DEVELOPMENT OF RECOMBINANT VACCINES AGAINST FOOT-AND-MOUTH DISEASE

By

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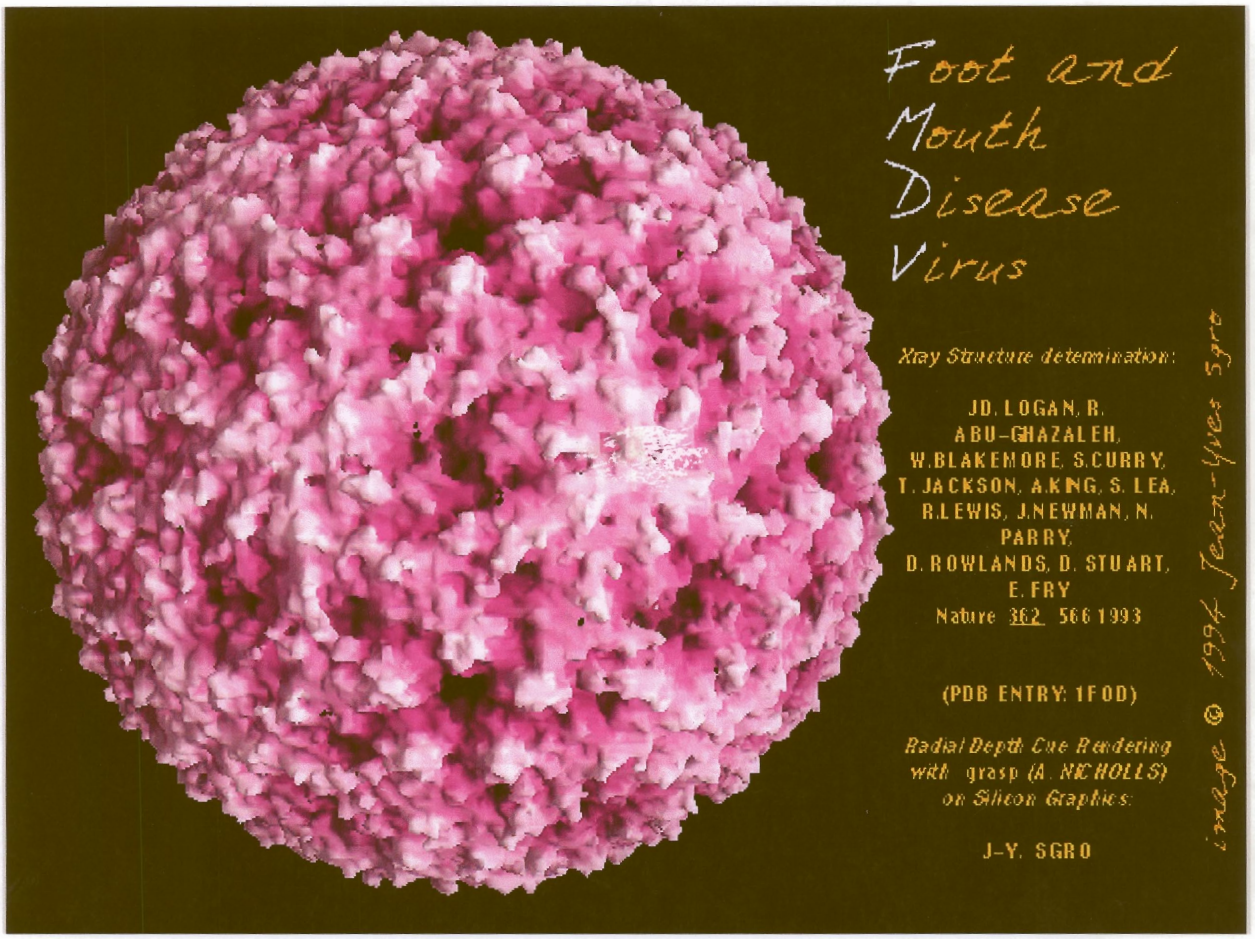

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"In the temple of science are many mansions ... and various indeed are they that dwell therein and the motives that led them there.

"Many take to science out of a joyful sense of superior intellectual power; science is there own special sport to which they look for vivid experience and the satisfaction of ambition;
many others are to be found in the temple who have offered the products of their brains on this altar for purely utilitarian purposes.

Were an angel of the Lord to come and drive all the people belonging to these two categories out of the temple, it would be noticeably emptier but there would still be some men of both present and past times left inside ... If the types we have just expelled were the only types there were, the temple would never have existed any more than one can have a wood consisting of nothing but creepers ...

... and those who have found favor with the angel ... are somewhat odd, uncommunicative, solitary fellows, really less like each other than the hosts of the rejected.

"What has brought them to the temple ... no single answer will cover ... escape from everyday life, with its painful crudity and hopeless dreariness, from the fetters of one's shifting desires. A finely tempered nature longs to escape from this noisy cramped surroundings into the silence of the high mountains where the eye ranges freely through the pure still air and fondly traces out the restful contours apparently built for eternity."

This is an excerpt from a speech given in 1918 by a young German scientist called Albert Einstein.

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ABBREVIATIONS

BEA – bromoethylamine hydrobromide

BGH – bovine growth hormone

BHK – baby hamster kidney

CMV – cytomegalovirus

CTE – C-terminal extention

DEAED – diethylaminoethyldextran

EIF – eukaryotic initiation factor

ELISA – enzyme linked immunosorbent assay

FLC – full-length clone

FMDV – foot-and-mouth disease virus

IB-RS-2 – Instituto Biologico Rim Suino

IRES – internal ribosome entry site

KNP – Kruger National Park

MEGA – molecular evolutionary genetics analysis

MOI – multiplicity of infection

NCR – non coding region

NTP – nucleotide phosphate

OIE – Office des Epizooties

ORF – open reading frame

PBS – phosphate buffered saline

PCR – polymerase chain reaction

PK – pig kidney

PTB – polypyrimidine tract-binding

RPMI – Roswell Park Memorial Institute

RT-PCR – reverse-transcribed polymerase chain reaction

SAT – South African Territories

TCID – tissue culture infectious dose

UTR – untranslated region

VNT – virus neutralization test

VP – viral protein

VPg – viral genome-linked protein

SUMMARY

The South African Territories (SAT) types of foot-and-mouth disease virus (FMDV) show marked genomic and antigenic variation throughout sub-Saharan Africa. This variation is to a large extent geographically linked and requires therefore the use of custom-made vaccines. Adaptation of field isolates as vaccine strains is cumbersome, time consuming and expensive. A possible means of circumventing the adaptation process is to construct recombinant or chimeric FMD viruses, followed by the production of conventional, inactivated vaccine utilizing these viruses. The advantage of such a strategy would be the ability to manipulate the antigenicity of these viruses by substituting the antigenic coding regions (i.e. structural proteins) of a full-length cDNA clone of a suitable strain.

Towards this objective the structural-protein-coding region (P1) of a SAT 2 vaccine strain, ZIM/7/83/2, was determined and compared with two other known SAT 2 P1 regions. Five hypervariable regions were identified of which four are situated in VP1. The cleavage sites for proteolytic processing and especially the regions adjacent to these sites, differ between types A and SAT 2. The genetic heterogeneity of two FMDV proteinases, the Leader and 3C, of representatives of six different serotypes, was subsequently investigated. The results revealed these genomic regions of the SAT viruses originating from southern Africa to be distinct from types A, O and C. Interestingly, it was also seen that the Leader and 3C proteinases of the SAT types are less variable than their European counterparts. These results were in contrast to that obtained for the structural proteins, which showed the SAT 2 P1 region to be at least 2-3 times more variable than that of types A, O and C. Despite the observed differences in the proteinases, a three-dimensional structural model for the Lb form of the ZIM/7/83/2 Leader proteinase predicted the three-dimensional fold of the enzyme to be conserved.

A chimeric cDNA clone between types A and SAT 2 was constructed by inserting the external capsid-coding region of ZIM/7/83/2 into the genetic backbone of the A_{12}

cDNA clone. The subsequent evaluation of the resulting recombinant FMD virus indicated the virus to be immunogenically identical to the wild type ZIM/7/83/2. However, the recombinant virus was found to be a slower antigen producer and less stable than the wild type SAT 2. These characteristics make the recombinant FMD virus constructed in this study unsuitable for conventional vaccine production. Alternative means, such as the use of a SAT 2 cDNA clone, should be investigated.

CHAPTER 1

The past, present and future of foot-and-mouth disease vaccinology: A Literature Review

1.1 General introduction

Foot-and mouth disease (FMD) is a highly contagious acute viral infection, affecting all cloven-hoofed animals. Its host range in domestic animals include cattle, sheep, goats and pigs, while a number of wild life species such as antelope, both water and African buffalo, camels, llama and giraffe are also susceptible. Although mortality rates are generally low (less than 5%), FMD has a major economic impact on the livestock industry. The direct losses are mainly due to reduced production of meat, milk and other animal products during an outbreak. Spread of the disease is usually rapid with a short incubation period of ± 2 to 8 days (Thomson, 1994).

Transmission of the disease is either direct or indirect. The most general route is direct contact between infected animals that excrete the virus, and susceptible animals. FMD virus is present in all secretions and aerosols derived from the respiratory tract during the infectious period. Indirectly, the disease can be transmitted by viral contaminated objects or materials, such as tissues and animal products (e.g. milk and meat), while humans and vehicles have also been implicated in the mechanical transmission thereof (Sellers, 1971). Although airborne transmission occurs very rarely and is very much dependent on climate, several outbreaks occurring by this means have been reported in northern Europe (Donaldson, 1979; Fogedby *et al.*, 1960; Gloster *et al.*, 1982).

The FMD virus can persistently infect ruminants for periods ranging from a few weeks to years. In such cases, virus can be isolated from the oesophagus and throat fluids by probang extractions (van Bekkum *et al.*, 1959). In cattle, this carrier status has been shown to be responsible for outbreaks of the disease up to two years post infection

(Thomson, 1996). The African buffalo (*Syncerus caffer*), known to maintain the virus for five years in an individual animal and at least 24 years in an isolated herd (Condy *et al.*, 1985), can transmit the disease to cattle (Hedger & Condy, 1985; Dawe *et al.*, 1994; Vosloo *et al.*, 1996). The mechanism of transmission is however not clearly understood, but recent reports indicate that sexual transmission of the disease between buffalo and cattle is a possibility (Bastos *et al.*, 1999).

Early clinical signs include fever, dullness and a fall in milk yield. These signs are however rapid in onset and are followed by the development of lesions in the mouth and on the feet (Figure 1.1). The lesions result in lameness and discomfort in the mouth such as grinding of the teeth. Young domestic animals infected with FMD virus, may die suddenly due to myocarditis (Thomson, 1994).

Initially, control of FMD involves containment of the disease, although the ultimate aim should be the total eradication thereof. A systematic approach in endemic areas usually starts with mass vaccination, followed by the control of sporadic outbreaks. The status of "freedom of disease with vaccination" and consequently the status of "freedom of disease without vaccination" is then awarded to free areas by the Office des Epizooties (OIE). The International Animal Health Code of the OIE defines each of these stages which also entails specific international trading implications for live animals and animal products. Although a very important aspect in the control of FMD, vaccination is only one of several zoo-sanitary measures essential for successful control. Other measures include the control of animal movement, prohibition of importation of susceptible animals and animal products from high risk areas, appropriate diagnostic testing of susceptible animals prior to importation and the application of quarantine measures as well as the slaughtering of infected and susceptible, in-contact animals, depending on the overall epizootiological situation. The authority responsible for effective control should also have access to specific fields of expertise. These include epidemiology, animal health legislation, risk analysis, logistics, training and education (Garland, 1999).

B

A

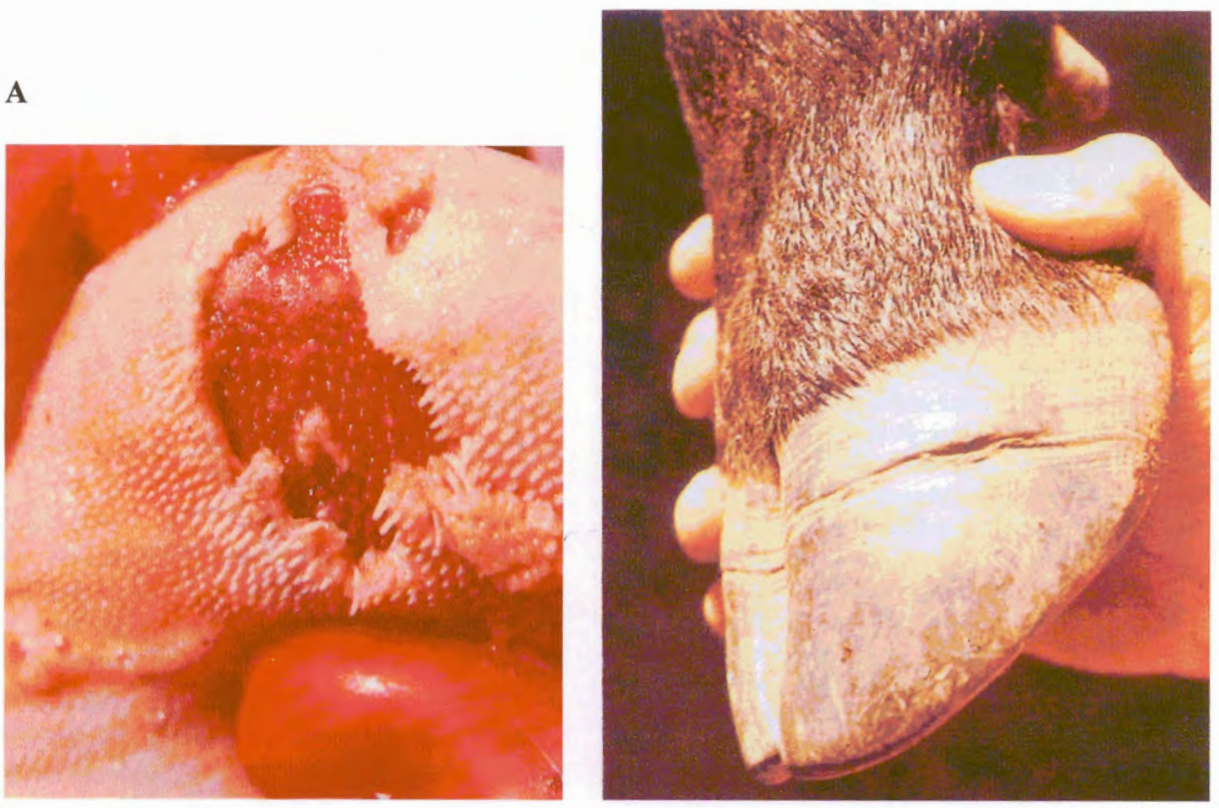

Figure 1.1: Examples of foot-and-mouth disease lesions. A: Ruptured vesicle on the tongue of an ox. B: A faulty hoof of an ox that has recovered from foot-and-mouth disease (Taken from Thomson, 1994).

An integral part of control is the rapid and accurate diagnosis of FMD. Due to the highly infectious nature of the virus, handling thereof as well as diagnosis is restricted to high-security laboratories. Diagnosis involves clinical evaluation, identification of virus and viral antigens during early stages of infection and serology in cases where the infection is lasting longer than two weeks (Thomson, 1994).

Positive viral identification entails viral isolation from clinical specimens such as lesion material and probang extractions on primary pig kidney cells (PK) as well as the detection of viral RNA through the polymerase chain reaction (PCR). As it is a relatively new introduction, the use of PCR is however restricted to only a limited number of laboratories. Enzyme linked immunosorbent assays (ELISAs) and the virus neutralization tests (VNT) are used for antigen and antibody identification and assay (Thomson, 1994; Garland, 1999).

1.2 A historical perspective of foot-and-mouth disease outbreaks

The first descriptions of foot-and-mouth disease were given by Fracastorius in 1546. He described a "contagious affection" which occurred in cattle in 1514 in what is now known as Italy that was characterized by "a certain rawness and small pustules in the mouth" (Bulloch, 1927). Frequent occurrence of the disease was reported during the 17^{th} and 18^{th} centuries in France, Germany and Italy, but was recorded in the United Kingdom for the first time only in 1839. FMD appeared for the first time in South America in Argentina in 1871 (Brown, 1986), probably introduced from Europe. During the first half of the 20^{th} century, pandemic outbreaks of the disease raged through Europe. Through extensive vaccination programs, FMD was gradually controlled and later eradicated from western Europe (Brown, 1992). In southern Africa the first reports on FMD date back to 1780 when le Vaillent described a disorder in cattle which "attacked the feet of oxen. In some cases the hoofs dropped off". The first official report was obtained in 1892, although older inhabitants of the region described a disease which resembles FMD long before the official recordings were made. Due to the rinderpest outbreak of 1896, FMD "disappeared" until 1931 when an outbreak re-occurred in Zimbabwe (Thomson, 1994).

Although prophylactic vaccination has been stopped in 1991 in the European Union, its disease-free status is often threatened. Examples of outbreaks include Italy (1993), Greece (1994, 1996 & 2000[1]) and east European countries such as Bulgaria (1991, 1993 & 1996) and Turkish Thrace (1995 & 1996). In 1997 the only outbreaks reported were in Armenia and Georgia (Kitching, 1998; Kitching 1999; Leforban, 1999). Several reports involving type O outbreaks in Georgia and Kazakhstan were however, received from May to July 2000[1].

FMD in the Middle East, Central and east Asia is wide-spread. Outbreaks occur frequently in dairy herds in Saudi Arabia, probably introduced from Asia and Africa. Just recently, an outbreak of SAT 2 has been confirmed in these dairy herds[1]. This is the first incidence of SAT 2 outside the continent of Africa as far as could be determined. The epizootic subsequently spread to Kuwait as well. In countries such as India, parts of China, Thailand and Cambodia FMD is endemic. Control is complicated in certain countries through unstable political situations, lack of supporting legislation and religious considerations (Kitching, 1998; Kitching, 1999).

A very serious outbreak of type O occurred in Taiwan in 1997. The pig-adapted strain swept through the entire island within four months (Dunn *et al.*, 1997). Taiwan had been free of foot-and-mouth disease since 1929. It was subsequently controlled through extensive vaccination and slaughtering. The total economic loss in Taiwan is estimated at approximately US$ 380 million (Yang *et al.*, 1999). Following the disastrous 1997 outbreak in Taiwan, the countries of the Far East reviewed their national management policies regarding surveillance, diagnostic vaccine stocks, legislation etc. to be better prepared for future emergencies (Yagasaki, 1999). Despite these actions, South Korea has had numerous outbreaks of the disease during 1999 and 2000, while Japan reported two incidents of FMD recently – the first in 92 years[1].

Due to the significant improvement in the control program in South America, Uruguay was declared free of disease in 1995, while Argentina, Paraguay and the southern states of Brazil are free with vaccination. Recent reports of FMD outbreaks have, however,

[1] See Disease Information Webpage of the OIE (http://www.oie.int/Info/a_dsum.htm)

been received from these free-zone areas in Paraguay, Argentina and Brazil[2]. Outbreaks were also reported in Bolivia, Colombia and Ecuador. Despite these recent outbreaks, the situation has vastly improved in South America (Kitching, 1998; Kitching 1999).

An outbreak of type O swept through northern Africa during 1989 and 1990, starting in Tunisia and spreading to Algeria and Morocco (Samuel *et al.*, 1999). Currenly, Morocco is free of the disease. As serotype O is endemic to Egypt and Libya, outbreaks in the region and neighboring countries occur frequently. FMD is endemic in sub-Saharan Africa, with the exception of Madagascar. Countries such as Ivory Coast, Mali, Rwanda, Senegal, Tanzania, Uganda, Malawi and Kenya[2] reported several outbreaks in recent years. This is however, not a true reflection of the epidemiology of the disease. Poor surveillance and diagnostic facilities are possibly responsible for the poor reporting of the disease in the region (Kitching, 1998; Kitching, 1999).

In southern African countries, the disease is mainly restricted to game parks where it persists in African buffalo populations. Occasionally, it spreads from wildlife to domestic cattle populations. Such an outbreak of SAT 2 occurred in 1997 in Zimbabwe where impala or kudu jumping fences, were implicated as intermediaries in transmission between buffalo and cattle (Thomson, 1999). The role of antelope in the transmission from buffalo to cattle was again reported with the independent outbreaks of SAT 1 and SAT 3 in Zimbabwe in 1999[2]. Buffalo were also involved in the 2000 outbreak of SAT 1 in cattle on an experimental farm in the controlled area of South Africa (A. D. S Bastos, personal communication). This was the first outbreak of disease in domestic animals since 1983 in the country. A much more threatening outbreak of FMD was reported during September 2000 in the disease-free zone of South Africa[2]. This type O virus, previously exotic to South Africa, forms part of the Pan Asian topotype, which has caused numerous outbreaks in the nineties throughout the Middle East, Asia and the Far East[2] (O. Sangare, personal communication). This outbreak emphasized the need for stricter movement control of animals and animal products world-wide.

[2] See Disease Information Webpage of the OIE (http://www.oie.int/Info/a_dsum.htm)

1.3 Foot-and-mouth disease virus properties

1.3.1 Classification

The causative agent of FMD is the foot-and-mouth disease virus (FMDV) which belongs to the family *Picornaviridae* (Ruekert, 1996). This family of positive strand RNA viruses contains nine genera, namely *Entero-*, *Rhino-*, *Cardio-*, *Aphtho-*, *Hepato-*, *Parecho-*, *Erbo-*, *Kobu-* and *Teschoviruses* (Stanway *et al.*, 2000). Together with equine rhinitisvirus A (Li *et al.*, 1996), FMDV make up the genus *Aphthovirus*. Identified by Loeffler and Frosch in 1897 as the first filterable agent, FMDV played an important role in virus research. Seven distinct serological types were identified using cross-protection experiments. Types A, O and C were recognized in the 1920's by French and German scientists, while the South African Territory types 1, 2 and 3 as well as Asia-1 were identified at the Animal Virus Research Institute at Pirbright, England, in the late 1940's and early 1950's respectively (Brooksby, 1982).

1.3.2 Physical properties

A well-known characteristic of FMDV is its lability below pH6. The acid instability varies however with strains, isolates and passage history (Domingo *et al.*, 1990). This characteristic of *aphthoviruses* is shared by the *rhinoviruses*, while the *entero-* and *cardioviruses* can survive pH as low as 3 (Ruekert, 1996). FMD virions have a diameter of 27-28nm, are spherical in shape and exhibit icosahedral symmetry. Seventy percent of the virion consist of protein, 30% RNA and a small amount of lipid (Putnak & Phillips, 1981). The sedimentation constant of virions in sucrose gradients (146S), another well-known characteristic of FMDV, is widely used in vaccine manufacture for the determination of intact virions (Barteling & Vreeswijk, 1991).

1.3.3 Genome organization and viral structure

The FMD viral genome is a positive sense, single stranded RNA genome approximately 8 500 nucleotides in length. A schematic representation indicating the characteristics of the genome as well as the encoded proteins, is shown in Figure 1.2.

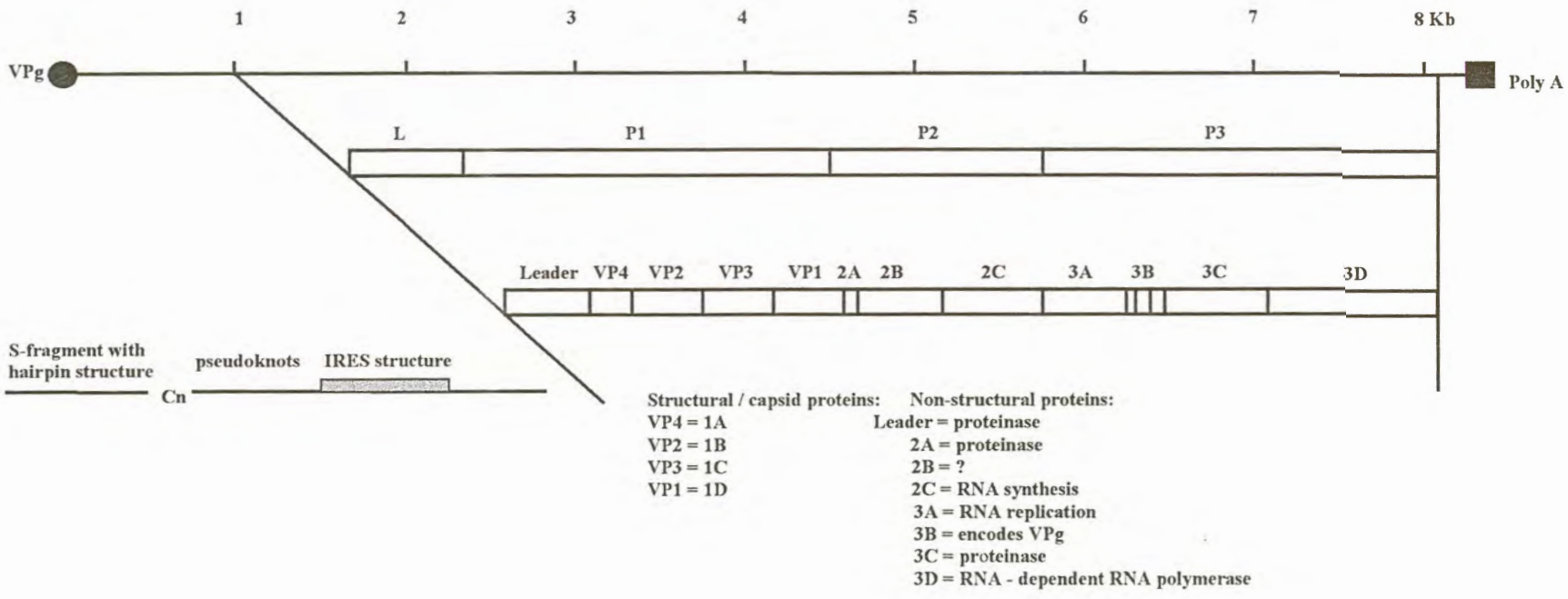

Figure 1.2: Schematic representation of the foot-and-mouth disease virus genome. The VPg-linked RNA strand, the four polyprotein precursors (L, P1, P2, P3), the mature viral proteins as well as the location of the 5' UTR secondary structures are indicated.

Covalently linked to the 5' untranslated region (5' UTR) of the genome, is a small virus-encoded protein, VPg (viral genome-linked protein). The gene (3B) encoding VPg is located in the P3 region of the open reading frame (ORF). Although the exact function of the protein is not clear, it has been implicated in RNA synthesis and possibly virion formation (Falk *et al.*, 1992). A poly(C) tract of unknown function is located approximately 150 to 370 bases from the 5' end (Harris *et al.*, 1976; Rowlands *et al.*, 1978). Although initial reports suggested that this region play a role in virulence, it was subsequently shown that even a virus with a poly (C) tract of 2 residues, was virulent in mice (Rieder *et al.*, 1993). The region, 100-420 bases in length (Black *et al.*, 1979; Escarmis *et al.*, 1992), separates the small (S) fragment of the genome from the large fragment (L). No open reading frames exist in the S fragment. Secondary structure predictions suggest the sequence to fold into a hairpin structure (Newton *et al.*, 1985).

The L fragment contains a series of pseudoknots (Clarke *et al.*, 1987a), the internal ribosome entry site (IRES) and an ORF. To the 3' end of the pseudoknots, the highly structured IRES is found. This region contains extreme secondary structures and directs cap-independent internal initiation of protein synthesis (Jackson *et al.*, 1990). The ORF encodes a polyprotein that is cleaved during translation by viral encoded proteinases. Several protein precursors (P1, P2 and P3) are produced that are then further processed into structural and non-structural proteins. The proteinases responsible for these cleavages, the Leader, 2A and 3C, are discussed in more detail in Chapter 3. The four viral proteins, VP1, VP2, VP3 and VP4, are structural proteins and form the capsid of the virus.

The exact function of the 2B region (Figure 1.2) is unknown, while it has been implicated in host range determination in *rhinoviruses* (Lomax & Yin , 1989). Due to the presence of three NTP-binding domains (GXXXXGK, DXXG, NKXD) in the 2C protein, it is evident that this region interacts with nucleic acids and is therefore also implicated in RNA synthesis (Dever *et al.*, 1987; Hodgman, 1988). According to Gorbalenya and co-workers (1989a & 1989c), the protein exhibits motifs characteristic of the helicase superfamily. The 3A-gene product has been shown to be membrane

associated, a prerequisite for RNA replication and is involved in the initiation of vesicle formation (Weber *et al.*, 1996). Recently, a 3A protein containing a deletion, was implicated in viral virulence (Beard & Mason, 2000). As mentioned previously, the 3B gene encodes three forms of the VPg protein. The 3D gene encodes a RNA-dependent RNA polymerase and is highly conserved in the picornavirus family (Stanway *et al.*, 1984). The functional domain includes the GDD (Gly-Asp-Asp) motif which shows similarity to the YXDD (Tyr-Xxx-Asp-Asp) motif found in other RNA viral polymerases (Kamer & Argos, 1984). Downstream of the 3D gene, is the 3' UTR and the poly (A) tract, both variable in length.

The P1 polyprotein precursor undergoes several proteolytic processing events to produce VP0 (VP4 + VP2), VP3 and VP1 (Domingo *et al.*, 1990; Belsham, 1993). The N-terminus of the precursor is myristoylated (Chow *et al.*, 1987) which has been shown to be essential for capsid assembly/stability. Following proteolytic processing, the VP0 precursor has a myristate moiety attached to the N-terminus. VP0 is cleaved possibly through auto-catalysis and the four viral proteins (VP1-4) form the virus particle. The icosahedral capsid (Figure 1.3) consists of 60 copies of each of the four proteins. VP1-3 are exposed, while VP4 is located internally and can also be seen as an N-terminal extension of VP2 (Acharya *et al.*, 1989). The N-terminal region of VP1 and the C-terminus of VP4 are packed together at the three-fold symmetry axis and interact with the viral RNA which induces protein-protein interaction, thereby contributing capsid stability (Curry *et al.*, 1997).

Five copies of VP1 are situated around the five-fold symmetry axis, while VP2 and VP3 are situated at the two-and three-fold axes of symmetry, respectively. Together they form the protomeric subunit. The spatial arrangement of VP1-3 corresponds with other picornaviruses, although the surface of the particle is smoother, lacking the peaks and depressions of other picornaviruses (Acharya *et al.*, 1989; Fry *et al.*, 1990). Due to the smaller size of VP1 with respect to other picornaviruses and the fact that it is being directed towards the five-fold axis, a hole is formed that leads from the surface into the interior through the β annulus. The latter is formed by the N-terminal

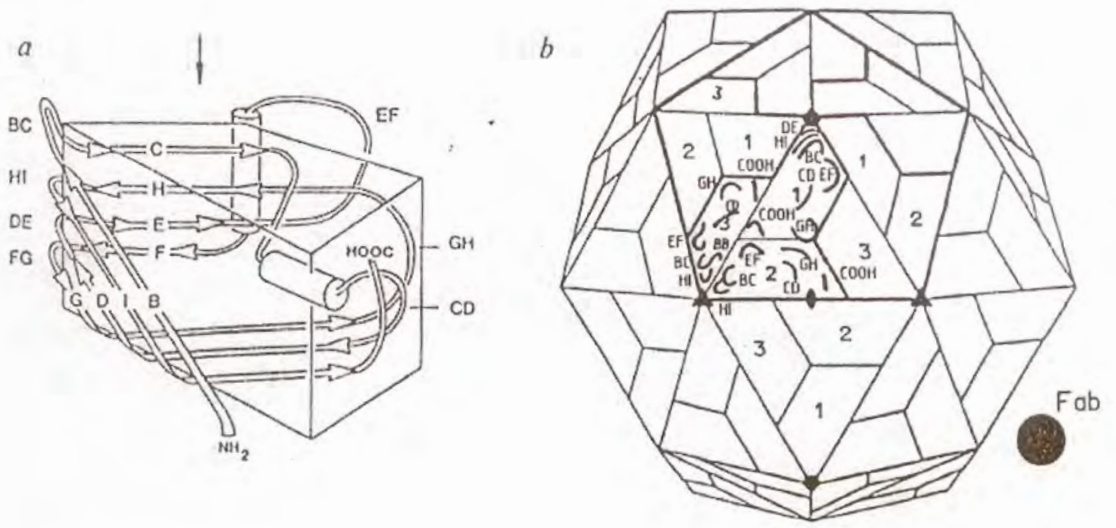

Figure 1.3: Structural representation of the picornavirus capsid proteins. A: The core of each protein (VP1, VP2, VP3) is an 8-stranded β-sandwich. The β-strands are indicated as B, I, D, G, C, H, E and F and are joined through connecting loops. Black arrow indicates the capsid surface. B: The capsid is formed by 60 biological protomers. Each protomer is composed of one copy of VP1, VP2 and VP3. The icosahedral symmetry axes are labaled 5*x* pentagon, 3*x* triangle and 2*x* oval. Most surface loops of VP1, VP2 and VP3 are indicated. The area of an antibody footprint is also indicated (Fab) (Taken from Mateu, 1995).

extensions of 5 VP3 molecules. The residues of VP3, forming this region, are highly conserved.

Characteristic of FMDV is its acid lability. At pH 6.8, the viral particle is disrupted and the pentameric subunits are produced with the release of VP4 and the RNA. From the structure, the high density of histidine residues on the pentamer interface is evident. As the pK of the histidine side chain correlates with the pH of pentamer dissociation, Acharya and co-workers (1990) speculated about the involvement of the histidine residues in the dissociation of the capsid. It has subsequently been shown that His-142 in VP3 is linked to the acid-induced disassembly of the capsid (Ellard *et al.*, 1999).

1.3.4 Overview of infectious cycle

Viral multiplication occurs in the cytoplasm of the host cell. Initiation of the infectious cycle (Figure 1.4) involves attachment of the virion to a specific cell receptor. The cell attachment site of the virus has been shown to be located on a highly mobile G-H loop on the surface of the VP1 protein containing a conserved Arg-Gly-Asp (RGD) sequence (Fox *et al.*, 1989; Logan *et al.*, 1993; Lea *et al.*, 1994; Mason *et al.*, 1994). The RGD consensus is also present in other proteins known to bind to the cell surface receptor family, the integrins (Hynes, 1992; Ruoslahti & Pierschbacher, 1987). Several integrin receptors have been implicated in the cell adhesion of FMDV. These include not only the vitronectin receptor (integrin $\alpha_v\beta_3$) (Berinstein *et al.*, 1995; Jackson *et al.*, 1997; Neff *et al.*, 1998), but also the integrins expressed in epithelial cells, $\alpha_5\beta_1$ (Villaverde *et al.*, 1996; Jackson *et al.*, 2000a) and $\alpha_v\beta_6$ (Jackson *et al.*, 2000b). Field isolates of FMDV have been shown to utilize the integrin receptors for internalization, whereas several cell cultured adapted viruses have been shown to utilize heparin sulfate proteoglycans (Jackson *et al.*, 1996; Sa-Carvalho *et al.*, 1997).

The receptor position the virion favorable for entering the cell. The mechanism of RNA entry into the host cell for FMDV is not exactly clear, although the involvement of VP4 in the release of the viral genome, has been shown by Knipe and co-workers (1997). Contrary to the case with cellular mRNA, picornaviral RNA is not capped, but contains a small protein, VPg. This protein is covalently linked to the 5' end of the

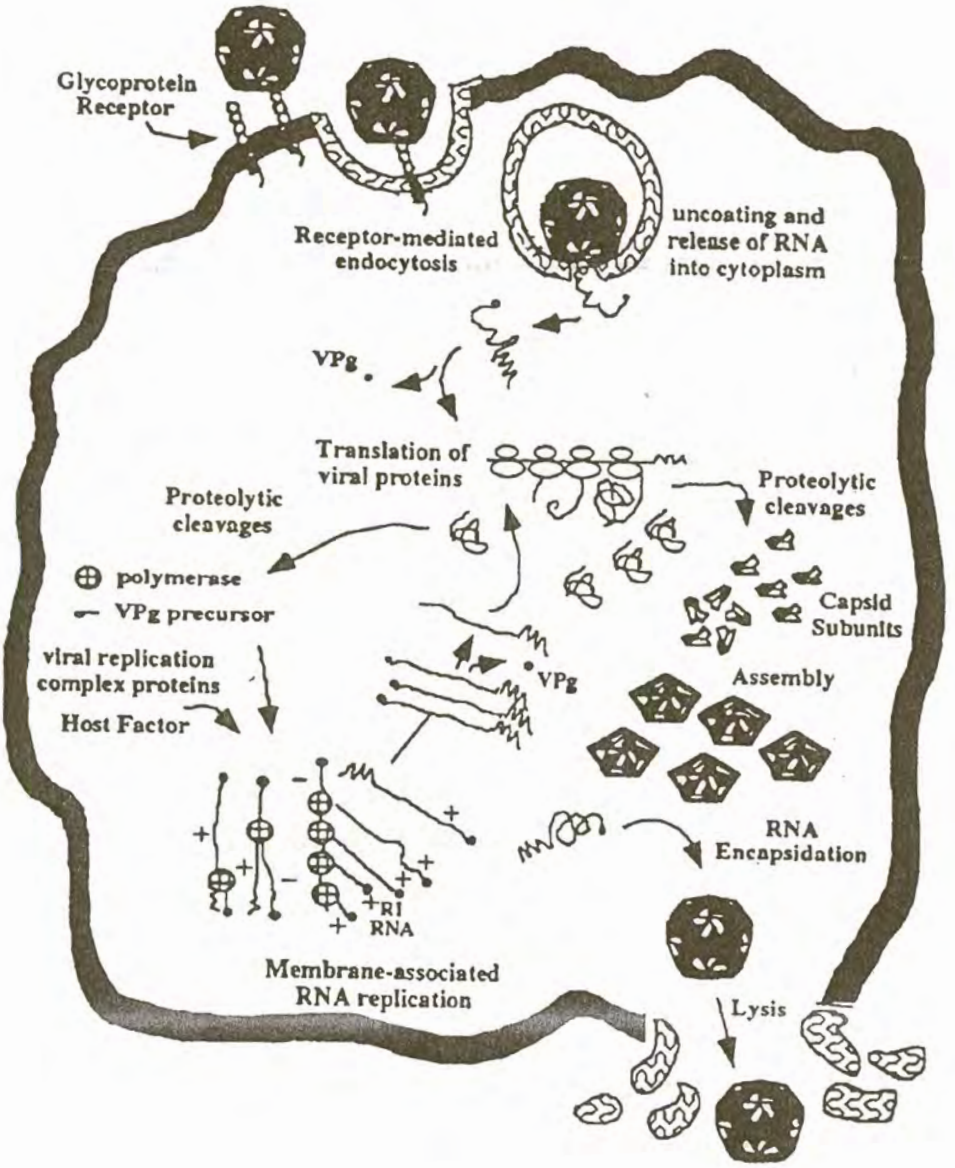

Figure 1.4: Overview of the poliovirus infectious cycle. (Taken from Ansardi *et al.*, 1996)

viral RNA and is removed in the cytoplasm (Lee *et al.*, 1977). Synthesis of the polyproteins is therefore initiated cap independent at the internal ribosome entry site (IRES). Characteristic of the picornaviral IRES structure, is the presence of an oligopyrimidine tract at the 3' border of the structure, followed by an AUG triplet. Translation is usually initiated at this AUG for *cardio-* and *aphthoviruses* (Pelletier *et al.*, 1988).

Several translation initiation factors, involved in cap-dependent initiation, have also been implicated in the translation initiation at the picornaviral IRES structure. For FMDV, these include the eukaryotic initiation factor (eIF) 4B (Meyer *et al.*, 1995) as well as the cellular polypyrimidine tract-binding protein (PTB) (Niepmann, 1996). The latter forms part of the 48S and 80S ribosomal initiation complexes formed with the IRES structure and plays an important role in the stimulation of internal translation initiation (Niepmann *et al.*, 1997). The RNA strand directs synthesis of the polyprotein which is cleaved into individual proteins as synthesis progress (Rueckert, 1996).

The Leader proteinase is the first protein to be synthesized and cleaves itself from the rest of the polyprotein whilst the latter is still being synthesized. The Leader then cleaves eIF-4G which results in the inhibition of cap-dependent mRNA translation of the host cell and the selective advantage of the uncapped viral RNA translation (Devaney *et al.*, 1988; Belsham & Brangwyn, 1990; Ohlmann *et al.*, 1995). It was also shown that the cleavage of eIF-4G by the Leader proteinase supports internal initiation by the IRES elements (Ohlmann *et al.*, 1996; Ohlmann *et al.*, 1997; Ziegler *et al.*, 1995). With the progression of polyprotein synthesis, the polyprotein is cleaved into viral proteins, mainly by the 3C proteinase. The latter is also responsible for the cleavage of the nuclear protein histone H3, which could be related to the host cell transcription shutoff (Falk *et al.*, 1990; Tesar & Marquardt, 1990). The cleavages of eIF-4G and histone H3 are therefore implicated in an almost complete break down of host cell functions during viral infection.

Synthesis of the positive sense viral RNA occurs on the smooth endoplasmic reticulum and is initially extremely rapid to generate many positive-sense copies. With the

expansion of the positive sense copies, a greater fraction is translated, processed and packaged into virions (Ruekert, 1996). Virion assembly involves the formation of protomers, five of which then assemble into pentamers (12S), followed by the packaging of plus-stranded VPg-RNA to form provirions. Twelve pentamers associate together to form either empty capsids (lacking RNA) with a sedimentation coefficient of 75S or provirions (146S) (Grubman *et al.*, 1985). The final step in mature virion formation is the cleavage of VP0 to form the mature four-chain subunits. Viral particles are released from the host cell by infection-mediated disintegration of the host cell (Ruekert, 1996).

1.3.5 Antigenic properties

Initial experiments suggested the presence of a major immunodominant site for FMD located within the G-H loop of VP1 (Bachrach *et al.*, 1975; 1979). Subsequent research demonstrated the involvement of multiple sites independent of the G-H loop for FMD (Domingo *et al.*, 1990 and references therein. Table 1.1 summarizes the immunodominant sites determined for types A, O and C.

Table 1.1: Description of immunologically important secondary structure elements (taken from Mateu, 1995)

AXIS	SECONDARY STRUCTURE ELEMENTS	TYPE O	TYPE C	TYPE A_{10}	TYPE A_{12}	TYPE A_{22}	TYPE A_5
5X	VP1 B-C	3					
	VP1 H-I			4	1		
2X	VP1 G-H	1a,5	A	1	2	1	
	VP2 E-F	2					
3X	VP2 B-C	2	D	3			1
	VP3 B-B	4	D	5			
	VP3 B-C			3			
	VP3 H-I			3			
	VP1 C_T	1b	C,D	2	3,4		2
	VP3 E-F			3			
	VP3 G-H				4		

Kitson and co-workers (1990) identified four independent immunodominant sites for serotype O (O_1Kaufbeuren). The positions of these sites have been located on the related strain O_1BFS (Acharya *et al.*, 1989). Site 1 involves the G-H loop and C-terminus regions of VP1, while sites 3, 2 and 4 are located on loops of VP1, VP2 and VP3, respectively. Another functionally independent site was described later, although its physical position might overlap with site 1 (Crowther *et al.*, 1993a). Three independent sites for serotype C (C-S8c1) have been determined and located on the structure of the same strain (Mateu *et al.*, 1989; Mateu *et al.*, 1990; Lea *et al.*, 1994). Site A is located on the G-H loop of VP1 and site C on the C-terminus region. Site D involves regions from VP1, VP2 and VP3. Multiple sites have been identified for different subtypes of serotype A. Sites identified for A_{10} (Thomas *et al.*, 1988) include the G-H loop (site 1), C-terminus region (site 2), another site in VP1 and several regions in VP3. Baxt and co-workers (1989) identified four immunodominant sites for A_{12}, also involving the G-H loop and C-terminus regions as well as a further epitope on VP1 and a fourth on VP1 and VP3.

The antigenic structure of the site present on the G-H loop of VP1 display a high level of complexity. This includes the involvement of overlapping and non-overlapping epitopes as well as the fact that some of these epitopes are conformational-dependant or discontinuous epitopes (Domingo *et al.*, 1990). The latter by definition implies the involvement of amino acid residues on different protein segments and is therefore dependent on the conformation of the protein. Continuous epitopes involve residues located in a single protein segment although strictly speaking, most of these epitopes will be discontinuous as not every residue is necessarily involved in antibody binding. Continuous epitopes are however distinguished from discontinuous ones by their ability to be mimicked by peptides (Mateu, 1995). Following the above description of continuous and discontinuous epitopes, serotypes A and C display continuous epitopes, while serotype O contains discontinuous epitopes as well (Mateu, 1995).

1.4 Vaccination against Foot-and-mouth Disease

The effectiveness and consequently success of vaccination against human and animal infectious diseases are well known. In fact, vaccination or immunization has been responsible for increasing the life-span of humans more than any other 20th century medicine (Plotkin, 1993). The contribution of active vaccination has been crucially important in the prevention and control of major infections such as diphteria, mumps, rubella, tetanus, poliomyelitis, measles, yellow fever as well as the eradication of smallpox (Moxon, 1990). Due to it's very important impact on the economics of livestock and poultry industry, vaccination also remains to be the most cost-effective method to manage the onslaught of infectious diseases in the veterinary field (Babiuk, 1999).

Yearly vaccination programs against foot-and-mouth disease were introduced in 1952 in the Netherlands and soon followed by other European countries. The number of outbreaks decreased drastically and in 1992 Western Europe was declared free of the disease by the OIE. Eradication of FMD in South America is also progressing well with several countries in the region being free of disease (see section 1.2). The success in South America is mainly due to extensive vaccination programs, co-ordinated by the Pan-American Centre for FMD in Rio de Janeiro (Barteling & Vreeswijk, 1991; Brown, 1992).

Several important factors ensuring vaccine efficacy should be considered during a vaccination campaign. These include satisfactory potency and safety as well as the implementation of an acceptable quality assurance system (Doel, 1999). According to the European Pharmacopoeia, potency testing involves the vaccination of three groups of five cattle with three dilutions of the vaccine. These animals are then challenged 2-4 weeks post vaccination with a virulent strain from the same type as the vaccine. A 50% protective dose (PD50) of 3 or more is required (Van Oirshot, 1999). Due to the nature of FMD vaccines (killed or inactivated virus), it is imperative that a high standard of safety is maintained. Previously, several outbreaks of FMD in Europe were reportedly linked to vaccination (King *et al.*, 1981; Beck & Strohmaier, 1987). The preservation

of the cold chain, a long shelf-life and vaccine strain selection are other important factors to consider. The vaccine should also be used as prescribed by the manufacturer (Doel, 1999).

A protective immune response to FMDV entails the induction of neutralizing antibodies and macrophage activity, thus involving both B- and T helper-lymphocyte dependent responses (McCullough *et al.*, 1992 and references therein). These facts should be taken into consideration during vaccine design. Furthermore, to obtain and sustain rapid and long-lasting herd immunity, the frequency of vaccine administering is essential. Usually, annual and biannual vaccination is required. Other factors influencing the immune response, include the quality of antigen, type of adjuvant used during formulation and physical condition (age and health) of animals (Doel, 1999; Van Oirschot, 1999).

1.4.1 Conventional, inactivated vaccines

The availability of high concentrations of viral antigen, properly inactivated viral preparations and the addition of non-toxic adjuvants to stimulate the desired immune response are essential for inactivated viral vaccine production (Barteling & Vreeswijk, 1991). In the 1920's Vallée and his co-workers showed that FMDV could be killed or inactivated by formaldehyde without losing its antigenicity. However, it was only during the early 1950's when Frenkel developed the large-scale *in vitro* production of natural host tissue to cultivate the virus, that mass vaccination against FMD was initiated (Barteling & Vreeswijk, 1991; Brown, 1999). Aided by the discovery of antibiotics, Frenkel cultivated virus on bovine tongue epithelium, followed by viral harvest, adsorption onto aluminium hydroxide and inactivation with formaldehyde (Gillespie & Frenkel, 1955). The use of aluminium hydroxide as an adjuvant was discovered during the 1930's first by Schmidt and later by Waldmann. Although this was a simple production system and there was also no need for viral adaptation to the culture system, a low level of bacterial and fungal contamination was ever present. It was also impossible to increase production during outbreak situations (Barteling & Vreeswijk, 1991).

1.4.1.1 FMD viral production in cell lines

Following the development of tissue culture technology, the growth of FMDV on primary cells from pigs and cattle was investigated. It was soon shown that baby hamster kidney cells (BHK 21, clone 13) was better host cells for production of the virus (Mowat & Chapman, 1962). Production of FMDV on BHK cells in monolayer (roller bottle system) can easily be used for large-scale production, but this is a labour-intensive and prone to contamination.

Alternatively, different types of "multi-surface systems" have also been investigated over the years. These include the Girogen system, which is made up of long glass tubes fixed around a shaft inside a cylindrical fermenter (Girard *et al.*, 1979). Cells grow over the inner and outer surfaces of the tubes. The use of glass beads packed in a stationary fermenter has also been shown to be effective in the production of FMDV on BHK monolayer, although the method has presumably not been applied on an industrial scale (Spier & Whiteside, 1976). Micro-beads in suspension is well known in the production of inactivated vaccines for human use, such as polio vaccine (Van Wezel, 1967). FMDV production using this system was attempted experimentally, but probably due to financial implications was not used for vaccine production (Barteling & Vreeswijk, 1991).

With the adaptation of BHK 21 cells to grow in suspension culture (Capstick *et al.*, 1962), large-scale production of cultures was possible. Although FMDV production on BHK monolayer is considered to be better than production in suspension culture, most vaccine factories today use the latter technology. This is probably due to the fact that it is less labour intensive, less prone to contamination and much easier for large-scale production of vaccine.

1.4.1.2 Inactivation of FMDV

Viral inactivation is the most crucial step in the production of vaccine. As mentioned previously, several outbreaks in Europe have been linked to improperly inactivated virus particles present in the vaccine. The safety of a vaccine batch is usually determined with inactivation kinetics. The minimal accepted safety level should be

below $-5\log_{10}$ plaque forming units for a 100 liter batch (Barteling & Vreeswijk, 1991). Following the historical method of formaldehyde inactivation, $Al(OH)_3$ gel is adsorbed to the viral particle. This gel is however toxic for cells, making proper *in vitro* inactivation kinetics determination impossible. This problem was solved by ultracentrifugation of the virus – $Al(OH)_3$ complex. During this procedure, the virus and the gel are separated, enabling one to collect and titrate the virus and subsequently determining inactivation kinetics (Barteling & Woortmeijer, 1984).

Inactivation using aziridines has been shown to be a better option than formaldehyde as inactivation is rapid with first order kinetics enabling extrapolation to predict the safety of the batch (Brown *et al.*, 1963; Bahnemann, 1973; Barteling & Vreeswijk, 1991). However, these compounds are highly toxic. A substitute chemical, bromoethylamine hydrobromide (BEA), is available and is transformed into the active substance ethylenimine at pH > 8 (Bahnemann, 1975). It is still necessary to check for residual live viral particles in the vaccine batch.

1.4.1.3 Vaccine formulation

To ensure a proper immune response, the inactivated antigen must be adsorbed to an immune stimulant or adjuvant. Only three adjuvants have been applied to FMD vaccine production: the aqueous adjuvants $Al(OH)_3$ and saponin, and oil emulsion. With the adsorption of $Al(OH)_3$ to the antigen, followed by formaldehyde inactivation, initial monovalent vaccines had a dose volume of 60 ml. Following the concentration of the antigen – $Al(OH)_3$ complex and the addition of purified saponin, a final dose volume of 5 ml was obtained for a trivalent vaccine (Barteling & Vreeswijk, 1991).

Problems were however encountered with the vaccination of pigs. With the addition of diethylaminoethyldextran (DEAED) to the antigen – $Al(OH)_3$ complex, efficient protection was obtained. Due to the high price of DEAED, this is however not a viable financial option for vaccine manufacturers (Leeuw *et al.*, 1979). Improved protection of pigs was also obtained using oil emulsion vaccines. According to this method the antigen is emulsified in a mineral oil in the presence of an emulsifying agent (Freund & Thompson, 1945). Injected intramuscularly, cattle developed adverse reactions. By

reduction of the dose volume and with subcutaneous administration, the problem could be solved. An advantage of oil emulsion vaccines is the longer lasting immune response in comparison to the aqueous vaccines (McKercher & Graves, 1977). When the water in oil emulsion is emulsified once more, a double emulsion (water in oil in water) is obtained (Herbert, 1965). The advantage of the double emulsion is its lower viscosity during injection.

An exciting new development in the field of vaccine technology is the controlled-release microsphere formulations, which mimic repeated vaccinations. The antigen is contained in a polymer and is released as a pulse 1-6 months post injection. The pulse is dependent on the rate of polymer degradation, which is determined by the composition and molecular weight of the polymer (Cleland, 1999). An example of the application of microspheres containing antigen in viral vaccines is the work done by Hilbert and co-workers (1999) with influenza A. Eight weeks post injection of mice with a microsphere formulation, the same level of neutralizing antibodies could be detected as for two administrations four weeks apart with conventional vaccine. This new development holds promise for single administration, providing prolonged protection.

*1.4.2 **Alternative vaccines***

The technology used to produce killed or attenuated conventional vaccines has not changed much over the past 200 years (Babiuk, 1999). With the advances in immunology and especially molecular biology, new techniques are being developed to address emerging infectious diseases, improve current vaccines and to address diseases that previously evaded conventional vaccines, including malaria and HIV (Ada, 1997). New approaches to vaccinology include: (i) heterologous gene expression in recombinant vectors; (ii) attenuated vectors containing deleted genes; (iii) combination vaccines containing multivalent antigens to protect against more than one disease simultaneously; (iv) DNA vaccination; (v) regulation of the immune response and (vi) new microcarrier systems for immunomodulation delivery (Kurstak, 1999). Another very important application of new technologies would be the design and refining of

vaccines to produce the "ideal" vaccine. In table 1.2 a list of parameters for a so-called "ideal" vaccine is given.

Table 1.2 Parameters of an "ideal" vaccine (taken from Babiuk, 1999)

1. Efficacy greater than 90%
2. Effective after a single dose
3. Long-lived immunity (preferably life-long)
4. Effective when given orally (no need for injections)
5. Induces a wide range of appropriate responses (mucosal, humoral, cellular)
6. Low cost
7. Compatible with local management practices
8. Compatible with co-administration of other vaccines
9. Stable (genetically/thermally)
10. High safety

Evidence for FMD outbreaks originating from improperly inactivated viral particles present in conventional vaccine preparations, prompted researchers to explore alternative means of vaccination against FMD. Several different options were and are still being investigated and include heterologous gene expression, using different expression systems, DNA vaccination as well as the construction of synthetic peptides.

1.4.2.1 Heterologous gene expression

The development of recombinant DNA technology provided the means for the expression of FMD proteins. One of the first reports described the cloning and expression of VP1 (Figure 1.2) in *Escherichia coli*, but only partial protection against the homologous strain was achieved (Kleid *et al.*, 1981). The VP1 region was targeted due to the presence of the major immunogenic site. Despite the enthusiasm generated by this and other reports (Boothroyd *et al.*, 1981; Küpper *et al.*, 1981), immunological levels induced were 100-1000 times lower than equivalent amounts of the intact virion (Domingo *et al.*, 1990). This suggested the presence of important antigenic regions on other parts of the P1 region. Other systems used to express the VP1 protein include hepatitis B core proteins (Clarke *et al.*, 1987b) and bacterial fusion proteins (Broekhuijsen *et al.*, 1987; Winther *et al.*, 1986).

Several systems for the expression of P1 have been tested. *Escherichia coli* expression of P1-2A, part of 2B and 3C, resulted in the efficient synthesis and processing of the structural protein precursor as well as the formation of empty capsids (Lewis *et al.*, 1991; Grubman *et al.*, 1993). In spite of this, the capsid assembly process was found to be inefficient. This is probably because no myristoylation occurs in *E.coli*, and the toxic effect of the 3C proteinase. Expressing the cassette P1-2A+3C in insect cells using a baculovirus system, resulted in the efficient myristoylation of VP0 and the processing of capsids proteins (Roosien *et al.*, 1990; Belsham *et al.*, 1991). The same results were obtained with expression of this cassette in mammalian cells, showing that myristoylation of the capsid protein precursors occurs independently of other viral proteins (Belsham *et al.*, 1991).

The same construct (P1-2A+3C) was also expressed constitutively in a vaccinia virus system, but no empty capsids were obtained (Abrams *et al.*, 1995). Upon expression in an inducible system, the efficient processing of the capsid proteins was obtained as well as the formation of empty capsids. The myristoylation region on VP4 was mutated and the mutated cassette was again expressed in the constitutive system. Although the correctly processed capsid proteins were expressed, the formation of empty capsids could still not be obtained. This result is probably due to 3C toxicity. Recently, it was shown that protection with homologous challenge in mice could be obtained using a recombinant vaccinia virus expressing only the P1 region of FMDV strain C3Arg85 (Berinstein *et al.*, 2000), thereby avoiding the 3C proteinase toxicity.

Recently the expression of the VP1 protein in transgenic plants (*Arabodopsis* and Alfafa) has been demonstrated (Carrillo *et al.*, 1998; Wigdorovitz *et al.*, 1999a). Although protective immune responses were obtained in mice, the production of recombinant antigen was very low. A tobacco mosaic virus-based vector was subsequently used for the expression of the VP1 region in plants. Mice injected with foliar extracts from infected leaves, developed an immunogenic response to the VP1 protein and were protected against virulent challenge (Wigdorovitz *et al.*, 1999b).

1.4.2.2 DNA vaccination

DNA vaccination involves the direct introduction by needle innoculation or particle bombardment of host tissue with plasmid DNA. The result is expression of antigenic proteins by transfected cells (Whalen, 1996). DNA vaccination resembles a viral infection since the biosynthetic machinery of the host is used for protein synthesis. Due to the ease of production and stability of DNA, any desirable virus may be used to produce a potential DNA vaccine, side-stepping the difficult strain selection process for the production of conventional inactivated vaccines. Additional advantages include the ability to elicit cell-mediated immune responses and the elimination of risks associated with the use of live agents. DNA vaccination may potentially render protection against diseases that previously evaded conventional vaccination. The amount of antigen produced can be low, resulting in a lower humoral immune reponse in comparison to traditional antigen-containing vaccines (Robinson *et al.*, 1993; Xiang *et al.*, 1994).

Several safety concerns regarding DNA vaccination exist. Although no evidence for the integration of foreign DNA into the host genome has been obtained yet, it does remain a possibility. In addition, production of anti-DNA antibodies may also lead to auto-immune disease. Again, no evidence for the production of such antibodies has been obtained thusfar (Manickan *et al.*, 1997). Several DNA vaccine trials in the livestock and poultry industry have been carried out (Beard & Mason, 1998), with the DNA vaccine developed against avian influenza being the most successful. The hemaglutinin protein is expressed under the control of the cytomegalovirus (CMV) promotor. Upon heterologous challenge, higher levels of protection were obtained with the DNA vaccine than with the conventional inactivated vaccine (Kodihalli *et al.*, 1997). Despite these encouraging results, problems with delivery systems are generally encountered. The real challenge for DNA vaccination in the veterinary field involves therefore the improvement of delivery and potency of low doses of DNA in order to achieve similar levels of efficacy as obtained for conventional vaccines.

Although empty FMD capsids could be obtained with the expression of regions of the FMDV genome in different systems, the antigen yield remained low due to the toxic

effect of the 3C proteinase on the different host cells. To address this problem, DNA vaccines were designed for the production of empty capsids in animals.

One approach entails the construction of the cassette P12X3C using the pcDNA3 plasmid, which contains the strong type II promoter from the CMV immediately early gene as well as the bovine growth hormone (BGH) polyadenylation site. The P1/2A region as well as the 3C proteinase-coding region was inserted into this vector to produce the P12X3C cassette (Chinsangaram *et al.*, 1998a). It was shown that the expected viral proteins (VP0, VP3, VP1 and 3C) could be processed. Several variations of this cassette were also constructed. The plasmid (pP12X3C-mut) contains a point mutation at the active site of the 3C proteinase. Plasmid piP12X3C contains an intron, but piIP12X3C contains an intron and the FMDV IRES structure, upstream of the P1 region (Chinsangaram *et al.*, 1998a). The intron was included for enhanced expression in mammalian cells as well as immunogenicity (Beard *et al.*, 1999). The addition of the FMDV IRES structure was included to investigate whether cap-independent translation would have an effect on protein expression levels. These constructs were evaluated in mice and administered through gene gun inoculations. Plasmid piP12X3C was shown to be more immunogenic than pP12X3C. The addition of the IRES structure did not seem to have an effect on the immunogenicity. Both pP12X3C and pP12X3C-mut were able to elicit an antibody response, but only pP12X3C was able to produce neutralizing antibodies. The importance for the production of empty capsids to maintain conformational epitopes, was therefore confirmed again (Chinsangaram *et al.*, 1998a).

Another approach in the development of FMD DNA vaccines involves the engineering of the genome to produce attenuated FMD virions. To be able to manipulate the FMDV genome, an infectious cDNA clone, $pRMC_{35}$, was constructed (Rieder *et al.*, 1993). Attenuation was subsequently achieved through the deletion of the coding region of the Leader proteinase (Piccone *et al.*, 1995a). This proteinase is responsible for the inhibition of translation of the host-cell mRNA during infection. It was shown that the Leader-less virus is less virulent than the wild type virus (Brown *et al.*, 1996). Upon evaluation of the construct as a vaccine in cattle, two of the three animals did not

develop lesions, but did show signs of infection post challenge (Mason *et al.*, 1997). Partial protection was also observed upon evaluation in swine (Chinsangaram *et al.*, 1998b).

Attenuation was also achieved through the deletion of the receptor-binding site RGD (McKenna *et al.*, 1995). The virus is consequently unable to spread between normal cells, but can be propagated in cells containing a novel, genetically engineered receptor (Rieder *et al.*, 1996). Cattle vaccinated with the RGD-deleted mutants did not show signs of infection and were protected upon challenge (McKenna *et al.*, 1995). The RGD-deleted mutants were subsequently incorporated into the pcDNA3 vector containing the CMV promotor as well as the BGH site (Ward *et al.*, 1997). This construct, pWRMHX, produced non-infectious FMDV particles in cells and animals. Swine inoculated with this replicating DNA vaccine did not show any signs of disease and produced neutralizing antibodies to FMDV. Through the insertion of an intron onto the 3D region of the genome (pWRMHiX), infectivity was enhanced (Beard *et al.*, 1999). Evaluation of pWRMHX, pWRMHiX, piP12X3C and piIP12X3C in swine through gene gun inoculations showed that neutralizing antibodies could be obtained for all the DNA vaccine candidates. However, levels were much lower than preliminary studies in mice. Only pWRMHX and pWRMHiX were able to render significant protection upon challenge (Beard *et al.*, 1999). Although promising results were obtained, neutralizing antibody titres are much lower than that elicited with conventional inactivated vaccines. Improvements in plasmid design as well as alternative delivery systems could increase infectivity and therefore improve immunogenicity in animals (Beard *et al.*, 1999).

Recently, a recombinant replication-defective human adenovirus serotype 5 containing the P1-2A region of FMDV as well as the 3C region (Ad5-P12X3CWT) was constructed and investigated as a potential DNA vaccine candidate (Mayr *et al.*, 1999). Viral proteins VP0, VP3 and VP1 could be detected upon infection of human 293 cells. Swine inoculated intramuscularly with this construct developed neutralizing antibodies and were partially protected upon challenge. A variation of this construct, which contains a mutation in the proteinase, was not able to elicit neutralizing antibodies in

mice (Mayr *et al.*, 1999). In a similar study, only the P1 region was incorporated into the adenovirus-based vector (Sanz-Parra *et al.*, 1999). Partial protection in cattle was obtained only after a second round of combined subcutaneous and intranasal vaccination. Improvement of potency as well as the efficacy of the Ad5-P12X3CWT construct, are currently being examined (Mayr *et al.*, 1999).

1.4.2.3 Synthetic peptide vaccines

The production of synthetic peptides as a means to vaccination has the advantage of being chemically stable and defined. Due to the simple structure of these peptides, manipulation thereof can be readily accomplished. Incorporating of both B and T cell epitopes in the design of synthetic peptides, an appropriate immune response might be obtained (Brown, 1993; Sobrino *et al.*, 1999).

Following the elucidation of the viral capsid structure of FMDV, peptide vaccine candidates were constructed against the major immunodominant site (residues 140-160) located in the G-H loop of the VP1 protein. Initial evaluation of these peptides in guinea pigs, mice and cattle seemed promising (Bittle *et al.*, 1982). Although a peptide comprising of residues 140-160 of VP1 as well as residues 200-213 at the C-terminus end of the protein was able to protects upon challenge, immunogenicity was substantially lower than that elicited by conventional vaccines (DiMarchi *et al.*, 1986).

A large-scale evaluation of peptide vaccines directed against an Argentinean serotype C isolate was carried out recently (Taboga *et al.*, 1997). Four peptides containing B and T cell epitopes present in VP1 were tested. These peptides were defined as A (site A), AT (site A as well as a T-cell epitope), AC (site A as well as the C-terminus region) and ACT which included all three linearised epitopes. Upon challenge with the homologous virus, the highest level of protection was obtained with the more complex ACT peptide, although protection levels were still lower than 40%. The lack of solid protection reflects the difficulty in reproducing the immunogenicity of the entire viral capsid through the much simpler synthetic peptides. This is probably due to the inadequacy of T-cell epitopes to provide help to B-cell epitopes, the inefficient

presentation of the peptides to the immune system as well as the highly variable nature of the RNA virus (Sobrino *et al.*, 1999).

A better understanding of the B-and T-cell interaction as well as the inclusion of T helper epitopes will lead to improved strategies for synthetic peptide vaccines (Sobrino *et al.*, 1999). A T-cell epitope on VP4 has been shown to be recognised by T lymphocytes from vaccinated cattle (Van Lierop *et al.*, 1995) and swine (Sobrino *et al.*, 1999). This epitope, when positioned in tandem with the immunodominant site (B cell epitope) in VP1, rendered help to the B cell epitope and could therefore be a likely candidate to provide T cell help (Blanco *et al.*, 2000).

Improvement of peptide stability and presentation are also important factors to take into consideration. Promising results have been obtained recently through the use of retro-inverso peptides. These peptides are more resistant to proteinase degradation, thus enhancing immunogenicity. An all-D retro peptide corresponding to the immunodominant region of type A_{12} was linked to activated keyhole limpet haemocyanin and oil-adjuvanted before the inoculation of swine. These animals were challenged eleven days post-vaccination with the homologous virus. Only one animal from nine was completely unprotected, two developed small lesions, while the remaining six animals exhibited no clinical signs of disease (Nargi *et al.*, 1999).

1.5 Vaccination in sub-Saharan Africa – Aim and Scope of this study

As discussed earlier in this review (see sections 1.2 and 1.4), several parts of the world have been declared free of FMD by the International Animal Health Code of the OIE. These regions include the countries in the European Union, Uruguay and Paraguay in South America as well as several disease-free zones. Such an internationally recognized FMD-free zone status was given to South Africa in 1996 by the OIE. This status is of immense value to the country as animal and other agricultural products to the amount of approximately US$ 3.2 billion are exported annually (Agricultural News, 1998). The free-zone in South Africa include major parts of the country, with the exception of the northeastern region of the country (inclusive of the Kruger National

Park), while similar zones have also been awarded to Namibia and Botswana. Eradication of FMD in sub-Saharan Africa is however, highly unlikely due to the presence of large numbers of the free-living maintenance host, the African buffalo (*Syncerus caffer*). These animals provide a potential source of infection for domestic livestock (Dawe *et al.*, 1994) and other wildlife, such as impala (*Aepyceros melampus*) (Bastos *et al.*, 2000).

At present, the disease is controlled in sub-Saharan Africa essentially through vaccination, restriction of animal movement and frequent inspections of animals in the controlled areas (Hunter, 1998). However, the inactivated vaccines currently in use have several disadvantages. These include the inability to adequately cover the extent of antigenic variation within the SAT types in different regions. Antigenic and genetic characterization of field isolates have revealed that these viruses evolve independently and rapidly in different geographical areas, across international borders (Esterhuysen, 1994; Vosloo *et al.*, 1995; Bastos *et al.*, 2001). Vaccines therefore need to be custom-made to specific geographic areas to be effective. However, the adaptation of field isolates as high-antigen producing and stable vaccine strains is presently a cumbersome, time consuming and expensive process.

As an alternative method to address the problem of antigenic variation in different geographic areas, a combined approach between conventional inactivated vaccine technology and recombinant molecular biology, is proposed. The strategy entails the development of recombinant FMD viruses with the construction of a full-length cDNA clone of a suitable vaccine strain. The production of such recombinant viruses has been achieved previously for the A_{12} serotype (Sa-Carvalho *et al.*, 1997; Almeida *et al.*, 1998; Beard & Mason, 2000). The antigenic characteristics of such recombinant viruses can then be manipulated by exchanging the genetic determinants for antigenicity, i.e. specific regions of the genes encoding structural proteins. The recombinant viruses will then be applied in the manufacturing of conventional, inactivated vaccines.

It is therefore envisaged to develop custom-made vaccines to foot-and-mouth disease, employing recombinant FMD viruses, in quick response to an outbreak situation in a specific problematic area. The aim of this study was therefore to:

(I) investigate factors that could possibly influence the construction of such recombinant or chimeric viruses between the European and SAT types;

(II) the construction of a chimeric virus and the subsequent evaluation thereof, utilizing parameters important in commercial vaccine manufacturing.

CHAPTER 2

Molecular characterization of the structural-protein-coding region of ZIM/7/83/2, a SAT 2 type foot-and-mouth disease virus[1]

2.1 Introduction

Six of the seven serotypes of FMDV (South African Territories (SAT) 1, 2 and 3 as well as serotypes A, O and C), occur in sub-Saharan Africa. Sporadic reports of the occurrence of the SAT types outside this region have been documented before. These include the detection of SAT 1 in Northern Africa and the Middle East (Pereira, 1981) as well as the recent outbreak of SAT 2 in Saudi Arabia. Despite this, the SAT types occur almost exclusively in sub-Saharan Africa. Types A, O and C occur frequently in Western, Eastern and Central Africa where they are endemic in cattle populations and unlike the SAT types, are not established in wildlife (Hedger *et al.*, 1973). Historically, SAT 2 serotype has been responsible for more FMD outbreaks (48%) in domestic animals in southern Africa than any other type (Thomson, 1994). The SAT 2 serotype was therefore selected to be employed together with the type A_{12} genetic backbone in the construction of a recombinant chimeric virus. Towards this objective, the structural-protein-coding (P1) region of the SAT 2 vaccine strain, ZIM/7/83, was amplified using RT-PCR from partially purified virus, cloned into an appropriate vector, molecularly characterized and compared with known FMDV SAT 2 isolates.

2.2 Materials and Methods

2.2.1 Viral and bacterial strains

The FMD vaccine strain, ZIM/7/83/2 (passage history: B1BHK5B1), was used during this study. This bovine outbreak strain originates from western Zimbabwe and was obtained from the Botswana Vaccine Institute in Gabarone. RHO/1/48/2 (passage

[1] Parts of the results presented here have been published in *Virus Genes* 1999; 19 (3): 229 – 233.

history: BTY2RS2), also a bovine outbreak strain, originates from Zambia and was obtained from Dr. Nigel Ferris at the World Reference Laboratory for Foot-and-mouth Disease, Pirbright, UK. *Escherichia coli* JM109 (genotype: *rec*A1, *end*A1, *gyr*A96, *thi*, *hsd*R17 (r_K-,m_K+), *rel*A1, *sup*E44, delta(*lac-pro*AB), [F', *tra*D36, *pro*AB, *lac*I^qZdeltaM15]) was used for transformation experiments and was obtained from Promega.

2.2.2 Oligonucleotides

All oligonucleotides used in this study are summarized in Table 2.1. The specific region on the FMDV genome where the oligonucleotides bind, are indicated according to the KEN/3/57/2 (AJ251473) sequence, unless indicated differently. NCR1 was designed from the full-length nucleotide sequences of A_{10} (X00429), A_{12} (M10975), A_{22} (X74812), O_1 (X00871) and KEN/3/57/2 (AJ251473).

2.2.3 Partial purification of viruses and RNA extraction

Viruses were twice passaged on "Instituto Biologico Rim Suino" or IB-RS-2 cells (a pig kidney cell line), first at a low multiplicity of infection (m.o.i), followed by a high m.o.i. A 30% (w/v) sucrose gradient together with high-speed ultracentrifugation (150 000 x *g* for 3h at 4°C) was used to pellet the viral particles. RNA was extracted from the pellet which was resuspended in 1 x TE and sonicated for 3 to 5 min. Sodium dodecyl sulfate (SDS) and 2-mercaptoethanol (1% each) were added to the sonicated pellet followed by a phenol:chloroform (1:1) extraction. This was followed by phenol-(1:1) and diethylether (2:1) extractions. The supernatant was subsequently precipitated with 1/10 volume 3M sodium acetate pH 5.2 and 2 volumes ice cold 100% ethanol for 2 h at -20°C, dried *in vacuo* and stored at -70°C (Vosloo, 1992).

2.2.4 cDNA synthesis

Using AMV-RT (Promega), the viral RNA was reverse transcribed with the 2B208R oligonucleotide. This primer targets the 2B region of the genome (Table 2.1; Figure 2.1). The reaction was carried out at 42°C for 2h in the presence 2% DMSO.

Table 2.1 Summary of oligonucleotides

Oligonucleotide	Sequence	Purpose	Reference	Binding nucleotides
2B208R (20 mer)	5'-ACAGCGGCCATGCACGACAG-3'	cDNA synthesis	N. Knowles (pers. comm.)	3753 – 3772
NCR1 (22 mer)	5'-TACCAAGCGACACTCGGGATCT-3'	PCR amplification and nucleotide sequencing	This study	549 – 570
WDA (21 mer)	5'-GAAGGGCCCAGGGTTGGACTC-3'	PCR amplification and nucleotide sequencing	Beck & Strohmaier (1987)	3531 – 3551
WUS (23 mer)	5'-CCACRTATTACTTYTGTGACCTG-3'	Nucleotide sequencing	A.D.S. Bastos (pers. comm.)	3055 – 3074
VP4U (20 mer)	5'-TCTGGCAAYACTGGTAGCAT-3'	Nucleotide sequencing	A.D.S. Bastos (pers. comm)	1314 – 1333
VP4D (20 mer)	5'-CTACCAGTATTGCCAGATTG-3'	Nucleotide sequencing	This study	1311 – 1330
SEQ1 (18 mer)	5'-CGTCGATGAGCCACTCTT-3'	Nucleotide sequencing	This study	854 – 871
SEQ2 (17 mer)	5'-CATCAAAGGCACTGAAC-3'	Nucleotide sequencing	This study	1049 – 1110
SEQ3 (18 mer)	5'-ACAACACGACACGGTACC-3'	Nucleotide sequencing	This study	1572 – 1589
SEQ4 (19 mer)	5'-TTGTGCGAAGCGTGGTTGT-3'	Nucleotide sequencing	This study	3138 – 3156
SEQ5 (18 mer)	5'-CACCAGCACGCAGTTCAA-3'	Nucleotide sequencing	This study	1859 – 1876
SEQ6 (17 mer)	5'-GTTCGTGTTCGCCAAGG-3'	Nucleotide sequencing	This study	3089 – 3105
SEQ7 (16 mer)	5'-GGTAGCAGTGGGCYGC-3'	Nucleotide sequencing	This study	3604 – 3619
SEQ8 (18 mer)	5'-ACGTTSGTCGGNGCKATG-3'	Nucleotide sequencing	This study	2134 – 2152
SEQ9 (20 mer)	5'-GCKTAACCGTAGGTGAYKCC-3'	Nucleotide sequencing	This study	1617 – 1636
SEQ10 (17 mer)	5'-GACCCBAAGACCGCAGA-3'	Nucleotide sequencing	This study	2235 – 2251
SEQ11 (18 mer)	5'-GGGAYACAGGAYTGAACT-3'	Nucleotide sequencing	This study	2630 – 2646
SEQ12 (15 mer)	5'-CGTAGATMCCCTTGT-3'	Nucleotide sequencing	This study	1785 – 1800
SEQ13 (17 mer)	5'-GCGACGTCCAACAKGTT-3'	Nucleotide sequencing	This study	2310 – 2326
SEQ14 (19 mer)	5'-CAACGGTGAGTGCAAKKAC-3'	Nucleotide sequencing	This study	3179 – 3197
SEQ15 (19 mer)	5'-GTMMTTGCACTCACCGTTG-3'	Nucleotide sequencing	This study	3179 – 3197
pUC/M13 FOR (17 mer)	5'-GTTTTCCCAGTCACGAC-3'	Nucleotide sequencing	Messing (1983)	
pUC/M13 REV (17 mer)	5'-CAGGAAACAGCTATGAC-3'	Nucleotide sequencing	Messing (1983)	

2.2.5 PCR amplification

The structural-protein-coding region (VP1-VP4) was amplified with the Expand™ Long template PCR system (Roche) using two oligonucleotides which prime in the 5'-non coding region (NCR1) and on the 2A/2B junction (WDA), respectively (Table 2.1; Figure 2.1). The reaction was carried out in the presence of 0.5 mM deoxynucleotides, 0.25 μM of each primer, 3 mM $MgCl_2$ and detergents (reaction buffer 3). After an initial denaturation step of 2 min at 95°C, thirty cycles of 30 sec denaturation at 94°C, annealing at 58°C for 30 sec and elongation for 60 sec at 68°C, were performed. To achieve a higher yield, the elongation time of each cycle was extended with 20 sec after 10 cycles and continued for the remaining 20 cycles. The resulting amplicon was analysed by agarose gel electrophoresis and recovered from the agarose gel with Cleanmix kit (Talent) according to the specification of the manufacturer.

2.2.6 T/A cloning

The purified amplicon (structural-protein-coding region) was cloned into the pGEM®-T Easy vector system (Promega) by means of T/A cloning (Figure 2.1). Ligation reactions were carried out for at least 48h at 4°C. Transformation reactions were carried out according to Tang and co-workers (1994). Plasmid isolations were prepared with the QIAprep Miniprep kit from QIAGEN.

2.2.7 Nucleotide sequencing and analysis

The nucleotide sequence of the cloned region was subsequently determined with T7 DNA polymerase (sequenase version 2.0, USB) as well as the ABI PRISM 377 DNA Sequencer from Perkin Elmer Applied Biosystems. Sequencing of the region was completed in both directions using internal primers (Figure 2.1), while analysis of the sequencing data was performed using the DAPSA version 2.9 program (Harley, 1994). Amino acid hypervariable plots were constructed with MEGA version 1.0.

2.3 Results and Discussion

2.3.1 Cloning and characterization of ZIM/7/83/2 P1

Using the oligonucleotides NCR1 and WDA, a 3kb fragment was amplified from the viral cDNA template of ZIM/7/83/2. The fragment (L-P1-2A) was subsequently cloned into the pGEM®-T Easy vector by means of T/A cloning. This vector contains 3'-T overhanging at the insertion site and greatly improves the efficiency of ligation with PCR products. The Expand long template PCR system generated PCR products have mainly 3'-A overhanging analogs and are therefore suitable for T/A cloning. Cloning and nucleotide sequencing strategies are explained in Figure 2.1. The nucleotide sequence of the cloned region is shown in Figure 2.2 together with the deduced amino acid sequence of the Leader and 2A proteinases as well as the structural proteins (VP1-4). The nucleic acid and deduced amino acid sequence data reported here for the P1 region, have been submitted to the GenBank nucleic acid sequence database (accession number AF136607).

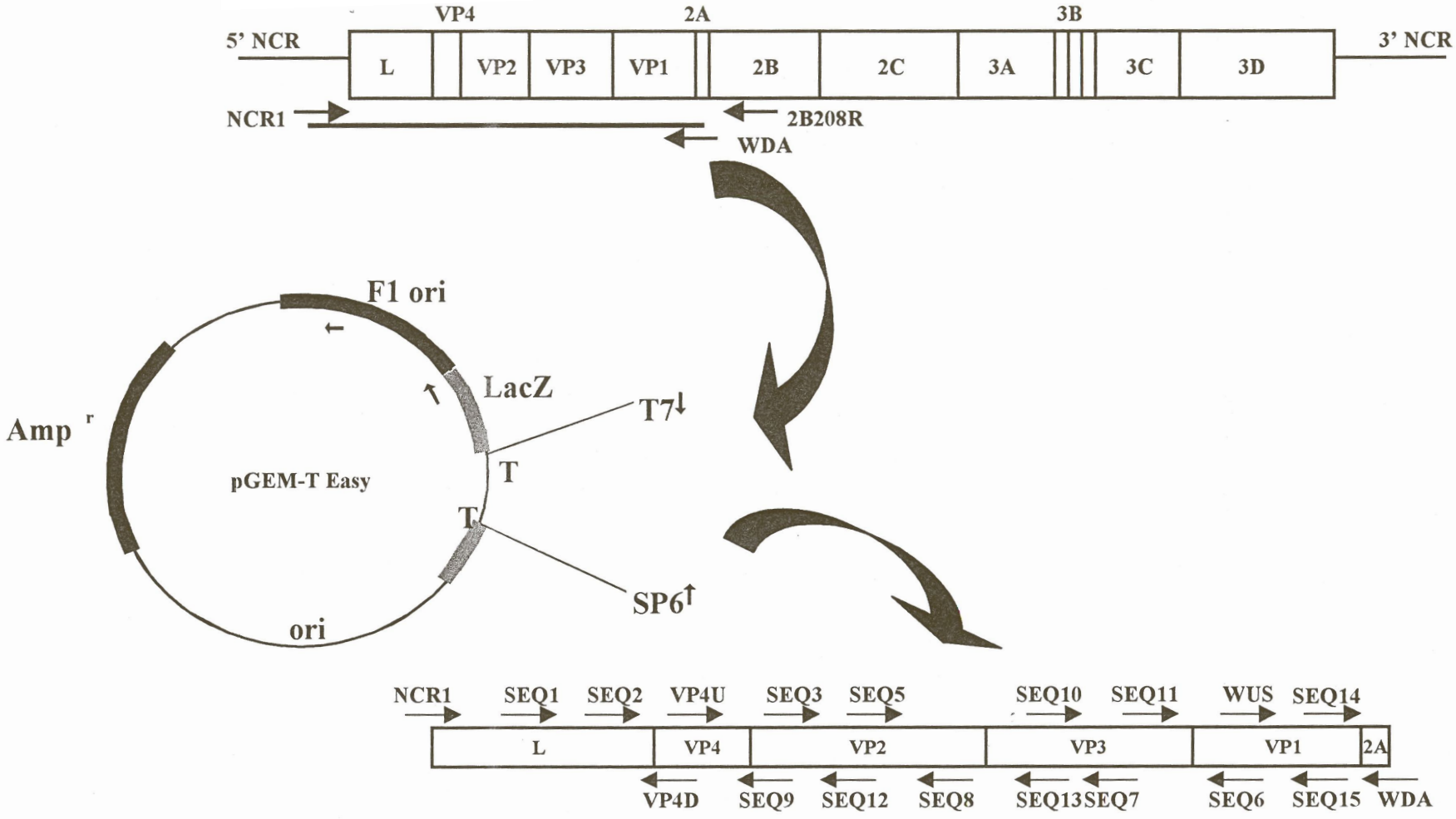

Figure 2.1: Schematic representation of the amplification of ZIM/7/83/2 P1, cloning of the amplified region into pGEM®-T Easy vector as well as the sequence strategy applied.

NCR1

1 **taacaagcgacaatcgggatct**gagaaggggaccaggagttctatcaaactgcccggtttaaaaagcttctatgcctggata

LEADER

M K T T D C F N V L

83 ggtgaccggagccggcacctttttcctttatttaaactcacttt ATG AAG ACA ACT GAC TGT TTC AAC GTT TTG

L E I I Y R F R H T F K T D R K M E F T L

156 CTC GAG ATC ATT TAC AGG TTC AGG CAC ACG TTT AAA ACA GAC AGG AAG ATG GAA TTC ACA CTC

Y N G E K K T F Y S R P N K H G N C W L N

219 TAC AAC GGA GAA AAG AAG ACC TTC TAC AGC AGG CCC AAC AAA CAC GGG AAC TGT TGG CTC AAC

S L L Q L F R Y V D E P L F E S E Y L S P

282 TCA CTT CTG CAG CTC TTT CGA TAC GTC GAT GAG CCA CTC TTT GAG TCT GAG TAC CTG TCA CCT

E N K T L D M I K Q L S D Y T K L D L S D

345 GAA AAC AAA ACA CTG GAC ATG ATC AAA CAG CTA TCT GAT TAC ACC AAA TTG GAC CTG TCA GAC

G G P P A L V L W L I K D C L Q T G V G T

408 GGA GGG CCC CCC GCT CTC GTT CTT TGG CTG ATC AAA GAT TGT CTT CAG ACT GGC GTT GGC ACC

S T R P S E I C V I N G V V M T L A D F H

471 AGC ACT CGC CCC AGC GAG ATC TGT GTC ATC AAC GGG GTT GTC ATG ACC CTG GCT GAT TTC CAC

A G I F I K G T E H A V F A L N T S E G W

534 GCC GGC ATT TTC ATC AAA GGC ACT GAA CAC GCC GTG TTC GCC CTC AAC ACA TCC GAG GGC TGG

Y A I D D E V F Y P W T P D P E N V L A Y

597 TAT GCC ATT GAT GAT GAG GTG TTC TAC CCT TGG ACA CCC GAC CCT GAA AAC GTA CTC GCG TAC

V P Y D Q E P L D V D W Q D R A G L F L R

660 GTC CCC TAC GAC CAG GAA CCA CTG GAC GTA GAC TGG CAA GAT CGC GCG GGT CTG TTC CTC CGT

VP4

G A G H S S P V T G S Q N Q S G N T G S I

723 GGA GCA GGC CAC TCA TCA CCT GTC ACA GGG TCA CAA AAC CAA TCT GGC AAT ACT GGT AGT ATC

I N N Y Y M Q Q Y Q N S M D T Q L G D N A

786 ATC AAC AAT TAC TAC ATG CAA CAG TAC CAG AAT TCA ATG GAC ACC CAA CTT GGC GAC AAC GCC

I S G G S N E G S T D T T S T H T N N T Q

849 ATC TCG GGT GGG TCC AAC GAG GGC AGC ACT GAC ACC ACG TCT ACC CAC ACA AAC AAC ACG CAG

N N D W F S K L A Q S A I S G L F G A L L

912 AAC AAT GAT TGG TTT TCA AAA TTG GCC CAG TCA GCG ATC TCG GGG CTT TTC GGA GCC CTC CTC

VP2

A D K K T E E T T L L E D R I V T T R H G

975 GCA GAC AAA AAG ACA GAG GAA ACC ACT CTG CTC GAG GAC CGC ATC GTC ACA ACA CGA CAC GGT

T T T S T T Q S S V G I T Y G Y A D A D S

1038 ACC ACC ACC TCC ACC ACA CAG AGT TCC GTT GGC ATC ACC TAC GGT TAC GCT GAC GCT GAC TCT

```
     F   R   P   G   P   N   T   S   G   L   E   T   R   V   E   Q   A   E   R   F   F
1101 TTC CGC CCC GGA CCC AAC ACA TCG GGC CTG GAG ACG CGT GTG GAA CAA GCA GAG CGG TTC TTC

     K   E   K   L   F   D   W   T   S   D   K   P   F   G   T   L   Y   V   L   E   L
1164 AAG GAA AAG CTT TTT GAT TGG ACA TCA GAC AAA CCA TTT GGC ACG CTG TAT GTT TTG GAA TTG

     P   K   D   H   K   G   I   Y   G   S   L   T   D   A   Y   T   Y   M   R   N   G
1227 CCC AAG GAC CAC AAG GGG ATC TAC GGC AGC CTG ACC GAC GCG TAT ACT TAC ATG CGC AAC GGT

     W   D   V   Q   V   S   A   T   S   T   Q   F   N   G   G   S   L   L   V   A   M
1290 TGG GAC GTC CAG GTT TCC GCC ACC AGC ACG CAG TTC AAC GGC GGG TCA CTC CTT GTG GCC ATG

     V   P   E   L   C   S   L   K   D   R   E   E   F   Q   L   S   L   Y   P   H   Q
1353 GTG CCG GAG CTG TGC TCG CTC AAG GAC AGA GAG GAG TTT CAA CTC TCT CTC TAC CCA CAC CAG

     F   I   N   P   R   T   N   T   T   A   H   I   Q   V   P   Y   L   G   V   N   R
1416 TTT ATC AAC CCA AGG ACC AAC ACC ACA GCA CAC ATC CAG GTG CCC TAC CTC GGT GTG AAC AGG

     H   D   Q   G   K   R   H   Q   A   W   S   L   V   V   M   V   L   T   P   L   T
1479 CAC GAT CAG GGC AAG CGC CAC CAG GCG TGG TCC CTG GTC GTC ATG GTC CTC ACG CCT CTC ACC

     T   E   A   Q   M   Q   S   G   T   V   E   V   Y   A   N   I   A   P   T   N   V
1542 ACC GAG GCA CAA ATG CAA TCC GGG ACT GTT GAG GTT TAC GCC AAC ATC GCC CCG ACG AAC GTC

                                    VP3
     F   V   A   G   E   K   P   A   K   Q   G   I   I   P   V   A   C   F   D   G   Y
1605 TTC GTT GCT GGC GAA AAG CCT GCG AAA CAG GGC ATC ATT CCA GTT GCC TGT TTC GAC GGC TAT

     G   G   F   Q   N   T   D   P   K   T   A   D   P   I   Y   G   Y   V   Y   N   P
1668 GGT GGA TTC CAA AAC ACC GAC CCG AAG ACC GCA GAT CCC ATC TAC GGT TAC GTG TAC AAC CCG

     S   R   N   D   C   H   G   R   Y   S   N   L   L   D   V   A   E   A   C   P   T
1731 TCT CGC AAC GAT TGT CAC GGC AGG TAC TCC AAC CTG TTG GAC GTC GCC GAG GCG TGC CCC ACT

     F   L   N   F   D   G   K   P   Y   V   V   T   K   N   N   G   D   K   V   M   T
1794 TTC CTG AAC TTT GAT GGT AAG CCC TAC GTC GTC ACC AAG AAC AAC GGC GAC AAG GTC ATG ACC

     C   F   D   V   A   F   T   H   K   V   H   K   N   T   F   L   A   G   L   A   D
1857 TGT TTT GAT GTG GCA TTC ACG CAC AAA GTT CAC AAG AAC ACG TTT CTT GCG GGC CTA GCG GAT

     Y   Y   A   Q   Y   Q   G   S   L   N   Y   H   F   M   Y   T   G   P   T   H   H
1920 TAC TAC GCC CAG TAC CAG GGT TCG CTG AAC TAC CAC TTC ATG TAC ACA GGT CCT ACT CAC CAT

     K   A   K   F   M   V   A   Y   I   P   P   G   I   E   T   D   R   L   P   K   T
1983 AAA GCA AAG TTC ATG GTT GCC TAC ATC CCA CCA GGC ATT GAG ACT GAC AGA CTG CCC AAG ACA

     P   E   D   A   A   H   C   Y   H   S   E   W   D   T   G   L   N   S   Q   F   T
2046 CCC GAG GAC GCA GCC CAC TGC TAC CAC TCG GAG TGG GAC ACA GGA CTG AAC TCC CAG TTC ACG

     F   A   V   P   Y   V   S   A   S   D   F   S   Y   T   H   T   D   T   P   A   M
2109 TTC GCC GTC CCA TAC GTC TCT GCA AGT GAC TTC TCC TAC ACA CAC ACT GAC ACC CCC GCA ATG

     A   T   T   N   G   W   V   A   V   F   Q   V   T   D   T   H   S   A   E   A   A
2172 GCA ACC ACC AAC GGC TGG GTG GCG GTG TTC CAG GTG ACT GAC ACC CAT TCG GCC GAA GCC GCT

     V   V   V   S   V   S   A   G   P   D   L   E   F   R   F   P   V   D   P   V   R
2235 GTG GTT GTG TCG GTG AGC GCT GGA CCC GAC CTG GAG TTC AGG TTC CCG GTT GAC CCA GTG CGC
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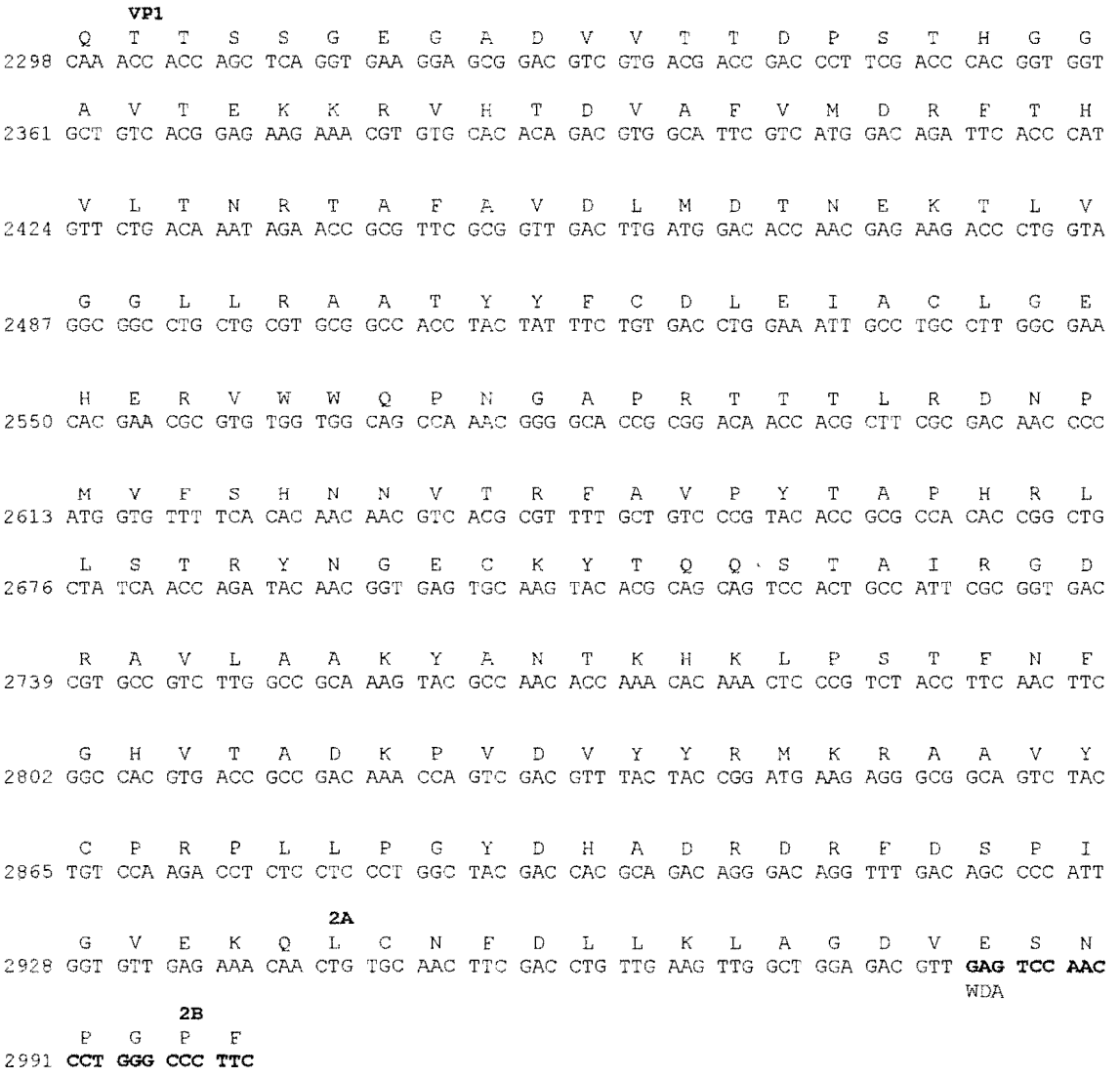


Figure 2.2: Nucleotide and deduced amino acid sequences of the LEADER and 2A proteinases as well as the structural proteins (VP1-4) of ZIM/7/83/2. The NCR1 and WDA primers used during amplification are indicated in bold.

2.3.2 Comparison of ZIM/7/83/2 P1 with known P1 regions

The deduced amino acid sequence of ZIM/7/83/2 was compared to two known SAT 2 isolates, RHO/1/48 and KEN/3/57 (Figure 2.3). Approximately 200 nucleotides on the 5'-end of VP4 of RHO/1/48 were also determined during this study. The same PCR amplification steps and T/A cloning procedures were followed as described for ZIM/7/83/2. Nucleotide sequencing of the region in question was performed with the SEQ1, SEQ2, VP4U oligonucleotides. These three viruses, all bovine outbreak strains, originate from different countries, namely Zimbabwe (ZIM/7/83), Zambia (RHO/1/48)

and Kenya (KEN/3/57), and represent different genetic lineages within the SAT 2 serotype (A.D.S. Bastos, personal communication).

Intratypic variation calculated for the individual structural proteins VP4, VP3, VP2 and VP1 of the three SAT 2 isolates amounts to 3%, 11%, 12% and 30% respectively. The VP1 region is therefore the most variable region within P1. When the intratypic variation for the P1 region and VP1 of the SAT 2 serotype is compared to that of serotypes A, O and C (Table 2.2), it is evident that the former is considerably more variable than its European counterparts. Intratypic variation for the VP1 region within the SAT 2 type (30%) is approximately two to three times higher than the variation within serotypes A, O and C.

2.3.3 Identification of hypervariable regions within the SAT 2 serotype

Field isolates of FMDV enter cells via receptor-mediated endocytosis and internalization occurs due to interaction between a protein ligand (Arg-Gly-Asp or RGD) and cell surface receptors of the integrin superfamily. Integrin receptors that have been shown to be involved in this process, include $\alpha_v\beta_3$ (Berinstein *et al.*, 1995; Jackson *et al.*, 1997; Neff *et al.*, 1998), $\alpha_5\beta_1$ (Villaverde *et al.*, 1996; Jackson *et al.*, 2000a) and $\alpha_v\beta_6$ (Jackson *et al.*, 2000b). The RGD triplet is conserved among all seven serotypes and is situated on a highly immunogenic loop in VP1. Interesting to note is the presence of an arginine residue directly after the RGD in all three SAT 2 virus sequences (Figure 2.3). This observation is in agreement with results obtained by Jackson and co-workers (2000a), who showed that this arginine residue usually follows the RGD in type SAT 2 and that these viruses have a low binding affinity for the $\alpha_5\beta_1$ receptor. In other serotypes a leucine or methionine residue usually follows the RGD and is linked to the higher binding affinity for the receptor.

The G-H loop also contains major immunodominant epitopes. Using monoclonal antibodies, these immunodominant epitopes were previously determined for RHO/1/48 (Crowther *et al.*, 1993b). One of these, valine-675 (Figure 2.3), is involved in both

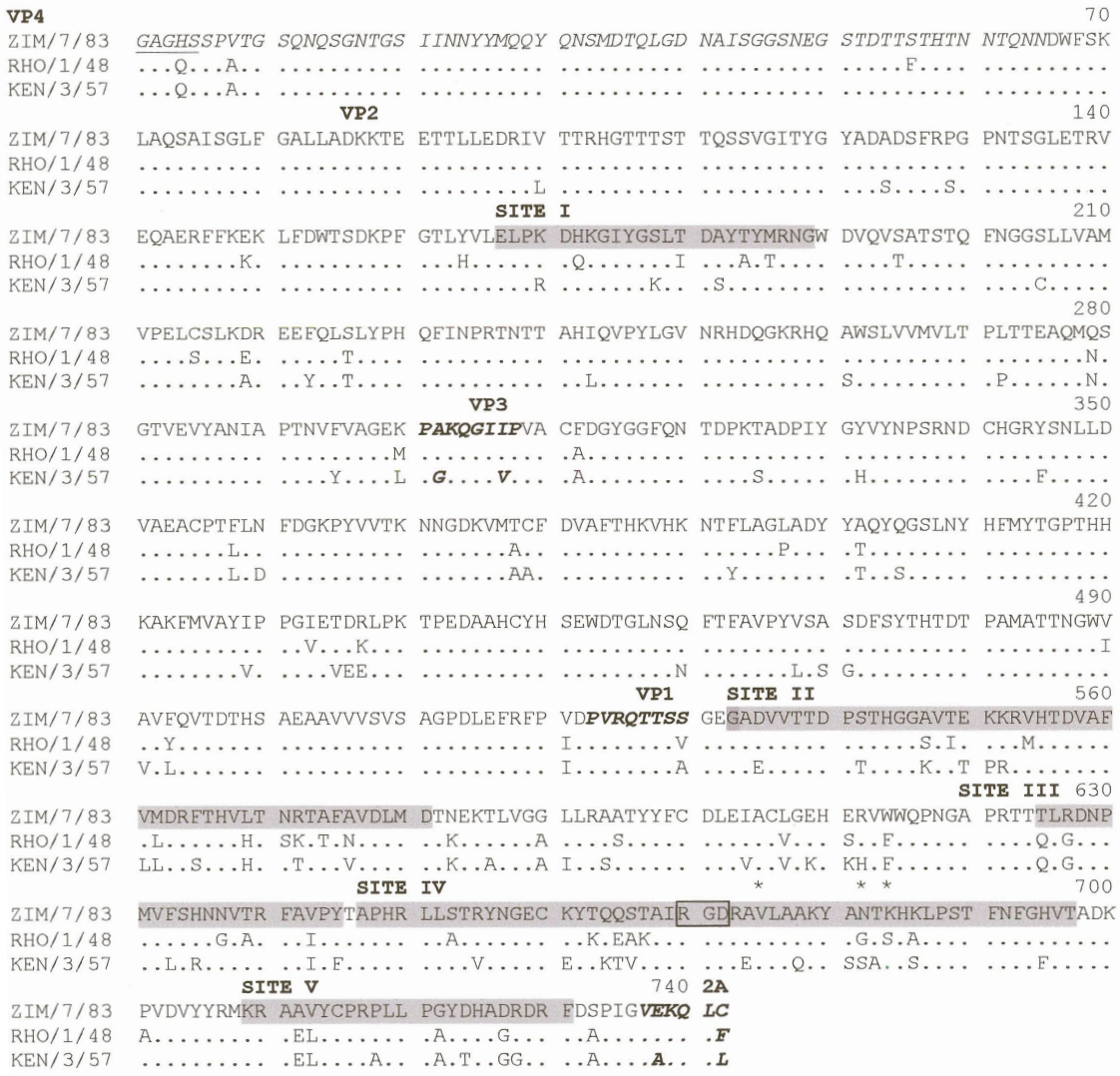


Figure 2.3: Deduced amino acid sequence comparison of ZIM/7/83 (GenBank Acc. No. AF136607) with KEN/3/57 (AJ251473) and RHO/1/48 (AJ251475). The region completed in VP4 for RHO/1/48 is indicated in italics. The protein ligand RGD is boxed. Antigenic sites for RHO/1/48 are indicated with asterisks (*). Proteolytic cleavage sites between VP2/VP3, VP3/VP1 and VP1/2A are indicated in bold and italic. The myristate binding consensus sequence in VP4 is underlined. Hypervariable regions are indicated in gray shadowing.

Table 2.2: Comparison of intraypic variation between serotypes SAT 2, A, O, and C

	Serotype SAT 2[a]		Serotype A[b]		Serotype O[c]		Serotype C[d]	
	Amino acid comparison	% variation	Amino acid comparison	% variation	Amino acid comparison	% variation	Amino acid comparison	% variation
P1	[1]ZIM/7/83+RHO/1/48	8	A_{10} + A_{12}	6	O_1Kaufbeuren + O_1Caseros	5	Cs-8c1 + C_2Uru	4
	RHO/1/48+KEN/3/57	13	A_{12} + A_{22}	8	O_1Caseros + O_2 Brescia	5	Cs-8c1+C_1Germany	7
	ZIM/7/83+KEN/3/57	13	A_{10} + A_{22}	10	O_1Kaufbeuren + O_2 Brescia	5	C_1Germany+ C_2Uru	7
	[2]Intratypic	**16**	**Intratypic**	**11**	**Intratypic**	**7**	**Intratypic**	**9**
VP1	[1]ZIM/7/83+RHO/1/48	13	A_{10} + A_{12}	12	O_1Kaufbeuren + O_1Caseros	8	Cs-8c1 + C_2Uru	9
	RHO/1/48+KEN/3/57	23	A_{12} + A_{22}	11	O_1Kaufbeuren + O_2 Brescia	10	Cs-8c1+C_1Germany	10
	ZIM/7/83+KEN/3/57	24	A_{10} + A_{22}	15	O_1Caseros + O_2 Brescia	13	C_1Germany+ C_2Uru	10
	[2]Intratypic	**30**	**Intratypic**	**18**	**Intratypic**	**14**	**Intratypic**	**10**

[1]Pair-wise comparisons of isolates.
[2]Total intratypic variation within serotype.
[a]SAT 2 intratypic variation calculated for ZIM/7/83 (AF136607), RHO/1/48 (AJ251475) and KEN/3/57 (AJ251473).
[b]Serotype A intratypic variation calculated for A_{10} (X00429), A_{12} (M10975) and A_{22} (X74812).
[c]Serotype O intratypic variation calculated for O_1Kaufbeuren (X00871), O_1 Caseros (U82271) and O_2Brescia (M55287).
[d]Serotype C intratypic variation calculated for Cs-8c1 (M60118), C_1Germany/26 (M90368), C_2Uruguay/44 (M90367).

conformational and non-conformational epitopes and is conserved in all three strains. In contrast, an immunodominant site present at residues 682 and 684 was found to be highly variable within the SAT 2 serotype.

Using an amino acid hypervariable plot constructed with MEGA 1.0, five independent hypervariable regions were determined. The region in VP2 (167–189) corresponds with immunodominant site 2 of serotype O (Kitson *et al.*, 1990) as well as immunodominant site D of serotype C (Mateu *et al.*, 1990). The second hypervariable region (533-581) corresponds with immunodominant site 3 of serotype O (Kitson *et al.*, 1990). Residues 647 to 697 contain the G-H loop, while residues 709 to 731 contain the C-terminus of VP1. Both regions have previously been shown to contain major immunodominant sites for the other serotypes (Mateu, 1995). This is however only an indication of hypervariability within the SAT 2 serotype and the actual immunodominant sites need to be determined empirically, e.g. through escape mutant studies.

2.3.4 Description of ZIM/7/83/2 cleavage sites

Although the 3C proteinase cleavage sites for the FMDV P1 region will be discussed in more detail in Chapter 3, it is interesting to note some differences and similarities between types A and SAT 2. The P1 region is cleaved by the 3C proteinase to produce VP0 (E/G), VP3 (Q/T) (Belsham, 1993) and VP1 (Q/) (N. Knowles, personal communication), according to the A_{12} sequence (Robertson *et al.*, 1995; Baxt *et al.*, 1989). The cleavage site between VP0 and VP3 of A_{12} contains an acidic glutamic acid (E), while a polar glutamine (Q) residue is found instead in the SAT 2 serotype. The VP3/VP1 as well as the VP1/2A junction sites of the SAT 2 serotype corresponds with that of A_{12}. It is interesting to note that the flanking regions, which could play a role in the recognition of the cleavage site by the proteinase, are not highly conserved within the SAT 2 serotype. These differences could alter the structure of the cleavage site and have implications in the construction of recombinant viral particles considering that a 3C proteinase from a particular serotype may not be able to optimally process the structural proteins of a different viral serotype.

2.4 Conclusions

As has been shown previously and verified again in this study, the VP1 region is the most variable within the P1 region (Palmenberg, 1989). Therefore, an alternative option in the construction of chimeric viruses could be to exchange the VP1 region only. Advantages would be the exclusion of proteolytic cleavage sites, as well as the simplification of the PCR-cloning steps due to the smaller size of the domain to be exchanged. Retaining all the other structural proteins may also decrease the likelihood of the recombinant particle being less stable than the wild type vaccine isolate. However, it is known that both conformational and non-conformational epitopes exist for FMDV (Crowther *et al.*, 1993a; Mateu, 1995). Thus, the expected immune response may not be achieved if only VP1 is exchanged, due to altering of immunodominant sites upon assembly of the virion. Towards the construction of infectious chimeric viruses, it is imperative to investigate the heterogeneity in the FMDV proteinases and thus to understand the role of proteolytic variation and cleavage site differences in isolates originating from different geographical localities.

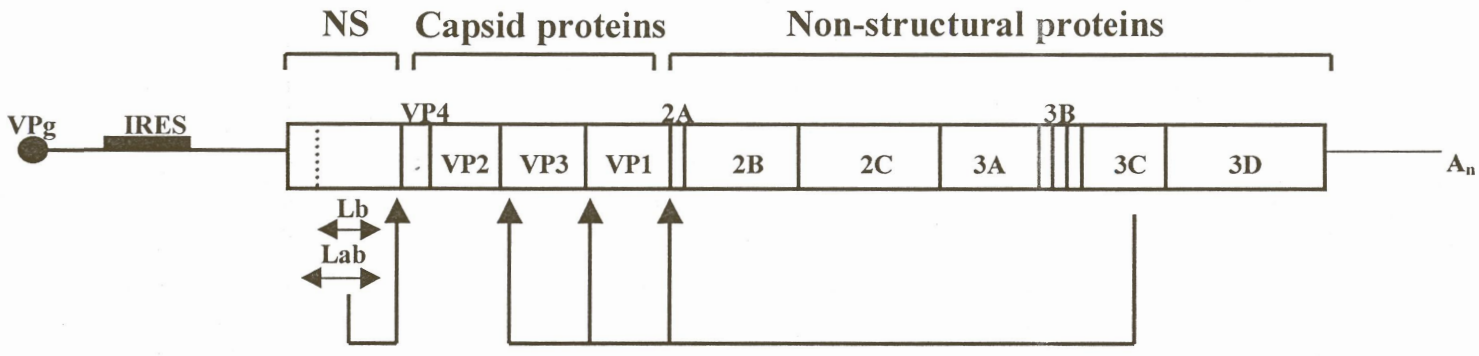
CHAPTER 3

Genetic heterogeneity in the foot-and-mouth disease virus leader and 3C proteinases

3.1 Introduction

The positive strand RNA genome of the foot-and-mouth disease virus is translated into a polyprotein which undergoes several proteolytic processing events to produce three polyprotein precursors P1, P2 and P3 (Domingo *et al.*, 1990; Belsham, 1993). The capsid proteins, which determine the antigenic characteristics of the virus, form part of the P1-2A polyprotein precursor (Figure 1.2, Chapter 1). The precursor is subsequently processed to produce the mature structural or capsid proteins, VP1, VP2, VP3 and VP4, which are assembled into virions (Figure 3.1). The precursor is obtained following cleavage at the L/P1 junction by the Leader proteinase (L^{pro}) (Strebel & Beck, 1986) as well as the 2A/2B junction by the 2A proteinase (Ryan *et al.*, 1991). The mechanism of the latter cleavage mediated by the 18 amino acid 2A region, is however not clear. The P1-2A precursor is then cleaved by the 3C proteinase ($3C^{pro}$) to produce VP0, VP3 and VP1. In addition to the viral protein processing, the $3C^{pro}$ of FMDV cleaves the host-cell protein histone H3 and may be involved with shut-off process of host-cell transcription (Falk *et al.*, 1990; Tesar & Marquardt, 1990).

The Leader proteinase acts both intra-and intermolecularly. Intramolecularly cleavage involves, as mentioned before, the cleavage of the L/P1 junction (RKLK↓GAGN) (Kirchweger *et al.*, 1994). During virus replication, L^{pro} specifically cleaves a host cell protein, eukaryotic initiation factor 4G (eIF-4G) (Devaney *et al.*, 1988). This event impairs the ability of the host cell to translate its own capped mRNAs. Characteristic of L^{pro} and conserved in all seven serotypes is the presence of two initiation sites (Sanger *et al.*, 1987). Both forms Lab and Lb, exhibit the processing functions (Medina *et al.*, 1993; Cao *et al.*, 1995). The latter function (eIF-4G processing) is



Structural / capsid proteins: **Non-structural proteins:**

VP4 = 1A **Leader = proteinase**

VP2 = 1B **2A = proteinase**

VP3 = 1C **3C = proteinase**

VP1 = 1D **3D = RNA - dependent RNA polymerase**

Figure 3.1: Proteolytic processing of the foot-and-mouth disease vial P1 region. Cleavage by the Leader (L/P1) and 3C proteinases (VP2/VP3; VP3/VP1; VP1/2A) are indicated.

executed in other picornaviruses (e.g. entero- and rhinovirus) by the 2A proteinase (Ryan & Flint, 1997). Different cleavage sites are used to cleave the eIF-4G by the Leader and 2A proteinases and are only seven amino acids apart (Kirchwegen *et al.*, 1994). Evidence for the cleavage of eIF-4G as well as eIF-4A by the 3C proteinase was recently obtained (Belsham *et al.*, 2000).

It was previously proposed, through amino acid sequence alignments, that the Leader proteinase of FMDV is a cysteine proteinase (Gorbalenya *et al.*, 1991). This hypothesis was confirmed by site-directed mutagenesis (Piccone *et al.*, 1995b; Roberts & Belsham, 1995) indicating that Cys^{51} and His^{148} are the active site residues. Recently, the crystal structure of the FMDV Leader proteinase from O_1Kaufbeuren was determined (Guarné *et al.*, 1998), confirming Cys^{51} and His^{148} to be the catalytic residues, situated on top of a deep cleft. Asp^{163} was observed to be the third member of the catalytic triad and is responsible for the correct orientation of His^{148} with respect to the nucleophilic cysteine. The structure of the FMDV Leader proteinase catalytic domain is reminiscent of that of the cysteine proteinase superfamily. The corresponding residues forming the catalytic triad in the cysteine proteinase, papain, are Cys^{25} – His^{159} – Asn^{175} (Ryan & Flint, 1997). It was also seen that the Leader proteinase contains a C-terminal extension, which is positioned in relation to the catalytic domain to support the intramolecular processing (Guarné *et al.*, 1998).

Using amino acid sequence alignments, it could be shown that picornaviral 3C proteinases belongs to the family of sub-class (trypsin-like) cellular serine proteinases, indicating a chymotrypsin-like fold (Ryan & Flint, 1997 and references therein). The catalytic triad was predicted to be (according to the nomenclature presented here): His^{46}-Asp^{84}-Cys^{163}. The most interesting finding was that of the nucleophilic residue being a cysteine residue, rather than a serine residue (Gorbalenya *et al.*, 1989b). This catalytic triad was confirmed for FMDV with site-directed mutagenesis (Grubman *et al.*, 1995). The crystal structures for human rhino virus-14 (Matthews *et al.*, 1994) and hepatitis A virus (Allaire *et al.*, 1994; Bergmann *et al.*, 1997) 3C proteinases confirmed the chymotripsin-like fold for the picornaviral 3C proteinases.

It is a well established fact that RNA viruses, due to their error-prone RNA replication, have a high mutability rate and therefore have a high level of genomic variation (Holland *et al.*, 1982; Domingo *et al.*, 1990). Genetic characterization of the capsid proteins, especially the VP1 protein, has been the focus of attention. This protein contains important determinants for FMDV neutralization, the G-H loop and C-terminus regions and has been established as a useful tool in the determination of genetic relationships. Previously, it could be shown with amino acid sequence comparisons of the capsid proteins (Palmenberg, 1989) that the South African Territories (SAT) type viruses are genetically different from the European (A, O and C) types. These viruses also show a higher level of intratypic variation than the European types as was shown and discussed in Chapter 2.

Genetic characterization of the non-structural regions of the genome, specifically the Leader and 3C proteinases, are limited and restricted to only a few isolates (Ryan & Flint, 1997). To understand the gene heterogeneity of the Leader and 3C proteinases and their potential impact on the construction of chimeric viruses, representatives of the six FMDV serotypes that occur on the African continent (A, O, C, SAT 1, 2 and 3), were investigated.

3.2 Materials and Methods

3.2.1 Viruses investigated

A list of viruses used in this study is given in Table 3.1. Those indicated in bold were used to generate additional sequencing data needed for the investigation. Viral isolations were performed on oesophageo-pharyngeal specimens (probangs) according to standard procedures at the Onderstepoort Veterinary Institute – Division for Exotic Diseases. These probangs were obtained from buffalo (*Syncerus caffer*) during routine surveys in game parks in southern and eastern Africa. Additional viruses were obtained from the World Reference Laboratory at Pirbright as well as the Botswana Vaccine Institute in Gabarone, Botswana.

Table 3.1: Description of viruses used to assess genetic heterogeneity in the Leader and 3C proteinases

Viruses	Country of origin	Specie	Year of isolation	GenBank Acc. No. (L)	GenBank Acc. No. (3C)
A_{12}	United Kingdom	Bovine	1932	M10975	M10975
A_{10}*	Holland	Bovine	1942	**AF283430**	NA
A_{22}	USSR	Bovine	1965	X74812	X74812
K/37/84/A*#	Kenya	Bovine	1984	**AF283433**	**AF283447**
KEN/1/76/A*	Kenya	Bovine	1971	**AF283432**	NA
GAM/51/98/A*	Gambia	NA	1998	**AF283431**	**AF283446**
O_1Kaufbeuren	Germany	Bovine	1966	X00871	X00871
O_1Campos	Brazil	Bovine	1958	M95781	NA
O_5India*	India	Bovine	1962	**AF283435**	**AF283449**
KEN/1/91/O	Kenya	Bovine	1991	NA	**AF283450**
BKF/2/92/O*	Burkina Faso	Bovine	1992	**AF283434**	**AF283448**
C_1Oberbayen	Germany	NA	1966	X00130	NA
C_1Noville	Switzerland	NA	1965	L19600	**AF283451**
C_1-S8	Spain	Swine	1970	L19601	NA
C_3Resende*	Brazil	Bovine	1965	NA	**AF283452**
ZIM/3/88/1*	Zimbabwe	Buffalo	1988	**AF283437**	**AF283454**
ZIM/3/96/1‡	Zimbabwe	Buffalo	1996	**AF283438**	**AF283455**
KNP/196/91/1‡	South Africa	Buffalo	1991	**AF283436**	**AF283453**
UGA/1/97/1‡	Uganda	Buffalo	1997	**AF283439**	**AF283456**
TAN/1/99/1	Tanzania	Bovine	1999	NA	**AF283457**
KEN/3/57/2	Kenya	Bovine	1957	AJ251473	AJ251473
ZIM/7/83/2†	Zimbabwe	Bovine	1983	**AF283442**	**AF283459**
KNP/19/89/2‡	South Africa	Buffalo	1989	**AF283440**	**AF283458**
ZIM/14/90/2‡	Zimbabwe	Buffalo	1990	**AF283441**	NA
BEC/1/65/3*	Botswana	Bovine	1965	**AF283443**	**AF283460**
KNP/10/90/3‡	South Africa	Buffalo	1990	**AF283444**	**AF283461**
ZAM/4/96/3*	Zambia	Buffalo	1996	**AF283445**	NA

NA – Not available

*World Reference Laboratory, Pirbright, UK

†Botswana Vaccine Institute, Gabarone

‡Onderstepoort Veterinary Institute – Exotic Diseases Division

#Nomenclature used by Embakasi Laboratory, Nairobi, Kenya

3.2.2 RNA extraction, cDNA synthesis and PCR amplification

A modified guanidinium thyocianate/silica method from Boom and co-workers (1990) was used for the rapid extraction of viral RNA. To facilitate the amplification of the region encoding the Leader proteinase, the viral RNA was reverse transcribed using AMV-RT (Promega) and an oligonucleotide (5'-CTACCAGTATTGCCAGATTG-3') which targets the 5' region of VP4 (VP4D). The Leader coding region was subsequently amplified with the VP4D and NCR1 oligonucleotides (Table 2.1), using DynaZyme (Finnzymes). cDNA synthesis for the 3C genomic region was performed using an oligonucleotide situated in the 3D region, binding to nucleotides 6287 to 6304 on KEN/3/57/2 (AJ251473) (3Cminus): 5'-CGCTCTTCMACATCTCT-3'. Amplification of the 3C genomic region was obtained with 3Cminus and an oligonucleotide situated in the 3B region, binding to nucleotides 5574 to 5593 on KEN/3/57/2 (AJ251473) (3Cplus: 5'-CCRGTGAAGAAGCCTGTCGC-3'), again using DynaZyme. Oligonucleotides, 3Cminus and 3Cplus, were designed using nucleotide sequences of A_{10} (X00429), A_{12} (M10975), A_{22} (X74812), O_1 (X00871) and KEN/3/57/2 (AJ251473). Reactions were carried out in the presence of 1.5 mM $MgCl_2$ and nucleotide and oligonucleotide concentrations of 0.2 mM and 0.5 pmol, respectively. After an initial denaturing step of 2 minutes at 94°C, thirty cycles of denaturing at 95°C for 45 sec, followed by an annealing step at 55°C for 30 sec and an elongation step at 72°C for 90 sec, were performed. Altered annealing temperatures for a few isolates had to be used to refine amplification. The resulting fragments were analysed by agarose gel electrophoresis and recovered from the agarose gel with Cleanmix kit (Talent) according to the specification of the manufacturer to be characterized molecularly.

3.2.3 Nucleotide sequence determination and analysis

The nucleotide sequence of the 750bp (Leader proteinase) and 680bp (3C proteinase) fragments were determined using the ABI PRISM 377 DNA Sequencer from Perkin Elmer Applied Biosystems and T7 DNA polymerase (sequenase version 2.0, Amersham). Analysis of sequencing data was performed with the DAPSA version 2.9 program (Harley, 1994). Amino acid sequence alignment of the 3C proteinases with hepatitis A 3C proteinase (GenBank Acc. No.X70845) was done with CLUSTALW.

All nucleotide sequences determined in this study have been submitted to GenBank under the accession numbers indicated in Table 3.1.

3.2.4 Determination of gene relationships for FMDV Lab and 3C proteinase coding regions

The analysis for the Lab^{pro} and $3C^{pro}$ coding regions was carried out on the full-length nucleotide region (L=618 nt; 3C=639 nt). The corresponding regions of equine rhinitis type A virus (GenBank Acc. No. X96870) and hepatitis A virus (GenBank Acc. No. X70845) were selected as outgroups for Lab^{pro} and $3C^{pro}$ analysis, respectively. Gene relationships were established for the full data set (Lab^{pro}: N=25 and $3C^{pro}$: N=21) using the neighbor-joining method (*p*-distances) included in MEGA version 1.0 (Kumar *et al.*, 1993).

3.2.5 Structural modeling of FMDV Lb proteinase

ZIM/7/83/2 Lb amino acid sequence was aligned with that of O_1Kaufbeuren with ClustalX. This alignment was used for homology modeling of the ZIM/7/83/2 structure employing MODELLER version 4.0 (Sali & Blundell, 1993) on a Silicon Graphics Power Indigo2 Extreme. The approach is based on the satisfaction of spatial restraints. The co-ordinates for the crystal structure of O_1Kaufbeuren Lb^{pro} was generously provided by Dr. Alba Guarné (NIH, Washington) and used as template for homology modeling. The modeled structure was verified with PROCHECK version 3.4.4 (Laskowski *et al.*, 1993) and compared to the template with GRASP version 1.3.6 (A. Nicholls, Columbia University) and WHAT-IF (Vriend, 1990).

3.2.6 Determination of FMDV P1 cleavage sites mediated by the 3C proteinase

Representatives of the six serotypes were chosen to compare the 3C proteinase cleavage sites within the P1 region. Amino acid sequence data were obtained from GenBank with the exception of KNP/196/91/1, determined as discussed in Chapter 2, and KNP/10/90/3 (L.E. Heath, unpublished results).

3.3 Results and Discussion

3.3.1 Gene heterogeneity and gene relationships of Lab proteinases

Twenty four representatives of the six FMDV serotypes that occur on the African continent, were chosen for this study (Table 3.1). Representatives for types A, O and C were taken from Europe, Asia, Brazil, West and East Africa while representatives of the SAT types originate from southern and eastern Africa. As expected, the catalytic triad of the proteinase (Cys52-His149-Asp164), as well as the Lab and Lb forms of the protein (Figure 3.2) are conserved. A difference in length in the Lab form was detected. A type A isolate from Kenya (K/37/84) is 202 amino acids in length, whilst another type A isolate from Kenya (KEN/1/76) is the same length (201 residues) as the European type A, O and C's. All the SAT types are three residues shorter (199), the same length as the type A and O isolates from West Africa.

In Figure 3.2 the secondary structural motifs of Lb^{pro} are given as previously indicated by Guarné and co-workers (1998). Alpha helices 1 and 4 are well conserved, while helices 2 and 3 display a higher level of variation. With the exception of β4, the β-sheets are generally conserved. A high level of variation is observed in the C-terminus extension (CTE), though. The SAT types vary considerably from types A, O and C in this region, although these differences seem to be clustered. The two isolates from East Africa (UGA/1/97/1 and KEN/3/57/2) differ in the CTE region not only from types A, O and C, but also from the SAT type viruses. The cleavage site with VP4 (Lys/Gly) of these two isolates corresponds however with types A, O and C, while that of the southern African SAT types differ: Arg/Gly.

A neighbor-joining analysis depicting Lab proteinase gene relationships (Figure 3.3) between the six different serotypes, generated a phylogenetic tree with 2 major lineages. The first major group (lineage I, blue) comprises of types A, O and C as well as two SAT isolates from East Africa. The European types O and C group according to serotype, while the two isolates from Asia (A_{22} and O_5India) group with a type A isolate (KEN/1/76) from Kenya and a type O (BKF/2/92) isolate from West Africa to form a sub-cluster within lineage I. Another very interesting and unexpected grouping

```
                                            ββ1ββ   ββ2ββ          αααα1ααα
                Lab                         Lb                        *   60
O1Kaufbeuren    MNTTDCFIALVQAIREIKALFLSRTTG-KMELTLYNGEKKTFYSRPNNHDNCWLNAILQL
O1Campos        ......................P.....................................
O5India         .T........LH.L....T......Q.....F.......................T....
BKF/2/92/O      ..................L.LKGLK--....F................N......T....
A12             ....N......H.....R.F....A......F......R................T....
A10Holland      ....N......YL.....T..R.........F..H....................T....
A22             ..........LY.L.....FL....Q..............................T....
K/37/84/A       ...........R....F.I..SL.PLAR...F.....I.........K............
KEN/1/76/A      ..........LR.F....T.....VR.....F............................
GAM/51/98/A     .................RL.LKGSK--....F..H............R.......T....
C1Noville       .........V.N.....R....P........F..HD....V..............T....
C-s8c1          .........V.N..K.VR....P..A.....F..HD....V..............T....
C1Oberbayen     .......T.V.N.....R....P........F..HD....V.......Q......T....
ZIM/3/88/1      .K.....DV.LEIFHRFRQT.K---.DR...F...............T.G.....SL...
ZIM/3/96/1      .K..G..DV.IEIAHRLRQ.NK---.DR...F...............T.G.....SL...
KNP/196/91/1    .K.....SV.FEIFHRLRHT.K---.ER...F......R........K.G.....SL...
UGA/1/97/1      ..................S..R.S---R...F.......................T....
KEN/3/57/2      ..................L..KGI---R..K................S.......T....
ZIM/7/83/2      .K.....NV.LEI.YRFRHT.K---.DR...F...............K.G.....SL...
ZIM/14/90/2     .K.....NV.AEIFHRL.QINK---.DR...F..H............T.G.....SL...
KNP/19/89/2     .K.....DV.FEIFHRFRHT.K---.ER...F...............K.G.....SL...
BEC/1/65/3      .K.....NV.FEIFHRFGQT.K---ADR...F...............T.G.....SL...
KNP/10/90/3     .K.....NV.LETFHRFRNV.K---.DR...F.....D.........T.G.....SL...
ZAM/4/96/3      .K.....D..LE.FHRLRQT.K---.DR...F...............R.G.....SL...


                αααα     αα2αα     αααααα3αααααα       ααα4ααα         ββ3ββ
                                                                         120
O1Kaufbeuren    FRYVEEPFFDWVYSSPENLTLEAIKQLEDLTGLELHEGGPPALVIWNIKHLLHTGIGTAS
O1Campos        ..............T.............................................
O5India         ....D........D..........R...EI..............................
BKF/2/92/O      ....D........N..........D...KV..............................
A12             ....D........N.......A......E.......................Q.......
A10Holland      ....D........N.......D....VGK.H.....................Q.......
A22             ....D........D......C...R...EI..............................
K/37/84/A       ....D........E......IQ..G...E....D.R........V...............
KEN/1/76/A      ....D....E...D......V...R...E..........................V....
GAM/51/98/A     ....D........D.......Q..E...E..........................V.S..
C1Noville       ....D........N..............E......R........................
C-s8c1          ....D........N..............E......R........................
C1Oberbayen     ....D........N..............E......R........................
ZIM/3/88/1      ....D..L.ESE.L....K..DM....S.Y.K.D.SD.......L.L..DC.Q..V..ST
ZIM/3/96/1      ....D..L.ESE.L....K..DM....S.Y.K.D.SD.......L.L..DC.Q..V..ST
KNP/196/91/1    ....D..L.ESE.L....K..DM....S.Y.K.D.SD.......L.L..GC.Q..V..ST
UGA/1/97/1      ....D........N.......Q..E...E.......................Y.......
KEN/3/57/2      ....D........N.......R..E...E......R................Y.......
ZIM/7/83/2      ....D..L.ESE.L....K..DM....S.Y.K.D.SD.......L.L..DC.Q..V..ST
ZIM/14/90/2     ....D..L.ESE.L....K..DM....S.Y.K.D.SD.......L.L..DC.Q..V..ST
KNP/19/89/2     ....D..L.ESE.L....K..DM....S.Y.K.D.SD.......L.L..DC.Q..V..ST
BEC/1/65/3      ....D..L.ESE.L....K..DM....S.Y.K.D.SD.......L.L..DC.Q..V..ST
KNP/10/90/3     ....D..L.ESE.L....K..DM..R.S.Y.K.D.SD.......L.L..DC.Q..V..ST
ZAM/4/96/3      ....D..L.ESE.L....K..DM....S.Y.K.D.SD.......L.L..DC.Q.....ST
```

```
                    ββ4ββ        βββ5βββ    βββ6βββ/ββ7βββ  βββ8ββ      βββ9
                                            *                *              180
O1Kaufbeuren        RPSEVCMVDGTDMCLADFHAGIFLKGQEHAVFACVTSNGWYAIDDEDFYPWTPDPSDVLV
O1Campos            ..............................................................
O5India             ..........................................K...................
BKF/2/92/O          ..........................H...................................
A12                 ..........................E...................................
A10Holland          ..........S............M.............D.......................
A22                 ..................................D.......................
K/37/84/A           ..................................E.......................
KEN/1/76/A          ......................V...............................
GAM/51/98/A         ..........................V.......L...........E.............
C1Noville           ........................M.....................................
C-s8c1              ........................M..R..................................
C1Oberbayen         ........................M.....................................
ZIM/3/88/1          ....I.VIN.VT.T.........I..T......LN..E........V........EN..A
ZIM/3/96/1          ....I.VIN.VV.T.........I..T......LN..E........V........EN..A
KNP/196/91/1        ....I.VIN.VT.T.........I..T......LN..E........V........EN..A
UGA/1/97/1          ..........................T.......L..D.....................C
KEN/3/57/2          ........................M..T......LL..D.....................C
ZIM/7/83/2          ....I.VIN.VV.T.........I..T......LN..E........V........EN..A
ZIM/14/90/2         ....I.VIN.VT.T.........I..T......LN..E........V........EK..A
KNP/19/89/2         ....I.VIN.VT.T.........I..T......LN..E........V........EN..A
BEC/1/65/3          ....I.VIN.VV.T.........I..T......LN..E........V........EN..A
KNP/10/90/3         ....I.VIN.VV.T.........I..T......LN..E........V........EN..A
ZAM/4/96/3          ....I.VIN.VV.T.........I..T......LN..E........V........EN..A

                    βββ↛CTE
                                                                VP4
O1Kaufbeuren        FVPYDQEPLNGEWKAKVQRKLK 201                  GAGQ
O1Campos            ...................... 201                  ....
O5India             ..................KR.. 201                  .T.R
BKF/2/92/O          ..............T...KR.. 199                  ....
A12                 ............G...N...... 201                  ....
A10Holland          ............D..TL...... 201                  ....
A22                 ..................KR.. 201                  ....
K/37/84/A           ..............T...K.... 202                  ....
KEN/1/76/A          ...................R.. 201                  ...H
GAM/51/98/A         .........S....T...K.... 199                  ...R
C1Noville           ..........EG...N...... 201                  ....
C-s8c1              ..........EG...S...... 201                  ....
C1Oberbayen         ..........EG...N...... 201                  ....
ZIM/3/88/1          Y........DVD.QDRAGLF.R 199                  ....
ZIM/3/96/1          Y........DVD.QDRAGLF.R 199                  ....
KNP/196/91/1        Y........DVD.QGRAGLF.R 199                  ....
UGA/1/97/1          .....M..F..DAIV.ATAYV. 199                  ....
KEN/3/57/2          .....M..F..SSI..ATAYV. 199                  ....
ZIM/7/83/2          Y........DVD.QDRAGLF.R 199                  ...H
ZIM/14/90/2         Y........DVD.QDRAGLF.R 199                  ....
KNP/19/89/2         Y........DVD.QDRAGLF.R 199                  ....
BEC/1/65/3          Y........DVD.QDRAGLF.R 199                  ....
KNP/10/90/3         Y........DVD.QDRAGLF.R 199                  ....
ZAM/4/96/3          Y........DVD.QDRAGLF.R 199                  ....
```

Figure 3.2: Amino acid sequence alignment of Leader proteinases. Both the Lab and Lb forms of the proteinase are indicated as well as the first four amino acid residues of VP4. The catalytic residues are indicated with an asteriks (*). Secondary structural motifs (α-helices and β-sheets are indicated according to Guarné and co-workers (1998)).

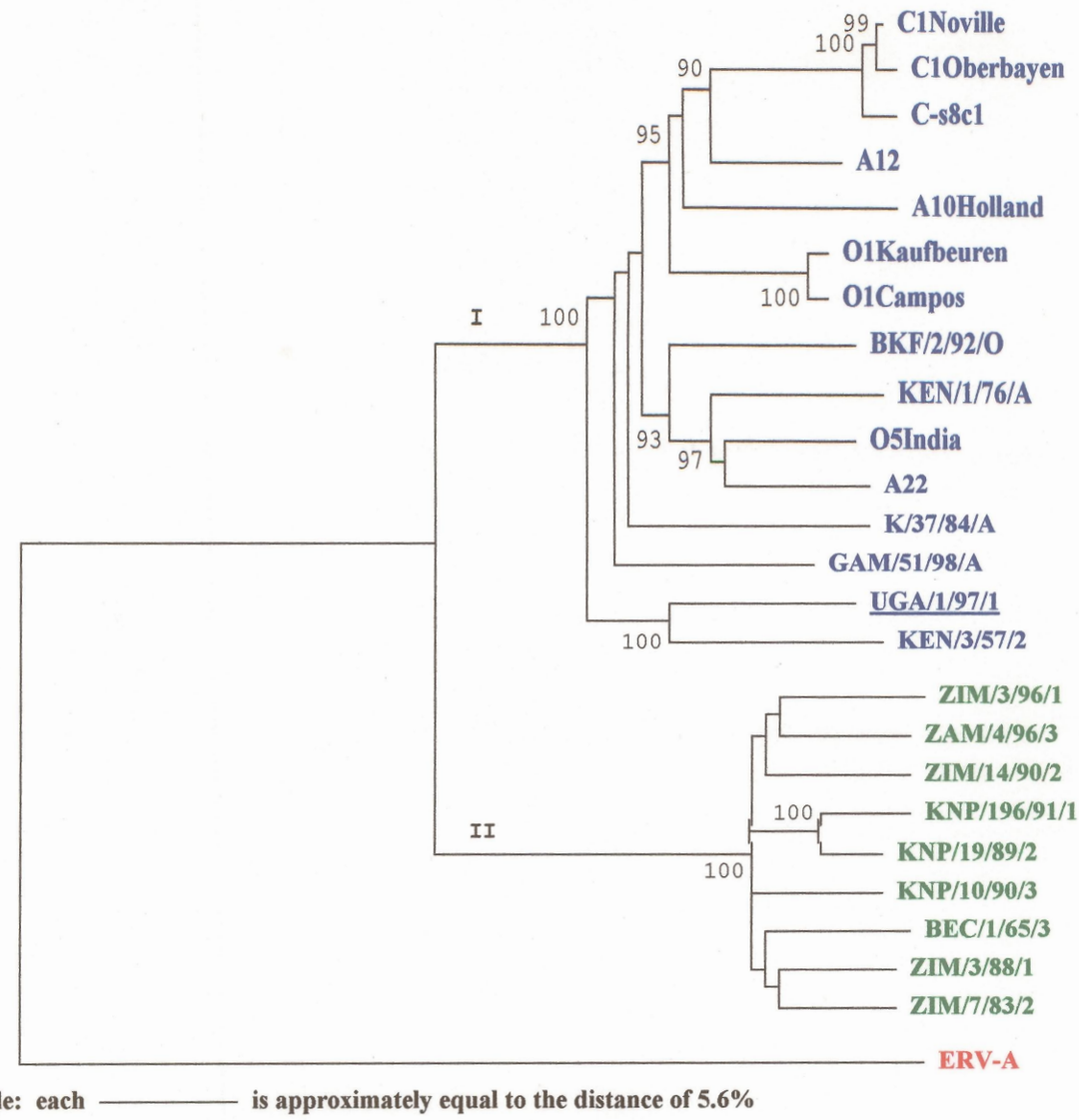


Figure 3.3: Neighbor-joining tree (*p*-distances) depicting Lab^{pro} gene relationships. Bootstrap values > 90 and based on 1000 bootstrap replications are shown with the equine rhinitis virus type A (ERV-A) Leader proteinase selected as outgroup. The two different lineages (I and II) are indicated in blue and green, respectively.

in this lineage, is that of the two East African SAT types (UGA/1/97/1 and KEN/3/57/2). Lineage II (green) contains all the southern African SAT types with no apparent phylogenetic sub-structuring, except for the grouping of a SAT 1 isolate (KNP/196/91) and a SAT 2 isolate (KNP/19/98) from the Kruger National Park, South Africa. The amino acid variation in lineage I (36%) was found to be much higher than the variation in lineage II (10%). This observation is unexpected, as the structural proteins of the SAT types are two to three times more variable than that of types A, O and C. Overall amino acid identity for the 24 viruses investigated, amounts to 58%.

3.3.2 Structural model for ZIM/7/83/2 Lb proteinase

The impact of the amino acid differences between lineages I and II (Figure 3.3) on the structure of the Leader proteinase, was investigated by constructing a three-dimensional model for a representative of lineage II (ZIM/7/83/2) (F. Joubert, Dept. Biochemistry, UP). A comparison with the previously elucidated crystal structure of O_1Kaufbeuren Leader proteinase is given in Figure 3.4. Despite an identity on amino acid level of only 64% between the two proteinases, the three-dimensional fold is completely conserved. The only difference between the two structures appears to be in the C-terminus extension (CTE). It is evident from amino acid comparisons in this region (Figure 3.2) that a high level of substitutions is present between the two lineages. This was also observed for the two East African SAT isolates (UGA/1/97/1 and KEN/3/57/2) within lineage I.

Previously RKLK↓GAGN was established to be the cleavage site of L^{pro} from types A, O and C. Results generated in this study indicated that the corresponding cleavage site for L^{pro} from southern African SAT types differs from the other types and is cleaved at: LFLR↓GAGN. The processing site for UGA/1/97/1 was found to correspond with the previously determined KEN/3/57/2: AYVK↓GAGN. In general these differences in the CTE region are not expected to have major implications on the three-dimensional structure of the proteinase, which are confirmed with the ZIM/7/83/2 L^{pro} model. A possible exception is the presence of the phenylalanine residue in the SAT isolates from

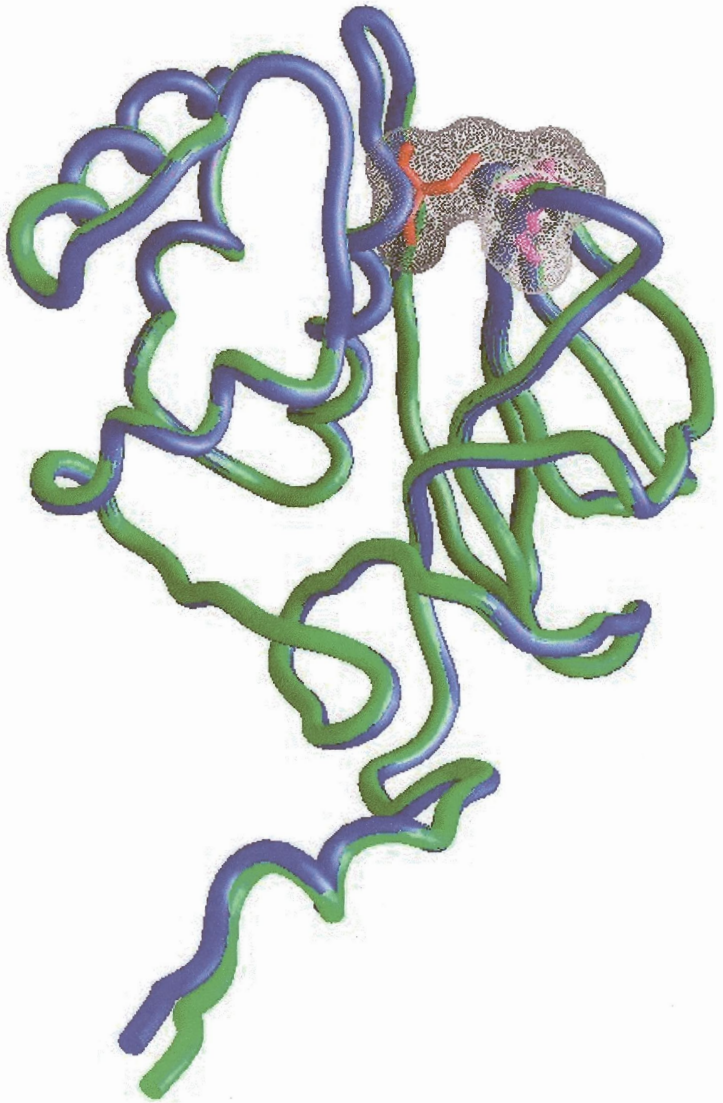


Figure 3.4: Superimposed C_α traces of O_1Kaufbeuren Lb^{pro} (blue) and ZIM/7/83/2 Lb^{pro} (green). The catalytic Cys-51 (red) and His-148 (purple) residues, which are situated on top of the deep cleft, are indicated as well as the electron density displayed by these residues.

southern Africa (T. Skern and A. Guarné, personal communication). The effect (if any) of these differences on self-processing are now investigated using biological assays.

3.3.3 Gene heterogeneity and gene relationships of 3C proteinase

Representatives (21 isolates) of serotypes A, O, C and SAT 1, 2 and 3 were chosen to investigate the gene heterogeneity in the 3C proteinase (Table 3.1). Amino acid sequence alignment of the isolates shows the complete conservation of the active site residues (His46-Asp84-Cys163), as expected (Figure 3.5). Alignment of the FMDV $3C^{pro}$ regions with that of hepatitis A virus (HAV) using CLUSTALW also shows the conservation of the active site residues. The three-dimensional structure of HAV (Allaire *et al.*, 1994; Bergmann *et al.*, 1997) indicates a chymotripsin-like fold, comprising of two anti-parallel β-barrels – each barrel consisting of six β-sheets. The secondary structural motifs as previously predicted for picornavirus 3C proteinases (Gorbalenya *et al.*, 1989) are indicated for the FMDV isolates in Figure 3.5 and shows the conservation of the 12 β-sheets. Overall the sheets are well conserved, although some differences can be seen in βE, βG and βJ. The C- and N-terminus α-helices are well conserved.

A neighbor-joining tree depicting $3C^{pro}$ gene relationships between the different serotypes is shown in Figure 3.6. Similar phylogenetic groupings were obtained for $3C^{pro}$ as was observed for Lab^{pro}. The group indicated in blue is inclusive of isolates form types A, O and C from Europe, Asia, West and East Africa as well as a single isolate from Brazil. In this analysis, it was interesting to observe that the SAT 2 virus KEN/3/57 grouped with two isolates from West Africa (GAM/51/98/A and BKF/2/92/O) and forms a sub-cluster within the first lineage. The group in green (Figure 3.6) represents SAT viruses from southern Africa. UGA/1/97/1 grouped with lineage II during this analysis instead of lineage I as previously found (Figure 3.3). This was the only difference obtained for the Lab^{pro} and $3C^{pro}$ phylogenetic analyses. Again, amino acid variation for lineage I (16%) was found to be higher than for lineage II (12%).

```
                    αaCαα   ββAββ          βββBββ ββββCββββ     βDββ
                                                          *          60
O1Kaufbeuren SGAPPTDLQKMVMGNTKPVELILDGKTVAICCATGVFGTAYLVPRHLFAEKYDKIMVDGR
O5India      ....................................S.................L...
BKF/2/92/O   ............A...........I.............................I...
KEN/1/91/O   ....................................................L...
A12          ....................................................L...
A22          ....................................................L...
K/37/84/A    ....................................................L...
GAM/51/98/A  ............A.............V.........................I...
C1Noville    ....................................V....G..........L...
C3Resende    ....................................................L...
KNP/196/91/1 ..C.........A.V...........L.........................L...
ZIM/3/88/1   ..C.........A.V...........L.........................L...
ZIM/3/96/1   ..C.........A.V...........L.........................L...
TAN/1/99/1   ..C.........A.V...........L.........................L...
UGA/1/97/1   ............A.V.........I..L.........................L...
KEN/3/57/2   ............A.............................................I...
ZIM/7/83/2   ............A.V...........L.........................L...
KNP/19/89/2  ..C.........A.V...........L.........................L...
KNP/10/90/3  ..C.........A.V...........L.........................L...
BEC/1/65/3   ..C.........A.V...........L.........................L...
HAV          .TLEIAG.VRKNLVQFGVE.--KN.CVRWVMN.L..KDDWL...S.AYKFEK.YE.MEFY
             αααCααα  βββA1ββ        βββB1βββββ  ββC1ββ          βββD1

                    βββββEββββ          βββFβββ                  βββGββ
                                           *                        120
O1Kaufbeuren AMT-DSDYRVFEFEIKVKGQD-MLSDAALMVLHRGNRVRDITKHFRDTARMKKGTPVVGV
O5India      ....................................................................
BKF/2/92/O   .....R.F..................V...........A.....C.Q...R.........
KEN/1/91/O   ....................................................................
A12          ....................................................................
A22          ....................................................................
K/37/84/A    ....................................................................
GAM/51/98/A  .....R.F.....................................Q.K.R.........
C1Noville    .L.....................................................V.............
C3Resende    ..................................T...........V.............
KNP/196/91/1 .L.....F......V..................S......L.G.....MKLS..S.....
ZIM/3/88/1   .LK....F......V..................S......L.G.....MKLS..S.I...
ZIM/3/96/1   .L.....F......V..................S......L.G.....MKLS..S.I...
TAN/1/99/1   .L.....F......V..................T......L.G.....MKLS..S.....
UGA/1/97/1   .L..NG.F......V.................N..Q......A.....V.VA..N.....
KEN/3/57/2   .....R.F.....................................R....Q...R.........
ZIM/7/83/2   .L.....F......V..................S......L.G.....MKLS..S.....
KNP/19/89/2  .L.....F......V..................S......L.G.....MKLS..S.....
KNP/10/90/3  .L...G.F......V..................S......L.G.....MKLS..S.....
BEC/1/65/3   .L.....F......V..................S......L.G.....MKLS..S.....
HAV          FNRGGTYYSISAGNVVIQSL.VGFQ.VV..KVPTIPKF..I.Q..IKKGDVPRALNRLAT
             ββ   βE1aβ     βE1bβ      βF1β                        ββA2
```

```
                β         ββββH    ββ                ββIβ          ββββJββββ
                                                                         *   180
O1Kaufbeuren    INNADVG--RLIFSG-----EALTYKDIVVCMDGDTMPGLFAYRAATK--AGYCGGAVLA
O5India         ............................................P...............
BKF/2/92/O      ............................................................
KEN/1/91/O      ............................................................
A12             V.....................................S....K................
A22             ...........................................K................
K/37/84/A       ............................................................
GAM/51/98/A     .........K..................................................
C1Noville       ...........................................K................
C3Resende       ...........................................K................
KNP/196/91/1    V...................D......L.................G....V....A....
ZIM/3/88/1      V...................D......L.................G....V....A....
ZIM/3/96/1      V...................D......L.................G....V....A....
TAN/1/99/1      V...................D......L.................G....V....A....
UGA/1/97/1      V...................D........................G....V....A...T
KEN/3/57/2      V....RW....................................K................
ZIM/7/83/2      V...................D......L.................G....V....A....
KNP/19/89/2     V...................D......L.................G....V....A....
KNP/10/90/3     V...................D......L.................G....V....A....
BEC/1/65/3      V...................D......L.................G....V....A....
HAV             LVTTVNGTPM..SE.PLKMEERA..VHKKNDGTTVDLTVDQ.WRGKGEGLP.M..G.LVS
                ββββ  ββββββB2ββββββ ββaβ    ββbβ  ββC2ββ          βD2β
```

```
                β      ββββKβββ/βLβ   αααNααα
O1Kaufbeuren    KDG-ADTFIVGTHSAGGNGVGYCSCVSRSMLLKMKAHIDPEPHHE    213
O5India         ..........................................L..    213
BKF/2/92/O      .....E.....................K....Q....V.......    213
KEN/1/91/O      .....................................V.......    213
A12             ...........................K....R....V....Q..    213
A22             .............................................    213
K/37/84/A       .....................................MH......    213
GAM/51/98/A     ....PE..........................Q....V..D..S.    213
C1Noville       .............................................    213
C3Resende       ....PE.......................................    213
KNP/196/91/1    .....K.V........................Q.......P..T.    213
ZIM/3/88/1      .....K.V........................Q.......P..T.    213
ZIM/3/96/1      .....K.V........................Q.......P..T.    213
TAN/1/99/1      .....K.V........................Q.......P..T.    213
UGA/1/97/1      .S..SQ.V.I.................K...DQ.......A..T.    213
KEN/3/57/2      .....E.....................K....Q....V.......    213
ZIM/7/83/2      .....K.V........................Q.......P..T.    213
KNP/19/89/2     .....K.V........................Q.......P..T.    213
KNP/10/90/3     .....K.V........................Q.......P..T.    213
BEC/1/65/3      .....K.V........................Q.......P..T.    213
HAV             SNQSIQNA.L.I.V....SILVAKL.TQE.FQNIDKKIESQ----    219
                        ββE2ββ      βF2β αNα
```

Figure 3.5: Amino acid sequence alignment of FMDV $3C^{pro}$ indicating differences between southern African types and types A, O and C. The conservation of the putative catalytic residues (His-46; Asp-84; Cys-163) in the isolates investigated as well as hepatitis A virus (HAV) , is indicated with an asteriks (*). Secondary structural motifs for FMDV as previously predicted by Gorbalenya and co-workers (1989) are indicated as well as for HAV (Ryan & Flint, 1997).

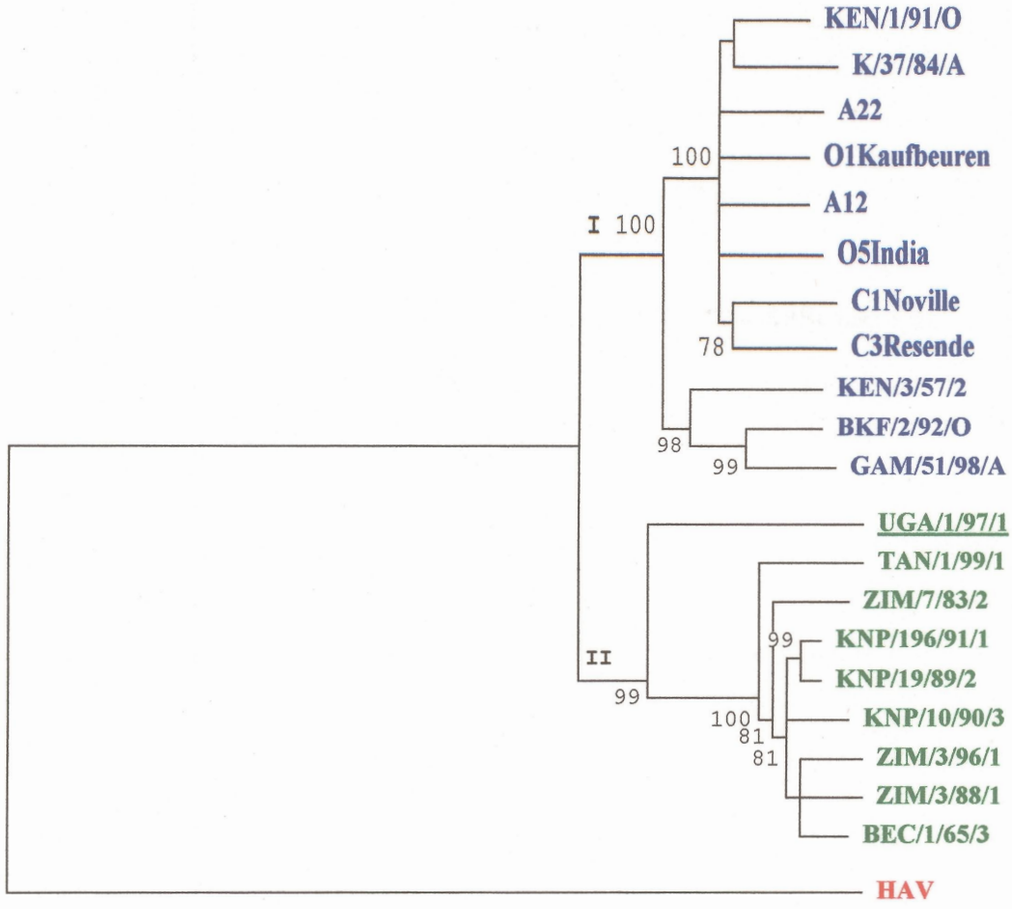


Figure 3.6: Neighbor-joining tree (*p*-distances) depicting $3C^{pro}$ gene relationships. Bootstrap values > 75 and based on 1000 replications are indicated with hepatitis A (HAV) 3C proteinase selected as outgroup. Lineage I is presented in blue and lineage II in green.

3.3.4 Comparison of FMDV P1 cleavage sites mediated by the 3C proteinase

The polyprotein precursor is proteolytically processed by viral proteinases. The primary cleavage site of the FMDV $3C^{pro}$ is at the 2B/2C junction (Ryan & Flint, 1997). The proteinase is, however, also responsible for a series of secondary cleavages. Preferred processing sites of the $3C^{pro}$ for another picornavirus member, poliovirus, are Gln-Gly (Q/G) (Bazan & Fletterick, 1988). Inspection of the P1 cleavage sites of the different FMDV serotypes (excluding Asia-1), revealed variations between the different types and poliovirus (Figure 3.7). The Q/G processing site is conserved in the SAT types for the VP0/VP3 junction, but is replaced by an acidic glutamic acid (E) residue in types A, O and C. The equivalent of this site in HAV and human rhino virus type 14 (HRV14) is Q/M and Q/G, respectively (Seipelt *et al.*, 1999). The Gln residue is conserved in the VP3/VP1 site in all the serotypes except type O, where it is again replaced by a glutamic acid. The Gly residue is however replaced in the FMDV types by a more bulky Thr residue. The corresponding VP3/VP1 site in HAV is Q/V and in HRV14 E/G (Seipelt *et al.*, 1999). In the VP1/2A junction site, the Gln residue in completely conserved. Variation occurs however on the 2A side of the junction site. Type O contains the neutral and polar Thr residue, but this residue is replaced in the other types by a hydrophobic Leu (L) residue. BEC/1/65/3 is an exception and contains a methionine residue (also hydrophobic) instead of Thr in this position.

The recognition of these processing sites is dependent on their position within the polyprotein. The regions flanking these sites might therefore play an important role in the recognition process. Although it is evident from Figure 3.7 that these regions are not highly conserved between the European and SAT types, one would assume the three-dimensional structure of these regions to be conserved. The efficient conservation of the proline residue in the –4 position from the cleavage site, may be of importance. The structural role this residue might play, due to its aromatic nature has been implicated before for both FMDV and encephalomyocarditis (EMCV) (Palmenberg *et al.*, 1984). Exceptions observed in this study entail the presence of an alanine residue in this position for types O and C at the VP3/VP1 cleavage site as well as the occurrence of a valine residue in the –4 position at the VP1/2A cleavage site for

	VP0 VP3	VP3 VP1	VP1 2A	
O1Kaufbeuren	NVHVAGEFPSKE GIFPVACSDG	ELRLPVDARAE TTSAGESADPV	KQKIVAPVKQ TLNFDLLKLAG	X00871
O1Campos	..........E G.....	.....E T.....	.....Q T.....	M95781
A12	Y.....E G.....	.....I.P.SQ T.AT.....	....I..G.Q L.....	M10975
A22	H.....L...E G.V.....	.....I.P.SQ T..T.....	....I..A.Q L.....	X74812
C1Oberbayen	..Y....L...E G..S.....	.....QQ T.TT.....	..PL...A.Q L.....	X00130
C3Resende	.....L...E G.....-...	.....LQ T.TT.....	..RLI..A.Q LS.....	M90381
BOT/1/68/1	..Y....K.V.Q G.L...V...	.F.M.ISPSRQ T.....G....	.TTL.K.A.Q LS.....	Z98203
KNP/196/91/1	..Y....K.A.Q G.L...V.V.	.F.M.ISPSRQ T.....G.E..	RTA.TK...Q LC.....	AF283429
KEN/3/57/2	..Y....L.G.Q G.V....A..	.F.F.I.PVRQ T.....G.EV.	FDAPIGVA.Q L.....	AJ251473
RHO/1/48/2	..F....M.A.Q G.I....A..	.F.F.I.PVRQ T.AV..G..V.	FDAPIGVE.Q LF.....	AJ251475
ZIM/7/83/2	..F....K.A.Q G.....F..	.F.F...PVRQ T..S..G..V.	FDSPIGVE.Q LC.....	AF136607
BEC/1/65/3	..YR...R...Q G.....N..	.....INPATQ T.....G..V.	.TPL.K.D.Q MC.....	M28719
KNP/10/90/3	..Y....K.T.Q G.V....H..	.F...INPV.Q T.....GG.V.	.T.L...D.Q LC.....	**Unpublished results**

Figure 3.7: P1 cleavage sites (VP0/VP3; VP3/VP1; VP1/2A) processed by $3C^{pro}$ are indicated as well as regions adjacent to the cleavage site. Amino acids identical to the cleavage site of poliovirus are indicated in blue, while those that differ are indicated in red. GenBank Accession numbers (except KNP/10/90/3) are given.

SAT 2 (Figure 3.7). However, in both cases a proline residue is present in the –7 position.

3.4 Conclusions

The genetic heterogeneity of the L^{pro} and $3C^{pro}$ regions of representatives of six FMDV serotypes originating from different geographical localities was investigated. The study revealed that the L^{pro} and $3C^{pro}$ genomic regions of the SAT type viruses from southern Africa are distinct from that of types A, O and C. Interestingly, results indicated that the amino acid sequences of L^{pro} and $3C^{pro}$ of the SAT types are less variable than the corresponding amino acid sequences of types A, O and C. This is unexpected as it has been shown previously that the structural proteins of SAT 2 are more variable than that of types A, O and C (see Chapter 2). Phylogenetic analysis of the L^{pro} and $3C^{pro}$ genomic regions revealed similar structuring, although different from what was previously obtained for the structural proteins (Bastos, 1998). In the latter case, isolates grouped strictly according to serotype.

An interesting observation made during analysis of the L^{pro} and $3C^{pro}$ gene relationships is the different groupings obtained for the East African strain, UGA/1/97/1. This strain was found to group with lineage I with L^{pro} analysis, but with lineage II with $3C^{pro}$ analysis. These findings strongly suggest the occurrence of viral recombination. This phenomenon has previously been shown in different laboratories for both poliovirus (Ledinko, 1963; King, 1988) and FMDV (Pringle, 1965; King *et al.*, 1985; Giraudo *et al.*, 1988). Due to the high number of recombination sites detected in the RNA genome of FMDV, it was concluded that recombination is a general process (King *et al.*, 1985). Despite these findings, no reports, as far as could be determined, on the occurrence of FMDV recombination in nature had previously been reported. The SAT 1 Ugandan strain was isolated from buffalo (Table 3.1) in the Queen Elizabeth National Park in Uganda. As multiple viral infections for individual buffaloes occur frequently in nature (Hedger, 1972), it could create the ideal physical conditions for viral recombination to occur.

The heterogeneity in the L^{pro} and $3C^{pro}$ genomic regions emphasize, yet again, the distinctiveness of the SAT type viruses. Nevertheless, it was shown in this study that the overall three-dimensional fold for L^{pro} is completely conserved. The biological significance of the observed differences in the CTE region will subsequently be determined in a biological assay system. Together with the heterogeneity observed in the $3C^{pro}$ P1 cleavage sites as well as the regions adjacent to the cleavage sites, these differences could possibly impact on the construction of viable chimeric viruses between different types, e.g. types A and SAT 2. This aspect will be addressed in the following chapter.

CHAPTER 4

Construction of a chimeric foot-and-mouth disease virus between serotypes A and SAT 2: Comparison with wild type SAT 2 in terms of antigenicity, growth properties and thermal stability[1]

4.1 Introduction

Effective vaccination in sub-Saharan Africa requires the use of custom-made vaccines for specific geographic localities, due to the genetic and antigenic variability of the SAT types of FMDV. Comparison of r-values (expression of antigenic relationships between viruses) determined for ZIM/7/83/2 (a west Zimbabwean strain) and KNP/19/89/2 (originating from the Kruger National Park, South Africa) in relation to isolates from the Kruger National Park, revealed much lower r-values for ZIM/7/83/2 than the KNP strain (Esterhuysen, 1994). The serological data demonstrated therefore the closer relatedness of the KNP strains. A similar study investigating the antigenic relationships of isolates originating from northern and southern Zimbabwe, indicated these isolates to belong to antigenically different groups (Bauman & Esterhuysen, 2000). These results were confirmed genetically which clearly showed the closer gene relationships of isolates originating in the same geographical region (Vosloo *et al.*, 1995; Bastos *et al.*, 2001). Taken together, these results are indicative of the independent evolution of FMDV in different geographical regions and argue thus for the use of custom-made vaccines.

The use of such vaccines implies unfortunately the screening of various field strains, usually buffalo isolates. Adaptation of these viruses to baby hamster kidney (BHK) cells have proven to be a tedious process. In addition, these viruses do not produce high amounts of stable antigen. The screening process is also a cumbersome, labor intensive and therefore an expensive process. It is thus proposed to develop chimeric

[1] Parts of the work presented here have been performed at the Plum Island Animal Disease Center (USA) under the supervision of Dr. P.W. Mason and sponsored by the International Atomic Energy Agency.

or recombinant viruses, which would facilitate the manipulation of the antigenicity of a particular virus to be used in the production of custom-made, but conventional, inactivated vaccines.

The construction of full-length cDNA copies for the foot-and-mouth disease virus was at first hampered due to the presence of the poly (C) tract in the FMDV genome. However, in the early 1990's such a full-length cDNA copy was constructed for type O containing 32C residues in the poly (C) tract (Zibert *et al.*, 1990). Since it was previously shown that picornaviral RNA is infectious (Alexander *et al.*, 1958; Colter *et al.*, 1957), synthetic RNA's were transcribed from the cDNA copy and used to infect cells. Subsequently, the construction of such cDNA copies of the foot-and-mouth disease virus proved to be a powerful and successful tool for manipulating the characteristics of the virus.

Using a full-length cDNA clone containing the genetic backbone of type A_{12} and a poly (C) of 35 (Rieder *et al.*, 1993), several chimeric viruses were constructed. These include the exchange of the immunodominant site, the G-H loop, of A_{12} with that of O_1BFS and C_3Resende. Inactivated vaccines prepared from these chimeric viruses induced antibodies in guinea pigs which neutralized both serotype A and either types O or C (Rieder *et al.*, 1994). The external capsid regions (partial P1 region) for two different type O isolates, O_1Campos (Sa-Carvalho *et al.*, 1997; Almeida *et al.*, 1998) and OTai (Beard & Mason, 2000) were also successfully inserted into the A_{12} infectious cDNA clone, yielding infectious FMDV. The construction of such chimeric viruses employing the SAT type viruses has, however, not yet been attempted.

In this study the construction of a chimeric virus between serotypes A and SAT 2 is described by exchanging the external capsid protein coding region of the A_{12} cDNA clone with that of ZIM/7/83/2. The viable chimeric virus was cultivated in BHK cells and subsequently compared to the wild type ZIM/7/83/2 virus in terms of immunogenicity, growth properties, antigen production and thermal stability.

4.2 Materials and Methods

4.2.1 Viral and bacterial strains

The SAT 2 strain described in Chapter 2 (ZIM/7/83/2 - passage history: B1BHK5B1) was used in this study. *Escherichia coli* MAX Efficiency DH5α™ competent cells (genotype: F⁻ϕ80d*lac*ZΔM15Δ(*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) was obtained from Life Technologies and used in the transformation experiments.

4.2.2 RNA extraction and cDNA synthesis

RNA was extracted from the tissue culture sample (250 µl) using the phenol-based Trizol reagent (750 µl) (Life Technologies) according to the manufacturer's specifications, followed by a chloroform extraction and isopropanol precipitation. The extracted RNA (5 µl of 20 µl)) was subsequently used as template for cDNA synthesis. MMLV RT (Life Technologies) and an antisense oligonucleotide located on the 2A/2B-junction site (Figure 4.1), cDNA-2A (5'-CGCCCCGGGGTTGGACTCAACGTCTCC-3') (binding nucleotides 2055 to 2088 on MAWT12), was used for the reverse transcription reaction carried out for 1h at 42°C.

4.2.3 PCR amplification

The external capsid coding region (VP2, VP3,VP1) of ZIM/7/83/2 was amplified using the AdvanTaq™ DNA Polymerase available from Clontech. The reaction was carried out in the presence of 0.2 mM deoxynucleotides as well as 0.4 µM of oligonucleotides: cDNA-2A and cDNA-VP2 (5'-CGGAATATTGACAACACGACACGGTACAA CCAC-3') (binding nucleotides 4002 to 4025 on MAWT12) (Figure 4.1). Both cDNA-2A and cDNA-VP2 were designed using data generated for ZIM/7/83/2 P1 region (Chapter 2). Reaction conditions comprised of an initial denaturing step of 60 sec at 94°C followed by 15 cycles of 30 sec at 94°C and 8 min at 68°C. A final extension step of 50 min at 68°C was followed by a cool-down step of 90 min at 15°C. The resulting amplicon was analysed by agarose gel electrophoresis and recovered from the agarose gel using a freeze-thaw method. This method involved the freezing and thawing (three times) of the gel slice in the presence of 100µl STE buffer (100mM

Tris-HCl, pH 7.5; 100mM NaCl; 1mM EDTA) and 0.01% SDS followed by buthanol extractions (twice) and an ethanol precipitation.

4.2.4 Cloning of SAT 2 external capsid coding region into A_{12} infectious cDNA clone

The two oligonucleotides, cDNA-VP2 (*SspI*) and cDNA-2A (*XmaI*), were designed with restriction enzyme sites to facilitate the cloning of the amplified external capsid coding region into the A_{12} full-length cDNA clone, MAWT12 (Rieder *et al.*, 1993). Following the restriction enzyme digestion of the purified amplicon and MAWT12 (treated with alkaline phosphatase, Roche), ligation reactions were carried out using the Rapid DNA Ligation kit from Roche according to the specifications of the manufacturer. The ligation mixture (3µl) was transformed into 20 µl of *E. coli* MAX Efficiency DH5α™ competent cells. Large-scale preparations of putative positive full-length clones (FLC) were isolated from 20ml *E. coli* cultures using the Wizard Plasmid Prep kit (Promega). Construction of the FLC was verified by sequencing of the restriction site using an ABI PRISM 377 DNA Sequencer from Perkin Elmer and a sense oligonucleotide in the VP4 region (5'-TCAACACACACAACCAACACTCA-3') (P307) as well as an antisense oligonucleotide in the 2B region (5'-GCATCTGGTTGATTGTGTCTACC-3') (P308) (P.W. Mason, personal communication).

4.2.5 RNA synthesis

To facilitate RNA transcription, 5 µg of the purified plasmid DNA was linearized with *Not I* to facilitate RNA synthesis . This step was followed by a proteinase K digestion, phenol and chloroform extractions as well as a final ethanol precipitation. RNA transcription was carried out with the MEGAscript™ T7 kit from Ambion at 39°C for 2 h. The quality of the RNA was analyzed on agarose gel electrophoresis (1% agarose gel) and the concentration of the newly synthesized RNA was determined by comparison to a known standard. All reactions were carried out using only RNA-grade reagents.

4.2.6 Transfection of BHK cells with infectious viral RNA

Baby hamster kidney (BHK) cells were transfected with the transcribed RNA by means of either electroporation or lipofectin reagent (Life Technologies). Approximately 15 μg infectious RNA together with 7.5 x 10^6 cells were used in the electroporation experiment. The electroporated cells were subsequently transferred to basal medium eagle (BME) (Life Technologies) containing 10% bovine calf serum (Hyclone). Five milliliters of the cell-medium mixture was transferred to a 35 mm well and incubated for 5h at 37°C in CO_2 incubator after which the cells were rinsed with BME containing 1% calf serum and incubated overnight with BME + 1% calf serum. Cells were frozen and thawed the next day and passed further on BHK cells. Aliquots were stored following each step. For transfection by means of lipofectin, RNA was diluted with opti-MEM (Life Technologies) followed by a 15-20 min incubation at room temperature with the lipofectin reagent. This lipofectin-RNA complex was then overlayed on pre-prepared cells (6 x 10^5 cells / 35 mm well) and incubated for 5 h at 37°C in a CO_2 incubator. The RNA containing medium was then replaced with 0.6% gum tragacanth in MEM (modified eagles medium) containing 1% calf serum and incubated in the 37°C incubator for 48-72h. The wells were stained with crystal violet.

4.2.7 Viral titrations on IB-RS-2 cells

The newly synthesized chimeric virus between types A and SAT 2 was passaged four times on BHK cells and stored at -70°C to be used in the subsequent characterizing experiments. The determination of viral titrations for the A12/SAT2 chimera (BHK4) and the wild type ZIM/7/83/2 (B1BHK5B1BHK1) was carried out in flat-bottomed microtitre plates (Nunc). Roswell Park Memorial Institute (RPMI) medium (Sigma) was used to prepare 0.5$\log_{10}$ dilutions of the viral stocks. RPMI medium (50μl) together with 50μl of the virus dilutions were plated out per well and incubated for 1h at 37°C. The test was performed in 8 fold. Following the incubation period, 100μl of a cell suspension (0.3 x 10^6 / ml IB-RS-2, a pig kidney cell line in RPMI medium containing 5% fetal calf serum (Delta Bioproducts)) was placed out per well. The microtiter plates were incubated at 37°C for 72h in a CO_2 incubator. Results were read directly with an inverted microscope. The calculated viral titers were expressed as $TCID_{50}$/50μl (tissue culture infectious doses) (Esterhuysen, 1994).

4.2.8 Plaque titrations on IB-RS-2 and BHK cells

Petri-dishes (50 mm diameter) were prepared with IB-RS-2 and BHK cells with a concentration of 5 x 10^6 cells / dish and incubated at 37°C to be confluent in 48h. The two viruses were diluted in RPMI medium using $\log_{10}$ dilutions. After the media was removed from the dishes containing the confluent cells, 200 µl of the viral dilutions were added and incubated for 30 min at 37°C. A RPMI medium-agarose mixture (5 ml), containing 5% normal bovine serum and 0.1% agarose (Merck) was added to each dish, left to set and incubated for 48h at 37°C and CO_2. All titrations were done in duplicate. Plaques were stained using a methylene blue solution (1% w/v methylene blue dissolved in absolute EtOH, added to an equal volume of formaldehyde and 8 x PBS).

4.2.9 Virus neutralization test

The two viruses were tested against cattle sera that were raised against the wild type ZIM/7/83/2, KNP/19/89/2 and a mixture of the two sera. The sera were generously provided by J. J. Esterhuysen. These sera were diluted in serial twofold dilutions (50µl) in 8 columns across a microtitre plate. The viral dilutions used ($0.5\log_{10}$ apart) were calculated to straddle the 10^2TCID_{50} dose. 50µl of the virus dilutions were plated out on the microtitre plate in three fold and incubated for 1h at 37°C. Subsequently, 100µl / well of cell suspension (0.3 x 10^6 IB-RS-2 cells in RPMI medium containing 5% fetal calf serum) was added and incubated for 72h at 37°C and CO_2. Results were read using an inverted microscope. A virus titration for each test was included to be able to determine the actual virus titer and dose for the specific experiment. Serum titers were expressed as the $\log_{10}$ reciprocal of the dilution, which protected 50% of cultures from that dose of virus. The final endpoint titer of the serum was determined as the log reciprocal of the dilution which protected 50% of cultures from 10^2TCID_{50} of virus (Esterhuysen, 1994).

4.2.10 Determination of 146S content

To determine the concentration (µg/ml) of the 146S viral particle, 0.5 ml of a sample was layered carefully on top of a 15 – 45% sucrose gradient. These gradients were

subsequently centrifuged in an ultracentrifuge at 285 000 x *g* and 6°C for 52 minutes. The protein content was then detected on an UV-detector and chromatographically registered on a recorder. The 146S concentration was calculated from the surface area of the relevant peak multiplied by an emission constant, $E_{254}^{1\%} = 73$ (Lei, 1978).

4.2.11 Determination of growth kinetics

In order to determine the relative growth rates and antigen production of wild type ZIM/7/83/2 and A12/SAT2 on BHK cells, an inoculum of 1 virus particle per 100 cells were used. Virus titers determined with plaque titrations (section 4.2.8) on BHK cells were used. Roller bottles containing 10^8 BHK cells / ml were incubated for 1h with the virus at 37°C after which 80 ml of RPMI (containing no fetal calf serum) was added and incubated at 37°C. Samples of the supernatant were taken at different time intervals. The viral titers and 146S content of these samples were subsequently determined.

4.2.12 Thermal stability testing

The same infection rate used in section 4.2.11 was applied to determine the thermal stability of the two viruses investigated. Viruses were grown till their highest point of antigen production as determined during the growth kinetics. Each virus was harvested and stirred for 1h in the presence of 1% chloroform at 4°C. This mixture was centrifuged for 10 min at 2 000 rpm and the supernatant was divided into 9 aliquots for each virus. Four of the aliquots were left at 4°C for 21 days and the rest at 37°C for the same duration. One sample was directly tested for its 146S content, whilst the remaining samples were determined in 7 day intervals.

4.3 Results and Discussion

4.3.1 Construction of a chimeric virus between serotypes A and SAT 2

The 2.2 kb external capsid protein coding region (VP2-VP3-VP1), of the SAT 2 isolate, ZIM/7/83, (B1BHK5B1) was amplified from cDNA and cloned into the *SspI* and *XmaI* sites of the A_{12} full-length cDNA clone, MAWT (constructed by Rieder *et al.* (1993)) (Figure 4.1). As ZIM/7/83/2 does not contain the *SspI* and *XmaI* restriction enzyme

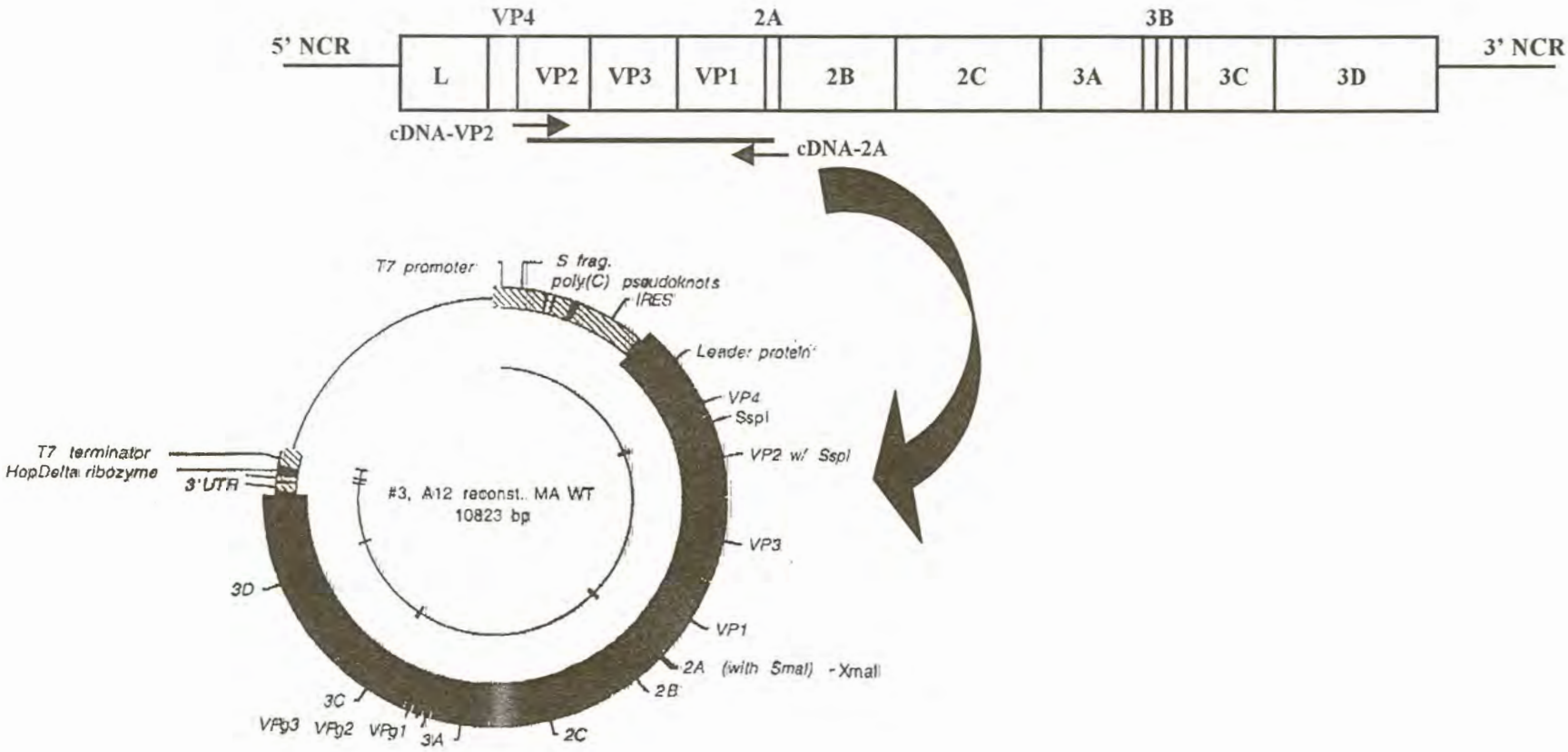


Figure 4.1: Schematic representation of the construction of the chimeric virus between serotype A and SAT 2. The external capsid protein coding region amplified by cDNA-VP2 and cDNA-2A, was cloned into the SspI and XmaI sites of MAWT, which contains the FMDV genome, T7 propmoter and terminator regions as well as the HepDelta ribozyme.

sites, these sites had to be engineered during the design of oligonucleotides cDNA-VP2 and cDNA-2A. In order to achieve this, a single amino acid change (Val (GTC)-Leu (TTG), both polar) was introduced in the VP2 region of ZIM/7/83/2 (Figure 4.2). The corresponding external capsid-coding region of the A_{12} clone was removed by restriction enzyme digestion and that of ZIM/7/83/2 was successfully inserted into the cDNA clone, creating a chimeric construct between types A and SAT 2. The successful construction of the chimeric clone, pA12/SAT2, was verified with nucleotide sequencing (Figure 4.2). The sequence of the newly constructed pA12/SAT2 corresponds to that of a computerized version of the construct, verifying the type A genetic backbone as well as the SAT 2 external capsid-coding region. The receptor-binding region (RGD) of the foot-and-mouth disease virus was also found to be unaltered.

In vitro-synthesized RNA's generated from several pA12/SAT2 clones were used to transfect BHK cells (Figure 4.3). Several clones were screened before infectious viral particles could be obtained (results not shown). A flow-diagram (Figure 4.4) indicates the subsequent steps followed to obtain an infectious A12/SAT2 chimeric virus. The viability of the RNA derived from #14A12/SAT2 was confirmed through plaque titrations on BHK monolayer. Viral particles generated from this clone (#14) were therefore harvested. The partial nucleotide sequence of the external capsid-coding region of the chimeric virus was again determined, verifying the isolation of the correct virus (results not shown). The ability of the chimeric #14A12/SAT2 virus to form plaques on BHK cells following transfection (Figure 4.4), suggests that the SAT 2 structural proteins were compatible with the type A 3C proteinase.

It is worthy to note that viable chimeric virus could not be obtained following the initial construction of a chimeric virus between types A and SAT 2. This construct was derived from the pGEMZIM/7/83/2 plasmid generated previously (Chapter 2). Direct amplification from cDNA using a high fidelity polymerase did however yield the viable chimeric virus, #14A12/SAT2. These results together with the sequence differences indicated in the VP1 region (Figure 4.2), emphasize the need to determine the nucleotide sequence of the structural-protein-coding region of clone #14. Since no

Figure 4.2: The nucleotide sequence comparison of a computerized construction of the A12/SAT2 chimera (A12ZIM/7/83/2), the wild type ZIM/7/83/2 P1 region (WTZIM/7/83/2) as determined previously (Chapter 2) and the nucleotide sequence as determined for the newly constructed pA12/SAT2 (clone #14) with oligonucleotides P307 (A) and P308 (B). The two oligonucleotides used for cloning, cDNA-VP2 (A) and cDNA-2A (B) as well as the junction sites are indicated in bold with the restriction enzyme sites used during cloning, indicated in bold and in italics. Differences obtained in VP1 between pA12/SAT2 and the wild type SAT 2, are indicated in italics. The receptor-binding region (ArgGlyAsp) in VP1 is also indicated in bold and underlined.

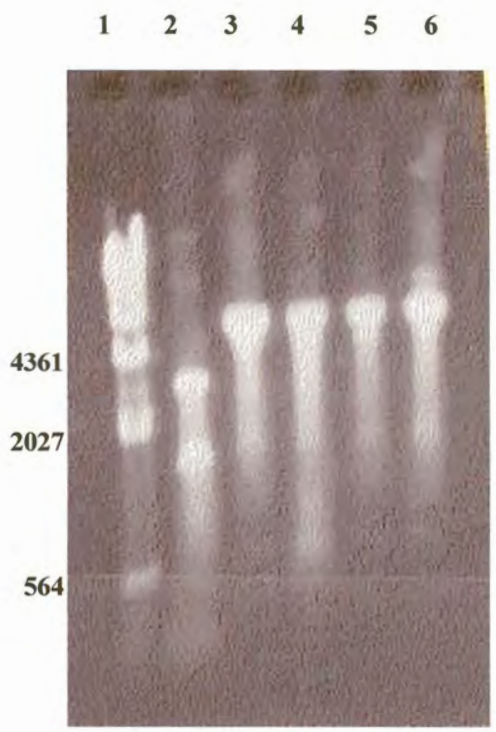


Figure 4.3: Agarose (1%) RNA gel indicating the *in vitro*-synthesized RNA's generated from the different pA12/SAT2 clones. Lane 1 containes the molecular weight marker (λ-DNA digested with *Hind III*), lane 2 RNA standard of known concentration and lanes 3-6, clones #7, #14, #15 and #21 derived from pA12/SAT2.

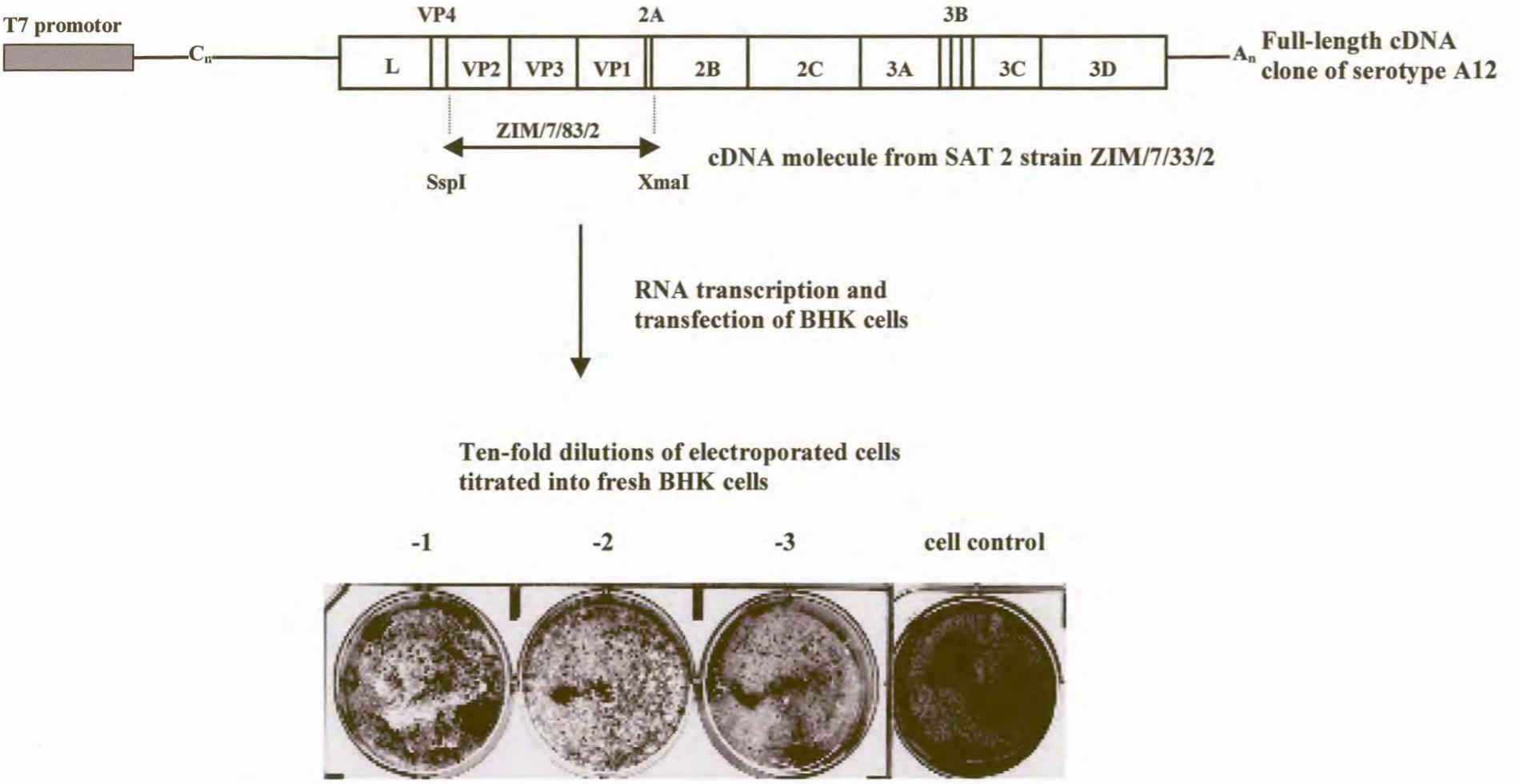


Figure 4.4: A flow-diagram indicating the construction of the chimeric virus between types A and SAT 2. The viability of clone #14 is shon with ten fold dilutions, clearly indicating the foci formed.

proof-reading enzyme was used, PCR artifacts could have been generated during the initial amplification of the region since no proof-reading enzyme was used.

4.3.2 Comparison with wild type SAT 2

The chimeric or recombinant virus, #14A12/SAT2, was compared to the wild type SAT 2 virus, ZIM/7/83/2, in terms of immunogenicity, growth properties, antigen production and thermal stability.

4.3.2.1 Immunogenicity

The unavailability of monoclonal antibodies raised against SAT 2 and ZIM/7/83/2 in particular, necessitate the use of the virus neutralization test (VNT) to investigate the immunogenicity of the chimeric virus. The wild type ZIM/7/83/2 and the recombinant virus were tested against sera raised against ZIM/7/83/2, another SAT 2 virus originating from the Kruger National Park, KNP/19/89/2, representing a different lineage, as well as a mixture of the two sera. The results indicate similar antigenic profiles for the two viruses, with the serum titer obtained against the KNP/19/89/2 strain being the lowest (WT ZIM/7/83/2 = $10^{2.2}$; #14A12/SAT2 = $10^{2.3}$) (Figure 4.5). Although it is not possible to conclude that the epitopes on the viral capsids of the two viruses are identical, the results obtained from the serological assays indicate the immunogenicity of the wild type and recombinant viruses to be similar.

4.3.2.2 Growth properties and antigen production

The growth properties of the recombinant virus were investigated by studying both plaque formation and growth kinetics and comparing it to that of the wild type virus. Plaque titrations were carried out on BHK and IB-RS-2 (pig kidney) cells (Figure 4.6). For both, ZIM/7/83/2 and #14A12/SAT2, plaques obtained on BHK cells were much smaller than on IB-RS-2 cells. Although the plaques obtained for the chimeric virus were extremely small compared to that of wild type ZIM/7/83/2, the plaque morphology of the viruses seemed to be similar. The similarity in morphology is expected since the receptor-binding region (RGD) of #14A12/SAT2 was shown to be intact (Figure 4.2). It was previously shown that with the exchange of only the G-H loop containing the RGD region of O_1BFS and C_3Resende, plaque morphologies

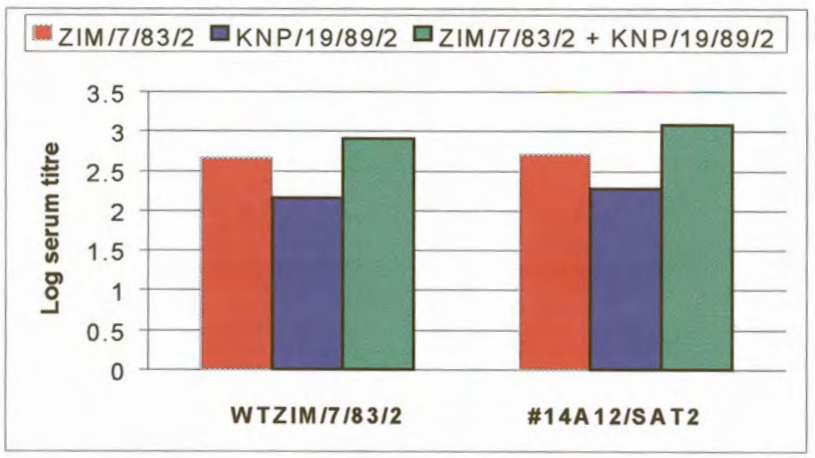


Figure 4.5: Immunogenic profile of wild type ZIM/7/83/2 and #14A12/SAT2 chimeric virus as determined with the virus neutralization test using sera raised against ZIM/7/83/2, KNP/19/89/2 and a combination thereof.

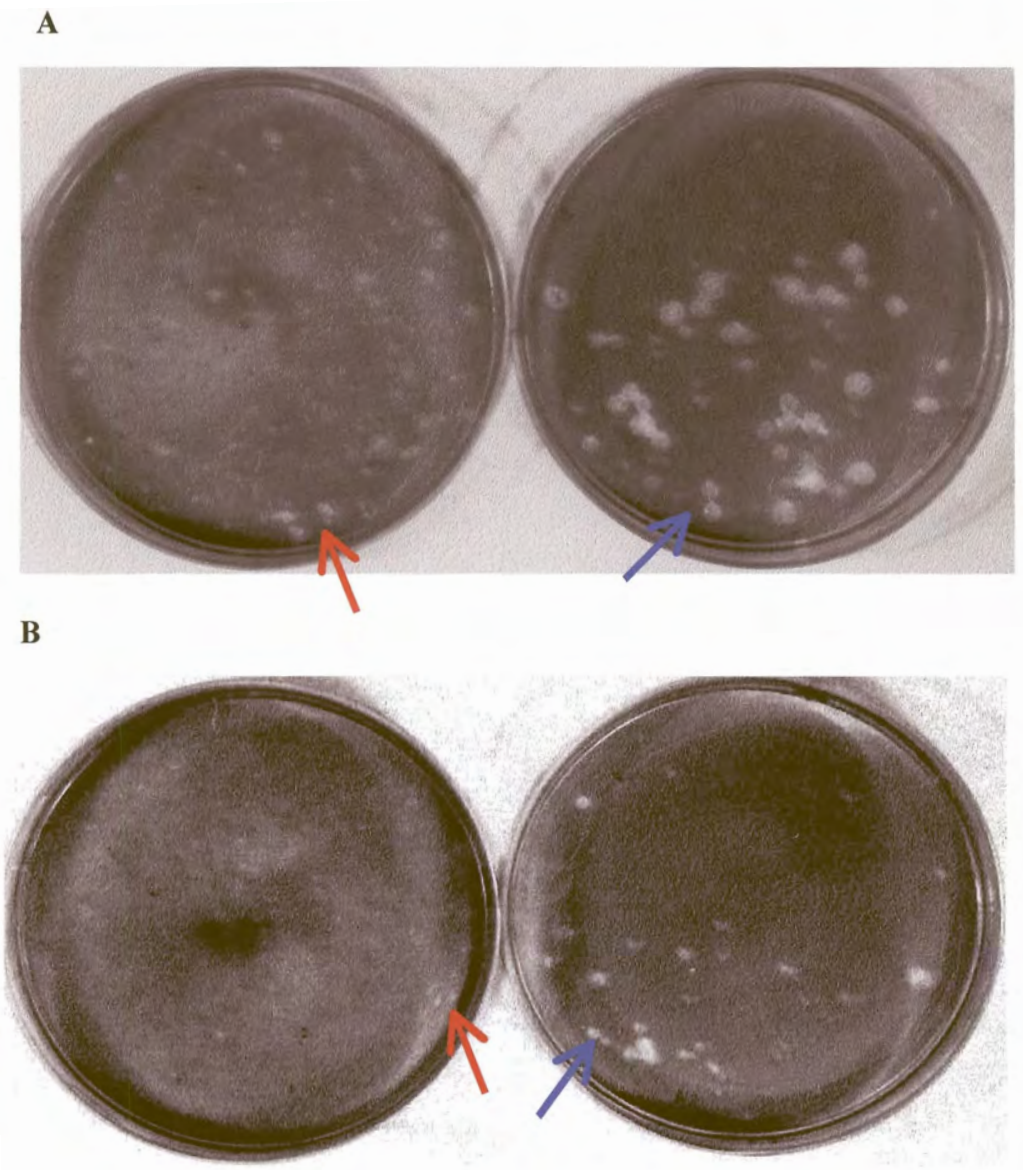


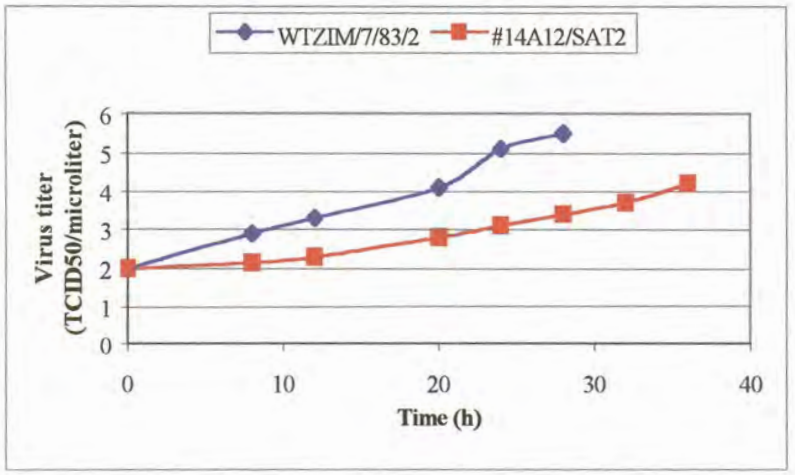
Figure 4.6: Plaque morphology as determined for WT ZIM/7/83/2 (A) and #14A12/SAT2 (B). The red arrows indicate foci on BHK cells and the blue arrows on IB-RS-2 cells.

similar to that of the wild type O_1BFS and C_3Resende were obtained (Rieder *et al.*, 1994).

A reduction in growth rate could explain the extremely small plaques obtained for the recombinant virus and was subsequently confirmed with growth studies (Figure 4.7). Using titers as determined with the plaque assays on BHK cells as well as a high multiplicity of infection (m.o.i) rate, the comparative growth rate of the wild type ZIM/7/83/2 and chimeric virus, #14A12/SAT2 was investigated. It was previously observed that ZIM/7/83/2 reaches high titers after 24 – 28h (results not shown) and therefore the experiment for this virus was discontinued after 32h. It was however found that #14A12/SAT2 reaches high titers after only 36h. The rate of antigen production was also seen to be reduced (Figure 4.7). The highest concentration of 146S content for ZIM/7/83/2 (1.64 µg/ml) was obtained after 28h, whilst the highest 146S yield for #14A12/SAT2 (1.29 µg/ml) was obtained after only 36h.

A reduced growth rate for a recombinant virus (A_{12}) had been reported previously (Rieder *et al.*, 1993). In this instance the recombinant in question had a poly (C) tract of only 2 residues. Plaques of less than 1 mm were obtained for this virus, whereas plaques of 5 to 8 mm were obtained for a recombinant containing a poly (C) tract of 35 residues. The latter virus ($A_{12}C_{35}$) also displayed a similar growth rate in BHK cells than the wild type A_{12} virus, achieving high titers within 24h (Rieder *et al.*, 1993). In the current study the A_{12} genetic backbone contains a poly (C) tract of 35 residues. Following the transfection of BHK cells with the synthetic RNA, the chimeric #14A12/SAT2 virus were passed an additional 4 times on BHK cells to allow the poly (C) tract to elongate. Previously, chimeras containing the identical A_{12} genetic background (poly (C) tract of 35 residues) were passed only twice on BHK and achieved high titers within 24h (Sa-Carvalho *et al.*, 1997). Nevertheless, since the actual length of the poly (C) tract of the #14A12/SAT2 chimera has not been determined, the role it might play in the slower growth properties observed for this virus can not be ruled out.

A



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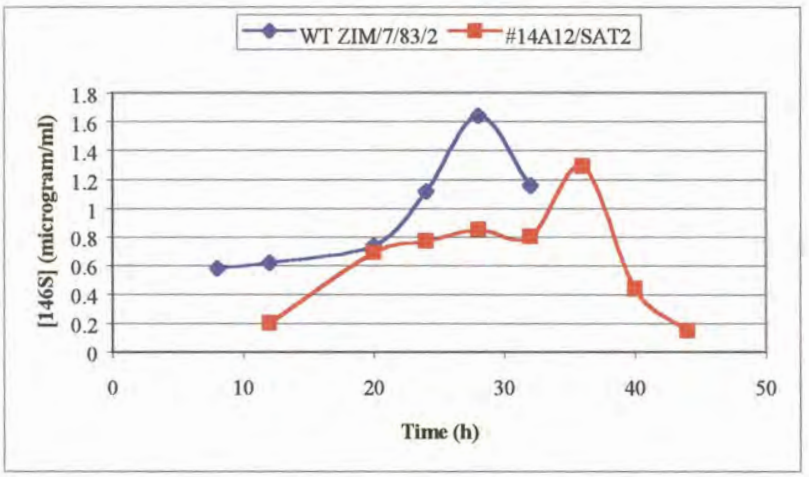

Figure 4.7: Comparative growth characteristics of wild type ZIM/7/83/2 and #14A12/SAT2 chimeric virus. (A) Virus growth as determined for ZIM/7/83/2 (28h) and #14A12/SAT2 (36h). (B) Antigen production (146S particle) as determined for ZIM/7/83/2 (28h) and #14A12/SAT2 (36h).

Similar growth properties for the wild type A_{12} and the recombinant virus derived from a full-length cDNA clone of the A_{12} genome containing 35 C residues were observed, reaching high titers within 24h (Rieder *et al.*, 1993). Although not performed under the same laboratory conditions, the growth rate as determined for the wild type ZIM/7/83/2 correlate well with that of A_{12}. Ideally, the recombinant #14A12/SAT2 virus should therefore display similar growth properties. The extended growth rate of 8h observed for the chimera could thus be indicative of inefficient replication of the SAT 2 capsid region by the A_{12} nonstructural proteins. Although only the VP0/VP3 cleavage site differs between A_{12} and ZIM/7/83/2, the regions adjacent to the P1 cleavage sites differ quite extensively between the two viruses (see Chapter 3, Figure 3.7). These junction sites might be important in the recognition of the processing site by the 3C proteinase. Another explanation for the slower growth rate observed for the chimeric virus could therefore be sub-optimal processing of the SAT 2 P1 region by the A_{12} 3C proteinase.

4.3.2.3 Thermal stability

The same infection rate (high m.o.i) employed during the growth studies were used to investigate the thermal stability of the chimeric virus. The chimeric virus was harvested after 36h, and the wild type after 28h. Incubation in the presence of 1% chloroform was carried out to lyse the cells and therefore to release cell-associated virus particles. Following centrifugation the supernatants of the two viruses were incubated at 4°C and 37°C for 21 days. From Figure 4.8 it is evident that the wild type ZIM/7/83/2 is stable at 37°C, exhibiting little variability in the 146S yield. The virus also seemed to be stable at 4°C. However, following a very low initial 146S yield for #14A12/SAT2, a 4 to 8 fold increase in 146S yield was obtained at 4°C and 37°C after 7 days. This result can be explained in terms of the aggregation of the viral particles. These viral particles aggregate with one another and therefore escape the UV-photometric measurement (Lei, 1978). The formation of these complexes may be caused by either electrostatic forces or hydrophobic interaction between viral particles, or both (S. J. Barteling, personal communication). Once the aggregates start to dissociate, the yield in 146S might increase drastically. In this case, higher yields for the chimeric virus were obtained after 7 days, but a decrease in 146S concentration followed over the next two weeks.

These results are indicative of the #14A12/SAT2 chimeric being less stable than the wild type ZIM/7/83/2. The stability of the A_{12} virus derived from the A_{12} cDNA clone has however not been determined under these conditions. It is therefore unclear at this stage whether the #14A12/SAT2 chimeric virus display similar thermal stability than the A_{12} virus or not. In addition, it is important to note that the thermal stability testing was carried out on the cell harvest and not on BEA/formaldehyde inactivated virus. The chemical inactivation process might also influence capsid stability. The results presented here may therefore not be a true reflection of the overall capsid stability of the viruses investigated.

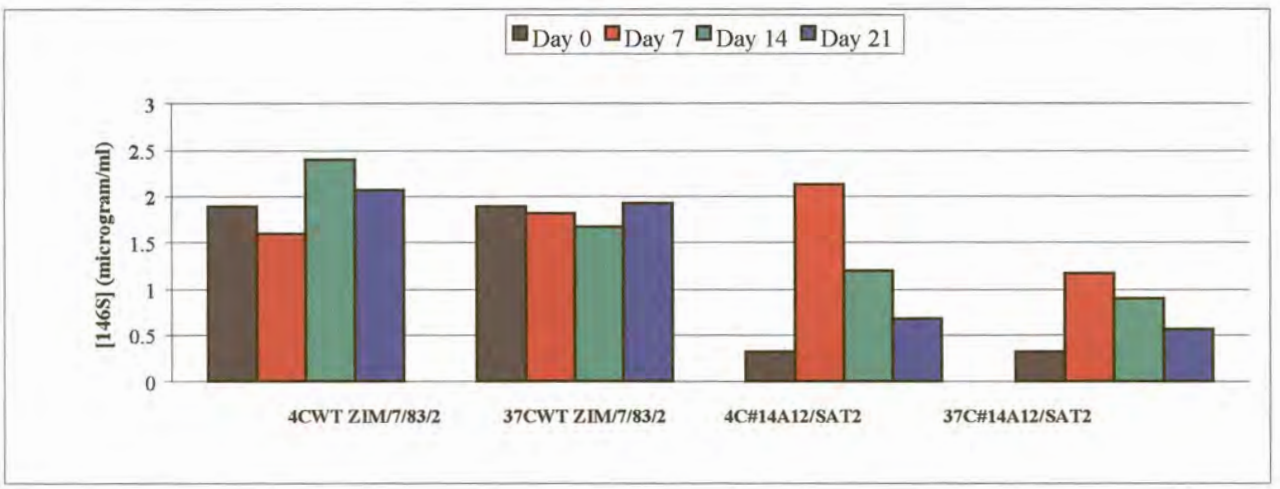

Figure 4.8: Thermal stability as determined for the WT ZIM/7/83/2 and #14A12/SAT2 chimeric virus at 4°C as well as 37°C, was carried out over a 21 day period. The amount of intact virions [146S] is presented in µg/ml.

4.4 Conclusions

In this chapter the construction of a chimeric cDNA clone between serotypes A and SAT 2 was described. The resulting recombinant virus was derived from infectious RNA transcribed from clone #14. This clone was obtained following the insertion of the external capsid-coding region of ZIM/7/83/2 cDNA into the A_{12} full-length cDNA clone, replacing the A_{12} structural-protein-coding region with that of ZIM/7/83/2. The resulting recombinant virus, #14A12/SAT2, was subsequently compared to the wild type SAT 2, ZIM/7/83/2. The results obtained indicated that the two viruses display almost identical immunogenicity, although the intactness of the epitopes could not be compared due to the unavailability of monoclonal antibodies against SAT 2. Comparisons in growth rate between the chimera and the wild type SAT 2 virus, ZIM/7/83/2, indicated the chimera to be a slower antigen producer, although comparative antigen yields could be obtained. Despite these yields, the chimeric virus constructed in this study between types A and SAT 2 is possibly not an ideal candidate for inactivated vaccine manufacturing due to the lack of stability displayed by this virus. The reason for the observed thermal instability is however unclear.

Although a viable chimeric virus could be constructed between the two serotypes, it is possible that the A_{12} replicating cDNA clone is not optimal for the construction and subsequent production of chimeric viruses employing the SAT type viruses originating from southern Africa. Alternatives should therefore be investigated.

CHAPTER 5

Concluding remarks: What does the future hold?

Custom-made vaccines, produced in response to specific outbreaks of FMD, may be achieved through the construction of recombinant viruses. In such recombinants, the antigenic determinants of a stable, high antigen producing and well-characterized FMDV strain may be manipulated to mimic the antigenicity of outbreak strains, leading to the production of conventional, but custom-made vaccines. Although a number of crucial factors, which may influence the success of such a strategy, were addressed in the current study, there are still many questions in need of answering. These include the protective roles of conformational and non-conformational epitopes as well as the role of proteolytic variation and cleavage site differences in isolates from different geographical localities.

Following the study on the genetic heterogeneity of the L^{pro} and $3C^{pro}$ of FMDV, it was seen that these genomic regions of the SAT type viruses originating from southern Africa, are distinct from that of types A, O and C. Although the three-dimensional structure of the ZIM/7/83/2 Lb^{pro} is completely conserved upon comparison to the Lb^{pro} crystal structure of O_1Kaufbeuren, the differences detected on the L/P1 cleavage site could have implications for intertypic processing. These implications (if any) will subsequently be investigated with the expression of Leader proteinases of SAT type representatives in bacteria. The purified proteinases will be assayed on oligopeptides corresponding to the cleavage site of the Leader proteinases of the European types. The activity of the proteinases to cleave the host cell protein eIF4G will also be examined. These biological assays might shed some light on the flexibility of FMDV proteolytic activity.

Despite the unknown factors, an infectious chimeric or recombinant virus between types A and SAT 2 was successfully constructed. This recombinant virus (#14A12/SAT2) contains the genetic background of type A_{12} as well as the external capsid region of ZIM/7/83/2. The subsequent evaluation of #14A12/SAT2 with respect

to the wild type SAT 2 virus revealed the chimera not only to be less stable, but also exhibited a slower growth rate. These characteristics make the current recombinant virus unsuitable for commercial vaccine manufacturing. Alternative means should therefore be explored to be able to produce stable and high antigen producing chimeric SAT viruses in order to address the antigenic variation in these viruses and the subsequent problems associated with vaccine production.

One such alternative option entails the construction of a full-length cDNA clone suitable for the southern African SAT type viruses. Such a cDNA clone has been constructed for the SAT 2 virus, ZIM/7/83/2 (Mason & van Rensburg, unpublished results). An exchange-cassette strategy (Figure 5.1) for the construction of this clone was followed. This approach entailed the exchange of certain regions of the original A_{12} full-length cDNA clone with the corresponding regions on the SAT 2 genome. These regions were obtained through PCR amplification from cDNA using a high fidelity enzyme and oligonucleotides, specifically designed with the required restriction enzyme sites to facilitate the cloning. During the construction of the cDNA clone, the 5' UTR was molecularly characterized. Despite low nucleotide sequence identity in this region (including a 37 nucleotide deletion in the S-fragment obtained for the SAT 2) between A_{12} and ZIM/7/83/2, similar folded structures could be obtained for the four pseudoknots and the S-fragments (hairpin-structure) of these viruses (results not shown).

Through the subsequent evaluation of these chimeric viruses (Figure 5.1) and comparison to the wild type SAT 2 virus, indications with regard to regions on the genome responsible for the reduced growth properties and lack of stability, might be obtained. The SAT 2 full-length cDNA clone will also be used to construct SAT 1 and SAT 3 chimeric viruses and together with similar constructs employing the A_{12} cDNA clone, be evaluated in terms of growth properties and stability. Such a strategy will, hopefully reveal whether the SAT 2 replicating cDNA clone is indeed a viable alternative in the construction and production of SAT chimeric viruses.

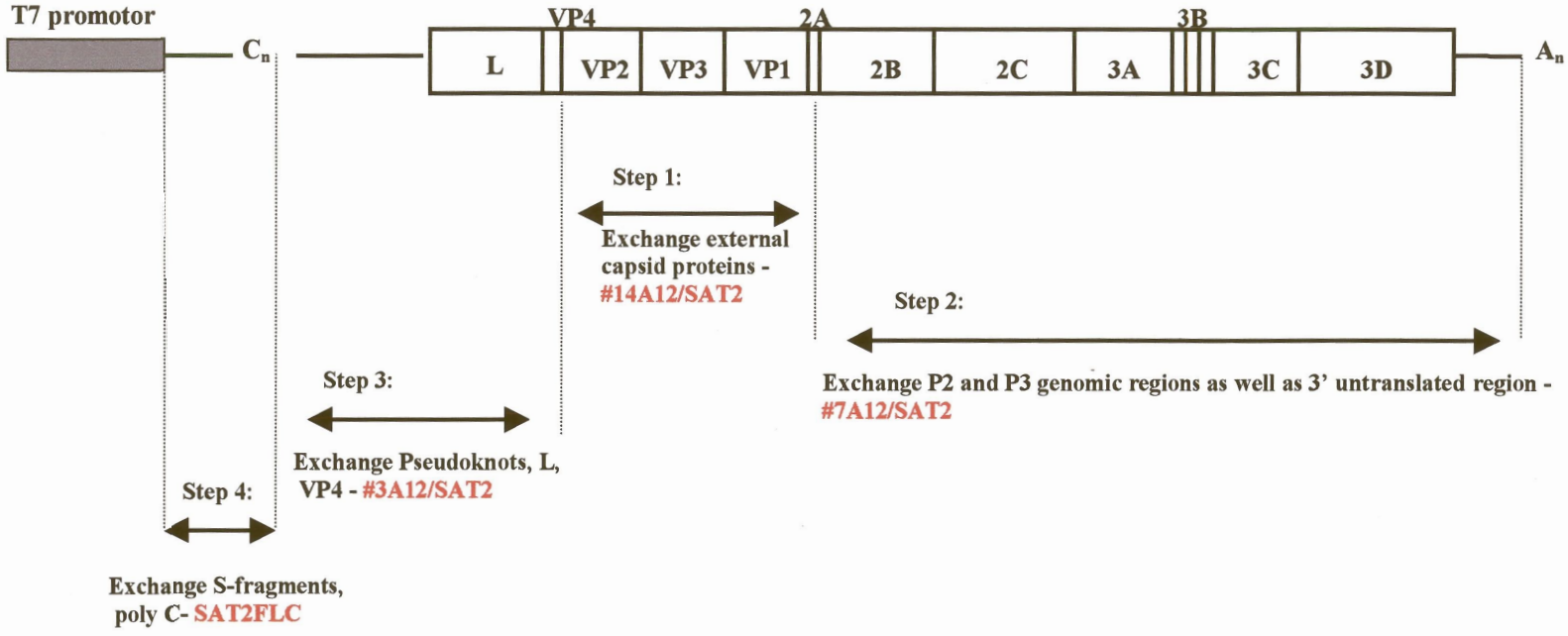

Figure 5.1: Schematic representation of the construction of the SAT 2 full-length cDNA clone (SAT2FLC). The chimeric SAT viruses obtained

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LIST OF CONGRESS CONTRIBUTIONS

H.G. van Rensburg, F. Joubert & L.H. Nel. Genetic heterogeneity in the leader and 3C proteinases of foot-and-mouth disease virus. EUROPIC 2000, Baia delle Zagare, Mattinata, Italy, 25-31 May 2000. (Talk)

H.G. van Rensburg & P.W. Mason. Construction of a chimeric virus between seotypes A and SAT 2 of foot-and-mouth disease virus. EUROPIC 2000, Baia delle Zagare, Mattinata, Italy, 25-31 May 2000. (Poster)

L. Heath, **H.G. van Rensburg**, W Vosloo & L.H. Nel. Genetic characterisation of the structural-protein-coding region of several South Afican type Foot-and-Mouth Disease viruses. 11th Biennial congress of the South African Society for Microbiology BioY2k Combined Millenium meeting, Grahamstown, 23-28 January 2000. (Talk)

H.G. van Rensburg, L.E. Heath, L.H. Nel & P.W. Mason. Important aspects in the development of recombinant vaccines against foot-and-mouth disease. 80th Annual Meeting of the Conference of research workers in Animal Diseases, Chicago, United States of America, 7 – 9 November, 1999. (Poster)

H.G. van Rensburg, L.H. Nel & L.E. Heath. Towards the development of a recombinant vaccine against foot-and-mouth disease. Xith International Congress of Virology, Sydney, Australia, 9-13 August, 1999. (Poster)

H. G. van Rensburg & L.H. Nel. Characterization of the structural-protein-coding region of foot- and-mouth disease virus serotype SAT2. 2nd FASBMB/15th SASBMB, Potchefstroom, 30 September - 3 October 1998. (Poster)

H.G. van Rensburg & L.H. Nel. Amino acid sequence comparison of foot-and-mouth disease viral proteases from different serotypes. 2nd FASBMB/15th , SASBMB, Potchefstroom, 30 September - 3 October 1998. (Poster)

H.G. van Rensburg & L.H. Nel. Characterization of the structural-protein-coding region of foot- and-mouth disease virus scrotype SAT2. EUROPIC '98, Jena, Germany, 5- 11 September 1998. (Poster)

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H.G. van Rensburg & L.H. Nel (1999) Characterization of the structural-protein-coding region of SAT2 type foot-and-mouth disease virus. **Virus Genes** 19 (3), 229 – 233.