CHAPTER 2

CLONING OF THE GLYCOPROTEIN GENES AND EVALUATION OF THE CONSTRUCT AS A VACCINE.

1. INTRODUCTION

The teratogenic and abortifacient properties of the Smithburn vaccine (Smithburn, 1949; Barnard, *et al.*, 1977) are the main reasons why it is necessary to develop an alternative vaccine. When used to immunise cattle, the vaccine seems to elicit low levels of neutralising antibodies (Barnard, *et al.*, 1977). This led to the development of the formalin inactivated viral vaccine. The inactivated vaccine is used to vaccinate pregnant sheep and cattle however, it is necessary to administer booster doses annually and the immune responses elicited in sheep is inconsistent (Barnard, *et al.*, 1977). Due to the rapid onset of the disease, administering the inactivated vaccine is ineffective in giving sufficient immediate protection to vaccinated animals. A third major problem that is being encountered seems to be that the immunity induced by the vaccine in a female animal, transferred to the immediate progeny via feeding (collostral immunity), is not sufficient to protect the animal against heavy infection immediately after birth. Due to the impractical implications of administering neutralising antibodies immediately after birth, passive immunity is not a solution to this problem. Because some of the animals are vaccinated during an outbreak, it is possible to spread the disease in or between herds during a vaccination procedure by using the same needle for injection. All of these advocate the need for a vaccine that confers high levels of life-long immunity as soon as possible after vaccination to all or most of the susceptible animal species without any adverse effects.

Economic implications of a Rift Valley fever (RVF) outbreak necessitate vaccination of susceptible animals and the generation of a vaccine that is fully protective without any side effects. The outbreak that occurred in Saudi Arabia and Yemen in 2000 underlines the need for a vaccine that confers life-long protection immediately after vaccination, thus limiting spread of the disease to regions not endemic for the disease agent. Since Saudi Arabia as well as Yemen is not RVF endemic, control measures to

keep the virus from spreading in this region are required. A vaccination program using a safe and effective vaccine would be useful in restricting the virus to endemic areas and stop outbreaks before reaching epidemic proportions or spread into non-endemic regions.

Differentiation between vaccinated and naturally infected animals is possible through the use of marker vaccines. Serology, in specific Enzyme-linked immunosorbent assay (ELISA), as well as Polymerase chain reaction (PCR) based assays may be employed in the differentiation process. Marker vaccines include in the case of DNA vaccines, a non-related gene additional to the gene for the protective antigen. This allows for the detection of such an additional gene or gene product for differentiation between vaccinated and infected animals. An example of such a marker would be the gene of the Enhanced Green Fluorescent Protein (EGFP; Chalfie, *et al.*, 1994). If included in the DNA vaccine construct, cells transfected with the construct would show EGFP fluorescence. This would enable the calculation of transfection efficiency of the plasmid construct during development of the vaccine. In addition, antibodies against EGFP would allow detection of the protein in vaccinated animals. DNA vaccinated animals or animals vaccinated with a vaccine containing such a marker gene, can be differentiated from naturally infected animals.

The genes for the protective immunogenic determinants of Rift Valley fever virus (RVFV) have been shown to be located on the M segment of RVFV (Dalrymple, *et al.*, 1989; Besselaar, *et al.*, 1991). The two glycoproteins induced neutralising antibody responses (Schmaljohn, *et al.*, 1989; Besselaar, *et al.*, 1992) and conferred protection to mice upon a lethal challenge (Schmaljohn, *et al.*, 1989). Injection with only the G2 expressed protein also resulted in the induction of a protective immune response and upon passive immunisation of naive mice with sera from the protected mice, protection from a lethal challenge was obtained. This indicates that a humoral response may be sufficient in protection against the virus in mice (Schmaljohn, *et al.*, 1989).

A better vaccine against RVF that gives long-lasting protection soon after vaccination has to be developed due to the described adverse effects associated with the currently used vaccines against the disease and to enable one to identify immunised individual animals. As an alternative to the classical vaccines in use, a strategy involving DNA vaccines was considered (Konishi, *et al.*, 2000). Although a DNA based

vaccination program would not eliminate all the mentioned problems associated with the Smithburn or killed vaccines, some adverse effects of the vaccine may be addressed.

DNA vaccines offer advantages over the current vaccines for RVF. DNA vaccines could eliminate associated abortions and teratology and it may confer life-long or long-lasting immunity against virus disease (Konishi, *et al.,* 2000) to vaccinated animals including higher protection levels. In addition, it has been shown that in using a DNA vaccine, an immune response could be elicited in large animals (Romito, *et al.,* 1999; van Drunen Little-van den Hurk, *et al.,* 2000) indicating the potential to elicit an immune response against RVF in sheep.

In developing a DNA construct to be used for vaccination purposes, the plasmid vector backbone has to consist of the necessary control elements for mammalian expression. Into the plasmid vector backbone a suitable gene has to be cloned, that upon expression in the mammalian cell, can induce the appropriate immune response. In this study, the genes of the protective glycoproteins were cloned into the mammalian expression vector, pCI. The Enhanced Green Fluorescent Protein (EGFP) gene (Chalfie, *et al.*, 1994) under the control of the Cytomegalovirus (CMV) immediate-early enhancer/promoter was included as marker gene in this construct. Expression and subsequent fluorescence of this marker protein facilitated in the evaluation of the construct in a DNA vaccine context. When excited with blue light, the Green Fluorescent Protein (GFP) was found to emit green light with a very stable fluorescence. In cloning this gene, Chalfie, *et al.* (1994) have demonstrated that it will be expressed in prokaryotic and eukaryotic systems. This protein was chosen, because it can be used in a system where no exogenous reagents have to be added and availability of substrates is no limitation. In addition, EGFP specific antibodies are available that can be used in a serological assay to detect EGFP after vaccination of animals to identify vaccinated animals. It can serve as an indication of transfection, monitoring gene expression and protein localisation and has been widely used as a marker gene and a subsequent marker or reporter protein (Viljoen, *et a.,* 1997; Chalfie, M. *et al.,*(1994; Cheng, *et al.*, 1996; Cha, *et al.*, 1997; Kwon, *et al.*, 1998 and Stretton, *et al.*, 1998).

A plasmid DNA construct was generated containing a fragment of the M segment of the RVFV coding for both glycoprotein genes and the pCI mammalian expression

vector as backbone. The DNA fragment was obtained from the pSCRV-6 plasmid. The genes were situated downstream of the Cytomegalovirus (CMV) immediate-early enhancer\promoter and an intron. In parallel, the EGFP gene fragment also under the control of a CMV immediate-early enhancer\promoter was included in this plasmid construct to function as marker gene. Transient expression of the glycoprotein genes was shown using indirect immunofluorescence. By monitoring the EFGP fluorescence, the transfection efficiency was determined for the vaccine construct.

The aim was to generate a novel DNA vaccine against RVFV that would elicit an appreciable immune response to confer life-long protection to susceptible vaccinated animals. Mice were used as small animal model to evaluate the plasmid construct. The mice were injected i.m. with the DNA vaccine. Booster doses followed and the mice were challenged using a virulent strain of RVFV. A delay in death was observed in the animals of the group that was vaccinated with the DNA vaccine. The generated plasmid construct was subsequently evaluated in sheep. Booster doses were administered before the sheep were challenged with a virulent strain of RVFV. The DNA vaccine was evaluated in terms of the ability to elicit neutralising antibodies, reduce clinical signs of the disease (biphasic temperature) as well as limiting hepatic necrosis due to virus activity, as measured by liver enzyme levels.

2. MATERIALS AND METHODS

2.1 Subcloning of the RVFV glycoprotein genes

Into construct pSCRV-6, generated by Kakach, *et al.*, (1988) was subcloned the cDNA that contains the part of the M segment of the virus RNA encoding the glycoprotein genes, G2 and G1, of the Zagazig human strain (ZH-501) isolated in Egypt (Collet, *et al.,* 1985). This part of the M segment represents sequences from the fourth in-phase translation initiation codon through nucleotide 3767 of the open reading frame (See Addendum I). This was subcloned into the *Sma* I site of the pSC 11 vaccinia virus transfer vector after excision from the pSP76-6 construct to generate the pSCRV-6 construct (Fig. 1). This plasmid has been used previously in studying protein expression of the M segment in a vaccinia virus system (Kakach, *et al.*, 1988).

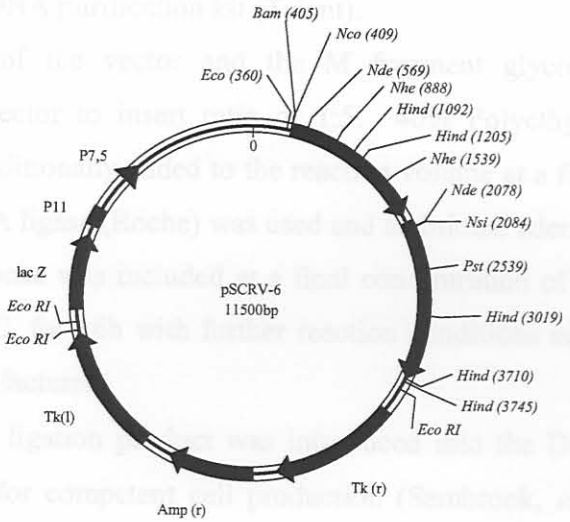


Figure 1. pSCRV-6 plasmid map indicating the relevant restriction sites and sequence information. pSCRV-6 originated from the pSP76-6 intermediate plasmid and the vaccinia transfer vector pSC11 (Kakach, et al., 1988; by permission from C.S. Schmaljohn and M.S. Collett).

The pSCRV-6 plasmid was digested with *Eco* RI restriction endonuclease using the buffer and reaction conditions according to the manufacturer's specification (Roche) to obtain the fragment containing the glycoprotein genes. The 3749 basepair (bp) DNA fragment (G2G1) was excised from a 0.8 % Tris-Acetic acid-Ethylenediaminetetraacetic acid (EDTA) (TAE) agarose gel using a sterile scalpel blade under long ultra violet (UV) wavelength (366 nm). The DNA fragment was purified from the agarose gel slice using the Geneclean (BIO 101) gel purification kit according to the protocol provided.

The purified fragment was inserted into the *Eco* RI site of the mammalian expression vector pCI (Promega Technical Bulletin, no. 206, 1994; Fig.2), located at position 1063 of the multiple cloning site (MCS) of the vector, to generate the pCI-G2G1 construct. The pCI vector was treated after linearisation, with 1unit (U) calf intestinal alkaline phosphatase (CIAP) for 1h at 37 °C (Roche) to remove 5′ phosphate groups. The reaction was terminated and the CIAP enzyme inactivated by incubation of the reaction mix for 15 minutes at 65 °C. The linearised, dephosphorylated pCI vector was purified using the Talent DNA purification kit (Talent).

Ligation of the vector and the M fragment glycoprotein gene insert was performed at a vector to insert ratio of 1:5. 40% Polyethylene glycol (PEG) 8000 (Promega) was additionally added to the reaction volume at a final concentration of 15% (v/v). 1U T4 DNA ligase (Roche) was used and additional adenosine triphosphate (ATP) (25 mM) from Roche was included at a final concentration of 5mM. The reaction was incubated at 15 °C, for 16h with further reaction conditions according to specifications given by the manufacturer.

The entire ligation product was introduced into the DH 5α *E.coli* strain using calcium chloride for competent cell production (Sambrook, *et al.,* 1989). Ampicillin resistant transformants were screened by plasmid DNA preparation from selected cultures using the alkaline lysis method (Sambrook, *et al.*, 1989). Comparison of wild-type pCI vector (4 kb) with recombinant plasmid pCI-G2G1 (7.8 kb) and determination of the orientation of the inserted M fragment was possible after restriction enzyme digestions of the prepared DNA.

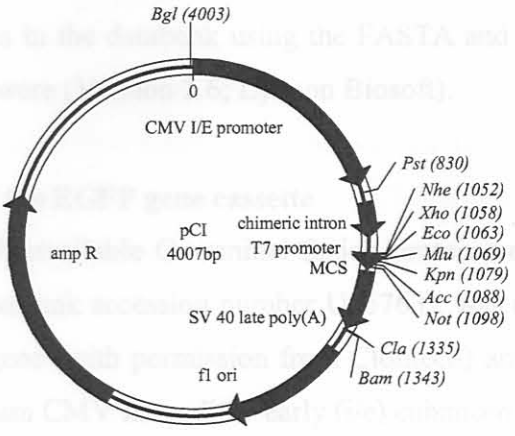


Figure. 2 - Plasmid map of the pCI mammalian expression vector. Multiple cloning site data indicated (Promega).

2.2 DNA sequence analysis of the M fragment insertion site

Integrity of the insertion site of the M fragment into the pCI plasmid vector and of the translation initiation codon of the glycoproteins had to be confirmed. The pCI-G2G1 plasmid template was sequenced using dideoxy chain terminating based reactions (Sanger, *et al.* 1977). The primer used was the pCI-primer ordered from Gibco BRL that anneals in the region between the chimeric intron of the vector and the T7 promoter sequence at position 990-1016 (5′- GTGTCCACTCCCAGTTC - 3′) of the pCi mammalian expression vector. Double stranded plasmid was purified using the Qiaquick Gel extraction kit (Qiagen). The sequencing reactions were done according to the proposed method described in the Big Dye Terminator cycle sequencing kit supplied by PE Biosystems. The kit was used according to the manufacturer's specifications for sequencing of a plasmid template. The chain termination sequencing reactions were done by employing cycle sequencing in a Perkin Elmer 2400 Thermocycler (PE Biosystems). Automatic reading of the sequence and generation of the electropherogram was done using ABI PRISM 377 automated DNA sequencer (PE Biosystems) and Wisconsin Genetics Computer Group (GCG) programs (Devereux, *et al.*, 1983) were used in

subsequent analysis of the obtained sequences for editing thereof. DNA comparisons were done with entries in the databank using the FASTA and BLAST programs as well as the DNAMAN software (Version 2.6; Lynnon Biosoft).

2.3 Subcloning of the EGFP gene cassette

A commercially available C-terminal fusion protein vector from Clontech (Palo Alto), pEGFP-C1 (GenBank accession number U55763), was used in this experiment as source of the EGFP gene (with permission from Clontech) and it's concomitant control sequences - the upstream CMV immediate-early (i/e) enhancer/promoter (pCMV) and the downstream poly-adenylation (poly (A)) signal (Fig. 3).

To facilitate the subcloning of this cassette, the multiple cloning site of the vector, located between the EGFP gene and the polyadenylation signal, had to be excised from the plasmid to remove these restriction sites. Digestions were performed with *Bam* HI and *Bgl* II (both supplied by AEC-Amersham). The linearised plasmid's ends were religated using 1U of T4 DNA ligase (Roche) and reaction conditions according to the manufacturer. The pCMV-EGFP-poly (A) cassette was excised from this modified pEGFP-C1 by restriction enzyme digestion of the plasmid sequentially with *Mlu* I and *Nsi* I (Eco T22 I isoschizomer) both supplied by AEC-Amersham.

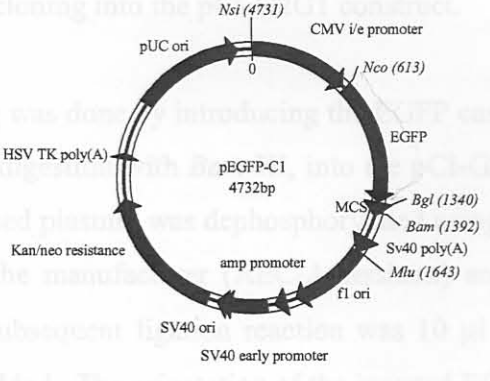


Figure. 3 pEGFP-C1 plasmid map showing the relevant restriction sites (Clontech).

The resultant 1.6 kb DNA fragment obtained from this digestion was subcloned directionally into the 2.8 kb pMTL 24 vector (Public Health Laboratory Services, Salisbury, Wiltshire; Chambers, *et al.,* 1988), an intermediate vector, into the *Pst* I/*Mlu* I (both enzymes supplied by New England Biolabs) linearised plasmid. This was done to facilitate subsequent cloning of the fragment into pCI-G2G1 by supplying the restriction endonuclease recognition sites (*Bam* HI) necessary for subcloning into pCI-G2G1. The multiple cloning site of the pMTL 24 vector has the following restriction sites occurring in this order, with restriction endonuclease sites used in this experiment indicated in bold:

5′ - *Eco* RI, *Sst* I, *Kpn* I, *Sma* I, ***Bam* HI**, *Xba* I, *Mlnc* II, *Acc* I, *Sal* I, *Aat* II, ***Mlu* I**, *Nco* I, *Bgl* II, *Xho* I, *Stu* I, ***Pst* I**, *Sph* I, *Hind* III, *Sph* I, ***Pst* I**, *Mlnc* II, *Acc*I, *Acc* I, *Sal* I, *Xba* I, ***Bam* HI**, *Sma* I, *Kpn* I, *Sst* I, *Eco* RI – 3′

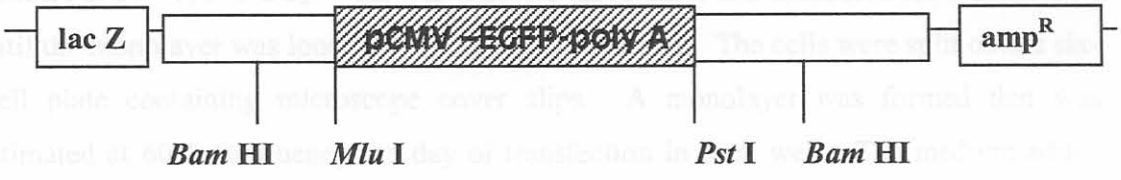


Figure. 4 – Subcloning of the EGFP gene cassette into the pMTL 24 intermediate vector to facilitate subcloning into the pCI-G2G1 construct.

The subcloning was done by introducing the EGFP cassette from the intermediate pMTL 24 vector after digestion with *Bam* HI, into the pCI-G2G1 construct's *Bgl* II site. The pCI-G2G1 linearised plasmid was dephosphorylated using 1U CIAP according to the specifications set by the manufacturer (AEC-Amersham) and as previously discussed. Total volume of the subsequent ligation reaction was 10 µl with an amount of 1U T4 DNA ligase (Roche) added. The orientation of the inserted EGFP cassette in the resultant pCI-G2G1-EGFP plasmid was confirmed by restriction enzyme digestion of the plasmid DNA. Restriction enzymes used in identifying the correct recombinant plasmids include

Cla I, *Mlu* I as well as *Nco* I (AEC-Amersham), all reaction conditions as described by the supplier.

2.4 Fluorescent microscopy and indirect immunofluorescence of pCI-G2G1-EGFP encoded proteins

2.4.1 Transfection of lamb fetal testis cells with pCI-G2G1-EGFP

pCI-G2G1-EGFP was transfected into lamb fetal testis (LFT) cells (results not shown) and fibroblastic African Green Monkey Kidney cells (BSC 40 cells). The cells were recovered after being stored in liquid nitrogen by quick thawing at 37 °C and resuspension in Ham's F12 and Dulbecco's Modified Eagle's Medium (DMEM; Highveld Biological) mixed in a 1:1 ratio and supplemented with 20% fetal calf serum. Penicillin/streptomycin (10mg/ml, 100x stock) was added at a final concentration of 1 µg/ml. At confluency of more than 80%, the BSC 40 cell monolayers were split. The cells were trypsinated by adding 0,1% trypsin to the monolayer and incubating for 5 minutes at 37 °C, 5% CO_2. This was repeated three times and incubated for the last time until the monolayer was loosened and the cells separated. The cells were split onto a six-well plate containing microscope cover slips. A monolayer was formed that was estimated at 60% confluency the day of transfection in each well. The medium added was also Ham's F12 and Dulbecco's Modified Eagle's Medium (DMEM) in a 1:1 ratio but supplemented with 10% Fetal Calf Serum (FCS), antibiotics (Penicillin and Streptomycin) and antimycotics (Fungizone) added both at a final concentration of 100 µg/ml. All the reagents were supplied by Highveld Biological Products. After the cells reached confluency of 60-80%, the transfections were done using the Effectene transfection reagent supplied by Qiagen according to the protocol recommended by the manufacturer. Briefly, 400ng of the plasmid DNA to be transfected was used per well at a minimum concentration of 100ng/µl. The volume of sample was adjusted to 100µl by adding the appropriate amount of buffer EC supplied. After the addition of the 3.2µl Enhancer reagent, the DNA sample was incubated for 5 minutes at room temperature. The non-liposomal lipid-based transfection reagent (Effectene) was added and 10 minutes incubation time was allowed for complex formation of the DNA with Effectene during which the cells were washed once using phosphate buffered saline (PBS) without calcium

and magnesium. 1.6ml Growth medium was then added to the cells. The same medium that was used for culturing the cells was used during the transfection experiment. The medium was added to the DNA-Effectene complexes and the suspension immediately added to the cells on the microscope coverslips.

As positive control, a virulent virus isolate from mosquitoes (Mosq NIV AR 20368, passaged in Vero cells, eight times in Madin-Darby bovine kidney (MDBK) cells and twice in hamsters in amplification and identification of the isolate) was used to infect the BSC 40 cells at a multiplicity of infection (m.o.i.) of 1. A positive control using the virus was included in the experiment to verify if the serum is specific for RVFV and that the Immunofluorescence (IF) procedure was followed correctly. The negative control for EGFP fluorescence was uninfected/untransfected cells. However, for the IF, the pEGFP-C1 transfected cells also acted as a negative control to verify if the acetone fixation eliminate EGFP background fluorescence. The cells in the test and control wells were incubated at 37 °C, 5% CO_2 for up to 48h before the expression of the proteins were examined.

2.4.2 Fluorescent microscopy

The transfected cells were incubated at 37 °C and after 24h and 48h examined for EFGP fluorescence. The cover slips with the cells were carefully removed from the wells and placed into a small petridish containing enough PBS without calcium or magnesium to cover the cells. The cells were examined using a fluorescent microscope with the 10x or 4x magnification lens and fluorescence could be seen after the EGFP was exited by a UV light source. An Olympus BH-2 microscope with SPlan magnification lenses was used. Photographs were taken of the cells showing fluorescence using an Olympus C-35 AD camera using the SPlan 4x lens with automatic magnification of the camera of 1.2 times. A Fujichrome Sensia ASA 400 slide film was used for photographic purposes.

2.4.3 Indirect immunofluorescence

After examining the cells for stable expression of EGFP, the cells on the coverslips were washed carefully in PBS and the coverslips were placed in a glass petridish. Cells on the microscope coverslip were fixed for 15 minutes using ice-cold

acetone after which it was rinsed in PBS once. A 1:100 dilution of sheep-α-RVFV polyclonal serum (originating from animals vaccinated with a Vaccinia virus recombinant and challenged using a virulent RVFV isolate) was prepared in a 2% milk powder solution (Elite, low fat; Clover, Roodepoort, SA). The serum was applied to the coverslips and incubated for 30 minutes at room temperature. The coverslips were washed four times using PBS. The secondary antibody conjugated to the fluorescing Fluorescein isothiocyanate (FITC) (Sigma) was added to the cells in the dark. The conjugate, an α-sheep-FITC antibody was used as a 1:50 dilution in 2% milk powder with Evans blue to counter stain cell background, added to a final concentration of 0.01%. The conjugate was added to the cells and incubated for 30 minutes in the dark after which the coverslips were washed four times using PBS and a final rinse with water. The coverslips were blotted to dry and left to air-dry after which it was mounted onto a microscope slide using a glycerol based mounting fluid (pH 5). Fluorescence could be detected by examining the immunofluorescent stained cells using the 50x magnification lens of Leitz-Wetzlar microscope. Photographs were taken of the obtained results using a Fujichrome Sensia ASA 400 slide film in a Wild MP551 camera fitted onto the microscope.

2.5 Preparation of the DNA to be used in the vaccine trial

In order to obtain sufficient quantities of DNA to inoculate experimental animals, plasmid DNA was prepared from a 6*l* culture using the Qiagen Endofree Giga plasmid purification kit (Qiagen). The plasmid DNA preparation was done according to the proposed method described in the product manual supplied by the manufacturer. The DNA was purified employing anion exchange column chromatography using the supplied Diethylamino ethane (DEAE) column. The plasmid DNA was eluted using the elution buffer (pH 8.5) included in the purification kit. After isopropanol precipitation of the eluted DNA, the purified plasmid was dissolved in 10mM Tris-EDTA (TE) buffer. An amount of 2,5 ml DNA with a concentration of 6.3 mg/ml was obtained to use as DNA vaccine in subsequent trials. The DNA was quantitatively checked on an agarose gel. The quality of the obtained plasmid DNA was determined by measuring the absorbancy values at 280 nm and 260 nm using the GeneQuant RNA/DNA calculator (Pharmacia)

spectrophotometer after diluting the DNA 1:100. The ratio of $A_{260\ nm}$/ $A_{280\ nm}$ was determined to give an indication of the purity of the DNA. A value of <1.8 would indicated the possible presence of protein or aromatic compounds, while a value of >2 gives an indication of possible RNA contamination. Restriction digestion of the obtained DNA was done to confirm the identity of the plasmid obtained during the preparation.

2.6 Vaccination of experimental animals with pCI-G2G1-EGFP

2.6.1 Use of mice as a small animal model

Four groups of three mice each were used in this experiment. Mice were obtained from SA Vaccine Producers (Edenvale, Johannesburg, South Africa). The vaccination schedule is presented in Table 1. The mice that received plasmid DNA were all injected intramuscularly (i.m) with 100 µg of plasmid DNA suspended in 10 mM TE at pH 8.5, 50 µg in each hind leg, in the *anterior tibialis* muscle (Cardoso, *et al.,* 1996). The positive control group received 0.2 ml intraperitoneally (i.p.) of the Smithburn live attenuated virus vaccine ($10^{6.5}$ mouse LD_{50} /ml) while the negative control group received no vaccination at all. The plasmid control group received pCI vector at the same dosage as the pCI-G2G1-EFGP DNA vaccine. All mice were given booster doses of the different vaccines and plasmid DNA at weeks three and six. Mice that were vaccinated had to be anaesthetised. In this experiment a mixture of Ketamine and Xylazine was administered i.p. according to the prescribed dosage indicated on the product information sheet by the manufacturer. The mice were bled from the tail veins before inoculation and booster doses were given (week 3). RVFV-specific antibody titres were checked using an ELISA based assay with the whole virus as antigen. The microtitre plates were coated with 1/1000 dilution of whole virus (sucrose gradient purified) as antigen. Albumin was used as blocking reagent at a concentration of 10% and serum was added after 1/10 dilution. Serum was not diluted further, because low levels of RVFV-specific antibodies are expected for DNA vaccinated animals. Peroxidase labelled Protein G was used for detection of the bound serum antibodies. The horseradishperoxidase (HRP) labelled Protein G was added at 1/8000 dilution to allow for detection of the RVFV-specific antibodies.

All mice groups were challenged with 0.3 ml i.p of a $10^{-4,4}$ dilution of a virulent RVFV isolate (mosq NIV AR 20368, passaged 8 times in MDBK cells and twice in hamsters, i.p.) at a 100 mouse LD_{50}. The concentration of the challenge material was $10^{6,8}$ log_{10} LD_{50} per ml. The mice were monitored for a period of 14 days for signs of disease and subsequent death (OIE Manual, 1994). Virus isolation was done from the liver and brain of animals that died during the trial to confirm death due to the viral infection. This was carried out by the Virology section at Onderstepoort Veterinary Institute. In short, organs from infected animals were homogenized, injected into suckling mice and monitored for 24-28 hours. Death is known to occur within 24-48 hours and the causative agent can then be confirmed to be RVFV using a complement fixation (CF) test.

Table 1 - Vaccination schedule for the injection of mice with the pCI-G2G1-EGF plasmid construct against Rift Valley fever (RVF). The negative control animals in group 1 received no vaccination, plasmid vector control group received only 100 µg pCI vector, the animals in the DNA vaccine group received 100 µg of the plasmid pCI-G2G1 construct and the positive control group's animals received 2 x $10^{5.5}$ mouse LD_{50} of the live attenuated Smithburn vaccine. The same doses were repeated at each of the booster injections.

Group	Vaccine received	Dosage	Route	Booster (weeks 3, 6)	Challenge (week 9)
1- Negative controls	none	-	-	-	100xLD_{50} virulent RVFV
2 - pCI controls	pCI	100 µg	i.m.	100 µg i.m.	100xLD_{50} virulent RVFV
3 - DNA vaccine	pCI-G2G1-EGFP	100 µg	i.m.	100 µg i.m.	100xLD_{50} virulent RVFV
4 - Positive controls	Smithburn vaccine	2x$10^{5.5}$ LD_{50}	i.p.	0,2 ml	100xLD_{50} virulent RVFV

2.6.2 Vaccination of sheep

The experimental animals used in this trial were sero-negative Merino sheep susceptible to RVF, considered a natural susceptible host of the virus (Barnard, *et al.*, 1977). The negative control animals (3004 and 3670, with reference to Table 2) were not vaccinated and the positive control group received live, **naturally** attenuated vaccine (Clone 13 (Muller, *et al*, 1995); personal communication Dr. Pamela Hunter, Onderstepoort Biological Products). The animals that were to receive DNA vaccine were divided into three groups (A, B and C) to examine three different scenarios with respect to route of vaccination and presence of the adjuvant Quil A (Sjölander, *et al.*,1997) in the vaccination strategy (Table 2). Animals were bled pre-inoculation, pre-challenge and at days 7 and 14 after challenge.

Table 2. The vaccination strategy used for the vaccination of sheep with the pCI-G2G1-EGFP construct against RVF. Group A received DNA in saline via the intramuscular (i.m.) route, group B received the DNA in saline with Quil A saponin as adjuvant and group C received the DNA intradermally (i.d.) without any adjuvant. 3004 and 3670 are the negative control animals that received no vaccination.

Groups	**Animals numbered**	**Route administered**	**Dosage**	**Adjuvant**
A	3283	i.m.	500 µg	none
	3345	i.m.	500 µg	none
	3287	i.m.	500 µg	none
B	3383	i.m.	500 µg	Quil A (saponin)
	3368	i.m.	500 µg	Quil A (saponin)
	3372	i.m.	500 µg	Quil A (saponin)
C	3347	i.d.	500 µg	none
	3313	i.d.	500 µg	none
	3373	i.d.	500 µg	none
Negative controls	3004	lamb	-	none
	3670	negative control	-	none

3 weeks post-inoculation, the animals received booster doses of the DNA vaccine. The DNA was prepared in the same way as the inoculation dose (Table 2). The challenge dose consisted of a virulent isolate of the virus at a titre of 10^5 $TCID_{50}$. The isolate used, originated from the outbreak among buffalo in the Kruger National Park early in 1999 (Dr. Pamela Hunter, personal communication; www.who.int). The challenge material was administered i.m. at week 6.

The clinical signs and symptoms of the disease were monitored for 14 days. Clinical signs that were monitored included temperature of the individual animals (indication of a sometimes biphasic fever), disease or death of the two young animals (lambs). Abortion would not be observed because none of the ewes was pregnant at the challenge date. Liver enzyme assays were performed to obtain signs of loss in liver function (hepatic necrosis) due to viremia in the individual animals. The assays were performed on a Technicon RA system in the Departement of Companion Animal Medicine, Veterinary Faculty (Onderstepoort), University of Pretoria. Kits and reagents were supplied by Bayer (Bayer Corporation) for the aspartate amino-transferase (AST) and gamma-glutamyl transferase (GGT) enzyme tests and Roche supplied the kit for glutamate dehydrogenase (GLDH) test. For the GGT assay, the rate of formation of p-nitroaniline used as indicator can be measured at 405 nm at 25 °C. The absorbance of nicotinamide adenine dinucleotide (NADH) is measured at 340 nm in the AST assay. Wavelengths that can be used in detection for the GLDH assay are 365nm, 340nm and 334 nm with higher accuracy obtainable when using 340nm or 334 nm (Product manual of GLDH assay, Roche).

3 RESULTS

3.1 Generation of the pCI-G2G1-EFGP construct and sequencing of the G2G1 insertion site

Upon *Eco* RI digestion of pSCRV-6 (Fig. 5), the 3.7 kb fragment containing the glycoprotein genes of RVFV was gel-purified and subcloned into the 4.0 kb mammalian expression vector pCI. The resultant recombinant plasmids were screened for correct orientation by restriction endonuclease digestion (Fig. 6). The map of the resultant construct pCI-G2G1 is represented in Fig. 7.

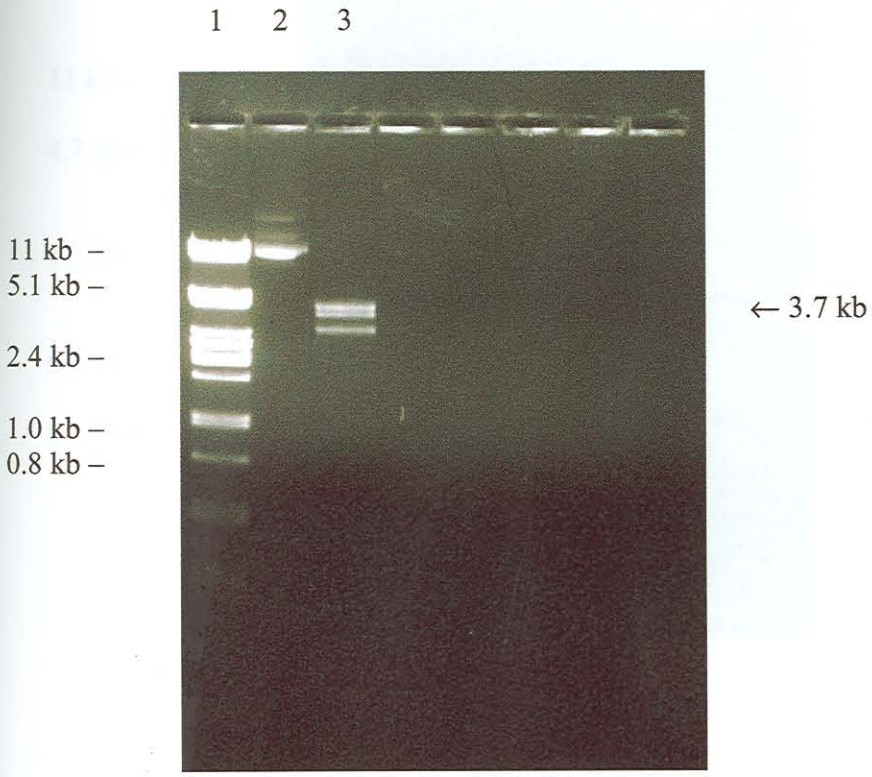


Figure. 5 Electrophoretic profile on an ethidium bromide stained 0.8% agarose gel of pSCRV-6 upon *Eco* RI restriction endonuclease treatment (lane 3) and the lambda DNA digested with *Pst* I to serve as molecular weight marker (lane 1). The 3.7 kb fragment contains the part of the M segment of the RVFV glycoproteins that was subcloned into the pCI mammalian cell expression vector obtained from Promega (Madison). Lane 2 - undigested pSCRV-6 control.

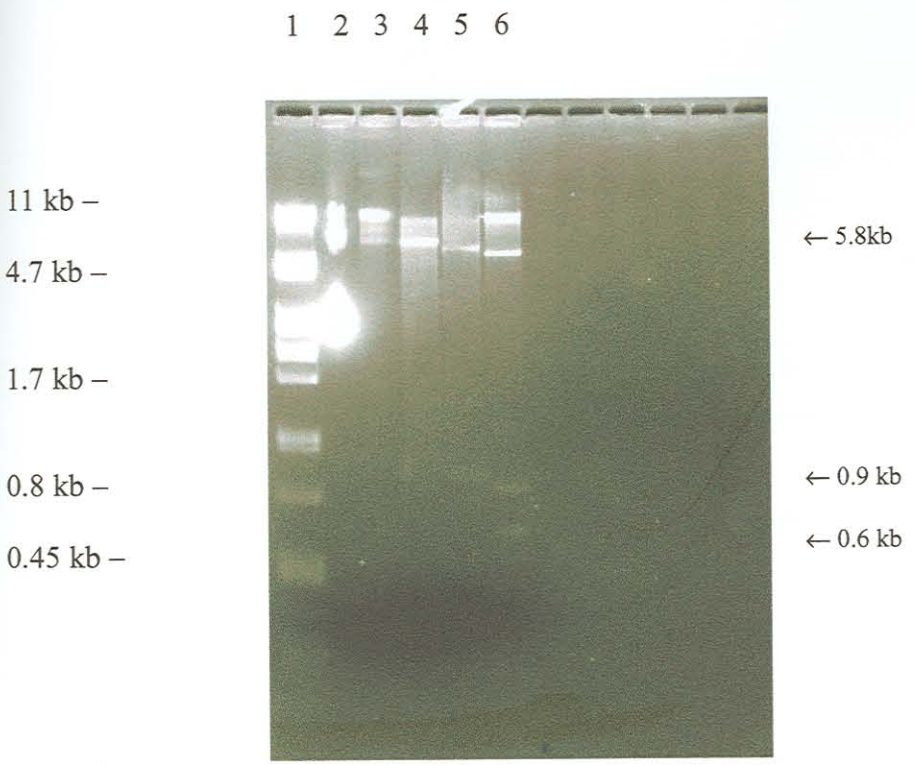


Figure. 6 pCI-G2G1 restriction digestion profile obtained on a ethidium bromide stained 1 % agarose gel. Restriction endonuclease digestion was done of the recombinant plasmids (pCI-G2G1) to screen for correct orientation of the G2G1 fragment. *Cla* I and *Nhe* I (AEC-Amersham) as well as *Nco* I (New England Biolabs) digestions were done. The obtained fragment sizes confirmed the correct profile obtained upon digestion. (lane 1) Molecular weight marker; (lane 2) undigested control; (lane 3) pSCRV-6; (lane 4) *Cla* I digestion; (lane 5) *Nco* digestion; (lane 6) *Nhe* I digestion.

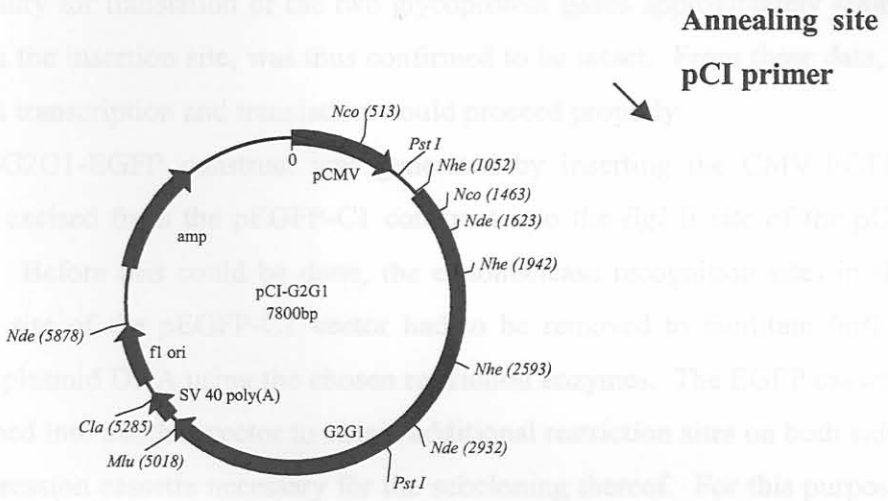


Figure. 7 Diagrammatic representation of the pCI-G2G1 construct indicating positions of restriction endonuclease recognition sites and sequence regions important in the subcloning of the G2G1 fragment. The position of the annealing site of the pCI primer is indicated.

The sequences obtained after chain termination sequencing of the pCI-G2G1 plasmid, using the pCI primer (supplied by GIBCO BRL) confirmed that the *Eco* RI site where the G2G1 fragment was inserted into was intact (Addendum II, p.100). The fragment that was inserted also contained sequences upstream of the RVFV specific sequences that were found to be vaccinia virus specific. These originated from the pSCRV-6 plasmid construct that was designed to function in a vaccinia virus expression system (Kakach, *et al.*, 1988) and served as the source of the glycoprotein genes. Downstream of the vaccinia virus specific sequences were the ATG representing the fourth in-phase translation initiation codon of the RVFV, followed by the glycoprotein genes. As was expected, the ATG and the glycoprotein genes were confirmed to be in-frame. The obtained sequence data revealed that the 5′ 50 base pairs were vector (pCI) specific sequences, while the region from position 51-428 were vaccinia virus specific

sequences originating from pSCRV-6. The remaining part of the sequence obtained was confirmed to be the 5′ sequences of the RVFV M segment starting at position 409 (Addendum III, p.101). The insertion site of the G2G1 fragment as well as the start codon site necessary for translation of the two glycoprotein genes approximately 400bp downstream from the insertion site, was thus confirmed to be intact. From these data, it was expected that transcription and translation would proceed properly.

The pCI-G2G1-EGFP construct was generated by inserting the CMV-EGFP-poly(A) cassette excised from the pEGFP-C1 construct into the *Bgl* II site of the pCI-G2G1 construct. Before this could be done, the endonuclease recognition sites in the multiple cloning site of the pEGFP-C1 vector had to be removed to facilitate further digestions of the plasmid DNA using the chosen restriction enzymes. The EGFP cassette had to be subcloned into another vector to obtain additional restriction sites on both sides of the EFGP expression cassette necessary for the subcloning thereof. For this purpose, the pMTL24 vector (Public Health Laboratory Services, Salisbury, Wiltshire; Chambers, *et al.*, 1988) was selected. After cloning the *Nsi* I/*Mlu* I restriction enzyme fragment containing the EGFP gene (Fig. 8) into pMTL 24, it was again excised, this time with Bam HI and cloned into the pCI- G2G1 construct.

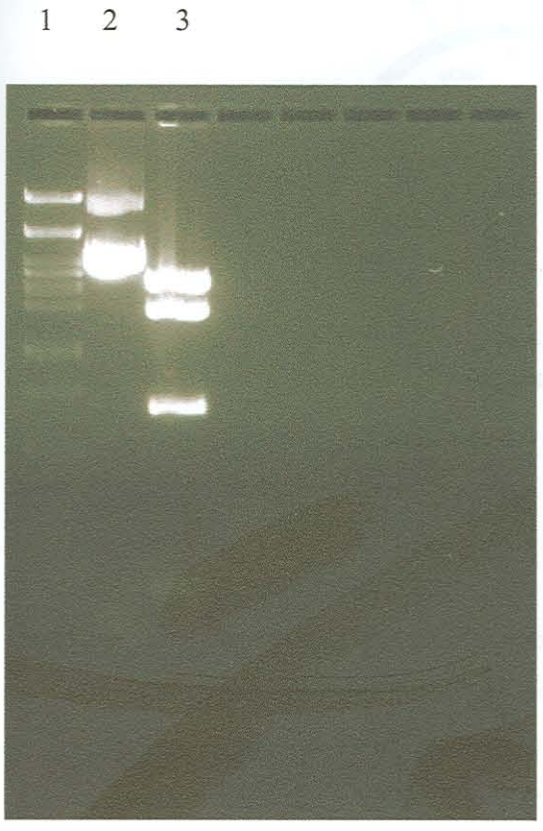


Figure. 8 Electrophoretic profile of the pEGFP-C1 plasmid upon *Mlu* I and subsequent *Nsi* I (Eco T22 I isoschizomer) restriction digestion (lane 3) on an ethidium bromide stained 0.8 % agarose gel. The 1.6 kb gelpurified fragment containing the EGFP gene was used in the subsequent cloning steps. Lane 1 represents the molecular weight marker – lambda DNA digested with *Hind* III, lane 2 - undigested pEGFP-C1 control.

Because the G2G1 and EGFP genes have the same promoter sequences and poly-adenylation signal sequences, it was necessary to insert the fragments in opposite orientations (Fig. 9). This would minimize the possibility of homologous recombination to occur that could lead to a subcloned gene cassette to be removed from the plasmid.

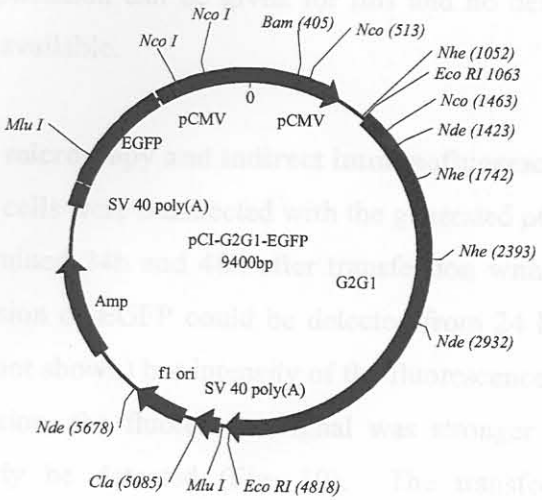


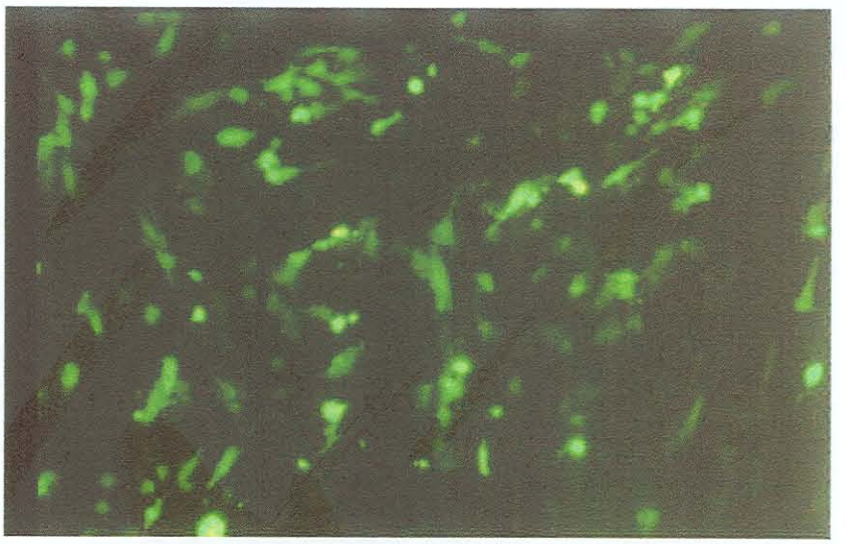
Figure 9 The pCI-G2G1-EGFP construct indicating the relevant restriction endonuclease recognition sites, fragments inserted and the orientations of the coding segments. For a diagrammatic summary of the cloning strategy and events, please see - Addendum 1 (p. 98).

It was observed that growth of bacterial cells containing the pCI-G2G1 and pCI-G2G1-EGFP was slower than cells containing no plasmid or cells transformed with the vectors (pCI or pEGFP-CI, results not shown). Absorbancy measurements taken at 600 nm wavelength after the same time of incubation for the different bacterial cultures containing the different plasmid vectors, indicated double the absorbancy value (cell density) for the slower growing culture (containing pCI-G2G1-EGFP plasmid). However, the inoculum (5 ml culture grown overnight) for all the cultures originated from a single colony. The duration to reach the same cell density (absorbancy values) was twice the time it took the cultures containing pCI or pEGFP-C1. Plasmid DNA yield were much lower for the slower growing cultures than for pCI or pEGFP-C1 transformed cells according to quantitative analysis done on an agarose gel. Since the slower growth of the bacterial cells containing the pCI-G2G1-EGFP plasmid was not further

investigated, no explanation can be given for this and no definite conclusion could be made from the data available.

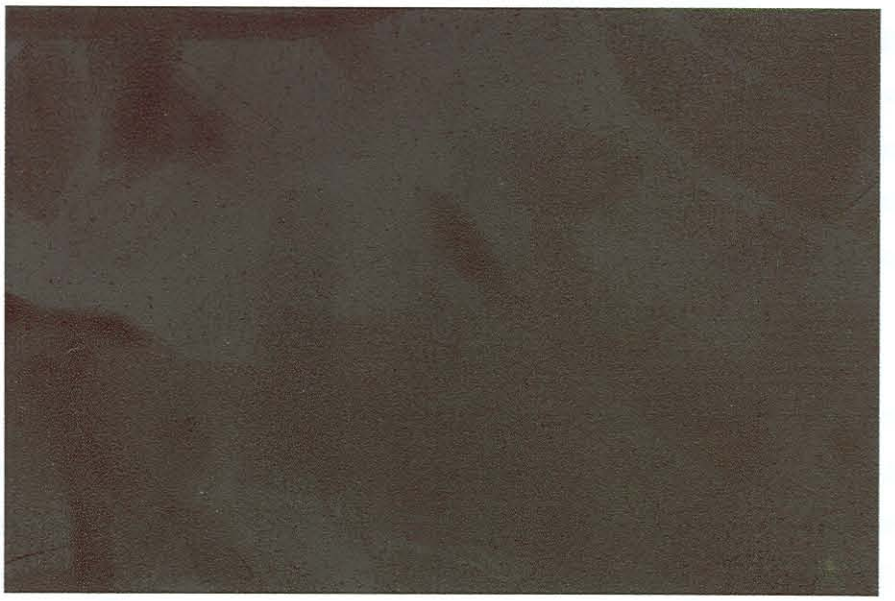
3.2 Fluorescent microscopy and indirect immunofluorescence

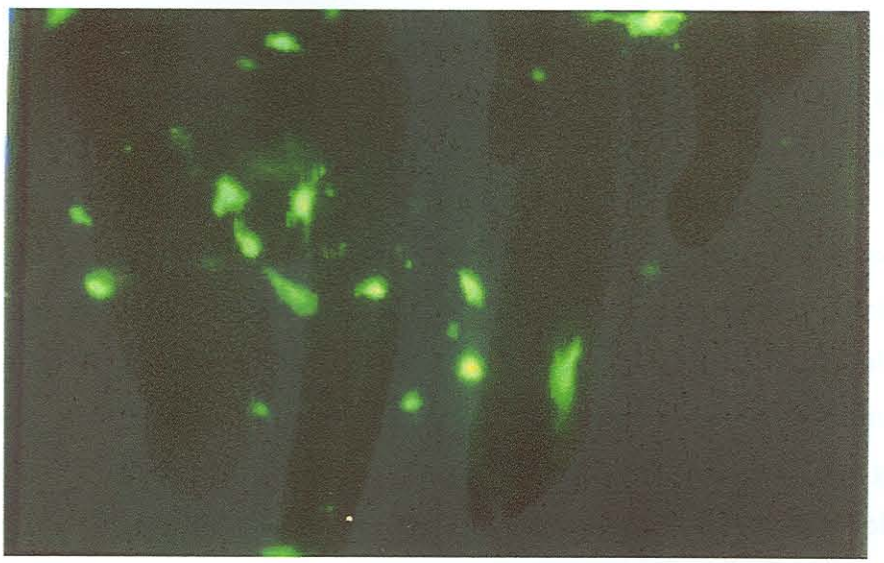
The BSC 40 cells were transfected with the generated pCI-G2G1-EGFP construct. The cells were examined 24h and 48h after transfection with plasmid DNA for EGFP expression. Expression of EGFP could be detected from 24 hours after transfection in this system (results not shown) but intensity of the fluorescence was low. However, after 48 hours of incubation, the fluorescent signal was stronger and cells expressing the protein could clearly be detected (Fig. 10). The transfection efficiency in cells transfected with pEGFP-C1 could be estimated by using the number of EGFP expressing cells as an indication of the amount of cells that received some plasmid DNA. It was estimated that the transfection efficiency of the cells was 70% on average (Fig. 10A). However, transfection efficiency was lower in cells transfected with pCI-G2G1-EGFP using the intensity of EGFP fluorescence as an indication. Transfection efficiency could be estimated in this case at 40 – 50 % (Fig. 10C).



A

B





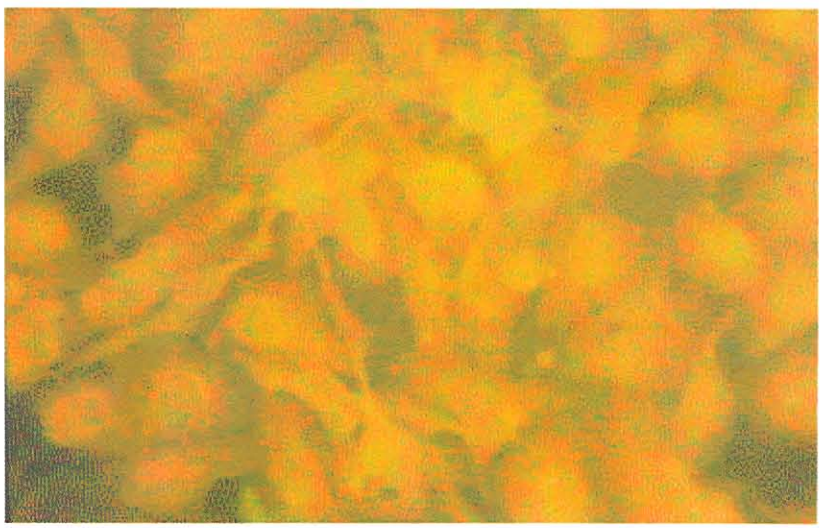
C

Figure. 10- Stable expression of EGFP after 48 hours of transfection of BSC 40 cells with pCI-G2G1-EGFP (C, 10 mm = 21 µm). As a positive control, BSC 40 cells were transfected with pEGFP-C1 (A, 10 mm = 21 µm) and to check for endogenous expression of the cells and background, untransfected cells were used as the negative control (B). An Olympus BH-2 microscope with the 4x magnification lens was used. The eyepiece added another 10x magnification. Olympus C-35 AD camera was used for photographic purposes with additional 1,2x automatic magnification of the camera. Exposure time was 16 seconds using a Fujichrome Sensia ASA 400 film.

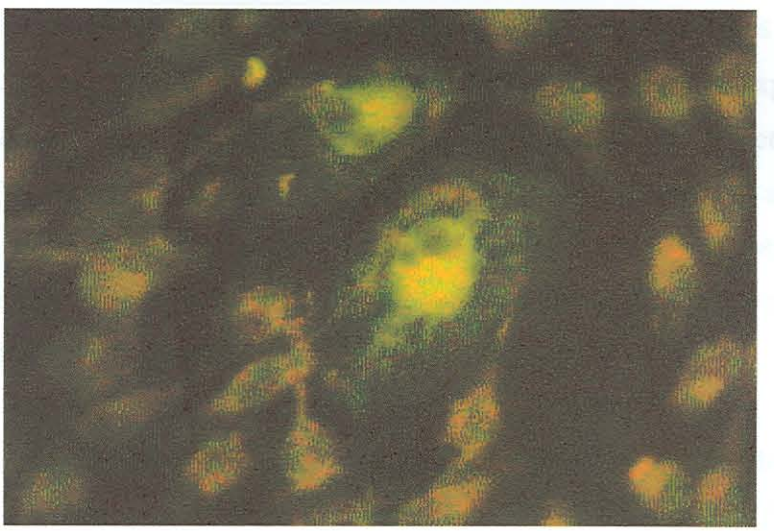
After detection of the EGFP in the transfected cells to confirm successful transfection and integrity of the subcloned EGFP gene cassette, indirect immunofluorescence was done to detect expressed glycoprotein. Both genes are in the same construct, therefore the amount of cells fluorescing due to expressed EGFP should theoretically be the same as cells showing fluorescence after indirect staining of the

expressed glycoproteins with the fluorescently labelled conjugate. Although FITC fluorescence could be seen in pCI-G2G1-EGFP transfected cells, the amount of cells in which FITC fluorescence could be detected was 20 % in comparison to the 40 –50 % cells showing EGFP fluorescence. The fluorescent signal was much less intense in comparison to the positive control (Fig. 11 B, C). In the positive control, (Fig. 11 C) the polyclonal RVFV specific sheep serum used for detection of the expressed proteins recognized the viral antigen to give a strong positive fluorescing signal. This is an indication of the specificity of the serum for RVFV. No background fluorescence (non-specific recognition by secondary antibody) was observed in the negative control (Fig. 11 A). The expression detected in the pCI-G2G1-EGFP transfected cells was observed as a less intense fluorescence signal that might be due to factors having an influence on the level of mRNA processing or at the level of protein synthesis. Both the glycoprotein genes and the EGFP gene cloned into the pCI vector were under the control of a CMV promoter. Transcription is expected to proceed at the same efficiency for both the genes. EGFP expression could clearly be detected as a strong fluorescence signal and an intense FITC signal for immunofluorescence was expected. However, such a strong fluorescence signal was not obtained. The number of cells fluorescing with EGFP was more in comparison with the number of cells showing immunofluorescent staining. The intensity of the signal was also lower in the latter.

A



B



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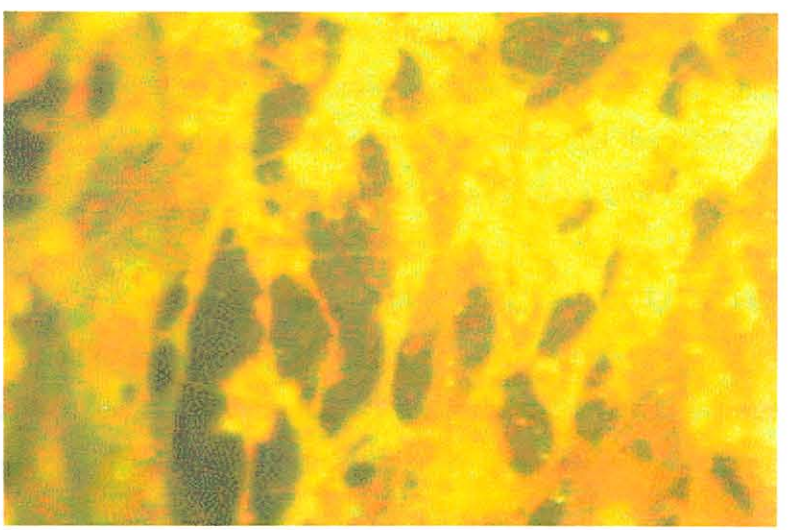

Figure. 11 - Transient expression of the RVFV glycoproteins after indirect immunofluorescent staining of BSC 40 cells with FITC conjugated secondary antibody (B, 7 seconds exposure time, 10 mm = 15 µm). Cells transfected with the pEGFP-C1 construct served as the negative control (A, 7 seconds exposure time, 10 mm = 15 µm) while cells infected with the whole virus served as the positive control (C, 3 seconds exposure time, 10 mm = 15 µm). Expression could be detected 48 hours post transfection/infection using the 50x objective lens of the Zeiss-Wetzler microscope. The magnification of the eyepiece as well as the magnification of the camera added another 10x and 1.3x respectively. A Fujichrome Sensia ASA 400 film and Wild MP551 camera was used photographic purposes.

3.3 Preparation of the DNA vaccine using a Giga plasmid purification kit

The culture volume of 6*l* gave a total plasmid DNA yield of 15,75mg DNA (6.3 mg/ml) according to absorbance values obtained after spectrophotometric readings of a 1:100 dilution of the DNA. The culture volume was larger than the protocol suggests compensating for the low DNA yield obtained from bacterial cells transformed with pCI-G2G1-EGFP and the slower growth rate observed as discussed earlier. To estimate the purity of the DNA obtained, the absorbany values at 260 nm and 280 nm were compared and the ratio was found to be 1.83. This indicates that the DNA preparation contains no protein or RNA. From this and according to analysis on an agarose gel as well as restriction enzyme digest profiles, it could be concluded that the DNA was of sufficient quality and quantity to be injected into animals as a DNA vaccine.

3.4 Vaccination of experimental animals with the pCI-G2G1-EGFP construct

3.4.1 Vaccination of mice with pCI-G2G1-EGFP

Unspecified deaths occurred in two of the mice after anaesthetizing before injecting the plasmid DNA. It was suspected that the mice were sensitive to the anaesthetic used in this trial (Ketamine/Xylazine). It was decided to keep three mice in the group destined to receive the DNA vaccine. One mouse was taken from each of the negative control group and the pCI control group rearing two animals in each of those

groups. The two additional mice were added to the remaining mouse in the group receiving the DNA vaccine. The mice were vaccinated i.m. with 100 µg of plasmid DNA, 50 µg in each *anterior tibialis* (calf) muscle. The target cells (myocytes in the *anterior tibialis* muscle) have the least amount of connective tissue in the mouse, thereby making the transfer of the DNA in the tissue between cells easier (Davis, *et al.*, 1995). DNA was administered using a fine (29 gauge) needle to prevent leakage of the injected DNA from the site of injection (H.L. Davis and R.G. Whalen, The Paris-Ottawa DNA Immunisation Handbook, http://www.genweb.com/Dnavax/dnavax.html). The Smithburn vaccine was administered i.p. in a 200 µl volume of the reconstituted vaccine. Booster doses at weeks six and nine after primary inoculation followed. Mice were bled from the tail vein pre-inoculation and before injecting the booster dose. One week after primary inoculation, a mouse from the positive control group died of unspecified causes. This left the positive control group also with only two mice. Serum prepared from collected blood, was used in an ELISA assay to determine the presence of RVFV specific antibodies after seroconversion due to vaccination. Antibody titres were expected to be very low, therefor a dilution of 1/10 was chosen for the serum in the ELISA based assay (Addendum IV). Limited amounts of blood could be collected from individual mice, limiting the amount of serum that was available for serological assays. As expected for that for RVFV, the antibody levels can be very low in mice after one inoculation i.p.. This did not discourage us to examine the possible protective effects the DNA vaccine might have. Although antibody titre plays a role in the protection against RVF, it has been observed that animals with low titres were still protected (Barnard, 1979, Collet, *et al.*, 1987).

After challenge with 100 LD_{50} of a virulent RVFV isolate, the mice in the negative control group all died within three days (Fig. 12). Mice in the pCI control group all died within five days. However, mice vaccinated with the DNA vaccine construct, survived until day 9. All mice in the positive control group survived the challenge. Thus, a delay in death (some "protective interference") was observed in mice from the group vaccinated with pCI-G2G1-EGFP in comparison to the negative and plasmid control groups. Virus isolation done at the Virology section at the Onderstepoort

Veterinary Institute were made from the spleen and liver of the mice that died, indicating that virus replication did occur in these organs and that death could be ascribed to RVF.

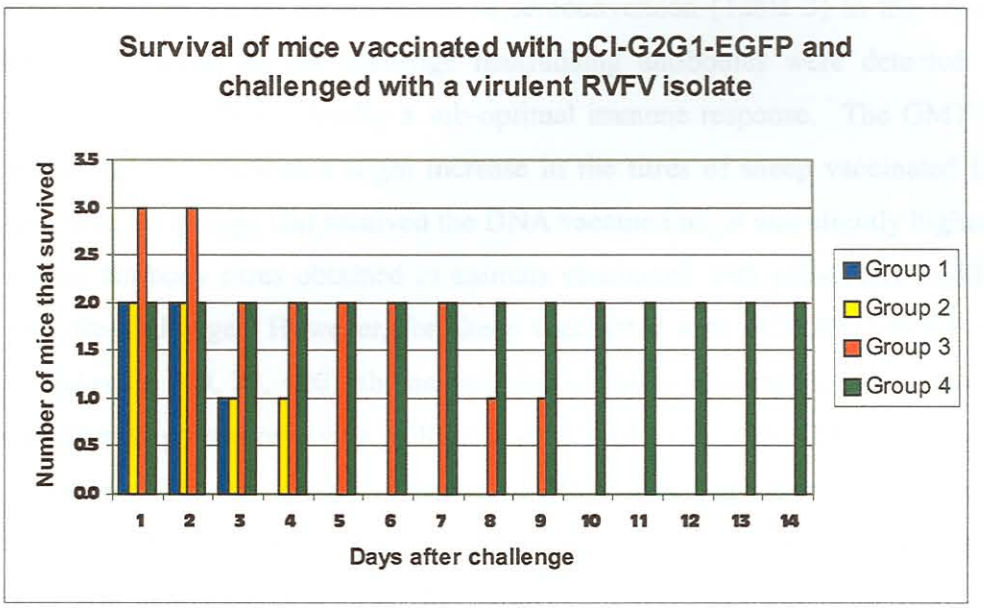

Figure 12 Survival of mice after immunisation with pCI-G2G1-EGFP and subsequent challenge with a virulent isolate of RVFV. The mice were observed for 14 days after injection with the virus. Group 1 – negative control, group 2 – pCI control, group 3 – DNA vaccinated mice, group 4 – positive control vaccinated with the naturally attenuated "clone 13".

3.4.2 Vaccination of sheep with pCI-G2G1-EGPF

Nine Merino sheep was injected with 500 µg of plasmid DNA in PBS via different routes with, or without adjuvant. During inoculation, a 29 gauge needle was used to ensure maximum amounts of DNA entering the site of injection, limiting leakage from the site (Davis, H.L and Whalen, R.G., The Paris-Ottowa DNA immunisation handbook, http:// www.genweb.com/Dnavax/dnavax.html). Blood samples were collected before inoculation, before administering and after administering the challenge dose (cell lysate) prepared from a virulent isolate from buffalo originating from the

outbreak that occurred in the Kruger Park in 1999. Serum was prepared to determine the titre of serum neutralising antibodies. This Plaque Reduction neutralisation test ($PRNT_{50}$) was performed by Research and Development, Viral (Onderstepoort Biological Products). This would be an indication of seroconversion (Table 3) in the vaccinated animals. Low levels of pre-challenge neutralising antibodies were detected in the individual animals. This indicates a sub-optimal immune response. The GMT values depicted in Table 3 showed a slight increase in the titres of sheep vaccinated i.d. In comparison to the groups that received the DNA vaccine i.m., it was slightly higher. The neutralising antibody titres obtained in animals vaccinated with pCI-G2G1-EGFP were very low pre-challenge. However, for sheep vaccinated with pCI-G2G1-EGFP via the intradermal route (40, 20, <20), the neutralising antibody titre pre-challenge was higher in comparison to groups receiving pCI-G2G1-EGFP i.m. (20, <20, <20). It is known for DNA vaccines that low antibody titres are often observed after vaccination. The neutralising antibody titres at 7 days post-challenge of the i.d. vaccinated group (40, 60, 30) where still higher than the group that received the DNA i.m. adjuvanted with Quil A (<20, 20, 40). However, at day 14 post-challenge, all three groups displayed titres that were, on average, comparable. Animals that developed levels of neutralising antibodies after vaccination (Table 3, nr. 3313, 3347) are likely to develop higher levels of neutralising antibodies post-challenge due to a further boosting effect during challenge. Neutralising antibodies of the animals that served as positive controls (vaccinated with naturally attenuated clone 13) had much higher titres at 7 and 14 days post-challenge (>78,5 and >160 respectively) than any of the plasmid DNA vaccinated animals. This is likely due to a strong secondary immune response after administering the challenge material. The neutralisation titres after challenge of the negative controls were on average comparable with the titres observed in the DNA vaccinated animals. The differences observed were insignificant. Because the sheep were seronegative and had no history of prior vaccination, the presence of maternal antibodies against RVFV could be ruled out in the lambs.

Table 3. Plaque reduction neutralisation test ($PRVT_{50}$) obtained from sheep at T_0 (pre-inoculation) with pCI-G2G1-EGFP DNA vaccine construct, pre-challenge with a virulent field strain of Rift Valley fever virus, day seven and day 14 after challenge (GMT-geometric mean titre, i.m.-intramuscular, i.d.-intradermal).

				Post challenge		GMT			
Route of inoculation	Animal nr.	Pre-inoculation	Pre-challenge	day 7	day 14	Pre-inoculation	Pre-challenge	day 7	day 14
i.m	3283	<20	20	80	120	<20	<20	20-40	>40
	3345	<20	<20	20	40				
	3287	<20	20	<20	20				
Quil A	3383	<20	<20	<20	40	<20	<20	<20	>40
	3368	<20	<20	20	30				
	3372	<20	20	20	120				
i.d.	3347	<20	40	40	60	<20	20-40	>40	>40
	3313	<20	20	60	80				
	3373	<20	<20	30	20				
Negative	3760			40	60			>20	20-40
orphan lamb	3010			<20	20				
lamb	3004			60	120			60	120
positive controls (on average)								40-80	>160

After challenging the animals, clinical signs that must be monitored for 14 days in a RVF case include a biphasic fever, abortions, death in neonatal animals and abnormal liver enzyme levels. However, since no pregnant animals were included in this trial, only

temperature (sometimes biphasic, reaching up to 41 °C), death in the very young animals and liver enzyme levels were monitored. A fever (Fig. 13) was evident in all the DNA vaccinated animals during the 9 days of temperature monitoring. However, the temperatures of the "clone 13 " vaccinated group were on average much lower than the DNA vaccinated animals (values not available). The maximum temperature recorded was 41.5 °C (often observed in a RVFV infection) (Addendum V) that developed in the negative control group and one in the group vaccinated with pCI-G2G1-EGFP i.m. with Quil A as adjuvant. The negative control group had a fever that unlike the other groups developed from day 1, with no signs of a biphasic trend. High temperatures developed for the DNA vaccinated sheep from day 2. These temperatures indicate fever due to viremia (virus replication). The temperatures normalised again after day 5 for most of the animals. The temperatures of animals in the negative control had on average the highest temperature readings, however as mentioned, temperatures can subside in very sick animals just before death. A decline in the temperatures of the negative controls does not indicate protection levels against RVF. Inconclusive results were thus obtained after vaccinating with the DNA vaccine against RVF in terms of the clinical signs and symptoms that were observed during this challenge experiment. However, it can be stated that the DNA vaccine was not effective in protecting against RVF in comparison to the positive control group as indicated by the observed clinical signs and symptoms.

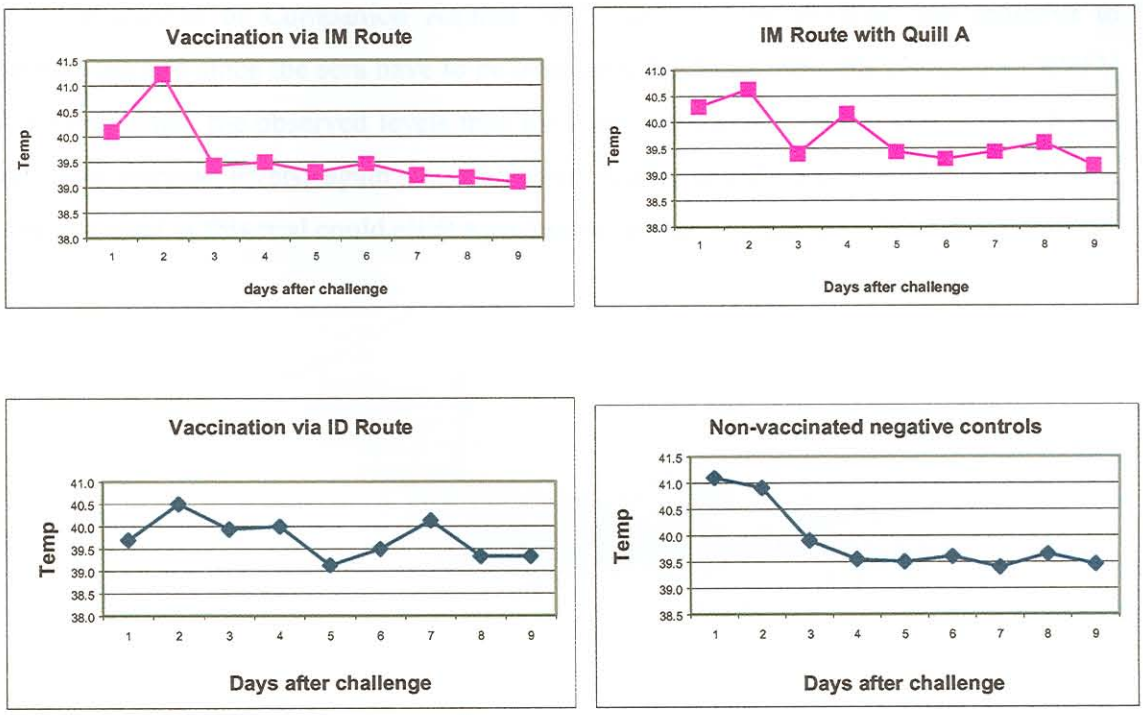

Figure. 13 - Average temperatures of vaccinated sheep taken daily post-challenge with a virulent RVFV strain (exact values shown in Addendum V). Different routes of inoculation were intradermally and intramuscularly (with or without Quil A used as adjuvant), (i.m –intramuscular, i.d – intradermal).

The levels of the liver enzymes in a healthy animal are 5-60 U/l for AST, 2-32 U/l for GGT and 0-2 U/l for GLDH. Serum samples were collected pre-challenge (basal value) and on days 2, 3 and 6 after challenge. Overall, the values observed in a number of the sheep gave an indication of hepatic necrosis (hepatopathy, Fig. 14; Addendum VI). However, many of the sheep already had abnormal enzyme levels, evident from the basal samples (Addendum VI). Since the pathology report of an ewe that died shortly before the trial indicated possible copper toxicosis, it can be speculated that this might be consistent with the elevated enzyme levels. Levels of enzymes obtained in the serums of

the positive controls were also abnormal in the basal samples. Extreme fluctuations can be seen in most of the individual enzyme levels (Addendum VI). It was commented by the Department of Companion Animal Medicine, that the enzymes are sensitive to irradiation and since the sera have to be irradiated due to the possible presence of RVFV after challenge, the observed levels may have been influenced by the irradiation. Based on liver enzyme levels, again no conclusive results could be obtained that the DNA vaccine used in this trial could elicit a protective immune response.

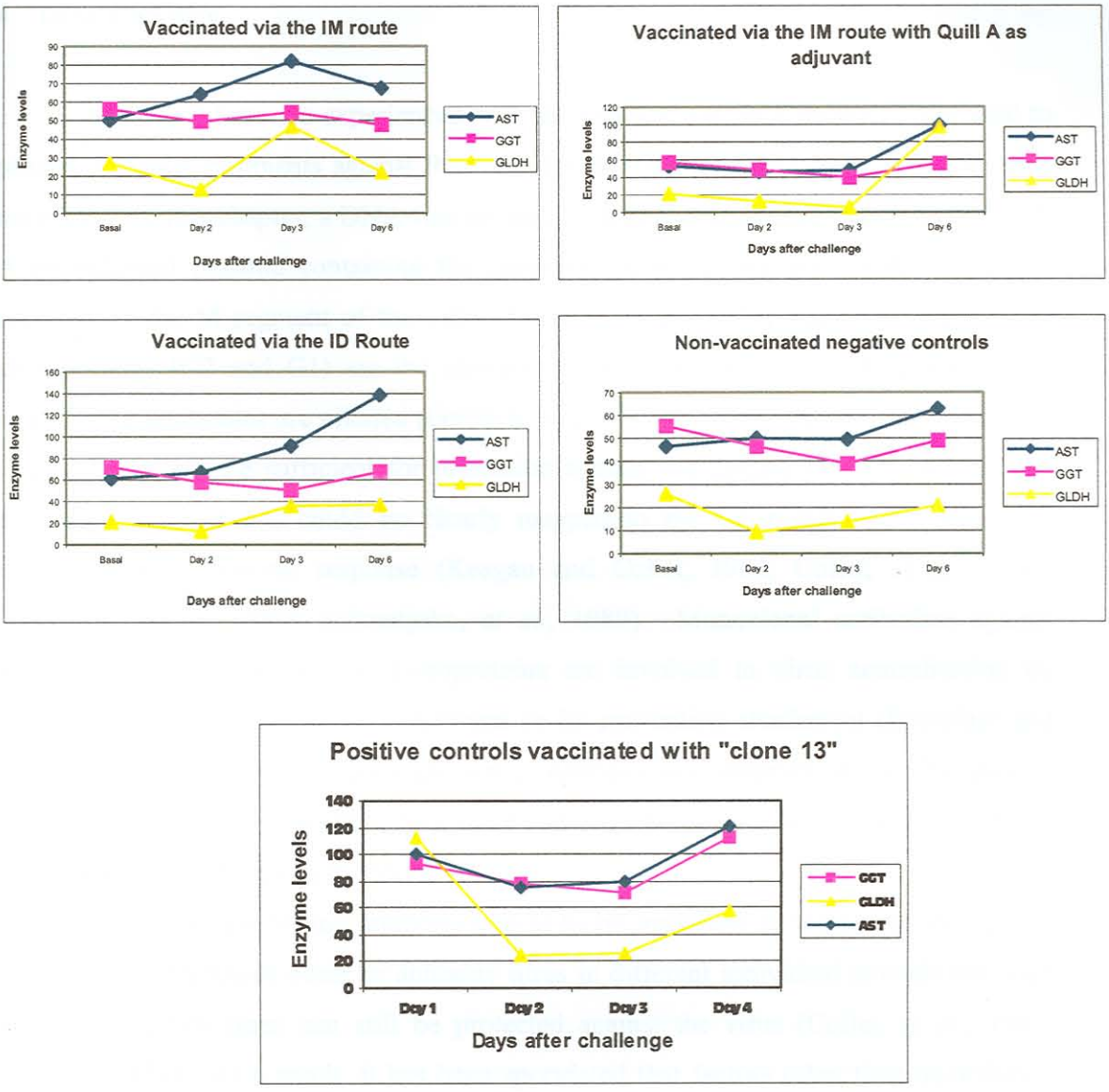

Figure. 14 - Liver enzyme levels of sheep over a six-day period post-challenge with a virulent strain of RVFV. Routes of inoculation were intramuscular (with or without Quil A as adjuvant) and intradermal. (AST – aspartate amino transferase, GGT – gamma-glutamyl transferase, GLDH – glutamate dehydrogenase).

4 DISCUSSION

Since problems are experienced with the classical vaccines produced and used to protect domestic ruminants against RVF the development of a new vaccine has become necessary. In this chapter, a DNA vaccine against RVFV was constructed and evaluated. A recombinant plasmid containing the genes of the protective glycoproteins that are encoded by the M segment of the virus (Gentsch, *et al.*, 1979) was constructed. The glycoproteins (G2 and G1) are the structural components of the virus against which neutralising antibodies are elicited (Gentsch, *et al.,* 1980). It was shown that antibodies against G2 alone are sufficient for protection against the disease in mice and that the protective determinants could be clearly mapped to the G2 protein with protection mediated by a humoral response (Keegan and Collet, 1986; Collet, *et al.*, 1987; Dalrymple, *et al.*, 1989; Schmaljohn, *et al.*, 1989). Monoclonal antibodies against antigenic determinants on the glycoproteins are involved in virus neutralisation by inhibiting infection after virus attachment or by preventing attachment (Besselaar and Blackburn, 1992). Four antigenic clusters of epitopes were mapped on the glycoprotein G1 and two domains containing four neutralising or hemagglutination determinants on G2 (Besselaar and Blackburn, 1991).

Although neutralising antibodies seem to be important in the protection against the disease, differences occur in antibody titres in different individual animals although those having low titres can still be protected against the virus (Collet, *et al.*, 1987; Barnard, 1979). As a result, it has been speculated that factors other than neutralising antibodies play a role in protection, even though neutralising antibodies have been used as a measure of immunity in RVF (Barnard, *et al.*, 1977). Sera from protected mice could confer protection to mice upon passive immunisation (Schmaljohn, *et al.,* 1989).

The gene fragment (containing the glycoprotein genes) that was subcloned in this study, include nucleotides 411 to 3767 (Kakach, *et al.*, 1988), representing sequences from the fourth translation initiation codon. In a vaccinia virus expression system, it has been observed that optimal expression of the glycoproteins is obtained if the fourth translation initiation codon on the M segment is used (Kakach, *et al.*, 1988).

The sequence data obtained indicated the presence of an additional 400 bp vaccinia virus-specific sequence upstream of the start codon of the G2 glycoprotein gene. This sequence is part of the fragment inserted into pCI and originates from the pSC11 vaccinia virus transfer vector that was a backbone of the pSCRV-6 plasmid. These additional bases could inhibit translation due to the presence of additional in-frame start- or stop codons. The translation might proceed less efficient resulting in less protein being expressed correctly from the desired translation initiation codon explaining the low levels of expression observed.

Upon transfection of LFT cells (results not shown) and BSC 40 cells, EGFP fluorescence could clearly be detected and used as a measure for transfection efficiency. However, transfection efficiency of 40-50 % of the cells transfected with pCI-G2G1-EGFP was much lower than the cells transfected with pEGFP-C1 (70%). This could be explained because of the different sizes of the constructs, resulting in lower molar quantities of the larger plasmid being transfected. It was expected that the same amount of cells would show EGFP and IF signal after transfection with pCI-G2G1-EGFP. However, the indirect immunofluorescent staining of the glycoproteins expressed by the mammalian cells indicated only 20 % of the cells expressed the glycoproteins at a detectable level. The signal obtained was significantly less intense when compared to the positive virus infected control. It can be speculated that in addition to low expression levels, low transfection levels were obtained.

Transcription of G2, G1 and EGFP is under the control of a CMV promoter. Transcription levels from both promoters are therefore expected to be the same. However, because of the gene cassettes occurring on the same plasmid construct, competition for polymerase binding may lead to less efficient transcription. However, if the transcription from both promoters takes place at the same efficiency, it can be assumed that translation of the mRNA plays a role in the inhibition of expression. In an *in vitro* transcription-translation system, (results not shown) RNA of the correct molecular weight could be obtained from the plasmid DNA. The amount of protein subsequently obtained was however low. It is thought that the RNA might be unstable due to improper processing (polyadenylation or 5′ capping) of the mRNA in the cell. In addition, the possibility exists that the translation product from the M segment mRNA

might not be post-translationally cleaved to yield both glycoproteins (Suzich, *et al.*, 1988). However, the expression of both glycoprotein genes in a vaccinia and baculovirus system lead to seemingly correct processing of the mRNA to yield proteins that was immunogenic and/or protective (Schmaljohn, *et al.*, 1989). The reasons for obtaining lower levels of the IF signal in our hands remain unclear.

In addition, it was noticed that in subcloning the G2G1 gene fragment, bacterial cultures transformed with the recombinant plasmids took twice as long to reach the same cell densities than cells transformed with pEGFP-C1 or pCI alone (results not shown). This was also noticed when the plasmid DNA was prepared for vaccination purposes. The G2G1 gene fragment inserted into pCI or the expression products had a significant influence on the transformed cells. The additional sequences originating from pSCRV-6 may also contain bacterial transcriptional cis-acting elements that may lead to expression of the cloned genes in a prokaryotic expression system (Tamarin, 1993). Although this is not expected, a small amount of gene products obtained after expression of the gene in the bacterial culture might be toxic to the cells (Konishi, *et al.*, 2000). Such a toxic effect can only be sufficiently explained and proved after thorough investigation. This as yet indeterminate expression problem might also be part of the reason for low expression levels in mammalian cells.

A delay in death observed in the mice vaccinated with pCI-G2G1-EGFP was the motivation for proceeding with the vaccine trial in the host animals. The results obtained with the ELISA assay were inconclusive due to high background observed (Addendum IV). Because of limited amounts of serum obtained after collection of blood samples, the assay could not be repeated. It is known however, that low antibody titres specific for RVFV are evident after vaccination of mice against the disease and after periferal injection of the virus. A difference in time before death was noticed between mice of the negative groups (naïve and pCI vaccinated) and the group of mice vaccinated with pCI-G2G1-EGFP. A member of the group of mice that received the DNA vaccine survived until day 10, while members of the group that received control plasmid pCI survived five days at the most in comparison to the mice in the negative control group that survived for three days only. Longer survival in the pCI plasmid control group in comparison to the group that received no vaccination might be as result of the non-specific immune

response elicited by the plasmid DNA, with the CpG sequences having a non-specific immunomodulating effect.

To optimise transfection efficiencies of the plasmid DNA into the target cell, a positive sense RNA can be used for immunisation purposes (Ward, *et al.*, 1997). Altering (truncation) of the G2G1 gene fragment or adding additional leader sequences to the cloned fragment at the 5′ terminus, may lead to better secretion of the proteins. This may lead to an enhanced antibody response being elicited. Cellular localisation and retention of the RVFV glycoproteins seem to be at the Golgi complex as a result of the preglycoprotein signal sequences (Wasmoen, *et al.*, 1988). However, it was observed that both G2 and G1 were localised to the Golgi complex in a vaccinia virus expression system. Examination of the existing sequences and corresponding changes of such sequences could enhance secretion of the expression products as well as the subsequent immune response elicited against such products.

Low levels of RVFV-specific antibodies in sheep obtained in the $PRNT_{50}$ assay after vaccination with pCI-G2G1-EGFP (Table 3) indicate some expression of the glycoproteins G1 and G2 from the DNA construct in the mammalian system. Antigen could only have been presented to the immune systems via the injected DNA vaccine since the animals were seronegative. The serum samples used for liver enzyme assays were irradiated. An adverse effect on the outcome of the assay had been observed in serum samples that had been irradiated (results not shown). From the liver enzyme assays (Fig. 14), it could be deduced that hepatopathy is evident in most of the sheep. However, basal levels of liver enzyme activity were also found to be abnormally high. This might be ascribed to copper toxicosis that the animals might have had prior to the challenge with the virulent RVFV isolate. A ewe died prior to challenge due to suspected copper toxicosis. This raised suspicions on the cause of elevated liver enzyme activity.

Mild viremia was expected after challenge with a virulent virus strain. However, after vaccination with a vaccine that could elicit a protective immune response, these reactions should be limited. It was previously found that even though neutralising antibody titres were high and sheep were likely to be protected, mild viremia occurred after virus infection or challenge (Barnard and Botha, 1977). An elevated fever has been observed in the sheep vaccinated with the DNA vaccine according to clinical signs (Fig.

13). However, as mentioned, observed symptoms of RVF are not always indicative of the severity of the disease. The severity of the disease also depends on the virus strain and the particular individual animal that is infected with the virus. Despite RVF's virulence during natural outbreaks, it was found that induction of the disease after peripheral administration in the laboratory was frequently unsuccessful (Swanepoel, *et al.,* 1986). Results obtained by Swanepoel, *et al.,* (1986), indicated that unvaccinated control sheep developed apparent mild infection symptoms after challenge with a virulent virus and that this was not a reflection of the underlying severity of the disease. Thus, the low level of symptoms of viremia observed in the sheep in the negative control group is not unique in challenge experiments when RVFV is involved. The obtained results only enable one to reach the conclusion that the DNA vaccine did not protect the vaccinated sheep.

To conclude: small differences in clinical signs and obtained values from the liver enzyme assays between the negative controls and the vaccinated groups gave little indication of immunological activity due to vaccination of the animals. The delay in death observed in vaccinated mice corroborates this observation. It is known that low levels of antibodies elicited by a vaccine can still lead to a protective response against RVF, even though antibodies may be indicative of how protected an animal may be against RVF. The clinical signs indicated viremia in the sheep after the challenge procedure. The temperature of an animal in the negative control group seemed to be higher than the sheep in the other groups, but since there were only three animals per group, this observation has no statistical relevance. The observations made from the DNA vaccination trial in small animals (mice) indicated that the generated plasmid construct had an immunological effect based on the time delay before death. We therefore decided to test the construct as vaccine against RVF in sheep. Optimisation of the construct and vaccination strategy to improve on the level of protection obtained in the target animal (sheep) is a future objective to further improve and evaluate a DNA vaccine against RVF.