An evaluation of the vaccine-vector potential of thymidine kinase-disrupted recombinants of lumpy skin disease virus (South African vaccine)

by

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Dedicated to the Lord,

May His will be done.

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SUMMARY

An evaluation of the vaccine-vector potential of thymidine kinase-disrupted recombinants of lumpy skin disease virus (South African vaccine).

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The aim of this study was to investigate the feasibility of developing the South African vaccine strain of the capripoxvirus, lumpy skin disease virus (LSDV), as a vector for recombinant vaccines to various diseases of veterinary importance in Africa using the viral thymidine kinase (TK) gene as the site of foreign gene insertion.

The first part of the study involved the development of a DNA transfer vector (pLSTK7.5) specific for the South African vaccine strain of LSDV containing a multiple cloning site, viral promoter and viral flanking sequences for the insertion of foreign genes (initially visual reporter genes, and subsequently genes from pathogenic viruses which are immunogenic) into the viral TK gene and for the expression of these genes leading to a protective immune response.

In order to evaluate the proposed recombination strategy, a visual marker gene, the *Escherichia coli* β–galactosidase gene (lacZ), was inserted into the multiple cloning site in pLSTK7.5 and a TK-deficient cell line of bovine kidney cells (BU100) was obtained. However, using the TK-negative selection strategy commonly used for selecting other poxvirus recombinants, it was impossible to recover stable LSDV recombinants. The strategy was then modified to include the *E. coli* guanine phosphoribosyl transferase (gpt)

positive selectable marker gene, which resulted in the selection of stable, homogeneous recombinants.
In order to improve the cloning and selection process, the pLSTK7.5 transfer vector was streamlined by the removal of extraneous sequences and the enhanced green fluorescent protein (EGFP) visual marker gene was introduced, giving rise to the new transfer vector, pLSEG.

The structural glycoprotein genes of bovine ephemeral fever virus (BEFV) and Rift Valley fever virus (RVFV), that encode proteins that can elicit protective immunity, were inserted separately into the pLSEG transfer vector and recombinants were generated and selected for homogeneity.
Expression of the glycoproteins under control of the early/late vaccinia virus P7.5K promoter was shown using immunofluorescence and the ability of the recombinants to induce both humoral and cell-mediated immune responses was demonstrated.
In protection studies, the LSDV-BEFV recombinant construct was unable to provide effective protection to cattle against virulent BEFV challenge most probably due to an over-challenge of virulent virus, although high levels of neutralising antibodies were produced which serve as an indicator for protection, whereas the LSDV-RVFV recombinant conferred complete protection to mice and at least partial protection to sheep. An attempt to demonstrate the dual protective nature of the vaccine against sheeppox virus in sheep was unsuccessful as the sheep failed to react to the challenge strain of sheeppox virus.

The results of this study indicate that the South African vaccine strain of LSDV shows good potential as a vector for recombinant vaccines using the viral TK gene as the site for foreign gene insertion.

Keywords: poxvirus, recombinant, lumpy skin disease, capripoxvirus, vaccine vector, homogeneity, thymidine kinase, lacZ, selection

ABBREVIATIONS USED IN TEXT:

A	adenine
ATCC	American type cell collection
ATP	adenosine triphosphate
BEF	bovine ephemeral fever
BEFV	bovine ephemeral fever virus
BEM	Basal Eagle's Medium
bp	base pair
BTV	bluetongue virus
BUdR	5-bromo-2'-deoxy-uridine
C	cytosine
CAM	chorioallantoic membrane
°C	degrees Celsius
CEF	chicken embryo fibroblast
CFK	calf foetal kidney
cm	centimetre
CO_2	carbon dioxide
cpe	cytopathic effect
Da	Dalton
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
EDD	Exotic Diseases Department
EDTA	ethylene diamine tetra-acetic acid
EGFP	enhanced green fluorescent protein
ELISA	enzyme-linked immunosorbant assay
EtBr	ethidium bromide
EtOH	ethanol
F	fusion
FBT	foetal bovine testes
FCS	foetal calf serum
ffu	focus forming units
FITC	fluorescein isothiocyanate
g	gram or gravitational force
G	guanine
gfp	green fluorescent protein
GP	glycoprotein

gpt	guanine phosphoribosyl transferase
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HI	haemagglutination inhibition
I-ELISA	indirect enzyme-linked immunosorbant assay
IF	immunofluorescence
IgG	immunoglobulin G
ID	intradermal
IM	intramuscular
IP	intraperitoneal
IV	intravenous
k	kilo
kbp	kilobase pair
kb	kilobase
KC	Kenya cattle
kDa	kiloDalton
kg	kilogram
KS	Kenya sheep
lacZ	β-galactosidase
LSD	lumpy skin disease
LSDV	lumpy skin disease virus
M	Molar
MCS	multiple cloning site
MDBK	Madin Darby bovine kidney
mg	milligram
µg	microgram
µl	microlitre
µM	micromolar
mA	milliamperes
ml	millilitre
mM	millimolar
mmol	millimoles
MOI	multiplicity of infection
MPA	mycophenolic acid
mRNA	messenger RNA
MVA	modified vaccinia Ankara
MW	molecular weight
N	normal
nAb	neutralising antibody
NaCl	sodium chloride

ng	nanograms
nm	nanometer
NaOH	sodium hydroxide
OBP	Onderstepoort Biological Products
O/N	overnight
OD	optical density
ORF	open reading frame
OVI	Onderstepoort Veterinary Institute
PCR	polymerase chain reaction
PBMC	peripheral blood mononucleocytes
PBS	phosphate buffered saline
pfu	plaque forming units
pH	negative $\log_{10}$ of the hydrogen concentration
pi	post infection or post inoculation
PP	percentage positive
PPRV	peste des petits ruminants virus
R.E.	restriction enzyme
rLSDV	LSDV recombinant
RNA	ribonucleic acid
rpm	revolutions per minute
RPV	rinderpest virus
RR	ribonucleotide reductase
RT	room temperature
RVF	Rift Valley fever
RVFV	Rift Valley fever virus
SC	subcutaneous
SDS	sodium dodecyl sulphate
SI	stimulation index
Sn	supernatant
SN	serum neutralisation
T	thymine
TAE	Tris acetate EDTA
TE	Tris EDTA
TK	thymidine kinase
Tris	Tris-(hydroxymethyl)-aminomethane
tRNA	transfer RNA
U	units or uracil
UV	ultra-violet
V	volts

VN	virus neutralisation
VV	vaccinia virus
v/v	volume per volume ratio
wt	wild type
wtLSDV	wild type LSDV
w/v	weight per volume ratio
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactoside

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CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION TO POXVIRUSES

Poxviruses are large DNA-containing viruses which infect both vertebrate and invertebrate hosts and were the first viruses to be visualised by light microscopy (as reviewed by Moss, 2001). An important characteristic of poxviruses is their ability to replicate in the cytoplasm of infected cells. The family *Poxviridae* is divided into two subfamilies, namely the *Chordopoxvirinae* (vertebrate poxviruses) and the *Entemopoxvirinae* (insect poxviruses). These in turn consist of a number of genera (Table 1.1). The *Orthopoxvirus* genus contains the two most well-known poxviruses; variola, which was the causative agent of smallpox and its benign relative, vaccinia virus, which was used successfully as a vaccine against variola and played an important role in the global eradication of smallpox. However, laboratory stocks of variola virus still exist and the potential threat of its use in bio-terrorism has resulted in renewed preparations for mass-vaccination programmes, especially in the USA. Avipoxviruses have been isolated from many avian species and the type member, fowlpox virus, has caused considerable economic losses to the poultry industry (as reviewed by Ghildyal *et al.*, 1989). Capripoxviruses, infecting the livestock species of cattle, sheep and goats, are also economically important (as reviewed by Hunter and Wallace, 2001; Gershon and Black, 1988; Rao and Bandyopadhyay, 2000).

In southern Africa a number of members of the *Poxviridae* family occur. Lumpy skin disease virus (LSDV) of cattle is the only member of the *Capripoxvirus* genus known to occur in South Africa (Weiss, 1968), although all three members of the genus (including sheeppox and goatpox virus) are found throughout the rest of Africa. Avipoxviruses have been isolated from a number of species of southern African birds (Kow, 1992).

Besides vaccinia virus, many poxviruses have been attenuated by serial passage in either cell cultures or on the chorioallantoic membranes of embryonated chicken eggs to serve as live attenuated vaccines (Van Rooyen *et al.*, 1969; Kirmse, 1969; as cited by Taylor and Paoletti, 1988; Winterfield and Reed; 1985).

The development of a recombinant vaccinia virus provided a new strategy for vaccine development (Sam and Dumbell, 1981). Foreign genes could be inserted into non-essential regions of the poxvirus genome using naturally occurring homologous recombination. Expression of the genes, under control of poxvirus promoters, was obtained and it was shown that protective immune responses could be elicited against the expressed proteins. Other

TABLE 1.1 : CLASSIFICATION OF POXVIRUS GENERA*

SUBFAMILIES	GENERA	SELECTED MEMBER VIRUSES
CHORDOPOXVIRINAE (vertebrate poxviruses)	ORTHOPOXVIRUS	vaccinia, variola
	AVIPOXVIRUS	fowlpox, canarypox
	CAPRIPOXVIRUS	lumpy skin disease, goatpox, sheeppox
	PARAPOXVIRUS	orf
	LEPORIPOXVIRUS	shope fibroma
	SUIPOXVIRUS	swinepox
	YATAPOXVIRUS	tanapox
	MOLLUSCIPOXVIRUS	Molluscum contagiosum
ENTEMOPOXVIRINAE (insect poxviruses)	A	*Melontha melontha*
	B	*Amsacta moori*
	C	*Chironimus luridus*

(* - adapted from Moss, 2001)

poxviruses were also then developed as vectors for recombinant vaccines, especially due to concerns arising from complications experienced with vaccinia virus in immunocompromised persons and its broad host-range (as cited by Cadoz *et al.*, 1992). Avipoxviruses and capripoxviruses have been investigated as alternatives (Taylor *et al.*, 1988; Cadoz *et al.*, 1992; Romero *et al.*, 1993), as well as highly attenuated strains of vaccinia virus which are missing a number of genes, especially those associated with virulence and host-range (Scheiflinger *et al.*, 1998). Avipoxviruses have found tremendous favour as they are even

able to express foreign antigens in mammalian cells though unable to complete a productive infection cycle (Taylor and Paoletti, 1988; Stannard *et al.*, 1998).

Capripoxviruses, and in particular strains of LSDV, are being investigated as vaccine vectors for use in the veterinary field for a number of reasons: they are highly host-range restricted (Weiss, 1968), stable (Wallace, 1994), and a number of effective attenuated vaccines have been developed from virulent field isolates (Van Rooyen *et al.*, 1969).

Where they are to be used as vectors for recombinant vaccines in animals which they naturally infect, they also have potential as dual vaccines: a northern African vaccine strain of LSDV (Kenya Sheep-1) expressing either the fusion protein or haemagglutinin protein of rinderpest virus protected cattle against both virulent rinderpest and LSDV challenge (Ngichabe *et al.*, 1997).

Although recent sequence data comparisons have shown that a virulent northern African isolate of LSDV and a virulent southern African isolate share a high degree of sequence homology (Kara *et al.*, 2003), it has been reported that northern African isolates are able to infect more than one ruminant host species (Kitching *et al.*, 1987; Kitching *et al.*, 1989; Gershon and Black, 1989a), whereas there have been no reports of natural capripoxvirus infections in any animals in southern Africa besides cattle (Weiss, 1968). Although the northern African vaccine strains of LSDV have proven safe and effective thus far, there are no guarantees that under the right conditions they might not be able to revert to virulence and infect other ruminant species. There is evidence that at least one isolate of capripoxvirus was formed by a recombination event during its evolutionary history (Gershon *et al.*, 1989b). For these reasons it is inadvisable to use northern African isolates of LSDV as a vector for recombinant vaccines in southern Africa.

Restriction endonuclease studies on the DNA of a number of southern African isolates of LSDV collected over a 40 year period from different geographical locations have shown that the isolates are stable and highly conserved (Wallace, 1994). Thus, the high degree of host-range specificity of the southern African isolates of LSDV makes them prime candidates for development as vectors for recombinant vaccines for use in southern Africa, as well as in other countries where use of a highly host-range restricted vector is a necessity.

The southern African vaccine strain of LSDV was developed from a virulent field isolate and has been in use for over 40 years (Van Rooyen *et al.*, 1969). This highly attenuated strain has proven safe and effective and provides long-term immunity, although the original assertion that protection is life-long has been challenged recently and more frequent vaccinations are now recommended (as reviewed by Weiss, 1968; Hunter and Wallace, 2001).

1.2 AIMS OF THIS INVESTIGATION

In lieu of the need for alternative vectors for poxvirus-vectored recombinant vaccines, the high degree of host-range restrictiveness, the potential for use as a dual vaccine and long history of safe and effective use in the field, the South African vaccine strain of LSDV is the obvious choice for development as a vector for recombinant vaccines for use in the veterinary field in southern Africa. In the late 1980's work began in this regard. A number of the earlier studies concentrated on mapping the virus (Perlman, 1993), the use of selectable marker genes for recombinant virus selection (Brand, 1993), identification of unique restriction enzyme sites as potential insertion sites (Wallace, 1994) and identification of potential non-essential genes or intergenic regions as insertion sites (Cohen and Cox, personal communication). More recently Fick (1998) studied transcriptional control and cloned and characterised a bi-directional LSDV promoter (Fick and Viljoen, 1994; Fick and Viljoen, 1999).

The results of these studies provided essential information for developing the virus as a vector. Two independent studies were then initiated to investigate the best route for generating and selecting recombinants. The first study (undertaken at the University of Cape Town's Medical School) concentrated on the use of the ribonucleotide reductase gene as the insertion site and the *Escherichia coli* guanine phosphoribosyltransferase (gpt) gene for dominant selection (Aspden *et al.*, 2002).

The second study, which forms the basis for this doctoral thesis, undertook to investigate the feasibility of using the viral thymidine kinase (TK) gene as the insertion site and on the use of the resulting thymidine kinase-negative phenotype of the virus as the means for recombinant virus selection. The aim of this study was thus as follows:

To evaluate LSDV (type SA vaccine) as a vector for recombinant vaccines against economically important livestock diseases using the viral TK gene as the insertion site.

However, in order to achieve this aim a number of areas had to be addressed, as follows:

(a) Develop a LSDV transfer vector suitable for the insertion of foreign genes into the viral thymidine kinase gene.

(b) Obtain, or develop, a LSDV-permissive cell line suitable for use in thymidine kinase selection of recombinant viruses.

(c) Develop and evaluate a suitable method for generating and selecting recombinants.

(d) Select the recombinants to homogeneity and evaluate stability.

(e) Generate recombinants expressing genes of veterinary importance.

(f) Evaluate the recombinants in animals for their ability to induce a protective immune response.

By way of introduction, the following literature survey provides a general overview of poxvirus biology, with special reference to all aspects of LSDV and the development of poxviruses as vectors for recombinant vaccines.

1.3 BIOLOGY OF POXVIRUSES

Vaccinia virus has been extensively characterised and thus serves as the best model to describe general poxvirus characteristics.

1.3.1 Virion structure

Vaccinia virions appear by electron microscopy to be oval or brick-shaped bodies approximately 300-400 x 170-260 nanometers (nm) (Westwood *et al.*, 1964). Early studies of electron microphotographs of vaccinia virus revealed the presence of two types of particles; the one form, thought to be the complete form, consisted of a nucleoid with surrounding envelope material and the other, incomplete form, consisted of envelope material with or without nucleoid components (Nagington and Horne, 1962). Thin-sections of virions reveal a lipoprotein bilayer, called the outer membrane, surrounding a central core that encases the viral DNA. The core is surrounded by a palisade layer of rod-shaped molecules (Dales and Pogo, 1981) (Figure 1.1). In vertebrate poxviruses, the core appears biconcave with two structures, called lateral bodies, nestled in the concavities. The lateral bodies are attached to the outer membrane and are ellipsoidal in shape (Medzon and Bauer, 1970). The outer surface of the outer membrane is studded with randomly arranged surface tubule elements (MW 58,000) which give the virion its textured surface (Dales, 1963; Stern and Dales, 1976). Vaccinia virus particles released naturally from cells are called extracellular enveloped virions as they contain an additional lipid-bilayer, called the envelope (Roos *et al.*, 1996). The outer membrane and envelope contain a number of virus-encoded proteins, some of which are important in eliciting an immune response in infected hosts (Payne and Kristensson, 1985).

1.3.2 Genomic structure and organisation

Poxvirus genomes consist of a linear, double-stranded DNA molecule which is covalently cross-linked at the terminals (Berns and Silverman, 1970; Gershelin and Berns, 1974; Black *et al.*, 1986). These terminal sequences have been shown to contain repeat sequences which are inverted with respect to one another (Wittek *et al.*, 1978). The inverted terminal repeats of the vaccinia virus genome (strain WR) are each 10 kilobase pairs (kbp) in length (Baroudy *et al.*, 1982; Winters *et al.*, 1985). The termini are incompletely based-paired (bp) giving rise to 104 bp inverted loops which are A-T rich. The termini consist of two sets of 70 bp tandem direct repeats. The first set, consisting of 13 direct repeats, is disrupted by a 325 bp region of unique sequence, and is then followed by the second set of 18 direct repeats.

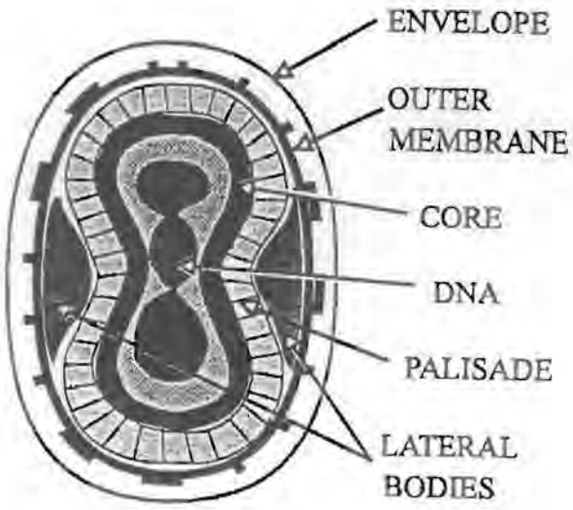


Figure 1.1 Schematic representation of a poxvirus virion. (Adapted from Dales and Pogo, 1981)

The genomes of poxviruses are large. The genome length of vaccinia virus (Copenhagen) is 191.6 kilobases (kb), potentially coding for 263 proteins greater than 65 amino acids in length (Goebel *et al.*, 1990). At least 30 of these are structural proteins, and the majority are viral enzymes concerned with nucleic acid synthesis and processing (e.g. a multisubunit DNA-

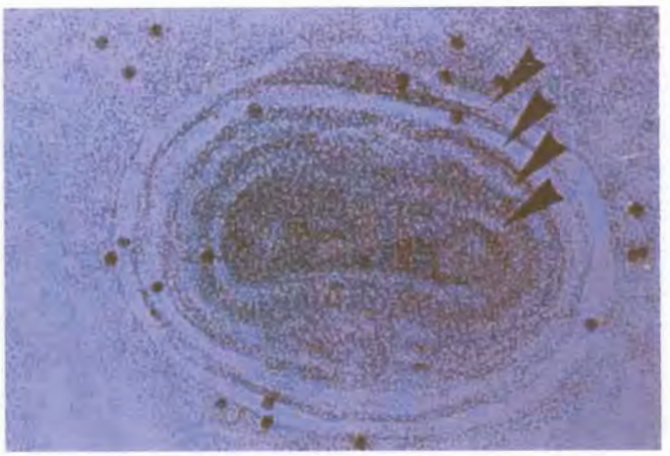


Figure 1.2. Electronphotomicrograh of a cryosection through a vaccinia virus EMV (x 80 000) (from Schmelz *et al.*, 1994). Arrowheads indicate membranous layers.

dependant RNA polymerase and a DNA polymerase) (Broyles and Moss, 1986; Challberg and Englund, 1979).

To date 26 complete genome sequences of various poxviruses have been elucidated (Gubser *et al.*, 2004). Chordopoxvirus genomes were found to be considerably more divergent toward their termini than in their central regions. Host-range, virulence and genes associated with interactions with the host immune systems have been mapped towards the termini of poxvirus genomes whereas genes coding for structural proteins and enzymes are placed more centrally (Gillard *et al.*, 1985; Moss, 2001).

1.3.3 Virus cell entry

Electron micrographs revealed attachment of enveloped and non-enveloped vaccinia virus to the plasma membrane at the surface of the cell (Dales and Kajioka, 1964). A number of mechanisms have been proposed for virus cell entry. The results of morphological studies suggest that vaccinia may obtain entry by endocytic mechanisms and by fusion of the viral envelope with the host plasma membrane. Experiments using methylamine and chloroquine [lysomotropic agents (weak bases which have the ability to raise the pH of endosomes, thus blocking proteolysis)] showed that these agents increased the *infectivity of vaccinia virus* (Janeczko *et al.*, 1987). Subcellular fractionation studies also revealed the absence of viral polypeptides from endosomes, whereas an abundance of these proteins co-migrated with the plasma membrane fraction. Since plaque formation is not inhibited by lysomotropic agents, pH-independent fusion with the plasma membrane may be the most important entry

mechanism. The exact mechanism of entry now appears to rely upon the type of virion particle: IMVs entering via surface membrane fusion and EEVs by fusion and entry in endosomes with the loss of the outer membrane (Vanderplasschen *et al.*, 1998).

Once inside the cell, the virus undergoes at least two discrete stages of disassembly (Pedley and Cooper, 1987): The first stage is associated with the loss of virion coat proteins and lipids, although the genome is still protected within the core (Holowczak, 1972; Sarov and Joklik, 1972). During the second stage the genome becomes accessible to DNase. A putative, viral-encoded uncoating protein of 23 kDa with trypsin-like activity is thought to be involved in uncoating (Pedley and Cooper, 1987).

1.3.4 Gene expression

Almost immediately after entering the cell, and after uncoating, transcription takes place within the core particles producing functional and capped polyadenylated messenger RNA (mRNA) (Brakel and Kates, 1974). ATP is required for the extrusion of transcripts into the cytoplasm where they bind ribosomes for translation. RNA-DNA hybridisation studies revealed that about one-half of the genome is transcribed prior to DNA replication (review by Moss, 2001). Transcription factors have been identified which bind to, and initiate transcription from viral promoters (Li and Broyles, 1993). Although the large genomes are able to code for most of the enzymes required for gene expression, the host cell nucleus is required for the production of infectious virions (Yuwen *et al.*, 1993). Vaccinia virus encoded proteins have been located in the nucleus and one of these (E3L gene product) is able to bind double-stranded RNA. Viral induction of the synthesis of host factors has been reported (Moss and Filler, 1970). These factors are thought to be required for the translation of viral mRNA. During the infection cycle, however, the virus eventually causes the complete inhibtion of host protien synthesis.

Vaccinia virus gene expression is temporal, with classes of early, intermediate and late genes being identifiable (Moss, 2001). Early genes are expressed from 20-100 minutes post infection, intermediate genes from 100-120 minutes post infection and after the onset of DNA replication, whereas late genes are expressed only after replication (140 minutes post infection) (Baldick and Moss, 1993). A number of regulatory mechanims, controlling the switch from early to late gene expression, have been proposed (Moss, 2001). One of these suggests the involvement of regulatory factors which might suppress early gene transcription by binding to transcription factors or early gene promoters (McAuslan, 1963). In addition, early and intermediate gene transcripts have short half lives (30 minutes) providing the basis

for rapid changes in gene expression (Baldick and Moss, 1993). Early protein synthesis is terminated once late gene products are being synthesised, unless the early genes contain late promoter sequences in addition to their early promoters (Cochran *et al.*, 1985). Late gene products consist mainly of structural proteins and enzymes to be packaged within the progeny virions.

The functional elements of promoter sequences are conserved between poxvirus genera; evidence of this is provided by studies demonstrating the ability of the TK promoter of fowlpox virus to function in a recombinant vaccinia virus and of vaccinia virus promoters to function in a recombinant fowlpox virus (Coupar *et al.*, 1990; Prideaux *et al.*, 1990).

A class of early promoters has been identified (Davison and Moss, 1989). They are A-T rich and contain a critical region for activity which corresponds to the downstream site of transcription initiation (AAAAATGAAAAAAAA). Probably the most important enzyme required for transcription is the vaccinia virus RNA polymerase and subunits (Broyles and Pennington, 1990). Another important gene is the DNA topoisomerase (Fogelsong and Bauer, 1984). Inhibition of the activity of this enzyme results in inhibition of transcription of viral genes from intact core particles. Vaccinia virus also codes for an early transcription factor (VETF) which consists of two subunits (70 and 83 kDa) and has ATPase activity required for early gene transcription (Broyles *et al.*, 1988; Broyles and Moss, 1988; Li and Broyles, 1993). An 85 kDa protein (called RAP94) has been identified which is tightly associated with the RNA polymerase, and along with the VETF is important for transcription of early genes.

The 5' ends of early mRNA transcripts are capped by a viral-encoded capping enzyme (Moss *et al.*, 1976) and the 3' ends are polyadenylated by a poly(A) polymerase (Nevirs and Joklik, 1977). A transcription termination factor (VTF) was found associated with the capping enzyme (Shuman *et al.*, 1987). Termination signals (TTTTTNT) were discovered 20 to 50 bp upstream of the transcription termination sites (Yuen and Moss, 1987).

Expression of these genes is dependant upon DNA replication and requires transcription factors coded for by early genes (Wright and Moss, 1989). There appear to be two classes of intermediate genes, viz. early intermediate (consisting of at least two genes) (Vos and Stunnenberg, 1988) and true intermediate (consisting of three genes coding for late gene transcription factors; A1L, A2L and G8R) (Baldick and Moss, 1993). The promoter sequences of the true intermediate genes have been found to contain two critical regions for gene expression (AAATAA and TAAA) (Baldick *et al.*, 1992).

Late gene expression is dependant on replication of the viral genome (Belle *et al.*, 1981) and translation of the three late gene transcription factors. Most late genes occur in the centre region of the genome and code for structural proteins and many of the enzymes destined for packaging within progeny virions. There are two classes of late genes; one which is expressed immediately after DNA replication and the other which is delayed. The promoter sequences of late genes consist of a critical region with the consensus sequence: TAAATG (Weir and Moss, 1984).

A major difference between early and late mRNA transcripts is that late transcripts do not have defined 3' ends and continue downstream through early genes, not recognising early gene termination sequences (Mahr and Roberts, 1984). The 5' capped ends of the late mRNAs also differ from those of early transcripts (Boone and Moss, 1977) and include poly(A) tracts which are thought to be important for ribosomal binding (Ahn and Moss, 1989).

Vaccinia late transcription factor (VLTF-1), VETF and RNA polymerase have been identified in cell extracts purified late in the infection cycle.

Proteolytic maturation of some vaccinia virus proteins (e.g. late gene core proteins 4a, 4b and 25K) occurs via cleavage of precursor polypeptides (Van Slyke *et al.*, 1991). A conserved "cleavage signal" sequence (Alanine-Glycine-Alanine) has been identified.

For LSDV not much work has been performed on gene expression. Sequence comparisons have revealed that the overall genomic organisation is very similar to that of other chordopoxviruses such as suipoxvirus, yatapoxvirus and leporipoxvirus (Tulman *et al.*, 2001). What has been established is that the synthesis of early LSDV mRNA is initiated immediately after infection and continues for nine hours. The transition to late gene transcription occurs approximately 10 hours post-infection and requires DNA replication (Fick and Viljoen, 1994). The identification and characterisation of a LSDV bi-directional promoter showed substantial structural similarities with other poxvirus promoters (Fick and Viljoen, 1999). In transient expression assays the temporal nature of the promoter was verified.

1.3.5 DNA replication

Poxviruses replicate in the cytoplasm of infected cells (Dales and Pogo, 1981; Harford *et al.*, 1966). Most, or all of the genes required for DNA replication are encoded by the virus as it is still able to replicate in enucleated cells, although the nucleus is required for the production of

infectious particles (Pennington and Follet, 1974; Yuwen *et al.*, 1993). Replication begins within the first three hours after infection (Harford *et al.*, 1966). Discrete "virus factories" have been identified which are electron dense regions in the cytoplasm where the virus replicates (Moss, 2001). No specific origins of replication have been found in the genome and it is speculated that replication occurs via nicking of the DNA at random points, followed by self-priming and replication giving rise to large concatemeric-branched structures. These might be resolved into unit genomes at a later stage. Concatemeric forms have been isolated when inhibitors of late protein synthesis are added to infected cells. Resolution occurs upon reversal, however, indicating that the concatemers are replicative intermediates. A number of viral enzymes are required to facilitate the high levels of DNA synthesis. Two of these include the vaccinia virus single unit DNA polymerase (MW 110,000) (Challberg and Englund, 1979; Traktman *et al.*, 1984) and a DNA ligase (Moss, 2001). Another enzyme involved in replication is a thymidine kinase (TK) enzyme. It is 20 kDa in size and appears to exist as a tetramer. The TK is important for nucleotide metabolism and is thus needed early in infection, especially in non-replicating infected cells with a low level of thymidylic acid. Other viral enzymes important for replication include a ribonucleotide reductase, which converts ribonucleotides into DNA precursors and a serine/threonine kinase (Banham and Smith, 1992).

Recombination of poxvirus DNA has been linked to replication and could explain the considerable amount of variation in the terminal regions of poxvirus genomes (as reviewed by Buller and Palumbo, 1991). Genetic recombination between orthopoxvirus genomes has been well documented (Ball, 1987; Sam and Dumbell, 1981) and it is thought that a member of the *Capripoxvirus* genus arose by genetic recombination between two other members (Gershon *et al.*, 1989b). The ability of poxvirus genomes to recombine, and to replicate after regions of their genomes have been deleted and replaced with foreign DNA, has resulted in the development of recombinant vector vaccines (Taylor *et al.*, 1988; Cadoz *et al.*, 1992; Taylor *et al.*, 1992).

1.3.6 Virus assembly and release

Many of the assembly proteins undergo proteolytic processing prior to assembly. Assembly is initiated in the cytoplasm and the first step is the appearance of crescent-shaped shells with a border of "spicules" on the convex surface and granular material adjacent to the concave surface (Dales and Pogo, 1981). These crescent-shaped shells were thought to consist of a double lipid bilayer membrane derived from the intermediate compartment

between the cellular endoplasmic reticulum and the golgi stacks of the Golgi apparatus (Sodeik *et al.*, 1993; Griffiths *et al.*, 2001), however high resolution electron microscopy points to a single lipid bilayer membrane independent of intermediate compartments (Hollinshead *et al.*, 1999). The spicules (MW 65,000) are thought to provide a scaffolding for the viral assembly (Essani *et al.*, 1982). The immature envelope circularises and the nucleoproteins and DNA genome enter just before it is completely sealed (as reviewed by Buller and Palumbo, 1991). The immature virion then undergoes internal differentiation producing the core and lateral bodies. The surface spicules are replaced by surface tubular elements and the virion attains its brick-like morphological appearance. Mature virions are transported to the cell periphery either in vacuoles or bound in a double membrane (or envelope). There is much controversy over the origin of this second envelope as evidence exists to support an early endosomal origin (Tooze *et al.*, 1993) and an equal degree of evidence exists to support a trans Golgi network origin (Schmelz, 1994). A viral-encoded 42 kDa glycoprotein has been shown to be nesessary for this second enveloping event (Wolffe *et al.*, 1993). Virions may also become associated with a proteinaceous A-type inclusion body which is thought to protect the virus from the external environment (Ichihashi *et al.*, 1971). The virions are released from the cell either by fusion of the surrounding membrane with the plasma membrane or via budding from the cell surface, especially at sites on the microvilli.

1.3.7 Host interactions

In evolutionary terms poxviruses are ancient and due to their complexity it is not surprising that they have evolved various mechanisms to interact with their hosts including evading detection by the host's immune system (Gubser *et al.*, 2004).

Shortly after infection viral factors are responsible for shutting down host protein synthesis (Pedley and Cooper, 1984) and DNA replication (Jungwirth and Launer, 1968). Viral homologues of epidermal growth factor actually have a stimulatory effect on cell growth (Buller *et al.*, 1988). However, it is the effects of a broad class of poxviral proteins which help the virus to evade or suppress the host's immune system which have been receiving the most attention in recent years. Poxviruses code for homologues of cellular chemokines, chemokine receptors and intracellular proteins that are involved in signalling pathways and possibly anti-viral activities (Smith *et al.*, 2000; Cao *et al.*, 2002; Harte *et al.*, 2003; as reviewed by Johnston and McFadden, 2003). The proteins have been divided roughly into three classes: virostealth, virotransduction and viromimicry (Nash *et al.*, 1999). Virostealth is characterised by masking of the visible signals associated with virus infection, for example, by reducing the capacity of effector leukocytes to recognise and eliminate infected cells. Virotransducers are intracellular viral proteins that inhibit innate antiviral pathways, such as apoptosis,

proinflammatory cascades, or the induction of the antiviral state. Virotransducers can also target host signal transduction pathways that influence host range.

Viromimicry proteins consist of the virokines and viroreceptors, which are virus-encoded proteins which mimic host cytokines or their receptors respectively. These proteins block extracellular communication signals and promote a protected microenvironment for the virus within normally immuno-exposed tissues.

As the functions of these viral proteins have been elucidated, especially with the use of gene knockout technology (Johnston and McFadden, 2004), their potential for use in immunosuppressive medicine and other therapies has been realised (Dabbagh *et al.*, 2000; Anderson *et al.*, 2003; Essajee and Kaufman, 2004).

1.4 – LUMPY SKIN DISEASE VIRUS (LSDV)

1.4.1 History and epidemiology

In Zambia in 1929 a new disease of cattle was first noted which manifested itself as large skin nodules (Fig. 1.3) (Morris, 1931). The disease then spread rapidly north and southwards until by the late fifties it was well characterised throughout sub-Saharan Africa (including Madagascar) (von Backstrom, 1945; Thomas and Mare, 1945; Haig, 1957; Burdin and Prydie, 1959; MacOwan, 1959; Ramisse *et al.*, 1969; Nawathe *et al.*, 1978; as reviewed by Woods, 1988). By the late eighties lumpy skin disease (LSD) had spread via Egypt into the Middle East and was noted in an Arabian oryx in Saudi Arabia in 1989 (Ali *et al.*, 1990; House *et al.*, 1990; Abraham and Zissman, 1991; Davies, 1991; Khalafalla *et al.*, 1993; Greth *et al.*, 1992a; Yeruham *et al.*, 1995). The disease is of major economic importance and during one of the first large-scale outbreaks in South Africa, over 8 million cattle were affected with a mortality rate of over 75% (Diesel, 1949). Although more recent outbreaks generally show lower mortality rates, long-term debilitating effects are frequent (Green, 1959; Kitching el al., 1989).

Many attempts were made to isolate the causative agent of LSD. A number of different viruses were isolated from skin lesions on cattle displaying LSD-like symptoms (Alexander *et al.*, 1957; Haig, 1957) and eventually a virus belonging to the poxvirus family was identified as causing true LSD in cattle (Alexander *et al.*, 1957; Munz and Owen, 1966). One of the first purified isolates was a South African isolate named the Neethling isolate, and, thus the virus became known throughout Africa as lumpy skin disease virus (LSDV) (type-Neethling) (Alexander *et al.*, 1957; Weiss, 1963).

LSDV belongs to the *Capripoxvirus* genus (along with goatpox and sheeppox viruses) (Matthews, 1982). Although LSDV is a very recent disease of cattle, sheep and goatpox viruses have been known for centuries and their distribution was widespread, ranging throughout Europe, the Middle East, central Asia and northern Africa. Strict eradication programmes have since removed them from Britain and parts of Europe, and a number of live attenuated vaccine strains have helped control their spread in other regions, although they continue to cause significant economic losses (Kitching, 1983; Kitching, 1986a; Kitching, 1986b; Carn, 1993; Bhanuprakash *et al.*, 2004). Due to the close relatedness of capripoxviruses it has been suggested that they be referred to as one viral species with regional adaptations to specific host species. However, restriction endonuclease analysis, cross-hybridisation studies and, more recently, genomic sequence analysis reveals that the viruses can be distinguished from one another (Davies and Otema, 1981; Kitching and Taylor, 1985; Black *et al.*, 1986; Gershon and Black, 1988; Tulman *et al.*, 2002; Kara *et al.*, 2003).

The three viruses share a common major precipitating antigen (Kitching *et al.*, 1986) making them difficult to distinguish on the basis of serology alone (Davies and Otema, 1981), but, at the same time allowing for the use of heterologous virus for protection (Kitching and Taylor, 1985). For example, in Kenya an attenuated sheeppox virus isolate is used for controlling LSD in cattle (Coakley and Capstick, 1961; Carn, 1993). This phenomenon about LSDV raises some interesting questions as to its origins and natural host reservoirs. LSD is a relatively recent disease of cattle (Morris, 1931). In southern Africa LSDV has only been isolated from cattle and the sub-continent is free from goat and sheeppox viruses (Weiss, 1968). However, in parts of Africa all three capripoxviruses occur simultaneously and in Kenya isolates of LSDV have been recovered from both cattle and sheep (Davies, 1982 – with reference to Davies, 1976; Gershon and Black, 1987; Gershon and Black, 1989a). Serological surveys of game animals in Kenya and Tanzania detected antibodies to LSDV in buffalo (*Syncerus caffer*), greater kudu (*Tragelaphus strepsiceros*), waterbuck (*Kobus ellipsiprymnus* and *K. defassa*), reedbuck (*Redunca arundinum*), impala (*Aepyceros marsupialis*) and giraffe (*Giraffa camelopardalis*) (Davies, 1982; Hedger and Hamblin, 1983; Hamblin *et al.*, 1990; as cited by Greth *et al.*, 1992a). However, it took until 1989 for the first isolation of LSDV from a naturally infected game animal, namely the Arabian oryx (*Oryx leucoryx*) (although antibodies had been detected in captive animals during an earlier survey) (Greth *et al.*, 1992a; Greth *et al.*, 1992b). It is not surprising that this took so long as diseased animals would prove easy prey for large predators such as lions or leopards. Experimental infection of game animals by Young *et al.* (1970) showed a high susceptibility of giraffe and impala to LSDV, whereas buffalo and wildebeest (*Connochaetes gnou*) showed no adverse effects, suggesting their possible role as long-term maintenance hosts.

1.4.2 Diagnosis

Diagnosis of LSD is not always easy as the symptoms often resemble those caused by bovine herpes mammalitis virus (originally called "Allerton virus") (Haig, 1957; Burdin, 1959; Capstick, 1959; Prydie and Coakley, 1959; Davies *et al.*, 1971; Woods *et al.*, 1996; as reviewed by Hunter and Wallace, 2001). A pyrexia of 40.0-41.5 °C often occurs quickly after infection, lasting anywhere from one to 10 days (Davies, 1991). This is accompanied by lachrymation, increased nasal and pharyngeal secretions, anorexia, dysgalactia, depression and a disinclination for moving. Within a couple of days large nodules (5-50 mm in diameter) appear on the skin. These may cover the entire body, but this is not always the case (Carn and Kitching, 1995a). Regional lymph nodes become enlarged and may be oedematous. Lesions may also develop in the larynx and trachea, throughout the alimentary tract and especially in the abomasums, where they eventually become necrotic and ulcerate. Mucopurulent nasal discharges, persistent dribbling of infected saliva, coughing and distressed respiration, inflammatory and oedematous swellings of the limbs and genitalia often result and conjunctivitis and keratitis commonly occurs. The skin lesions also become necrotic, and, while some remain *in situ*, where they are recognizable for at least a year, others slough away to leave holes in the skin. This is where the risk of secondary bacterial infection becomes high. Lesions on the udder and teats may cause a mastitis and oedema with secondary infections and can result in sloughing of mammary tissue. Pneumonia is a common and often fatal complication of LSD (de Boom, 1947). Abortion frequently follows the acute infection in cows, and infertility has been a problem in the months succeeding an outbreak. Bulls may have painful lesions of their genitalia, which can prevent them from mounting females and they may remain infertile for four to six months after onset of disease symptoms.

Virus can be isolated from the skin lesions, semen, nasal, lachrymal and pharyngeal secretions, milk and blood (Thomas and Mare, 1945; Henning, 1949; Weiss, 1968; Irons *et al*; 2005). Electron microscopy of skin biopsy samples (Figure 1.4), growth in cell culture and fluorescent antibody staining are just some of the techniques routinely used to detect the presence of virus and antibodies in infected animals (Plowright and Witcomb, 1959; De Lange, 1959; Davies *et al.*, 1971; Prozesky and Barnard, 1982; Binepal *et al.*, 2001). More recently molecular-based tests, including Western-blot analysis, indirect enzyme-linked immunosorbant assays (I-ELISAs) and a diagnostic PCR test have been developed for detecting capripoxviruses allowing for more sensitive and specific detection of viral antibodies and DNA respectively (Chand *et al.*, 1994; Carn *et al.*, 1994a; Carn, 1995; Ireland and Binepal, 1998; Heine *et al.*, 1999; Markoulatos *et al.*, 2000; Hosamani *et al.*, 2004).

Figure 1.3 Young calf showing undisrupted lumps due to lumpy skin disease virus infection (Photograph by D. Wallace).

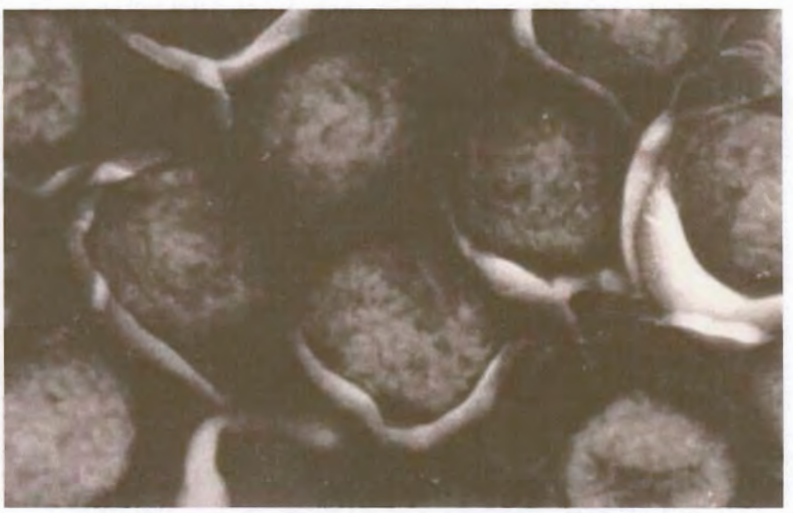


Figure 1.4 Electronphotomicrograph of clustered lumpy skin disease virus particles (x 75 000). (Photograph kindly supplied by L.M. Stannard, Medical School, University of Cape Town)

1.4.3 Transmission

During the first outbreaks of LSD in South Africa in 1944 there was speculation that the transport of cattle was responsible for the spread of the disease, however, once watercourses were reached it spread rapidly along low-lying areas (Thomas and Mare, 1945), suggesting that insects might also be playing an important role. However, many new outbreaks of LSD in sub-Saharan Africa have been related to cattle movements both within a country and from one country to another (Davies, 1991). The introduction of LSD to Egypt followed the importation of cattle from Somalia, where there had been recent outbreaks of LSD. Infected animals might not have shown clear symptoms of the disease, thus avoiding detection by inspectors.

Weiss (1968) described the isolation of virus from the biting fly, *Stomoxys calcitrans* and from *Biomyia fasciata*. Natural capripox infections occur throughout the endemic areas as a result of contact between diseased and susceptible animals (Davies, 1976), however, transmission studies suggest that the most prevalent method for the spread of LSD is mechanical transmission by insects such as *S. calcitrans* (Kitching and Mellor, 1986; Carn and Kitching, 1995b; as reviewed by Carn, 1996), and *Aedes aegypti* mosquitoes (Chihota *et al.*, 2001). Mosquitoes that had fed upon lesions of LSDV-infected cattle were able to transmit virus to susceptible cattle over a period of two to six days post-infective feeding. Virus was isolated from the recipient animals in five out of seven cases. The clinical disease recorded in the animals exposed to the infected mosquitoes was generally of a mild nature, with only one case being moderate.

The outbreak of LSD in Israel in 1989 is thought to have been caused by the aerial spread of LSDV-infected *S. calcitrans* from foci of the disease at El Arish in northern Sinai, or at Ismailiya and the Nile delta in Egypt, a minimum distance of over 85 kms (Yeruham *et al.*, 1995). Epizootics of LSD are associated with high temperatures and high humidity. Cold weather reduces its rate of spread, whereas in warm, moist conditions the disease spreads rapidly (Kitching and Mellor, 1986).

However, not all mechanical insect transmission studies have successfully shown transmission from infected to susceptible cattle suggesting that the other mechanisms might also be involved (Chihota *et al.*, 2003). Attempts to obtain transmission by biting lice (*Hydrotea irritans*) and suckling lice (*Damalina* spp.) were also unsuccessful (Kitching and Mellor, 1986). Carn and Kitching (1995a) attempted to obtain infection by inoculation of LSDV onto the conjunctival sac of susceptible British cattle – however, this also failed. Intradermal inoculation produced local lesions in 80% of animals and generalised infection in the

remaining 20%. By contrast, the intravenous route produced generalised lesions in almost 80% of animals, supporting the role of intravenously feeding arthropods in disease spread.

1.4.4 Control

Effectual control in limiting outbreaks of LSD is possible as a number of live, attenuated vaccine strains of LSDV are available. Over 40 years ago a South African field isolate (the "Neethling" isolate) of LSDV was attenuated by serial passage in the chorio-allantoic membranes of embryonated hen's eggs (Weiss, 1963; van Rooyen *et al.*, 1969). Cattle inoculated with this attenuated virus only developed mild local reactions and produced antibodies which persisted upwards of 3 years and were protected from virulent challenge (Weiss, 1968). Originally it was accepted that protection was life-long, however, more recently there have been reports of vaccine failures and annual immunisation is now recommended (Hunter and Wallace, 2001). Not all cattle produce circulating antibodies after vaccination, but they are still resistant to challenge demonstrating the protective role of cell-mediated immunity (Andrew *et al.*, 1989). Those animals that don't sero-convert are unable to confer collostral antibody protection to suckling calves and there are reports of vaccinated dams giving birth to calves with full-blown LSD (Hunter and Wallace, 2001).

Capstick and Coackley (1961) describe the tissue culture production of the Kedong Valley strain of sheeppox virus for use as a vaccine for protecting cattle against LSDV. Conversely, Davies (1976) (as cited by Davies, 1982 and Kitching *et al.*, 1987) describes the isolation of the 0240 capripox virus isolate from a sheep. Attenuation of this isolate gave rise to a vaccine to control sheep and goatpox (called Kenya sheep and goatpox virus, later re-named Kenya Sheep-1 [KS-1]). Sequence analysis of KS-1 revealed that it was almost identical to Kenya Cattle-1 (KC-1), a cattle isolate of LSDV (Gershon and Black, 1989b; Gershon *et al.*, 1989a).

Carn *et al.* (1994b) investigated the possibility of using purified capripoxvirus P32 antigen as a subunit vaccine for priming the immune response of exotic animals from capripoxvirus-free countries before introduction into capripoxvirus-endemic regions. Their results showed favourable responses, although they concluded that complete immunity would best be obtainable with conventional live vaccines.

Except in Israel due to the rapid response of the authorities in the diagnosis and slaughtering of all diseased and in-contact cattle, LSD has not been eradicated from any country in which it has appeared (Davies, 1991). LSD is classified as a group A infectious disease by the OIE and thus countries at risk are encouraged to follow certain guidelines to control the spread of the disease. Annual vaccination is recommended with newly born calves receiving vaccine within 10 days of birth. As the natural host reservoirs of LSDV are unknown, and, as farmers don't always vaccinate their animals, especially during years in which there is disease

quiescence, sporadic outbreaks continue to occur. Until stricter control measures are enforced and more is learnt about the disease, LSD will continue to be of major economic importance.

1.4.5 Genomic characterisation

Over the past 20 years molecular tools have been used to compare the genomes of capripoxviruses using restriction enzyme analysis and sequence data (Black *et al.*, 1986; Gershon and Black, 1987; Gershon and Black, 1988). *Hind*III, *Pst*I, *Ava*I and *Sal*I restriction enzyme maps of the genomes of cattle, sheep and goat capripoxvirus isolates have been compared with each other and with those of isolates from related animals from different geographical regions. The results of these comparisons have allowed the division of the capripoxviruses into 5 groups: group 1 isolates are restricted to sheep (e.g. India sheep-1), group 2 isolates to goats (e.g. Iraq goat-1), group 3 isolates infect sheep, goats and cattle, but, are restricted to Africa (e.g. KS-1), group 4 isolates infect either sheep or goats in Africa or the Middle East and group 5 isolates are restricted to cattle in southern Africa (e.g. LSDV-Neethling) (Gershon *et al.*, 1989a).

A comparison of the whole genome organisation of the sheep LSDV isolate, KS-1, with the Western Reserve (WR) strain of vaccinia virus (VV) using cross-hybridisation studies revealed a similar pattern of gene arrangement as observed within the *Orthopoxvirus* genus (Esposito and Knight, 1985; Gershon *et al.*, 1989a) with housekeeping genes constituting the majority of genes found within the highly conserved central regions and host-range and virulence genes situated towards the more divergent termini. KS-1 lacks approximately 45 kb of DNA found in the terminal regions of VV making it one of the smallest poxvirus genomes along with the parapoxviruses (Robinson *et al.*, 1987). Early estimates of the genome lengths of northern African isolates of capripoxviruses, including KC-1, using pulse-field gel electrophoresis technology arrived at an average genome length of 148 kbp (Gershon and Black, 1987; Gershon and Black, 1988). Using similar methodology, the genome length of the South African vaccine strain of LSDV was estimated to be 152,6 kbp (Perlman, 1993). Recently, however, the genomes of the 2490 Kenyan field isolate of LSDV (Tulman *et al.*, 2001), the South African vaccine strain, and a field isolate from an outbreak of LSD in 2001 in the Northern Province in South Africa (Warmbaths) (Kara *et al.*, 2003) have been sequenced to completion (except for the terminal hairpin loops). The assembled sequences yielded genome lengths of 150,8 kbp, 150,5 kbp and 150,8 kbp respectively consisting of 156 putative genes. The sequences of the geographically distinct virulent field isolates were highly conserved with only 18% of the genes containing between one and a maximum of three amino acid changes. On the other hand, the highly cell-passaged and attenuated SA vaccine

strain contains amino acid changes in 76% of its genes compared to the Warmbaths isolate. A thorough investigation of these changes may help to elucidate some of the mechanisms of attenuation which might help in developing an even more effective LSDV vaccine.

From the genome sequences, LSDV has been shown to most closely resemble leporipoxviruses in gene content and organisation, although it also contains homologues of interleukin-10 (IL-10), IL-1 binding proteins, G protein-coupled CC chemokine receptors, and epidermal growth factor-like proteins which are found in other poxviruses (Gershon and Black, 1989a; Cao *et al.*, 1995, Tulman *et al.*, 2001).

1.5 – POXVIRUSES AS VECTORS FOR RECOMBINANT VACCINES

1.5.1 Introduction

A number of poxviruses from different genera are being developed as potential vectors for recombinant vaccines for use in the medical and veterinary fields. Poxviruses are able to present foreign antigens on the surface of infected cells, which in turn induce cellular and humoral immune responses depending upon the specific poxvirus promoter controlling their expression (Coupar *et al.*, 1986; Andrew *et al.*, 1989).

VV was the first poxvirus to be developed as a eukaryotic cloning and expression vector, and thereafter as a vector for recombinant vaccines (Mackett *et al.*, 1982; Smith *et al.*, 1983a; Smith *et al.*, 1983b). One of its most successful early applications was as a vector for a recombinant rabies vaccine developed for oral vaccination of foxes against rabies in central Europe (Kieny *et al.*, 1984; Blancou *et al.*, 1986; Brochier *et al.*, 1991). Yet there is concern over the safety of VV, especially in immunocompromised persons - in September 2000 a pregnant woman in Ohio (USA) was bitten by her dog while attempting to remove a VV-rabies recombinant vaccine bait from its mouth (Rupprecht *et al.*, 2001). The woman subsequently developed fever, swelling of the bitten arm (with blistering) accompanied by inflammation. Electron microscopy, polymerase chain reaction (PCR) and serology confirmed the presence of the recombinant vaccine from various tissue samples. She fortuitously recovered fully and there were no apparent ill effects to her unborn child. Thus in the veterinary field other poxviruses, including avipoxviruses and capripoxviruses (refer to next section), are being developed as alternatives to VV as vectors as they are more host-range restrictive and have already been used as effective and safe live attenuated vaccines in animals for many years (van Rooyen *et al.*, 1969; Cadoz *et al.*, 1992; Romero *et al.*, 1993; Romero *et al.*, 1994a; Romero *et al.*, 1994b; Wade-Evans *et al.*, 1996; Fries *et al.*, 1996; Karaca *et al.*, 1998;

Amano *et al.*, 1999). A fowlpox virus (FPV) recombinant co-expressing chicken type I interferon and Newcastle disease virus (NDV) hemagglutinin-neuraminidase (HN) and fusion (F) genes protected chickens against challenge with virulent FPV and NDV (Karaca *et al.*, 1998). In a similar study by Viljoen *et al.* (2003) it was shown that expression of just the F gene offered the highest levels and most consistent protection when administered via the wing-web route.

For safer human vaccines a number of poxviruses are being investigated, including avipoxviruses as their ability to undergo early stage gene expression in mammalian cells allows for the induction of an immune response although they are unable to complete a productive infection cycle (Stannard *et al.*, 1998; Vazquez-Blomquist *et al.*, 2002). A canary poxvirus-rabies recombinant vaccine injected into human volunteers induced presumptive protective levels of rabies-specific antibodies (Fries *et al.*, 1996).

Highly attenuated strains of VV lacking virulence and host-range genes, such as modified vaccinia Ankara (MVA) and NYVAC, are also being considered for human use (Blanchard *et al.*, 1998; Hel *et al.*, 2002).

For the development of poxviruses as vectors for recombinant vaccines a number of important factors were taken into consideration including the large size of their DNA genomes (ranging from 140-300 kbp), the lack of infectivity of isolated viral DNA, the packaging of viral enzymes necessary for transcription within the infectious virions, poxvirus-specific promoters, and the cytoplasmic site of virus transcription and replication (Mackett *et al.*, 1982). The largest technological problems which had to be overcome involved insertion of DNA into the large genome, efficient expression of heterologous genes and selection of recombinant virus.

1.5.2 Strategies for generating recombinants

Homologous recombination was the first (and still most widely used) approach for inserting the foreign DNA into specific sites in the poxviral genome using transfection of either viral DNA (Sam and Dumbell, 1981) or plasmids containing viral DNA sequences (Weir *et al.*, 1982) into virus-infected cells. Falkner and Moss (1990) provide a useful schematic to describe this process (Fig. 1.5 A).

The coding sequence for the foreign protein to be expressed is inserted downstream of a poxvirus promoter flanked by sequences homologous to a nonessential region of the poxvirus genome (Fig. 1.5 B). The original transfection methods involved the use of calcium-phosphate to precipitate the viral and plasmid DNA (Weir *et al.*, 1982), however, as technology has

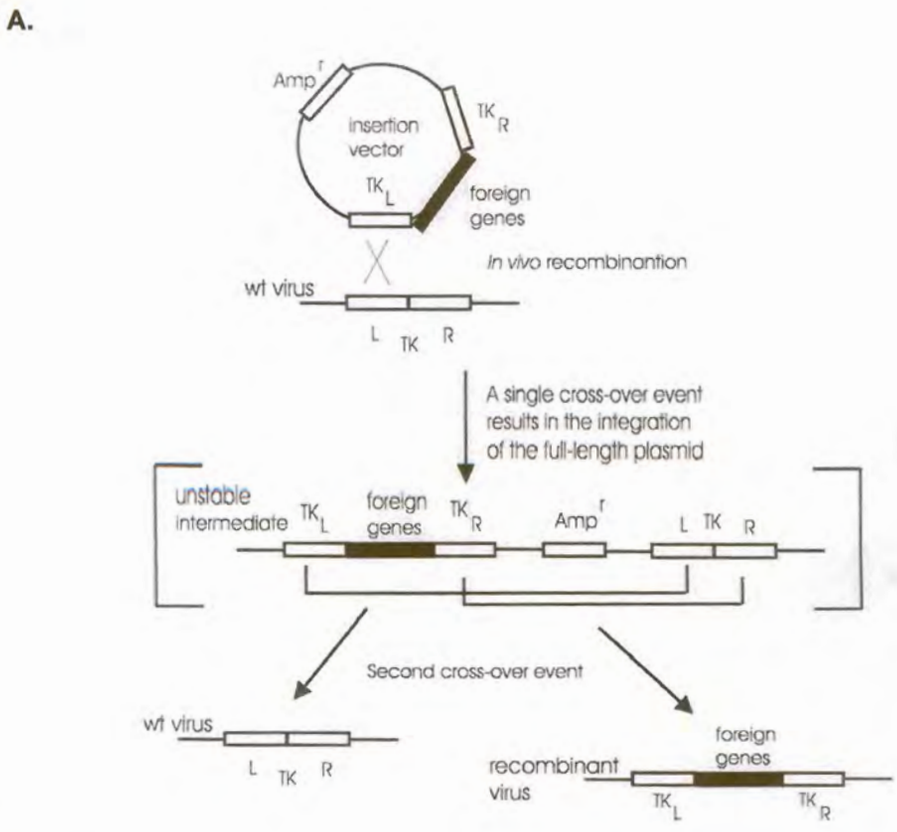


Figure 1.5 Schematic representation of homologous recombination between viral and plasmid DNA. **A** - The insertion vector/plasmid contains sequences homologous to a non-essential region of the viral genome (e.g. the TK gene) interrupted by the foreign genes (selectable marker genes, reporter genes and immunogenic genes under control of poxvirus promoters – **B**) for insertion into the viral genome. After transfection of virus-infected cells with the insertion vector, homologous recombination occurs between the homologous regions of the viral and plasmid DNA during viral DNA replication. This results in integration of the insertion vector into the viral genome. A second round of recombination then occurs due to the presence of direct repeat sequences, resulting in either wild type (wt) virus, or recombinant virus containing the desired foreign genes (Adapted from Falkner and Moss, 1990).

advanced, better and more convenient methods have been developed e.g. the use of liposomal-based delivery systems (Romero *et al.*, 1993).

In vitro direct ligation using a helper virus for recovery of recombinants is another method used for the insertion of foreign genes into the poxviral genome (Merchlinsky and Moss, 1992). Modifications of this method have involved the insertion of unique restriction enzyme sites into the poxviral genome preceded by strong poxvirus promoters to drive efficient expression of the inserted genes (Pfleiderer *et al.*, 1995; Merchlinsky *et al.*, 1997). Although these techniques allow for the insertion of large pieces of DNA, do not require drug selection nor intermediate bacterial plasmid vectors, they have not found general favour due to the difficulties involved in manipulating the large DNA and the need for helper viruses.

A method for overcoming this problem has been to combine *in vitro* ligation with homologous recombination and to use psoralen and long-wave UV irradiation to "create" helper viruses (Timiryasova *et al.*, 2001). This method generates an extremely high percentage (> 90 %) of homologous recombinants without the need for drug selection.

For the expression of the inserted/foreign genes naturally occurring and synthetic poxvirus promoters are inserted upstream to drive transcription (Mackett *et al.*, 1982; Davison and Moss, 1990). It has also been found that due to a high degree of conservation of the structural domains of poxvirus promoters, even between different genera, it is possible to drive expression of foreign genes using promoters from heterologous poxvirus hosts (Prideaux *et al.*, 1990).
As for the selection of sites for the insertion of foreign genes into the poxviral genome, Dubbs and Kit (1964) described the natural occurrence of a number of VV mutants deficient in thymidine kinase (TK) activity, and the selection of these TK-negative mutants in mouse cells using 5-bromo-2'-deoxy-uridine (BUdR). Once the location of the TK gene in the viral genome had been mapped (Weir *et al.*, 1982) it became possible to insert foreign genes into the viral TK gene and to select for recombinants on the basis of their TK-negative phenotype (Mackett *et al.*, 1985). However, TK gene activity does not appear to be non-essential for all poxviruses (Nazerian and Dhawale, 1991; Letellier, 1993; Scheiflinger *et al.*, 1997), and, thus, alternative insertion sites have been utilized (Lorenzo and Blasco, 1998).

1.5.3 Selection of recombinants

Besides the insertional inactivation of the viral TK gene as a means for selecting recombinants, a number of other methods have been developed – the common aim of all of them being the eventual selection of a homogeneous pool of recombinants, free from contaminating wild-type (or, parental) virus. The introduction of dominant selectable marker genes, such as the *Escherichia coli* guanine phosphoribosyl transferase (gpt) gene, under control of poxvirus promoters into the DNA insertion vectors, is one such method. Mycophenolic acid (MPA), an inhibitor of purine metabolism, has been shown to block the replication of VV in normal cell lines. However, recombinant viruses expressing gpt are able to replicate in selection medium containing MPA, xanthine and hypoxanthine (Falkner and Moss, 1988). Transient dominant selection allows for the serial construction of recombinants containing several foreign genes; the gpt gene is cloned outside of the viral insertion segments resulting in its loss after selection pressure is removed (Falkner and Moss, 1990). An improvement upon this procedure is called transient marker stabilization in which the marker genes are inserted within the viral flanking regions, however they are flanked by tandem repeated DNA sequences. Thus, when selection pressure is lifted the marker gene is rapidly lost by recombination (Scheiflinger *et al.*, 1998). This method is highly efficient and requires only a few plaque purification steps to select marker-free recombinant virus. The loss of the marker genes from the recombinants is a highly desirable situation, especially for recombinants targeted for vaccine development due to concerns over the release of genetically modified organisms containing antibiotic resistance genes.

Visual markers are also used for selection. The *E. coli* β–galactosidase gene has been widely used due to its property of producing blue plaques in the presence of the chromophore, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) when expressed from recombinant viruses (Chakrabarti *et al.*, 1985; Panicali *et al.*, 1986). Insertion of foreign genes into the β–galactosidase gene provides a further means of selection as plaques then lose their ability to stain blue. A more recent approach utilises the green fluorescent protein gene from the jellyfish, *Aequorea victoria*, as a visual marker by fusing the gene with an antibiotic resistance gene to produce a bifunctional enzyme (BiZyme) (Hansen *et al.*, 2002).

Host-range genes have also been exploited. Their presence confers the ability of the virus to multiply and/or spread in certain additional cell types although their exact functions may be quite diverse (Spehner *et al.*, 1988; Perkus *et al.*, 1989; Smith *et al.*, 1993; Lanar *et al.*, 1996). The VV K1L host-range gene is necessary for growth of the virus in a rabbit kidney cell line (RK-13), but not for growth in monkey kidney cells (Smith *et al.*, 1993). A VV mutant was constructed with parts of this gene deleted. An insertion plasmid containing the hepatitis B surface antigen gene and the complete K1L gene was then used to generate and select recombinant viruses on RK-13 cells.

1.5.4 Future prospects

Poxviruses as vehicles for gene expression and recombinant vaccines have come a long way in almost 30 years. This period has seen vast improvements in selection strategies, vaccination regimes and applications (Kwak *et al.*, 2004). Now that the genomes of many poxviruses have been sequenced it has become possible to assign functionality to most poxvirus genes. This has opened up new avenues for the design of improved vectors and for some of these genes to be utilised in areas of immunotherapy and cancer therapy (Jackson *et al.*, 2001; Boyle *et al.*, 2004). Concerns over the use of smallpox virus as a weapon for bioterrorism have accelerated efforts to design better antiviral drugs (Painter and Hostetler, 2004) and recombinant poxviruses are being developed for the testing of these drugs in primates (Jahrling *et al.*, 2004). Poxviruses are even being used as delivery vehicles for gene and anti-tumor therapy (Zhi *et al.*, 2002; Flanagan *et al.*, 2004).

1.5.5 Development of capripoxviruses as vectors for recombinant vaccines

Attenuated vaccine strains of capripoxviruses have been in use for many years. These have proven safe, reliable and provide long-term protection, although there have been reports of severe adverse reactions to vaccination of dairy cattle with the Kenyan KS-1 (0240) vaccine strain of LSDV (Yeruham *et al.*, 1994). Capripoxviruses are highly host-range restricted, although certain strains appear able to cross-infect the three host livestock species. These characteristics have made the vaccine strains ideal candidates as vectors for recombinant vaccines against other livestock diseases, with the added advantage of the potential for dual protection. A number of recombinant vaccines using the KS-1 isolate of LSDV as vector have been developed and tested in field trials in Kenya (Romero *et al.*, 1994a; Romero *et al.*, 1994b). Recombinants expressing either the fusion or haemagglutinin genes of rinderpest virus were used successfully in field trials in Kenya to protect cattle against both rinderpest and LSD (Ngichabe *et al.*, 1997), whereas KS-1 recombinants expressing the major core structural protein (VP7) of bluetongue virus (BTV) only provided partial protection to sheep against a virulent heterotypic BTV challenge (Wade-Evans *et al.*, 1996).

Although both LSDV (Neethling) and KS-1 fall within the *Capripoxvirus* genus, they exhibit a number of significant differences in their epidemiological characteristics, geographical distribution and genomic structure (Weiss, 1968; Black *et al.*, 1986). The Neethling isolate of LSDV was discovered in the North Western Province of South Africa in 1944 (Thomas and Mare, 1945). Since then, epidemics have occurred every few years in South Africa, as well as in neighbouring countries. An early field isolate was attenuated by passage on the

chorioallantoic membrane of chicken embryos to produce an effective vaccine (van Rooyen *et. al.*, 1969). Genomic studies on the vaccine and field isolates from southern Africa have revealed minor differences in their terminal regions, whereas comparative studies with isolates from Kenya showed more major differences (Gershon and Black, 1988; Perlman, 1993, Wallace, 1994). These differences might also be reflected in the host-range restrictiveness of LSDV (Neethling), and closely related strains, to natural infection in only cattle, whereas the Kenyan strains are able to infect cattle, sheep and goats (Weiss, 1968; Gershon and Black, 1988).

Sequencing of a virulent field isolate of LSDV from Kenya and comparison with a field isolate from South Africa and the South African vaccine strain has revealed that only minor differences occur between the field isolates, whereas the vaccine strain exhibits many deletions by comparison (Tulman *et al.*, 2001; Kara *et al.*, 2003). It will be interesting to determine which of the genetic differences between the northern and southern field isolates are responsible for the differences in the degree of host-range restrictiveness.

Due to these differences independent studies were initiated to develop both the northern and southern vaccine strains of LSDV as vectors for recombinant vaccines.

For the development of the first capripoxvirus-vectored recombinant vaccines the TK gene of KS-1 was chosen as the insertion site as this gene was shown to be non-essential for VV (Romero *et al.*, 1993). The fusion (F) gene of rinderpest virus (RPV) was inserted under control of the VV late P11K promoter and the *E. coli* gpt gene was used for positive selection of recombinant viruses. Resulting recombinants were confirmed for the presence of the F and gpt genes by Southern transfer and radioactive probe hybridisation. Expression of the F gene was confirmed using immunofluorescence with an anti-F monoclonal antibody. Virus was then grown to high titres and cattle were immunised. Three weeks later they were challenged with virulent RPV and three weeks after that with virulent LSDV. All the vaccinated animals survived both challenges proving the effectiveness of the KS-1 strain of LSDV as a recombinant vaccine vector able to offer dual protection (Romero *et al.*, 1994a).

A similar recombinant vaccine was then developed expressing the haemagglutinin (H) gene of RPV also under control of the VV P11K promoter. In this trial it was shown that although most cattle were protected from virulent RPV challenge, the presence of neutralising antibodies was not always enough for protection, thus suggesting that cell-mediated immunity plays an important role (Romero *et al.*, 1994b). A mixture of both recombinants provided long-term protection against both RPV and LSDV challenge in cattle (Ngichabe *et al.*, 1997).

These constructs were also shown to protect goats against challenge with virulent peste des petits ruminants virus (PPRV) (Romero *et al.*, 1995), proving more effective than a mixture of live, attenuated vaccines against PPRV and capripox (Martrenchar *et al.*, 1997).

For an even more efficient KS-1 vectored recombinant vaccine against PPRV, the F gene of PPRV was inserted under control of the VV P7.5K early/late promoter (Berhe *et al.*, 2003). Proteins under control of this promoter are known to stimulate both a humoral and cell-mediated immune response (Coupar *et al.*, 1986; Andrew *et al.*, 1989). Using the sonication and PCR testing method (as developed and described in this thesis, Chapter 3) they were able to obtain pure recombinants which conferred protection to goats against virulent PPRV challenge at a low dose of 0.1 plaque forming units (pfu).

Work on developing the southern African vaccine strain of LSDV as a recombinant vaccine vector has been ongoing since 1988. As mentioned earlier ("Aims of this investigation"), initial efforts were aimed at mapping the viral genome, identifying suitable insertion sites, selection markers and promoters. While the direction taken by ourselves at the Onderstepoort Veterinary Institute was that of the viral TK gene as an insertion site (as presented in this thesis), our colleagues at the University of Cape Town's Medical School (under the leadership of Professor Keith Dumbell and Dr Anna-Lise Williamson) investigated alternative insertion sites (Cohen and Cox, personal communication). They identified the ribonucleotide reductase (RR) gene and an intergenic region as potential insertion sites. The RR gene was inserted into a transfer plasmid interrupted by the *E. coli* gpt positive selectable marker gene, the lacZ visual marker gene and the rabies virus glycoprotein gene under control of the fowlpox virus early/late pAF promoter (Aspden *et al.*, 2002). Recombinants were generated and selected and inoculated into cattle once expression of the glycoprotein gene had been confirmed. They were able to show that both humoral and cell-mediated immune responses were elicited against the glycoprotein. They were also able to show the potential of the recombinant to be used as a vaccine in non-ruminant hosts (Aspden *et al.*, 2003). One drawback of their work, however, was that their recombinant was not purified to homogeneity, and thus upscale growth of the recombinant virus should be performed under selection pressure. The development of a repeatable method for selecting recombinants to homogeneity forms an important part of this doctoral thesis.

In the following chapters methods for generating recombinants using the TK gene of LSDV (Neethling) as the insertion site are described as well as a method for selecting the resulting recombinants to homogeneity. Recombinants expressing immunogenic genes of viruses of veterinary importance were then generated and their ability to protect animals against virulent challenge evaluated.

CHAPTER 2

GENERATION AND SELECTION OF TK-DISRUPTED LSDV RECOMBINANTS

2.1 INTRODUCTION

A number of methods have been developed over the years for the generation and selection of poxvirus recombinants (Mackett *et al.*, 1982; Mackett *et al.*, 1985; Chakrabarti *et al.*, 1985; Evans *et al.*, 1988; Guo *et al.*, 1989; Falkner and Moss, 1990; Merchlinsky and Moss, 1992; Cadoz *et al.*, 1992; Scheiflinger *et al.*, 1998; Timiryasova *et al.*, 2001; Boyle *et al.*, 2004).

In order to generate LSDV recombinants using the SA vaccine strain of LSDV our laboratory decided to follow the approach most commonly used to generate VV recombinants (Mackett *et al.*, 1985; Boyle and Coupar, 1986), viz. foreign gene insertion into the viral thymidine kinase (TK) gene making use of naturally occurring homologous recombination with selection on the basis of the resulting TK-negative viral phenotype (generally referred to as the TK-negative selection method). This method was possible as the TK gene of VV was found to be non-essential for normal growth of the virus (Dubbs and Kit, 1964). The same situation has been found for most, but not all poxviruses (Nazerian and Dhawale, 1991). For example with the avipoxvirus, pigeonpox virus, insertion into the TK gene resulted in the generation of unstable recombinants (Letellier, 1993). A similar situation was found for certain highly attenuated strains of fowlpox virus (Scheiflinger *et al.*, 1997). For both poxviruses other non-essential regions had to be utilised for generating stable recombinants.

Recombinants have been successfully generated from the northern African KS-1 strain of LSDV using the viral TK gene for insertion (Romero *et al.*, 1993; Wade-Evans *et al.*, 1996) and thus this site should prove suitable for use with the South African vaccine strain of LSDV.

A distinct advantage of using the TK-negative selection approach is that selection is on the basis of the resulting TK-negative phenotype, and thus does not rely upon the inclusion of a selectable antibiotic marker gene (such as the E. *coli* gpt gene), which hold certain environmental concerns. However it does rely upon the availability of a TK-negative cell line for the selection of recombinants. It is possible to use certain mutagens in cell culture medium to induce and select for mutants which are deficient in TK activity, but fortuitously for this study it was possible to acquire a Madin-Darby bovine kidney (MDBK) cell line (from Dr L.J. Bello, University of Pennsylvania, USA) which is resistant to high levels (up to 100 μg/ml) of the TK selection drug, BUdR. These cells, renamed BU100 cells, were shown to contain less than 5% of the TK activity found in the normal parental MDBK cells (Bello *et al.*, 1987). BUdR is a thymidine analogue which results in strand breakage when phosphorylated and incorporated into newly synthesized

DNA by active TK. Thus only viruses which have had their TK genes inactivated, and for which TK activity is non-essential, are able to survive in the presence of BUdR (Dubbs and Kit, 1964).

As poxviruses code for most of their own genes, including regulatory genes, expression of foreign genes needs to be under control of poxvirus promoters. The expression of poxvirus genes is temporal and is dependant on the "class" of promoter which regulate their expression (Coupar *et al.*, 1986; Kumar and Boyle, 1990). Promoters can be divided into early, intermediate, late and early/late depending on when they are activated during the infection/replication cycle. A number of synthetic promoters have been developed containing optimised sequences for the crucial regulatory regions (Chakrabarti *et al.*, 1997). It has also been found that the different "classes" stimulate different types of immune responses: early promoters stimulate a cell-mediated immune (CMI) response and late promoters a humoral response (Coupar *et al.*, 1986).

In developing a vaccine system it would be an advantage if both arms of the immune response were stimulated, and thus a promoter active early and late in the infection cycle would be ideal. The P7.5K promoter of VV is an early/ate promoter (Cochran *et al.*, 1985) and has been used successfully in a number of potential vaccinia virus-vectored recombinant vaccines in which good levels of immunity were induced (Smith *et al.*, 1983a; Collett *et al.*, 1987), including the highly protective VV-rabies recombinant vaccine used to control rabies in foxes in Europe and in raccoons in the USA (Kieny *et al.*, 1984; as reviewed by Paoletti, 1996). As the functional elements of poxvirus promoters have been shown to be active in heterologous poxviruses (Coupar *et al.*, 1990; Prideaux *et al.*, 1990), this promoter was chosen to drive the expression of foreign genes in this study.

The calcium phosphate coprecipitation method (Drillien and Spehner, 1983) was decided upon for transfection as this method has proven successful for the generation of many other poxvirus recombinants (Weir *et al.*, 1982; Smith *et al.*, 1983a; Kieny *et al.*, 1984) and has been used successfully in our laboratory for generating VV recombinants. As the rates of the infection-cycles of LSDV and vaccinia virus differ markedly (Fick and Viljoen, 1999), minor adaptations were implemented to help maximize the chances for successful homologous recombination (Fig. 2.1).

To assist with the selection process for potential recombinants the *E. coli* lacZ reporter gene was inserted into the LSDV transfer vector. Expression of this gene results in the production of a blue colouration by utilising the chromogenic substrate, X-gal (Panicali *et al.*, 1986).

After many attempts at generating LSDV recombinants using this approach, as well as introducing modifications such as transfecting the cells 12 hours after infection which would synchronise the time of the DNA (viral and plasmid) entering the cells with the early stages of viral replication (thus enhancing the chances for homologous recombination), no evidence for recombinants could be found. In a parallel study, 4 VV recombinants (each containing either the VP2, 3, 5 or 7 genes of African horse-sickness virus type 3) were successfully generated and

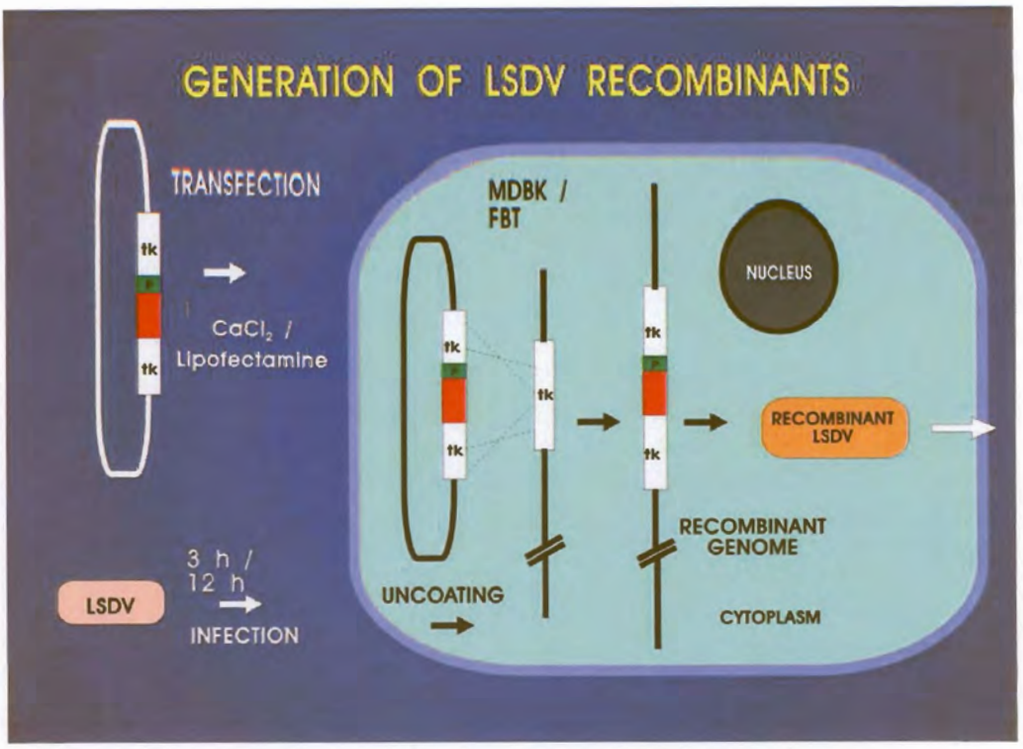


Figure 2.1 Schematic representation of the processes involved in generating LSDV recombinants using homologous recombination and the viral TK gene as the insertion site. Cells are infected with wtLSDV. A number of hours later, coinciding with the early stages of viral DNA replication in the cytoplasm, they are transfected with plasmid DNA containing the necessary viral insertion sequences (tk) flanking a foreign gene under control of a poxvirus promoter (P). Homologous recombination occurs between the viral and plasmid DNA resulting in incorporation of the foreign gene into the newly synthesised viral DNA. This DNA is then packaged and released from cells as recombinant virus. Schematic prepared by Marco Romito.

selected using the same basic protocol, thus demonstrating that the reagents and general methodology were correct.

An alternative approach was thus attempted, incorporating the *E. coli* gpt gene as a dominant selectable marker and following the methodology of Romero *et al.* (1993) with minor adaptations. This time success was achieved in generating and selecting homogeneous LSDV recombinants which were shown to be stable.

2.2. MATERIALS AND METHODS

2.2.1 Viruses and cells:

Freeze-dried virus of the South African vaccine strain of LSDV (also referred to as "Neethling-type virus" or "parental control LSDV" or "wild-type LSDV") was reconstituted in sterile distilled water and passaged 5 times in Madin-Darby bovine kidney (MDBK) cells (obtained from the American Type Tissue Culture Collection, USA). Stocks were prepared from clarified cell lysates, and were stored at -20 °C. Aliquots were removed for titration on MDBK cells as described (Wallace, 1994).

Primary cultures of foetal bovine testis (FBT) cells were prepared using standard procedures (Freshney, 1987) and were used for the generation and selection of recombinant viruses.

BU100 cells, a derivative of MDBK cells, which are tolerant of BUdR to a concentration of 100 μg/ml, were kindly supplied by L.J. Bello (University of Pennsylvania, USA).

All cells were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 nutrient supplement (Highveld Biological Products, South Africa), 10% foetal calf serum (FCS) (Highveld Biological Products, South Africa) and antibiotics (100 μg/ml penicillin, 100 μg/ml streptomycin and 250 μg/ml amphotericin) (Highveld Biological Products, South Africa).

2.2.2 Plasmid DNA analysis, cloning and purification:

Restriction enzyme digestions

Restriction enzyme (R.E.) digestions were carried out at optimal temperatures and in appropriate buffers as specified by the manufacturer (Roche, Germany). Routine digestions were performed for one to three hours, however, when complete digestion was required for cloning purposes they were performed overnight. Double-digests were performed in the same buffer where

possible, or, the first digest in the buffer requiring the lowest salt concentration and then with the addition of salt to increase the final concentration to that required for the second enzyme. Where buffers were totally incompatible the DNA was digested with the first enzyme, run on an agarose gel, the appropriate fragment excised, purified and digested with the second enzyme. Partial DNA digestions were optimized according to each individual application by varying combinations of enzyme concentration, incubation temperature and duration of digestion.

Analysis and/or separation of DNA

Digested DNA was analysed or separated using horizontal agarose (0.8 %) gel electrophoresis using a mini-sub DNA cell (7x10 cm) (Pyramid, SA) and a EC 105 power-pack (E-C Apparatus Corporation, USA). Gels were prepared in a 1X TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) (Sambrook *et al.*, 1989) containing 0.5 µg/ml ethidium bromide and were run at a constant rate of 7 V/cm in order to avoid heat build-up and distortion of the DNA bands. DNA molecular weight markers were prepared from digestion of phage lambda DNA (Promega, USA) with either *Pst*I or *Hind*III enzymes. DNA fragments were viewed under 254 nm wavelength UV light on a transilluminator (UVP, USA). Where necessary, images were captured using a Lumi-Imager (Roche, Germany) gel documentation system and printed on thermal paper (UPP-110HG, Sony, Japan) from a Sony UP-D895 Digital Graphic Printer (Sony, Japan).

Excision and purification of DNA fragments

Gels containing DNA fragments for excision and purification were viewed under 366 nm wavelength UV light to avoid strand damage using a hand-held transilluminator (Camag, UK). The relevant fragments were excised from gels using sterile scalpel blades and the DNA extracted and purified using a Qiaex II Gel Extraction Kit (Qiagen, Germany). DNA concentrations were determined by running aliquots on agarose gels alongside controls of known concentration (pBR322, Promega, USA).

Ligation of DNA fragments

For the ligation of DNA fragments with incompatible R.E. digested termini, the termini were first blunted using the Klenow fragment of *E. coli* DNA polymerase I (USB, USA). Termini of vector DNA were prevented from re-ligating upon themselves by dephosphorylation using calf intestinal phosphatase (Roche, Germany).

Sticky-end ligations were routinely carried out at 4-8 °C for 18 hours using a 1:2 vector to insert molar ratio using T4 DNA ligase (Roche, Germany) and blunt-end ligations were routinely carried out at 23 °C for 18 hours (or longer) using a 1:4 vector to insert molar ratio. However, when problematic ligations were encountered a range of incubation temperatures, incubation times and vector to insert ratios were applied.

Transformation of competent *E. coli* cells with ligated DNA

Stocks of competent DH5α *E. coli* cells were prepared according to the technique of Inoue *et al.* (1990) and were stored in 100 µl aliquots at – 80 °C (Ultra-low temperature freezer, Specht Scientific, South Africa). When required, an aliquot was thawed slowly on ice and approximately 20 ng of ligated DNA was added. The cell-DNA mixture was incubated on ice for one hour followed by a 30 second heat-shock at 42 °C. The mixture was then returned to ice for 2 minutes and then 800 µl of room temperature SOB medium was added and the cell mixture shaken vigorously for one hour at 37 °C. The mixture was then divided up equally and spread onto four LB-agar plates (containing 100 µg/ml ampicillin [Sigma, USA]) for overnight incubation at 37 °C. Single colonies were picked using sterile tooth-picks and inoculated into 2 ml LB medium for overnight incubation and plasmid DNA extraction. Using this technique it was possible to routinely obtain transformation efficiencies of 2 x 10^8 transformants/ µg of plasmid pBR322 DNA.

Plasmid purification

Plasmid DNA was extracted from transformed bacterial cells using the modified alkaline lysis method of Ish-Horowicz and Burke (1981) (original method by Birnboim and Doly [1979]). Air-dried DNA was resuspended in sterile distilled water and stored at – 20 °C. For larger scale plasmid preparations and where plasmid DNA of high purity was required (such as for use in transfections) 100 ml bacterial cultures were grown and the DNA purified through a gravity-flow column from a Qiagen Plasmid Midi Kit (Qiagen, Germany). DNA concentrations were determined by measuring the absorbance at 260 nm (1 OD_{260} = 50 µg/ml DNA) and the purity by determining the OD_{260}/OD_{280} absorbance ratio.

2.2.3 Construction of LSDV transfer vector, pLSTK7.5:

The DNA plasmid, pLSDTK3c (Figure 2.2), was obtained from Dr Anna-Lise Williamson (University of Cape Town). This plasmid contains the 2.5 kbp *Hind*III fragment of LSDV in which is found the entire viral TK gene, and was used as the base plasmid for the construction of the transfer vector. The VV P7.5K early/late promoter and a restriction enzyme multiple cloning site (MCS) were excised as a single fragment from the VV transfer vector, pGVRW1, using *Nde*I and *Eco*RI (Figure 2.3) and blunt-end inserted into the *Kpn*I site of the LSDV TK gene in pLSDTK3c.

2.2.4 Insertion of lacZ reporter gene into pLSTK7.5:

To assist with monitoring the generation and selection of the first TK-disrupted LSDV recombinants the lacZ chromogenic reporter gene was utilized. The lacZ gene was inserted into the pLSTK7.5 transfer vector as follows:

The full-length lacZ gene (3.7 kbp) was removed from pSV-β-gal (Invitrogen, USA) using *Hind*III and *Eco*RI, blunted using Klenow, and inserted into the blunted (and dephosphorylated) *Sal*I site in the MCS of pLSTK7.5 to produce the insertion plasmid, pLSTK7.5-lacZ (Figure 2.7).

2.2.5 Transient expression of lacZ and X-gal staining:

MDBK cells were seeded on 6-well cell culture dishes (Nunclon, Denmark) and when 90% confluent they were infected with wtLSDV at an MOI = 1.0. One hour later the cells were washed with PBS and co-transfected with 5 µg of ultra-pure plasmid DNA using the DOTAP (Roche, Germany) liposomal-based transfection reagent. Twenty four hours later the cells were fixed in 4% paraformaldehyde (Merck, Germany) for 5 minutes, washed in PBS and stained with an X-gal stain (1 mg/ml X-gal [Biosolve Ltd, Netherlands], 5 µg/ml potassium ferrocyanate [Sigma, USA], 5 µg/ml potassium ferricyanate [Sigma, USA], and 5 µg/ml magnesium chloride [Sigma, USA]). The fixed cell monolayer was incubated at 37 °C (5% CO_2, Forma Scientific, model 3164, USA) until the blue colouration of cells expressing lacZ became visible.

2.2.6 Insertion of the positive selectable marker gene, gpt, into pLSTK7.5-lacZ:

When the TK-negative selection method failed to deliver selectable LSDV recombinants, the use of the *E. coli* gpt dominant positive selectable marker gene was investigated as an alternative method for selecting recombinants. This gene was inserted into the pLSTK7.5-lacZ insertion vector as follows (Figure 2.9):

The 1.3 kbp E. *coli* gpt gene was excised from the plasmid pSelp(HS)G_1G_2 (supplied by Dr Anna-Lise Williamson, University of Cape Town) using *Eco*RI, blunted and was inserted into the dephosphorylated *Sma*I site of pLSTK7.5-lacZ to produce the insertion plasmid, pLS(g)lac.

2.2.7 Generation and selection of LSDV recombinants:

TK-negative selection method

The method was adapted from that described by Drillien and Spehner (1983). In brief, MDBK cells (90% confluency) were infected with LSDV (SA-Neethling vaccine) at a MOI = 1 focus

forming units per cell (ffu/cell). At 12 hours post-infection (to coincide with the early onset of LSDV DNA replication) (Fick and Viljoen, 1999) calcium phosphate-precipitated LSDV DNA (2.5 μg) and 0.5 μg pLSTK7.5-lacZ were added. The cells were then glycerol-shock treated and incubated until 80 % displayed cytopathic effect (cpe) (2-3 days post-infection). These cells were then harvested, freeze-thawed three times, clarified by low-speed centrifugation and the supernatant fraction titrated serially on BU100 cells in the presence of 100 μg/ml BUdR (Sigma, USA).

The infected cells were incubated for 4 days, and areas of the cell monolayer which resembled viral foci were collected in a 50 μl volume using a 200 μl filter pipette tip. Samples were divided into two equal aliquots: one set was freeze-thawed at –20 °C three times and transferred to individual wells of a 24-well cell culture dish (Nunclon, Denmark) containing BU100 cells under TK selection. An X-gal agarose overlay (1% agarose containing 200 μg/ml X-gal) was placed over the cells for the evaluation of lacZ expression (Panicali *et al.*, 1986). The other set of aliquots were prepped for DNA extraction and polymerase chain reaction (PCR) analysis (2.2.5).

MPA-selection method (adapted from Romero *et al.*, 1993):
A 6-well microtitre plate (Nunclon), was seeded with FBT cells. The cells were incubated at 37 °C in a 5% CO_2 incubator until they reached 80% confluency. They were then infected with LSDV (SA vaccine) at a MOI = 0.1 ffu per cell in diluent (0.1 mM Hepes, DMEM) for 60 minutes at 37 °C in a 5% CO_2 incubator. During this period, the transfection mixes were prepared as follows: 5 μl of the liposomal-based transfection reagent LipofectAMINE (Gibco-BRL, Scotland) was mixed with 95 μl of OPTIMEM (Gibco-BRL) in a 10 ml polystyrene test tube. 5 μg of ultra-pure pLS(g)lac DNA was added to OPTIMEM to a final volume of 100 μl in a separate tube. The solutions were then mixed and left at room temperature (24 °C) for 20 minutes. The volume was made up to 1 ml with DMEM and gently pipetted over the cells once the inoculum had been removed. The cells were returned to the incubator for 7 hours after which time 1 ml of DMEM (10% FCS, no antibiotics) was added to each well, and incubation was continued for 17 hours. The transfection mixture was then replaced with DMEM (10% FCS, including antibiotics) and the cells were incubated for a further 4 days, or until almost 100% of them showed cpe. Cells were freeze-thawed alternately at -20 °C and room temperature three times. A 10-fold serial dilution was made of the cell lysate and an aliquot of each dilution was placed onto fresh FBT cells pre-incubated for 24 hours in the presence of gpt selection medium (20 μg/ml xanthine [Sigma, USA], 20 μg/ml hypoxanthine [Sigma, USA], 30 μg/ml mycophenolic acid [Sigma, USA], DMEM, 2.5% FCS). Cells were incubated under standard conditions (37 °C, 5% CO_2), with the medium being replaced every 48 hours until cpe became visible (usually 4-5 days post-inoculation). The cells were washed in PBS and individual foci were picked. These were then freeze-thawed as

described and were inoculated onto fresh cells under selection in 6-well or 12-well culture dishes (Nunclon). Cells were incubated until foci appeared. Foci were again picked, except, this time, only half a focus was removed. The position of each focus in relation to the well was carefully noted. The cells were then fixed and stained with the X-gal stain as described (2.2.5). It was thus possible to determine which foci contained viruses expressing the lacZ enzyme. A number of these foci were then selected for DNA extraction and PCR analysis.

2.2.8 Extraction of viral DNA for PCR analysis:

Foci were diluted 1:1 in lysis buffer consisting of 1% SDS [Sigma, USA], 20 mM β–mercaptoethanol [Merck, Germany], and 20 mM EDTA. These were then digested with Proteinase K (Roche, Germany)(100 µg/ml) at 56 °C for 2 hours in order to release viral DNA (approximately 1 ng per sample). Calf liver tRNA (0.5 µl of a 10 mg/ml stock) (Roche, Germany) and 1/10th volume 3 M sodium acetate (pH 5.3) were added and the DNA was extracted with phenol/chloroform [Sigma, USA], and then ethanol precipitated. After air-drying, the DNA was resuspended in 50 µl sterile, ultra-pure water and stored at –20 °C for PCR analysis.

2.2.9 PCR analysis:

Two primers were designed, P1 and P2 (Gibco-Brl, Scotland), which bind to the wtLSDV TK gene flanking the *Kpn*I restriction enzyme site used for the insertion of the foreign genes (as shown in Fig. 2.13 A).

P1 - CACCAGAGCCGATAAC
P2 - GTGCTATCTAGTGCAGCTAT
P3 - GAAGTGTCCCAGCCTG
P4 – CTCGCAAGCCGACTGATGCC
P5 - GATCCCCCACCCGCTTTTTATAGTAAGTTTTTCACA

These primers were then used to help distinguish wt from recombinant virus using a GeneAmp 2400 thermal cycler (Perkin-Elmer, USA) and Taq DNA polymerase. Primers P3 and P4 (Gibco-Brl, Scotland), binding to the gpt and lacZ marker genes, were used as internal controls. Primer P5 binds to the VV P7.5K promoter and was used to confirm insertion of this promoter (along with the MCS) into the LSDV transfer vector, pLSTK7.5.

A 50 µl reaction volume was used consisting of 5 µl 10X PCR buffer (containing 20 mM $MgCl_2$) (Takara Biomedicals, Japan), 4 µl 2.5mM dNTPs (Takara Biomedicals, Japan), 0.5 U Taq DNA

polymerase (TaKaRa Ex Taq™, Takara Biomedicals, Japan), 1µl of each primer [20 pmoles each], 1 µl template DNA (~ 0.1 ng) and 38 µl sterile distilled water. Template DNA was denatured for 45 seconds at 93 °C, primer annealing was carried out at 53 °C for 45 seconds, and strand extension was at 72 °C for 5 minutes (repeated through 35 amplification cycles).

2.2.10 Purification of LSDV:

Preparation of routine virus stocks

The infected FBT cells were harvested, either by shaking the flask vigorously to dislodge the cells (only possible when the cells were heavily infected) or by scraping the cells loose using a "rubber policeman" or by removing them gently using 0.1% trypsin (Difco Laboratories, USA). The medium containing the loose cells was dispensed into 20 ml glass bottles. These bottles were balanced and centrifuged at low speed in a benchtop centrifuge at 1500 rpm (250g) for 10 minutes at 4 °C. The cell pellets were lysed in 2 ml McIlvain's hypotonic buffer (Lennette and Schmidt, 1969) and left on ice for 10 minutes. The lysed cells were centrifuged at 2000 rpm (450g) for 10 minutes and the supernatant fluid was collected. The cell debris was resuspended in McIlvain's buffer and was again centrifuged at 2000 rpm (450g) for 10 minutes. The supernatant fluid was collected and pooled with the first virus-containing supernatant fluid and an aliquot was removed for virus titre determination (Wallace, 1994). The rest of the virus suspension was diluted in glycerol to produce a final concentration of 70 % glycerol and was dispensed into glass ampoules and frozen at -70 °C.

Preparation of pure LSDV for DNA extraction (Esposito, 1981)

The culture medium was removed from flasks containing infected cells exhibiting 90 % cpe. The infected cells were harvested by using trypsin to release them from the flasks. The cells were pooled with the medium, dispensed into centrifuge tubes and 0.5 ml of 36 % sucrose (in PBS) was layered at the bottom of each tube. The virus/cell suspension was centrifuged at 11 000 rpm (19000g), 60 minutes, 4 °C in a Beckman J2-21 (Beckman Instruments, USA) high speed centrifuge in a JS13-1 (Beckman Instruments, USA) rotor. The pellet was resuspended in 9 ml of McIlvain's buffer (4 mM) and left on ice for 10 minutes. ß-Mercaptoethanol (26 µl) and 1 ml of 10 % Triton X-100 (Merck, Germany) (in McIlvain's buffer) were then added and the suspension was further incubated on ice for 10 minutes to disrupt the cells. The cell debris was removed by centrifugation at 2000 rpm (450g) for 5 minutes, 4 °C in a benchtop centrifuge and the supernatant fluid was collected. The cell debris was resuspended in McIlvain's buffer and the centrifugation was repeated (2000 rpm (450g) for 5 minutes, 4 °C in a benchtop centrifuge). The supernatant fluid was collected and pooled with the first virus-containing supernatant fluid. This fluid was transferred to a centrifuge tube and 0.5 ml of 36 % sucrose [prepared in TE (10 mM

Tris, 1 mM EDTA, pH 9.0)] was layered beneath. Virus was then pelleted [centrifuged at 11 000 rpm (19000g), 60 minutes, 4 °C in a Beckman J2-21 high speed centrifuge in a JS13-1 rotor]. The pellet was resuspended in 0.5 ml of TE buffer. Lysis buffer (450 µl 4% sodium lauroylsarcosinate, 54% sucrose, 100 mM Tris (pH 7.8) mixed with 50 µl 200 mM β-Mercaptoethanol, 100 mM Tris (pH 7.8)) and Proteinase K (Roche, Germany) (final concentration = 100 µg/ml) were then added and the virus mixture was incubated for 3 hours at 56 °C (Massung and Moyer, 1991).

2.2.11 Purification of viral DNA (adapted from Sambrook *et al.* (1989):

The viral DNA was subjected to one round of phenol extraction, followed by one round of phenol and chloroform extraction (24:1 chloroform:isoamylalcohol) (Sigma, USA), and finally, one round of chloroform extraction. DNA was precipitated by addition of $1/10^{th}$ volume sodium acetate (3 M, pH 5.2) and 2.5 volumes 96% ethanol, followed by a wash in 70% ethanol. Resuspension of the DNA was in sterile distilled water or TE buffer and storage was at -20 °C.

2.2.12 Southern transfer and radioactive ^{32}P-labelled probe hybridisation:

Purified DNA from wtLSDV and LSDV-lacZ recombinant (from purified virions and from the cellular debris fraction after low speed centrifugation) were cut with *Hind*III and run on a 0.8% agarose gel. The separated DNA fragments were then Southern transferred to a Hybond^{TM}N^{+} nylon membrane (Amersham, USA) using alkaline buffer for transfer (Sambrook *et al.*, 1989).
A ^{32}P-dATP labelled DNA probe was prepared from the *Hind*III fragment of LSDV containing the TK gene using nick translation (Nick Translation Kit, Promega, USA). The probe was then hybridised to the membrane, washed under stringent conditions (Sambrook *et al.*, 1989) and the washed membrane was then exposed to Kodak Biomax^{TM} MS-1 Scientific Imaging Film (Sigma, USA) at –70 °C. The film was then developed and the resulting autoradiograph analysed.

2.3 RESULTS

2.3.1 Construction of the LSDV DNA transfer vector, pLSTK7.5:

In order to insert foreign genes into the TK gene of LSDV it was necessary to construct a transfer vector containing the viral TK sequence interrupted by a restriction enzyme MCS, into which the foreign genes could be inserted. A poxvirus promoter is required to drive expression of the foreign genes. For this purpose the VV early/late P7.5K promoter was chosen. A distinct

advantage of using a dual early/late promoter to drive the expression of foreign genes is that it has been shown that proteins expressed early and late in the course of poxviral infections are able to stimulate both humoral and cell-mediated immune responses in animals (Andrew *et al.*, 1989). Recombinant LSDVs resulting from double cross-over homologous recombination between the disrupted plasmid TK gene and the intact parental viral genomic TK gene would thus contain an inactive TK gene. The TK-negative phenotype of resulting recombinants formed the basis for selection in the TK-selection method.

A plasmid containing the full-length LSDV TK gene (pLSDTK3c) was utilized as the base plasmid for the construction of the transfer vector (with kind permission of AL Williamson, UCT). A convenient *Kpn*I site is located close to the middle of the TK gene (Figure 2.2) and this site was used for the insertion of a MCS downstream of the VV P7.5K promoter.

The MCS and promoter were excised as a single 250 bp fragment from the VV transfer vector (pGVRW1) using *Nde*I and *Eco*RI and blunt-end ligated into the blunted *Kpn*I site in the TK gene in pLSDTK3c (Figure 2.3). Competent DH5α cells were transformed with the ligation mixture and plasmid DNA was extracted from 24 transformed bacterial colonies growing on the ampicillin-agar plates. DNA was then cut with *Hind*III and the resulting fragments were separated using agarose gel electrophoresis (Figure 2.4 A). Although difficult to see on the photograph, a number of DNA patterns looked correct by direct visualization on a UV transilluminator through a protective Perspex screen. One of these preps (#13) was then compared with a *Hind*III digest of pLSDTK3c (Figure 2.4 B). *Hind*III cuts pLSDTK3c to produce two fragments of 3.4 and 2.5 kbp (Figure 2.2). However, with the insertion of the 250 bp DNA fragment containing the P7.5K promoter and MCS from pGVRW1, the additional *Hind*III site in the MCS will result in three fragments of 3.4, 1.9 and 0.85 kbp. These fragment sizes are obtained for prep #13 cut with *Hind*III (Figure 2.4B and Figure 2.5A). Prep #13 was then cut with *Pst*I for further confirmation and the expected fragment sizes of approximately 2.85, 1.90 and 1.35 kbp were obtained (Figure 2.5A).

To be certain that the P7.5K promoter was included in the insert in prep #13, a PCR was performed (including a number of other preps showing the correct *Hind*III pattern) using a primer (P5) binding specifically to the P7.5K promoter and a primer (P1) which binds towards the right end of the LSDV TK gene (refer to figure 2.13 A). PCR conditions were as described (2.2.9) except that extension was 60 seconds.

An amplification product of 450 bp would be produced if the promoter was in the correct orientation. In Figure 2.5 (B) it can be seen that for a number of the preps (#13, 16, 11, 20 and 3) this amplification product is produced, although not for prep #5 (lane 7), nor for pLSDTK3c (lane 8) which lacks the P7.5K promoter. Thus it is probable that for prep #5 the insert is in the opposite orientation to the other preps.

It can be concluded from the results of these experiments that the P7.5K promoter and the MCS were successfully inserted into the LSDV TK gene in pLSDTK3c to produce a LSDV transfer vector (pLSTK7.5, Figure 2.6) with a number of unique R.E. sites for the insertion of foreign genes.

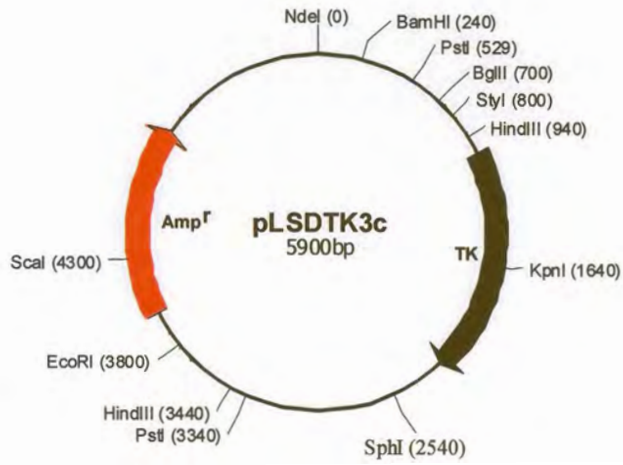


Figure 2.2 Restriction enzyme map of plasmid pLSDTK3c (supplied by Dr Anna-Lise Williamson, University of Cape Town).

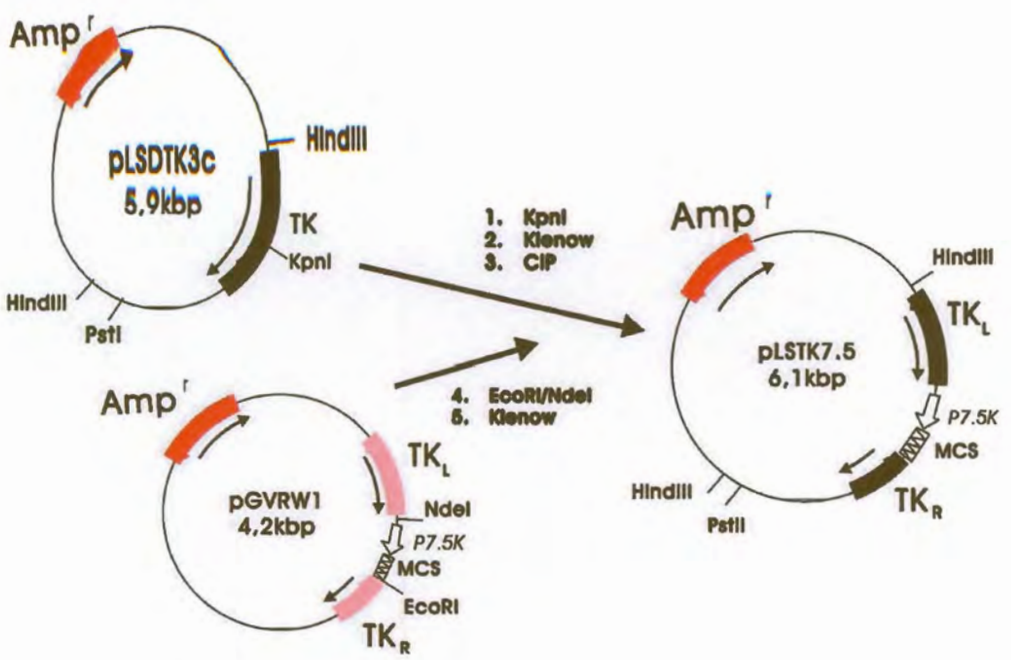
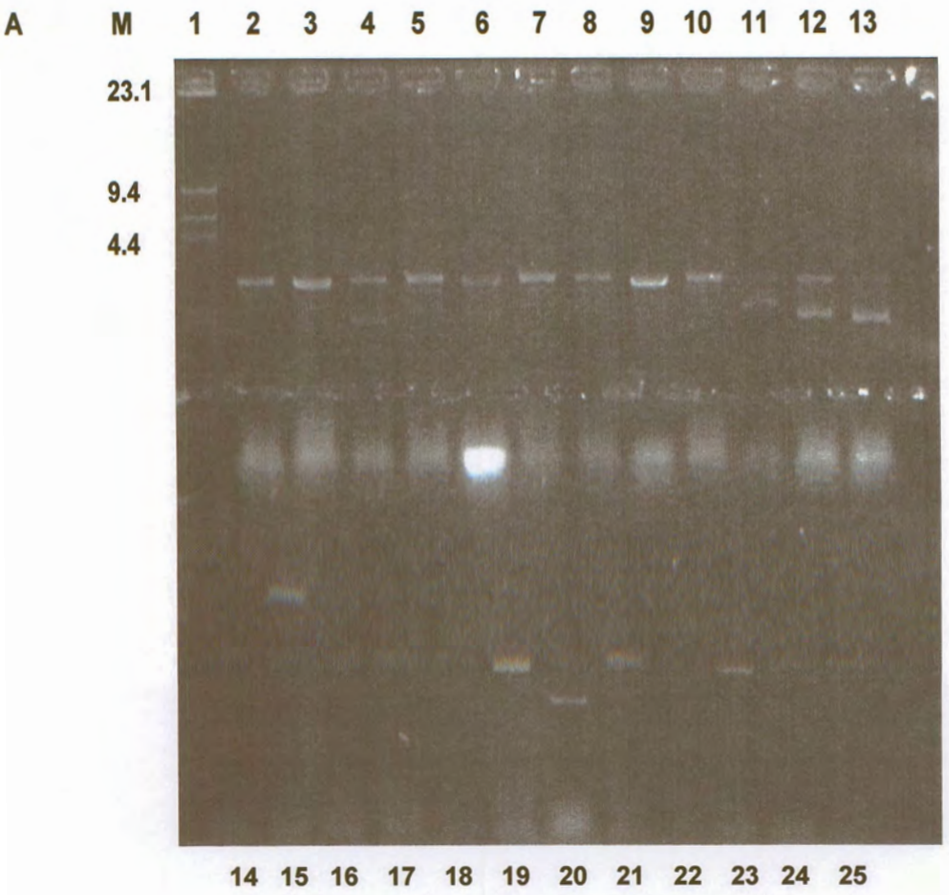


Figure 2.3 Construction of the LSDV transfer vector, pLSTK7.5. The VV early/late P7.5K promoter and downstream multiple cloning site (MCS) were removed from the VV transfer vector, pGVRW1, and inserted into the KpnI site of the LSDV thymidine kinase (TK) gene in pLSDTK3c. The resulting LSDV transfer plasmid, pLSTK7.5, contains an inactive TK gene with convenient restriction enzyme sites for the insertion of foreign genes under control of the P7.5K promoter.



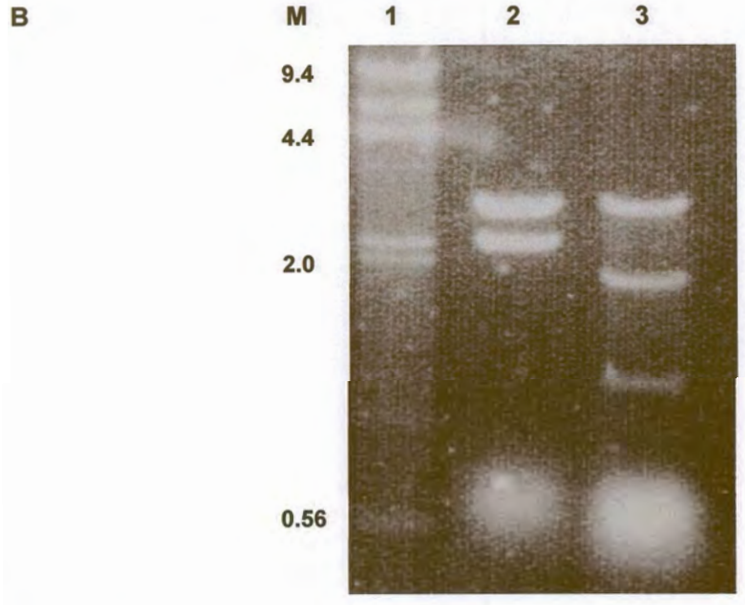


Figure 2. 4 Agarose gel electrophoretic analysis of miniprep DNA for the construction of the LSDV transfer vector, pLSTK7.5. **A** – miniprep DNA cut with *Hind*III (lanes 2 to 25). Lane 1 = lambda *Hind*III marker. **B** – *Hind*III cut pLSDTK3c (lane 2) and miniprep #13 (lane 3). Lane 1 = lambda *Hind*III marker. M – sizes of DNA fragments in kbp.

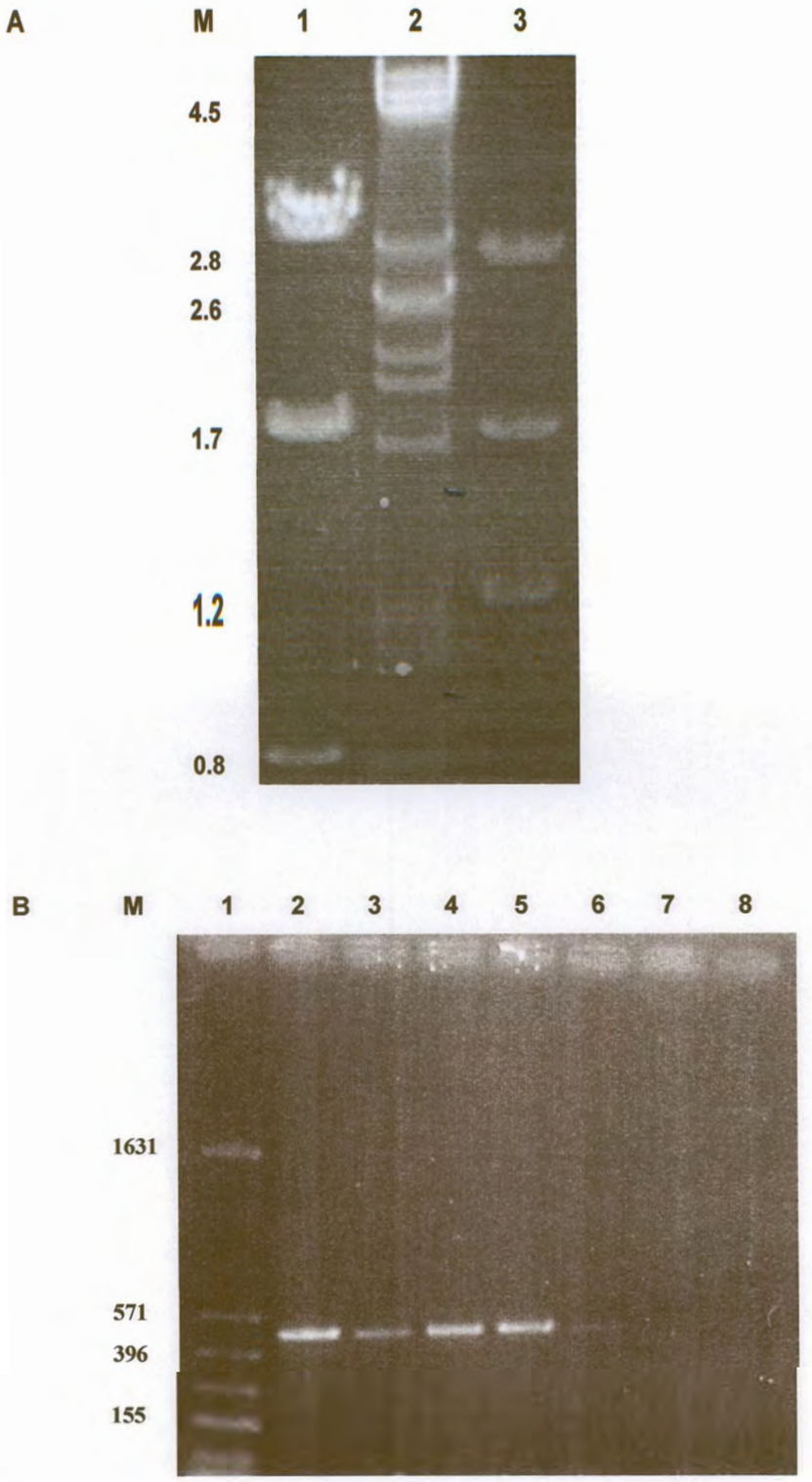


Figure 2.5 Agarose gel electrophoretic analysis of miniprep DNA for the construction of the LSDV transfer vector, pLSTK7.5. **A** – *Hind*III (lane 1) and *Pst*I (lane 3) cut prep #13 with a lambda *Pst*I DNA marker (lane2). **B** – PCR-amplified DNA from preps #13 (lane 2), #16 (lane 3), #11 (lane 4), #20 (lane5), #3 (lane 6), #5 (lane 7) and pLSDTK3c (lane 8) using primers P1 and P5. Lane 1 = pAT153 *Hin*fI DNA marker. M = DNA fragment sizes in kbp.

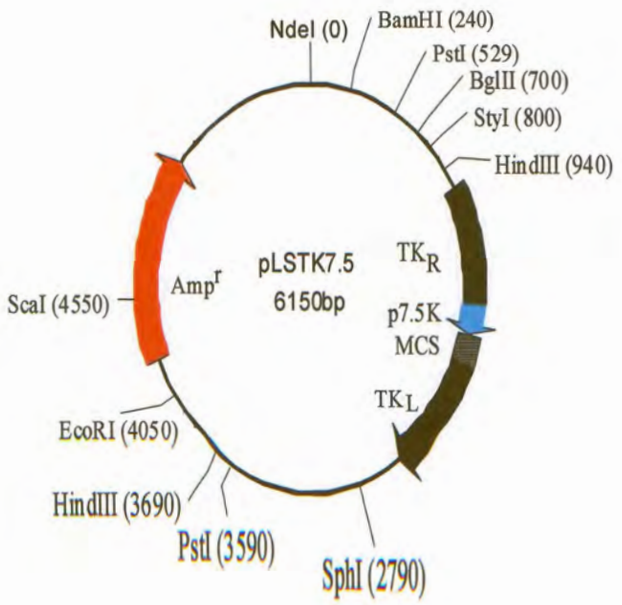


Figure 2.6 R.E. map of the LSDV transfer vector, pLSTK7.5 showing the restriction enzyme sites in the MCS (R.E. sites starting from P7.5K promoter: - *Bgl*II, *Pst*I, *Sal*I, *Bam*HI, *Hind*III, *Xba*I, *Kpn*I, *Sph*I, *Eco*RV, *Sac*I, *Xma*I, *Ava*I, *Sma*I, *Eco*RI -)

2.3.2 Insertion of lacZ reporter gene into the LSDV transfer vector, pLSTK7.5:

With the construction of a suitable LSDV transfer vector, it was then possible to insert foreign genes into the vector for the generation of TK-disrupted LSDV recombinants expressing the foreign genes. However, as the chosen approach (TK-negative selection) for generating and selecting the recombinants was unproven for LSDV, the first recombinants were to be generated expressing a reporter gene to allow easier evaluation of the selection strategy. For this purpose the *E. coli* lacZ reporter gene was selected as a means for colour screening of recombinants and was to be inserted into the pLSTK7.5 transfer vector under control of the P7.5K promoter.

A copy of the lacZ gene was obtained from the pSV-ß-gal mammalian expression vector (Promega, USA). The gene was removed using *Hind*III and *Bam*HI (a 3.7 kbp fragment) and was inserted blunt-ended into the *Sal*I site (also blunt-ended) in the MCS of pLSTK7.5 (Figure 2.7). As the gene was inserted as a blunt-ended product, it was important to determine its orientation with respect to the P7.5K promoter. Plasmid mini-preps were cut with *Sac*I and the DNA fragments were separated using agarose gel electrophoresis (Figure 2.8). The lacZ gene contains a *Sac*I site 1.6 kbp from its 3' end and the pLSTK7.5 vector contains a *Sac*I site

downstream of the *SalI* site (Figure 2.6). Thus, for the correct orientation, cutting with *SacI* should produce two bands of 8.2 kbp and 1.6 kbp – as is the case for preps in lanes 2, 5 and 6 (Figure 2.8). The rest of the preps contain the gene in the wrong orientation. Prep #2 was then cut with *EcoRI*, *PstI*, *SacI* and *SmaI* and run on an agarose gel to confirm that the construct was correct. The expected fragment patterns were obtained (results not shown) and the plasmid (now named pLSTK7.5-lacZ) was grown to large scale and purified through a Qiagen Plasmid Midi Kit gravity-flow column for storage at – 20 °C.

In order to ensure that the P7.5K promoter and lacZ gene contained no mutations which could interfere with lacZ expression, standard practice would have been to sequence the expression cassette. However, it was quicker to evaluate the construct by co-transfecting it into LSDV-infected MDBK cells and monitoring for transient expression of lacZ by fixing and staining the cells with X-gal. MDBK cells were co-transfected as described (2.2.5) with pLSTK7.5-lacZ (or, pLSTK7.5 as a negative control) and were then fixed and stained with an X-gal stain 24 hours post-infection. Transient expression of lacZ was observed in cells co-transfected with the pLSTK7.5-lacZ construct as evidenced by the production of a strong blue colouration. (Figure 2.12 B). However, no such colouration was observed for cells co-transfected with pLSTK7.5 (Figure 2.12 A) proving that it was expression of the lacZ gene from pLSTK7.5-lacZ that was responsible for the production of the blue colouration.

Thus the pLSTK7.5 transfer vector was modified by the insertion of the lacZ reporter gene under control of the VV P7.5K promoter. This construct could now be used in the generation of TK-disrupted LSDV recombinants and the lacZ gene would prove a useful marker for assisting with the evaluation of the selection process.

2.3.3 Generation and selection of LSDV recombinants using the TK-negative selection method:

The construction of an insertion vector (pLSTK7.5-lacZ) containing the lacZ reporter gene under control of a poxvirus promoter and flanked by viral TK sequences made it possible to assess the TK-negative selection method as a means to generate and select TK-disrupted LSDV recombinant viruses. The availability of a LSDV-permissive cell line (BU100 cells) able to tolerate the BUdR selection drug further assisted with this process.

For the generation of recombinants, MDBK cells were infected with wtLSDV at an MOI = 1 and were co-transfected 12 hours later with calcium-phosphate precipitated LSDV genomic DNA and pLSTK7.5-lacZ as described (2.2.7). Once the cells displayed 80% cpe they were harvested, freeze-thawed three times, clarified by low-speed centrifugation and the supernatant fraction titrated serially on BU100 cells in the presence of 100 µg/ml BUdR. Four days later areas of the

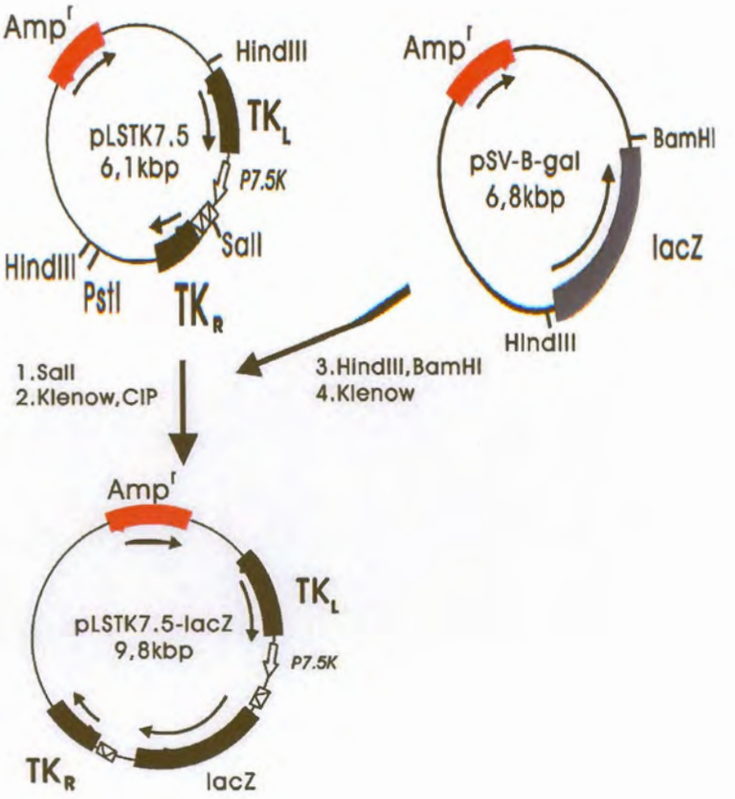


Figure 2.7 Construction of the LSDV insertion vector, pLSTK7.5-lacZ. The complete β–galactosidase gene (lacZ) was removed from the mammalian expression vector, pSV-β-gal, and blunt inserted into the *Sal*I site in the MCS of pLSTK7.5. The lacZ gene serves as a useful reporter gene to monitor the progression and efficiency of poxviral recombinant generation and selection.

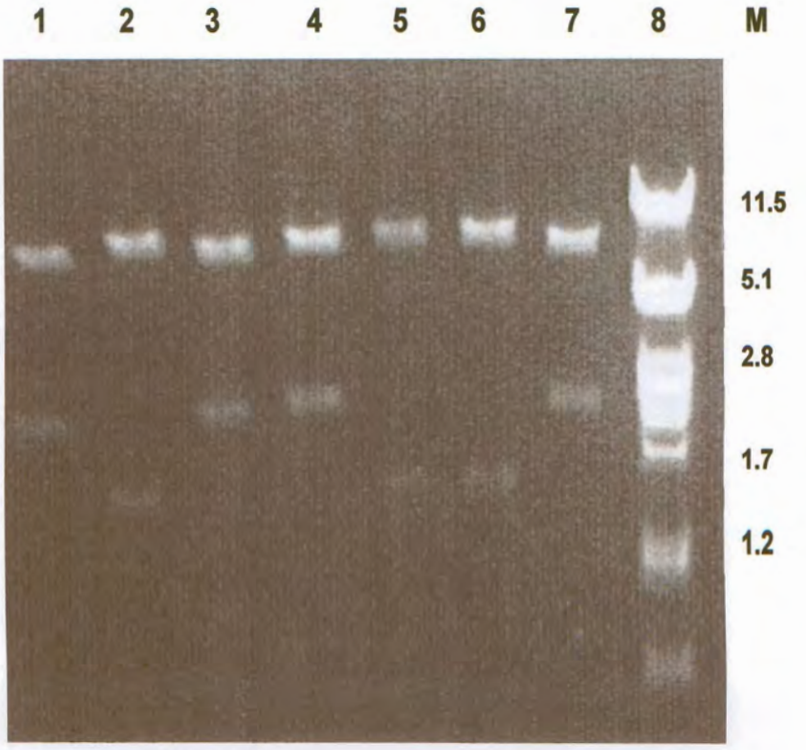


Figure 2.8 Agarose gel electrophoresis analysis of miniprep DNA for the insertion of the lacZ reporter gene into pLSTK7.5. DNA was cut with *Sac*I. Lanes 1-7 contain *Sac*I cut miniprep DNA and lane 8 a lambda PstI marker. M = DNA fragment sizes in kbp.

cell monolayers which resembled viral foci were picked, divided into two equal aliquots and the one half was treated as described for lacZ expression. Surprisingly none of the aliquots placed under an X-gal agarose overlay produced any clear indication of lacZ expression.

The other half of the aliquots from which DNA had been extracted were then tested for viral DNA (both recombinant and wild type) using PCR analysis (Pasamontes *et al.*, 1991). The primer pair P1 and P2 bind specifically to regions flanking the insertion site in the LSDV TK gene and can be used to distinguish wt from recombinant viral DNA (Fig. 2.13 A). As internal controls, primer P3 which binds to the 3' end of the gpt gene and P4 which binds to the central region of the lacZ gene, were designed. Even though the plasmid controls worked, no PCR products were produced from DNA extracted from the "foci" (data not shown).

A number of modifications were then introduced including increasing the amounts of input viral and plasmid DNA, varying the times between infection and co-transfection of DNA, and leaving out the agarose overlay and fixing and staining the cells directly for lacZ expression (results not shown). However, none of modifications yielded recombinants. Fick (1998) attempted a similar approach to generating LSDV recombinants expressing the VP2 gene of African horsesickness

virus (type 3) and also failed. In parallel experiments, generation of VV recombinants via the TK-negative selection strategy were successful, confirming technical competence (data not shown). This approach therefore totally failed to produce recombinant viruses which were selectable on the basis of their TK-negative phenotype and therefore a change in strategy was required. The MPA selection method had been successfully used by Romero *et al.* (1993) to select TK-disrupted recombinants of the KS-1 strain of LSDV, and therefore this method was now attempted for the South African vaccine strain of LSDV.

2.3.4 Insertion of the *E. coli* gpt dominant selectable marker gene into pLSTK7.5-lacZ:

The MPA selection method requires the presence of the *E. coli* gpt dominant selectable marker gene in the recombinant virus during the selection stages. It can either be retained in the final recombinant construct if inserted between the TK flanking sequences in the insertion vector, or, allowed to be deleted once selection pressure has been removed if inserted outside the TK flanking sequences. For the purposes of this study it was decided to insert the gene inside the TK flanking sequences of the insertion vector, pLSTK7.5-lacZ.

The gpt gene, under control of the VV P7.5K promoter, was provided by Dr Anna-Lise Williamson (University of Cape Town) within the plasmid, pSelp(HS)G1G2. The gene was then removed from the plasmid (including the poxvirus promoter) using *Eco*RI and the 1.3 kbp DNA fragment was blunt-end inserted into a unique *Sma*I site downstream of the lacZ gene in pLSTK7.5-lacZ, giving rise to pLS(g)lac (Fig. 2.9). In this construct it was important to ensure that the two P7.5K promoters were in opposite orientations to each other or else there would be the risk of an unwanted recombination event occurring which would drop out the lacZ gene. Thus making use of an internal *Sac*I site in the lacZ gene and a *Sac*I site at the 5' end of the gpt expression cassette (Figure 2.11) miniprep DNA was cut with *Sac*I to enable the orientation of the P7.5K promoter-gpt cassette to be determined. The desired orientation would produce three bands of 8.5, 1.5 and 1.3 kbp. In Figure 2.10 (A) it can be seen that preps in lanes 4, 5, 6, 9 and 11 produced this pattern. Prep #11 was then chosen for further characterization using a number of different restriction enzymes (Figure 2.10 B) – from the R.E. map (Figure 2.11) the expected patterns were produced and large amounts of highly pure DNA were prepared – the insertion plasmid was now called pLS(g)lac.

Further evidence for the integrity of the pLS(g)lac vector is provided in figure 2.13 (B) whereby PCR amplification of certain regions of the vector using different primer pairs (lanes 1 and 4) resulted in amplification products of the expected sizes (5.5 kbp and 1.7 kbp respectively).

Now that the gpt gene had been inserted into the lacZ insertion vector it was possible to attempt the MPA selection strategy for generating and selecting LSDV recombinants.

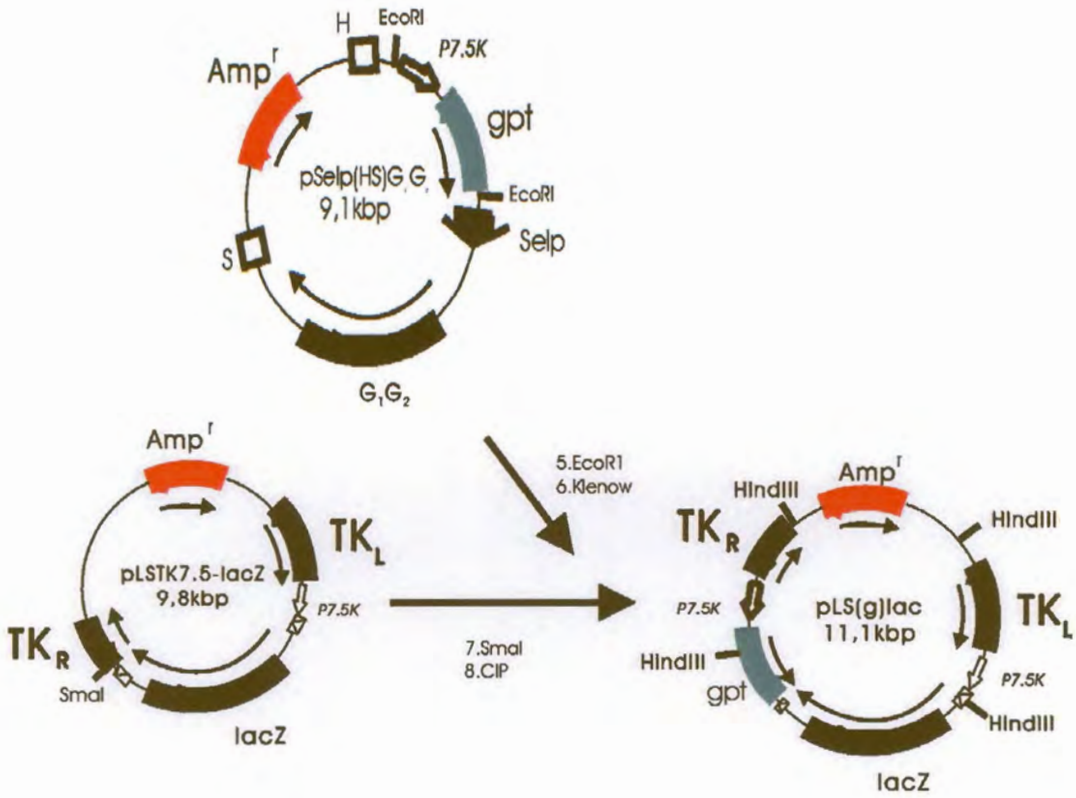


Figure 2.9 Construction of the insertion vector, pLS(g)lac. The *E. coli* gpt gene (1.3 kbp) was excised from pSelp(HS)G_1G_2 using *Eco*RI and blunt-end inserted into the *Sma*I site of the remaining MCS of pLSTK7.5-lacZ. The gpt gene is a positive selectable marker gene and allows for the selection of recombinant virions (expressing the gene) over non-expressing virions when grown in MPA selection medium.

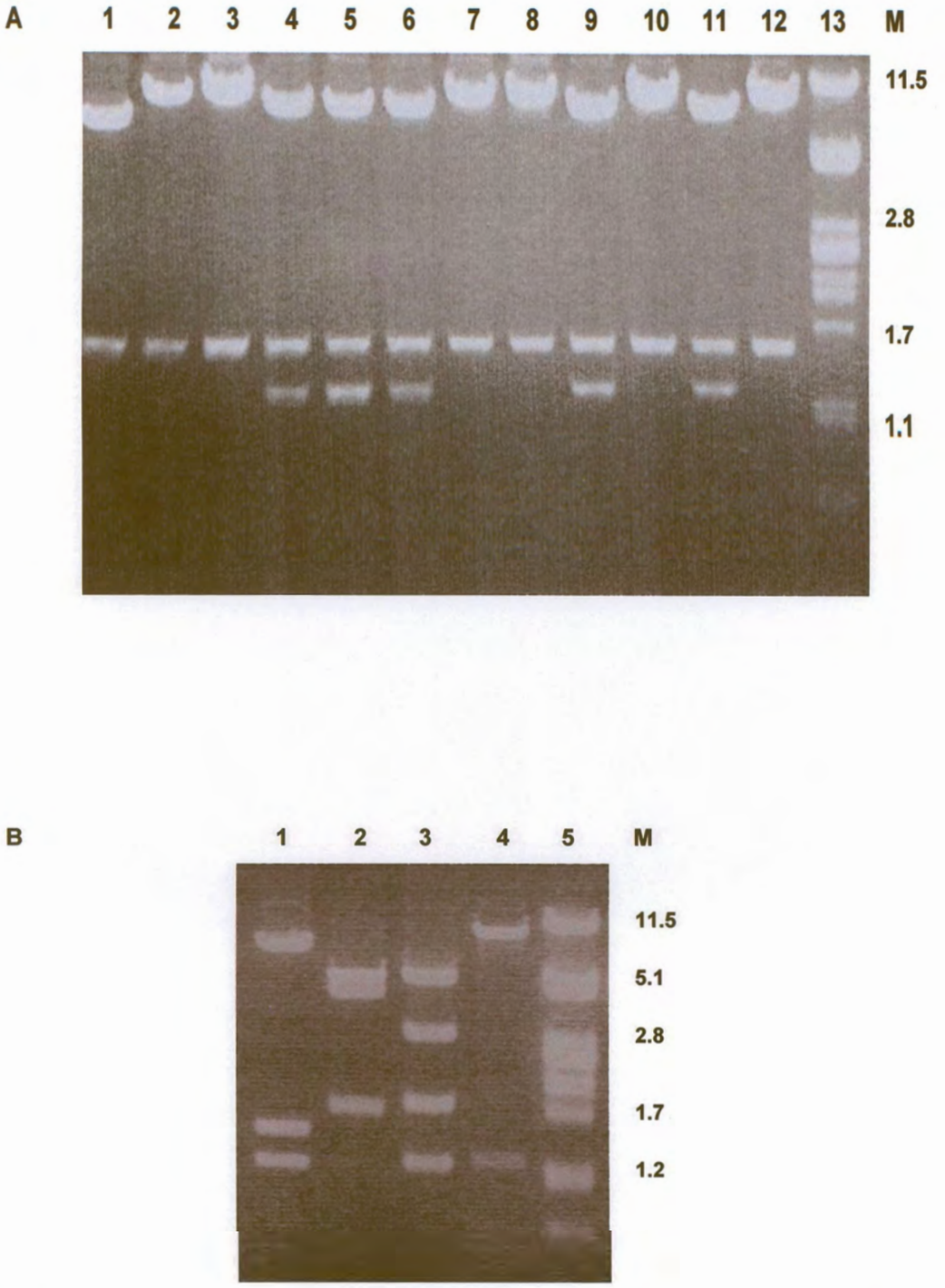


Figure 2.10 Agarose gel electrophoresis analysis of R.E. digests of pLS(g)lac. **A** – Minipreps digested with *Sac*I (lanes 1-12). Lane 13 = lambda *Pst*I marker. **B** – Prep #11 cut with a number of R.E.s Lane 1 = *Sac*I digest, lane 2 = *Nde*I, lane 3 = *Pst*I, lane 4 = *Sma*I and lane 5 = lambda *Pst*I marker. M = DNA fragment sizes in kbp.

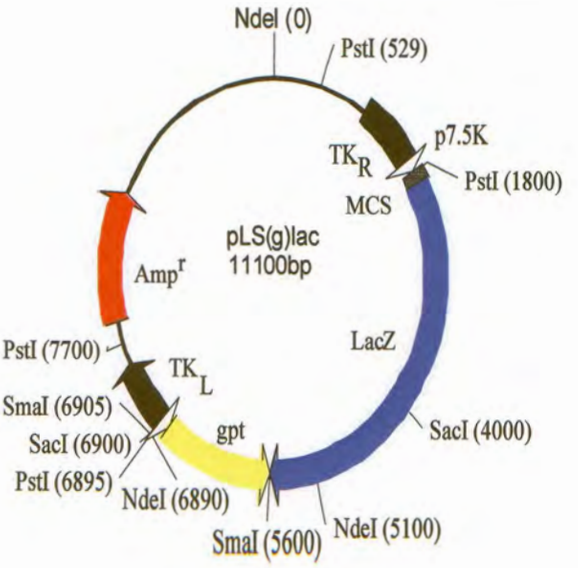


Figure 2.11 R.E. map of pLS(g)lac.

2.3.5 Generation and selection of LSDV recombinants using the MPA selection method:

This approach was based on the method of Romero *et al.* (1993) and makes use of positive dominant selection in the form of the *E. coli* gpt gene. Before commencing with the generation and selection of recombinants, lacZ expression from pLS(g)lac was first confirmed using a transient expression assay in FBT cells and LipofectAmine as transfecting reagent (2.2.5).

Transient expression of lacZ was observed in LSDV-infected FBT cells co-tranfected with pLS(g)lac and fixed and stained 24 hours post-infection (Figure 2.12 D). No blue colouration was observed in wells in which LSDV-infected FBT cells were transfected with pLSTK7.5 (Figure 2.12 C).

FBT cells were then tested for tolerance to the MPA selection medium. No adverse effects were observed (data not shown).

Cells were also infected with wt LSDV in the presence of selection medium and were observed for the development of viral foci. No viral foci were visible in control wells in which FBT cells were infected with wt LSDV under MPA selective pressure. However, when this approach was used for the generation and selection of LSDV recombinants (2.2.7), progeny virions released

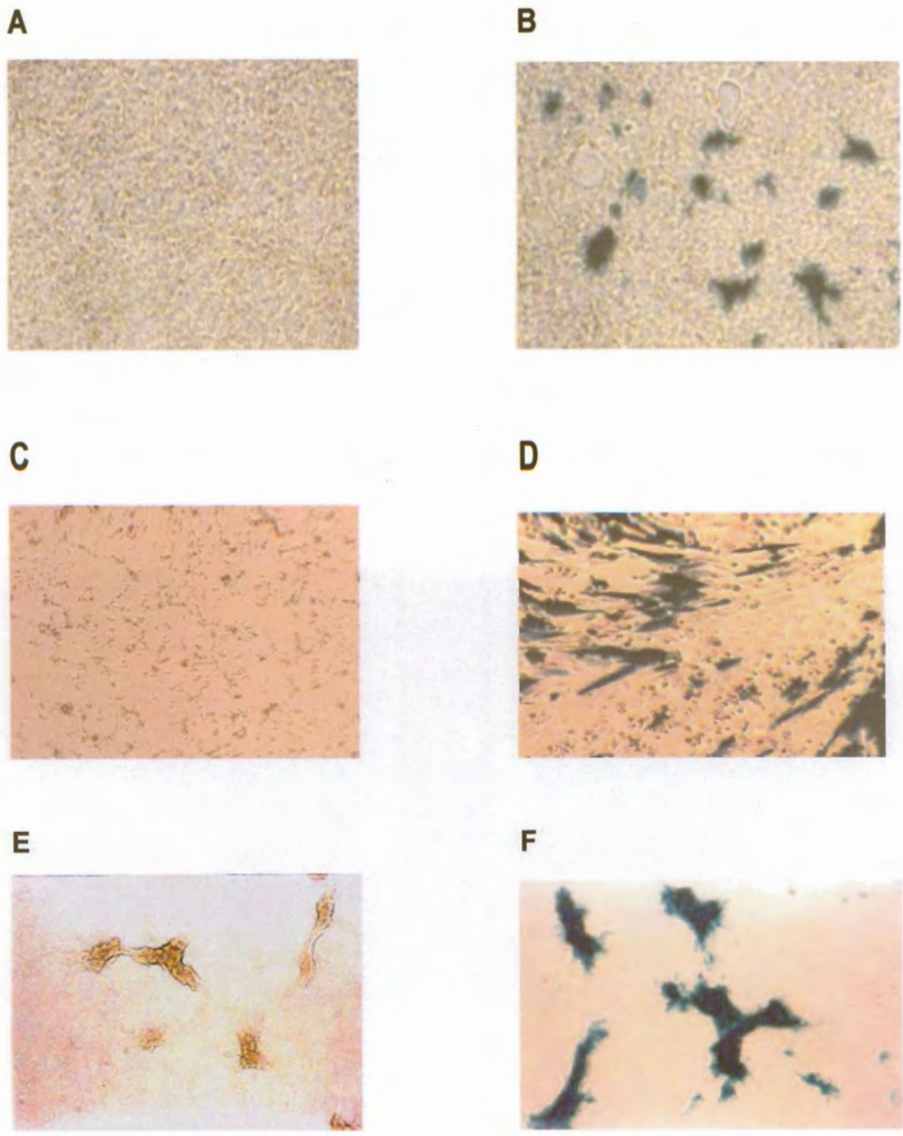
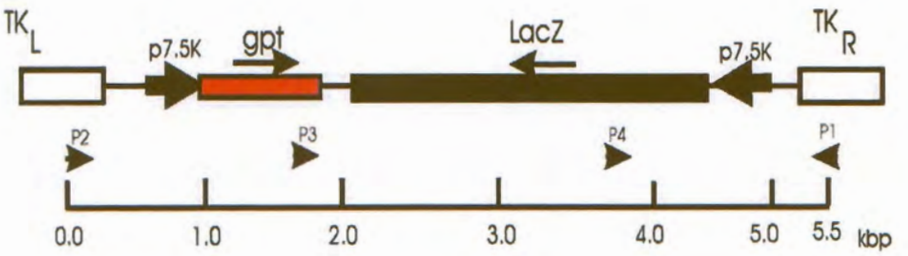


Figure 2.12 LSDV-lacZ expression: transient and from LSDV-lacZ recombinants. All cells were fixed and stained with an X-gal stain as described (2.2.5). **A** – MDBK cells co-transfected with pLSTK7.5 and wtLSDV (MOI = 1). **B** - MDBK cells co-transfected with pLSTK7.5-lacZ and wtLSDV (MOI = 1). **C** - FBT cells co-transfected with pLSTK7.5 and wtLSDV (MOI = 1). **D** - FBT cells co-transfected with pLS(g)lac and wtLSDV (MOI = 1). **E** – wtLSDV foci on MDBK cells (4 days post-infection). **F** – LSDV-lacZ recombinant virus on MDBK cells (4 days post-infection). Magnification: 250X.

A



B

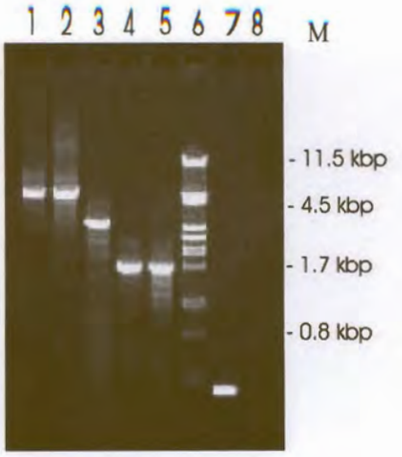


Figure 2.13 PCR analysis of LSDV-lacZ recombinants. (**A**) Schematic representation of the TK insertion region in the LSDV genome showing the gpt and lacZ genes and their p7.5K promoters. The primers used to analyse the recombinants are shown, including their direction of amplification (arrows). Primer sequences are given in section 2.2.9.

(**B**) Agarose gel of the PCR-amplified products separated by electrophoresis. Lane 1 – pLS(g)lac (primers P1 and P2), lane 2 – recombinant LSDV-lacZ virus (primers P1 and P2), lane 3 – recombinant LSDV-lacZ virus (primers P1 and P3), lane 4 – pLS(g)lac (primers P1 and P4), lane 5 – recombinant LSDV-lacZ virus (primers P1 and P4), lane 6 – lambda *Pst*I DNA marker, lane 7 – wtLSDV (primers P1 and P2), and lane 8 – dH_2O (primers P1 and P2). M – DNA marker sizes in kbp.

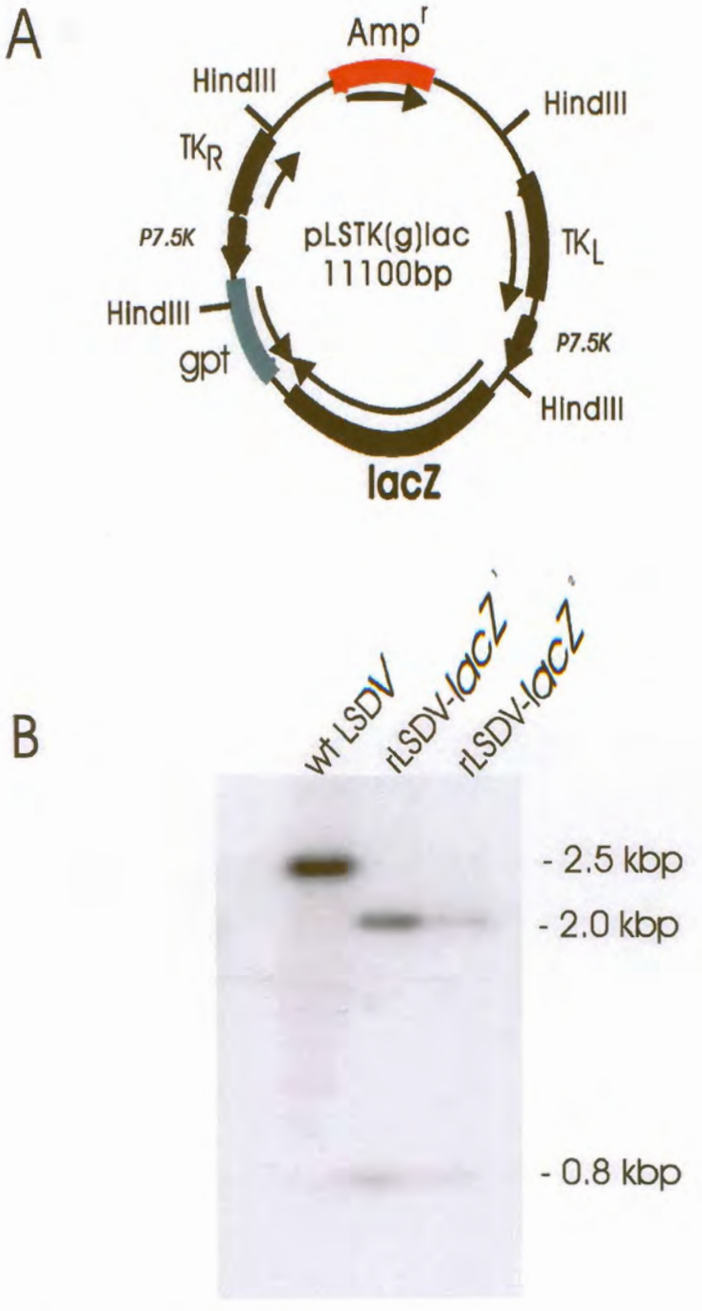


Figure 2.14 Autoradiograph of wtLSDV and lacZ recombinant *Hind*III digested DNA. **A** - detailed plasmid map of pLS(g)lacZ showing the location of the *Hind*III RE sites. **B** - an autoradiograph of *Hind*III cut wtLSDV DNA and LSDV-lacZ recombinant DNA run on an agarose gel, Southern transferred to a HybondN$^+$ nylon membrane and probed with a ^{32}P-labelled LSDV TK gene probe. Sizes of the labeled fragments are indicated in kbp on the right.
[1] – DNA from purified virions, [2] – whole DNA extract from infected cells.

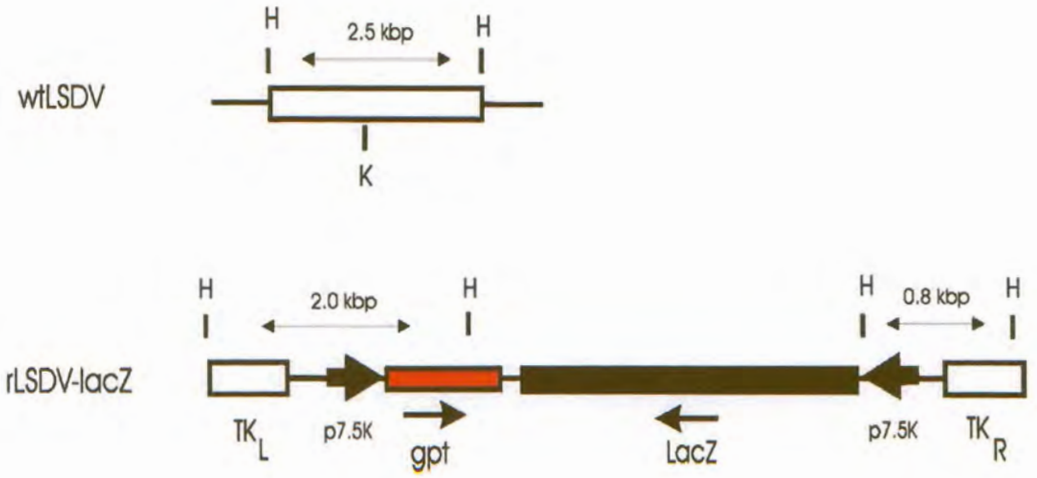
from FBT cells co-transfected with wt LSDV and pLS(g)lac produced distinct viral foci in fresh cells during the second round in selection medium in wells from the lowest dilutions. Media was collected from these wells, treated as described (2.2.7) and titrated further. Cells were then fixed and stained using X-gal (2.2.5). Approximately 90% of the foci stained blue, a strong indication for the presence of recombinants (Figure 2.12 F), whereas no blue colouration was observed for cells infected with wtLSDV (Figure 2.12 E).

An important aspect of the generation and selection of poxvirus recombinants for vaccine purposes is to ensure that they are homogeneous (free from wt parental virus). Mixed populations of wt and recombinant virus would necessitate the constant use of selection media for large-scale virus propagation which would hugely elevate the cost of vaccine production.

Two molecular-based methods were employed to help evaluate the ability of the modified Romero method (Romero *et al.*, 1993) to yield homogeneous recombinants, namely PCR and DNA hybridisation.

PCR was utilised as follows: the DNA from a number of viral foci which appeared homogeneous for the expression of lacZ was isolated and subjected to PCR using the TK primers P1 and P2 as described (2.2.9). For at least one of these foci, an amplification product of the full-length insertion region (5.5 kbp) was obtained (Figure 2.13 B, lane 2), providing further support for the recombinant's homogeneity. The presence of an undisrupted TK gene would have produced a band of 450 bp, as can be seen in lane 7 for wtLSDV DNA. Combinations of the internal control primers (P1 and P3, and, P1 and P4) also gave the expected product sizes for DNA amplified from both the pLSTK(g)lac insertion plasmid (lane 4) and this recombinant viral focus (lanes 3 and 5).

Recombinant virus was propagated to high titres from this focus (under selection) and DNA was extracted for additional confirmation of homogeneity using the DNA hybridisation method as follows: DNA from wtLSDV and the recombinant virus was cut with *Hind*III, and the resulting DNA fragments were separated on a 0.8% agarose gel using electrophoresis. The fragments were then Southern transferred to a Hybond$^{TM}N^{+}$ nylon membrane (Amersham, USA) and hybridised with a ^{32}P-labelled wtLSDV-specific TK gene probe (as described, 2.2.12).
The intact LSDV TK gene is located on a 2.5 kbp *Hind*III DNA fragment. However, as can be seen from figure 2.14 (A) and the schematic below, interruption of the TK gene by insertion of the gpt-lacZ cassette in the *Kpn*I site results in the viral TK gene being split between two *Hind*III fragments (2.0 and 0.8 kbp in size). Thus a radioactive probe produced from the entire viral TK gene would be expected to bind to the 2.5 kbp *Hind*III fragment for wtLSDV DNA and to the 2.0 and 0.8 kbp fragments for rLSDV-lacZ DNA.



As can be seen from the autoradiograph (Figure 2.14 B) this is the case, and, no evidence for the wt TK gene (2.5 kbp fragment) can be seen for the lanes containing the recombinant viral DNA, thus confirming homogeneity.

Another pre-requisite for a pox-vectored recombinant vaccine is that the resulting recombinant is stable. In order to test the stability of the LSDV-lacZ recombinant, selection pressure was removed by growth of the recombinant in normal growth medium (DMEM/Ham's F12, 10% FCS) (no MPA) for 10 passages. DNA was isolated from virus recovered after the 10^{th} passage and tested via PCR using the TK primers P1 and P2. Only the recombinant PCR amplification product of 5.5 kbp was obtained (data not shown).
LacZ expression was also tested for progeny virions produced during the 10^{th} passage without selection using the X-gal stain. All foci still stained blue.
Collectively, all these results thus confirm that the LSDV-lacZ recombinant was stable and absolutely homogeneous.

2.4 DISCUSSION

The aim of this part of the study was to evaluate the viral TK gene of the South African vaccine strain of LSDV as a suitable insertion site for the generation and selection of recombinants. Of the many techniques available for generating and selecting poxvirus recombinants (Mackett *et al.*, 1982; Mackett *et al.*, 1985; Chakrabarti *et al.*, 1985; Guo *et al.*, 1989; Falkner and Moss, 1990; Mason *et al.*, 1991; Cadoz *et al.*, 1992; Merchlinsky and Moss, 1992; Scheiflinger *et al.*, 1992; Ravanello and Hruby, 1994; Stone-Marschat *et al.*, 1996; Scheiflinger *et al.*, 1998;

Timiryasova *et al.*, 2001; Boyle *et al.*, 2004), one of the first and most successfully used methods involves disruption of the viral TK gene with the insertion of foreign genes under control of a poxvirus promoter (Mackett *et al.*, 1985; Boyle and Coupar, 1986). This method has a double advantage as the resulting TK-negative phenotype can provide a means for selection of recombinants and the resulting recombinants are more environmentally acceptable (as no antibiotic resistance markers are present in the final construct). This route was thus the obvious choice for attempting the generation and selection of recombinants of the South African vaccine strain of LSDV earmarked for use as a vector for recombinant vaccines. The success of this method does however reply upon the TK gene being non-essential for growth of the virus. Dubbs and Kit (1964) demonstrated that in mouse fibroblast cells infected with wild type VV there was an induction of TK activity and that the virus encodes a TK gene. They also demonstrated the existence of mutant VV viruses lacking TK activity which were relatively stable. This is also the case for most, but not all poxviruses – TK-disrupted recombinants of pigeonpox virus proved unstable and it was surmised that the gene was essential for certain viral functions (Letellier, 1993). The viral TK gene has been shown to be non-essential for the Kenyan KS-1 vaccine strain of LSDV (Romero *et al.*, 1993), however, this vaccine strain was developed along a different route (and originally for use in sheep and goats) to that of the South African vaccine strain and thus it cannot be assumed that what holds true for one will necessarily hold true for the other (Weiss, 1968; Davies and Otema; 1981; Kitching and Taylor, 1985; Kitching *et al.*, 1987; Gershon and Black; 1989b; Kitching *et al.*, 1989).

Another prerequisite for the TK-negative method of selection is a cell line permissible for growth of LSDV and which is able to tolerate the TK selection drug, BUdR (Boyle and Coupar, 1986). As LSDV is highly host-range restricted (making it ideal for development as a recombinant vaccine vector targeted for use in specific animal species) there are very few cell types which support its growth (Prydie and Coackley, 1959). Surprisingly, attenuation of the South African vaccine strain of LSDV was achieved via repeated passage of a virulent field isolate on the CAMs of embryonated hen's eggs, although subsequent growth in chicken embryo fibroblasts is poor (van Rooyen *et al.*, 1969; Wallace, 1994). An Indian isolate of goatpox virus has been adapted for growth in cells of monkey origin (Vero cells), but, these cells do not support growth of the South African vaccine strain of LSDV (Wallace, 1994; Hosamani *et al.*, 2004). The best growth for LSDV is obtained in cells of bovine and ovine origin (Weiss and Geyer, 1959; Wallace, 1994). It was thus extremely fortuitous to find a bovine kidney cell line (BU100) which had been adapted to tolerate high levels of BUdR (Bello *et al.*, 1987). In preliminary experiments these cells were infected with wild type LSDV (vaccine strain) and observed for cytopathic effects (data not shown) – no cytopathic effects ever developed. However, in the absence of selection pressure, virus grew to levels comparable with growth in normal bovine kidney cells (MDBK cells) (Wallace

and Viljoen, 2002). These results suggest that the viral TK gene was active and propagation of the virus was totally suppressed by the addition of BUdR in the BU100 cells.

A transfer vector which would allow insertion of foreign genes under control of a poxvirus promoter into the LSDV TK gene also had to be developed. pLSTK7.5 was constructed for this purpose with the VV P7.5K early/late promoter upstream of a MCS flanked by the viral TK gene. To assist with monitoring the success rate for the generation and selection of the recombinants the lacZ visual marker gene was included in the insertion cassette. This marker provided a convenient means to rapidly assess whether or not recombinant viruses had been generated, without the need to extract DNA and test via hybridization or PCR. With the construction of the pLSTK7.5-lacZ insertion vector containing the viral TK gene interrupted by the lacZ reporter gene under control of the VV P7.5K promoter it was possible to begin evaluating the TK-negative selection method for generating and selecting recombinants. The calcium phosphate co-precipitation method was used for transfection of the viral and plasmid DNA as this method was being successfully used to generate VV recombinants in our laboratory. However, after many attempts to generate and select recombinants, even after optimising the transfection conditions to match the replication cycle of LSDV, altering the MOI and the amounts of transfecting viral and plasmid DNA, no recombinants could be selected using this approach. At first the reasons for this failure were unknown. Different transfection reagents were even tested (data not shown), but, without success. The only logical explanation was that viral TK activity was important for growth of the virus, but, at this stage it was not possible to test this hypothesis.

Eventually an alternative approach was attempted using positive selection in the form of the *E. coli* gpt gene, however still retaining the viral TK gene as the insertion site – as this approach was found to work for the northern African KS-1 strain of LSDV (Romero *et al.*, 1993). The method does not rely upon a TK-negative cell line for selection. This time success was achieved and it was possible to generate and select LSDV recombinants expressing the lacZ reporter gene, which was evident from the blue colouration of viral foci stained with X-gal.
A single recombinant was selected and was shown to be homogeneous via PCR and DNA hybridisation and indications were that it is highly stable, as removal of the MPA selection drug for 10 passages in FBT cells failed to give any evidence of revertance to wt form (data not shown).

Now that a homogeneous TK-negative LSDV was available, it was possible to evaluate its growth characteristics in a number of different cell types, including the TK-negative BU100 cells. As expected, the recombinant virus was unable to grow in the BU100 TK-negative cell line with or without selection, whereas it was able to grow to high titres in normal TK-producing MDBK, or

FBT cells (Wallace and Viljoen, 2002). These results strongly suggest that some form of TK activity is required for normal growth of LSDV and helps to explain the failure of the TK-negative selection method to yield recombinant viruses. Letellier (1993) found that TK-disrupted recombinants of pigeonpox virus were highly unstable and had a distinct growth disadvantage as compared to TK-positive virus. The importance of TK activity for growth of pigeonpox virus (and fowlpox virus) is highlighted by the inability of others to isolate naturally occurring TK-negative mutants of either virus under BUdR selection (Nazerian and Dhawale, 1991; Letellier, 1993), whereas this is possible for VV (Dubbs and Kit, 1964). Scheiflinger *et al.* (1997) described their inability to isolate TK-negative fowlpox recombinants using a highly attenuated virus strain and they suggest that for such attenuated strains an intact TK gene is required. A similar situation was found for the highly attenuated MVA strain of VV (Scheiflinger *et al.*, 1996), and in this case restoration of viral TK activity by insertion of a functional fowlpox virus TK gene allowed the isolation of recombinants. As the strain of LSDV used in these studies is also a highly attenuated strain, it is not surprising that TK activity is important for viral growth - even if this activity is cellular derived.

Once a strategy had been developed for generating and selecting LSDV recombinants which were shown to be homogeneous and stable, the following step was then to replace the lacZ reporter gene with genes of veterinary importance, as described in the next chapter.

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Once a strategy had been developed for generating and selecting LSDV recombinants which were shown to be homogeneous and stable, the following step was then to replace the lacZ reporter gene with genes of veterinary importance, as described in the next chapter.

CHAPTER 3

GENERATION AND SELECTION OF LSDV RECOMBINANTS EXPRESSING GENES OF VETERINARY IMPORTANCE

3.1 INTRODUCTION

Once a system had been developed for generating and selecting homogeneous LSDV recombinants expressing a reporter gene, it was possible to generate and select LSDV recombinants expressing genes of veterinary importance for use as pox-vectored recombinant vaccines.

However, before commencing further, two areas were identified where the current system could be improved upon, viz. the LSDV transfer vector, and, the strategy for selecting homogeneous recombinants. The LSDV transfer vector was streamlined by removal of non-essential sequences, and, a number of unique restriction enzyme (R.E.) sites were restored in the MCS after their uniqueness was lost due to introduction of the gpt positive selectable marker gene (along with a number of R.E. sites).

Although it was possible to select homogeneous LSDV recombinants using the modified MPA-selection strategy outlined in chapter 2, it needs to be mentioned that this strategy is not deemed to be perfect and early attempts to select for homogeneous LSDV recombinants expressing immunogenic genes of veterinary importance (in place of the lacZ reporter gene) were highly frustrated by the presence of contaminating wild-type (parental) LSDV. The lacZ reporter gene provided a useful visual basis for assisting with the selection process, however it was found that attempting to retain this gene in the transfer vector with the addition of immunogenic genes from viruses of veterinary importance led to logistical problems such as size (insertion plasmids would have been greater than ~ 14 kbp in size).

In order to overcome this problem the gene of the enhanced green fluorescent protein (EGFP) (originally isolated from the jellyfish, *Aequorea victoria*) (Cheng *et al.*, 1996) was substituted for the lacZ gene and was incorporated into the improved LSDV transfer vector. Not only is this gene much smaller in size than the lacZ gene (1 kbp, as opposed to 3.7 kbp), but it also allows for the *in vivo* selection of recombinants. The enhanced form of the green fluorescent protein produces a stable continuous emission of visible green light at a wavelength of 511 nm when excited with UV light in the 490 nm range (which coincides with the range used in most laboratory fluorescence microscopes and flow cytometers).

The EGFP gene was placed under control of the VV late P11K promoter. Recombinant LSDVs were found to express the protein within 12 hours post-infection making it easier to select for viral foci homogeneous for recombinant virus over foci containing a mixture of both wild-type and recombinant viruses when the cells were viewed under UV light. Contaminating wtLSDV still proved to be a problem though, and other modifications were introduced to the selection process to attempt to overcome this problem (described in the Materials and Methods).

For the next stage of the evaluation process, the immunodominant genes of bovine ephemeral fever virus (BEFV) and Rift Valley fever virus (RVFV) were chosen for insertion into the improved LSDV transfer vector, as these viruses cause diseases in cattle (BEFV and RVFV) and sheep (RVFV) of economic importance throughout Africa where LSDV is also endemic. The resulting recombinant vaccines should elicit a dual protective response not only in cattle (against LSD and bovine ephemeral fever, or Rift Valley fever), but also in sheep and goats (against sheep-and-goatpox and Rift Valley fever) as sheep-and-goatpox virus share a common surface antigen with LSDV (Kitching *et al.*, 1986).

BEFV is an arthropod-borne single-stranded negative sense RNA virus belonging to the family *Rhabdoviridae* (genus *Ephemerovirus*) (Della-Porta and Brown, 1979; Venter *et al.*, 2003). It causes an acute febrile disease in cattle and water buffalo (as reviewed by Nandi and Negi, 1999, and by Walker, 2005). Neutralising antibodies have been found in other species of African wildlife (Davies *et al.*, 1975). Bovine ephemeral fever (BEF) is widespread throughout Africa, Australia, and parts of the Far East and is commonly known as "ephemeral fever" or "three-day stiffness sickness" due to the immobilisation of infected animals for 3-5 days following the height of viraemia and fever (Bevan, 1912; as reviewed by St George, 2004). Although recovery may be complete, mortality occurs in 2-3% of cases and a permanent drop in milk production in cows and reduced fertility in bulls often occurs resulting in heavy economic losses (MacFarlane and Haig, 1955; Basson *et al.*, 1970; as reviewed by St George, 2004). A fluorescence antibody test revealed cross-reactions between BEFV isolates from Japan, Australia and South Africa (Theodoridis, 1969). There is a need to replace current live-attenuated virus vaccines due to heat sensitivity, especially in Africa where maintenance of a continuous cold-chain is extremely difficult.

Structurally, BEFV virions resemble those of other mammalian rhabdoviruses, containing five structural proteins (Walker *et al.*, 1991a; Walker *et al.*, 1991b). One of these is a membrane glycoprotein (GP) on which 6 neutralisation sites have been identified by competitive binding of GP monoclonal antibodies (Cybinski *et al.*, 1990). A vaccinia virus recombinant expressing the GP of an Australian strain of BEFV (the BB7721 field isolate from a cow in 1968, passaged in calves and in suckling mice before adaptation to cell culture in BHK and Vero cells) was shown

to induce neutralising antibody production in rabbits and protected cattle against experimental BEFV infection (Hertig *et al.*, 1995). A cDNA clone of this GP gene was kindly supplied to us by Dr Peter Walker (CSIRO, Australia).

Rift Valley fever (RVF) is a zoonosis with symptoms in humans ranging from mild influenza-like illness to severe complications such as ocular sequelae, encephalitis or haemorrhagic disease, sometimes resulting in death (Easterday, 1965; as reviewed by Swanepoel and Coetzer, 2004). In ruminants the disease is usually mild but may cause severe disease in cattle, sheep and goats, especially in neonates. Generally considered a disease of sub-Saharan Africa, in more recent years it has spread throughout northern Africa to Saudi Arabia and Yemen (Meegan *et al.*, 1979; Shoemaker *et al.*, 2002). In South Africa RVF has not as yet posed a major threat, although sporadic outbreaks have occurred within the boundaries of the Kruger National Wildlife Park.

RVFV belongs to the *Phlebovirus* genus of the family *Bunyaviridae* and consists of a three-segmented, single-stranded negative-sense RNA genome (Bishop *et al.*, 1980). A number of live-attenuated and formalin-inactivated vaccines against RVFV have been in use for many years, although in a proportion of pregnant sheep vaccinated with the live-attenuated Smithburn vaccine, the vaccine appears to be responsible for causing abortions or teratology of the foetus and *hydrops amnii* and prolonged gestation in the dam (Coetzer and Barnard, 1977). A number of other potential RVFV vaccine candidates have been evaluated more recently in animal trials and found to provide good levels of protection (Moussa *et al.*, 1986; Morrill *et al.*, 1987; Takehara *et al.*, 1989; Schmaljohn *et al.*, 1989; Saluzzo and Smith; 1990; Morrill *et al.*, 1991; Anderson *et al.*, 1991; Morrill *et al.*, 1997a; Morrill *et al.*, 1997b) , although none of them appear yet to be in commercial use (Davies and Martin, 2003; as reviewed by Swanepoel and Coetzer, 2004). Collet *et al.* (1987) cloned and sequenced a cDNA copy of the genomic M segment RNA of RVFV containing the G_1 and G_2 viral glycoproteins. These were then cloned into a bacterial expression vector and into a VV transfer vector for the generation of a VV-vectored recombinant expressing the glycoproteins. A high percentage (> 90%) of mice inoculated with the resulting recombinant and challenged with RVFV, survived. However, the concerns over the use of VV as a vector for recombinant vaccines have already been detailed, and thus LSDV should prove a suitable alternative.

This chapter describes the modification of the LSDV transfer vector into which the EGFP visual marker gene was inserted, and the use of this vector for the generation and selection of two separate, homogeneous LSDV-vectored recombinants expressing the structural glycoproteins of BEFV and RVFV.

3.2 MATERIALS AND METHODS

3.2.1 Cells and viruses:

Primary cultures of FBT cells were prepared using standard procedures (Freshney, 1987) and were used for the generation and selection of recombinant viruses.
Cells were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 nutrient supplement (Highveld Biological Products, South Africa), 10% foetal calf serum (FCS) (Highveld Biological Products, South Africa) and antibiotics (100 µg/ml penicillin, 100 µg/ml streptomycin and 250 µg/ml amphotericin) (Highveld Biological Products, South Africa).

Freeze-dried virus of the South African vaccine strain of LSDV (type Neethling) was reconstituted in sterile distilled water and passaged 5 times in Madin-Darby bovine kidney (MDBK) cells (obtained from the American Type Tissue Culture Collection, USA). Stocks were prepared from clarified cell lysates, and were stored at -20 °C. Aliquots were removed for titration on MDBK cells as described (Wallace, 1994).

3.2.2 Plasmid DNA analysis, cloning and purification:

As described in the Materials and Methods, chapter 2 (2.2.2).

3.2.3 Construction of pHGS7-E:

This plasmid was constructed for the insertion of the EGFP visual marker gene under control of the VV P11K promoter.
The African horsesickness virus VP2 gene in the VV insertion plasmid, pGVRW1(g)VP2, was truncated using partial *Nde*I digestion and the vector was re-ligated (re-named, pGVRW1-g-V). The EGFP gene (without its ATG start-codon) was then removed from the mammalian expression vector, pEGFP-C1 (Promega, USA) using *Nco*I and inserted into the *Nco*I site of pGVRW1-g-V giving rise to pGVRW1-EGFP. The VP2 leader sequence and approximately 50% of the EGFP gene were excised from pGVRW1-EGFP using *Eco*RI and inserted into the *Eco*RI site of pHGS7 (supplied by Gerrit Viljoen, OVI) resulting in the plasmid, pHGS7-5'E. A full-length copy of the EGFP gene was then excised using *Nco*I from pBB-EGFP-C1 (supplied by Anel Espach, OVI) and inserted into the *Nco*I site in pHGS7-5'E resulting in the plasmid pHGS7-E.

3.2.4 Construction of a new, streamlined LSDV transfer vector containing the EGFP gene:

The pLS(g)lac insertion plasmid was chosen as the starting template as it already contained a number of convenient R.E. sites which helped to facilitate the procedure. The gpt and lacZ genes were removed from pLS(g)lac using partial *Pst*I digestion and the vector was re-ligated giving rise to pLS-Pst. Then a non-essential *Nde*I-*Sty*I section was removed using Klenow to blunt the DNA ends and the vector was re-ligated giving rise to pLS-A. This was followed by removal of a non-essential *Eco*R1-*Sph*I section using partial digestion of *Eco*RI resulting in pLS-Aa. The *Eco*R1 site at the 3' end of the TK gene (in TK_R) was then destroyed using partial *Eco*RI digestion and Klenow giving rise to pLS-B.

The *E. coli* gpt gene was then excised from pSelp(HS)G_1G_2 (provided by Dr Anna-Lise Williamson, University of Cape Town) using *Eco*RI and re-introduced into the remaining *Eco*RI site in pLS-B to produce pLS-C. The next step would have been to blunt-end insert the EGFP gene from pHGS7-E into the *Spe*I site in pLS-C located between TK_R and P7.5K promoter, however difficulties were experienced with the partial digestions (required due to the presence of an additional *Spe*I site in the MCS) and thus an extra step was introduced whereby the 3' end of the gpt gene and the MCS were removed using *Bgl*II – this produced pLS-BglII. The EGFP gene was then removed from pHGS7-E using *Xmn*I and *Mlu*I and blunt-end inserted into the remaining *Spe*I site in pLS-BglII producing pLS-EGFP-B. The BglII-BglII gpt+MCS fragment was then re-inserted into pLS-EGFP-B using *Bgl*II resulting in the new LSDV transfer vector, pLSEG.

3.2.5 Insertion of the BEFV and RVFV GPs into pLSEG:

The structural GP gene of BEFV was removed from pG1.A6 (supplied by Dr Peter Walker, CSIRO, Australia) using *Bgl*II and inserted into the unique *Bam*HI site in the MCS of pLSEG. Similarly, the two structural GP genes of RVFV were excised as a single fragment from pSCRV6 (supplied by Drs Connie Schmalljohn and Mark Collett, US Army) using *Bam*HI and inserted into the dephosphorylated *Bam*HI site of pLSEG.

3.2.6 Sequencing of expression cassettes:

Before commencing with the generation of the recombinants the insertion plasmid constructs were sent for sequencing (Sequencing Lab, OVI) to ensure that no mutations had resulted which might affect the expression of the BEFV and RVFV GPs. Sequencing was performed

using an ABI Prism 3100 Genetic Analyser (Hitachi, Japan) using BigDye^{Tm} chemistry from a BigDye^{Tm} Terminator cycle sequencing kit (Applied Biosystems, USA).

3.2.7 Transient expression assay:

FBT cells were propagated on sterile microscope glass coverslips in 6-well cell culture dishes (Nunclon, Denmark) until approximately 90 % confluent. Wells were then inoculated with wt LSD virus at an MOI = 1.0. Five hours later the cells were co-transfected with highly pure plasmid DNA using the Effectine (Qiagen, Germany) transfection reagent according to the manufacturer's instructions. Cells were then returned to the CO_2 incubator for 48 hours. They were then either, fixed in acetone and treated further for immunofluorescence (IF) or, viewed directly under UV light for EGFP expression.

3.2.8 Immunofluorescence:

FBT cells were propagated on sterile microscope glass coverslips in 6-well cell culture dishes (Nunclon, Denmark) until approximately 90 % confluent. Some wells were then inoculated with recombinant virus, while others were inoculated with wt virus at an MOI = 0.1. Cells were then returned to the CO_2 incubator. After approximately four days, or when cpe were visible, the cells were fixed in ice-cold acetone for 20 minutes. The acetone was removed and the cells were washed once in PBSA (PBS without magnesium or calcium). Cells were then flooded in pre-absorbed polyclonal antibody for BEFV (obtained from a naturally infected bovid) or polyclonal antibody for RVFV (sheep anti-RVFV polyclonal serum supplied by Mara Schoeman, Virology Department, OVI) diluted in blocking reagent (2% low fat milk powder [Elite, South Africa] diluted in PBSA) (the optimal dilution factor having been determined previously) and incubated for 30 minutes at RT. Incubation was then followed by 4 washes in PBSA (each wash lasting 10 minutes) with gentle rocking on a STR6 Platform Shaker (Stuart Scientific, UK). Rabbit anti-bovine immunoglobulin type-G (IgG) conjugated to fluorescein isothiocyanate (FITC) (Sigma, USA) was diluted 1:80 in blocking reagent (containing 0.01% Evans Blue counterstain [Merck, Germany]) for BEFV detection (and donkey anti-sheep IgG-FITC conjugate (Sigma, USA) diluted 1:100 for RVFV detection), and was added to the cells for a period of 30 minutes at RT. The four PBSA washes were repeated, followed by a final 10 minute wash in distilled water. The samples were then air dried, mounted on glass microscope slides in mounting fluid (1:1 glycerol:PBS, pH = 7.4) and viewed under UV light using a Leica DM IL inverted fluorescence microscope (Germany).

3.2.9 Generation and selection of recombinants:

Although the technique was similar to that described in chapter 2 for the generation and selection of LSDV recombinants expressing the lacZ reporter gene using the MPA-selection approach, a number of modifications were introduced to assist with the selection for homogeneous recombinants. The co-transfection procedure was the same, except that in place of LipofectAmine™, Effectine (Qiagen, Germany) was used according to the manufacturer's instructions. After co-transfecting the LSDV-infected FBT cells with either pLSEG-BEFV, or pLSEG-RVFV, the cells were incubated until cpe were visible. The cells were then freeze-thawed three times and the solution cleared by low speed centrifugation. A 10-fold serial dilution was made of the supernatant fluid and an aliquot of each dilution was placed onto fresh FBT cells pre-incubated for 24 hours in the presence of gpt selection medium (20 μg/ml xanthine, 20 μg/ml hypoxanthine, 25 μg/ml mycophenolic acid, DMEM/Ham's F12, 2.5% FCS). Cells were incubated under standard conditions (37 °C, 5% CO_2), with the medium being replaced every 48 hours. During this period, cells were visualised under UV light for expression of EGFP from distinct viral foci – once these were visible (usually 4-5 days post-inoculation) the freeze-thawing, serial dilutions, and incubation under standard conditions on fresh FBT cells were repeated once more.

After 4-5 days, once foci were visible expressing EGFP, the selection medium was removed from the cells and dead cells were pelleted at low-speed (200g) in a bench-top centrifuge. The supernatant fluid was then subjected to sonication for 15 minutes in a cooled waterbath-sonicator (Sonorex TK52, Bandelin, Germany). It was then filtered through a 0.45 μm Millex-GV filter (Millipore, France). The filtrate was diluted to end-point using a 10-fold serial dilution, and placed onto fresh FBT cells under selection (as described above). The supernatant fluid from the well exhibiting foci at the highest dilution was again removed, separated from dead cells, sonicated, filtered, and titrated. For the 3rd cycle the cells in the well exhibiting foci at the highest dilution were washed in PBS and those foci appearing homogeneous for the expression of EGFP were picked. These were then divided into two equal aliquots, one half being returned to individual wells of a 12-well culture dish containing fresh FBT cells, and the other half being treated for the extraction of viral DNA for PCR analysis (2.2.9). Foci which were shown to be homogeneous via PCR were then evaluated for expression of their respective glycoprotein genes using immunofluorescence. One focus from each recombinant which showed good expression was then grown to high titres for immunological studies in animals (chapter 4).

3.3 RESULTS

3.3.1 Insertion of EGFP gene under control of the VV P11K promoter:

As was described in the introduction to this chapter, retaining a visual reporter gene in recombinant virus constructs was a desirable feature. However, due to the large size of the lacZ reporter gene it was decided to substitute this gene for the smaller EGFP visual marker gene. In order to obtain expression of the EGFP gene in recombinant viruses the gene would need to be inserted under control of a poxvirus promoter. As the LSDV insertion vector already contained two copies of the P7.5K promoter (in opposite orientations) it was not possible to use this promoter. The VV P11K promoter is a strong late promoter and would prove a suitable alternative (Romero *et al.*, 1994b).

The plasmid pHGS7 (supplied by Gerrit Viljoen, OVI) contained a copy of this promoter with an upstream *Xmn*I R.E. site and a downstream *Eco*RI R.E. site and could thus be used for the insertion of the EGFP gene under control of the promoter. However, 5' of the *Eco*RI site in pHGS7 is an ATG start codon (as shown below), and, therefore the EGFP gene would need to be inserted in-frame into the *Eco*RI site. A copy of the EGFP gene was obtained from the mammalian expression vector, pEGFP-C1 (Clontech, USA). Unfortunately, though, there was not a convenient *Eco*RI site at the 5' end of the gene – however, the ATG start codon is contained within an *Nco*I site. Thus, if a DNA sequence could be found with an *Nco*I site flanked by *Eco*RI sites, and, if at least one of the *Eco*RI sites was in-frame with the *Nco*I site, then, the EGFP gene could be removed with *Nco*I from pEGFP-C1 and inserted into the *Nco*I site, and then removed with *Eco*RI and inserted in-frame into the *Eco*RI site in pHGS7. A short stretch of DNA which would allow this in-frame insertion was located in the 5' region of the African horsesickness virus (type 3) VP2 gene (as shown below):

pHGS7	5'	NNNN- **p11K promoter** -TAA **ATG** AAT TCN NNN 3'
		EcoRI
AHSV3 VP2	5'	NNNN CGG AAT TCC CGA AGC C**AT G**GNNN 3'
		EcoRI NcoI

A copy of the VP2 gene (originally supplied by Frank Vreede, OVI) was located in the VV TK insertion vector, pGVRW1(g)VP2 (constructed by myself for generating VV recombinants

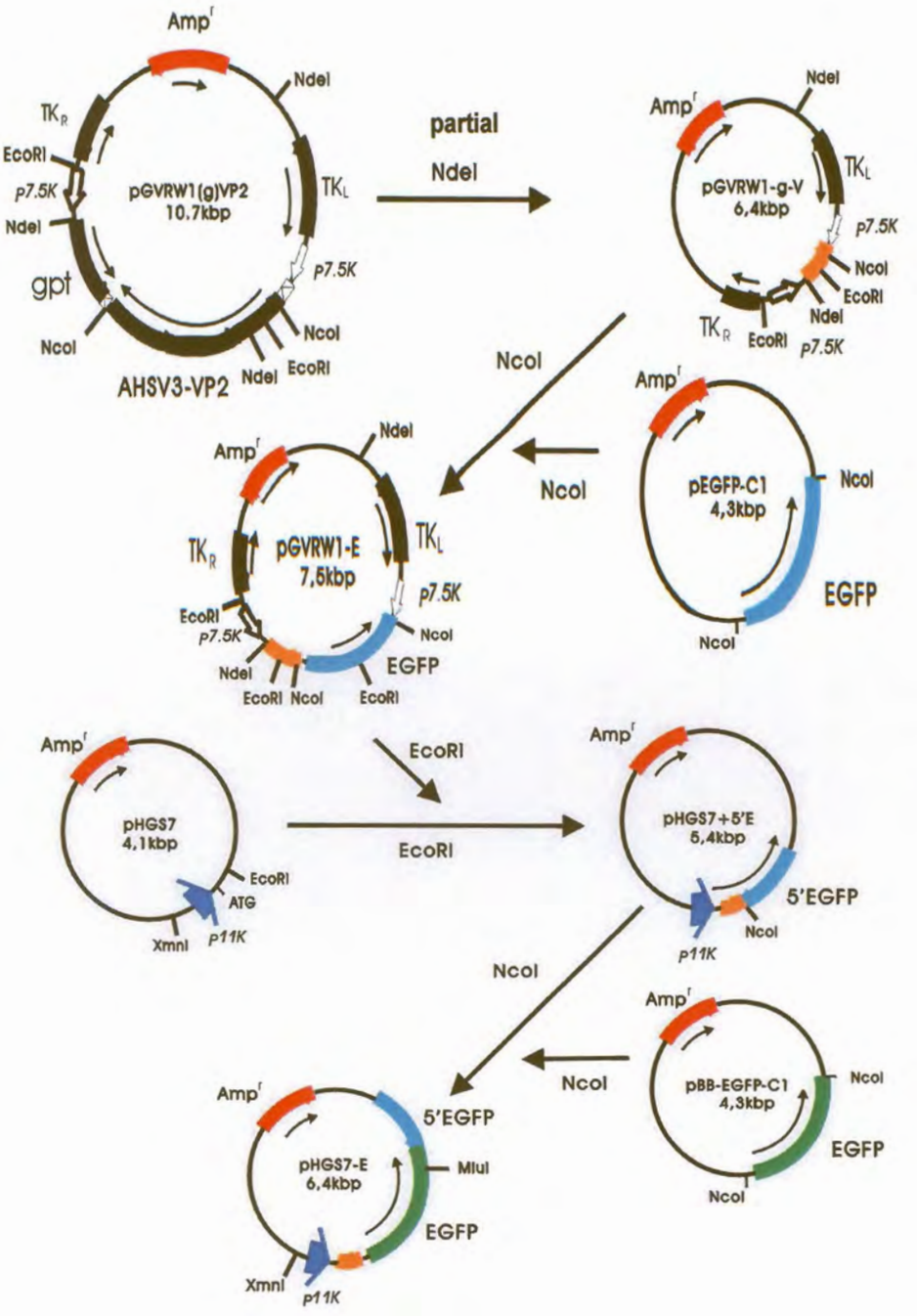


Figure 3.1 Insertion of EGFP under control of VV P11K late promoter. The EGFP gene was first inserted upstream of the AHSV3-VP2 gene to create an in-frame sequence with the ATG start codon located downstream of the P11K promoter in pHGS7. An *Eco*RI-*Eco*RI fragment containing this sequence and the 5' 1/3 of the EGFP gene was then excised and inserted into pHGS7, after which the full-length EGFP gene was inserted into the *Nco*I site resulting in pHGS7-E. The EGFP gene could then be excised, along with the P11K promoter, using *Xmn*I and *Mlu*I.

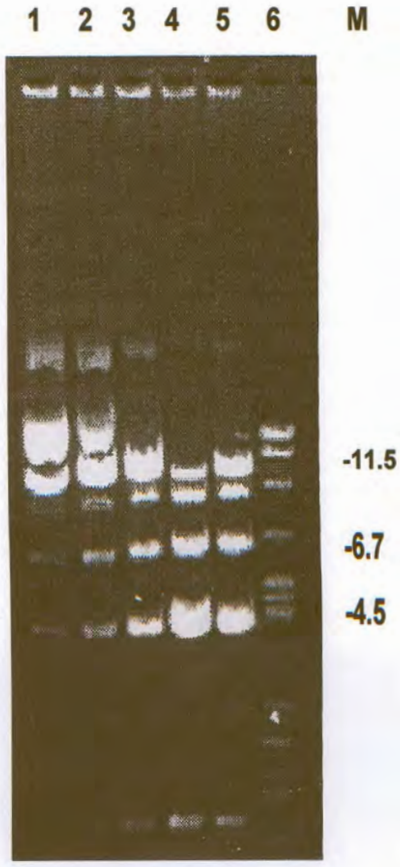
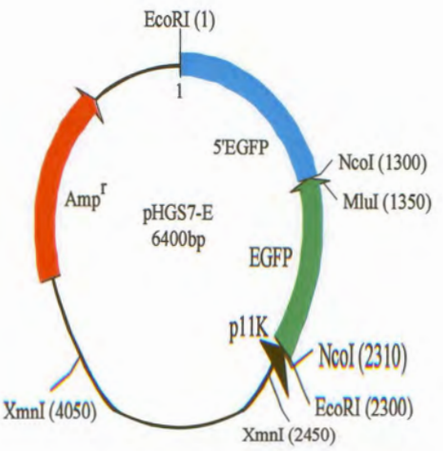


Figure 3.2 Agarose gel electrophoresis analysis of partial *Nde*I restriction enzyme digestions of pGVRW1(g)VP2. lane 1 = 1 U, lane 2 = 2 U, lane 3 = 4 U, lane 4 = 6 U, lane 5 = 7 U. Lane 6 = combined lambda *Pst*I and *Hind*III DNA marker. M = DNA fragment sizes in kbp.

expressing VP2). However, before inserting the EGFP gene into the *Nco*I site in VP2, most of the VP2 gene (and the gpt gene) was removed using partial *Nde*I digestion to allow easier manipulation (as the plasmid was already 10.7 kbp in size) (Figure 3.1). For the partial digestions, five eppendorf tubes were prepared containing 1 µg each of pGVRW1(g)VP2, and 1, 2, 4, 6 and 7 units of *Nde*I enzyme. The tubes were then incubated overnight at 4 °C. Cutting of the correct two *Nde*I sites would produce a DNA fragment of approximately 6.4 kbp. Analysis of the reactions (Figure 3.2) indicates that the digestion performed with 6 units of enzyme produced the highest quantity of the correct DNA fragment. Ten eppendorf tubes were then prepared, each containing 6 units of enzyme and 1 µg plasmid DNA, and after overnight incubation at 4 °C the preps were pooled on ice, the DNA fragments separated on an agarose gel and the 6.4 kbp fragment excised, purified and subjected to self-ligation. The plasmid was now called pGVRW1-g-V (Figure 3.1).

The EGFP gene was then removed from pEGFP-C1 using *Nco*I and inserted into the *Nco*I site (in the correct orientation) of pGVRW1-g-V giving rise to pGVRW1-EGFP (data not shown).

A



B

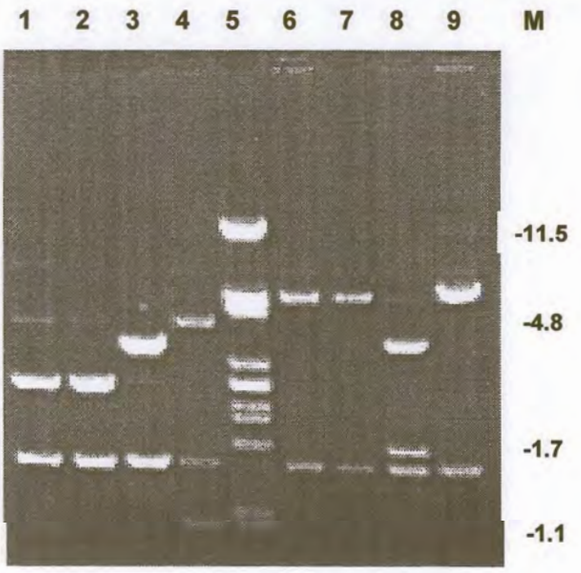


Figure 3.3 R.E. plasmid map of pHGS7-E (**A**) and agarose gel electrophoresis analysis of R.E. digests of pHGS7, including EGFP inserts (**B**). lane 1 – pHGS7 cut with *Xmn*I, lane 2 – pHGS7 cut with *Xmn*I and *Mlu*I, lane 3 – pHGS7+5'E cut with *Xmn*I and *Mlu*I, lane 4 - miniprep #12 of pHGS7-E cut with *Xmn*I and *Mlu*I, lane 5 – lambda *Pst*I DNA marker, lanes 6-9 – minipreps #18, 19, 20 and 21 of pHGS7-E cut with *Xmn*I and *Mlu*I. M – Marker DNA sizes in kbp.

The following step would have been to excise the intact EGFP gene using *Eco*RI, however, there was no downstream *Eco*RI site available. Thus an internal *Eco*RI in the EGFP gene had to be utilized. The VP2 leader sequence and most of the EGFP gene were then excised from pGVRW1-EGFP using *Eco*RI and inserted into the *Eco*RI site of pHGS7 (in the correct orientation) resulting in the plasmid, pHGS7+5'E (data not shown).

The final step would be to insert a full-length copy of the EGFP gene into the *Nco*I site in pHGS7+5'E. For this purpose a modified form of the EGFP gene was utilised from which a short internal in-frame *Bam*HI-*Bgl*II sequence containing a number of R.E. sites had been removed from the 3' end of the gene (making those sites available as unique sites in the MCS of the new transfer vector, pLSEG – Figure 3.7). The modified EGFP gene was excised from pBB-EGFP-C1 (obtained from Anel Espach, OVI) using *Nco*I and inserted into the *Nco*I site in pHGS7-5'E. Restriction enzyme digestion was used to confirm the insertion and orientation of the gene. From the calculated R.E. map of the expected plasmid (to be called pHGS7-E) (Figure 3.3 A) a *Xmn*I plus *Mlu*I digestion should produce three bands of 3.7, 1.6 and 1.1 kbp. Miniprep #12 produced the correct pattern (lane 4, Figure 3.3 B). As a final confirmation a highly pure preparation of the plasmid was made and tested for transient expression of EGFP in LSDV-infected FBT cells (as described in 3.2.7). Expression of EGFP was clearly evident when LSDV-infected cells were co-transfected with pHGS7-E and were observed under UV light (Figure 3.14 A), thus confirming that the construct was correct and that EGFP was being expressed under control of the P11K promoter.

3.3.2 Construction of a new LSDV transfer vector, pLSEG:

With the change in selection strategy from TK-negative to MPA selection it became necessary to modify the original transfer vector, pLSTK7.5, by addition of the gpt dominant selectable marker gene. In addition it was deemed an advantage to include a visual reporter gene to assist with the selection of recombinants – due to its small size and *in vivo* screening advantage, the EGFP gene was chosen. Assessment of the LSDV transfer vector, pLSTK7.5, indicated that there were areas of the plasmid which could probably be safely removed without introducing deleterious effects. This would have the advantage of reducing the size of the vector, allowing for larger inserts, and, would enhance the versatility of the MCS by increasing the number of available unique R.E. sites. Thus a strategy was devised to construct a new streamlined transfer vector containing the gpt and EGFP genes (as outlined in Figures 3.4 and 3.6). R.E. digestion and analysis of the resulting DNA fragments separated using agarose gel electrophoresis was

used to confirm the intermediate and final constructs in each stage of the cloning procedures, although only results for a number of the constructs will be shown.

For the reconstruction of the transfer vector, the pLS(g)lac insertion plasmid was chosen as the starting template due to the presence of a number of convenient R.E. sites. The gpt and lacZ genes were first removed using partial *Pst*I digestion and the vector was re-ligated giving rise to pLS-Pst. For the partial digestions a range of different incubation temperatures, incubation times and enzyme concentrations had to be evaluated as there were four *Pst*I sites present in the plasmid and only the two flanking the lacZ and gpt genes were required to cut (Figure 3.4). Finally it was found that cutting 1 µg of DNA using 0.5 units of *Pst*I enzyme at 4 °C overnight produced a faint band of the correct size (6.1 kbp) (data not shown).

A non-essential *Nde*I-*Sty*I section was then removed from pLS-Pst giving rise to pLS-A (data not shown). This was followed by removal of a non-essential *Eco*RI-*Sph*I section by first cutting 1 µg of DNA fully with *Sph*I and then adding 4 units of *Eco*RI enzyme and cutting for 4 hours on ice. The 3.9 kbp fragment was isolated, Klenow-treated to produce blunt ends and the ends were re-ligated to produce pLS-Aa. Unfortunately the re-ligation re-formed an *Eco*R1 site at the 3' end of the TK gene (in TK_R) (Figure 3.4). Thus the site had to be destroyed using partial *Eco*RI digestion. The best conditions were found by cutting 1 µg of DNA with 4 units of enzyme overnight at 4 °C. The ends were then blunted and re-ligated. The ligated DNA was transformed into DH5α *E. coli* cells and colonies were picked, cultivated overnight in LB medium and plasmid DNA was extracted (as described, 2. 2.2). The preps were cut with *Eco*RI and a few of them produced a single 3.9 kbp band when analysed using agarose gel electrophoresis (data not shown). One of these preps (#2) was then cut with a number of R.E. enzymes and analysed (Figure 3.5). The expected patterns were obtained (Figure 3.5 B), although the DNA did not cut fully for the *Eco*RI/*Sca*I double-digestion, and a partial digestion product of 3.9 kbp is present (lane 6). The lower 0.21 kbp band is present, but, is very faint. The plasmid was called pLS-B (Figure 3.5 A).

With the removal of all extraneous DNA sequences and the destruction of unwanted R.E. sites, it was now possible to insert the gpt and EGFP genes as outlined in figure 3.6.

The 1.3 kbp *E. coli* gpt gene (including the VV P7.5K promoter) was excised from pSelp(HS)G_1G_2 using *Eco*RI and re-introduced into the remaining *Eco*RI site in pLS-B to produce pLS-C. For the insertion of the EGFP gene, a *Spe*I site located between TK_R and the P7.5K promoter (controlling the gpt expression) was chosen as the insertion site. However, another *Spe*I site is located within the MCS. Partial *Spe*I digestion was attempted to destroy this site, but problems were encountered and the wrong site appeared to cut preferentially. This necessitated a change in strategy. A solution was found by removal of most of the gpt gene and the MCS as a single 800 bp fragment using *Bgl*II and to close up the vector producing pLS-BglII.

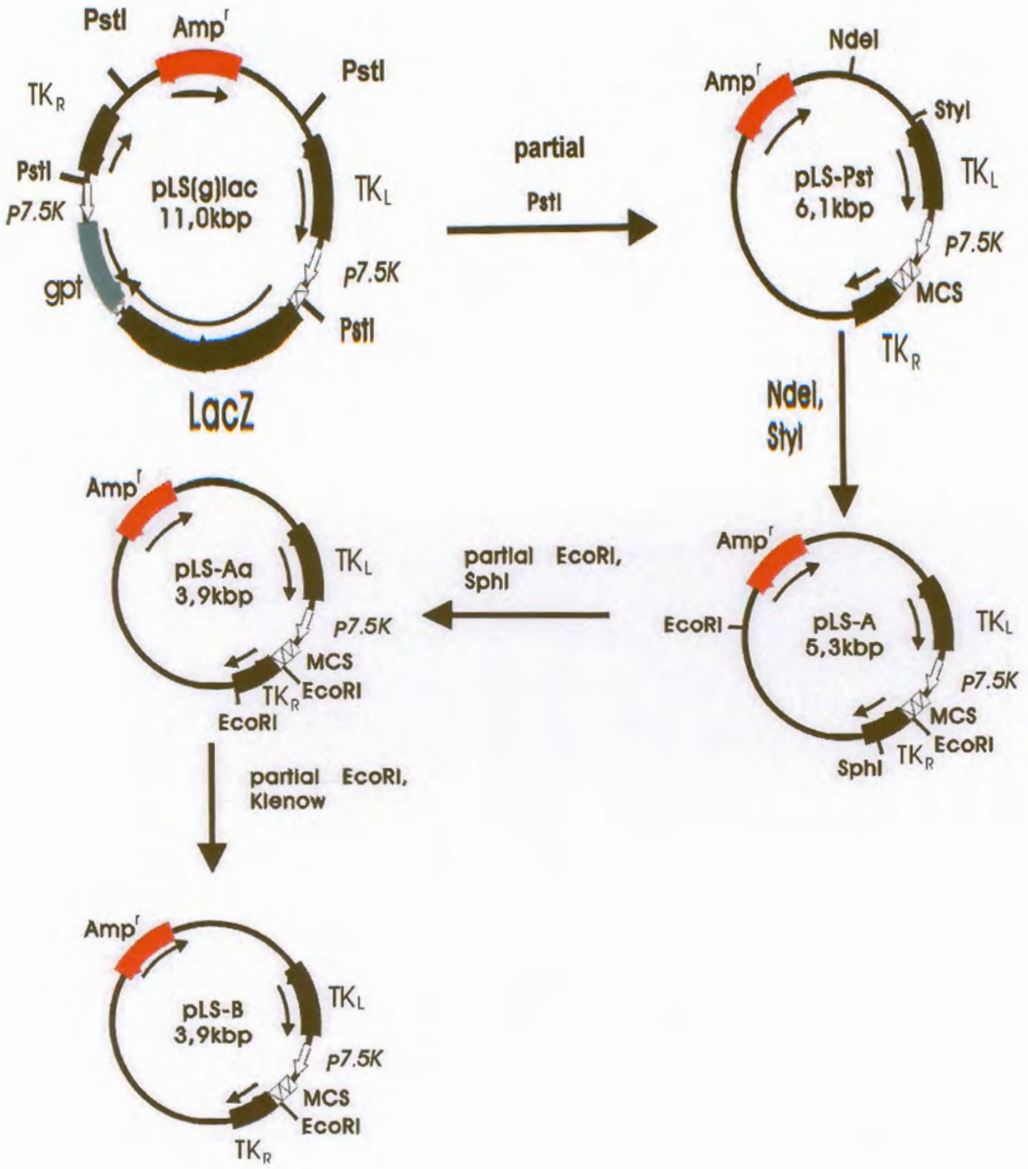
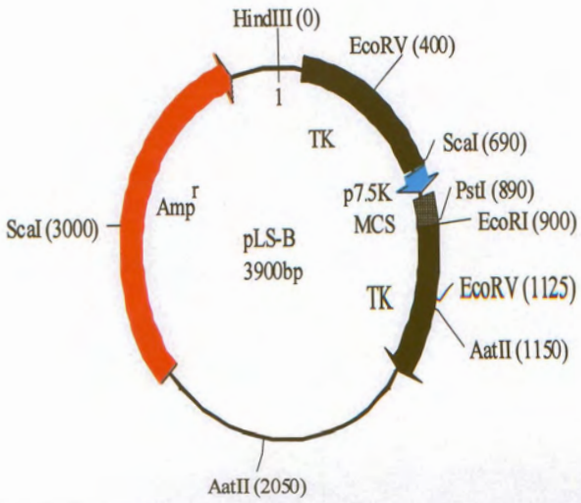


Figure 3.4 Construction of the improved LSDV transfer vector, pLSEG – phase 1. pLS(g)lac was used as the basal plasmid and extraneous sequences were removed using partial *Pst*I, then *Nde*I and *Sty*I and finally *Eco*RI (partial) and *Sph*I to produce pLS-Aa. Then the *Eco*RI site at the 3' end of the TK gene was destroyed using *Eco*RI and Klenow resulting in pLS-B.

A



B

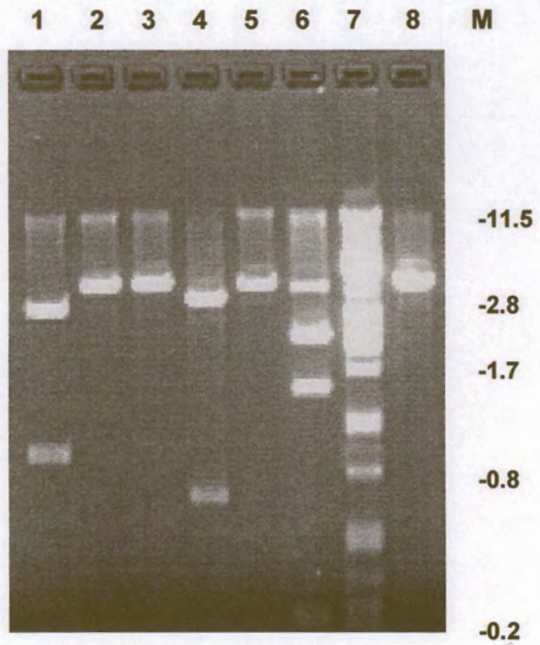


Figure 3.5 R.E. plasmid map (**A**) and agarose gel electrophoresis analysis of R.E. digests of pLS-B (**B**). Miniprep #2 cut with *Aat*II (lane 1), *Eco*RI (lane 2), *Pst*I (lane 3), *Eco*RV (lane 4), *Hind*III (lane 5), *Eco*RI/*Sca*I (lane 6), and *Eco*RI/*Pst*I (lane 8). Lane 7 contains a lambda *Pst*I DNA marker. M – DNA marker sizes in kbp.

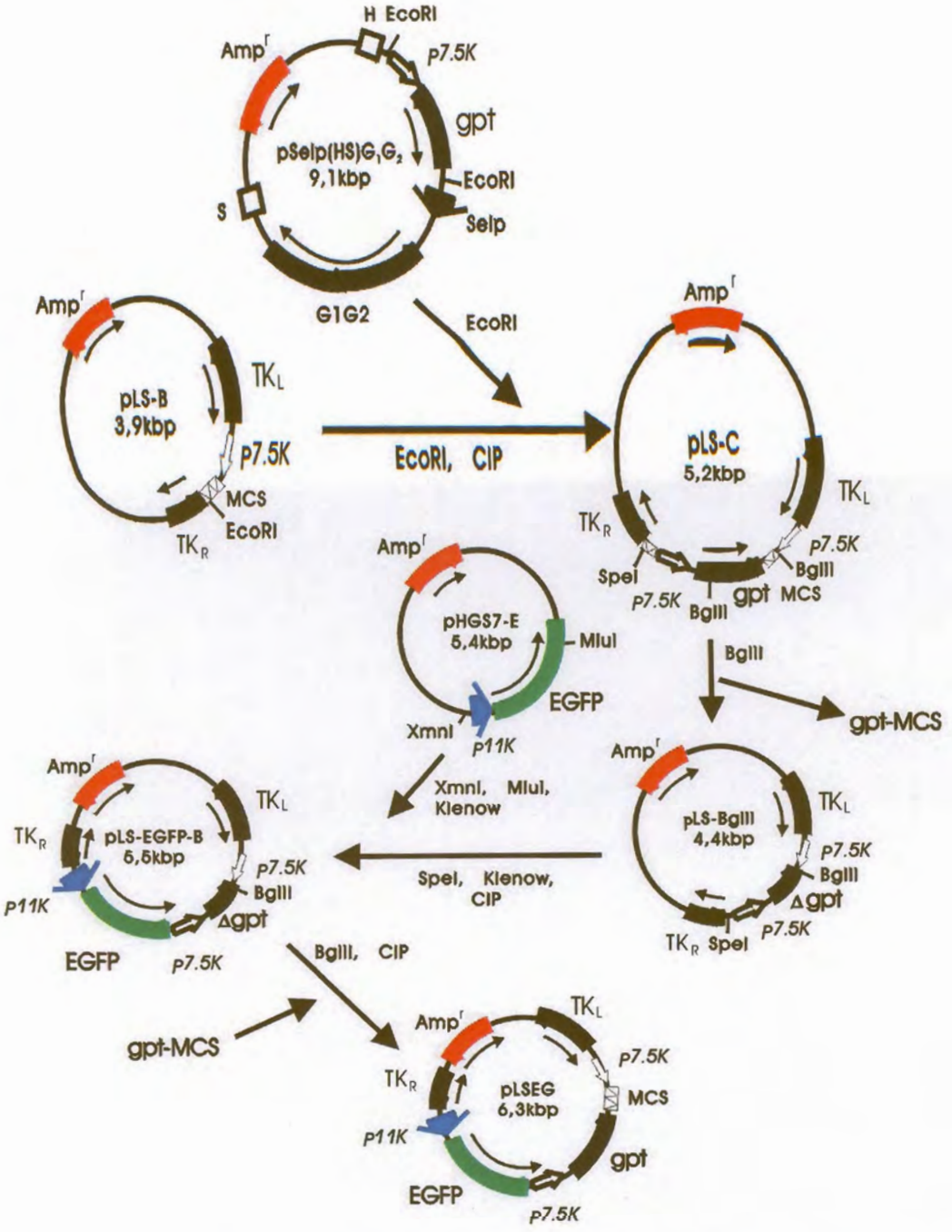


Figure 3.6 Construction of improved LSDV transfer vector, pLSEG – phase 2. For the reconstruction phase the gpt gene (from pSelp(HS)G_1G_2) was first inserted using *Eco*RI (pLS-C). Part of the gpt gene and the MCS (containing an SpeI site) were then removed using *Bgl*II so that the remaining *Spe*I site could be used for the blunt insertion of the EGFP gene from pHGS7-E (pLS-EGFP-B). The missing gpt portion and MCS were re-inserted using *Bgl*II completing the new transfer vector (pLSEG).

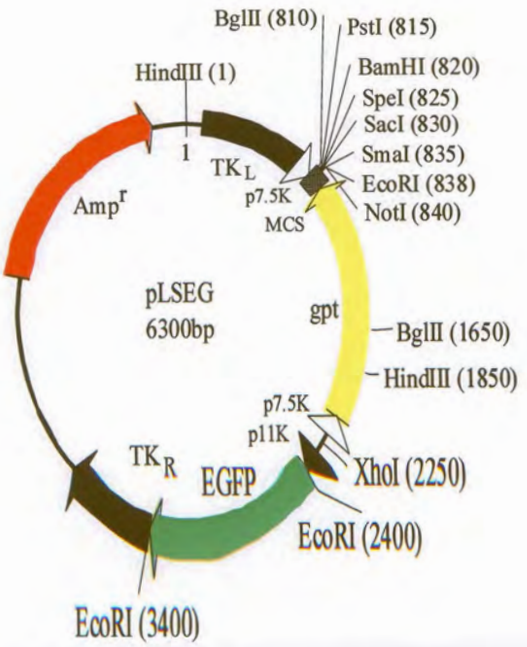


Figure 3.7 R.E. plasmid map of pLSEG. Except for the *Bgl*II and *Eco*RI sites in the MCS, the rest of the sites are unique.

It was now possible to insert the blunted EGFP gene (removed from pHGS7-E using *Xmn*I and a *Mlu*I site located at the extreme 3' end of the EGFP gene) into the remaining *Spe*I site in pLS-BglII producing pLS-EGFP-B. The 800bp *Bgl*II-*Bgl*II gpt+MCS fragment was then re-inserted into pLS-EGFP-B using *Bgl*II. Miniprep plasmid DNA was then double-digested with *Xho*I and *Pst*I to ensure that the fragment was in the correct orientation. From the R.E. map (Figure 3.7) it can be seen that for the correct orientation two fragments of 5.0 kbp and 1.3 kbp were expected. From figure 3.8 (A) preps #6 (lane 6), 14 (lane 15), 21 (lane 23) and 22 (lane 24) were correct. The remaining preps contain the fragment in the wrong orientation. The correct preps were characterised further with *Eco*RI, *Hind*III and *Bam*HI. Digests checked out perfectly with *Eco*RI producing three bands of 3.7, 1.6 and 1.0 kbp, *Hind*III producing two bands of 4.45 and 1.85 kbp, and, *Bam*HI producing a single band of 6.3 kbp (Figure 3.8 B).

Prep #21 (now called pLSEG) was then used in a transient expression assay to confirm expression of EGFP. In figure 3.14 (C) over 90% of the co-transfected FBT cells exhibit EGFP expression as compared to the negative control cells which were co-transfected with plasmid DNA lacking the EGFP gene (D).

With the completion of the new streamlined LSDV transfer vector, pLSEG, containing an improved MCS and the gpt and EGFP selectable marker genes, it was possible to insert genes of veterinary importance for the generation and evaluation of LSDV-vectored recombinant vaccines.

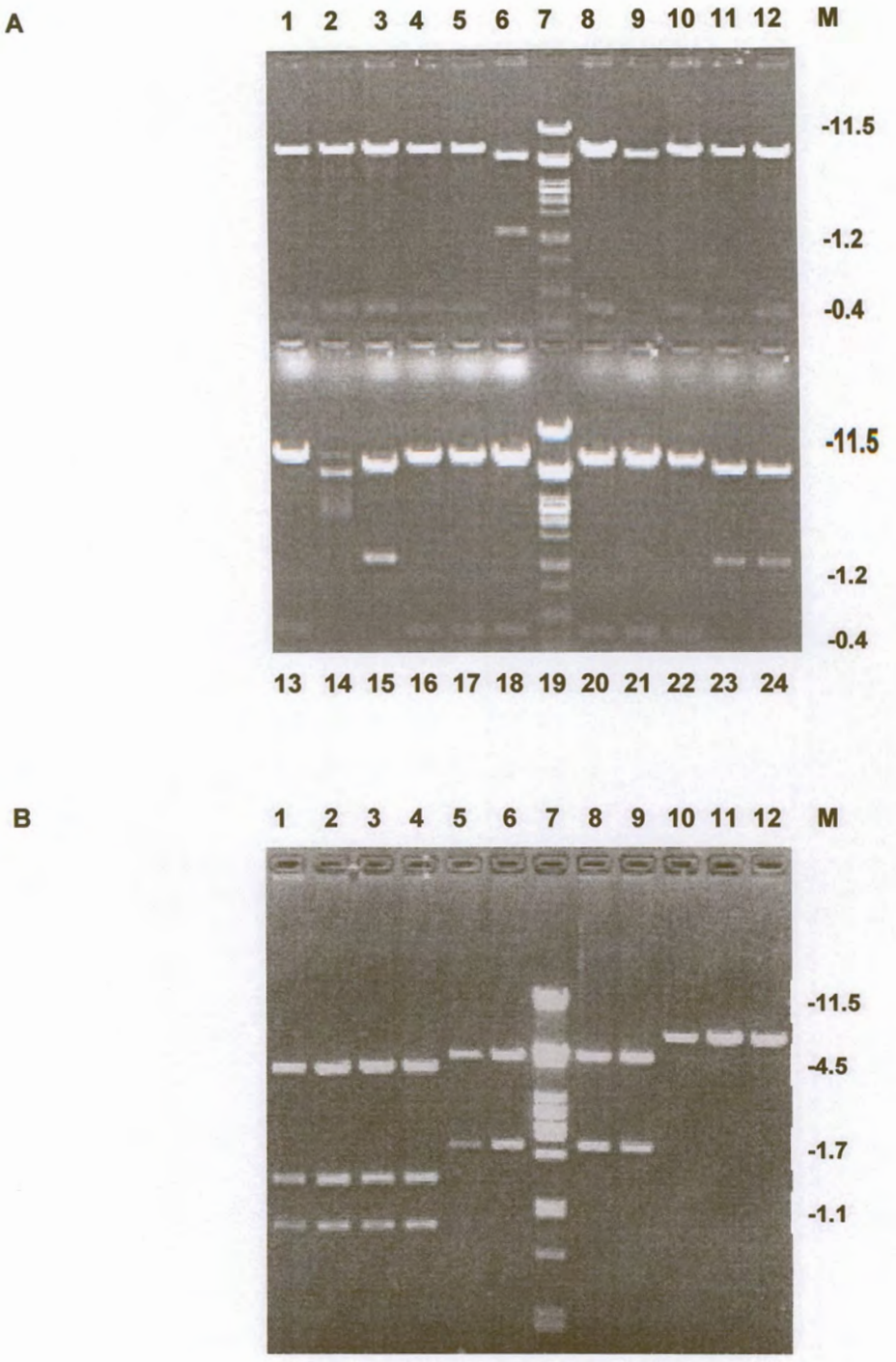


Figure 3.8 Agarose gel electrophoresis analysis of R.E. digestions of minipreps of pLSEG. **A** - Preps cut with *Xho*I/*Pst*I (lanes 1-6, 8-18, and 20-24). Lanes 7 and 19 – lambda *Pst*I DNA marker. **B** - preps #6, 14, 21 and 22 cut with *Eco*RI (lanes 1-4), *Hind*III (lanes 5, 6, 8 and 9) and *Bam*HI (lanes 10-12). Lane 7 – lambda *Pst*I DNA marker. M – DNA marker sizes in kbp.

3.3.3 Insertion of BEFV and RVFV GPs into pLSEG:

For the generation of the first TK-disrupted LSDV-vectored recombinant vaccines two viral pathogens were targeted, namely BEFV and RVFV, as there is a need for improved vaccines against these pathogens. The structural GP of BEFV and the two GPs of RVFV have been shown previously to induce protective neutralising antibody production in animals immunised with VV-vectored recombinant vaccines (Hertig *et al.*, 1995; Collet *et al.*, 1987). These were thus the genes of choice to be inserted into the new LSDV transfer vector for generation of LSDV-vectored recombinant vaccines.

The structural GP of BEFV was removed as a 1.8 kbp fragment from the plasmid pG1A6 (kindly provided by Dr Peter Walker, CSIRO, Australia) using *Bgl*II and inserted into the *Bam*HI site in the MCS of pLSEG (Figure 3. 9). Transformed DH5α cells were incubated on LB-agar plates overnight, single colonies were picked, grown, and plasmid DNA extracted. In order to orientate the GP gene, miniprep DNA was cut with *Sal*I as an internal *Sal*I site lies within the GP gene and one lies downstream of the P7.5K promoter driving expression of the gpt gene (Figure 3.10). Two bands of 5.85 and 2.25 kbp would be produced for the correct orientation. Preps #4, 9, 10, 13, 18, 19 and 22 produce this pattern (Figure 3.11 A). These preps, along with pLSEG, were then cut with *Bam*HI and *Eco*RI and the resulting fragments separated and analysed (Figure 3.11 B). Insertion of a *Bgl*II cut DNA fragment into a *Bam*HI site destroys the *Bam*HI, therefore the preps only linearise with *Bam*HI due to the internal *Bam*HI site in the BEFV GP gene. For the *Eco*RI digests there are three *Eco*RI sites in pLSEG and one in the BEFV GP gene – the extra 1.45 kbp band generated from insertion of the GP gene runs as a doublet with the existing 1.5 kbp band from pLSEG. For both the *Bam*HI and *Eco*RI digests, a distinct upward shift in the largest fragment can be seen for the preps compared to the pLSEG digests supporting insertion of the GP gene. For final confirmation pLSEG and the preps were cut with *Eco*RV and the expected fragments were obtained (results not shown). Prep #4 (now called pLSEG-BEFV) was then chosen for large-scale DNA preparation.

The RVFV GPs were excised as a single 3.4 kbp *Bam*HI fragment from pSCRV6 (kindly supplied by Dr Connie Schmalljohn and Dr Mark Collett, US Army, USA) (Figure 3.9) and inserted into the unique *Bam*HI site in pLSEG. Miniprep DNA prepared from single colonies of transformed DH5α cells were cut with *Pst*I due to an internal *Pst*I site allowing for orientation of the genes relative to the P7.5K promoter. The correct orientation would produce two bands of 7.7 and 2.0 kbp (Figure 3.12 A). Prep #19 produced the expected bands (results not shown), and it was characterised further using a number of restriction enzymes (Figure 3.12 B). The bands produced matched those expected from the R.E. map (Figure 3.12 A) and a large-scale DNA preparation was made as described (2.2.2). The plasmid was now called pLSEG-RVFV.

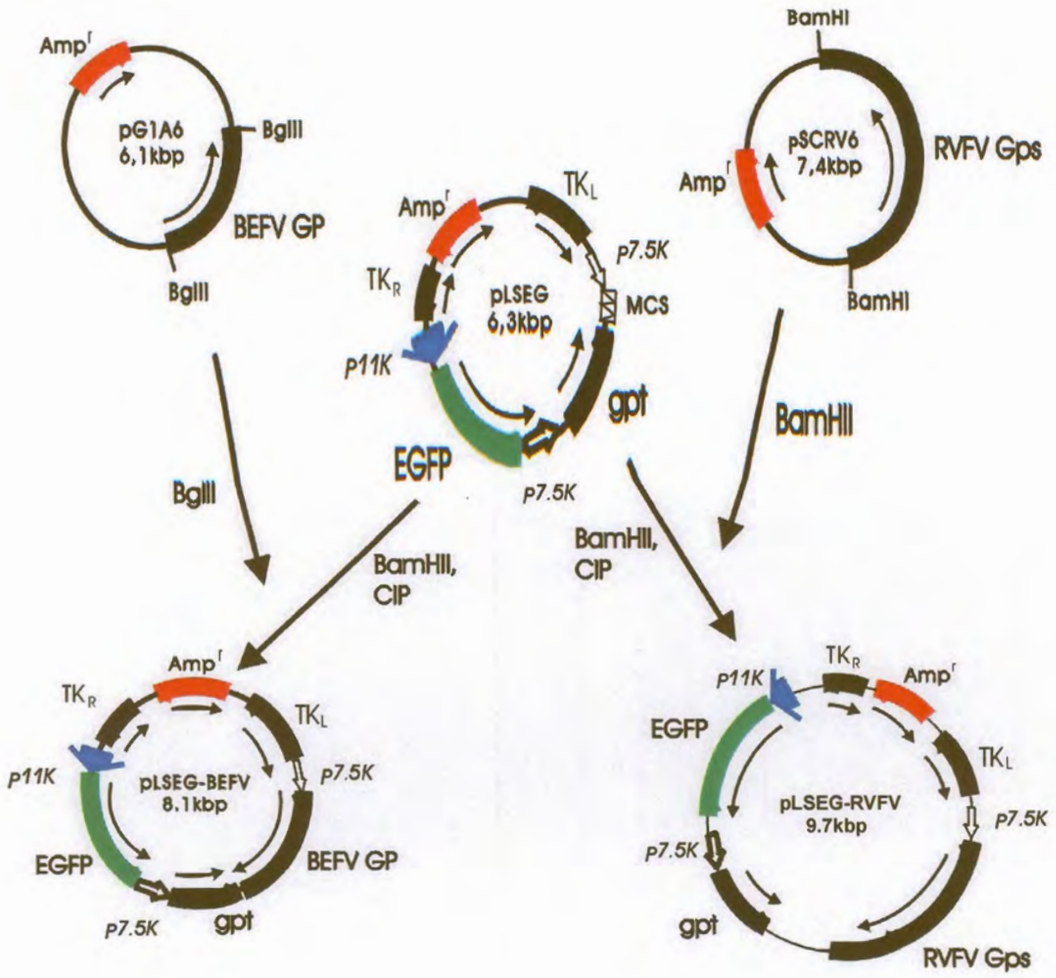


Figure 3.9 Insertion of BEFV and RVFV GPs into pLSEG. The structural GP gene of BEFV was excised from pG1A6 using *Bgl*II and was inserted into the unique *Bam*HI site in pLSEG (pLSEG-BEFV) and the G1G2 GPs of RVFV were excised from pSCRV6 using *Bam*HI and were also inserted into the *Bam*HI site in pLSEG (pLSEG-RVFV). Both sets of GPs were under control of the VV P7.5K promoter.

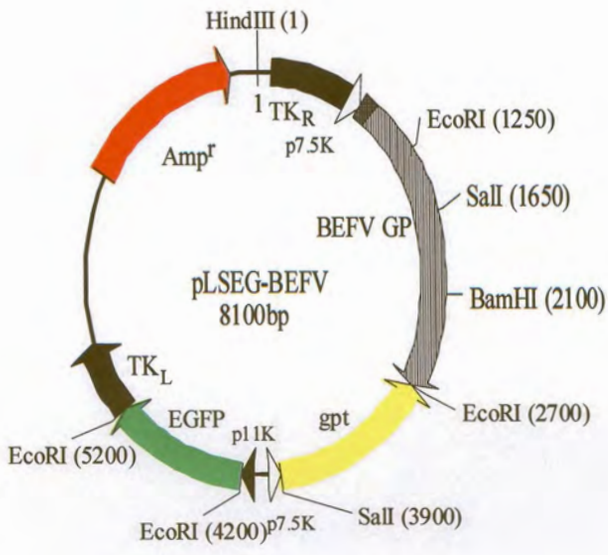


Figure 3.10 R.E. plasmid map of pLSEG-BEFV.

3.3.4 Sequencing of the promoter region and GPs:

In order to ensure that the expression cassettes contained no potentially deleterious mutations which might have arisen during the cloning procedures, the P7.5K promoter region and 5' ends of the GPs in pLSEG-BEFV and pLSEG-RVFV constructs were sent for sequencing using an automated sequencer and primer P1 as the sequencing primer (data not shown). Sequencing of the entire genes was contemplated, although it was decided that confirmation of expression using IF would suffice.

No deleterious mutations were encountered and the constructs were then subjected to IF in transient expression assays to confirm expression.

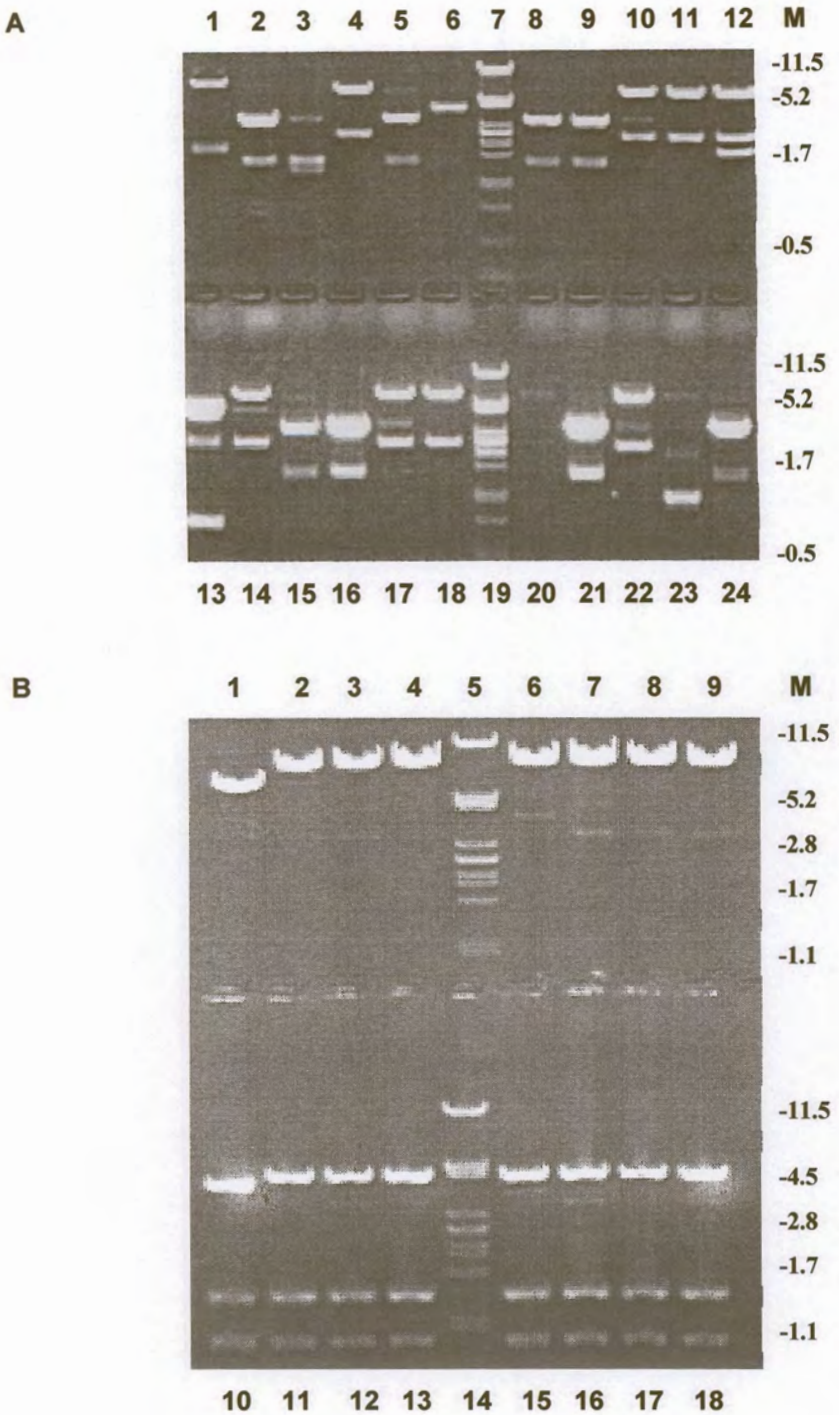
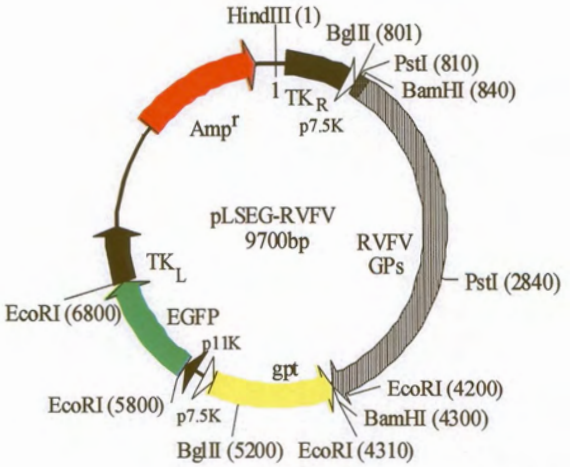


Figure 3.11 Agarose gel electrophoresis analysis of R.E. digests of minipreps of pLSEG-BEFV. **A** – minipreps cut with *Sal*I (lanes 1-6, 8-18, 20-24). Lanes 7 and 19 – lambda *Pst*I DNA marker. **B** – minipreps #4, 9, 10, 13, 18, 19 and 22 cut with *Bam*HI (lanes 2-4, 6-9) and *Eco*RI (lanes 11-13, 15-18). Lane 1 – pLSEG cut with *Bam*HI, lane 10 – pLSEG cut with *Eco*RI. Lanes 5 and 14 – lambda *Pst*I DNA marker. M – DNA fragment sizes in kbp.

A



B

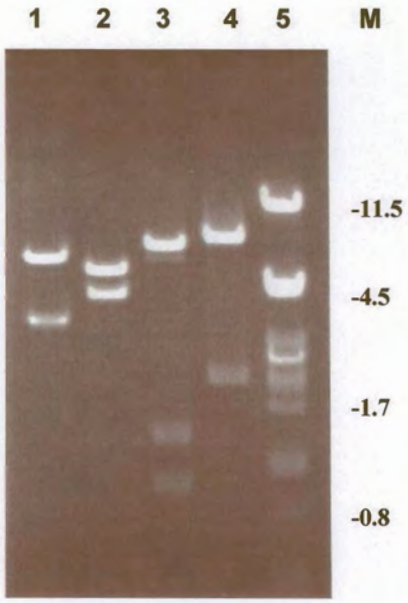


Figure 3.12 R.E. plasmid map (**A**) and agarose gel electrophoresis analysis of R.E. digests of pLSEG-RVFV (**B**). Lane 1 – *Bam*HI, lane 2 – *Bgl*II, lane 3 – *Eco*RI, lane 4 – *Pst*I, and lane 5 – lambda *Pst*I DNA marker. M – DNA fragment sizes in kbp.

3.3.5 Transient expression assays for BEFV and RVFV GP expression using IF:

Expression of the BEFV and RVFV GPs under control of the VV early/late P7.5K promoter was confirmed using transient expression assays and IF. The pLSEG-BEFV and pLSEG-RVFV plasmid constructs were co-transfected (separately) into FBT cells cultured on sterile glass coverslips infected with wtLSDV (MOI = 1.0). After 48 hours the cells were fixed in acetone and prepared for IF staining using a polyclonal serum from a naturally infected bovid for BEFV and a polyclonal serum from a RVFV-infected sheep for RVFV as primary antibodies. A clear indication for transient expression of the GPs is seen in figure 3.13. No fluorescence is visible in control cells (A and C), while there is strong specific fluorescence in cells co-transfected with either the pLSEG-BEFV or pLSEG-RVFV constructs (B and D respectively). These results confirmed that the GPs were being expressed and that they were ready for insertion into LSDV.

3.3.6 Generation and selection of LSDV recombinants expressing the GPs of BEFV and RVFV:

With the construction of an improved LSDV transfer vector, pLSEG, containing a positive selectable marker gene (gpt) and a visual marker gene (EGFP), and with the insertion of the immuno-protective GP genes of BEFV and RVFV into this transfer vector, it was now possible to generate and select recombinant LSD viruses expressing these genes for evaluation as recombinant vaccines. The procedure was essentially that described for generating and selecting the LSDV-lacZ recombinant, but with minor modifications. LSDV-infected FBT cells were co-transfected with either the pLSEG-BEFV or the pLSEG-RVFV constructs and treated as described (3.2.9).

For both constructs, after the second round of MPA selection, foci were clearly visible on the FBT cells. Potential recombinants were treated in a similar manner as described in chapter 2 except where modifications are indicated, however, this time, the introduction of the EGFP visual marker gene helped facilitate the *in vivo* selection of homogeneous recombinants. Foci were viewed under visible and UV light using a Leica DM IL inverted microscope (with fluorescence) (Germany), and only those foci appearing homogeneous for expression of EGFP were selected for further rounds of focus picking. In figure 3.14 (E) the FBT cell monolayer is illuminated with both visible and UV light simultaneously. The cells are infected with the LSDV-BEFV recombinant (rLSDV-BEFV) during the early stages of selection. As can be seen from the roughly circular focus on the right (arrows), the cells are already exhibiting cpe and yet only about 5% of them are actively expressing EGFP as is evidenced when the same focus is only

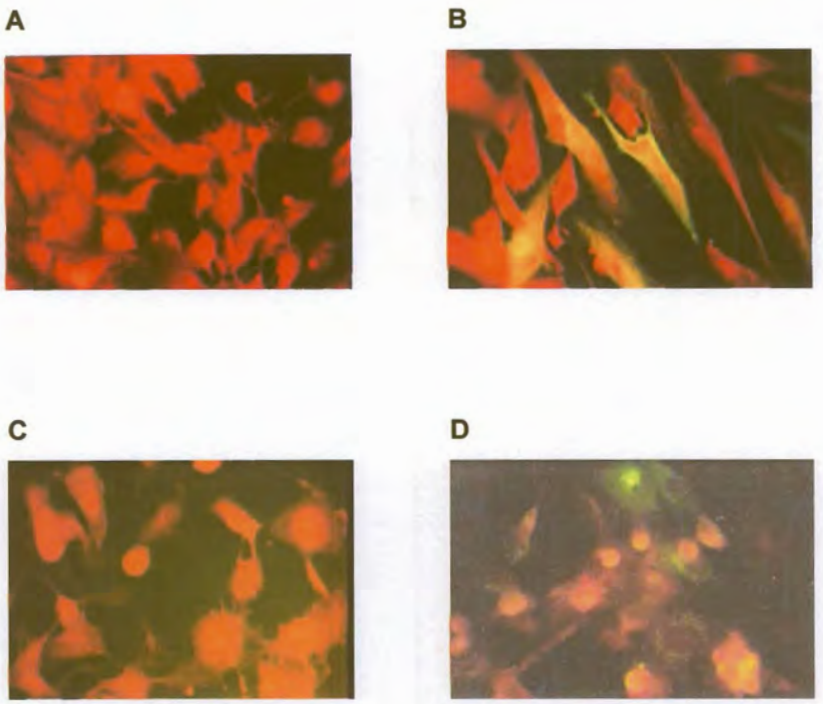


Figure 3.13 Immunofluorescence for the detection of transiently expressed BEFV and RVFV GPs from LSDV insertion vectors. A – FBT cells co-transfected with wtLSDV and pLSEG and incubated with a polyclonal anti-BEFV cattle serum. B – FBT cells co-transfected with wtLSDV and pLSEG-BEFV and incubated with a polyclonal anti-BEFV cattle serum. C – FBT cells co-transfected with wtLSDV and pLSEG and incubated with a polyclonal anti-RVFV sheep serum. D – FBT cells co-transfected with wtLSDV and pLSEG-RVFV and incubated with a polyclonal anti-RVFV sheep serum. The red/orange colour of the cells is due to the Evans Blue counterstain and the yellow/green colour is the light emitted from UV-irradiated FITC. Magnification: 500X.

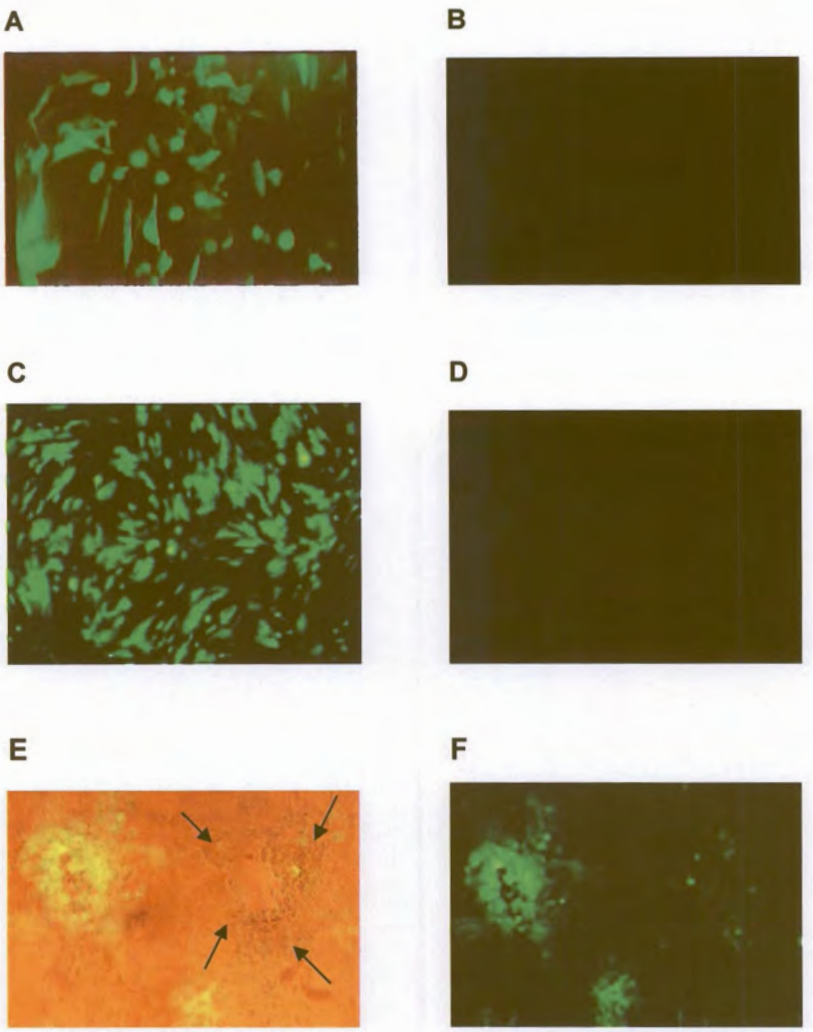


Figure 3.14 EGFP expression. **A** - transient expression of EGFP in FBT cells co-transfected with pHGS7-E and wtLSDV, 24 hours post-transfection. **B** – negative control – as in "A", but using pLS-A in place of pHGS7-E. **C** - transient expression of EGFP in FBT cells co-transfected with pLSEG and wtLSDV, 24 hours post-transfection. **D** – negative control – as in "C", but using pLS-A in place of pLSEG. **E** – rLSDV-BEFV infected FBT cells illuminated with visible and UV light. **F** –as in "E", but only illuminated with UV light. The arrows indicte a viral focus. Magnification: 250X for A, B, C, D, and 100X for E and F.

illuminated with UV light (Figure 3.14 F), indicating the presence of contaminating wtLSDV. The focus to the left of this one, however, already appears almost homogeneous for EGFP expression. Only foci such as this one were picked for subsequent rounds of selection.

Foci which appeared homogeneous for EGFP expression were then tested using PCR and the TK primer pair, P1 and P2 (2.2.9). In figure 3.15 amplification products for a number of such foci from the early stages of selection for each recombinant are shown (lanes 1 to 3 for rLSDV-BEFV and and lanes 4 to 6 for rLSDV-RVFV). Only products of ~ 450 bp are evident, clearly indicating the presence of wtLSDV TK DNA. No products are visible in lanes 5 and 6 probably due to the amount of viral DNA purified from the foci being below the cut-off amount for detectable amplification. The extra bands (~ 2.8 and 2.6 kbp) in lane 4 are possibly due to an unstable, intermediate form of the recombinant resulting from a single-crossover event during recombination. 0.1 ng each of plasmids pLSEG-BEFV DNA (lane 7) and pLS-A DNA (lane 10) were included in the PCR as positive controls. The correct amplification products of 4.7 and 0.7 kbp were obtained, however, another faint band of 2.6 kbp is also present in lane 10 – this band is possibly due to contamination.
After a few more rounds of selection and focus picking, foci were obtained which produced amplification products suggestive of homogeneous recombinants. In lanes 11 and 12 are the full-length amplification products (~ 4.7 and 6.3 kbp) for one rLSDV-BEFV focus and one rLSDV-RVFV focus respectively which were grown for more than five passages without selection pressure. The absence of the 450 bp wtLSDV TK band provides clear evidence that both the foci were homogeneous for recombinant virus. In lane 12 there are also a number of faint, smaller amplification products – these bands are possibly due to mis-priming of the P1 and/or P2 primers from the LSDV DNA.

With the generation and selection of both recombinants to homogeneity IF could now be used to confirm continued expression of the GPs from each recombinant.

3.3.7 Immunofluorescence detection of GP expression:

The homogeneous rLSDV-BEFV and rLSDV-RVFV foci were then tested for expression of their respective GPs using IF. FBT cells were infected with the respective recombinant viruses and once foci appeared they were fixed and stained as described (3.2.8). In figure 3.15 there is no evidence for fluorescence in the negative control samples (A and C), however, expression of the GPs is evident from the strong specific fluorescence visible in the cells infected with the

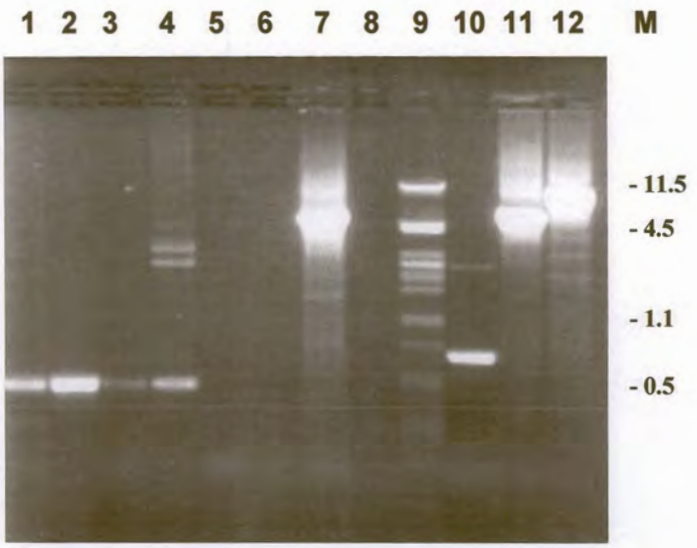


Figure 3.15 Agarose gel of PCR amplification products of LSDV-BEFV and LSDV-RVFV recombinants separated by electrophoresis (including plasmid controls and lambda *Pst*I marker DNA). Lanes 1-3: three early stage LSDV-BEFV recombinant foci, lanes 4-6: three early stage LSDV-RVFV recombinant foci, lane 7: pLSEG-BEFV positive control, lane 8: dH_2O negative control, lane 9: lambda *Pst*I DNA marker (kbp), lane 10: 0.1 ng pLS-A DNA (as internal positive control, figure 3.4), lane 11: late stage LSDV-BEFV recombinant virus focus and lane 12: late stage LSDV-RVFV recombinant virus focus. The primer pair P1 and P2 were used as described (chapter 2.2.9). M - marker DNA sizes in kbp.

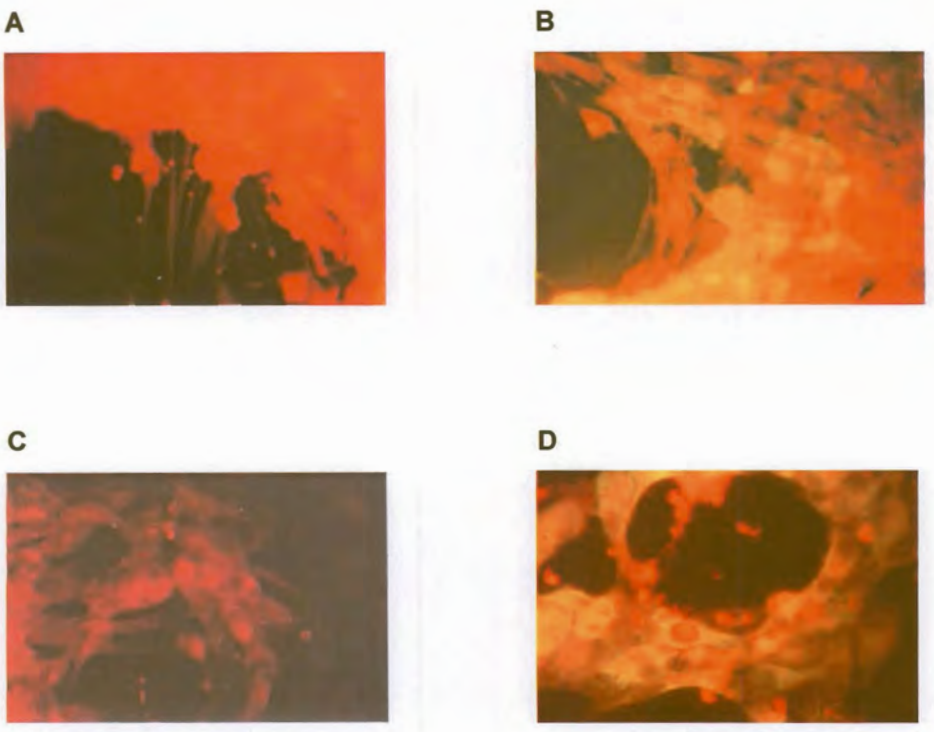


Figure 3.16 Immunofluorescence for the detection of BEFV and RVFV GPs expressed from LSDV recombinants. A – FBT cells infected with wtLSDV and incubated with a polyclonal anti-BEFV cattle serum. B – FBT cells infected with LSDV-BEFV recombinant virus and incubated with a polyclonal anti-BEFV cattle serum. C – FBT cells infected with wtLSDV and incubated with a polyclonal anti-RVFV sheep serum. D – FBT cells infected with LSDV-RVFV recombinant virus and incubated with a polyclonal anti-RVFV sheep serum. The red/orange colour of the cells is due to the Evans Blue counterstain and the yellow colour is the light emitted from UV-irradiated FITC. Magnification: 500X.

recombinants and incubated with primary and secondary (FITC-bound) antibodies specific for either BEFV or RVFV (B and D respectively).
The foci were then grown to high titres for use in animal trials – chapter 4.

3.4 DISCUSSION

For the generation and selection of TK-disrupted LSDV recombinants, the finding that some form of TK activity was needed for viral growth necessitated a change in selection strategy. Success was achieved by adopting a dominant selectable marker (*E. coli* gpt) approach. Inclusion of a lacZ reporter gene greatly assisted with this process, however, as the gene is large (3.7 kbp) it was not convenient to retain it in the insertion vectors once immunogenic genes from pathogens of veterinary importance had been included. Thus a more streamlined LSDV transfer vector was constructed (pLSEG) in which the lacZ gene was substituted with the smaller EGFP visual marker gene (1.0 kbp). The EGFP gene has an additional advantage over the lacZ gene as its expression and visual effects can be monitored *in vivo* – this allows for the direct selection of recombinants without having to fix the cells and virus, as is the case for lacZ. EGFP expression was also found to greatly enhance the process for the selection of recombinant viruses. It made it possible to determine whether or not a high enough percentage of cells were co-transfected to allow for selection of recombinant viruses. On average it was found that greater than 10% of cells were required to show EGFP expression within 48 hours after co-transfection for it to be possible to successfully select a recombinant (data not shown). EGFP also helped with selecting for foci which were close to homogeneity – foci showing almost 100% of cells expressing EFGP were preferentially selected over foci showing a lower percentage of expression. Using this technique it was possible to reduce the number of passages required to select a homogeneous recombinant by approximately half (data not shown).

The insertion of the EGFP gene under control of the VV P11K late promoter and the streamlining and reconstruction of the LSDV TK transfer vector proved challenging. For the EGFP gene it was necessary to insert the gene in-frame into the *Eco*RI R.E. site downstream of the p11K promoter in the plasmid pHGS7 due to the presence of an ATG start codon upstream from the *Eco*RI site. Initial attempts made use of Mung Bean nuclease to digest the 5' end of the EGFP gene for an in-frame blunt-end insertion into the *Eco*RI site – however, when this proved unsuccessful, an alternative approach was sought. The 5' end of the African horsesickness

virus (type 3) VP2 gene contains an *Eco*RI site and *Nco*I site which would allow the in-frame insertion of the EGFP gene. This short stretch of DNA was then utilized for this purpose and the EGFP was successfully inserted under control of the p11K promoter.
For the improvement of the transfer vector a number of partial R.E. digestions were performed, the first of which necessitated the specific selective cutting of two out of four *Pst*I sites. A number of different combinations of digestion conditions had to be evaluated before success was achieved. At a stage of the re-construction of the vector where a partial *Spe*I digestion was required, the wrong site cut preferentially and an alternative approach had to be utilized. Once these problems had been overcome it was possible the complete the pLSEG vector which was more streamlined, contained an improved MCS and the gpt and EGFP marker genes.

Two pathogens of livestock (BEFV and RVFV) were targeted for development of the first TK-disrupted recombinant vaccines using the SA vaccine strain of LSDV as vector. The reasons being that these pathogens cause economically important diseases of livestock in regions endemic for LSDV, thus allowing for the potential use of the recombinant vaccines as dual vaccines, there is a need for new and improved vaccines against these pathogens, and, previous studies had already laid much of the groundwork by identifying the immuno-protective antigens from these pathogens (Van der Westhuizen, 1967; Weiss, 1968; Collett el al., 1987; as reviewed by Swanepoel and Coetzer, 2004; Hertig el al., 1995).

With the construction of the relevant insertion vectors (pLSEG-BEFV and pLSEG-RVFV), two separate recombinants, one expressing the structural glycoprotein gene of BEFV (rLSDV-BEFV), and the other the two structural glycoprotein genes of RVFV (rLSDV-RVFV), were generated. Homogeneity of the recombinants was confirmed via PCR analysis after removal of the selection pressure for more than five passages and expression of the glycoproteins was confirmed using immunofluorescence (IF).

One of the major problems encountered in this part of the study, even with the help of the EGFP visual marker, was the ability to select for a homogeneous population of recombinant viruses, completely free from contaminating wtLSDV, in a reasonable time period. As has been shown in previous studies, over 99% of progeny LSDV virions remain cell-associated (Wallace, 1994). In the early stages of the selection process, the ratio of wtLSDV to recombinant virus is high therefore in order to select for recombinant virus it is necessary to select from the cell-associated pool of viruses. Electron photomicrographs of cell-associated LSDV particles indicate that they are surrounded by a membranous material, forming loosely bound clumps (Figure 1.4). These "clumps" probably contain a mixture of wt and recombinant viruses making it

necessary to thoroughly disassociate individual particles from one another in order to select a single homogeneous recombinant (Dumbell, personal communication). The problem is further compounded by the use of a positive dominant selectable marker system for recombinant selection, especially as the gene of choice, the *E. coli* gpt gene, is an enzyme, which, by its very nature can exert a wide-ranging effect. In other selection strategies such as the TK-negative strategy, any viruses which have an active TK gene are knocked out under BUdR selection. It is relatively quick and easy to select homogeneous recombinants where the use of this strategy is possible, such as for the selection of VV recombinants (personal observation). However, Wallace and Viljoen (2002) have provided evidence that the highly attenuated South African vaccine strain of LSDV has a dependence on TK activity (whether viral or cellular) for growth and thus an alternative strategy had to be chosen. In the MPA-selection strategy, using the gpt dominant selectable marker gene, viruses expressing gpt (and probably viruses in close proximity which are non-expressers) are able to survive in gpt selection medium, making this strategy more prone to the influence of co-contaminating wt virus.

In order to overcome this problem a number of techniques were then investigated to ensure the complete disaggregation of the virus particles, including alternate cycles of freeze-thawing, sonication and filtration. Three cycles of freeze-thawing between -20 °C and room temperature (~ 20 °C) were first used to lyse the infected cells. Mild sonication, using a water-bath sonicator, is routinely used with poxviruses to assist with the disaggregation of particles (Mackett *et al.*, 1985). For LSDV we found that it was possible to sonicate a virus suspension in a Sonorex TK52 water-bath sonicator (35 kHz) (Bandelin, Germany) for up to 15 minutes before a noticeable loss in infectivity resulted (data not shown). Trypsinisation is another method used for some poxviruses, however, for LSDV it has been found to completely destroy infectivity (Mackett *et al.*, 1985; Black, personal communication). Another idea formulated in our laboratory was to force the viral particles through the pores of a membrane. If the correct pore size could be chosen, then only single viral particles would be able to pass through a pore at a time – unless particles were joined to form perfectly symmetrical "chains" able to pass through the pores length-wise. Pore sizes of 0.22 and 0.45 µm were evaluated using single-use syringe filters (Millipore, France). The 0.22 µm filters excluded the passage of all virus particles, whereas sufficient numbers of particles passed through the 0.45 µm pores to allow for the next round of selection (data not shown).

When each method was used on its own it took many rounds of selection to select for a homogeneous recombinant virus, however, when used in combination, the required time period was greatly reduced.

Once the ratio of recombinant virus to wt virus had been greatly enhanced after a number of rounds of selection, it was possible to subject virus released into the selection medium to these

methods. In theory, this released virus should have been easier to select to homogeneity, however this did not prove to be the case. This suggests that the problem is not as simple as viral clumping, unless released viral particles have a high affinity for each other. Another possibility is that for a high percentage of progeny virions a mixture of wt and recombinant genomes are being packaged into single viral particles. It could prove advantageous for recombinant viruses to co-package wt genomes due to the added growth advantage offered by the active TK gene encoded by the wt genome. Although there is no direct proof to support this hypothesis, it would explain why it takes a number of rounds of vigorous disaggregation and chemical selection to select for a single homogeneous recombinant virus.

Some evidence also exits to suggest that single cross-over recombination events could be responsible for indicating the presence of wt virus when recombinant foci are analysed using PCR. The extra PCR amplification products obtained for a rLSDV-RVFV focus (Figure 3.15, lane 4) might be explained by such an event. Nazerian and Dhawale (1991) describe the generation of unstable intermediate forms of recombinant fowlpox virus caused by single cross-over events. They were able to select for stable recombinants by passage of the intermediate recombinants in chicken embryo fibroblast cultures treated with BUdR. However, our findings have shown that selection using BUdR is not possible for selecting LSDV recombinants.

Whatever its shortcomings, the selection method described in this chapter has proven reproducible and successful in the hands of others (Berhe *et al.*, 2003) and at present remains the method of choice for selecting recombinant LSDV viruses in our laboratory. With slight modifications to the methodology described in section 3.2.9 it has been possible to select a homogeneous recombinant virus within one month. Advances in selection strategies, which promise to make the selection for homogeneous recombinants even more efficient, such as described by Timiryasova *et al.* (2001), are currently under investigation for use with LSDV. Alternative insertion sites are also being investigated, especially where these involve genes with probable immuno-suppressive properties and if these genes prove to be non-essential for growth of LSDV, then the problems encountered with selecting for homogeneous LSDV recombinants using viral TK insertion might eventually cease to exist.

The combined use of PCR and removal of selection pressure for a number of passages were found to be crucial tests to ensure that a recombinant was indeed homogeneous. In one instance a LSDV-BEFV recombinant focus, which at first appeared homogeneous via PCR when cultivated under selection pressure, reverted back to producing the wtLSDV TK amplification product (using the P1 and P2 primers) of 450 bp within three passages of selection pressure being removed, thus showing that the recombinant was not homogeneous. This result

also indicated that wtLSDV has a distinct growth advantage over TK-disrupted recombinants, as is the case for other poxviruses (Mackett *et al.*, 1985), although given enough time, the recombinants are still able to grow to high titres in cell culture (Wallace and Viljoen, 2002).

The two recombinant constructs were grown to high titres and were purified for the next stage of this study – an evaluation of their ability to protect animals against challenge with the respective pathogens.

CHAPTER 4

IMMUNE RESPONSES AND PROTECTION STUDIES IN ANIMALS

4.1 INTRODUCTION

One of the final testing stages in the development of any new vaccine system is an evaluation of the ability of the potential vaccines to elicit a protective immune response. Poxviruses as vectors for recombinant vaccines have proven extremely valuable in this regard. They can accommodate large amounts (up to 25 kb) of extra DNA (Smith and Moss, 1983), are generally thermostable (Weiss, 1968), safe (Cadoz *et al.*, 1992; Fries *et al.*, 1996), provide long-term immunity (Inui *et al.*, 1995), are able to elicit both humoral and cell-mediated immune responses (Coupar *et al.*, 1986) and serve as excellent boosters when used in combination with DNA vaccines (Richmond *et al.*, 1997; Caver *et al.*, 1999).

The VV-rabies recombinant vaccine used successfully for many years to control rabies in foxes and racoons in Europe and the USA respectively has shown conclusively that VV is suitable as a vector for recombinant vaccines (Kieny *et al.*, 1984; as reviewed by Paoletti, 1996). However, concerns over its use in immunocompromised persons and the accidental infection of a pregnant woman with the VV-rabies recombinant in the USA recently (Rupprecht *et al.*, 2001) necessitated the development of other poxviruses as vectors. A canarypox-rabies recombinant has shown tremendous potential in human trials (Fries *et al.*, 1996). Although avipoxviruses are unable to undergo a complete infection cycle in mammalian cells, they readily infect nonavian cells and express foreign genes which can induce immune responses (Taylor and Paoletti, 1988; Taylor *et al.*, 1988; Taylor *et al.*, 1992). Highly attenuated strains of VV which are host-range restricted, such as MVA and NYVAC, have also been targeted (Perkus *et al.*, 1991; Konishi *et al.*, 1992; Lanar *et al.*, 1996; Scheiflinger *et al.*, 1998). There has been concern that even these restricted vaccinia viruses might not prove suitable as vectors, especially in cases where there is a pre-existing immunity in persons vaccinated against smallpox (renewed recently due to the threat of bio-terrorism) (Ramirez *et al.*, 2000). However, studies have revealed that although certain aspects of the immune responses are affected, using alternative vectors, or first priming using nonviral DNA vaccines, effective immune responses are still elicited (Yang *et al.*, 2003).

For the capripoxviruses (which includes LSDV) a number of potential recombinant vaccines have been developed and tested in animals, many with promising results. The northern African KS-1 vaccine strain of LSDV has served as a vector for recombinant rinderpest vaccines

(Romero *et al.*, 1993; Romero *et al.*, 1994a; Romero *et al.*, 1994b; Ngichabe *et al.*, 1997), and a recombinant BTV vaccine (Wade-Evans *et al.*, 1996). Varying levels of protection were obtained, and cattle vaccinated with the rinderpest recombinant vaccines were also protected against challenge with virulent LSDV. The recombinant rinderpest vaccines were also used to protect goats against PPRV, although no detectable levels of specific antibodies to PPRV were produced prior to challenge (Romero *et al.*, 1995).

Recent work using the ribonucleotide reductase (RR) gene of the SA vaccine strain of LSDV as the insertion site to generate a recombinant LSDV-rabies vaccine produced high levels of neutralising antibodies and a cell-mediated immune response in cattle. Expression of the rabies virus GP gene was under control of a fowlpox virus early/late promoter (pAF) (Aspden *et al.*, 2002). However, in this work the recombinant virus was not purified to homogeneity and thus it is uncertain whether or not the RR gene is non-essential for LSDV, or whether the recombinant generated using their approach is stable, and thus potentially suitable as a commercially viable vaccine.

The southern African strain of LSDV is highly host-range restricted and natural infection has only been observed in cattle (Weiss, 1968). The vaccine strain was prepared by repeated passage on the CAMs of embryonated chicken eggs and in ovine cells (van Rooyen *et al.*, 1969). Besides growth in ovine and bovine cells, Weiss (1968) reports on the propagation of LSDV in monkey, rabbit, hamster and chicken cells. What is not clear from Weiss' observations is whether virulent or attenuated virus was used. In a number of recent studies using the attenuated vaccine strain of LSDV it has been shown that the virus is unable to undergo a productive infection in cells other than of ovine, bovine, or chicken origin (Wallace, 1994), although gene expression from both early/late and late promoters in non-permissive cells does occur (personal observation; Aspden *et al.*, 2003). Growth in chicken cells is limited (Wallace, 1994) and inoculation into one day old chicks produced no noticeable ill-effects and no neutralising antibodies were detectable (personal observation). How LSDV is able to propagate in chicken cells still remains unclear. As such there is no small animal model for testing the SA vaccine strain of LSDV, although LSDV-specific antibodies have been raised in rabbits (Davies *et al.*, 1971).

Rabbits were thus chosen to evaluate the ability of the LSDV-BEFV and LSDV-RVFV recombinants generated in this study to elicit an immune response to the BEFV and RVFV GPs in the form of neutralising antibodies (Wallace and Viljoen, 2005). For protection studies in laboratory animals, the only model available for BEFV is the use of a BEFV strain which is neurotropic for mice (Gaffar Elamin and Spradbrow, 1979; Young and Spradbrow, 1981). However, such a strain does not exist in South Africa, and attempts to establish a mouse

neutrotropic strain by sequential passage of a field isolate in suckling mouse brain failed (personal observation). Thus it was necessary to evaluate the protective abilities of the LSDV-BEFV construct directly in cattle. For RVFV it is possible to use mice for a challenge study (Collett *et al.*, 1987) and thus mice were chosen for evaluating the protective abilities of the LSDV-RVFV construct. Once this construct was shown to protect mice against virulent RVFV challenge, a sheep challenge study was undertaken to test the dual protective abilities against RVFV and sheeppox virus.

This chapter describes these protection studies.

4.2 MATERIALS AND METHODS

4.2.1 Viruses:

BEFV

The commercial BEFV vaccine is a live attenuated strain of BEFV and was supplied as freeze-dried material by Onderstepoort Biological Products (SA) (Theodoridis *et al.*, 1973; Cameron *et al.*, 1987). Unusually for a live vaccine it requires addition of an adjuvant.

The BEFV cattle challenge material was untitrated virus-infected blood passaged once in a cow from a field isolate collected near Skeerpoort (border of Gauteng and North-West Province of South Africa) (isolate 1741) (kindly provided by Onderstepoort Biological Products, SA).

The BEFV strain used to prepare antigen for the lymphocyte proliferation assay was a field isolate (no. 8) passaged twice in CEF cells (ATCC) and twice in the brains of suckling mice (kindly provided by Roelf Greyling, Virology Department, Onderstepoort Veterinary Institute, SA).

LSDV

The commercial LSDV vaccine is the South African vaccine strain of LSDV and was supplied as freeze-dried material by Onderstepoort Biological Products (SA) (van Rooyen *et al.*, 1969).

RVFV

The RVFV Smithburn vaccine strain was supplied by Onderstepoort Biological Products (SA) (Smithburn, 1949).

The RVFV challenge strain was a mosquito isolate (no. AR 20368) passaged 8 times in Madin-Darby bovine kidney cells and twice in hamsters (intra-peritoneal route) (kindly provided by Roelf Greyling). The isolate was then titrated in suckling mouse brains (by Roelf Greyling) to

determine a 50% lethal dose for mice (MLD_{50}) for the mouse challenge. For the sheep challenge, the virus was grown and titrated in MDBK cells.

Sheeppox virus

The sheeppox virus vaccine strain was provided by Onderstepoort Biological Products as freeze-dried material. This vaccine strain (KS-1) is derived from a virulent field isolate from Kenya (0240 isolate) after one passage in LT cells, two passages in baby hamster kidney cells and a further 4 passages in LT cells, and has since been shown to be a strain of LSDV (Davies and Otema, 1981; Kitching *et al.*, 1987; Gershon and Black, 1989a).

The sheeppox virus challenge strain was a Yemen isolate obtained freeze-dried from Tom Barrett at the Pirbright Animal Health Institute, Woking, UK. The material was re-constituted in cell culture growth medium (DMEM), passaged twice in LT cells, and then titrated (in LT cells) before use.

4.2.2 Neutralising antibody production in rabbits against BEFV and RVFV:

Five 14-week old New Zealand White rabbits were inoculated with $1x10^7$ infectious units of either parental control LSDV (vaccine strain) or the recombinant viruses (rLSDV-BEFV or rLSDV-RVFV) via the routes shown in Table 4.1. They were boosted at 73, 130 and 150 days post-inoculation (pi) with $3x10^7$ infectious units and blood samples were drawn from an ear vein 21 days after each boost. Blood sera were tested for antibodies to BEFV (by Anita Engelbrecht, OBP) and RVFV (by Katy Roos, Virology, OVI) using serum neutralisation (SN) (Vanselow *et al.*, 1985) and haemagglutination inhibition (HI) (Paweska *et al.*, 2003a) testing respectively (the HI assay for detecting RVFV antibodies was used as the rabbit sera were found to be toxic for the Vero cells used for the RVFV assays at low dilutions in a SN test).

The mean neutralising antibody (nAb) titres were calculated from the post-boost bleeds for each rabbit according to the respective tests.

4.2.3 Protection studies:

4.2.3.1 BEFV (cattle)

Twelve 1-year old Bonsmara-Nguni crossbreed cattle (both sexes) were bled and their sera tested for antibodies to BEFV (Vanselow *et al.*, 1985) and LSDV (Timoney, 1996) using SN assays. Eight animals were found to be negative for antibodies to both viruses and these were divided equally into four groups and housed in an insect-proof stabling facility.

Table 4.1 Antibody responses of rabbits to BEFV and RVFV inoculated with wild-type LSDV, rLSDV-BEFV and rLSDV-RVFV.

Rabbit No.	Virus	Inoculum Day0	Inoculum Boost	Route[a]	Mean SN titre (BEFV)	Mean HI titre (RVFV)
#1	LSDV (vac)	$1x10^7$	$3x10^7$	IM/SC[b]	neg	neg
#2	rLSDV-BEFV	$1x10^7$	$3x10^7$	IM	1:16	neg
#3	rLSDV-BEFV	$1x10^7$	$3x10^7$	SC	1:12	neg
#4	rLSDV-RVFV	$1x10^7$	$3x10^7$	IM	neg	1:50
#5	rLSDV-RVFV	$1x10^7$	$3x10^7$	SC	neg	1:60

[a] – IM= intramuscular; SC = subcutaneous [b] – 50% inoculated IM and 50% SC.
neg - negative

Groups #1 (cattle #7956 and #7963) and #2 (cattle #7901 and #7968) were inoculated subcutaneously (SC) (according to the manufacturer's instructions) with the locally produced commercial BEFV (2 x 10^5 pfu/dose) and LSDV (1.6 x 10^5 pfu/dose) vaccines respectively (prepared by Onderstepoort Biological Products, South Africa) (Table 4.2). Group #3 (cattle #7884 and #7983) were inoculated SC with $1x10^7$ focus-forming units (ffu) of the LSDV-BEFV recombinant virus. Group #4 consisted of one negative control animal (unvaccinated) (bovid #7973) and one animal to be used to boost the virulent BEFV titre immediately prior to challenge (bovid #7971). Animals were monitored for reactions to the vaccinations, and rectal temperatures and blood samples were taken at regular intervals throughout the trial period. At 3, 6 and 12 weeks pi the cattle were boosted using the same protocols as in the initial inoculations. Ten days after the second boost blood samples were withdrawn in 10 ml EDTA-coated blood-collection tubes (Becton, Dickinson and Company, UK) from all six vaccinated cattle for a lymphocyte proliferation assay (by Dr Mirinda van Kleef, Molecular Biology, OVI) (van Kleef *et al.*, 2000). Antigen was obtained from clarified suckling mouse-brain homogenate (infected with a virulent field strain of BEFV). The challenge material provided for this study was an untitrated stock of BEFV-infected cattle blood stored for 8 years at – 70 °C. Due to the variable levels of responses of indigenous breeds of South African cattle to BEFV and to the observed instability of the virus (Aitchison, personal communication), in order to ensure a response it is necessary to boost the challenge virus titres in a naïve animal immediately prior to challenge.

Ten weeks after the cattle had received their third boost bull #7971 was injected intraveneously (IV) with 5 ml of the challenge material. At the height of viraemia blood was removed and mixed

Table 4.2 Neutralising antibody titres and symptom scores of cattle vaccinated with different regimes of BEFV vaccines and challenged with BEFV-infective blood.

Cattle Group	Cattle ID no	LSDV SN titres	BEFV SN titres pre[a]	BEFV SN titres post[b]	Symptoms[c]
#1 - OBP BEFV vaccine	7956	< 1:4	1:600	1:65 536	+
	7963	1:4	1:600	1:4 096	-
#2 - OBP LSDV vaccine	7901	1:6	1:10	N/A	N/A
	7968	1:6	1:10	N/A	N/A
#3 - rLSDV-BEFV	7884	1:12	1:100	1:4 096	++
	7983	1:24	1:200	1:32 768	++
#4 – unvaccinated control	7973	< 1:4	1:10	1:256	+++

[a] – 6 weeks before challenge [b] – 14 days post-challenge

[c] – symptom severity scores according to Van der Westhuizen (1967), Table 4.3

N/A – not applicable

Table 4.3 Symptom severity scores of BEFV challenged cattle[a]

Designated Scale	Distinguishing symptoms
+	Fever of short duration and inappetence
++	Fever, inappetence, slight shivering, nasal and/or eye discharge, slight stiffness
+++	Fever, inappetence, nasal and eye discharges, shivering, moderate stiffness with dejection
++++	Fever, inappetence, nasal and eye discharges, shivering with severe lameness and stiffness, sometimes paretic, usually recumbent. Severe depression.

[a] – from Van der Westhuizen (1967)

1:1 with sterile OCG (0.5% phenol, 0.5% potassium oxalate, 50% glycerol) and 5ml of this mixture was then inoculated IV into each of the cattle in groups #1, #3 and the unvaccinated negative control (#7973). Cattle were then monitored for BEF-related symptoms (Table 4.3) (Van der Westhuizen, 1967).

4.2.3.2 RVFV (mice)

Six-week old Balb/c mice (5 mice per group) were inoculated with either the Smithburn vaccine strain of RVFV, recombinant LSDV-RVFV (rLSDV-RVFV), recombinant LSDV-BEFV (rLSDV-BEFV) or saline solution (the latter two groups as negative controls) via the routes shown in Table 4.4. The mice were boosted 21 days pi. Blood samples were removed from the tail veins at 0, 21 and 31 days pi. The samples were pooled between mice within each group and tested for RVFV-specific antibodies using HI testing (by Katy Roos, Virology) and an indirect enzyme-linked immunosorbent assay (I-ELISA) (by Shirley Smith, Virology) developed for the detection of RVFV-specific IgG antibodies (Paweska *et al.*, 2003b).

Ten days after the boost (at 31 days pi) all mice were challenged via the intramuscular (IM) route with 100 mouse lethal dose 50% (MLD_{50}) of virulent RVFV. Mice were monitored for reaction to RVFV challenge and the results are summarised in Table 4.4.

Table 4.4 RVFV antibody responses and protection studies in mice

Mouse Group#	Virus	Inoculum Day0	Inoculum Boost	Route[a]	Mean RVFV HI titres	Protection/ Survival[d]
#1	Smithburn	[b]	[b]	IP	1:80	5 (5)
#2	rLSDV-RVFV	$1x10^7$	$1x10^7$	SC/IM[c]	1:160	5 (5)
#3	rLSDV-BEFV	$1x10^7$	$1x10^7$	SC/IM[c]	neg	1 (5)
#4	Saline only	100 µl	100 µl	SC/IM[c]	neg	1 (5)

[a] – IP = intraperitoneal, IM = intramuscular; SC = subcutaneous

[b] – 50x equivalent protective dose in sheep

[c] – prime SC, boost three weeks later 50% SC and 50% IM.

[d] – number of mice surviving of total challenged (number in brackets)

4.2.3.3 RVFV/sheeppox (sheep)

Eighteen one-year old Merino sheep (both sexes) were purchased from the Eastern Cape Province and housed in an insect-free stabling facility at Onderstepoort. All were tested for antibodies to RVFV and sheeppox/LSDV using the serum neutralisation assay (SN). They were then divided equally into two groups – one group for RVFV vaccination and challenge (Group A), and the other group for sheeppox virus vaccination and challenge (Group B). Each group was housed in a separate stable. The sheep were sheared and a 100 cm^2 area close-clipped on their left and right flanks. Rectal temperatures were taken and the animals were vaccinated in the close-clipped area on their left flanks according to the schedule in Table 4.5. At three weeks post-vaccination they were boosted in their right flanks using the same regime as for priming. Rectal temperatures were recorded daily and the animals were observed for reactions to the vaccine. Blood was drawn on a weekly basis from the jugular vein for SN testing for antibodies to RVFV (by Shirley Smith, Virology) and sheeppox virus (by Roelf Greyling, Virology). Blood was also drawn into EDTA-coated collection tubes for a lymphocyte proliferation assay (by Ndavhe Tshikhudo, Immunology, OVI) prior to vaccination and at ten days post-boost.

At nine days post-boost whole blood was collected from sheep boosted with the recombinant vaccine and skin biopsies taken at the boost site of the same animals for PCR testing for the presence of recombinant virus. The sheep were then transferred to the high containment stabling facilities at Onderstepoort (EDD) for the challenge phase of the trial (as both RVF and sheep- pox are exotic diseases to South Africa).

At 14 days post-boost the Group A sheep were challenged IM with $1x10^7$ pfu virulent RVFV, and the Group B sheep were challenged IV with $1x10^6$ pfu virulent sheeppox virus. Twice daily rectal temperatures were taken and daily bleeds for antibody determination. The animals were observed daily for symptom development. At 14 days post-challenge the Group A animals were euthanased and organs removed for histopathological evaluation (by Dr Comfort Phiri, EDD). The Group B animals were treated in a similar manner on day 28 post-challenge.

4.3 RESULTS

4.3.1 Neutralising antibody production in rabbits against BEFV and RVFV:

Before conducting challenge trials in laboratory or large animals the recombinant viruses were inoculated into rabbits to monitor for the production of either BEFV or RVFV neutralising antibodies to give an indication whether or not the constructs would be protective. Neutralising antibodies against BEFV have been shown to be a direct indicator for protection (Uren *et al.*, 1994), although for RVFV cell-mediated immunity is also deemed to be important (Hunter, personal communication). Rabbits were inoculated and boosted as described (4.2.2) and blood

Table 4.5 Vaccine regimes for sheep vaccinated against RVFV and sheepox virus

Sheep Group	Sheep ID no.	Vaccine	Route[a]	Dose[b]
Group A	957	Smithburn vaccine	SC	one std dose
(RVF)	959	Smithburn vaccine	SC	one std dose
	667	rLSDV-RVFV	ID	1 x 10^6 pfu
	677	rLSDV-RVFV	ID	1 x 10^6 pfu
	680	rLSDV-RVFV	ID	1 x 10^7 pfu
	760	rLSDV-RVFV	ID	1 x 10^7 pfu
	841	rLSDV-RVFV	ID	1 x 10^7 pfu
	662	PBS	ID	0.2 ml
	674	PBS	ID	0.2 ml
Group B	813	KS-1 vaccine	ID	1x10^3 $TCID_{50}$
(sheeppox)	990	KS-1 vaccine	ID	1x10^3 $TCID_{50}$
	659	rLSDV-RVFV	ID	1 x 10^6 pfu
	668	rLSDV-RVFV	ID	1 x 10^6 pfu
	703	rLSDV-RVFV	ID	1 x 10^6 pfu
	721	rLSDV-RVFV	ID	1 x 10^7 pfu
	807	rLSDV-RVFV	ID	1 x 10^7 pfu
	704	PBS	ID	0.2 ml
	743	PBS	ID	0.2 ml

[a] – SC – subcutaneous, ID – intradermal

[b]- $TCID_{50}$ – 50% tissue culture infective dose

samples were periodically taken for subjection to SN testing for BEFV and HI testing for RVFV. Neutralising antibody and HI titres (for BEFV and RVFV respectively) obtained after priming and boosting rabbits with recombinant viruses or wild-type virus (negative control) are shown in Table 4.1. For parental LSDV (the vaccine strain), no detectable levels of neutralising antibodies to either BEFV or RVFV were elicited, whereas for rLSDV-BEFV inoculated via either the IM or SC routes, SN titres for BEFV of 1:16 and 1:12 were obtained respectively. For rLSDV-RVFV inoculated via the same routes, neutralising RVFV HI antibody titres of 1:50 and 1:60 were obtained.

These results indicate that both constructs were expressing their respective GPs which were able to induce the production of neutralising antibodies and should thus afford protection in a challenge study.

4.3.2 Protection studies of the constructs in laboratory and target animals:

Once the constructs had been shown to induce neutralising antibody production in laboratory animals, they were ready for evaluation of their protective abilities in challenge studies. The results of these studies would determine whether or not the constructs would have the potential to be effective recombinant vaccines against BEFV and RVFV. Ideally challenge studies in laboratory animals should have been performed first for both constructs, although this was not possible for BEFV. Thus for BEFV a small-scale challenge study had to performed in cattle. For RVFV it was possible for evaluation in mice, before performing a challenge study in sheep.

An added aim of these studies was also to test the dual protective abilities of the constructs. For the rLSDV-BEFV construct this would be against both BEFV and LSDV, and, for the rLSDV-RVFV construct it would be against RVFV and sheeppox virus.

4.3.2.1 BEFV (cattle)

In order to test the protective ability of the BEFV construct the standard procedure would have been to first test it in a laboratory animal protection study. However, for BEFV a neurotropic strain for challenging mice is not available in South Africa and the only alternative was thus a small-scale trial in cattle. Of the twelve cattle made available for the study, only eight of them were found to be free from antibodies to either LSDV or BEFV. These were divided equally into four groups and treated as described in the Materials and Methods (4.2.3.1) and in Table 4.2.

One day after the initial inoculations, cow #7956 developed a round swelling 3.5 cm in diameter at the site of inoculation. This increased to 5 cm by day seven and persisted for another 14 days before subsiding. Cow #7963 developed a slightly smaller swelling on day seven which also subsided after approximately three weeks. Cow #7983 developed a mild fever (40.8 °C) on day

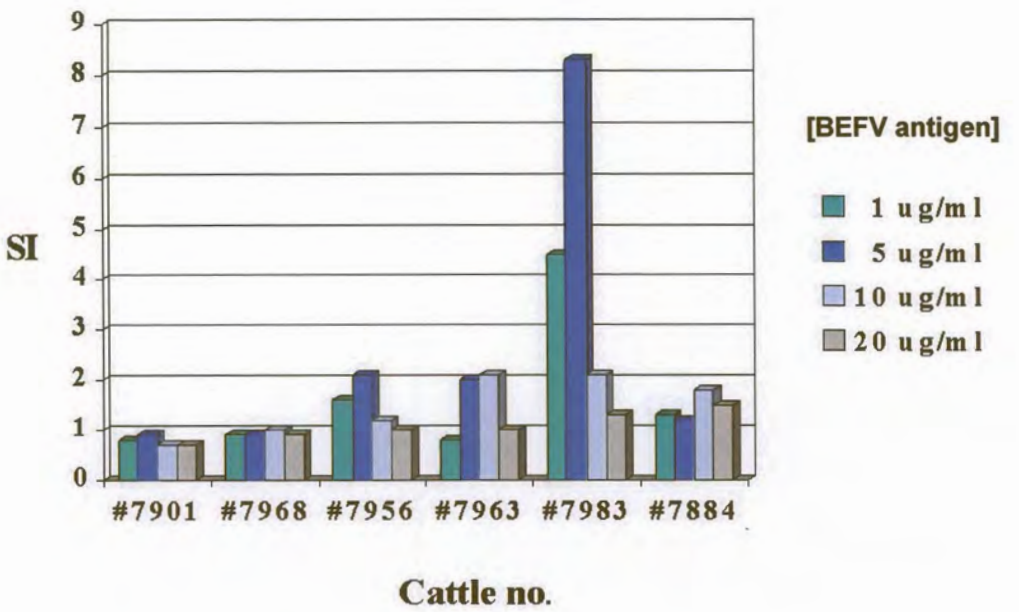


Figure 4.1 Lymphocyte proliferation assay of LSDV and BEFV vaccinated cattle. PBMCs isolated from the commercial LSDV vaccinated cattle (#7901 and #7968) (as negative controls), commercial BEFV vaccinated cattle (#7956 and #7963) (as positive controls) and the LSDV-BEFV recombinant vaccinated cattle (#7983 and #7884) were stimulated with mouse-brain purified BEFV antigen (at different concentrations) and the resulting proliferation was determined by [methyl-^{3}H]thymidine incorporation. Results are presented as a stimulation index (SI) where SI = mean counts per minute of stimulated sample/mean counts per minute of unstimulated sample. An SI > 1.0 was considered to be an indication of antigen-specific proliferation.

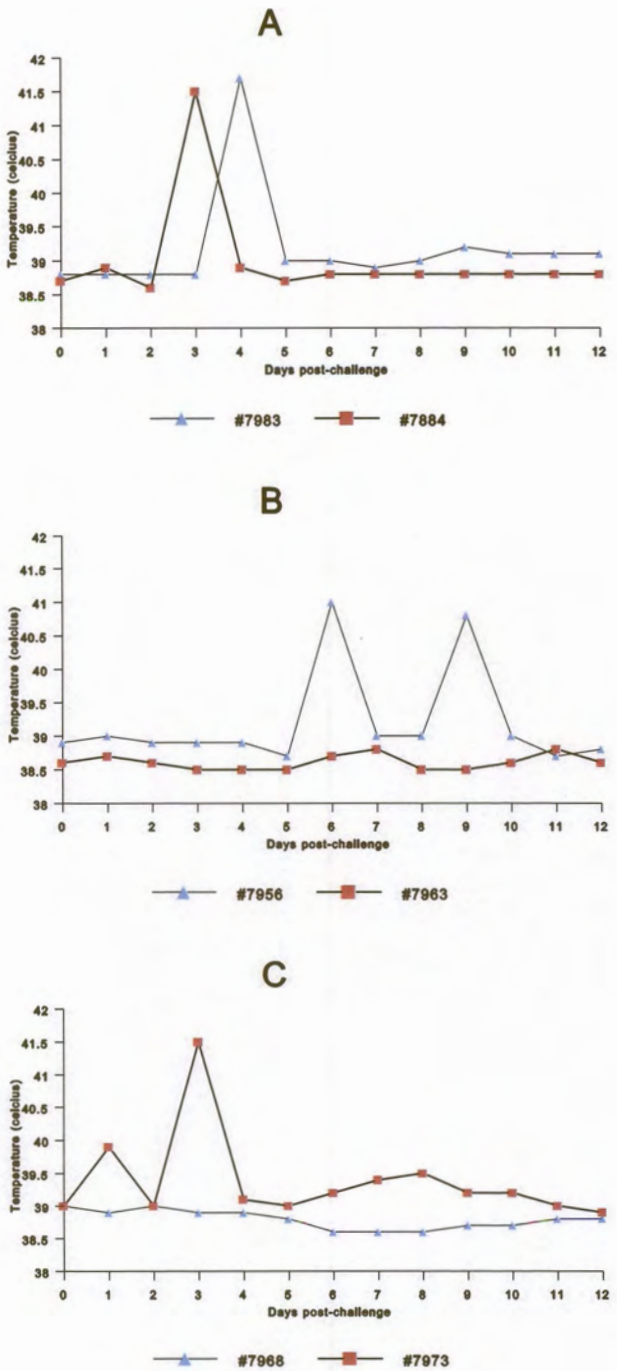


Figure 4.2 Rectal temperature recordings of the cattle after challenge with virulent BEFV. **A**. Cattle vaccinated with rLSDV-BEFV. **B**. Cattle vaccinated with the commercial live attenuated BEFV vaccine. **C**. Unvaccinated, challenged cow (#7973) and unchallenged, negative control cow (#7968).

seven after inoculation for a period of 48 hours before returning to the daily average temperature for the cattle of 38.8 °C (data not shown).

Neutralising antibodies to both LSDV and BEFV were detectable in blood from the relevant cattle. LSDV titres appeared from 14 days post-inoculation and BEFV titres only seven days after the 1st boost (Table 4.2).

In a lymphocyte proliferation assay using purified BEFV as antigen on peripheral blood mononucleocytes (PBMCs) isolated from the vaccinated cattle a stimulation index (SI) of 1.0 or below was taken as negative (Figure 4.1). SI values for the commercial LSDV vaccinated cattle (#7901 and #7968) (negative controls) were below this cut-off value (highest values of 0.9 and 1.0 respectively), whereas for the two cattle vaccinated with the commercial BEFV vaccine (#7956 and #7963) both had highest SI values of 2.1, an indication of low level stimulation. However, for the two cattle inoculated with the LSDV-BEFV recombinant construct (#7983 and #7884) highest SI values of 8.3 and 1.8 were obtained, indicating that for at least one of them (#7983) there was a high level of stimulation.

Ten weeks after the 3rd boost the cattle were prepared for challenge. Bull #7971 (unvaccinated) was injected IV with 5ml with the challenge BEFV as described (in Materials and Methods). Rectal temperature readings were taken at eight-hourly intervals. At 72 hours post-challenge its rectal temperature rose to 40.5 °C. Blood was drawn and mixed 1:1 with OCG and 5 ml was then injected immediately IV into each of the test cattle as described. The cattle were then closely monitored for symptom development. Blood samples and rectal temperatures were taken at regular intervals. The results are summarised in Table 4.2 and Figure 4.2. The rectal temperatures of the unchallenged control animal (cow #7968) remained at a fairly constant temperature throughout the challenge period (~38.8 °C) (Figure 4.2 C), whereas the challenged unvaccinated control (#7973) showed a slight fever on day one post-challenge (40.0 °C) and a high fever on day three (41.5 °C). On day two post-challenge this animal began displaying symptoms of BEF (stiffness in limbs and sensitivity to touch) which had progressed to severe symptoms the following day (inappetence, shivering, nasal discharge and lameness) (Note: all animals displaying symptoms were treated with the anti-inflammatory, phenylbutazone, to relieve suffering). Symptoms fully subsided by day five. The two rLSDV-BEFV vaccinated cattle (#7884 and #7983) (Figure 4.2 A) developed high fevers on day three and four respectively and relatively severe BEF symptoms (inappetence, shivering, nasal discharge and lameness) starting on day four and were fully recovered by day six. Of the two cows vaccinated with the commercial BEF vaccine, one of them (#7956) displayed a high fever on days six and nine, although neither of them exhibited any symptoms (Figure 4.2 B).

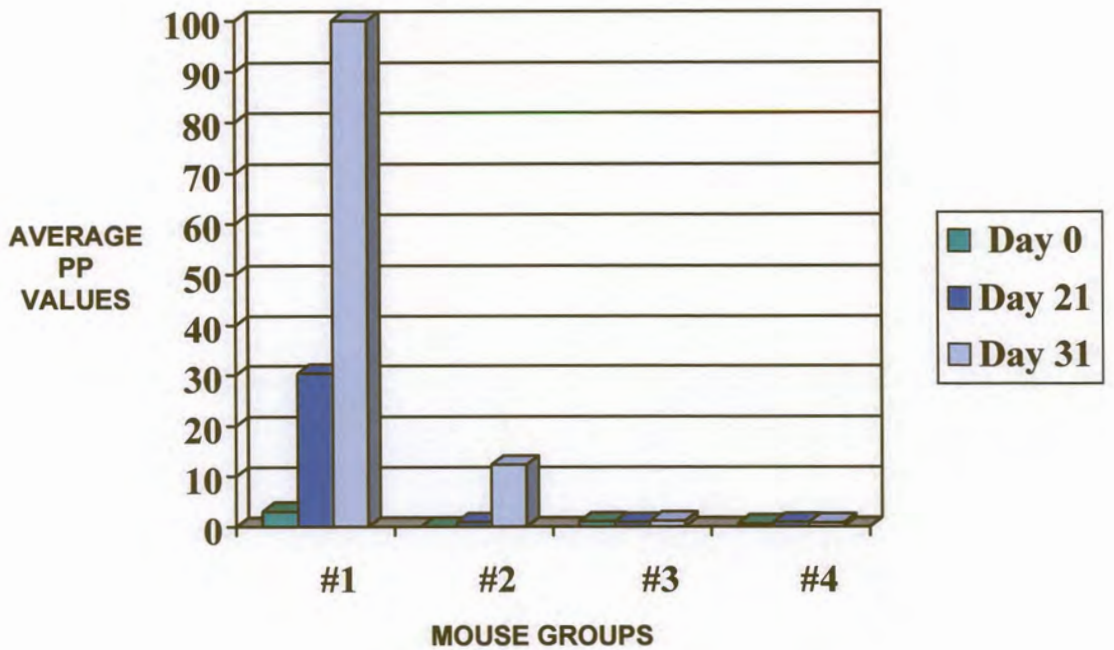


Figure 4.3 I-ELISA assay for RVFV-specific IgG in Balb/c mice. Five Balb/c mice per group were inoculated with either the live attenuated Smithburn vaccine of RVFV (group #1), rLSDV-RVFV (group #2), rLSDV-BEFV (group #3) or saline (group #4). Mice were boosted 21 days post-inoculation and challenged at 31 days post-inoculation. Blood samples were removed from the tail veins of each mouse at 0, 21 and 31 days post-inoculation and the pooled samples (from each group) were subjected to an I-ELISA according to Paweska *et al.* (2003b). Values are presented as a percentage positive (PP) of the ELISA value obtained for the Smithburn vaccine inoculated mice at 31 days post-inoculation.

High titres of BEF neutralising antibodies were detected in the blood of all the challenged cattle at 14 days post-challenge, especially in those primed and boosted beforehand (Table 4.2).

The findings of this study were that although the cattle inoculated with the rLSDV-BEFV construct produced relatively high titres of BEFV-specific neutralising antibodies they were not fully protected against virulent challenge. This was also the case for one of the cattle inoculated with the commercial BEFV vaccine, suggesting that the cattle might have been over-challenged.

4.3.2.2 RVFV (mice)

A laboratory animal evaluation of the protective abilities of the rLSDV-RVFV construct was possible as mice, especially Balb/c mice, are highly susceptible to RVFV. Twenty six-week old Balb/c mice were divided into four equal groups and were inoculated as described (4.2.3.2). Blood samples were taken periodically and HI titres determined. Neutralising HI antibody titres to RVFV were obtained in the blood of the RVFV-vaccinated mice (group #1 and #2; 1:80 and 1:160 respectively), but not in the negative control mice (group #3 and #4) (Table 4.4). Pooled blood samples from each group were also assayed in an I-ELISA test to detect RVFV-specific IgG antibodies (Figure 4.3). Values were presented as a percentage positive (PP) of the ELISA value obtained for the Smithburn vaccine inoculated mice at 31 days post-inoculation (PP = 100). The two negative control groups (#3 and #4) inoculated with rLSDV-BEFV and saline showed no detectable levels of RVFV-specific IgG antibodies (PP values < 1.0), whereas the mice inoculated with the rLSDV-RVFV construct showed moderate levels of antibody at 31 days post-inoculation (PP = 11).

All of the mice were then challenged with 100 MLD_{50} virulent RVFV and monitored for reactions (Table 4.4). The mice inoculated with the Smithburn vaccine (group #1) and those inoculated with the rLSDV-RVFV construct (group #2) displayed no symptoms and were fully protected, but all the negative control mice displayed signs of lethargy and inappetence starting from four days post-challenge, and only 20% of them survived.

This study clearly showed the ability of the rLSDV-RVFV construct to protect mice against virulent challenge with RVFV.

4.3.2.3 RVFV/sheeppox (sheep)

The encouraging results obtained from the mouse challenge study for RVFV with the rLSDV-RVFV construct prompted a challenge study in sheep, the target animals for a new RVFV vaccine. The aim of this study was to test the dual protective ability of the construct – against both RVFV and sheeppox virus challenge. Although sheep are susceptible to RVFV, the older an animal becomes, the more refractory they become to displaying clinical symptoms. Thus

ideally, very young lambs should have been used in this study, although this was not possible due to funding constraints. Eighteen one-year old Merino sheep were donated for the trial. They were tested for existing antibodies to RVFV and sheeppox and were then divided equally into two groups according to the results of the SN testing. None of them were seropositive for sheeppox (or LSD), however a number of them were positive for RVF (data not shown) – these animals were placed in Group B for the sheeppox virus challenge, except for the animal showing the highest titre (#841) – this animal was placed in Group A for RVF challenge in order to monitor the booster effects of the recombinant vaccine and to provide high-titre RVFV-specific serum for reference purposes. The average daily temperatures for the sheep prior to vaccination were 39.2 °C (data not shown). All the animals were vaccinated according to the routes and titres indicated in Table 4.5. None of the animals showed a significant rise in temperature post-vaccination (highest recorded temperature was 39.9 °C at two days post-vaccination in sheep #703, returning to 39.0 °C the following day). Only sheep #721 showed any clinical reaction with the appearance of a round swelling 2.0 cm in diameter at the inoculation site 2 days after vaccination and subsiding 5 days later.

Twenty one days after vaccination the animals were boosted (using the same regimes and routes as indicated in Table 4.5), this time with inoculation in the cleared area on their right flanks. No clinical reactions were noted post-boost, although one-day post-boost sheep #677 and #703 had rectal temperatures of 40.5 °C and 40.0 °C respectively, and, sheep #743 and #841 had rectal temperatures of 40.1 °C and 40.2 °C respectively 5 days post-boost (all temperatures returned to normal one day later).

Nine days after boosting skin biopsies and blood samples were taken from all sheep receiving the recombinant vaccine (a pre-requisite from the Department of Agriculture for the movement of animals having received a genetically modified vaccine). DNA was extracted and the samples were tested via PCR using the LSDV-specific TK primers, P1 and P2 (Chapter 2; 2.2.9). None of the samples showed the presence of recombinant virus (besides the appropriate controls) (data not shown) and thus it was deemed safe to move the animals to the high-containment stabling facilities at Onderstepoort for the challenge phase of the trial.

At ten days post-boost blood was removed in EDTA-coated collection tubes for a lymphocyte proliferation assay (van Kleef *et al.*, 2000). PBMCs were purified, divided into 96-well cell culture dishes (Nunclon, Denmark) and stimulated with either LSDV, or inactivated RVFV. Unfortunately the cells (including the interleukin 2 positive controls) failed to proliferate (reasons unknown), and as the sheep had already been challenged when this result became known, it was impossible to repeat the experiment.

At eleven days post-boost all of the sheep were moved to the high-containment stables (Group A in one stable and Group B in another) and three days later they were challenged with

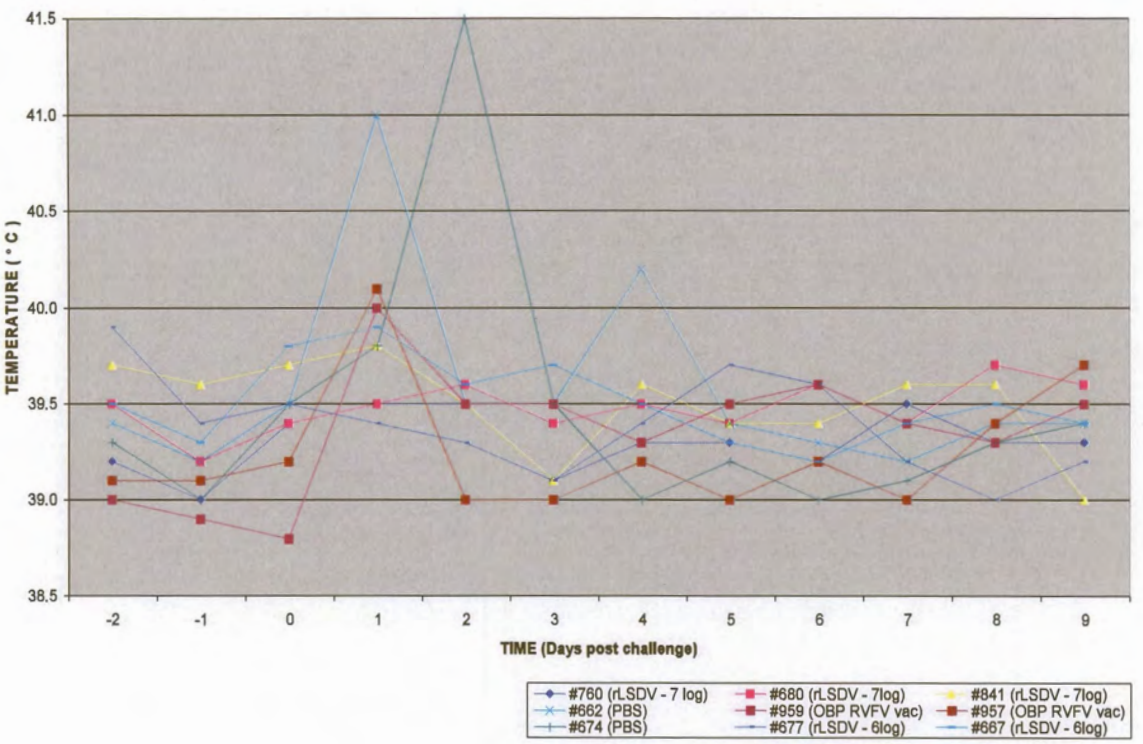


Figure 4.4 Rectal temperatures of sheep challenged with virulent RVFV. Rectal temperatures were taken from two days pre-challenge. The vaccine regime inoculated into each sheep is shown in brackets in the legend. 6log = $1x10^6$ pfu and 7log = $1x10^7$ pfu

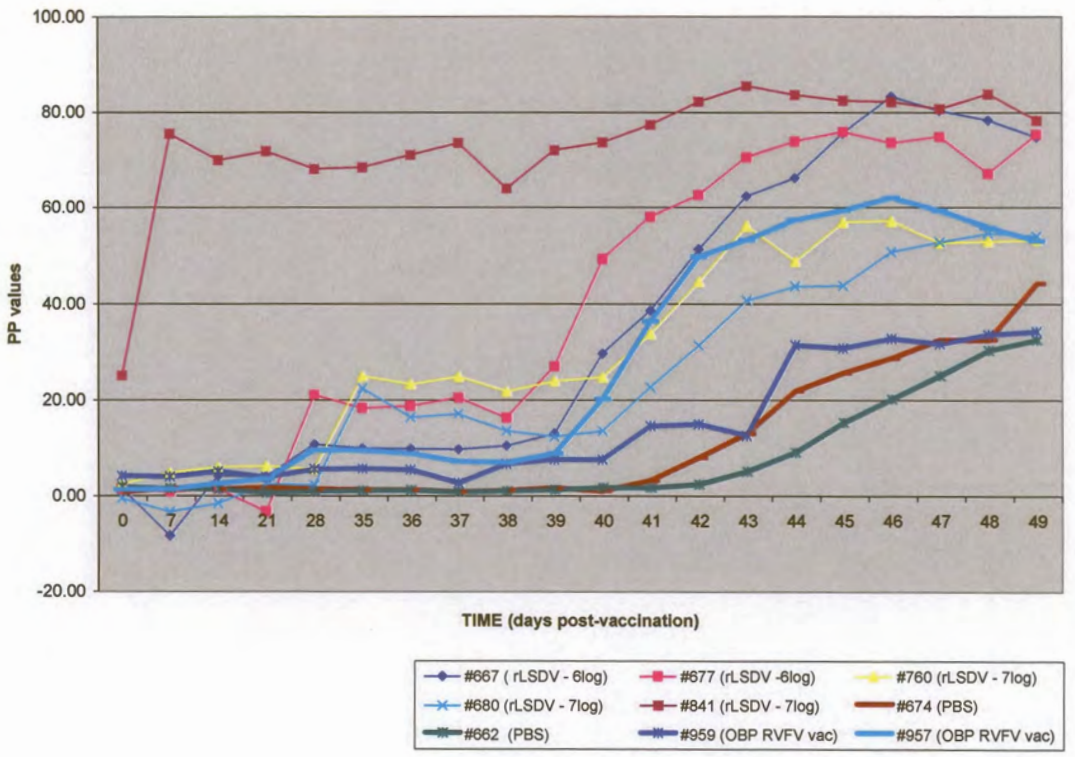


Figure 4.5 I-ELISA results of RVFV-specific antibody responses in sheep vaccinated with different RVF vaccine regimes. The ELISA readings are presented as a percentage of a strongly positive reference serum (PP). 6log = $1x10^6$ pfu and 7log = $1x10^7$ pfu

either virulent RVFV (Group A) or virulent sheeppox virus (Group B) (as described in Materials and Methods; 4.2.3.3). They were monitored closely for symptoms.

None of the Group A animals showed any symptoms typical of RVFV infection, although both of the negative control animals (#662 and #674) had fevers with temperatures above 40.1 °C (41.0 °C 24 hours post-challenge and 41.5 °C 48 hours post-challenge respectively) (Figure 4.4). The highest temperature for a vaccinated animal (#957) was 40.1 °C at 24 hours post-challenge – this animal was vaccinated with the commercial OBP RVFV vaccine.

Antibody levels to RVFV from the sheep sera collected during the trial period were measured using the RVFV-specific IgG I-ELISA (Paweska *et al.*,2003b). These results are summarised in Figure 4.5. Of note is the strong booster effect induced by the recombinant LSDV-RVFV vaccine within 7 days post-vaccination in the sheep with pre-existing antibodies to RVFV (#841). For the rest of the vaccinated sheep significant antibody levels are only observed after boosting (21 days post-vaccination), with a major booster effect from 4 days post-challenge (challenge was on day 35). Antibody levels for the sheep vaccinated with recombinant vaccine (#667, #677, #760 and #660) are consistently higher than those vaccinated with the attenuated Smithburn vaccine (#959 and #957). There are no significant differences in antibody levels between the sheep vaccinated with $1x10^6$ pfu of recombinant virus (#667 and #677) as opposed to those vaccinated with $1x10^7$ pfu (#760 and #680). No antibodies were detectable in the negative control sheep (#674 and #662) until approximately 8 days after challenge.

Fourteen days after challenge the Group A animals were euthanased and a post-mortem performed on the organs for signs of RVFV-induced damage (Table 4.6). One of the rLSDV-RVFV vaccinated sheep (#677) showed mild lesions on its spleen, whereas both animals vaccinated with the commercial RVFV vaccine (#957 and #959) showed lesions on their spleen and liver respectively. Only one of the negative control animals had indications of lesions on its spleen (#674).

Liver samples were also removed for liver enzyme testing by an accredited laboratory – however, due to the exotic nature of RVF in South Africa, permission was refused for these samples to be released from the high containment facilities.

The sheeppox virus challenge of the Group B animals yielded no useful data. None of the animals showed any significant increase in temperatures due to challenge and displayed no symptoms – not even the negative control animals (data not shown). Nor was there any increase in antibody titres in any of the challenged animals (data not shown). The sheep were euthanased 28 days post-challenge and an autopsy yielded no internal lesions typical of sheeppox virus infection.

Table 4.6 Pathologies noted in organs of sheep challenged with RVFV

Sheep no.	Vaccine regime	Pathological changes
#667	rLSDV-RVFV ($1x10^6$ pfu)	None
#677	rLSDV-RVFV ($1x10^6$ pfu)	Mild limited splenic petechiae
#680	rLSDV-RVFV ($1x10^7$ pfu)	None
#760	rLSDV-RVFV ($1x10^7$ pfu)	None
#841	rLSDV-RVFV ($1x10^7$ pfu)	None
#957	OBP RVFV vaccine	Severe splenomegally
#959	OBP RVFV vaccine	Mild limited hepatic petechiae
#662	PBS control	None
#674	PBS control	Moderate splenomegally with limited focal petechiae

PCR was used to confirm the presence of sheeppox virus in the challenge virus stock using the LSDV P1 and P2 primer pair (a high degree of sequence homology exists for the TK genes of sheeppox and LSDV viruses warranting use of this primer pair) (data not shown). Dr Paul Kitching (personal communication), who has vast prior experience with the Yemen isolate of sheeppox virus used in the trial, was consulted and he could find no fault with the experimental procedures followed. It has thus been concluded that the virus lost pathogenicity for the Merino sheep during the two passages in LT cells for preparation of the challenge virus stock.

This sheep trial aimed to evaluate the dual protective ability of the rLSDV-RVFV construct in target animals. Unfortunately, though, due once again to funding constraints, and the need for high containment due to the exotic nature of the diseases to South Africa, the trial parameters were not ideal. However, useful data was still generated and the sheep did produce high levels of neutralising antibody against RVFV and they appeared to be protected against virulent RVFV challenge.

The failure of the virulent strain of Yemen sheeppox virus to induce symptoms in the negative control animals was of major concern and requires further investigation.

4.4 DISCUSSION

The yardstick by which any vaccine is measured is its ability to elicit a protective immune response. Foreign antigens expressed by poxviruses under control of the appropriate poxvirus promoters have been shown to elicit high levels of neutralising antibodies and cell-mediated immune responses which are protective and of long duration (Smith *et al.*, 1983a; Smith *et al.*, 1983b; Kieny *et al.*, 1984; Andrew *et al.*, 1989; Brochier *et al.*, 1991; Brochier and Pastoret, 1993; Hertig *et al.*, 1995; Fries *et al.*, 1996; Paoletti and Plotkin; 1996; Caver *et al.*, 1999; Aspden *et al.*, 2002).
The final stage of this study was to test whether foreign antigens of veterinary importance expressed from viral TK-insertion recombinants of the South African vaccine strain of LSDV would be able to elicit a protective immune response in animals. The two recombinants expressing the structural glycoprotein genes of either BEFV or RVFV (as described in the previous chapter) were utilised for this purpose.

The vaccinia virus P7.5K early/late promoter used to drive expression of the BEFV and RVFV glycoproteins has been shown to stimulate both a humoral and cell-mediated immune response to antigens expressed under its control (Coupar *et al.*, 1986). For most pathogenic viruses immunity appears biased towards one type of immune response (Uren *et al.*, 1994; Vanselow *et al.*, 1995; Kitching, 1996), although there is a growing amount of evidence to suggest that all aspects of the immune response play a role. It is thus a distinct advantage to use a promoter which is able to stimulate both arms of the immune response. For the LSDV constructs evaluated in this study, both were shown to elicit the production of neutralising antibodies to their respective foreign glycoproteins in laboratory animals. In addition, the LSDV-BEFV construct in cattle was shown to stimulate a cell-mediated immune response by way of a lymphocyte proliferation assay. These results indicate that the VV P7.5K promoter is suitable for use in LSDV recombinant constructs.

The inoculation route is also an important factor in determining the extent of induced immune responses (Andrew *et al.*, 1989). In this study two different routes were evaluated for the recombinant constructs in rabbits; the IM and SC routes. As concerns humoral responses, neither route appeared better than the other, and due to ease of administration for future animal work most of the inoculations were performed SC (unless otherwise stated).

In order to test the protective abilities of the LSDV recombinant constructs, a number of challenge studies were performed in laboratory and target animals. The only laboratory animal model available for testing BEFV protection is to use a strain of BEFV neurotropic for mice

(Gaffar Elamin and Spradbrow, 1979; Young and Spradbrow, 1981) – however, such a strain was unavailable for use in this study, and, thus it was necessary to test the rLSDV-BEFV construct directly in cattle. Due to severe funding limitations, only a small-scale pilot trial could be initiated using indigenous breeds of southern African cross-bred cattle. These animals were bred at the Onderstepoort-owned Kaalplaas farm and were kindly donated for the trial.

Within a number of weeks after priming and boosting animals with the rLSDV-BEFV construct, high levels of BEFV-specific neutralising antibodies were produced prior to challenge, comparable with a commercial live attenuated BEF vaccine. There is an apparent relationship between neutralising antibody response and the level of protection for BEF, suggesting that the recombinant vaccine should provide protection to the cattle upon challenge (Uren *et al.*, 1994; Vanselow *et al.*, 1995).

However, upon challenge, the cattle vaccinated with the rLSDV-BEFV construct developed a fever and relatively severe symptoms associated with BEF, although recovery was rapid when compared to an unvaccinated control animal. One of the cattle vaccinated with the commercial live attenuated BEF vaccine also developed fever, even though its levels of neutralising antibodies were three times that of the rLSDV-BEFV vaccinated cattle prior to challenge. These results suggest that the cattle were over-challenged, although the fever reaction in the bovid vaccinated with the commercial vaccine might have been due to sensitisation related to the immune response. Under ideal conditions a range of challenge doses would have been administered, but due to the limited number of cattle available for this trial, this was not possible. It would also have been appropriate to have carefully regulated the challenge dose – however, there is evidence to suggest that BEFV is highly unstable, and, in order to ensure clinical responses upon challenge, it has become common practice at Onderstepoort to first boost challenge virus titres in an untreated animal and at the height of viremia (measured by the onset of fever) to remove blood, mix it with OCG, and then to use this mixture immediately to challenge the test animals – without having first determined the challenge virus titre (as reviewed by St George, 2004; Aitchison, personal communication). Although generally effective, this practice provides no control over the titre of challenge virus administered to each animal. It is also not possible to prepare the challenge virus stock in mice as it has been shown that virus prepared in this manner rapidly loses pathogenicity for cattle (Van der Westhuizen, 1967). In addition, the use of indigenous breeds of cattle for this trial was not ideal as they tend to be more resistant to challenge with BEFV than exotic breeds (Verwoerd, personal communication).

The potential use of the LSDV recombinant constructs as dual vaccines is evident in the high LSDV neutralising antibody titres obtained in the cattle. It is even foreseeable that they will be useful in protecting goats and sheep against goat and sheeppox virus infection as the three viruses (goatpox, sheeppox, and LSDV) share a common surface antigen (Kitching *et al.*, 1986)

and there is evidence that LSDV replicates in both sheep and goats (Weiss, 1968). To this end a number of sheep were vaccinated with the rLSDV-RVFV construct and challenged with the virulent Yemen isolate of sheeppox virus. The animals did show sero-conversion to LSDV (data not shown). However, none of the sheep reacted to the challenge, even though correct experimental procedures were followed. Attempts are currently being made to repeat this work using the virulent Nigeria strain of sheeppox virus due to commercial interest in the rLSDV-RVFV construct.

In mice, the rLSDV-RVFV construct was able to afford complete protection against a potentially lethal challenge of virulent RVFV. The construct also protected sheep against virulent RVFV challenge although some lesions were noted in spleens or livers in a number of the vaccinated animals.

This study has also shown the potential application of the constructs as recombinant vaccines in non-permissive hosts. Neither rabbits nor mice showed any adverse reactions to inoculation with high titres of the recombinant viruses (personal observation), and yet neutralising antibodies were produced to the foreign glycoproteins. The expression of foreign antigens under control of a late poxvirus promoter in LSDV has been observed in cells of monkey origin (personal observation). It is thus surmiseable that this LSDV-vector system has potential for use in humans.

The large animal trials conducted in this study were not performed under ideal conditions. In most instances too few animals were used to produce statistically relevant results and the correct breeds were not available. The main reason for this was a cost factor - limited funding and the cost of housing and maintaining large animals in high-level containment facilities severely restricted the number of animals and choice of breeds that could be utilised. However, these and other studies (Aspden *et al.*, 2002) have shown the tremendous potential of the southern African vaccine strain of LSDV for use as a host-restricted vaccine vector. It is therefore hoped that in the future the appropriate funding will be made available to overcome these problems. Interest already exists for further development of both recombinant vaccine constructs into commercially viable vaccines. Due to environmental concerns over the use of genetically modified organisms in the field, it is proposed that the gpt antibiotic resistance marker gene should be removed and work in this regard is already in progress.

CHAPTER 5

CONCLUDING REMARKS

In just under 25 years since the first use of poxviruses as molecular tools to monitor gene expression in mammalian cells, their repertoire of applications has expanded to include almost every facet of molecular-based disease control, including cancer and gene therapy (as reviewed by Mountain, 2000). Perhaps the most important of these to date has been their development as vectors for recombinant vaccines. The release of a VV recombinant expressing the rabies glycoprotein gene to successfully control rabies in foxes in central Europe (Brochier *et al.*, 1991; Brochier and Pastoret, 1993) showed the tremendous potential of VV as a recombinant vaccine vector. However, as concerns arose over its use in immunocompromised persons alternative poxviruses were sought which were more host-range restricted (Baxby and Paoletti, 1992). A number of avipoxviruses were investigated, including fowlpox virus (Taylor and Paoletti, 1988) and canarypox virus (Taylor *et al.*, 1992) for use in the veterinary and medical field. Capripoxviruses have also been investigated for use in the veterinary field, especially as dual vaccines in regions where they cause diseases of economic importance (Romero *et al.*, 1993).

The highly attenuated South African vaccine strain of the capripoxvirus, LSDV, is currently being investigated as a recombinant vaccine vector as southern African strains of the virus appear more host-range restricted than northern African isolates (Weiss, 1968; Gershon and Black, 1988). A number of potential insertion regions have been identified and these are being evaluated (Wallace and Viljoen, 2002; Aspden *et al.*, 2003).

The overall aim of this study was to evaluate the vaccine-vector potential of TK-disrupted recombinants of the South African vaccine strain of LSDV. The choice of an insertion site is an important one. Historically the viral TK gene was chosen as in some poxviruses, such as VV, this site was shown to be non-essential, although TK-positive VV grows to higher titres *in vivo* and stimulates higher humoral and cell-mediated immune responses than TK-negative recombinant virus (Andrew *et al.*, 1989). Another important consideration when choosing an insertion site is its level of conservation which potentially impacts upon the stability of resulting recombinants. Genes found within the central regions of poxvirus genomes generally code for "housekeeping" proteins, and these tend to be highly conserved. Thus genes found in the central regions to be non-essential tend to make better insertion sites (Scheiflinger *et al.*, 1996; Scheiflinger *et al.*, 1997).

Selection for the TK-disrupted recombinants was to be on the basis of the resulting TK-negative phenotype. For this to be possible a suitable transfer vector was required. The pLSTK7.5 transfer vector was constructed containing the viral TK gene interrupted by a MCS downstream of the VV p7.5K early/late promoter. In addition, TK-negative selection for LSDV recombinants required a

TK-negative cell line which is permissible for growth of the virus. Once such a cell line was acquired (BU100 cells) it was possible to proceed with the generation and selection of the recombinants. The calcium phosphate co-precipitation method (Drillien and Spehener, 1983) for generating recombinant poxviruses was adapted for LSDV. In parallel studies in our laboratory this method was successfully used to generate VV recombinants expressing various structural genes of African horsesickness virus.

However, this approach failed to yield selectable LSDV recombinants and it was discovered that some form of TK activity is required for growth of the virus (Wallace and Viljoen, 2002), making it impossible to use the TK-negative phenotype as a means for selection. The selection strategy was then modified to include the *E. coli* gpt dominant selectable marker gene according to the method of Romero *et al.* (1993). Using some modifications to this strategy it was possible to select LSDV recombinants expressing the lacZ reporter gene. The recombinant virus was purified to homogeneity and was subsequently shown to be stable.

Selecting a homogeneous stock of recombinant virus was however not easy. LSDV, like most poxviruses is predominantly cell-associated (Weiss, 1968). Electron micrographic studies of cell-associated LSDV particles shows a membranous material surrounding them (personal observation). In the early stages of selection a mixed population of wt and recombinant viruses exists and as the gpt gene is an enzyme its effect is wide-ranging. It is thus possible that wt viral particles in close proximity to recombinant viruses are able to survive selection pressure. Modifications to the selection procedure were thus introduced to ensure maximal separation of wt from recombinant viruses. These included sonication, filtration, and end-point titrations. Using this modified approach it was possible to select all recombinants generated to homogeneity.

The final stage of the study was to generate recombinants expressing genes from viruses of veterinary importance and to evaluate their protective abilities in animal trials. However, before commencing further, the pLSTK7.5 transfer vector was first streamlined by removal of extraneous DNA sequences and a number of unique R.E. sites were restored to the MCS. The EGFP visual marker gene was then inserted into the new transfer vector to further assist with the *in vivo* selection of homogeneous recombinants. Two separate recombinants were then generated and selected to homogeneity, one expressing the structural glycoprotein gene of BEFV and the other the glycoprotein genes of RVFV. Both recombinants were able to elicit the production of neutralising antibodies in rabbits and the RVFV construct conferred complete protection to RVFV-challenged mice. Due to the unavailability of a neurotropic mouse strain for BEF challenge, it was necessary to conduct a challenge study directly in cattle. Financial constraints severely limited the number of animals available for the trial and it was thus impossible to use a range of challenge doses, nor to determine the minimal vaccine dose able to confer protection against known challenge titres. As only cross-breeds of indigenous cattle were made available for the trial, and

indigenous breeds tend to be more resistant to BEFV than exotic breeds (MacFarlane and Haig, 1955), it was necessary to challenge the cattle with the maximal dose of challenge virus possible, after first boosting virus titres in a non-vaccinated animal. Cattle inoculated with the LSDV-BEFV construct did produce high levels of neutralising antibodies and a cell-mediated immune response was induced, although they were not fully protected against virulent challenge. Future trials with the appropriate number and species of animals will hopefully overcome these problems.

The recombinant vaccines developed in this study have dual vaccine potential. The LSDV-BEFV construct has the potential to protect cattle against BEF and LSD, and the LSDV-RVFV construct has the potential to protect cattle against RVF and LSD. This construct also has the potential to be used in sheep and goats to protect them against RVF and sheep and goat pox due to the sharing of a major surface antigen between all three capripoxviruses (Kitching *et al.*, 1986). A pilot study was conducted in sheep to test the dual protective potential of the construct against RVFV and sheeppox virus. The sheep were almost completely protected against the RVFV challenge, although not even the negative control animals reacted to the sheeppox virus challenge. It was surmised that the sheeppox virus challenge stock had lost immunogenicity and pathogenicity during the cell culture passage steps to prepare a high virus titre for challenge. However, as there is commercial interest in the LSDV-RVFV construct this work will need to be repeated at a later stage using a different virulent isolate of sheeppox virus, including the use of pregnant ewes and young lambs to test the safety of the recombinant vaccine.

The results of this study have shown that the TK gene is a suitable insertion site for development of the SA vaccine strain of LSDV as a vector for recombinant vaccines of veterinary importance.

Although work has already been described on the potential use of the viral RR gene as an insertion site (Aspden *et al.*, 2002; Aspden *et al.*, 2003) the authors made it clear that their recombinant still contained contaminating wild type virus. This would make it unsuitable for commercialisation due to the need to propagate the virus in the presence of expensive selection medium. In addition, the presence of contaminating wild type virus made it impossible for them to evaluate the stability of their recombinant which is also of paramount importance for commercialisation.

On the other hand the TK-insertion recombinants described in this study were shown to be pure and stable, thus demonstrating their suitability for commercialisation. Both TK-insertion recombinants have now been grown in excess of 20 passages in cell culture in the absence of selection pressure and have been shown via PCR to be stable at the genomic level and via IF to continue to express their respective foreign glycoproteins (data not shown).

The only way to accurately assess which of the two insertion sites, TK or RR, is the most suitable for use in the future would be to insert identical gene cassettes into both sites, select the resulting recombinants to homogeneity and to evaluate them side-by-side in an animal protection study.

As the SA vaccine strain of LSDV is highly attenuated, and the genome contains many mutations compared to field isolates (Kara *et al.*, 2003), it is possible that the choice of alternative insertion sites, such as an intergenic site, or restoration of viral TK activity by insertion of a heterologous TK gene (Scheiflinger *et al.*, 1996), might prove more suitable.

Work is currently in progress to generate deletion mutants from the virulent Warmbaths field isolate (Kara *et al.*, 2003). Virulence and host-immunomodulatory genes are being targeted for deletion with the aim to develop an improved vaccine able to stimulate a better humoral immune response providing a longer duration of protection (as reviewed by Johnston and McFadden, 2004a; as reviewed by Johnston and McFadden, 2004b). Approximately 10% of cattle immunised with the current SA LSDV vaccine fail to mount a humoral response although they are protected from virulent challenge (Weiss, 1968; Hunter and Wallace, 2001). Those cows that fail to produce neutralising antibodies are unable to confer protection to their calves through colostral antibody secretion and this possibly explains reports of "vaccine failure" in young calves (less than six months of age) of vaccinated dams. An improved vaccine able to stimulate a humoral response in all vaccinated animals will hopefully overcome this problem.

Due to environmental concerns over the use of antibiotic resistance markers in genetically modified organisms (such as poxvirus-vectored recombinant vaccines) the selection process for generating the LSDV deletion mutants will make use of transient dominant selection (Falkner and Moss, 1990) which will result in the loss of the marker gene in the final stages of selection. This will also enable the generation of multiple deletion mutants from different regions of the genome as the same marker gene will be reusable for the deletion of each gene or genomic region.

As for the level of immunity induced in the animal work in this study, the P7.5K promoter used to drive expression of the GP genes is a moderate strength promoter derived from VV (Cochran *et al.*, 1985). The fowlpox virus promoter used in the RR insertion construct appears to be a stronger promoter as high levels of rabies-specific antibodies were produced in mice, even when inoculated with relatively low levels of recombinant virus (Aspden *et al.*, 2003). It is also possible that insertional inactivation of the RR gene had a beneficial effect on the ability of the mice to elicit an immune response to the expressed rabies glycoprotein.

A bi-directional promoter from LSDV has been identified and characterised (Fick and Viljoen, 1999) and it is possible that this promoter will prove more suitable for use in future constructs. The use of an in-house promoter will also solve the problem of patent rights as many of the poxvirus promoters are protected by international patents.

In conclusion, a number of studies, including this one, have demonstrated the recombinant vaccine vector potential of the highly host-range restricted SA vaccine strain of LSDV. In this study, using the viral TK gene for insertion of foreign genes (both reporter genes and genes of

veterinary importance) it was possible to generate and select recombinants which are homogeneous and stable – important factors when considering commercialisation and environmental concerns. The two recombinants evaluated in animal trials were able to elicit the production of neutralising antibodies and at least one of them was able to confer complete protection in a challenge study (rLSDV-RVFV in mice). However, more extensive animal trials are still required to evaluate the recombinant vaccines properly.

As sequence data has become available on complete genomes of LSDV isolates and environmental concerns have arisen over the release of organisms expressing antibiotic resistance markers, a new generation of LSDV-vectored vaccines are being developed. The knowledge gained from this study will prove valuable in the generation and selection of homogeneous recombinants and it is still possible that the viral TK gene might be used as an insertion site. Up to 5 kbp of foreign DNA was inserted into the TK gene in this study with no noticeable deleterious effect on viral growth, although in a mixed population recombinant viruses were shown to grow slower than wt virus. With the generation of deletion mutants it is probable that it will be possible to insert even larger amounts of foreign DNA. One recombinant construct might be able to confer protection to a range of microbial pathogens. The LSDV vaccine vector system developed and evaluated in this study has applications in the veterinary field, but, like avipoxviruses, it is not unreasonable to envisage future constructs targeted against human pathogens.

Parts of the results presented in this thesis have been published:

Reviewed Journals:

Wallace, D.B. and Viljoen, G.J. (2002). Importance of thymidine kinase activity for normal growth of lumpy skin disease virus (SA-Neethling). *Archives of Virology*, **147**, 659-663.

Wallace, D.B. and Viljoen, G.J. (2005). Immune responses to recombinants of the South African vaccine strain of lumpy skin disease virus generated by using thymidine kinase gene insertion. *Vaccine*, **23**, 3061-3067.

Parts of the results presented in this thesis have been presented at scientific meetings:

Thirteenth Congress of the South African Biochemical Society. Bloemfontein, UOFS. 2-5 April, 1995.
Title: Construction and generation of recombinant lumpy skin disease viruses expressing African horsesickness virus VP2.
Fick, W.C., Wallace, D.B. and Viljoen, G.J.

Thirteenth Congress of the South African Biochemical Society. Bloemfontein, UOFS. 2-5 April, 1995.
Title: Generation of recombinant vaccinia viruses expressing African horsesickness virus genes.
Wallace, D.B., Fick, W.C. and Viljoen, G.J.

Twelfth International Poxvirus and Iridovirus Symposium, St Thomas Island, Bahamas, 9-12 May. 1998.
Title: Generation and characterisation of a stable lumpy skin disease virus (Neethling) recombinant expressing B-galactosidase.
Wallace, D.B. and Viljoen, G.J.

Thirteenth International Poxvirus and Iridovirus Symposium, Montpellier, France, 2-6 September. 2000.
Title: Generation and characterisation of a homogeneous lumpy skin disease virus recombinant expressing the structural glycoprotein of bovine ephemeral fever virus.
Wallace, D.B. and Viljoen, G.J. (2000).

Fourteenth International Poxvirus and Iridovirus Symposium, Lake Placid, USA, 20-24 September. 2002.

Title: Lumpy skin disease virus (SA-Neethling) recombinants elicit neutralising antibody production and protective immune responses in small-animal trials.

Wallace, D.B. and Viljoen, G.J.

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