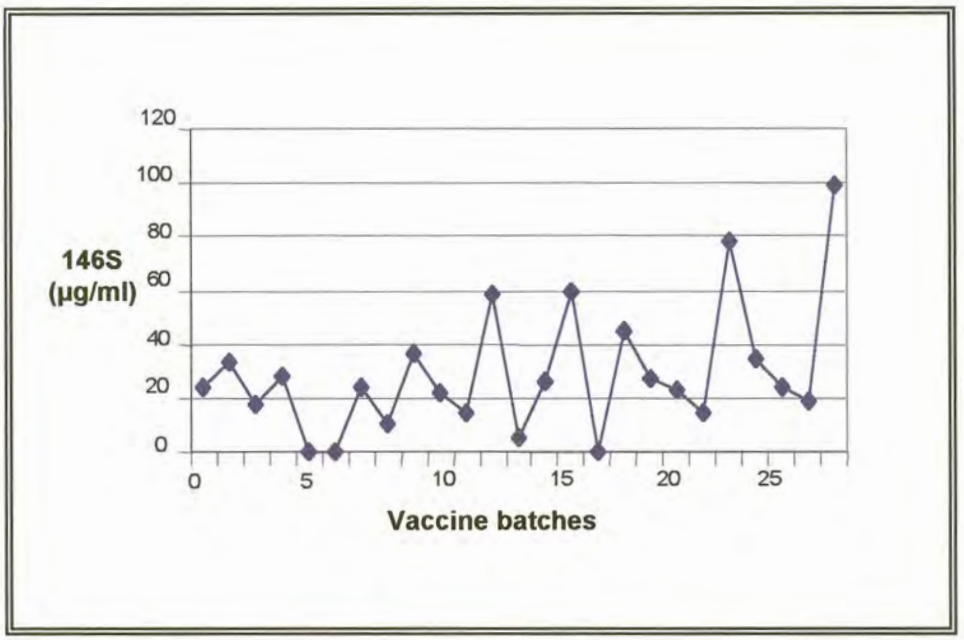

Fig. 2.1. Variability in the yield of SAU/6/00 antigen for several vaccine production batches.

2.2 MATERIALS AND METHODS

2.2.1 Viral and bacterial strains

The SAT2/SAU/6/00 strain (passage history: BTY_1RS_1), used in this study, was obtained from Dr Nigel Ferris at the World Reference Laboratory for Foot-and-Mouth Disease, Pirbright, UK. *Escherichia coli* MAX EFFICIENCY® DH5α™ competent cells (genotype: F⁻ϕ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *deo*R *rec*A1 *end*A1 *hsd*R17 (r_K^-, m_K^+) *pho*A *sup*E44 λ⁻ *thi*-1 *gyr*A96 *rel*A1) were obtained from Life Technologies and used in cloning experiments as transformation host.

2.2.2 Oligonucleotides

Oligonucleotides used in cDNA synthesis, PCR and nucleotide sequencing procedures were custom-synthesized by Invitrogen and Inqaba Biotechnical Industries, and are described in Table 2.1.

2.2.3 RNA extraction

RNA was extracted from aliquots of cell culture samples (200 µl) using a modified guanidinium thyocianate (GuSCN)/silica method (Boom *et al.*, 1990). The cells were lysed with a L6 lysis buffer containing GuSCN (Appendix) and an aliquot of silica suspension (Appendix) that served as a carrier of the released nucleic acids. The silica-bound nucleic acid was pelleted by centrifugation and washed with L2 wash buffer (Appendix), 70% EtOH and acetone, respectively. The pellet was vacuum-dried and the nucleic acid eluted from the silica matrix at 56°C for 2 min in 1 x TE buffer (Appendix). The eluate, containing viral RNA, was stored at –80°C in 1 x TE buffer containing RNasin® ribonuclease inhibitor (Promega).

2.2.4 cDNA synthesis

Viral RNA was reverse-transcribed in a reaction mixture containing 5 µl RNA, 0.23 µM of the antisense oligonucleotide 2B208R (Table 2.1), 0.42 U RNasin (Promega), 1 x AMV-RT buffer, 0.34 mM dNTPs, 2% (v/v) DMSO and 4.55 µM of a random hexanucleotide mixture (Roche). Following incubation for 3 min at 70°C and 2 min on ice, the first strand cDNA was synthesized at 42°C for 2 h by the addition of 0.6 U AMV-Reverse Transcriptase (Promega). The enzyme

Table 2.1. Oligonucleotides used in this part of the study

Oligonucleotide	Nucleotide sequence*	Reference	Purpose
2B208R	5'-ACAGCGGCCATGCACGACAG-3'	N. Knowles, unpublished	cDNA synthesis (2B)
NCR1	5'-TACCAAGCGACACTCGGGATCT-3'	Van Rensburg and Nel (1999)	PCR amplification, nucleotide sequencing (5' UTR)
WDA	5'-GAAGGGCCCAGGGTTGGACTC-3'	Beck and Strohmaier (1987)	PCR amplification, nucleotide sequencing (2A)
FOR	5'-GTAAAACGACGGCCAGT-3'	Messing (1983)	Nucleotide sequencing (pUC/M13)
REV	5'-GTTTTCCCAGTCACGAC-3'	Messing (1983)	Nucleotide sequencing (pUC/M13)
LINT	5'-GWTACGTCGATGARCC-3'	Van Rensburg, unpublished	Nucleotide sequencing (Leader)
VP4M	5'-GTGTCCATKGAGTTYTG-3'	Van Rensburg, unpublished	Nucleotide sequencing (1A)
NCR2	5'-GCTTCTATGCCTGAATAGG-3'	Van Rensburg, unpublished	Nucleotide sequencing (5' UTR)
SAU1	5'-CACTCTMTACCCACACCAG-3'	This study	Nucleotide sequencing (1B)
SAU2	5'-GTGTGTKGAWGGGTCSGTGG-3'	This study	Nucleotide sequencing (1D)
SAU3	5'-CTGTCTACAAYGGTGAGTG-3'	This study	Nucleotide sequencing (1D)
SAU4	5'-GAGGACTITTACCCATGGAC-3'	This study	Nucleotide sequencing (1A)
SAU5	5'-CACTCACCK TTGTAGAYKGT-3'	This study	Nucleotide sequencing (1D)
SAU6	5'-GCTGGGTCGCGAGGCAGTGC-3'	This study	Nucleotide sequencing (Leader)
SAU8	5'-CCACGACCTCCACCACGC-3'	This study	Nucleotide sequencing (1B)
SAU9	5'-GACCCCGTGCGACAGACCAC-3'	This study	Nucleotide sequencing (1D)
SAU10	5'- GACTGCCACGGACGGTTCTC-3'	This study	Nucleotide sequencing (1C)
SAU11	5'-GACTGCCACGGACGGTTCTC-3'	This study	Nucleotide sequencing (1C)
SAU12	5'-CCATSACAACCAGKGACC-3'	This study	Nucleotide sequencing (1B)
SAU13	5'-CCAAMACKTAMAGCGTGC-3'	This study	Nucleotide sequencing (1B)
SEQ11	5'-GGGAYACAGGAYTGAACT-3'	Van Rensburg, unpublished	Nucleotide sequencing (1C)

* Abbreviations representing ambiguities are Y (C/T), M (A/C), K (A/G), W (A/T), S (C/G) and K (T/G)

was subsequently inactivated by heating to 80°C for 2 min and the reaction mixture stored at 4°C until required.

2.2.5 PCR amplification and analysis

The capsid-coding region was amplified using the Expand Long Template PCR system (Roche), as previously described (Van Rensburg and Nel, 1999). The reaction mixture contained 3 µl of the first strand cDNA reaction mixture, 0.3 µM of oligonucleotides NCR1 and WDA (Table 2.1), 2.5 U Expand Long Template DNA polymerase, 1 x Expand buffer, 2 µM dNTPs and 0.75 mM $MgCl_2$. Thermal cycling was performed in a Hybaid PCR Sprint thermocycler. The cycling profile consisted of initial denaturation at 94°C for 2 min, followed by 10 cycles of denaturation at 95°C for 30 s, oligonucleotide annealing at 53°C for 30 s and elongation at 68°C for 60 s. For the next 20 cycles, the cycling profile was identical, except that the elongation time was increased by 20 s per cycle to ensure that full-length amplicons were obtained. The PCR-amplified products were visualized by electrophoresis on a 1% (w/v) agarose gel containing 0.5 µg/ml EtBr, using 1 x TAE (Appendix) as electrophoresis buffer. The size of the amplified DNA fragment was estimated against a DNA molecular weight marker (phage λ DNA digested with *Hind*III; Promega). The amplicon was purified from the agarose gel using the Nucleospin® Extract kit (Macherey-Nagel) according to the specification of the manufacturer.

2.2.6 Cloning of the capsid-coding region into pGEM®-T Easy

The purified amplicon was cloned into the pGEM®-T Easy vector (Promega) (Fig. 2.2). Since a single non-template specific deoxyadenosine is added to the 5' ends of the amplicon during PCR amplification, cloning of the amplicon into the pGEM®-T Easy vector, which contains single 3' thymidine overhangs at the insertion site, is greatly facilitated. Approximately 250 ng of purified amplicon was ligated with 25 ng of the pGEM®-T Easy vector in the presence of 3 U T4 DNA ligase and 1 x ligation buffer. Following incubation at 4°C for 48 h, 20 µl of the ligation reaction mixture was transformed into 50 µl of competent *E. coli* MAX EFFICIENCY® DH5α™ cells, as described by Sambrook and Russell (2001), with the following modifications. Transformation of the competent cells was accomplished by incubation at 42°C for 40 s and following addition of

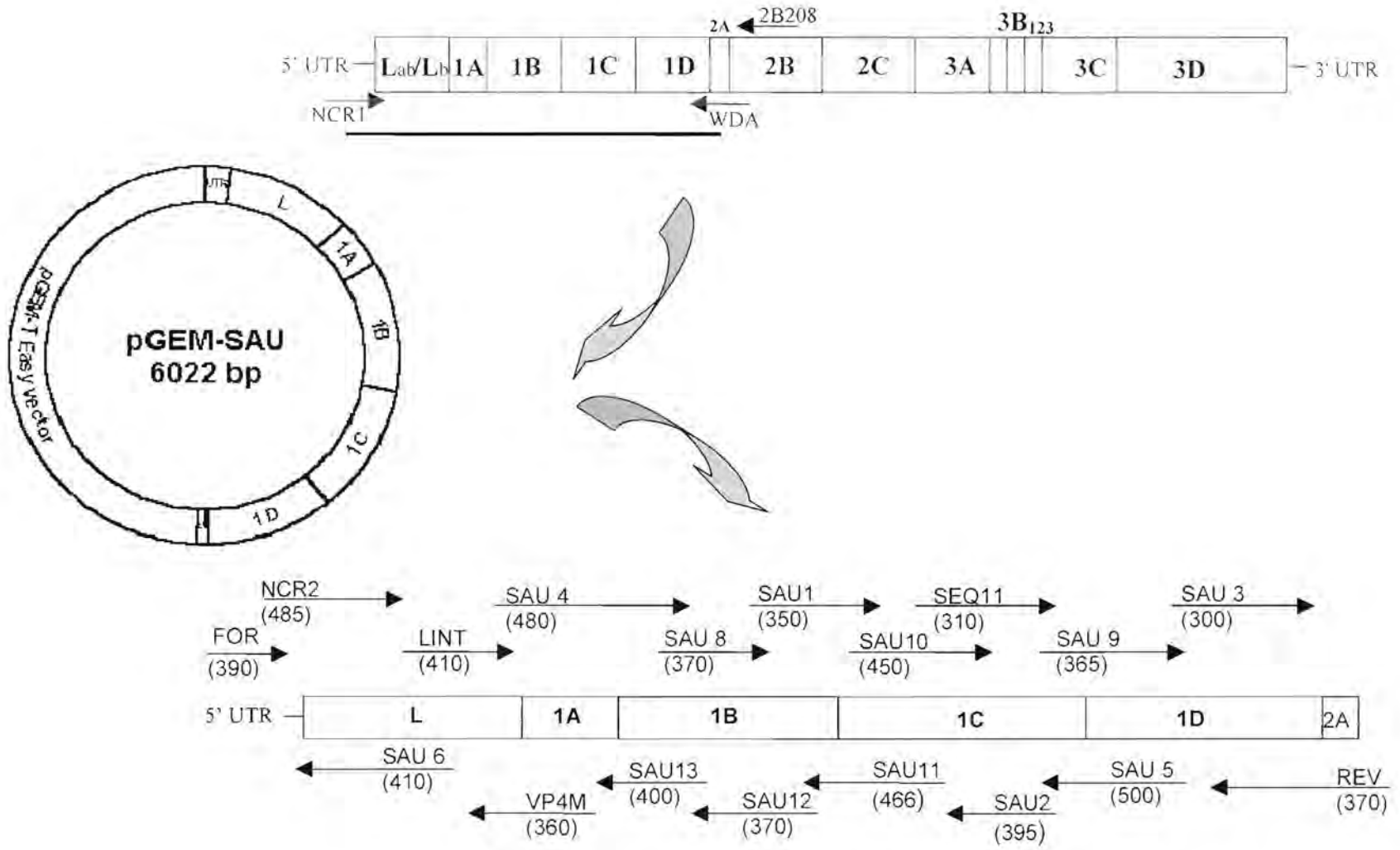

Fig. 2.2. Schematic representation of the capsid-coding region of SAU/6/00 cloned into pGEM®-T Easy and the sequencing strategy used to determine the nucleotide sequence. FOR, NCR2 etc. are the oligonucleotides that were used; arrows indicate the direction and () indicate the length of the nucleotide sequences obtained by the individual oligonucleotides.

150 µl of SOC medium (Appendix), the cells were allowed to recover by incubation at 37°C for 1 h. The transformed cells were selected by plating the transformation mixtures onto LB agar plates (Appendix), supplemented with 50 µg/ml ampicillin (Roche), in the presence of 0.2 g/ml IPTG (Roche) and 30 µg/ml X-Gal (Roche) to allow for blue-white selection. The agar plates were incubated at 37°C overnight.

2.2.7 Plasmid isolation

Plasmid DNA was extracted from putative recombinant clones using the QIAprep® Spin Miniprep Kit (Qiagen) according to the specification of the manufacturer. To confirm successful cloning of the capsid-coding region into the pGEM®-T Easy vector, PCR amplification using oligonucleotides NCR1 and WDA (Table 2.1) was performed, as described in Section 2.2.5.

2.2.8 Nucleotide sequence determination and analysis

The nucleotide sequence of cloned insert DNA was determined in both directions using an ABI PRISM™ Big Dye™ Terminator Cycle Sequencing Ready Reaction kit v3.0 (Applied Biosystems). In addition to the universal pUC/M13 forward and reverse sequencing oligonucleotides, several insert-specific oligonucleotides were designed (Table 2.1) from the sequence obtained and used to determine the sequence of the full-length insert and to obtain good overlaps in both strands. The annealing position of the oligonucleotides and direction of sequencing are indicated in Fig. 2.2. After cycle sequencing, the extension products were purified and resolved on an ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems). The sequencing data was analyzed using DAPSA version 2.9 (Harley, 2001), BioEdit and MEGA version 2.1. Other FMDV isolates included in the analysis, together with the GenBank accession numbers, are indicated in Table 2.2. The nucleotide sequence of the P1-coding region from SAT2/SAU/6/00 has been submitted to GenBank under the accession number AY297948.

2.3 RESULTS AND DISCUSSION

2.3.1 Cloning of the P1 region from SAU/6/00 into pGEM®-T Easy

To enable amplification of the capsid-coding region from SAU/6/00, the viral RNA was extracted and reverse-transcribed using the antisense oligonucleotide 2B208R, situated in the 2B region. The L-P1-2A coding region was subsequently PCR-amplified using the Expand Long Template DNA polymerase and oligonucleotides NCR1, which anneals at the 5' UTR, and WDA, which anneals at the 2A/2B junction (Table 2.1, Fig. 2.2).

An aliquot of the reaction mixture was analyzed by agarose gel electrophoresis (Fig. 2.3) and an amplicon of the expected size (*ca.* 3 kb) was observed. Following gel-purification of the amplicon and ligation into pGEM®-T Easy, competent *E. coli* MAX EFFICIENCY® DH5α™ cells were transformed and recombinant transformants with a *lacZ* phenotype were selected and cultured in LB broth supplemented with ampicillin. The extracted plasmid DNA was analyzed for the presence of cloned insert DNA by PCR analysis using oligonucleotides NCR1 and WDA. A recombinant plasmid, designated pGEM-SAU, from which a 3-kb amplicon could be amplified was selected and used in subsequent investigations.

2.3.2 Molecular characterization of the P1 region from SAU/6/00

The nucleotide sequence of both strands of the 3-kb capsid-coding region of SAU/6/00 was determined through primer walking, using existing and newly constructed oligonucleotides (Table 2.1). The obtained sequences were aligned and then assembled to obtain the full-length nucleotide sequence from which the amino acid sequence of the viral proteins (1A-1D) was deduced (Fig. 2.4).

2.3.2.1 Intratypic variation of the P1 region between types A, O and SAT2

The percentage intratypic variation between the full-length capsid protein (P1), as well as individual viral capsid proteins (1A-1D), was determined for viruses belonging to serotypes SAT2, A and O by pair-wise sequence alignments (Table 2.3). For this purpose, four viruses from each of the three different serotypes were chosen of which the full-length P1 nucleotide sequences are available (Table 2.2).

Table 2.2. FMD viruses used in comparative studies of the P1-coding region

Serotype	Virus	Country of origin	Specie	Year of isolation	GenBank accession number
O_1*	Caseros/Argentine/67	Argentina	N/A#	1982	U82271
O_1	Kaufbeuren/FRG166	Germany	Bovine	1966	X00871
O_2	Brescia/Italy/47	Italy	N/A	1947	M55287
O*	SAR/19/2000	South Africa	Swine	2000	AJ539140
A_{10}	Argentine/61	Argentina	N/A	1961	V01130
A_{12}*	119/Kent/UK	UK	Bovine	1932	M10975
A_{22}	Azerbaijan/USSR/65	USSR	Bovine	1965	X74812
A_{24}	Cruzeiro/Brazil/55	Brazil	N/A	N/A	AJ251476
C_1	Germany/26	Germany	N/A	N/A	M90368
C_1	Santa Pau/Spain/70	Spain	N/A	N/A	AJ133357
C_2	Pando/Uruguay/44	Uruguay	N/A	N/A	M90367
SAT1	BOT/1/68	Botswana	N/A	1968	Z98203
SAT1	KNP/196/91	South Africa	Buffalo	1991	AF283429
SAT1	UGA/1/97	Uganda	Buffalo	1997	AY043300
SAT2*	RHO/1/48	Zambia	Bovine	1948	AJ251475
SAT2*	ZIM/7/83	Zimbabwe	Bovine	1983	AF136607
SAT2*	SAU/6/00	Saudi Arabia	Bovine	2000	AY297948
SAT2*	KEN/3/57	Kenya	Bovine	1957	AJ251473
SAT3	BEC/1/65	Botswana	Bovine	1965	M28719
SAT3	KNP/10/90	South Africa	Buffalo	1990	AF286347

* Viruses used to assess the intratypic variation of the P1 regions and viral proteins (1A-1D)
# Not available

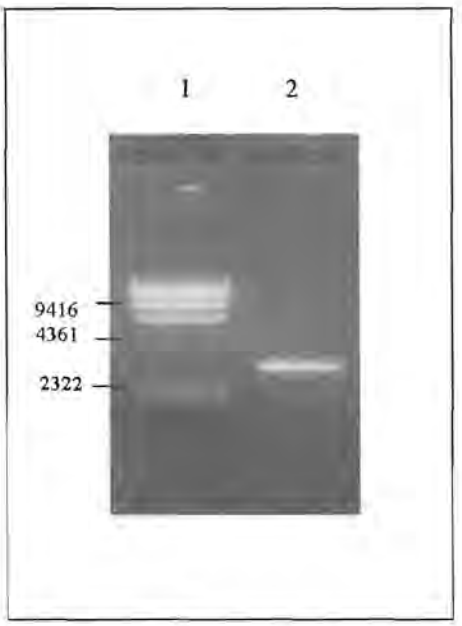

Fig. 2.3. Agarose gel electrophoretic analysis of the 3-kb L-P1-2A fragment obtained by PCR amplification. Lane 1, DNA molecular marker; lane 2, PCR amplicon. The sizes of the DNA molecular weight marker, phage λ DNA digested with *Hind*III, are indicated to the left of the figure.

The intratypic variation of the P1 nucleotide and amino acid sequences between the SAT2 type viruses was 18.3% and 19%, respectively. Whereas the highest amino acid sequence variation (13%) was observed between the SAU/6/00 and ZIM/7/83 vaccine strains, the least variation (8%) was observed between ZIM/7/83 and RHO/1/48. These results are in agreement with the phylogenetic tree presented in Fig. 2.5, indicating that the SAT2 vaccine strains are not genetically closely related. Comparatively, the intratypic variation in types A and O viruses was lower. The intratypic amino acid sequence variation between the P1 regions of type A viruses was 14.1% (12% on nucleotide level) and that of type O viruses was 9.3% (9.2% on nucleotide level). In all serotypes, the greatest sequence variation was in the 1D protein. Whereas the intratypic amino acid sequence variation of the 1D protein was 33.3% for SAT2, it was lower, but similar for types A and O (21.5% and 20.2%, respectively). By contrast, the 1A protein displayed the least variation in all three serotypes. The greatest intratypic variation (5.9%) was observed for type A viruses and the lowest (2.9%) for type O viruses. The percentage intratypic

variation for the 1B and 1C proteins was intermediatory to those observed for the 1D and 1A proteins (Table 2.3).

The high level of intratypic sequence variation observed for the SAT2 serotype is typical of the SAT type viruses (Domingo *et al.*, 1990). Several reports have indicated that the SAT types evolve rapidly and independently in different geographical areas and that field isolates are characterized by a high degree of antigenic and genetic heterogeneity (Bastos *et al.*, 2001). Furthermore, the variation displayed by the individual capsid proteins appears to correspond to their location in the viral capsid. The 1D protein, which is the most immunogenic of the FMDV capsid proteins, is located at the surface of the capsid and therefore subject to immunological selection pressure. By contrast, protein 1A is located internally (Acharya *et al.*, 1989), which corresponds with the observation that it displays the least variation. Proteins 1B and 1C both form part of the external capsid, and although they contribute to the antigenicity of the virus, these proteins only display intermediate variation, possibly due to the absence of a structural protrusion such as the G-H loop which is situated in 1D.

2.3.2.2 Phylogenetic relationships

Phylogenetic analysis depicting relationships of the P1 nucleotide sequences between representatives of the six FMDV serotypes, generated a neighbour-joining tree with six serotype-specific lineages (numbers 1 to 6), which could be grouped into two supra-clades (I and II) (Fig. 2.5). Supra-clade I comprises virus isolates of types O (lineage 1), A (lineage 2) and C (lineage 3). Type O and A viruses are distributed widely in Africa, southern Asia, the Far East (excluding type A) and South America, whereas type C viruses are found mainly on the Indian sub-continent (Knowles and Samuel, 2003). Of interest is the Pan-Asian strain, SAR/19/2000, which caused the first type O FMD outbreak in South Africa (Sangare *et al.*, 2001).

Supra-clade II comprises the SAT type viruses found mainly in sub-Saharan Africa, with isolates belonging to SAT1 (lineage 4), SAT2 (lineage 6) and SAT3 (lineage 5) grouping together. The SAT types show greater variation compared to the European types, as supported by the longer branch lengths (Fig. 2.5) (Palmenberg, 1989; Knowles and Samual, 2003). These results are in

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5' UTR  TA ACA AGC GAC ACT CGG GAT CTG AGG AGG GGA CTG GGA CCT CTG TAA
                NCR1
   48   AAG TGC CCA GTT TAA AAA GCT TCT ATG CCT GAA TAG|GTG ACC GGA
                                                  Leader
   93   GGC CGG CAC CTT TTC TTT TTA CAC AGA AAT TAC TAC ATG AAC ACA
                                                   M   N   T
  138   ACT GAT TGT TTT ATC GCT TTG GTA GAA GCT ATC AGA GAG ATC AAA
          T   D   C   F   I   A   L   V   E   A   I   R   E   I   K

  183   TTT TTG TTT AAA CAC ACC AGA AAG ATG GAG TTC ACG CTG CAC AAC
          F   L   F   K   H   T   R   K   M   E   F   T   L   H   N

  228   GGC GAG AAA AAG ACT TTC TAC TCA AGG CCC AAC CGC CAC GAT AAC
          G   E   K   K   T   F   Y   S   R   P   N   R   H   D   N

  273   TGC TGG CTA AAC ACC ATC CTG CAA TTG TTC AGG TAC GTC GAT GAG
          C   W   L   N   T   I   L   Q   L   F   R   Y   V   D   E

  318   CCA TTC TTC GAC TGG GCC TAC AAT TCA CCT GAA AAC CTC ACG CTC
          P   F   F   D   W   A   Y   N   S   P   E   N   L   T   L

  363   CAG GCA ATT GAG CAG CTC GAG GAG CTC ACA GGC CTC AGC CTA CAC
          Q   A   I   E   Q   L   E   E   L   T   G   L   S   L   H

  408   GAG GGT GGG CCC CCC GCT CTC GTG ATT TGG AAC ATC AAA CAC TTG
          E   G   G   P   P   A   L   V   I   W   N   I   K   H   L

  453   CTG CAC ACC GGA ATC GGC ACT GCC TCG CGA CCC AGC GAG GTG TGC
          L   H   T   G   I   G   T   A   S   R   P   S   E   V   C

  498   ATG GTT GAC GGT ACT GAC ATG TGT CTT GCT GAT TTC CAC GCA GGA
          M   V   D   G   T   D   M   C   L   A   D   F   H   A   G

  543   ATC TTC CTC AAG GGT GCT GAA CAC GCC GTG TTC GCC TGT TTG ACC
          I   F   L   K   G   A   E   H   A   V   F   A   C   L   T

  588   TCC AAC GGA TGG TAC GCC ATC GAC GAC GAG GAC TTT TAC CCA TGG
          S   N   G   W   Y   A   I   D   D   E   D   F   Y   P   W

  633   ACT CCG GAT CCG TCC GAT GTC TTG TGT TTT GTC CCG TAC GAC ATG
          T   P   D   P   S   D   V   L   C   F   V   P   Y   D   M

  678   GAA CCG TTC AAC GGA AAC GCG ATT GCG AAA GCG ACC GCG TAC GTG
          E   P   F   N   G   N   A   I   A   K   A   T   A   Y   V
                IA
  723   AAG|GGA GCC GGG CAA TCC AGC CCA GCC ACT GGA TCG CAA GAT CAG
          K▼  G   A   G   Q   S   S   P   A   T   G   S   Q   D   Q

  768   TCA GGC AAC ACT GGT AGC ATT ATT AAC AAC TAC TAC ATG CAA CAG
          S   G   N   T   G   S   I   I   N   N   Y   Y   M   Q   Q

  813   TAC CAA AAC TCG ATG GAC ACA CAA CTT GGT GAC AAC GCC ATT AGT
          Y   Q   N   S   M   D   T   Q   L   G   D   N   A   I   S

  858   GGT GGT TCC AAC GAG GGG TCG ACA GAC ACT ACG TCG ACA CAC ACA
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        G   G   S   N   E   G   S   T   D   T   T   S   T   H   T

903     AAC AAC ACA CAG AAC AAT GAT TGG TTC TCC AAG TTG GCC CAA TCA
        N   N   T   Q   N   N   D   W   F   S   K   L   A   Q   S

                                                    1B
948     GCC ATC TCG GGG CTC TTC GGA GCT CTA CTG GCC | GAC AAG AAA ACA
        A   I   S   G   L   F   G   A   L   L   A ▼ D   K   K   T

993     GAG GAG ACC ACA CTG TTG GAG GAC AGA ATT TTG ACC ACA CGT CAC
        E   E   T   T   L   L   E   D   R   I   L   T   T   R   H

1038    GGA ACC ACG ACC TCC ACC ACG CAA AGT TCT GTG GGT GTG ACA CTT
        G   T   T   T   S   T   T   Q   S   S   V   G   V   T   L

1083    GGT TAC GCT GAT GCT GAC TCG TTT CGC CCA GGA CCC AAC ACC TCT
        G   Y   A   D   A   D   S   F   R   P   G   P   N   T   S

1128    GGG CTT GAG ACG CGT GTG CAA CAG GCA GAA CGC TTC TTT AAG GAG
        G   L   E   T   R   V   Q   Q   A   E   R   F   F   K   E

1173    AAA CTG TTT GAC TGG ACC AGT GAC AAA CCT TTC GGC ACG CTT TAC
        K   L   F   D   W   T   S   D   K   P   F   G   T   L   Y


1218    GTT TTG GAG TTG CCC AAA GAC CAC AAG GGC ATT TAC GGT AAA CTT
        V   L   E   L   P   K   D   H   K   G   I   Y   G   K   L

1263    ACC GAC TCC TAC ACG TAC ATG CGT AAC GGC TGG GAC GTA CAG GTC
        T   D   S   Y   T   Y   M   R   N   G   W   D   V   Q   V

1308    AGC GCA ACC AGC ACA CAG TTC AAC GGT GGA TCA CTG CTC GTA GCA
        S   A   T   S   T   Q   F   N   G   G   S   L   L   V   A

1353    ATG GTA CCA GAG CTG TCT AGT CTG AAA AGT AGA GAA GAA TTC CAG
        M   V   P   E   L   S   S   L   K   S   R   E   E   F   Q

1398    CTC ACT CTA TAC CCA CAC CAG TTC ATC AAC CCG CGC ACT AAC ACG
        L   T   L   Y   P   H   Q   F   I   N   P   R   T   N   T

1443    ACT GCA CAC ATA CAG GTC CCG TAC CTG GGT GTG AAC AGA CAC GAC
        T   A   H   I   Q   V   P   Y   L   G   V   N   R   H   D

1488    CAA GGC AAG CGC CAC CAT GCG TGG TCT CTG GTT GTG ATG GTG CTC
        Q   G   K   R   H   H   A   W   S   L   V   V   M   V   L

1533    ACG CCT CTC ACC ACC GAG GCG CAG ATG AAC AGC GGC ACC GTC GAG
        T   P   L   T   T   E   A   Q   M   N   S   G   T   V   E

1578    GTG TAC GCC AAC ATC GCA CCA ACA AAT GTA GTT GTG GCG GGT GAG
        V   Y   A   N   I   A   P   T   N   V   V   V   A   G   E

                                                1C
1623    CTG CCA GGC AAA CAG | GGT ATT GTG CCG GTC GCC GCC GCT GAC GGG
        L   P   G   K   Q ▼ G   I   V   P   V   A   A   A   D   G

1668    TAT GGT GGT TTC CAA AAC ACC GAC CCG AAA ACG GCC GAC CCC ATT
        Y   G   G   F   Q   N   T   D   P   K   T   A   D   P   I
1713    TAC GGG TAT GTG TAC AAC CCG TCC AGA AAC GAC TGC CAC GGA CGG
```

```
      Y   G   Y   V   Y   N   P   S   R   N   D   C   H   G   R

1758  TTC TCC AAT CTT TTG GAT GTC GCC GAG GCG TGT CCA ACA CTC CTG
        F   S   N   L   L   D   V   A   E   A   C   P   T   L   L

1803  GAT TTT GAT GGC AAG CCA TAT ATT GTG ACC AAG AAC AAC GGT GAC
        D   F   D   G   K   P   Y   I   V   T   K   N   N   G   D

1848  AAG GTG ATG ACA TCC TTT GAC GTC GCC TTC ACA CAC AAG GTG CAC
        K   V   M   T   S   F   D   V   A   F   T   H   K   V   H

1893  AGG AAC ACG TTT CTG GCG GGC TTG GCT GAC TAC TAC ACA CAG TAC
        R   N   T   F   L   A   G   L   A   D   Y   Y   T   Q   Y

1938  TCA GGC AGC CTA AAC TAC CAC TTC ATG TAC ACT GGA CCC ACA CAC
        S   G   S   L   N   Y   H   F   M   Y   T   G   P   T   H

1983  CAC AAG GCA AAG TTC ATG GTG GCA TAC GTG CCC CCT GGT GTT GAA
        H   K   A   K   F   M   V   A   Y   V   P   P   G   V   E

2028  ACT GCA CAA CTA CCT ACA ACA CCG GAG GAT GCC GCG CAC TGC TAT
        T   A   Q   L   P   T   T   P   E   D   A   A   H   C   Y

2073  CAC GCG GAA TGG GAC ACT GGA CTG AAC TCC TCC TTC TCG TTC GCG
        H   A   E   W   D   T   G   L   N   S   S   F   S   F   A

2118  GTG CCT TAC ATC TCC GCT GCG GAC TTC TCC TAC ACA CAC ACA GAC
        V   P   Y   I   S   A   A   D   F   S   Y   T   H   T   D

2163  ACG CCA GCC ATG GCC ACC ACC AAC GGC TGG GTG ATT GTA CTG CAG
        T   P   A   M   A   T   T   N   G   W   V   I   V   L   Q

2208  GTC ACC GAC ACG CAC TCT GCT GAA GCT GCC GTT GTG GTG TCA GTC
        V   T   D   T   H   S   A   E   A   A   V   V   V   S   V

2253  AGT GCT GGG CCA GAT TTG GAA TTT CGG TTC CCT ATC GAC CCC GTG
        S   A   G   P   D   L   E   F   R   F   P   I   D   P   V

                1D
2298  CGA CAG |ACC ACC TCA GCG GGA GAA AGC GCA GAT GTC GTC ACC ACG
        R   Q ▼ T   T   S   A   G   E   S   A   D   V   V   T   T

2343  GAC CCA TCT ACA CAC GGT GGA AAC GTG CAA GAG GGC CGA CGC AAA
        D   P   S   T   H   G   G   N   V   Q   E   G   R   R   K

2388  CAC ACC GAA GTT GCG TTC CTT CTT GAC CGC AGT ACA CAC GTC CAC
        H   T   E   V   A   F   L   L   D   R   S   T   H   V   H

2433  ACA AAC AAA ACA TCC TTT GTT GTG GAC CTC ATG GAC ACA AAG GAG
        T   N   K   T   S   F   V   V   D   L   M   D   T   K   E

2478  AAG GCA CTC GTG GGC GCA ATC CTG CGG GCT TCC ACC TAC TAC TTT
        K   A   L   V   G   A   I   L   R   A   S   T   Y   Y   F

2523  TGT GAC CTT GAG ATT GCA TGT GTG GGC GAC CAC ACA AGG GCC TTT
        C   D   L   E   I   A   C   V   G   D   H   T   R   A   F

2568  TGG CAG CCT AAC GGG GCG CCG CGG ACC ACC CAA CTT GGC GAC AAC
```

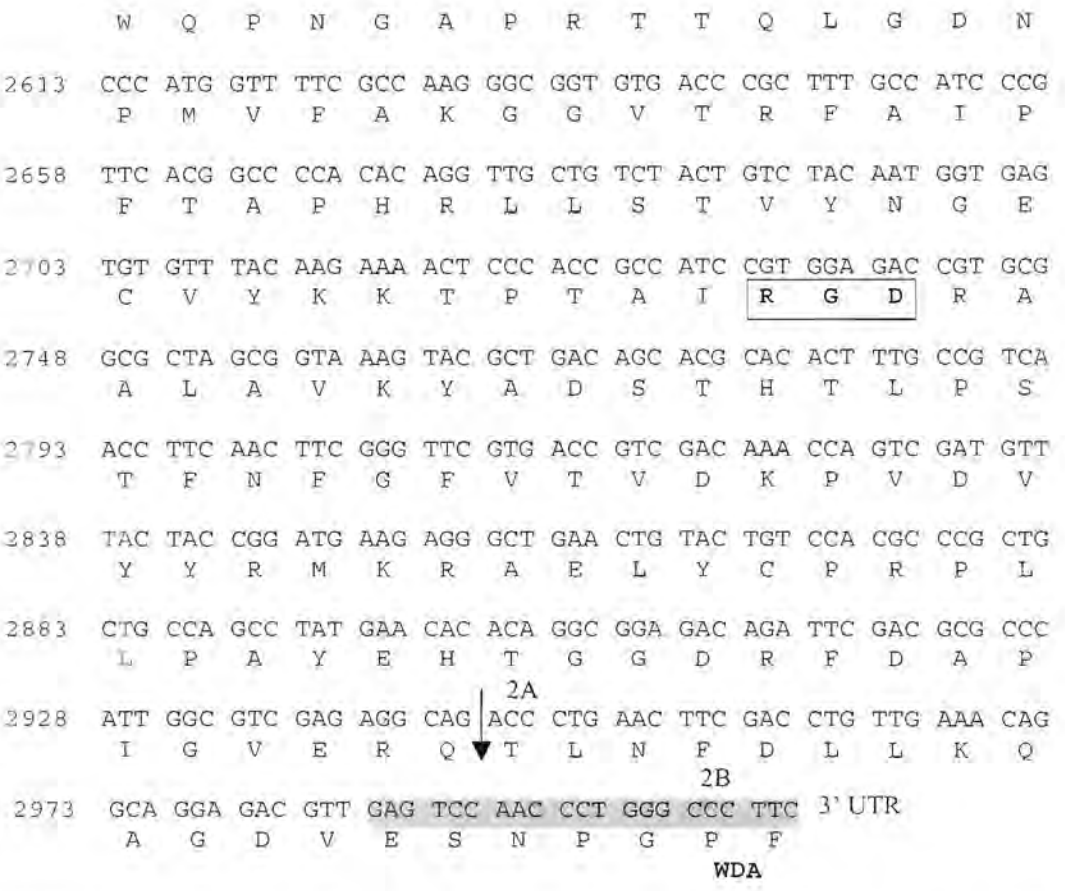


Fig. 2.4. Nucleotide and deduced amino acid sequence of the Leader protease, capsid-coding region (1A-1D), 2A and part of 2B of SAU/6/00. The 5' untranslated region is indicated in italics, the second initiating methionine residue is indicated in bold and the RGD is boxed. The protease cleavage sites are indicated by arrows and the NCR1 and WDA oligonucleotides used for PCR amplification are shaded.

Table 2.3. Intratypic amino acid and nucleotide sequence variation in the capsid protein (P1) and viral proteins (1A-1D), as determined for serotype SAT2, type A and type O

	Percentage variation					
Proteins	Serotype SAT2		Serotype A		Serotype O	
P1	SAU/6/00 + KEN/3/57	* 10.3	A_{10} + A_{12}	* 6.2	O_2 Brescia + O_1 Kaufbeuren	* 4.7
	SAU/6/00 + RHO/1/48	11.5	A_{10} + A_{22}	10.3	O_2 Brescia + O_1 Caseros	5.4
	SAU/6/00 + ZIM/7/83	12.9	A_{10} + A_{24}	7.3	O_2 Brescia + SAR/19/2000	5.7
	KEN/3/57 + RHO/1/48	12.8	A_{12} + A_{22}	8.8	O_1Kaufbeuren + O_1Caseros	4.7
	KEN/3/57 + ZIM/7/83	12.3	A_{12} + A_{24}	7.3	O_1Kaufbeuren + SAR/19/2000	5.5
	RHO/1/48 + ZIM/7/83	7.9	A_{22} + A_{24}	8.8	O_1 Caseros + SAR/19/2000	6.2
	Intratypic (aa)	19	Intratypic (aa)	14.1	Intratypic (aa)	9.3
	Intratypic (nt)	18.3	Intratypic (nt)	12	Intratypic (nt)	9.2
1D	SAU/6/00 + KEN/3/57	* 18.7	A_{10} + A_{12}	* 11.5	O_2 Brescia + O_1 Kaufbeuren	* 7.7
	SAU/6/00 + RHO/1/48	21.1	A_{10} + A_{22}	14.8	O_2 Brescia + O_1 Caseros	11.5
	SAU/6/00 + ZIM/7/83	25.3	A_{10} + A_{24}	12.0	O_2 Brescia + SAR/19/2000	12.4
	KEN/3/57 + RHO/1/48	22.9	A_{12} + A_{22}	11	O_1 Kaufbeuren + O_1 Caseros	10.5
	KEN/3/57 + ZIM/7/83	22.9	A_{12} + A_{24}	10.5	O_1Kaufbeuren + SAR/19/2000	11.9
	RHO/1/48 + ZIM/7/83	15.5	A_{22} + A_{24}	15.8	O_1 Caseros + SAR/19/2000	14.3
	Intratypic (aa)	33.3	Intratypic (aa)	21.5	Intratypic (aa)	20.2
	Intratypic (nt)	32.8	Intratypic (nt)	17.7	Intratypic (nt)	19.6
1B	Intratypic (aa)	13.3	Intratypic (aa)	11.8	Intratypic (aa)	3.7
1C	Intratypic (aa)	14.4	Intratypic (aa)	10.9	Intratypic (aa)	7.3
1A	Intratypic (aa)	4.7	Intratypic (aa)	5.9	Intratypic (aa)	2.4

* Pair-wise comparisons were determined using the amino acid sequences of the respective viruses

agreement with those based on comparisons of the complete P1 (Van Rensburg and Nel, 1999) and partial 1D nucleotide sequences (Bastos *et al.*, 1998). Notably, the SAU/6/00 virus grouped with virus isolates from serotype SAT2 and is most closely related to the east-African strain KEN/3/57, corresponding to previous findings that the virus possibly originated from north-eastern Africa (Bastos *et al.*, 2003).

2.3.2.3 Comparison of the P1 regions between different SAT2 viruses

- ***Hypervariable regions***

The amino acid sequence of the P1 region from SAU/6/00 was compared to that of three different SAT2 FMD viruses in order to delineate hypervariable regions within the structural proteins, which may be indicative of possible antigenic sites (Fig. 2.6). Previous studies have identified several antigenic sites in the P1 region of the European type FMD viruses (Table 1.1). For type O, site 1 consists of the G-H loop and the C-terminus of the 1D protein, whereas sites 2, 3 and 4 consist of loops in proteins 1B, 1D and 1C, respectively (Xie *et al.*, 1987; Pfaff *et al.*, 1988; Aggarwal and Barnett, 2002). A fifth conformational epitope has been identified for type O_1 and is associated with amino acid residue His-149 on the 1D protein (Crowther *et al.*, 1993a). This His residue plays an important role in maintaining the three-dimensional structure in the G-H loop region, or between the loop region and amino acids in the 1B and 1C proteins (Parry *et al.*, 1990). The C-terminus of the 1D protein constitutes an independent antigenic site in type A and type C viruses and is termed site C (Mateu *et al.*, 1990).

The 1D protein of FMDV contains a G-H loop, which is not seen in other Picornaviruses. Using bovine sera of convalescent or vaccinated animals, Francis and co-workers (1990) reported that 50% of the neutralizing antibodies were directed at this site. This major antigenic site is expressed on the surface of the FMDV virion (Bachrach, 1977; Grubman *et al.*, 1985) and contains the amino acid sequence RGD (Arg-Gly-Asp). Despite the 1D protein displaying the greatest intratypic sequence variation (Table 2.3), the RGD sequence is completely conserved in this immunodominant hypervariable region (Fig. 2.6). The RGD motif interacts with integrin cellular receptors α_vB_1 (Jackson *et al.*, 2002), α_vB_3 (Berinstein *et al.*, 1995) and α_vB_6 (Jackson *et al.*, 2000) for cell binding (Fox *et al.*, 1989). Following the RGD motif in the 1D protein of SAU/6/00 is an Arg-673 amino acid (Fig. 2.6), which is also conserved in other SAT2 viruses

(Crowther *et al.*, 1993b). By contrast, other FMDV serotypes have a Leu or Met amino acid at this position (Bastos *et al.*, 2001; Mateu *et al.*, 1990; Sangare *et al.*, 2001), which is associated with a higher binding affinity for integrin receptors (Jackson *et al.*, 2000).

For SAU/6/00, the hypervariable amino acid regions 661-691 and 718-739 (Fig. 2.6) in the 1D protein correspond to the G-H loop and C-terminus of this protein, respectively. The residues involved in the major immunodominant site of the SAT2 type virus RHO/1/48 has been determined and reported to be located on the G-H loop (Crowther *et al.*, 1993b). The amino acids at these sites, however, are different for SAU/6/00 (Ala-675, Asp-682 and Thr-684) compared to those of RHO/1/48 (Val-675, Gly-682 and Ser-684) (Fig. 2.6). The amino acid Val-675 has been reported to be involved in maintaining the three-dimensional structure of the protein (Crowther *et al.*, 1993b). In addition, this region in the SAT2 type viruses has three extra amino acids compared to European type sequences. This highly variable region of SAU/6/00 corresponds to the major antigenic site (site 1) for type O_1 Kaufbeuren (Kitson *et al.*, 1990) and site A for type C-S8c1 (Logan *et al.*, 1993). The amino acid Cys-660 in SAU/6/00 corresponds to Cys-134 in the 1D protein of type O, which forms a disulfide bond with Cys-130 in the 1B protein, thus promoting the formation of a conformational epitope in the G-H loop of type O FMD viruses (Acharaya *et al.*, 1990; Parry *et al.*, 1990).

In protein 1B of SAU/6/00, a hypervariable region (amino acids 168-189) was identified that correlates with antigenic site 2 in type O_1 (Crowther *et al.*, 1993a) and site D in type C_1. In these serotypes, the antigenic site comprises a discontinuous epitope involving amino acid residues in proteins 1B, 1C and 1D (Mateu *et al.*, 1990). The region between amino acids 541-562 in protein 1D of SAU/6/00 corresponds to antigenic site 3 in type O_1, whilst the region between amino acids 355-389 of the 1C protein corresponds to site 4. Both the antigenic sites 3 and 4 are conformation-dependent in type O_1 (Kitson *et al.*, 1990).

- ***Protease ($3C^{pro}$) cleavage sites***

The 3C viral protease ($3C^{pro}$) of FMDV cleaves at the 1AB/1C, 1C/1D and 1D/2A junctions of the P1 polyprotein. The $3C^{pro}$ recognizes a conformational determinant and is not able to process

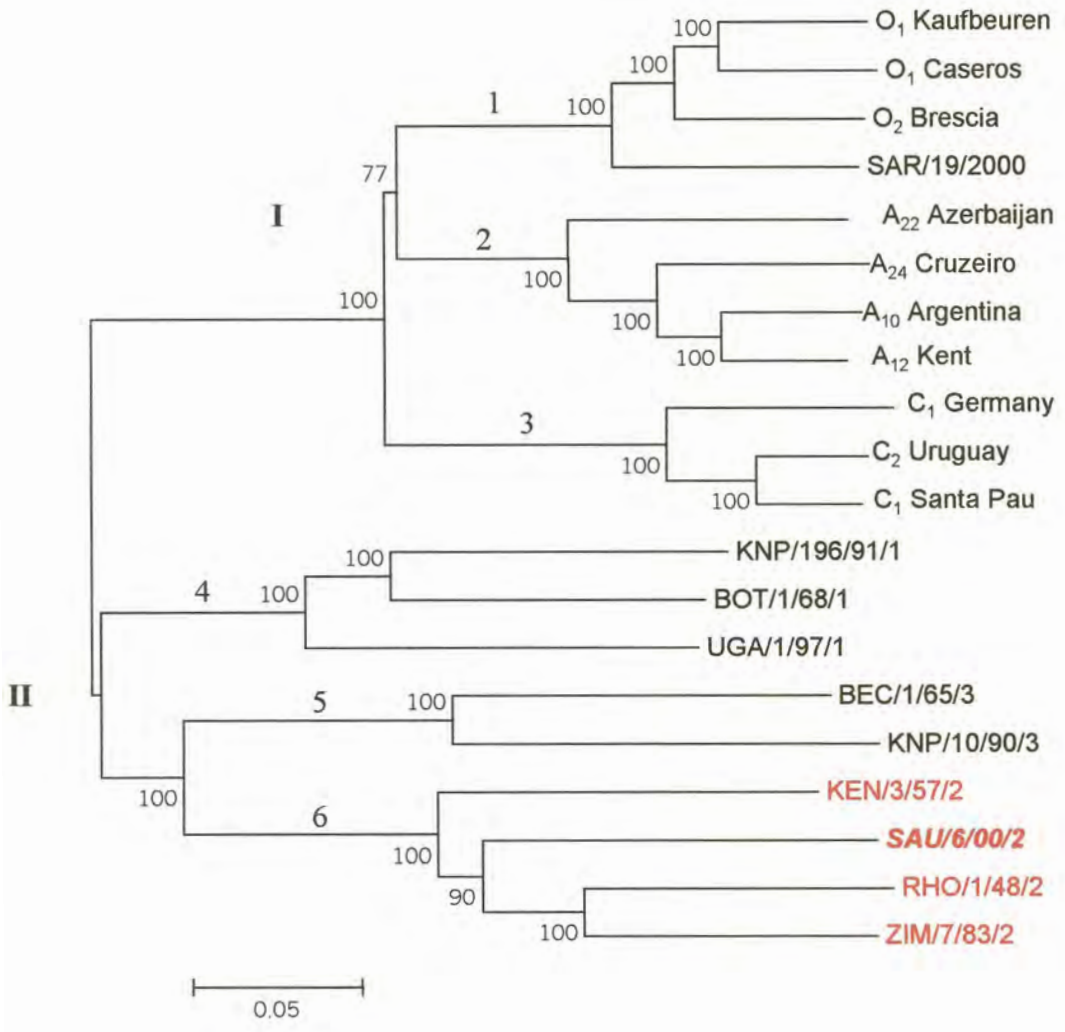


Fig. 2.5. Neighbour-joining tree depicting the relationships between nucleotide sequences of the P1 regions of six FMDV serotypes. The SAT2 viruses are indicated in red and SAU/6/00 in bold and italics. For this analysis, P-distance and 1000 bootstrap replications were applied.

```
                1A
KEN/3/57/2 GAGQSSPATG SQNQSGNTGS IINNYYMQQY QNSMDTQLGD NAISGGSNEG STDTTSTHTN 60
SAU/6/00/2 .......... ..D..... .......... .......... .......... .......... 
RHO/1/48/2 .......... .......... .......... .......... .......... .....F.... 
ZIM/7/83/2 ...H...V.. .......... .......... .......... .......... .......... 

                                      1B
KEN/3/57/2 NTQNNDWFSK LAQSAISGLF GALLADKKTE ETTLLEDRIL TTRHGTTTST TQSSVGITYG 120
SAU/6/00/2 .......... .......... .......... .......... .......... ......V.L. 
RHO/1/48/2 .......... .......... .......... .........V .......... .......... 
ZIM/7/83/2 .......... .......... .......... .........V .......... .......... 

                                                                  Site 2
KEN/3/57/2 YADSDSFRSG PNTSGLETRV EQAERFFKEK LFDWTSDKPF GTLYVLELPR DHKGIYGKLT 180
SAU/6/00/2 ...A....P. .......... Q......... .......... .........K .......... 
RHO/1/48/2 ...A....P. .......... ........K. .......... ...H.....K .Q.....S.I 
ZIM/7/83/2 ...A....P. .......... .......... .......... .........K .......S.. 

KEN/3/57/2 DSYTYMRNGW DVQVSATSTQ FNGGCLLVAM VPELCSLKAR EEYQLTLYPH QFINPRTNTT 240
SAU/6/00/2 .......... .......... ....S..... ....S...S. ..F....... .......... 
RHO/1/48/2 .A.A.T.... ....T..... ....S..... ....S...E. ..F....... .......... 
ZIM/7/83/2 .A........ .......... ....S..... ........D. ..F..S.... .......... 

KEN/3/57/2 AHLQVPYLGV NRHDQGKRHQ SWSLVVMVLT PPTTEAQMNS GTVEVYANIA PTNVYVAGEL 300
SAU/6/00/2 ..I....... .........H A......... .L........ .......... ....V..... 
RHO/1/48/2 ..I....... .......... A......... .L........ .......... ....F....M 
ZIM/7/83/2 ..I....... .......... A......... .L......Q. .......... ....F....K 

                1C
KEN/3/57/2 PGKQGIVPVA CADGYGGFQN TDPKSADPIY GHVYNPSRND CHGRFSNLLD VAEACPTLLD 360
SAU/6/00/2 .......... A......... ....T..... .Y........ .......... .......... 
RHO/1/48/2 .A....I... .......... ....T..... .Y........ ....Y..... .........N 
ZIM/7/83/2 .A....I... .F........ ....T..... .Y........ ....Y..... .......F.N 

           Site 4
KEN/3/57/2 FDGKPYVVTK NNGDKVMAAF DVAFTHKVHK NTYLAGLADY YTQYSGSLNY HFMYTGPTHH 420
SAU/6/00/2 ......I... .......TS. ..........R ..F....... .......... .......... 
RHO/1/48/2 .......... ........C. .......... ..F...P... ....Q..... .......... 
ZIM/7/83/2 .......... .......TC. .......... ..F....... .A..Q..... .......... 

KEN/3/57/2 KAKFMVAYVP PGIEVEELPK TPEDAAHCYH SEWDTGLNSN FTFAVPYLSS GDFSYTHTDT 480
SAU/6/00/2 .......... ..V.TAQ..T .......... A........S .S.....I.A A......... 
RHO/1/48/2 ........I. ..V.TDK... .......... .........Q .......V.A S......... 
ZIM/7/83/2 ........I. ....TDR... .......... .........Q .......V.A S......... 

                                                1D
KEN/3/57/2 PAMATTNGWV VVLQVTDTHS AEAAVVVSVS AGPDLEFRFP IDPVRQTTSA GEGAEVVTTD 540
SAU/6/00/2 .......... I......... .......... .......... .......... ..S.D..... 
RHO/1/48/2 .........I A.Y....... .......... .......... .........V ....D..... 
ZIM/7/83/2 .......... A.F....... .......... .......... V........S ....D..... 

           Site 3
KEN/3/57/2 PTTHGGKVTT PRRVHTDVAF LLDRSTHVHT NTTAFVVDLM DTKEKALVGA ILRSATYYFC 600
SAU/6/00/2 .S....N.QE G..K..E... .......... .K.S...... .......... ...AS..... 
RHO/1/48/2 .S....S.IE KK.M...... V...F..... SK.T.N.... .....T.... L..AS..... 
ZIM/7/83/2 .S....A..E KK........ VM..F...L. .R...A.... ..N..T...G L..A...... 
```

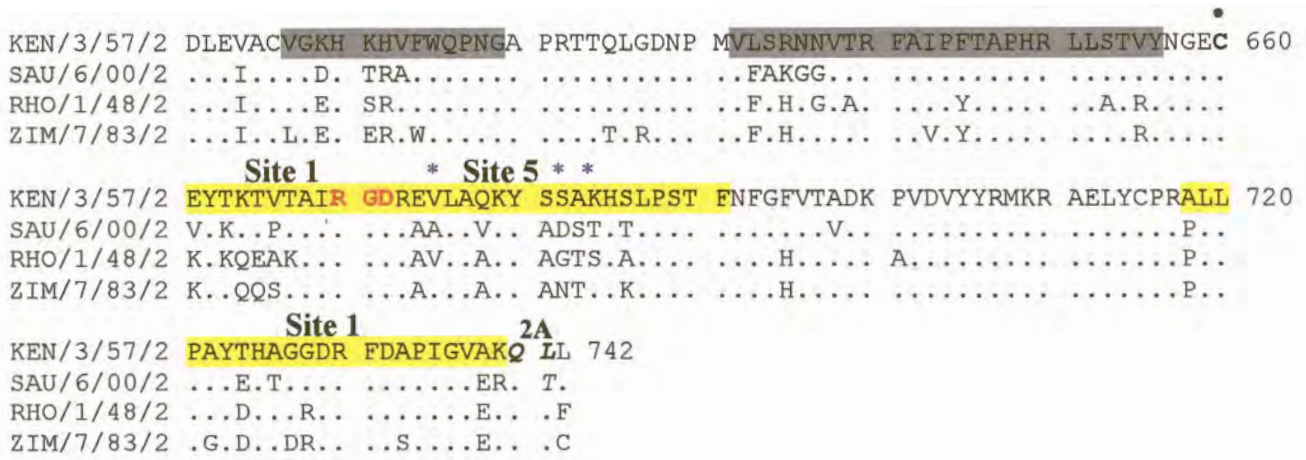


Fig. 2.6. Amino acid sequence alignment of P1 regions of four SAT2 FMD viruses. The start positions of the individual viral proteins (1A-1D) are indicated. Identical amino acid residues are indicated by dots. The hypervariable regions for these SAT2 viruses are highlighted in grey and yellow. The immunodominant sites (Sites 1-5), previously determined for FMDV type O, are indicated and the hypervariable regions that are similar to these sites are highlighted in yellow. The protease cleavage sites are indicated in italics, whilst the RGD sequence is indicated in red. The RHO/1/48/2 epitopes are indicated with (*) and the Cys-660 residue with (•).

the structural protein when it is incomplete (Clarke and Sanger, 1988). For *Picornaviridae* the dipeptide Gln-Gly is most prevalent at $3C^{pro}$ cleavage sites (Seipelt *et al.*, 1999). However, the FMDV $3C^{pro}$ can cleave different sites and is not limited only to Gln-Gly dipeptides (Hanecak *et al.*, 1982). In addition, it has the ability to cleave proteins of heterologous serotypes (Ryan *et al.*, 1989).

For SAU/6/00 (Fig. 2.6), the 1AB/1C cleavage site is Gln-Gly, which is identical to that of the other SAT viruses, whereas the European types have Glu-Gly (Van Rensburg *et al.*, 2002). At the 1C/1D junction, the cleavage site is Gln-Thr for all serotypes, as well as for SAU/6/00. At the 1D/2A junction, the cleavage site is Gln-Leu for most serotypes. However, the SAU/6/00 virus has Gln-Thr at this cleavage site, as also reported for type O_1 Kaufbeuren (Van Rensburg *et al.*, 2002). The amino acid Thr has a polar, uncharged side chain and Leu has a non-polar side chain. Although there is a difference in amino acids at this cleavage site for SAU/6/00 compared to other viruses, the Gln-Thr site is recognized and cleaved successfully by the 3C protease (Clarke and Sanger, 1988).

2.4 CONCLUSIONS

In this chapter, the nucleotide sequence of the capsid-coding region of the SAT2/SAU/6/00 strain was determined and compared to the P1 regions of other FMD viruses. Phylogenetic analysis of the P1 regions indicated that although the Saudi Arabian outbreak strain SAU/6/00 clustered with the SAT2 type viruses, it was genetically distantly related to the SAT2 vaccine strain ZIM/7/83. Furthermore, it was observed that the P1 region and specifically the 1D protein of the SAT2 type viruses display considerable intratypic variation (Vosloo *et al.*, 1992; Van Rensburg and Nel, 1999). Since the 1D protein contains the immunodominant site, it emphasizes the need for a vaccine strain with antigenic properties representative of that of the field strain virus.

The problem of SAU/6/00 not being a stable, high antigen-producing strain may be addressed through the engineering of a chimeric FMD virus, which would contain the antigenic determinants of the SAT2/SAU/6/00 strain in the genetic backbone of the stable, tissue cultured-

adapted vaccine strain ZIM/7/83. Although FMDV contains several antigenic sites, the immunodominant site is located in the G-H loop of the 1D protein (Strohmaier *et al.*, 1982). The antigenic sites are not known for the SAT types, but hypervariable regions corresponding to the antigenic sites of FMDV serotypes were identified by multiple amino acid sequence alignments. Although these results are merely predictive of the presence of antigenic sites, the true nature and identity of the SAT type antigenic sites are subject to further investigation by making use of, for example, antibody escape mutants. Since the five antigenic sites identified for type O_1 are conformation-dependent and/or discontinuous (Mateu, 1995), the efficient processing of the capsid proteins and assembly of infectious chimeric virus particles would be of great importance for the correct presentation of such epitopes to the host immune system.

Comparative analysis of the $3C^{pro}$ cleavage sites indicated that the cleavage sites at the 1AB/1C and 1C/1D junctions are conserved between the SAU/6/00 strain and different SAT2 viruses. Although the cleavage site at the 1D/2A junction of the SAU/6/00 virus is different to that of other SAT2 type viruses, it is identical to that reported for type O_1 Kaufbeuren (Van Rensburg *et al.*, 2002). Since the $3C^{pro}$ has the ability to cleave proteins of heterologous serotypes (Ryan *et al.*, 1989) and its cleavage is not limited to Gln-Gly sites only (Hanecak *et al.*, 1982), it was concluded that the different cleavage site at the 1D/2A junction would not have a negative impact on the engineering of a viable chimeric virus. The engineering of a chimeric virus between two SAT2 types, namely SAU/6/00 and ZIM/7/83, will be described in the following chapter.

CHAPTER 3

ENGINEERING OF A CHIMERIC SAT2 VIRUS CONTAINING THE EXTERNAL CAPSID-CODING REGION FROM SAU/6/00

3.1 INTRODUCTION

Knowledge regarding picornaviral nucleotide sequences and genome functions has advanced greatly over the past three decades. In the case of poliovirus, studies on the virus proteolytic pathways have been facilitated through the ability to derive infectious viral particles from a genome-length cDNA copy of the viral genome (Racaniello and Baltimore, 1981). By contrast, despite it having been reported that FMDV genomic RNA is infectious in the absence of other viral components (Belsham and Bostock, 1988), the construction of similar complete infectious cDNA clones of *Aphthoviruses* was initially hampered due to the presence of a poly(C) tract at the 5' end of the UTR in the FMDV genome.

Zibert and co-workers (1990) reported the first successful construction of an infectious genome-length cDNA clone for FMDV using type O_1 Kaufbeuren. Synthetic RNA transcribed *in vitro* from the genome-length cDNA clone, which contained a synthetic poly(C) tract, was subsequently used to transfect BHK cells and resulted in the production of viable virus particles. The construction of genome-length cDNA clones of FMDV has provided a valuable tool for the manipulation or exchange of viral genetic material (Rieder *et al.*, 1993; Beard and Mason, 2000). Not only have chimeric FMD viruses derived from such cDNA clones assisted investigations regarding the process of cell receptor binding (Sa-Carvalho *et al.*, 1997; Neff *et al.*, 1998; Baranowski *et al.*, 1998; 2000; 2001; Zhao *et al.*, 2003), but it also represents a novel approach whereby attenuated vaccines can be produced (Rieder *et al.*, 1996; Mason *et al.*, 1997; Almeida *et al.*, 1998; Chinsangaram *et al.*, 1998). In addition, this approach may also lead to the successful development of genetically engineered vaccines (Rieder *et al.*, 1994; Baranowski *et al.*, 2001; Van Rensburg and Mason, 2002; Van Rensburg *et al.*, 2004).

Due to the genetic and antigenic variability of the SAT type FMD viruses (Esterhuysen, 1994; Vosloo *et al.*, 1995; Bastos *et al.*, 2001; 2003), the development of custom-made vaccines for specific geographic regions has been investigated (Van Rensburg and Mason, 2002). For this purpose, the external capsid-coding region of the A_{12} genome-length cDNA clone was replaced with the corresponding region of the SAT2 vaccine strain, ZIM/7/83. The viable chimeric vSAT2/A12 virus, however, displayed poor growth characteristics in comparison to the parental ZIM/7/83 vaccine strain when propagated in BHK cells. Consequently, a genome-length cDNA clone from the parental SAT2 type virus, ZIM/7/83, has been constructed by a cassette-exchange strategy (Van Rensburg *et al.*, 2004) and termed pSAT2. The ZIM/7/83 virus was chosen for this purpose, as it is a stable vaccine strain and grows to high titers in BHK cells (Esterhuysen *et al.*, 1985). Subsequent characterization of the virus derived from the SAT2 genome-length cDNA clone indicated that vSAT2 has similar growth properties in BHK cells than the parental ZIM/7/83 vaccine strain. Comparison of the growth properties of the chimera vSAT2/A12 indicated it is inferior to that of the ZIM/7/83 vaccine strain and vSAT2. However, the chimera vSAT2/A12 had similar growth properties to that of the parental type A_{12} virus (Van Rensburg *et al.*, 2004). Therefore, the characteristics of the backbone used to construct a chimera can be transferred to such a recombinant virus.

As the current production of the SAU/6/00 antigen is characterized by low, variable yields and instability of the antigen, it necessitates that an alternative approach to the production of the inactivated vaccine be investigated. In this study, the SAT2 genome-length cDNA clone was therefore used to engineer a chimeric virus whereby investigations regarding its usefulness as a vaccine strain could be undertaken. To this extent, the external capsid-coding region of SAU/6/00, which contains the primary protective determinants, was inserted into pSAT2 of which the corresponding region had been removed. The chimeric construct was used for *in vitro* RNA synthesis and following transfection of BHK cells with the synthetic RNA transcripts, viable chimeric virus was recovered through serial passage in BHK cells.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial strains and plasmids

Escherichia coli MultiShot™ Top10 competent cells (genotype: F⁻ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G) were obtained from Invitrogen and used in cloning experiments as the transformation host. The SAT2 genome-length cDNA clone, pSAT2 (Van Rensburg *et al.*, 2004), was used as the genetic backbone in the construction of a chimeric genome-length cDNA clone.

3.2.2 PCR amplification of the external capsid-coding region of SAU/6/00

3.2.2.1 Oligonucleotides

The oligonucleotides used in the PCR assays to amplify the external capsid-coding region (1B-1D) of SAU/6/00 were designed using the nucleotide sequence data generated for the P1 region of SAU/6/00 (Chapter 2) and ZIM/7/83 (Van Rensburg, 2001; GenBank accession number AF136607), respectively. To facilitate cloning of the amplicon, unique restriction endonuclease recognition sites were included at the 5' terminus of the respective oligonucleotides. Thus, oligonucleotide SAUcDNA (5'-CGGAATATTGACCACACGTCACGGAACCACGAC-3') contained an unique *Ssp*I site (underlined) and oligonucleotide cDNA-2A (5'-CGCCCCGGGGTTGGACTCAACGTCTCC-3') contained an unique *Xma*I site (underlined) (Fig. 3.1). These oligonucleotides anneal to the 1B and 2A (3' end) regions on the viral genome, respectively. The oligonucleotides were custom-synthesized by Inqaba Biotechnical Industries and Invitrogen.

3.2.2.2 PCR amplification

The external capsid-coding region (1B-1D-2A) was amplified using the Expand™ High Fidelity PCR system (Roche) and the previously prepared reverse-transcribed viral RNA of the SAU/6/00 virus (Section 2.2.4) as template. The reaction mixture (50 µl) contained 3 µl of the cDNA template, 2.6 U of Expand™ High Fidelity DNA polymerase, 0.4 µM of each the SAUcDNA and cDNA-2A oligonucleotides, 1 x Expand buffer, 1.75 mM $MgCl_2$ and each dNTP at a concentration of 0.2 mM. The reaction tubes were placed in a Hybaid PCR Sprint thermal

cycler. Following initial denaturation at 94°C for 2 min, the reactions were subjected to 25 cycles of denaturation at 95°C for 30 s, annealing of oligonucleotides at 55°C for 30 s and elongation at 72°C for 2 min. Following the last cycle, an extended elongation step at 72°C for 7 min was included to ensure complete synthesis of all strands. An aliquot of the PCR reaction was analyzed by electrophoresis on a 1% (w/v) agarose gel in the presence of an appropriate DNA molecular size marker (phage λ DNA digested with *Hind*III; Promega). The amplicon was subsequently purified from the agarose gel using the Nucleospin® Extract kit (Macherey-Nagel) according to the specifications of the manufacturer.

3.2.3 Restriction endonuclease digestions

DNA was digested in the presence of 1 x restriction endonuclease buffer for the specific endonuclease (using the buffer supplied by the manufacturer) and 5 U of endonuclease per µg of DNA. The reaction mixtures were incubated at 37°C, except for *Sma*I (New England Biolabs) and *Swa*I (New England Biolabs), which were incubated at 25°C. When digestion entailed the use of two endonucleases requiring different salt concentrations for optimal activity, the DNA was first digested with the endonuclease requiring a lower salt concentration. The salt concentration was then adjusted by the addition of 1 M NaCl to a final concentration of 0.02 M, after which the second endonuclease was added. Following incubation, the endonucleases were inactivated by the addition of 2.5 µl RE STOP (consisting of 1 part 0.5 M EDTA, RNase A and 8 parts loading buffer; Promega) and incubated at room temperature for 5 min, after which the reaction mixture was stored at 4°C. The digestion products were analyzed on a 1% (w/v) agarose gel in the presence of an appropriate DNA molecular size marker. The DNA fragments of interest were purified from the agarose gel using the Nucleospin® Extract kit (Macherey-Nagel), prior to being used in cloning experiments.

3.2.4 Construction of the pSAU6/SAT2 chimeric clone

Plasmid pSAT2, comprising of the genome-length cDNA clone of the SAT2 type virus ZIM/7/83, was digested with both *Xma*I and *Ssp*I to excise the external capsid-coding region. Following digestion, the pSAT2 vector was dephosphorylated by treatment with 0.5 U alkaline phosphatase (Roche) for 30 min at 37°C to prevent self-ligation of the vector DNA (Turner *et al.*, 1997). The amplicon resulting from PCR amplification of the external capsid-coding region

using SAU/6/00 cDNA (Section 3.2.2) as template was similarly digested and then ligated into pSAT2. The ligation reactions were carried out using the Rapid DNA Ligation kit (Roche). Each reaction mixture consisted of 50 ng pSAT2 vector DNA, 150 ng insert and 1.25 U T4 DNA ligase in the presence of 1 x ligation buffer. Following incubation at room temperature for 10 min, aliquots (3 µl) of the ligation reaction mixtures were transformed into 25 µl of *E. coli* MultiShot™ Top10 competent cells (Invitrogen) using the heat shock method, as described previously (Section 2.2.6). Transformed cells were selected by plating the transformation mixtures onto LB agar plates supplemented with 50 µg/ml ampicillin and were incubated overnight at 37°C. The cloning strategy is indicated diagrammatically in Fig. 3.1.

3.2.5 Characterization of recombinant plasmid DNA

For rapid screening of a large number of transformants, plasmid DNA was extracted using a STET-boiling minilysate method (modified from a method obtained from Dr P.W. Mason, PIADC). Ampicillin-resistant colonies were randomly selected, inoculated into 1 ml of LB broth (Appendix) supplemented with 50 µg/ml ampicillin and grown overnight at 37°C with shaking. Cells from the overnight cultures were collected by centrifugation for 2 min at 13 000 rpm and the pellet suspended in 250 µl of STET buffer (Appendix) containing 0.25 mg/ml lysozyme (Roche). The bacterial cell suspension was boiled for 1 min at 100°C to facilitate cell lysis and then centrifuged immediately for 8 min at 13 000 rpm to pellet the bacterial debris. The pellet was removed and plasmid DNA was precipitated from the supernatant by the addition of 250 µl isopropanol. The precipitated plasmid DNA was pelleted by centrifugation for 8 min at 13 000 rpm, vacuum-dried and then resuspended in 20 µl 1 x TE. Plasmid DNA constructs were subsequently characterized by restriction endonuclease digestion using agarose gel electrophoresis.

A recombinant plasmid, designated pSAU6/SAT2, was further characterized by nucleotide sequencing (Section 3.2.8) to verify successful cloning of the external capsid-coding region into the pSAT2 genome-length cDNA clone. The nucleotide sequence flanking the cloning sites was determined using a sense oligonucleotide p622 (5'-GCACTGACACCACGTCTAC-3') and an

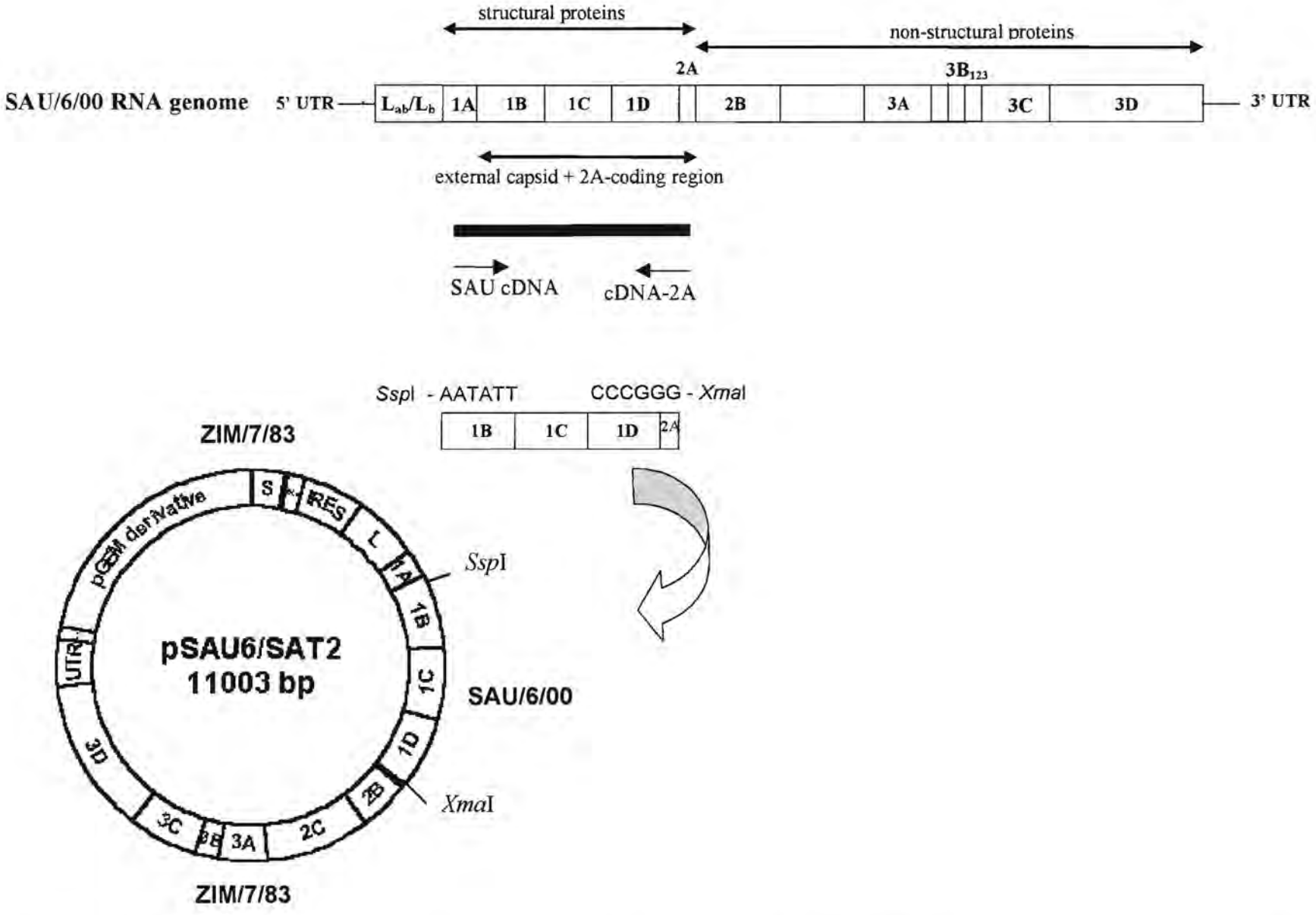


Fig. 3.1. Schematic representation of the cloning strategy used for the construction of the pSAU6/SAT2 chimeric construct. The external capsid-coding region (1B-1D) of SAU/6/00 was used to replace the corresponding region in the pSAT2 genome-length cDNA clone derived from SAT2/ZIM/7/83.

antisense oligonucleotide p621 (5'-GGACATATCTTGTTGCATG-3'), which are situated in the 1A and 2B regions, respectively, of the pSAT2 genome-length cDNA clone (Van Rensburg, unpublished results).

3.2.6 *In vitro* RNA synthesis

3.2.6.1 Preparation of template DNA

The QIAprep® Spin Miniprep kit (Qiagen) was used for plasmid extraction to obtain plasmid DNA of high purity for use as template in *in vitro* transcription reactions. The purified recombinant plasmid pSAU6/SAT2 was prepared for use in RNA transcription reactions by first linearizing 5 µg of the purified plasmid DNA with 0.2 U *Swa*I (New England Biolabs) at 25°C for 10 h. The pSAT2 genome-length cDNA clone was included as a control and linearized by digestion with 0.2 U *Not*I (New England Biolabs) at 37°C for 10 h. The liniarized DNA constructs were subsequently incubated at 55°C for 15 min with 20 ng proteinase K in the presence of 1 x STE (Appendix), 10 ng oyster glycogen (Sigma®), 0.5 M EDTA and 0.2% SDS. Following inactivation of the endonucleases by heating to 80°C for 15 min, the reaction mixtures were deproteinized by phenol and chloroform extraction. For this purpose, an equal volume (100 µl) of Tris-saturated phenol (pH 8) was added to the sample, mixed and the organic and aqueous phases separated by centrifugation for 2 min at 13 000 rpm. The upper aqueous phase was removed and extracted with an equal volume chloroform. The DNA was precipitated from the recovered aqueous phase by the addition of 2.5 volumes of 100% ethanol and incubation for 15 min at –80°C. The DNA was pelleted by centrifugation at 13 000 rpm for 15 min, washed with 70% EtOH and dried under vacuum before being resuspended in 10 µl RNase-free H_2O and stored at –20°C until required. Since this template was used to obtain *in vitro*-synthesized RNA transcripts for transfection of BHK cells in order to recover a viable chimeric SAT2 virus, all chemicals used were RNA-grade.

3.2.6.2 RNA transcription

In vitro RNA synthesis was carried out using the MEGAscript™ T7 kit from Ambion. The transcription reaction mixture contained 1 µg of the purified linearized plasmid DNA, 2 µl of the

supplied enzyme mix and 7.5 mM of each of the four ribonucleotides. The reaction mixtures were incubated for 2 h at 39°C. The quality of the RNA was analyzed on a 1% (w/v) agarose gel following electrophoresis in 0.5 x TBE buffer (Appendix).

3.2.7 Transfection of BHK cells and virus recovery

Baby hamster kidney (BHK) cells strain 21, clone 13 (ATCC CCL-10), were maintained in Eagle's basal medium (BME; Invitrogen) supplemented with 10% (v/v) tryptose phosphate broth (TPB; Sigma®) (Appendix), 1 mM L-glutamine (Invitrogen), antibiotics and 7% (v/v) fetal calf serum (FCS; Delta Bioproducts).

Transfection reactions were performed using Lipofectamine Plus™ reagent (Invitrogen) as a carrier for the synthetic RNA into the BHK cells. Monolayers of BHK cells in 35-mm-diameter wells were prepared the day before to reach 80% confluence (1 x 10^6 cells/ml) within 24 h of incubation at 37°C in the presence of 5% CO_2. For transfection, 3 µg of RNA was diluted in 84 µl D-MEM (Invitrogen) and pre-complexed with 16 µl Plus reagent by incubation at room temperature for 15 min. In a separate well, 8 µl of the Lipofectamine reagent was diluted in 92 µl D-MEM. Just prior to transfection, the two solutions were mixed and incubated for 15 min at room temperature. In the meantime, the medium was removed from the monolayers and the dead cells were removed by washing with serum-free D-MEM containing 0.1 mM non-essential amino acids (NEAA), but lacking antibiotics. After addition of 800 µl D-MEM to the cells, the cells were overlayed with the RNA-Lipofectamine Plus™ complexes and incubated for 4 h at 37°C in the presence of 5% CO_2. The medium was then aspirated and replaced with 3 ml BME, containing 25 mM HEPES (Invitrogen) and 1% FCS, to buffer the virus particles and sustain cell growth, respectively. The transfection plates were incubated at 37°C for 48 h in a CO_2 incubator, whereafter the transfected cell cultures were frozen and then thawed to lyse the cells. Aliquots of the supernatant were stored at -80°C. Mock-transfected cells, treated as above, were included as controls. Ten percent of the thawed supernatant (300 µl) was used to infect freshly prepared BHK monolayers in 35-mm wells, in the presence of 25 mM HEPES and 1% FCS, and observed for CPE. The same procedure was followed for serial passage of the viruses in BHK cells and the cells were monitored for CPE up to 48 h post-infection.

3.2.8 Characterization of recovered chimeric viruses

The recovered chimeric virus (vSAU6/SAT2) was characterized by RT-PCR of the external capsid-coding region, inclusive of the cloning sites, followed by partial nucleotide sequencing of the amplicon. RNA was extracted from the tissue culture supernatant by a modified guanidinium thiocyanate/silica method (Boom *et al.*, 1990) 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. All of these procedures were performed in accordance with the methods described previously (Sections 2.2.3 and 2.2.4). The external capsid-coding region was subsequently amplified by performing two separate PCRs. Oligonucleotides p622 (Section 3.2.5) and SAU12 (Table 2.1), and oligonucleotides p621 (Section 3.2.5) and VP3-AB (5'-CACTGCTACCACTCRGAGTG-3') (Bastos *et al.*, 2003), situated in the 1C region, were used to amplify parts of the 1A-1B and 1C-2B genomic regions spanning the insertion sites, respectively. The PCR reaction mixtures contained 3 µl cDNA, 2.5 U Taq DNA polymerase (Roche), 0.4 µM of each the sense and antisense oligonucleotides, 1 x Taq buffer containing $MgCl_2$ and 2.5 mM dNTPs. The reaction tubes were placed in a Hybaid PCR Sprint thermal cycler and following an initial denaturing step of 2 min at 94°C, the reactions were subjected to 30 cycles of amplification using the following conditions: denaturation at 95°C for 30 s, oligonucleotide annealing at 56°C for 30 s, elongation at 72°C for 1 min, followed by an additional elongation step at 70°C for 30 s. The PCR-amplified products were analyzed in the presence of an appropriate DNA molecular size marker by electrophoresis on a 1% (w/v) agarose gel.

The amplicons were subsequently purified from the agarose gel with the Nucleospin® Extract kit (Macherey-Nagel). The nucleotide sequence of the purified amplicons was determined using an ABI PRISM™ Big Dye™ Terminator Cycle Sequencing Ready Reaction kit v3.0 (Applied Biosystems) with an ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems). The amplicons were sequenced using the oligonucleotides that had been used in the above-described PCR reactions. The obtained nucleotide sequences were compared to that of the capsid-coding region of the SAT2/SAU/6/00 virus (Chapter 2) using the DAPSA version 2.9 software programme (Harley, 2001).

3.3 RESULTS AND DISCUSSION

3.3.1 Construction of the chimeric cDNA clone pSAU6/SAT2

3.3.1.1 PCR amplification of the external capsid-coding region from SAU/6/00

The external capsid-coding region (1B-1D) of SAU/6/00 was amplified from cDNA using oligonucleotides SAUcDNA (containing a *Ssp*I site) and cDNA-2A (containing a *Xma*I site). The High Fidelity DNA polymerase, with 3'→5' exonuclease proofreading activity, was used in order to minimize misincorporation errors during PCR amplification. Since no amplicon could initially be obtained with the PCR, the reaction conditions were optimized using a method described by Cobb and Clarkson (1994). For this purpose, plasmid pGEM-SAU, containing a copy of the capsid-coding region from SAU/6/00 cloned into pGEM®-T Easy (Section 2.3.1), was used as template in the PCR reactions. The $MgCl_2$, dNTP and template DNA concentrations were varied, as described in the legend to Fig. 3.2. In addition, changes in the annealing temperature and the use of DMSO to effectively disrupt base pairing were also investigated (results not shown). Using the optimized PCR conditions, as described under Materials and Methods (Section 3.2.2), an amplicon of the expected size (1.9 kb) could be obtained using cDNA as template in the PCR (Fig. 3.2, lanes 2-6). By contrast, no amplification products were observed in the negative control in which template DNA was omitted (Fig. 3.2, lane 9).

3.3.1.2 Cloning of the external capsid-coding region from SAU/6/00 into pSAT2

Towards construction of the recombinant clone pSAU6/SAT2, the pSAT2 genome-length cDNA clone (Van Rensburg *et al.*, 2004) was digested with both *Ssp*I and *Xma*I to excise the *ca.* 2-kb external capsid-coding region from the pSAT2 clone. The larger of the two DNA fragments (*ca.* 8 kb) was purified from the agarose gel, dephoshorylated and used in subsequent ligation reactions. The insert DNA was prepared similarly by digestion of the amplicon with the endonucleases *Ssp*I and *Xma*I, gel-purified and ligated to the prepared pSAT2 vector DNA. Following transformation of *E. coli* Top 10 competent cells with the ligation reaction mixture, plasmid DNA was extracted from the ampicillin-resistant transformants and characterized by agarose gel electrophoresis and by restriction endonuclease digestion.

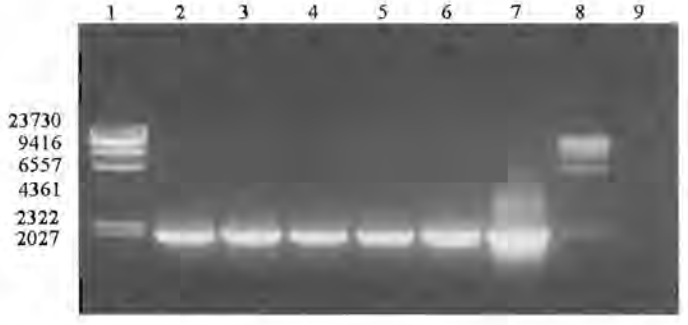


Fig. 3.2. Agarose gel electrophoretic analysis of the amplicons obtained by RT-PCR amplification of the external capsid-coding region of SAU/6/00 using oligonucleotides SAUcDNA and cDNA-2A. Lanes 1 and 8, DNA molecular marker; lanes 2-4, amplicons obtained using cDNA from SAU/6/00 as template (50, 75 and 100 ng), 0.1 mM dNTPs and 1.5 mM Mg^{2+}; lane 5, 50 ng cDNA, 0.2 mM dNTPs, 1.5 mM Mg^{2+}; lane 6, 50 ng cDNA, 0.2 mM dNTPs, 1.75 mM Mg^{2+}; lane 7, positive control (pGEM-SAU plasmid DNA as template); lane 9, negative control (PCR reaction mixture lacking template DNA). The sizes of the DNA molecular weight marker, phage λ DNA digested with *Hind*III, are indicated to the left of the figure.

Digestion of the plasmid DNA with *Sal*I, which cuts once in 1D of SAU/6/00 and once in 3A and twice in 3D of pSAT2 (Fig. 3.3), yielded bands corresponding to 7.56, 1.95, 1.27 and 0.23 kb, in the case of recombinant plasmid DNA (Fig. 3.4, lanes 4-10). The 0.23 kb fragments are not visible due to their small size and poor resolution of the gel. By contrast, non-recombinant plasmid yielded different sized DNA fragments (Fig. 3.4, lanes 2 and 3). The presence of a *Sal*I site in 1D of pSAU6/SAT2 results in these different sized DNA fragments. To further confirm the successful construction of plasmid pSAU6/SAT2, the recombinant plasmid DNA was digested with both *Ssp*I, which cuts once in 1B, and *Sma*I, which cuts once in 2A (Fig. 3.3). Although an *Xma*I site was incorporated in the cDNA-2A oligonucleotide used for PCR amplification, *Sma*I is an isoschizomer of *Xma*I and thus digests DNA within the same recognition site in an identical manner. Agarose gel electrophoresis of the digested products indicated the presence of two DNA fragments of *ca.* 2 kb and 8 kb. The size of the DNA fragments corresponded to the size of the cloned external capsid-coding region of SAU/6/00 and the size of the pSAT2 vector DNA, respectively (results not shown).

The integrity of the cloned insert DNA in pSAU6/SAT2 was verified by nucleotide sequencing of the cloning sites and flanking sequences, prior to it being used in further manipulations (Fig. 3.6). The nucleotide sequence of the newly constructed clone was identical to the corresponding region of the parental SAU/6/00 virus, except for two nucleotide dissimilarities. The nucleotides were incorporated into the oligonucleotides to create the desired restriction endonuclease recognition sequences required for cloning of the external capsid-coding region of SAU/6/00 into pSAT2 (Fig. 3.1). However, the observed nucleotide substitutions did not result in a change in the corresponding amino acid residues.

3.3.2 Generation of a viable chimeric virus, vSAU6/SAT2

The strategy for obtaining an infectious vSAU6/SAT2 chimeric virus involved transfection of BHK cells with *in vitro*-synthesized RNA transcripts prepared from linearized recombinant plasmid pSAU6/SAT2. As a control, RNA transcripts derived from pSAT2 were also used to transfect BHK cells. The supernatants obtained from transfected cells were subsequently used to infect BHK cells and the cells were monitored for signs of CPE by comparison to mock-infected

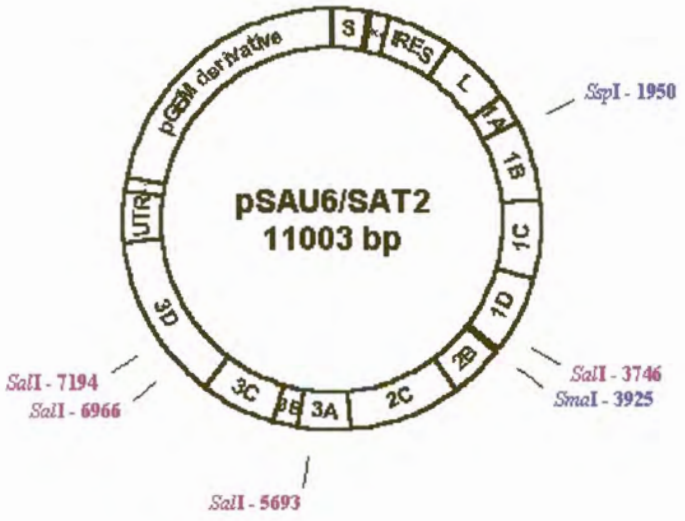


Fig. 3.3. Schematic representation of the restriction endonuclease recognition sites for *Sal*I, *Ssp*I and *Sma*I on the chimeric construct pSAU6/SAT2.

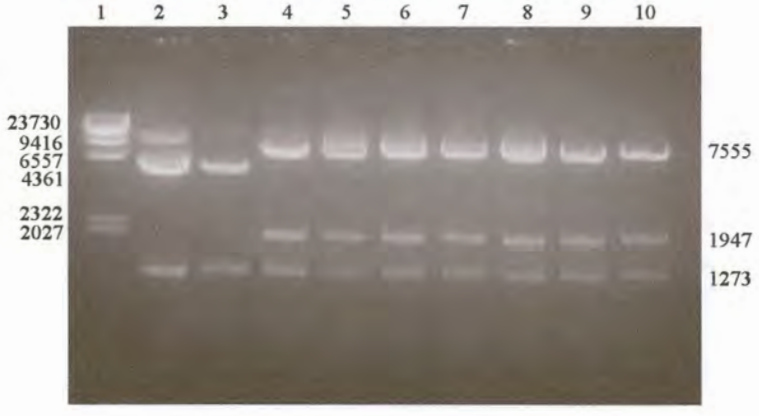


Fig. 3.4. Agarose gel electrophoretic analysis of plasmid DNA from ampicillin-resistant transformants following digestion with *Sal*I. Lane 1, DNA molecular marker; lanes 2-10, *Sal*I-digested plasmid DNA. The sizes of the DNA molecular weight marker, phage λ DNA digested with *Hind*III, are indicated to the left of the figure. The sizes of restriction fragments indicative of recombinant plasmids are indicated to the right of the figure.

BHK cells. The results obtained following repeated passaging in BHK cells are shown in Table 3.1.

The cells transfected with RNA derived from pSAT2 displayed 100% CPE after 24 h of incubation upon the first round of passage, which corresponds to results obtained previously for the same virus (Van Rensburg *et al.*, 2004). By contrast, no CPE was initially observed for cells transfected with RNA derived from the chimeric pSAU6/SAT2 clone. This may have been due to a limited amount of synthetic RNA being successfully transfected into the cells, thereby resulting in a low titre of viable virus. In addition, the parental virus SAU/6/00 is characterized by slow propagation in tissue culture and this characteristic may be reflected in the slower recovery rate compared to vSAT2. Upon subsequent passaging in BHK cells, thus resulting in increasingly higher titres of the virus, more severe CPE could be observed. Therefore, following the initial lack of CPE observed for vSAU6/SAT2 in BHK cells, the amount of CPE increased on each successive round of passage, until CPE of 80% to 90% was observed after 48 h of incubation on the fourth passage. As no CPE was observed for mock-infected BHK cells, it was concluded that the observed cell lysis might be due to infectious viral particles having been generated from the RNA derived from the pSAT2 and chimeric pSAU6/SAT2 clones.

Table 3.1. CPE observed during serial passage of the chimeric vSAU6/SAT2 virus and vSAT2 virus in BHK cells transfected with synthetic RNAs

	CPE observed *		
Passage	**Mock-infection**	**vSAU6/SAT2**	**vSAT2**
BHKp1	-	-	++++
BHKp2	-	++	++++
BHKp3	-	+++	++++
BHKp4	-	++++	++++

* No CPE observed; (-)
** CPE observed; (++) 40-60%, (+++) 65-90%, (++++) 100%

3.3.3 Confirmation of recovered vSAU6/SAT2

To verify that a chimeric virus between the two SAT2 type viruses, consisting of the external capsid-coding region of SAU/6/00 in the pSAT2 genome-length cDNA clone, had indeed been recovered, viral RNA was extracted from the supernatant of BHK cells infected with vSAU6/SAT2 and reverse-transcribed. The regions of the external capsid-coding region surrounding the cloning sites were subsequently amplified in two separate PCR reactions.

Oligonucleotides p622 and SAU12 were used to amplify a 0.65-kb product comprising the region flanking the *Ssp*I cloning site at the 5' end of the cloned external capsid-coding region, whereas oligonucleotides VP3-AB and p621 were used to amplify a 0.9-kb product comprising the region flanking the *Xma*I cloning site at the 3' end of the cloned region. The respective products were produced when cDNA derived from the chimeric virus and the control recombinant pSAU6/SAT2 plasmid DNA were used as templates in the PCR reactions. Despite the amplification of non-specific products, probably due to sub-optimal PCR conditions, amplicons of the expected size were the most prominent bands in the agarose gel (Fig. 3.5, lanes 2, 3 and 6). No amplification products were observed in the control reactions from which template DNA had been omitted (Fig. 3.5, lanes 4 and 7).

The amplicons corresponding to the expected sizes were subsequently purified from the agarose gel and their nucleotide sequence determined (Fig. 3.6). Analysis of the partial nucleotide sequence of the capsid-coding region of the chimeric virus confirmed recovery of the correct virus. Except for the two nucleotide substitutions incorporated in oligonucleotides SAUcDNA and cDNA-2A, there were no mutations in the sequence of the chimera vSAU6/SAT2.

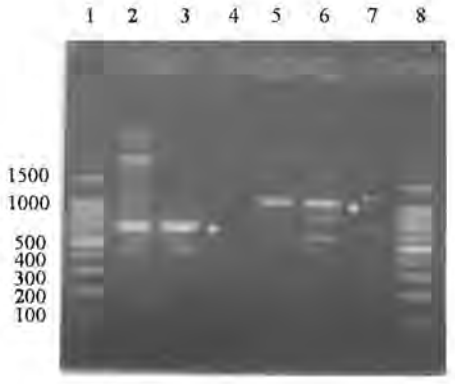


Fig. 3.5. Agarose gel electrophoretic analysis of the amplification products obtained following PCR amplification of cDNA prepared from the chimeric vSAU6/SAT2 virus (lanes 2 and 5) and the chimeric pSAU6/SAT2 plasmid construct (lanes 3 and 6), using oligonucleotides p622 and SAU12 (lanes 2 to 4) and oligonucleotides VP3-AB and p621 (lanes 5 to 7). Control reactions lacking template DNA were included (lanes 4 and 7). The sizes of the 100-bp ladder molecular weight marker (Promega) (lanes 1 and 8) are indicated to the left of the figure. Amplicons of the expected size are indicated by asterisks.

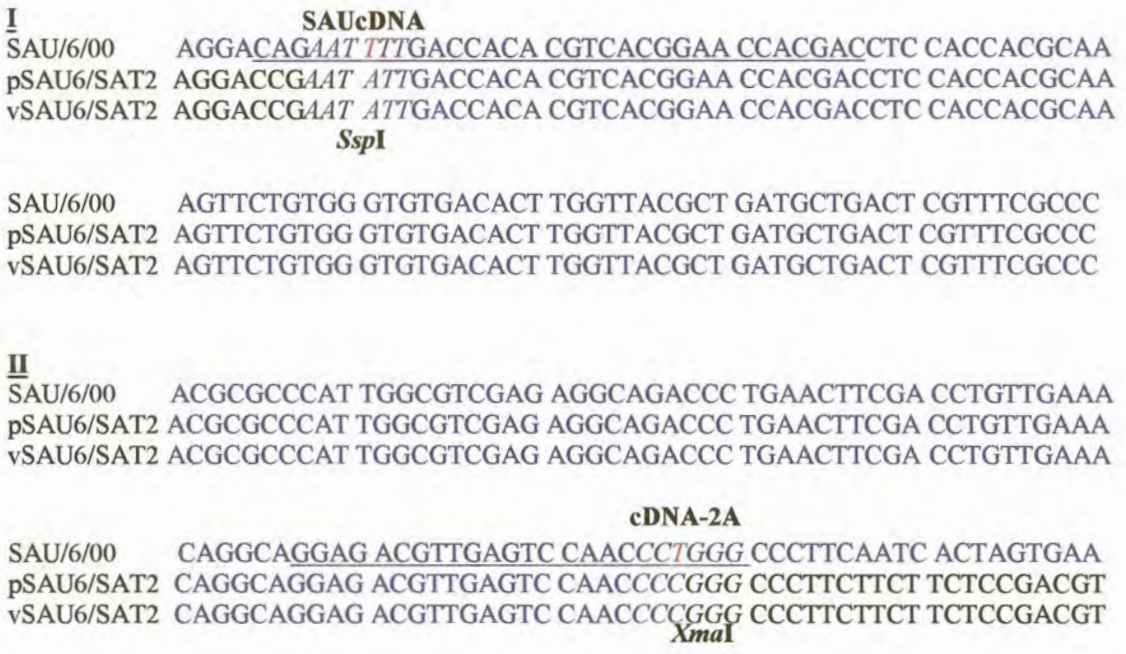


Fig. 3.6. Nucleotide sequence alignment of the parental virus (SAU/6/00), recombinant construct (pSAU6/SAT2) and chimeric virus (vSAU6/SAT2). Partial nucleotide sequences are indicated; the SAU/6/00 sequence is in blue and the pSAT2 sequence is in black. The *Xma*I and *Ssp*I restriction endonuclease sites used in cloning are indicated in italic. The oligonucleotides SAUcDNA (I) and cDNA-2A (II) used in PCR amplification are underlined. Nucleotide sequence differences obtained are indicated in red.

3.4 CONCLUSIONS

In this part of the study, an infectious chimeric virus between two different SAT2 type viruses has been engineered using a reverse genetics approach. A recombinant clone pSAU6/SAT2 was obtained following cloning of a cDNA copy of the external capsid-coding region from SAU/6/00 into the ZIM/7/83 genome-length cDNA clone pSAT2, thereby replacing the ZIM/7/83 structural protein-coding region with that of SAU/6/00. Viable chimeric virus was derived from clone pSAU6/SAT2 by transfection of BHK cells with the *in vitro*-transcribed RNA. The resultant chimeric virus vSAU6/SAT2 was characterized by partial nucleotide sequencing of the external capsid-coding region. Based on the ability of the chimeric virus to replicate in BHK cells, as witnessed by an increase in CPE upon successive passaging in BHK cells, it could be concluded that the RGD sequence in the 1D protein of the chimeric virus bound to integrin receptors on the BHK cell surface for internalization and that the external capsid region was maintained upon subsequent passaging in BHK cells. These results are consistent with those previously reported for type A_{12} chimeric viruses containing the external capsid-coding regions from type O_1 or type C_3 (Rieder *et al.*, 1994), or the SAT2 strain ZIM/7/83 (Van Rensburg and Mason, 2002).

The ability to manipulate and exchange genome regions between different FMD viruses using the above reverse genetics technology has greatly facilitated studies regarding the development of inactivated vaccines (Rieder *et al.*, 1994; Baranowski *et al.*, 2001) and improved genetically engineered vaccines (Van Rensburg *et al.*, 2004). The successfully recovered vSAU6/SAT2 chimera will subsequently be characterized by comparison with the parental strain SAT2/SAU/6/00 with regards to properties important in FMD vaccinology, as detailed in the following chapter.

CHAPTER 4

COMPARISON OF THE SAT2 TYPE CHIMERA vSAU6/SAT2 WITH THE PARENTAL SAU/6/00 VACCINE STRAIN IN TERMS OF GROWTH PROPERTIES, TEMPERATURE STABILITY AND ANTIGENIC PROFILE

4.1 INTRODUCTION

Chemically inactivated vaccines used for FMD have greatly contributed to its eradication and control in many parts of the world (Sutmoller *et al.*, 2003). Production of efficient 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 (Rweyemamu, 1978). In order to produce such vaccines, the immunizing antigen of FMDV has been studied and characterized well. Tissue culture harvests of FMDV contain four immunogenic, as well as non-immunogenic virus-specific antigens, that comprise of the complete nucleocapsid with a sedimentation coefficient of 146S, RNA-free empty capsids (75S), degraded capsid subunits (12S) and non-structural virus infection-associated antigen (Grubman *et al.*, 1985). Both the 146S and 75S components of FMDV stimulate significant amounts of neutralizing antibody and confer immunity to animals (Wild and Brown, 1968; Bachrach, 1975).

The high degree of antigenic variability in FMDV and presence of seven serotypes, including several subtypes, has important implications for vaccine strain selection. Vaccine candidates should be closely related to field strains (Doel, 1996) and induce a response with a broad immunological cross reactivity (Esterhuysen *et al.*, 1988) for appropriate protection against subtypes (Pay, 1983). FMDV particles are relatively unstable with respect to temperature and pH, characteristics which could influence vaccine efficiency (Doel and Baccarini, 1981). The dissociation of the infective particle and subsequent loss of immunogenetic properties (Brown and Wild, 1966) has implications on the efficacy and shelf-life of a vaccine. The SAT types have proven more labile, which might affect the stability and immunological properties of the capsid (Doel and Baccarini, 1981).

In addition to the above-mentioned, the selection of a suitable FMD vaccine strain is complicated by the fact that not all field strains can be adapted to large-scale production in BHK cell cultures and do not necessarily produce desirable amounts of stable antigen. The production of successful SAT vaccines has been recorded as difficult as it may take considerable time to adapt and validate a new strain for commercial production (Preston *et al.*, 1982). In such a study where SAT2 viruses were investigated as possible vaccine strains only one out of 27 produced acceptable titres (>10^6 pfu/ml within the third round of passage) (Pay *et al.*, 1978). Recently, in 2003, seventeen SAT1 and SAT2 FMDV field strains were investigated as possible vaccine strains and adapted to BHK cells. Five of these viruses were chosen for stability studies on the basis of acceptable titres in BHK cells and 146S content. Four of the viruses that were tested for stability failed and one strain is still under investigation (Dr M. Cloete, pers. comm.).

Whilst the intact virus particle elicits the most neutralizing antibody activity of the multiple antigenic sites on the viral capsid (Mateu *et al.*, 1995), the primary protective determinants are located on the trypsin-sensitive 1D protein with the G-H loop and C-terminus being highly exposed regions (Logan *et al.*, 1993; Jackson *et al.*, 2003). The significant involvement of 1D in eliciting an immune response in host animals led to studies utilizing this region as synthetic peptides (DiMarchi *et al.*, 1986) and DNA vaccines (Wong *et al.*, 2000), resulting in partially protective immunity in cattle and swine, although less than inactivated complete virus vaccines. For such a vaccine to ensure immunogenic potency equivalent to the complete virus particle, it must have comparable high structural integrity and similar presentation to the immune system (Kleid *et al.*, 1981; Kitching, 1992). Despite all its potential and although it has proven useful in FMDV research, biotechnology applied in vaccine development has not yet replaced conventional tissue culture-derived vaccines.

An alternative approach in the development of inactivated vaccines involves the engineering of chimeric FMD viruses of which the antigenic properties can be readily manipulated. Using a genome-length cDNA clone of type A_{12}, Rieder and co-workers (1994) engineered chimeric viruses of which the G-H loop region of A_{12} was exchanged with that of type O (O_1BFS) and type C (C_3RES). Chemically inactivated vaccines prepared from the chimeric viruses induced neutralizing antibodies against the homologous serotype A and either types O or C viruses in

guinea pigs and pigs (Rieder *et al.*, 1994). The external capsid-coding region (1B-1D) of the SAT2 isolate ZIM/7/83 has subsequently been cloned into the type A_{12} cDNA clone and yielded viable chimeric FMDV. By engineering such a chimeric virus, a possible alternative to the conventional inactivated vaccine production of the SAT type viruses was investigated for the development of custom-made inactivated FMD vaccines (Van Rensburg and Mason, 2002).

Due to problems associated with the current production of the SAT2/SAU/6/00 antigen, a chimeric virus vSAU6/SAT2 has been engineered whereby the desirable properties of the ZIM/7/83 vaccine strain was combined with the antigenicity required for a SAU/6/00-specific vaccine (Chapter 3). The constructed chimera has the same external capsid as the parental SAU/6/00 strain and therefore the same determinants for antigenicity. In this part of the study, the new chimera vSAU6/SAT2, parental SAU/6/00 vaccine strain and vSAT2 were grown to high titers in BHK cells, whereafter these viruses were compared with regards to growth properties in BHK cells, temperature stability and the antigenic profile of the viral particles.

4.2 MATERIALS AND METHODS

4.2.1 Viruses

Viruses recovered previously (Chapter 3) were of the following passage history: vSAU6/SAT2 (BHK_4) and vSAT2 (BHK_4). Following a second passage in IB-RS-2 cells, the vaccine strain had the following passage history: SAU/6/00 (BTY_1RS_2). These viruses were used in all subsequent experiments.

4.2.2 Viral amplification

The vaccine strain SAU/6/00, chimera vSAU6/SAT2 and control vSAT2 viruses were amplified in BHK cells. Monolayers in T-150 flasks were prepared the day before to reach 90% confluence (1.5×10^7 cells) within 24 h. The cell density was not 100%, as this could inhibit virus propagation (Sarma, 1985). The spent medium and dead cells were removed and the cells washed with BME (1% FCS, 25 mM HEPES and antibiotics). Each of the recovered viruses (500 µl), together with 3 ml BME, was added and incubated for 1 h at 37°C with gentle rocking.

Following initial infection, 7 ml BME (1% FCS, 25 mM HEPES and antibiotics) was added and incubation at 37°C continued. Viruses were harvested with CPE 90% to 100% and clarified by centrifugation for 10 min at 1 000 rpm in the presence of HEPES to buffer the virus particles. The supernatant was stored as 1-ml aliquots at –80°C.

4.2.3 Plaque assay

Plaque titration assays were performed on BHK, IB-RS-2 (a pig kidney cell line) and Chinese hamster ovary (CHO) cells. The cells were seeded to achieve 80% confluence within 24 h in 35-mm wells and incubated overnight at 37°C in a CO_2 incubator. BHK cells were maintained in BME medium (10% FCS), IB-RS-2 cells in RPMI medium (5% FCS) and CHO strain K1 cells (ATCC CCL-61) in Ham's F-12 medium (10% FCS).

BME medium (25 mM HEPES, 1% FCS and antibiotics) was used to prepare $\log_{10}$ dilutions for each virus. The medium was aspirated from the cell monolayers and 200 µl of each dilution added to the cells. Following incubation for 1 h at 37°C, with periodic gentle shaking, 2 ml of tragacanth overlay (Appendix) was added to each well and incubated in a CO_2 incubator for 48 h. The cell monolayers were stained with 2 ml of 1% (w/v) methylene blue stain and investigated for plaque morphology. All plaque assays were performed in duplicate (Grubman *et al.*, 1979).

4.2.4 Single-step growth studies

The release of virus particles from infected cells was determined by infecting BHK cell monolayers (80% confluent) with virus at a MOI of 2. The growth medium was aspirated from the cell monolayers and the virus (in a total volume of 500 µl BME containing 1% FCS and antibiotics) added and incubated for 1 h at 37°C with shaking to allow the virus to absorb. Residual extracellular virus was eliminated by an acid wash step with 5 ml MBS (MES-buffered saline) (Appendix), followed by washing with BME containing 1% FCS, after which 5 ml of BME, containing 1% FCS, was added to sustain cell growth. At different time intervals, 2% of the supernatant was removed and added to BME to prepare 10-fold dilutions. Following centrifugation for 5 min at 5 000 rpm to clarify the virus, the supernatant was stored at –80°C. The samples were used for titration in BHK plaque assays, performed as described in Section 4.2.3.

4.2.5 Investigation of antigen stability

4.2.5.1 Virus amplification

BHK cells were prepared in BME, containing 10% FCS, on T-265 flasks (80% confluent) and grown overnight at 37°C. The medium was aspirated and the monolayers infected with virus (MOI of 1) in 3 ml BME, containing 1% FCS, followed by incubation at 37°C for 1 h with gentle rocking. Thereafter, 7 ml BME was added to each flask and incubated at 37°C. After 26 h, the cell harvest was treated with 1% chloroform for 1 h at 4°C to lyse the cells and centrifuged for 10 min at 5 000 rpm (4°C) for clarification of the virus. Samples of 1.4 ml were dispensed into tubes and used for thermal stability studies.

4.2.5.2 Heat treatment of viruses

The stability of the viruses was determined at temperatures of 42°C (to represent the acute phase of FMDV infection) and 56°C (the temperature used for heat inactivation) (Thomson, 1994), respectively. One sample of each virus was kept at 4°C for the duration of the experiment. Three samples of each virus were incubated at 42°C and removed at 3, 6 and 24 h, respectively. Similarly, samples were incubated at 56°C and removed after 30 min, 1 and 3 h. During the heating period, the samples were agitated followed by rapid cooling on ice and used in subsequent studies to asses the level of viral antigen.

4.2.5.3 Indirect sandwich ELISA

The indirect sandwich enzyme-linked immunosorbent assay (ELISA) was used for the detection and quantification of FMD viral antigens. Of each sample, 100 µl was added (in triplicate) to the first row of a 96-well microplate (Nunc™ Maxisorp) coated with trapping antibody (rabbit antiserum specific for SAT2) with a final dilution of 1/2500. The rest of the wells were filled with 50 µl 0.5% (w/v) casein to block non-specific protein binding reactions. The ZIM/7/83 virus (100 µl) was added as a positive control and 100 µl casein as a negative control. The samples were diluted across the plate producing 1/2, 1/4, 1/8, etc. dilutions. The microplates were incubated overnight at 4°C to allow for the binding of the virus to the trapping antibodies and washed for four cycles with 0.05% (v/v) PBS-Tween in an automated microplate washer (Bio-Tek Instruments). Guinea pig-detecting serum for SAT2 was diluted in casein to a final

concentration of 1/100 and 50 µl added to all the wells. The plates were then incubated for 1 h at 37°C and washed five times. Antispecies conjugate (50 µl) was added to all the wells and the plates incubated for 1 h at 37°C and washed four times to remove unbound labeled conjugate. Bound antibodies were visualized by σ-phenylenediamine and H_2O_2 as the substrate/chromogen, of which 100 µl was added to each well. After incubation for 10 min at room temperature, the colour reaction was stopped by the addition of 50 µl of 1 M H_2SO_4. The absorbance at 492 nm of the contents of each well was determined using a Labsystems Multiscan Plus photometer (OIE Manual of Standards).

4.2.5.4 Plaque assays

Plaque assays were performed on BHK cells to determine the titre of the vSAU6/SAT2, SAU/6/00 and vSAT2 viruses at different intervals following heat treatment at 42°C and 56°C. The assays were performed as described in Section 4.2.3.

4.2.5.5 146S quantification

The 146S content (µg/ml) was determined by quantitative 146S determination where a 500-µl sample was layered on top of a 10 to 25% (w/v) sucrose gradient and centrifuged in an Beckman ultracentrifuge at 45 000 rpm (6°C) for 44 min. Antigen content was detected on a UV-detector (optical density 254 nm) and chromatographically registered on a recorder. The 146S concentration was calculated from the surface area (mm^2) of the relevant peak multiplied by a constant (0.0116 µg/ml) (under standard conditions, $1mm^2$ represents 0.167 x 0.0694 µg/ml = 0.0116 µg/ml) (Mr N. Ismail-Cassim, pers. comm.).

4.2.6 Antigenicity determination of the viruses using the virus neutralization test

4.2.6.1 Titration of viruses on IB-RS-2 cells

The tissue culture infective doses (TCID) of the viruses were determined using IB-RS-2 cells in flat-bottomed microtitre plates (Nunc™) to establish which virus dilutions should be used in the virus neutralization test (VNT). Each virus was used to prepare 0.5 log_{10} dilutions in RPMI medium containing antibiotics. RPMI medium (50 µl) was added to each well, after which 50 µl of the virus dilutions were added (in 8-fold) and the plates incubated for 1 h at 37°C in a CO_2

incubator. IB-RS-2 cells were prepared in a suspension of 3 x 10^5 cells/ml in RPMI medium containing 5% FCS, and 100 µl of the suspension culture was added to each well, followed by incubation for 72 h at 37°C in the presence of 5% CO_2. The plates were read with an inverted microscope and the amount of wells with CPE was used to determine the virus titre expressed as $TCID_{50}$/50 µl (Esterhuysen *et al.*, 1985).

4.2.6.2 Virus neutralization test

The virus titres determined in Section 4.2.6.1 were used to calculate which four virus dilutions to use in the virus neutralization test. The vSAU6/SAT2, SAU/6/00 and vSAT2 viruses were tested against two SAT2 type sera that were obtained through infection of cattle with the SAU/6/00 and ZIM/7/83 viruses. The cell suspension was prepared at 3 x 10^5 cells/ml in RPMI medium (5% FCS). RPMI medium containing antibiotics (50 µl) was added to each well, except the first row. The serum (100 µl) was added to the top row and serial dilutions were made across the microtitre plate. Each serum was tested against four dilutions of the different viruses (0.5 $\log_{10}$ apart) and calculated to straddle the $\log_{10}^{2.0}$ $TCID_{50}$ dose. Each virus dilution (50 µl) was added in 3-fold starting at the highest dilution. The plates were incubated for 1 h at 37°C, followed by the addition of 100 µl of the IB-RS-2 cell suspension per well and further incubation for 72 h at 37°C in a CO_2 incubator. The results were read with an inverted microscope (Esterhuysen *et al.*, 1985). A regression line was calculated from the results and the 50% serum end-point titre at the $\log_{10}^{2.0}$ $TCID_{50}$ level established (Esterhuysen *et al.*, 1988). Serum titres were expressed as the logarithm of the reciprocal of the final serum dilution present in the serum/virus mixture at the 50% end-point, as calculated by the method of Kärber (1931).

4.3 RESULTS

4.3.1 Plaque morphology on different cell lines

The virus titres were determined on BHK, IB-RS-2 and CHO cells and the results are presented in Table 4.1. These titres are indicative of the ability of the viruses to produce plaques on these cells and should not be taken as an indication of their growth kinetics.

Virus titres observed were the highest on IB-RS-2 cells, followed by slightly lower titres on BHK cells. In BHK cells, the chimera vSAU6/SAT2 and parental virus SAU/6/00 had similar titres of 3.5×10^7 pfu/ml and 1.25×10^7 pfu/ml, respectively. In comparison, the control vSAT2 had the highest titre of 5×10^8 pfu/ml. In IB-RS-2 cells, the parental SAU/6/00 produced a titre of 1.25×10^8 pfu/ml, the chimera vSAU6/SAT2 produced a titre of 5×10^7 pfu/ml, whilst the vSAT2 produced the highest titre of 8×10^8 pfu/ml. vSAT2, derived from a tissue culture-adapted virus, was the only virus that produced plaques on CHO cells, as also seen previously (Van Rensburg *et al.*, 2004).

The plaque morphologies on these different cell lines are indicative of the ability of the capsids of the different viruses to interact with the cells (Fig. 4.1). On BHK cells, both the chimera and parental virus formed small turbid plaques, whereas the control, vSAT2, formed larger clear plaques. On IB-RS-2 cells, the plaques were not as distinctly different. The vSAT2 virus formed smaller clear plaques on the CHO cells and is characteristic of viruses that use heparan sulfate proteoglycans as receptors. The vSAU6/SAT2 and SAU/6/00 viruses were unable to produce plaques on CHO cells, which is indicative of these viruses not being able to utilize heparan sulfate as cellular receptor. The lack of plaques on CHO cells thus indicates that the parental and chimeric viruses containing the SAU/6/00 external capsid, only use intergrin as a cellular receptor.

Both viruses with the external capsid of SAU/6/00 displayed plaque morphology on BHK cells and an inability to form plaques on CHO cells. This is similar to what has been reported for virus recovered from bovine, which had been inoculated with a high dose of virus with mutations in the capsid to remove the ability to bind heparan sulfate (vCRM8) (Sa-Carvalho *et al.*, 1997). By contrast, the vSAT2 control produced clear plaques on BHK and small, clear plaques on CHO cells, also seen for the heparan-binding and tissue culture-adapted virus (vCRM4) (Sa-Carvalho *et al.*, 1997). Therefore, the chimera has the same plaque morphology on BHK and IB-RS-2 cells and a similar inability to replicate in CHO cells as the parental virus.

Table 4.1. Viral titres (pfu/ml) determined for vSAU6/SAT2, SAU/6/00 and vSAT2 on BHK, IB-RS-2 and CHO cells

	vSAU6/SAT2	SAU/6/00	vSAT2
BHK	3.5×10^7	1.25×10^7	5×10^8
IB-RS-2	5×10^7	1.25×10^8	8×10^8
CHO *	-	-	2×10^6

* (-) No titre could be determined

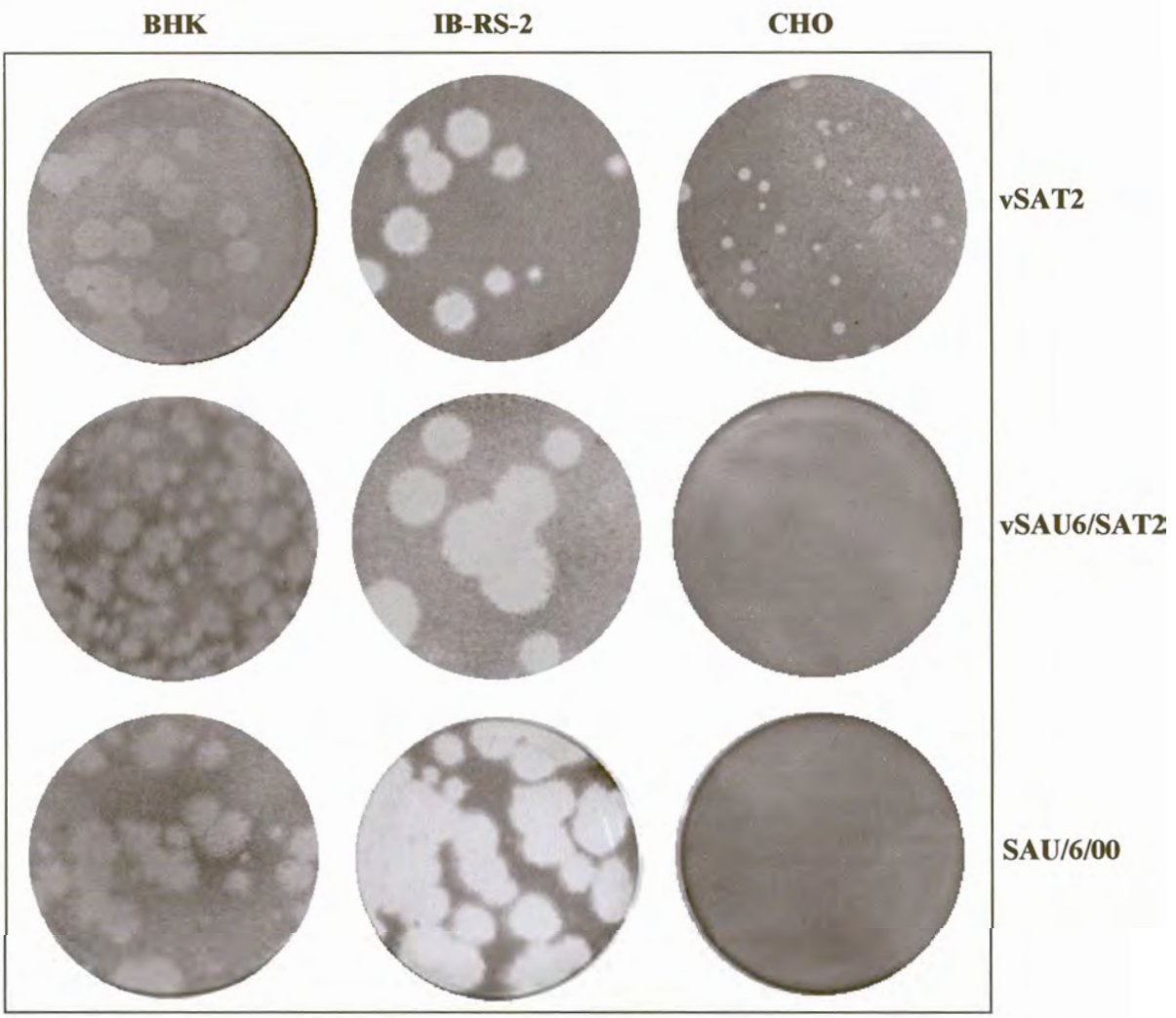


Fig. 4.1. Plaque morphology of the vSAU6/SAT2, SAU/6/00 and vSAT2 viruses on BHK, IB-RS-2 and CHO cells.

4.3.2 Growth kinetics in BHK cells

Single-step growth studies in BHK cells indicated differences for vSAU6/SAT2 and SAU/6/00 compared to vSAT2 (Fig. 4.2). The chimera vSAU6/SAT2 showed increased replication capacity in BHK cells upon comparison with the parental SAU/6/00. Viral replication between 4 and 6 hours was initially high, wherafter it continued at a lower rate after 6 h post-infection. At 10 h post-infection, vSAU6/SAT2 (1.85×10^4 pfu/ml) had a titre 3-fold higher than SAU/6/00 (6.75×10^3 pfu/ml). For the chimera, a peak infectivity titre of 5×10^4 pfu/ml was observed at 26 h in comparison to 2.25×10^4 pfu/ml observed for the parental virus.

The peak infectivity titre for vSAT2 (2×10^5 pfu/ml) was observed at 10 h post-infection. This was 10-fold higher than for vSAU6/SAT2 (1.85×10^4 pfu/ml) and 30-fold higher than for SAU/6/00 (6.75×10^3 pfu/ml). The titre for vSAT2 declined with an increase in the incubation period (after 10 h). This could be due to an inactivating effect of the incubation temperature on the virus (Sarma, 1985). Moreover, Doel and Collen (1984) also suggested that viruses should be harvested shortly after the highest infectivity is reached to prevent the effect of proteolytic enzymes released during virus culture.

4.3.3 Antigen stability

4.3.3.1 Indirect sandwich ELISA

The indirect sandwich ELISA was used to measure the presence of intact virus particles following heat treatment at 42°C and 56°C. This ELISA test does not allow for the determination of a titre and therefore the OD value is the only way to compare virus quantity.

The absorbency measured for the vSAU6/SAT2, SAU/6/00 and vSAT2 viruses at different time intervals of heat treatment at 42°C indicated that a distinct differentiation could not be made (Fig. 4.3a). If an absorbency of 1 OD at 3 h is taken as a constant, the corresponding dilution factor for vSAU6/SAT2 was 1/80 compared to 1/36 for SAU/6/00 and 1/16 for vSAT2, respectively. This could indicate that at OD=1 there was a higher antigen concentration for the chimera than for the parental virus. Similar results were obtained for the ELISA performed on

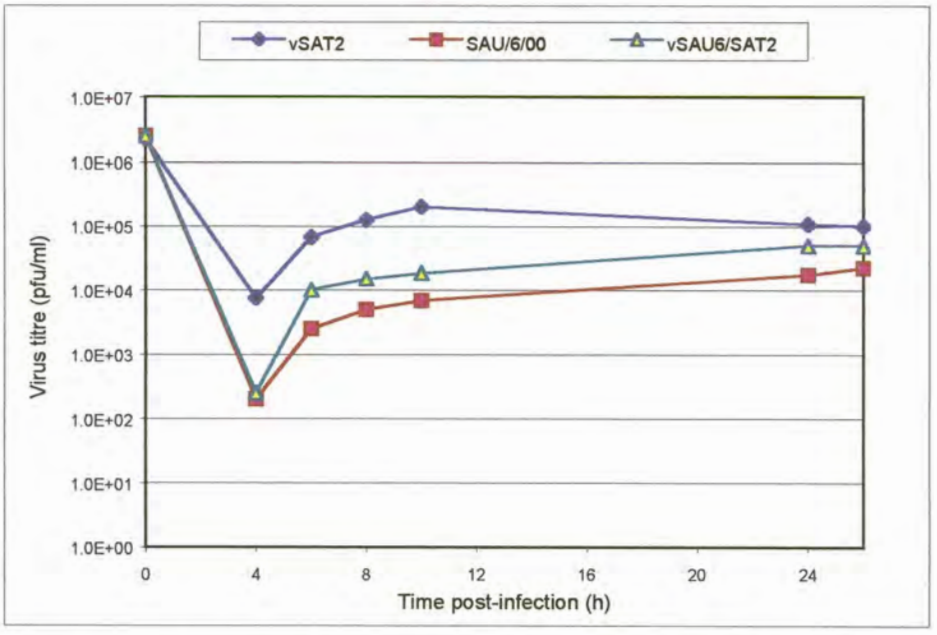


Fig. 4.2. Single-step growth kinetics of vSAU6/SAT2, SAU/6/00 and vSAT2 viruses in BHK cells.

samples treated at 56°C. However, OD values at different time intervals could not clearly be distinguished (Fig. 4.3b). At an absorbency of 1 OD, the corresponding dilution factor was similar for vSAU6/SAT2 (1/68) compared to 1/64 for SAU/6/00 and 1/60 for vSAT2, respectively. The antibodies included in the ELISA were raised against a crude virus extract of ZIM/7/83 and would therefore not be reactive only with complete 146S particles, but also detect subunits such as 75S empty capsids and 12S protein present in cytoplasmic extracts from infected cells (Grubman *et al.*, 1985). The 12S particles detected could also have resulted from the disruption of the 146S component by heat treatment at temperatures above 50°C (Brown and Cartwright, 1961).

4.3.3.2 Plaque assays in BHK cells

Following heat treatment at 42°C and 56°C, viral titres of the vSAU6/SAT2, SAU/6/00 and vSAT2 viruses were determined in BHK cells. Incubation of the viruses at 42°C (Fig. 4.4a) indicated the presence of viable virus for all three viruses used in this study at 6 h post-infection, and the control after 24 h. The vSAU6/SAT2 chimera produced a 5-fold higher titre (9.25×10^4 pfu/ml) after 3 h in comparison to 2×10^4 pfu/ml for the parental SAU/6/00. Viable virus could be detected following 6 h of heat treatment with titres of 4×10^4 pfu/ml and 5.8×10^3 pfu/ml for the chimera and parental virus, respectively. No titres could be detected for vSAU6/SAT2 and SAU/6/00 at 6 to 24 h. The control vSAT2 virus was more stable. This virus had the highest titres on BHK cells at 6 h (3.8×10^5 pfu/ml) and viable virus was still detected after 24 h at 42°C.

Heat treatment of the viruses at an elevated temperature of 56°C (Fig. 4.4b) indicated that the chimera produced a titre 6-fold higher (1.5×10^2 pfu/ml) after 30 min than the parental virus (2.5×10^1 pfu/ml). The two viruses, containing the external-capsid coding region of SAU/6/00, were still viable after treatment at 56°C for 1 h, but not after 3 h. By contrast, the vSAT2 control produced higher titres of 2.5×10^4 pfu/ml after 30 min of inactivation. Viable virus could be detected following incubation at 56°C for 3 h. It is evident from the aforementioned, that comparison of the heat stability of the chimera vSAU6/SAT2 and parental SAU/6/00 virus at 42°C and 56°C was complicated by the difference in input concentrations of these viruses that were used.

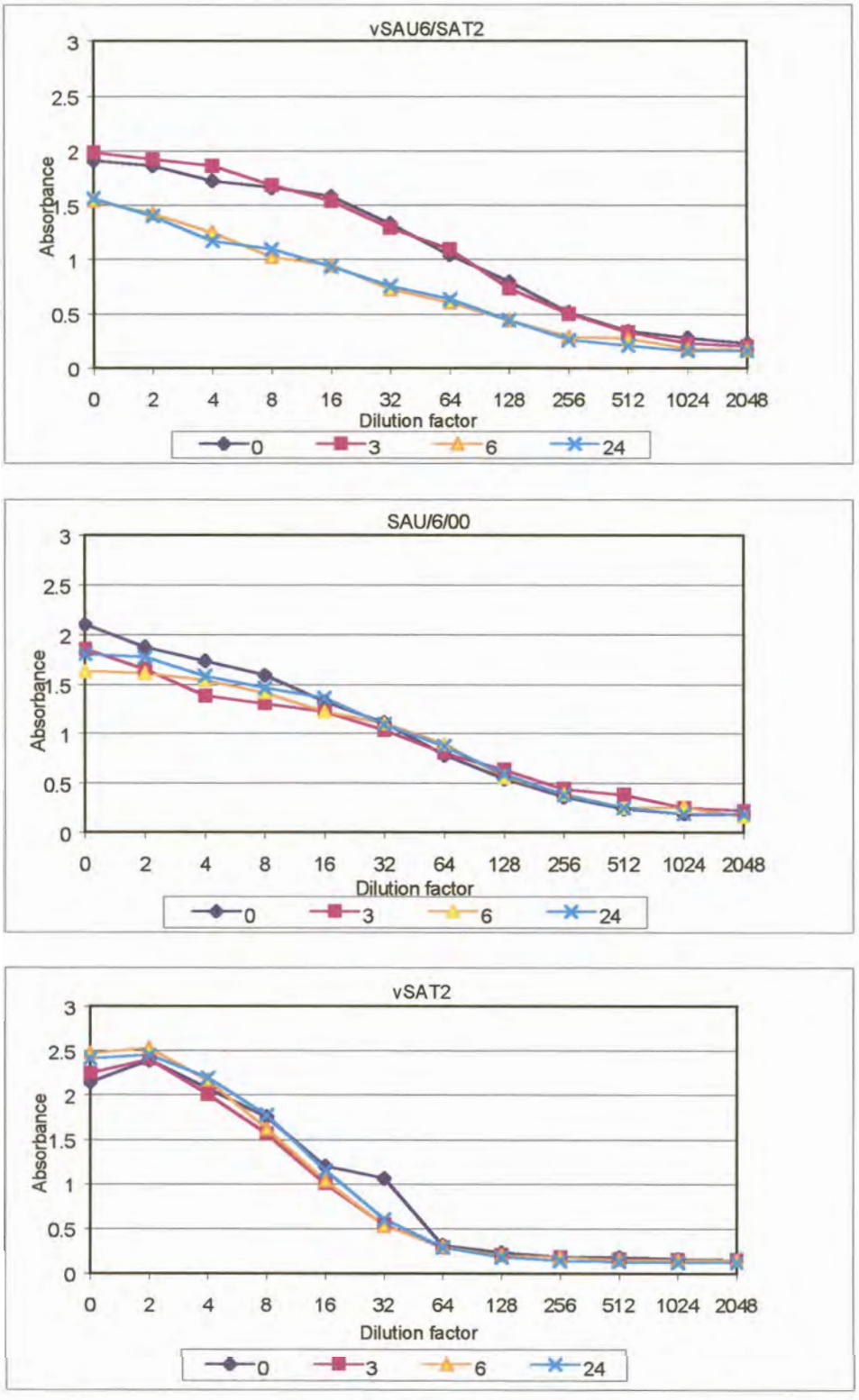


Fig. 4.3a. Antibody reactivity with vSAU6/SAT2, SAU/6/00 and vSAT2 viruses detected with the indirect sandwich ELISA test following heat treatment at 42°C for 3, 6 and 24 h.

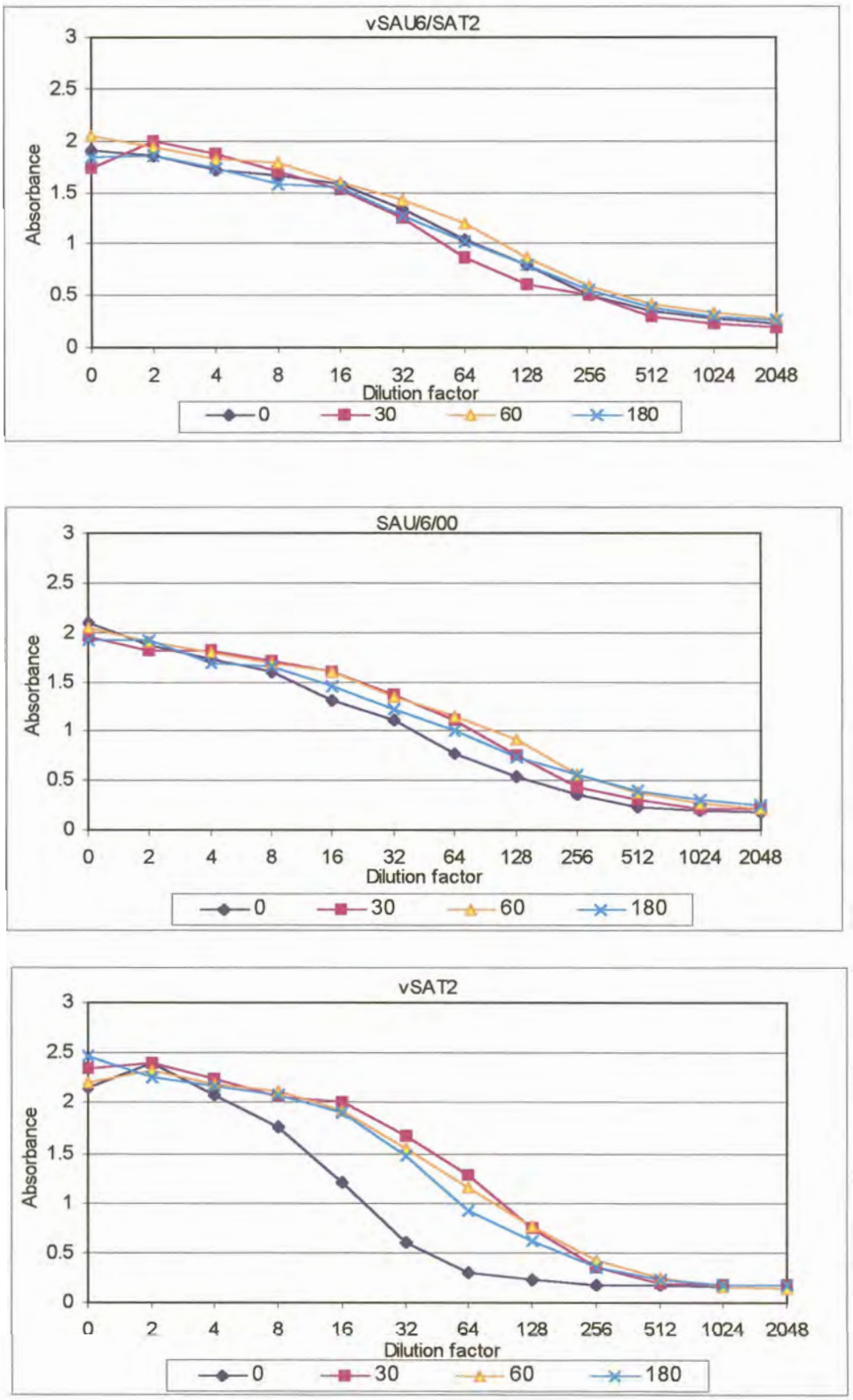


Fig. 4.3b. Antibody reactivity with vSAU6/SAT2, SAU/6/00 and vSAT2 viruses detected with the indirect sandwich ELISA test following heat treatment at 56°C for 30, 60 and 180 min.

4.3.3.3 Stability of 146S particles

No 146S particles could be detected for the majority of the samples and very low concentrations ($\leq$ 0.5 µg/ml) were determined for a few samples (results not shown), suggesting that the scale on which the experiment was performed was too small.

4.3.4 Antigenic profile

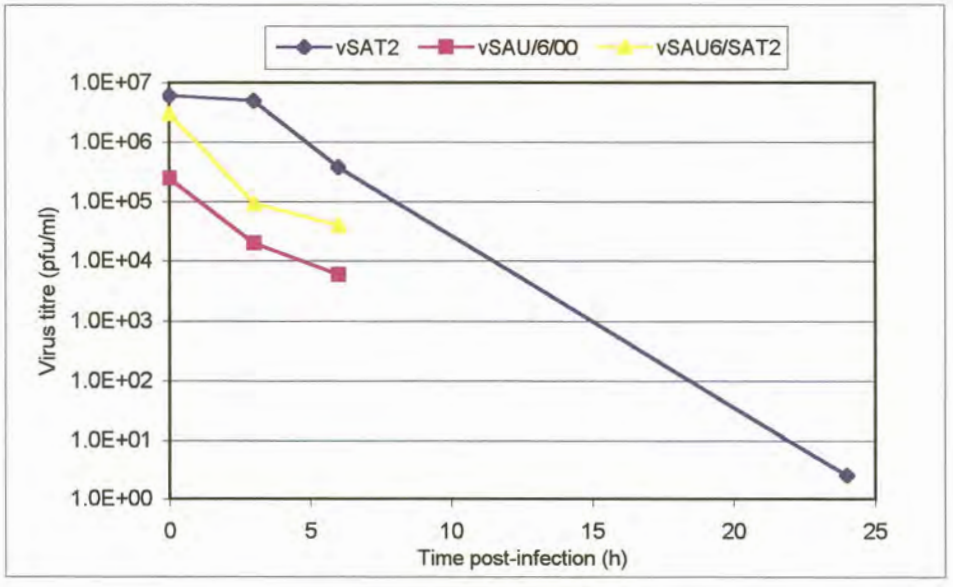
The antigenic profile obtained for the viruses indicated that the chimera vSAU6/SAT2 and parental SAU/6/00 reacted different to the vSAT2 control (Fig. 4.5). Both viruses produced higher titres against the SAU/6/00 serum than the ZIM/7/83 serum, which is genetically more distantly related (Chapter 2). By contrast, the vSAT2 control had a different profile and resulted in a less positive reaction for the SAU/6/00 serum than the ZIM/7/83 homologous serum.

4.4 DISCUSSION

In this study, a genetically engineered SAT2 type virus containing the external capsid-coding region of SAT2/SAU/6/00 was characterized with regards to properties important in FMD vaccinology. This is the first report of the engineering of a chimera between two different SAT2 viruses. Recombinant DNA technology has proven useful for genetically engineered European type viruses (Rieder *et al.*, 1994; Beard *et al.*, 1999) and for the construction of chimeric viruses to produce attenuated FMDV vaccines (Almeida *et al.* 1998; Chinsangaram *et al.*, 1998). Following the slower growth rate of a chimera containing the external capsid of a SAT2 strain in the A_{12} full-length cDNA clone (Van Rensburg and Mason, 2002), a SAT2 full-length infectious clone was constructed and evaluated (Van Rensburg *et al.*, 2004). This reverse genetics technology was used to construct the SAT2 type genetically engineered virus, vSAU6/SAT2, and is the first study of a chimeric SAT2 type foot-and-mouth disease virus.

The vaccine strain virus SAT2/SAU/6/00 used in this study (passage history BTY_1RS_2) had to be adapted to BHK cells; the tissue culture cell line of choice for experiments as vaccine antigen is prepared in BHK suspension cells. The plaque phenotype of the chimera was similar to that of

a



b

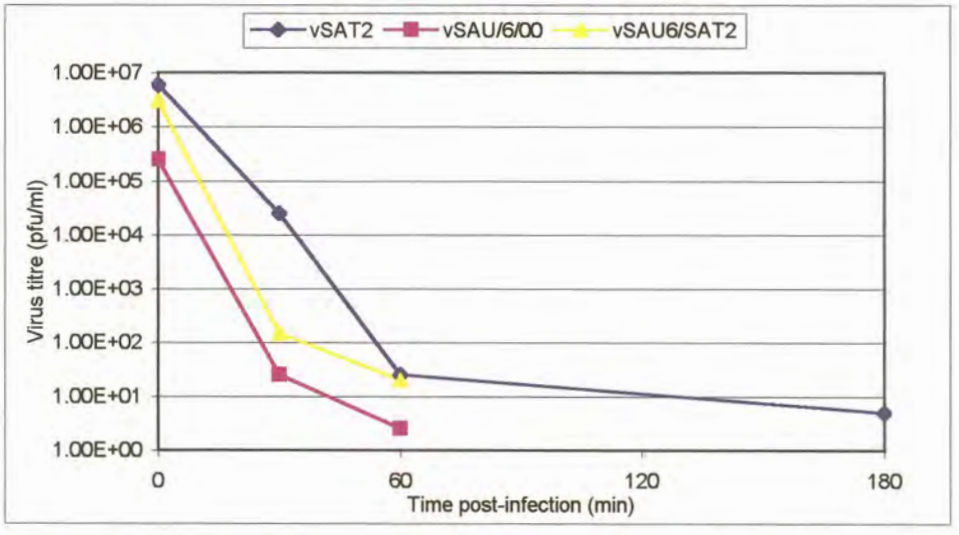


Fig. 4.4. Virus titres in BHK monolayers following heat treatment at (a) 42°C and (b) 56°C.

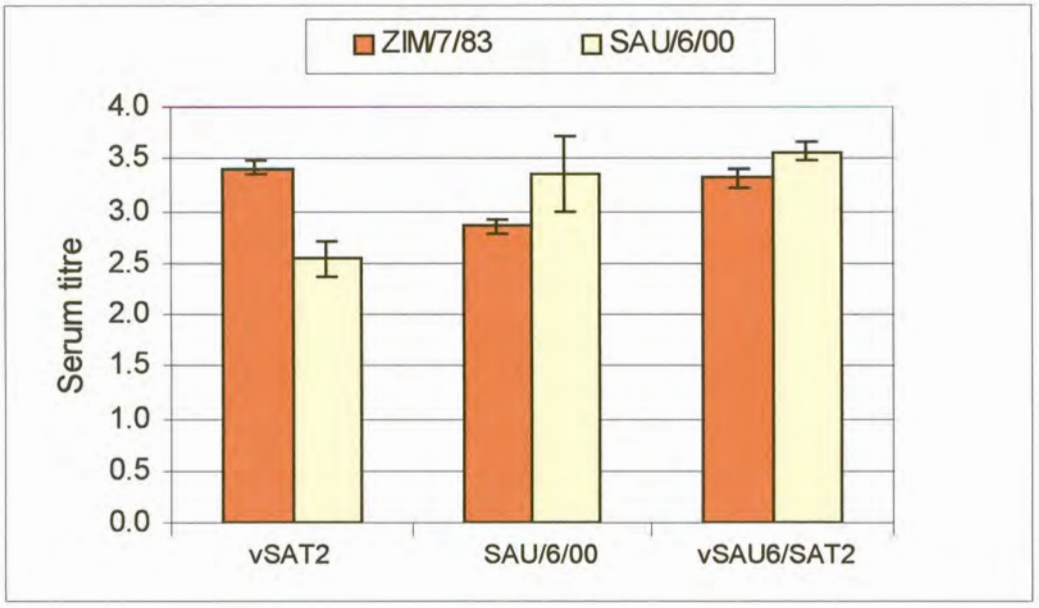


Fig. 4.5. Antigenic profiles of vSAT2, SAU/6/00 and vSAU6/SAT2 viruses tested against bovine-raised SAT2 sera in the virus neutralization test.

the parental virus, indicating that the external capsid region for both viruses conferred the ability to replicate in cells and influenced plaque morphology (Sa-Carvalho *et al.*, 1997). Integrin receptors present in BHK cells are mostly utilized by FMD viruses for internalization through the integrin-binding motif (RGD) (Berinstein *et al.*, 1995; Jackson *et al.*, 2000). Both the chimera and parental virus use integrins, which are believed to be the cellular receptors used to cause natural infection (McKennna *et al.*, 1995). The vSAT2 control is a tissue culture-adapted virus derived from the ZIM/7/83 vaccine strain (Van Rensburg *et al.*, 2004), capable of binding integrin and heparan sulfate proteoglycans. Such tissue culture-adapted viruses have been observed to produce CPE at a faster rate in cell culture, with increased virulence and the capability to infect an expanded range of cultured cells (Jackson *et al.*, 2003). Although large plaque variants have been considered characteristic of the parental virus producing disease in animals, plaque size could not be considered a measure of the suitability as vaccine antigen (Preston *et al.*, 1981). The similar plaque morphology observed for the chimera and parental viruses confirmed that the characteristics of SAU/6/00 are present in vSAU6/SAT2.

The vSAU6/SAT2 chimera showed improved growth properties compared to the parental SAU/6/00 vaccine strain. The chimera and parental virus titres indicated that the SAT2 non-structural proteins of ZIM/7/83 could functionally replicate the SAU/6/00 external capsid, as also seen for the external capsid-coding region of type O_1 cloned into the A_{12} genome-length cDNA clone (Sa-Carvalho *et al.*, 1997). Although these studies were not performed on large-scale in BHK cells used for vaccine production, the results from the single-step growth curves correlated with suggested peak titres that should ideally be reached within 24 h (Rweyemamu, 1978). Virus variants with the ability to bind heparan sulfate also display enhanced virulence for BHK cells (Baranowski *et al.*, 1998), a possible explanation for the high titres produced by vSAT2, as also seen by Van Rensburg and co-workers (2004). The improved growth dynamics of vSAU6/SAT2 compared to the parental vaccine strain is desirable with regards to the characteristics of a good vaccine strain, *i.e.* rapid growth in tissue culture, as well as high antigen yields.

Investigation of antigen stability at elevated temperatures indicated that the chimera is not distinctly more heat-stable than the parental virus. No conclusive results could be obtained by the ELISA assays, as not only intact capsids were detected. Similar titration curves were also

obtained for an indirect sandwich ELISA test where 146S and 12S particles reacted in a 4:1 ratio (Abu Elzein *et al.*, 1979). No clear correlation could be made between the ELISA results and plaque assays. Plaques observed on BHK cells indicated that at a higher temperature, both vSAU6/SAT2 and SAU/6/00 viruses displayed a more rapid decrease in titres. The conservation of intact virus particles and the RGD cell-binding motif is necessary for cell attachment and entry (Fox *et al.*, 1989; Mason *et al.*, 1994). When heat alters the capsid protein by dissociation of the infective virus particle and release of RNA, initiation of infection of susceptible cells was prevented (Brown and Wild, 1966; Lasta *et al.*, 1992). Interestingly, the SAT type viruses have been described as more heat-labile than the European types (Pay *et al.*, 1978). It should be mentioned that cell lysates were used in these experiments and none of the viruses were treated or inactivated as would be done for vaccine production where formaldehyde is used to stabilize the virus (Anderson *et al.*, 1982). An important property for vaccine production is that the virus is more resistant to disruption by heating, as variation in temperatures in the field results in disruption of the virions (Anderson *et al.*, 1982).

The virus neutralization test (VNT) is an appropriate *in vitro* test for assessing intratypic antigenic variation, since it detects the antigenic determinants that induce protective antibodies. The VNT measures antibodies that confer immunity, as serum from convalescent animals neutralizes the virus. In addition, there is a good correlation between serum neutralization antibody titre (SNT), as determined in the VNT, and protection from challenge (Rweyemamu, 1984; Pay, 1983). In this study, the chimera and parental virus displayed a similar antigenic profile. Although the chimera has not been tested *in vivo*, these results suggest that the necessary antigenic properties of SAU/6/00 are most probably present. A vaccine strain should raise antiserum, which is highly reactive with many virus strains of the same type that are derived from different parts of the world (West *et al.*, 1983). This was evident in the relatively high level of reactivity for SAU/6/00 and vSAU6/SAT2 with ZIM/7/83 serum.

Comparing outbreak strains with existing subtypes is a complicated and time-consuming process and these isolates may not be suitable as vaccine strains with regards to cell adaptation, stability and antigenicity (Kitching, 1992). The production of chimeric virus vaccines will exclude the time-consuming cell culture adaptation-process needed for traditional chemically inactivated

vaccines, which might also result in antigenic variation produced by passage in cell cultures (Gonzáles *et al.*, 1992; Bolwell *et al.*, 1989). With the developments in recombinant DNA technology, existing genome-length clones could be altered relatively rapidly to include the external capsid-coding region of new field isolates and lead to the production of effective good quality vaccines in future.

CHAPTER 5

CONCLUDING REMARKS

This study reports the first successful engineering of a chimera between two SAT2 type viruses and characterization thereof in terms of properties important for FMD vaccine production. The SAT2 Saudi Arabian outbreak strain SAU6/00, isolated in 2000, is genetically distinct from other FMDV vaccine strains currently used in vaccine production. In the absence of an appropriate alternative vaccine strain, the SAU/6/00 antigen is currently being used in vaccine production. However, antigen harvests for vaccine batches are low and variable and the maximum antigen production necessitates an extended harvest time, thereby making it a less ideal vaccine strain.

Towards engineering of the desired SAT2 chimera, the capsid (P1)-coding region containing the antigenic determinants of SAT2/SAU/6/00, was molecularly characterized. The nucleotide sequence obtained strengthened the database for genetic analysis and comparison of SAT type viruses. In addition, data such as intratypic variation between viruses, hypervariable regions on the P1 region and the $3C^{pro}$ cleavage sites were invaluable in assessing whether a viable chimeric virus could be obtained. The greatest nucleotide and amino acid variation in the P1 and 1D regions was between SAU/6/00 and the SAT2 vaccine strain ZIM/7/83. Characteristically, the SAT2 type virus capsid-coding region displayed great variability on the nucleotide and amino acid level.

The sequencing data generated was used in the construction of the recombinant plasmid pSAU6/SAT2. Previously, the vSAT2/A12 chimera, consisting of the A_{12} genome-length cDNA clone, containing the external capsid-coding region (1B-1D) of ZIM/7/83, displayed poorer growth properties than the parental type A_{12} virus in BHK cells (Van Rensburg and Mason, 2002). Consequently, a SAT2 genome-length cDNA clone (pSAT2) was constructed from ZIM/7/83 and displayed good growth properties in BHK cells (Van Rensburg *et al.*, 2004). Utilizing this reverse genetics technology, the external capsid-coding region of SAU/6/00 was

cloned into pSAT2, of which the corresponding region was removed. *In vitro*-synthesized RNA was used to transfect BHK cells and a viable vSAU6/SAT2 chimera was recovered and passaged to high titres; thus indicating that the ability to replicate in BHK cells was conferred to the chimera. The successful recovery of viable vSAU6/SAT2 furthermore indicated that the recombinant pSAU6/SAT2 genome-length cDNA clone contained an intact reading frame and the complete genetic information essential for virus replication. Despite differences on the amino acid level between the pSAT2 construct and the cloned external capsid-coding region of SAU/6/00, the proteases could functionally cleave the viral proteins.

Compared to the parental SAU/6/00, the chimera vSAU6/SAT2 showed improved growth properties in BHK cells. Such improved propagation in tissue culture is desirable for a good vaccine strain. However, this was only investigated on an experimental scale and should in future be compared to large-scale commercial vaccine production. The plaque morphology of vSAU6/SAT2 and SAU/6/00 was comparable on several cell lines. This similar phenotype of vSAU6/SAT2 was conferred by the external capsid-coding region of SAU/6/00. Temperature stability of the chimera could not be assessed successfully, as the antigen quantity was insufficient for 146S determination and no conclusive results could be obtained by other techniques. Testing of the antigenicity of the chimera with VNT indicated a similar antigenic profile to that of the parental virus. However, the exact epitopes of SAU/6/00 would have to be determined empirically.

The results obtained in this investigation indicate that a vSAU6/SAT2 chimera, containing the antigenic determinants of the vaccine strain SAU/6/00 cloned into the pSAT2 genome-length cDNA clone, was successfully recovered and showed improved growth properties in BHK cells, as well as a similar antigenic profile to the parental SAU/6/00. To ascertain its true potential in vaccine production, vSAU6/SAT2 would have to be evaluated further for stability, large-scale tissue culture propagation, duration of immunity and for immunogenicity against a range of strains present in the field.