Improved method for the generation and selection of homogeneous lumpy skin disease virus (SA-Neethling) recombinants

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Abstract

Lumpy skin disease virus (LSDV) is being developed as a vector for recombinant vaccines against diseases of veterinary importance. A strategy for generating viral thymidine kinase (TK) gene-disrupted recombinants which are stable and homogeneous using the South African Neethling vaccine strain of LSDV as vector has been developed. To assist with the selection process, the *Escherichia coli* β-galactosidase (lacZ) visual marker gene was incorporated into the constructs. However, the use of lacZ has certain limitations. An improved strategy was then devised substituting lacZ with the enhanced green fluorescent protein (EGFP) under control of the vaccinia virus (VV) P11K late promoter. The EGFP marker was found to enhance the selection process, and with the inclusion of additional sonication and filtration steps the number of passages required to select recombinants to homogeneity has been reduced. In support of the improved method for generation and selection of recombinants described, three different LSDV

recombinants expressing the glycoprotein genes of bovine ephemeral fever virus, Rift Valley fever virus and rabies virus were prepared and characterised.

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1. Introduction

Lumpy skin disease virus (LSDV) belongs to the *Capripoxvirus* genus of the Poxviridae family (Buller et al., 2006) and is the etiological agent of an economically important disease of cattle throughout Africa. As for other members of the Poxviridae family, attenuated vaccine strains of LSDV are being developed as vectors for recombinant vaccines for use in the veterinary field. The Kenya Sheep-1 strain, KS-1 ([Kitching et al., 1987] and [Kitching et al., 1989]), has been utilised for the development of recombinant rinderpest, bluetongue and peste-des-petits-ruminants virus vaccines ([Romero et al., 1993], [Romero et al., 1994a], [Romero et al., 1994b], [Romero et al., 1994c], [Wade-

Evans et al., 1996], [Ngichabe et al., 1997] and [Berhe et al., 2003]). The southern African LSDV vaccine strain, SA-Neethling, was developed from a virulent field isolate, and is a highly host-restricted vaccine that offers long-term protection (Weiss, 1968). It is therefore considered as a prime candidate for development as a vector of recombinant vaccines, particularly for cattle, and has been investigated in several disease models including rabies, bovine ephemeral fever and Rift Valley fever ([Aspden et al., 2002], [Aspden et al., 2003], [Wallace and Viljoen, 2005] and [Wallace et al., 2006]).

The preparation of recombinant poxviruses has become standard laboratory practice and a wide range of techniques are available ([Falkner and Moss, 1990], [Mackett et al., 1985], [Merchlinsky and Moss, 1992], [Pfleiderer et al., 1995] and [Timiryasova et al., 2001]). For the generation and selection of LSDV recombinants our laboratory first attempted one of the approaches most commonly used to generate VV recombinants, viz. the insertion of foreign genes into the viral TK gene with selection relying on the TK-negative phenotype of the resulting recombinants (Mackett et al., 1985). The advantage of using such an approach is that the selection is "negative" thus making it easier to select recombinants which are homogeneous (free from parental virus)—an important factor for commercialization purposes. As the rates of the infection-cycles of LSDV and VV differ markedly (Fick and Viljoen, 1999), minor adaptations were implemented to maximize the chances for successful homologous recombination. In our study, the *Escherichia coli* lacZ visual marker gene was included in the DNA transfer vector to assist with the selection process. However, the TK-negative selection approach failed to yield stable LSDV recombinants due to a later finding that the SA-Neethling vaccine strain of LSDV has a dependence for growth on some source of TK activity (either of viral or cellular origin) (Wallace and Viljoen, 2002). The selection strategy was then modified to that of dominant positive selection ([Falkner and Moss, 1990] and [Romero et al., 1993]) with inclusion of the *E. coli* guanine phosphoribosyl transferase (gpt) gene. Following this protocol stable TK-disrupted recombinants expressing lacZ could be generated and isolated. The use of dominant positive selection made it possible to generate TK-disrupted recombinants expressing genes of veterinary importance for evaluation as vaccines ([Wallace and Viljoen, 2005] and [Wallace et al., 2006]). Unfortunately though,

contaminating parental virus proved to be a problem and thus a method had to be developed to separate parental from recombinant virus.

This paper describes the modifications made to the method of Romero et al. (1993) for the generation and selection of LSDV-vectored recombinants, with additional steps included to ensure homogeneity. The substitution of lacZ with EGFP (originally isolated from the jellyfish, *Aequorea victoria*) (Cheng et al., 1996) as a more convenient *in vivo* visual selectable marker is also described. The generation and characterisation of three homogeneous LSDV recombinants that effectively express the glycoprotein (GP) genes of bovine ephemeral fever virus (BEFV), Rift Valley fever virus (RVFV) and rabies virus (RABV), respectively, is described in support of the methods presented here.

2. Materials and methods

2.1. Viruses and cells

The South African Neethling vaccine strain of LSDV (referred to as wild-type [wt] LSDV) was used as parental virus for the generation of recombinants (Weiss, 1968). Primary cultures of foetal bovine testes (FBT) cells were prepared according to standard techniques (Freshney, 1994). Unless otherwise stated, cells were cultured in a 1:1 combination of Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F-12 medium (DMEM/F12) containing 8% foetal calf serum (FCS) and antibiotics (100 μg/ml penicillin, 100 μg/ml streptomycin and 250 μg/ml amphotericin) (Highveld Biological Products, South Africa).

2.2. Plasmid constructs

The insertion vector, pLS(g)lac (formerly called “pLSTK(g)lac”), was used for the generation of the LSDV–lacZ recombinant (Fig. 1A) (Wallace and Viljoen, 2002). The construction of the transfer vector, pLSEG (Fig. 1B), and the insertion vectors for BEFV and RVFV have been described elsewhere ([Wallace and Viljoen, 2005] and [Wallace, 2006]). A cDNA clone of a GP gene of a South African RABV isolate (ARC-OVI reference: m710/90), inserted in the BamHI and SacI sites of pLSEG, was acquired from one of the authors (Louis Nel, University of Pretoria).

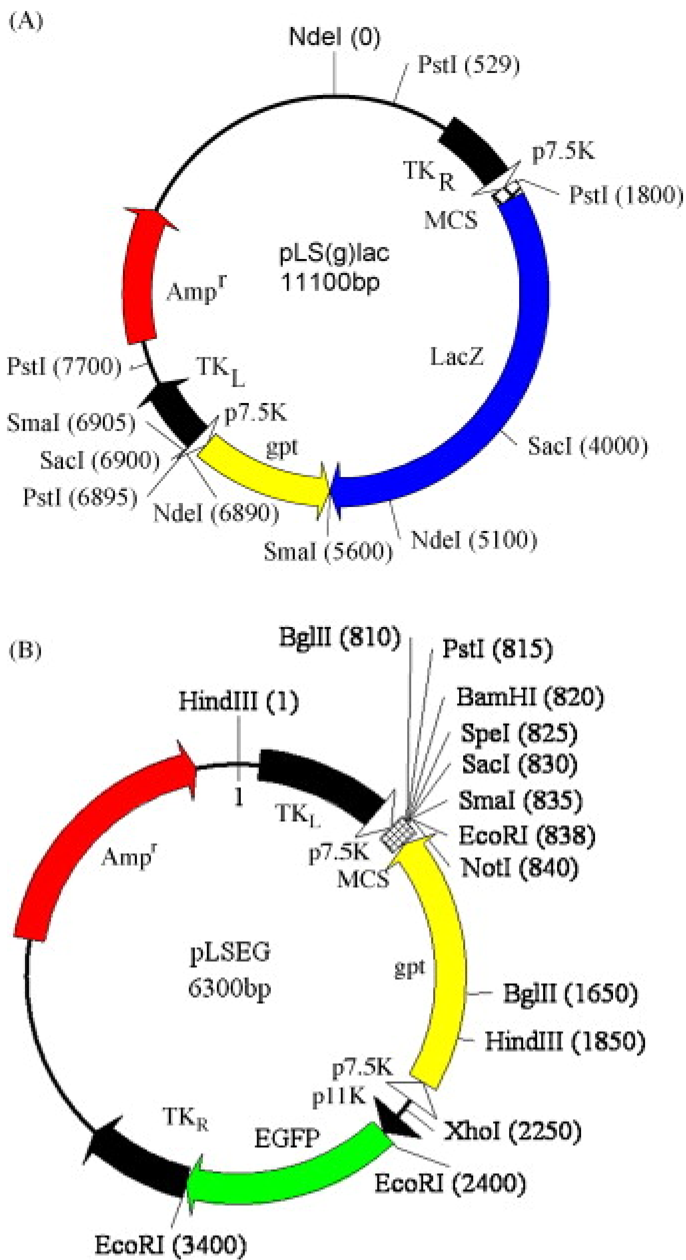

Fig. 1. Schematic representation of the DNA plasmids used for the generation of LSDV recombinants. (A) pLS(g)lac. The vector consists of the LSDV TK gene disrupted by the *E. coli* lacZ gene (under control of the VV P7.5K early/late promoter) and the *E. coli* gpt dominant selectable marker gene (also under control of the VV P7.5K early/late promoter). Arrows indicate the direction of transcription of the VV P7.5K early/late promoter, and the ampicillin resistance (Amp^r), thymidine kinase (TK), *E. coli* guanine

phosphoribosyl transferase (gpt) and β-galactosidase (lacZ) genes. (B) pLSEG. The vector is derived from pLS(g)lac with the substitution of lacZ with EGFP (under control of the VV P11K promoter), and removal of extraneous DNA sequences. A multiple cloning site (MCS) allows for insertion of immuno-protective genes under control of the VV P7.5K promoter. Arrows indicate the direction of transcription of the VV P7.5K early/late promoter, VV P11K late promoter, and the ampicillin resistance (Amp^r), thymidine kinase (TK), *E. coli* guanine phosphoribosyl transferase (gpt) and enhanced green fluorescent protein (EGFP) genes.

2.3. Generation and selection of recombinants expressing lacZ

Primary FBT cells were seeded in six-well cell culture dishes (Nunclon, Denmark) (1×10^5 cells per well in 3 ml growth medium). Once they attained 80% confluency they were infected with wtLSDV at a multiplicity of infection (MOI) of 0.1. Transfection complexes were prepared according to the manufacturer's instructions, but with the following modifications: 5 µl of the liposomal-based transfection reagent, LipofectAMINE™ (Gibco-BRL, UK) was added to 95 µl of OPTIMEM (Gibco-BRL, UK) in a 10 ml sterile polystyrene test tube. Five micrograms of highly pure insertion vector DNA, pLS(g)lac (purified using the Talent Cleanmix Kit, Italy, according to the manufacturer's suggestions) was added to OPTIMEM in a separate tube to give a final volume of 100 µl. The solutions were mixed and left at room temperature (23 °C) for 20 min. The volume was made up to 1 ml with antibiotic-free DMEM and gently pipetted onto the cells once the inoculum had been removed. The cells were returned to the incubator for a period of 7 h, 1 ml of antibiotic-free DMEM/Ham's F12 medium (including 10% FCS) was added to each well, and incubation was resumed for a further 17 h. The transfection mixture was then replaced with DMEM/F12 (with 10% FCS, and antibiotics), and the cells were incubated for another 4 days, or until complete cytopathic effect (CPE) was observed. Cells were then freeze-thawed three times and the solution cleared by centrifugation. A 10-fold serial dilution was made of the supernatant and an aliquot of each dilution was placed onto fresh FBT cells pre-incubated for 24 h in the presence of MXH selection medium (250 µg/ml xanthine, 15 µg/ml hypoxanthine, 25 µg/ml mycophenolic acid, DMEM/F12, 2.5% FCS and antibiotics). Cells were

incubated under standard conditions (37 °C, 5% CO_2), with replacement of the selection medium every 72 h until CPE became visible (usually 4–5 days post-inoculation). The freeze-thawing, serial dilutions, and incubation under standard conditions were repeated once more. After 4–5 days, once CPE appeared, the selection medium was removed from the cells and dead cells were pelleted at low speed (200 g, 5 min) in a bench-top centrifuge. The supernatant fluid was subjected to sonication at 35 kHz for 15 min in a water-bath sonicator (Sonorex TK52, Bandelin, Germany) and then filtered through a 0.45 µm pore acetate filter (Millipore, USA). 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 third cycle the cells in the well exhibiting foci at the highest dilution were washed in phosphate-buffered saline (PBS) and individual foci were picked using a 20 µl filtered pipette tip. These were then freeze-thawed as described, and half of the material collected from each focus was inoculated onto fresh cells under selection in 6-well or 12-well culture dishes (Nunclon), while the other half was stored at −20 °C. Once foci developed, the cells were fixed in 4% paraformaldehyde (Merck, Germany) for 5 min, washed in PBS and stained with an X-gal stain (1 mg/ml X-gal [Biosolve Ltd., The Netherlands]), 5 µg/ml potassium ferrocyanate, 5 µg/ml potassium ferricyanate, and 5 µg/ml magnesium chloride (all from Sigma, USA). The fixed cell monolayer was incubated at 37 °C until the blue coloration of cells expressing lacZ became visible. Those foci which were positive for expression of β-galactosidase were analysed for homogeneity using PCR analysis and DNA hybridization.

2.4. PCR analysis of viral DNA

Foci were diluted 1:1 in lysis buffer consisting of 1% sodium docecyl sulphate (Sigma, USA), 20 mM β-mercaptoethanol (Merck, Germany), and 20 mM EDTA (Merck, Germany). Proteinase K (Roche, Germany) was added (final concentration = 100 µg/ml) and incubation was carried out at 56 °C for 2 h in order to release viral DNA. Calf liver tRNA (0.5 µl of a 10 mg/ml stock) (Roche, Germany) and 1/10th volume 3 M sodium acetate (pH 5.3) were added and the DNA was extracted with phenol/chloroform (Sigma,

USA) and ethanol-precipitated (Sambrook et al., 1989). After air-drying, the DNA was resuspended in 50 µl sterile, ultra-pure water and stored at −20 °C for PCR analysis. Two primers, P1 (CACCAGAGCCGATAAC) and P2 (GTGCTATCTAGTGCAGCTAT), were designed to anneal to the wtLSDV TK gene flanking the KpnI restriction enzyme site used for the insertion of the foreign genes (Fig. 2A). These primers were used to help distinguish wt from recombinant virus while primers P3 (GAAGTGTCCCAGCCTG) and P4 (CTCGCAAGCCGACTGATGCC), binding to the gpt and lacZ marker genes, were used as internal controls. For the PCR reactions (GeneAmp 2400 thermal cycler—Perkin Elmer, USA), 50 µl reaction volumes were used consisting of 5 µl 10× PCR buffer (containing 20 mM $MgCl_2$) (Takara Biomedicals, Japan), 4 µl 2.5 mM dNTPs (Takara Biomedicals, Japan), 0.5 U Taq DNA polymerase (TaKaRa Ex Taq™, Takara Biomedicals, Japan), 20 pmol of each primer (Gibco-Brl, Scotland), template DNA (∼ 0.1 ng) and sterile distilled water to the final reaction volume. Template DNA was denatured for 45 s at 93 °C, primer annealing was carried out at 53 °C for 45 s, and strand extension was at 72 °C for 5 min (repeated through 35 amplification cycles).

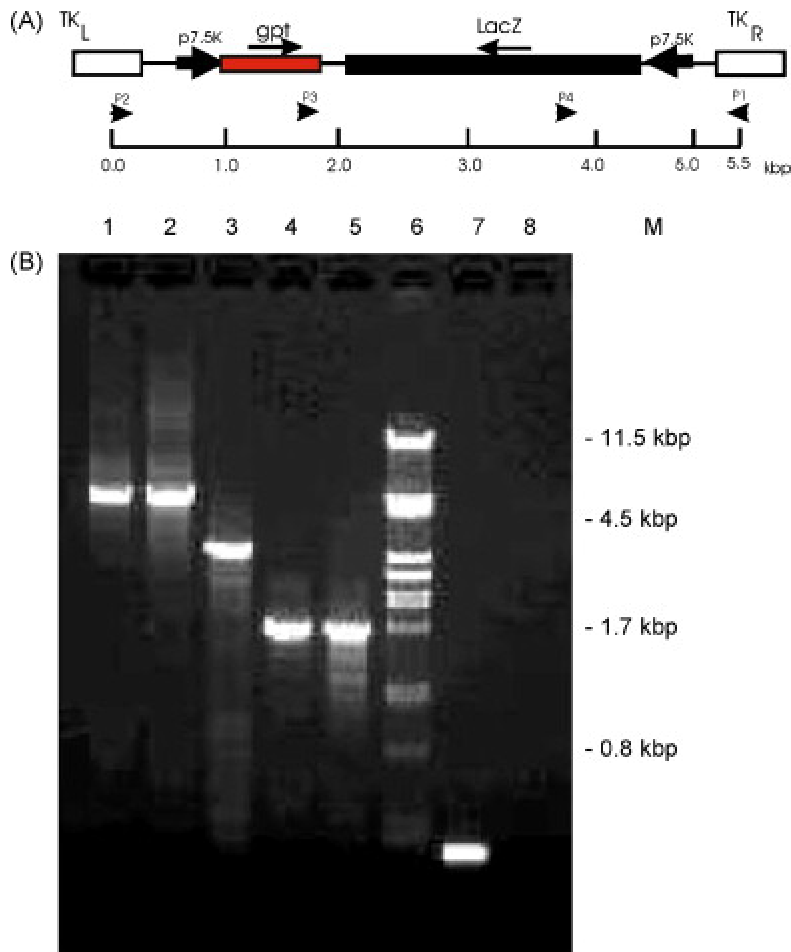

Fig. 2. (A) Schematic representation of the region between the left (TK_L) and right (TK_R) flanks of the LSDV TK gene within the pLS(g)lac insertion plasmid. A slide scale indicates the size of the insertion region and the relevant genes in kilo base pairs (kbp). Binding sites and direction (solid arrows) of the oligonucleotide primers P1 (CACCAGAGCCGATAAC), P2 (GTGCTATCTAGTGCAGCTAT), P3 (GAAGTGTCCCAGCCTG) and P4 (CTCGCAAGCCGACTGATGCC) are shown. (B) PCR analysis of the LSDV–lacZ recombinants. Thermocycling (as described in Section **2**) was used to amplify specific regions of the inserted genes in the rLSDV–lacZ construct. Amplification products were separated on an ethidium-bromide stained 1% agarose gel using electrophoresis: DNA from rLSDV–lacZ grown under MPA selection (primers P1 and P2) (lane 1) and for 10 passages without selection (lane 2); rLSDV–lacZ DNA (primers P1 and P3) (lane 3); rLSDV–lacZ DNA (primers P1 and P4) (lane 4); pLS(g)lac DNA (primers P1 and P4) (lane 5); phage lambda PstI-digested DNA marker

(lane 6); wtLSDV DNA (primers P1 and P2) (lane 7); sterile distilled water (primers P1 and P2) (lane 8).

2.5. Preparation of pure LSDV and viral DNA extraction (adapted from Esposito et al., 1981)

Ten 175 cm^2 cell culture flasks (Nunclon, Denmark) containing a 90% confluent monolayer of primary FBT cells were infected with virus at an MOI = 0.1. The culture medium was removed from the flasks once cells exhibited 90% CPE and the remaining cells were harvested using trypsin digestion. The cells were pooled with the medium, dispensed into centrifuge tubes and 0.5 ml of 36% sucrose (in PBS) was layered at the bottom of each tube. The virus/cell suspension was centrifuged at 11,000 rpm (19,000 × *g*), 60 min, 4 °C in a Beckman J2-21 (Beckman Instruments, USA) high speed centrifuge in a JS13-1 swing-out rotor (Beckman Instruments, USA). The pellet was resuspended in 9 ml of McIlvain's hypotonic buffer (4 mM, pH 7.4) (Lennette and Schmidt, 1969) and left on ice for 10 min. β-Mercaptoethanol (26 µl) and 1 ml of 10% Triton X-100 (Merck, Germany) (in McIlvain's buffer) were added and the suspension was further incubated on ice for 10 min to disrupt the cells. The cell debris was removed by centrifugation at 2000 rpm (450 × *g*) for 5 min at 4 °C in a benchtop centrifuge and the supernatant fluid was collected. The cell debris was resuspended in McIlvain's buffer and the centrifugation was repeated (2000 rpm [450 × *g*] for 5 min, 4 °C in a benchtop centrifuge). The supernatant fluid was collected and pooled with the first virus-containing supernatant fluid. This fluid was transferred to a centrifuge tube and 0.5 ml of a 36% sucrose solution (prepared in TE [10 mM Tris, 1 mM EDTA, pH 9.0]) was layered beneath. Virus was then pelleted (centrifuged at 11,000 rpm [19,000 × *g*], 60 min, 4 °C in a Beckman J2-21 high speed centrifuge in a JS13-1 rotor). The pellet was resuspended in 0.5 ml of TE buffer. Lysis buffer (450 µl 4% sodium lauroylsarcosinate, 54% sucrose, 100 mM Tris [pH 7.8] mixed with 50 µl 200 mM β-mercaptoethanol, 100 mM Tris [pH 7.8]) and Proteinase K (Roche, Germany) (final concentration = 100 µg/ml) were added and the virus mixture was incubated for 3 h at 56 °C (Massung and Moyer, 1991). The viral DNA was phenol/chloroform-extracted and ethanol-precipitated and was

resuspended in sterile distilled water or TE buffer and stored at −20 °C (adapted from Sambrook et al., 1989).

2.6. Southern transfer and radioactive ^{32}P-labelled probe hybridization

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

2.7. Generation and selection of recombinants using EGFP expression

The transfer vector, pLSEG, containing the EGFP gene under control of the VV P11K promoter (Fig. 1B) was constructed (Wallace, 2006) and the GP genes of BEFV, RVFV and RABV were inserted separately into the multiple cloning site (MCS) as described (Wallace and Viljoen, 2005).

Initial generation and selection of recombinants expressing the EGFP was performed along the same lines as for lacZ, except that the Effectene (Qiagen, Germany) transfection reagent was used in place of Lipofectamine™. In addition, at the stage where the first foci were picked, foci were observed *in vivo* under UV light and only those which appeared homogeneous for EGFP expression were selected. After the third round of focus picking and end-point dilution on fresh FBT cells, 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 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

(Section 2.4). Foci, which were shown to be homogeneous via PCR, were evaluated for expression of their respective GP genes using immunofluorescence (IF) (data not shown). One focus from each recombinant that showed good expression was then grown to high titres for immunological studies in animals ([Wallace and Viljoen, 2005], [Wallace et al., 2006] and [Weyer, 2006]).

3. Results

3.1. Generation and selection of recombinants expressing lacZ

Romero et al. (1993) described the development of a dual recombinant vaccine using the attenuated KS-1 sheep isolate of LSDV as a vector. Our approach was an adaptation of this method and included a number of additional steps to ensure that homogeneous recombinants were selected.

No viral foci were visible in control wells in which FBT cells were infected with wtLSDV under MXH selective pressure and the FBT cells proved tolerant of the selection medium. However, progeny virions released from cells co-transfected with wtLSDV and pLS(g)lac produced distinct viral foci in fresh cells during the second round in selection medium in wells from the lowest dilutions. Media was collected from these wells, treated as described and further titrated. Cells were then fixed and stained using X-gal, whereupon approximately 90% of the foci stained blue, a strong indication for the presence of recombinants (Fig. 3B). A number of foci were carefully picked and, after a further four passages using end-point titrations and focus picking, a few of them appeared homogeneous for lacZ expression. DNA was isolated from these foci and subjected to PCR using the TK primers P1 and P2. Only amplification products of the full-length insertion region were obtained (5.5 kbp), further supporting the recombinant's homogeneity (Fig. 2B). Combinations of the internal control primers (P1, P3 and P1, P4) also gave the expected product sizes for DNA amplified from both the pLS(g)lac insertion plasmid and the recombinant virus.

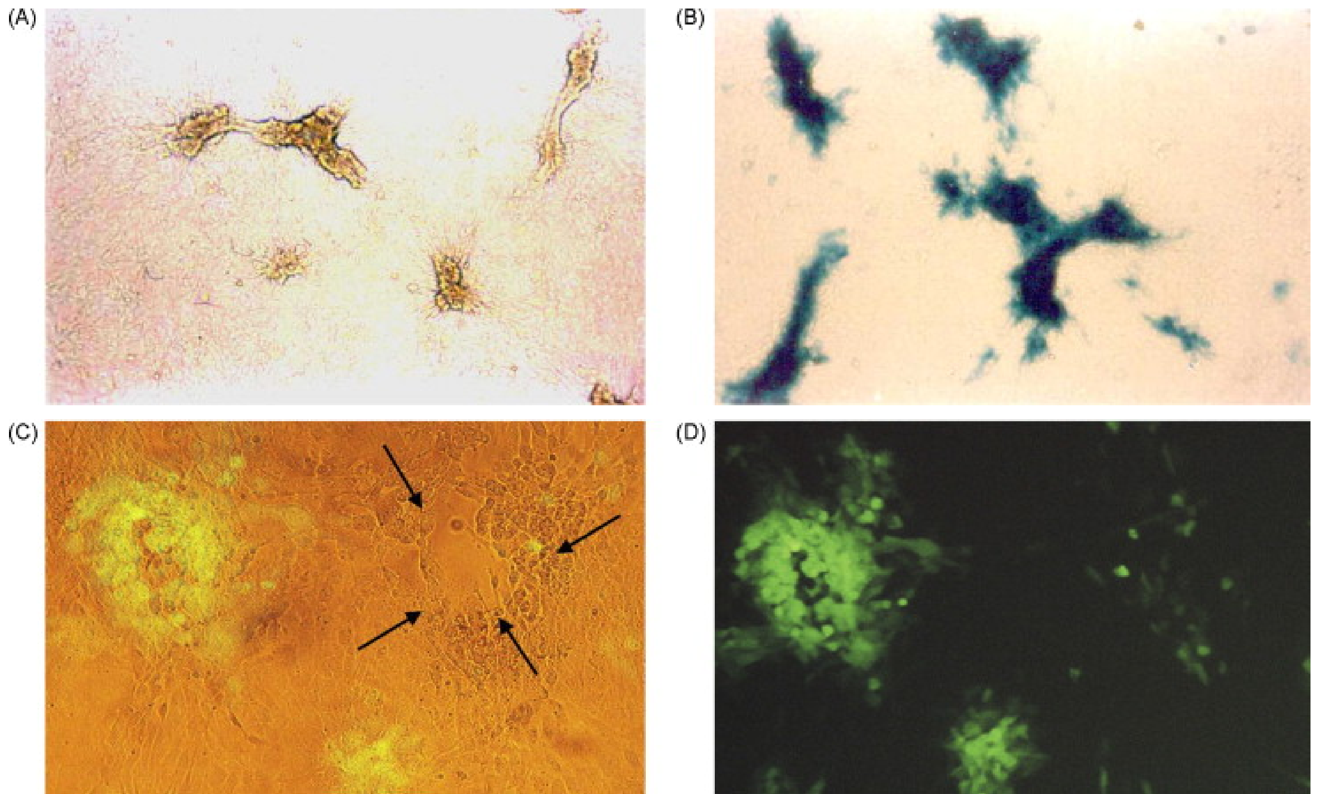

Fig. 3. (A) Madine–Darby bovine kidney (MDBK) cells infected with wtLSDV. (B) Expression of lacZ in MDBK cells infected with rLSDV–lacZ. Cells were fixed and stained as described in Section **2**. (C) rLSDV–BEFV infected FBT cells illuminated with visible and UV light. (D) As in "C", but only illuminated with UV light. The arrows indicate a viral focus. Magnification: 250×. (For interpretation of the references to colour in the text, the reader is referred to the web version of the article.)

The PCR data provided good evidence for the homogeneity of the recombinant virus. However, as this is the first LSDV-vectored recombinant study utilising the SA-Neethling vaccine strain as vector in which we are trying to prove homogeneity, we decided to include the use of DNA hybridization to provide further support to the PCR results. Purified and semi-purified recombinant viral DNA was digested with HindIII (along with DNA from purified wtLSDV). The resulting DNA fragments were separated on an agarose gel, Southern transferred to a Hybond™N^+ nylon membrane (Amersham, USA) and hybridised with a ^{32}P-labelled wtLSDV-specific TK gene probe. The intact wtLSDV TK gene is located on a 2.5 kbp HindIII fragment. Interruption of this gene with

the gpt-lacZ cassette results in the gene being split between two HindIII fragments of 2.0 and 0.8 kbp. As can be seen from the autoradiograph (Fig. 4) the 2.5 kbp wt TK-containing fragment from lane 1 is completely absent for the recombinant viral DNA (lanes 2 and 3), thus further supporting homogeneity.

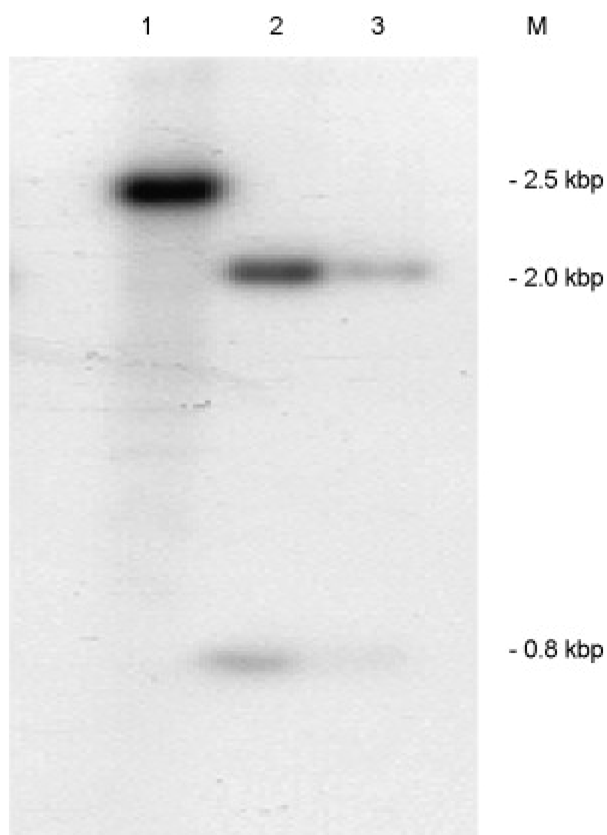

Fig. 4. Autoradiograph identifying agarose gel electrophoresis-separated HindIII digested fragments of wt and recombinant LSDV–lacZ labeled with a ^{32}P TK-specific DNA probe. 1: purified wt virion DNA; 2: purified LSDV–lacZ recombinant DNA; 3: total DNA extracted from FBT cells infected with LSDV–lacZ recombinant, M: fragment sizes in kbp.

In order to evaluate the stability of the recombinant, selection pressure was removed by growth of the recombinant in normal growth medium (DMEM/F12, 10% FCS, antibiotics) for 10 passages. DNA was isolated from virus recovered after the 10th passage and tested via PCR using the TK primers P1 and P2. Only the recombinant PCR amplification product of 5.5 kbp was obtained (Fig. 2B, lane 2). Additionally, the lacZ expression of progeny virions produced during the 10th passage without selection (as

above) was determined with the use of X-gal staining and all foci were found to stain blue. Cumulatively the above results provided evidence that the recombinants were stable and indeed homogeneous.

3.2. Selection of recombinants expressing EGFP

The procedure was essentially that as described for the generation and selection of the LSDV–lacZ recombinants, but with minor modifications. FBT cells were co-transfected separately with wtLSDV and each of the insertion vectors containing the GP genes of either BEFV, RVFV or RABV, and recombinants were selected. For each construct, after the second round of selection, foci were clearly visible on FBT cells—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 Fig. 3C, the FBT cell monolayer was illuminated with both visible and UV light simultaneously. These cells were 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 were already exhibiting CPE and yet only about 5% of them were actively expressing EGFP as is evidenced when the same focus is only illuminated with UV light (Fig. 3D). This result indicated the presence of contaminating wtLSDV. The focus to the left of this one, however, already appears almost homogeneous for EGFP expression and only foci such as this one were picked for subsequent rounds of selection.

Foci that appeared homogeneous for EGFP expression were tested using PCR and the TK primer pair, P1 and P2. In Fig. 5 amplification products for a number of such foci from each of the recombinants are shown. During the very early stages of selection (~4 passages) the 450 bp wt TK gene product was found to be predominant (A: lanes 1–4), whereas after more rounds of selection (~6–8 passages) a mixture of wt (450 bp) and full-length amplification products (4.5 kbp for the LSDV–RABV recombinant) were present (B: lanes 4–6, 8 and 9), although some foci still produced only wt product (B:

lane 10). Foci which were close to homogeneity were selected for further passage and within two to three rounds of selection most of these only produced full-length amplification products, indicative of homogeneity (A: lane 11 for a LSDV–BEFV recombinant focus [5.0 kbp] and lane 12 for a LSDV–RVFV recombinant focus [6.3 kbp]; B: lane 11 for a LSDV–RABV recombinant focus [4.5 kbp]). No products were visible in Fig. 5A (lanes 5 and 6) and B (lane 7), probably due to the amount of viral DNA purified from the foci being below the cut-off amount for detectable amplification. The additional bands (∼2.8 and 2.6 kbp) in lane 4 (Fig. 5A) were possibly due to mispriming or an unstable, intermediate form of the recombinant resulting from a single-crossover event during recombination. 0.1 ng of DNA from plasmids pLSEG–BEFV (Fig. 5A, lane 7) and pLS-A (an intermediate plasmid from the construction of pLSEG, data not shown) (Fig. 5A, lane 10) were included in the PCR as positive controls. The correct amplification products of 5.0 kbp (lane 7) and 0.7 kbp (lane 10), respectively, were obtained, including an additional faint band of 2.6 kbp in lane 10 (possibly due to PCR mispriming). Foci that were grown under selective pressure and that appeared homogeneous by PCR were further passaged in the absence of selection pressure for another five passages and were then re-evaluated by PCR for homogeneity. Once recombinants were shown to be homogeneous, IF was used to confirm expression of their respective GPs (data not shown). These recombinants were then grown to high titres in FBT cells and evaluated for their respective abilities to induce protective immune responses in a variety of appropriate animal models ([Wallace and Viljoen, 2005], [Wallace et al., 2006] and [Weyer, 2006]).

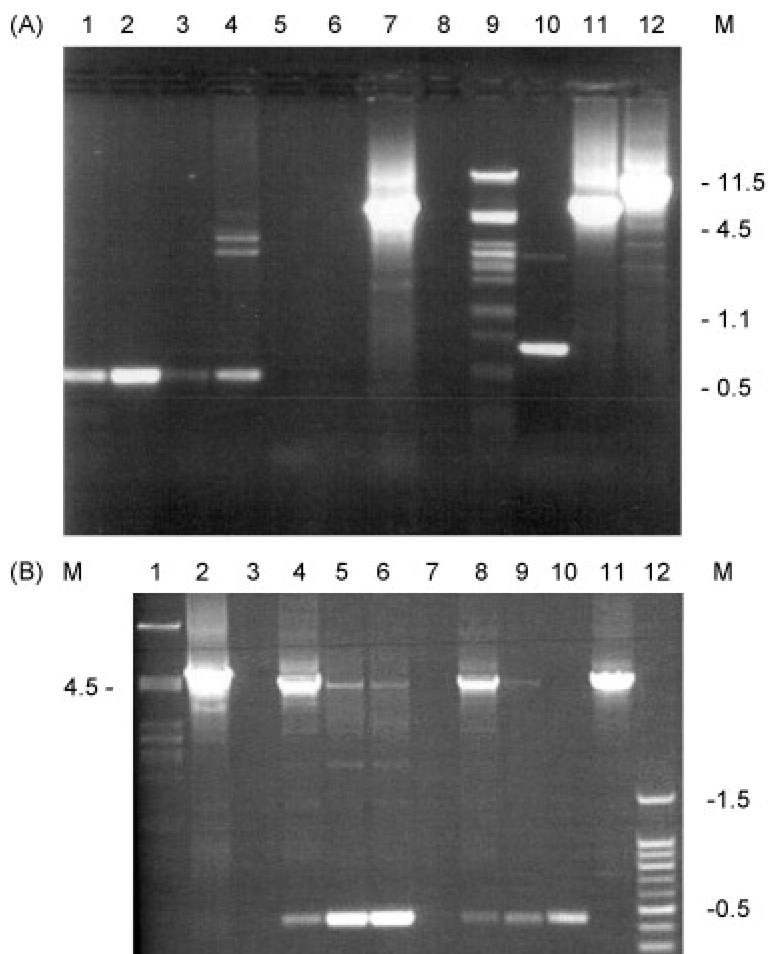

Fig. 5. (A) Agarose gel of PCR amplification products of LSDV–BEFV and LSDV–RVFV recombinants separated by electrophoresis (including plasmid controls and phage lambda PstI marker DNA). Lanes 1–3: three early stage LSDV–BEFV recombinant foci, lanes 4–6: three early stage LSDV–RVFV recombinant foci, lane 7: pLSEG–BEFV positive control, lane 8: sterile water negative control, lane 9: phage lambda PstI DNA marker (kbp), lane 10: 0.1 ng pLS-A DNA, lane 11: late stage LSDV–BEFV recombinant virus focus and lane 12: late stage LSDV–RVFV recombinant virus focus. The primer pairs P1 and P2 were used as described (Section **2.4**). M: marker DNA sizes in kbp. (B) Agarose gel of PCR amplification products of the LSDV–RabG recombinant separated by electrophoresis (including plasmid controls and phage lambda PstI marker DNA). Lanes 1 and 12: phage lambda PstI marker and 100 bp marker (Promega), respectively, lane 2: pLSEG–RABG positive control, lane 3: sterile water negative control and lanes 4–11: recombinant foci at different stages of homogeneity. M: marker DNA sizes in kbp.

4. Discussion

The use of LSDV (SA-Neethling, vaccine) as a safe and immunogenic carrier of pathogen genes has proved promising in a number of vaccine development studies. In particular, the development of dual veterinary vaccines against LSD as well as a second disease of interest could prove very useful. In this regard it is important to be able to isolate stable and homogeneous recombinant LSDV vaccine viruses quickly and efficiently. Here, different methods for the isolation of such recombinants were compared.

Since previous studies have shown that TK activity is necessary for the growth of TK gene-disrupted LSDV recombinants, a selection strategy other than TK-negative selection was required (Wallace and Viljoen, 2002). The use of a dominant selectable marker, *E. coli* gpt, was successfully employed. The selection process was further facilitated by the inclusion of a visual reporter gene (lacZ). However, the use of lacZ presented some limitations, including that the presence of endogenous lacZ activity in certain cell types complicates the identification of recombinant foci. With these factors in mind, EGFP was identified as a substitute. The use of EGFP abrogates the need for substrate chemicals and colorimetric assays, but does require the availability of an inverted microscope with a UV light source. In addition, at the outset of recombinant selection, the use of EGFP allows for an evaluation of the percentage of cells showing co-transfection towards determining whether the thresholds for successful selection of recombinants are maintained. Visualization of EGFP expression also facilitated the selection of foci which were close to homogeneity. Foci presenting 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. Although the use of EGFP as a visual marker facilitated the selection of recombinant foci, there are additional points to consider regarding the isolation of homogenous recombinants. As 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 and 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 (Wallace, 1994). These "clumps" probably contain a mixture of wt and recombinant viruses making it necessary to 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 that by its very nature can exert a wide-ranging effect. In other selection strategies such as the TK-negative strategy, viruses which have an active TK gene are knocked out under 5-bromo-2-deoxyuridine (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. However, as the highly attenuated South African vaccine strain of LSDV has a dependence on TK activity for growth an alternative strategy had to be chosen. In the MXH 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 MXH 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 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 (−22 °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 min 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 significantly reduce 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. 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 number of passages was reduced by approximately half. 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, although this did not prove to be the case. This finding 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. Evidence from earlier studies also suggested that single crossover recombination events could be responsible for the demonstration of the presence of wt virus when recombinant foci are analysed by PCR. Nazerian and Dhawale (1991) describe the generation of unstable intermediate forms of recombinant fowlpox virus caused by single crossover 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 effective in the selection of TK-disrupted LSDV recombinants.

The modifications described for the selection strategy were applied to the generation and selection of recombinant vaccine constructs against three pathogens of livestock (BEFV, RVFV and RABV). With the construction of the relevant insertion vectors, three separate recombinants were generated. Homogeneity was confirmed via PCR analysis after removal of the selection pressure for more than five passages and expression of the

glycoproteins was confirmed using IF. The combined use of PCR and removal of selection pressure for a number of passages were found to be crucial tests towards ensuring that a recombinant was indeed homogeneous. In one instance a LSDV–BEFV recombinant focus, which at first appeared homogeneous by 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 had a distinct growth advantage over TK-disrupted recombinants, as is the case for other poxviruses (Mackett et al., 1985). Nevertheless, given enough time, the recombinants are still able to grow to high titres in cell culture (Wallace and Viljoen, 2002).

The use of filtration and sonication to select for homogeneous LSDV recombinants has proven reproducible and successful in the hands of others (Berhe et al., 2003) and at present remains the method of choice in our laboratory. 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 gene insertion, might eventually cease to exist.

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