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

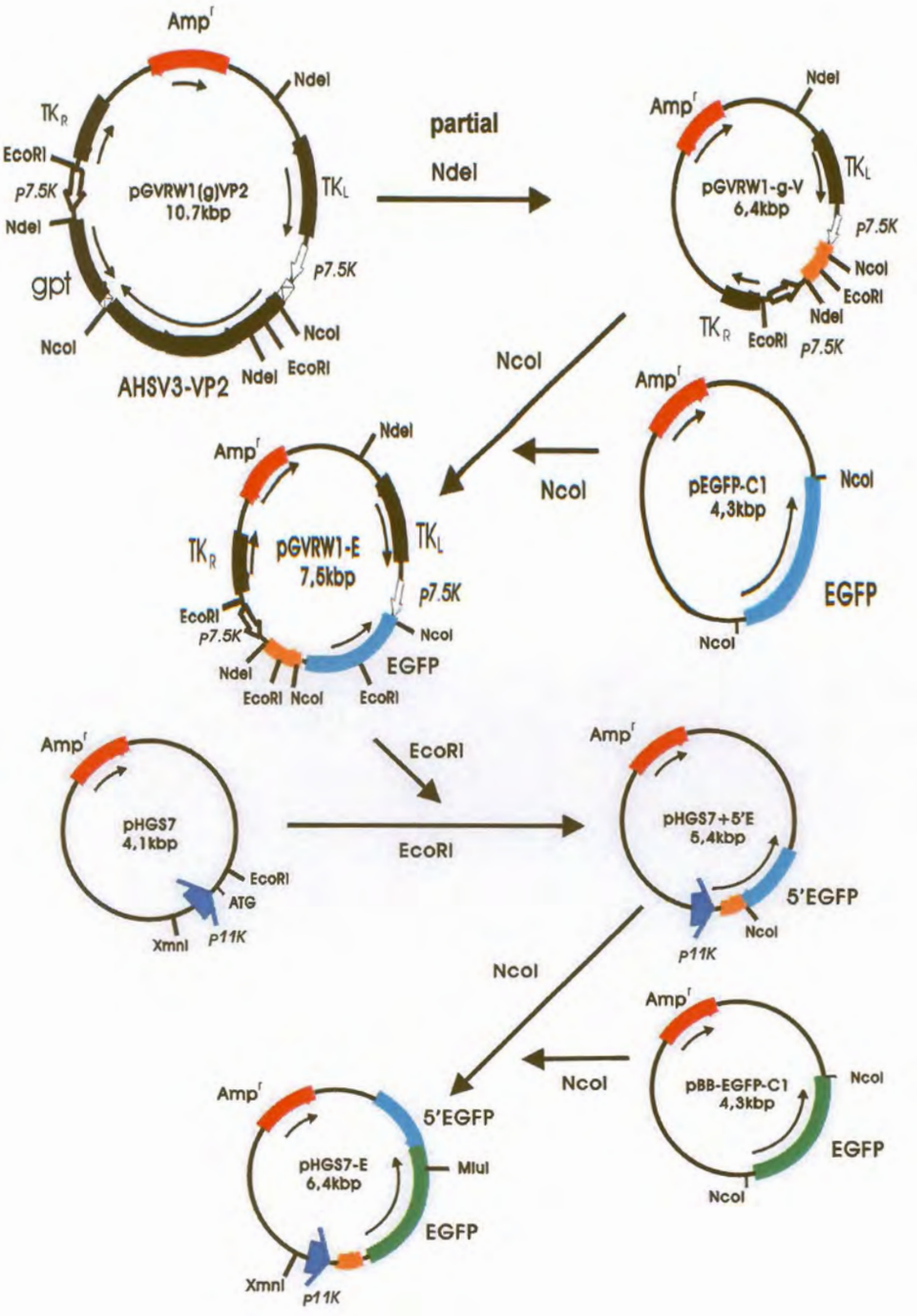


Figure 3.1 Insertion of EGFP under control of VV P11K late promoter. The EGFP gene was first inserted upstream of the AHSV3-VP2 gene to create an in-frame sequence with the ATG start codon located downstream of the P11K promoter in pHGS7. An *Eco*RI-*Eco*RI fragment containing this sequence and the 5' 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.

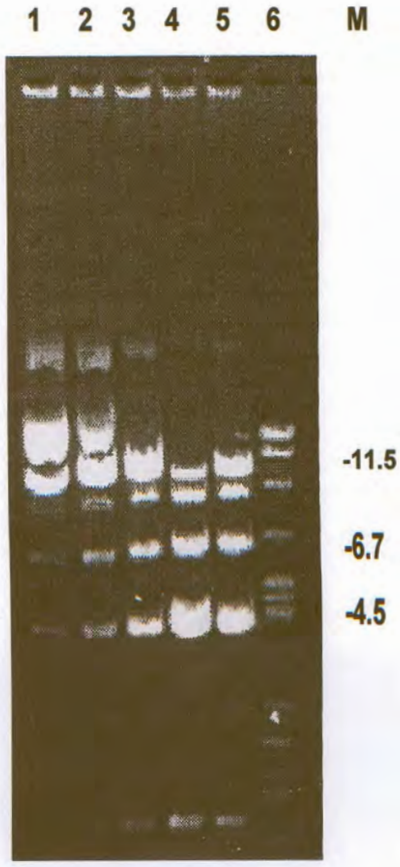
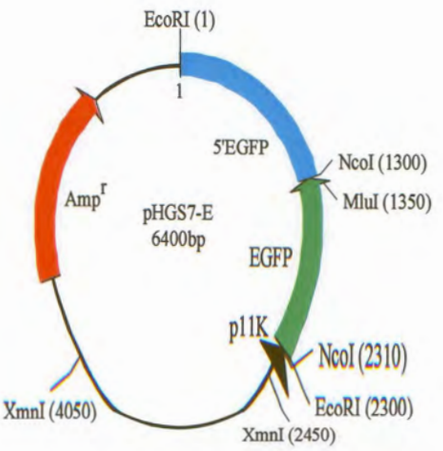


Figure 3.2 Agarose gel electrophoresis analysis of partial *Nde*I restriction enzyme digestions of pGVRW1(g)VP2. lane 1 = 1 U, lane 2 = 2 U, lane 3 = 4 U, lane 4 = 6 U, lane 5 = 7 U. Lane 6 = combined lambda *Pst*I and *Hind*III DNA marker. M = DNA fragment sizes in kbp.

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

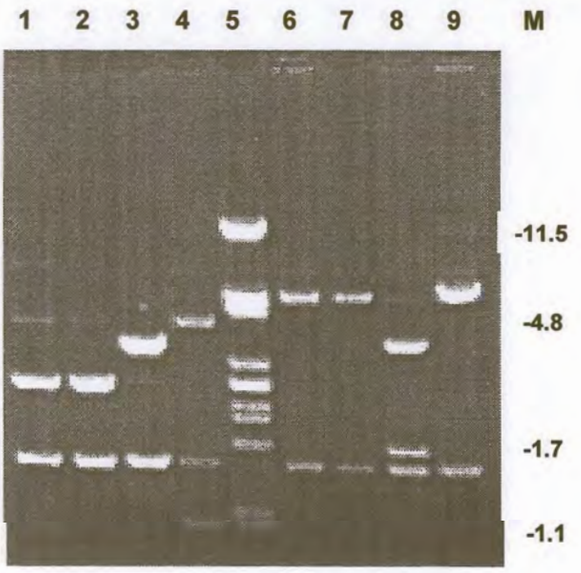


Figure 3.3 R.E. plasmid map of pHGS7-E (**A**) and agarose gel electrophoresis analysis of R.E. digests of pHGS7, including EGFP inserts (**B**). lane 1 – pHGS7 cut with *Xmn*I, lane 2 – pHGS7 cut with *Xmn*I and *Mlu*I, lane 3 – pHGS7+5'E cut with *Xmn*I and *Mlu*I, lane 4 - miniprep #12 of pHGS7-E 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.

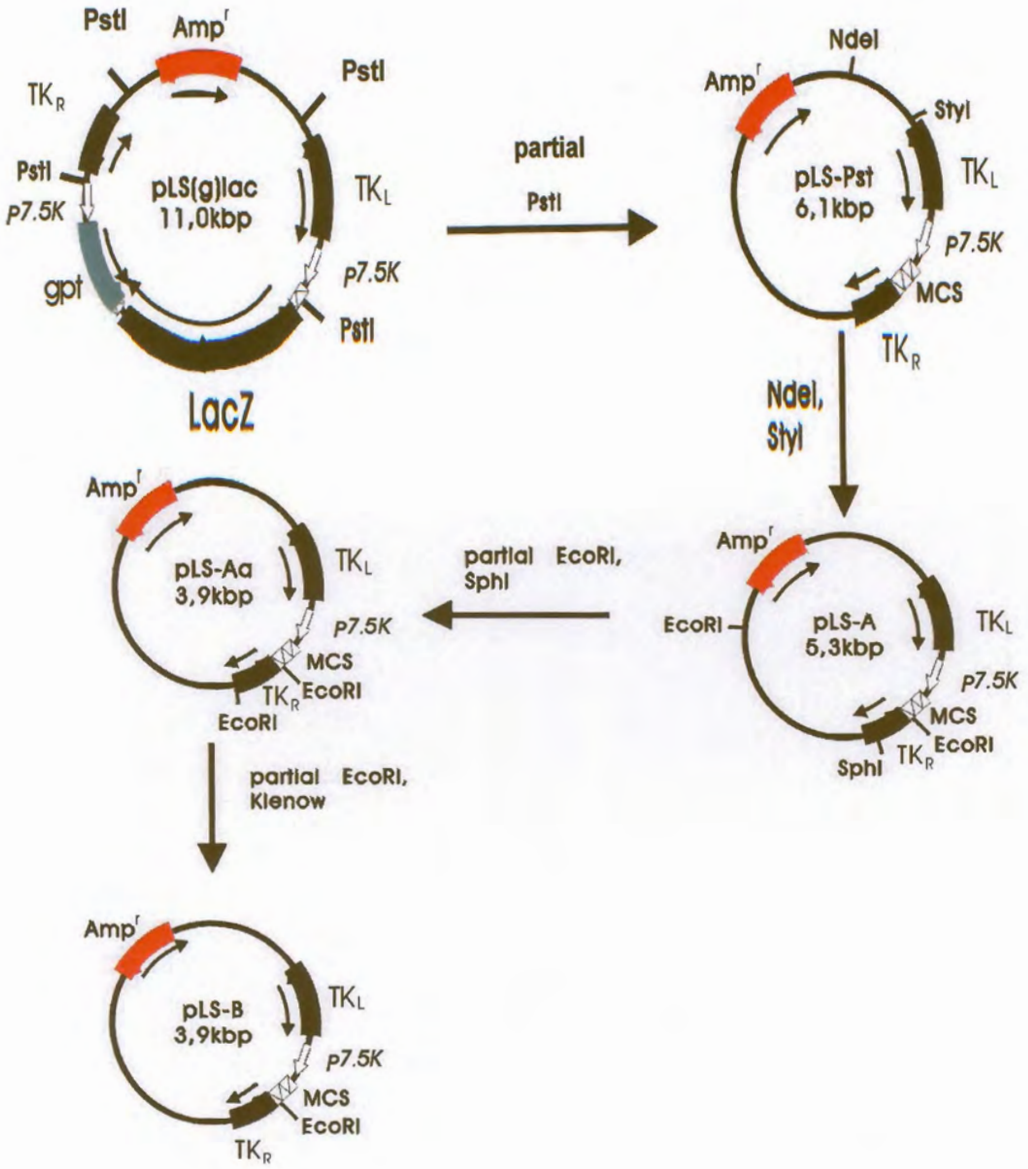
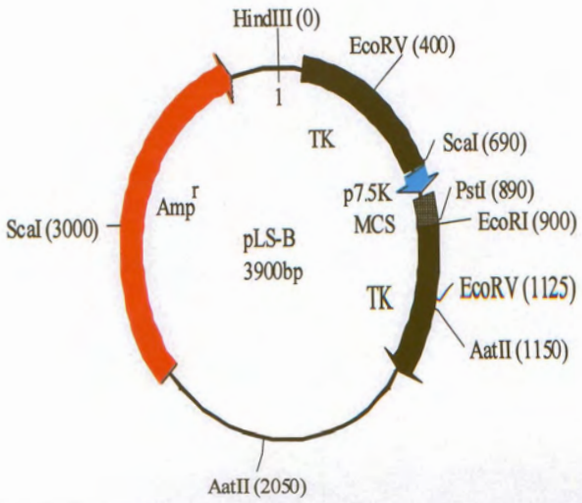


Figure 3.4 Construction of the improved LSDV transfer vector, pLSEG – phase 1. pLS(g)lac was used as the basal plasmid and extraneous sequences were removed using partial *Pst*I, then *Nde*I and *Sty*I and finally *Eco*RI (partial) and *Sph*I to produce pLS-Aa. Then the *Eco*RI site at the 3' end of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

A



B

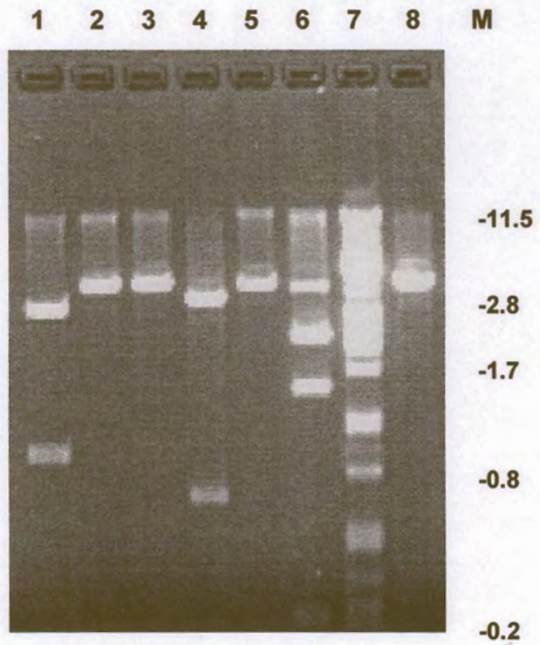


Figure 3.5 R.E. plasmid map (**A**) and agarose gel electrophoresis analysis of R.E. digests of pLS-B (**B**). Miniprep #2 cut with *Aat*II (lane 1), *Eco*RI (lane 2), *Pst*I (lane 3), *Eco*RV (lane 4), *Hind*III (lane 5), *Eco*RI/*Sca*I (lane 6), and *Eco*RI/*Pst*I (lane 8). Lane 7 contains a lambda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

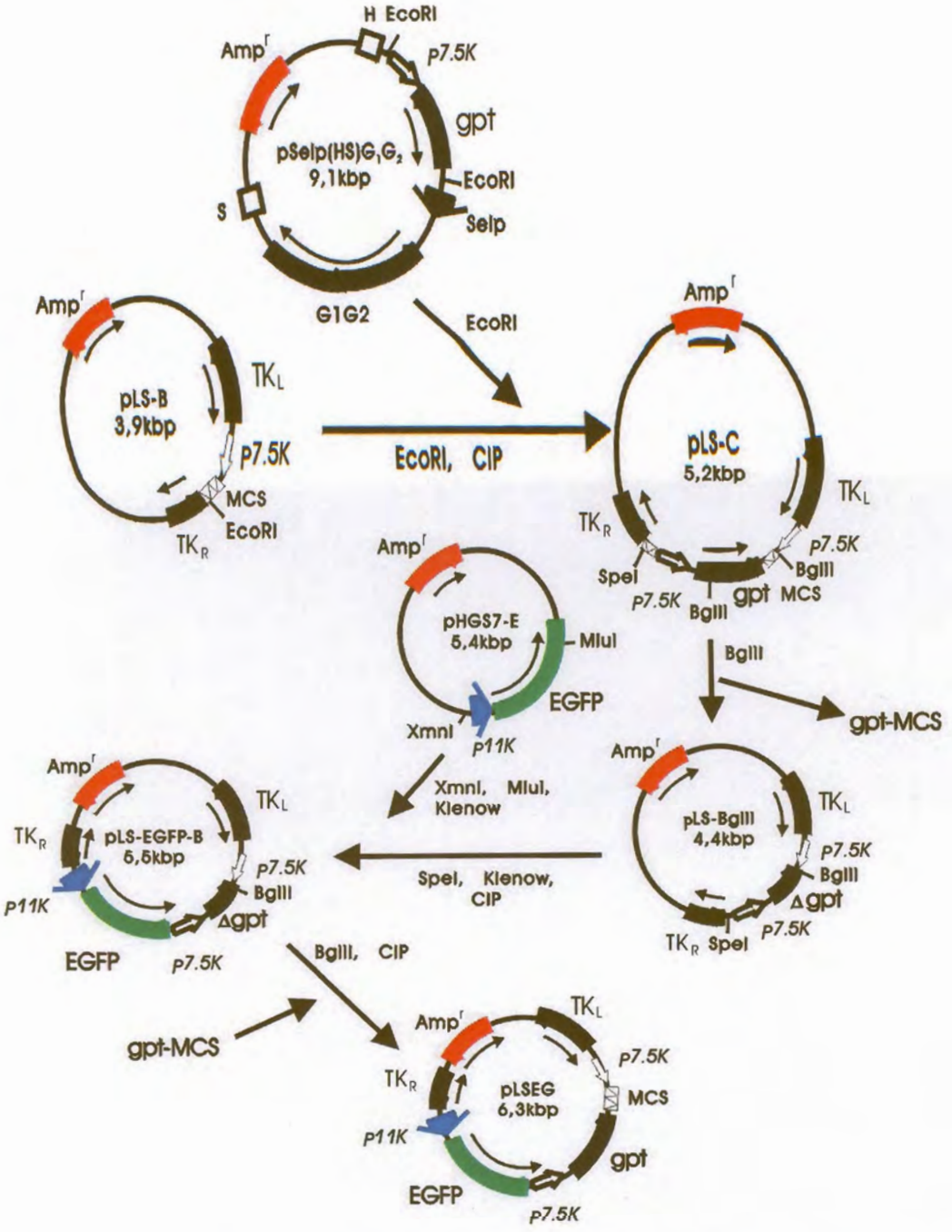


Figure 3.6 Construction of improved LSDV transfer vector, pLSEG – phase 2. For the reconstruction phase the gpt gene (from pSelp(HS)G_1G_2) was first inserted using *Eco*RI (pLS-C). Part of the gpt gene and the MCS (containing an SpeI site) were then removed using *Bgl*II so that the remaining *Spe*I 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).

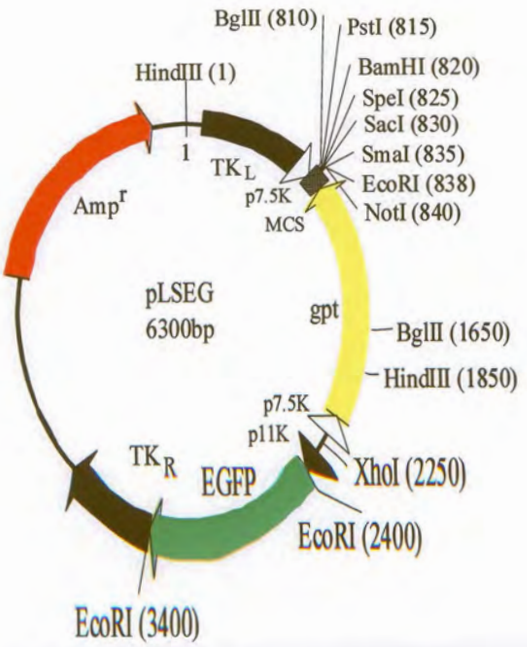


Figure 3.7 R.E. plasmid map of pLSEG. Except for the *Bgl*II and *Eco*RI sites in the MCS, the rest of the sites are unique.

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.

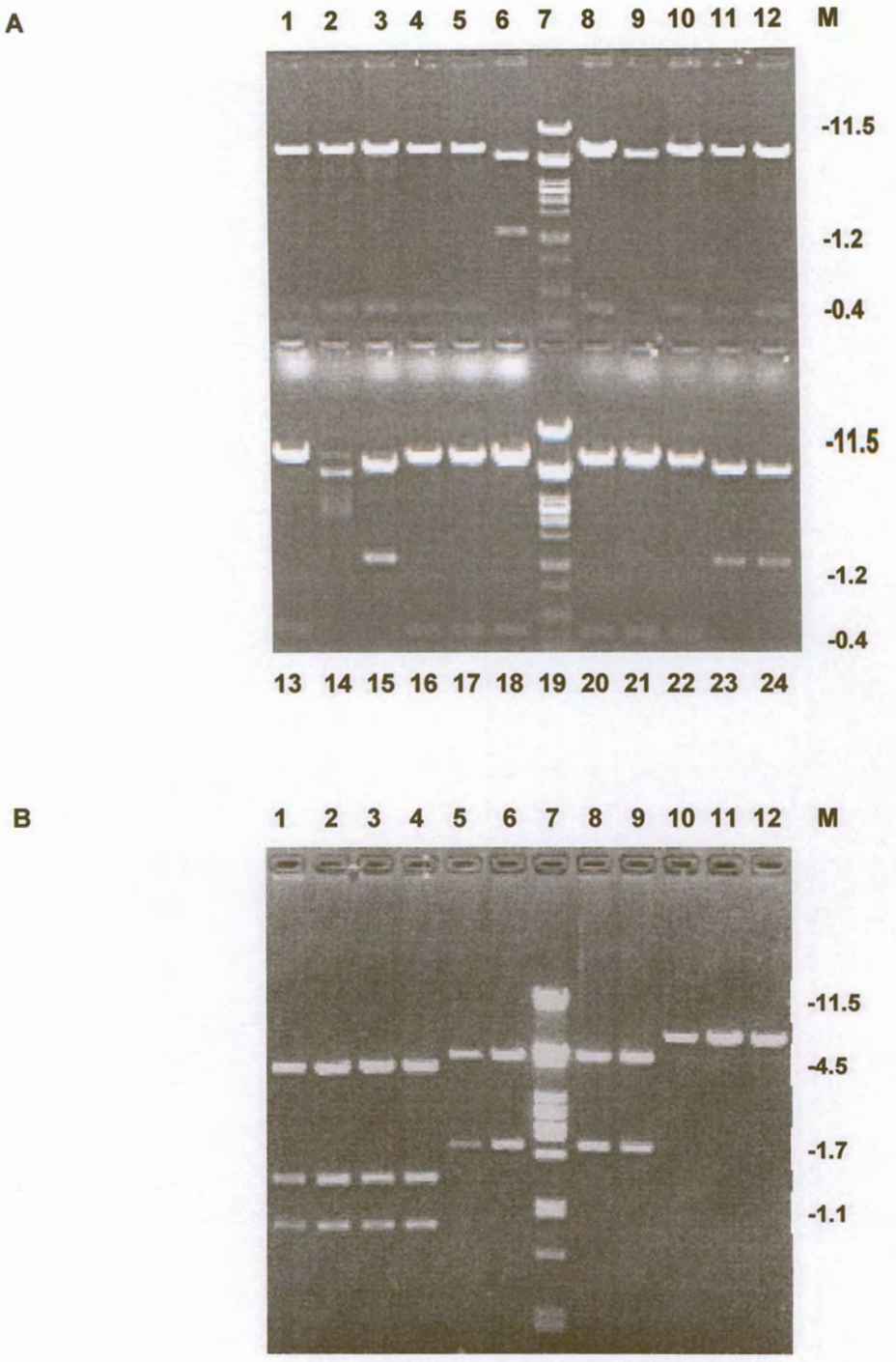


Figure 3.8 Agarose gel electrophoresis analysis of R.E. digestions of minipreps of pLSEG. **A** - Preps cut with *Xho*I/*Pst*I (lanes 1-6, 8-18, and 20-24). Lanes 7 and 19 – lambda *Pst*I DNA marker. **B** - preps #6, 14, 21 and 22 cut with *Eco*RI (lanes 1-4), *Hind*III (lanes 5, 6, 8 and 9) and *Bam*HI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

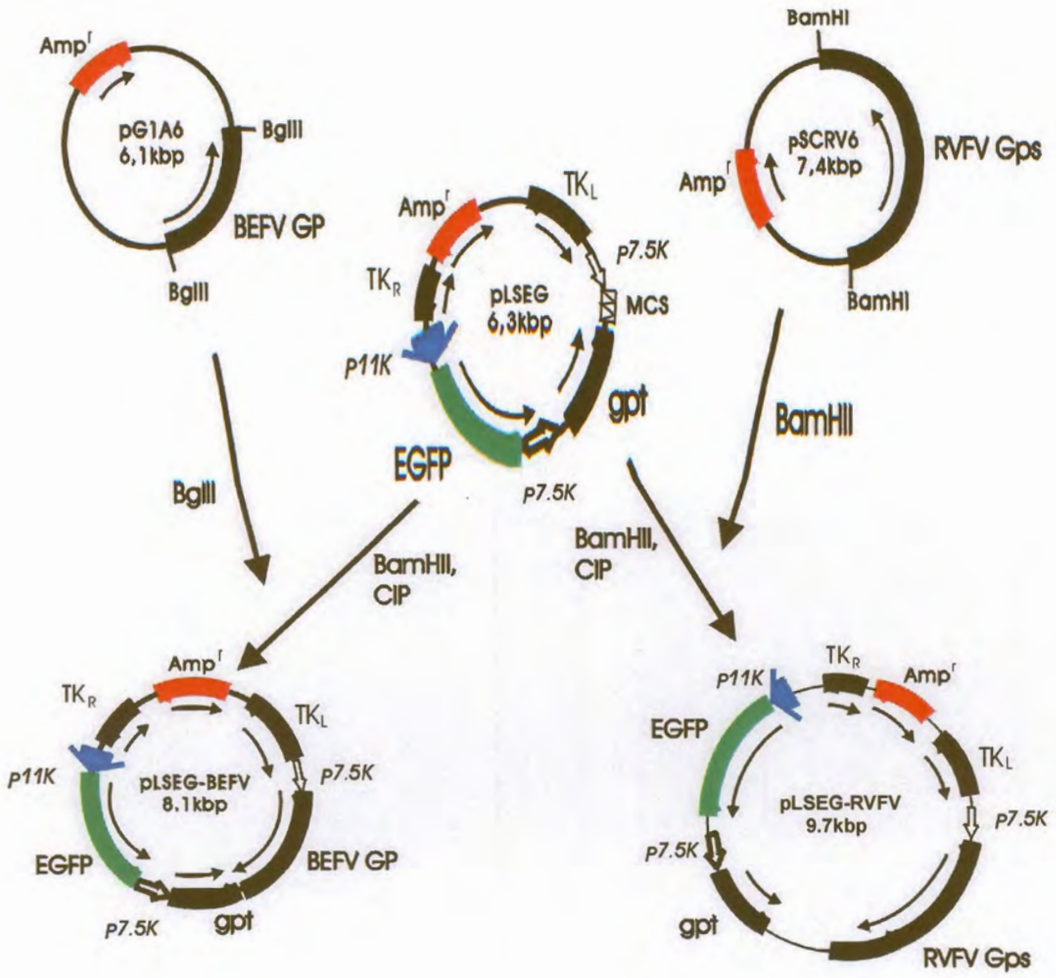


Figure 3.9 Insertion of BEFV and RVFV GPs into pLSEG. The structural GP gene of BEFV was excised from pG1A6 using *Bgl*II and was inserted into the unique *Bam*HI site in pLSEG (pLSEG-BEFV) and the G1G2 GPs of RVFV were excised from pSCRV6 using *Bam*HI and were also inserted into the *Bam*HI site in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

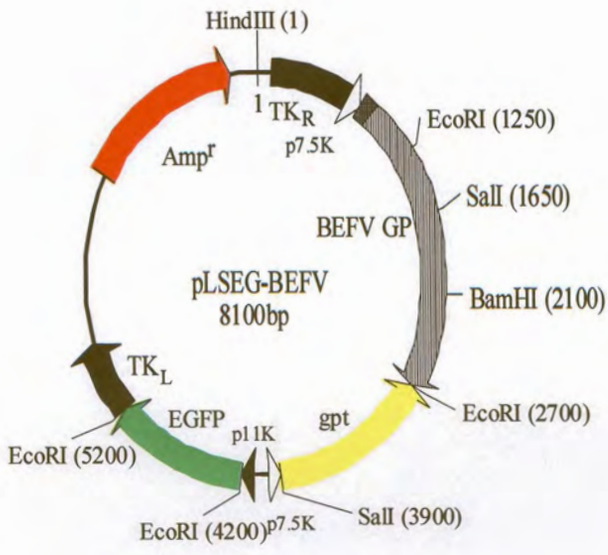


Figure 3.10 R.E. plasmid map of pLSEG-BEFV.

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.

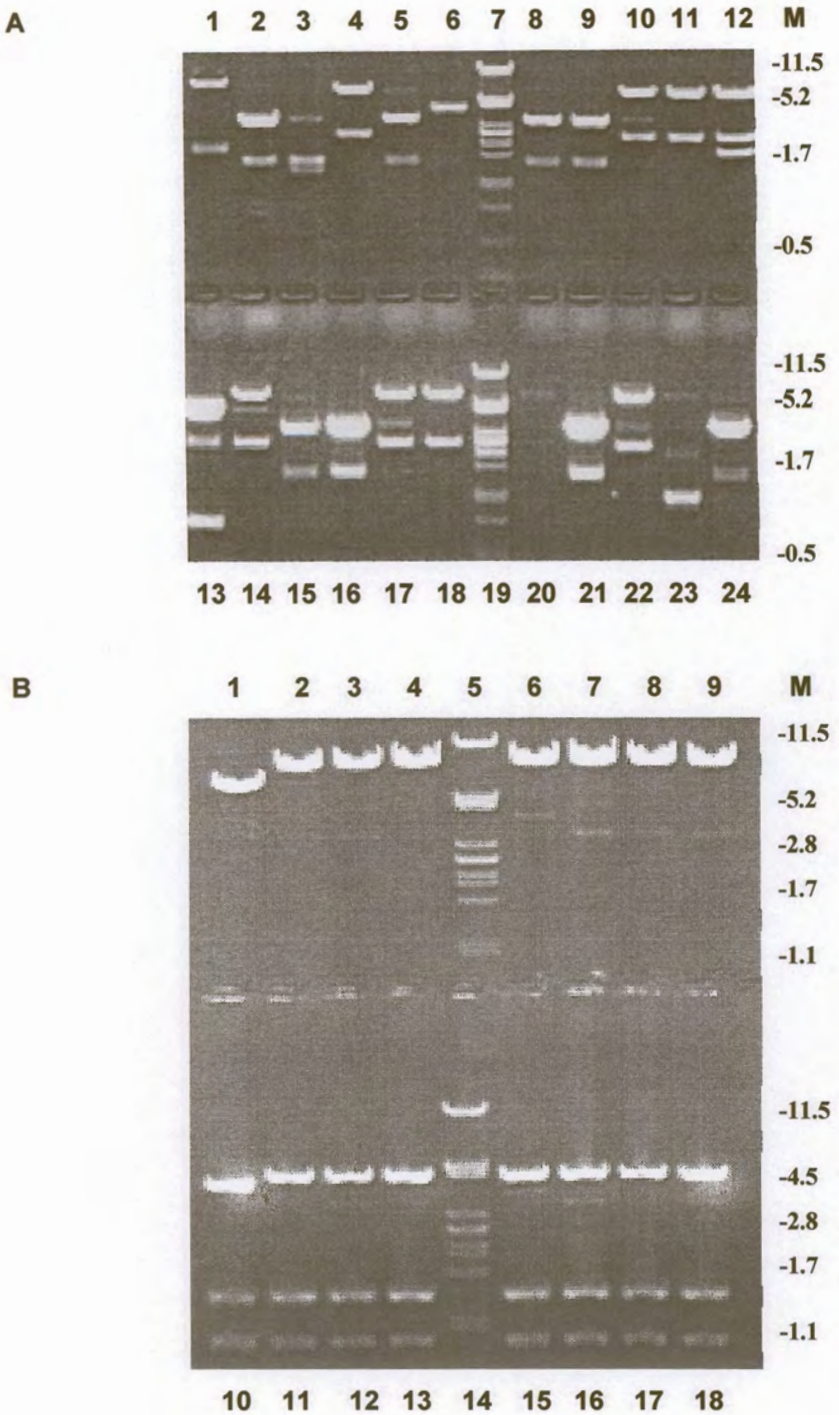
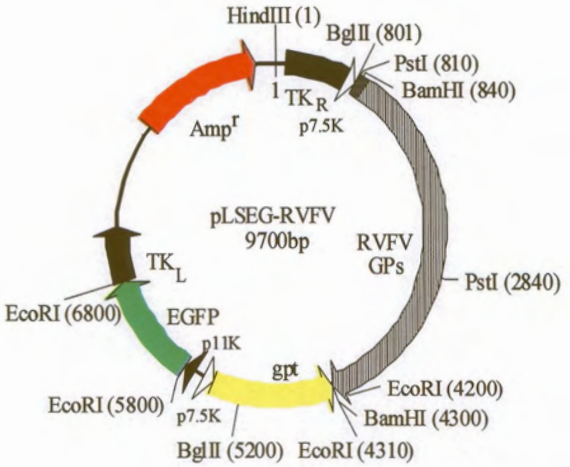


Figure 3.11 Agarose gel electrophoresis analysis of R.E. digests of minipreps of pLSEG-BEFV. **A** – minipreps cut with *Sal*I (lanes 1-6, 8-18, 20-24). Lanes 7 and 19 – lambda *Pst*I DNA marker. **B** – minipreps #4, 9, 10, 13, 18, 19 and 22 cut with *Bam*HI (lanes 2-4, 6-9) and *Eco*RI (lanes 11-13,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

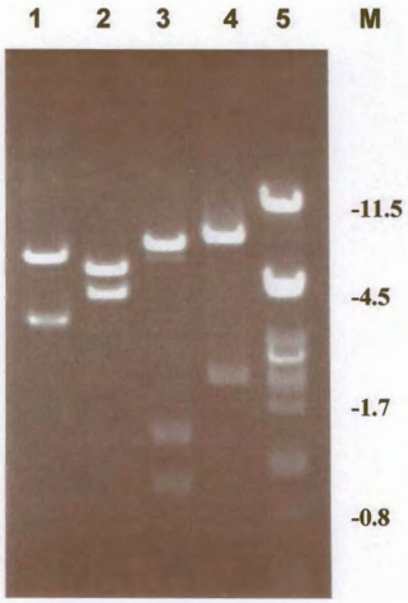


Figure 3.12 R.E. plasmid map (**A**) and agarose gel electrophoresis analysis of R.E. digests of pLSEG-RVFV (**B**). Lane 1 – *Bam*HI, lane 2 – *Bgl*II, lane 3 – *Eco*RI, lane 4 – *Pst*I, and lane 5 – lambda *Pst*I DNA marker. M – DNA fragment sizes in kbp.

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

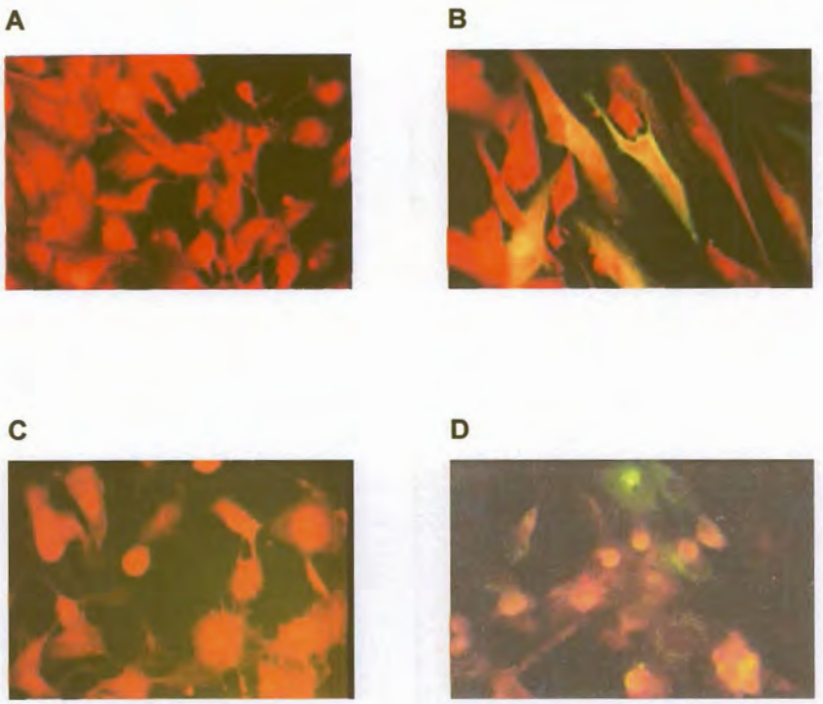


Figure 3.13 Immunofluorescence for the detection of transiently expressed BEFV and RVFV GPs from LSDV insertion vectors. A – FBT cells co-transfected with wtLSDV and pLSEG and incubated with a polyclonal anti-BEFV cattle serum. B – FBT cells co-transfected with wtLSDV and pLSEG-BEFV and incubated with 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.

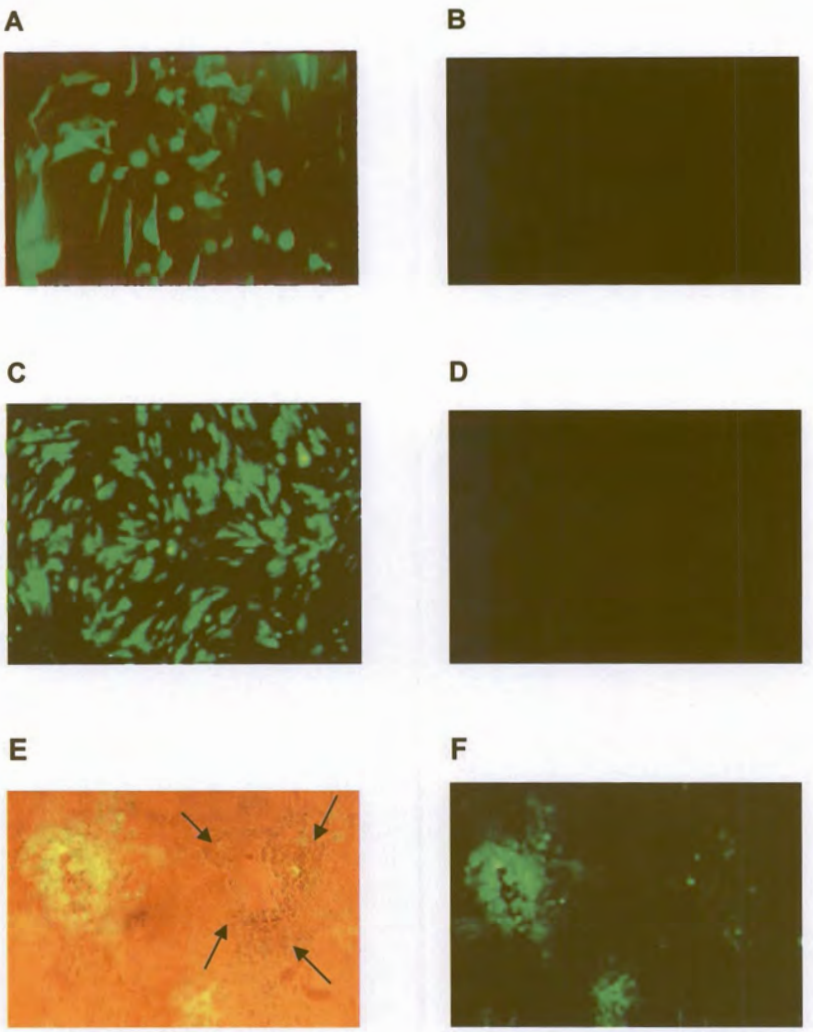


Figure 3.14 EGFP expression. **A** - transient expression of EGFP in FBT cells co-transfected with pHGS7-E and wtLSDV, 24 hours post-transfection. **B** – negative control – as in "A", but using pLS-A in place of pHGS7-E. **C** - transient expression of EGFP in FBT cells co-transfected with pLSEG and 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

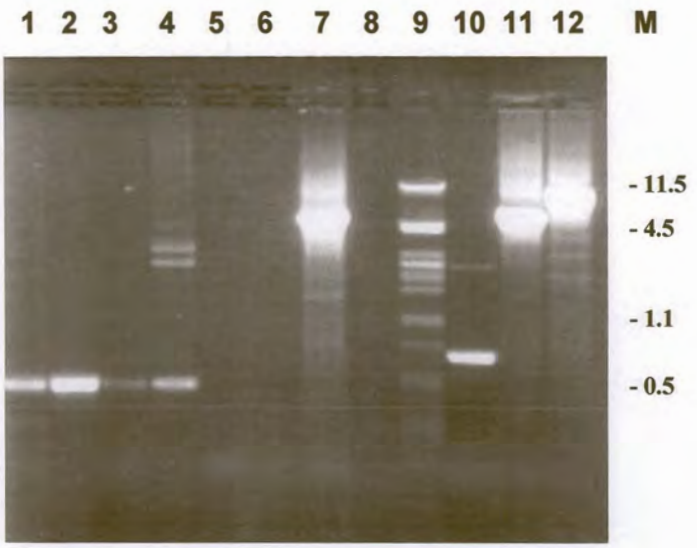


Figure 3.15 Agarose gel of PCR amplification products of LSDV-BEFV and LSDV-RVFV recombinants separated by electrophoresis (including plasmid controls and lambda *Pst*I marker DNA). Lanes 1-3: three early stage LSDV-BEFV recombinant foci, lanes 4-6: three early stage LSDV-RVFV recombinant foci, lane 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.

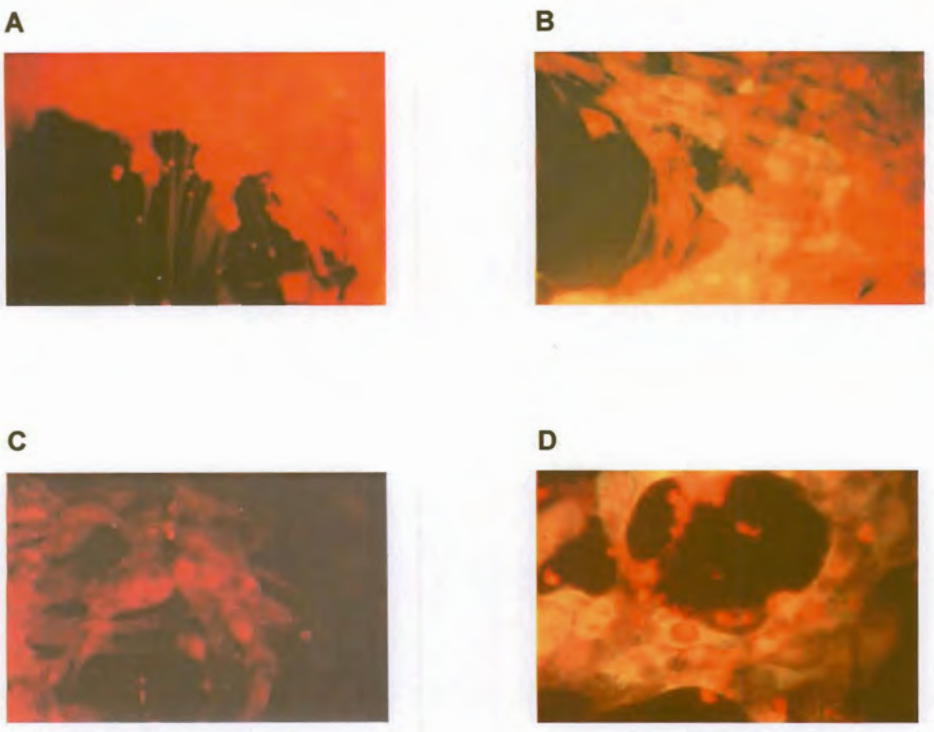


Figure 3.16 Immunofluorescence for the detection of BEFV and RVFV GPs expressed from LSDV recombinants. A – FBT cells infected with wtLSDV and incubated with a polyclonal anti-BEFV cattle serum. B – FBT cells infected with LSDV-BEFV recombinant virus and incubated with a polyclonal anti-BEFV cattle serum. 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

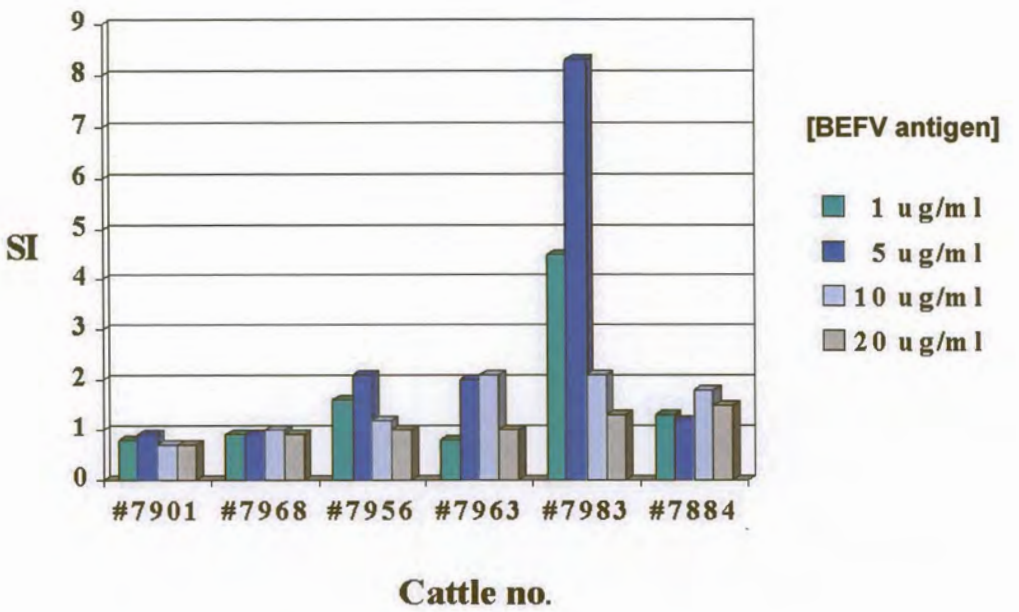


Figure 4.1 Lymphocyte proliferation assay of LSDV and BEFV vaccinated cattle. PBMCs isolated from the commercial LSDV vaccinated cattle (#7901 and #7968) (as negative controls), commercial BEFV vaccinated cattle (#7956 and #7963) (as positive controls) and the LSDV-BEFV recombinant vaccinated cattle (#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.

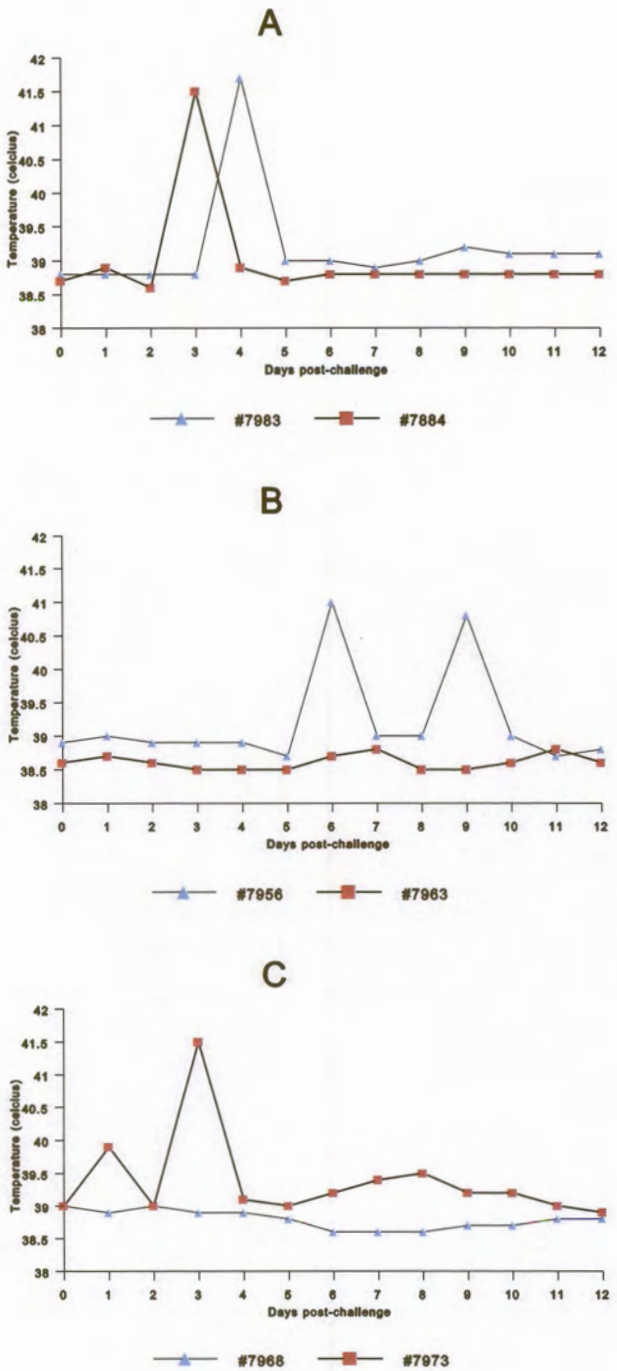


Figure 4.2 Rectal temperature recordings of the cattle after challenge with virulent BEFV. **A**. Cattle vaccinated with rLSDV-BEFV. **B**. Cattle vaccinated with the commercial live attenuated BEFV vaccine. **C**. Unvaccinated, challenged cow (#7973) and unchallenged, negative control cow (#7968).

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).

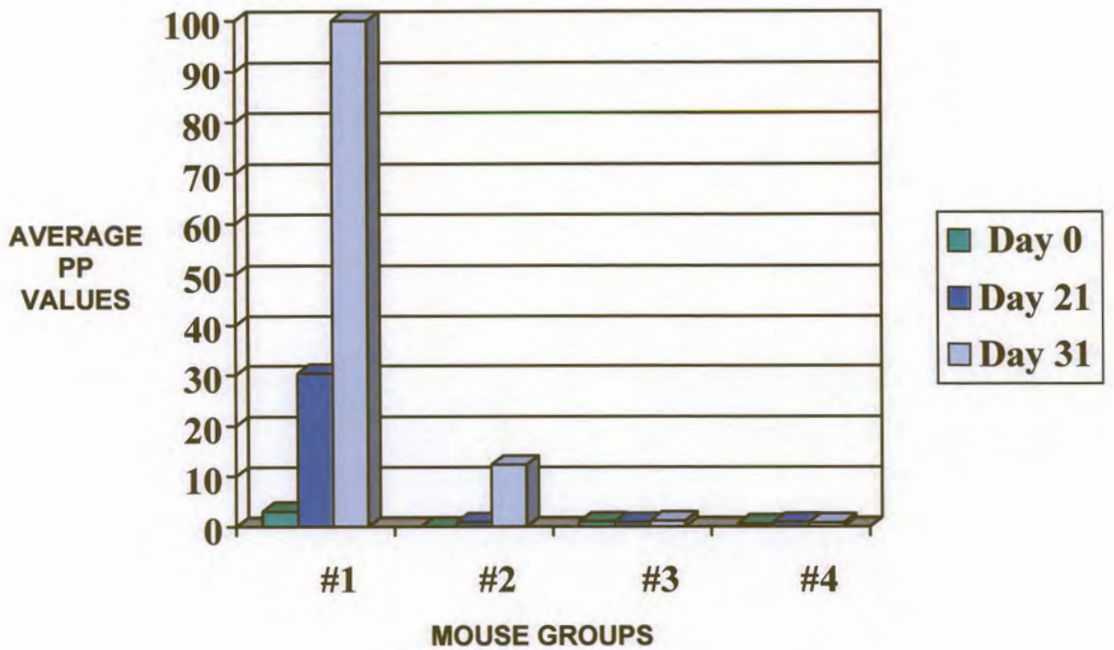


Figure 4.3 I-ELISA assay for RVFV-specific IgG in Balb/c mice. Five Balb/c mice per group were inoculated with either the live attenuated Smithburn vaccine of RVFV (group #1), rLSDV-RVFV (group #2), rLSDV-BEFV (group #3) or saline (group #4). Mice were boosted 21 days post-inoculation and challenged 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

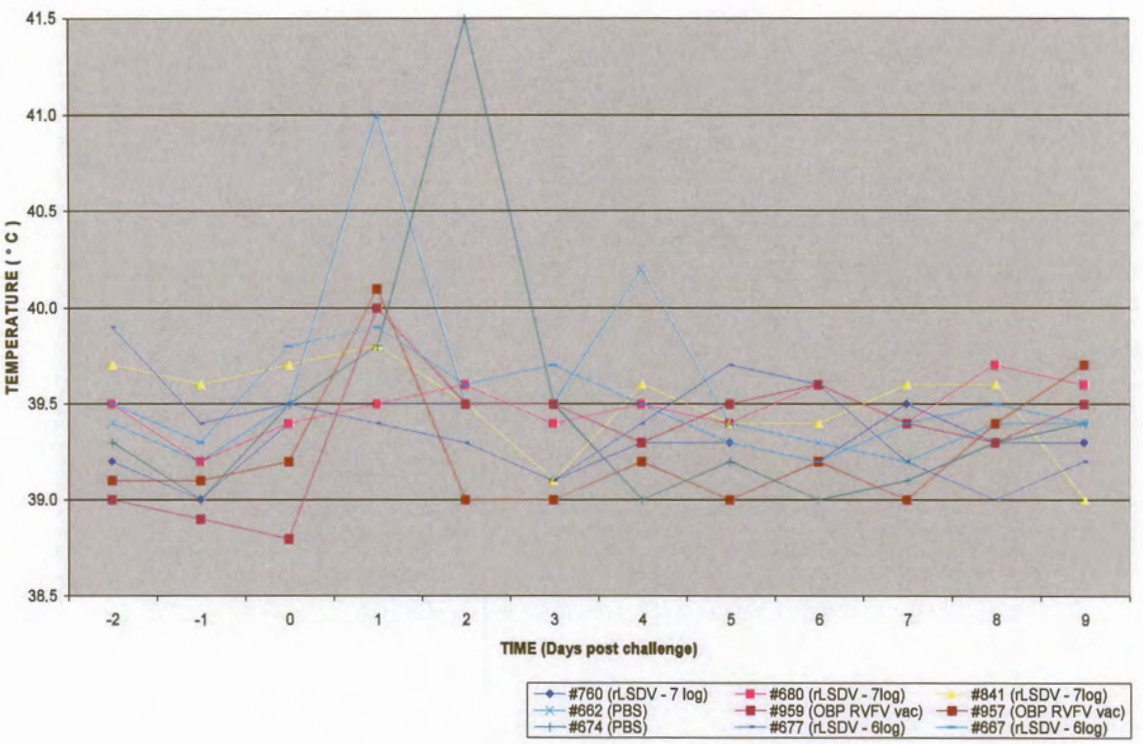


Figure 4.4 Rectal temperatures of sheep challenged with virulent RVFV. Rectal temperatures were taken from two days pre-challenge. The vaccine regime inoculated into each sheep is shown in brackets in the legend. 6log = $1x10^6$ pfu and 7log = $1x10^7$ pfu

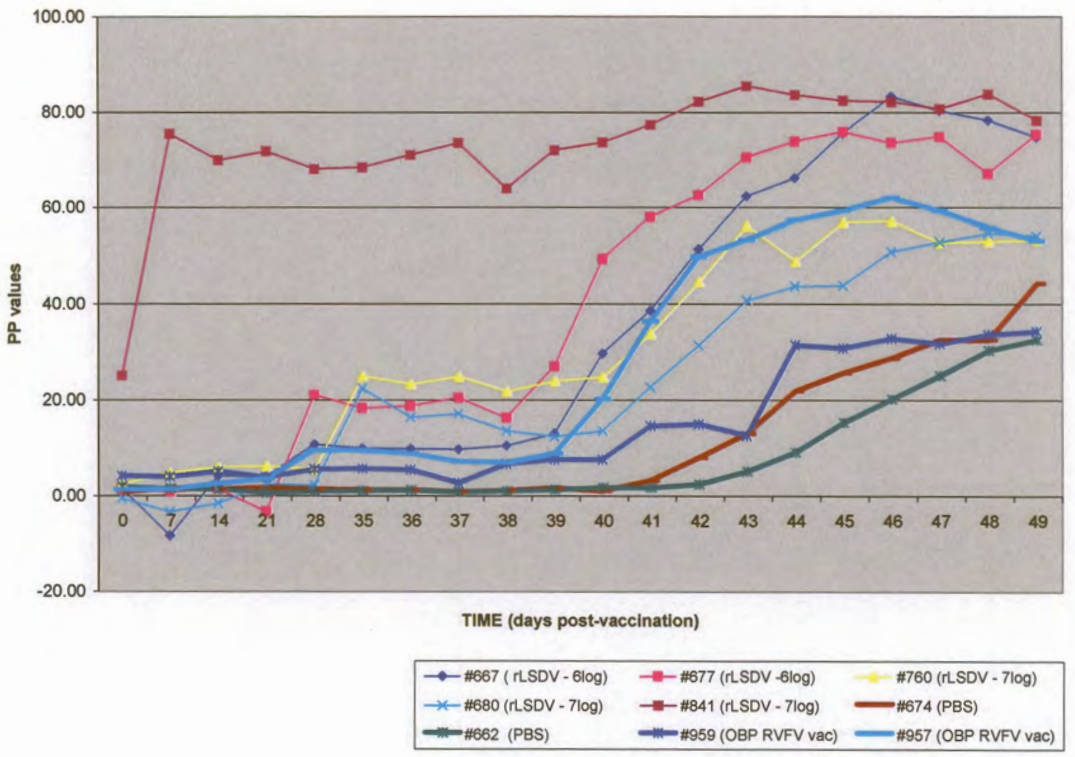


Figure 4.5 I-ELISA results of RVFV-specific antibody responses in sheep vaccinated with different RVF vaccine regimes. The ELISA readings are presented as a percentage of a strongly positive reference serum (PP). 6log = $1x10^6$ pfu and 7log = $1x10^7$ pfu

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.