

**Comparison of the two lumpy skin disease virus vaccines, Neethling and Herbivac,  
and construction of a recombinant Herbivac-Rift Valley fever virus vaccine**

Ruzaiq Omar

Supervisor: Professor Anna-Lise Williamson

Co-supervisor: Dr Nicola Douglass

University of Cape Town

Division of Medical Virology

Department of Clinical Laboratory Sciences

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## List of Abbreviations

BHK – Baby Hamster Kidney

CAMs – Chick Allantoic Membranes

CEV – cell associated extracellular virus

CPE – Cytopathic effect

EMV – extracellular mature virus

FBT – Foetal Bovine Testis

GPV – Goatpox virus

Herbivac – Herbivac®LS

Herbivac-LSDV – Recombinant LSDV expressing RVFV genes

HIV – human immunodeficiency virus

IEV – intracellular enveloped virus

iLSDV – Immunogenically improved lumpy skin disease virus strain

IMV – Intracellular mature virus

IV – Immature Virions

KS-1 – Kenyan strain sheeppox virus

LSD – Lumpy skin disease

LSDV – Lumpy skin disease virus

MDBK – Madin-Darby bovine kidney cells

MPA – Mycophenolic Acid

NC – Nucleocapsid

OIE – World Organization for Animal Health

RVF – Rift Valley fever

RVFV – Rift Valley fever virus

SPV – Sheeppox virus

tGn – truncated Gn

VLP – virus like particle

## Abstract

There are two broad aims to this project. The first aim is to compare and characterise two lumpy skin disease virus (LSDV) vaccines namely the vaccine based on attenuated Neethling LSDV (nLSDV) and Herbivac<sup>®</sup>LS (Herbivac). The second aim is to construct a recombinant LSDV expressing Rift Valley fever virus (RVFV) genes.

An LSDV vaccine is critical for sustainable control of lumpy skin disease (LSD). There are four commercially available live attenuated vaccines for LSDV, nLSDV, Herbivac, Lumpyvax and the Kenyan strain sheeppox virus (KS-1). In this study Herbivac was characterised by comparing it to its parent, nLSDV. Growth curves of the two viral strains were conducted in cell culture as well as in embryonated hens' eggs. No notable difference in the growth rate of the two strains could be detected when the viruses were grown in cell culture, however a notable difference was detected when the viruses were grown on the chick allantoic membranes (CAMs) of embryonated hens' eggs. When grown on CAMs a faster growth rate was observed for nLSDV compared to Herbivac. nLSDV also killed the embryos at 4 d.p.i where Herbivac did not. The two strains were then further characterised through histological analysis of CAMs after infection with each of the viruses. Overall, higher levels of hyperplasia and hypertrophy were observed in CAMs infected with either nLSDV or Herbivac compared to uninfected CAMs. Herbivac-infected CAMs resulted in thicker chorionic membranes and larger pocks compared to nLSDV.

RVFV and LSDV both contribute to the disease burden among cattle in Africa and the Arabian Peninsula. The main aim of this study was to construct a recombinant Herbivac which expresses immunogenic proteins of Rift Valley fever virus (Herbivac-RVFV).

Herbivac-RVFV was designed to express specific RVFV genes selected for their antigenic properties. The genes selected are also representative of the genes from recent viral outbreaks in the horn of Africa. The selection of outbreak relevant RVFV genes involved phylogenetic analysis of all full length M-segment and NC gene sequences available on Genbank. Phylogenetic trees were constructed for M-segments and NC genes and groups identified which were highly representative of sequences from recent outbreaks of the

virus. Consensus sequences were derived from these groups and included in the transfer vector. The phylogenetic analysis also revealed that the sequences of current RVFV vaccines are phylogenetically distant from viruses isolated from current outbreaks, although high levels of sequence conservation was maintained across all viral strains.

This is the first study in which the RVFV genes coding for proteins that will induce a protective immune response (Gn and Gc, as well as the nucleocapsid (NC) gene) were selected so as to be representative of current outbreak strains of the virus. These genes were inserted between LSDV ORFs 49 and 50, a novel insertion site. The transfer vector also contained an eGFP marker gene and an ECO-GPT selection gene, located outside of the LSDV flanking sequences. This meant a two-step isolation procedure, first to isolate the recombinant containing the entire transfer vector with eGFP and ECO-GPT, and then to isolate a recombinant with only the RVFV genes and not eGFP and ECO-GPT. Transient expression of RVFV proteins in cells infected with Herbivac and then transfected with the transfer vector was confirmed via western blotting and immunofluorescence. Here the proteins Gn, Gc and NC were shown to be expressed. In the present study, a single crossover Herbivac-RVFV recombinant was isolated through multiple passaging of cell lysates, originally obtained from Herbivac-infected FBT cells transfected with the transfer vector, in the presence of mycophenolic-acid selection medium.

This study provides evidence that Herbivac displays different characteristics to its parent, nLSDV. An immunogenically improved vaccine for the prevention of LSDV has economic implications in the cattle rearing industry. Additionally, the first crossover recombinant Herbivac-RVFV was isolated. Our final product is proposed to function as a dual vaccine to protect cattle against RVF and LSD, and to protect goats and sheep against goatpox and sheeppox respectively, as well as RVFV.

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## 1.1 Lumpy Skin Disease Virus

### 1.1.1 Lumpy Skin Disease and its Economic Importance

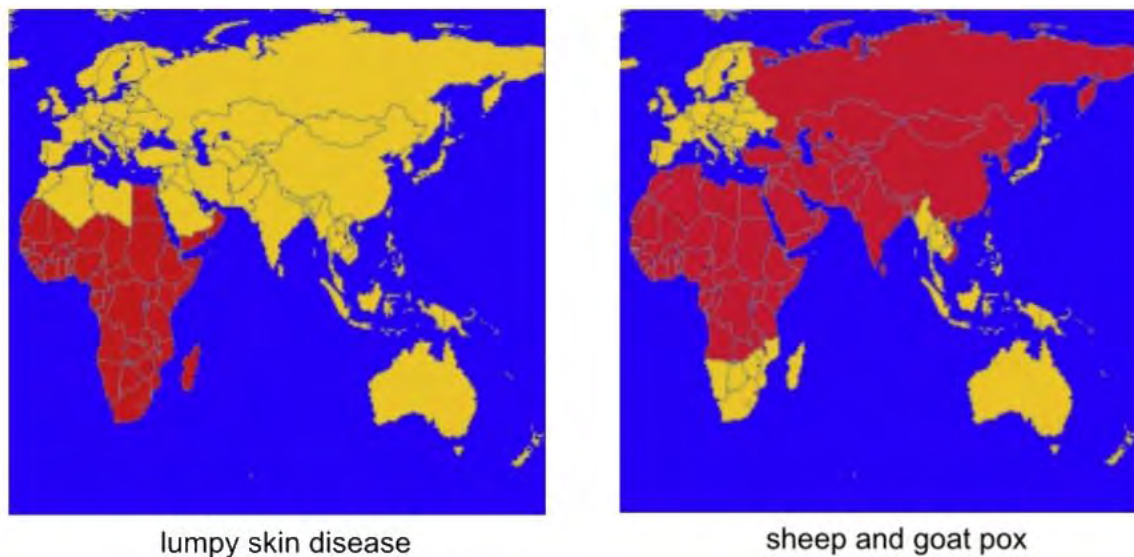
Lumpy skin disease virus (LSDV) is a member of the *Capripoxvirus* genus of the *Poxviridae* family of viruses and is the causative agent of lumpy skin disease (LSD) in cattle [1].

LSDV is endemic within Africa and is an emerging threat to Europe, the Middle East and Asia [2]. The morbidity and mortality rate of LSD varies from 2% to 12% and is largely dependent on the breed of cattle, where local breeds are less susceptible than European breeds [3]. Lactating cattle have been found to be the most susceptible to disease. The rate of morbidity is often higher during an outbreak and can rise to 85% [4], [5]. LSD in cattle ranges from subclinical to an acute infection and usually lasts for 2-5 weeks. The acute infection is characterised by fever and localised or disseminated nodules developing on the skin, where lesions are often found in the upper respiratory tract and a secondary bacterial infection often occurs [6]. Nodules have been reported to occur on the skeletal muscles and the mucosa of the oral and upper respiratory tract. Systemic effects include pyrexia, anorexia, dysgalactia and pneumonia [6]. Cows may lose reproductive ability and milk producing capacity for several months. Fever, anorexia and abortions are also common [6]. The drop in milk production in lactating cows along with temporary or permanent infertility in cows and bulls and skin lesions caused by the virus all impact on the economic effects of the disease [2]. Eradication campaigns and imposed trade restriction on live animals and animal products add to the financial toll of an outbreak. In Africa economic losses due to LSD are comparable to that of foot and mouth disease. This has resulted in the World Organization for Animal Health (OIE) recognising LSD as a notifiable agricultural disease [7].

### 1.1.2 Host range and viral transmission

LSDV is only able to naturally cause disease in cattle despite a high level of sequence identity shared with sheeppox virus (SPV) and goatpox virus (GPV) [8–10]. Some isolates of sheep and goatpox virus are able to infect both sheep and goats; however most isolates display host preference for either sheep or goats [8]. These host range characteristics are reflected in the independent distribution of the different *Capripoxvirus* species (shown in Figure 1.1) [11], [12]. Wild game animals such as giraffes and impala have been shown to be permissive

for wild-type LSDV where, during experimental infection, both animal species displayed pathology resembling that of LSDV infection in cattle [13].



**Figure 1.1** Geographic distribution of LSD, sheeppox and goatpox over a 25 year period (from 1987 to 2012). Affected areas are shown in red. Figure obtained from Boshra *et al.* (2012)[14].

Although experimental infection can be done subcutaneously, in the wild LSDV is believed to be spread via arthropod vectors [15], [16]. This finding is supported by a study involving experimental infection of cattle with LSDV. Here experimental infection of cattle could only be achieved via the intravenous route [1]. In a separate study, examining LSDV transmission, *Aedes Aegypti* female mosquitoes were shown to successfully transmit virus to naive cattle after feeding on infected cattle [12]. Earlier studies describe the isolation of LSDV from flies *Stomoxys calcitrans* and *Biomyia fasciata* although mechanical transmission was not investigated [18]. The arthropod transmission theory is further supported by records of LSD incidence being higher during wet periods when arthropod occurrence is higher and the incidence waning during dryer periods [17], [19].

### 1.1.3 Commercially available vaccines

LSDV is closely related to two other members of the *Capripoxvirus* genus, sheeppox virus and goatpox virus, the causative agents of sheeppox and goatpox respectively [20]. Sheeppox virus, goatpox virus and LSDV have been shown to be antigenically indistinguishable and vaccination with a vaccine against one of the *Capripoxvirus* strains is said to protect the vaccinated animal against all three strains [9], [21]. There are four commercially available vaccines for LSD, the Neethling strain LSDV (nLSDV), Herbivac, Lumpyvax and Kenyan strain sheeppox virus (KS-1) [7], [22].

nLSDV was developed in the 1950s, at the Onderstepoort Veterinary Institute in South Africa, through attenuation via 61 serial passages of a field isolate (Neethling strain) in lamb kidney cell culture monolayers. Further attenuation was carried out through 20 passages on the chorioallantoic membranes (CAMs) of embryonated hens' eggs followed by 3 passages in lamb kidney cell monolayers. At this point the virus was shown to be significantly attenuated and suitable for use as a vaccine in cattle. The vaccine was then passaged by Weiss (1968) a further 10 times in Madin-Darby bovine kidney cells, followed by 5 passages in primary bovine testis cells [23], [24]. Following this, the live attenuated vaccine has been grown for commercial purposes in primary lamb testis cells which has further increased its passage number. The KS-1 strain was isolated from sheep and attenuated through 16 passages in lamb testis cells [1]. Both vaccines are currently used in preventing LSD.

Despite the nLSDV vaccine being widely used it has been shown to have a 'take' as low as 40-60% (Personal communication with Dr Louis Maartens, Deltamune). A 'take' is the formation of a localised nodule at the site of injection. This low level of take may be due to the presence of existing anti-LSDV antibodies in the vaccinated animal, improper administration of the vaccine or reduced vaccine efficacy. It is also largely possible that the large number of serial passages of LSDV on CAMs and in primary cells followed by further passaging for vaccine production has attenuated the vaccine to the point of reduced efficacy.

Two sheeppox strains (the Romanian strain and RM-65) have also been used as prophylaxis against LSD in cattle. All agriculturalists rearing cattle are recommended to vaccinate their livestock against LSD and provide their cattle with annual booster vaccinations [25].

#### **1.1.4 Virus structure and life cycle**

Members of the *Poxviridae* family share numerous characteristics and research findings from one genus can often be extrapolated to other genera. Due to its instrumental function as a vaccine in the smallpox eradication campaign, vaccinia virus has become a focal point for research interest in poxviruses. The study of vaccinia virus has thus provided much information regarding the characteristics and general life cycle of poxviruses [26].

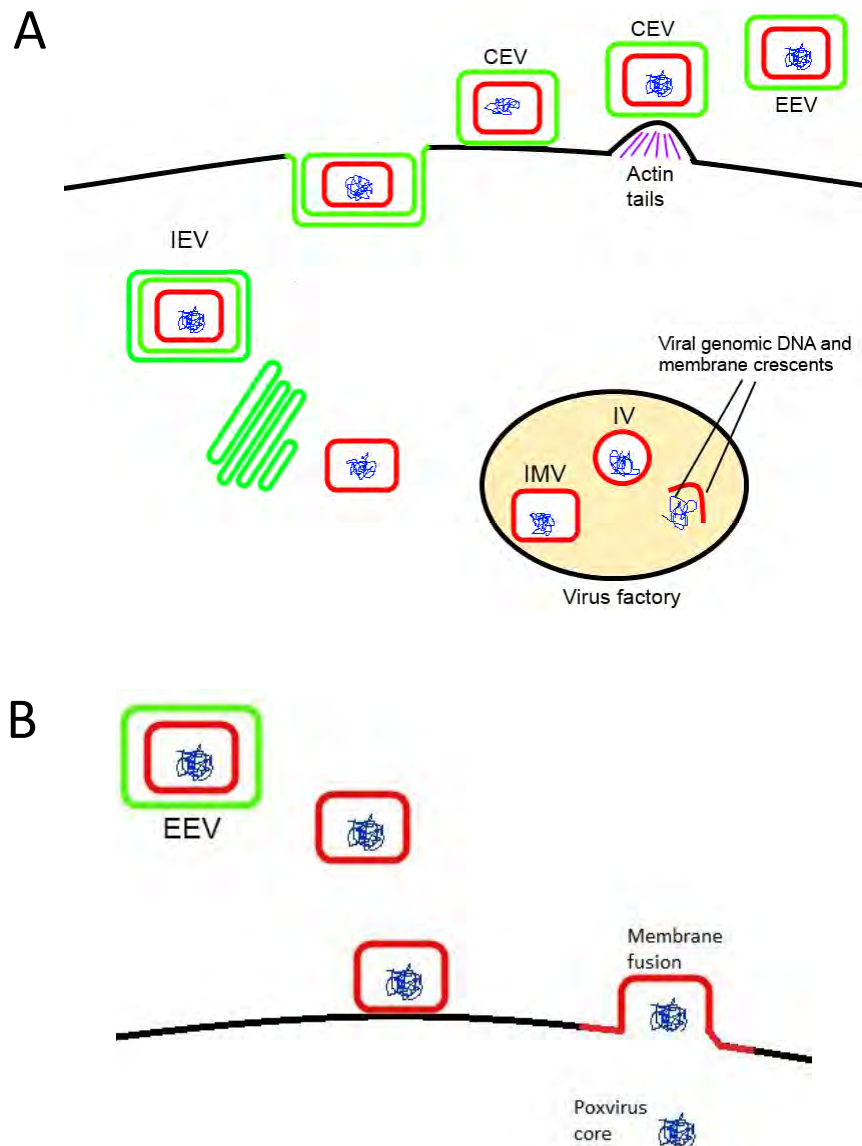
Poxviruses have large brick shaped virions approximately 250nm by 300nm in size and undergo DNA replication in the cytoplasm of host cells [27]. There are three key stages in the poxvirus lifecycle 1) attachment of the virus to the host cell membrane and release of the poxvirus core 2) viral DNA replication and transcription of viral genes followed by 3) assembly of infectious viral particles which are released through budding or cell lysing [26].

The poxvirus core enters the host cell initiating the infection process. The poxvirus core contains structural proteins, a linear double-stranded DNA genome and the enzymes required for early gene regulation [28]. The viral cores are transported along microtubules deeper into the cytoplasm [29]. Cores accumulate in the perinuclear regions of the cell, forming viral factories, where transcription of viral early mRNAs occurs using the virus associated DNA-dependent RNA polymerase [30], [31]. The replication process is then initiated, the stages of which are largely determined by the three groups of poxvirus genes; early, intermediate and late [32]. Early gene expression occurs within 2 hours post-infection where early gene products function mainly to modify the host cell environment and aid in viral escape from host immune responses [33]. Early gene expression is followed by poxvirus DNA replication [26]. During DNA replication, intermediate gene expression occurs. Intermediate genes are fewer in number compared to early genes and their main function is to regulate the expression of late genes. Late genes, in turn, generally encode final stage structural proteins responsible for virion formation, enzymes and transcription factors

required for the next round of replication [34]. Following late gene expression a crescent shaped structure forms. This structure consists of a host derived single layer lipid membrane and viral proteins. The formation of viral crescents marks the early stages of virion formation [35].

The infectious poxvirus occurs in four distinct forms. The forms are the intracellular mature virus (IMV), which makes up the majority of virus particles, the intracellular enveloped virus (IEV), the cell-associated extracellular virus (CEV) and the extracellular mature virus (EMV) [36]. During early morphogenesis viral crescents grow to form spherical shapes that encapsulate the virus core components to form what is known as immature virions (IV). These IVs then mature into infectious brick shaped IMV through the proteolytic cleavage of core proteins [37]. IMV is the first infectious form of the virus to occur and is encapsulated in a single lipid membrane. This form of the virus remains within the host cell until the cell lyses. Small subsets of IMV particles are enveloped by host cellular membranes (derived from endosomes or the Golgi network) to form IEVs [38]. IEVs are transported along microtubules from the viral factories toward the periphery of cells. Here at the outer membrane of the host cell the outer membrane of IEVs fuse with the plasma host membrane. The virus may remain associated with the extracellular cell surface forming CEV which makes use of cellular actin filaments to aid in cell to cell viral transmission. Particles are also released from the cell surface forming EMVs which are thought to aid in long range transmission of the virus [36], [38–41]. The wrapping of viral particles in host membrane(s) aid in viral evasion of the host immune response [26].

The IMV and EMV disseminate through the host. IMV infects uninfected host cells through the use of viral attachment transmembrane proteins [42]. For the attachment of EMV, the outer membrane is first shed; following this attachment proteins in the inner membrane attach to the host cell membrane. Viral membrane attachment is followed by fusion of the IMV membrane with the host membrane resulting in cellular entry of the poxvirus core [43]. Figure 1.2 indicates the poxvirus lifecycle described.



**Figure 1.2 (A)** Overview of poxvirus morphogenesis pathway. Viral factories are formed in the cytoplasm of host cells, here crescent membranes are synthesised which enclose viral DNA and proteins to form a circular immature virion (IV). Proteolytic cleavage of poxviral capsid proteins causes the transition from IV to a brick shaped intracellular mature virus (IMV). Subsequent Golgi membrane wrapping of the IMV outside of the viral factories results in the formation of the intracellular enveloped virus (IEV). IEV buds out of the cellular plasma membrane through exocytosis. Here the virus remains associated with the cell in the form of cell-associated enveloped virus (CEV) which forms actin tails in the cell's cytoplasm which aid in cell to cell transmission of the virus. Alternatively viral particles are released as extracellular enveloped virus (EEV). **(B)** Entry of EEV into host cell. Viral membrane attachment and fusion of the IMV membrane with the host membrane results in cellular entry of the poxvirus core. This Figure has been adapted from a publication by Smith *et al.* (2002).

### 1.1.5 The use of poxviruses as vaccine vectors

Through a deep understanding of the poxvirus lifecycle along with the advent of DNA manipulation techniques, it was found that foreign DNA could be stably inserted into the genome of vaccinia virus and other members of the *Poxviridae* family [33], [36], [44–46]. Foreign DNA insertion can be achieved through homologous recombination within poxvirus infected cells, where infected cells are transfected with plasmids containing the foreign DNA flanked by poxvirus DNA [33], [47]. In such an experiment during the cytoplasmic replication cycle of poxviruses, homologous recombination may occur between the native poxvirus DNA and the same sequence poxvirus DNA present in the plasmid. This homologous recombination will then result in the incorporation, at a specific site, of the foreign plasmid DNA contained between the two flanking poxvirus DNA sequences.

In order to produce a recombinant poxvirus capable of correctly expressing the foreign genes of interest an understanding of poxvirus transcriptional regulation is needed. This understanding will enable correct design of a plasmid containing the foreign gene(s). Poxviruses undergo DNA replication in the cytoplasm of infected cells and therefore make use of their own poxviral DNA and RNA polymerases [48]. This highlights the importance of designing foreign genes with a poxvirus promoter sequence upstream of the gene(s) start site with its own translation initiation codon (ATG) and stop codon (TAA) [49]. Various poxvirus enhancer elements have also been shown to affect foreign gene expression and the presence of poxvirus early transcription termination sequences (TTTTTNT) may disrupt gene expression [50]. There is a large amount of literature confirming poxvirus genomic stability and its ability to stably integrate up to 25Kbps of foreign DNA as well as synthesise foreign gene products that are correctly processed and immunogenic [51–60].

With the emergence of human-immunodeficiency virus (HIV) and the need for alternative vaccine vectors, a focus has been placed on the use of poxviruses as vaccine vectors for expressing HIV immunogens [61]. Clinical studies have thus far been conducted on a variety of poxvirus vectored HIV vaccines, the most notable vectors being canary poxvirus (ALVAC), modified vaccinia Ankara virus (MVA), NYVAC (an attenuated vaccinia virus strain) and fowlpoxvirus [62–65]. *Avipoxviruses* and MVA are especially favourable for construction of HIV vaccines as they undergo abortive replication in humans, thereby eliminating many of

the concerns relating to the use of live vectors in immunocompromised persons [66], [67]. The most well publicised use of an *Avipoxvirus* vectored vaccine is the phase III clinical trial evaluating the use of ALVAC expressing HIV gag, pol and nef genes along with recombinant gp120 protein in a four dose regime amongst participants in Thailand [65]. The trial involved 16402 healthy volunteers and showed 31.2% fewer infections ( $p=0.04$ ) occurring in the vaccine arm compared to the control arm after a three year follow-up period [65].

Poxvirus vectors have also been used extensively in the creation of recombinant veterinary vaccines. A highly successful field rabies veterinary vaccine, comprising a recombinant vaccinia virus expressing immunogenic rabies glycoprotein (VRG), was used to orally vaccinate wild foxes in Europe and Canada against rabies [68]. VRG was shown to be non-pathogenic in target species, non-target species and non-human primates [69], [70]. In Europe VRG was constituted in a thermo-stable bait and used successfully to eliminate sylvatic rabies in foxes from large areas of land [71], [72].

#### **1.1.6 LSDV as a vaccine vector**

Due to the extensive use of live attenuated LSDV vaccines and the genetic malleability of poxviruses, LSDV has been used as an effective vector for development of recombinant vaccines to provide protection against LSD and the virus from which the foreign genes were taken [56–60]. Table 1.1 shows recombinant LSDV vaccines made to date. This table shows the diverse range of antigens that have been inserted into LSDV. As can be seen in table 1.1, most antigens belong to viruses that infect hosts that are also permissive to LSDV, such as Bluetongue, rinderpest, rabies, Rift Valley fever or Peste des petits ruminants virus and *E. ruminantium* bacteria, thereby creating a dual veterinary vaccine. However others have inserted HIV antigens in order to use LSDV as a replication deficient vaccine vector for human use [73].

Choosing an attenuated LSDV as a vaccine vector for expressing foreign antigens in cattle is an appropriate choice as live attenuated LSDV is commercially used to vaccinate cattle against LSD. The attenuated virus is replication competent in cattle which means it may be able to express foreign proteins for longer periods of time compared to replication incompetent poxviruses; this will aid in its ability to induce both humoral and cellular

immunity [74–77]. Several LSDV recombinants have proven their efficacy in a wide range of permissive and non-permissive animals including mice, sheep, cattle, rabbits and goats. LSDV has also been proposed as a potential vector for human vaccines [22], [74], [78]. This will prove useful when conducting preliminary efficacy testing of the proposed recombinant vaccine in small animal models such as mice and rabbits. There is no evidence to suggest that humans are permissive to LSDV; this makes it a more attractive vaccine vector compared to vaccinia virus as vaccinia virus is able to cause disseminated disease in immunocompromised individuals [66]. This drawback of vaccinia virus is becoming increasingly significant as the incidence of HIV in developing countries is extremely high [79]. LSDV is host restricted to cattle, goats and sheep and the vaccine form is not transmitted horizontally from vaccinated to unvaccinated animals [80], [81].

Poxviruses have been successfully used as vaccine vectors for correctly expressing foreign antigens. Sometimes the recombinant vaccine functions as a dual vaccine and sometimes as a replication deficient viral vector delivering a specific antigen. Several attempts have been made at developing a recombinant nLSDV-RVFPV veterinary vaccine; however, multiple inoculations were necessary and/or the experiment could not clearly evaluate efficacy [14], [57], [82].

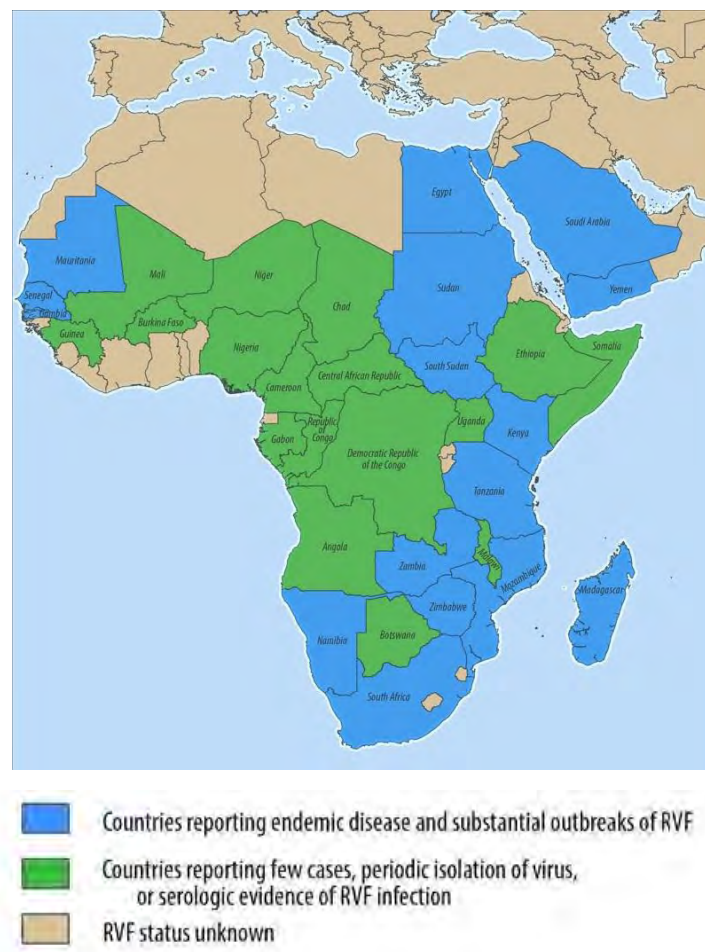
**Table 1.1 Previously constructed recombinant LSDV vaccines**

Antigen(s) expressed	Antigen insertion site	Promoter(s)	Summary of Key results	Reference
1H12 ORF of <i>E. ruminantium</i>	Ribonucleotide reductase gene	VV 7.5kDa early/late promoter	20% protection was obtained in sheep immunised with DNA prime, recombinant LSDV boost when subjected to natural tick challenge in the field. The immunisation strategy did not confer significant protection against field challenge.	[83]
Bluetongue Virus antigens. Two capsid proteins and two non-structural proteins of BTV serotype 2.	Thymidine kinase gene	VV Synthetic promoter	Sheep were immunised with recombinant <i>Capripoxviruses</i> individually expressing four different bluetongue virus genes. Partial protection of sheep was achieved when challenged with a virulent strain of bluetongue virus.	[60]
Hemagglutinin protein gene and Fusion protein gene of rinderpest virus	Thymidine kinase gene	VV late promoter p11	Cattle were vaccinated once with a mixture of recombinant LSDV expressing either the hemagglutinin protein or the Fusion protein of rinderpest virus. Approximately 50% of the cattle were protected from challenge with a lethal dose of virulent rinderpest virus 2 years after vaccination.	[84]
Gn and Gc glycoproteins of RVFV, structural glycoprotein of Bovine ephemeral fever virus	Thymidine kinase gene	VV 7.5kDa early/late promoter	Cattle were inoculated once with recombinant LSDV expressing bovine ephemeral fever virus structural glycoprotein. Cattle in vaccinated group showed similar symptoms to cattle in saline only group. The vaccine did not confer protection against symptoms.	[78]
Gn and Gc of Rift Valley fever virus	Thymidine kinase gene	VV 7.5kDa early/late promoter	Mice vaccinated with a recombinant lumpy skin disease Virus expressing RVFV glycoproteins developed neutralising antibodies and were fully protected when challenged. Further evaluation of the recombinant in a sheep model failed to show any significant difference upon RVFV challenge between vaccinated and unvaccinated sheep as a result of a failed challenge.	[57]
Gn and Gc glycoproteins of RVFV	Thymidine kinase gene	VV 7.5kDa early/late promoter	Mice vaccinated three times with recombinant LSDV expressing RVFV glycoproteins, were protected against challenge. Sheep vaccinated twice developed nAbs and were protected from challenge with RVFV and sheeppox virus.	[82]
Fusion gene of Peste des petits ruminants	Thymidine kinase gene	VV synthetic Early/late promoter pSS	A single dose regime protected goats against challenge with a virulent PPRV strain.	[85]
Hemagglutinin gene of peste des petits ruminants	Thymidine kinase gene	VV Synthetic promoter	Recombinant LSDV expressing peste des petits ruminants Hemagglutinin gene inoculated once provided protection against virulent peste des petits challenge.	[86]
Rabies glycoprotein gene	Ribonucleotide reductase gene	Fowlpoxvirus early/late promoter	Cattle were vaccinated with recombinant LSDV expressing rabies glycoprotein gene. The recombinant successfully induced both humoral and cellular immune responses to rabies glycoprotein.	[56]
HIV-1 subtype C Gag, reverse transcriptase (RT), Tat and Nef as a polyprotein (Grtn)	Ribonucleotide reductase gene	vaccinia virus early/late mH5 promoter	The rLSDV-grtn vaccine was immunogenic in mice particularly in prime-boost regimens with recombinant MVA or DNA as a prime. Mice developed HIV-specific cells producing IFN-gamma and IL-2. LSDV was demonstrated to be non-pathogenic in immunocompromised mice.	[73]

## 1.2 Rift Valley fever virus

### 1.2.1 Disease and socio-economic importance of disease

Rift Valley fever (RVF) is a notifiable enzootic agricultural disease found within Africa and the Arabian Peninsula (Figure 1.3). The disease affects cattle, goats and sheep and can be transmitted to humans. The World Bank reports agriculture to contribute more than 30% to gross domestic product of African countries, highlighting the significance of a disease such as RVF [87]. The recent emergence of RVFV in Saudi Arabia and Yemen has raised concerns about the emergence of RVFV in Europe, Asia, or the Americas. Such an introduction would result in significant economic damage as well as threaten human health and food security [88].



**Figure 1.3** Map showing countries and their RVF disease status as reported by the Centre of Disease Control and Prevention (CDC). Image taken from [www.cdc.gov](http://www.cdc.gov).

RVFV is of the *Bunyaviridae* family of the genus *Phlebovirus*. The first reported outbreak of the virus occurred in 1931 in the Kenya Rift Valley as an outbreak of necrotic hepatitis in a herd of ewes [89]. The virus has since spread and is endemic to most parts of Africa, including Madagascar, causing widespread infection and death amongst ruminants (sheep, goats, cattle, camels and water buffalo), causing losses of up to 20% of the ruminant population [89–93]. Sheep are often the worst affected by the virus which can cause death within 1 to 2 weeks post infection [14]. Sheep that do not die of infection often develop hepatic lesions, virus is detected within the blood within days of infection and horizontal transmission to uninfected sheep has been observed [94]. The high rate of mortality among ruminant foeti is mainly due to the viral induction of bloody diarrhoea, acute hepatitis, abortions in pregnant female lambs, foetus teratology and still births in sheep [89, 91, 95].

RVFV is an arbovirus and is spread between animals via insect vectors and passed on to humans through mosquito bites or by human exposure to bodily fluids of infected animals [95]. This includes the drinking of raw unpasteurised milk [96], [97]. Unlike many other arboviruses RVFV is not limited to a specific vector species and utilises several species of mosquito, and possibly ticks and flies to infect new hosts [15], [98].

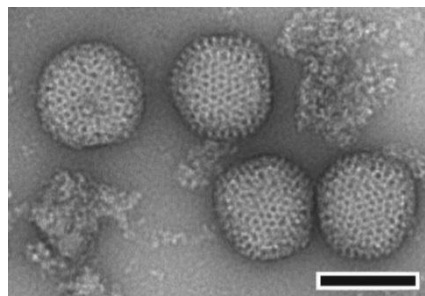
In addition to its effect on livestock, RVFV has also been shown to cause high levels of morbidity in humans [99], [100]. In humans the virus commonly brings about a self-limiting febrile illness [95]. In these cases the incubation period is typically between 4 and 6 days and symptoms include an elevated body temperature, severe chills, vomiting, diarrhoea, rigor and photophobia [89], [95], [101]. A small percentage of infected persons (1-2%) develop more severe conditions such as viral encephalitis, haemorrhagic fever, loss of vision and renal failure [95]. Among hospitalised patients the mortality rate can be up to 20-44%, the main cause of mortality relating to a haemorrhagic fever [97], [102–105].

The hospitalization of people, severe loss of livestock and reduced agricultural trade have resulted in significant economic losses. In Africa, the most severe outbreaks occurred in South Africa and Egypt in the 1950s and 1970s respectively [106]. The South African outbreak resulted in the deaths of 100,000 sheep and caused 500,000 live-stock abortions [107]. The outbreak in Egypt caused 623 human deaths and an estimated 18,000 persons

were infected. The outbreak also caused significant loss of livestock [108]. In 2000, the virus spread to the Arabian Peninsula (Northern Yemen and Gazan), where there was a total of 882 human cases with a 14% case fatality rate [97]. The presence of RVFV insect vectors in non-endemic countries and increasing international trade of animals and animal products gives RVFV the potential to become a global threat [14], [94], [109].

### 1.2.2 Virus structure, genetic organisation and life cycle

Early electron microscopy studies done during the 1977 RVFV outbreak in Egypt found particles to be between 90 and 100nm in diameter [110]. Later electron cryo-microscopy studies describe the NC protein coating genome segments to form viral ribonucleoproteins (RNPs). These RNPs are associated with numerous copies of the NC and viral RNA-polymerase and form the virion core [111]. Surrounding the RNP layer is a lipid bilayer and a shell of glycoproteins arranged in a heterodimer icosahedral lattice of approximately 220-222 glycoprotein capsomers [112], [113].



**Figure 1.4** Electron microscopy image of purified RVFV particles stained with 2% uranyl acetate. Image taken from Huiskonen *et al.* (2009). Scale bar, 100nm.

RVFV, along with all other *Bunyaviridae*, has a tripartite negative-stranded RNA genome [114] (Figure 1.5). The (L)arge segment contains a single open reading frame and encodes an RNA dependent RNA polymerase (or L protein). The (M)edium segment encodes a 78kDa protein which is a non-structural virulence factor (NSm) as well as two envelope glycoproteins (Gc and Gn) which are expressed as a fusion protein and later spliced [115], [116]. The multiple genes present on the M segment are differentially transcribed with multiple ATG start sites along the genome segment [17], [19], [119]. The (S)mall segment of the RVFV genome encodes a non-structural protein (NSs) and a nucleocapsid (NC) protein and makes use of an ambi-sense strategy to differentially express these two proteins [120].

The NC mRNA is transcribed in the negative sense of the S-segment and the NSs mRNA is transcribed in the positive sense [121].

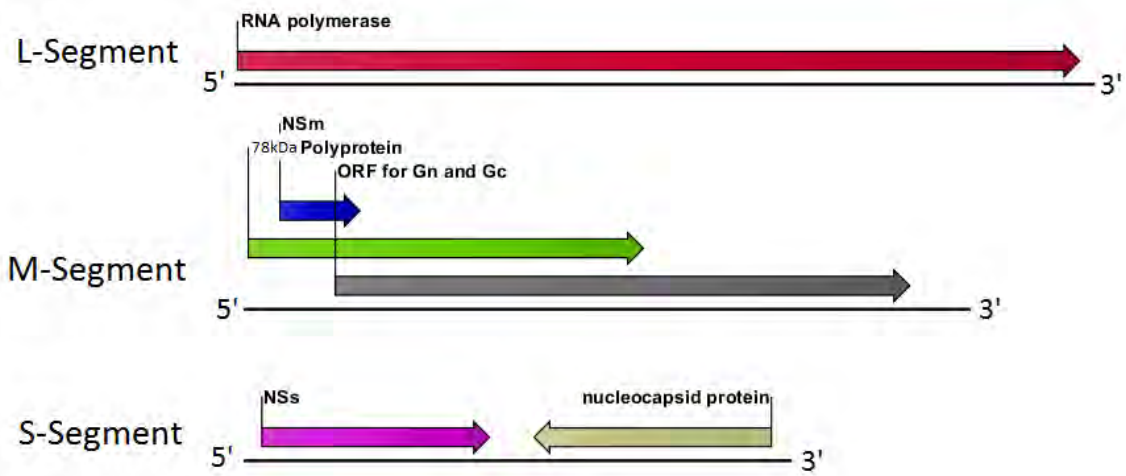


Figure 1.5 Diagram showing RVFV single stranded RNA genome. The tripartite genome is divided into three segments, the (L)arge, (M)edium and (S)mall segment. Arrows indicate the direction in which genes are transcribed.

The NC and L proteins are required for viral RNA synthesis, while Gc and Gn function as envelope glycoproteins. It has been shown that mutant RVFV unable to express the non-structural 78kDa protein is still able to replicate in cell culture [116], [119], and the role of the protein is uncertain. It is possible that the 78kDa protein may function as a decoy antigen as it contains the amino-acid sequence of the Gc and Gn glycoproteins. The decoy theory is further supported by the fact that the 78kDa protein is not found in purified particles [122]. The two non-structural proteins, namely NSm and NSs, while dispensable for viral replication in cell culture, have been shown to function as virulence factors [119], [123]. The NSs was found to aid in viral pathogenesis through the general suppression of transcription of host RNAs. NSs does this through interacting with the host p44 subunit of TFIIF, a transcription factor essential for the functioning of host RNA polymerase II [121], [124]. In addition; NSs specifically inhibits transcription of host IFN- $\beta$  by binding to Sin3A-Associated Protein 30 (SAP30) [125], [126]. SAP30 is important for maintaining the repressor complex on the IFN- $\beta$  transcription regulatory region, binding of NSs to SAP30 functions to maintain the repressor function early in infection [127]. NSs and NSm have also been shown to be involved in infection and dissemination in mosquito species, where mutant RVFV lacking both accessory proteins failed to infect and replicate in *Aedes aegypti* mosquitoes

(which are primary reservoirs and vectors for the virus)[128]. The NSm accessory protein was shown to reduce levels of apoptosis in infected cells through the inhibition of staurosporine induced cleavage of caspases 8 and 9, where the cleavage of caspases 8 and 9 function as pivotal junctions in the apoptosis pathway [116]. In a separate study it was demonstrated using a rat model that the NSm protein is non-essential for virulence and lethality, although RVFV lacking NSm showed delayed onset of neurologic disease in infected animals [95]. This gene may be involved in the neurologic symptoms sometimes seen in humans and experimentally infected animals [129–132].

The NC protein coats the L, M and S segments and associates with numerous copies of the L protein to form ribonucleoproteins (RNP) which constitutes the virion core [133], [134]. The tripartite RNA genome core is then enclosed by a lipid bilayer and a glycoprotein envelope made up of Gn and Gc heterodimers [135]. The glycoprotein envelope is essential for the proper fusion and entry of the virus particle into the host cell through the binding of yet unidentified host cellular receptors [136]. Fusion and entry of the virus particle allows the release of the RNPs into the cytoplasm [137]. The virus replicates in the cytoplasm and virions are assembled by budding into the lumen of the Golgi [138], [139].

Localization studies have shown that Gc and Gn are localised to the Golgi complex and that Gc expressed on its own localises to the endoplasmic reticulum, suggesting that the Golgi localization signals reside in Gn and that Gc migrates to the Golgi via physical association with Gn [139], [140]. Towards the end stages of infection the Golgi complex undergoes vacuolization and disperses vacuoles of varying sizes into the cytoplasm [141]. These vacuoles are then transported to the cellular membrane with which they fuse to allow release of mature viral particles into the extracellular space [141].

### **1.2.3 Currently available vaccines**

Since RVFV was first isolated in 1931 vaccines have been developed for the disease and the WHO recommends vaccination as the first line of defence against the virus [89], [100]. The first attempts at a vaccine were formalin inactivated and live attenuated versions of the virus which have been used extensively in Africa to control spread of disease [142]. Live attenuated vaccines circumvent the issues surrounding expensive multiple inoculation

regimes and are used extensively in livestock vaccination campaigns within endemic countries. Live attenuated vaccines also allow for a single inoculation to obtain protective immunity in animals [100]. Two commercially available live attenuated RVFV vaccines are discussed here.

#### ***1.2.3.1 Inactivated RVFV vaccine, TSI-GSD 200***

Formalin inactivated RVFV vaccines are easily made, provide a protective immune response and are commercially available for veterinary purposes [123], [143]. A formalin inactivated RVFV vaccine, TSI-GSD 200, was developed and found to be both safe and efficacious in human trials and is the only vaccine approved for human use [143], [144]. A total of 598 volunteers were vaccinated and after four subcutaneous inoculations nearly all vaccinee non-responders were converted to responders and achieved a protective anti-RVFV antibody titre [143]. A formalin inactivated veterinary vaccine used for the safe immunization of animals was derived from a South African RVFV strain which was passaged through mouse brain and cell culture [145]. This vaccine has been shown to have no adverse reactions and is safe for use in pregnant animals but requires two initial inoculations and annual boosters; in addition the vaccine is expensive to produce [99], [145]. Such inactivated vaccines are preferred in non-endemic countries at risk of RVFV introduction [99]. Despite their safety profile and efficacy, formalin inactivated vaccines are logistically impractical for large scale use in animals as they require up to three initial inoculations and annual boosters to maintain long term immunity, both of which add to the costs of vaccination campaigns [143].

#### ***1.2.3.2 Live attenuated MP-12 vaccine***

A wild type RVFV strain, wtZH548, was attenuated through 12 serial passages in the MRC5 cell line in the presence of 5-fluorouracil to obtain the vaccine strain MP-12 [146]. MP-12 is highly immunogenic and is under development as a human and veterinary vaccine [142]. Ewes in the middle stages of pregnancy (70-100 days) vaccinated with MP-12 developed antibodies which are passed onto progeny [147]. The antibody titre of the progeny increased from 10 at birth to greater than or equal to 80 after ingestion of colostrum [147]. However, when ewes were vaccinated in their early stages of pregnancy (28<sup>th</sup> day) the vaccine induced low levels of abortion (4%) and teratology (14%) [148]. The MP-12 vaccine

was also administered to in-utero foetuses and at time of delivery both the dams and new born calves had a protective serum neutralizing antibody titre [149]. The MP-12 vaccine was also given to calves as young as 2 days old where protective antibody titres were induced and calves were protected against virulent RVFV challenge [149]. The live attenuated MP-12 has been shown to contain an intact and functional NSs protein (a RVFV virulence factor) which may explain some of its retained pathogenicity [121], [150]. In other studies, the MP-12 vaccine was shown to be safe and efficacious for use in pregnant or lactating bovids and sheep [151], [152].

### ***1.2.3.3 Live attenuated Smithburn vaccine***

Another live attenuated vaccine known as the neurotrophic Smithburn vaccine was isolated from mosquitoes in Uganda and has been developed as a vaccine through 102 serial passages through sucking mice using the intracerebral route [153]. Sheep and cattle were found to respond well to the vaccine and nearly all animals were protected against challenge with the wild-type strain. However the vaccine did cause abortions in a low percentage of sheep and cattle [153], [154]. The vaccine strain is now grown in hamster kidney cells and has been used extensively in South Africa, Kenya and Egypt [100], [154]. The Smithburn vaccine requires only a single inoculation, is cheap to produce, provides life protection in animals and is widely used in endemic countries [99], [153].

As discussed above, inactivated RVFV vaccines maintain a good safety profile but are logistically impractical and expensive as they have low levels of immunogenicity and thus require multiple inoculation regimes. Highly immunogenic live attenuated vaccines circumvent the issues surrounding multiple inoculations but run the theoretical risk of reversion back to the pathogenic wild-type [100]. For this reason live-attenuated vaccines are only recommended for use in countries where RVFV is endemic so as to prevent introduction of the virus into disease free regions [123]. Furthermore live attenuated vaccines have retained pathogenicity and have been reported to induce neuropathological complications and cause abortions or teratology of the foetus [100], [148]. Clearly, there is a need to develop an alternative RVFV vaccine which is safe, affordable and highly immunogenic so that a single inoculation is sufficient for long term protection.

#### 1.2.4 Antigenic Determinants

Due to the costs and impracticality of whole killed vaccine and the risks and complications associated with live attenuated vaccines, more recent next generation vaccines are being developed to resolve these issues. For the development of such vaccines the correct antigenic determinants and appropriate immune response have to be identified.

The Gc and Gn RVFV envelope glycoproteins form the outer shell of the virus and are highly exposed to a humoral immune response. In addition the glycoproteins are responsible for virus-cell fusion and entry of the virus into the host cell explaining why mAbs binding to specific regions of Gc and Gn are able to neutralise the virus [155], [156]. Gc and Gn have been shown to induce a protective immune response and provide cross protection against most field isolates of RVFV as the genes encoding the two glycoproteins undergo minimal genetic change and different geographical isolates of RVFV have been found to be of a single serotype when evaluated using neutralization assays [157], [158]. Mapping of RVFV envelope glycoproteins Gc and Gn by Besselaar (1991) revealed four distinct antigenic domains on each envelope glycoprotein. In addition, Besselaar showed that Gc antigenic domains I, II and IV were involved in virus neutralization and haemagglutination [156]. Besselaar went on to show that Gc Ia and Gn Ia are associated with strong neutralization responses and epitopes Gc Ia and IIb and Gn Ia and IIb confer protection against virulent challenge in mice [156]. From the above it is clear that nAb responses are able to confer protection against the virus and that the envelope glycoproteins are able to induce cross-neutralizing antibodies.

Various studies have also been conducted to determine the antigenic determinants of the NC protein. Thus far no known neutralizing epitopes for the NC can be found and protection is thought to involve cell-mediated immunity [57]. Also, studies evaluating the immunogenicity of proteins produced by another filovirus, the Toscana virus, found that anti-NC antibodies had only partial neutralizing activity and the NC protein induced a CD8+ T-cell response in mice [159], [160]. In the case of RVFV, antibodies against the RVFV-NC protein can be identified in mice after challenge with RVFV. Further studies need to be conducted before one can conclusively say that there are no neutralizing anti-NC antibodies and to determine possible functions of the non-neutralizing antibodies [161]. The NC

protein induces a non-neutralizing antibody response and a cellular immune response. While immune responses to the NC protein may not confer full protection against the virus, we speculate that the added cellular and non-neutralizing antibody response on top of the nAb response to the envelope glycoproteins may allow for enhanced protection against the virus.

Advancements in molecular techniques along with additional knowledge of the antigenic determinants of RVFV have allowed for the development of alternative vaccines. Current experimental vaccines are discussed below.

### **1.2.5 Rational design of vaccines**

#### **Reverse engineered attenuated RVFV vaccine**

A more modern form of a live attenuated vaccine was made by Bird *et al.* in 2008. Bird used a reverse genetics system to generate RVFV mutants with deletions in the NSs and NSm genes (both virulence factors) [123]. The virus was highly attenuated in rats and a single dose induced a robust anti-RVFV antibody titre at 26 days post infection. Additionally no detectable viremia or clinical illness was observed and the vaccine showed safety and protection in pregnant ewes [123]. Furthermore, all vaccinated rats resisted a high dose of virulent RVF virus [123]. This positive advancement highlights the importance and practical implications of creating a safe, immunogenic, rationally designed vaccine.

Such a vaccine would be a viable replacement for currently available live attenuated vaccines as it has shown a good safety profile in rats. However the live vaccine would still allow for recombination with virulent RVFV within the vaccinated animal raising GMO ethical issues. There is a theoretical chance of reversion to virulence of live attenuated strains but the scientific proof of such events for RVFV have not been published [109].

### **Baculovirus expressed RVFV antigens**

The immunogenic properties of Gc and Gn were tested by Schmaljohn *et al.* in 1989. The proteins were produced in a baculovirus expression system and used to inoculate mice, which were subsequently challenged with RVFV [161]. A single inoculation with Gc and Gn lysates and a double inoculation containing only Gn lysate protected mice from lethal challenge with RVFV [161]. Survivors of the challenge developed antibodies to Gc, Gn envelope proteins and the RVFV nucleocapsid protein (NC). In addition, naïve mice were also protected by passive transfer of antibodies from immunised mice [161].

### **Bacterially expressed RVFV antigens**

The work done by Schmaljohn *et al.* was further corroborated by Keegan *et al.* who identified four antigenic domains in Gn, three of them neutralizing [162]. Keegan *et al.* went on to show that guinea pigs hyperimmunised with bacterially expressed RVFV GnGc produced antibody reactivity with three major proteins: Gc, Gn and the NC. Also, Gn is able to induce virus-neutralizing and protective antibodies when used by itself and mAbs against Gn are able to protect animals against lethal RVFV infection [162]. Lastly, Keegan *et al.* showed that a bacterially produced Gn polypeptide- $\beta$ -galactosidase fusion protein was immunogenic and maintained antigenicity. This has positive implications for recombinant vaccine technology where one desires the expression of RVFV Gn along with another immunogen [162].

In a separate study, the protective ability of bacterially expressed truncated Gn (tGn) protein was tested for its efficacy in mice when inoculated subcutaneously using a prime boost regime. The tGn protein used in this study contained three neutralizing epitopes and when a tGn lysate was used as an antigen, 100% of mice were protected from RVFV at 50% lethal dose for mice [57]. Following this, the immunogenicity of RVFV NC protein, bacterially expressed, was assessed in mice. 60% of mice inoculated with purified NC protein were protected from RVFV challenge (compared to 20% in the control) [57]. A further study evaluating the sole use of NC protein as a vaccine antigen made use of the subunit NC in combination with various adjuvants (ISA50, Alhydrogel, TiterMax Gold or SaponinQ) in a mouse model as well as in sheep [163]. In this study immunization with NP in combination

with Alhydrogel conferred 100% protection against morbidity, mortality and viral replication in mice, however sterilizing immunity could not be achieved in sheep with any NP/adjuvant combination. This study questions the use of the mouse model for reliably studying the protective ability of RVFV subunit vaccines [163].

### **DNA expressed RVFV antigens**

Another vaccination approach makes use of a recent technology of genetic immunization with cDNA encoding RVFV antigen. Genetic immunization is a cheap method but requires multiple immunizations to induce a protective antibody response and RVFV DNA vaccines have shown variable successes in animal models [164].

A study by Lagerqvist *et al.* (2009) aimed to identify antigenic determinants of the NC protein. They analysed serum from mice infected with RVFV and serum from mice vaccinated with cDNA encoding the NC gene. A strong uniform NC specific Ab response was found in all mice [165]. However, in the same study no antibody response was detected against truncated sections of the NC protein (amino-terminus, middle section and carboxy-terminus) [165]. Interestingly, the vaccinated group, but not the infected group, reacted to the truncated amino-terminus NC protein and other studies have shown strong antigenic determinants located near the amino-terminus of the NC protein of other viruses of the Bunyaviridae family [165], [166]. The study by Lagerqvist concluded the lack of reactivity against the truncated NC proteins was most likely due to distortion or disruption of conformational epitopes [165], thus a RVFV vaccine should include full length NC protein in order to maintain antigenicity.

In a separate study, a DNA vaccine expressing Gc and Gn glycoproteins, administered either alone, or as a prime followed by a recombinant LSDV-RVFV boost did not confer any protection in mice [57]. A study making use of a DNA vaccine expressing Gc and Gn glycoproteins found that after three inoculations of the vaccine all mice developed nAbs and survived challenge [167].

Different RVFV antigens for the genetic vaccination of mice were examined in a four dose regime [165]. One group of mice were given cDNA encoding the NC protein (cDNA-NC) and

another given the Gc and Gn glycoproteins (cDNA-GcGn). The cDNA-NC was shown to induce strong humoral and lympho-proliferative immune responses and protected approximately 50% of mice against challenge. Despite the strong anti-NC specific Ab response, no virus neutralizing Abs could be detected post cDNA-NC vaccination and protection was concluded to be due to cellular immunity. All mice vaccinated with cDNA-GcGn seroconverted and produced virus neutralizing antibodies. 50% of the mice were protected [165]. When vaccinated with cDNA encoding Gc only, only 50% of mice seroconverted [165]. These studies highlight the limited potential of first generation DNA vaccines to provide sterilizing immunity and show once again the protective function of the NC protein, which likely involves a T-cell mediated correlate of protection.

### **Rift Valley fever virus-like particles (VLPs)**

Several studies have researched the use of VLPs for the purpose of vaccinating of animals against RVFV [168–170]. Liu *et al.* reports the self-assembly of RVFV structural proteins, NC, Gc and Gn, to form VLPs exhibiting enveloped structures resembling the wild-type RVFV virion particle. Liu also reports the formation of nucleocapsid like particles by monomers of NC protein [168].

A VLP of RVFV was constructed by transfecting cells with cDNA plasmids expressing viral L, M and NC genes along with an RNA construct which functions as a genome which allows the VLP to express the luciferase reporter gene [170]. 11 out of 12 mice inoculated three times with  $1 \times 10^6$  RVFV-VLPs/dose were protected against challenge [170]. The RVFV-VLP was then re-engineered to express the NC gene instead of the reporter gene [169]. This time, a single inoculation of  $1 \times 10^6$  RVFV-VLPs/dose was able to protect 100% of mice from lethal challenge with RVFV [169]. This study confirms the added protection provided through the inclusion of the NC protein as a vaccine antigen along with the envelope glycoproteins.

### **Alphavirus replicon vectors for RVFV vaccines**

The alphavirus replicon vectors are a novel type of recombinant vaccine. They are able to express foreign genes in place of native viral structural genes. The replicons are thus able to produce large quantities of foreign protein but are unable to produce viral progeny [171]. A sindbis virus replicon expressing Gc and Gn was able to induce an antibody response and

protect 100% of mice from lethal RVFV challenge after two inoculations. The same recombinant replicon administered twice was able to induce RVFV-specific neutralizing antibody responses in sheep [171]. A similar study using the alpha virus, Venezuelan equine encephalitis virus, to express Gn was able to protect 100% of mice from challenge using only one inoculation [172].

### **Recombinant poxvirus vectored RVFV vaccines**

Recombinant pox-virus vectored vaccines have been made using a cDNA sequence originally constructed by Collett *et al.* from the RVFV GnGc RNA sequence [173]. The viral strain used was of the ZH-501 RVFV strain, isolated from the serum of a fatal human haemorrhagic fever victim in Egypt, during an epidemic in 1977 [174]. Two separate studies made use of vaccinia virus engineered to express Gc and Gn RVFV glycoproteins. The recombinants induced high levels of neutralizing Abs in mice and most mice survived challenge [173], [175]. Despite its success, the use of vaccinia virus recombinants is not recommended as it can cause disseminated disease in immunocompromised persons and has a wide host range [66]. For these reasons the use of attenuated poxviruses with a more limited host range, such as LSDV are more desirable [74]. LSDV is known to cause disease in cattle; thus a recombinant RVFV vaccine using attenuated LSDV as a vector will allow for the creation of a dual vaccine to protect cattle against both RVFV and LSDV.

Wallace *et al.* tested the efficacy of recombinant LSDV expressing Gc and Gn glycoproteins (LSDV-RVFV) in mice and sheep [57]. Mice inoculated subcutaneously with LSDV-RVFV produced neutralizing antibody titres and were protected against RVFV at 50% lethal dose for mice [57]. In the same study, mice primed with a DNA vaccine expressing RVFV GnGc and then boosted with LSDV-RVFV, deteriorated rapidly after challenge and 40% of mice survived. Sheep were then inoculated twice with LSDV-RVFV and were shown to induce antibody titres against RVFV and sheeppox virus (SPV) (a virus antigenically similar to LSDV). Sheep were then challenged with virulent RVFV or sheeppox virus (SPV). However, after challenge no significant disease was observed in the vaccinated or saline control animals for either RVFV or SPV challenge. In addition sheep organ damage was observed in both vaccinated and unvaccinated control animals after RVFV challenge. It was concluded that more virulent strains of RVFV and SPV were needed for challenge to properly measure the

efficacy of the vaccine [57]. It is also possible that the vaccinated animals had prior immunity to RVFV antigens.

A similar study was conducted by Soi *et al.* where KS-1 (a *Capripoxvirus* strain belonging to the LSDV group [176], [177]) was engineered to express Gc and Gn glycoproteins [82]. The recombinant virus was shown to protect 70% of mice when inoculated once. Sheep were vaccinated twice (three weeks apart) with the recombinant virus and were protected against RVFV viremia and fever. However as in the study conducted by Wallace *et al.* this study could also benefit from the use of a more virulent strain of RVFV for challenge [82].

Both LSDV recombinants discussed here required multiple inoculations and efficacy of the vaccines was not clear as the RVFV challenge strains used were of low virulence. In addition a more virulent strain of RVFV should be used for challenge so that a clear distinction can be made between disease in vaccinated and unvaccinated control animals. In both studies a 50% lethal dose was used for mouse challenge work. Additionally, using a 100% lethal dose of RVFV ( $1 \times 10^4$  50% tissue culture infective dose) as conducted in other studies [169], [178] would have produced a more efficacious result. Both studies claim protection to be due to a humoral neutralizing antibody response to the RVFV glycoproteins.

An ideal recombinant vaccine should be easy to produce, inexpensive, safe, highly immunogenic, induce both humoral and cellular immune responses and require only one inoculation for protection. This project aims at fulfilling these criteria.

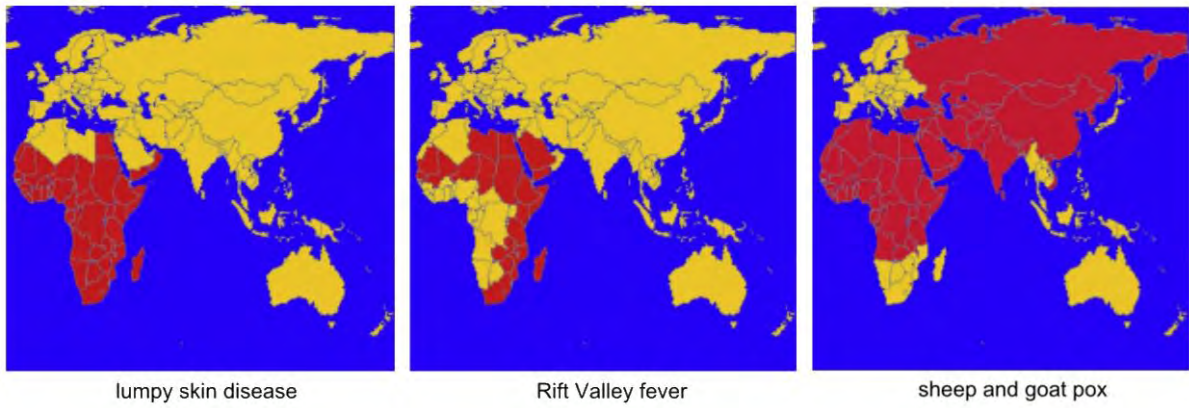
### **1.3 Project Rationale**

LSD, which infects ruminants, is a notifiable disease. The primary form of protection against the disease is the use of live attenuated vaccines, KS-1 and nLSDV. These currently available vaccines have a low “take” and more effective LSD vaccines are becoming a priority. We have available within our group an immunogenically improved vaccine strain (Herbivac) derived from the more widely used Neethling vaccine strain (nLSDV). This study has two aims. The first aim of the project is to compare and characterise two commercially available LSDV vaccines, nLSDV and Herbivac, in order to determine the more suitable vaccine strain for use in making a recombinant vaccine. The second aim of the project is to construct a

recombinant LSDV vaccine expressing appropriate RVFV genes coding for proteins that will induce a protective immune response. This will involve selection of genes for a RVFV vaccine, the design of a transfer vector for inserting these genes into LSDV, optimisation of conditions for selecting a recombinant, transient expression of foreign genes and isolation of a LSDV-RVFV recombinant.

Reviewing RVFV antigenic determinants and vaccine design it is evident that RVFV envelope glycoproteins Gc and Gn are able to induce a protective neutralizing antibody response in animals; Gn alone is also able to induce similar protective immunity. The protective immune response generated by the NC antigen is likely to be a cellular immune response. Animals infected with RVFV generate antibodies to the envelope glycoproteins as well as the RVFV nucleocapsid protein. It is also noted that the presence of both Gn and Gc glycoproteins (as opposed to Gn alone) allows for the development of tertiary structures which better resemble the RVFV virion. Studies have demonstrated that the expression of Gn, Gc as well as the NC protein result in VLPs resembling the RVFV virion. A highly efficacious vaccine should thus contain the RVFV envelope glycoproteins in order to induce a sterilizing nAb response and include the NC protein in order to activate a cellular immune response.

Our aim is to insert the RVFV envelope glycoprotein genes as well as the NC protein gene into the Herbivac vector for use as a recombinant RVFV vaccine. Expression of all three genes is expected to induce neutralizing antibody responses as well as a cellular immune response. This choice of LSDV as a viral vector will allow for the recombinant vaccine to function as a dual veterinary vaccine for cattle, sheep and goats, where these agriculturally important animals will be protected against LSD, sheeppox and goatpox respectively and be protected against RVFV. The wide-spread co-localization of these two agriculturally significant diseases in Africa (as seen in Figure 1.6) further emphasises the strategic advantage of using LSDV as a vector for expressing RVFV genes.



**Figure 1.6** Geographic distribution of LSD, RVF and Sheep and Goatpox disease over a 25 year period (from 1987 to 2012). Affected areas are shown in red. Figure obtained from Boshra *et al.* (2012) [14]

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## 2.1 Source of Viruses

*Herbivac*<sup>®</sup>*LS (Herbivac)*: An immunogenically improved strain of LSDV (Herbivac) was obtained from Dr. Louis Maartens (Dr Louis Maartens, Deltamune) and was annotated Herbivac. The Herbivac stock virus was stored at 4°C, in freeze-dried form with a mass of 0.4g per vial. Vials were made from glass and were closed using a rubber stopper and sealed using an aluminium seal to maintain a constant moisture content. Virus was reconstituted in phosphate buffered saline (PBS) prior to experimentation.

*Neethling strain LSDV (nLSDV)*: nLSDV was obtained from Onderstepoort Biologicals. The virus was passaged in bovine testes cells followed by further passages in embryonated hens' eggs.

## 2.2 Source of Cells

- A Madin-Darby bovine kidney (MDBK) adherent cell line (CCL-22) was obtained from the American Type Culture Collection.
- A baby hamster kidney (BHK) adherent cell line was obtained from the American Type Tissue Culture Collection.
- Adherent chicken embryo fibroblasts primary cells (CEF) were prepared using 6-day old embryonated hens' eggs (section 2.4.1).
- Adherent foetal bovine rete testes primary cells (FBT) were prepared using foetal bovine testes obtained from the Paarl abattoir in Cape Town (section 2.4.2).

## 2.3 Cell culture

Cell lines were incubated under standard growth conditions of 37°C, 5% CO<sub>2</sub> and 80% relative humidity.

### 2.3.1 Thawing and propagation of cell-lines

A single frozen vial containing  $1.5 \times 10^6$  MDBK cells in freezing medium (Appendix A) was removed from -80°C and thawed in a 37°C water-bath for 1 minute. The cells were resuspended in 10ml cDMEM (Appendix A) to dilute the dimethyl sulfoxide (DMSO) contained within the freezing medium. Cells were then pelleted by centrifugation at 1200rpm for 8 minutes using a Benchtop Megafuge 5810 (Eppendorf, USA). The supernatant

containing DMSO was removed and cells were resuspended in 5ml cDMEM. Media containing cells was transferred to a 25cm<sup>2</sup> tissue culture flask. Cells were incubated for 24 hours under standard growth conditions, after which a confluent monolayer was observed and cells were passaged according to maintenance protocol described in 2.3.2.

### **2.3.2 Maintenance of adherent cell lines**

Cells were incubated under standard growth conditions until a confluent monolayer was observed. The culture media was then aspirated and cells were washed once with 5ml of PBS (without Mg<sup>2+</sup> Ca<sup>2+</sup>), and then trypsinised with 2ml of 1x trypsin-EDTA (Lonza, USA). After a 2-5 minute incubation, the trypsinised cells were diluted in 3ml of cDMEM and 2 ml of the cell suspension was used to seed a 75cm<sup>2</sup> flask. The volume was made up to 10ml with cDMEM and cells were incubated for 2 days under standard growth conditions. All cell cultures were maintained by passaging 1/5 every 2-3 days.

### **2.3.3 Determining the cell count and viability of cells**

Cell counts were performed using the trypan blue staining method [179], where a two-fold dilution of the cell suspension is made in trypan blue. Ten microlitres of this mixture was then added to each chamber of a haemocytometer (Neubauer, Marienfeld) with coverslip in place. Non-viable cells have a permeable membrane and are stained blue by trypan blue solution while viable cells remain clear. Clear cells were counted on ten 1mm squares and an average was taken. To calculate the concentration of cells, the average was multiplied by the dilution factor of 2 and then by 10 000 to get number of cells per ml (a 1mm square on the haemocytometer contains a volume of 0.1µl).

### **2.3.4 Preparation of frozen stocks of cell-lines for liquid nitrogen storage**

A confluent monolayer of cells was trypsinised with 2ml trypsin-EDTA as described in 2.3.2. Cells were then resuspended in cDMEM (Appendix A) and transferred to a 15 ml conical tube. A cell and viability count was done as outline in 2.2.3.3 and cells were pelleted via centrifugation at 1500rpm for 5 minutes in a Benchtop Megafuge 5810 (Eppendorf). Following centrifugation, the supernatant was aspirated and the pelleted cells were resuspended in freezing media medium (Appendix A) to obtain cells at 1.5x10<sup>6</sup> cells/ml. One millilitre aliquots of the cell suspension were dispensed into cryogenic vials. Vials were then placed in a Mr Frosty (freezing container containing isopropanol to facilitate slow freezing)

and placed at -80°C for 48 hours. After 48 hours, frozen vials were ready for transfer to liquid nitrogen.

## **2.4 Preparation of primary cells**

### **2.4.1 Chick Embryo Fibroblasts (CEFs)**

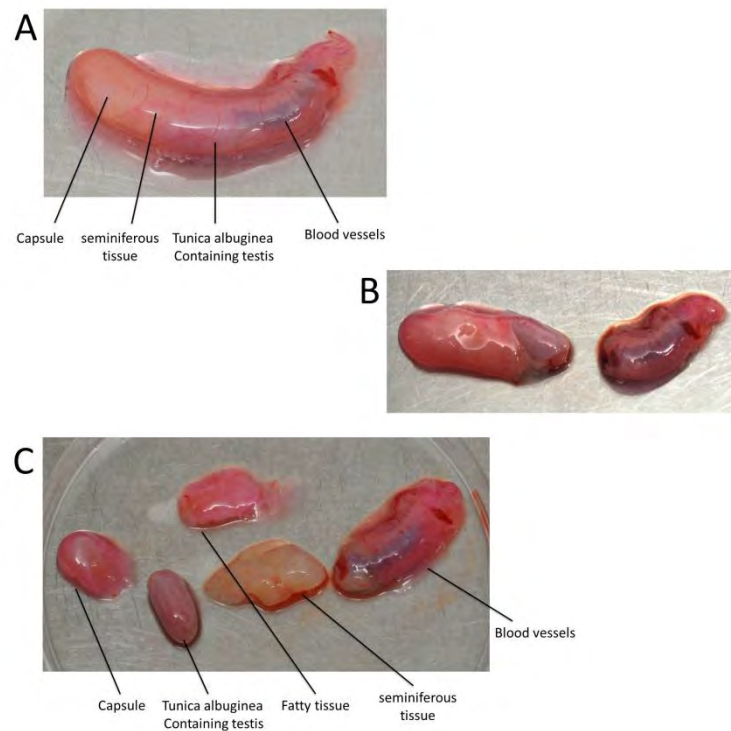
A method was adapted from Silim *et al.* for the preparation of primary CEFs [180]. Four 6-day old embryonated hens' eggs were used to isolate primary chick embryo fibroblasts for use in cell culture. The eggs were placed at 4°C for approximately 1 hour to kill the embryo. The eggs were then transferred to a class II biosafety cabinet where they were cleaned by wiping the shells with a 70% ethanol solution.

Surgical scissors were used to make an incision in the apex of the egg; egg shell, shell membrane and chorioallantoic membrane surrounding the point of incision was peeled away using forceps and the chick embryos were removed from the egg using forceps and placed onto a petri-dish. The head and limbs of the embryos were removed and the remaining portions washed by placing them in a 50ml conical tube containing 25ml of PBS. The portions were then transferred to a new 50ml conical tube containing 0.25% trypsin in PBS and incubated at 37°C for 10 to 15 minutes. After incubation the solution became cloudy indicating cells in suspension. The remainder of the chick embryo was discarded. The suspension was subjected to centrifugation at 2500g for 5min to pellet large clumps of tissue. The supernatant was aspirated and passed through a cheese cloth which allowed only single cells to pass through. A cell count was conducted on the filtered solution and 2 million cells were seeded per T75cm<sup>2</sup> tissue culture flask. These were incubated under standard growth conditions until monolayers were observed.

### **2.4.2 Foetal Bovine Testes (FBT) cells**

A method was adapted from P. Tung [181] for the preparation of FBT cells. Primary foetal bovine cells were isolated from foetal bovine testes obtained from a local abattoir. Testes were transported in DMEM containing 200U/ml penicillin, 200U/ml streptomycin and 2X fungin which was kept cold on ice. Testes were transferred to a class II biosafety cabinet where they were washed in DMEM and placed onto a sterile petri-dish (Figure 2.1 **A**). First the connective tissue and blood vessels were cut away from the rest of the testis (**B**). Next

the capsule, fatty tissue, tunica albuginea containing the testes and seminiferous tissue were separated from each other **(C)**. The tunica albuginea of the testis was incised and the rete testis removed (not shown). The testes were cut into 2 x 2mm pieces using a surgical blade and placed in 5ml 0.05% trypsin solution at 37°C for 1 hour, shaking occasionally. The trypsin solution became cloudy after the 1 hour incubation, indicative of cells in suspension. The trypsin solution was then filtered through a steel mesh and collected in 15ml tubes (approximately 3ml of liquid was collected per testis). The entire filtered solution was added to a T75cm<sup>2</sup> flask and the volume increased to 10ml with cDMEM + 10% FCS and incubated under standard growth conditions. Cell culture media was replaced with fresh media 24 hours post seeding to remove cells that did not adhere.



**Figure 2.1** Isolation of primary foetal bovine testis cells. **(A)** single detached testis. **(B)** Connective tissue and blood vessels separated from rest of testis. **(C)** isolated capsule, fatty tissue, tunica albuginea and seminiferous tissue.

## 2.5 Growth of LSDV

Both nLSDV and Herbivac were grown in cell culture and on chorioallantoic membranes of embryonated hens' eggs. Stocks were prepared by infecting MDBK cells with nLSDV or Herbivac, amplifying the viruses through two passages in MDBK cells and freeze (-

20°C)/thawing (37°C) the infected cells three times. The resulting cell lysate was stored at 4°C.

### **2.5.1 Growth of LSDV on chorioallantoic membranes of embryonated hens' eggs**

The chorioallantoic membrane (CAM) is a well vascularised membrane found beneath the egg shell in embryonated chicken eggs. This method for LSDV growth has been adapted from a method first described by Hoffstadt in 1938 [182]. A previous study by Van Rooyen *et al.* (1969) examined the conditions for growth of LSDV on CAMs. The optimal age at which to infect eggs was found to be between 5 and 7 days. Harvesting after 7 days incubation between 33.5°C and 35°C yielded the highest viral titres [23].

Embryonated hens' eggs were obtained from a commercial chicken farm and ordered through the University of Cape Town Health Sciences Animal Unit. Ethical approval for this study was obtained from the Faculty of Health Sciences Animal Research Ethics Committee (ethics approval number: 010/012). 7-day old eggs were used for growth of LSDV. Eggs were sprayed with 70% ethanol to prevent bacterial and fungal contamination and then candled to identify the air-sac and a well vascularised area of the chorioallantoic membrane (CAM). An egg pricker was used to create two holes in the egg shell: one hole at the location of the air-sac (antapex opening) and the other at a point at the well vascularised area (dorsal/ventral opening). The hole at the air sac was widened using a witch pen. Eggs were then placed laterally and a drop of phosphate buffered saline (PBS) containing penicillin, streptomycin and Fungin (Lonza, USA) (Appendix A) was placed on-top of the dorsal/ventral opening using a Pasteur pipette. The PBS was allowed to enter the egg by gentle pressure with the witch pen at the dorsal hole. Suction was created on the antapex opening using a pipette bulb. This suction caused the air-sac to be pulled towards the shell creating a vacuum which causes the CAM to drop away from the shell. A new space was created above the CAM and below the dorsal/ventral opening.

The eggs were then incubated at 34-37°C for 2 hours to allow the dropped CAMs to stabilize. Once the CAMs were stabilized, the virus was diluted in PBS containing penicillin and streptomycin (Appendix A) (if necessary) and inoculated (using a hypodermic needle)

into the space above the CAM through the dorsal/ventral opening. As an uninfected control, one egg was inoculated with PBS containing no virus.

To further prevent bacterial and fungal contamination the holes made in the egg shells were sealed using a molten mixture of candle wax and Vaseline (mixed in a 4:1 ratio of candle wax to Vaseline). The eggs were then sprayed with 70% ethanol and incubated at 37°C for 6 days or 34°C for 7 days. During this time the virus is allowed to grow on the surface of the CAM.

After incubation eggs were removed and placed at -20°C for 30 minutes to kill the embryo. The eggs were then cut along the lateral axis using surgical scissors and the CAMs were removed using sterile forceps and washed three times in physiological saline. At this point CAMs were spread out on Petri-dishes for photographing and visual analysis of pock morphology. Three CAMs at a time were added to a McCartney bottle which was kept on ice and filled quarter way with glass beads (Sigma-Aldrich, USA). Following this 1ml per membrane of McIlvain's standard buffer solution (Appendix A) was added to each McCartney bottle. CAMs were shaken in McCartney bottles for 2 minutes to break apart membrane and release virus into McIlvain's solution. Bottles were then centrifuged at 800rpm for 10 minutes (Boeco U-320, Germany) to pellet membrane debris. Supernatants containing virus were pooled into clean McCartney bottles. This process was repeated three times to extract as much virus from CAMs as possible. Pooled supernatants were centrifuged at 1500rpm for 30 minutes to remove debris (Boeco U-320, Germany). Resulting supernatants were pooled into sterile 50ml oakridge tubes (Sigma-Aldrich, USA) and 0.5ml of a 36% sucrose solution (Appendix A) was added to the base of the tube to cushion virus particles during centrifugation. Oakridge tubes were centrifuged at 11 000rpm (Sorvall, RC5C+ centrifuge) at 4°C for 1.5 hours to pellet virus. Supernatants were discarded and the pellets were reconstituted in 0.5ml of Tris-EDTA buffer (Appendix A) or PBS.

## **2.6 Growth curves**

Growth curves of the two LSDV vaccines were conducted in cell culture and on CAMs of embryonated hens' eggs. This was done to compare and contrast the growth of the two strains to each other and to themselves in different growth systems.

### **2.6.1 Growth curve of LSDV in cells**

Immediately after trypsinization T25cm<sup>2</sup> tissue culture flasks were infected in duplicate with Herbivac or nLSDV at an m.o.i. of 0.001. Two flasks for each strain were removed 2 hours post infection. Upon removal of flasks, flasks were photographed and placed at -20°C until titrated. Following this, two flasks for each strain were removed at 24 hour time points for the duration of the experiment and treated in the same way. Flasks for each time point were freeze/thawed three times in total and the resulting crude virus stock was titrated using the TCID<sub>50</sub> titration method (section 2.7.2). An average titre was taken for each time point.

### **2.6.2 Growth curve of LSDV on CAMs**

nLSDV or Herbivac was used to infect 7-day old embryonated hens' eggs in triplicate (see section 2.5.1). Eggs were infected with  $1.5 \times 10^3$  ffu per egg in a total of 100µl PBS. 1 uninfected control egg was used for each time point. Eggs were incubated at 34°C in an air ventilated incubator and removed at specific time points and placed at 4°C until harvest. Eggs were placed at 4°C 2 hours post infection and then at 24 hour intervals for 8 days. A crude virus harvest was then used where 2-3 membranes per time point were pooled and shaken once in McCartney bottles, containing glass beads and 1ml/membrane of McIlvain's solution, for 2 minutes. The resulting virus-containing McIlvain's solution was aspirated, frozen and titrated in duplicate using the TCID<sub>50</sub> method (described in 2.7.2).

## **2.7 Virus titration**

Virus titration techniques are used to determine the number of infectious viral units in a given volume, otherwise known as the titre.

### **2.7.1 Immunostaining**

A method has been adapted from Ludwig *et al.* (1970) for the detection of LSDV through immunostaining [183]. The method involves the use of a primary polyclonal bovine anti-LSDV antibody (1°Ab) produced by collecting serum from cattle previously inoculated with

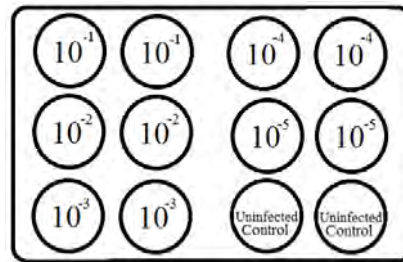
LSDV. The 1°Ab is used to detect the presence of LSDV in cells. A secondary antibody (2°Ab), rabbit anti-bovine peroxidase (Sigma-Aldrich), is then used to bind the 1°Ab. The 2°Ab contains a conjugated horseradish peroxidase enzyme which is able to produce an insoluble brown pigment upon the addition of o-dianisidine [184]. Brown patches of pigment are visible where LSDV has infected cells. These brown patches (or foci) are then counted using an inverted light microscope and used to calculate a titre expressed as foci forming units per ml. The method is described below.

A 75cm<sup>2</sup> flask containing a confluent monolayer of MDBK cells was trypsinised with 2ml trypsin-EDTA according to maintenance protocol (2.3.2). Cells were then counted as described in 2.3.3 and diluted in cDMEM to obtain a cell concentration of 1x10<sup>5</sup>cells/ml. 1ml of cell suspension was then added to each well of a flat bottom 12-well plate. Plates were incubated under standard growth conditions for 24 hours. After 24 hours the cells were 70-80% confluent and ready for infection. The medium was removed from each well and cells were washed with DMEM + 2% FCS (Appendix A). Cells were then layered with 150µl of DMEM + 2% FCS.

A ten-fold dilution series of the virus was prepared in DMEM +2% FCS (10<sup>-1</sup> to 10<sup>-5</sup>). A total of 100µl of each dilution was added to a designated well in duplicate as shown in Figure 2.2. An uninfected control was included in which 100µl of DMEM + 2% FCS (containing no virus) was added to two wells (Figure 2.2). Plates were then incubated at 37°C for 2 hours to allow the virus to adsorb. After 2 hours 750µl of DMEM +2% FCS was added to each well and plates were returned to the incubator under standard growth conditions for 72 hours for foci to form.

Culture medium was then removed from wells and cells were washed with 1ml/well PBS and left to dry overnight. Cells were fixed with 1ml/well of 1:1 acetone/methanol mixture (at -20°C) for 2 minutes. Fixative was removed and cells were washed again with 1ml/well PBS. A total of 400µl/well of 1° Ab (diluted 1:100 in PBS containing 3% FCS for blocking) was added to each well and plates were incubated at room temperature for 1 hour. Cells were then washed with PBS followed by incubation at room temperature with 400µl/well of 2° Ab (Sigma-Aldrich, USA) (1:100 dilution in PBS containing 3% FCS). After 2°Ab binding, 400µl of

peroxidase substrate (Sigma-Aldrich, USA) (Appendix A) was added to each well and incubated at room temperature for 10 minutes to allow for staining. 1ml sterile distilled water was added to each well and the number of foci counted. The titre was determined by the following formula: number of foci counted in a well multiplied by the dilution factor multiplied by 10 (because 100µl of virus dilution was added per well). Titre is expressed as foci forming units (ffu)/ml.



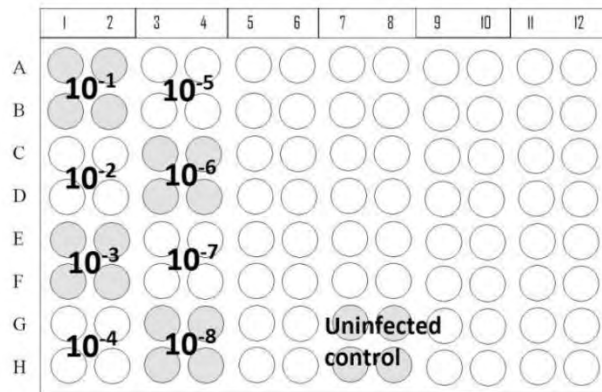
**Figure 2.2** Diagram showing standard tissue culture 12-well plate with the dilution of virus added to each well for immunostaining. Two wells were used as uninfected controls.

### 2.7.2 50% Tissue culture infective dose (TCID<sub>50</sub>)

The method determining viral end points was first described by Reed and Muench (1938) and has been adapted to include the use of 96-well plates to determine a TCID<sub>50</sub> titre [185]. The TCID<sub>50</sub> titre is found by determining the highest dilution of virus that allows for cytopathic effect to form in 50% of cell culture wells inoculated. The titre of the virus is then expressed as logTCID<sub>50</sub>/ml. The TCID<sub>50</sub> titre is not comparable to titres obtained by the other methods used in this project. A formula is thus needed to convert the TCID<sub>50</sub> titre to immunostain titre: (TCID<sub>50</sub> titre) x (0.69) = (immunostain titre, ffu/ml) [185].

A 75cm<sup>2</sup> flask containing a confluent monolayer of MDBK cells was trypsinised with 2ml trypsin-EDTA as described in 2.3.2. Cells were then counted (2.3.3) and diluted in cDMEM to obtain a cell concentration of 4x10<sup>5</sup> cells/ml. A 96-well plate was used and 100µl of cell suspension was added per well. A ten-fold dilution series of the virus was prepared using DMEM + 2% FCS (Appendix A). A total of 100µl of virus was added to each well; for each dilution 4 wells were infected (Figure 2.3). The plates were incubated under standard growth conditions until cytopathic effects were observed, approximately 72 hours post-infection.

The highest dilution of the virus in which cytopathic effect was observed in 2 out of 4 wells (50% of wells) was recorded. This dilution was then multiplied by 1/-1 (to give a positive number) and then multiplied by 10 to get the number of viral units per ml (because only 100µl of virus was added to each well). The final viral titre is expressed as logTCID<sub>50</sub>/ml.



**Figure 2.3** Dilutions of virus used to infect MDBK cells are indicated. A total of four wells are used for each dilution. Four wells were included as an uninfected control.

## 2.8 Histology

For histopathological analysis, 7-day old embryonated hens' eggs were inoculated (as described in 2.5.1) with  $2 \times 10^5$  ffu per egg, of either nLSDV or Herbivac. An uninfected control egg was inoculated with PBS only. Eggs were incubated for 7 days at 34°C in a ventilated incubator. Thereafter, virally infected CAMs were harvested (as described in 2.5.1), washed three times in saline and placed onto sterile petri dishes for photographing. CAMs were fixed in 10% buffered formalin (Appendix A). Highly infected portions of the CAMs were excised using a surgical blade and embedded in paraffin. The paraffin embedded tissues were cut into 4µm sections and stained with conventional Hematoxylin-Eosin (HE). Slides were examined and photographed using a light microscope (Zeiss AxioSkop 2M, Camera: Zeiss AxioCam HRc).

## 2.9 Transfer vector amplification and purification

A transfer vector was designed (see section 3.4.1), using DNA analysis software CLC-Bio, and ordered from GeneScript (China). The transfer vector was received from GeneScript as lyophilised DNA.

The lyophilised transfer vector DNA was resuspended in purified water (Sigma, USA). Ten to 100ng DNA was used to transform competent *E.coli* (DH5 $\alpha$ ) cells which were plated onto agar plates containing carbenicillin (100 $\mu$ g/ml) for selection (Appendix A). Plates were incubated at 37°C overnight. A single colony was then picked into 3ml Luria broth medium containing carbenicillin (100 $\mu$ g/ml) (Appendix A), which was incubated overnight at 37°C with gentle shaking. The following day 1ml of growth medium containing *E. coli* culture was transferred into 99ml Luria broth medium containing carbenicillin (100 $\mu$ g/ml) and incubated overnight at 37°C with gentle shaking. *E.coli* Glycerol stocks (Appendix A) were also prepared from the 3ml culture and stored at -80°C. A total of 100ml of overnight grown culture was used in a 'Pure Yield™ Plasmid Midiprep System' (Promega, USA) to extract plasmid DNA. Plasmid DNA was eluted in nuclease-free water and the concentration determined using a spectrophotometer ND-1000 (NanoDrop, USA).

## **2.10 Agarose gel electrophoresis**

Electrophoresis separates DNA products according to size and the size of each product is determined by comparing the position of the band of interest to a DNA marker containing bands of known sizes. Electrophoresis of DNA was carried out on agarose gels (20cm) stained with 0.5 $\mu$ g/ml ethidium bromide (10mg/ml). Gels were prepared by adding 1-2% agarose to tris-acetate-EDTA buffer (Appendix A). Gel electrophoresis was performed in tris-acetate-EDTA buffer under a constant voltage of 15V for approximately 20 hours. Gels were visualised using an ultraviolet transilluminator.

The marker DNA used for determining the sizes of PCR products was 'Gene Ruler 100bp DNA ladder plus' (Fermentas). 'Gene Ruler 1 kb DNA ladder' (Fermentas) was used for restriction enzyme digestion products. A 6X loading dye (Fermentas) was added to samples before loading into wells of agarose gel. The loading dye allowed for visual tracking of DNA along the gel.

## **2.11 Detection of transient expression**

The RVFV genes (GnGc and Nucleocapsid) and the eGFP from the transfer vector pRO1 are all under the regulation of poxvirus promoters. Thus transient expression of these genes

occurs when the transfer vector is present in an LSDV infected cell. In order to induce transient expression, LSDV infected cells are transfected with pRO1. Transfections were conducted using three different cell types; MDBK, BHK and FBTs.

### **2.11.1 Transfection**

Cells were trypsinised and counted as described in 2.3.2 and used to seed 6-well plates at  $3.5 \times 10^6$  cells/well. The final volume of medium was 2ml per well. While cells were still in suspension they were infected with Herbivac at an m.o.i. of 0.8 ffu/cell. Two wells per plate were left uninfected. Plates were incubated under standard growth conditions for 24 hours at which point the cells reached 70-90% confluency. Media was then removed from wells and cells were washed with 2ml DMEM. In order to prepare the transfection reaction; 500 $\mu$ l of room temperature DMEM was added to a sterile polypropylene tube. In order to achieve a DNA to transfection reagent ratio of 1:1; 2 $\mu$ g of plasmid DNA was added to the DMEM. The mixture was pipetted gently to mix, followed by the addition of 2 $\mu$ l of room temperature XtremeGeneHP (Roche). The resulting transfection mixture was incubated at room temperature for 30 minutes to allow for DNA-transfection reagent complexes to form. A total of 500 $\mu$ l of the mixture was added to each of 4 wells of the 6-well plate. An infected well and an uninfected well were left untransfected as controls. Plates were incubated under standard growth conditions for 4 hours followed by the addition of 1.5ml of DMEM containing 2% FCS to each well.

Forty-eight hours post transfection wells were viewed and photographed using a fluorescence microscope (Zeiss Axiovert 200M, Germany) or used to prepare a lysate for western blotting.

### **2.11.2 Western blotting**

After infection and transfection of BHK cells as described above (2.11.1), the medium was aspirated and the cells washed twice with PBS. An aliquot of 200  $\mu$ l 1X Glo Lysis Buffer (Promega, South Africa), supplemented with 1 $\times$  Complete EDTA-free Protease Inhibitor (Roche, USA) was then added to each well. Plates were left to incubate at room temperature for 5 minutes. The resulting lysates were removed from the wells and placed in single Eppendorf tubes which were incubated on ice for 2 minutes. The lysates were

centrifuged at 13000rpm for 10 minutes using a benchtop centrifuge, the supernatants were aspirated and aliquotted into new Eppendorf tubes at 50µl per tube and stored at -80°C for future use.

Samples were prepared for western blotting as follows: 28µl thawed lysate; 2µl β-mercaptoethanol reducing agent (BioRad, USA) and 10µl sample buffer (BioRad, USA). Samples were boiled for 5 minutes and then separated on a 7% SDS-PAGE gel subjected to 150V for 1 hour. The protein was then transferred onto a nitrocellulose membrane via semi dry electroblotting at 15V for 1 hour. This involved the placement of the gel on top of the nitrocellulose membrane both of which were sandwiched between two layers of extra thick blotting paper (BioRad, USA). The extra thick blotting paper and nitrocellulose membranes were pre-soaked in transfer buffer (appendix) for 20 minutes.

Membranes were then blocked using blocking/wash buffer (Appendix A) for 2 hours at room temperature with shaking. After blocking, membranes were incubated with pre-absorbed primary Ab (rabbit-anti-Gn-Ab, ProSci 4521; rabbit-anti-Gc-Ab, ProSci 4519 or rabbit-anti-NC-Ab, ProSci 7413) (1:1000 dil in blocking buffer) overnight at 4°C with gentle shaking. Pre-absorption of primary Ab involved incubation of the antibody with a BHK cell lysate in PBS for approximately 30 hours. The solution was then spun down at 1200rpm for 8 minutes using a Benchtop Megafuge 5810 (Eppendorf, Germany) to remove cell debris. The supernatant was removed and combined with blocking buffer for use.

Following overnight incubation membranes were washed using blocking buffer four times (15 minutes each) with shaking. Anti-rabbit horseradish peroxidase (HRP) conjugated secondary antibody (Sigma, USA) was added at a 1:10 000 dilution in blocking buffer and left to incubate at room temperature with shaking for 1 hour.

Membranes were then washed with blocking/wash buffer (appendix) four times (15 minutes each) with shaking. Finally bands were detected by adding 5 ml of NBT/BCIP solution (Roche, USA) for 5 minutes at room temperature in the dark.

### 2.11.3 Immunostaining

The method for the detection of proteins through the use of immunostaining has been adapted from an original method first described by Coons *et al.* in 1941 [186].

BHK cells were seeded into an 8-well permanox chamber slide (Lab-Tek, Australia) at  $8 \times 10^5$  cells per well in a total volume of 200 $\mu$ l 2% cDMEM (appendix). Immediately following seeding of cells HerbiVac was added to each well at an m.o.i. of 0.0025. A transfection mixture with a DNA to transfection reagent ratio of 1:1 was prepared as indicated above (2.11.1) and 100 $\mu$ l of transfection mixture was added per well. The slide was then incubated for 48 hours under standard growth conditions.

After incubation, medium was removed and cells fixed with the addition of 200 $\mu$ l per well of ice-cold acetone (-20°C) for 10 minutes. The acetone was removed and the slides left to air dry. Slides were then washed once with phosphate buffered saline (PBS) and blocked with 2% ovalbumin in PBS for 20 minutes. Slides were then washed twice (20 minutes each) with PBS followed by a 1 hour incubation with 100 $\mu$ l of the primary antibody (rabbit-anti-Gn-Ab, ProSci 4521; rabbit-anti-Gc-Ab, ProSci 4519 or rabbit-anti-NC-Ab, ProSci 7413) (1:300 dilution). Incubation was done at 37°C with shaking.

Slides were then washed twice (10 minutes each) with PBS followed by a 1 hour incubation with 100 $\mu$ l of the secondary antibody (FITC-conjugated donkey-anti-rabbit antibody). Incubation was done at 37°C with gentle shaking followed by a single 10 minutes wash with phosphate buffered saline. Cells were stained with 200 $\mu$ l per well of hoechst stain at 1 $\mu$ g/ml and washed twice for 10 minutes with PBS with gentle shaking. The slides were then mounted using mounting medium containing antifade (Life Technologies, USA) and viewed using a confocal microscope.

The same negative controls were used for the assay of each antigen. Two sets of controls were used. Set 1 tests the specific binding of the primary antibody (Ab) to the RVFV protein. Set 2 tests the specificity/nonspecificity of the secondary Ab. The negative controls are as follows:

**Set 1**

- Plasmid only with primary and secondary Ab.
- Virus only, with primary and secondary Ab.
- Cells only, with primary and secondary Ab.

**Set 2**

- Plasmid (pRO1) only with secondary Ab only.
- Virus only with secondary Ab only.
- Cells only with secondary Ab only.
- Experimental well with transfected and infected cells and secondary Ab only.

**2.12 Isolation of Herbivac-RVfV**

FBT cells, seeded into 6-well plates and infected with wild-type Herbivac, were transfected with pRO1 (as described in 2.11.1). Plates were incubated under standard growth conditions for three days. Next, plates were viewed using a fluorescence microscope and wells with high levels of fluorescence were subjected to three cycles of freeze/thawing (-20°C/37°C) in order to release virus. Serial dilutions were conducted on harvested virus and used to infect fresh MDBK cells. A single fluorescing focus was isolated in this manner and used to infect fresh MDBK cells, this time cells were incubated in the presence of MPA with xanthine and hypoxanthine selection medium (Appendix A). This process was repeated nine times in order to select for the recombinant virus through negative selection of wild type LSDV.

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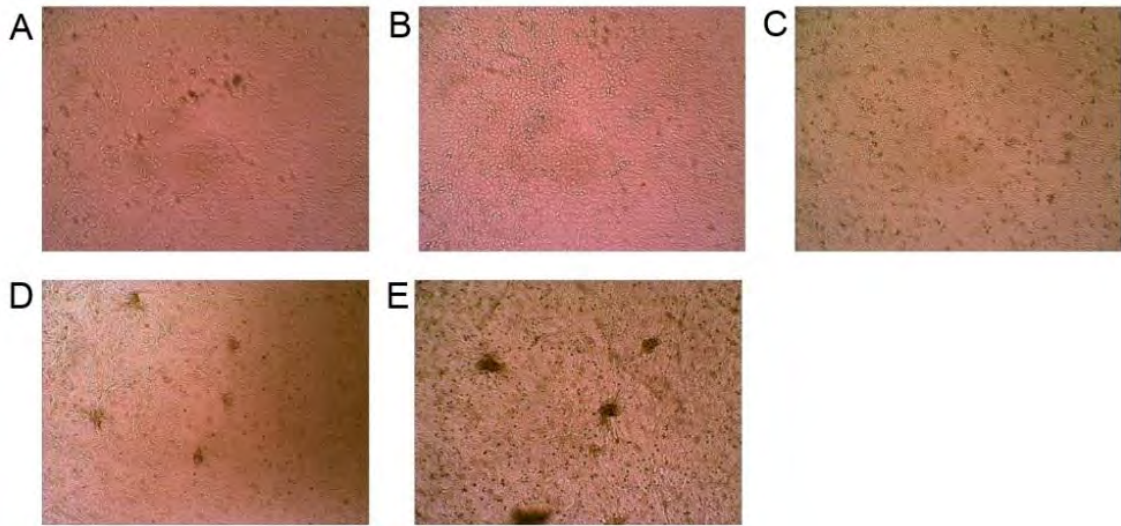
### **3.1 Characterization of Herbivac and nLSDV**

The growth of LSDV has been reported in various cell types such as foetal bovine testis (FBT) and lamb testes cells, foetal bovine muscle cells, primary kidney cells from calf, lamb and sheep and the Madin-Darby bovine kidney (MDBK) cell line [187–191]. LSDV has also been shown to grow on the chorio-allantoic membranes (CAMs) of embryonated hens' eggs [23]. To better characterise and compare the two LSDV vaccines, Neethling and Herbivac, their growth was assessed in MDBK cells and on CAMs of embryonated hens' eggs.

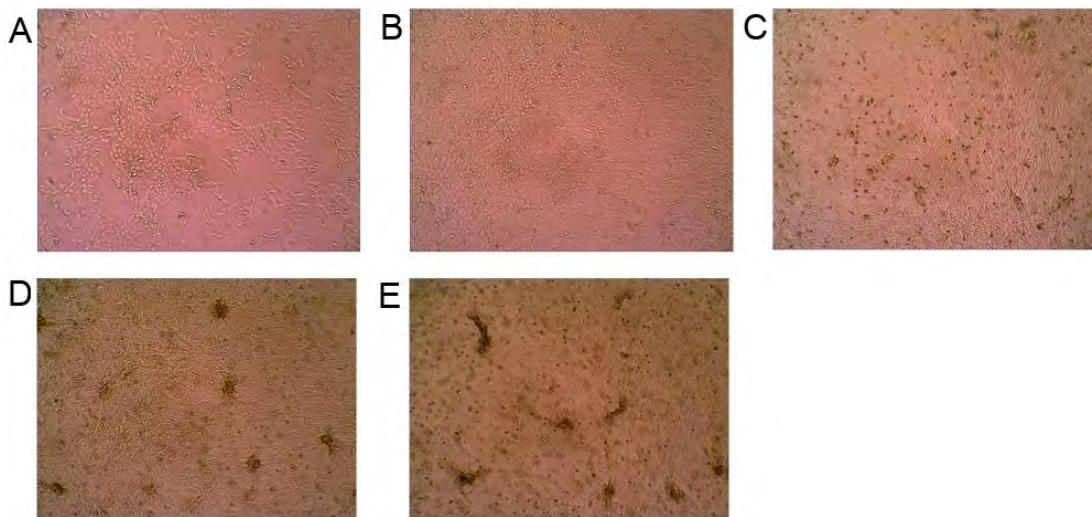
#### **3.1.1 Growth characterization in cell culture**

For a cell type to be used successfully in making recombinant viruses, the infecting virus must be able to replicate in the cell type. We therefore evaluated the growth of Herbivac in two additional cell types, a baby hamster kidney (BHK) cell line and primary chick embryo fibroblast cells (CEF) isolated from the foetuses of embryonated hens' eggs.

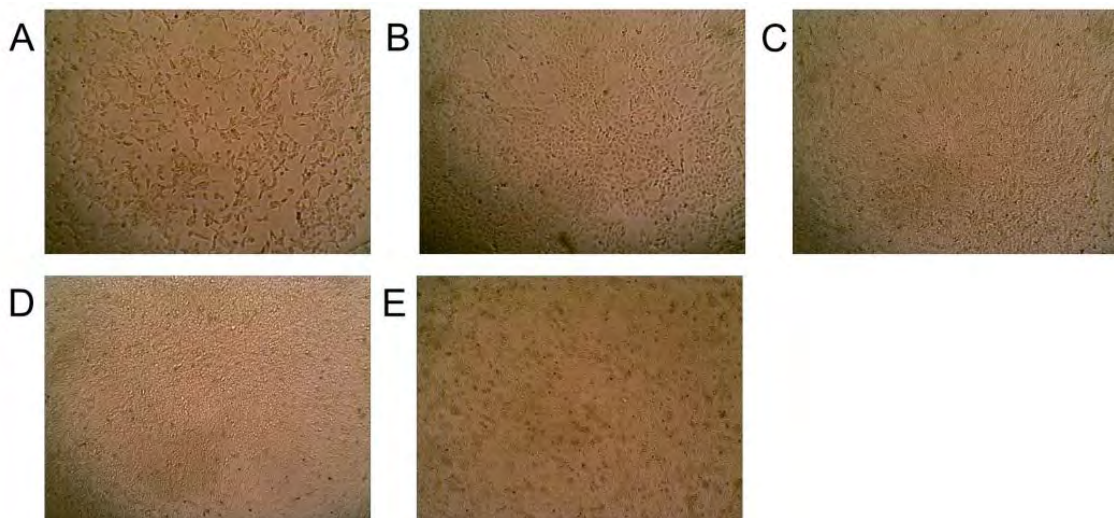
MDBK cells were infected with Herbivac or nLSDV at an m.o.i. of 0.001 as described in 2.6.1. Cytopathic effects (CPE) were observed from 3 days p.i. for both nLSDV and Herbivac (Figures 3.1 and 3.2 respectively). At day 3 small dense foci were observed which were larger and more defined at day 4. No noticeable difference in cell pathology could be observed between the two strains. CPE developed only in infected cells and not in uninfected control cells (Figure 3.3) indicating that CPE was indeed due to LSDV infection. After photographing, cell lysates from infected flasks were titrated in duplicate as described in 2.7.2. Figure 3.4 shows this growth curve.



**Figure 3.1** MDBK cells infected with nLSDV at an m.o.i. of 0.001. Cells as seen **(A)** 2 hours post infection **(B)** 1 day post infection (d.p.i.) **(C)** 2 d.p.i. **(D)** 3 d.p.i. **(E)** 4 d.p.i. (40X magnification).



**Figure 3.2** MDBK cells infected with Herbivac at an m.o.i. of 0.001. Cells as seen **(A)** 2 hours post infection **(B)** 1 day post infection (d.p.i.) **(C)** 2 d.p.i. **(D)** 3 d.p.i. **(E)** 4 d.p.i. (40X magnification).

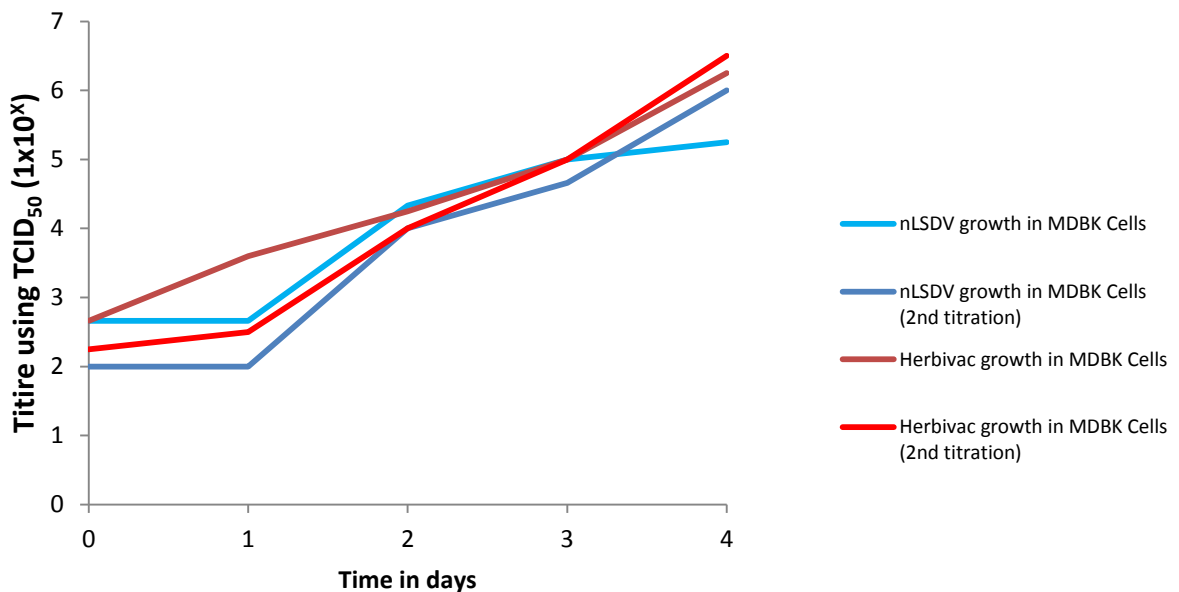


**Figure 3.3** MDBK cells left uninfected (40X magnification). Cells as seen **(A)** 2 hours post seeding **(B)** 1 day post seeding **(C)** 2 days post seeding **(D)** 3 days post seeding and **(E)** 4 days post seeding. (40X magnification).

**Table 3.1 nLSDV and Herbivac growth in MDBK Cells**

(log values obtained from TCID<sub>50</sub> titration)

day	nLSDV		Herbivac	
	titration 1	titration 2	titration 1	titration 2
0	2.66	2	2.66	2.25
1	2.66	2	3.6	2.5
2	4.33	4	4.25	4
3	5	4.66	5	5
4	5.25	6	6.25	6.5

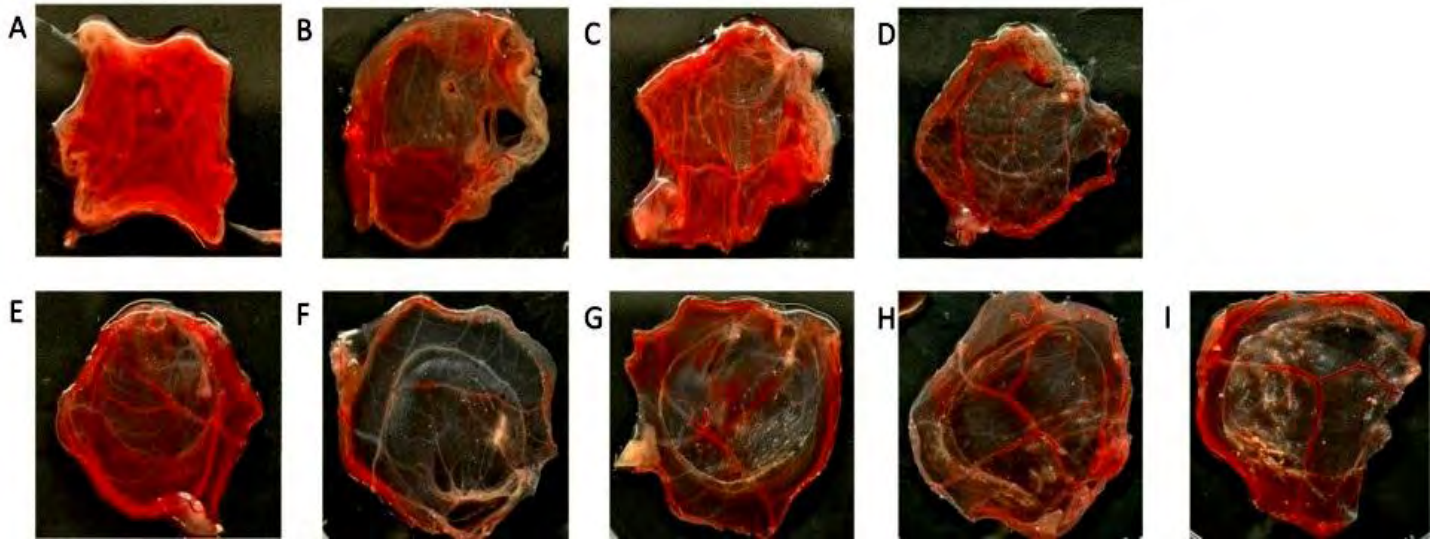


**Figure 3.4** Growth of nLSDV and Herbivac in MDBK cells. MDBK cells were infected with Herbivac or nLSDV at an m.o.i. of 0.001. At daily intervals a flask of each virus was harvested and the lysates titrated in duplicate.

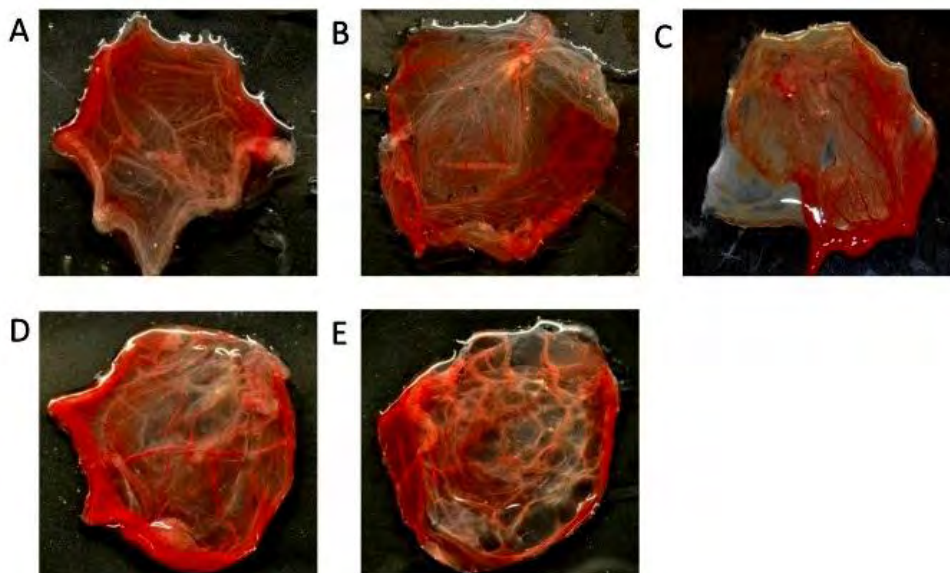
A slight lag phase can be observed for both viruses over the first 24 hours followed by a steady growth rate. Throughout the four day period, both viruses had similar growth rates (Figure 3.4 and table 3.1).

### 3.1.2 Growth characterization in embryonated hens' eggs

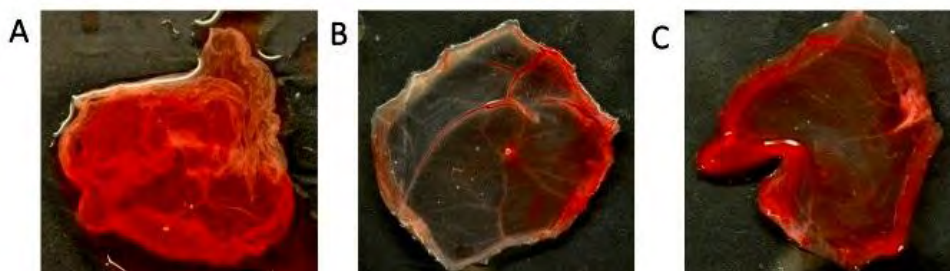
The growth of nLSDV and Herbivac was assessed in embryonated hens' eggs. 7-day old embryonated hens' eggs were infected in triplicate with either nLSDV or Herbivac (as described in 2.6.2). Eggs were placed at 4°C at different time points (2hrs, 1, 2, 3, 4, 5, 6, 7 and 8 days post infection) in preparation for harvest. Membranes were harvested and photographed (Figures 3.5, 3.6 and 3.7). Crude viral preparations were isolated from the membranes and titrated as described in 2.6.2. Figures 3.5 and 3.6 show growth of Herbivac and nLSDV respectively on chick CAMs. The titration results are graphically displayed in Figure 3.8.



**Figure 3.5:** Growth of Herbivac on CAMs. Membranes were first harvested at 2 hours post infection and then at 24 hour intervals over an 8 day period. **(A)** 2 hours post infection **(B)** 1d.p.i. **(C)** 2d.p.i. **(D)** 3d.p.i. **(E)** 4d.p.i. **(F)** 5d.p.i. **(G)** 6d.p.i. **(H)** 7d.p.i. **(I)** 8d.p.i.



**Figure 3.6:** Growth of nLSDV on CAMs. Membranes were first harvested at 2 hours post infection and then at 24 hour intervals. **(A)** 2 hours post infection **(B)** 1d.p.i. **(C)** 2d.p.i. **(D)** 3d.p.i. **(E)** 4d.p.i.

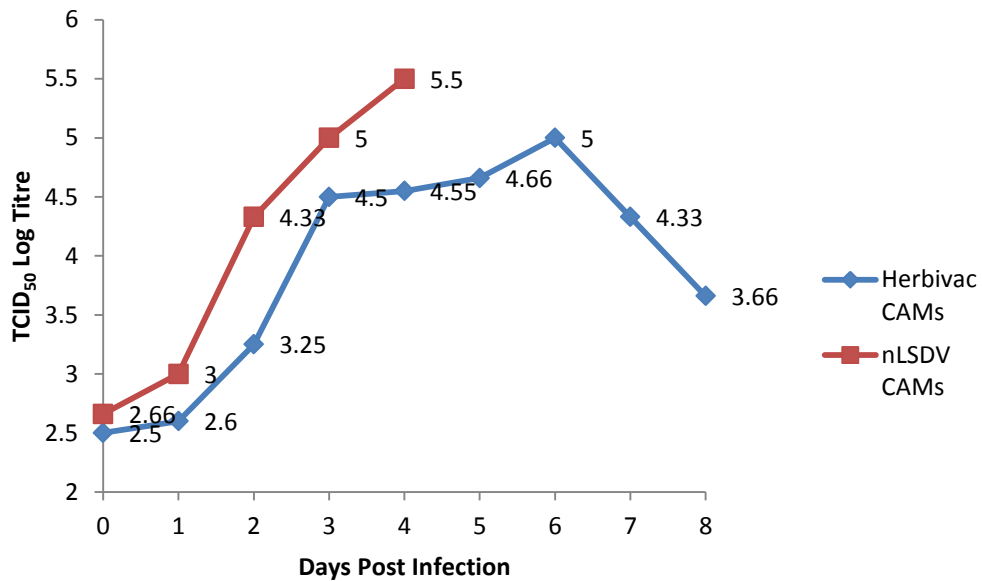


**Figure 3.7** Uninfected CAMs **(A)** 2h.p.i. **(B)** 5d.p.i. **(C)** 6d.p.i.

For Herbivac infected CAMS (Figure 3.5), a thin, fragile membrane was observed 2 hours post infection and 1 d.p.i. From 2 d.p.i. small, fine, white pocks could be seen along CAM blood vessels and along the edges of the infected area of the CAM. The membrane also became less fragile from 2 d.p.i. and notable thickening of the membrane was observed 3 d.p.i. On day 5 the membrane appeared significantly thicker compared to control membranes and a faint white haze of pocks could be seen on the CAM, in addition to a line of more defined pocks following the curvature of the air-sac produced when the CAM was dropped. On days 6, 7 and 8 increased thickening of the membrane was observed along with increase in the number and size of pocks.

Figure 3.6 shows membranes harvested at different time points after infection with nLSDV. nLSDV killed the live embryos from day 5 resulting in no membrane harvest after day 4. Infection of CAMs with nLSDV resulted in membrane thickening and a slight haze of pocks from 1 d.p.i. On days 2 and 3, a thicker film of pocks was seen and increased membrane thickening was observed. Pocks were amorphous, large and white in colour and occurred along the blood vessels of the CAM. Herbivac and nLSDV were each grown on CAMs several times. In general Herbivac produced more defined pocks than nLSDV and chick embryos survived longer after infection with Herbivac than nLSDV.

CAMs from uninfected control eggs remained fragile and thin for the duration of the experiment. No pock formation or general viral pathology was observed on these uninfected CAMs.



**Figure 3.8** Growth of Herbivac and nLSDV on CAMs of embryonated hens' eggs over an 8 day period. Note the embryos infected with nLSDV died at day 5.

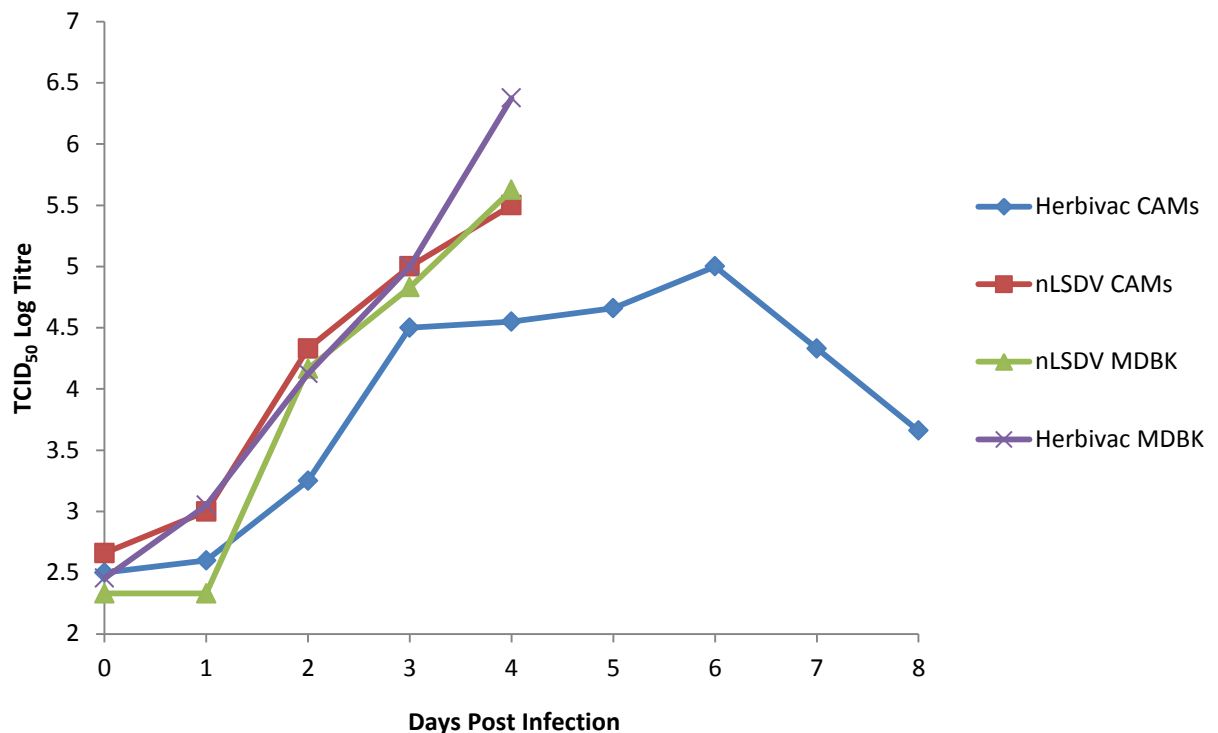
Figure 3.8 shows the growth curves of nLSDV and Herbivac for CAMs. nLSDV grew at a notably faster rate in embryonated eggs compared to Herbivac, with a 10-fold increase in titre at day 4 p.i. compared to Herbivac. Both the Herbivac and nLSDV grew rapidly between days 1 and 3. After day 3 there was a plateau in growth of Herbivac, with a peak on day 6 after which there was a drop in titre. Despite the Herbivac drop in titre after 6 d.p.i., increased viral pathology was observed on CAMs on days 7 and 8 (Figure 3.5).

The growth of nLSDV was rapid until day 4 at which point the virus killed the embryos. At each time point the titre of nLSDV was observed to be higher than that of Herbivac. nLSDV caused greater viral pathology of CAMs compared to Herbivac when the same titre of virus was present in the CAM (Figure 3.6 D and E compared to Figure 3.5 E and F respectively).

### 3.1.3 Comparison of nLSDV and Herbivac growth in MDBK cells and on chick CAMs

The comparative growth curves of nLSDV and Herbivac in MDBK cells and on CAMs are presented in Figure 3.9. The same amount of virus ( $10^3$  ffu) was added to each egg as was added per flask of MDBK cells. For nLSDV, no marked difference could be found between its growth in MDBKs and on CAMs. For Herbivac a faster rate of growth is observed when the viral strain is used to infect MDBK cells compared to CAMs. Herbivac growth in MDBK cells

results in a final titre of 6.4TCID<sub>50</sub>/ml 4d.p.i., whereas on CAMs the titre of Herbivac 4d.p.i. is 100 fold lower at 4.55TCID<sub>50</sub>/ml. Here the viral harvests for cells were prepared using freeze/thaw cycles of cells. The cell lysates were titrated without using a purification step. For chick CAMs debris was removed through centrifugation and the supernatant titrated (see section 2.6.2 for more details).



**Figure 3.9** Growth curves of Herbivac and nLSDV in MDBK cells and on CAMs of embryonated hens' eggs.

There is no notable different in growth between nLSDV and Herbivac in MDBK cells up to 3 days. The log increase in titre observed for Herbivac at day 4 cannot be interpreted to be statistically significant at this stage. A more pronounced difference was observed between the two viruses when grown in eggs. nLSDV grew at a faster rate compared to Herbivac and killed the embryos by day 5. Although it is accepted that the egg results may not be valid, in cattle it was hypothesised that Herbivac may be a safer alternative for vaccine use compared to nLSDV.

### 3.2 Histopathology of CAMs infected with either nLSDV or Herbivac

LSDV is known to infect and replicate on the chorio-allantoic membrane (CAM) of embryonated hens' eggs [23]. In order to compare and characterise possible histopathological differences between Herbivac and nLSDV, both viruses were used to infect CAMs of embryonated hens' eggs. 7-day old embryonated hens' eggs were inoculated with  $10^3$  pfu/egg of either Herbivac or nLSDV (as described in section 2.8). 7 days post infection CAMs were harvested and photographed (Figure 3.10).



**Figure 3.10** CAMs infected with  $10^3$  pfu/egg of either nLSDV or Herbivac and harvested 7 days post infection. **(A)** uninfected control CAM. **(B)** CAM infected with nLSDV. **(C)** CAM infected with Herbivac.

Sections of CAMs were isolated, prepared and stained for histopathological analysis (as described in 2.8). Figures 3.11, 3.12 and 3.13 show CAMs uninfected, infected with nLSDV and infected with Herbivac respectively. In Figure 3.11, the uninfected CAM, three distinct layers could be observed. The inner most of the three layers, the allantois-epithelium, was one cell thick in the uninfected membrane and is approximately  $6-9\mu\text{m}$ . The mesoderm layer runs down the middle of the outer and inner layers and its width ranges from  $70-130\mu\text{m}$ . The outer most membrane, the chorionic membrane (upon which the virus is inoculated) usually consists of two layers of cells. The chorionic-epithelium was approximately  $10-13\mu\text{m}$  thick in this uninfected CAM. The different cell layers in the uninfected membrane were all thin and the whole CAM itself was delicate.

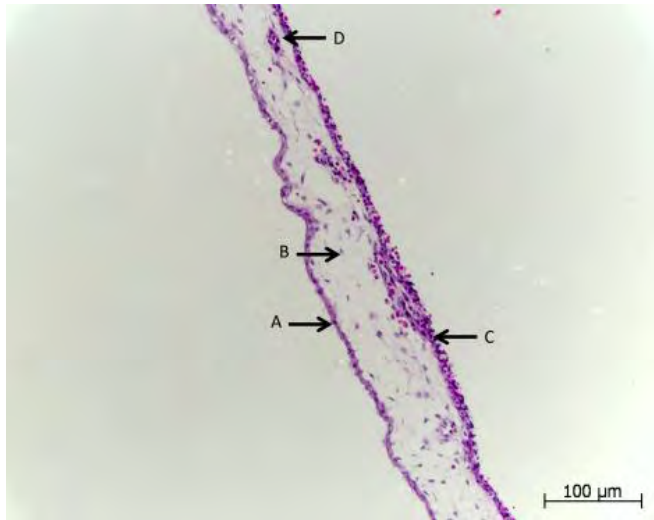
Figure 3.12, shows a CAM infected with nLSDV. Excessive hyperplasia (an increase in cell proliferation) and hypertrophy (increase in cell size) could be seen along the mesoderm layer. However the allantoic and chorionic epithelial layers remain similar to the control. Ballooning degeneration (Figure 3.12 and 3.16) as well as angiogenesis (Figure 3.12) was observed.

As with nLSDV infection of the CAM, Herbivac infection also caused substantial hyperplasia and hypertrophy along the mesoderm layer although this increase in mesoderm thickness was not as notable as in the nLSDV infected CAM (Figures 3.12 and 3.13).

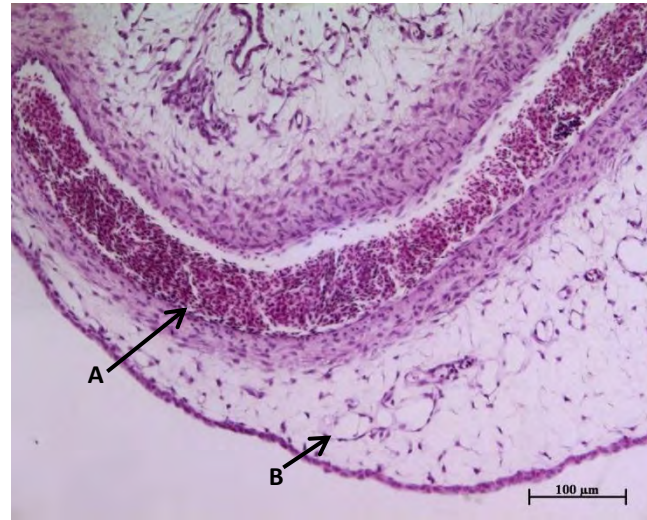
Figure 3.14 identifies a pock, on the chorionic membrane, caused by nLSDV infection of the CAM. At the outward facing edges of the pock, dead cells and sloughing off of infected cells could be seen. Viral infection caused the formation of intracytoplasmic eosinophilic inclusion bodies inside the mesoderm and chorionic epithelium of infected CAMs. The inclusion bodies are poxvirus factories which produce new infectious particles. Inclusion bodies are especially numerous in pocks and are identified by a darkly stained blue nucleus surrounded by a dark pinkish cytoplasm, the cytoplasm is stained pinkish due to the eosinophilic staining of the excess basic viral proteins contained in the cytoplasm of infected cells. A close up of the nLSDV infected membrane in Figure 3.16 shows vascularisation to the infected area. This is known as angiogenesis; where numerous capillaries are formed in response to infection. Angiogenesis has allowed for heterophil infiltration identified by the numerous darkly stained spots seen in the mesoderm.

In Figure 3.15, a single pock, caused by Herbivac infection, can be seen. Angiogenesis is indicated which has led to large amounts of heterophil infiltration both of the mesoderm layer and the chorionic epithelium. Herbivac infection was observed to have caused greater levels of angiogenesis and heterophil infiltration compared to nLSDV infection. Large amount of viral infection could be observed by the numerous inclusion bodies found in the centre and in localised regions in the chorionic epithelium. On the periphery of the pock late stage infected cells were found to be undergoing cell death and ballooning degeneration. Ballooning degeneration is where cells first increase in size before undergoing cell death. Figure 3.17 shows a higher magnification (40x) of the chorionic epithelium as seen in a pock. Sloughing off of cells can be clearly seen along with other responses such as ballooning degeneration which is occurring just below the surface cell layer. Below the layer of ballooning degenerating cells is a distinctive layer of inclusion bodies.

Overall, higher levels of hyperplasia and hypertrophy are seen in the mesoderm of nLSDV compared to Herbivac (Figures 3.12 and 3.12 respectively). However, larger pocks were observed on the chorionic membrane for Herbivac infected CAMs compared to nLSDV. This resulted in extensive hypertrophy and hyperplasia of the chorionic membrane in Herbivac infected CAMs. Overall the chorionic membrane was thicker for Herbivac infected CAMs compared to nLSDV infected CAMs (Figures 3.15 and 3.14 respectively). Extensive ballooning degeneration was noted in both Herbivac and nLSDV infected CAMs.



**Figure 3.11** (magnification 20X; HE stain). Cross-sectional view of an uninfected CAM. **(A)** The allantoic epithelium. **(B)** Mesoderm layer is the middle layer between the inner and outer membranes. An arrow points to the nucleus of a single mesodermal cell. **(C)** Chorionic epithelium. **(D)** Capillary containing erythrocytes.



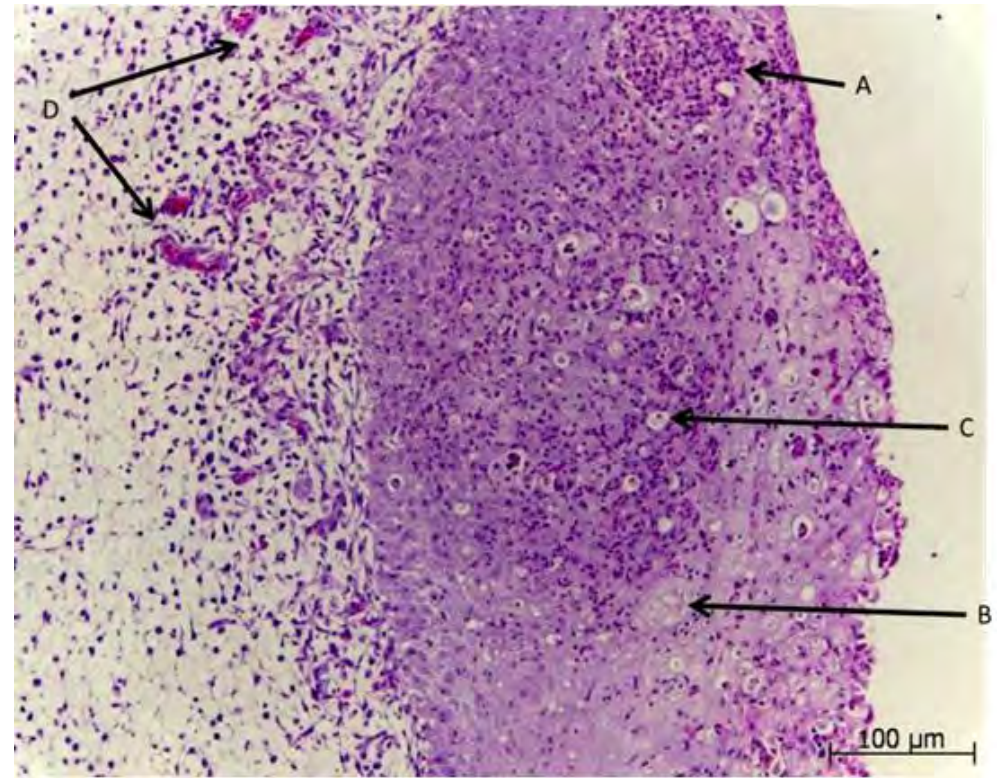
**Figure 3.12** (magnification 20X; HE stain). Cross sectional view of nLSDV infected CAM. **(A)** A blood vessel is seen running through a thickened mesoderm layer. **(B)** Ballooning degeneration of cells.



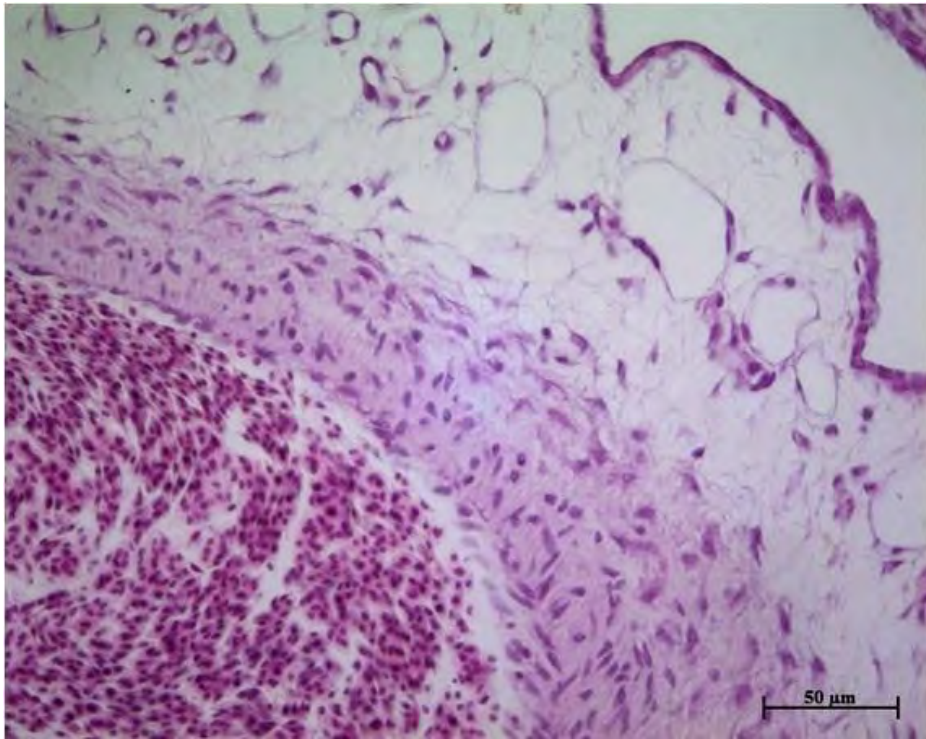
**Figure 3.13** (magnification 20X; HE stain). Cross section of a HerbiVac infected CAM. **(A)** A blood vessel is seen in the middle of the mesoderm layer. **(B)** Ballooning degeneration of cells. **(C)** Thickened chorionic epithelium.



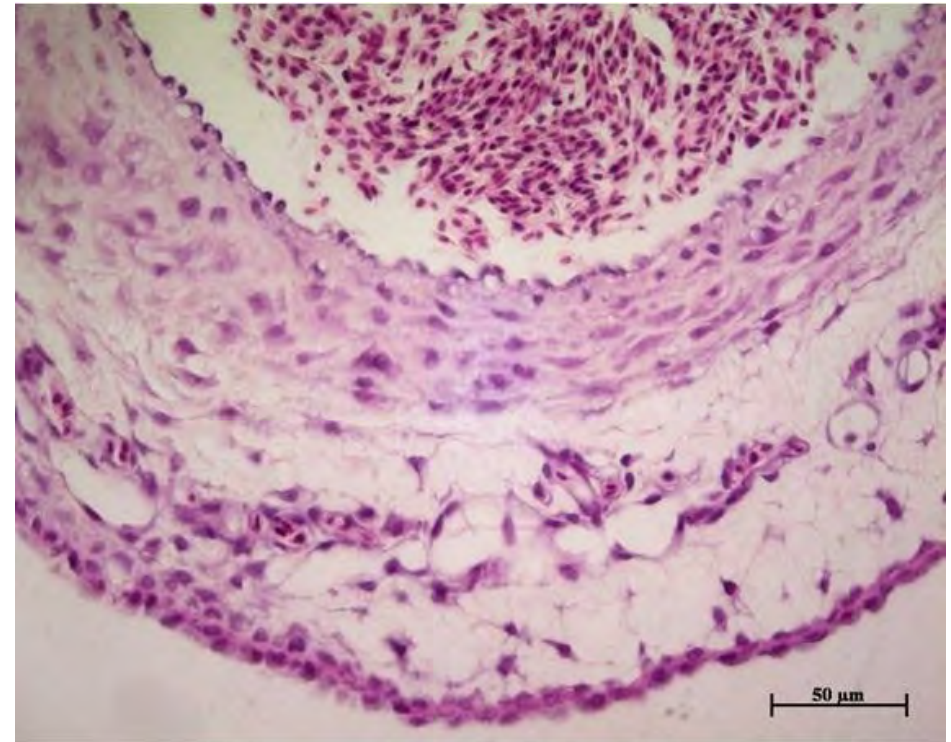
**Figure 3.14** (magnification 20X; HE stain). Cross-section of nLSDV infected CAM. The chorionic epithelium runs diagonally across the figure and consists of darkly stained cells. The mesoderm is seen beneath the chorionic membrane and is composed of lightly stained cells. A pock can be seen at the centre of the figure protruding from the chorionic epithelium. The pock contains large amounts of inclusion bodies as indicated by **(A)**. **(B)** points to the most outward part of the pock where sloughing off of cells can be seen.



**Figure 3.15** (magnification 20X; HE stain). Cross-section of pock caused by Herbivac infection of CAM. The chorionic epithelium is stained dark purple and is to the right of the figure. The mesoderm layer is stained light purple and is to the left of the figure. **(A)** region of viral inclusion bodies. **(B)** Cell undergoing ballooning degeneration. **(C)** Late stage of ballooning degeneration; shrinking to form an eosinophilic councilman body. **(D)** Regions of angiogenesis indicated.



**Figure 3.16** (magnification 40X; HE stain). Cross-sectional view of nLSDV infected CAM. The chorionic epithelium is identified toward the top right by a dark purple stain. The mesoderm is seen beneath the chorionic membrane and is composed of lightly stained cells. The mesoderm layer contains a blood vessel (bottom left).



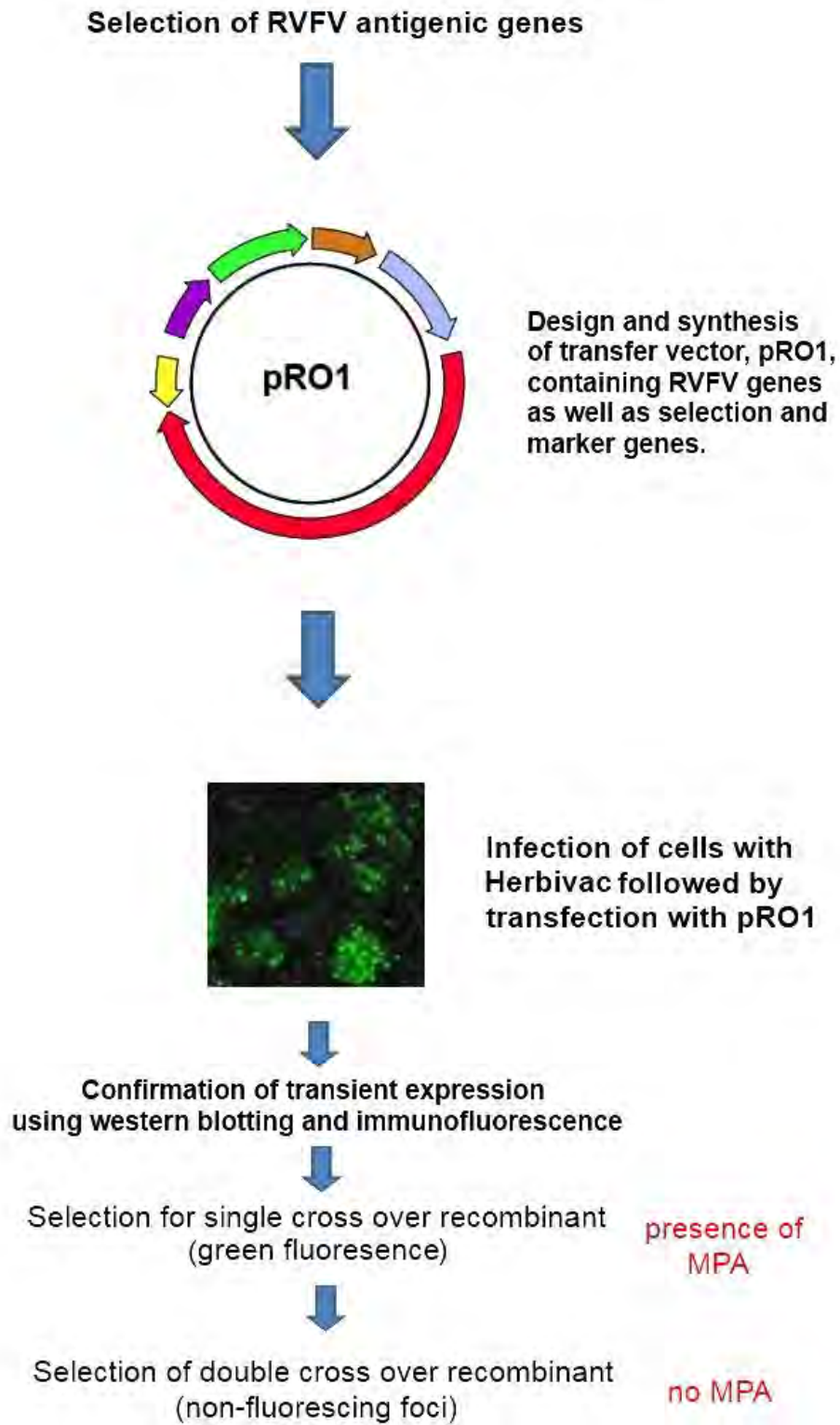
**Figure 3.17** (magnification 40X; HE stain). Cross-sectional view of Herbivac infected CAM. The chorionic epithelium is shown as the bottom most layer. A blood vessel is observed at the top of the image and the mesoderm layer is observed between the chorionic epithelium and viral inclusion bodies.

### **3.3 Construction of a recombinant LSDV expressing RVFV genes**

Herbivac was selected for use as a vaccine vector for expressing the RVFV genes coding for proteins that will induce a protective immune response. Histopathologically, compared to nLSDV, it showed a greater immune response where extensive hypertrophy and hyperplasia of the chorionic epithelium was observed in infected membranes. Herbivac also displayed a reduced level of pathogenicity in embryonated hens' eggs where nLSDV killed the embryos at day 4 and Herbivac did not. Compared to nLSDV, Herbivac has also been reported by Deltamune (South Africa) to have a higher percentage take in cattle (data not available). Herbivac was therefore chosen as a vector for the construction of a dual veterinary vaccine designed to express the RVFV coding for proteins that will induce a protective immune response, GnGc polyprotein and NC. Figure 3.18 shows a flow diagram of the experimental stages used to create the recombinant LSDV virus.

#### **3.3.1 Design of transfer vector required for construction of Herbivac-RVFV**

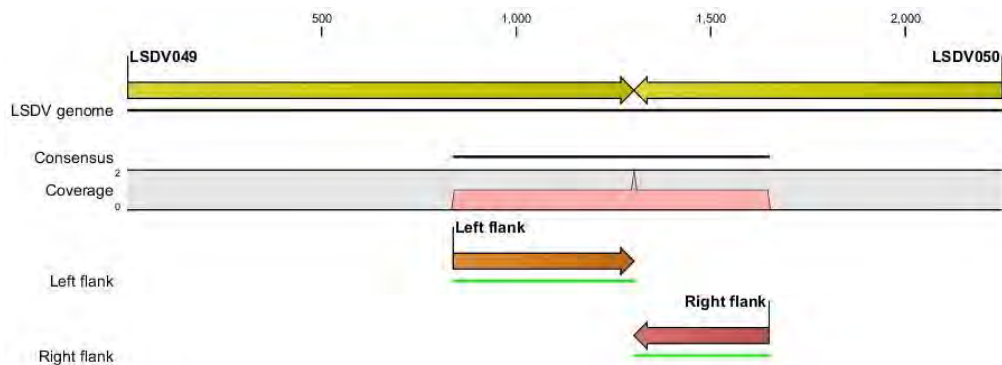
In order to isolate a recombinant virus a cell needs to be simultaneously infected with the parent virus and transfected with transfer vector DNA. The transfer vector is a circular plasmid which contains the foreign genes to be inserted (in this case the RVFV genes). Homologous recombination needs to occur between the viral DNA and the same DNA sequence contained within the transfer vector in order for the foreign DNA to be inserted into the viral genome. The transfer vector thus needs to contain segments of DNA which are homologous to the viral DNA. Once homologous recombination has occurred and the transfer vector DNA has been inserted into the viral genome the recombinant virus can be isolated. In order to isolate the recombinant virus marker and selection genes are included in the transfer vector. This allows one to differentiate between the recombinant and the wild-type viruses.



**Figure 3.18** Flow diagram of the experimental steps used to create the recombinant virus.

### 3.3.1.1 Site of insertion

For this study we selected parts of highly conserved LSDV genes for inclusion in the transfer vector. The gene segments selected were LSDV49 (vvI8R) and LSDV50 (vvG1L) [192]. Where the virion protein I8R has both DNA and RNA helicase activities and the G1L putative metalloproteinase is essential for viral replication in vivo [192]. Figure 3.19 shows a segment of the LSDV genome with the LSDV49 and LSDV50 regions showing the left and right flanks.



**Figure 3.19** Diagram showing site of insertion of foreign genes, between LSDV ORFs 49 and 50.

In this study, the transfer vector (pRO1) (Figure 3.19) was designed such that the left (LSDV49) and right (LSDV50) flanking DNA sequences are located at opposite ends of the RVFV genes. The cassette can be divided into two parts; one part contains the two RVFV genes coding for proteins that will induce a protective immune response, flanked on either side by the left and right flanks. The other part is located outside of the flanks and contains the marker and selection gene as well as the remainder of the plasmid DNA. Using such a design requires two homologous recombination events to take place in order to produce the final recombinant virus. The first recombination event occurs between one of the transfer vector flanks (LSDV49 or 50) and its corresponding DNA sequence in the poxvirus genome. This results in the first homologous recombination event to produce what is known as the single cross-over recombinant. This single cross-over event allows for the insertion of the entire transfer vector into the LSDV genome. The second recombination event will be between the other flanking sequences resulting in deletion of the reporter and selection genes and retention of the RVFV genes.

### **3.3.1.2. Reporter and selection genes**

The reporter gene allows for identification of the recombinant virus and the selection gene allows for selection of the recombinant as opposed to the wild-type parent virus. The reporter gene used here was the enhanced green fluorescent protein (eGFP) under the regulation of vaccinia virus synthetic early/late promoter [193], [194]. Recombinant poxvirus infected cells thus emit a bright green fluorescence when exposed to UV light [195]. The selection gene used here was the *E.coli* xanthine-guanine phosphoribosyltransferase (GPT) gene, coding for xanthine-guanine phosphoribosyltransferase.

GPT has been shown to function as an effective positive selection gene to isolate recombinant poxviruses [196]. This selection method involves the use of mycophenolic acid (MPA), an inhibitor of purine metabolism. MPA disrupts the purine metabolism pathway through the inhibition of inosine monophosphate (IMP) dehydrogenase. IMP dehydrogenase would normally convert IMP to xanthosine monophosphate (XMP), a vital precursor in guanine synthesis. Cells in the presence of MPA are thus unable to undergo normal synthesis of guanylic acid, an important intermediary for RNA and DNA synthesis. The *E. Coli* GPT gene product overcomes this block through a salvage pathway which utilises xanthine and hypoxanthine, whereby GPT converts xanthine to XMP [196].

In the presence of MPA, xanthine and hypoxanthine wild-type LSDV will not replicate. Recombinant LSDV containing GPT will grow in the presence of MPA, xanthine and hypoxanthine. Initially a recombinant is selected which has undergone only one cross-over event. This virus will produce fluorescence (eGFP) and grow in the presence of MPA.

Once a pure culture is obtained, the second cross-over event is allowed to occur through the removal of the MPA selection agent and results in the looping out of the selection and marker genes as well as the remainder of the plasmid. The selection of the final construct will be based on its non-fluorescing properties.

The second cross-over can occur in one of two ways. If the single cross-over homologous recombination occurred between the right flank and its corresponding region in the LSDV50 gene; then the second cross-over event will occur between the left flank and the LSDV49 gene. Conversely, if homologous recombination occurred on the left flank; then the second cross-over event will occur between the right flank and the LSDV50 gene. An alternative second cross-over event can occur between the viral DNA and the flanking DNA such that the entire cassette is removed resulting in wild-type virus.

### **3.3.1.3. Selection of RVFV antigens**

The recombinant LSDV has been designed to express three separate Rift Valley fever virus genes, two glycoprotein genes, namely Gc and Gn, as well as one nucleocapsid gene (NC). In order to gain a better understanding of RVFV diversity and current outbreaks new phylogenetic trees were constructed. Here we made use of all full length RVFV M-segment and NC sequences published on Genbank. This analysis also included the most recently sequenced strains of RVFV.

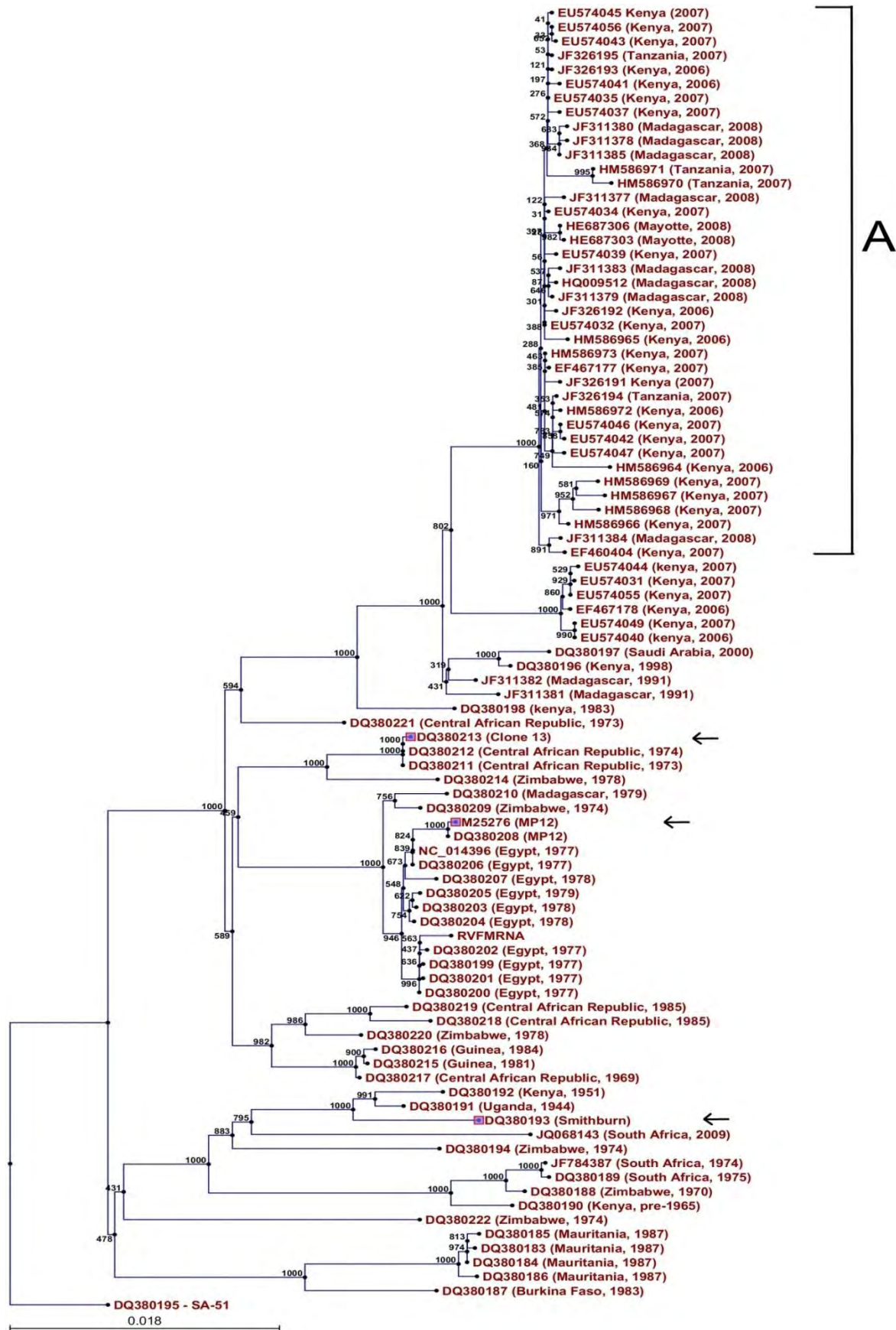
#### **3.3.1.3.1 GnGc polyprotein**

The gene coding for the GnGc polyprotein is contained within the RVFV M-segment. The M-segment comprises a total length of 3885 base pairs. A section of the M-segment consisting of 3594 bases codes for the polyprotein which is processed into the Gn and Gc envelope glycoproteins. This section excludes the translation initiation sites of the two other M-segment proteins, the 78kDa structural protein and the non-structural virulence factor 14kDa protein (NSm2). In order to design an appropriate GnGc gene a representative RVFV M-segment was designed. This M segment was then used to extract the GnGc gene sequence for inclusion in the transfer vector. The polyprotein sequence will ultimately allow for the expression of the RVFV Gn and Gc envelope glycoproteins by Herbivac.

In order to construct a representative M-segment, all full-length RVFV M-segments available on Genbank (108) were compiled in DNA analysis software, CLC bio. Duplicate M-segment sequences were then removed (duplicate sequences were defined as being 100% identical at the nucleotide level and derived from isolates found in the same country in the same

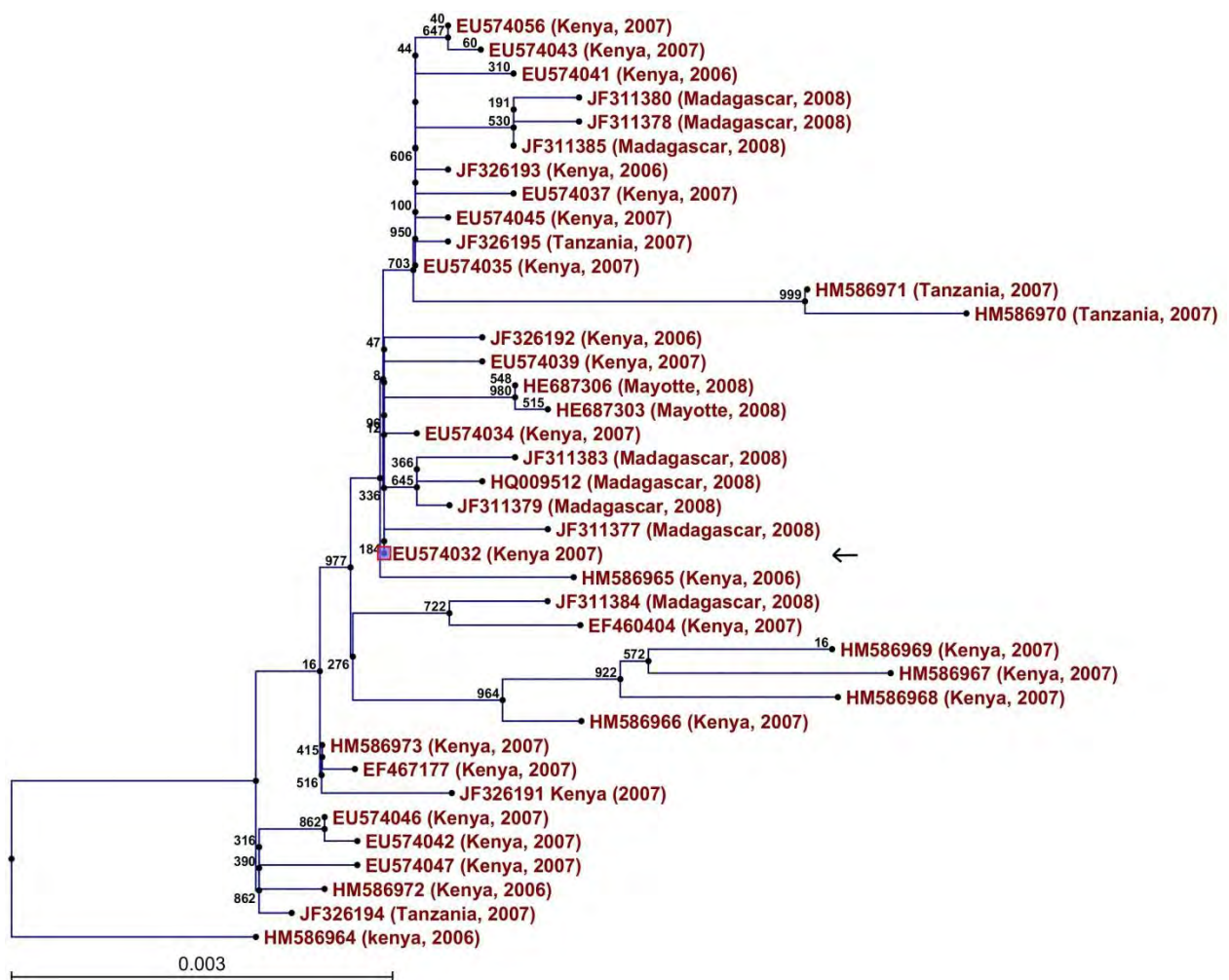
year.) This resulted in 16 sequences being removed leaving a total of 92 sequences available for analysis. All duplicates found were submitted as part of a study examining a RVFV outbreak in Kenya in 2007 [197].

A neighbour joining phylogenetic tree was constructed from all 92 full length M-segments, Figure 3.20. RVFV strain SA-51 has been used to root the tree as it forms its own lineage in trees constructed by *Grobbelaar et al.* [198] and *Bird et al.* [199].



**Figure 3.20** Neighbour joining phylogenetic tree constructed using all ‘non-duplicate’ full length Rift Valley fever virus M-segments available on Genbank. This tree includes strains from throughout Africa and the Arabian Peninsula from 1944 to 2009. Vaccine strains of RVFV have been highlighted using blue boxes and black arrows. Bootstrap replicates were set at 1000. A phylogenetically distant strain, DQ380195 SA-51, has been used to root the tree. Scale bar indicates nucleotide substitutions per site.

As can be seen in Figure 3.20, the most recently sequenced isolates of RVFV (2006 to 2008) group together geographically in Kenya and its neighbouring country Tanzania. This group has been designated Group A. Group A has been differentiated from the rest of the strains by a 1.5% different in nucleotide identity. Group A also includes isolates found in Madagascar and its neighbouring island Mayotte. Strains from Group A are appropriate for informing vaccine design as one would want vaccine antigens that are representative of the latest outbreaks in vaccine targeted regions. A new phylogenetic tree was thus constructed of all Group A sequences as shown in Figure 3.21.



**Figure 3.21** Neighbour joining phylogenetic tree of RVFV M-segment Group A sequences. This tree includes strains isolated in Kenya, Tanzania, Madagascar and Mayotte from 2006 to 2008. A sequence found to be 100% similar at a nucleotide level to a consensus sequence compiled from all sequences in group A has been highlighted using a blue box and an arrow. Bootstrap replicates set at 1000. Tree rooted with the most phylogenetically distinct sequence (HM586964, Kenya 2006). Scale bar indicates nucleotide substitutions per site.

A consensus sequence was compiled from all sequences in group A. This consensus sequence was then compared at an amino acid and a nucleotide level to other sequences in the group A. A single sequence was found to be 100% identical at a nucleotide level to the consensus sequence. This single sequence was obtained from an isolate in Kenya in 2007 (Genbank: EU574032) and is highlighted using an arrow in Figure 3.22. Several sequences in Group A showed 100% identity to the consensus sequence when compared at an amino acid level. A consensus sequence was then derived from all RVFV M-segments (i.e. M-segments from all strains shown in Figure 3.20) and compared to the consensus sequence Group A. A total of 15 differences were found at a nucleotide level and 4 differences found at an amino acid level between the consensus group A and consensus derived from all M-segments. There was no natural M-segment which was identical in nucleotide sequence to the consensus sequence derived from all M-segments.

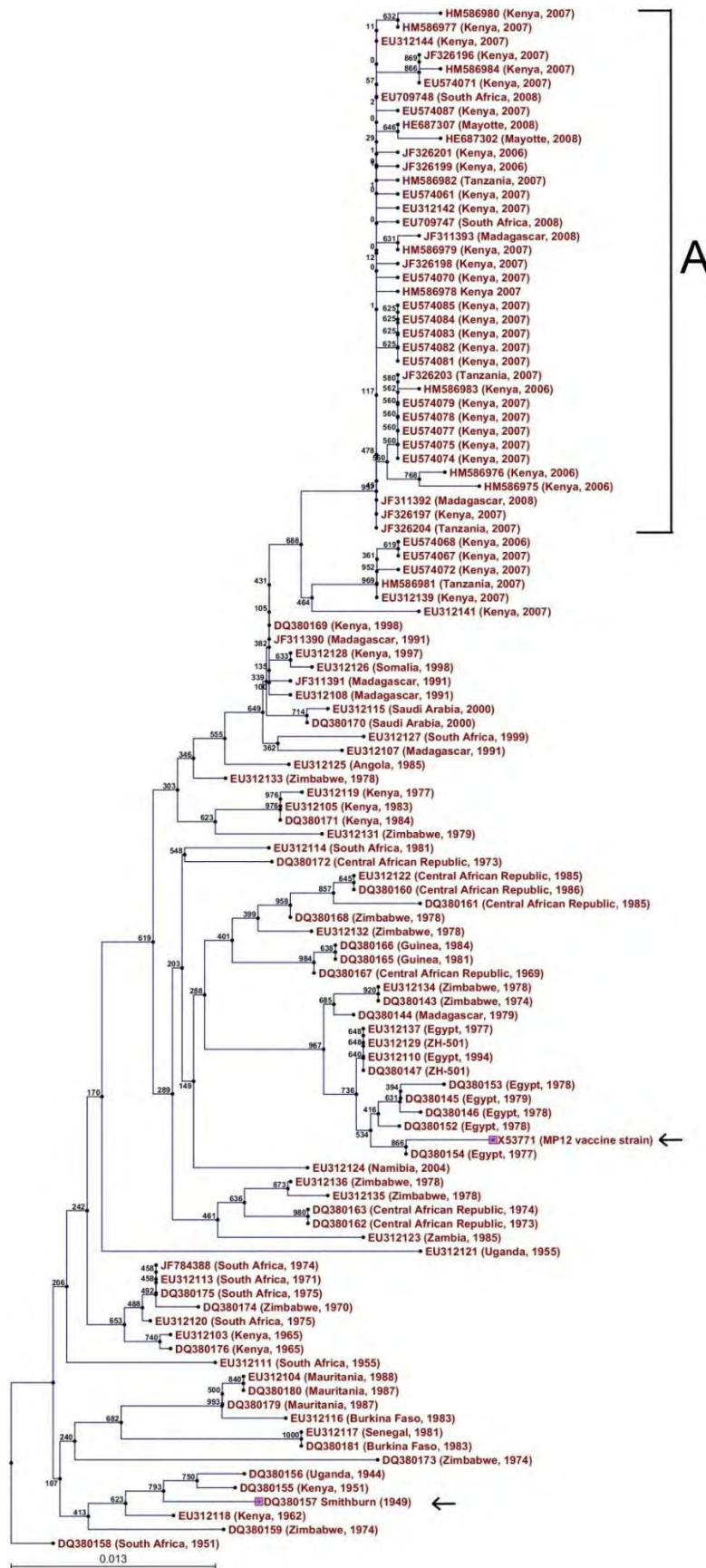
The consensus sequence derived from group A sequences is representative of current outbreaks, as group A is made up of the most recent outbreaks of RVFV. In addition the group A consensus sequence was found to have 100% identity at a nucleotide level to an existing RVFV sequence, indicating that this gene will encode a protein which would be antigenically the same as the natural virus. For these reasons the group A consensus sequence was used as the sequence from which the RVFV GnGc polyprotein gene sequence would be taken. A selection of the consensus Group A was made from the base pair position 411 (the 4<sup>th</sup> ATG start site) to base pair position 3614 (the position of the TAG stop-codon in frame with the 4<sup>th</sup> ATG start site). This has been shown to be the coding region for GnGc [167][168]. This selection was named consensus GnGc group A.

#### **3.3.1.3.2 Nucleocapsid gene**

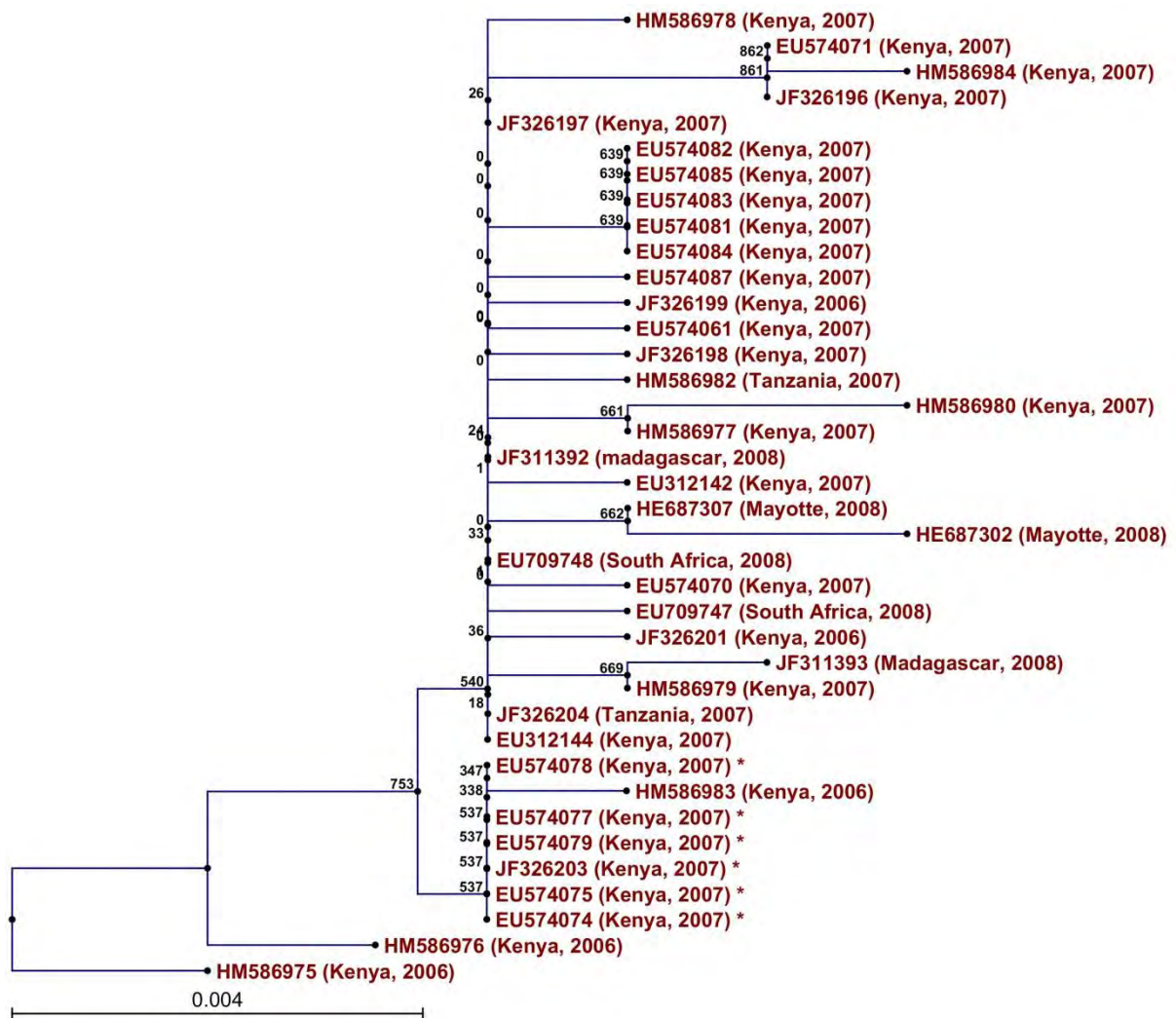
A similar approach as was used to define the consensus GnGc group A gene was used to define an appropriate synthetic nucleocapsid (NC) gene. The S-segment is approximately 1690bps long and encodes the nucleocapsid (NC) protein in the negative sense and the non-structural virulence factor (NSs) in the positive sense. All RVFV full length NC coding sequences (coding from 3' to 5' on the S-segment from nucleotide base pair position 1653 to 916) were extracted from Genbank and compiled in CLC bio. A total of 149 sequences

were obtained in this manner. From this, 38 duplicate sequences (as defined previously) were excluded and the remaining 111 sequences were used in the analysis. Only the coding regions of the NC gene were used and not the full length S-segment of RVFV. The NSs virulence factor and regulatory regions were excluded from the analysis.

A neighbour joining phylogenetic tree was constructed using all 111 sequences (Figure 3.22). Again, a group A was identified which contained sequences from the most recent outbreaks in Kenya, Mayotte, Tanzania, South Africa and Madagascar from 2006 to 2008. Again, group A was differentiated from the rest of the strains by a 1.5% difference in nucleotide identity. A second neighbour joining phylogenetic tree was constructed which contained sequences from group A only, (Figure 3.23).



**Figure 3.22** Neighbour joining phylogenetic tree of all RVFV non-duplicate nucleocapsid gene sequences available on Genbank. This includes strains from throughout Africa and the Arabian Peninsula from 1944 to 2008. The RVFV vaccine strains have been highlighted using a blue box and an arrow. Clone 13 vaccine is not included in this tree because a full length sequence could not be found. Bootstrap replicates were set at 1000. The most phylogenetically distant strain has been used to root the tree (DQ380158, South Africa 1951). Scale bar indicates nucleotide substitutions per site.



**Figure 3.23** Neighbour joining phylogenetic tree of all RVFV Group A NC gene sequences. This included strains isolated in Kenya, South Africa, Mayotte, Tanzania and Madagascar from 2006 to 2008. The most phylogenetically distant strain has been used to root the tree (HM586975, Kenya 2006). Bootstrap replicates were set at 1000. Scale bar indicates nucleotide substitutions per site. Sequences highlighted using an asterisk were found to be 100% identical at a nucleotide level to the consensus NC group A sequence.

A consensus sequence was derived from all group A sequences and was annotated 'consensus NC group A'. Using similar logic used when defining the consensus GnGc group A sequence, this consensus NC sequence is representative of current RVFV outbreak NC sequences; as group A is made up of the most recent outbreaks of RVFV. In addition the consensus NC group A sequence was found to have 100% identity at a nucleotide level to six existing sequences and would therefore encode a protein which would fold correctly (strains with asterisk in Figure 3.23). The consensus NC group A sequence was compared to a consensus sequence compiled from all 111 NC sequences. Here we found 6 nucleotide differences and no differences at an amino acid level. At a nucleotide level, no NC sequence

could be found that was 100% identical to the consensus sequence compiled from all RVFV NC sequences. For these reasons the consensus NC group A sequence was used in construction of the transfer vector.

#### **3.3.1.3.3 M-segment and NC gene sequence analysis**

A comparative analysis was conducted on all RVFV M-segments (excluding duplicates, as defined above). According to the analysis M-segment diversity was found to be relatively low, with a nucleotide identity of 95.3% and an amino identity of 99%. A comparative analysis was then conducted on all RVFV nucleocapsid genes (excluding duplicates). For the nucleocapsid gene an average identity of 97.5% at a nucleotide level and 99.8% at an amino acid level was found, indicating greater conservation of this gene.

#### **3.3.1.3.4 RVFV vaccine strain sequences**

The desired coding regions of the compiled consensus sequences were compared to the equivalent coding regions of RVFV vaccine strains. The ZH-501 GnGc coding sequence was also included in the Group A GnGc consensus sequence table as the ZH-501 GnGc coding sequence was used in the construction of several previous recombinant poxviruses [78][82][115]. Comparative nucleotide and amino-acid alignments were conducted and tabulated as shown in Figures 3.24 and 3.25.

A

	1	2	3	4	5
Consensus GnGc group A	1	96.79	96.75	95.01	86.27
ZH-501 recombinant vaccine	2	103	97.57	95.91	87.61
Clone 13 vaccine strain	3	104	78	96.07	86.86
Smithburn vaccine strain	4	160	131	126	85.89
MP12 vaccine strain	5	440	397	421	452

B

	1	2	3	4	5
Consensus GnGc group A	1	99.34	99.16	98.22	98.88
ZH-501 recombinant vaccine	2	7	99.25	98.22	99.34
Clone 13 vaccine strain	3	9	8	98.41	98.97
Smithburn vaccine strain	4	19	19	17	97.94
MP12 vaccine strain	5	12	7	11	22

**Figure 3.24** Nucleotide and amino acid comparison of RVFV GnGc coding regions showing percentage identity and number of differences. **(A)** The nucleotide sequence of GnGc of group A is compared to the equivalent nucleotide region in three live attenuated vaccine strains: Clone 13; Smithburn and MP12, as well as the ZH-501 sequence **(B)** The amino-acid sequence of GnGc of group A is compared to the equivalent sequence of the three live attenuated vaccine strains and recombinant poxvirus vectored vaccines. (1) Consensus GnGc group A (2) ZH-501 recombinant vaccine (3) Clone 13 vaccine strain (4) Smithburn vaccine strain (5) MP12 vaccine strain.

A

	1	2	3	4
Consensus NC group A	1	97.56	96.61	97.15
Clone 13	2	18	97.83	97.70
Smithburn	3	25	16	96.34
MP12	4	21	17	27

B

	1	2	3	4
Consensus NC group A	1	100.00	99.19	99.59
Clone 13	2	0	99.19	99.59
Smithburn	3	2	2	98.78
MP12	4	1	1	3

**Figure 3.25** Nucleotide and amino acid comparison of RVFV NC coding regions showing percentage identity and number of differences. **(A)** Nucleotide sequence of NC of Group A. **(B)** Amino-acid sequence of NC group A. (1) Consensus GnGc group A (2) ZH-501 recombinant vaccine (3) Clone 13 vaccine strain (4) Smithburn vaccine strain (5) MP12 vaccine strain.

#### ***3.3.1.4 Selection of poxvirus promoters for expression of foreign genes***

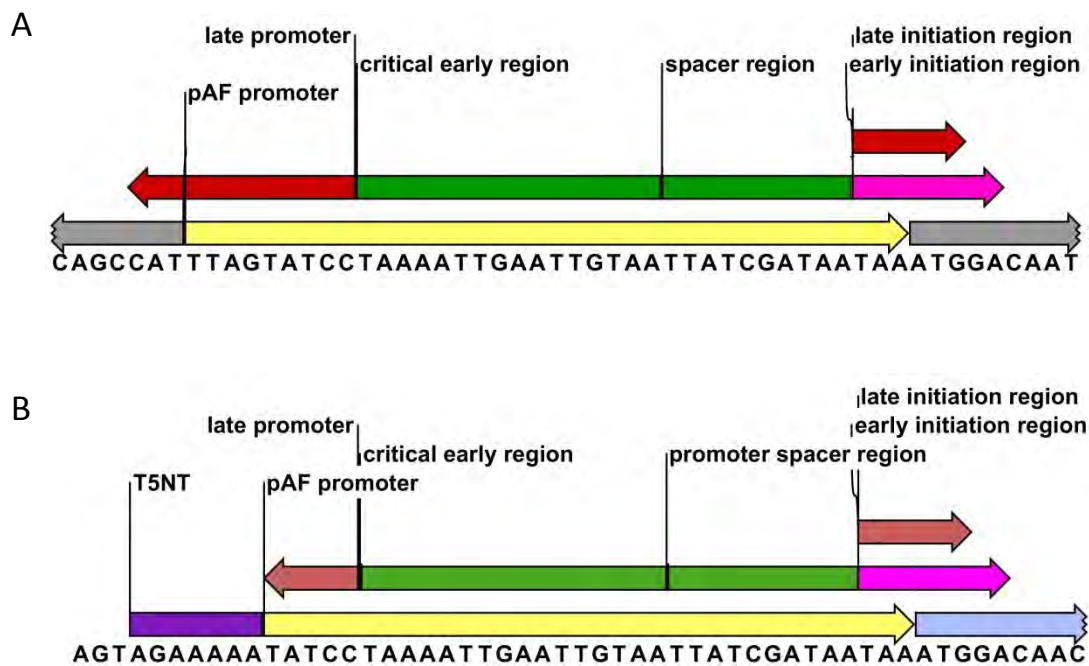
Poxvirus promoter elements were used to regulate the expression of all genes in the expression cassette contained within the transfer vector, pRO1. These genes are the two Rift Valley fever virus (RVFV) glycoprotein genes (Gc and Gn), the NC gene as well as an enhanced green fluorescence protein (eGFP) marker gene and guanine phosphotransferase (GPT) selection gene. The eGFP sequence was taken from CloneTech (USA) and the GPT sequence was taken from a portion of the plasmid pAF09 (provided by David Boyle of the CSIRO, Australia).

The Gc and Gn RVFV glycoproteins are expressed from a single gene sequence as a polyprotein which is then cleaved. Here the glycoprotein gene was successfully expressed under the regulation of a vaccinia virus modified H5 promoter (mH5) as shown in Figure 3.26.



**Figure 3.26** Vaccinia virus modified H5 promoter (yellow arrow) is placed upstream of RVFV GnGc gene (blue arrow). The promoter transcription initiation region is indicated by a red arrow.

The NC gene was successfully expressed while being regulated by a modified version of an early/late fowl pox promoter (pAF). Figure 3.27 **(A)** shows the promoter in its native form. For the purposes of this study we have modified the promoter to remove the antisense late promoter function. This has been done through the removal of CATTAG sequence from the 5' end of the original promoter. The CATTAG sequence has been replaced with a poxvirus translation termination signal in the antisense direction (AAAAATA). The resulting promoter sequence was renamed modified early/late fowl pox promoter (mFP) and is depicted in Figure 3.27 **(B)**.



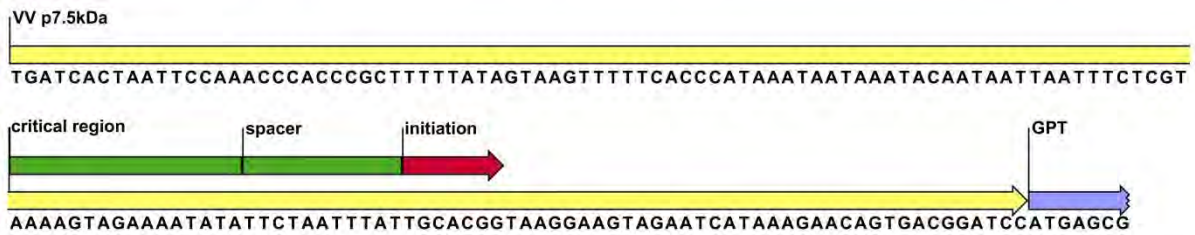
**Figure 3.27** Native and modified fowlpox virus promoter sequences are shown. Yellow arrows indicate the promoters. Pink arrows indicate early initiation regions and red arrows indicate late initiation regions. Other promoter elements are shown in green. (A) Fowlpox virus promoter (pAF) as seen in the fowlpox virus genome (genbank NC002188). The native pAF promoter is bi-directional with an early/late promoter function in the sense direction and late promoter function in the antisense direction. Coding genes belonging to the fowlpox virus genome are shown in grey. (B) Modified fowlpox promoter (mFP) as designed for transfer vector. Purple labelled region indicates the T5NT poxvirus early transcription termination sequence in the antisense direction.

The eGFP gene (Clontech, USA) was successfully expressed under the regulation of a synthetic vaccinia virus promoter named pSS (Figure 3.28) [200].

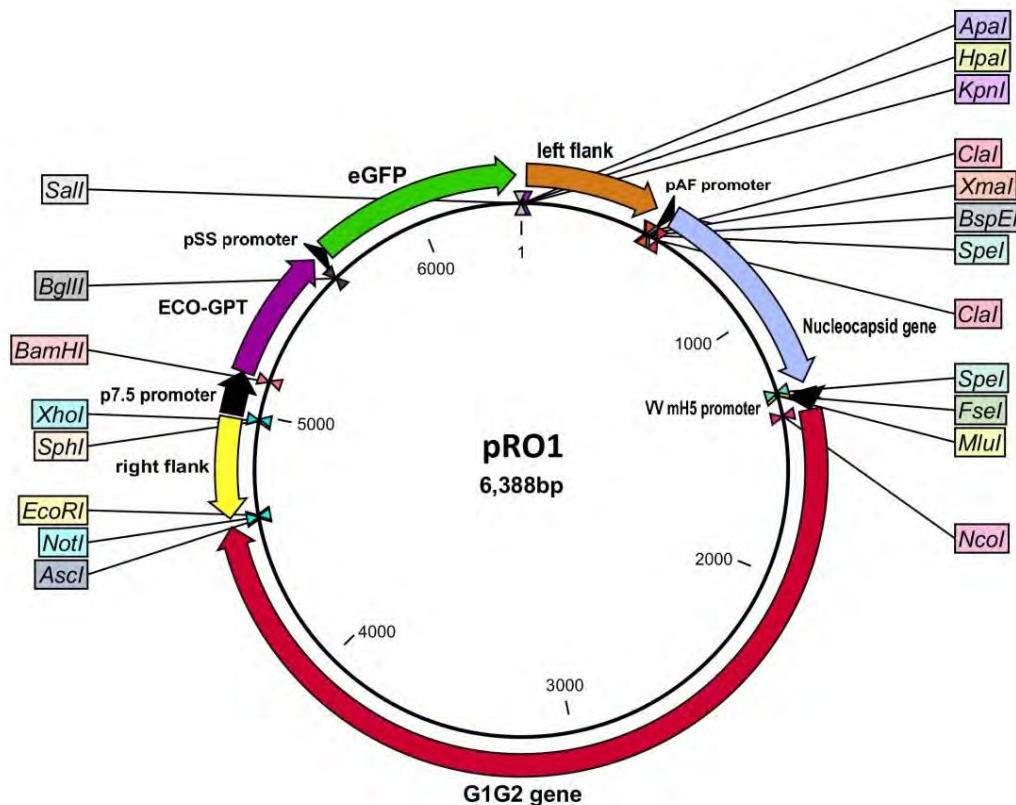


**Figure 3.28** Synthetic vaccinia virus early/late promoter (yellow) shown upstream of eGFP marker gene (blue). A green arrow indicates an essential promoter region, the tata box.

The promoter used for the expression of the GPT selection gene is the vaccinia virus 7.5kDa early/late promoter (VV p7.5kDa) (Figure 3.29).



**Figure 3.29** Vaccinia virus 7.5kDa early/late promoter (yellow) shown upstream of GPT selection gene (blue). Promoter initiation region of transcription is indicated by a red arrow and other promoter elements are indicated by green regions.



**Figure 3.30** The transfer vector, pRO1, drawn to scale. Promoter sequences are shown in black and cloning sites are indicated at their relative positions along the plasmid. Gene and flank sequences are indicated by arrows which point in the direction in which the gene is transcribed. The diagram does not show the pUC57 E.coli plasmid into which the designed insert was cloned. The insert was cloned into pUC57 between the Sal1 and Apa1 sites. G1G2 gene = GnGc.

The DNA transfer vector, pRO1 (see Figure 3.30), allows for the insertion of foreign DNA into the genome of Herbivac. It contains genes for the RVFV antigens GnGc and NC. In this study, each RVFV antigen sequence was carefully selected according to phylogenetic analysis of RVFV sequences (see section 3.3.1.3). Previously published data has either made use of a small number of sequences or a small section of the genome for the analysis [199][198].

### **3.3.2 Selection of optimal conditions for construction of recombinant Herbivac**

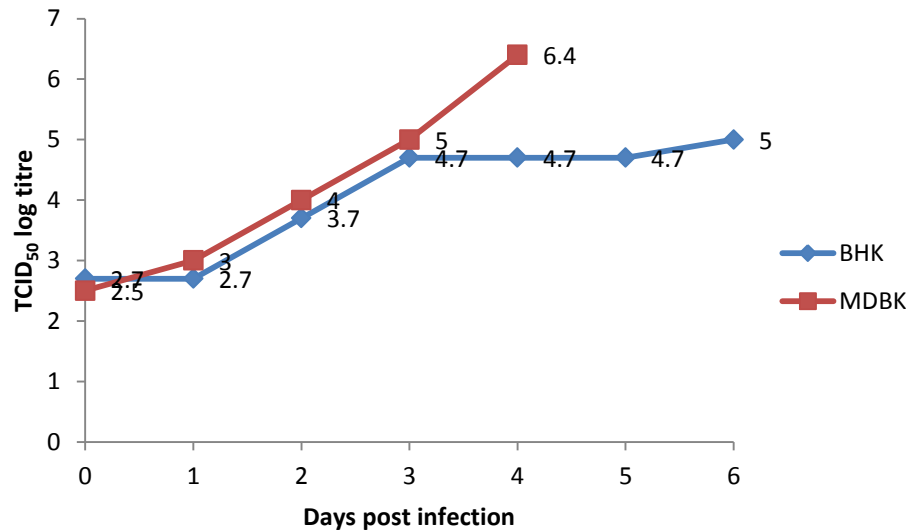
Successful isolation of recombinant poxviruses is complex. The virus and transfer vector are required to be present simultaneously in the same cell. The viral DNA then needs to undergo homologous recombination with the transfer vector during the DNA replication cycle of the virus. The homologous recombination needs to allow for stable insertion of the foreign transfer vector DNA into the parent virus resulting in a recombinant virus. This experiment requires the optimisation of a number of variables. The virus needs to replicate well in the cell line of choice. The cell line also needs to be highly susceptible to transfection by plasmid DNA. The amount of transfection reagent used in the experiment needs to be optimised so that it allows for efficient entry of the transfer vector DNA into the cells. The amount of transfection reagent and virus also needs to be optimised so that the cells do not die before recombination has taken place.

#### ***3.3.2.1 Selection of cell type for transfections***

It was shown that Herbivac grows in Madin-Darby bovine kidney (MDBK) cells (section 3.1.1) and this was the cell line initially selected for construction of a recombinant virus. However, the transfection efficiency in this cell line was so low (see section 3.3.2.2) it was necessary to select a different type of cell for the construction of a recombinant LSDV. Because BHK and CEF cells have been shown to be permissive for MVA, a host restricted vaccinia virus, the growth of Herbivac was also investigated in these cells.

##### **3.3.2.1.1 Herbivac growth in BHK and CEF cells**

Growth curves were generated to compare Herbivac growth in BHK and MDBK cells (Figure 3.31). The initial rate of viral growth for MDBK and BHK cells is similar for the first three days. At 4 days post infection (d.p.i.) the rate of viral growth in BHK cells plateaus while it increases at the same rate in MDBK cells for a further 24 hours resulting in a 1.7 log increase in viral titre in MDBK cells compared to BHK cells on day 4. Limited Herbivac growth is observed in BHKs from day 3 to day 7.



**Figure 3.31** Growth of Herbivac in BHK cells. BHK cells infected with Herbivac at an m.o.i. of 0.001. Virus was harvested from flasks at different time points and titrated. The average viral titre for each time point was plotted. For comparison, the growth curve of Herbivac in MDBK cells has been included.

Herbivac growth was further assessed in CEF cells. No signs of cell pathology were observed and no increase in viral titre could be detected over an infection period of 6 days.

We have shown BHK cells to be permissive to LSDV growth. BHK cells are readily transformable with plasmid DNA which makes this finding relevant for the construction of recombinant LSDV. In the past recombinant LSDV could only be made in primary sheep or bovine cells which could be passaged for a limited period of time. This fact combined with the laborious process of making the primary cultures is seen to be a disadvantage over the use of a cell line such as BHK.

These results show that BHK cells, and not CEF cells, can be included as an alternative cell line for use in transfections for making recombinant Herbivac LSDV.

### 3.3.2.1.2 Transfection optimisation in MDBK, FBT and BHK cells

Transfections were conducted as described in 2.11.1 in MDBK, BHK and FBT cells. Various ratios of pRO1 DNA to transfection reagent were used. The marker gene, eGFP, under the regulation of a synthetic vaccinia virus promoter, pSS, allowed for visualisation of infected

and transfected cells by the emission of green fluorescence under UV light (Figures 3.32 and 3.33).

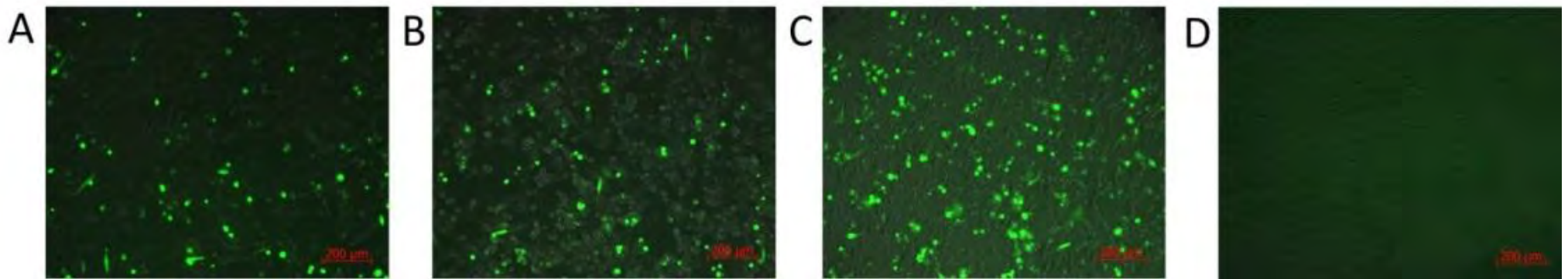
At day 3 MDBK cells displayed no fluorescence (not shown) whereas BHK and FBT cells showed increasing fluorescence with increasing amounts of pRO1 DNA added (Figure 3.32, 3.33). The highest level of fluorescence was observed when 3µg of DNA was added with 3µg of transfection reagent (a 1:1 ratio). FBT cells displayed higher levels of fluorescence compared to BHK cells and MDBK cells at each pRO1 DNA to transfection reagent ratio, indicating that FBT cells are the optimal cell type for use in transfections. These results also indicate the optimal DNA to transfection reagent ratio to be 1:1 using 3µg DNA and 3µl transfection reagent per 1ml reaction (Figure 3.32 C and 3.33 C). Therefore, although LSDV did grow in BHK cells, FBT cells were eventually selected to make recombinant LSDV because the transfections were more efficient.

As expected, all three cell types displayed no fluorescence in the no-virus control showing that expression of eGFP on pRO1 requires the presence of Herbivac. No fluorescence is detected in any of the three cell types when infected with Herbivac alone (not shown). FBT cells were thus used for conducting transfections needed to generate the recombinant. However FBT cells are not easily obtained and can only be passaged a few times (5-7 passages) thus passaging of transfections was conducted in both FBT cells and MDBK cells depending on availability (as the virus grows well in both cell types).

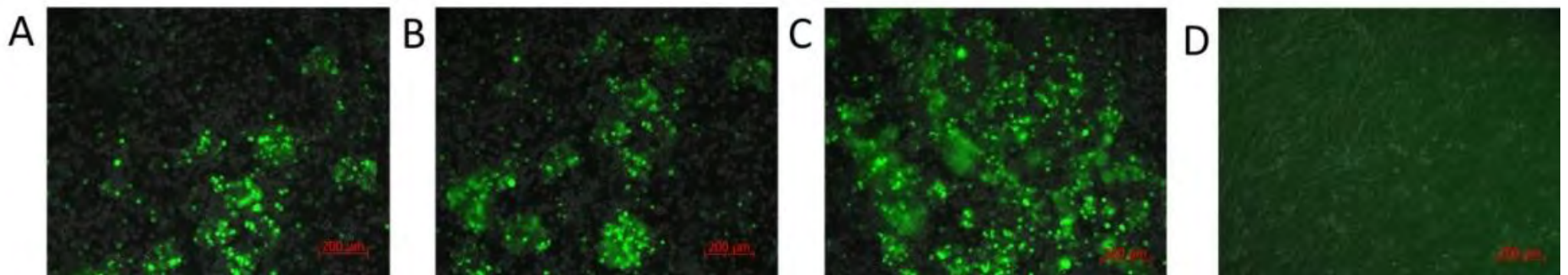
### ***3.3.2.2 MPA optimisation for selection of recombinant virus***

Mycophenolic acid (MPA) was used to select for the recombinant virus over the wild type virus. The principle behind this selection is described in detail in section 2.12. In order to use MPA as a selection agent the optimal concentration of MPA had to be determined. The optimal selection conditions would be the lowest concentration of MPA which hinders the growth of wild-type Herbivac while allowing for the growth of recombinant Herbivac; and causing the least amount of cell pathology.

MDBK cells were seeded into a 24-well plate and infected with Herbivac at various m.o.i.s and different concentrations of MPA were used (table 3.2). Three days post infection cells were photographed and cell pathology noted. No Herbivac cytopathic effects could be seen when an MPA concentration of 1.2µg/ml was used with an m.o.i. of 0.03 (table 3.2). This was determined to be the optimal MPA concentration used for selecting recombinant virus. An MPA concentration of less than 1.2µg/ml allowed for growth of Herbivac. Above 1.2µg/ml MPA caused the cells to lift.






**Figure 3.32** Transient expression of eGFP in BHK cells. BHK cells infected with Herbivac at an m.o.i. = 0.8 and transfected with pRO1 DNA using XtremeGeneHP (Roche, USA). Cells were visualised under UV light 3 d.p.i. **(A)** 1µg DNA, 3µl XtremeGeneHP **(B)** 2µg DNA, 3µl XtremeGeneHP **(C)** 3µg DNA, 3µl XtremeGeneHP **(D)** no virus control, transfected with 3µg DNA, 3µl XtremeGeneHP. (Zeiss Axiovert 200M, Germany. Image overlay; Excitation:BP 365/12 and Emission: BP 450-490).



**Figure 3.33** Transient expression in FBT cells. Primary FBT cells infected with Herbivac at an m.o.i.=0.8 and transfected with pRO1 using XtremeGeneHP (Roche, USA). Cells were visualised under UV light 3 d.p.i. **(A)** 1µg DNA, 3µl XtremeGeneHP **(B)** 2µg DNA, 3µl XtremeGeneHP **(C)** 3µg DNA, 3µl XtremeGeneHP **(D)** no virus control, transfected with 3µg DNA, 3µl XtremeGeneHP. (Zeiss Axiovert 200M, Germany. Image overlay; Excitation: BP 365/12 and Emission: BP 450-490).

**Table 3.2 MPA optimization to determine the minimum, non-toxic, MPA concentration needed to prevent the growth of Herbivac in MDBK cells**

Multiplicity of infection	no MPA	0.6µg/ml MPA	1.2µg/ml MPA	2.5µg/ml MPA	5µg/ml MPA	10µg/ml MPA
<b>Uninfected cells</b>	Confluent mono-layer. No cells lifting	Confluent mono-layer. No cells lifting	Patches of no-cells. Less confluent monolayer	Patches of no-cells. Less confluent monolayer	50% of base covered with cells. A few cells lifted	20% of base covered with cells. Many cells lifted
<b>0.0003</b>	Very few foci seen. Confluent monolayer.	Single developing focus seen. Confluent monolayer.	50% of base covered with cells. Many cells lifted	20% of base covered with cells. Many cells lifted	20% of base covered with cells. Many cells lifted	10% of base covered with cells. Many cells lifted
<b>0.003</b>	Few foci seen. Confluent monolayer.	No foci seen. Confluent monolayer.	Patches of no-cells. Cells rounding.	50% of base covered with cells. A few cells lifted	50% of base covered with cells. A few cells lifted	20% of base covered with cells. Many cells lifted
<b>0.03</b>	Several foci seen. Confluent monolayer. 	Several developing foci seen. Confluent monolayer. 	Confluent monolayer. No foci seen. 	50% of base covered with cells. A few cells lifted	20% of base covered with cells. Many cells lifted	20% of base covered with cells. Many cells lifted

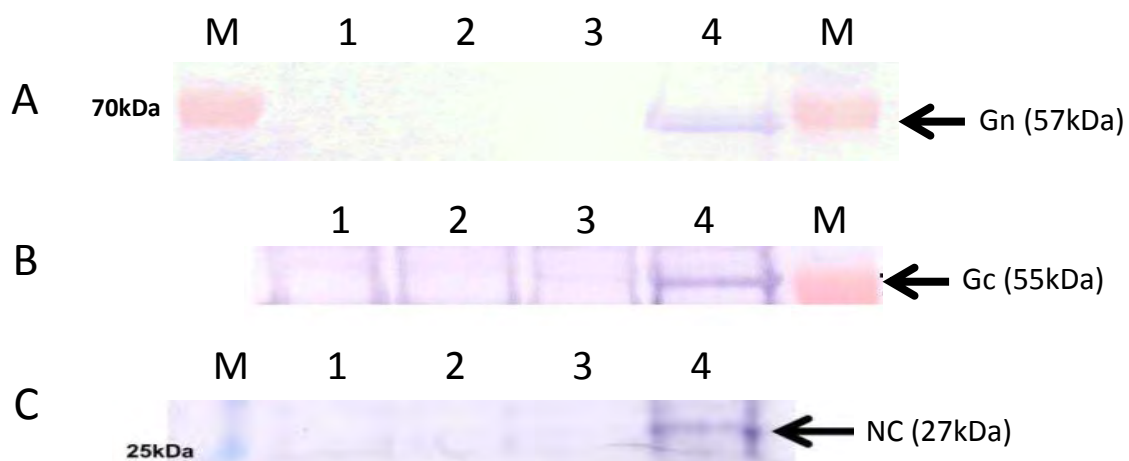
### 3.3.3 Confirmation of promoter activity and transient expression of RVFV genes from pRO1

For the construction of the recombinant Herbivac it is important to demonstrate the ability of Herbivac to recognise the vaccinia virus and fowlpox virus promoters (mH5 and mFP respectively) and express the desired RVFV proteins in their correct size and conformation. The mH5 promoter regulates the expression of RVFV Gn and Gc glycoproteins and the mFP promoter regulates the expression of the NC protein. Here western blotting and immunofluorescence were employed to assay for transient expression of RVFV genes and demonstrate Herbivac recognition of these foreign poxvirus promoters.

### 3.3.3.1 Western blotting

BHK cells were infected with Herbivac and then transfected with pRO1 (as described in 2.10.1) and a protein lysate prepared (as described in 2.10.2). BHK cells were used in this experiment instead of FBT due to the lack of availability of FBT cells. Protein lysates were subjected to western blot analysis to confirm the expression of full length RVFV antigens, Gn (57kDa), Gc (55kDa) and NC (27kDa) (lane 4 of Figure 3.34). Gn and Gc appear larger on the gel, possibly due to glycosylation. The NC protein migrated to the expected position on the gel.

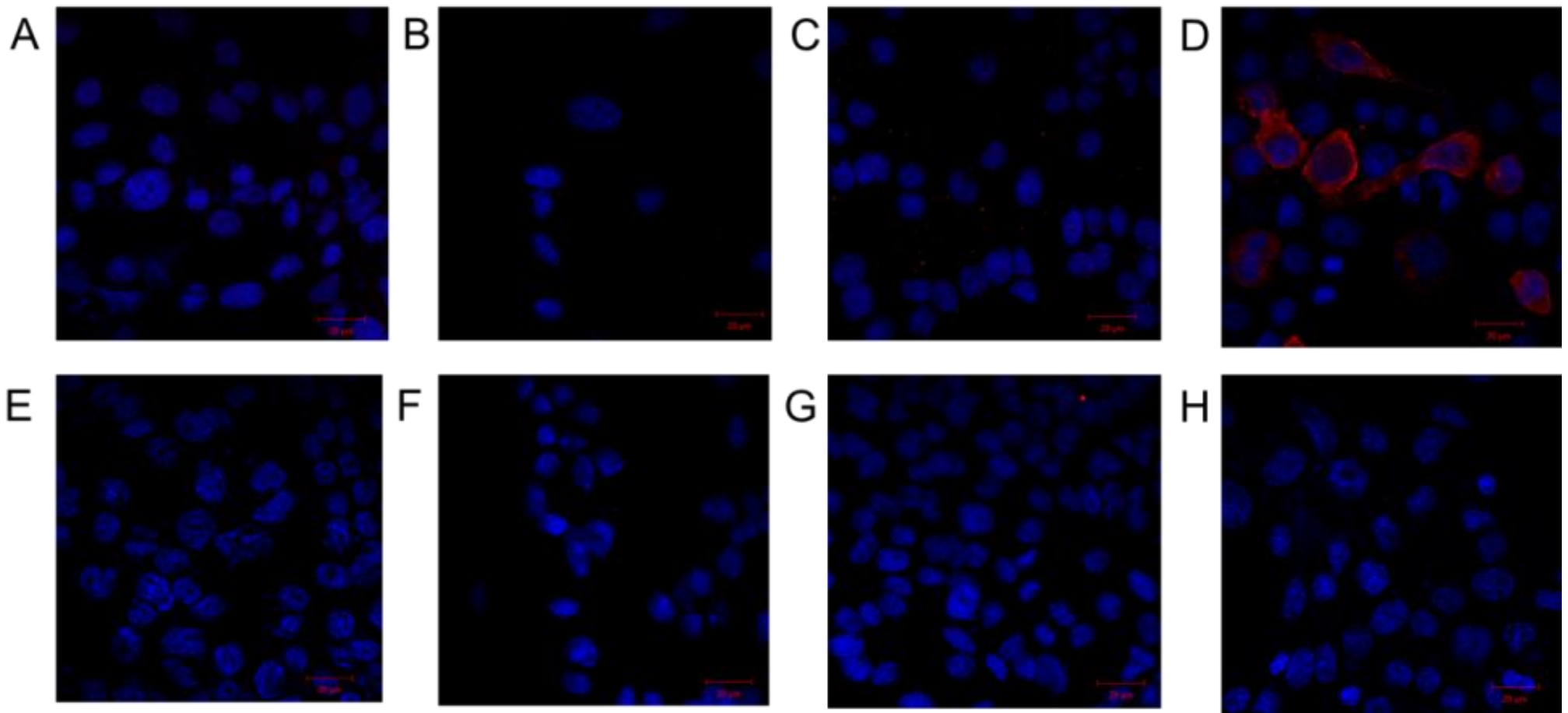
RVFV antigens approximating the expected sizes were only detected in protein lysates from cells transfected with pRO1 and infected with Herbivac. Transient expression of RVFV antigens was not detected in the negative controls which included cells only, pRO1 only and Herbivac virus only (lanes 1, 2 and 3 respectively of Figure 3.34). This demonstrates that Herbivac recognises the mH5 and mFP promoters and is able to express all three RVFV proteins.



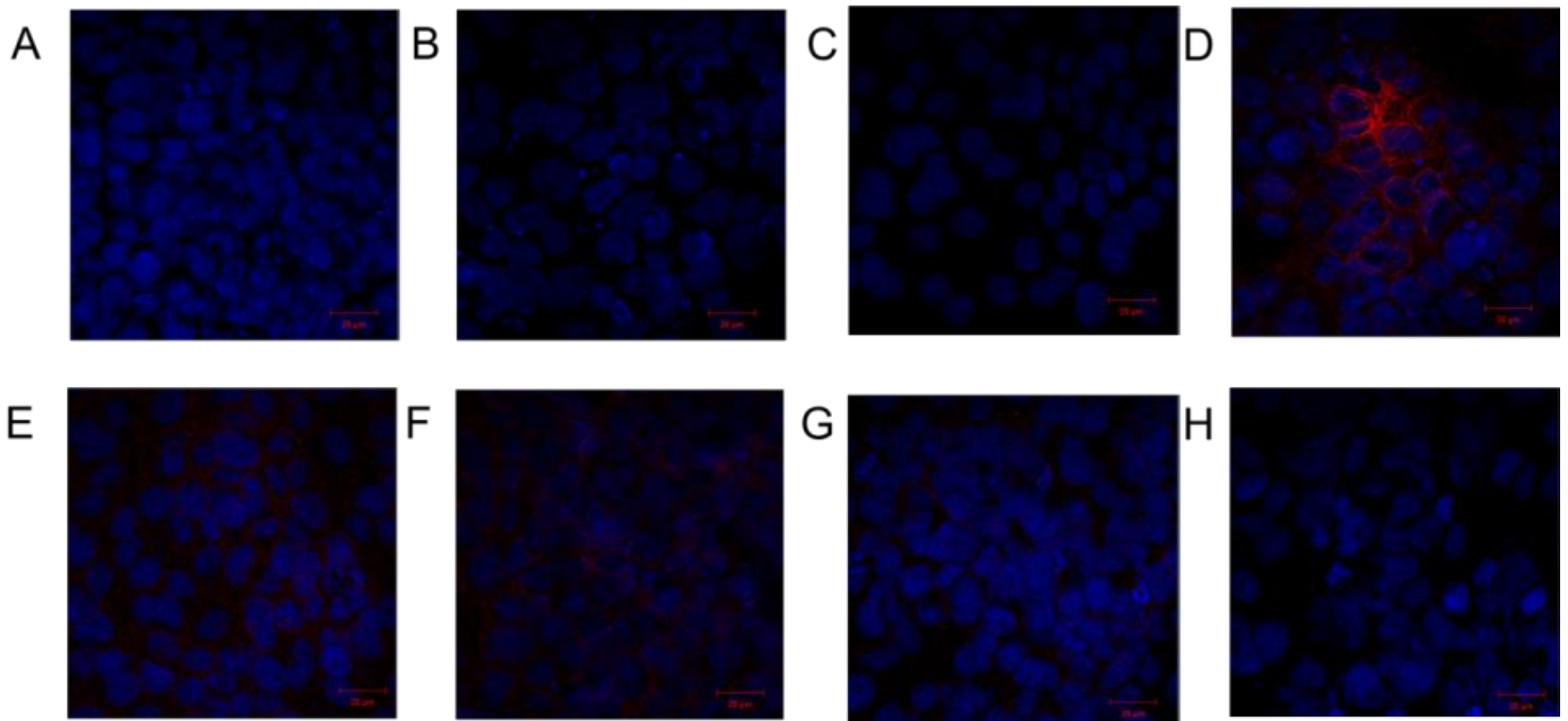
**Figure 3.34** Transient expression of RVFV proteins in BHK cells **(1)** Lysate of cells only **(2)** lysate from uninfected cells transfected with pRO1 **(3)** lysate from cells infected with Herbivac **(4)** experimental lysate from cells infected with Herbivac and transfected with pRO1. **(M)** Page Ruler prestained protein ladder #26616 (ThermoScientific, USA). Transient expression of **(A)** RVFV Gn glycoprotein (57kDa), **(B)** RVFV Gc glycoprotein (55kDa) and **(C)** transient expression of RVFV NC (27kDa) protein.

### **3.3.3.2 Immunostaining**

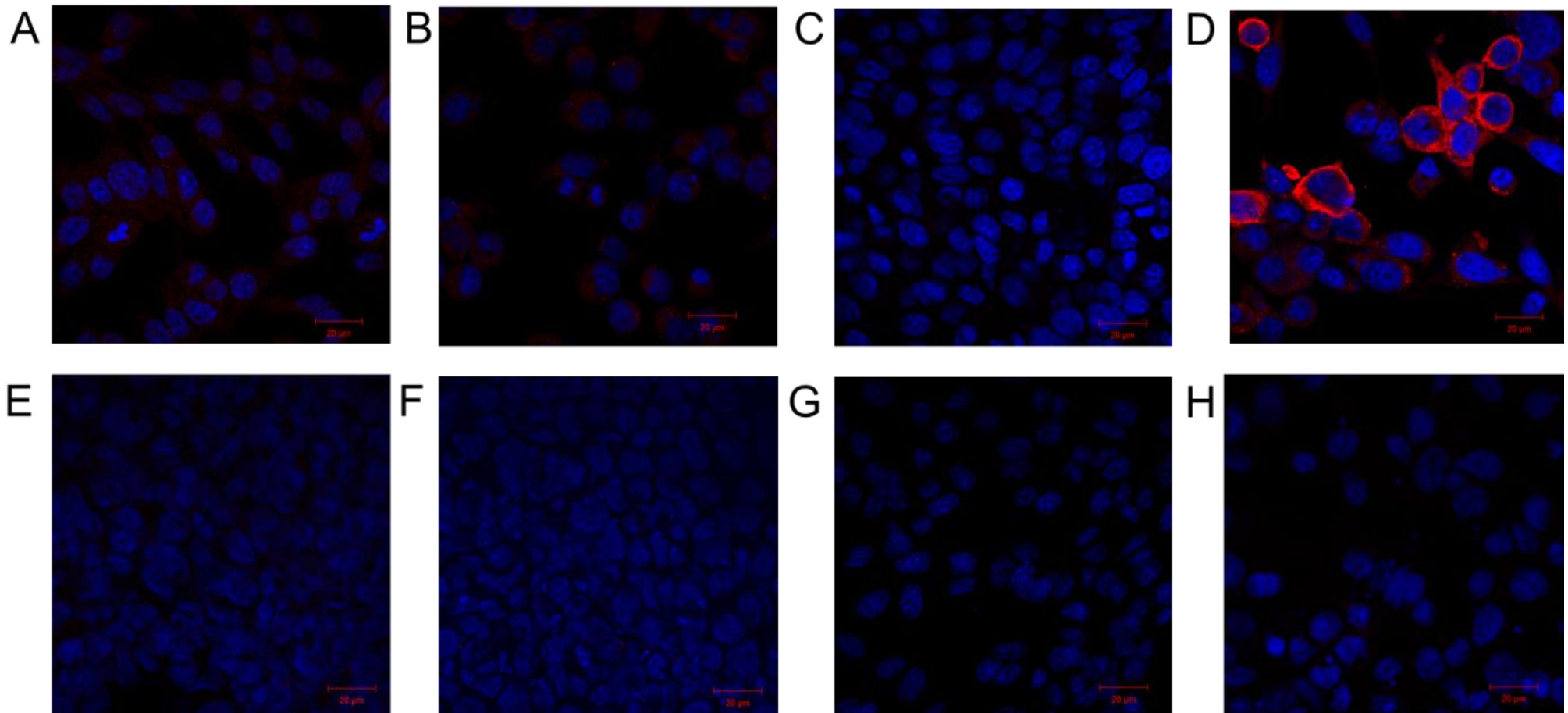
Transient expression of RVFV antigens was detected by immunofluorescence using three separate polyclonal rabbit antibodies against the three different antigens Gn, Gc and NC. Primary antibodies were then detected using Cy5-labeled donkey-anti-rabbit immunoglobulin (as described in 2.10.3). The transient expression of Gn, Gc and NC was detected as red fluorescence in Herbivac infected cells which were transfected with pRO1 (image D in Figures 3.35, 3.36 and 3.37 respectively). Negative controls included uninfected cells, infected and untransfected cells and transfected uninfected cells. In addition the experiment was repeated with no primary antibody for each. No fluorescence was detected in any of the control wells.



**Figure 3.35** Detection of the RVFV Gn Glycoprotein transient expression by immunofluorescence. Primary antibody is rabbit-anti-Gn glycoprotein (prosci #4521), secondary antibody is donkey-anti-rabbit-Cy3 (red). The blue stain is a hoechst nucleus stain. In the upper panel **A-D** both primary and secondary antibodies were used. In the lower panel only the secondary antibody was used. **A** and **E**: plasmid only; **B** and **F**: virus only; **C** and **G**: uninfected cells only; **D** and **H**: infected and transfected cells. (Zeiss 510 LSM Meta, Excitation: 561nm).



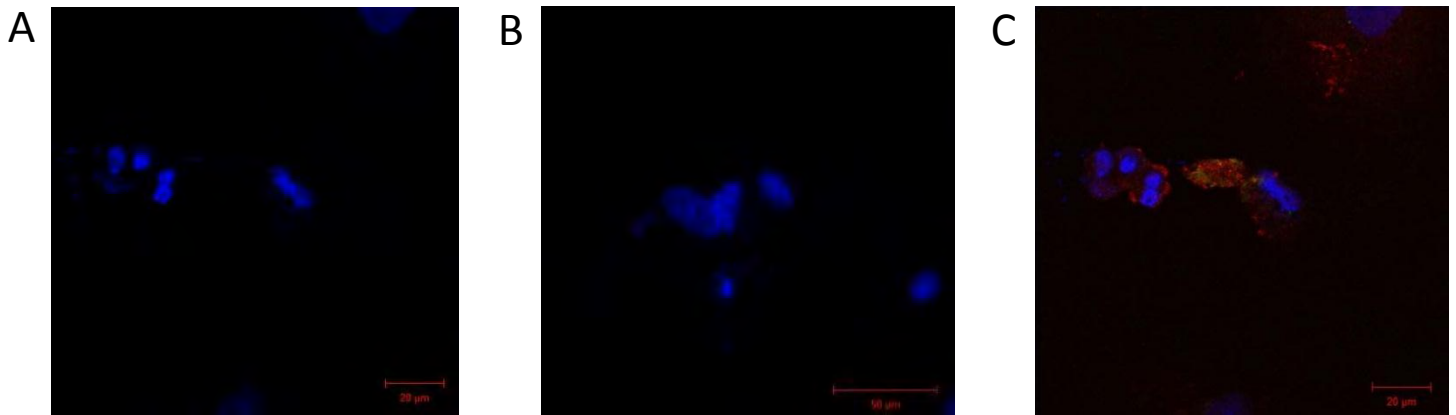
**Figure 3.36** Immunofluorescence staining for the detection of the RVFV Gc glycoprotein transient expression. Primary antibody is rabbit-anti-Gc glycoprotein (Prosci 4519), secondary antibody is donkey-anti-rabbit-Cy3 (red). The blue stain is a hoechst nucleus stain. In the upper panel (**A-D**) both primary and secondary antibodies were used. In the lower panel only the secondary antibody was used. **A** and **E**: plasmid only; **B** and **F**: virus only; **C** and **G**: uninfected cells only; **D** and **H**: infected and transfected cells. (Zeiss 510 LSM Meta, Excitation: 561nm).



**Figure 3.37** Immunofluorescence staining for the detection of the RVFV NC protein transient expression. Primary antibody is rabbit-anti-NC (prosci 7413), secondary antibody is donkey-anti-rabbit-Cy3 (red). The blue stain is a hoechst nucleus stain. In the upper panel (**A-D**) both primary and secondary antibodies were used. In the lower panel only the secondary antibody was used. **A** and **E**: plasmid only; **B** and **F**: virus only; **C** and **G**: uninfected cells only; **D** and **H**: infected and transfected cells. (Zeiss 510 LSM Meta, Excitation: 561nm).



to be confirmed by western blotting at each stage. Time constraints prevented the completion of these tasks.



**Figure 3.39** Immunostaining of Herbivac-RVFV infected primary FBT cells. Primary antibody is rabbit-anti-NC (Prosci 7413), secondary antibody is donkey-anti-rabbit-Cy3 (red). The blue stain is a hoechst nucleus stain. **(A)** Cells only control **(B)** wild-type Herbivac only **(C)** Herbivac-RVFV. (Zeiss 510 LSM Meta, Excitation: 561nm). Images taken by Nai-Chung Hu.

## Chapter 4 Discussion

There were two major aims to this body of research. The first aim was to evaluate possible differences between two commercial LSDV vaccines. And the second aim was to construct a recombinant LSDV expressing genes coding for proteins that will induce a protective immune response to RVFV.

### Aim 1: Compare and contrast two commercial LSDV vaccine strains

The Herbivac LSDV vaccine was received from a research collaborator, Dr. Louis Maartens (Deltamune, South Africa). Herbivac was derived from the nLSDV strain and has improved immunogenicity in cattle compared to its parent, nLSDV (personal communication, Louis Maartens). It is not known if the immunogenicity is due to the virus or the formulation of the vaccine.

Growth curves of Herbivac and nLSDV were conducted in MDBK cells. The lack of difference in growth and cell pathology between the two viral strains in cell culture is likely due to the absence of immune cells and lack of immune regulation pathways in the cell-line used. This may have allowed for little difference in immune regulation between the two viral strains.

We explored the use of the more immunogenically advanced embryonated hens' egg system for infection with nLSDV and Herbivac. The embryonated hens' egg contains a living embryo and a circulatory system that penetrates the chorioallantoic membrane providing it with a blood supply containing heterophils (avian neutrophilic leukocytes). Here the nLSDV strain grew at a faster rate compared to the Herbivac and maintained a higher titer on each day of the growth curve until 4 days post infection. After day 4, eggs infected with nLSDV died, while eggs infected with Herbivac did not. A faster rate of viral growth was observed in CAMs infected with nLSDV than in CAMs infected with Herbivac. The faster growth rate also correlated with the killing of the embryos with nLSDV infected CAMs. However, it is not known if the higher titre of nLSDV killed the embryos or if the nLSDV was more pathogenic than Herbivac. Increased levels of inflammation was observed as membrane thickening which occurred earlier in nLSDV infected CAM (1 d.p.i.) compared to Herbivac (3 d.p.i.).

The growth patterns on eggs confirmed that Herbivac has different properties to nLSDV. For nLSDV, no marked difference could be found between its growth in MDBKs and on CAMs. For Herbivac, however, a faster rate of growth is observed when the viral strain is used to infect MDBK cells compared to CAMs. From this direct comparison (between the cell-line which lacks immuno-regulatory functions compared to embryonated hens' eggs) we may be able to further clarify that the Herbivac strain may have some effect on immuno-regulation.

Histopathological studies of CAMs infected with the two strains of LSDV were conducted in order to gain a deeper understanding of the embryonated hens' egg immune response to the different strains. A higher level of inclusion bodies were observed in nLSDV infected CAMs compared to Herbivac. Inclusion bodies are formed as a result of viral factories which may contribute to a higher level of pathogenicity of nLSDV.

In conclusion, notable differences were observed between Herbivac and its parent, nLSDV. Growth curves of Herbivac and nLSDV in cell culture showed no difference in rate of viral growth. However when the two strains were grown on CAMs a faster growth rate was observed for nLSDV growth compared to Herbivac. nLSDV also killed the embryos at 4 days post infection and Herbivac did not. Histological studies of CAMs infected with either virus indicate that both Herbivac and nLSDV cause significant pathology of CAMs, however Herbivac causes higher levels of angiogenesis, hyperplasia and hypertrophy compared to nLSDV. Thus this study has provided evidence that Herbivac displays differing characteristics to nLSDV and that these differences are most likely attributed to altered immune regulation. This has implications for veterinary vaccine design as a vector with an improved immune response but reduced pathogenicity may result in a more efficacious vaccine.

Further studies are needed in order to gain an understanding of the altered immune responses. Future work should include a biological repeat of the growth curves of Herbivac and nLSDV in cell culture and CAMs of embryonated hens' eggs in order to obtain a statistically significant result. Histopathology work on CAMs infected with Herbivac or nLSDV should be repeated to test for reproducibility of the results.

## Aim 2: Construction of a recombinant LSDV expressing RVFV genes

A recombinant LSDV was designed to express RVFV genes coding for proteins that will induce a protective immune response which are representative of recent outbreak strains of the virus. An appropriate transfer vector was designed containing the relevant RVFV genes as well as suitable promoters to express foreign genes. Conditions were optimised for infection, transfection and selection of the recombinant virus. Before isolating a recombinant virus it was necessary to confirm that the foreign genes would indeed be expressed. Transient assays were performed to test for expression of the foreign genes.

Foreign genes were shown to be expressed from the poxvirus promoters. The RVFV glycoprotein gene was placed under the regulation of a vaccinia virus modified H5 promoter (mH5). The sequence of the mH5 promoter was taken from published sequences [201], [202].

The NC gene was regulated by a modified version of an early/late fowl pox promoter (pAF). The native pAF promoter was first mapped by Kumar and Boyle and found to be bi-directional with an early/late function in the sense direction and a late promoter function in the antisense direction (Figure 3.2 **(A)**) [203]. In the study by Kumar and Boyle the promoter was shown to have a strong early function which is often beneficial for high levels of expression of foreign genes in poxviruses [203]. A later study reported favourable expression levels when using pAF to transiently express reporter genes using LSDV [204]. The promoter was also successfully used within our research group for expressing the rabies virus glycoprotein gene in LSDV [56]. For the purposes of this particular study the pAF promoter was modified to remove its antisense late function. This represents the first successful use of a modified version of the pAF promoter. The eGFP gene (Clontech, USA) is under the regulation of a synthetic vaccinia virus promoter named pSS. pSS was developed by Chakrabarti *et al.* (1997) who described its use as a vaccinia virus promoter with strong early/late expression (Figure 3.3) [200].

The promoter used for the expression of the GPT selection gene is the vaccinia virus 7.5kDa early/late promoter (VV p7.5kDa). VV p7.5kDa has been used previously by our research group [56], [73] and other groups [22], [78], [82], [204–207] to successfully express foreign

genes in LSDV. For the purposes of this study a portion of this commonly used promoter was selected and placed upstream of the GPT gene start site.

When constructing a recombinant poxvirus it is critical to select an appropriate site for insertion of foreign genes. A common approach is to introduce the foreign genes into sites considered to be poxvirus non-essential regions or intergenic regions [53], [208]. The thymidine kinase gene has been used extensively as an insertion site for generating LSDV recombinants [78], [85], [204], [209–211], although it has also been shown that the thymidine kinase gene may be important, if not essential for normal growth of LSDV [206].

Another LSDV gene shown to be non-essential for LSDV growth, the ribonucleotide reductase gene, has been used within our research group for the construction of two separate LSDV recombinants [56], [73]. The insertion of foreign genes into existing poxvirus genes may be deleterious to vector growth even if the disrupted gene is assumed to be non-essential. In addition intergenic regions may have unknown regulatory and/or structural functions. Furthermore the insertion of foreign DNA products into non-essential and intergenic regions may result in an unstable recombinant where the vector excises the foreign genes as non-essential regions of DNA are associated with less DNA conservation. For these reasons our approach was to insert foreign DNA between two highly conserved LSDV genes without causing gene disruption.

The highly conserved genes selected for this study were LSDV49 and LSDV50; homologs of vaccinia virus I8R and G1L respectively. The equivalent insertion site has been used by Moss *et al.* (2007) in modified vaccinia Ankara (MVA) [212], but this is the first time this region has been used for cloning into LSDV. In addition to its favourable location between two highly conserved genes, the LSDV49/50 insertion site is located at the terminal points of two genes transcribed towards each other. By introducing foreign DNA into such a region, the chance of disrupting LSDV upstream regulatory regions is greatly reduced [212].

RVFV M-segments and NC genes were studied using phylogenetic analysis in order to construct RVFV genes coding for proteins that will induce a protective immune response relevant to current outbreaks of the virus. According to the analysis of all RVFV M-segments

published on Genbank, diversity was found to be relatively low, with a nucleotide genetic identity of 95.3% and an amino identity of 99%. For the nucleocapsid gene an average identity of 97.5% at a nucleotide level and 99.8% at an amino acid level was found. These phylogenetic studies include the largest number of RVFV M-segment and NC sequences compared to date. These results are relatively consistent with an earlier study by Bird *et al.* on the whole genome sequences of 33 ecologically representative RVFV strains. Bird found a nucleotide identity of 95% and an amino acid identity of 98% [199]. From these results it appears that nucleotide and amino acid identity is higher in the NC gene sequence compared to the M-segment. Here analysis of full M-segments (which includes the GnGc glycoprotein genes, a virulence factor and regulatory regions) is compared to the NC gene (excluding the virulence factor and regulatory regions found on the S-segment). It is possible that higher levels of nucleotide and amino acid diversity is observed in the M-segment as this segment consists largely of the GnGc viral surface glycoproteins which are exposed to the host immune system which may provide pressure for greater glycoprotein diversity.

Neighbour joining phylogenetic trees were constructed using RVFV M-segments and RVFV NC genes. The resulting trees (shown in Figures 3.9 and 3.11) indicate that current live attenuated and whole killed RVFV vaccine strains (highlighted using small blue boxes and arrow) are phylogenetically distant from the recent outbreak strains which were identified in Group A. Further analysis of Group A sequences allowed for the construction of consensus sequences (a consensus RVFV M-segment and consensus NC gene). These consensus sequences were found to be 100% similar to at least one other nucleotide sequence in the group, indicating that they are likely to fold into proteins of the correct conformation. These consensus sequences are highly representative of current outbreak strains of RVFV and are therefore suitable in the construction of a RVFV vaccine.

In order to better understand how these consensus sequences relate to the sequences of currently available RVFV vaccines, a comparative analysis was conducted. It was found that, for both GnGc genes and NC genes there are high levels of similarity between the Group A consensus sequences in the different RVFV vaccine strains, where the MP12 vaccine was found to be the most divergent. These tables show that, despite the RVFV vaccine strains being phylogenetically distant from current outbreak strains, overall low levels of nucleotide

and amino acid substitutions for GnGc and NC are maintained. Furthermore comparisons revealed no gaps, insertions or deletions for either coding region. The use of consensus sequences taken from recent outbreaks of RVFV will result in the construction of a more outbreak relevant recombinant vaccine. This study represents a first in the use of such logic to construct a recombinant RVFV vaccine.

In order to construct the recombinant Herbivac expressing the RVFV genes (Herbivac-RVFV) as well as assay for transient expression of those genes, the optimal transfection conditions needed to be found. It was shown, for the first time, that Herbivac grows in BHK cells, although at a reduced rate compared to its growth in MDBK cells. This does however, allow for BHK cells to be used as a cell line for conducting transfections. However in this study it was demonstrated that FBT cells could be transfected more efficiently and so these primary cells were chosen instead of BHK cells. It was established that transfection efficiency was highest when using primary FBT cells using a 1:1 ratio of extreme gene HP reagent to plasmid DNA. Transfection efficiency was assessed through observation of levels of expression of eGFP, which was placed under the pSS promoter. The observation of eGFP expression confirms the ability of Herbivac to recognise the pSS promoter and correctly initiate the expression of eGFP.

Transient expression of RVFV glycoproteins Gn and Gc as well as the expression of RVFV NC gene was observed by western blotting and immunofluorescence. Both mH5 and mFP promoters were recognised by Herbivac and expressed all three RVFV proteins of sizes approximating the expected size. This is the first demonstration of capripoxvirus recognition of, and expression via, the modified version of the fowlpox virus promoter. The expected sizes of RVFV proteins were Gn (57kDa), Gc (55kDa) and NC (27kDa) [171][113]. For the NC protein, western blot analysis identified a protein band of exactly the expected size, 27kDa. For the Gn and Gc glycoproteins western blot analysis identified specific bands which were larger than the expected size, 68kDa for Gn and 71kDa for Gc. These larger than expected sizes may be due to glycosylation of these glycoproteins in BHK cells. Transient expression of RVFV antigens was also detected through the use of immunofluorescence.

A single-crossover recombinant Herbivac-RVFPV was isolated. Transfections were conducted successfully in FBT cells and lysates were subjected to passaging in MDBK cells under optimised selection conditions. In this manner we were able to isolate fluorescing foci and increase the number of fluorescing foci (relative to non-fluorescing foci) through passaging of the viral lysates under conditions of selection. This increase in number of fluorescing foci indicated that we were successful in isolating a single-cross over recombinant virus.

Furthermore, a pure culture of the single cross-over recombinant virus was isolated in primary FBT cells. To test for the expression of foreign genes by the single cross-over recombinant, infected cells were immunostained and viewed using a UV microscope. Both GFP and NC protein expression could be detected, indicating expression of these two proteins by the recombinant virus. Expression of the Gn and Gc RVFPV glycoproteins from the single cross-over recombinant is still to be evaluated.

Following this, a second cross-over recombinant will need to be isolated in order to remove the selection and marker genes. This double cross-over recombinant will contain only the desired RVFPV genes and not contain selection and marker genes. Gene expression assays, akin to the transient expression assays used in this study, will need to be done on the double cross-over recombinant. Immunogenicity studies as well as challenge studies can be conducted in mice. Immunogenicity studies should assess both humoral and cellular immunity as the vaccine was designed to elicit both. Appropriate tests would thus include ELI-SPOT assays to test for specific T-cell responses and neutralisation assays where dilutions of sera are tested for ability to neutralise RVFPV; this assay will test for a neutralizing antibody response. Approval has been obtained to carry out RVFPV challenge studies in mice.

In conclusion, here we selected novel RVFPV glycoprotein and NC genes that are relevant to current outbreaks of RVFPV. The novel sequences were used in the construction of a transfer vector which was used in transient expression assays with Herbivac. Transient expression of RVFPV Gn and Gc glycoproteins as well as the NC protein was confirmed using western blotting and immunofluorescence. A recombinant virus was partially purified. Further

studies will involve further purification of the single cross-over and double cross-over recombinants followed by immunogenicity testing of the final recombinant in mice.

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## Appendix A

### Composition of Reagents:

#### Blocking/wash buffer (100 ml)

100 ml 1 × PBS

5 g Instant milk powder

1 ml 10 % Tween20

#### Buffered Formalin pH7.4

Formaldehyde (37-40%)

NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O (4g) Na<sub>2</sub>HPO<sub>4</sub> (anhydrous) (6.5g)

made up to 1 Litre sdH<sub>2</sub>O

pH solution to 7.4 using either 4nM citric acid (acid) or 0.2M Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O (base)

#### Complete DMEM (cDMEM)

DMEM, 4.5g/l glucose, L-glutamine (Lonza, Switzerland) supplemented with

10% heat-inactivated fetal calf serum (Sigma-Aldrich, USA)

1% penicillin/streptomycin (Invitrogen, USA)

#### *E.coli* Glycerol stocks

Make up 50% glycerol solution in HPLC H<sub>2</sub>O (autoclave)

Add 1ml bacterial culture to 0.5ml of 50% glycerol solution

#### Freezing medium

Solution made up heat-inactivated fetal calf serum (Sigma-Aldrich, USA)

10% Dimethyl sulfoxide (DMSO)

10% DMEM (BioWhittaker, USA)

#### Luria Broth media and agar containing ampicillin (or carbenicillin) 100mg/ml

Solution made up in 1L sdH<sub>2</sub>O

10g tryptone

5g yeast Extract

10g NaCl

100g ampicillin (or carbenicillin)

for Luria Broth agar plates add 1.5% Agar (Sigma-Aldrich, USA)

### **Lysis Buffer A**

Solution made up in sterile distilled nuclease free H<sub>2</sub>O.

100mM KCl

10 mM Tris-HCl pH8.3

2.5mM MgCl<sub>2</sub>

1.5% Tween-20

### **Lysis Buffer B**

Solution made up in sterile distilled nuclease free H<sub>2</sub>O.

10 mM Tris-HCl pH8.3

2.5mM MgCl<sub>2</sub>

1.5% Tween-20

### **McIlvain's standard buffer solution pH 7.4**

4nM citric acid, 0.2M Na<sub>2</sub>HPO<sub>4</sub>.12H<sub>2</sub>O, pH 7.4 made up as follows:

1.83ml of 0.1M citric acid

18.17ml of 0.2M Na<sub>2</sub>HPO<sub>4</sub>.12H<sub>2</sub>O

to 800ml sdH<sub>2</sub>O

pH solution to 7.4 using either 4nM citric acid (acid) or 0.2M Na<sub>2</sub>HPO<sub>4</sub>.12H<sub>2</sub>O (base).

### **MPA selection media stock (25µg/ml)**

10mg MPA powder added to 1ml NaOH (filter sterilise)

0.1g hypoxanthine to 10ml NaOH (hypoxanthine stock solution)

0.1g xanthine to 10ml NaOH (xanthine stock solution)

Add 25µl of 10mg/ml MPA, 14µl of hypoxanthine stock solution and 250µl xanthine stock solution to 10ml 2%DMEM.

### **PBS-PSF**

Solution made up in PBS (BioWhittaker, USA)

100µg/ml penicillin/streptomycin mixture

1µg/ml fungin

### **Peroxidase substrate**

Add a bead size di-anisidine substrate to Eppendorf tube

Add 500µl absolute ethanol

Vortex and warm for 10minutes

Centrifuge for 1minute

Add 120µl of di-anisidine supernatant to 6ml PBS and 6µl of >30% H<sub>2</sub>O<sub>2</sub>

### **36% sucrose solution**

36g sucrose added to 100ml of either Tris-EDTA pH9.0 or PBS

### **Transfer buffer (1X) (1 litre)**

1 × Tris base 5.82 g

Glycine 2.93 g

Methanol 200 ml

Water adjust to 1 litre

### **Tris-Acetate-EDTA buffer**

Made up in sterile distilled H<sub>2</sub>O

0.04M tris

128 mM EDTA

0.11% glacial acetic acid

### **Tris-EDTA pH9.0**

Solution made up in sdH<sub>2</sub>O

10mM Tris

1mM EDTA

### **Trypsin-EDTA**

Solution made up in distilled water pH7.8

0.3% NaCl

0.012% KH<sub>2</sub>PO<sub>4</sub>

0.02% KCl

0.091% Na<sub>2</sub>HPO<sub>4</sub>

0.05% glucose

0.02% EDTA

0.25% Penicillin/Streptomycin (Invitrogen, USA)

### **2%**c**DMEM**

DMEM, 4.5g/l glucose, L-glutamine (Lonza, Switzerland) supplemented with

2% heat-inactivated fetal calf serum (Sigma-Aldrich, USA)

1% penicillin/streptomycin (Invitrogen, USA)

## Appendix B

### RVFV transfer vector

SpeI sites have been placed flanking 'pAF-NC gene', this is to allow removal of the 'pAF-NC gene' segment of the plasmid at a later stage. The resulting plasmid can then be used to replace the glycoprotein gene with other genes using NcoI and AscI/NotI/EcoRI. No restriction site can be incorporated between pAF and the NC gene without destroying promoter function thus the NC gene cannot be replaced with other genes.

Apal-HpaI-KpnI-LSDV49(I8R)(left flank)-ClaI-XmaI-BspEI-SpeI-AGAAAAA-pAF-Consensus group A NC gene-TTTTTCT-SpeI-FseI-MluI-VVmH5-NcoI-Consensus group A Glycoprotein-TTTTTCT-AscI-NotI-EcoRI-LSDV50(G1L)(right flank)-SphI-XhoI-p7.5kDa-BamHI-ECO-GPT-TTTTTCT-BglII-pSS-eGFP-TTTTTCT-Sall-Sall

GGGCCCGGTACCATAGATTCAGAATTTTACATAATTACATTTTATATGAAAAATATTTAACTTATCATTACCA  
AACGATTTATTTGTGCAACCATCTAATTTAGATTTTTAAACAAAGTAAGTACTTATATAGACTCATTTTGTATC  
AGTGATGAAAAATGGACATTTATTATGTCTTCTTATTATGTTAATATTTTAGAATATGCAAAAAATACTCTAAG  
GGTGAAACGATGCATGTGCGTTGGATATTTTTGAAAGAGAAAATGTTTTAAATGATGATGCATTTAACGCAA  
TAAAGTTGTTGAATATGAAAGCAAAAATAATAAAGCACAGAAGATATAATAAATTATATGTATTAACGTGTAA  
ATTAATGTTTGGTGTATATTTGGTAAACATTTAACATTATACATAATCGACCATTATTTGGTTATATTACTAT  
GATAACAGATAGTACTTTTGTAACTGATGATTAATCGATCCCGGGTCCGGAAGTAGAGAAAATATCCTAA  
AATTGAATTGTAATTATCGATAATAAATGGACAACATCAAGAGCTTGCATCCAGTTTGTCTGCTCAAGCAGT  
GGACCGCAATGAGATTGAACAGTGGGTCCGAGAGTTGCTTATCAAGGGTTTGATGCTCGTAGGGTTATCGA  
ACTCTTAAAGCAGTATGGTGGGGCTGACTGGGAGAAGGATGCCAAGAAAATGATTGTTCTGGCTCTGACTCG  
TGGCAACAAGCCCCGGAGGATGATGATGAAAATGTGCAAGAAGGCAAACTGTGGAGGCTCTCATCA  
ACAAGTATAAGCTAAAGGAGGGGAATCCTCCGAGATGAGCTGACTCTATCACGAGTTGCTGCTGCCCTGGC  
TGGCTGGACATGCCAGGCTTTGGTCGTCTGAGTGAGTGGCTTCTGTCACTGGGACAACAATGGACGGTCTA  
TCCCCTGCATACCCGAGGCATATGATGCATCCAGCTTGTGTCATGGTGGACCCTTCTACCAGAAGACTA  
TCTAAGGGCAATATTAGATGCTCACTCTGTATCTGCTGCAGTTCTCCGAGTCATCAACCAAACCTCCGAG  
GTAGAACAAGGAGGAGGTGCTGCAACGTTACGCAGCCAATGAATGCAGCAGTGAATAGCAACTTTATAA  
GCCATGAGAAGAGGAGAGAATTTTTGAAAGCTTTGGGCTTGTGGATTCTAATGGGAAACCGTCAGCTGCTG  
TCATGGCAGTCTCAGGCCTACAAGACAGCAGCCTAAATTTTTCTACTAGTGGCCGACGCGTAAAAATTG  
AAAATAAATACAAAGTTCTTGAGGGTTGTGTTAAATTGAAAGCGAGAAATAATCATAAATAACCAUGGCA  
GGGAUUGCGAUGACAGUCCUCCAGCCUAGCAGUUUUUGCUUUGGCACCUGUUGUUUUUGCUGAAG  
ACCCCAUCUCAGAAACAGACCAGGGAAGGGGCACAACUACAUUGACGGGAUGACUCAGGAGGAUGCCA  
CAUGCAAACCUUGACAUUUGCUUGGGAUGUAGCAGUUUUGAUGUCUUGCUUGAAAAGGGAAAAUU  
UCCCCUUUCCAGUCAUUGCUCAUCAUAGAACUCUACUAGAGGCAGUUCACGACACCAUCAUUGCAAA  
GGCUGAUCCACCUAGCUGUGACCUUCAGAGUGCUCUUGGGAUCCUGUAUGAAAGAGAAACUUGUGA  
UGAAGACACAUUGUCCAAAUGACUACCAGUCAGCUCUUAACUCAACAUGACGGGAAAAUUGGCUUCAG  
UCAAGUGCCCACCUAAGUAUGAGCUCACUGAGGACUGCAACUUUUGUAGACAGAUAGACAGGUGCUAGC  
CUGAAGAAGGGGUCUUAUCCUCUCCAAGACUUAUUUUGUCAGUCAAGUGAGGAUGAUGGAUCAAAAAUU

GAAAACAAAAAUGAAAGGGGUCUGCGAAGUGGGGGUUCAAGCACUCAAAAAGUGUGAUGGCCAACUCA  
GCACUGCACAUAGAGGUUGUGCCCUUUGCAGUGUUUAAAAACUCAAGAAGGUUUUAUCUUGAUAAACUU  
GACCUCAAGACUGAGGAGAAUCUGCUACCAGACUCAUUUGUCUGCUUCGAGCAUAAGGGACAGUAUAA  
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