THE DEVELOPMENT OF RECOMBINANT LUMPY SKIN DISEASE VIRUS VACCINES FOR FOOT-AND-MOUTH DISEASE AND RIFT VALLEY FEVER

by

Nicolette Michelle Edith Johnston

THE DEVELOPMENT OF RECOMBINANT LUMPY SKIN DISEASE VIRUS VACCINES FOR FOOT-AND-MOUTH DISEASE AND RIFT VALLEY FEVER

Nicolette Michelle Edith Johnston

A dissertation submitted in fulfilment
of the requirements for the
degree of Masters of Science (Med) in the
Department of Medical Microbiology, University of Cape Town
February, 2001

CONTENTS

ACKNOWLEDGEMENTS

I would firstly like to thank my supervisor, Associate Professor Anna-Lise Williamson, for all she has taught me, for her encouragement, patience and inspiration. I would also like to thank her for proof-reading of this thesis.

Secondly I would like to express my sincere thanks to my Mom and the rest of my family and friends for their interest, encouragement and for always supporting and believing in me.

In addition, I would like to thank the following people:

All my lab partners for all their invaluable help and encouragement.
A special thanks to Kate for proof-reading my thesis.
Wilna Vosloo, for her help with acquiring some of the reagents.
Hiram Arendse and Noel Markgraph for the animal work which they did for this project.
Thanks to the Foundation for Research and Development and the Poliomyelitis Research Foundation for my bursary.

Finally, I would like to thank Darryl for all his love, patience, encouragement and help.

ABBREVIATIONS

A	adenine
ATV	activated trypsin versene
aa	amino acid(s)
β-gal	β-galactosidase
bp	base pair(s)
CO_2	carbon dioxide
°C	degrees celcius
C	cytosine
DNA	deoxyribonucleic acid
DMSO	dimethyl sulfoxide
ELISA	enzyme linked immunosorbent assay
E.coli	*Escherichia coli*
EDTA	ethylenediamine tetraacetic acid
FITC	fluorescein isothyocyanate
FCS	foetal calf serum
FMD	Foot-and-mouth disease
FMDV	Foot-and-mouth disease virus
F	fungizone
G	guanine
HRP	horse radish peroxidase
IPTG	isopropyl-β-D-thio galactosidase
kB	kilobases
kD	kilodalton
LSD	Lumpy skin disease
LSDV	Lumpy skin disease virus
$MgCl_2$	magnesium chloride
µg	micrograms
µl	microlitres
ml	millilitres
mM	millimolar
M	Molar
N	normal
ηg	nanograms
NaOH	sodium hydroxide
nt	nucleotide
OPD	1,2-phenylenediamine dihydrochloride

pmol	picomole
pfu	plaque forming units
Ponceau S	3-hydroxy-4-(2-sulfo-4-[sulfophenylazo]-phenylazo)-2,7-naphthalenedisulfonic acid
RNase	ribonuclease
RVF	Rift valley fever
RVFV	Rift valley fever virus
rpm	revolutions per minute
r.t.	room temperature
PS	penicillin/streptomycin
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
SDS	sodium dodecyl sulfate
SSC	sodium sodium citrate
T	thymidine
TAE	Tris-acetate
TBS	tris-buffered saline
TBST	tris-buffered saline tween
UCT	University of Cape Town
UV	ultraviolet
U	units
U	uridine
V	volts
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactosidase
YT	yeast-tryptone

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Lumpy skin disease virus (LSDV), with a host-range restricted to ruminants, causes disease in cattle. LSDV is the ideal vaccine vector as poxviruses are able to stimulate both humoral and cellular immunity, are heat stable and have a low cost of production. The Neethling strain of LSDV therefore, forms the basis for the construction of two LSDV recombinant vaccines, one against foot-and-mouth disease virus and the other against rift valley fever virus.

Rift valley fever (RVF) is an important arthropod-borne disease which affects mostly sheep, cattle and goats, were it causes an acute febrile illness, abortions and even death. The plasmid, pSelp-HS-G1G2-βgal, which encodes the RVFV glycoproteins, G1 and G2, was transfected into lamb testes cells infected with the Neethling strain of LSDV. Following selection procedures, the recombinant virus was isolated. Once expression of the G1 and G2 genes had been confirmed by immunofluorescence, the recombinant virus was inoculated into rabbits. ELISA tests, however, failed to detect an antibody response to the RVFV.

The second part of the study involved the construction of a recombinant vaccine against foot-and-mouth disease virus (FMDV) which is a highly contagious disease infecting ruminants and pigs. The FMDV P1 gene (from serotype A24) was amplified from a reference plasmid and subcloned into pGEM Easy Vector ®. The newly constructed plasmid was sequenced and the P1 gene cloned into the shuttle vector, pAFMCRR, to generate the recombinant plasmid, pAFMCRRP1. Following successful transfection of pAFMCRRP1 into LSDV (Neethling) infected lamb testes cells, isolation and purification of the recombinant virus was carried. Expression of the P1 gene was evaluated by Western blots and immunofluorescence. Southern hybridisation analysis revealed the P1 gene in an integrated form in the LSDV genome.

CHAPTER 1: LITERATURE REVIEW

1.1: LUMPY SKIN DISEASE VIRUS

1.1.1: Introduction

Lumpy skin disease (LSD) was first described as a skin disease in Northern Rhodesia (now Zambia) in 1929. The disease was referred to as pseudo-urticaria and was thought to be the result of an allergenic reaction to the bites of insects (McDonald, 1931). The disease continued to occur sporadically in other African countries over subsequent years. Although LSD was shown to be infectious and transmissible, the virus was only isolated in 1948 by Van Den Ende *et al* (1948). However, Van Den Ende *et al* (1984) failed to show an aetiological relationship between this isolate and the disease, LSD (Haig, 1957; Alexander *et al.*, 1957). It was only in 1959 that lumpy skin disease virus (LSDV) was ascribed to be the causal agent of lumpy skin disease (Neethling) by Prydie and Coackley, 1959, following isolation of the virus from skin lesions of diseased cattle and after passaging in tissue culture. Presently, the disease is found throughout sub-Saharan Africa, North Africa and the Middle East (Davies, 1991a and Carn, 1993).

Lumpy skin disease virus is a member of the family, *Poxviridae*. These are large complex DNA viruses which are capable of replicating in vertebrate and invertebrate cells (Moss, 1996a). LSDV specifically affects cattle and is characterised by fever and the appearance of skin nodules of between 5 to 50 mm in diameter, which usually undergo necrosis. The disease is thus of major economic importance as it can result in temporary or permanent infertility, anorexia, cessation of milk production, permanent damage to hides as well as abortion in pregnant cows (Henning, 1956 and Barnard *et al.*, 1994).

A number of measures have been implemented to control and prevent the spread of LSDV. Two live attenuated vaccines were developed in Kenya

and South Africa respectively to protect against LSDV. Both proved effective in protecting cattle from LSDV infections (Barnard *et al.*, 1994, Capstick *et al.*, 1961).

1.1.2: Classification

The *Poxviridae* family can be divided into two subfamilies, *Chordopoxvirinae*, which are vertebrate poxviruses and *Entomopoxvirinae*, which are insect poxviruses (Moss, 1996a). The genus capripoxvirus falls under the subfamily *Chordopoxvirinae* of which sheep pox, goat pox and LSDV are members (refer to table 1.1). Clearly being able to distinguish between these poxviruses had for many years been a problem as they shared a major precipitating antigen (Kitching *et al.*, 1986), very high nucleotide homology (Gershon and Black, 1988) and do not have absolute host specificity (Kitching and Taylor, 1985).

Black *et al.*, 1986, characterised a number of capripoxvirus strains according to the country and species from which each had originated. Evidence did suggest that at least two of these strains were recombinant viruses. The use of enzyme restriction analysis was employed to further determine genome homology between capripoxviruses. This was based on Schumperli and co-authors (1978) who had determined that with restriction endonuclease digests using a six nucleotide cutter, a genomic homology of greater than 80% can be inferred between two DNA species if their restriction patterns contain comigrating patterns. Black *et al.* (1986) and Kitching *et al.* (1989) used the *Hin*dIII restriction endonuclease to characterise a number of strains of capripoxviruses as it generated approximately 40 fragments with a wide range of sizes. They found that although all the capripoxviruses so far studied shared a close degree of relatedness, it was possible to distinguish and compare the various strains using this method. Restriction enzyme analysis also indicated that Kenyan sheep and cattle isolates are more closely related to one another than to isolates from goats (Gershon and

Black, 1988). This indicated that sheep and cattle isolates diverged from goat isolates prior to diverging from one another. Based on restriction mapping analysis of the genomes of 26 capripoxvirus isolates, the capripoxviruses have been divided into four types:

(1) isolates from sheep,

(2) isolates from goats,

(3) isolates restricted to Africa from sheep, goats and cattle and

(4) isolates from either sheep or goats in Africa or the Middle East (Gershon *et al.*, 1989).

However, neither sheep- nor goatpox have been diagnosed in southern Africa (Munz and Dumbell, 1994) and as such speculation exists on whether the above mentioned classification system is accurate (personal communication, Ass. Prof. A-L. Williamson).

Table 1.1: Classification of the family *Poxviridae*. Adapted from Moss, 1996.

SUBFAMILIES	GENERA	SELECTED MEMBERS
CHORDOPOXVIRINAE (Vertebrate poxviruses)	*CAPRIPOXVIRUS*	Lumpy skin disease virus, goatpox virus, sheeppox virus
	AVIPOXVIRUS	Fowlpox virus, pigeonpox virus, canarypox virus
	ORTHOPOXVIRUS	Vaccinia virus, variola virus, camelpox virus
	PARAPOXVIRUS	Orf virus
	LEPORIPOXVIRUS	Shope fibroma virus
	SUIPOXVIRUS	Swinepox virus
	YATAPOXVIRUS	Tanapox virus
	MOLLUSCIPOXVIRUS	*Molluscum contagiosum*
ENTOMOPOXVIRINAE (insect poxviruses)	*ENTOMOPOXVIRUS A*	*Melolontha melolontha*
	ENTOMOPOXVIRUS B	*Amsacta moorei*
	ENTOMOPOXVIRUS C	*Chironimus luridus*

1.1.3: Host range

Cattle are naturally infected by LSDV, in which all breeds and both sexes are susceptible (Weiss, 1968). The role of other animals in the epidemiology of LSD however, remains unclear. Davies (1980) found that many buffalo showed a high titre of LSDV antibodies and thus concluded that they could act as a reservoir host. However, other surveys failed to show infection in buffalo (Hamblin *et al.*, 1990). Experimental infection of buffalo with LSDV also failed to generate the disease, whereas giraffes (*Giraffa camelopardalis*) and impala (*Aepyceros melampus*) died of LSD following experimental infection with the virus (Young *et al.*, 1970). Although there is no supporting evidence to indicate that sheep may act as carriers of LSDV, Barnard *et al.*, 1994, found LSDV was able to replicate and be recovered from sheep inoculated with various field isolates of LSDV.

1.1.4: Transmission

The mode of transmission of LSDV has not been established with any certainty but evidence does suggest the role of biting insects in the spread of the disease. Carn and Kitching (1995) showed the transmission of LSDV to animals by intravenously feeding arthropod vectors. This is consistent with reports from field observations that outbreaks of LSDV remained contained in the absence of significant populations of biting flies. Furthermore, Carn and Kitching, 1995, showed that the transmission of LSDV was inefficient in the absence of arthropods as the uninfected cattle housed with infected cattle failed to seroconvert or show any signs of LSDV infection. The disease also diminished with the onset of the dry season which correlated with reduction of biting flies. The virus has also been recovered from the flies, *Stomoxys calcitrans* and *Biomyia fasciata* (cited by Tripathy *et al.*,1981). Other modes of transmission of LSDV have been implicated. Henning, 1956, claimed that the disease could be transmitted to suckling calves through infected milk,

although at this stage, there was some confusion as to the exact causal agent of LSD.

1.1.5: Virion and genome structure and organisation

The virions of poxviruses are larger than other viruses and are just visible by light microscopy. The mature capripoxviruses measures approximately 350 x 300 nm and are enveloped (Munz and Owen, 1966). Within the outer membrane, tubular protein filaments are irregularly located on the surface (refer to figure 1.1 for electron micrograph of LSDV particles).

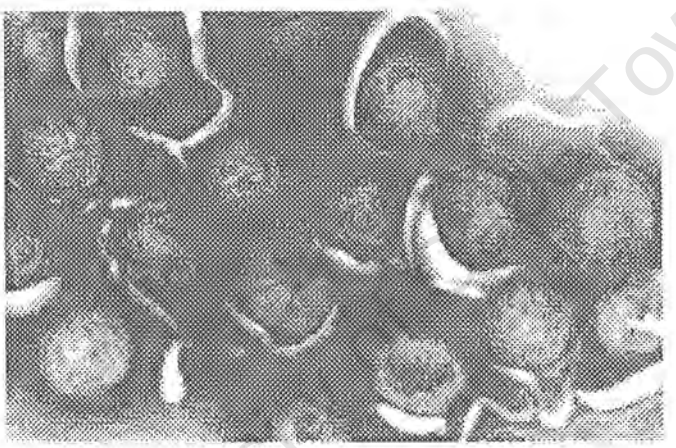


Figure 1.1: Electron micrograph of LSDV particles purified from infected bovine kidney cells. The envelope surrounding most of the virions has collapsed to reveal the tubular structure of the outer membrane. Magnification X75 000. Micrograph by Dr L. Stannard.

When negatively stained at pH6.5, the virus particles morphologically resemble the M-forms (describing virions covered in a cord-like structure or mulberry) of the vaccinia virus (Westwood *et al.*, 1964). When negatively stained at pH8.5, the virus particles do not show the thread-like surface structure as seen at pH6.5 but more a homogeneous granular mass enveloped by a capsule (described as the C-form) (Weiss, 1968). Both C-forms and M-forms are observed at pH7.0 in stain preparations, which is in accordance with other poxviruses. The virus does maintain infectivity

between pH6.6 and pH8.6 without significant decline in titre after exposure for 5 days at 37^{0}C (Tripathy *et al.*, 1981). The virus has also been shown to be relatively stable as it was shown to survive for 33 days in necrotic dry skin lesions and for 6 months in infected tissue culture fluid at 4^{0}C (Tripathy *et al.*, 1981). Weiss (1968) also demonstrated that LSDV could remain viable for 18 days in scrapings from lesions in air-dried portions of the hide held at room temperature and for 10 years under dry ice refrigeration in tissue culture fluid. The virus is sensitive to ether and chloroform (unlike orthopoxviruses) and is inactivated by the detergent sodium-dodecyl-sulphate, suggesting that a lipid is incorporated in the structure of the virus (Plowright and Ferris, 1959).

Although earlier literature states that the genome size of capripoxviruses ranges between 143-147 kilobases (kb) in length, experiments done by Perlman (1993) indicated that the genome size of LSDV Neethling is in fact 152.61kb in length. This was found to be some 9kb larger than the size specified by Gershon and Black, 1988, for the same strain of capripoxvirus (refer to figure 1.2 and 1.3).

The genomic organisation of capripoxviruses has been elucidated by methods of cross-hybridisation of mapped genome fragments with those of other poxviruses. Inverted terminal repeats have been found in all poxviruses examined and are identical but oppositely orientated at either end of the genome (sited by Moss, 1996a). Gershon *et al.* (1989) found that capripoxviruses are more divergent in sequence and size towards their termini than in the central region which is in keeping with the poxvirus genomes. Gershon and Black (1989) have also found that the centrally located 2.5K genomic fragment from the Kenya sheep-1 strain contains three complete and two incomplete open reading frames (ORFs). One of these ORFs has been shown to be the gene for thymidine kinase. The additional ORF located directly downstream of the thymidine kinase gene is not present on the vaccinia and fowlpoxvirus genomes but does match a sequence immediately downstream of the Shope fibroma virus genome, of the genus leporipoxvirus (Gershon and Black, 1989).

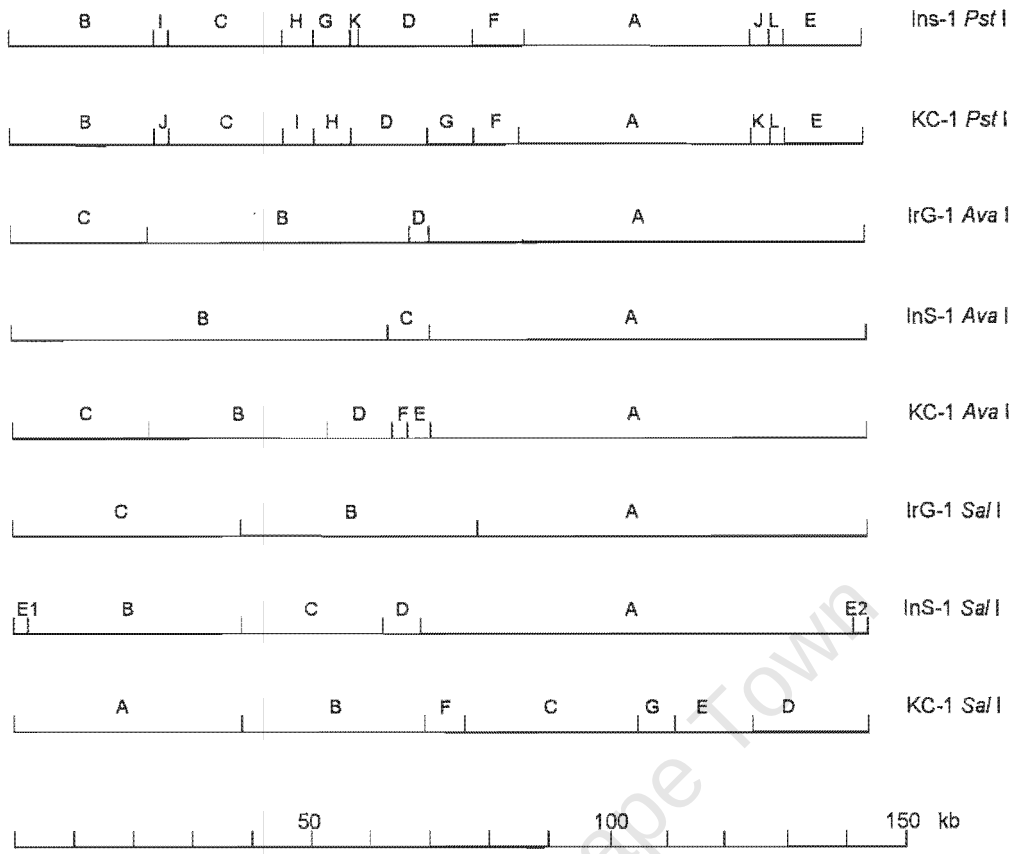


Figure 1.2: Restriction enzyme maps of the capripoxviruses India Sheep -1 (IS-1), Kenya cattle -1 (KC-1) & Iraqi Goat -1(IrG-1), using the restriction enzymes *Ava* I, *Sal* I and *Pst* I. Taken from Gershon and Black, 1988.

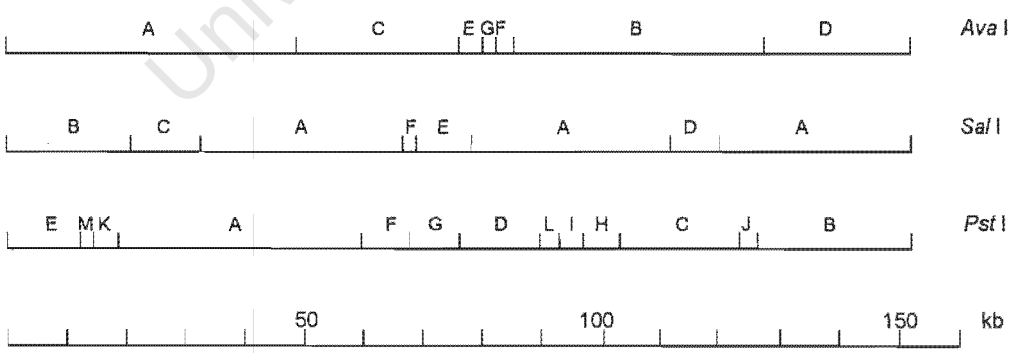


Figure 1.3: Restriction enzyme map of LSDV Neethling using the restriction enzymes *Ava*I, *Sal*I and *Pst*I. Taken from Perlman, 1993

1.1.6: Replication and gene expression

Little is known about the replication cycle of LSDV per se. However, much more is known about poxviruses replication and these basic features can be applied to other family members. Poxviruses differ from the majority of DNA viruses in that they replicate and transcribe their genome within the cytoplasm of infected cells and encode most of the proteins required for the synthesis of viral macromolecules (Moss, 1996a). Refer to figure 1.4 for diagrammatic representation of the replication cycle of vaccinia virus.

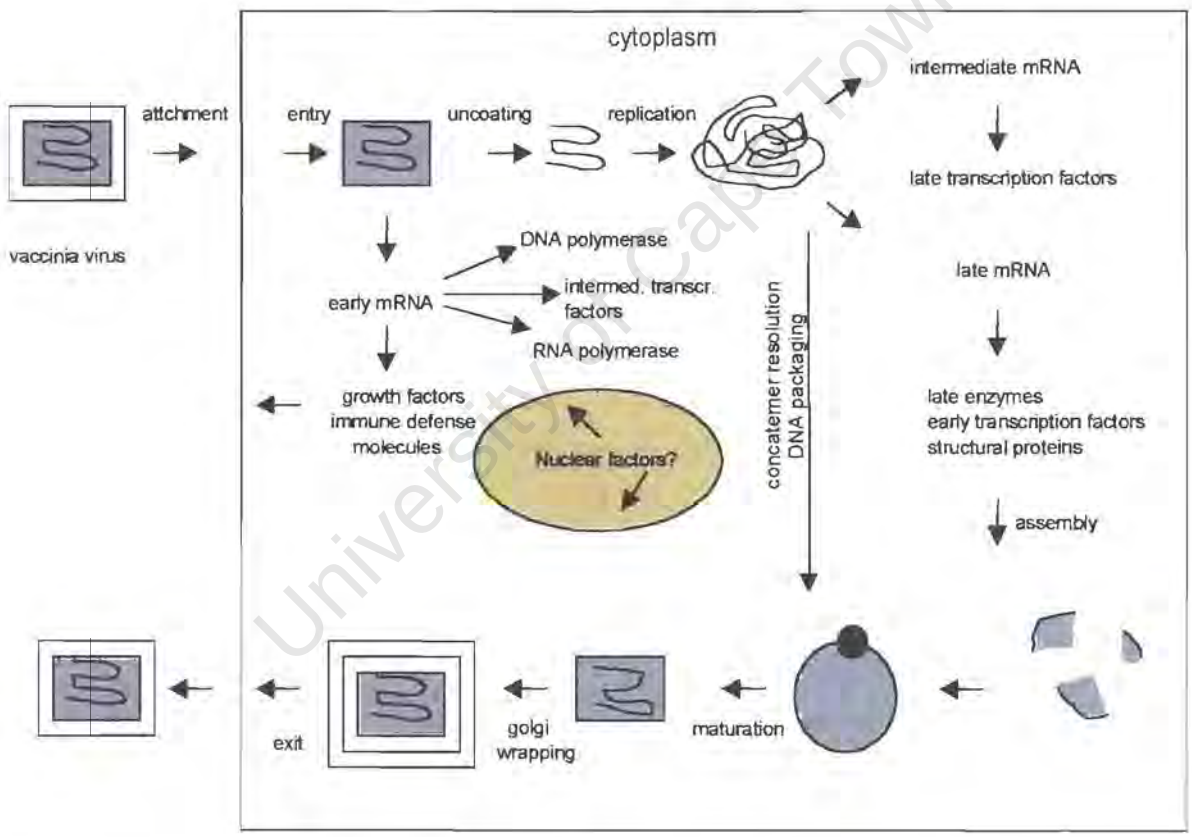


Figure 1.4: Replication cycle of vaccinia virus. Virions enter the cells by as yet unknown mechanism (see text for more detail), releasing cores into the cytoplasm. The cores synthesise early mRNAs that are translated into a variety of proteins. Uncoating occurs and the DNA is replicated. Intermediate genes are transcribed and translated. Assembly begins once the late mRNAs have been translated. The concatemeric DNA intermediates are resolved and packaged in immature virions. Maturation takes place to finally produce the infectious virus. Taken from Moss, 1996a.

The accepted view is that poxviruses enter cells by means of fusion at the plasma membrane (Doms *et al.*, 1990), or alternatively, via endophagocytosis (Dales and Kajioka, 1964). Experiments using lysomotropic agents (weak bases which can raise the pH of endosomes, thus blocking proteolysis) increased the infectivity of vaccinia virus (Janeczko *et al.*, 1987). Additionally, subcellular fractionation studies revealed that some virion proteins remain associated with fractions containing the plasma membrane and there was an absence of viral polypeptides from endosomes (Janeczko *et al.*, 1987; Mallon and Holowczak, 1985). The implication therefore, is that the plasma membrane may be the most important site of entry. Support was further obtained for a neutral pH, temperature-dependent process by directly monitoring the cell fusion of vaccinia virus intracellular mature virus (IMV) using fluorescence assay (Doms *et al.*, 1990). However in studies done by Chung *et al.*, 1998, vaccinia virus was shown to bind to cell surface heparan sulphate during infection. Since entry is often a multiple stage process, it is possible that heparan sulfate-virus interaction could induce conformation rearrangements that may enhance subsequent fusion events (Chung *et al.*, 1998). Furthermore, Chung *et al.*, 1998, also found that one viral membrane protein, A27L, could mediate virus attachment to the heparan sulfate moieties on cells. Although this data suggests an attachment-fusion type entry, studies by Pedersen *et al.*, 2000, argues against fusion as they found the viral remnants attached to the plasma membrane but not merged with it. Recent studies have also demonstrated a signal-dependent entry for the IMV but not the extracellular enveloped virus (EEV), both of which are infectious forms of the vaccinia virus (Krijnse Locker *et al.*, 2000).

Upon delivery of the viral core to the cytoplasm, viral uncoating occurs. The virus undergoes at least two stages of disassembly (Pedley and Cooper, 1987). The first stage involves the loss of virion coat proteins and lipids, with the viral genome still protected within the core (Holowczak, 1972; Sarov and Joklik, 1972). Here, core disassembly can be prevented by using the vaccinia virus early transcription inhibitor actinomycin, indicating that early

vaccinia virus protein synthesis is required for core uncoating (Holowczak, 1972; Sarov and Joklik, 1972). During the second stage the genome becomes accessible to DNase (Holowczak, 1972; Sarov and Joklik, 1972).

Poxviruses encodes most if not all of the genes required for replication in the cytoplasm, which begins within 3 hours after infection (Harford *et al.*, 1966). The genes can be divided into three classes which are expressed in succession (Pennington, 1974). Transcription of the early genes occurs immediately after infection and does not require *de novo* protein and DNA synthesis (Keck *et al.*, 1990). Transcription of the early genes are also regulated by A/T-rich promoters located upstream of the transcription initiation (Cochran *et al.*, 1985; Weir and Moss, 1987). Early genes transcribed include DNA polymerase and other proteins involved in transcriptional activity of late genes. Early mRNA transcripts are capped at the 5' end (Wei and Moss, 1975) and polyadenylated at the 3' end (Kates and Beeson, 1970). Cellular protein synthesis is shut down soon after infection, which is apparently due to viral protein(s) and/or the production of the polyadenylated nontranslated viral RNAs that affect ribosome function.

Only five vaccinia virus genes, which have intermediate promoters, have been identified thus far. One of these is a RNA helicase (Vos and Stunnenberg, 1988), the other a phosphoprotein, which interacts with ribonucleotide reductase (Davies and Mathews, 1993), and three late transcription factors (Keck *et al.*, 1990). At least 4 proteins are required for the transcription of the intermediate genes (*in vitro*) – RNA polymerase (Vos *et al.*, 1991), capping enzymes (Harris *et al.*, 1993), VITF-1, an unidentified cellular protein called VITF-2 (Rosales *et al.*, 1994) and VITF-3 (Sanz and Moss, 1999). Although all the viral proteins play a role in the biosynthesis of the three classes of mRNAs, VITF-3 seems to play a role in the regulation of transcription of the intermediate genes. Intermediate mRNAs are synthesised for a short period before transcription of late genes occurs which is approximately 6 hours after infection (Baldick and Moss, 1993). There are numerous late genes, which encode major structural proteins and other

components of viral particles, expression of which is dependent on DNA replication.

Replication of poxviruses is not well understood, but replication is presumed to begin from a nick introduced near one or both ends of the genome. This nick exposes a 3'OH group which serves as a primer for strand-displacement synthesis. This results in concatemer junctions as replication occurs through the hairpin. Concatemeric intermediates are resolved into unit-length genomes following the onset of late-stage transcription (Moss, 1996a).

The basic pathway of virion assembly was elucidated by using transmission electron microscopy. Assembly begins with the appearance of discrete structures deep within the virus factories which are the crescent-shaped membranes that develop into spherical particles and acquire a DNA nucleoid before sealing. A series of maturation steps occur, including proteolysis which gives rise to the IMV which represent the majority of infectious progeny (Moss and Rosenblum, 1973). Some IMV become enveloped by two membranes derived from the Golgi complex (Schmelz *et al.*, 1994) or early endosomes (Tooze *et al.*, 1993) to form intracellular enveloped virus (IEV) particles. However, recent studies by Hollinshead *et al.*, 1999, indicated that vaccinia virus IMV are surrounded by only a single lipid bilayer membrane. In addition, Hollinshead *et al.*, 1999, also indicated that the origin of this membrane was in question, but does not appear to be derived from host organelle membranes. The IEV particles can induce the polymerisation of actin (Cudmore *et al.*, 1995) and move to the cell surface where the outer membrane fuses with the plasma membrane, forming EEV. The EEV can either be released from the cell or retained at the cell surface as cell-associated enveloped virus (CEV). CEV and EEV are thought to be responsible for cell-to-cell transmission and long-range spread (Blasco and Moss, 1992; Payne, 1980). The electron micrographs documenting the morphogensis of poxviruses from different genera and subfamilies have been

very similar thus suggesting that these viruses all have the same number of membranes that form in similar ways (cited by Hollinshead *et al.*, 1999).

1.1.7: Prevention and control

In September 1989, the European Economic Community (EEC) identified LSDV as a notifiable animal disease, effectively compelling countries to report outbreaks of the disease within 24 hours to all other members of the Community. This measure was implemented to restrict and prevent LSDV infection in Europe. Other measures have also been taken in which countries that are Capripox-free impose restrictions on the import of livestock and animal products from LSDV affected areas (Carn, 1993). In countries which are remote from enzootic areas, these preventative measures coupled with radical slaughter policies in the event of an outbreak as well as radial vaccination policies has up to now ensured the control of LSDV in their countries.

In enzootic regions, vaccination policies are followed occasionally by slaughter of infected animals and in-contact animals during outbreaks which are well confined (Carn, 1993). The reliance on vaccination is sensible as attempts to control the disease by quarantine and movement control is impractical because transmission of LSDV is mainly due to arthropod vectors (Barnard *et al.*, 1994). In regions that have a high probability of LSD outbreak, vaccination of young stock before they enter the herd is generally followed.

The first work done on the development of a vaccine against LSD was carried out at the Onderstepoort Veterinary Institute in Pretoria, South Africa, following severe outbreaks of LSD in the 1940s and 1950s. The Neethling strain of LSDV was attenuated by passaging the virus in embryonated eggs (Van Rooyen *et al.*, 1959). The virus was further attenuated by Weiss (1968) following 60 passages in lamb kidney cells and 20 times on the chorio-

allantoic membrane of hen's eggs. Today the attenuated virus is propagated in cell culture. The vaccine is administered subcutaneously with a minimum protective dosage of $10^{3.5}$ $TCID_{50}$ (Davies, 1991a) One disadvantage of this vaccine is that it produces a localised lesion at the site of inoculation in approximately 50% of animals, but this usually regresses after a month (Weiss, 1968). There may also be a temporary decrease in milk production in dairy cattle.

A similar attenuated vaccine was developed in Chad in the late 1960's by passaging a Madagascan strain of LSDV 101 times in rabbit kidney cells and 5 times in fetal calf kidney cells (Ramisse *et al.*, 1969). However, this vaccine is not widely used.

Subsequent vaccines have been developed which rely on the use of a heterologous virus. The first was described by Capstick and Coackley (1961) who used the Kedong and Isiolo strains of a sheep poxvirus which was grown in lamb testes and kidney cells and used to protect cattle against LSD. This vaccine was used extensively in Kenya between 1958 and 1959. Care, however, has to be taken when using this vaccine as it can provide a source infection for susceptible sheep. More recently, the Romanian and RM65 strains of sheep pox were used in Egypt and Israel respectively in an attempt to contain outbreaks of LSD in these regions (Davies, 1991b). These sheep pox virus strains, used as vaccines, were shown to be immunogenic in the field with no reported complications (Carn, 1993).

Attempts have also been made to produce subunit vaccines. Carn *et al.*, 1994, showed that goats inoculated with a fusion protein consisting of the capripoxvirus group specific antigen (KS-1 P32) linked to glutathione S-transferase (GST) could illicit neutralising antibodies and reduced clinical signs on challenge. One of the benefits of using a subunit vaccine is that it could be used in countries where capripoxviruses are not endemic (Carn *et al.*, 1994).

1.1.8: Poxviruses as a live recombinant vaccine vector

Recombinant vaccines refer to viruses in which foreign genes have been inserted into the non-pathogenic virus genome by *in vivo* recombination. These recombinant viruses retain their infectivity and are able to express the foreign gene and elicit a humoral and cell-mediated immune response to the immunogenic antigens. In certain circumstances recombinant viruses can elicit protection against more than one disease at the same time if immunogens from more than one pathogen are inserted into the virus vector. Recombinant vaccines are generally safer to use than live attenuated vaccines, in which the possibility of reverting back to the pathogenic strain exists, as only immunogenic subunit or subunits of the pathogen is used.

The pioneering work on recombinant vaccines was done on the poxvirus, vaccinia virus (Yamanouchi *et al.*, 1998). This was made possible as much is known about the molecular biology of vaccinia virus. Furthermore, considerable experience has been gained from the development, production and use of the smallpox vaccine which could be applied to that of recombinant vaccinia virus vaccines. The vaccinia virus has a large genome which allows for the insertion of large fragments of foreign DNA of up to 25kb (Smith and Moss, 1983), this permits the insertion of different genes into the genome, allowing for the development of polyvalent vaccines. Insertion of the foreign gene occurs under specific conditions. The foreign gene has to be inserted into a region which is non-essential to the replicative cycle of the virus and has to contain its own start and stop codons to allow for the correct reading of the foreign gene. Furthermore, poxvirus RNA polymerase only recognises its own transcriptional control sequence (promoters) and thus the foreign gene has to be inserted downstream of an adjacent poxvirus promoter (Baxby, 1993). The other possibility is the insertion of the foreign gene with its own poxvirus promoter (Mackett *et al.*, 1982). The promoter may be an early promoter (pre-replicative) or a late promoter (post-replicative), which will indicate at which stage during the

replicative cycle the foreign gene is to be expressed. In order that the foreign gene be inserted into the poxvirus genome, homologous recombination has to occur. This is executed by means of a shuttle vector or insertional plasmid (refer to figure 1.5).

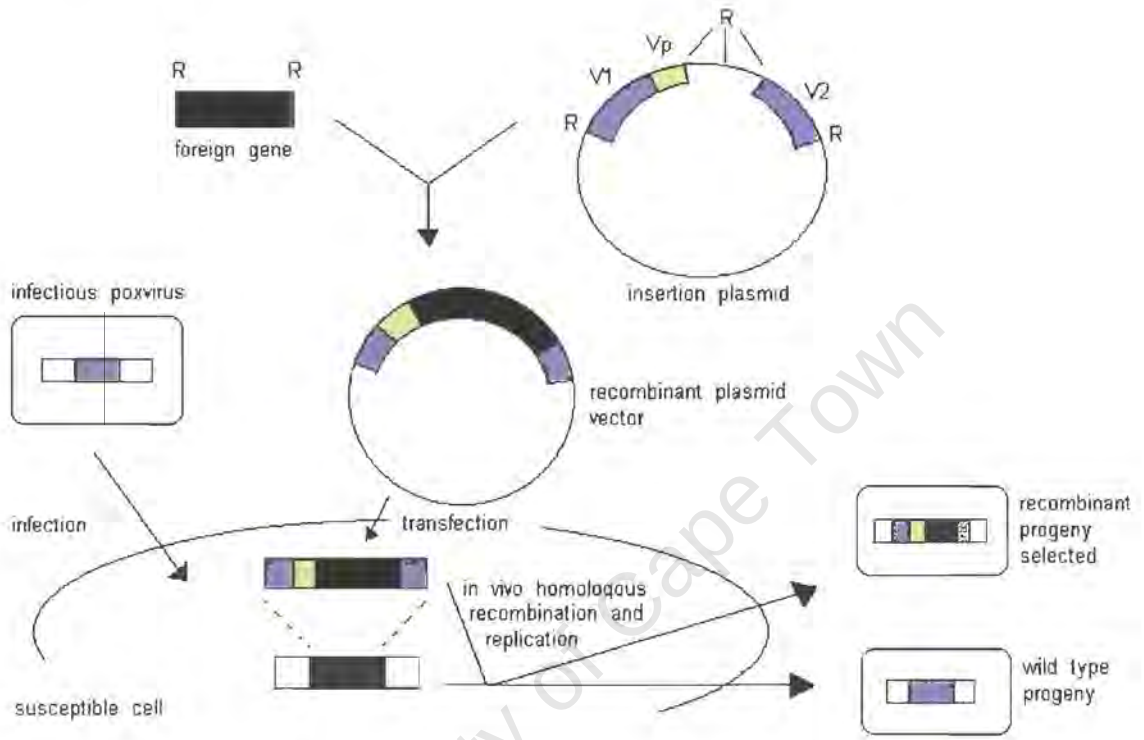


Figure 1.5: Production of recombinant vaccinia viruses. R = restriction sites, Vp = vaccinia promoter, V1 and V2 = two sections of a non-essential virus gene. Adapted from Baxby, 1993

The shuttle vector has the appropriate restriction sites to allow insertion of the foreign gene which are flanked by the non-essential region and the promoter. The shuttle vector is then transfected into susceptible cells which have been infected with the non-pathogenic virus. During replication of the virus, *in vivo* recombination can take place in which the foreign gene is incorporated into the vaccinia genome at the site dictated by the identity of the flanking non-essential sequences. Recombinant viruses can then be selected for by using the appropriate methods. Methods used for the

isolation of the recombinant virus are varied. The *E.coli lacZ* gene which can be incorporated into the virus via the shuttle vector allows for blue selection of recombinants when the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside is present. A number of other selection methods are available. A common strategy is the incorporation of the foreign gene into the thymidine kinase (TK) gene of the virus, thereby inactivating it. The thymidine kinase gene codes for an enzyme which is involved in the salvage pathway for the synthesis of thymidine nucleotides. A number of TK^- cell lines have been isolated from different mammalian species and these cell lines are able to grow in medium which contains the thymidine analog 5-bromodioxyuridine. Thus recombinants would be TK^- and can grow in TK^- cells in the presence of 5-bromodioxyuridine whereas non-recombinants are TK^+ and is lethal in the presence of 5-bromodioxyuridine. An additional method involves the use of mycophenolic acid (MPA), an inhibitor of purine metabolism. Cells are normally unable to synthesize guanylic acid (GMP) in a medium containing mycophenolic acid as this substance inhibits inosinate dehydrogenase (IMP) and so prevents the formation of xanthosine monophosphate (XMP). By the incorporation and expression of the *E.coli* (*gpt*) gene which codes for xanthine-guanine phosphoribosyl transferase (XGPRT) (Boyle and Coupar, 1988) an analog of the mammalian enzyme hypoxanthine-guanine phosphoribosyl transferase (hgprt), this block can be overcome in the presence of xanthine and hypoxanthine. Hypoxanthine and guanine are substrates for hgprt, while xgprt can use xanthine and hypoxanthine for the synthesis of XMP. The pathway has been illustrated in figure 1.6.

A number of immunogens from human and veterinary pathogens have been inserted into the vaccinia virus (Tartaglia *et al.*, 1990). Presently, a rabies recombinant vaccinia virus is in use as a veterinary oral vaccine against rabies (Yamanouchi *et al.*, 1998). This was achieved by inserting the rabies virus glycoprotein G gene into the Copenhagen strain of vaccinia virus (Blancou *et al.*, 1986).

Further research has consequently given rise to more attenuated strains of vaccinia virus. One of these includes the modified vaccinia Ankara strain (MVA) which was passaged 570 times in chicken embryo fibroblasts and has large deletions resulting in a genome length of 178kb. Thus, MVA does not productively infect mammalian cells and must be grown up in avian cells (Scheiflinger *et al.*, 1996). This makes MVA an ideal vaccinia virus strain for use as a live recombinant vaccine vector. Mice immunized with recombinant MVA viruses expressing the influenza haemagglutinin and nucleoprotein genes or parainfluenza type 3 virus antigens demonstrated protective immunity (Sutter *et al.*, 1994, Bender *et al.*, 1996 and Wyatt *et al.*, 1996). Other recombinant MVA virus have been developed including:

(1) recombinant MVA viruses expressing Japanese encephalitis virus (JEV) genes, with effective protection developed in mice challenged with a 10^5 LD_{50} JEV (Nam *et al.*, 1999).

(2) recombinant MVA viruses expressing respiratory syncytial virus genes, with increased levels of antibodies following priming and boosting (Wyatt *et al.*, 2000).

(3) recombinant MVA expressing malaria antigens, which, together with a malaria DNA vaccine prime, generated a strong CTL response against malaria in mice (Schneider *et al.*, 1998).

Recent developments have also suggested that recombinant MVA has considerable potential as a vaccine vector for human AIDS.

The techniques, pioneered in vaccinia virus, have been extended to incorporate other poxviruses. Many of these poxviruses have restricted host ranges and certain poxviruses do not cause productive infection in mammalian cells making them safer than vaccinia virus and thus ideal vaccine candidates. The fowlpox vaccine strain, has been used to incorporate the HA gene of avian influenza virus and was shown to protect chickens against challenge with virulent avian influenza virus (Taylor *et al.*, 1988). Other useful vaccines made using the fowlpox vaccine strain include recombinant vaccines against Newcastle Disease (Taylor *et al.*, 1991) and Gumboro disease (Bayliss *et al.*, 1991). Canarypox recombinant vaccines

have been developed against viral haemorrhagic disease in rabbits (Fischer *et al.*, 1997), against canine distemper in ferrets (Stephensen *et al.*, 1997) and rabies (Taylor *et al.*, 1991).

The LSDV vaccine (Neethling strain) has been shown to be a good recombinant vaccine candidate (Cohen *et al.*, 1999, Turner-Smith *et al.*, 1999). Like vaccinia virus, a number of foreign genes can be inserted into the LSDV genome and furthermore, the correct post-translational modifications of viral glycoproteins is generated. The LSDV vaccine strain is thermostable and the cost of production is low. LSDV has a limited host range and will only naturally infect cattle, sheep and goats and therefore poses no risk to humans, making it a safe bovine and ovine vaccine vector. In addition, the capripoxvirus vaccine is not transmitted horizontally from vaccinated to unvaccinated animals or vertically during pregnancy (Kitching *et al.*, 1986). The attenuated vaccine strain for LSDV was developed over thirty years ago and has proved safe and effective, adding to the benefits of selecting it as a candidate recombinant vaccine.

The use of other capripoxviruses as a successful recombinant vaccine has been documented. Romero *et al.*, 1994a, showed protection of cattle against virulent rinderpest and LSDV using a recombinant Kenya sheep-1 strain expressing the fusion (F) protein of rinderpest virus. Another Kenya sheep-1 recombinant vaccine, expressing the hemagglutinin (H) gene of the RBOK vaccine strain of rinderpest virus, was also developed by Romero *et al.*, 1994b. This recombinant produced significant amounts of the correctly folded H protein, to finally be expressed on the membrane of infected cells. This proved highly immunogenic as vaccinated cattle were protected against virulent rinderpest virus challenge. Goats, vaccinated with recombinant capripoxvirus expressing either the fusion gene or the haemagglutinin gene of rinderpest virus or a mixtures of the two, were protected from challenge with peste des petits ruminants virus (Romero *et al.*, 1995). Protection was also demonstrated in Kenyan cattle vaccinated with differing doses of an equal mixture of recombinant capripoxvirus expressing either the fusion gene

or the haemagglutinin gene of rinderpest virus (Ngichabe *et al.*, 1997). Here, the results indicated that, 1 month post-vaccination with a dosage of $2X10^4$ p.f.u. or greater of the combined recombinant viruses, cattle were fully protected against challenge with rinderpest virus and LSDV (Ngichabe *et al.*, 1997). A recombinant capripoxvirus, expressing the major core structural protein of bluetongue virus, provided partial protection in sheep against challenge with virulent heterotypic virus (Wade-Evans *et al.*, 1996).

The attenuated LSDV vaccine strain is thus a good candidate for the production of new recombinant vaccines with the added advantage of not only immunising against the foreign gene product but also against virulent LSDV.

1.2: PROJECT MOTIVATION

Foot and mouth disease is a highly contagious disease, significantly impacting free-trade in animals and animal products internationally. Rift valley fever too, is of major economic importance and a public health concern in Africa, where it infects ruminants and humans. Compounding these factors are the problems and concerns associated with currently available vaccines. Investigations into the development of recombinant poxviruses have shown that they are ideal vaccine vectors.

The aim of the project therefore, was to develop two live recombinant vaccines. One against RVFV and the other against FMDV. The RVFV glycoproteins G1 and G2 and the FMDV P1 were chosen as candidates for insertion into the LSD poxvirus genome as they have shown to be immunogenic (Collett *et al.*, 1987, Schmaljohn *et al.*, 1989) . In order that insertion can occur however, shuttle vectors had to be constructed containing the insertion sequence flanked by poxvirus sequences that direct recombination to the desired locus. The next step was transfection experiments allowing in vivo homologous recombination to occur. Finally, purification and isolation experiments, of the recombinant virus, was carried out. Evaluation of the constructed recombinant viruses was performed with comparisons carried out with wild type LSDV (Neethling) as well as uninfected lamb testes cells. RVF-LSDV was further tested as a candidate vaccine by the evaluation of the immune response in rabbits following vaccination with the recombinant virus.

CHAPTER 2: MATERIALS AND METHODS

2.1: INTRODUCTION AND STRATEGY

One of the distinctive features of poxviruses and African Swine Fever virus is their ability to entirely replicate within the cytoplasm of infected cells (Moss, 1996a). This factor makes it possible for homologous DNA recombination in infected cells to occur, a process that naturally occurs during the replication of poxviruses (Ball, 1987). As poxviruses use their own transcription system, poxvirus promoters and continuous open reading frames are needed for the expression of foreign genes. The level and time of gene expression can be controlled by the choice of an early, intermediate or a late promoters, with the highest level of expression obtained with strong natural or synthetic late or early/late promoters (Moss, 1996b). Methods for producing recombinant poxviruses employ plasmid transfer vectors. These vectors contain an expression cassette, which consists of a poxvirus promoter with adjacent restriction endonuclease sites for foreign gene insertion, flanked by poxvirus sequences, which directs the recombination event to the desired locus. Once homologous recombination has occurred, screening methods are used for the identification and isolation of recombinant virus. These screening procedures are possible as the transfer vector may allow integration of reporter and selection genes.

The basis for the construction therefore of a RVF-LSDV and a FMD-LSDV recombinant vaccine is very much the same. Both recombinant viruses employ the Neethling vaccine strain of LSDV, which has a number of advantages associated with its use, as a vehicle for the generation of recombinant vaccines (as discussed in 1.1.8). In this study, the genes of interest from RVFV and FMDV were inserted into the LSDV genome by means of a plasmid transfer vector, although different transfer vectors were used for each recombinant virus made.

The transfer vector, pAFMCRR (Appendix B), which was constructed by K.Aspden (unpublished data), was used for the insertion of the FMDV genes. This plasmid has the ability to replicate in bacterial *Escherichia coli* (*E.coli*) cells as it has an *E.coli* origin of replication. The plasmid has gene

sequences flanking the insertion site, which are homologous to the non-essential ribonucleotide reductase gene within the LSDV genome. This factor allows for the insertion of foreign genes into the LSDV genome. The plasmid also has a multiple cloning site, and although not essential, allows one greater flexibility. A bi-directional fowlpox promoter, is found just upstream of the multiple cloning site and is used for the expression of the reporter gene, *lacZ*, and the FMDV P1 gene. The selectable marker, *Ecogpt*, is under the control of the vaccinia virus P7.5 late promoter.

The pSelp-HS-G1-G2βgal plasmid (Appendix B), used for the construction of a RVF-LSDV recombinant virus, already contains the RVFV glycoprotein gene and was constructed by A.Cohen. The *Ecogpt* gene is under the control of the vaccinia virus P7.5 late promoter. This plasmid has a reporter gene, the *lacZ* gene, which is controlled by the elp1 promoter. The RVFV G1 and G2 genes are controlled by the pSelp promoter, which is a synthetic early/late poxvirus promoter. The site for insertion into the LSDV genome is an intergenic region which is reported to have some advantages over the use of so-called non-essential genes as insertion sites. A study by Letellier (1993) indicated that TK^+ recombinants viruses had a growth advantage over TK^- recombinants viruses. Therefore, disruption of non-essential regions in the LSDV genome may phenotypically alter the virus, affecting the growth of the virus. This in turn may not be desirable for the large-scale manufacture of a vaccine.

The process of construction of each of the LSDV recombinant vaccines has been illustrated in figure 2.1 and 2.2.

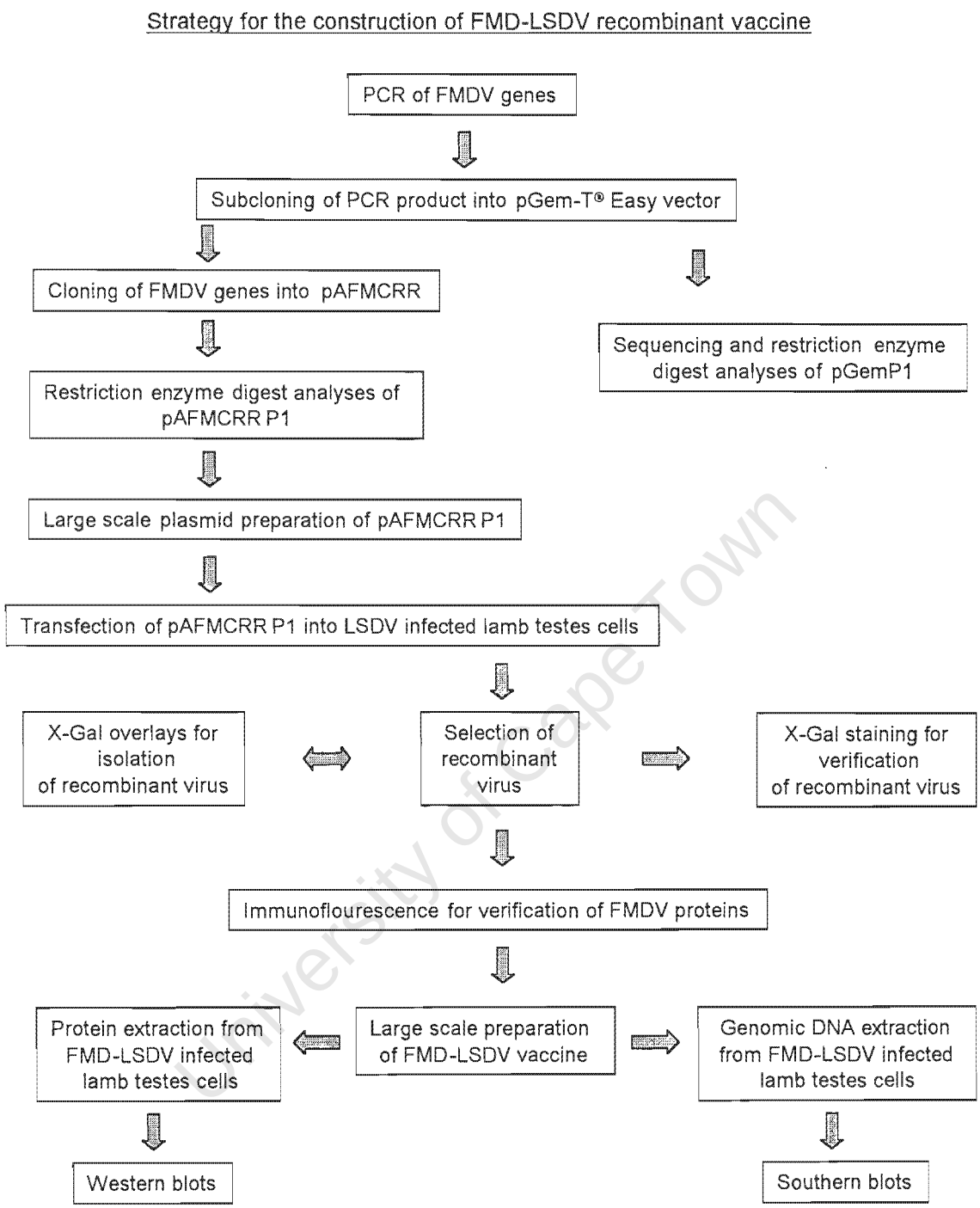


Figure 2.1: Outline of steps and techniques used for the generation of a FMD-LSDV recombinant vaccine.

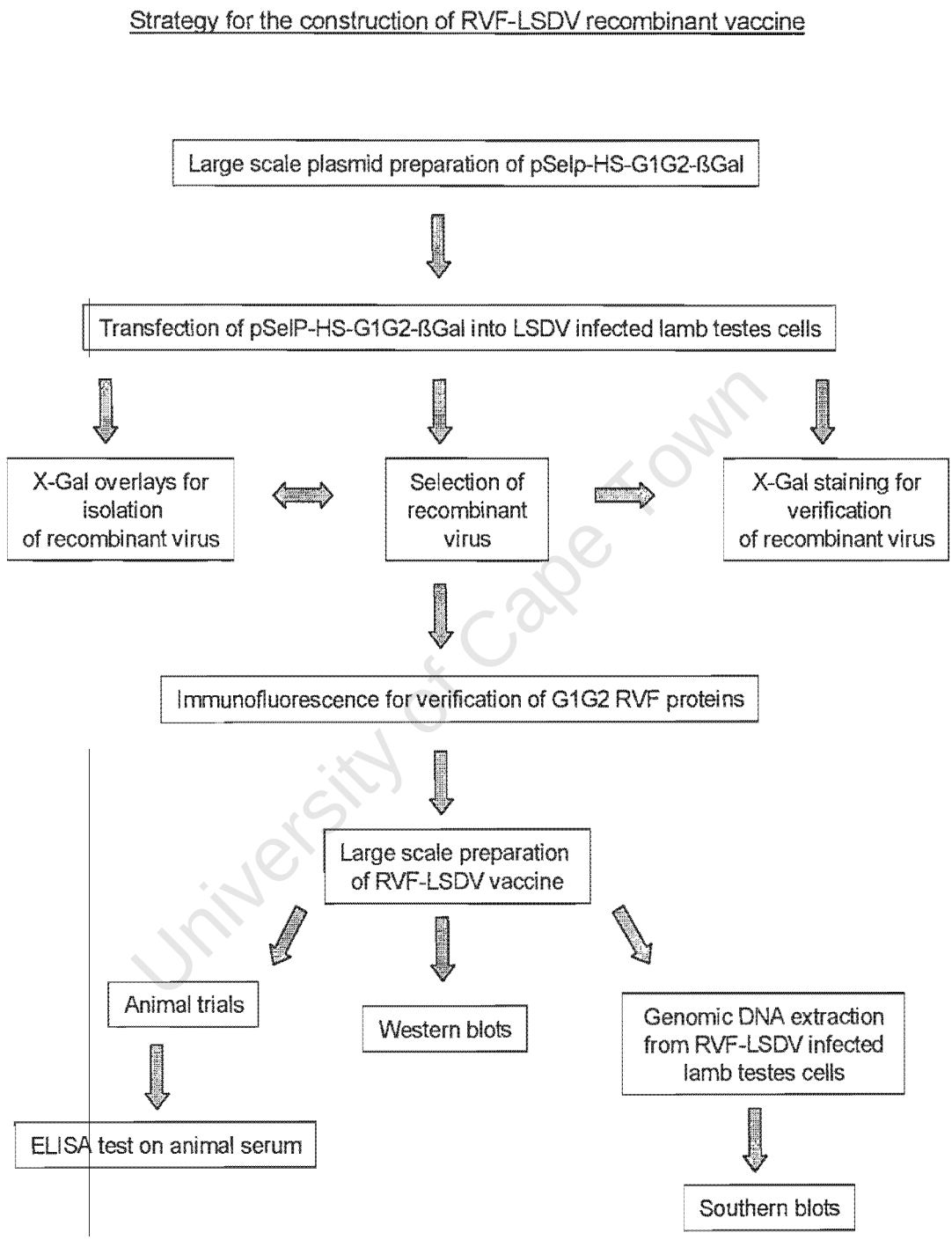


Figure 2.2: Outline of the steps and techniques used for the generation of a RVF-LSDV recombinant vaccine.

2.2: CONSTRUCTION OF THE FMD-LSDV RECOMBINANT PLASMID (pAFMCRRP1)

2.2.1: Polymerase chain reaction

The polymerase chain reaction (PCR) is used to exponentially amplify a region of DNA, which lies between two primers (Sambrook *et al.*, 1989). The primers are designed such that they are complementary to the sequence that lies on the opposite strand of the template DNA. The template DNA is first denatured in the presence of the primers, four deoxynucleoside triphosphates (dNTPs) and the DNA polymerase. The reactions are allowed to cool in order that the primers can anneal to the target sequence and extension can occur with the themostable *Taq* DNA polymerase.

The Expand High Fidelity PCR System was used as it also contains a Pwo proofreading polymerase, which was originally isolated from the thermophilic archaebacterium *Pyrococcus woesei* (Roche, Germany). This allows for an error rate of only 8.5×10^{-6} thereby offering a higher fidelity than *Taq* alone. All other reagents were also supplied by Roche, Germany. Reaction volumes were typically 50µl. Each reaction contained 2µl of 10mM PCR Nucleotide Mix (Roche, Germany), 35-40pmol of the forward and reverse primers, 5µl of 10X Expand HF buffer, 0.75µl of Expand™ High Fidelity PCR System enzyme mix and 10ηg template DNA. A varying amount of magnesium chloride ($MgCl_2$) was added to each reaction for optimisation of the PCR. The final volume of each reaction was made up to 50µl with distilled water. Negative controls consisting of PCR reactions without template DNA were included in all PCR reactions carried out to detect any contamination in the experiment.

The PCR was carried out using a Perkin Elmer GenAmp 9600 Thermocycler with the following designed cycle profile (refer to table 2.1) yielding the best results:

Table 2.1: Cycle profile for PCR, designed for the amplification of the FMDV P1 gene from a reference plasmid pCa24IRES.

	Denature	Anneal	Elongation
Cycle 1x	2minutes 94°C		
Cycle 35x	30seconds 94°C	45seconds 62°C	2.30minutes 72°C
Cycle 1x			6minutes 72°C

2.2.2: Preparation of plasmid DNA

2.2.2.1: Large scale DNA plasmid extractions

A single bacterial colony containing the plasmid to be isolated was inoculated into 2X yeast-tryptone (YT) broth. The antibiotic, ampicillin at a concentration of 50μg/ml, was included to prevent untransformed bacteria from growing. The bacteria were incubated at 37^{0}C overnight with shaking. The bacterial cells were harvested by centrifugation at 5000 revolutions per minute (rpm) in a JA-20 fixed angle rotor in a Beckman J2-21 centrifuge (Beckman, Irvine, USA) for 5 minutes at 4^{0}C. The plasmid DNA was extracted using the Nucleobond AX PC-kit 100 (Macherey-Nagel, Germany) according to the manufacturers' instructions. For plasmid DNA used for transfections the Qiagen Plasmid Midi Kit and Nucleobond AX PC-kit 100 was used.

Nucleobond AX is a silica-based anion exchanger and uses a technique similar to the alkaline/sodium dodecyl sulphate (SDS) lysis method. The bacterial pellet was gently resuspended in a buffer solution containing Ribonuclease A to remove the RNA. The bacterial culture was lysed by the addition of a sodium hydroxide (NaOH)/SDS solution. A potassium acetate solution was added which precipitates chromosomal DNA and other cellular compounds which was then pelleted at 10 000 rpm in a JA-20 rotor using a

Beckman in a F45-30-11 rotor in an Eppendorf Centrifuge 5417C. The plasmid DNA remains in solution and reanneals to form its supercoiled structure. The supernatant was removed and loaded on a Nucleobond cartridge which was equilibrated. The DNA binds to the silica matrix as the supernatant is eluted by gravity flow. A washing step removed contaminating substances and elution of the plasmid DNA was carried out. The elutant was collected in 2ml microfuge tubes (Eppendorf-Netheler-Ninz-GmbH, Germany) and the DNA precipitated by the addition of 0.8 volumes of isopropanol at room temperature and centrifuged at 14 000 rpm in a F45-30-11 rotor in a Eppendorf Centrifuge 5417C (Eppendorf-Netheler-Ninz-GmbH, Germany) for 30 minutes. The DNA pellet was washed with 70% ethanol, the microfuge tube re-orientated and centrifuged for 10 minutes at 14 000 rpm in a F45-30-11 rotor in a Centrifuge 5417C. The ethanol was removed and the DNA pellet dried under vacuum in a Speedvac concentrator (Lasec) and finally dissolved in 55µl of ultra pure water.

The Qiagen plasmid purification system uses an anion-exchange resin which interacts with the negatively charged phosphates of the DNA backbone. The protocol is similar to that employed by the Nucleobond system. Salt concentrations and pH conditions provided for by the buffers determine whether DNA is bound or eluted from the column. The bacterial colony was grown up overnight in Luria broth at 37^0C, pelleted and resuspended in a buffer solution. The cells were then lysed and centrifuged to remove cellular debris. The supernatant was loaded on a column and the bound DNA washed twice with the washing buffer supplied. The DNA was eluted, precipitated with isopropanol, washed with 70% ethanol, air-dried and resuspended in ultra pure water.

2.2.2.2: Small scale plasmid preparation

Small-scale isolation of DNA plasmids was carried out to enable one to analyse the recombinant plasmid. Sequencing was carried out on plasmid DNA isolated using the High Pure Plasmid Isolation Kit (Roche, Germany). A

quick lysis method was used when the plasmid had to be analysed by restriction digestion.

When using the High Pure Plasmid Isolation Kit (Roche, Germany), a bacterial colony was inoculated into 2 to 4ml of 2XYT broth and grown up overnight in a shaking 37°C incubator. A bacterial culture volume of between 0.5 and 4ml was pelleted in a 2ml tube (Eppendorf, Germany), resuspended in 250µl suspension buffer and mixed well. Lysis buffer (250µl) was added, the solution gently mixed and incubated for 5 minutes at room temperature (r.t.) before 350µl of chilled binding buffer was added, the solution gently mixed again and placed on ice for a further 5 minutes. The cell debris was collected by centrifugation at 14 000rpm in a F45-30-11 rotor in an Eppendorf Centrifuge 5417C for 10 minutes before the supernatant was loaded onto a High Pure filter tube and centrifuged for 30 to 60 seconds at maximum speed to allow the DNA to bind to the glass fibers. The flowthrough was discarded and a washing step carried out. The flowthrough was again discarded and an additional centrifugation step included to remove residual wash buffer. The DNA was eluted with a 100µl of water and centrifuged for 30 seconds at 14 000rpm in a F45-30-11 rotor in an Eppendorf Centrifuge 5417C.

The quick lysis method, which is described by Berghammer and Auer (1992), has been shown to be very fast, however, degradation of the DNA does occur and therefore storage of the DNA is not possible. A single bacterial colony was grown up in 2 to 4ml of 2XYT broth at 37°C in a shaking incubator. The culture was pelleted in a 2ml tube (Eppendorf, Germany). The cells were resuspended in 35µl lysis buffer A (Appendix A) and left shaking at r.t. for 5 minutes. The solution was placed in boiling water for 1 minute and thereafter immediately placed on ice for a further 1 minute. The solution was centrifuged for 10 minutes at 14 000rpm in a F45-30-11 rotor in an Eppendorf Centrifuge 5417C, the supernatant (containing the plasmid DNA) removed and analysed by restriction enzyme digestion.

2.2.3: Restriction endonuclease digestion

Restriction enzymes are able to bind to a specific site, termed the recognition site, were they cleave the double-stranded DNA within or adjacent to the site. All restriction enzymes and buffers were supplied by Roche Molecular Biochemicals (Cape Town). Each enzyme reaction was carried out with the correct buffers containing the necessary salt and other components for optimal activity. Typically 1 unit (U) of enzyme completely digests 1μg of DNA and the amount of enzyme included in the reaction was therefore adjusted according to the amount of DNA in the reaction. The enzyme volumes never exceeded $^{1}/_{10}$th of the total reaction volume as the glycerol inhibited the reaction. The reaction volume was generally 20μl. Digestions were usually carried out at 37^{0}C in a water bath for 60 to 120 minutes, unless the suppliers recommended different incubation temperatures. In the case of large quantities of DNA that had to be digested, such as genomic DNA, the reaction volume was adjusted proportionally and incubation was for carried out for 5 hours or overnight at the appropriate temperature.

Multiple digestions were carried out simultaneously when the temperature and buffer requirements were the same. If reaction conditions were not the same, they were done sequentially, starting with the enzyme needing the lowest salt requirements.

The enzyme reactions were stopped by the addition of 10X stop buffer (Appendix A) and the digested fragments analysed by electrophoresis on an agarose gel. A molecular weight marker of known fragment sizes was included on the gel.

2.2.4: Agarose gel electrophoresis

DNA can migrate in agarose when an electric field is applied, with the rate of migration dependent on the size of the DNA (Ausubel *et al.*, 1987). An intercalating fluorescent dye, ethidium bromide, is added to the agarose to

allow detection of the DNA within the gel. The agarose gel electrophoresis was performed using a submerged horizontal slab gel system (Sambrook *et al.*, 1989). Agarose (Techcomp LTD., Hong Kong) was dissolved in Tris-acetate buffer (TAE) (Appendix A), with the concentration of the agarose dependent on the sizes of the DNA to be separated. The agarose was dissolved by heating the solution to boiling temperature in a microwave (Sharp Carousel). The solution was then cooled to approximately 56°C and ethidium bromide (Appendix A) added to a final concentration of 0.5μg/ml. Once cooled the agarose was poured into a gel mould and allowed to solidify with a comb in place.

The gels were placed in a tank containing TAE buffer and the comb removed. Loading buffer (Appendix A) was added to the samples which serves three purposes: the buffer contains sucrose which increases the density of the sample allowing the DNA to drop evenly in the wells, the dye allows one to monitor migration of the DNA and the ethylenediamine tetraacetic acid (EDTA) chelates the Mg^{2+} ions thereby inhibiting enzymatic activity. An amount of 5μl to 20 μl was loaded per well. Molecular weight markers were included for all agarose gel electrophoresis performed and chosen to cover the range of fragments being examined. Electrophoresis of minigels was carried out at 60 to 95 V and a constant current. For fragments such as those generated from digestion of genomic DNA was electrophoresed overnight at 10 to 13 V. Gels were viewed on a 254nm ultraviolet (UV) transilluminator and photographed using a DC120 Zoom Digital Camera (Eastman Kodak Company, New York)

2.2.5: Cloning

The principle of cloning involves ligation of vector DNA with insert or foreign DNA having compatible ends (Sambrook *et al.*, 1989). The resultant plasmid is then transformed into competent cells and resultant bacterial colonies screened for recombinant plasmid (Sambrook *et al.*, 1989). Sub-cloning refers to the procedure of cloning into a vector, removing of the

inserted DNA and cloning the insert once again into another vector. This enables one to generate different restriction sites on either end of the insert DNA or to facilitate the cloning of PCR products. The PCR product generated from the amplification of the FMDV P1 gene was subcloned into the pGEM®-T Easy Vector, excised using *Bam*HI and *Sal*I and finally cloned into pAFMCRR. Refer to figure 2.3 for an illustration of the cloning strategy of the FMDV P1 gene.

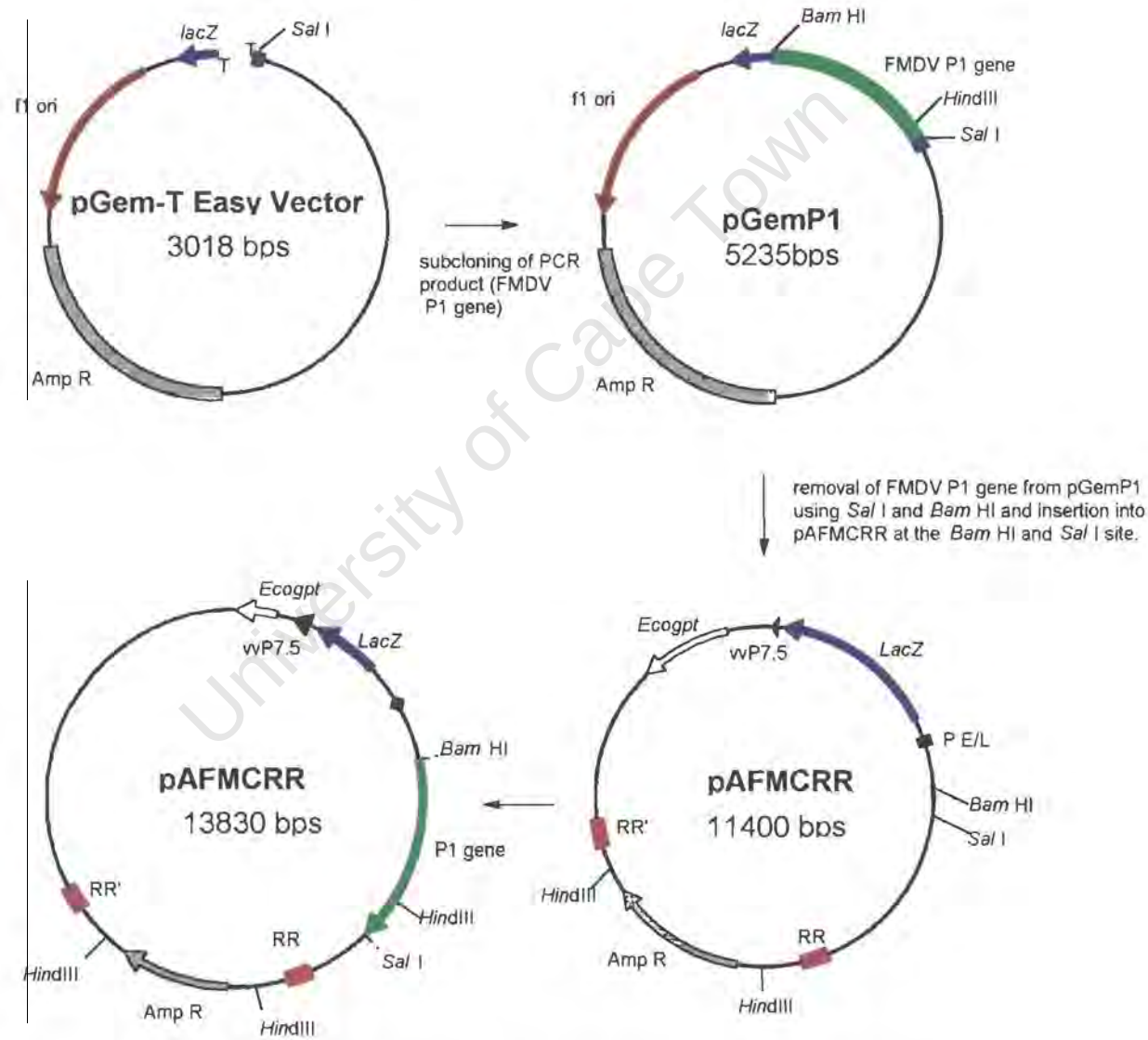


Figure 2.3: Cloning strategy for the construction of pAFMCRRP1. RR and RR' represents the ribonucleotide reductase gene. AmpR represents ampicillin resistance gene. Plasmid maps not drawn to scale.

2.2.5.1: Subcloning of PCR product into pGEM®-T Easy Vector

2.2.5.1.1: Preparation of vector and insert DNA

The PCR product was subjected to electrophoresis in agarose without ethidium bromide, the gel briefly stained and the PCR product visualised under long wave UV light. The band was excised from the agarose and the Cleanmix Kit (Talent, Italy) used for purification of the DNA, according to the manufacturers' specifications. An aliquot of the resultant DNA was electrophoresed in agarose to determine the concentration before been sub-cloned using the pGEM®-T Easy Vector System I (Promega, USA). The vector DNA, supplied with the pGEM®-T Easy Vector System, has 3'-T overhangs at the insertion sites. This greatly improves the efficiency of ligation of PCR product by preventing recircularisation of the vector and providing a compatible overhang for PCR products generated by the polymerase.

2.2.5.1.2: Ligation

An optimal ratio of vector DNA to insert DNA exists which allows for the greatest amount of correctly ligated vector and insert DNA. A ratio of 3:1 of vector to inset was used with a ligation reaction. The T4 DNA ligase (1µl) (Promega, USA) and 5µl of 2Xrapid ligation buffer (Promega, USA) was added with the final volume made up to 10µl with dH_2O. The ligation was carried out at 16°C overnight and then transformed into competent *E.coli* DHα cells as described in 2.2.5.4. Control ligations were also included which consisting of vector DNA only.

2.2.5.2: Cloning of FMDV P1 gene into pAFMCRR

2.2.5.2.1: Preparation of vector and insert DNA

The pAFMCRR plasmid was digested with the restriction enzymes *Bam*HI and *Sal*I (as described in section 2.2.3) to allow compatible termini for

ligation with the FMDV P1 gene. The stuffer fragment was removed by electrophoresing the digest in agarose and the appropriate fragment was cut out. The Cleanmix kit (Talent, Italy) was used for purification of the fragment according to the manufacturers' instructions.

The insert fragment (i.e. FMDV P1 gene) was released from the pGemP1 plasmid by digestion with *Bam*HI and *Sal*I as described in section 2.2.3. The digest was subjected to electrophoresis in agarose; the fragment containing the FMDV P1 gene cut out and purified using the Cleanmix kit according to the manufacturers' instructions.

2.2.5.2.2: Ligation

For optimal ligation, 10ηg of insert was ligated with 50ηg of vector. The reaction volume was 20μl with the addition of 2μl of 10Xligase buffer and 0.5μl T4 ligase (1U/μl; Roche, Germany). The reaction was incubated overnight at 16°C and then transformed into competent *E.coli* DHα cells as described below. Control ligations were included consisting of:

(a) vector, no insert, no ligase
(b) vector, no insert, ligase,
(c) insert, no vector, no ligase
(d) insert, no vector, ligase

2.2.5.3: Preparation of competent *E.coli* cells

Competent *E.coli* strains of DHα and LKIII cells were prepared using the dimethyl sulfoxide (DMSO) method (Chung and Miller, 1988). A single bacterial colony was picked and grown up in 5ml of 2XYT overnight at 37°C with shaking. The overnight culture was then diluted $^{1}/_{100}$ in 100ml of 2XYT and allowed to grow to early log phase (OD_{600} = 0.35). The culture was transferred to ice-cold polypropylene tubes and collected by centrifugation at 4°C at 5000rpm in a JA-20 rotor in a Beckman J2-21 centrifuge for 10 minutes. The cells were gently resuspended in 10ml of ice cold

transformation and storage buffer (Appendix A) and held on ice for 10 minutes before transformation. Cells not transformed, were dispensed into 100µl aliquots, snap-frozen in a dry-ice/ethanol bath and stored at –70^{0}C.

A transformation efficiency of 10^6 to 10^7 was obtained per µg of plasmid DNA, as assayed by transformation with dilutions of DNA.

2.2.5.4: Transformation of competent *E.coli* cells

Competent cells stored at –70^{0}C were thawed on ice and transformed by the heat-shock method of Cohen *et al.*, 1972. Approximately 1-10ng of DNA or $^1/_5$ of the ligation reaction was mixed with 100µl of competent cells and incubated on ice for 10-30 minutes. The mixture was heat-shocked at 42^{0}C for 90 seconds before 0.9ml of 2XYT was added. The mixture was incubated for 30-60 minutes at 37^{0}C to allow the expression of the antibiotic resistance gene. Typically 200µl of transformation mixture was plated on YT agar plates containing 100µl of ampicillin (50mg/ml) per 100ml agar. When the *lacZ* gene was used as a selectable marker, X-gal and isopropyl-β-D-thio galactosidase (IPTG) was added (0.5ml of 20mg/ml X-gal and 50µl of 200mg/ml of IPTG per 100ml agar) to the YT agar before the cells were plated. Once the cells were spread on the agar, the plates were allowed to dry in the laminar flow hood before incubation at 37^{0}C overnight in an inverted position.

2.2.5.5: Screening for recombinants

There are a number of methods used for screening bacterial colonies for recombinant plasmid.

The addition of the ampicillin to the growth media selected for transformed cells as the ampicillin resistance was conferred on the transformants by the plasmid.

Both vectors, the pGem and pAFMCRR, carries a segment of DNA derived from the *lac* operon of *E.coli* that codes for the amino-terminal fragment of β-galactosidase. The gene, *lacZ*, is induced by IPTG and the α-fragment is capable of intra-allelic (α) complementation with the carboxyl terminal form of β-galactosidase produced by the bacteria. Thus a functional form of the enzyme, β-galactosidase, is produced and is able to hydrolyse the chromogenic substrate X-gal to yield galactose and soluble indoxyl molecules. The indoxyl molecules are oxidised to form bromochloroindole, an insoluble blue dye, giving the colonies a blue appearance. Cloning into the pGem vector causes the inactivation of the *lacZ* gene and thus β-galactosidase cannot be produced and the bacterial colonies are white in colour. Therefore, white colonies were selected for and the presence of the insert further verified by restriction analysis with *Bam*HI and *Sal*I following isolation of the DNA. Cloning into pAFMCRR, however, does not inactivate the *lacZ* gene and therefore blue colonies form. The presence of the insert in pAFMCRR was verified by restriction analysis with *Bam*HI and *Sal*I.

2.2.6: Sequencing

DNA sequencing was carried out by D.James (Microbiology, UCT), using an automated sequencer (Pharmacia™ ALFexpress® sequencer). This eliminates the use of radioactive labels and rather uses fragments labelled with fluorescent dyes. Primers were designed so as to sequence both strands and all regions of the PCR product subcloned into pGem. Furthermore, the reference plasmid, pCA24IRES, was also sequenced using the same primers except the M13 forward and M13 reverse primers. All DNA sequenced was first isolated using the High Pure Isolation Kit (Roche, Germany) as described in 2.2.2.2.

2.3: SOURCE OF THE RVF-LSDV RECOMBINANT PLASMID (pSelP-HS-G1G2-βGal)

The pSelp-HS-G1G2-βGal was constructed by A.Cohen and contains the RVFV glycoproteins, G1 and G2. This plasmid as is (refer to figure 2.4 for plasmid map), is ready for transfection into LSDV infected lamb testes cells. However, a large-scale plasmid isolation was carried out in order to obtain sufficient amounts of plasmid DNA (as described in section 2.2.2.1) for the transfection.

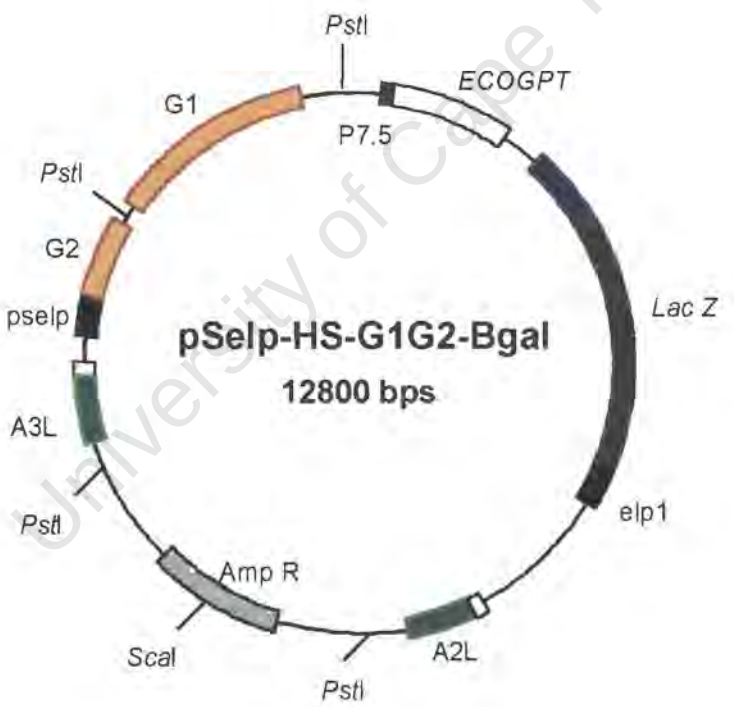


Figure 2.4: Plasmid map of pSelp-HS-G1G2-βgal containing the Rift Valley fever virus glycoproteins G1 and G2. Expression of G2 and G1 is under the control of a synthetic early/late promoter, pselp. The *lacZ* gene is under the control of the elp1 promoter and the *ECOGPT* gene under the control of the vaccinia virus P7.5 late promoter. Plasmid map not drawn to scale.

2.4: GENERATION OF RECOMBINANT FMD-LSDV AND RVF-LSDV

2.4.1: Sterile technique for cell culture

All tissue culture work was done in a class 2 biological safety laminar flow hood (Laminaire, Model-4BH, Bino Instrumentation, Bellville, South Africa). The work area was kept free of any nonessential items and the circulator fan switched on before and during any work undertaken in the hood. The area was swabbed down with 70% alcohol and irradiated with UV light for 10 minutes before use. Any items that were to be used in the hood were also cleaned with 70% alcohol. The operator's hands and arms were cleaned with soap and water and then swabbed with 70% alcohol to ensure sterility. After any work was completed in the hood, all nonessential items were removed, the area washed with 70% alcohol and irradiated with UV light for 10 minutes.

2.4.2: Preparation of primary cells

Primary lamb testes cells were prepared according to the method by Freshney (1987). Fresh foetal lamb testes (LT) were collected from the State Abattoir in Maitland and placed in ice-cold phosphate buffered saline (PBS) (Appendix A). When possible, the lamb testes were obtained from embryonic tissue as these gives a higher yield of proliferating cells as opposed to newborn or adult tissue. The outer tissue, the fat and the capsule were removed. The remaining tissue was rinsed in PBS and cut into small pieces with a sterile scalpel. The pieces were rinsed in PBS to remove excess blood and finally rinsed once with 0.25% trypsin (Appendix A). The tissue was transferred to a sterile conical flask with a magnetic bar and 1% trypsin was added. The mixture was heated to 30^0C for a minute and placed on a stirrer (Fried Electric, Haifa, Israel) for 30 minutes at room temperature. The trypsin was then discarded, fresh 1% trypsin added and the stirring was repeated as before. The trypsin was collected after 30 minutes and additional fresh 1% trypsin added to the remaining tissue. Stirring was repeated as before, the trypsin collected and diluted 1:1 in tissue culture medium, containing

Dulbecco's modified Minimum Essential Medium (DMEM) (Appendix A), 10% fetal calf serum (FCS), 1% fungizone (F) and 0.5% penicillin/streptomycin (PS) (Appendix A). The cells were pelleted at 1000rpm in a 215 rotor in a IEC HN-SII Centrifuge (International Equipment Company, Boston, USA) for 10 minutes and then resuspended in DMEM supplemented with 10% FCS and 0.5% PS. Any trypsin left would be inactivated by the serum in the medium. The cells were counted using a haemocytometer as described in 2.4.3 and seeded in a culture flask to a concentration of approximately $1x10^5$ per cm^2. The cells were incubated at 37°C in a 5% carbon dioxide (CO_2) incubator (Queue, USA) until confluent before they were passaged as described in 2.4.4.

2.4.3: Quantitation of cells

A cell count allows one to determine the status of the culture at a specific time. This is essential when subculturing or assessing the effects of experimental procedures on the cells.

An aliquot of 0.1ml was taken from a cells suspended in tissue culture medium and added to 0.15ml of physiological saline and 0.25ml of trypan blue solution (Appendix A) and left at r.t. for 10 minutes. Live cells exclude the blue dye, whereas dead cells are permeable and will take up the stain. The surface of the haemocytometer (Neubauer, Brand, Blaubrand, Germany) was cleaned with 70% alcohol as well as the coverslip. Approximately 2 drops was then placed on the edge of the haemocytometer with the coverslip in place and capillary action allowed for distribution of the fluid in the counting area. Cells are counted in two $1mm^2$ areas and the average of the two are taken. To calculate the number of viable cells in 1ml; the average number of cells counted was multiplied by the dilution factor and with 10^4 (the volume of the $1mm^2$ area with the coverslip in place is 0.1µl).

2.4.4: Growth and maintenance of cell cultures

Cells were grown in a incubator with 5% CO_2 at 37^{0}C until the cells were confluent. The tissue culture medium was then removed and the monolayer washed with sterile PBS followed by activated trypsin versene (ATV) (Appendix A). A fresh amount of ATV was added to the monolayer and the cells incubated at 37^{0}C which removes the cells from the flask. Once the cells have detached from the flask, the activity of the trypsin is stopped by the addition of serum-containing medium (serum contains the trypsin inhibitor α-1-antitrypsin). The tissue culture medium was added to a predetermined final volume and the cells separated by pipetting a few times till no clumps were visible. The cells were split into amounts which would allow monolayers to form within 3-4 days. The cells were kept in medium to a depth of 4mm at 37^{0}C in a 5% CO_2 incubator. The tissue culture medium was changed at regular intervals. Confluent cells monolayers were maintained with tissue culture medium but the FCS was reduced to a concentration of 2-4%, although the confluent cell monolayer was not maintained for longer than a week on maintenance medium.

2.4.5: Storage of cells

Cryopreservation involves the storage of cells at very low temperatures, which allows for very little metabolic/enzymatic activity. Cells are grown to a late log phase, trypsinised and centrifuged at 1000rpm in a 215 rotor in a IEC HN-SII Centrifuge for 10 minutes. The cells were then suspended in 90% FCS and 10% DMSO to a concentration of 1X10^6 cells/ml. The freezing mix was then aliquoted into one ml amounts and placed in cryo-vials (Nunc CryoTubes^{TM} Vials, Nalge Nunc International, Denmark). The cells were cooled at a rate of 1^{0}C/minute to -70^{0}C. Thereafter, they were placed in liquid nitrogen at a temperature of -196^{0}C.

When cells were required from the liquid nitrogen storage facility, they were thawed rapidly at 37^{0}C (once removed from the liquid nitrogen). Warm

tissue culture medium was slowly added to the cells to avoid osmotic damage to the cells and centrifuged at 1000rpm for 10 minutes. The cells were resuspended in fresh tissue culture medium and seeded into a tissue culture flask at a 10-fold higher concentration than was normally done for routine subculturing. The cells were placed in a 5% CO_2 incubator at 37^0C

2.4.6: Propagation and concentration of virus in mammalian cells

Primary lamb testes cells, were selected for the propagation of the vaccine strain (Neethling) of LSDV which was obtained from the Ondersterpoort Veterinary Institute, Pretoria. Lamb testes cells were grown to a confluence of 80%. The tissue culture medium was removed and the cells washed 3X with PBS to remove all traces of substances which could inhibit viral infectivity. The cells were infected with $1X10^6$ plaque forming units (p.f.u.) LSDV, diluted in virus diluent (Appendix A) and allowed an adsorption time of 1 hour. Thereafter, the cells were washed with diluent and replaced with 4% tissue culture medium (Appendix A). Following three days post infection, the cells were disrupted by three cycles of freeze/thawing to allow release of virions. The debris was then pelleted by low speed centrifugation in a swing out 215 rotor in a IEC HN-SII Centrifuge for 2-5 minutes and the supernatant recovered. The virus was passaged a number of times before all the supernatant was pooled, placed in a Beckman centrifuge tube with a 1ml, 36% sucrose cushion (Appendix A) at the bottom and centrifuged at 11000 rpm in a JA-20 rotor in a Beckman J2-21 centrifuge for 2 hours at 4^0C. The viral pellet was resuspended in a small volume of McIlvain's buffer, pH 7.4 (Appendix A) and stored at -20^0C. For long-term storage at -70^0C, glycerol was added to a concentration of 80%.

2.2.7: Determination of virus concentration

A 24-well tissue culture plate was seeded with lamb testes cells and grown to a confluence of 80%. Serial 10-fold dilutions were made of the virus in virus diluent (Appendix A) and each well was infected with 200µl of the viral

dilution from $10^{-2} - 10^{-7}$. Each virus dilution infectivity was done in triplicate. After one hour adsorption at 37^{0}C, the viral inoculum was removed and the cells washed with virus diluent (Appendix A). One ml of 4% tissue culture medium was added and the cells incubated for 3 days at 37^{0}C in a 5% CO_2 incubator. The concentration was then determined as follows:

f.f.u. (foci forming units)/ml = average amount of foci per dilution X dilution factor X volume of the inoculum per well

2.2.8: Transfection and infection of cells

Nuclei acid can be incorporated into cells by the technique of transfection. DOTAP, a cationic liposome reagent (Boehringer Mannheim, Germany) was used for the incorporation of DNA into the cells. DOTAP is a synthetic cationic lipid which forms unilamellar vesicles, liposomes, in an aqueous solution. These positively charged liposomes interact with the negatively charged DNA to form stable complexes. The complexes adhere to the cell surface, fuse with the cell membrane and release the DNA into the cytoplasm.

The DNA, to be transfected, was prepared using the Qiagen Plasmid Midi Kit and the Nucleobond AX PC-kit 100 as described in 2.2.2.1. Lamb testes cells were grown in a 6-well tissue culture plate to a confluence of 60% to 80%. The cells infected with 0.01 p.f.u. of the Neethling strain of LSDV, allowed to adsorb for one hour, washed twice with PBS and 1ml 10% tissue culture medium was added per well. For each well, 2.5μg to 10μg of DNA was used and diluted to a final volume of 50μl in Hepes buffer (Appendix A). The amount of DOTAP used per well was dependent on the amount of DNA added. The DNA/DOTAP was diluted to 100μl with Hepes buffer, gently mixed together in a polystyrene tube and left at r.t. for 15 minutes (polystyrene tubes were used as they do not attract DNA as much as glass or plastic). The DOTAP/DNA mixture was then added drop wise while gently swirling the cells and then incubated overnight at 37^{0}C. The transfection medium was removed and replaced with fresh 10% tissue culture medium and

incubated for a further 3 days. The cells with the medium were freeze/thawed three times and centrifuged at 1000rpm in a swing out 215 rotor in a IEC HN-SII Centrifuge for 2-5 minutes. The supernatant was recovered and stored -20^{0}C. Controls were also performed involving mock-transfections of uninfected and LSDV infected cells.

Duplicate transfection experiments were carried out in order that one could be used for testing for transfection efficiency by X-gal staining. The other sample was used for selection and isolation of recombinant virus as described below.

New lamb testes cells were grown to a confluency of 70-80% and infected with approximately 0.5ml of the medium harvested from the transfection reaction. Selection medium (Appendix A) was added to the cells and incubated for 3 days at 37^{0}C. The selection medium contains mycophenolic acid, hypoxanthine and xanthine and consequently allows only recombinant virus to survive as the purine salvage pathway is selected. The cells with the medium were freezed/thawed 3X, the debris pelletted at 1000rpm for 5 minutes and the supernatant kept for reinfecting new cells as described above. In total three rounds of passaging was carried out before x-gal overlays performed for isolation of recombinant virus.

2.2.9: X gal staining

X-gal stain was performed in order to rapidly determine the presence of recombinant virus which contains the *E.coli lacZ* reporter gene.

Cells infected for 3 days with medium containing the recombinant virus were washed 3X with PBS and then fixed with 4% paraformaldehyde (Appendix A) for 5 minutes at r.t. The cells were washed once again with PBS after which the substrate solution (Appendix A) was added and left for 30 minutes to 18 hours. The development of blue cells was visualized with the aid of a microscope.

The sodium ions, which are provided by PBS, activates the β-gal enzyme. Potassium ferrocyanide and potassium ferricyanide increase the rate of conversion of indoxyl molecules to the indigo form and thereby enhances the colour.

2.2.10: X-gal overlays

X-gal overlays were carried out in order to isolate and purify the recombinant virus. The basis for this technology is similar to that described in the above section. Lamb testes cells infected with the recombinant virus will appear blue as the *E.coli lacZ* reporter gene contained on the viral genome will be expressed. Lamb testes cells were grown to a confluency of 80-90% and then infected with recombinant virus (LSDV (Neethling) infected cells and uninfected cells were included as controls). The cells were infected for an hour, the virus inoculum removed and selection medium (Appendix A) added. Three days post-infection, the cells selection medium was removed and retained for possible further use. The cells were washed with PBS and overlayed with 1% low melting point agarose containing selection medium as well as x-gal (50μg/μl). The cells were incubated at 37°C for a further 12 hours or until blue foci were visible. Pasteur pipettes were used to pick the blue foci, which were then suspended in virus diluent and stored at –20°C until needed.

2.2.11: Immunofluorescence

Indirect immunofluorescence was used for detection of specific proteins expressed by recombinant virus. The method of Lyerla and Forest (1979) was followed. LT cells were grown on coverslips and infected with recombinant virus with the inclusion of non-recombinant virus and uninfected controls. Following 3 days of infection, the cells were washed with PBS, fixed with ice-cold acetone for 10 minutes at 4^0C and air-dried. The cells were incubated at 37^0C with the appropriate dilution of primary antibody for 1 hour and then washed 2X PBS for 10 minutes each. The cells were air-dried and

incubated with the appropriate dilution of fluorescein isothiocyanate (FITC)-conjugated secondary antibody and Evans blue (Appendix A). The cells were washed 2XPBS for 10 minutes, air-dried and the coverslips mounted on glass slides and viewed under a Leitz Wetzlar fluorescent microscope.

2.2.12: Extraction of total DNA from cell culture

The method used was adapted from Bailey and Possee, 1991. Almost confluent lamb testes cells, grown in a 35mm diameter dish, were infected with the recombinant virus (with the inclusion of LSDV infected cells and uninfected cells as controls). The cells were infected for one hour at 37^{0}C, the inoculum removed and selection medium added. Incubation was carried out for 24 hours at 37^{0}C, the medium removed and the cells washed 3X with PBS. Lysis buffer B (Appendix A) was added to the cells (500µl per 35mm diameter dish). Once the cells were lysed, vigorous pipetting or shaking was avoided to prevent shearing of the DNA. RNase A was added to a final concentration of 40µg/ml and the lysate incubated for one hour at 37^{0}C before been transferred to an Eppendorf tube. Proteinase K was added to a final concentration of 100µg/ml and incubated at 55^{0}C for three to four hours or overnight at 42^{0}C. The lyaste was extracted twice with equal volumes of pre-warmed phenol (37^{0}C) and once with chloroform:isoamyl (24:1). The DNA was precipitated with the addition of sodium acetate (to 0.3M), two volumes of absolute ethanol and incubated at -20^{0}C overnight. Centrifugation was carried out at 14 000rpm in a F45-30-11 rotor in an Eppendorf Centrifuge 5417C for 15 minutes and the resultant DNA pellet washed twice with cold 70% alcohol. The pellet was stored in an open tube at r.t. until the ethanol had evaporated. The DNA was resuspended in ultra pure water at 4^{0}C overnight.

2.2.13: Southern blot

DNA can be analysed by Southern Blot hybridisation, a process described by Southern (1975). The technique involves restriction endonuclease digestion of genomic DNA, separation by electrophoresis,

immobilisation on a membrane and hybridisation to a labelled probe for detection of specific sequences.

Genomic DNA was digested with restriction enzymes as described previously in section 2.2.3. The DNA fragments were separated by agarose gel electrophoresis with the inclusion of molecular weight markers and positive controls. Electrophoresis was performed for 16 hours at 15V to prevent smearing of the fragments.

Prior to capillary transfer of the fragments to a nitrocellulose membrane, unused areas of the gel was trimmed away and the corner cut so as to orientate the gel in subsequent steps. The gel was soaked in 0.25M HCl for 10 minutes with gentle agitation, which causes nicks to the DNA by depurination, thereby assisting the transfer of larger fragments. The DNA was denatured by soaking the gel for 45 minutes in 1.5M NaCl and 0.5N NaOH with gentle agitation. The gel was briefly washed in water and soaked in several volumes of neutralisation solution (1M Tris (pH7.4), 1.5M NaCl) for 30 minutes followed by a further 15 minutes with shaking.

The gel was inverted and placed on a plastic support covered with a wick extending into a reservoir of 5XSSC (Appendix A). The nitrocellulose membrane was cut to the same dimensions as the gel, briefly wetted with 5XSSC and carefully placed on top of the gel to ensuring that there was no air bubbles trapped. Cling film was used to surround the gel, serving as a barrier to prevent liquid from flowing directly from the reservoir to the paper towel placed on top of the gel. Three pieces of Whatmann 3MM paper, wetted in 5XSSC, cut to the same dimensions as the gel, was placed on top of the membrane. All bubbles were smoothed out with a glass rod. A stack of dry paper towels was placed on top with a final weight following that. The capillary transfer has been illustrated in figure 2.5. The transfer was allowed to processed overnight.

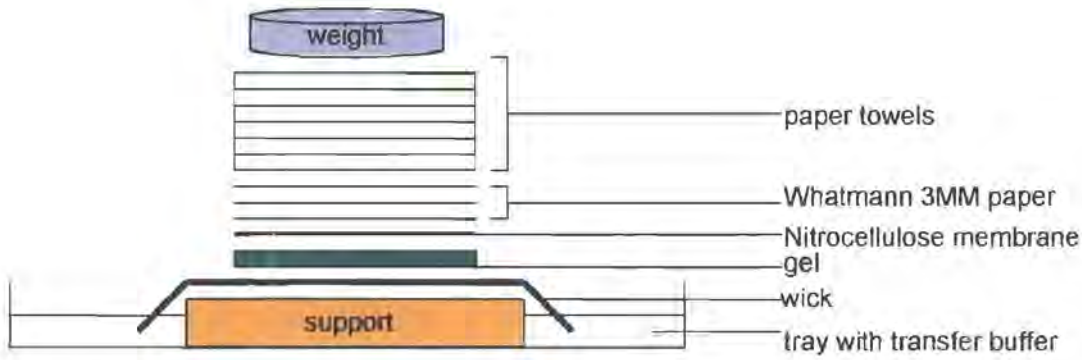


Figure 2.5: Diagrammatic representation of the Southern blot capillary transfer of DNA from a gel to a nitrocellulose membrane.

Following transfer, the membrane was removed briefly washed in 2XSSC, air-dried and the DNA irreversibly fixed onto the membrane by a UV-crosslinker (Hoefer Scientific Instruments, CA, USA). If the membrane was not used immediately, it was stored in a sealed plastic bag and stored at 4°C.

2.2.14: Probe-labelling, hybridisation and detection

The pAFMCRRP1 and pSelp-HS-G1G2-βgal plasmids were digested with *Hin*dIII and *Pst*I respectively and used as probes on the respective membranes. The DNA probes were gel purified and the concentration determined first. The DNA was labelled using α^{32}P-dCTP and the Multiprime DNA labelling system (Amersham, UK) according to the manufacturer's instructions. The Multiprime DNA labelling system uses random sequence hexanucleotides to prime DNA synthesis on denatured template DNA at various sites along its length. High specific activity radiolabelled probes are generated once radiolabelled dNTPs and the DNA polymerase I Klenow fragment is added. The DNA to be labelled (20-25ηg) was denatured by heating for 2-3 minutes at 95-100°C, immediately placed on ice and then centrifuged briefly. Solutions supplied with the kit was added to the denatured DNA as instructed, together with the appropriate amount of α^{32}P-dCTP. The reaction was incubated for 30 minutes at 37°C or overnight at rt.

The membrane to be probed was hybridised overnight at 42°C in a Hybridiser HB-1D oven (Techne, Cambridge) with hybridisation solution (2X SSC, 0.25% blocking buffer and 1% SDS). This allows the blocking of sites to which the probe may bind non-specifically to, resulting in a high background. The labelled probe was denatured at 95-100°C for 2 minutes, chilled on ice and then added to the hybridisation solution. Incubated was carried out overnight at 68°C with rolling. Once hybridisation was complete, the solution was removed and stored at –20°C for re-use.

The membrane was washed with 2XSSC and 0.1% SDS for 5 minutes at r.t. and then 2X 30 minutes with 0.5XSSC and 0.5% SDS at 68°C. The membrane was briefly washed with 0.1% SSC, sealed in plastic and exposed to a Curix RP1 (Agfa, Germany) X-ray film in a X-ray cassette with an intensifying screen and stored at –70°C. Exposure time was 2 days or longer. The autoradiographs were developed by exposing the film to developer for 3 minutes, immersion in stop solution (2% acetic acid) for 1 minute and in fixer for 2 minutes. The film was briefly washed in water and air-dried for 30 minutes.

2.2.15: Total protein extraction from cell culture

Two methods of proteins extraction were carried out on lamb testes cells infected with the recombinant virus. LSDV infected cells and uninfected cells were included as controls.

The first method was adapted from Sambrook, 1989. Cells which had been infected for 3 to 4 days with recombinant virus, wild type virus and uninfected cells were trypsinised and collected by centrifugation in an Eppendorf tube. Following two washes with PBS, hot (85°C) 2XSDS gel loading buffer (Appendix A) was added and the cells placed in boiling water for 10 minutes. The DNA released from the cells, which causes the solution to become viscous, was broken up by passing the solution through a fine-needle syringe five times. The lysate was clarified by centrifuged and the supernatant collected and stored at -20°C. Prior to use, the proteins were

denatured by boiling the samples for 5 minutes before resolving the proteins using SDS-polyacrylamide gel electrophoresis.

A second method of protein extraction was used, involving the isolation of glycoproteins from tissue culture. This method incorporates the use of Igepal, which is a nonionic detergent, and phenylmethylsulfonyl fluoride (PMSF), which is a protease inhibitor. Cells, which had been infected for 3 to 4 days with recombinant virus, wild type virus, and uninfected cells were washed twice with PBS. The cells were lysed with 500µl/well of lysis buffer C (Appendix A) and the cells rocked on ice for 30 minutes. The cell lysates were frozen at -70^0C for later use. Prior to use the samples were incubated with 2X SDS gel loading buffer for 30 minutes and the proteins resolved using SDS-polyacrylamide gel electrophoresis.

2.2.16: SDS-polyacrylamide gel electrophoresis

Protein extracted from tissue culture was separated by electrophoresis using the modified disconituous SDS system (Laemmli, 1970). The strong anionic detergent, SDS, denatures the proteins by wrapping around the polypeptide backbone, thereby conferring a net negative charge to the polypeptide in proportion to its length. Together with a reducing agent the polypeptides have an equal charge density or charge per unit length and can thus separate according to molecular weight. The disconituous SDS system allows for the establishment a nonrestrictive large-pore stacking gel, which is layered on top of a resolving or running gel. The stacking gel contains Tris-Cl at a pH of 6.8, the resolving gel Tris-Cl at a pH 8.8 while the tank buffer Tris-glycine at a pH of 8.3. Upon electrophoresis, the proteins and ions migrate through the acrylamide with the chloride ions of the stacking gel forming the leading edge and the glycine ions of the tank buffer the trailing boundary. The protein are thus concentrated between these two boundaries or stacked in between. Once the resolving gel is reached, the higher pH causes ionisation of the glycine molecules, which moves through the protein stack, and migrate through the resolving gel immediately behind the chloride ions. Thus the

protein-SDS complex is free to migrate through the resolving gel with uniform voltage and pH and are therefore separated according to size.

The Mighty Small dual gel caster (Hoefer Scientific Instruments, San Francisco, CA, USA) was used for pouring the polyacrylamide gel into vertically set up slabs, which were sealed and clamped at the sides and bottom to form a liquid tight box. A 12.5% resolving gel (Appendix A) was poured to a level of approximately 4cm from the top. A small quantity of water saturated butanol was layered on top to prevent the formation of a meniscus and to minimise exposure to oxygen which may inhibit polymerisation. Once the gel was polymerised, the water-saturated butanol was poured off and the gel rinsed with distilled water. The comb was inserted and the stacking gel (Appendix A) poured and left to polymerise. The gel sandwich was removed from the casting stand and clamped to a SE 250/260 gel electrophoresis unit (Hoefer Scientific Instruments). The comb was removed and the wells washed with tank buffer in preparation for the protein samples.

Protein samples, depending on the preparation used, were either boiled for 5 minutes or the incubated with 2X SDS gel loading buffer for 30 minutes before loading into the wells. Molecular weight markers (Pharmacia, USA), containing proteins of known sizes, were boiled for 5 minutes following the addition of treatment buffer (Appendix A). Duplicate gels were run and electrophoresis was allowed to commence at 40mA until the dye front had been eluted from the gel. Once electrophoresis was complete, the apparatus was disassembled and the polyacrylamide gels removed. The duplicate gels were processed in different ways. The one gel was fixed and stained while the other gel was used for transfer of the proteins to a nitrocellulose membrane for Western blotting.

2.2.17: Coomassie staining

The non-specific binding of Coomassie blue dye to proteins is referred to as Coomassie blue staining. The Coomassie blue dye or Coomassie Brilliant Blue R250 is a triphenylmethane textile dye which together with the

concentrated methanol/acetic acid solution of the dye, fixes and stains the polypeptides. Excess dye is allowed to diffuse from the gel during a prolonged period of destaining of the gel to leave blue bands on a clear background. The commercially available Coomassie stain, PhastGel Blue R (Pharmacia, Sweden) was used according to the manufacturers specification to stain the gels. The gel was immersed in the staining solution for4 hours to overnight with shaking. Destaining was performed by first soaking the gel in destaining solution I (40% methanol, 7% acetic acid) for 30 minutes and then in several volumes of destaining solution II (5% methanol, 7% acetic acid) overnight with gentle agitation. For accelerated staining and destaining the solution were heated to 45^0C. Following destaining, the gel was stored in 20% glycerol to prevent cracking and distortion.

2.2.18: Western blotting, Ponceau S staining and immunodetection

Western blotting refers to the transfer of electrophoretically separated proteins from a gel to a solid supports and probing with antibodies that are capable of recognising particular antigenic epitopes (Towbin *et al.*, 1979). A semi-dry electric transfer unit was used to transfer the proteins from the acrylamide gel onto a nitrocellulose membrane (Hybond-P, Amersham, UK). The stacking gel was first removed and the resolving gel soaked in Towbin transfer buffer (Appendix A) for 5 minutes. The nitrocellulose membrane was cut to the same size as the gel, briefly soaked in methanol before been transferred to Towbin transfer buffer for a few minutes while the absorbent towels were soaked for 30 minutes. The transfer apparatus was set up as illustrated in figure 2.6.

Transfer was carried out at 200mA for 2 hours before the apparatus was disassembled and the gel soaked in Coomassie stain to verify transfer.

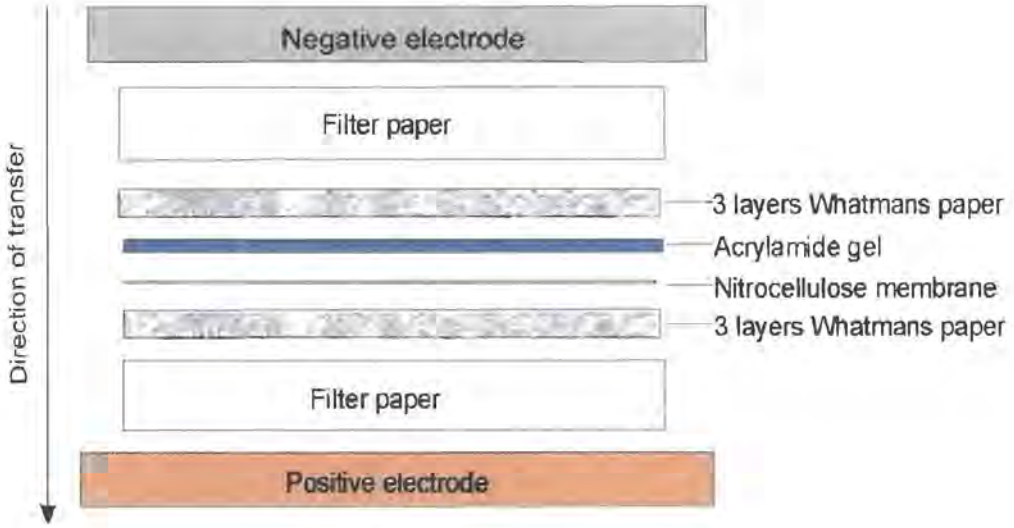


Figure 2.6: Diagrammatic representation of electrophoretic transfer of protein from a polyacrylamide gel to a nitrocellulose membrane.

Further verification of transfer was carried out by staining the membrane with 3-hydroxy-4-(2-sulfo-4-[4-sulfophenylazo]-phenylazo)-2,7-naphthalenedisulfonic acid or more commonly, Ponceau S (Sigma). The staining also allowed one to locate the position of the molecular weight marker. Ponceau S is water-soluble and is easily removed by washing the membrane in water. The membrane was incubated with Ponceau S (Appendix A) for a minute and then membrane briefly washed with water. The protein bands appear red and the positions of the molecular weight standards marked with a pencil. The membrane was then washed in water several times to completely remove the stain, as residual staining can interfere with the antigenicity of the proteins, before immunodetection was carried out.

Immunodetection was carried out using the Chemiluminescence Western Blotting Kit (Roche, Germany) according to the manufacturers' specifications. The system is designed to use a peroxidase-labeled secondary antibody and the chemiluminescent substrate luminol, a cyclic diacylhydrazide. The horseradish peroxidase (HRP) catalyzes the oxidation the luminol, in the presence of hydrogen peroxide, to form an activated intermediate product, which then decays to a ground state by emitting light.

Light emission is enhanced by the addition of 4-iodophenol, which acts as a radical transmitter between the formed oxygen and luminol. The light emission is detected on autoradiography film.

The nitrocellulose membrane was briefly washed in TBS (Appendix A) and then incubated in 1% blocking solution overnight at 4^0C to lessen non-specific binding of the antibody to the membrane. Primary sheep antibody and primary bovine antibody was diluted 1:1000 in 0.5% blocking solution and added to the membrane in separate experiments. Incubation was carried out for 1 hour at room temperature or overnight at 4^0C. The membrane was washed twice with TBST (Appendix A) for 10 minutes each followed by two washes with 0.5% blocking solution for 10 minutes each. Horseradish peroxidase conjugated secondary anti-sheep or anti-bovine was diluted 1:2000 in 0.5% blocking solution and then added to the respective membranes and incubated for 30 minutes at r.t. Four washing steps of 15 minutes each were carried out using TBST. The membrane was incubated for 1 minute with detection solution provided with the kit, the detection solution removed and the membrane exposed to an autoradiograph for a few seconds to 10 minutes. The autoradiograph was processed as previously described.

2.2.19: Immunisation of animals with recombinant RVF-LSDV

Eleven outbred rabbits were obtained from the Animal Unit, UCT, following an application and approval to perform procedures on the animals by UCT Animal Research Review Committee (project reference number 98/024). All animal work was in accordance with the University's "Code of Ethics and Procedures for the Use of Animals in Teaching and Research". The animals were housed in the UCT Animal Unit, with all procedures performed by trained personnel.

Four rabbits (A,B,C and D) were inoculated intradermally with 0.25ml of RVF-LSDV recombinant ($1.5X10^6$ p.f.u. per ml) while two rabbits (E and F) were inoculated intradermally with 0.125ml of RVF-LSDV recombinant. Rabbits, G and H were vaccinated subcutaneously with 1ml of the OBP RVFV

vaccine, which served as a positive control. Other control rabbits included were:

(1) rabbit, I, unvaccinated

(2) rabbit, J, inoculated with tissue culture medium

(3) rabbit, K, inoculated with $1X10^6$ p.f.u. of LSDV (Neethling)

Blood was taken from each of the rabbits every second week. The samples were left for one day at 4°C to coagulate, thereafter the serum collected and stored at –70°C for further manipulation.

2.2.20: Enzyme-linked immunosorbent assay (ELISA)

Rabbit serum was investigated for anti-RVF antibodies by sandwich ELISA. Sandwich ELISA involves the attachment of a coating antibody to a solid surface by passive adsorption followed by the addition of an antigen. The sera is then applied and specific labelled anti-IgG added, which is capable of yielding a colour change on the addition of a substrate

Rabbit serum was tested for antibodies to RVFV antigen by using the anti-RVF IgG ELISA supplied by the Special Pathogens Unit, National Institute for Virology, Johannesburg. Nunc immunoplates F96 Maxisorp plates were coated with coating antibody (anti-RVF hyperimmune mouse ascitic fluid) diluted 1:2000 in PBS. The plates were incubated overnight at 4^0C with lids. The plates were washed 3X with washing buffer (0.1% Tween 20/PBS) using a LP35 automatic plate washer (Sanofi, Diagnostics Pateur). The wells were blocked with 200µl/well of blocking buffer (10% skimmed milk in PBS) and incubated for 1 hour at 37°C in a moist chamber with lids. Thereafter, the plates were once again washed 3X with washing buffer. The wells were then either incubated with 100µl of RVFV or 100µl control antigen, both diluted 1:400 in diluent (2% skimmed milk in PBS), at 37^0C in a moist chamber with lids for 1 hour. Following three washes, the control and test serum was added to the appropriate wells, diluted in diluent, and incubated for 1 hour in a moist chamber at 37^0C. The plates were washed 3X with washing buffer and 100µl of HRP conjugated anti-sheep IgG or anti-rabbit IgG, diluted 1:1000 added to

according to the manufacturers specification. OPD serves as a chromogenic substrate for HRP, yielding a yellow colour upon hydrolysis. A volume of 100µl of the substrate solution was added to each well. When a suitable yellow colour had developed, the enzymatic reaction was stopped with the addition of 100µl of 0.5M H_2SO_4. The absorbance was measured using an ELISA plate reader (PR 2100, Sanofi Diagnostics, Pasteur) at 490nm with a reference filter of 620nm.

All samples were done in duplicate with positive and negative controls included in all ELISA tests performed. Ovalbumin was also used as a blocking reagent in place of skimmed milk to reduce cross-reactivity and the high background absorbance readings obtained when using skimmed milk.

Sera was also obtained from rabbits vaccinated with a similar recombinant virus construct, expressing the G1G2 RVFV glycoproteins made by A.Cohen (unpublished data). The sera was tested with the negative and positive antigens also using the anti-RVF IgG ELISA kit as described above.

2.2.21: Haemaglutination inhibition

Haemeglututination inhibition is a direct measurement of antibody binding to antigen. When sufficient amounts of antibody are mixed with soluble antigens, a visible precipitate forms, consisting of large aggregates of antigen cross-linked by antibody molecules. The amount and ratio of the antigen to antibody determines the amount of precipitate.

The haemaglutination inhibition tests were performed at the National Institute of Virology (NIV). The sera tested was obtained from rabbits A-K, and sent via cold chain.

CHAPTER 3: DEVELOPMENT OF A RIFT VALLEY FEVER LUMPY SKIN DISEASE VIRUS VACCINE

3.1: LITERATURE REVIEW

3.1.1: Introduction

Rift Valley fever is an important arthropod-borne disease of both animals and humans in sub-Saharan Africa. Infection in animals, of which mostly sheep, cattle and goats are affected, may lead to acute febrile illness, abortions and even death (Meegan and Bailey, 1989). Humans suffer a variety of symptoms from an influenza-like illness to more severe clinical disease including haemorrhagic syndrome, encephalitis, blindness and deaths (Van Velden *et al.*, 1977 and Laughlin *et al.*, 1979). The aetiological agent of this disease is the Rift Valley Fever Virus (RVFV).

Rift Valley fever virus was first isolated in 1930 (Daubney *et al.*, 1931) following investigations of an epidemic among ewes and lambs in the Rift Valley in Kenya, after which the virus was named. Major outbreaks continued to occur thereafter, with the disease first appearing in southern Africa in the late 1950s. During an epidemic in South Africa in this period, an estimated 100 000 sheep died and 500 000 aborted with smaller losses occurring in cattle (Schulz, 1951). Extensive epidemics continued to occur in various other African countries such as Zimbabwe, Zambia, Sudan, Senegal River basin, Mauritania and Egypt with major losses occurring in many of these counties. In the 1977-1978 epidemic along the Nile and estimated 18,000 to 200,000 human cases occurred with some 600 deaths (Meegan and Bailey, 1989). These epidemics demonstrate the potential of this disease to escape the normal endemic area and to establish itself as a threat world-wide.

3.1.2: Classification

Rift Valley fever virus had for many years been an unclassified arbovirus (Gonzalez-Scarano and Nathanson, 1996). In 1973, Rift Valley Fever Virus was shown to be morphologically similar to the bunyaviruses (Murphy *et al.*, 1973), while molecular based studies (Rice *et al.*, 1980) and

serological tests (Shope *et al.*, 1980) indicated that it belonged to the genus *Phlebovirus*. The use of serological methods for the identification of unknown isolates in the family *Bunyaviridae* is preferred as serological cross-reactivity has not been found among the different genera of this family (Schmaljohn, 1996).

3.1.3: Epidemiology and transmission

Epidemics of RVF have occurred throughout sub-Saharan Africa and Egypt since the disease was first observed to occur in lambs and humans at the turn of the century. During an outbreak which occurred in 1997-1998 in the Garissa District, a remote area of north-eastern Kenya, over 300 people were reported to have died as well as affecting a number a domestic animals such as goats, sheep, cattle and camels (World Health Organisation (WHO), http://www.who.int/emc/outbreak_news/n1998/jan/n6jan1998b.htm). Similar outbreaks have also been reported from Somalia during this period (WHO, http://www.who.int/emc/outbreak_news/n1998/jan/n6jan1998b.htm). The most recent outbreak in South Africa occurred in 1999 in and around the Kruger National Park, which was reported by the National Institute of Virology in Johannesburg (WHO, http://www.who.int/emc/outbreak_news/n1999/feb/n10feb1999.htm). The Center for Disease Control and Prevention (CDC), which forms part of the WHO Collaborating Center, continues to monitor outbreaks of RVFV. Distribution maps (refer to figure 3.1 for maps) have been issue by CDC indicating the countries which have lesser outbreaks of RVF and countries which have substantial outbreaks of RVF in Africa (CDC, http://www.cdc.gov/ncidod/dvrd/spb/mnpages/dispages/rvfmap.htm).
However, in September 2000, cases of Rift Valley fever were reported outside the traditionally affected areas of North and sub-Saharan Africa (CDC, http://www.cdc.gov/travel/other/rvf-saudi-yemen.htm). The affected areas were found to be Saudi Arabia and the bordering nation of Yemen, with a number of human deaths reported in both countries (WHO, http://www.who.int/disease-outbreak-news/n2000/october/26october2000.html). These outbreaks illustrate the potential of RVFV to spread to unaffected regions.

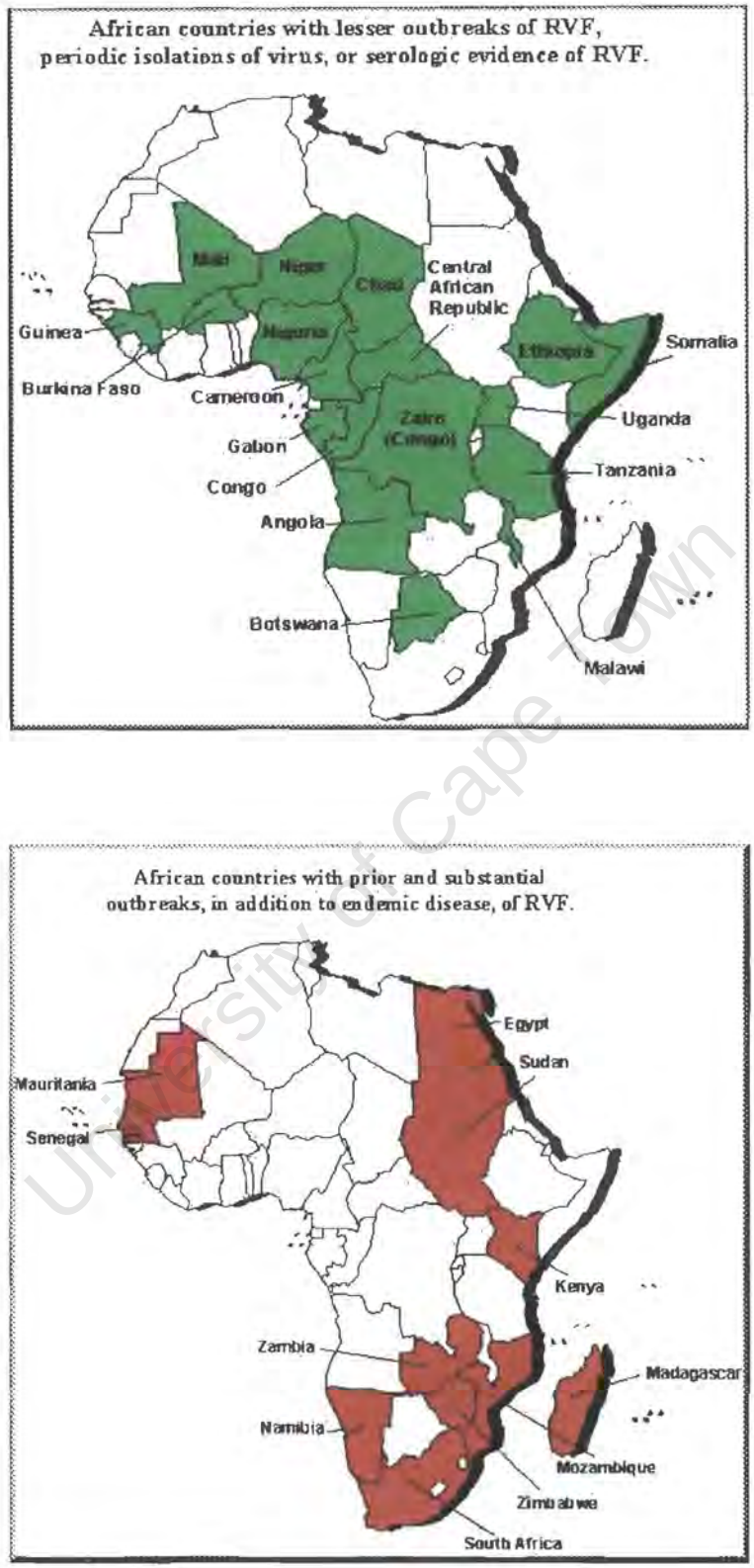


Figure 3.1: Maps representing outbreaks of RVFV. Taken from Center for Disease Control, internet page: http://www.cdc.gov/ncidod/dvrd/spb/mnpages/dispages/rvfmap.htm

The primary puzzle in the epidemiolgy of RVF has been the fate of the virus during inter-epidemic periods. A number of laboratories have as yet failed to show that the virus is maintained in birds, monkeys, baboons and other veterbrate species. Indeed, observations have been made in which the advent of the disease coinciding with periods of above average rainfall (cited by Swanepoel and Coetzer, 1994), although this relationship is certainly not straightforward. The final breakthrough in the understanding of the epidemiology of RVF was made when the virus was isolated from unfed male and female *Aedes mcintoshi* mosquitoes during inter-epidemic periods in 1982 and 1984 (Swanepoel and Coetzer, 1994). This clearly indicated that the virus is maintained by transovarial transmission, therefore, new generations of mosquitoes may hatch already infected from their eggs. This has proved to be a major contributing factor in the epidemiology of RVF as the eggs of mosquitoes may survive for periods up to several years in dry conditions.

RVFV can be transmitted by a wide variety of mosquito species. Once a mosquito has become infected from a bloodmeal, the virus replicates in the midgut from were the virus is disseminated via the haemolymph to replicate in the salivary glands and other organs. Transmission of the virus may increase as numerous attempts, by certain infected mosquito species, are made to feed due to reduced fecundity and inefficient feeding (Swanepoel and Coetzer, 1994). During an epizootic episode, virus not only circulates among arthropod vectors but the virus is also significantly amplified in livestock, such as sheep and cattle (Sall *et al.*, 1999).

Humans become infected with RVFV either by being bitten by infected mosquitoes or through contact with blood, body fluids or infected tissues. Generally persons at highest risk of contracting RVF are those closely associated with infected animals, such as farmers, veterinarians and abattoir workers. Aerosols have also been shown to play a role in transmission, leading to infections in laboratory workers as well some people during the Egyptian epidemic (cited by Swanepoel and Coetzer, 1994).

The segmented nature of the genome of RVFV may very well allow for RNA reassortment, as was demonstrated experimentally in tissue culture (Saluzzo and Smith, 1990) and mosquitoes that were dually infected (Turell *et al.*, 1990). Various field isolated strains of RVFV were also tested for reassortment and findings by Sall *et al.*, 1999, suggested that some of these strains had arisen as a result of reassortments with strains of the two main major lineages (West-Africa or Central-East Africa). Reassortment in nature implies the presence of two different strains in the same host. Furthermore, once generated, these chimeric viruses have to remain viable and not negatively selected against. Because reassortment in nature has been shown to occur in RVFV, this has consequences on the evolution and epidemiology of the disease (Sall *et al.*, 1999). The implications of these findings also suggest safety concerns for the use of live attenuated strain of RVFV as a means of vaccination as there may be a possibility of reassortment between wild type and attenuated vaccine strains.

3.1.4: Virus structure and genomic organisation

Rift Valley Fever Virus is typical morphologically and physiochemically similar to other Bunyaviruses. It is spherical in shape with a diameter of 90nm and a host-derived lipid bilayered envelope approximately 5nm thick from which virus coded glycoprotein spikes project. The virus contains three-segmented, single-stranded, negative-sense RNA designated as large (L), medium (M) and small (S) which is complexed with the nucleocapsid (Rice *et al.*, 1980). In contrasted to other bunyaviruses, phleboviruses has a S segment with bi-directional coding and codes for the nucleocapsid (N) protein as well as a nonstructural (NS_S) protein. In RVFV, the NS_S protein has been found to be phosphorylated and to accumulate in the nuclei of infected cells (Struthers and Swanepoel, 1982), although the function of this protein is at yet not defined. The L segment codes for an L protein which was found to be a component of the viral transcriptase (Keegan and Collett, 1986) as well as being responsible for generating the capped primers needed for transcription (Jin and Elliott, 1993). The M segment codes for the glycoproteins, G1 and G2, and the non-structural M (NS_M) protein in a single open reading frame

(ORF) in circular RNA (cRNA) (Schmaljohn, 1996). These envelope glycoproteins have proved important for viral adsorption and penetration. Besselaar and Blackburn (1991) showed, by means of topological mapping of the antigenic sites on the G1 and G2 glycoproteins, at least 7 neutralising/haemagglutinin sites on the G1 glycoprotein and at least 4 on the G2 glycoprotein. This, therefore, indicates that the G1 and G2 glycoproteins serve as important neutralising and haemagglutinin antibody targets. Further evidence for this was sited by Keegan and Collett, 1986, in which polyclonal and monospecific antisera to either G2 or G1 of RVFV was able to neutralise RVFV infectivity in an *in vitro* plaque-reduction neutralisation test. Likewise, Besselaar and Blackburn, 1992, demonstrated that certain monoclonal antibodies to different antigenic regions on G1 and G2 was able to neutralise RVFV, either through neutralisation by inhibiting the infection process after virus attachment or through neutralisation by preventing virus attachment to cells. Furthermore, monoclonal antibodies raised against G2 protected against RVFV challenge *in vivo* (Gonzalez-Scarano and Nathanson, 1990). The immunogenic properties of the G1 and G2 RVFV glycoproteins was further illustrated by Schmaljohn *et al.*, 1989, who inoculated mice with a recombinant baculovirus expressing the G1 and G2 RVFV proteins. Here, mice were protected from lethal challenge with RVFV following immunization with the lysates of cells infected with the recombinant virus. Collett *et al.*, 1987, also demonstrated that vaccinia virus expressing G1 and G2 RVFV proteins was able to protect mice from challenge with RVFV.

3.1.5: Prevention and control

In the field of prevention and control, a number of measures have been pursued to deal with RVF. These include the slaughter of infected animals, the movement of stock to well-drained wind-swept pastures, and biological or chemical control of the vector. However, the most effective measure remains vaccination to protect livestock. The Smithburn strain of RVF, used at the 106 intercerebral mouse passage level, has been used as a vaccine in Kenya since 1960 (sited by Swanepoel and Coetzer, 1994). However, there has been problems associated with this live attenuated vaccine in as much as it

been problems associated with this live attenuated vaccine in as much as it induces abortions or teratology of the foetus and *hydrops amnii* and prolonged gestation in a proportion of pregnant animals (Coetzer & Barnard, 1977). Furthermore, the Smithburn RVF vaccine induces a poor antibody response in cattle (Barnard, 1979). Another live attenuated virus was developed called MP12 (Caplen *et al.*, 1985). This vaccine proved promising as it had attenuation markers in all three segments and thus has a low probability of reversion (Saluzzo and Smith, 1990; Vialet *et al.*, 1997). Although the RVF-MP12 vaccine was shown to be safe and immunogenic in humans, the vaccine had a teratogenic effect when inoculated into ewes during the first trimester of pregnancy (Sall *et al.*, 1998).

In South Africa, a formalin-inactivated vaccine has been used and proved safe in pregnant animals (Swanepoel and Coetzer, 1994). Nevertheless, this vaccine is expensive to produce, requires multiple inoculations and only provides short-lived immunity (Swanepoel and Coetzer, 1994). This vaccine is not licensed or commercially available, although it has been used to protect veterinary and laboratory workers at high risk of exposure to RVFV.

3.2: RESULTS

3.2.1: Transfection of recombinant plasmid

Transfection experiments were carried out in order that homologous recombination could occur between the LSDV (Neethling) genome and the plasmid DNA. The lamb testes cells were therefore infected with 0.01 p.f.u. of the LSDV (Neethling) first, followed by the transfection experiment. The experiment was done in duplicate. Control experiments were also carried out simultaneously involving mock-transfections of uninfected and LSDV infected cells. Following three days post-transfection, X-gal stains were performed on the controls as well as on one of the duplicates in order that efficiency of transfection could be evaluated. The results showed no blue foci on the control plates for at least 2 days following the X-gal stain. A few faint blue cells were, however, visible thereafter, indicating the presence of endogenous β-galactosidase activity. The LSDV-infected cells transfected with the plasmid displayed a high number of blue foci within 1 hour after X-gal staining. Optimisation of transfection conditions was also necessary. The recommended amount of DNA for a transfection reaction per well was 2.5μg, the amount recommended for DOTAP by Roche. However, it was found that 2.5μg plasmid DNA yielded a low rate of transfection efficiency, with an increase in transfection rate proportional to the amount of DNA added up to 10μg plasmid DNA. The transfection rate was estimated to be approximately 50-60% for cells transfected with 10μg of plasmid DNA. Furthermore, transfections carried out using DNA isolated using the Qaigen kit gave a better rate of transfection as opposed to DNA isolated using the Nucleobond kit. The other duplicate experiment was used for freeze/thawing in order that the recombinant virus could be released from the infected cells. It was evident that the medium from the cells suspected of containing recombinant virus proved less effective as opposed to collecting the medium plus freeze/thawing the cells when trying to propagate virus.

3.2.2: Isolation and purification of recombinant virus

The process of purification and isolation of recombinant virus is reliant on the presence of the *Ecogpt* gene and the *Lac Z* gene. The *Ecogpt* gene, in which the purine salvage pathway is selected, allows selection in MPA, xanthine and hypoxanthine. The material obtained following transfection was passaged 4X in lamb testes cells in this selection medium. Following each passage, the cells were freeze/thawed. X-gal staining and x-gal overlay were then performed, with the development of blue foci 1 hour later (x-gal staining) and blue plaques 12 hours later (x-gal overlays). The x-gal staining (refer to figure 3.2) gives rapid result, indicating the presence of the *LacZ* gene and therefore the presence of recombinant virus. However, the virus is no longer viable, therefore, x-gal overlays are performed (refer to figure 3.3) which allows additional purification steps to be included. Controls were also done involving uninfected cells and LSDV-infected cells. There was no development of blue plaques or foci on the controls. A number of blue plaques were picked which underwent three rounds of passaging in selection medium. X-gal staining was carried out in conjunction with X-gal overlays again with the development of blue foci and plaques. The blue plaques were isolated, underwent another 2 rounds of passaging in selection medium, after which the last X-gal overlay was performed. The blue foci picked then underwent at least 5X passaging in selection medium with x-gal staining been performed on the last passage for additional conformation of the presence of the recombinant virus (RVF-LSDV) by the development of blue foci.

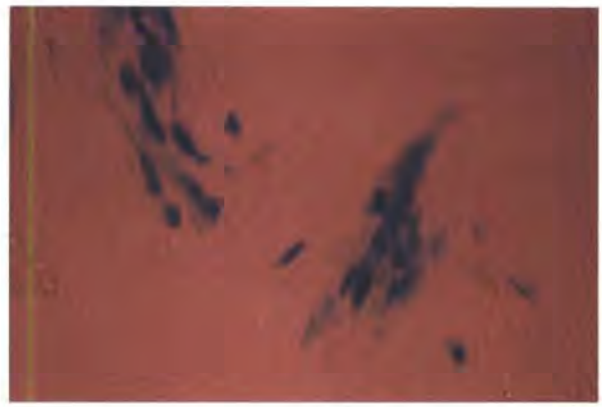


Figure 3.2: X-gal stain of lamb testes cells infected with RVF-LSDV. Blue foci are indicative of the presence of the *LacZ* gene.

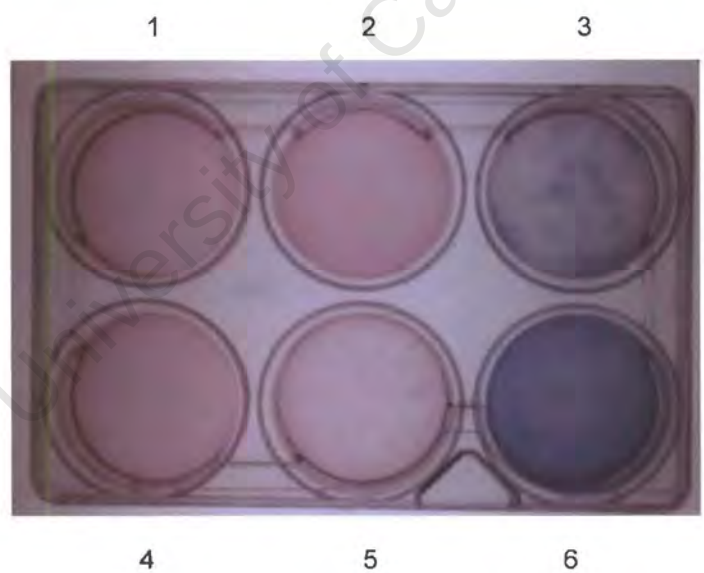


Figure 3.3: X-gal overlays. Well 1 and 4 are uninfected lamb testes cells, well 2 and 5 are wild type LSDV (Neethling) infected lamb testes cells. Wells 3 and 6 contain RVF-LSDV

3.2.3: Characterisation of RVF-LSDV

3.2.3.1: Southern blot hybridisation

Southern blot hybridisation analysis of genomic DNA extracted from RVF-LSDV infected lamb testes cells, wild type LSDV (Neethling) infected lamb testes cells and uninfected lamb testes cells, digested with *Pst*I, was carried out. This was done to determine if a double cross-over or a single cross-over event had occurred between the transfer plasmid and the LSDV genome, as well as to determine if any wild type virus is present in the recombinant virus preparation. Refer to figure 3.4 for a diagram of the predicted consequences of either a double cross-over or a single cross-over event between the LSDV genome and pSelp-HS-G1G2-βGal.

The results of the Southern blot hybridisation are shown in figure 3.5b. A positive control was included in lane 3 which contains pSelp-HS-G1G2-βgal plasmid DNA digested with *Pst*I. The probe (pSelp-HS-G1G2-βgal digested with *Pst*I) bound to 4 bands in this lane of sizes 5.8kb, 2.6kb, 2.5kb and 1.2kb, as expected. This indicated that the experiment was functioning and effective. No signal is visible in lane 5 which represents uninfected lamb testes DNA digested with *Pst*I, when probed. This was as expected as no LSDV was present in this negative control. The probe hybridised to a 12.3kb fragment in the DNA extracted from wild type infected cells (lane7). This band identifies A2L, A3L and the intergenic region in the LSDV genome used as the insertion site for the recombinant plasmid. Positive hybridisation signals were detectable in DNA extracted from RVF-LSDV infected cells (lane 9). In total 5 bands are visible although the sizes of these bands do not correspond to the sizes expected if a single cross-over event had occurred. The five bands are of the sizes 18kb, 12.3kb, 2.7kb, 3.9kb and 1.2kb. As shown in figure 3.4, a single cross-over would give five band sizes of 18kb, 2.6kb, 2.5kb, 1.2kb and another band of unknown size as the exact positions of the *Pst*I sites in the LSDV genome have a yet not been mapped exactly. The one additional band of size 12.3kb in lane 9 indicated the presence of wild type LSDV

(Neethling) in the recombinant preparation. The band intensity also indicates a large amount of wild type LSDV present. The other unexpected band of size 3.9kb most likely represents partially digested DNA. In fact, the 3.9kb band seems to be a partial digest of the two bands, 1.2kb and 2.7kb, as is evident by the size (ie. 2.7+1.2=the 3.9kb).

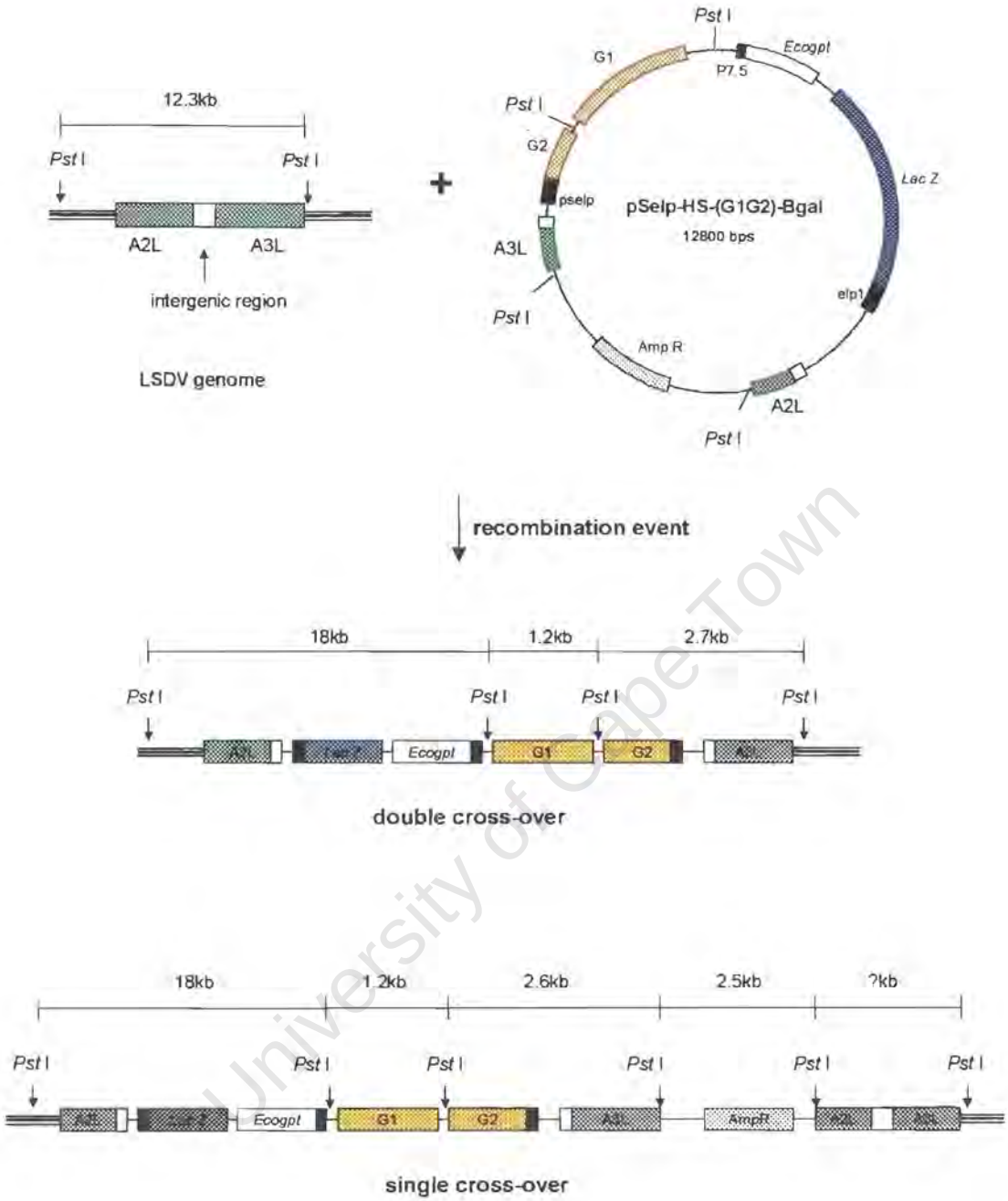


Figure 3.4: Diagrammatic representation of homologous recombination between LSDV genome and pSelp-HS-G1G2-βgal. A double cross-over would result in three bands if DNA was digested with *Pst*I. A single cross-over would give rise to 5 bands if the DNA was digested with *Pst*I.

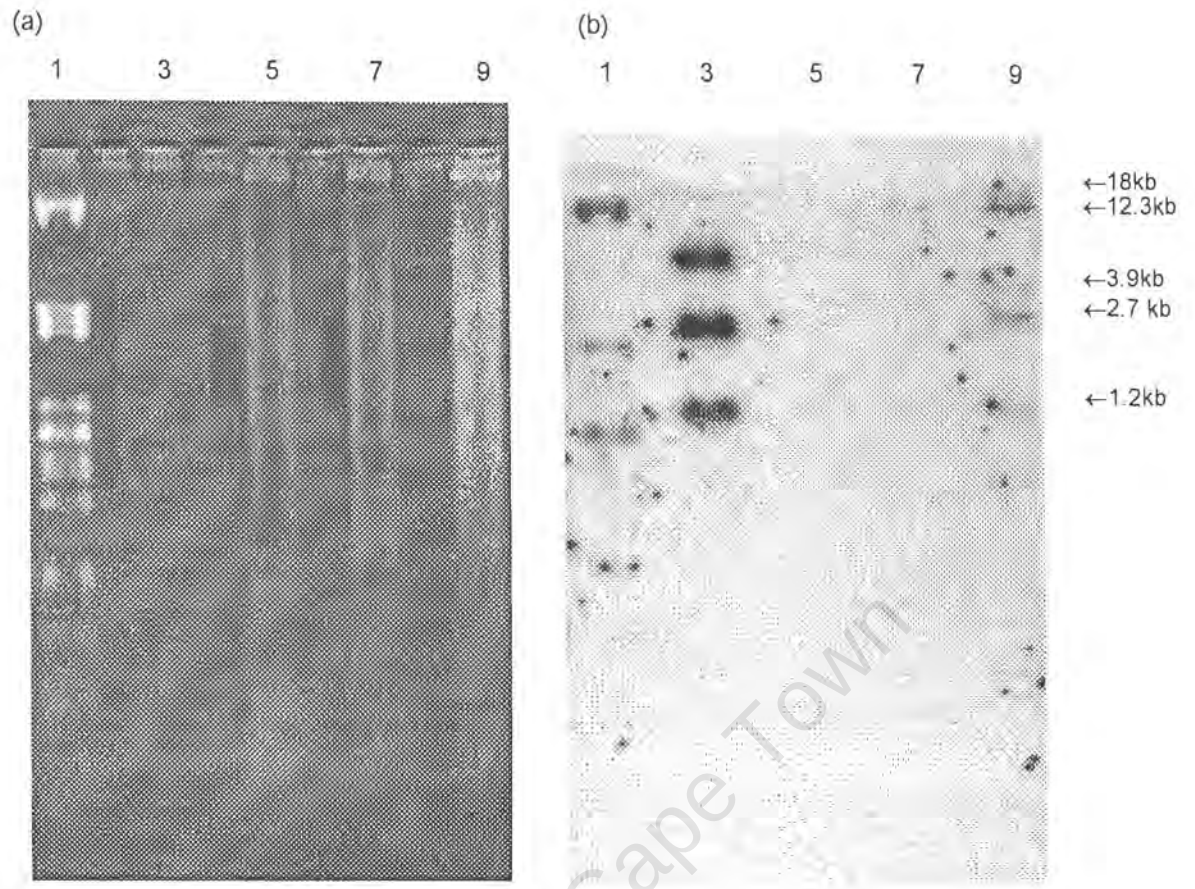


Figure 3.5: Identification of the RVF-LSDV.

(a) Agarose gel electrophoresis of genomic DNA. Lane 1, λ DNA digested with *Pst*I. Lane 3, pSelp-HS-G1G2-βgal digested with *Pst*I. Lane 5, DNA extracted from uninfected lamb testes cells digested with *Pst*I. Lane 7, DNA from wild type LSDV (Neethling) infected lamb testes cells digested with *Pst*I. Lane 9, DNA from RVF-LSDV infected lamb testes cells digested with *Pst*I.

(b) Autoradiograph of the gel in figure 3.6a probed with pSelpHS-G1G-βgal digested with *Pst*I. Lane 1, λ DNA digested with *Pst*I. Lane 3, pSelp-HS-G1G2-βgal digested with *Pst*I. Lane 5, DNA extracted from uninfected lamb testes cells digested with *Pst*I. Lane 7, DNA from wild type LSDV (Neethling) infected lamb testes cells digested with *Pst*I. Lane 9, DNA from RVF-LSDV infected lamb testes cells digested with *Pst*I.

3.2.3.2: Protein analysis

Expression of the RVFV glycoprotein genes was evaluated by detecting the products of these genes. This was carried out to ensure that the proteins were produced by the recombinant virus. *In situ*

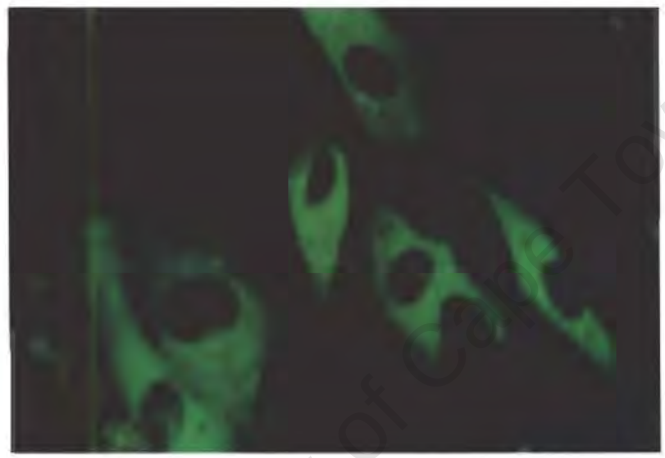
immunoflourescence staining of lamb testes cells infected with RVF-LSDV was performed once CPE had developed. A polyclonal sheep antisera was used for the detection of the RVFV glycoproteins, as the sheep had been infected with RVFV. No immunofluorescence was detectable from uninfected lamb testes cells (results not shown) and wild type LSDV (Neethling) infected lamb testes cells (figure 3.6a). Both these were included as negative controls, giving negative results as expected.

Cells infected with RVF-LSDV showed fluorescence in isolated foci (figure 3.6b) on cell culture monolayers. It was also apparent that the RVFV glycoproteins are not produced in the nucleus as seen in figure 3.6b and 3.6c, as the nucleus is emitting no fluorescence.

Western blots analysis on proteins isolated from RVF-LSDV infected cells failed to detect the G1 and G2 proteins using the polyclonal antibodies obtained from RVFV infected sheep and polyclonal antibodies obtained from RVFV infected bovine(results not shown). Optimisation was carried out in which various amounts of primary antibody was used as well as various amounts of proteins loaded on the gels. However, these optimisation experiments failed to detect the G1 and G2 proteins.

(a)

(b)



(c)

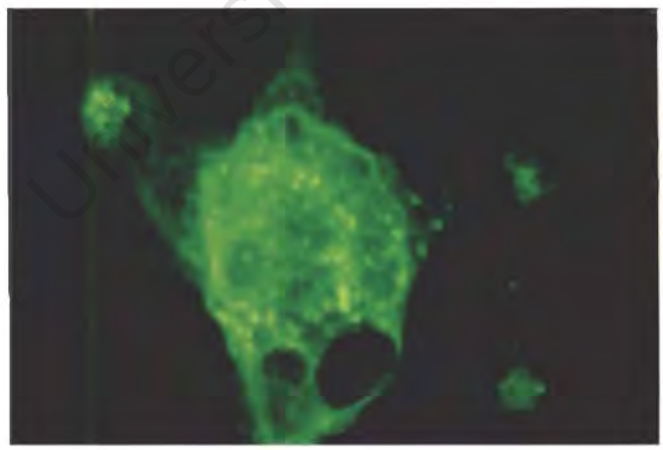


Figure 3.6: Immunofluorescence assay

(a): lamb testes cells infected with wild type LSDV (Neethling). Magnified 16x.

(b): lamb testes cells infected with RVF-LSDV. Magnified 16X.

(c): lamb testes cells infected with RVF-LSDV. Magnified 40X.

3.2.4: Evaluation of immune response to RVFV glycoproteins in rabbits

Eleven rabbits were vaccinated according to the schedule described in section 2.2.19. The humoral response was evaluated by reacting the sera from each rabbit with the negative and positive antigen supplied with the Anti-RVF IgG ELISA kit (Special Pathogens Unit, NIV, Johannesburg). The negative antigen was derived from homogenised mouse brains (personal communication R. Swanepoel) and the positive antigen is the RVFV antigen. Sera from rabbits A, B, C and D (vaccinated with RVF-LSDV), which had received the same vaccination schedule, showed a similar pattern of response. The OD_{492} values from rabbits A, B, C and D were pooled for easy of evaluation and the mean values calculated, including the standard deviations, as shown in figure 3.7. There was no difference in reaction on the ELISA between the negative and the positive (RVFV) antigens. The same pattern of response is also seen in rabbits E and F (vaccinated with RVF-LSDV), were no difference in response on the ELISA between the negative and positive antigens (figure 3.8). Rabbits G and H, which had received the OBP vaccine, had elicited an immune response to the negative and the positive antigen. However, the absorbance readings were much higher for reaction to the positive antigen than the negative antigen (figure 3.9). This indicated a definite specific response to the RVFV antigens thus demonstrating the presence of RVFV antibodies. Furthermore, this is prove that the ELISA was functional.

Rabbit I, which was the unvaccinated control displayed no response to the negative and positive antigens (table 3.1). Rabbit J, vaccinated with tissue culture medium exhibited a response to both the negative and positive antigen (figure 3.10), which was similar in value. The wild type LSDV (Neethling) vaccinated rabbit (rabbit K) elicited no response to the negative and positive antigen (table 3.2).

The high background readings obtained for the majority of rabbits on reacting the sera to the negative antigen illustrated cross-reactivity to a component found within this material. Thus the sera activity of rabbit C, E and

H were further investigated following absorption of the sera with lamb testes cell extract and tissue culture components. Furthermore, a dilution factor of 1:20 was used as opposed to 1:100, in order that a more sensitive detection could be applied. A marked decrease can be seen on comparison of the OD values between sera not absorbed and preabsorbed with the cell debris and tissue culture medium as shown in figure 3.11 and 3.12. The OD values for sera preabsorbed (figure 3.13) on reacting to the negative and positive antigen was reduced to essentially zero. The same pattern is evident for rabbit E (figures 3.14 – 3.16). However, there is a slight increase at week 8 (figure 3.16) which exists in both the negative and positive antigen. The non-specific reaction seen in rabbit H was markedly reduced following absorption of the sera with cell debris and tissue culture medium, most noticeable seen in figure 3.17.

Haemaglutination inhibition tests were performed at NIV using sera from rabbits A-K. Results (not shown) revealed no antibodies to RVFV using this method except those rabbits vaccinated with the OBP RVFV vaccine.

Immunoflourescence assays, using sera from rabbits A-F, also revealed no antibodies present as no fluorescence was detectable in lamb testes cells infected with RVF-LSDV or LSDV (Neethling). Sera from rabbits vaccinated with the OBP RVFV vaccine, however, displayed flourescence.

Sera from rabbits vaccinated with a recombinant LSDV constructed by A.Cohen was obtained. This construct is similar to the RVF-LSDV construct made in this project, except that A.Cohen's earlier construct lacks the *LacZ* gene. The sera was tested for an immune response to the RVFV antigens using the Anti-RVF IgG ELISA kit. This was done as previous ELISA performed by A.Cohen had not included negative controls. The results (not shown) revealed a remarkably similar pattern to rabbits A, B, C, D, E and F, in that a response was seen on reaction with the negative antigen. Once the sera was preabsorbed with tissue culture medium and cell debris, the OD readings were reduced to virtually zero on reaction with the negative and positive antigens (results not shown).

(a)

		Time (weeks)						
		0	2	4	6	8	10	12
Negative antigen	Mean OD	0.0259	0.38	0.073	0.034	1.0615	0.209	0.064
	Std dev.	0.023432	0.294193	0.050553	0.021389	0.462907	0.123476	0.040844
Positive antigen	Mean OD	0.024	0.367	0.074	0.03	1.026	0.205	0.062
	Std dev.	0.018195	0.293228	0.051391	0.020087	0.440271	0.113394	0.032895

(b)

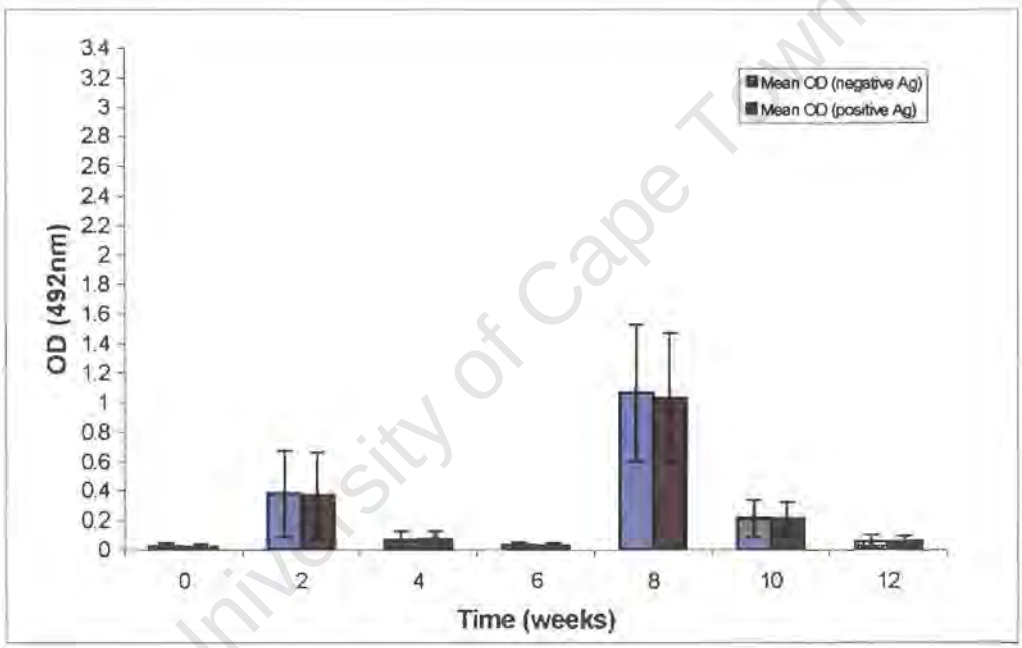


Figure 3.7: Results representing mean values taken from serum of rabbits A,B,C and D (vaccinated with RVF-LSDV) tested with the negative and positive antigen. Serum dilution factor 1:100. OD values weretaken at 492nm. Std dev. = standard deviation. (a) Results represented in a tabular form. (b) Results represened in a graphic form.

(a)

		Time (weeks)						
		0	2	4	6	8	10	12
Negative antigen	OD	0.029	0.154	0.05	0.04	1.422	0.519	0.228
	Std dev.	0.017678	0.088035	0.015203	0.013435	0.698975	0.376534	0.197283
Positive antigen	OD	0.02325	0.0145	0.055	0.02	1.323	0.489	0.219
	Std dev.	0.013789	0.082731	0.025102	0.002475	0.639225	0.351079	0.190212

(b)

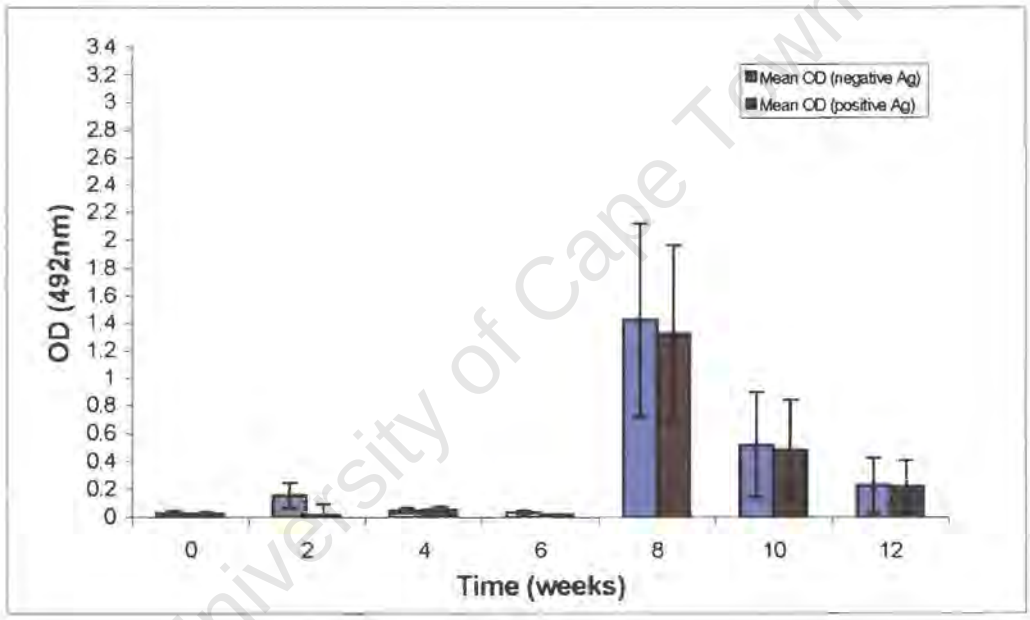


Figure 3.8: Results representing mean values taken from serum from rabbits E and F (vaccinated with RVF-LSDV) tested with the negative and positive antigen. Serum dilution factor 1:100. OD values read at 492nm. Std dev. = standard deviation.
(a) Results represented in tabular form. (b) represented in a graphic form.

(a)

		Time (weeks)						
		0	2	4	6	8	10	12
Negative antigen	OD	0.01175	1.82225	0.85325	0.588	0.62675	0.7945	0.49325
	Std dev.	0.001061	0.511592	0.166524	0.05374	0.053387	0.065761	0.209657
Positive antigen	OD	0.01175	2.50375	2.11075	1.7245	1.53975	1.6185	1.1525
	Std dev.	0.001061	0.764736	0.452195	0.231931	0.09157	0.2885	0.36911

(b)

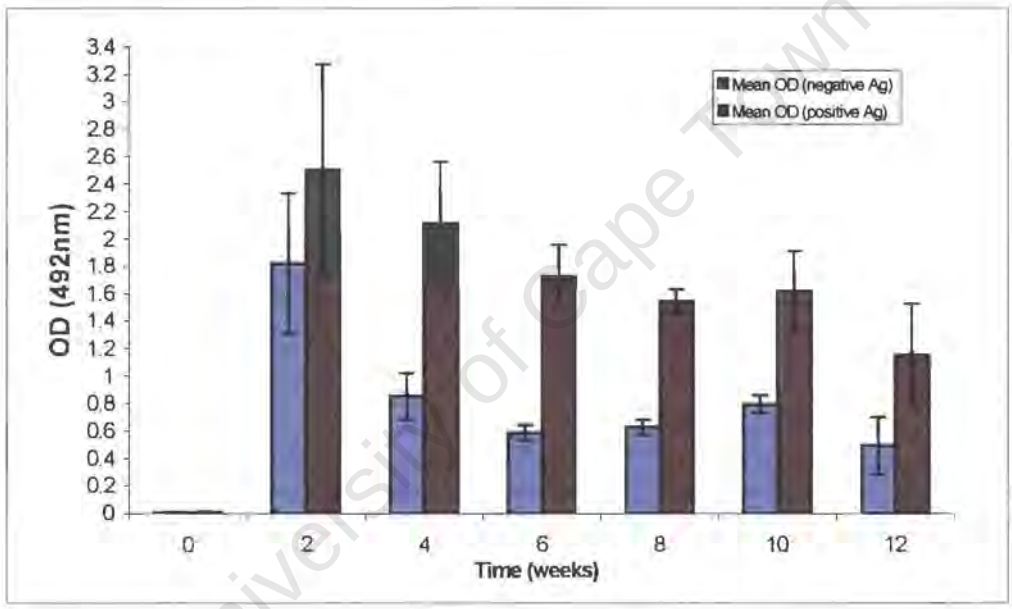


Figure 3.9: Results representing mean values taken from serum from rabbits G and H (vaccinated with OBP vaccine) tested with the negative and positive antigen. Serum dilution factor 1:100. OD values read at492nm. Std dev. = standard deviation.
(a) Results represented in tabular form. (b) Results represented in graphic form.

Table 3.1: Results of serum from rabbit I (unvaccinated) tested with the negative and positive antigens. Serum dilution factor 1:100. OD values read at 492nm.

	Time (weeks)				
	0	2	4	6	8
OD (negative Ag)	0.0015	0.002	0.001	0.001	0.0015
OD (positive Ag)	0.001	0.0015	0.002	0.002	0.002

(a)

	Time (weeks)				
	0	2	4	6	8
OD (negative Ag)	0.001	0.14	0.039	0.0065	0.005
OD (positive Ag)	0.002	0.1275	0.0375	0.008	0.0055

(b)

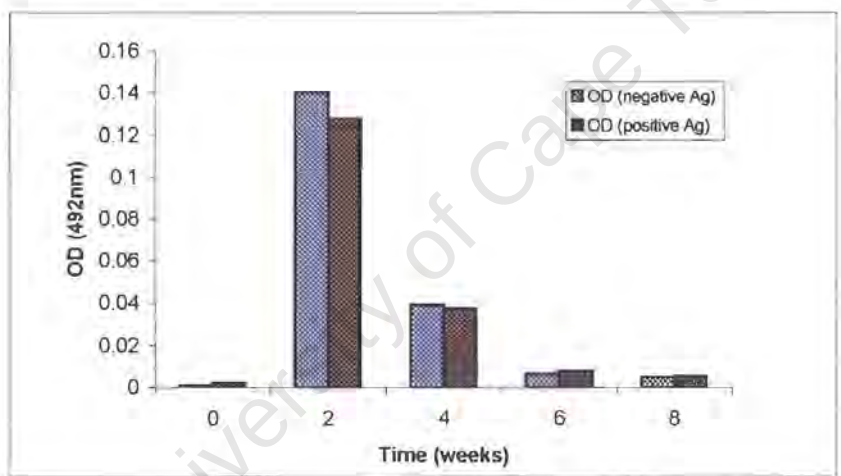


Figure 3.10: Results of serum from rabbit J (inoculated with tissue culture medium) tested with the negative and positive antigen. Serum diluted 1:100. OD values read at 492nm. Results in tabular form (a) and graphic form (b).

Table 3.2: Results of serum from rabbit K (vaccinated with wild type LSDV) tested with the negative and positive antigen. Serum diluted 1:100. OD values read at 492nm.

	Time (weeks)						
	0	2	4	6	8	10	12
OD (negative Ag)	0.002	0.007	0.0015	0.001	0.0165	0.0015	0.001
OD (positive Ag)	0.003	0.008	0.0015	0.002	0.014	0.001	0.0015

(a)

Time (w)	OD	OD (abs)
0	0.0145	0.0175
2	0.473	0.043
4	0.0915	0.021
6	0.0235	0.0205
8	1.481	0.0605
10	0.3005	0.029
12	0.087	0.0245

(b)

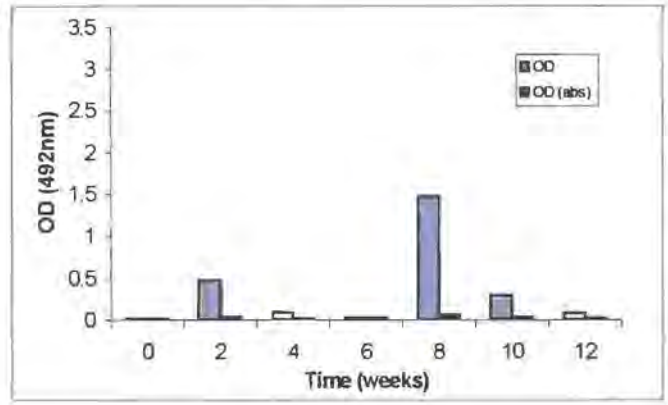


Figure 3.11: Results of serum from rabbit C tested with the negative antigen, represented in a tabular form (a) and graphic form (b). Serum diluted 1:20. OD values read at 492nm. OD (abs) = OD readings using sera preabsorbed with cell debris and tissue culture medium.

(a)

Time (w)	OD	OD (abs)
0	0.0115	0.024
2	0.419	0.05
4	0.091	0.0295
6	0.022	0.0235
8	1.3785	0.0655
10	0.287	0.034
12	0.0845	0.0285

(b)

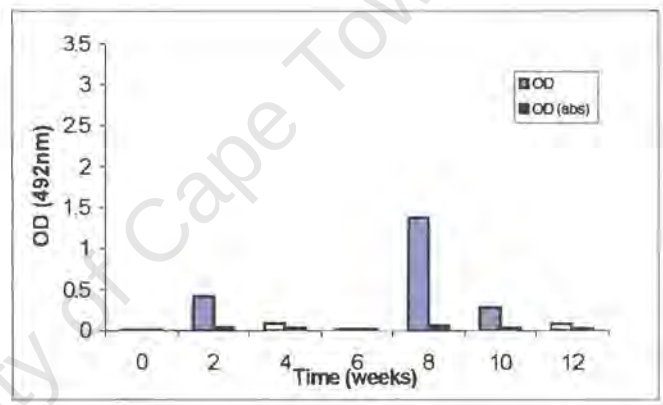


Figure 3.12: Results of serum from rabbit C tested with the positive antigen, presented in a tabular form (a) and graphic form (b). Serum diluted 1:20. OD readings at 492nm. OD (abs) represents OD readings using sera preabsorbed with cell debris and tissue culture medium.

(a)

Time (w)	Negative antigen OD(abs)	Positive antigen OD(abs)
0	0.0175	0.024
2	0.043	0.05
4	0.021	0.0295
6	0.0205	0.0235
8	0.0605	0.0655
10	0.029	0.034
12	0.0245	0.0285

(b)

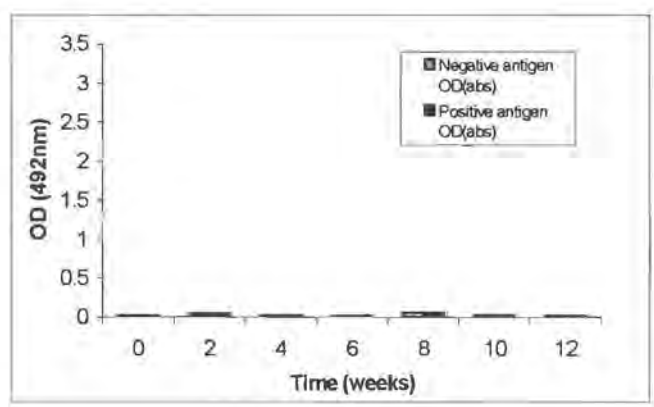


Figure 3.13: Results of serum, preabsorbed with cell debris and tissue culture medium, from rabbit C, tested with negative and positive antigen. Serum diluted 1:20. Results in tabular form (a) and graphic form (b).

(a)

Time (w)	OD	OD (abs)
0	0.00165	0.0145
2	0.092	0.031
4	0.039	0.019
6	0.0295	0.019
8	1.916	0.5235
10	0.785	0.3155
12	0.367	0.0885

(b)

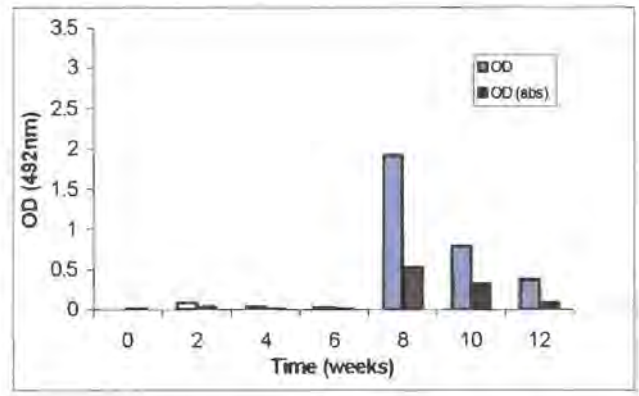


Figure 3.14: Results of serum from rabbit E tested with the negative antigen, represented in a tabular form (a) and graphic form (b). Serum diluted 1:20. OD values read at 492nm. OD (abs) = OD readings using sera preabsorbed with cell debris and tissue culture medium.

(a)

Time (w)	OD	OD (abs)
0	0.033	0.0265
2	0.0905	0.0465
4	0.0037	0.0265
6	0.018	0.0285
8	1.7745	0.508
10	0.7375	0.297
12	0.353	0.0915

(b)

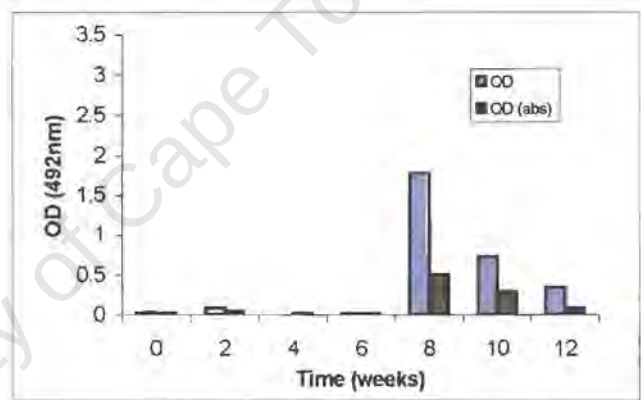


Figure 3.15: Results of serum from rabbit E tested with the positive antigen, presented in a tabular form (a) and graphic form (b). Serum diluted 1:20. OD readings at 492nm. OD (abs) represents OD readings using sera preabsorbed with cell debris and tissue culture medium.

(a)

Time (w)	Negative antigen OD(abs)	Positive antigen OD(abs)
0	0.0145	0.0265
2	0.031	0.0465
4	0.019	0.0265
6	0.019	0.0285
8	0.5235	0.508
10	0.3155	0.297
12	0.0885	0.0915

(b)

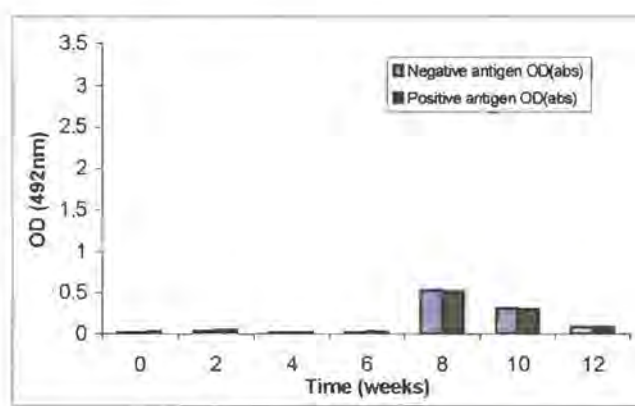


Figure 3.16: Results of serum, preabsorbed wih cell debris and tissue culture medium, fom rabbit E, tested with negative and positive antigen. Serum diluted 1:20. Results in tabular form graphic form (b).

(a)

Time (w)	OD	OD (abs)
0	0.0125	0.034
2	2.184	0.581
4	0.971	0.3955
6	0.626	0.207
8	0.589	0.154
10	0.748	0.14
12	0.6415	0.098

(b)

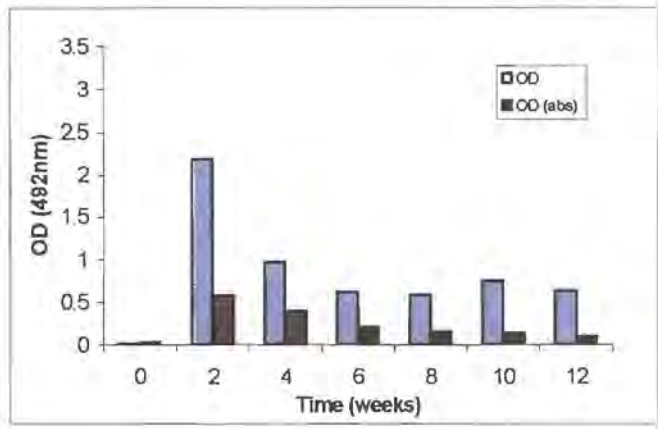


Figure 3.17: Results of serum from rabbit H tested with the negative antigen, represented in a tabular form (a) and graphic form (b). Serum diluted 1:100. OD values read at 492nm. OD (abs) = OD readings using sera preabsorbed with cell debris and tissue culture medium.

(a)

Time (w)	OD	OD (abs)
0	0.0125	0.039
2	3.0445	2.366
4	2.4305	2.2915
6	1.8885	2.181
8	1.6045	2.0695
10	1.4145	1.889
12	1.4135	1.7225

(b)

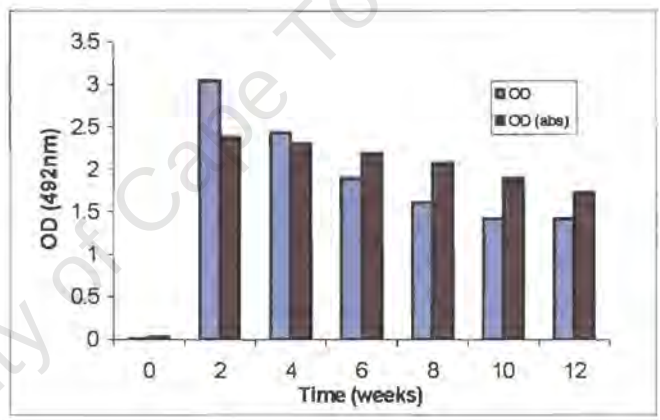


Figure 3.18: Results of serum from rabbit H tested with the positive antigen, presented in a tabular form (a) and graphic form (b). Serum diluted 1:100. OD readings at 492nm. OD (abs) represents OD readings using sera preabsorbed with cell debris and tissue culture medium.

(a)

Time (w)	Negative antigen OD(abs)	Positive antigen OD(abs)
0	0.034	0.039
2	0.581	2.366
4	0.3955	2.2915
6	0.207	2.181
8	0.154	2.0695
10	0.14	1.889
12	0.098	1.7225

(b)

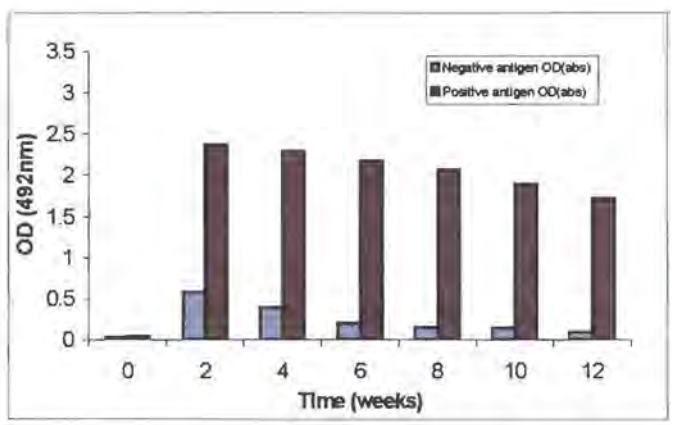


Figure 3.19: Results of serum, preabsorbed wih cell debris and tissue culture medium, from rabbit H, tested with negative and positive antigen. Serum diluted 1:100. Results in tabular form (a) and graphic from (b).

3.3: DISCUSSION

A suitable transfer vector was constructed by A.Cohen containing the RVFV glycoprotein G1 and G2. This transfer vector, pSelp-HS-G1G2-βgal, was successfully transfected into wild type LSDV (Neethling) infected lamb testes cells. Initially, the efficiency of transfection was judged by performing X-gal stains following the transfection experiments. This illustrates both transient expression as well stable recombination. A higher efficiency of transfection was demonstrated by increasing the concentration of the plasmid DNA. Luo and Saltzman (2000) demonstrated that increasing the DNA complexes at the cell surface in the transfection experiments increased the efficiency. However, the increase in the DNA complex must not be accompanied by increased toxins which can interfere with the transfection (Luo and Saltzman, 2000). These toxic substances can arise from the method of plasmid preparation as well as from the transfection reagents themselves. Plasmid DNA prepared using the Nucleobond AX PC-kit 100 and used in transfection experiments yielded a low rate of transfection even when 10μg of DNA was used. In comparison, plasmid DNA prepared using the Qiagen Plasmid Midi Kit and used in transfections yield a very high rate of tansfection particularly when the concentration of the DNA was increased to 10μg. The Qiagen Plasmid Midi Kit is reported to have reduced levels of endotoxins and it probably for this reason that the rate of transfection was improved, together with the quantity of DNA used. Thus the method of DNA preparation is important when using the DNA for transfections.

Following further purification steps of the recombinant virus, the continual presence of the blue foci after X-gal staining and X-gal overlays indicated the functionality of the *lacZ* gene. The blue foci indicates that the cells are harbouring the recombinant virus. Furthermore, the cells infected with recombinant virus displayed high levels of β-galactosidase activity when compared to uninfected and LSDV (Neethling) infected cells. The recombinant virus infected cells developed blue foci within 1 hour of the X-gal staining whereas the control cells (uninfected and LSDV Neethling infected

cells) showed only a few very faint blue cells 3-4 days post X-gal staining. According to MacGregor *et al.* (1991), some tissues such as kidney and testes exhibit higher endogenous β-galactosidase activity than others which is due to the presence of lysosomal β-galactosidases.

The RVF-LSDV was further tested for expression of the G1G2 RVFV glycoproteins by infecting lamb testes cells with the recombinant virus and performing immunoflourescence assays. The development of fluorescence indicated that the RVF glycoproteins G1 and G2 was expressed as the polyclonal antibody used was from sheep infected with RVFV. Furthermore, it was clear that the G1 and G2 glycoproteins was produced in the cytoplasm as fluorescence was exclusively seen at this site. This is in keeping with the proposed replication cycle of LSDV which occurs in the cytoplasm. However, the inability to recognise the G1 and G2 proteins on the Western blots may indicate that either the process of isolating of G1 and G2 from the infected lamb testes cells may not be optimal, or the polyclonal antibodies used are unable to recognise the linear epitopes.

Southern blot analysis of DNA extracted from RVF-LSDV infected cells revealed that a double crossover event had occurred between the flanking sequences on the transfer vector and the intergenic region found in the LSDV genome. However, the DNA analysis also revealed the presence of some wild type LSDV. A similar plasmid construct to the one used in this project, but it lacked the *lacZ* gene, was made by A.Cohen. Recombinant virus made from this construct also yielded wild type LSDV in the final preparations and it was for this reason that the construct used in this project was made. The rational behind this was that the incorporation of the *lacZ* gene would facilitate purification of the recombinant virus. However, as shown here, wild type LSDV was still present in the recombinant virus preparation (when employing the pSelp-HS-G1G2-βgal vector), even though two selectable processes was used. It is therefore evident that although the *lacZ* gene is useful in helping to distinguish the recombinant virus through X-gal overlays and X-gal staining, the number of purification steps may be vital. Thus additional rounds of

purification are necessary. To ensure that no wild type LSDV (Neethling) is present it may be useful to include Southern blot hybridisation experiments after each purification step to ensure a continual reduction in the wild type virus until none is detectable. Furthermore, PCR can be included, whereby the insertion site used in the LSDV genome for homologous recombination is amplified. The rational behind this is that if the complete insertion site is amplified, then wild type is still present as insertion into this site would disrupt the site.

Although it is clear that additional rounds of purification is necessary to obtain pure recombinant virus, it was surprising that wild type virus was able to withstand several purification procedures. These include 14 passages in selection medium and three plaque purification steps. The reason for this may very well be depletion or breakdown of the MPA in the selection medium. Consequently wild type LSDV is able to replicate, in which case it may be useful to change the selection medium regularly when passaging the recombinant virus in selection medium together with additional purification steps.

The rational behind vaccinating rabbits with RVF-LSDV was as a consequence of previous work done by A.Cohen in which a similar recombinant virus construct was also used to vaccinate rabbits. A.Cohen demonstrated that the rabbits, which had been vaccinated intramuscularly or subcutaneously with $1X10^6$ p.f.u., elicited a humoral response to the G1G2 RVFV glycoproteins (unpublished data). Thus the RVF-LSDV construct made in this project was inoculated into outbred rabbits to evaluate the immune response elicited by the G1 and G2 glycoproteins. Outbred animals reflect more accurately the genetic diversity within each species than do inbred animals although inbred animals do allow a more comparative analysis. The results indicated a high background binding indicating non-specific binding. Further investigations revealed that traces of tissue culture medium and cell debris was present in the vaccine preparations and this was eliciting an immune response. These antibodies in turn are reacting to components in the negative antigen as well as the positive antigen as preabsorbing of the sera to

tissue culture medium and lamb testes cell debris reduced the OD readings to nearly zero when tested with the negative and positive antigens. Although the dilution factor was lowered from 1:100 to 1:20 in the ELISA, there was continual low absorbance readings on reaction of the sera to the positive antigen. This exemplifies the lack of an immune response to the RVFV G1 and G2 protein antigens. The positive results achieved when the OBP vaccine antiserum was tested using the same system proves that the Anti-RVF IgG ELISA test used is indeed sensitive and correct. Furthermore, the negative and positive control included in the ELISA kit was also tested, both of which was shown to work. Rabbit antiserum was also tested for non-specific binding by applying the antiserum directly to coated and blocked plates, however no absorbance readings were found. In addition tests on the conjugate indicated no non-specific binding to the plates.

Other methods of evaluating the immune response in the vaccinated rabbits were performed. Haemaglutination inhibition tests were carried out at NIV and the results were shown to be negative. Immunofluorescence assays carried out using the sera from rabbits vaccinated with RVF-LSDV revealed no fluorescence, indicating no antibodies to G1 and G2. Immunofluorescence assay performed using the sera from rabbits vaccinated with OBP vaccine showed fluorescence, indicating the assay was effective.

Further experimental work was carried out on the sera obtained from rabbits vaccinated intramuscularly or subcutaneously with the recombinant virus made by A.Cohen at a dosage of $1X10^6$ p.f.u. Although as stated A.Cohen's revealed an immune response to the RVFV glycoproteins, the sera was never tested with the negative antigen and therefore the ELISA was redone. The results revealed that although there was a high reading observed on reacting the sera to the positive antigen, the same level of response was seen when tested with the negative antigen. This indicates a high background and thus the sera was preabsorbed with tissue culture medium and cell debris. The OD readings then dropped to nearly zero for both the negative and positive antigens. This illustrates the importance of including negative controls. Moreover, these experiments revealed that no

antibodies to G1G2 RVF glycoproteins was present in animals vaccinated intramuscularly or subcutaneously with recombinant virus by the method of ELISA. Additionally, those rabbits vaccinated subcutaneously showed no RVFV specific antisera as was revealed by immunofluorescence and haemaglutination inhibition (A.Cohen, unpublished data). However, it must be stated that animals vaccinated intramuscularly did show specific antibodies to RVFV using immunofluorescence and haemaglutination inhibition (A.Cohen, unpublished data). However, the sera used for testing in the immunoflourescence and haemaglutination inhibition assays were not preabsorbed with tissue culture medium and cell debris. Thus the positive results seen in these tests may very well be false positives in light of the results seen in the ELISA tests.

The lack of a specific immune response to the RVFV antigens seen in the RVF-LSDV vaccinated rabbits could reflect the host specificity of the LSDV. Rabbits are not permissive to LSDV and it may very well be possible that the recombinant virus is not infecting the rabbit cells in which case the G1 and G2 proteins would not be expressed. No immunoflourescence on lamb testes cells infected with LSDV (Neethling) was visible when sera from rabbits vaccinated with RVF-LSDV was used. This indicates no antibody response to LSDV, implying that RVF-LSDV was not infectious in vaccinated animals. Experiments performed by D.Wallace, 1994, showed that LSDV was unable to replicate in cells of rabbit, hamster or monkey origin (RK-13, Don, CV-1 or Vero cells). Other authors have also demonstrated the host-specificity of capripoxviruses , which was unable to grow in cells other than goats, sheep or cattle (Gershon and Black, 1988). However, Weiss *et al.*, 1968, reported experimental induction of LSDV infection of a number of ovine and bovine cell lines including foetal rabbit kidney and skin cells, chick embryo fibroblasts, adult vervet monkey kidney cells and baby hamster kidney cells. In any event, the continual passaging of the recombinant virus in lamb testes cells, may cause loss or rearrangement of genes concerned with host-range specificity, resulting in attenuation of the virus. This strong selective pressure may make the virus less permissive in other hosts. It therefore may be necessary to test the vaccine (RVF-LSDV) in a permissive host. Interestingly,

the recombinant virus constructed by A.Cohen was further tested by vaccinating sheep, a permissive host, with the recombinant virus and challenge experiments performed. The results illustrate that partial protection was generated (A.Cohen *et al.*, 1999), indicating that the recombinant virus, which generated no antibody response in rabbits (as tested by ELISA) was able to generate an antibody response in sheep.

Although, the non-permissiveness of the host to LSDV is the most likely reason for the lack of an immune response to the recombinant virus, it may be due to other reasons as well. The lack of an immune response to the RVFV antigens may be caused by the low dosages of the recombinant virus (RVF-LSDV) given to the rabbits. Previous work in the laboratory had revealed an effective dosage of $1X10^6$ p.f.u. of recombinant virus, however, only $3.75X10^5$ p.f.u. for rabbits A, B, C and D was given, while rabbits E and F received only $1.875X10^5$ p.f.u.

CHAPTER 4: DEVELOPMENT OF A FOOT AND MOUTH DISEASE LUMPY SKIN DISEASE VIRUS VACCINE

4.1: LITERATURE REVIEW

4.1.1: Introduction

Foot-and-mouth disease is a highly contagious acute viral infection which affects ruminants and pigs (refer to figure 4.1). In livestocks it is characterised by high morbidity, low mortality and by eruption of vesicles around the mouth and hoofs (Thomson, 1994). Although the virus is transferable to humans, it is characterised by only a mild and temporary infection.

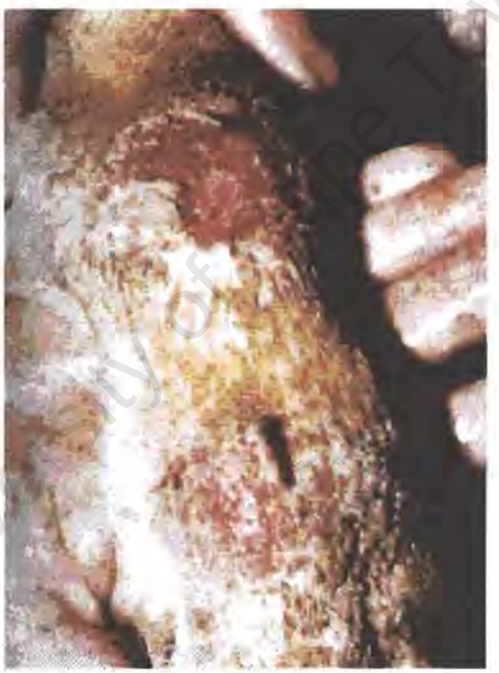


Figure 4.1: Pig infected with foot-and-mouth disease virus, showing erosive dermatitis affecting the carpal and metacarpal skin. Picture taken from Thomson, 1994

The history of Foot and Mouth disease virus only goes back to 1546 when Hieronymus Francastorius first described an outbreak in cattle (cited by Thomson, 1994). In 1898, Loeffler and Frosch discovered that the causative agent of Foot and Mouth disease was able to pass through Berkfield filters and

was therefore much smaller than other micro-organisms known to transmit diseases (Rueckert, 1996). This was the first filterable agent to be identified (Rueckert, 1996). The disease was rarely mentioned between the 16th and 20th centuries (Brooksby, 1982) and has only become noteworthy since the 20th century when significant outbreaks occurred.

The disease is present in many countries and in South Africa alone, an outbreak has been estimate to cost one billion US dollars as a result of lost production and loss of export markets (Hunter, 1998). In the 1989 outbreak in Zimbabwe, the country lost an estimated 30 million US dollars in beef exports (Vosloo, *et al.*, 1992). In September 2000, an outbreak of FMDV type O occurred on a pig farm in the Camperdown region in KwaZulu-Natal in South Africa. The resulting consequences included slaughter of thousands of animals to prevent the spread of the disease, loss of revenue worth millions of rands to South Africa and removal of South Africa, by the World Organisation for Animal Health, from a list of countries free of Foot-and-mouth disease (Mail and Guardian: http://www.mg.co.za/mg/za/archive/2000nov/07novpm-news.html). Foot-and-mouth disease is consequently regarded as the most important disease of livestock not only in southern Africa but in many other countries as well. The preventative measures and control of foot-and-mouth disease outbreaks is complicated, following various programmes of quarantine, slaughter, disinfection and/or vaccination. However, some authors have indicated that the benefit:cost ratio is always in favour of a vaccination programme (cited by Hunter, 1998).

4.1.2: Transmission and epidemiology

The distribution of Foot and Mouth Disease is almost world wide except in North and Central America, Australia, New Zealand, Great Britain and Scandinavia (Agriculture and Agri-Food Canada, 1994). These countries maintain rigid quarantine strategies and import restrictions to prevent the introduction of FMD. Incidences of FMD in northern Europe, have been quite rare over the past 10-20 years (sited by Thomson, 1994) and the majority of outbreaks herein have been ascribed to improperly inactivated vaccines or to

escapes from laboratories (Beck and Strohmaier, 1987). This, in itself, has proved to be a major problem and countries such as the USA have declined to produce the vaccine as a result of the associated safety concerns (McKenna *et al.*, 1996).

Seven serotypes have been identified and these appear to be confined to certain geographical regions. Types A, O and C are prevalent in South America, Europe, Africa and the Middle and Far East, whereas, Asia 1 is restricted to the Middle and Far East as well as the Balkans. SAT type 1, 2, and 3 are confined to sub-Saharan Africa, with SAT-2 showing the highest incidence in domestic animals in this century (cited by Thomson, 1994).

FMD is a highly contagious disease, which can spread over great distances with the movement of infected animals and products. The virus can be transmitted by direct or indirect contact (Hyslop, 1970). A significant source of infection, is virus which is shed by animals before any clinical signs are evident (Hyslop, 1970). Furthermore, in viraemic animals, virus is found in almost all physiological fluids and thus potentially in nearly all excretions and secretions (Hyslop, 1970). Cattle, sheep, goats, and other wildlife that have recovered from infection, however, do show persistent virus (Brooksby, 1982 and Hyslop, 1970). Whether these animals serve as carriers for a period of time and therefore a reservoir for further infection and spread of the disease is still debatable. It has also been well documented that domestic pigs shed vast amounts of FMDV, with a reported 1500 times more virus been shed into the atmosphere than sheep or cattle within a 24 hour period (Donaldson, 1983). These animals thus provide an important source of infection for other animals although pigs are not intensively farmed in southern Africa (Thomson, 1994).

One of the major obstacles in the eradication of FMDV is the continual maintenance of the virus in African buffalo (*Syncerus caffer*) populations (Hedger, 1976) which occur throughout the African continent. These buffalo are considered one of the main sources of infection for cattle in southern Africa. The role of other wild life in the maintenance and transmission of

FMDV to cattle is unclear although there is little evidence is suggest an important role (Thomson, 1994).

4.1.3: Classification

FMDV belongs to the family, *Picornaviridae*, which are amongst the smallest ribonucleic acid containing viruses. The family is presently divided up into nine genera of which FMDV forms part of the aphthoviruses genera. FMDV has been shown to be very labile and rapidly inactivated at pH less than seven and in alkaline conditions of greater than ten. Although the virus is inactivated at these pH's, under certain conditions, the virus is able to survive. Blackwell, 1984, found that FMDV could survive in casein from skim milk at pH4.6, in butter ripened with lactic acid cultures at pH4.6, in cheese at pH5.1 and in sweet whey at pH5.2. Furthermore, notwithstanding this sensitivity to pH, aphthoviruses are regarded as one of the most contagious viruses known. As regards heat inactivation, FMDV has been found to be relatively resistant, however, there is some variation among virus types and strains (Doel and Baccarini, 1981).

4.1.4: Virus structure and genome

FMDV is a non-enveloped virus, having a 25-35nm capsid of icosahedral symmetry consisting of 60 copies of four proteins VP1 – VP4 (cited by Samina *et al.*, 1998). The viral genome is positive sense RNA consisting of 8,450 bases. The RNA is polyadenylated at the 3' end but the function of this poly (A) is not yet known although RNA molecules with short poly (A) tracts have a lower specific infectivity (cited by Rueckert, 1996). There is a virion protein (VPg) covalently attached to the 5' end, which is reported to play an important role in RNA synthesis. Refer to figure 4.2 for a diagrammatic representation of the FMDV genome.

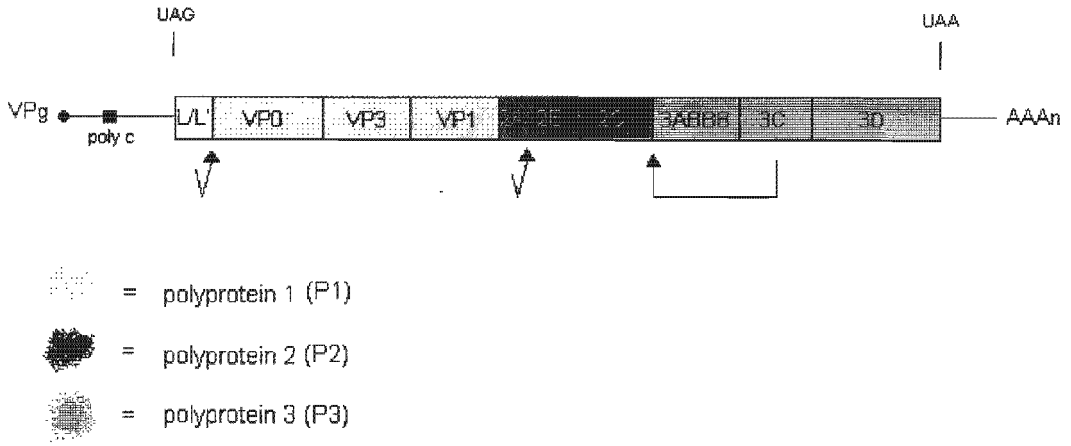


Figure 4.2: FMDV polyprotein processing. The translation products L, P1-2A, 2BC and P3 are the primary polypeptide products of polyprotein processing. Arrows indicate cleavages of the nascent polyprotein by at least three proteinases L/L', 2A and 3C.

The RNA has a single open reading frame, which codes for a long polyprotein, which undergoes autoproteolysis upon translation to yield mature viral products (Abrams *et al.*, 1995). The precursor capsid polyprotein, P1, contains the four structural proteins VP0 (VP2 + VP4), VP3 and VP1. The leader (L) protein is a papain-like proteinase (Piccone *et al.*, 1995) which autocatalytically cleaves itself from the polyprotein (Strebel and Beck, 1986). Furthermore, the L protein also cleaves p220, a subunit of the cap-binding protein complex involved in the intitation of translation at the 5' end of most eukaryotic mRNA (Kirchweger *et al.*, 1994). Cleavage of p220 results in shut-off of cap-dependent host protein synthesis (Etchison *et al.*, 1982), although viral mRNA translation is cap-independent and is therefore not affected in FMDV-infected cells (Almeida *et al.*, 1998). Findings by Brown et al., (1996) have shown that a lack of the leader protein in clone-derived FMDV have led to avirulence when compared to wild-type virus, as the virus was unable to spread to neighbouring cells. As mentioned, FMDV RNA is translated by a cap-independent process. This process is directed by a region termed the

internal ribosome entry site (IRES) found within the 5' non-coding region. Myristoylation occurs at the amino terminus of P1-2A and is essential for efficient capsid assembly (Abrams *et al.*, 1995).

4.1.5: Prevention and control

Presently, measures to control FMD are aimed at reducing the incidence of infection, of disease severity and of disease incidence and prevalence (Garland, 1999). These objectives are achieved using policies of slaughter, embargoes, disinfection and mass vaccination. However, the ultimate aim is to totally eradicate FMD to such an extent as to declare "freedom from disease without vaccination", a progression known as "the OIE pathway". This can only be attained though various stages which is not totally reliant on vaccination and revaccination policies alone. The other important factors includes national centralised planning and control; the availability of epizootiological intelligence based on adequate diagnostic capability and ongoing immunological surveillance; cleaning and disinfection of premises, vehicle and personnel; control of animal movement; recording; ongoing economic outcome and benefit analysis and finally training and retraining of all relevant persons (Garland, 1999). These complex strategies are not always attainable, especially in third world countries.

Presently, only whole virulent FMDV inactivated with an imine is used as a vaccine against FMD (Ward *et al.*, 1997). This vaccine is poorly immunogenic and has to be emulsified with aluminium hydroxide-saponin or an oil adjuvant (Doel, 1999). There are a number of other problems associated with making this vaccine, one is the impurities and large quantities of cellular proteins and media constituents which may be present in the vaccine (Doel, 1999). These components can affect the potency and efficacy of the vaccine as impurities may affect the quality of the FMDV antibodies induced by the vaccine (Doel, 1999). Furthermore proteases may also cleave certain sites in the VP1 protein, thereby reducing the ability of the vaccine to stimulate neutralising antibodies (Doel, 1999). The many strains of FMDV all have varying growth characteristics in BHK cells and due to a lack of expertise to

make good FMD vaccines, the potency requirements may not always be met (Doel, 1999). The other more important factor is the residual infectivity of the vaccine post inactivation and accidental release of live virus from vaccine factories. This problem has been linked to outbreaks of FMDV (Beck *et al.*, 1987). The vaccine only has a shelf life of one year and must also be maintained under refrigeration ($4 \pm 2\ ^{0}C$) so as to retain the antigenic potency, thus the vaccine must be kept in a cold chain from the time of manufacture to the point of inoculation (Thomson, 1994). In addition, it is essential that the virus strains incorporated into the vaccine will immunise against virus strains circulating in the region (Thomson, 1994), as infection with one strain does not necessarily confer immunity to other strains post-infection. Finally, the vaccine only provides short-lived immunity and regular boosting has to be carried out. These problems highlight the need to develop a more effective and safer vaccine against FMD.

Prevailing literature has indicated a number of FMD vaccines which has been constructed and tested over the past few years. In 1995, Piccone *et al.* developed an attenuated FMDV type A12 (A12-LLV2) which lacked the coding region for the leader (L) proteinase. This variant was shown to be less virulent in cattle and was recommended for candidacy as a live-attenuated vaccine against FMDV (Brown *et al.*, 1996). Evaluation of A12-LLV2 as a vaccine by Mason *et al.*, 1997 however, showed protection in only two out of three animals vaccinated and challenged. Further experimental evidence by Almeida *et al.*, 1998, also showed little success with the attenuated FMDV (vLLCRM8), which was also leaderless, as virulence was detected in swine post-inoculation with this candidate vaccine. Further attenuation of the vLLCRM8 virus showed avirulence but a very weak anti-FMDV immune response, which failed to protect (Almeida *et al.*, 1998).

The use of DNA vaccines as a FMDV vaccine has yielded variable results. Ward *et al.*, 1997, showed protection from challenge in only 20% of animals vaccinated with the pWRMHX plasmid. Chinsangaram *et al.*, 1998 showed that more than two inoculation of their DNA vaccine was needed for

the animals to seroconvert. Furthermore, the high cost of the delivery system used for DNA vaccines, which is the gene gun, makes this type of vaccine impractical in a third world scenario.

Synthetic peptides, which have been used to produce FMDV vaccines, have been shown to present a limited subset of viral immunogens, and although they may induce a high antibody titres, they may not protect livestock (McKenna *et al.*, 1996). The failure of these subunit vaccines could be due to the fact that they only represent a limited repertoire of sites from the virus (McKenna *et al.*, 1996). Other peptides have also displayed similar characteristics. Attempts have been made to overcome this flaw. Volpina *et al.*, 1999, enhanced the immune response to the main immunogenic 135-158 (T- and B-epitope) region of the FMDV VP1, which had previously been tested by Volpina et al 1996 and shown not to protect cattle, by linking this sequence with the 170-188 (T-epitope) peptide. Enhanced immunogenic and protective activity was seen using this new construct in the animals tested and challenged, emphasising the important role of including sites responsible for effective immunity and FMDV (Volpina *et al.*, 1999). However caution has to be taken with these peptides with respect to developing vaccines. Although Volpina *et al.*, 1999, has overcome the problem of limited epitopes by including more epitopes, the problem of proteolytic enzymes which act on these peptides may still be relevant. Briand *et al.*, 1997 and Nargi *et al.*, 1999, try to overcome this problem by using pseudopeptides or peptide mimetics that possess the –NH-CO- bond between amino acid residues rather than the –CO-NH- bond without success. However, these subunit or peptide vaccines do provide us with useful information on regions of the FMDV, which may or may not play a role in eliciting an immune response in animals tested.

Live recombinant vaccines have also been produced against FMDV. Sanz-Parra *et al.*, 1998, Sanz-Parra *et al.*, 1999, testing the B and T cell response in guinea pigs induced using recombinant vaccinia and adenoviruses expressing FMDV structural P1 and VP1, found that both CD4+ and CD8+ FMDV-specific T cells were induced. Both responses have been found to be desirable as recent evidence indicates that they both play a role in FMDV

protection (Sanz-Parra *et al.*, 1998). However, although both responses were induced, there was a lack of high levels of neutralising anti-FMDV antibodies. This factor has been attributed to the fact that the recombinant virus is preferentially expressing FMDV antigens in the cytoplasm of infected cells resulting in a lack of adequate presentation to the B cells and/or insufficient amount of soluble antigen in the extracellular medium (Sanz-Parra *et al.*, 1998). Nevertheless, these results are promising as regards the use of live recombinant vaccines as there is a possibility of inducing a long-lasting immunity against FMDV with the induction of the cellular response.

Several studies have identified regions of the FMDV, which elicit an immune response in infected animals. Moreover, VP1 has been shown to have major antigenic sites within the flexible surface-exposed G-H loop, which has been termed site A or site1 (Saiz *et al.*, 1994). However, this site has been shown to be genetically and antigenically hypervariable and thus vaccines based on this region alone may prove ineffective as single substitutions at critical residues can alter the antigenic specificity (Mateu *et al.*, 1994 and Rieder *et al.*, 1994). A number of other sites have been identified, displaying continuous and discontinuous epitopes which have not only be found on other regions of VP1 but on VP2 and VP3 too. The use of complete polyprotein (VP1, VP2 and VP3) in a vaccine to yield empty capsids is not recommend as the 3C protease, which cleaves the polyprotein, has been found to be toxic to certain cells (sited by Saiz *et al.*, 1994). Consequently certain vaccines have been developed which lack this 3C region. Saiz *et al.*, 1994 developed a recombinant baculovirus expressing VP1 and part of the VP2 regions of the polyprotein. Their results showed monoclonal neutralising antibodies recognising continuous epitopes of the major antigenic site A as well as complex discontinuous epitopes. This indicates that the capsid precursor may fold in such a manner as to maintain discontinuous epitopes involved in virus neutralisation, opening up the possibility of using the unprocessed capsid precursor as a basis for a noval vaccine.

4.2: RESULTS

4.2.1: Construction of the recombinant plasmid

4.2.1.1: Primer design

Primers were designed to amplify the VP1-4 region (P1 region) of the foot-and-mouth disease virus genome from a reference plasmid, pCa24IRES (Abrams, *et al.*, 1995), which was kindly supplied by A. King from the Institute for Animal Health, Pirbright Laboratory, Pirbright, England. The sequence CNNAUG does not conform to Kozak's optimum sequence of A/GNNAUG. These factors were taken into account when designing the primers with the addition of a stop codon at the end of the gene as well as the addition of restriction sites, *Bam*HI and *Pst*I, on either end of the gene to facilitate cloning. Refer to figure 4.3 for primers designed for the amplification of P1.

Primer 1 (forward primer)

5' AA GGA TCC ATT ATG GGG GCC GGG CAA TCC AGT C 3'

GGATCC = *Bam*HI site
ATT = Kozak sequence
ATG = Start codon

Primer 2 (reverse primer)

5' CG CTG CAG TCA CAG AAG CTG CTT TGC TGG TG 3'

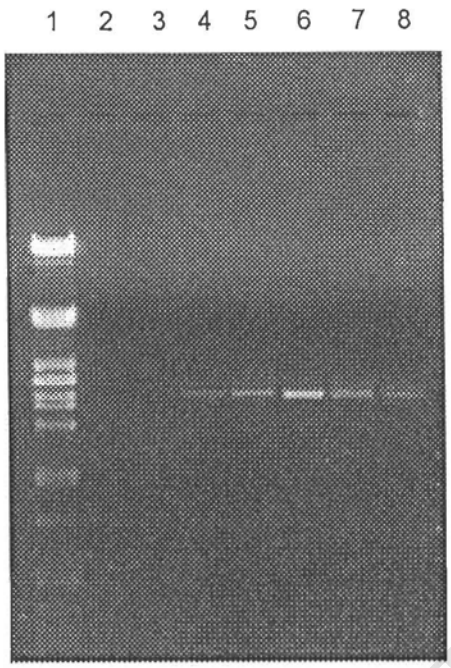
TCA = Stop codon
CTGCAG = *Pst* I site

Figure 4.3: Representation of the forward and reverse primers designed for the amplification of the FMDV P1 gene from a reference plasmid pCa24IRES.

4.2.1.2: Amplification of FMDV P1 gene

PCR was used for the amplification of the FMDV P1 gene from the reference plasmid, pCA24IRES. This plasmid contains the P1 coding region composed entirely of the strain A24 Cruzeiro sequences.

Optimisation of the PCR was necessary as non-specific bands were generated when using annealing temperatures of 57°C and 60°C. On increasing the annealing temperature to 62°C, the FMDV P1 gene was successfully amplified without the presence of non-specific bands, as shown in figure 4.4. The PCR product was found to be 2200bps in length, which corresponds to the size expected. Varying amounts of $MgCl_2$ was used for optimisation of the PCR product. Final concentrations of between 1.75-2.75mM $MgCl_2$ in the reaction mix yielded a PCR product of similar concentrations (refer to figure 4.4 lanes 5, 6 and 7). The negative control, which contained all relevant elements for PCR except the template DNA yielded no band as expected (lane 2, figure 4.4).



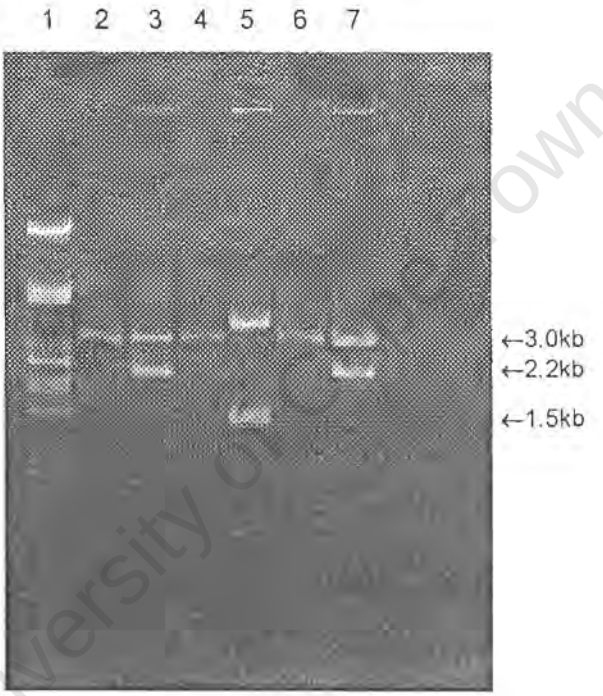
lane 1: DNA molecular weight marker - Lambda *Pst* I (refer to Appendix C for sizes)
lane 2: Negative control (no template included, 2.25mM $MgCl_2$)
lane 3: PCR sample 1 (0.75mM $MgCl_2$)
lane 4: PCR sample 2 (1.25mM $MgCl_2$)
lane 5: PCR sample 3 (1.75mM $MgCl_2$)
lane 6: PCR sample 4 (2.25mM $MgCl_2$)
lane 7: PCR sample 4 (2.75mM $MgCl_2$)
lane 8: PCR sample 4 (3.25mM $MgCl_2$)

Figure 4.4: Agarose gel electrophoresis of the PCR product generated, with the expected base pair size of 2200, following amplification of the FMDV P1 gene from pCA24IRES.

4.2.1.3: Cloning and sequencing of FMDV P1 gene

The PCR product generated following amplification of the FMDV P1 gene from pCA24IRES was successfully sub-cloned into pGem®-T Easy Vector (Appendix B) to generate the plasmid, pGemP1 (Appendix B). This was verified using restriction digestion analysis (figure 4.5). The pGem®-T Easy Vector digested with *Eco*RI yielded a fragment of 3000bp (lane 1, figure 4.5). The pGem®-T Easy Vector is received as a linearised plasmid with the two *Eco*RI sites adjacent to the terminal ends (refer to Appendix B). The clone,

pGemP1, digested with *Eco*RI generated two fragments, a 3000bp and a 2200bp fragment as expected. Two *Sac*I sites (at a nucleotide position of 509 and 2129) are found within the P1 gene whereas the pGem®-T Easy Vector has only one *Sac*I sites. Digestion of pGemP1 with *Sac*I yielded two visible fragments of 1570 and 3649bp (lane5, figure 4.5). The third fragment of approximately 140bp was not visible on the 0.8% agarose gel. Digestion of pGemP1 with *Bam*HI and *Sal*I released the P1 gene from the pGem®-T Easy Vector (lane 7, figure 4.5)



lane 1: DNA molecular weight marker - Lambda *Pst* I (refer to Appendix C for sizes)
lane 2: pGem®-T Easy Vector cut with *Eco*RI
lane 3: pGemP1 cut with *Eco*RI
lane 4: pGem®-T Easy Vector cut with *Sac*I
lane 5: pGemP1 cut with *Sac*I
lane 6: pGem®-T Easy Vector cut with *Sal*I and *Bam*HI
lane 7: pGemP1 cut with *Sal*I and *Bam*HI

Figure 4.5: Agarose gel electrophoresis of restriction enzyme analysis of the plasmids, pGem-T Easy Vector and pGemP1. Digestion of the clone, pGemP1, with *Bam*HI and *Sal*I released the 2.2kb P1 gene as expected (lane 7).

The fidelity of the PCR was confirmed by sequencing both strands of pGemP1. Primers were designed (refer to table 4.1) to sequence the P1 gene, although the M13 forward and M13 reverse primers were commercially available.

Table 4.1: Oligonucleotides designed and used for sequencing. * = commercially available primers.

Oligonucleotides	Sequence (5' to 3')	Reference
M13 forward*	GTAAAACGACGGCCAGT	Sambrook et al. (1989)
M13 reverse*	CAGGAAACAGCTATGAC	Sambrook et al. (1989)
NJ1 forward	GACTGGACAACGGACAAGGCA	N. Johnston
NJ2 forward	AGCTCCGACCTATGTTCACGTGG	N. Johnston
NJ3 forward	AATCCAGAGACGTCACCAC	N. Johnston
NJ4 reverse	GTCTGTCGTCACCAATCCTCC	N. Johnston
NJ5 reverse	TGTTGGTGAGTCTGCATGAGG	N. Johnston

The resultant nucleotide sequence of pGemP1 was compared with the reference sequence. Figure 4.6, shows the alignment of this reference sequence with that of pGemP1. Sequence variation was detected at a number of positions, indicated in blue and red in figure 4.6. The nucleotides indicated in blue were expected, arose as a consequence of the primer sequence and was intentionally inserted. These changes resulted in the generation of:

(1) A start codon (ATG) at position 3
(2) An altered Kozak sequence (CTC to ATT) at position 1
(3) A stop codon (TGA) at position 2158

(refer to Appendix E for nucleotides coding for amino acids).

The nucleotides (four changes in total) indicated in red in figure 4.6 were unexpected. To determine whether this represented mutations in the template plasmid, pCA24IRES, or a PCR artefact, pCA24IRES was sequenced. The results thereof were aligned with the nucleotide sequence of pGemP1 and the reference sequence (figure 4.6). The sequence of pCA24IRES and the FMDV

gene in pGemP1 shared 100% homology except for those nucleotides, which had been intentionally changed (indicated in blue in figure 4.6). Comparison, however, of the reference sequence with that of pCA24IRES indicates a lack of homology in certain areas (nucleotides indicated in red in figure 4.6). This was unexpected as the reference sequence was given as the *bona-fide* sequence of pCA24IRES.

```
Ref. sequence     CTCAAGGGGGCCGGGCAATCCAGTCCGGCGACCGGCTCGCAGAACCAATCTGGCAACACT   60
pCA24IRES         CTCAAGGGGGCCGGGCAATCCAGTCCGGCGACCGGCTCGCAGAACCAATCTGGCAACACT   60
pGemP1            T A GGGGGCCGGGCAATCCAGTCCGGCGACCGGCTCGCAGAACCAATCTGGCAACACT   60
                   * * ************************************************************

Ref. sequence     AGCAGCATAATTAACAACTACTACATGCAGCAATACCAGAACTCCATGGACACACAGTTG   120
pCA24IRES         AGCAGCATAATTAACAACTACTACATGCAGCAATACCAGAACTCCATGGACACACAGTTG   120
PGemP1            AGCAGCATAATTAACAACTACTACATGCAGCAATACCAGAACTCCATGGACACACAGTTG   120
                  ************************************************************

Ref. sequence     GGAGACAATGCCATCAGTGGAGGCTCCAACGAGGGCTCCACGGACACAACTTCAACACAC   180
pCA24IRES         GGAGACAATGCCATCAGTGGAGGCTCCAACGAGGGCTCCACGGACACAACTTCAACACAC   180
pGemP1            GGAGACAATGCCATCAGTGGAGGCTCCAACGAGGGCTCCACGGACACAACTTCAACACAC   180
                  ************************************************************

Ref. sequence     ACAACCAACACTCAAAACAATGACTGGTTCTCGAAGCTCGCCAGTTCAGCTTTTACCGGT   220
pCA24IRES         ACAACCAACACTCAAAACAATGACTGGTTCTCGAAGCTCGCCAGTTCAGCTTTTACCGGT   220
pGemP1            ACAACCAACACTCAAAACAATGACTGGTTCTCGAAGCTCGCCAGTTCAGCTTTTACCGGT   220
                  ************************************************************

Ref. sequence     CTGTTCGGTGCACTGCTCGCCGACAAGAAGACAGAGGAAATGACACTTCTTGAGGACCGC   260
pCA24IRES         CTGTTCGGTGCACTGCTCGCCGACAAGAAGACAGAGGAAATGACACTTCTTGAGGACCGC   260
pGemP1            CTGTTCGGTGCACTGCTCGCCGACAAGAAGACAGAGGAAATGACACTTCTTGAGGACCGC   260
                  ************************************************************

Ref. sequence     ATCCTCACCACCCGCAACGGGCACACCACCTCGACGACCCAATCGAGTGTGGGTGTCACA   300
pCA24IRES         ATCCTCACCACCCGCAACGGGCACACCACCTCGACGACCCAATCGAGTGTGGGTGTCACA   300
pGemP1            ATCCTCACCACCCGCAACGGGCACACCACCTCGACGACCCAATCGAGTGTGGGTGTCACA   300
                  ************************************************************

Ref. sequence     CACGGGTACTCCACAGAGGAGGACCACGTTGCTGGGCCCAACACATCGGGCCTGGAGACG   360
pCA24IRES         CACGGGTACTCCACAGAGGAGGACCACGTTGCTGGGCCCAACACATCGGGCCTGGAGACG   360
pGemP1            CACGGGTACTCCACAGAGGAGGACCACGTTGCTGGGCCCAACACATCGGGCCTGGAGACG   360
                  ************************************************************

Ref. sequence     CGGGTGGTGCAGGCAGAGAGATTCTACAAAAAGTACTTGTTTGACTGGACAACGGACAAG   420
pCA24IRES         CGGGTGGTGCAGGCAGAGAGATTCTACAAAAAGTACTTGTTTGACTGGACAACGGACAAG   420
pGemP1            CGGGTGGTGCAGGCAGAGAGATTCTACAAAAAGTACTTGTTTGACTGGACAACGGACAAG   420
                  ************************************************************
```

```
Ref. sequence     GCATTTGGACACCTGGAAAAGCTGGAGCTCCCGTCCGACCACCACGGTGTCTTTGGACAC   480
pCA24IRES         GCATTTGGACACCTGGAAAAGCTGGAGCTCCCGTCCGACCACCACGGTGTCTTTGGACAC   480
pGemP1            GCATTTGGACACCTGGAAAAGCTGGAGCTCCCGTCCGACCACCACGGTGTCTTTGGACAC   480
                  ************************************************************

Ref. sequence     TTGGTGGACTCGTACGCCTATATGAGAAATGGCTGGGATGTTGAGGTGTCCGCTGTTGGC   540
pCA24IRES         TTGGTGGACTCGTACGCCTATATGAGAAATGGCTGGGATGTTGAGGTGTCCGCTGTTGGC   540
pGemP1            TTGGTGGACTCGTACGCCTATATGAGAAATGGCTGGGATGTTGAGGTGTCCGCTGTTGGC   540
                  ************************************************************

Ref. sequence     AACCAATTCAACGGCGGGTGCCTCCTGGTGGCCATGGTACCTGAATGGAAGGAATTTGAC   600
pCA24IRES         AACCAGTTCAACGGCGGGTGCCTCCTGGTGGCCATGGTACCTGAATGGAAGGAATTTGAC   600
pGemP1            AACCAGTTCAACGGCGGGTGCCTCCTGGTGGCCATGGTACCTGAATGGAAGGAATTTGAC   600
                  ***** ******************************************************

Ref. sequence     ACACGGGAGAAATACCAACTCACCCTTTTCCCGCACCAGTTTATTAGCCCCAGAACTAAC   660
pCA24IRES         ACACGGGAGAAATACCAACTCACCCTTTTCCCGCACCAGTTTATTAGCCCCAGAACTAAC   660
pGemP1            ACACGGGAGAAATACCAACTCACCCTTTTCCCGCACCAGTTTATTAGCCCCAGAACTAAC   660
                  ************************************************************

Ref. sequence     ATGACTGCCCACATCACGGTCCCCTACCTTGGTGTGAACAGGTATGATCAGTACAAGAAG   720
pCA24IRES         ATGACTGCCCACATCACGGTCCCCTACCTTGGTGTGAACAGGTATGATCAGTACAAGAAG   720
pGemP1            ATGACTGCCCACATCACGGTCCCCTACCTTGGTGTGAACAGGTATGATCAGTACAAGAAG   720
                  ************************************************************

Ref. sequence     CATAAGCCCTGGACATTGGTTGTCATGGTCGTGTCGCCACTTACGGTCAACAACACTAGT   780
pCA24IRES         CATAAGCCCTGGACATTGGTTGTCATGGTCGTGTCGCCACTTACGGTCAACAACACTAGT   780
pGemP1            CATAAGCCCTGGACATTGGTTGTCATGGTCGTGTCGCCACTTACGGTCAACAACACTAGT   780
                  ************************************************************

Ref. sequence     GCGGCACAAATCAAGGTCTACGCCAACATAGCTCCGACCTATGTTCACGTGGCCGGTGAA   840
pCA24IRES         GCGGCACAAATCAAGGTCTACGCCAACATAGCTCCGACCTATGTTCACGTGGCCGGTGAA   840
pGemP1            GCGGCACAAATCAAGGTCTACGCCAACATAGCTCCGACCTATGTTCACGTGGCCGGTGAA   840
                  ************************************************************

Ref. sequence     CTTCCCTCGAAAGAGGGGATTTTCCCGGTTGCATGTGCGGACGGGTACGGAGGATTGGTG   900
pCA24IRES         CTTCCCTCGAAAGAGGGGATTTTCCCGGTTGCATGTGCGGACGGGTACGGAGGATTGGTG   900
pGemP1            CTTCCCTCGAAAGAGGGGATTTTCCCGGTTGCATGTGCGGACGGGTACGGAGGATTGGTG   900
                  ************************************************************

Ref. sequence     ACGACAGACCCGAAGACAGCTGACCCTGCTTATGGCAAGGTGTACAACCCGCCTAGGACT   960
pCA24IRES         ACGACAGACCCGAAGACAGCTGACCCTGCTTATGGCAAGGTGTACAACCCGCCTAGGACT   960
pGemP1            ACGACAGACCCGAAGACAGCTGACCCTGCTTATGGCAAGGTGTACAACCCGCCTAGGACT   960
                  ************************************************************

Ref. sequence     AACTACCCTGGGCGCTTCACCAACCTGTTGGACGTGGCCGAAGCGTGTCCCACTTTCCTC  1020
pCA24IRES         AACTACCCTGGGCGCTTCACCAACCTGTTGGACGTGGCCGAAGCGTGTCCCACTTTCCTC  1020
pGemP1            AACTACCCTGGGCGCTTCACCAACCTGTTGGACGTGGCCGAAGCGTGTCCCACTTTCCTC  1020
                  ************************************************************
```

```
Ref. sequence     TGCTTTGACGACGGGAAACCGTACGTCACCACGCGGACGGATGACACCCGACTTTTGGCC   1080
pCA24IRES         TGCTTTGACGACGGGAAACCGTACGTCACCACGCGGACGGATGACACCCGACTTTTGGCC   1080
pGemP1            TGCTTTGACGACGGGAAACCGTACGTCACCACGCGGACGGATGACACCCGACTTTTGGCC   1080
                  ************************************************************

Ref. sequence     AAGTTTGACCTTTCCCTTGCCGCAAAACATATGTCCAACACATACCTGTCAGGGATTGCT   1140
pCA24IRES         AAGTTTGACCTTTCCCTTGCCGCAAAACATATGTCCAACACATACCTGTCAGGGATTGCT   1140
pGemP1            AAGTTTGACCTTTCCCTTGCCGCAAAACATATGTCCAACACATACCTGTCAGGGATTGCT   1140
                  ************************************************************

Ref. sequence     CAGTACTACACACAGTACTCTGGCACCATCAATTTGCATTTCATGTTCACAGGTTCCACT   1200
pCA24IRES         CAGTACTACACACAGTACTCTGGCACCATCAATTTGCATTTCATGTTCACAGGTTCCACT   1200
pGemP1            CAGTACTACACACAGTACTCTGGCACCATCAATTTGCATTTCATGTTCACAGGTTCCACT   1200
                  ************************************************************

Ref. sequence     GATTCAAAGGCCCGATACATGGTGGCCTACATCCCACCTGGGGTGGAGACACCACCGGAC   1260
pCA24IRES         GATTCAAAGGCCCGATACATGGTGGCCTACATCCCACCTGGGGTGGAGACACCACCGGAC   1260
pGemP1            GATTCAAAGGCCCGATACATGGTGGCCTACATCCCACCTGGGGTGGAGACACCACCGGAC   1260
                  ************************************************************

Ref. sequence     ACACCTGAAAGGGCTGCCCACTGCATTCACGCTGAATGGGACACTGGACTAAACTCCAAA   1320
pCA24IRES         ACACCTGAAAGGGCTGCCCACTGCATTCACGCTGAATGGGACACTGGACTAAACTCCAAA   1320
pGemP1            ACACCTGAAAGGGCTGCCCACTGCATTCACGCTGAATGGGACACTGGACTAAACTCCAAA   1320
                  ************************************************************

Ref. sequence     TTCACTTTCTCAATCCCGTACGTATCCGCCGCGGATTACGCTTACACAGCGTCTGACACG   1380
pCA24IRES         TTCACTTTCTCAATCCCGTACGTATCCGCCGCGGATTACGCTTACACAGCGTCTGACACG   1380
pGemP1            TTCACTTTCTCAATCCCGTACGTATCCGCCGCGGATTACGCTTACACAGCGTCTGACACG   1380
                  ************************************************************

Ref. sequence     GCAGAAACAATCAACGTACAGGGATGGGTCTGCATCTACCAAATTACACACGGGAAGGCT   1440
pCA24IRES         GCAGAAACAATCAACGTACAGGGATGGGTCTGCATCTACCAAATTACACACGGGAAGGCT   1440
pGemP1            GCAGAAACAATCAACGTACAGGGATGGGTCTGCATCTACCAAATTACACACGGGAAGGCT   1440
                  ************************************************************

Ref. sequence     GAAAATGACACCTTGGTCGTGTCGGTTAGCGCCGGCAAAGACTTTGAGTTGCGCCTCCCG   1500
pCA24IRES         GAAAATGACACCTTGGTCGTGTCGGTTAGCGCCGGCAAAGACTTTGAGTTGCGCCTCCCG   1500
pGemP1            GAAAATGACACCTTGGTCGTGTCGGTTAGCGCCGGCAAAGACTTTGAGTTGCGCCTCCCG   1500
                  ************************************************************

Ref. sequence     ATTGACCCCCGCCAGCAGACCACGACTACCGGGGAATCAGCAGACCCGGTCACCACCACC   1560
pCA24IRES         ATTGACCCCCGCCAGCAGACCACCGCTACCGGGGAATCAGCAGACCCGGTCACCACCACC   1560
pGemP1            ATTGACCCCCGCCAGCAGACCACCGCTACCGGGGAATCAGCAGACCCGGTCACCACCACC   1560
                  ************************  ***********************************

Ref. sequence     GTGGAGAACTACGGCGGTGAGACACAAATCCAGAGACGTCACCACACGGACATTGGTTTC   1620
pCA24IRES         GTGGAGAACTACGGCGGTGAGACACAAATCCAGAGACGTCACCACACGGACATTGGTTTC   1620
pGemP1            GTGGAGAACTACGGCGGTGAGACACAAATCCAGAGACGTCACCACACGGACATTGGTTTC   1620
                  ************************************************************
```

```
Ref. sequence     ATCATGGACAGATTTGTGAAGATCCAAAGCTTGAGCCCAACACATGTCATTGACCTCATG   1680
pCA24IRES         ATCATGGACAGATTTGTGAAGATCCAAAGCTTGAGCCCAACACATGTCATTGACCTCATG   1680
pGemP1            ATCATGGACAGATTTGTGAAGATCCAAAGCTTGAGCCCAACACATGTCATTGACCTCATG   1680
                  ************************************************************

Ref. sequence     CAGACTCACCAACACGGTCTGGTGGGTGCCTTGCTGCGTGCAGCCACGTACTACTTTTCT   1740
pCA24IRES         CAGACTCACCAACACGGTCTGGTGGGTGCCTTGCTGCGTGCAGCCACGTACTACTTTTCT   1740
pGemP1            CAGACTCACCAACACGGTCTGGTGGGTGCCTTGCTGCGTGCAGCCACGTACTACTTTTCT   1740
                  ************************************************************

Ref. sequence     GACCTGGAAATTGTTGTACGGCACGAAGGCAATCTGACCTGGGTGCCCAACGGCGCCCCT   1800
pCA24IRES         GACCTGGAAATTGTTGTACGGCACGAAGGCAATCTGACCTGGGTGCCCAACGGCGCCCCT   1800
pGemP1            GACCTGGAAATTGTTGTACGGCACGAAGGCAATCTGACCTGGGTGCCCAACGGCGCCCCT   1800
                  ************************************************************

Ref. sequence     GAATCAGCCCTGTTGAACACCAGCAACCCCACTGCCTACAACAAGGCACCATTCACGAGA   1860
pCA24IRES         GAATCAGCCCTGTTGAACACCAGCAACCCCACTGCCTACAACAAGGCACCATTCACGAGA   1860
pGemP1            GAATCAGCCCTGTTGAACACCAGCAACCCCACTGCCTACAACAAGGCACCATTCACGAGA   1860
                  ************************************************************

Ref. sequence     CTCGCTCTCCCCTACACTGCGCCGCACCGTGTGCTGGCAACAGTGTACAACGGGACGAGT   1920
pCA24IRES         CTCGCTCTCCCCTACACTGCGCCGCACCGTGTGCTGGCAACAGTGTACAACGGGACGAGT   1920
pGemP1            CTCGCTCTCCCCTACACTGCGCCGCACCGTGTGCTGGCAACAGTGTACAACGGGACGAGT   1920
                  ************************************************************

Ref. sequence     AAGTATGCTGTGGGTGGTTCAGGCAGAAGAGGCGACATGGGGTCTCTCGCGGCGCGAGTC   1980
pCA24IRES         AAGTATGCTGTGGGTGGTTCAGGCAGAAGAGGCGACATGGGGTCTCTCGCGGCGCGAGTC   1980
pGemP1            AAGTATGCTGTGGGTGGTTCAGGCAGAAGAGGCGACATGGGGTCTCTCGCGGCGCGAGTC   1980
                  ************************************************************

Ref. sequence     GTGAAACAGCTTCCTGCTTCATTTAACTACGGTGCAATCAAGGCCGACGCCATCCACGAA   2040
pCA24IRES         GTGAAACAGCTTCCTGCTTCATTTAACTACGGTGCAATCAAGGCCGACGCCATCCACGAA   2040
pGemP1            GTGAAACAGCTTCCTGCTTCATTTAACTACGGTGCAATCAAGGCCGACGCCATCCACGAA   2040
                  ************************************************************

Ref. sequence     CTTCTCGTGCGCATGAAACGGGCCGAGCTCTACTGCCCCAGACCGCTGCTGGCAATAGAG   2100
pCA24IRES         CTTCTCGTGCGCATGAAACGGGCCGAGCTCTACTGCCCCAGACCGCTGTTGGCAATAGAG   2100
pGemP1            CTTCTCGTGCGCATGAAACGGGCCGAGCTCTACTGCCCCAGACCGCTGTTGGCAATAGAG   2100
                  ********************************************** ***********

Ref. sequence     GTGTCTTCGCAAGACAGGCACAAGCAAAAGATCATTGCACCAGCAAAGCAGCTTCTGAAT   2160
pCA24IRES         GTGTCTTCGCAAGACAGGCACAAGCAAAAGATCATTGCACCAGCAAAGCAGCTTCTGAAT   2160
pGemP1            GTGTCTTCGCAAGACAGGCACAAGCAAAAGATCATTGCACCAGCAAAGCAGCTTCTG[illegible]   2160
                  ************************************************************
```

Figure 4.6: Sequence alignment of the reference sequence, pCA24IRES and pGemP1. Nucleotides indicated in blue represent expected base changes, intentionally inserted. Nucleotides indicated in red were unexpected.

The sequence of pCA24IRES, pGemP1 and the reference sequence were further analyzed using the computer program, DNAMAN. Figure 4.7, shows the alignment of the amino acid sequence of pCA24IRES, pGemP1 and the reference sequence. Those amino acids indicated in blue are expected (refer to Appendix D and E for codon usage) and were intentionally inserted as mentioned before. The four unexpected base changes gave rise to only one amino acid change at position 548 (refer to table 4.2). The amino acid change was non-conserved.

```
Ref. sequence   LKGAGQSSPATGSQNQSGNTSSIINNYYMQQYQNSMDTQLGDNAISGGSNEGSTDTTSTH   60
pCA24IRES       LKGAGQSSPATGSQNQSGNTSSIINNYYMQQYQNSMDTQLGDNAISGGSNEGSTDTTSTH   60
pGemP1          I GAGQSSPATGSQNQSGNTSSIINNYYMQQYQNSMDTQLGDNAISGGSNEGSTDTTSTH   60
                  ************************************************************

Ref. sequence   TTNTQNNDWFSKLASSAFTGLFGALLADKKTEEMTLLEDRILTTRNGHTTSTTQSSVGVT   120
pCA24IRES       TTNTQNNDWFSKLASSAFTGLFGALLADKKTEEMTLLEDRILTTRNGHTTSTTQSSVGVT   120
pGemP1          TTNTQNNDWFSKLASSAFTGLFGALLADKKTEEMTLLEDRILTTRNGHTTSTTQSSVGVT   120
                ************************************************************

Ref. sequence   HGYSTEEDHVAGPNTSGLETRVVQAERFYKKYLFDWTTDKAFGHLEKLELPSDHHGVFGH   180
pCA24IRES       HGYSTEEDHVAGPNTSGLETRVVQAERFYKKYLFDWTTDKAFGHLEKLELPSDHHGVFGH   180
pGemP1          HGYSTEEDHVAGPNTSGLETRVVQAERFYKKYLFDWTTDKAFGHLEKLELPSDHHGVFGH   180
                ************************************************************

Ref. sequence   LVDSYAYMRNGWDVEVSAVGNQFNGGCLLVAMVPEWKEFDTREKYQLTLFPHQFISPRTN   240
pCA24IRES       LVDSYAYMRNGWDVEVSAVGNQFNGGCLLVAMVPEWKEFDTREKYQLTLFPHQFISPRTN   240
pGemP1          LVDSYAYMRNGWDVEVSAVGNQFNGGCLLVAMVPEWKEFDTREKYQLTLFPHQFISPRTN   240
                ************************************************************

Ref. sequence   MTAHITVPYLGVNRYDQYKKHKPWTLVVMVVSPLTVNNTSAAQIKVYANIAPTYVHVAGE   300
pCA24IRES       MTAHITVPYLGVNRYDQYKKHKPWTLVVMVVSPLTVNNTSAAQIKVYANIAPTYVHVAGE   300
pGemP1          MTAHITVPYLGVNRYDQYKKHKPWTLVVMVVSPLTVNNTSAAQIKVYANIAPTYVHVAGE   300
                ************************************************************

Ref. sequence   LPSKEGIFPVACADGYGGLVTTDPKTADPAYGKVYNPPRTNYPGRFTNLLDVAEACPTFL   360
pCA24IRES       LPSKEGIFPVACADGYGGLVTTDPKTADPAYGKVYNPPRTNYPGRFTNLLDVAEACPTFL   360
pGemP1          LPSKEGIFPVACADGYGGLVTTDPKTADPAYGKVYNPPRTNYPGRFTNLLDVAEACPTFL   360
                ************************************************************

Ref. sequence   CFDDGKPYVTTRTDDTRLLAKFDLSLAAKHMSNTYLSGIAQYYTQYSGTINLHFMFTGST   420
pCA24IRES       CFDDGKPYVTTRTDDTRLLAKFDLSLAAKHMSNTYLSGIAQYYTQYSGTINLHFMFTGST   420
pGemP1          CFDDGKPYVTTRTDDTRLLAKFDLSLAAKHMSNTYLSGIAQYYTQYSGTINLHFMFTGST   420
                ************************************************************
```

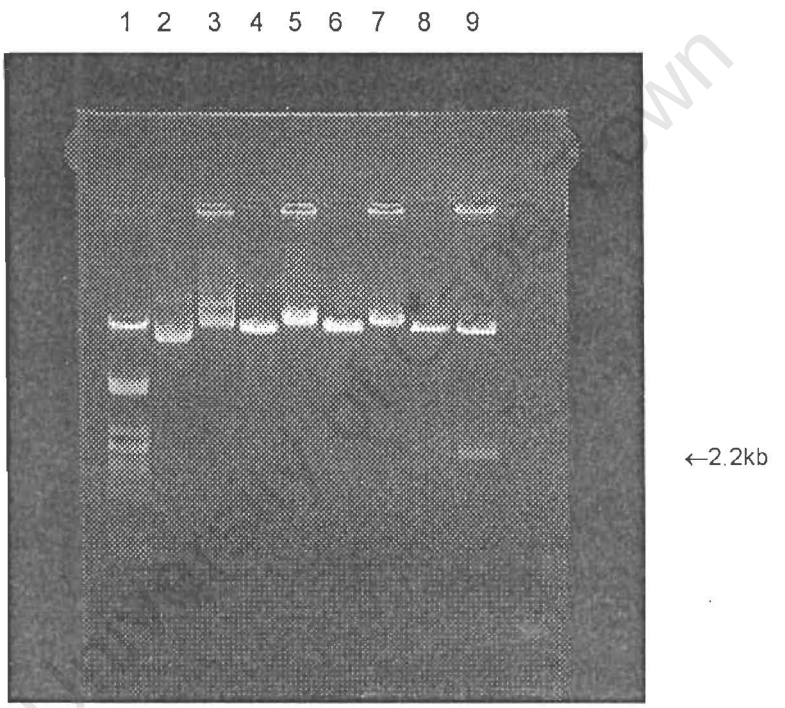
```
Ref. sequence   DSKARYMVAYIPPGVETPPDTPERAAHCIHAEWDTGLNSKFTFSIPYVSAADYAYTASDT   480
pCA24IRES       DSKARYMVAYIPPGVETPPDTPERAAHCIHAEWDTGLNSKFTFSIPYVSAADYAYTASDT   480
pGemP1          DSKARYMVAYIPPGVETPPDTPERAAHCIHAEWDTGLNSKFTFSIPYVSAADYAYTASDT   480
                ************************************************************

Ref. sequence   AETINVQGWVCIYQITHGKAENDTLVVSVSAGKDFELRLPIDPRQQTTTTGESADPVTTT   540
pCA24IRES       AETINVQGWVCIYQITHGKAENDTLVVSVSAGKDFELRLPIDPRQQTTATGESADPVTTT   540
pGemP1          AETINVQGWVCIYQITHGKAENDTLVVSVSAGKDFELRLPIDPRQQTTATGESADPVTTT   540
                ************************************************.***********

Ref. sequence   VENYGGETQIQRRHHTDIGFIMDRFVKIQSLSPTHVIDLMQTHQHGLVGALLRAATYYFS   600
pCA24IRES       VENYGGETQIQRRHHTDIGFIMDRFVKIQSLSPTHVIDLMQTHQHGLVGALLRAATYYFS   600
pGemP1          VENYGGETQIQRRHHTDIGFIMDRFVKIQSLSPTHVIDLMQTHQHGLVGALLRAATYYFS   600
                ************************************************************

Ref. sequence   DLEIVVRHEGNLTWVPNGAPESALLNTSNPTAYNKAPFTRLALPYTAPHRVLATVYNGTS   660
pCA24IRES       DLEIVVRHEGNLTWVPNGAPESALLNTSNPTAYNKAPFTRLALPYTAPHRVLATVYNGTS   660
pGemP1          DLEIVVRHEGNLTWVPNGAPESALLNTSNPTAYNKAPFTRLALPYTAPHRVLATVYNGTS   660
                ************************************************************

Ref. sequence   KYAVGGSGRRGDMGSLAARVVKQLPASFNYGAIKADAIHELLVRMKRAELYCPRPLLAIE   720
pCA24IRES       KYAVGGSGRRGDMGSLAARVVKQLPASFNYGAIKADAIHELLVRMKRAELYCPRPLLAIE   720
pGemP1          KYAVGGSGRRGDMGSLAARVVKQLPASFNYGAIKADAIHELLVRMKRAELYCPRPLLAIE   720
                ************************************************************

Ref. sequence   VSSQDRHKQKIIAPAKQLLN                                          740
pCA24IRES       VSSQDRHKQKIIAPAKQLLN                                          740
pGemP1          VSSQDRHKQKIIAPAKQLL                                           740
                ********************
```

Figure 4.7: Alignment of the amino acid sequence of the reference sequence, pCA24IRES and pGemP1. Amino acids indicated in blue were intentionally inserted and those indicated in red were unexpected.

Table 4.2: Summary of the amino acid changes observed in pGemP1 and pCA24IRES when compared to the reference sequence obtained from Pirbright, England.

Position of nucleotide	Codon change	Position of amino acid	Amino acid change	Nature of change
603	CAA → CAG	201	Glu → Glu	silent
1581	ACG → ACC	527	Thr → Thr	silent
1582	ACT → GCT	528	Thr → Ala	non-conserved
2146	CTG → TTG	716	Leu→Leu	silent

The FMDV P1 gene was cloned from pGemP1 into the shuttle vector, pAFMCRR (refer to Appendix B), to generate the plasmid, pAFMCRRP1. Small scale DNA preparations were digested with the restriction endonucleases *Bam*HI and *Sal*I to determine whether the insert was present. Figure 4.8 represents the results thereof. Linearised pAFMCRR is present in lane 4 and 6, with a clear increase in plasmid size in lane 5 and 7 (represents linearised pAFMCRRP1). Restriction endonuclease digestion of the recombinant plasmid, pAFMCRRP1, with *Sal*I and *Bam*HI, released the 2,2kb FMDV P1 gene (lane 9).



lane 1: DNA molecular weight marker - Lambda *Pst* I (refer to Appendix C for sizes)
lane 2: pAFMCRR uncut
lane 3: pAFMCRRP1 uncut
lane 4: pAFMCRR cut with *Bam* HI
lane 5: pAFMCRRP1 cut with *Bam* HI
lane 6: pAFMCRR cut with *Sal* I
lane 7: pAFMCRRP1 cut with *Sal* I
lane 8: pAFMCRR cut with *Bam* HI and *Sal* I
lane 9: pAFMCRRP1 cut with *Bam* HI and *Sal* I

Figure 4.8: Agarose gel electrophoresis of restriction enzyme analysis of pAFMCRR and PAFMCRRP1. Lane 9 clearly indicates the successful cloning of FMDV P1 gene into pAFMCRR as the insert was cut out generating an expected band size of 2200bp. This is the size of the FMDV P1 gene.

4.2.2: Construction of recombinant virus (FMD-LSDV)

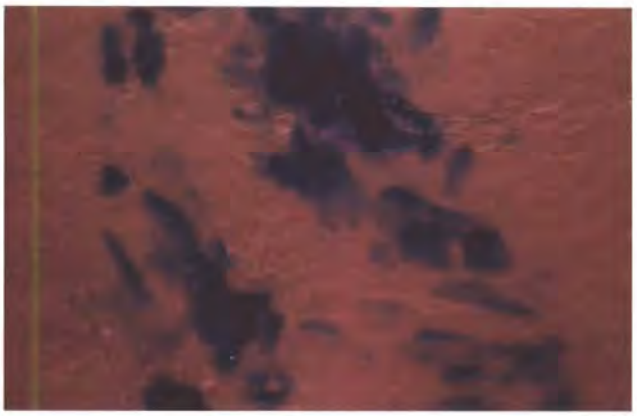
4.2.2.1: Transfection of recombinant plasmid

The recombinant plasmid, pAFMCRRP1, was transfected into Neethling LSDV infected lamb testes cells (controls were included consisting of wild type LSDV Neethling infected lamb testes cells and uninfected lamb testes cells). The efficiency of transfection was measured by X-gal staining 3 days post-transfection. The best transfection was obtained when the recombinant plasmid used was isolated by Qaigen kit as apposed to the Nucleobond kit. Furthermore, plasmid concentrations of 10μg, used per transfection, yielded higher efficiencies of transfection than when less plasmid DNA was used per reaction. These observations were also made when constructing RVF-LSDV.

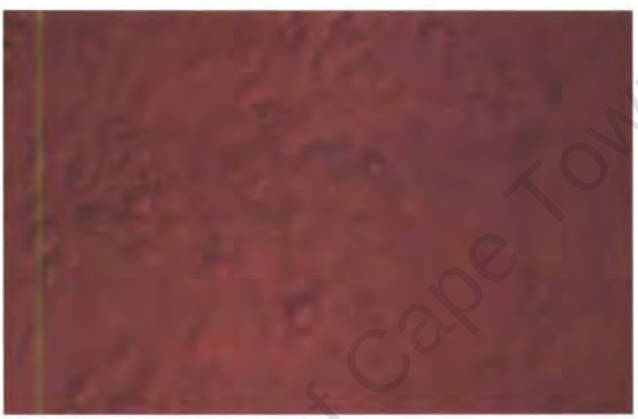
4.2.2.2: Isolation and purification of recombinant virus

Experiments carried out involving X-gal staining *in situ*, following passaging of the recombinant virus in selection medium, confirmed the presence of the *Lac*Z gene and the recombinant virus (figure 4.9). Recombinant virus was further selected for picking blue foci (after X-gal overlays, refer to figure 4.10) four times, with three rounds of passaging in selection medium between each plaque purification step.

(a)



(b)



(c)

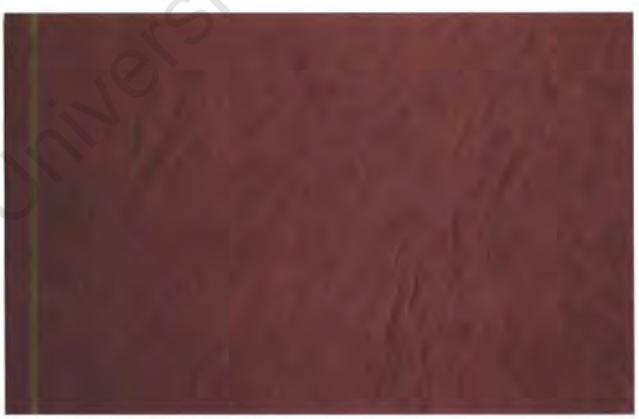


Figure 4.9: X-gal staining

(a) lamb testes cells infected with FMD-LSDV. Blue foci indicating the presence and expression of the *LacZ* gene found in the recombinant virus only.

(b) lamb testes cells infected with wild type LSDV (Neethling stain).

(c) uninfected lamb testes cells.

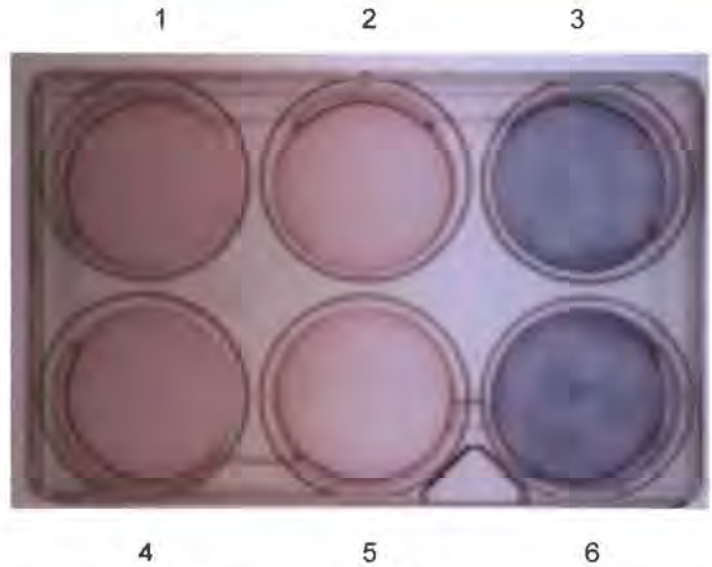


Figure 4.10: X-gal overlays. Well 1 and 4 are uninfected lamb testes cells. Well 2 and 5 are wild type LSDV (Neethling) infected lamb testes cells. Well 3 and 6 are FMD-LSDV infected lamb testes cells

4.2.3: Characterisation of FMD-LSDV

4.2.3.1: Southern blot

To demonstrate that recombination had occurred between the shuttle vector, pAFMCRRP1, and the LSDV genome, DNA from lamb testes cells infected with the recombinant FMD-LSDV, was digested with *Hin*dIII and analysed by Southern hybridisation (figure 4.12(a) and figure 4.12(b)). A double cross-over event would result in the incorporation of only one *Hin*dIII site, refer to figure 4.11. This would yield to two bands when recombinant viral DNA, digested with *Hin*dIII, is probed. Moreover, this specific *Hin*dIII site is contained in the FMDV P1 gene, further confirming the presence of this gene in the viral genome. Conversely, a single cross-over event would result in the incorporation of three additional *Hin*dIII sites (refer to figure 4.11). This in turn would give rise to four bands when recombinant viral DNA, digested with *Hin*dIII, is probed. It is therefore clear that a double cross-over event has occurred as two bands are visible (figure 4.12(b), lane 6). The 6.4kb and 4.6kb bands correlates with the expected sizes.

A band of corresponding to 4.6kb was observed in lane 4 in figure 4.12(b), representing wild type LSDV DNA. This band identifies the ribonucleotide reductase gene within the wild type LSDV genome. Unfortunately the *Hin*dIII site on either end of the ribonucleotide reductase gene yielded a fragment of the same size as genomic DNA from FMD-LSDV infected cells (cut with *Hin*dIII) that seen in lane 6, figure 4.12(b). Thus we cannot say with any certainty that some breakthrough wild type LSDV had not occurred during selection and isolation of recombinant virus. However, as similar processedures have been followed when constructing the RVF-LSDV recombinant vaccine, which, through southern hybridisation blots, indicated the presence of wild type LSDV in the final preparation, most likely there would also be some wild type LSDV present in the final preparation of the FMD-LSDV recombinant vaccine. One may also speculate that the 4.6kb band intensity as seen in figure 4.12(b) lane 6 is the same as the 6.4kb band intensity, or at least very close in intensity. If wild type virus was present in large amounts in the recombinant preparation, then most likely the 4.6kb fragment would yield a much darker band than the 6.4kb fragment. One may speculate that there probably is wild type present but not in vast amounts. No signal could be detected in lane 2, figure 4.12(b) containing DNA harvested from lamb testes cells, as expected.

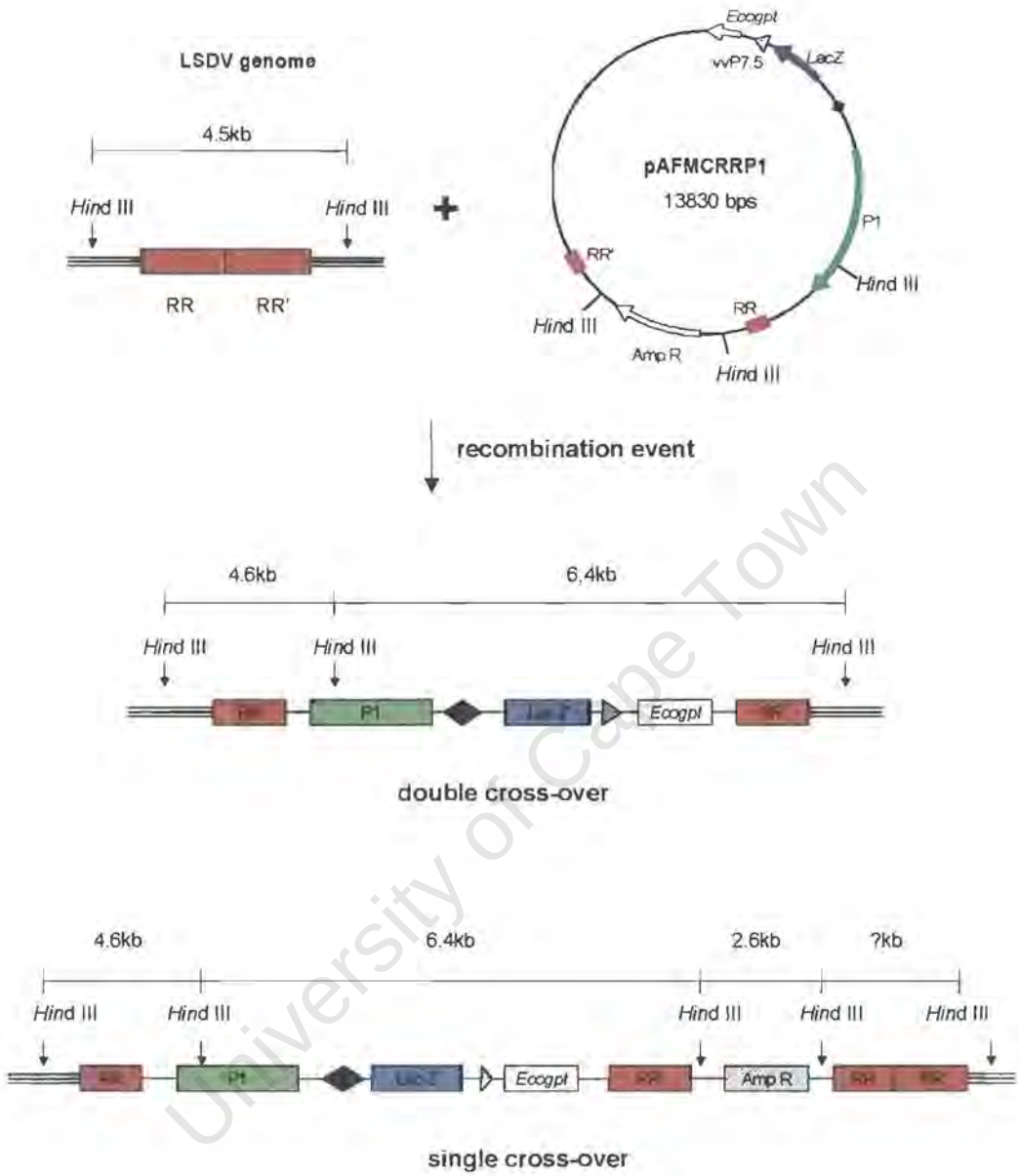


Figure 4.11: Diagrammatic representation of a double and single cross-over event between pAFMCRRP1 and LSDV genome. RR and RR' represents ribonucleotide reductase gene.

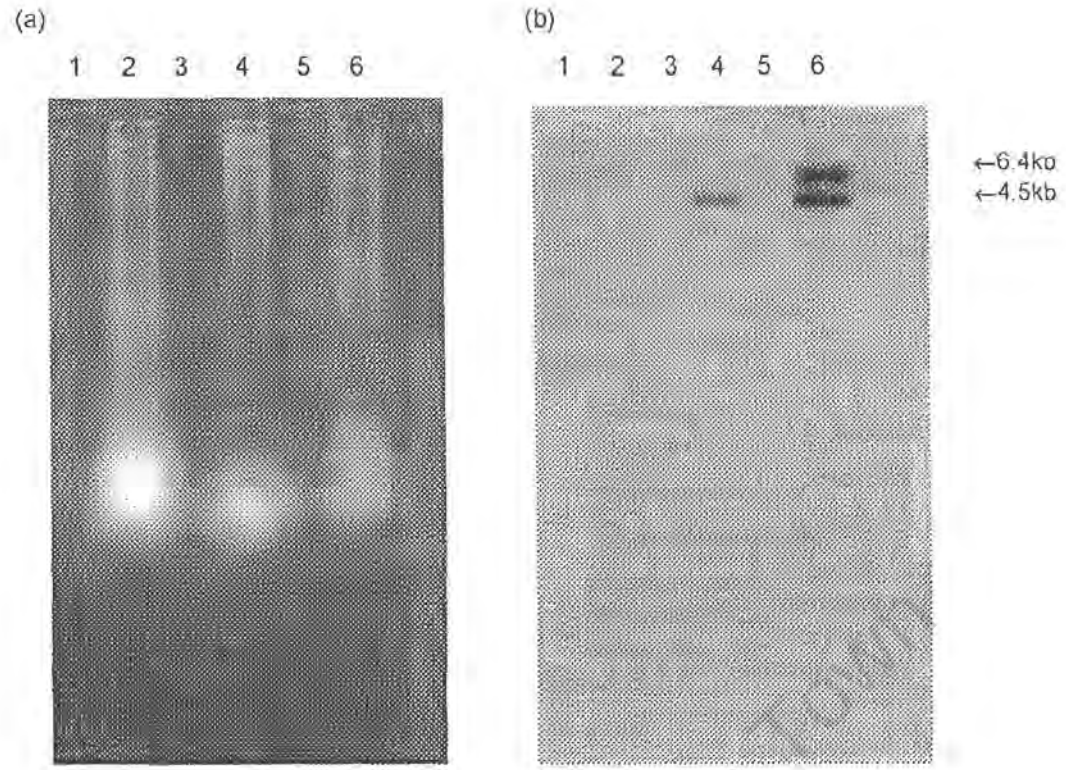


Figure 4.12: Identification of FMD-LSDV.

(a) Agarose gel electrophoresis of genomic DNA digested with *Hin*dIII. Lane 2, DNA from uninfected lamb testes cells. Lane 4, DNA extracted from wild type LSDV (Neethling) infected cells. Lane 6, DNA extracted from FMD-LSDV infected cells.

(b) Autoradiograph of gel in figure 4.13(a) probed with pAFMCRRP1 digested with *Hin*dIII. Lane 2, DNA from uninfected lamb testes cells. Lane 4, DNA extracted from wild type LSDV (Neethling) infected cells. Lane 6, DNA extracted from FMD-LSDV infected cells.

4.2.3.2: Protein analysis

The FMD-LSDV recombinant used to infect lamb testes cells after which the protein was extracted was subjected to SDS-PAGE, Coomassie staining and Western blots. Coomassie staining confirmed the presence of the proteins, although the P1 protein was not visible (results not shown) as no variation could be seen between proteins from wild type LSDV (Neethling) and recombinant virus infected cells. Ponceau S staining of the membrane confirmed the successful transfer from the gel to the membrane. Probing for

confirmed the successful transfer from the gel to the membrane. Probing for the P1 protein was carried out using a primary polyclonal antibody to type A5. The activity of the conjugate was verified. Various amounts of proteins were loaded onto different gels and various amounts of primary and secondary antibody were used for probing in an attempt to optimise the western blots. However, western blot analysis failed to detect the P1 protein (results not shown).

Further analysis of protein expression was carried out *in situ* using immunoflorescence experiments. No fluorescence could be detected in cells infected with FMD-LSDV using the monoclonal antibody to serotype A (results not shown).

4.3: DISCUSSION

A recombinant plasmid, pAFMCRRP1, containing the FMDV P1 gene was successfully constructed. However, one amino acid variation from a threonine to an alanine was detected at position 528 in the P1 gene when compared to the reference sequence received from Pirbright. This reference sequence was given as the *bona-fide* sequence of the plasmid, pCA24IRES, on which the FMDV P1 gene in pAFMCRRP1 was based. However, this proved incorrect as the same amino acid variation found in the recombinant plasmid, pAFMCRRP1 exists in pCA24IRES. This therefore indicates that the reference sequence given was not the same as pCA24IRES and that the fidelity of the PCR, used for the amplification of the P1 gene from pCA24IRES, was precise.

The appearance of blue foci on X-gal staining following transfection of the transfer plasmid, pAFMCRRP1, into LSDV infected lamb testes cells, indicated the presence and expression of the *lacZ* gene in the cells. Selection of the recombinant virus was successfully carried out using selection medium and X-gal overlays. This was evident by the continual presence of CPE and blue foci on infection with recombinant virus.

The Southern blot hybridisation analysis showed that a double cross over event had occurred between the flanking sequences in the transfer vector and the ribonucleotide reductase gene in the LSDV genome. The fragments observed in the Southern blot also confirmed the presence of the FMDV P1 gene in an integrated form. The restriction endonuclease digest could not reveal the presence of wild type LSDV in the final preparation of the recombinant virus. However, it may be possible to determine the presence of wild type LSDV in the final preparation by digesting the genomic DNA isolated from FMD-LSDV infected cells with other restriction enzymes such as *Sal*I. However, there are a number of problems associated with this strategy. Digestion with *Sal*I will yield two bands in both a double and a single cross-over event. Furthermore, the entire LSDV genome has not been mapped and thus the exact *Sal*I sites within the LSDV genome are not known. Therefore it

is extremely difficult to determine the sizes of these bands when the DNA is digested with *Sal*I or some of the other enzymes. One other method of determining if wild type LSDV is still present in the final preparation of FMD-LSDV is by amplifying the ribonucleotide reductase gene by designing primers at each end of the gene. This would generate a band of approximately 4.3kb (the size of the full length ribonucleotide reductase gene) if wild type LSDV is present.

The detection of the FMDV P1 protein was unsuccessful. Immunoflourescence staining techniques could not detect the P1 protein in FMD-LSDV infected lamb testes cells using a monoclonal antibody, which recognised a conformational epitope. A polyclonal antibody, which recognises linear epitopes, was used in the Western blot, which also proved unsuccessful. The inability to detect the P1 protein could be the result of a number of factors:

1: The possibility exists that the antibodies used are unable to recognise the epitopes on the P1 protein (from the FMDV A24 strain) produced by the recombinant virus. This is likely as the polyclonal antibody used is to the A5 strain of FMDV and the monoclonal antibody is to the A serotype. It is well known that the genome of FMDV, especially the type A virus, evolves rapidly (Brooksby, 1982). This gives rise to a large number of viral subtypes that have a limited ability to elicit cross-neutralising antibodies (Weddell *et al.*, 1985). Furthermore, a high degree of variation is observed between codons 130 and 171, which is an important immunogenic site on the viral surface (Bachrach et al., 1982). There is further evidence to support the argument of limited cross-reactivity within subtypes. Barnett *et al.*, 1996, developed a monoclonal antibody to the A40 peptide which is based on the published sequence of the VP1 protein of strain A24 Cruzeiro, but with additional amino acids. This peptide contains two regions which had previously shown to elicit virus neutralising antibodies. Furthermore, the minimum binding region was found to be the 141-158 region, which corresponds to the 149-154 residues of VP1. This binding region was found to be fairly conserved in other A serotypes virus examined and therefore the monoclonal antibody tested with these strains. Nonetherless, only viruses with a sequence that

encompassed the same minimum binding 'footprint', namely A24 Cruzeiro Brazil/55 (homologous), A27 Cundinamarca Colombia/76, A Argentina/79 and A Venceslau Brazil/76 reacted with similar affinity. Other strains tested yielded no reactivity, eg., A5 Westerwald, A5 Parma 1962, A Modena 11/84, etc. Of the 17 different type A strains tested with the monoclonal antibody only three cross-reacted.

Unfortunately no information was forthcoming as to the precise epitopes which the antibodies used in this part of the project recognised and the exact strains to which the antibodies were raised. Therefore, it is difficult to argue the point made above as to why the P1 protein is not been detectable.

2: It may be possible that the amino acid change at position 528 could alter the structure of the protein in such a way as to hide the epitopes which are recognised by the monoclonal antibody used. Amino acid substitutions outside an epitope can also sterically affect antibody recognition (Barnett *et al*, 1996) and thus affect binding of the antibody. This could thus affect the efficacy of the antibodies used.

3: In FMDV, translation initiation is known to be leaky and producing two forms of the L protein (King, personal communication). Although the L gene has not been included in the LSD-FMDV construct, the literature does give evidence for truncated VP0 in L deletants, indicating that initiation is also leaky from the same AUG when L is not there (Abrams *et al.*, 1995). Consequently, the Kozak sequence was altered to accommodate this fact, however, it may be entirely possible that the efficiency of initiation was not made any less leaky. Therefore, truncated forms of P1 could be produced, which the antibodies used for P1 protein detection, are unable to recognise.

CHAPTER 5: CONCLUSIONS

The infectious nature of FMDV and RVFV and the threat these diseases pose to the economy of countries necessitates the need for more effective vaccines. The availability of an attenuated strain of LSDV, which has been used as a successful cattle vaccine, provides the basis for a ideal viral vector for constructing live recombinant vaccines. LSDV has all the advantages of other recombinant poxviruses, with the ability to insert a number of foreign genes, low cost of production, stability of the vaccine and the ability to elicit an immune response. Furthermore, the vaccine would be able to yield long lasting immunity to more than one disease simultaneously.

A shuttle vector containing the RVFV glycoproteins genes, G1 and G2, was transfected into LSDV (Neethling) infected lamb testes cells. The shuttle vector also contains the *E.coli lacZ* gene and the *E.coli Gpt* gene for use as selectable markers. The site used for insertion of foreign genes into the LSDV genome is an intergenic region. Southern blot hybridisation analysis indicated that a double cross-over event had occurred and the glycoprotein genes were in an intergrated form. Contamination with parental virus was evident, indicating that selection procedures was not complete, however, the presence of the *lacZ* gene provides an easy tool for further purification steps. The presence of the glycoproteins, was revealed by immunoflourescence staining. Furthermore, the RVFV glycoproteins were predominantly expressed on the surface membrane of the lamb testes cells. Small animal trails were carried out, however, no specific immune response to RVFV could be detected in rabbits. It may therefore be possible that the recombinant LSDV is not able to reach a productive stage in rabbits as a consequence of the non-permissiveness of the host to LSDV. In light of these results, it may be necessary to test the recombinant virus in a more permissive host.

In this project, the P1 gene from FMDV was amplified by means of PCR, subcloned and sequenced. No PCR artefacts were detected by sequence analysis when compared to the prototype. The P1 gene was then

cloned into a shuttle vector. This shuttle vector contained flanking sequences homologous to the LSDV ribonucleotide reductase gene, thereby directing insertion to the desired locus in the LSDV genome. A reporter gene system (employing the *E.coli lacZ* gene) was used for the isolation of recombinant virus, together with the selectable marker, *E.coli Gpt* gene. Expression of the P1 gene by protein analysis was investigated but detection of the P1 protein was unsuccessful with the antibodies used. However, Southern blot hybridisation analysis clearly showed that a double cross-over event had occurred, with the incorporation of the P1 gene into the LSDV genome. The recombinant vaccine constructed here is ready for testing in animals.

In conclusion, two stable recombinant LSDV have been developed, one containing the P1 gene from FMDV and the other the G1 and G2 glycoprotein genes from RVFV. The future for veterinary vaccines based on LSDV is promising although fine-tuning is needed in relation to tests on protein expression and antibody responses in a variety of animals.

APPENDICES

APPENDIX A: BUFFERS, MEDIA AND SOLUTIONS

A1: Activated trypsin

10X Trypsin base	10ml
5% Trypsin stock solution	1ml
PS	0.5ml

The above solutions were made up to 100ml with dH_2O and stored at 4°C

A2: Dulbecco's Modified Eagle's Essential Medium (DMEM)/ Hams F12

DMEM powder	67.75g
Hams F12 powder	53.55g
$NaHCO_3$	24.4g

The above chemicals was mixed with 10 000ml of dH_2O and the pH adjusted to 7.1 with 3M HCl. The solution was sterilised using a 0.2μm filter, with two 5ml amounts taken at the start and end of the filtration process so that sterility tests could be carried out. The solution was aliquoted into 1000ml amounts and stored at 4°C.

A3: Hepes (1M)

HEPES	23.8g
NaOH(0.3M)	80ml

The HEPES was dissolved in the NaOH solution and the pH adjusted to 7.2. the final volume was made up to 100ml with dH_2O and passed through a 0.2μm filter. The buffer was kept at 4°C.

A4: Hypoxanthine (stock solution)

Hypoxanthine	0.1g
NaOH (0.1N)	10ml

The above solution was mixed, filter sterilised through a 0.2μm filter and stored at 4°C.

A5: Lysis buffer A

Tris-HCl (1M, pH8)	0.5ml
EDTA (0.5M)	0.1ml
Sucrose	15%wt/vol
Lysozyme	0.1g
RNase	0.01g
BSA	0.005g

The final volume was made up to 50ml with dH_2O, stored in 2ml aliquots at –20°C.

A6: Lysis buffer B

Tris-HCl (1M, pH7.8)	25ml
NaCl (5M)	5ml
EDTA (0.5M)	2.5ml
SDS (10%)	25ml

The final volume was made up to 250ml with dH_2O, filter sterilised and stored at r.t. in 10ml aliquots.

A7: Lysis buffer C

NaCl	0.35g
Tris-HCl (1M)	0.4ml
EDTA	0.0297g
Nonidet P-40 (NP-40)	0.4ml

The volume was adjusted to 40ml with dH_2O and 1mg/ml of BSA and 3µg/µl of phenylmethylsulfonyl fluoride (PMSF) added just before use.

A8: Mycophenolic acid (stock solution 10mg/ml)

Mycophenolic acid	0.1g
NaOH (0.1N)	10ml

The above solution was mixed and filter sterilised through a 0.2µm filter, aliquoted into 100µl and stored in the dark at –20°C.

A9: Resolving gel (12.5%)

Monomer solution	12.5ml
4X Running gel buffer	7.5ml
SDS (10%)	0.3ml
ddH_2O	9.6ml
Ammonium persulphate (10%)	150µl
TEMED	10µl

A10: Resolving gel buffer (4X)

Tris 36.3g

Add 150ml ddH_2O and adjust the pH to 8.8 with HCl. Adjust the final volume to 200ml with ddH_2O.

A11: Paraformaldehyde (4%)

Gloves and a mask was worn during the preparation of the paraformaldehyde solution. All preparation was also carried out in a fume hood. 4g of paraformaldehyde was dissolved in 80ml of PBS. The solution was heated to 60°C, 1M NaOH added till the solution cleared and the pH was 9.8. The final volume was made up to 100ml.

A12: Penicillian – Streptomycin (PS)

Penicillian (Novo Nordisk)	$1X10^6$ units
Streptomycin (Novo Nordisk) (1g/3ml)	3ml

The above antibiotics was made up to 20ml with physiological saline and filtered through a 0.2µm filter. The solution was stored at –20°C in 2ml aloiquots.

A13: Phenol

The phenol was melted at 68°C and hydroxyquinoline added to a final concentration of 0.1%. An equal volume of 0.5M Tris-HCl pH8 was added, mixed and the upper phase removed. An equal volume of 0.1M Tris-HCl pH8 was added, the solution mixed and the upper phase removed. The extractions was repeated until the pH of phenolic phase was greater than 7.8. A final overlay of 0.1 volume of 0.1M Tric-HCl pH8 containing 0.2% β-mercaptoethanol was added and the phenol solution stored at –20°C.

A14: Phenol Red (0.4%)

Phenol Red	0.4g
NaOH (0.05N)	60ml
dH_2O	to 100ml

The above solution was filter sterilised, dispense into 20ml aliquots and stored at 4°C.

A15: Phosphate Buffered Saline

NaCl	8.0g
KCl	0.2g
KH_2PO_4	0.12g
Na_2HPO_4	0.91g
or	
$Na_2HPO_4.2H_2O$	1.14g
or	
$Na_2HPO_4.12H_2O$	2.28g

The above ingredients were dissolved in 900ml of dH_2O and the pH adjusted to 7.5. The final volume was made up to 1000ml, the solution autoclaved and stored at r.t.

A16: Ponceau S

Ponseau S	1g
Acetic acid	50ml

Adjust to 1000ml with dH_2O

A17: Selection medium

MPA (stock solution)	25µl
Xanthine (stock solution)	250µl
Hypoxanthine (stock solution	15µl
FCS	1ml
PS	0.05ml
F	0.1ml
DMEM/Hams F12	to 10ml

A18: 2X SDS gel loading buffer

TrisCl (pH 6.8)	1ml
Dithiotheitol	2ml
SDS 10%	4ml
Bromophenol blue	0.02g
Glycerol	2ml

Adjust volume to 10ml with dH_2O.

A19: Stacking gel buffer (4X)

Tris	3g

Add 40ml ddH_2O and adjust pH to 6.8 with HCl. Adjust final volume to 50ml with ddH_2O.

A20: Stacking gel

Monomer solution	1.33ml
4X Stacking gel buffer	2.5ml
SDS (10%)	0.1ml
ddH_2O	6.0ml
Ammonium persulphate (10%)	50µl
TEMED	5µl

A21: Stop/Loading buffer (6X)

Bromophenol blue	0.025g
EDTA (0.5M, pH8)	0.4ml
Sucrose	4g
dH_2O	to 10ml

A22: Substrate solution

PBS	9.2ml
X-Gal (50mg/ml)	200µl
Potassium ferricyanide (50mM)	200µl
Potassium ferrocyanide (50mM)	200µl
$MgCl_2$	200µl

A23: Tissue culture medium (10% or 4%)

Fetal Calf serum	100ml or 40ml
PS	5ml
F	10ml
DMEM/ Hams F12	to 1000ml

A24: Towbin transfer buffer

Tris	30.3g
Glycine	144.1g
SDS	10g
Methanol	200ml
Adjust volume to 10 000ml with dH_2O	

A25: Transformation and storage buffer

Peptone	1.6g
Yeast extract	1g
NaCl	0.5g
PEG (mw 3350-4000)	10g
DMSO	5ml
$MgCl_2$	1ml
$MgSO_4$	1ml
H_2O	to 100ml

A26: Treatment buffer

Stacking gel buffer	2.5ml
SDS (10%,w/v solution)	4ml
Glycerol	2ml
2-mercaptoethanol	1ml
H_2O	0.5ml

A27: Tris-acetate buffer (50X)

Tris	242g

Glacial acetic acid	57.1ml
EDTA (0.5M, pH8)	100ml
dH_2O	to 1000ml

A28: Tris buffered saline (TBS) pH 7.5

Tris base	6.05g
Sodium chloride	8.76g
dH_2O	800ml

Adjust the pH to 7.5 with HCl and adjust to 1000ml with dH_2O.

A29: TBS-Tween (TBST)

Dilute 1ml of Tween® 20 in 1000ml of TBS

A30: Trypan blue

Trypan Blue Powder	5g
Physiological Saline	100ml

The powder was dissolved in the saline solution and filtered through Whatman number 1 paper. The dye was finally filtered again through a 0.2µm filter, dispensed into 2ml aliquots and stored at 4°C.

A31: Trypsin base (10X)

NaCl	30g
KH_2PO_4	1.2g
KCl	2g
Na_2HPO_4	9.1g
or	
$Na_2HPO_4.2H_2O$	11.2g
Glucose	5g

The above chemicals were dissolved in 700ml dH_2O. 2g of EDTA, dissolved in 100ml dH_2O was added to the above solution. 25ml of phenol red (0.4% stock) was added, the solution stirred and the pH adjusted to 7.8 with 1N NaOH, until a clear red colouration was obtained. The final volume was adjusted to 1000ml with dH_2O, the solution filtered through a 0.2µm filter, then dispensed into 20ml aliquots and stored at –20°C.

A32: Trypsin stock solution (5%)

10X Trypsin base	10ml
Trypsin powder	5g

The trypsin base was added to 90ml of dH_2O and 0.5ml of PS. HCl (1N) was added dropwise until the solution turned yellow. The solution was heated to 37°C, the trypsin powder added carefully and left to dissolve by either letting it stand at r.t. for a few hours or overnight at 4°C. The solution was finally sterilised by passing it through a 0.2μm filter, dispensed into 1ml aliquots and stored at the –20°C.

A33: Virus diluent

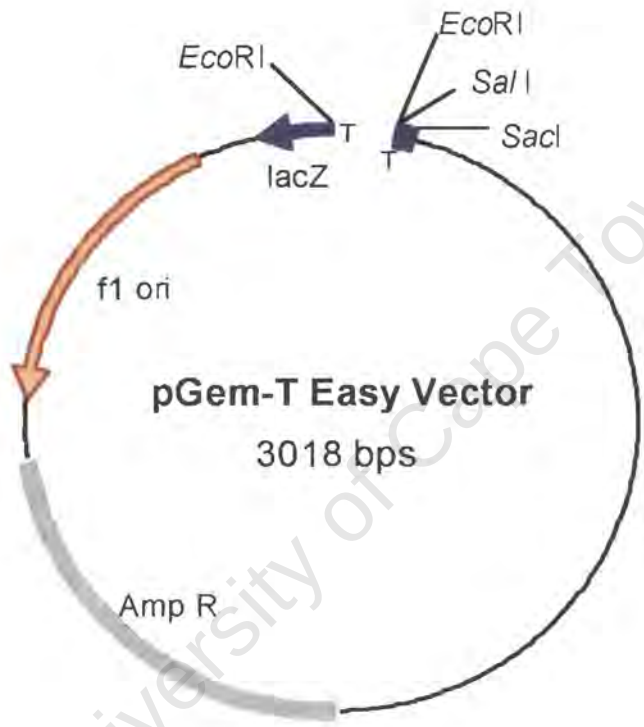
Hepes (1M)	1ml
PS	0.5ml
F	1ml
DMEM/ Hams F12	to 100ml

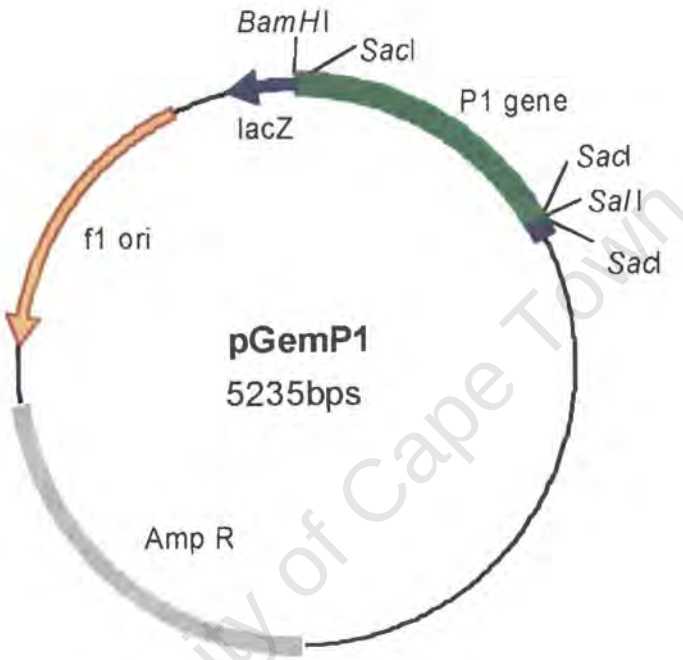
A34: Xanthine (stock solution 10mg/ml)

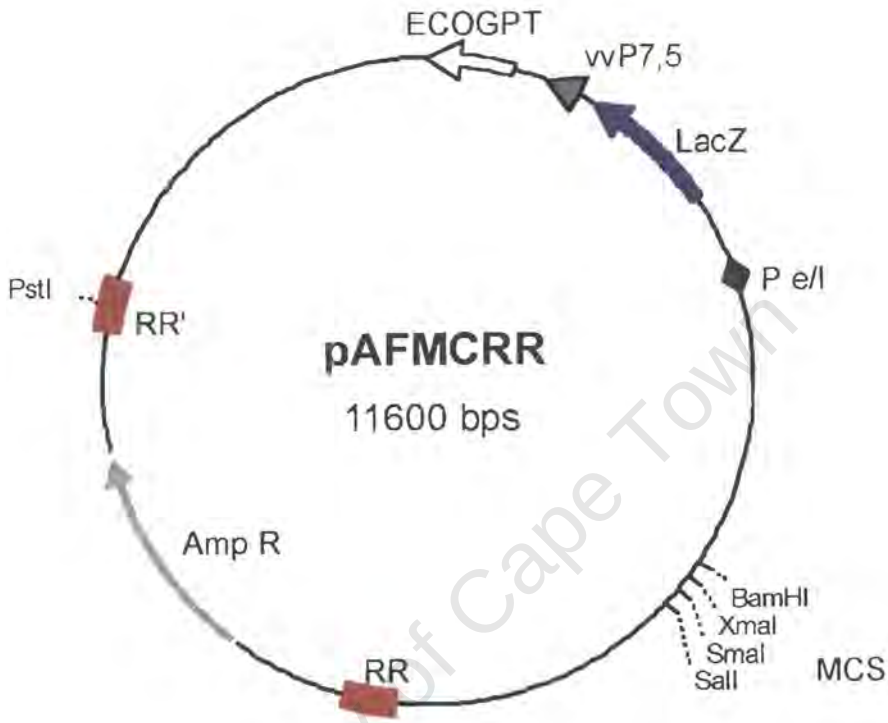
Xanthine	0.1g
NaOH (0.1N)	10ml

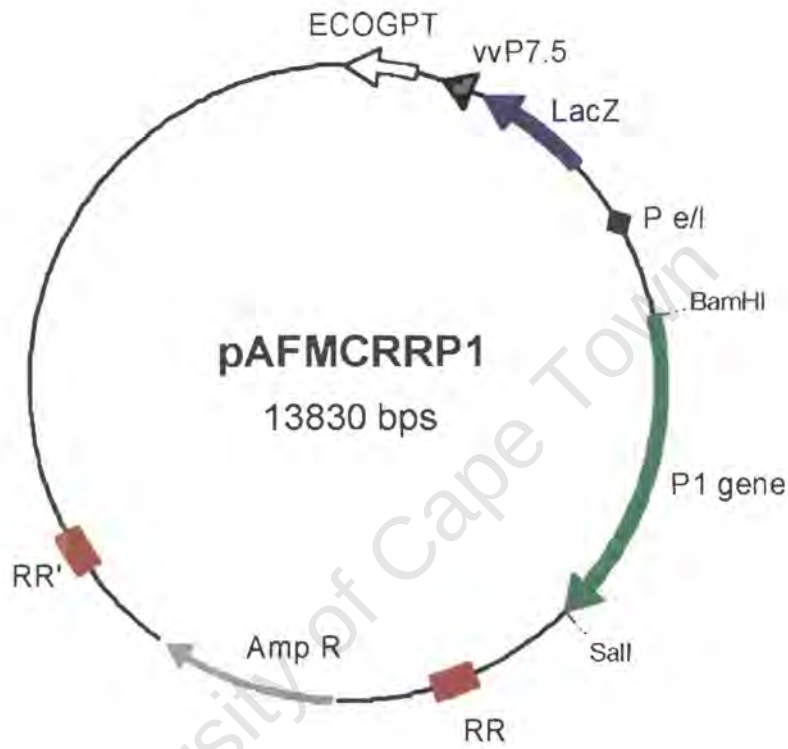
The above solution was mixed, filter sterilised through a 0.2μm filter and stored at 4°C.

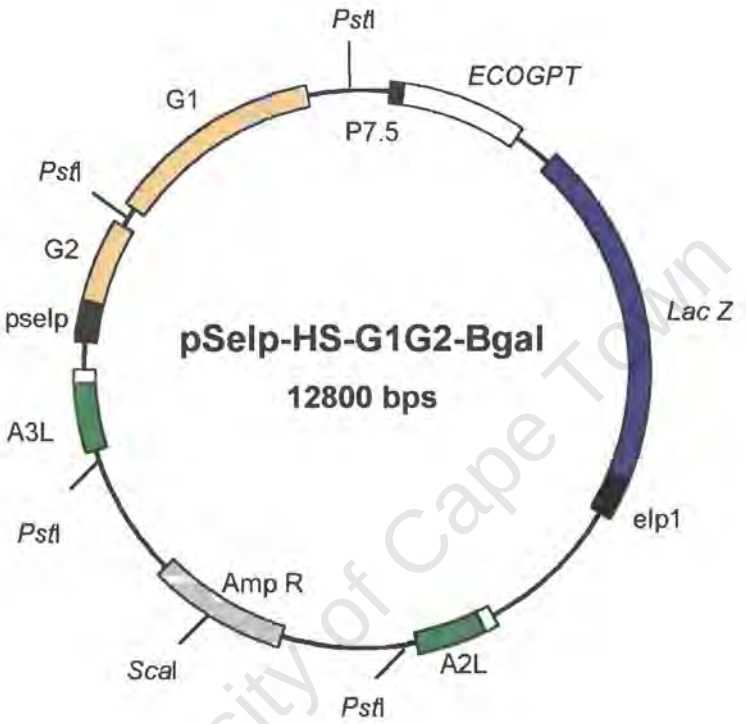
APPENDIX B: PLASMID MAPS











APPENDIX C: SIZES OF DNA MOLECULAR WEIGHT MARKERS

Lambda *Pst* I Marker

Fragment	Size	Fragment	Size	Fragment	Size
1	11497	11	1700	21	216
2	5077	12	1159	22	211
3	4749	13	1093	23	200
4	4507	14	805	24	164
5	2838	15	514	25	150
6	2560	16	468	26	94
7	2459	17	448	27	87
8	2443	18	339	28	72
9	2140	19	264	29	15
10	1986	20	247		

APPENDIX D: ABBREVIATIONS OF AMINO ACIDS

Alanine	Ala	A	Leucine	Leu	L
Arginine	Arg	R	Lysine	Lys	K
Asparagine	Asn	N	Methionine	Met	M
Aspartic acid	Asp	D	Phenylalanine	Phe	F
Cysteine	Cys	C	Proline	Pro	P
Glutamine	Gln	Q	Serine	Ser	S
Glutamic acid	Glu	E	Threonine	Thr	T
Glycine	Gly	G	Tryptophan	Trp	W
Histidine	His	H	Tryosine	Tyr	Y
Isoleucine	Ile	I	Valine	Val	V

APPENDIX E: GENETIC CODE

Codon	Amino acid	Codon	Amino acid	Codon	Amino acid	Codon	Amino acid
AAA	K	CAA	Q	GAA	E	TAA	*
AAC	N	CAC	H	GAC	D	TAC	Y
AAG	K	CAG	Q	GAG	E	TAG	*
AAT	N	CAT	H	GAT	D	TAT	Y
ACA	T	CCA	P	GCA	A	TCA	S
ACC	T	CCC	P	GCC	A	TCC	S
ACG	T	CCG	P	GCG	A	TCG	S
ACT	T	CCT	P	GCT	A	TCT	S
AGA	R	CGA	R	GGA	G	TGA	*
AGC	S	CGC	R	GGC	G	TGC	C
AGG	R	CGG	R	GGG	G	TGG	W
AGT	S	CGT	R	GGT	G	TGT	C
ATA	I	CTA	L	GTA	V	TTA	L
ATC	I	CTC	L	GTC	V	TTC	F
ATG	M	CTG	L	GTG	V	TTG	L
ATT	I	CTT	L	GTT	V	TTT	F

REFERENCES

Abrams, C.C., King, A.M.Q., Belsham, G.J. 1995. Assembly of foot-and-mouth disease virus empty capsids synthesized by a vaccinia virus expression system. J. Gen. Virol., 76, pp3089-3098.

Alexander, R.A., Plowright, W., Haig, D.A. 1957. Cytopathogenic agents associated with lumpy skin disease of cattle. Bull. epizoot. Dis Afr., 5, pp489-492.

Almeida, M.R., Reider, E., Chinsangaram, J., Ward, G., Beard, C., Grubman, M.J., Mason, P. 1998. Construction and evaluation of an attenuated vaccine for foot-and-mouth disease: difficulty adapting the leader proteinase-deleted strategy to the serotype O1 virus. Virus Res., 55, pp49-60.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K., Eds. 1987. Current protocols in molecular biology. John Wiley and Sons, NY, USA.

Bachrach, H.L., Morgan, D.O., McKercher, P.D., Moore, D.M., Robertson, B.H. 1982. Foot-and-mouth disease virus: immunogenicity and structure of fragments derived from capsid protein VP and of virus containing cleaved VP. Vet. Microbiol., May 7(2), pp85-96.

Bailey, M.J. and Posse, R.D. 1991. Manipulation of baculovirus vectors. Chapter 13, pp147-168. In methods in molecular biology, volume 7: Gene transfer and expression protocols. Murray, E.J. (ed). The Humana Press Inc., Clifton, NJ.

Baldick, C.J.Jr., and Moss, B. 1993. Characterisation and temporal regulation of mRNAs encoded by vaccinia virus intermediate stages genes. J. Virol., 67, pp3515-3527.

Ball, L,A. 1987. High-fequency homologous recombination in vaccinia virus DNA. J. Virol., 61, pp1788-1795.

Barnard, B.J.H. 1979. Rift Valley fever vaccine – antibody and immune response in cattle to a live and an inactivated vaccine. J. S. A. Vet. Ass., 50, pp155-157.

Barnard, B.J.H., Munz, E., Dumbell, K., Prozesky, L. 1994. Infectious diseases of livestock. Volume 1, pp605-612. Editors: Coetzer, J.A.W., Thomson, G.R., Tustin, R.C. Oxford University Press.

Barnett, P.V., Pullen, L., Staple, R.F., Lee, L.J., Butcher, R., Parkinson, D., Doel, T.R. 1996. A protective anti-peptide antibody against the immunodominant site of the A_{24} Cruzeiro strain of foot-and-mouth disease virus and its reactivity with other subtype viruses containing the same minimum binding sequence. J. Gen. Virol., 77, pp1011-1018.

Baxby, D. 1993. Recombinant poxvirus vaccines. Rev. Med. Micro., 4, pp80-88.

Bayliss, C.D., Peters, R.W., Cook, J.K.A., Reece, R.L., Howes, K., Binns, M.M., Boursnell, M.E.G. 1991. A recombinant fowlpox virus that expresses the VP2 antigen of infectious bursal disease virus induces protection against mortality caused by the virus. Arch Virol.;120(3-4), pp193-205.

Beck, E and Strohmaier, K. 1987. Subtyping of European foot-and-mouth disease virus strains by nucleotide sequence determination. J. Virol., 61, pp1621-1629.

Belsham, G.J., Ryan, M.D., Brangwyn, J.K. 1989. Expression of self-processing foot-and-mouth disease virus capsid precursors by vaccinia virus recombinants. In Vaccines '89'. R. Chanock, H.S. Ginsberg, R.A. Lerner, F. Brown (Eds.). Cold Spring Harbour Laboratory, Cold Spring Harbour, NY.

Bender, B.S., Rowe, C.A., Taylor, S.F., Wyatt, L.S., Moss, B., Small, P.A. 1996. Oral immunisation with a replication-deficient recombinant vaccinia virus protects mice against influenza. J. Virol., 12, pp1032-1040.

Berghammer, H and Auer, B. 1992. "Easypreps": fast and easy plasmid minipreparation for analysis of recombinant clones in *E.coli*. Biotechniques, 14, pp527-528.

Besselaar, T.G. and Blackburn, N.K. 1991. Topological mapping of antigenic sites on the Rift Valley fever virus envelope glycoproteins using monoclonal antibodies. Arch. Virol., 121, pp111-124.

Besselaar, T.G. and Blackburn, N.K. 1992. The synergistic neutralization of Rift Valley fever virus by monoclonal antibodies to the envelope glycoproteins. Arch. Virol., 125, pp239-250.

Black, D.N., Hammond, J.M., Kitching, R.P. 1986. Genomic relationship between capripoxviruses. Virus Res., 5, pp277-292.

Blackwell, J.H. 1984. Foreign animal disease agent survival in animal products: Present developments. J A. Vet. Ass., 184, pp674-679.

Blancou, J., Kieny, M.P., Lathe, R., Lecocq, J.P., Pastoret, P.P., Soulebot, J.P., Desmettre, P. 1986. Oral vaccination of the fox against rabies using a live recombinant vaccinia virus. Nature, 322, pp373-375.

Blasco, R. and Moss, B. 1992. Role of cell-associated enveloped vaccinia virus in cell-to-cell spread. J. Virol., 66, pp4170-4179.

Boyle, D.B. and Coupar, B.E.H. 1988. A dominant selectable marker for the construction of recombinant poxviruses. Gene, 65, pp123-128.

Briand, J.P., Benkirane, N., Guichard, G., Newman, J.F.E., Van Regenmortel, M.H.V., Muller, S. 1997. A retro-inverso peptide corresponding to the GH loop of foot-and-mouth disease virus elicits high levels of long-lasting protective neutralizing antibodies. Proc. Natl. Acad. Sci. USA., 94, pp12545-12550.

Brooksby, J.B. 1982. Portraits of viruses: Foot-and-mouth disease virus. Intervirology, 18, pp1-23.

Brown, C.C., Piccone, M.E., Mason, P.W., McKenna, T.S.-C., Grubman, M.J. 1996. Pathogenesis of wild-type and leaderless foot-and-mouth disease virus in cattle. J. Virol., 70, pp5638-5641.

Caplen, H., Peters, C.J., Bishop, D.H.L. 1985. Mutagen-directed attenuation of Rift Valley fever virus as a method for vaccine development. J. Gen. Virol., 66, pp2271-2277.

Capstick, P.B. and Coackley, W. 1961. Protection of cattle against lumpy skin disease. Res. Vet. Sci., 2, pp362-368.

Carn, V.M. 1993. Control of capripoxvirus infections. Vaccine, 11 (13) pp1275-1279.

Carn, V.M. and Kitching, R.P. 1995. An investigation of possible routes of transmission of lumpy skin disease virus (Neethling). Epiderm. Infect., 114 pp219-226.

Carn, V.M., Timms, C.P., Chand, P., Black, D.N., Kitching, R.P. 1994. Protection of goats against capripox using a subunit vaccine. The Vet. Rec., 135, pp434-436.

Chinsangaram, J., Beard, C., Mason, P.W., Zellner, M.K., Ward, G., Grubman, M.J. 1998. Antibody response in mice inoculated with DNA expressing foot-and-mouth disease virus capsid proteins. J. Virol., 72(5), pp4454-4457.

Choppin, P.W. and Scheid, A. 1980. The role of viral glycoproteins in adsorption, penetration and pathogenicity of viruses. Rev. Infect. Dis., 2, pp40-61.

Chung, C-S., Hsiao, J-C., Chang, Y-S., Chang, W. 1998. A27L protein mediates vaccinia virus interaction with cell surface heparan sulfate. J. Virol., 72(2), pp1577-1585.

Chung, C.T. and Miller, R.H. 1988. A rapid and convenient method for the preparation and storage of competent bacterial cells. Nucleic Acids Res., 16, pp3580.

Cochran, M.A., Puckett, C., Moss, B. 1985. In vitro mutangenesis of the promoter region for a vaccinia virus gene: evidence for tandem early and late regulatory signals. J. Virol., 54, pp30-37.

Coetzer, J.A.W. and Barnard, B.J.H. 1977. *Hydrops amnii* in sheep associated with hydranencephaly and arthrogryposis with wesselsbron disease and Rift Valley fever viruses as aetiological agents. Onderstepoort J. Vet. Res., 44, pp119-126.

Cohen, A., Williamson, A-L., Paweska, J., Swanepoel, R., Burt, F., Jardine, J., Van Dijk, A. 1999. Evaluation of the vaccine potential of a Rift Valley fever-lumpy skin disease virus recombinant in sheep. Abstract. XIth International Congress of Virology, pp81.

Cohen, S.N., Chang, A.C.Y., Hsu, L. 1972. Nonchomosomal antibiotic resistance in bacteria: Genetic transformation of *E.coli* by R factor DNA. Proc. Natl. Acad. Sci. USA, 69, pp2110-2114.

Collett, M.S., Keegan, K., Hu, S-L., Sridhar, P., Purchio, A.F., Ennis, W.H., Dalrymple, J.M. 1987. Protective subunit immunogens to Rift Valley fever virus from bacteria and vaccinia virus. Biology of negative strand viruses. Pp321-329. Editors: Mahy and Kolakofsky. Elsevier Science Publishers.

Cudmore, S., Cossart, P., Griffiths, G., Way, M. 1995. Actin-based motility of vaccinia virus. Nature, 378, pp636-638.

Dales, S. and Kajioka, R. 1964. The cycle of multiplication of vaccinia virus in Earle's strain L cells. 1. Uptake and penetration. Virology, 24, pp278-294.

Daubney, R., Hudson, J.R., Garnham, P.C. 1931. Enzootic hepatitis or Rift Valley fever. An undescribed virus disease of sheep, cattle and man from East Africa. J. Pathol. Bacteriol., 34, pp545-579.

Davies, F.G. 1980. The possible role of wildlife as maintenance hosts for some african insect-borne diseases. Wildlife Disease Research and Economic Development (Workshop Proceedings).

Davies, F.G. 1991a. Lumpy skin disease, an african capripox virus disease of cattle. Br. Vet. J., 147 pp489-503.

Davies, F.G. 1991b. Lumpy skin disease of cattle: a growing problem in Africa and the Near East. World Animal Review, 68, pp37-42.

Davies, R.E. and Mathews, C.K. 1993. Acidic-C terminus of vaccinia virus DNA-binding protein interacts with ribonucleotide reductase. Proc. Natl. Acad. Sci. USA, 90, pp745-749.

Doel, T.R. and Baccarini, P.J. 1981. Thermal stability of foot-and-mouth disease virus. Arch. Virol., 70, pp21-32.

Doel T.R. 1999. Optimisation of the immune response to foot-and-mouth disease vaccine. Vaccine, 17, pp1767-1771.

Doms, R.W., Blumenthal, R., Moss, B. 1990. Fusion of intra- and extracellular forms of vaccinia virus with the cell membrane. J. Virol., 64, pp4884-4892.

Donaldson, A.I. 1983. Quantitative data on airborne foot-and-mouth disease virus: Its production, carriage and deposition. Philosophical Transactions of the Royal Society, London, B302, pp529-534.

Etchison, D., Milburn, S.C., Edery, I., Sonenberg, N., Hersher, J.W.B. 1982. Inhibition of Hela cell protein synthesis following poliovirus infection correlates with the proteolysis of a 220 000-dalton polypeptide associated with eucaryotic initiation factor 3 and a cap binding protein complex. J. Biol. Chem., 257, pp14806-14810.

Falkner, F.G. and Moss, B. 1988. *Escherichia coli gpt* gene provides dominant selection for vaccinia virus open reading frame expression vectors. J. Virol., 62(6), pp1849-1854.

Fischer, L., Le Gros, F.X., Mason, P.W., Paoletti, E. 1997. A recombinant canarypox virus protects rabbits against a lethal rabbit hemorrhagic disease virus (RHDV) challenge. Vaccine, 15, 90-96.

Freshney, R.I. 1987. Culture of animal cells: A manual of basic techniques. Alan R. Liss. NY.

Garland A.J.M. 1999. Vital elements for the successful control of foot-and mouth disease by vaccination. Vaccine, 17, p1760-1766.

Gershon, P.D. and Black, D.N. 1988. A comparison of the genomes of capripoxvirus isolates of sheep, goats and cattle. Virology, 164, pp341-349.

Gershon, P.D. Ansell, D.M., Black, D.N. 1989. A comparison of the genome organisation of capripoxvirus with that of the orthopoxviruses. J. Virol., 63(11), pp4703-4708.

Gershon, P.D. and Black, D.N. 1987. Physical characterization of the genome of a cattle isolate of capripoxvirus. Virology, 160, pp473-476.

Gershon, P.D. and Black, D.N. 1989. The nucleotide sequence around the capripoxvirus thymidine kinase gene reveals a gene shared specifically with leporipoxvirus. J. Gen. Virol., 70, pp525-533.

Gonzalez-Scarano, F. and Nathanson, N. 1996. Bunyaviridae. Fields Virology. 3rd edition. Chapter 48, pp1473-1504. Editors: Fields, B.N., Knipe, D.M., Howley. P.M. Lippincott-Raven Publishers, Philadelphia.

Haig, D.A. 1957. Lumpy skin disease. Bull. Epizoot. Dis. Afr., 5, pp421-430

Hamblin, C., Anderson, E.C., Jago, M., Mlengeya, T., Hirji, K. 1990. Antibodies to some pathogenic agents in free living wild species in Tanzania. Epidermiol. Infect., 105, pp585-594.

Harford, C.G., Hamlin, A., Riders, E. 1966. Electron microscopic autoradiography of DNA synthesis in cells infected with vaccinia virus. Exp. Cell Res., 42, pp50-57.

Harris, N., Rosales, R., Moss, B. 1993. Transcription initiation factor activity of vaccinia virus capping enzyme is independent of mRNA guanylylation. Proc. Natl. Acad. Sci. USA., 90, pp2860-2864.

Hedger, R.S. 1976. Foot-and-mouth disease in wildlife with particular reference to the African buffalo *Syncerus caffer*. Wildlife Disease. Editor: Page, LA. Plenum Publishing Corporation: New York.

Henning M.W. 1956. Animal Diseases in South Africa. 3rd edition. C.N.A. Cape Town: Central News Agency.

Hollinshead, M., Vanderplasschen, A., Smith, G.L., Vaux, D.J. 1999. Vaccinia virus intracellular mature virions contain only one lipid membrane. J. Virol., 73(2), pp1503-1517.

Holowczak, J.A. 1972 Uncoating of poxviruses. I. Detection and characterisation of subviral particles in the uncoating process. Virology, 50, pp216-232.

Hunter, P. 1998. Vaccination as a means of control of foot-and-mouth disease in sub-saharan Africa. Vaccine, 16 (2/3), pp 261-264.

Hyslop, N.St.G. 1970. The epizootiology and epidemiology of foot-and-mouth disease. Adv. Vet. Sci., 14, pp261-307.

Janeczko, R.A., Rodriguez, J.F., Estaban, M. 1987. Studies on the mechanism of entry of vaccinia virus in animal cells. Arch. Virol., 92, pp135-150.

Jin, H. and Elliiott, R.M. 1993. Characterisatiion of Bunyamwera virus S RNA that is transcribed and replicated by the L protein expressed from recombinant vaccinia virus. J. Virol., 67, pp1396-1404.

Kates J. and Beeson, J. 1970. Ribonucleic acid synthesis in vaccinia virus. II. Synthesis of polyriboadenylic acid. J. Mol. Biol., 50, pp19-23.

Keck , J.K., Baldick, C.J., Moss, B. 1990. Role of DNA replication in vaccinia virus gene expression: a naked template is required for transcription of three late trans-activator genes. Cell, 61, pp801-809.

Keegan, K. and Collett, M.S. 1986. Use of bacterial expression cloning to define the amino acid sequences of antigenic determinants on the G2 glycoprotein of Rift Valley fever virus. J. Virol., 58(2), pp263-270.

Kirchweger, R., Ziegler, E., Lamphear, B.J., Waters, D., Liebig, H.-D., Sommergruber, W., Sobrino, F., Hohenadl, C., Blass, D., Rhoads, R.E., Skern, T. ,1994. Foot-and-mouth disease virus leader proteinase: purification of the Lb form and determination of its cleavage site on eIF-4γ. J. Virol., 68, pp5677-5684.

Kitching, R.P., Hammond, J.M., Black, D.N. 1986. Studies on the major common precipitating antigen of capripoxvirus. J. Gen. Virol., 67, pp139-148.

Kitching, R.P., Hammond, J.M., Taylor, W.P. 1986. A single vaccine for the control of capripox infection in sheep and goats. Res. Vet. Sci., 42, pp53-60.

Kitching, R.P., Bhat, P.P., Black, D.N. 1989. The characterization of african strains of capripoxvirus. Epidermiol. Infect., 102, pp335-343.

Kitching, R.P. and Taylor, W.P. 1985. Clinical and antigenic relationship between isolates of sheep and goat pox viruses. Trop. Anim. Health and Prod., 17, pp64-74.

Krijnse Locker, J., Kuehn, A., Schleich, S., Rutter, G., Hohenberg, H., Wepf, R., Griffiths, G. 2000. Entry of the two infectious forms of vaccinia virus at plasma membrane is signalling-dependent for IMV but not the EEV. Mol. Biol. Cell., 11(7), pp2497-2511.

Laemmli, U.K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature, 227, pp680-685.

Laughlin, L.W., Meegan, J.M., Strausbaugh, L.W., Morens, D.M., Watten, R.H. 1979. Epidemic Rift Valley fever in Egypt: observations of the spectrum of human illness. Trans. R. Soc. Trop. Med. Hyg., 73, pp630-633.

Letellier, C. 1993. Role of TK+ phenotype in the stability of pigeonpox vius recombinant. Arch. Virol., 131, pp431-439.

Luo, D and Saltzman, W.M. 2000. Enhancement of transfection by physical concentration of DNA at the cell surface. Nat. Biotecnol., 18, pp893-895

Lyerla, H.C. and Forrester, F.T. 1979. Immunofluorescence methods in virology. US Department of Health, Education and Welfare, Atlanta, GA.

MacDonald, R.A.S. 1931. Pseudo-urticaria of cattle. Northern Rhodesia Department of Animal Health. Annual Report. pp 20-21.

MacGregor, G.R., Nolan, G.P., Fiering, S., Roedere, M., Herzenberg, L.A. 1991. Use of E.coli lacZ (β-galactosidase) as a reporter gene. Methods in molecular biology. Volume 7: Gene transfer and expression protocols. Chapter 17, pp 217-235. Murray E.J. (ed.). The Humana Press Inc., Clifton, NJ.

Mackett, M., Smith, G.L., Moss, B. 1982. Vaccinia virus: A selectable eukaryotic cloning and expression vector. Proc. Natl. Acad. Sci. USA, 79, pp7415-7419.

Mallon, V. and Holowczak, J.A. 1985. Vaccinia virus antigens on the plasma membrane of infected cells. I. Viral antigens transferred from infecting virus particles and synthesised after infection. Virology, 141, pp201-220.

Mason, P.W., Piccone, M.E., McKenna, T. St.C., Chinsangaram, J., Grubman, M.J. 1997. Evaluation of a live-attenuated foot-and-mouth disease virus as a vaccine candidate. Virology, 227(1), pp96-102.

Mateu, M.G., Hernández, J., Martínez, M.A., Feigelstock, D., Lea, S., Pérez, J.J., Giralt, E., Stuart, D., Palma, E.L., Domingo, E. 1994. Antigenic heterogeneity of a foot-and-mouth disease virus serotype in the field is mediated by very limited sequence variation at several antigenic sites. J. Virol., 68(3), pp1407-1417.

McKenna, T. St.C., Reider, E., Lubroth, J., Burragw, T., Baxt, B., Mason, P.W. 1996. Strategy for producing new foot-and-mouth disease vaccines that display complex epitopes. J. Biotechnol., 44, pp83-89.

Meegan, J.M. and Bailey, C.L. 1989. The Arboviruses: Epidermiology and Ecology. Vol.IV, pp51-76. Editor: Monath, T.P. Boca Raton, Florida: CRC Press, Inc.

Meuth, M. and Harwood, J. 1991. Selection of cells defective in pyrimidine (TK-) and purine (APRT- and HPRT-) salvage. In Methods in Molecular Biology. Volume7: Gene transfer and expression protocols. Chapter 20, pp257-267. Editor: Murray, E.J. The Humana Press., Clifton, NJ.

Moss, B. 1996a. Poxviridae: The viruses and their replication. Fields Virology. 3rd edition. Chapter 83, pp2637-2671. Lippincott-Raven Publishers, Philadelphia.

Moss, B. 1996b. Genetically engineered poxviruses for recombinant gene expression, vaccination and safety. Proc. Natl. Acad. Sci. USA, 93, pp11341-11348.

Moss, B. and Rossenblum, E.N. 1973. Protein cleavage and poxvirus morphogenesis: tryptic peptide analysis of core precursors accumulated by blocking assembly with rifampicin. J. Mol. Biol., 81, pp267-269.

Munz, E. and Dumbell, K. 1994. Infectious diseases of livestock. Volume 1, pp613-615. Editors: Coetzer, J.A.W., Thomson, G.R., Tustin, R.C. Oxford University Press.

Munz, F.K. and Owen, N.C. 1966. Electron microscopic studies on lumpy skin disease virus type "Neethling". Ondersterpoort J. Vet. Res., 33(1), pp3-8.

Murphy, F.A., Harrison, A.K., Whitfield, S.G. 1973. Bunyaviridae: Morphological and morphogenetic similarities of Bunyawera serological supergroup viruses and several other arthropod-borne viruses. Intervirology, 1, pp297-316.

Nam, J-H., Wyatt, L.S., Chae, S-L., Cho, H-W., Park, Y-K., Moss, B. 1999. Protection against lethal Japanese encephalitis virus infection of mice by immunization with the highly attenuated MVA strain of vaccinia virus expressing JEV prM and E genes. Vaccine, 17, pp261-268.

Nargi, F., Kramer, E., Mezencio, J., Zamparo, J., Whetstone, C., Van Regenmortel, M.H., Briand, J.P., Muller, S., Brown, F. 1999. Protection of swine from foot-and-mouth disease with one dose of an all-D retro peptide. Vaccine, 17, pp2888-2893.

Ngichabe, C.K., Wamwayi, H.M., Barrett, T., Ndungu, E.K., Black, D.N., Bostook, C.J. 1997. Trail of a capripoxvirus-rinderpest recombinant vaccine in African cattle. Epidemiol. Infect., 118, pp63-70.

Payne, L.G. 1980. Significance of extracellular virus in the in vitro and in vivo dissemination of vaccinia irus. J. Gen. Virol., 31, pp147-155.

Pedersen, K., Snijder, E.J., Schleich, S., Roos, N., Griffiths, G., Krijnse Locker, J. 2000. Characterisation of vaccinia virus intracellular cores: implications for viral uncoating and core structure. J. Virol., 74(8), pp3525-3536.

Pedley, C.B. and R.J. Cooper. 1987. The assay, purification and properties of vaccinia virus-induced uncoating protein. J. Gen. Virol., 68, pp1021-1028.

Pennington, T.H. 1974. Vaccinia virus polypeptide synthesis: sequential appearance and stability of pre- and post-replicative polypeptides. J. Gen. Virol., 25, pp433-444.

Perlman, L.J. 1993. MSc thesis. Molecular characterisation of the lumpy skin disease virus genome. University of Cape Town, South Africa.

Piccone, M.E., Reider, E., Mason, P.W., Grubman, M.J. 1995. The foot-and-mouth disease virus leader proteinase gene is not required for viral replication. J. Virol., 69, pp5376-5382.

Plowright, W. and Ferris, R.D. 1959. Ether sensitivity of some mammalian pox viruses. Virology, 7, p357.

Prydie, J., Coackley, W. 1959. Lumpy skin disease: tissue culture studies. Bull. epizoot. Dis. Afr., 7, pp37-49.

Ramisse, J., Serres, H., Rakotondramary, E. 1969. Adaptation aux cellules renales de lapin de virus associés a la dermatose nodulaire. Rev. Elev. Med. Vet. Pays Trop., 22(3), pp363-371.

Redfield, R.R., Wright, D.C., James, W.D., Jones, T.S., Brown, C., Burke, D.C. 1987. Disseminated vaccinia in a military recruit with human immunodeficiency virus (HIV) disease. N. Engl. J. Med., 316, pp673-676.

Reider, E., Baxt, B., Lubroth, J., Mason, P.W. 1994. Vaccines prepared from chimeras of foot-and-mouth disease virus (FMDV) induce neutralizing antibodies and protective immunity to multiple serotypes of FMDV. J. Virol., 68(11), pp7092-7098.

Rice, R.M., Erlick, B.J., Rosato, R.R. Eddy, G.A., Mohanty, S.B. 1980. Biochemical characterisation of Rift Valley fever virus. Virology, 105, pp256-260.

Romero, C.H., Barrett, T., Kitching, R.P, Carn, V.M., Black, D.N. 1994a. Protection of cattle against rinderpest and lumpy skin disease with a recombinant capripoxvirus expressing the fusion protein gene of rinderpest virus. Vet. Rec., August 13, pp152-154.

Romero, C.H., Barrett, T., Chamberlain, R.W., Kitching, R.P., Flemming, M., Black, D.N. 1994b. Recombinant capripoxvirus expressing the hemagglutinin protein gene of rinderpest virus: protection of cattle against rinderpest and lumpy skin disease viruses. Virology, 204, pp425-429.

Romero, C.H., Barrett, T., Kitching, R.P., Bostook, C., Black, D.N. 1995. Protection of goats against peste des petits ruminants with recombinant , capripoxviruses expressing the fusion and haemagglutinin protein genes of rinderpest virus. Vaccine, 13 (1), pp36-40.

Rosales, R., Sutter, G., Moss, B. 1994. A cellular factor required for transcription of vaccinia viral intermediate stage genes. Proc. Natl. Acad. Sci. USA., 91, pp3794-3798.

Rueckert, R.R. 1996. Picornaviridae: The viruses and their replication. Fields Virology 3rd Edition. Editors: Fields, B.N., Knipe, D.M., Howley, P.M. Lippincott-Raven Publishers, Philadelphia.

Sáiz, J-C., Cairó, J., Medina, M., Zuidema, D., Abrams, C., Belsham, G.J., Domingo, E., Vlak, J.M. 1994. Unprocessed foot-and-mouth disease virus capsid precursor displays discontinuous epitopes involved in viral neutralization. J. Virol., 68(7), pp4557-4564.

Sall, A.A., Zanotto, P.M.A., Sène, O.K., Zeller, H.G., Digoutte, J.P., Thiongane, Y., Bouloy, M. 1999. Genetic reassotment of Rift Valley fever virus in nature. J. Virol., 73(10), pp8196-8200.

Sall, A.A., Zanotto, P.M.A., Vialat, P., Sène, O.K., Bouloy, M. 1998. Molecular epidemiology and emergence of Rift Valley fever. Mem. Inst. Oswaldo Cruz., 93(5), pp609-614.

Saluzzo, J.F. and Smith, J.F. 1990. Use of reassortants viruses to map attenuating and temperature sensitive mutations of the Rift Valley fever virus MP12 vaccine. Vaccine, 8, pp369-375.

Sambrook, J., Fritsch, E.F., Maniatis, T. 1989. Molecular cloning: A laboratory manual, 2nd ed., Cold Spring Harbour Lab. Press, NY.

Samina I., Zakay-Rones Z., Weller J.I., Peleg B-A. 1998. Host factors affecting the homologous and heterologous immune response of cattle to FMDV: genetic background, age, virus strains and route of administration. Vaccine, 16 (4): 335-339.

Sanz, P. and Moss, B. 1999. Identification of a transcription factor, encoded by two vaccinia virus early genes, that regulates the intermediate stage of viral gene expression. Proc. Natl. Acad. Sci. USA., 96 (6), pp2692-2697.

Sanz-Parra, A., Blasco, R., Sobrino, F., Ley, V. 1998. Analysis of the B and T cell response in guinea pigs induced with recombinant vaccinia expressing foot-and-mouth disease virus structural proteins. Arch. Virol., 143, pp389-398.

Sanz-Parra, A., Jimenez-Clavero, M.A., Garcia-Briones, M.M., Blanco, E., Sobrino, F., Ley, V. 1999. Recombinant virus expressing the foot-and-mouth disease virus capsid precursor polypeptide (p1) induce cellular but not humoral antiviral immunity and partial protection in pigs. Virology, 259, pp129-134.

Sarov, I and Joklik, W.K. 1972. Characterisation of intermediates in the uncoating of vaccinia virus DNA. Virology, 50, pp593-602.

Scheiflinger, F., Falkner, F.G., Dorner, F. 1996. Evaluation of the thymidine kinase (tk) locus as an insertion site in the highly attenuated vaccinia MVA strain. Arch. Virol., 141, pp663-669.

Schmaljohn, C.S. 1996. Bunyaviridae: The viruses and their replication. Fields Virology. 3rd edition. Chapter 47, pp14471471. Editors: Fields, B.N., Knipe, D.M., Howley. P.M. Lippincott-Raven Publishers, Philadelphia.

Schmaljohn, C.S., Parker, M.D., Ennis, W.H., Dalrymple, J.M., Collett, M.S., Suzich, J.A., Schmaljohn, A.L. 1989. Baculovirus expression of the M genome segment of Rift Valley fever virus and examination of antigenic and immunogenic properties of the expressed proteins. Virology, 170(1), pp184-192.

Schmelz, M., Sodeik, B., Ericsson, M., Wolffe, E.J., Shida, H., Hiller, G., Griffiths, G. 1994. Assembly of vaccinia virus: the second wrapping cisterna is derived from the trans Golgi network. J. Virol., 68, pp130-147.

Schneider, J., Gilbert, S.C., Blanchard, T.J., Hanke, T., Robson, K.J., Hannan, C.M., Becker, M., Sinden, R., Smith, G.L., Hill, A.V. 1998. Enhanced immunogenicity for CD8+T cell induction and complete protective efficacy of malaria DNA vaccination by boosting with modified vaccinia Ankara. Nat. Med., 4, pp397-402

Schulz, K.H. 1951. Rift Valley fever in South Africa. Special Report 5/51, Union Department of Health.

Schumperli, D., Lagadec, D., Muller, H.K. 1978. DNA sequence homology estimation by combinatorial analysis of endonuclease restriction data. J. Gen Virol., 38(1), pp161-166.

Shope, R.E., Peters, C.J., Walker, J.S. 1980. Serological relationship between Rift Valley fever virus and viruses of phlebotomus fever serogroup. Lancet, 1, pp886-887.

Smith, G.L. and Moss, B. 1983. Infectious poxvirus vectors have capacity for at least 25,000 base pairs of foreign DNA. Gene, 25, pp21-28.

Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol., 98, pp503-517.

Stephensen, C.B., Welter, J., Thaker, S.R., Taylor, J., Tartaglia, J., Paoletti, E. 1997. Canine distemper virus (CDV) infection of ferrets as a model for testing morbillivirus vaccine strategies: NYVAC- and ALVAC-based CDV recombinants protects against symptomatic infection. J. Virol., 71, pp1506-1513.

Strebel, K. and Beck, E. 1986. A second protease of foot-and-mouth disease virus. J. Virol., 58, pp893-899.

Struthers, J.K. and Swanepoel, R. 1982. Identification of a major non-structural protein in the nuclei of Rift Valley fever virus-infected cells. J. Gen. Virol., 60, pp381-384.

Sutter, G., Wyatt, L.S., Foley, P.L., Bennink, J.R., Moss, B. 1994. A recombinant vector derived from the host range-restricted and highly attenuated MVA strain of vaccinia virus stimulates protective immunity in mice to influenza virus. Vaccine, 12, pp1032-1040.

Swanepoel, R. and Coetzer, J.A.W. 1994. Infectious diseases of livestock. Volume I, p688-717. Editors: Coetzer, J.A.W., Thomson, G.R., Tustin, R.C. Oxford University Press.

Tartaglia, J., Pincus, S., Paoletti, E. 1990. Poxvirus-based vectors as vaccine candidates. Crit. Rev. Immunol., 10, p13.

Taylor, J., Edbauer, C.E., Rey-Senelonge, A., Bougue, J.F., Noroton, E., Goebel, S., Desmettre, P., Paoletti, E. 1990. Newcastle disease virus fusion protein expressed in a fowlpox virus recombinant confers protection in chickens. J. Virol., 64, pp1441-1450.

Taylor, J., Trimarchi, C., Weinberg, R., Languet, B., Guillemin, F., Desmettre, P., Paoletti, E. 1991. Efficacy studies on a canarypox-rabies recombinant virus. Vaccine, 9, pp190-193.

Taylor, J., Weinberg, R., Kawaoka, K., Webster, R.G., Paoletti, E. 1988. Protective immunity against avian influenza induced by a fowlpox virus recombinant. Vaccine, 6, pp504-508.

Thomson, G.R. 1994. Infectious diseases of livestock. Volume I, pp825-852. Editors: Coetzer, J.A.W., Thomson, G.R., Tustin, R.C. Oxford University Press

Tooze, J., Hollinshead, M., Reis, B., Radsak, K., Kern, H. 1993. Progeny vaccinia and human cytomegalovirus particles utilize early endosomal cisternae for their envelopes. Eur. J. Cell. Biol., 60, pp163-178.

Towbin, H., Staehelin, T., Gordon, J. 1979. Electrophoretic transfer of proteins from polyacryl gels to nitrocellulose sheets: procedure and some applications. PNAS, 76, pp4350-4354.

Tripathy, D.W., Hanson L.E., Crandell, R.A. 1981. Comparative diagnosis of viral diseases. Volume 3, pp267-346. Editors: Kurstak, E. and Kurstak, C. London Academic Press.

Turell, M.J., Saluzzo, F., Tammariello, R.F., Smith, J.F. 1990. Generation and transmission of Rift Valley fever viral reassortants by the mosquito culex pipiens. J. Gen. Virol., 71, pp2307-2312.

Turner-Smith, K., Bingham, J., Van Dijk, A., Cox, D., Williamson, A-L. 1999. Evaluation of the immune response induced in non-ruminant hosts by a rabies virus – lumpy skin disease virus recombinant. Abstract. XIth International Congress of Virology, pp212.

Vialat, P., Muller, R., Vu, T.H., Prehaud, C., Bouloy, M. 1997. Mapping of the mutations present in the genome of Rift Avlley fever virus attenuated Mp12 strain and their putative role in attenuation. Virus Res., 52, pp43-50.

Van Den Ende, M., Alexander., R.A., Don, P., Kipps, A. 1948. Isolation in chick embryos of a filterable agent possibly related etiologically to lumpy skin disease of cattle. Nature (London) 161, p526

Van Rooyen, P.J., Kumm, N.A.L., Weiss, K.E., Alexander, R.A. 1959. A preliminary note on the adaptation of a strain of lumpy skin disease virus to propagation in embryonated eggs. Bull. Epizoot. Dis. Afr., 7, pp79-85.

Van Velden, D.J.J., Meyer, J.D., Oliver, J., Gear, J.H.S., McIntosh, B. 1977. Rift Valley fever affecting humans in South Africa. S. A. Med. J., 51, pp867-871.

Volpina, O.M., Surovoy, A.Y., Zhmak, M.N., Kuprianova, M.A., Koroev, D.O., Chepurkin, A.V., Toloknov, A.S., Ivanov, V.T. 1999. A peptide construct containing B-cell and T-cell epitopes from the foot-and-mouth disease viral VP1 protein induces efficient antiviral protection. Vaccine, 17, pp577-584.

Volpina, O.M., Yarov, A.V., Zhmak, M.N., Kuprianova, M.A., Chepurkin, A.V., Toloknov, A.S., Ivanov, V.T. 1996. Synthetic vaccine against foot-and-mouth disease based on a palmitoyl derivative of the VP1 protein 135-159 fragment of the A22 virus strain. Vaccine, 14, pp1375-80.

Vos, J.C., Sasker, M., Stunnenberg, H.G. 1991. Promoter melting by a stage-specific vaccinia virus transciption factor is independent of the presence of RNA polymerase. Cell, 65, pp105-114.

Vos, J.C., Stunnenberg, H.G. 1988. Derepressionof a noval class of vaccinia virus genes upon DNA replication. EMBO J., 7, pp3487-3492.

Vosloo, W., Knowles, N.J., Thomson, G.R. 1992. Genetic relationship between South African SAT 2 isolates of FMDV. Epiderm. Infect., 109, pp547-558.

Wade-Evans, A.M., Romero, C.H., Mellor, P., Takamatsu, H., Anderson, J., Thevasagayam, J., Fleming, M.J., Mertens, P.P.C., Black, D.N. 1996. Expression of the major core structural protein (VP1) of bluetongue virus, by a recombinant capripox virus, provides partial protection of sheep against a virulent heterotypic bluetongue virus challenge. Virology, 220, pp227-231.

Wallace, D.B. 1994. MSc thesis. Genomic characterisation of southern African isolates of capripox and avipox viruses. University of Cape Town, South Africa

Ward G., Rieder E., Mason P.W. 1997. Plasmid DNA encoding replicating foot-and-mouth disease virus genomes induces antiviral immune response in swine. J. Virol., 71(10), pp7442-7447.

Wei, C.M. and Moss, B. 1975. Methylated nucleotides block 5'- terminus of vaccinia virus mRNA. Proc Natl. Acad. Sci. USA, 72, pp318-322.

Weir, J.P and Moss, B. 1987. Determination of the promoter region of an early vaccinia virus gene encoding thymidine kinase. Virology, 158, pp206-210.

Weiss, K.E. 1963. Lumpy skin disease. Emerging diseases of animals. FAO Agricultural Studies, 61, pp179-201.

Weiss, K.E. 1968. Lumpy skin disease virus. Virology Monographs, 3, pp111-131.

Weddell, G.N., Yansura, D.G., Dowbenko, D.J., Hoatlin, M.E., Grubman, M.J., Moore, D.M., Kleid, D.G. 1985. Sequence variation in the gene for the immunogenic capsid protein VP1 of foot-and-mouth disease virus type A. Proc. Natl. Acad. Sci. USA, 82, pp2618-2622.

Westwood, J.C.N., Harris, W.J., Zwartouw, H.T., Titimus, D.N.J., Appleyard, G. 1964. Studies on the structure of vaccinia virus. J. Gen. Microbiol., 34, pp67-78.

Wyatt, L.S., Shors, S.T., Murphy, B.R., Moss, B. 1996. Development of a replication-deficient recombinant vaccinia virus vaccine effective against parainfluenza viru 3 infection in an animal model. Vaccine, 14 (15), pp1451-1458.

Wyatt, L.S., Whitehead, S.S., Venanzi, K.A., Murphy, B.R., Moss, B. 2000. Priming and boosting immunity to respiratory syncytial virus by recombinant replication-deficient vaccinia virus MVA. Vaccine, 18, pp392-397.

Yamanouchi, K., Barrett, T., Kai, C. 1998. New approaches to the development of virus vaccines for veterinary use. Rev. Sci. Tech. Off. Int. Epiz. 17 (3) pp 642-653.

Young, E., Basson, P.A., Weiss, K.E. 1970. Experimental infection of game animals with lumpy skin disease virus (Prototype strain Neethling). Onderstepoort J. Vet. Res., 37, pp79-88.