The cloning and expression of the Rift Valley Fever G genes for the development of a DNA vaccine.

by

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Dedicated to my Heavenly Father, Herman and my parents.

I am grateful for undeserved mercy and support.

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SUMMARY

The cloning and expression of the Rift Valley Fever G genes for the development of a DNA vaccine.

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Rift Valley fever is an acute or peracute zoonotic disease of domestic ruminants and it is caused by a single serotype of the Rift Valley fever virus, a member of the Bunyaviridae family of the *Phlebovirus* genus. Vaccines that are currently used against this disease include a live attenuated virus (the Smithburn vaccine strain) and an inactivated virus vaccine. Several disadvantages are associated with both vaccines. For the attenuated vaccine, abortions in pregnant ewes, anatomical abnormalities in neonatal lambs and low antibody response in cattle are observed. In the case of the inactivated vaccine, it is necessary to produce large quantities of the virus antigen and only short term immunity can be obtained after vaccination. Third generation vaccines include DNA vaccines that offer numerous advantages above the currently used vaccines. These advantages include the possibility of long term and even lifelong immunity as well as eliciting of humoral and cellular immune responses after vaccination. Problems

encountered with the vaccines in use for Rift Valley fever can potentially be addressed by investigating a DNA vaccination strategy.

A mammalian expression vector backbone (pCI) was used in the construction of the DNA vaccine plasmid construct pCI-G2G1-EGFP. This construct includes the protective, immunogenic Rift Valley fever virus glycoprotein genes, G2 and G1 as well as the Enhanced Green Fluorescent Protein (EGFP) gene, both under the control of a Cytomegalovirus (CMV) enhancer-promoter, the latter acting as a marker gene during the construction, development and evaluation of the DNA vaccine. The insertion site of the glycoprotein gene fragment as well as the transcriptional start site of the G2 gene localized at the 5' end of the fragment was shown to be correct using chain terminating sequencing. Expression of the cloned genes was shown in BSC 40 cells with the use of indirect immunofluorescence to detect glycoproteins and fluorescent microscopy to detect EGFP. The plasmid construct was subsequently tested in a mouse model, where the mice were vaccinated intramuscularly with the DNA vaccine. Due to the delayed deaths observed in the groups vaccinated with DNA vaccine in comparison to mice in the non-vaccinated negative control group, we decided to use this construct in a sheep model. In the DNA vaccination trial the sheep were vaccinated either intradermally or intramuscularly with the pCI-G2G1-EGFP plasmid construct with or without adjuvant. Higher titres of neutralising antibodies were seen in the neutralising antibody assays of the animals vaccinated with the DNA vaccine than the negative control animals. However, clinical signs and symptoms (temperatures and liver enzyme levels) of DNA vaccinated animals after challenge with a virulent strain of Rift Valley fever virus indicated viremia. In comparison to animals in the negative control group, clinical signs and symptoms of the group vaccinated with the DNA vaccine were not significantly different indicating no detectable levels of protection after vaccination with the DNA vaccine.

Several of the diagnostic tests for Rift Valley fever are time-consuming, expensive, complicated and require handling of whole virus in antigen preparation. The replacement of the whole virus as antigen by a recombinant protein would eliminate this risk. The glycoprotein gene fragment has been subcloned into a bacterial expression vector and the bacterial vector system was evaluated for the expression of the glycoproteins. A product could only be obtained after truncation of the G2 gene. Diagnostic methods that limit

health risk by eliminating handling of the virus, save time, are less expensive and would be useful in assisting the diagnosis of RVF. The RT-PCR method would conform to the requirements and it is sensitive and specific. A one-step RT-PCR specific for Rift Valley fever virus was developed evaluated and validated. Primers were designed to anneal in the glycoprotein gene G2 of the virus at the 3' end of the G2 gene and the non-translated region between the G2 and G1 genes upstream of the G1 gene. It is foreseen that the RT-PCR could be used as a rapid diagnostic test and as a method for the determination of the viral load during production of classical vaccines in the future.

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ABBREVIATIONS

A	-	adenine
ALT	-	alanine aminotransferase
APC	-	antigen presenting cells
AST	-	aspartate amino-transferase
ATP	-	adenosine tri-phosphate
AU	-	absorbancy units
bp	-	base pairs
BSA	-	bovine serum albumine
BSC-40	-	fibroblast like African Green Monkey kidney cells
C	-	cytosine
°C	-	degrees Celsius
CAT	-	chloramphenicol acetyltransferase
CD	-	clusters of differentiation
CF	-	complement fixation
CIAP	-	calf intestinal alkaline phosphatase
CMI	-	cell mediated immunity
CMV	-	Cytomegalovirus
CPE	-	cytopathic effect
CpG	-	cytosine –phosphodiester linkage-guanine
CTL	-	cytotoxic T-lymphocyte
DEPC	-	Diethyl pirocarbonate
DEAE	-	Diethylaminoethane
DMEM	-	Dulbecco's modified Eagle's medium
DMSO	-	Dimethylsulphoxide
DNA	-	Deoxyribonucleicacid
dNTP	-	deoxynucleotide triphosphate
ds	-	double-stranded
DTT	-	Dithiotreitol
EDTA	-	Ethylene diaminetetraacetic acid

EGFP	-	Enhanced Green Fluorescent Protein
ELISA	-	Enzyme linked immunosorbent assay
et al.	-	et alii (and others)
FCS	-	foetal calf serum
FITC	-	Fluorescein isothiocyanate
G	-	guanine
GFP	-	Green Fluorescent Protein
GGT	-	gamma-glutamyl transferase
GLDH	-	glutamate dehydrogenase
GMT	-	geometric mean titre
h	-	hour
HAI	-	haemagglutination inhibition
hGH	-	human growth hormone
HPRI	-	Human placental ribonuclase inhibitor
HRP	-	horseradish peroxidase
i.d.	-	intradermal
ID	-	immunodiffusion
IF	-	immunofluorescence
IFNγ	-	interferon gamma
Ig	-	immunoglobulin
IL	-	interleukin
i.p.	-	intraperitoneal
IP	-	immunoperoxidase
IPTG	-	Isopropyl - β-D-thiogalactopyranoside
kb	-	kilobases
kDa	-	kiloDalton
lac Z	-	β - galactosidase gene
LB	-	Luria-Bertani medium
LD_{50}	-	lethal dose 50
LFT's	-	lamb fetal testis cells
M	-	molar

mAbs	-	monoclonal antibodies
MCS	-	multiple cloning site
MDBK	-	Madin-Darby bovine kidney cells
mg	-	milligram
MHC	-	major histocompatibility complex
min.	-	minutes
ml	-	millilitre
mM	-	millimolar
m.o.i.	-	multiplicity of infection
mRNA	-	messenger RNA
MW	-	molecular weight
NaAc	-	Sodium acetate
ng	-	nanogram
NK	-	natural killer cells
nm	-	nanometer
Ns	-	non-structural
OD_{590}	-	optical density at a wavelength of 590 nm
OIE	-	Office International des Epizooties
PAGE	-	Polyacrylamide gelelectrophoresis
p.i.	-	post infection
PBS	-	phosphate buffered saline
pCMV	-	Cytomegalovirus promoter
PCR	-	polymerase chain reaction
PEG	-	Polyethylene glycol
pfu	-	plaque forming units
poly(A)	-	poly adenylation
PRNT	-	plaque reduction neutralisation test
Pu	-	purine
Py	-	pyrimidine
RIA	-	radio-immunoassay
rpm	-	revolutions per minute

RNA - ribonucleic acid
RT-PCR - reverse transciption PCR
RVF - Rift Valley fever
RVFV - Rift Valley fever virus
SDH - sorbitol dehydrogenase
SPF - specific pathogen free
SDS - Sodium dodecyl sulphate
ssRNA - single-stranded RNA
T - tyrosine
TAE - Tris-acetate-EDTA
$TCID_{50}$ - 50% Tissue culture infective dose
TE - Tris-EDTA
Th - T-helper cells
Tris - Tris(hydroxymethyl)aminomethane
U - units
µl - microlitre
µg - microgram
UV - ultra violet
X-gal - 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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CHAPTER 1

LITERATURE REVIEW

1. RIFT VALLEY FEVER

Rift Valley fever (RVF) is an acute or peracute zoönosis primarily affecting domestic ruminants (sheep, goats and cattle) in Africa and it is caused by a single serotype of the mosquito-borne Rift Valley fever virus (RVFV) (OIE Manual 1996). An undescribed virus disease of sheep, cattle and man from East Africa was reported in 1931 (Daubney, *et al.,* 1931). The disease was called enzootic hepatitis or Rift Valley fever and caused heavy mortalities among neonatal lambs on a farm in the Rift Valley in Kenya. About 95% mortality in very young lambs was reported and mosquitoes were suspected to be the vectors for this virus. Previously the virus was classified in a natural group with the viral agents of yellow fever and dengue of man however, it has been reclassified since. Since mosquitoes are the vectors of RVFV, climatic conditions, which favour mosquito breeding, are associated with outbreaks of RVF (Linthicum, *et al.,* 1999).

1.1 Epidemiology

Epidemics occur mainly in eastern and southern Sub-Saharan African countries and Madagascar. Countries in which major outbreaks and epidemics have occurred include Kenya, South Africa, Namibia, Mozambique, Zimbabwe, Zambia, Sudan, Egypt, Mauritania and Senegal (the first documented outbreak in West Africa, 1987). Smaller outbreaks, isolations and serological evidence have been recorded in Angola, Botswana, Burkina Faso, Cameroon, Central African Republic, Chad, Gaboon, Guinea, Madagascar, Malawi, Mali, Nigeria, Somalia, Tanzania, Uganda and Zaire (http:/www.cdc.gov/travel/).

The most recent outbreak in South Africa was in the Kruger National Park in 1999 where abortions and death occurred among buffalo, the virus could be isolated from other wild ruminants and disease occurred in humans (www.who.int). One of the most recent epizootics/epidemics in Africa occurred in East Africa in late 1997 and early 1998

(Linthicum, *et al.,* 1999) in Kenya and Somalia following heavy rains. In this outbreak, 300 people died in Kenya while an unknown amount of deaths occurred in Somalia. An extensive outbreak occurred in 1950-1951 in Kenya where 100 000 sheep died of the disease, accentuating the economic implications that a RVF outbreak might have. Major outbreaks occurred in South Africa in 1950-51 (Schulz, 1951) and an extensive epidemic in 1974-76 (www.who.int). In September 2000, an outbreak occurred in Saudi Arabia and Yemen. Within three months, 1100 human cases were reported of whom 11% died. This RVF epidemic represents the first documented transmission of the disease outside Africa. It was found that the virus isolated in the Yemen/Saudi Arabic epidemic is similar to the virus isolated in Africa in 1998 during the outbreak in Senegal (www.who.int).

Most of these outbreaks coincided with above average rainfall at irregular intervals, thus favouring the increase in mosquito populations (Davies, *et al*., 1985). An exception to the general rule of climate that plays a role has been an outbreak in Zimbabwe in 1969, which indicates that the association of wetter periods and RVF is unanimous and that other factors are involved. Because of wetter conditions prevailing over endemic and adjacent non-endemic areas, outbreaks can spread easily to non-endemic areas. Herd immunity that occurs among animals in endemic areas, the onset of colder conditions that suppress vector activity and an increase in predators of mosquitoes might lead to the disease to subside during an epidemic.

Epidemics such as the epidemic in Egypt in 1977 (598 deaths) as well as the outbreaks in Senegal in 1987 (224 deaths) and Saudi Arabia, Yemen in 2000 (outbreaks in countries not endemic to RVF) emphasize the importance of efficient control, diagnosis and surveillance of the disease. The Senegal epidemic was the first confirmed outbreak not alone of the disease in West Africa, but also of the disease in humans in West Africa. Egypt was "virgin" territory until an outbreak in Sudan in 1976 spread to Egypt causing the epidemic in 1977. These and other areas may be receptive for the disease if the vector is abundant and the concentration of domestic animals is high enough to facilitate in the amplification of the vector and virus during the transmission cycle (Shope, *et al*., 1982). The high titres obtained with plaque reduction neutralisation tests and Enzyme-linked Immunosorbent Assay (ELISA) to detect RVFV specific

immunoglobulin M (IgM) and immunoglobulin G (IgG) antibodies among camel sera collected from Niger, indicates that camel may be a sensitive and useful indicator of the presence of the virus in these regions (Mariner, *et al.,* 1995).

The fate of the virus during inter-epidemic periods and the reservoir of RVFV are still uncertain. It is thought that the virus exists in forests in a natural cycle (McIntosh, *et al.*, 1980) although no maintenance in transmission cycles in birds, monkeys, baboons or other wild vertebrates could be proven. Wild ruminants could play a role in the transmission cycle since positive diagnosis has been made of RVF in wild ruminants, i.e. during the outbreak in the Kruger Park in 1999. In South Africa, isolation of the virus from male and female aedine and mosquitoes confirmed that the virus is endemic to areas where livestock usually occur (McIntosh, *et al.*, 1980) and that maintenance of the virus takes place through transovarial transmission. Venereal transmission from transovarially-infected males to females could possibly also occur. The infected livestock and wild ruminants serve as a source of virus for feeding mosquitoes after initial infection. Ticks don't seem to play a role in the epidemiology of the disease (McIntosh, *et al.*, 1980) and contagion is not considered to be of importance. In contrast, non-vectorial transmission is important in humans, since blood, bodily fluids and organs (spleen, liver, and brain) of an infected animal is highly infectious (Shope, *et al.*, 1980). Laboratory and abattoir workers as well as veterinarians are thus at risk. Virus transfer from animal to animal has been reported during the epidemic in South Africa in 1974-76 upon vaccination with the same needle (Coetzer, *et* al., 1994).

1.2 Experimental infection

Virulence of the RVFV viral strain and susceptibility of the host animal determine the morbidity and mortality of RVF after natural infection. Discrepancies in disease patterns in experimental infection can be explained in terms of differences in the challenge rate, age, acquired immunity and reproductive status of the host (Shope, *et* al., 1982). The disease could not always be reproduced in non-pregnant animals after peripheral administration of the virus (Swanepoel, *et al.,* 1986), even though a high dosage of a virulent virus strain was used in such experimental infections.

1.3 Manifestations of RVF

Some variation occurs in disease patterns between groups of animals and epidemics. During an epidemic RVF can predominate in cattle or sheep at a stage or location that may change as the epidemic continues. An example of the variation is the difference in disease patterns between the disease described in 1931 (Daubney, *et al.*, 1931) and the epidemic that occurred in South Africa in 1951 (Schulz, 1951). During the epidemic in 1951, cattle were affected to a lesser extent than what was observed in 1931. Pathological differences were evident in the spleen, lungs and adrenal glands. During an epidemic, abortions and disease in ruminants along with disease in humans tend to be characteristic of an outbreak of RVF, although clinical signs of the disease tend to be non-specific (Shope *et al.*, 1982).

Major manifestations of RVF are known to be abortion in pregnant animals (related to the febrile illness of the dam) as well as disease of adult and neonatal animals (Coetzer, 1982). Colostral immunity against RVF can be conferred upon neonatal lambs by immune ewes. However, irreversible infection can occur if the lambs are attacked by sufficient quantities of mosquitoes. Humans become infected by handling of infected animal tissue or by mosquito bites. This results in symptoms of fatal hepatic disease, encephalitis or nonfatal, sometimes influenza-like infection. Aerosol and intranasal infection is very rare and exceptional, but have been demonstrated (Bishop, *et al.*, 1980).

RVFV is spread from the initial site of replication (lymph nodes) to the critical organs (spleen, liver and brain) where viremia is caused by the release of the virus. Damage is done due to the lytic effects of the virus (Coetzer, 1982). Incubation times of RVF in lambs and kids are from 12 hours and onset of the disease is characterized by a fever (often showing bi-phasic trends) exceeding 41°C with a 12-18 hour remission, subsiding prior to death (OIE Manual, 1996). Listlessness, disinclination to move or feed, abdominal pain and rapid and abdominal respiration are clinical symptoms during the peracute stage of RVF. Viremia may be demonstrable within 16 hours post infection and it persists until death, 36-42 hours later in lambs less than one week old. Maximum titres recorded of viremia are the highest in lambs and kids. In older animals, viremia is demonstrable 1 to 2 days post infection and it may persist for up to 7 days. Highest intensity of viremia occurs between the second and fifth day of the disease. RVFV was

shown to persist in the spleen of infected cattle for up to 31 days after a virulent challenge (Yedloutschnig, *et al.,* 1981).

The disease may be acute (mostly occurring under field conditions), peracute with sudden death or unapparent. Acute disease has an incubation time of 24-72 hours, a fever of up to 42°C lasting for 24-96 hours, anorexia, weakness, listlessness, an increased respiratory rate, at times foetid diarrhoea, blood-tinged nasal discharge and icteric symptoms with death occurring 2 to 8 days post infection. Pregnant animals can abort autolysed foetuses at any stage of gestation. Disease in calves resembles the described situation in lambs (Coetzer, 1982). Infection in adult cattle is sometimes unapparent or otherwise acute with a fever of 24-96 hours duration, symptoms of anorexia, a staring coat, lachrymation, salivation, nasal discharge, dysgalactia and foetid diarrhoea. Cows may also abort an autolysed foetus at any stage of gestation. Symptoms in humans are unapparent or influenza-like with ocular lesions, encephalitis or severe haemorrhagic hepatic disease occurring in some cases. Less than 1 % of the patients develop the haemorrhagic or encephalitic forms of the disease (Shope, *et al.,* 1982).

1.4 Immune response and resistance to RVF

RVFV is able to replicate in almost all cell types *in vivo,* with the liver and spleen being the major sites of virus replication. After infection, due to the destruction of the lymphoid tissue by the lytic effects of the virus, interferon and antibody responses are delayed and the hepatic effect of the virus cannot be controlled by the host responses (Coetzer, *et al.*, 1994). Antibodies were seen to become demonstrable within 2 to 3 days after infection (Swanepoel, *et al.,* 1986). During recovery, antibodies and macrophages facilitate clearance of the infection, the latter by production of interferon and induction of cell-mediated immune responses.

Resistant flocks or herds might be bred because of resistance being inherited in a simple Mendelian gene (Coetzer, *et al.,* 1994). Different species and even breeds of susceptible animals are known to differ in their relative susceptibility to RVF. Breeds of domestic ruminants exotic to Africa or areas where the virus is not endemic seem to be more susceptible to the disease (OIE Manual, 1996).

1.5 Significance of liver enzymes

An increase in serum levels of enzymes such as sorbitol dehydrogenase (SDH) and glutamate dehydrogenase (GLDH) occur early in the disease. GLDH levels increase significantly from the second day post-infection and 1 week after infection, levels decline to pre-infection levels, while SDH values increase from 4 days post-infetion (Swanepoel, *et al.,* 1986). GLDH is responsible for the conversion of α-ketoglutarate to L-glutamate (Brobeck, 1978). The variation in these enzyme levels is indicative of liver necrosis or necrotic hepatitis. Gamma-glutamyl transferase (GGT) levels remain the same during the disease (Swanepoel, *et al.,* 1986), indicating that the biliary tract is not affected during RVF. Aspartate aminotransferase (AST) catalyses the conversion of α-ketoglutarate and aspartate to oxalacetate and glutamate (Brobeck, 1978). Increasing levels of aspartate aminotransferase (AST) later on in the disease suggest hepatocyte degeneration. Alanine aminotransferase (ALT) levels show irregular and non-significant fluctuations confirming the fact that this enzyme test is of no use in herbivores (Swanepoel, *et al.,* 1986; Coetzer, 1982).

1.6 Pathology

The main pathological feature of RVF is hepatic lesions of diagnostic significance (Coetzer, 1982). The lesions (confined primary foci of necrosis) found in sheep and cattle are similar in infected animals and humans, with the most severe lesions occurring in aborted sheep foetuses and neonatal lambs. Lesions in adult sheep are less severe and not as widespread, although icterus occurs more evidently. Although the lesions seem to be similar among species, there are significant differences among different age groups, where younger animals have massive hepatic necrosis. Haemorrhages in the abomasum lead to the presence of free blood in the intestines. The spleen tends to be slightly enlarged with haemorrhages occurring in the capsule (Coetzer, 1982). The most striking microscopic feature is hepatic necrosis in all infected individuals, from there the description enzootic hepatitis (Schulz, 1951).

2. RIFT VALLEY FEVER VIRUS

2.1 Virus classification

Rift Valley fever virus is classified among the Bunyaviridae family of viruses in the *Phlebovirus* genus (Shope, *et al.*, 1980; Levy, *et al.,* 1994). There are five genera within the family Bunyaviridae: *Bunyavirus, Hantavirus, Phlebovirus, Nairovirus* and *Uukuvirus* (Levy, *et al.,* 1994). These viruses are arthropod-borne with a negative single-stranded RNA (ssRNA) genome. The Bunyaviridae family comprises more than 200 viruses (Bishop, *et al.* 1980), with the prototype virus of the family the Bunyamwera virus (Porterfield, *et al.*, 1975/76). The wild-type virus can be described as hepatotropic, viscerotropic or pantropic depending on the cellular tropism of the viral strain (Coetzer, *et al.* 1994).

2.2 Virus morphology

Characteristic of viruses belonging to this family is the uniform, spherical shape with a diameter of approximately 80-110 nm and a unit membrane envelope (Holmes, 1971). After glutaraldehyde fixation of tissue preparations containing the virus, the structural glycoproteins can be seen as 8-10 nm surface projections under the electron microscope (Donets, *et al.*, 1977; Obijeski, *et al.*, 1977). The particles contain an electron dense area of membrane surrounding the less electron dense space containing the nucleocapsid (Holmes, 1971).

2.3 Expression strategy

Although the Bunyaviridae family is considered as a family of negative-sense RNA viruses, some of these viruses - including the *Phlebovirus* RVFV - have a replication cycle that employs an ambisense expression strategy. An ambisense expression strategy involves transcripts and proteins that are found within the virus-infected cell thus suggesting positive- and negative-sense expression strategies (Levy, *et al.,* 1994). This implies that positive-sense RNA can directly act as mRNA, or these positive-sense RNA transcripts may act as templates for transcription of negative-sense transcripts that in turn can code for mRNA's or positive-sense viral genome species. For

example, the Punta Toro virus and the Sandfly Fever Sicilian virus, both *Phleboviruses,* was shown to employ negative-sense expression strategies (Ihara, *et al.*, 1984; Marriott, *et al.,* 1989).

2.4 Virus release

The virus is released from the infected cell by lysis or exocytosis (Obijeski, *et al.,* 1977). It was shown by electronmicroscopy that viral particles are associated within the cisternae and vesicles of the Golgi complex (Donets, *et al.,* 1977) and the viral proteins accumulate in the cytoplasm and at cellular membranes (Bishop, *et al.,* 1980). The virus develops in the cytoplasm of infected cells and maturation occurs by budding into the smooth-surfaced vesicles in the region of the Golgi system (Porterfield, *et al.,* 1975/76).

2.5 Virus characteristics

The host-cell derived lipid membrane of the RVFV particle renders it sensitive to lipid solvents such as ether, sodium deoxycholate and non-ionic detergents. The virus is more stable at pH 7-9 than at acidic pH values and it is rapidly inactivated at a temperature of 56°C, while at 4°C, the virus is stable in serum for several months and when stored in a freeze-dried state or at -60°C, even more so (Bishop, *et al.*, 1980; Levy, *et al.*, 1994). By using proteolytic enzymes to remove the exterior glycoproteins, the infectivity of the virus can be reduced significantly and the virus is inactivated rapidly by using ultraviolet light. Low concentrations of formalin and pH values below 6.8 also result in loss of infectivity. RVFV grows and produces cytopathic effects (CPE) and plaques on almost all primary cell cultures and continuous lines, with the exception of primary macrophages and lymphoblastoid cell lines (Swanepoel, *et al.*, 1986; Coetzer, *et al.* 1994).

2.6 Genome organization and expression products

The RVFV genome consists of three separately encapsidated single-stranded RNA segments, designated S, M and L according to the relative sizes of the segments (Robeson, *et al.*, 1979; Bishop, *et al.*, 1980; Levy, *et al.,* 1994). Genomic RNA segments have the potential to be organized in non-covalently closed circles (Bishop, *et al*, 1980;

Levy, *et al.,* 1994) because of the existence of complementary, genus specific conserved terminal sequences on the 5' and 3' termini (Suzich, *et al.,* 1990). The different sizes of the segments are respectively 0.3-9kb, 3.2-5kb and 6.5-14.4kb for the S, M and L segments (Levy, *et al.* 1994). The sedimentation coefficients of the genome segments are for the different segments L: 27-31S; M: 22-26S; S: 16-20S and the molecular weights are L: 2.6-2.8 x 10^6; M: 1.8-2.2 x 10^6; S: 0.7-0.8 x 10^6 (Bishop, *et al.*, 1980). The segmented genome of members of the Bunyaviridae family confers the capacity to the virus for antigenic shift. This occurs via segment reassortment of the genome as evidenced by naturally occurring reassortant viruses (Ushijima, *et al.*, 1981; Levy, *et al.*, 1994).

The S (small) segment's expression proceeds via the ambisense strategy (Suzich, *et al.,* 1990) thus both gene products from the S segment are synthesized from different templates by means of bi-directional coding. The nucleocapsid protein (N), a 20-30kDa protein, is synthesized from virus-complementary mRNA (Cash, *et al.,* 1979), while the much smaller non-structural Ns_s protein, is synthesized from a viral-sense mRNA. However, both the L (large) and the M (medium) segments have a negative-sense expression strategy (Suzich, *et al.,* 1990).

A viral RNA-dependent RNA polymerase has been found to be associated with the virus (Ranki, *et al.,* 1975). This enzyme is encoded by the L segment, while the M segment encodes for the two unique structural glycoproteins G1 and G2 of 55-70 kDa and 50-60 kDa respectively (Gentsch, *et al.*, 1979; Suzich, *et al.* 1990). In addition, it was also shown that two other non-structural proteins that have sizes of 14 kDa and 78 kDa each are also being expressed from the M segment.

2.7 Significance of the glycoproteins

The glycoproteins are formed by post-translational cleavage of a primary viral gene product. This implies that the M segment RNA is first translated into a polyprotein, which is subsequently cleaved to yield the different protein species (Obijeski, *et al.,* 1977). Many biological functions can be assigned to the M segment gene products (glycoproteins). These include attachment, fusion, hemagglutination, neutralisation and virulence, while the other proteins encoded by the genome may have modulating effects

on the viral virulence (Levy, *et al.*, 1994). With the use of monoclonal antibodies (mAbs), four antigenic determinants on the G2 glycoprotein have been identified of which three were shown to be involved in virus neutralisation (Gentsch, *et al.*, 1980; Keegan, *et al.*, 1986). After denaturation of the antigen (G2), three of the four mAbs still showed reactivity to the G2 glycoprotein, indicating that the corresponding epitopes are displayed in the employed expression strategy in a manner similar to the authentic glycoprotein. The sequences involved in antibody recognition were subsequently identified after cloning different regions of the G2 glycoprotein and using a bacterial system to express the proteins. In mapping the glycoproteins antigenically, it was shown that the identified epitopes had biological functions that correlated to antibody neutralisation and hemagglutination-inhibition (Besselaar, *et al.*, 1991). The epitopes that were recognised by the neutralising antibodies were thus accessible in a manner similar to the native proteins on the virus. The G1 glycoprotein plays an important role in pathogenesis and infectivity of the virus, although it is not protective. Antigenic mapping of G1 demonstrated four clusters of possibly interlinked epitopes.

The glycoproteins, G1 and G2 are synthesized in a seemingly similar way in Bunyavirus infected cells for several hours after infection, starting at about 4h post infection (p.i.). During this time, no evidence for proteolytic cleavage of the polyprotein encoded by the M segment could be shown. G2 and G1 are made for several hours and then the rate of synthesis declines. The G1 protein synthesis started about 4 hours p.i. which is rather late in the virus growth cycle, while synthesis of the G2 could not be properly followed because of co-migration with cellular proteins during electrophoresis (Pennington, *et al.*, 1977).

Nucleotide sequence differences of 0-4.5% and amino acid sequence differences of 0-2,4% for sections of the glycoprotein genes and proteins respectively, indicate little genetic variation between RVFV isolates, although distinctions could be made between isolates using oligonucleotide fingerprinting (Cash, *et al.*, 1981). The virus isolated during an outbreak in South Africa in 1951, did not differ from the isolate that caused the disease in 1931 in Kenia (Schulz, 1951) indicating the conserved nature of the recognised structural glycoproteins. Due to the conserved nature of these viral structural proteins, it can serve in a vaccine strategy against all the isolates.

3. DIAGNOSIS OF RIFT VALLEY FEVER

Diagnosis can be made from blood (preferably in an anti-coagulant), plasma, serum or organ samples by doing virus isolation or demonstrating virus specific antibodies (OIE Manual, 1996). Virus can be isolated either from blood during the febrile stage of the disease, from liver, spleen or brain of animals that died of the disease or from aborted foetuses. Suckling or weaned mice or hamsters are used extensively for virus isolation although not for identifying the virus. When infecting a cell line, CPE can be detected 1 to 5 days after inoculation or infection of the cells.

Viral antigen can be detected in infected tissue using several methods. Primary virus isolation can be done in small animals (hamsters or mice) or on cell cultures (OIE Manual, 1996). Immunofluorescence (IF) gives results in 24 hours or sooner. Complement fixation (CF), immunodiffusion (ID) and immunoperoxidase (IP) staining can also be used to detect viral antigen in tissue. However, results can't be obtained as soon as with IF. In serum of infected animals antigen can be detected by reversed passive heamagglutination or ELISA (OIE Manual, 1996; Coetzer, *et al.* 1994).

Virus specific antibodies can be demonstrated using CF, ID, ELISA, indirect IF, heamagglutination-inhibition (HAI), reversed passive HAI, radio-immunoassays (RIA), neutralisation of CPE, plaque reduction neutralisation (PRNT) and neutralisation tests in mice (OIE Manual, 1996; Swanepoel, *et al.,* 1986). Virus neutralisation is performed most commonly in detecting virus specific antibodies, since antibodies become demonstrable within three days. The 80% plaque reduction neutralisation titre ($PRNT_{80}$) test is recommended as the most specific and sensitive serological assay for RVF (Swanepoel, *et al.*, 1986; Mariner, *et al.,* 1995). This test is able to detect the earliest response (three days p.i.) against RVFV. Tests such as the virus neutralisation test, where live virus is used to detect the antibodies, are not recommended for use in countries where RVF is not endemic. By employing a capture ELISA the presence of virus specific antibodies could be demonstrated at 6-7 days p.i., distinguishing between recently infected animals and infections at an earlier stage on the basis of IgM presence (Niklasson, *et al.*, 1984). However, these ELISA systems also employ inactivated, whole virus as antigen.

Due to the effects of possible antigenic cross-reaction between different *Phlebovirus*es, neutralisation tests that may be the least affected by this would be the most suitable in diagnosis of the virus infection (OIE Manual 1996). Cross-reactions occur in HAI tests between all *Phlebovirus*es, while CF, ID and neutralisation tests proved monospecific for the different serotypes. Cross-reactivity in serological assays of other *Phlebovirus* specific sera for RVF was found to be negligible and it could be concluded that when comparing the pathogenicity and cross-reactivity of *Phlebovirus*es with RVFV, none are likely to cause disease or elicit antibodies that may be confused with RVF during diagnosis (Swanepoel, *et al.,* 1986).

4. CURRENTLY EXISTING CONTROL MEASURES AGAINST RIFT VALLEY FEVER.

Efforts to control the spread of RVF include biological and chemical control of the vector, moving the livestock to different areas that are well-drained, wind-swept and on a higher altitude, confining the animals to insect proof stables and immunisation. Of all these, immunisation is the only effective control measure, because the others are implemented too late or is impractical and expensive.

4.1 The live, attenuated Smithburn vaccine

4.1.1 Development of the Smithburn vaccine

The Smithburn vaccine strain (Smithburn, 1949) originated from the virulent Entebbe strain, which was isolated from mosquitoes captured in Uganda and subsequently inoculated in mice. The virus was passaged serially in mice in the process of identifying it. This passaged virus isolate lost tropism for the liver, but the neurotropic properties were retained (Smithburn, 1949). Smithburn then studied the possibility of using the modified virus as an immunizing agent. The neurotropic virus was found to be suitable as a vaccine even for use in newborn lambs. Inherited immunity could be demonstrated in newborn lambs for 5 months after birth following immunisation of the pregnant ewes. Although a decline in antigenic potency was observed with serial passaging of the virus, sufficient antigenic potency was retained to elicit high levels of

antibodies. The live vaccine is being produced today from the stock antigen derived from Smithburn's original neurotropic strain. Durable immunity in sheep can be obtained after a single inoculation with this vaccine, although problems are associated with the Smithburn vaccine.

4.1.2 Problems encountered with the Smithburn vaccine

The Smithburn vaccine may cause teratology and a percentage of the inoculated pregnant animals abort (Barnard, *et al.,* 1977). The cerebral hemispheres of the brain of a foetus are often absent and replaced by sacs filled with cerebrospinal fluid (hydranencephaly) and limited motion in multiple joints (arthrogryposis) occur in foetuses. These conditions are associated with *hydrops amnii* (edema of the amniotic sac) and prolonged gestation in ewes (Coetzer, *et al.,* 1977) when ewes are immunised with the live Smithburn vaccine strain at 42-74 days of gestation. Inoculation before this time (first trimester of pregnancy) could lead to early loss of the conceptus and teratology and inoculation at a later stage may lead to abortion, stillbirth or birth of viremic progeny (Coetzer, *et al.,* 1977). The maternal antibodies developed after vaccination at a late stage in gestation might give some degree of protection in the neonatal lamb in the form of colostral immunity. Although antibody levels obtained after vaccination of cattle with the Smithburn vaccine is lower in comparison to antibody levels obtained after vaccination with the killed vaccine, protection can still be obtained when vaccinating with the Smithburn vaccine (Barnard, 1979; Shope, *et al.,* 1982).

4.2 Inactivated virus vaccine

4.2.1 Development of the inactivated vaccine

Because low levels of antibodies are observed after vaccination with the Smithburn vaccine, the use of a formalin-inactivated (killed) vaccine is advised in vaccination of cattle (Shope, *et al.,* 1982). A wild-type highly immunogenic field-strain is used in the production of formalin-inactivated RVF vaccines (Barnard, *et al.*, 1977; Coetzer, *et al.,* 1977; Coetzer, *et al.,* 1994; OIE Manual, 1996). The virus can be inactivated using formalin, formaldehyde or beta-propiolactone (Barnard, 1979; OIE Manual 1996). The inactivated vaccine is used in pregnant sheep, cattle and in countries

where RVFV is not endemic. The inactivated vaccine differs from the virus used for the attenuated strain in that it is lethal when injected intraperitoneally in adult mice (OIE Manual, 1996).

4.2.2 Problems encountered after vaccination with the inactivated vaccine

Although the inactivated virus is safe to use in pregnant animals, large quantities of viral antigen need to be prepared, making it expensive to produce. Another disadvantage of the killed vaccine is that it induces short-lived immunity (Barnard, 1979). Inconsistent antibody titres are elicited after vaccination of sheep and annual vaccination with booster doses is necessary for sufficient protection against RVF (OIE Manual, 1996).

4.3 Immune response of animals vaccinated with the classical vaccines

Both vaccines are stored in lyophilised form with a titre of at least $10^{6,5}$ mouse LD_{50} per ml (OIE Manual 1996). The primary response in cattle when injected with both vaccines is similar (Barnard, 1979). After administration of a booster dose of the live vaccine, the antibody response is poor and only animals responding to the first dose will respond to the booster dose. Low immunogenicity in cattle vaccinated with the Smithburn vaccine is observed although immunisation occasionally was done successfully against RVF with this vaccine despite low neutralising antibody levels (Barnard, 1979). A booster dose of the inactivated vaccine stimulates antibody response in cattle significantly. Significant antibody titres could still be detected in cattle after 9 months (Barnard, *et al.,* 1977) using the inactivated vaccine. A variation in the antibody response in vaccinated sheep was observed after vaccination with the inactivated vaccine and despite a low serum-neutralising index, most of the inoculated sheep could resist infection of the virus. Sheep vaccinated with the Smithburn vaccine developed significant antibody titres and long-term protection (Coetzer, *et al.,* 1994; Morrill, *et al.,* 1997).

4.4 Vaccination of humans at risk

An experimental formalin-inactivated cell culture adapted RVFV vaccine (TSI-GSD-200) produced in the USA has been used for protection of humans at risk, i.e. laboratory workers and veterinarians, while the MP-12 chemically inactivated vaccine is an alternative not yet commercially available (OIE Manual, 1996; Pittman, *et al.,* 2000). Because of limited availability, the TSI-GSD-200 vaccine is not recommended for use in a public vaccination strategy (Shope, *et al.,* 1982; OIE Manual, 1996). Multiple doses are required for sufficient protection, however, the TSI-GSD-200 inactivated vaccine gives protection after multiple doses to vaccinees for approximately 6 years (Pittman, *et al.,* 2000). In situations such as epidemics, rapid protection is required. Single dose vaccination and high immunogenicity of the vaccine would then be desirable.

4.5 Other control measures

Due to the disease potential in humans and problems associated with the vaccines against RVF, the disease situation needs to be monitored continually in an efficient surveillance program. It is necessary to advance in the search for a safe vaccine as well as practical control measures to prevent outbreaks and spread of the virus in endemic areas as well as to areas free of the disease.

Israel implemented quarantine measures for imported livestock to prevent the disease from entering the country (Shope, *et al.,* 1982). Although quarantine measures are expensive, in an effort where most of the preventative measures are being taken in combination with each other, successful control might be obtained. However, the impracticality of such measures makes quarantine not an obvious choice in disease control. Livestock can also be moved to drier, wind-swept, higher altitudes where mosquito populations are smaller and high rainfall does not lead to the formation of temporary ground pools (dambos). Protecting animals from insects, specifically mosquitoes, would ensure less amplification of the virus in a transmission cycle.

5. DNA VACCINES

5.1 DNA vaccines - an overview

Definition: Genetic or DNA immunisation is the process of *in vivo* transfection, in which plasmid DNA that encodes protein based antigens is administered to an organism to raise a required immune response against the desired protein antigens (Kucerova, 1998). In this, eukaryotic expression vectors are used as plasmid backbones to produce the target proteins in the vaccinee. Genetic vaccines employ DNA (as plasmids) or RNA (as mRNA) as source of the genetic information that has to be transferred into the cells.

5.2 Development of DNA vaccination

In 1990 J.A. Wolff and colleagues set the direction of DNA vaccine research by reporting on a study done on mouse muscle, where RNA and DNA expression vectors containing genes for chloramphenicol acetyltransferase (CAT), luciferase and β-galactosidase where introduced into the skeletal muscle *in vivo*. Injection of the pure DNA or RNA directly into the skeletal muscle was shown to result in the expression of the cloned reporter genes at significant levels (Wolff, *et al.,* 1990). The study was aimed at directly introducing genetic information for gene therapy, instead of indirect introduction through changes in target cells. Functional CAT could be detected in extracts of the skeletal muscle. β-galactosidase activity could be detected after staining of sections of the muscles indicating the site of expression. A dose-response effect could also be observed when different amounts of luciferase reporter gene constructs where injected. The luciferase activity could still be detected after 60 days and it was reported that the DNA present in the cell is in a non-replicating circular extra-chromosomal form indicating that at that stage the exogenous DNA has not been integrated into the cellular genetic material.

Exact mechanism of entry of the polynucleotides into the cells remains uncertain, but very low amounts of the reporter gene products could be detected in other tissues as well. Muscle cells were successful in taking up the constructs because of the specific structural features and the low amount of connective tissue present. The DNA enters the

post-mitotic cell nuclei through the nucleopore in the same way as karyophilic macromolecules enter the cell (Dowty, *et al.,* 1995). The introduced gene has to be transferred across the cell membrane and entrance into the nucleus has to take place before the onset of transcription. For this purpose, modified recombinant viruses can be used as vectors to introduce the genetic information into the cells (Davis, *et al.,* 1993) although direct transfer was shown to work (Wolff, *et al.*, 1990). Entry of DNA into tissue did not seem to be the result of damage to the cells, because in cells at a distance from an injection site, β-galactosidase activity could also be detected when included for use as a reporter. (Danko, *et al.,* 1994).

5.3 Choice of an expression vector

The desired gene must be introduced into a mammalian expression vector containing the necessary control elements to enable stable expression of the protein in mammalian cells. These control elements that need to be included in a plasmid vector for vaccine production, are transcriptional control elements (promoter and enhancer, intron, polyadenylation signals), a replicon and a selectable marker (antibiotic resistance markers) for growth in bacterial cells (Danko, *et al.*, 1994). It was shown that after injection of a linear plasmid encoding the luciferase gene, luciferase activity could still be detected after 4 months (Danko, *et al.*, 1994), but by using other control elements in the same plasmid, the stable expression persisted for up to 180 days. The stability of infection was further influenced by the mitotic rate of the cells.

5.4 Delivery methods

DNA delivery methods include intramuscular injection (Wolff, *et al.,* 1990) and making use of jet injection systems, i.e. the gene-gun and particle bombardment (Fynan, *et al.,* 1993) that deliver the plasmid DNA intradermally. DNA can also be delivered by intravenous injection, inhalation and instillation - where the DNA will reach the mucosal surfaces. In delivering the DNA ballistically or via a jet injection system (gene-gun delivery), the DNA is precipitated onto a particle, i.e. gold-beads, and forced into cells with a high airpressure device (Leitner, *et al.,* 2000). On the other hand, cationic liposomes are used to enhance the delivery of DNA to each cell by complexing the DNA

with the liposomes to form microcapsules containing target plasmid DNA. Delivery of the DNA to more of the target cells will result in elevated levels of proteins being expressed.

The choice of a delivery method is influenced by the total amount of protein that can be produced, efficiency and stability of gene expression in the target cell and the repeatability as well as safety of the procedure (Danko, *et al.,* 1994). The stability of infection is influenced by the mitotic rate of the cells. Muscle cells can contain DNA even a year after infection without any sign of integration into chromosomal DNA (Wolff, *et al.*, 1990).

Improvement of the efficiency of cell-to-cell transfer of the delivered DNA or target gene may be obtained by using regenerating muscle as target tissue. Adeno- and retroviral vectors have been used to introduce DNA into the regenerating or mature cells along with plasmid DNA. The plasmid DNA gene transfer gave better results in both mature and regenerating cells in comparison to the other vector systems used (Davis, *et. al.,* 1993). In addition, cellular infiltration by the viral vectors was disadvantageous in comparison to plasmid DNA alone which had no such deleterious effect. Upon introduction of the plasmid DNA encoding the human growth hormone (hGH) DNA directly into the skin of mice with microprojectiles coated with plasmid DNA, it could be concluded that an immune response could be elicited by subsequent boosts and that this method of eliciting antibodies proves to be simple and time saving (Tang, *et al.,* 1992).

As mentioned, genetic vaccination can also include RNA as source of genetic information. The advantages of delivering RNA into the cell, is that RNA is transient in nature, it will not integrate into the chromosome and it will not cause insertional mutagenesis. However, the disadvantages of RNA are the instability, higher costs of production and storage and shorter term of expression leading to the need for booster injections for long-term protection (Waine, *et al.*, 1995).

The choice of tissue would depend on the route that needs to be followed. An intramuscular route seems to be the most efficient if the DNA is injected as an aqueous solution, but if the DNA is coated onto gold particles, the intradermal route seems to be the best (Davis, *et al.*, 1995). In targeting the post-mitotic muscle cells, the DNA is not being diluted out after several divisions and this could mean longer expression that can

result in a vaccination regime without booster doses being necessary (Dowty, *et al.*, 1995). Because some cells synthesize and secrete antigen more efficiently than others, the need for post-translational modifications to the protein will dictate the choice of target tissue. It is important to keep in mind that the method of injection has to be acceptable to the vaccinee and it has to be easy to administer.

Although seroconversion takes place after DNA immunisations, the amount of antibodies being produced varies with different models. However, it seems possible that antibody titres comparable to those elicited in traditional vaccines can be obtained. It has been found that even if antibody titres may be low, the seroconversion obtained in many instances can be sufficient for virus neutralisation and even protection against a lethal challenge (Dowty, *et al.*, 1995).

5.5 Immune response elicited by DNA vaccination

Structure of the plasmid backbone, amount of plasmid delivered into the cell, expression levels of the antigen, immunisation schedule, route of immunisation, target-tissue, number of immunisations, introns in front of the gene and toxicity of the antigen to the transfected cells are all factors that influence the immunogenicity of DNA vaccines. The strain of species to be immunised and the age of animals must also be considered as influencing factors on the immunogenicity of the DNA vaccine (Leitner, *et al.*, 2000). Efficacy of the antibody response is influenced by the expression vector, the method of DNA delivery and form of the expressed antigen.

Immunisation with DNA expressing HIV-1 genes could elicit humoral and cellular immune responses sufficient to protect naive chimpanzees from a challenge with HIV (Bagarazzi, *et al.*, 1998). The same constructs were found to immunologically potentiate infected humans (Ugen, *et al.*, 1998). Genetic immunisation has been done with constructs containing the measles virus hemagglutination and nucleoprotein genes. It was found that both humoral and cellular responses were elicited after immunisation with DNA constructs encoding the hemagglutination and nucleoprotein genes of the measles virus (Cardosso, *et al.*, 1996). However, the magnitude of the overall response could not be increased by additional injections. Humoral and cellular responses were obtained after vaccination of mice with DNA constructs encoding genes of the Bovine

Parainfluenza Virus type 3 glycoproteins when the genes were under the control of the human cytomegalovirus immediate-early promoter followed by intron A for stabilization of the RNA transcript (Van Drunen Little-van den Hurk, *et* al., 1999). The fact that DNA vaccines are able to elicit antibodies as well as raising a cell-mediated response means that invading pathogens can be blocked by the antibody and the pathogen infected cells can be destroyed by cytotoxic T-lymphocytes (CTL). This represents an ideal vaccine situation.

In DNA vaccination the antigen can be synthesized from the plasmid *in vivo* in the targeted cell, processed and subsequently presentated on the major histocompatibility complex (MHC) class I molecules on the surface of the target cell. $CD8^+$ T cells are stimulated by antigen presented on the MHC class I molecules on the surface of the target cell. On the other hand, a T-helper response (CD 4+ cell stimulation) has also been observed after plasmid immunisation. The exogenous antigen expressed and secreted from the targeted cell is thus presented on the MHC class II molecule on the surface of the APC (i.e. dendritic cells, Casares, *et al.,* 1997; Robinson, 1997) after endocytic uptake of the antigen by the APC. The following of MHC-I and MHC-II pathways and subsequent induction of CD8+ and CD4+ T-cell subsets, mimic the situation of a live virus vaccine or infection.

Although muscle cells are not professional antigen presenting cells, some antigens that seem to be immunogenic enough to elicit a strong immune response, are expressed and presented to the T cells (Leitner, *et al.,* 2000). Thus, the possible mechanism of induction of immunity by muscle cells is the production of antigen as a protein or peptides for antigen presenting cells (APC). On the other hand, the APCs such as dendritic cells may be transfected directly and these cells then migrate to the lymph nodes where B and T cell activation occurs. In the situation where the inoculation was through the skin, the dendritic cells are stimulated by the antigen and it migrates to the lymph nodes for eliciting an immune response.

Gene-gun immunisation of mice compared well to the situation of sub-lethal influenza challenging of mice as a vaccination strategy, in that plateau levels of antibody persisted for more than one year (Boyle, *et al.*, 1996). It was concluded that the long-term antibody persistence that was seen with the DNA plasmid vaccination was

associated with the presence of antibody secreting cells in the bone marrow and that the initiation of responses were facilitated by the inguinal lymph nodes. The protective antibody response was associated with much lower levels of antibodies when animals were vaccinated with the plasmid DNA construct than in the situation with a natural infection.

5.6 Significance of CpG motifs

In a natural situation, a microbe has surface molecules that act as immunostimulatory factors or adjuvants during infection and although DNA plasmids are administered without such adjuvants, the immune response that is induced is quite strong. The reason for this is the presence of immunostimulatory sequences, the so-called CpG motifs, in the DNA itself (Krieg, *et al.*, 1995; Hartmann, *et al.*, 1999; McCluskie, *et al.*, 1999; Leitner, *et al.*, 2000). A CpG motif consist of a cytosine molecule bound to a guanine molecule via a phosphodiesther linkage. These non-methylated, palindromic, dinucleotide sequences in DNA can activate monocytes, natural killer cells (NK), dendritic cells and B-cells in an antigen-independent manner. These sequences occur in the order of 20 times more in bacterial DNA than in DNA of higher eukaryotes and it consist of purine-purine-CpG-pyrimidine-pyrimidine (Pu-Pu-CpG-Py-Py) sequences (Krieg, A.M. *et al.*, 1995). The non-specific adjuvant activity of CpG sequences (Hassett, D.E., *et al.*, 1999) causes cytokine production - including that of interferon-gamma (IFNγ). Cytokines are important in virus infection control by stimulation of monocytes and macrophages (Robinson, H.L., *et al.*, 1997). Although IFNγ is needed for the induction of IgG_{2a} antibodies after intramuscular plasmid administration, the antigen specific CD8 + T cell responses could still be stimulated by DNA immunisation and CTL activity could be primed in the absence of IFNγ (Hassett, *et al.*, 1999). The virus-specific IgG could be induced and maintained, but the antibody response was skewed after DNA immunisation in the absence of IFNγ. This could be explained by the fact that IFNγ is involved in IgG class switching, inhibiting IgG_1 and enhancing IgG_{2a} responses.

5.7 Dosage requirements

The amount of DNA necessary for intradermal inoculation is much more than required for inoculation using the gene-gun because the transfection efficiency is much higher for the latter scenario, where the DNA is shot inside the cell compared to the former in which the DNA is introduced in the needle wound and not directly into the cell (Fynan, 1993). Dosage requirements for mice is 1 to 100 µg, for monkeys and calves 10 µg to 1 mg (when injecting intradermally or intramuscularly), while in the event of a gene-gun inoculation, only 10 ng to 10 µg of DNA is required. The amount of DNA needed is almost independent of the size of the animal that needs to be vaccinated (Robinson, *et al.*, 1997).

By injecting large amounts of plasmid and immunostimulatory sequences, the immune response is a biased Th1-type response. Smaller amounts of plasmid DNA or a low amount of DNA that is able to enter the target cell, such as when using the gene-gun inoculation method, will lead to a Th2-type response with the IgG_1 playing a dominant role (Barry, *et al.*, 1997; Leitner, *et al.*, 2000).

5.8 Optimisation of DNA vaccination strategies

As DNA vaccination strategies need optimisation, enhancement can be achieved by modifying the antigens (Rice, *et al.*, 1999). This can achieved either by deleting domains functioning in targeting the protein (Xiang, *et al.,* 1995; Cardoso, *et al.*, 1996; Lewis, *et al.,* 1996) or by adding sequences to target the antigen specifically (i.e. to either the class-I or class-II pathway). Specific epitopes can be used as minigenes or it can be included in a highly immunogenic sequence. In this way, B- or T- cell specific epitopes can be chosen according to the requirements for a specific vaccine.

Cytokines can be included in a vaccination strategy to direct or enhance a desired response (Lewis, *et al.*, 1997; Kim, *et al.*, 1998; Leitner, *et al.*, 2000). The cytokines can be included exogenously or the cytokine-encoding gene can be included in the plasmid construct. Because the DNA is usually injected into the muscle cells that are poor APC's and thus poor initiators of an immune response, the co-administration of immunostimulatory molecules could engineer the muscle cells sufficiently to act as APC's. In this way, the induction of a T-cell immune response can be facilitated by the

transfected muscle cells. It is important that the administered cytokines are specific for the type of response or direction of the response, because the different cytokines facilitate in different arms of the immune response (Lee, *et al.,* 1999). Physical linkage of the cytokine to the antigen can further enhance the immune response by ensuring the stimulatory effect in the local environment of the antigen where the responses occur. Chow, *et al.* (1997) found that IL-2 could stimulate the immune response to a Hepatitis B surface antigen a 100-fold when it is co-expressed in a plasmid vector.

A heterologous prime-boost-regimen has certain advantages over homologous boosting (Leitner, *et al.,* 2000). The efficacy of a virus-based vaccine may be hampered by an anamnestic response to the virus-based carrier, if given repeatedly. To include DNA immunisation in a vaccination strategy could prevent this, in addition to the fact that the immune response can be shifted in a Th1 direction. It may be important to administer the antigenically simplest vector first and the more complex vector or more potentially powerful antigen last. The HIV envelope protein genes were used in a recombinant DNA molecule as well as a recombinant vaccinia virus (Caver, T.E., *et al.*, 1999). Both these constructs and purified envelope proteins were used in a heterologous prime-boost regimen where the immune response could be enhanced a 100-fold in relation to a strategy where a DNA construct was used as booster.

Another way of optimising a DNA vaccination strategy could be to facilitate an increase in the replication of the DNA in the mammalian cell. A useful characteristic of members of the Alphavirus genus is the feature that the RNA encodes it's own RNA replicase, thus after infecting the cell, the replicase complex is being translated and this facilitates the replication of the viral RNA that encodes the genes of the structural proteins (Levy, *et al.*, 1994; Polo, *et al.,* 1998). This implicates that the structural protein genes can be replaced by that of the desired antigen, establishing a self-replicating DNA vaccine construct. Because replication takes place in the cytoplasm of the host cell, it is independent of the host cell's replication machinery. The advantages of a DNA vaccine with these features, are a broad host range of cells that can be "infected", much higher levels of expression and cytoplasmic replication (Leitner, *et al.*, 2000). This can lead to efficient MHC-I presentation due to the high amount of antigen present in the cell and the presence of ds RNA that may cause the system to trigger the release of cytokines such as

interferon molecules. When the transfected cells release antigen after transfection, it may be taken up by the local APC's leading to an enhancement of antigen being presented to the immune system. The self-replication also means that less of the vaccine can be administered to achieve protection if compared to conventional DNA vectors.

Targeting the DNA to specific tissue is possible, i.e. by adding asialoglycoprotein receptor ligands to the liposome-DNA complex for targeting hepatocytes or Kupffer cells (Templeton, *et al.*, 1997) or by expressing CD4A fusion proteins that target the expressed protein to antigen presenting cells (APC) (Deliyannis, *et al.*, 2000).

5.9 Limitations of currently used vaccines

Currently used vaccines - subunit vaccines, live attenuated vaccines and killed vaccines, have the following limitations:

Subunit vaccines:

- Inability of subunit vaccines to elicit CD8+ CTL response (induce long term immunity), thus necessitate the need for boosters.
- The production of the antigen is complicated and expensive.
- The antigen may be of low immunogenicity.
- Vaccine is not heat stable.
- High production cost.
- Purification is difficult.
- Incorrect folding of the antigen

Live attenuated vaccines:

- Live attenuated vaccines may revert to virulence.
- Vaccine is often not heat stable.

Inactivated or killed vaccines:

- Killed vaccines are unable to induce a CD8+ CTL response.
- There is a possibility of infection due to incomplete inactivation and loss of immunogenicity due to denaturation following inactivation in the production process of inactivated vaccines.

5.10 Advantages of DNA vaccines

An ideal vaccine should be safe, easy to administer, inducing the full range of immune responses, have a long-lasting effect, easy and inexpensive to manufacture, heat stable and the quality control should be simple yet accurate. According to Davis, *et al.*, (1995), DNA vaccines may have the following advantages:

- There is no risk of an attenuated organism mutating back to a virulent form.
- Potential long-lived gene expression, leading to sustained presentation of the antigen to the immune system.
- Sustained presentation would prevent the need for booster injections.
- Because of the *in vivo* synthesis of the antigen, the antigen is presented on the cell surface by MHC -I, leading to cell mediated immunity (CMI) due to the activation of CD8+ cytotoxic T-lymphocytes (CTL).
- No infectious agents are used in vaccination, eliminating the risk for infection in immunocompromised individuals.
- Production is easier, less expensive and it is simple to maintain quality control.
- DNA is heat stable, eliminating use of the cold chain.
- Multi disease vaccines can be produced by the inclusion of antigens from different infectious agents in the DNA construct or one can include different epitopes of the same or different antigens in the same construct.
- Immunostimulatory or immunomodulatory molecules can be administered to enhance the efficacy of the vaccine.
- The possibility that maternal antibodies may clear the antigen before the immune system has been stimulated doesn't exist in this scenario. Usually this leads to an unresponsiveness and higher susceptibility to tolerance.
- Advantages over problems associated with recombinant viruses such as pre-existing immunity, insertion mutagenesis, loss of attenuation and the occasional spread of inadvertant infection can be overcome by employing a DNA vaccination strategy.

5.11 Disadvantages of DNA vaccines

Disadvantages of DNA vaccines include the possibility of homologous recombination to occur between the genomic DNA of the vaccinee and the plasmid DNA injected (Davis, *et al.,* 1995; Prljic, *et al.,* 1999). This would result in foreign sequences being incorporated into the genome of the vaccinee. The possibility exist that disadvantageous consequences like auto-immunity or tolerance to an antigen may be induced by administration of the DNA vaccine or genes encoding an antigen in particular. Although these concerns have been raised (Beard, *et al.,* 1998), experimental evidence of these adverse effects has not been shown (Bagarazzi, *et al.,* 1998).

6. AIMS OF THIS STUDY

The most important aspect of any vaccine is the ability to induce a protective immune response. Protection against a wide variety of diseases and organisms has been obtained using DNA immunisation (Sedegah, et al., 1994; Barry, *et al.*, 1995; Xiang, et al., 1995; Van Drunen Littel-van den Hurk, et al., 1999) indicating that DNA vaccines have a potential as an alternative to classical vaccines.

Problems associated with classical vaccines currently used to protect animals against RVF can be summarised as:

- Abortions in sheep after vaccination with the live attenuated Smithburn vaccine.
- Low antibody titres in cattle after vaccination of the cattle with the live attenuated Smithburn vaccine.
- Teratology caused by the live attenuated Smithburn vaccine after vaccination of pregnant ewes.
- Only short term immunity induced by the inactivated vaccine in cattle.
- Large quantities of the antigen are required for the killed vaccine.

A variety of glycoprotein genes have been used in experiments where these genes were cloned into a mammalian expression vector and used as DNA vaccines (Ugen, et al., 1998; Van Drunen Littel-van den Hurk, et al., 1999). The results obtained from these

different experiments indicate that protective glycoprotein genes have been used successfully as target genes in a DNA vaccination strategy.

The advantages of DNA vaccines and the mentioned disadvantages or limitations of the currently used vaccines against RVFV, indicate that DNA vaccines might prove to be useful in developing a vaccine to protect susceptible animals against RVF. DNA vaccines have been tested successfully for protection against viral (Xiang, et al., 1995), bacterial (Barry, et al. 1995) and parasitic pathogens with (Sedegah, et al., 1994) and without intracellular phases (Waine, et al., 1995). It has been shown that manipulation and modulation of the immune response is possible by the addition of the genes of immunomodulatory molecules and optimisation of the plasmid backbone sequence (Leitner, *et al.*, 2000).

The aims of this study was to:

- Generate a plasmid DNA construct containing the protective glycoprotein genes of the Rift Valley fever virus and the gene encoding the Enhanced Green Fluorescent protein.
- The generated construct's performance as a DNA vaccine where mice (as a small animal model) and sheep (as a large animal model) were vaccinated against Rift Valley fever have to be evaluated in terms of neutralising antibody titres, evaluation of clinical signs, serum levels of liver enzyme and overall protection against RVF.
- In addition, subcloning of the glycoprotein genes into a bacterial vector system to evaluate expression of the glycoproteins. Expression products obtained could function as antigens in an ELISA based diagnostic assay to replace the whole virus that is being used as antigen. Development of an ELISA based assay that is more sensitive (less false negatives), less time-consuming, more affordable, less complicated and makes use of a recombinant protein as antigen would be the eventual outcome after a useful expression product could be obtained.
- A final objective of this study was to develop and evaluate a one-step RT-PCR. Such an assay would be advantageous in diagnosis of the disease

and during quality control in development of classical vaccines. Depending on the type of vaccine used in the future, the RT-PCR could assist in differentiating between vaccinated animals and naturally infected animals. The RT-PCR would assist in the timely diagnosis of RVF, be useful in a surveillance program and would reduce the risk in handling of whole virus.

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).

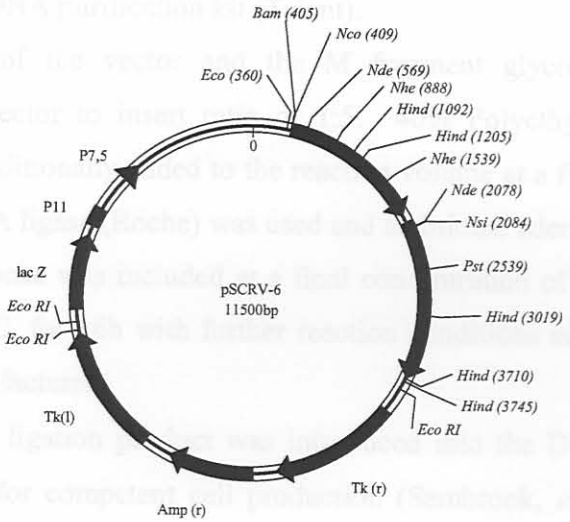


Figure 1. pSCRV-6 plasmid map indicating the relevant restriction sites and sequence information. pSCRV-6 originated from the pSP76-6 intermediate plasmid and the vaccinia transfer vector pSC11 (Kakach, et al., 1988; by permission from C.S. Schmaljohn and M.S. Collett).

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.

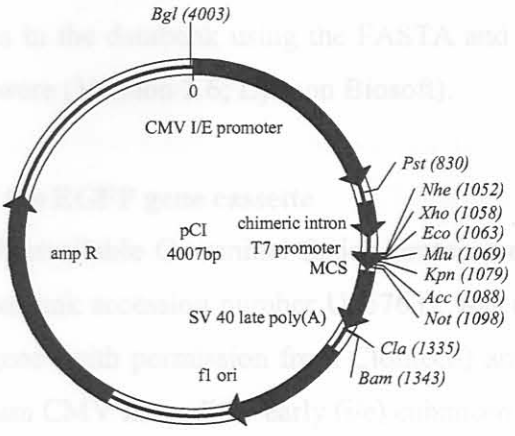


Figure. 2 - Plasmid map of the pCI mammalian expression vector. Multiple cloning site data indicated (Promega).

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.

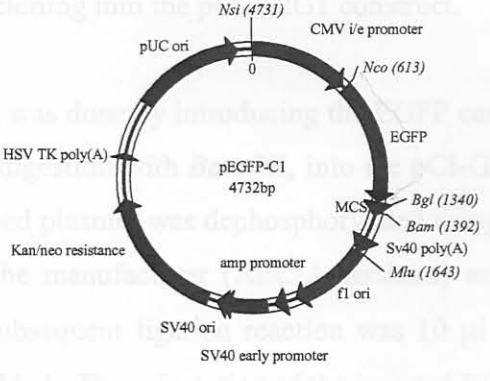


Figure. 3 pEGFP-C1 plasmid map showing the relevant restriction sites (Clontech).

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′

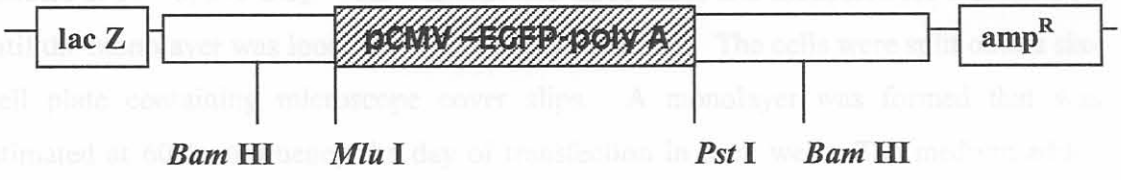


Figure. 4 – Subcloning of the EGFP gene cassette into the pMTL 24 intermediate vector to facilitate subcloning into the pCI-G2G1 construct.

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.

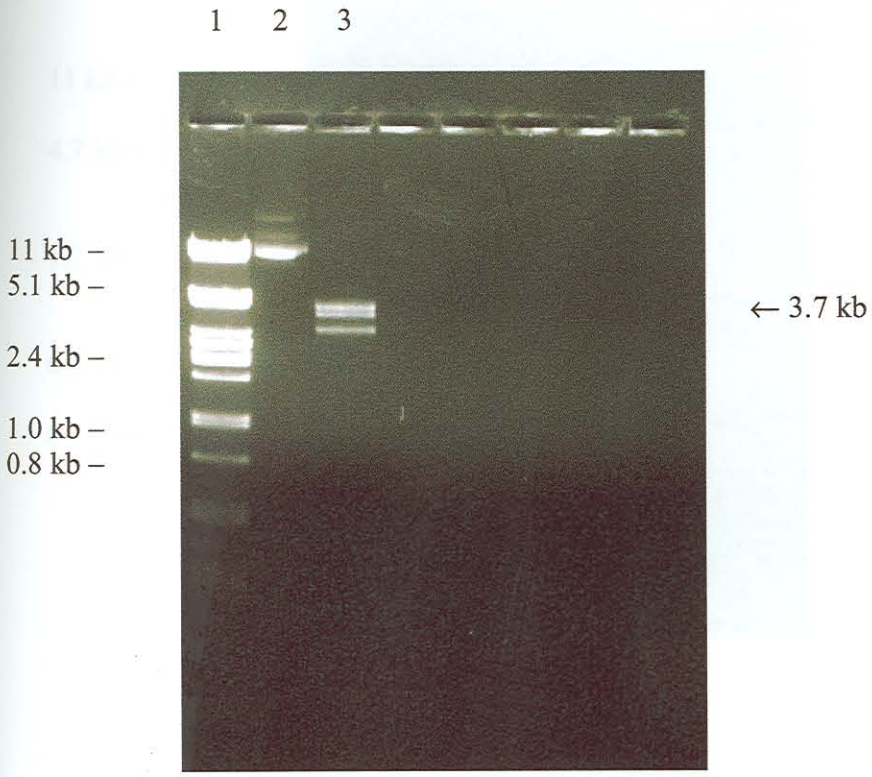


Figure. 5 Electrophoretic profile on an ethidium bromide stained 0.8% agarose gel of pSCRV-6 upon *Eco* RI restriction endonuclease treatment (lane 3) and the lambda DNA digested with *Pst* I to serve as molecular weight marker (lane 1). The 3.7 kb fragment contains the part of the M segment of the RVFV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

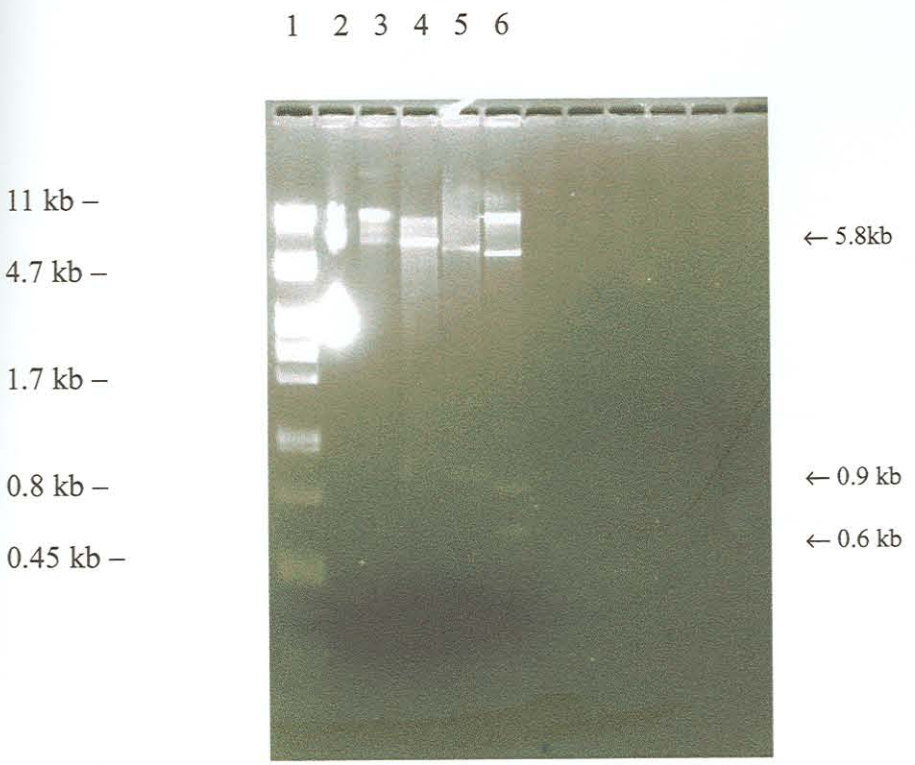


Figure. 6 pCI-G2G1 restriction digestion profile obtained on a ethidium bromide stained 1 % agarose gel. Restriction endonuclease digestion was done of the recombinant plasmids (pCI-G2G1) to screen for correct orientation of the G2G1 fragment. *Cla* I and *Nhe* I (AEC-Amersham) as well as *Nco* I (New 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.

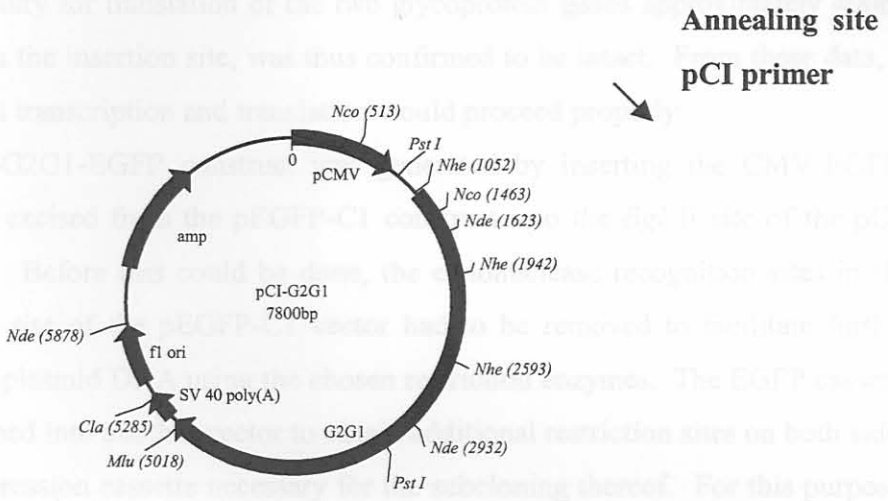


Figure. 7 Diagrammatic representation of the pCI-G2G1 construct indicating positions of restriction endonuclease recognition sites and sequence regions important in the subcloning of the G2G1 fragment. The position of the annealing site of the pCI primer is indicated.

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.

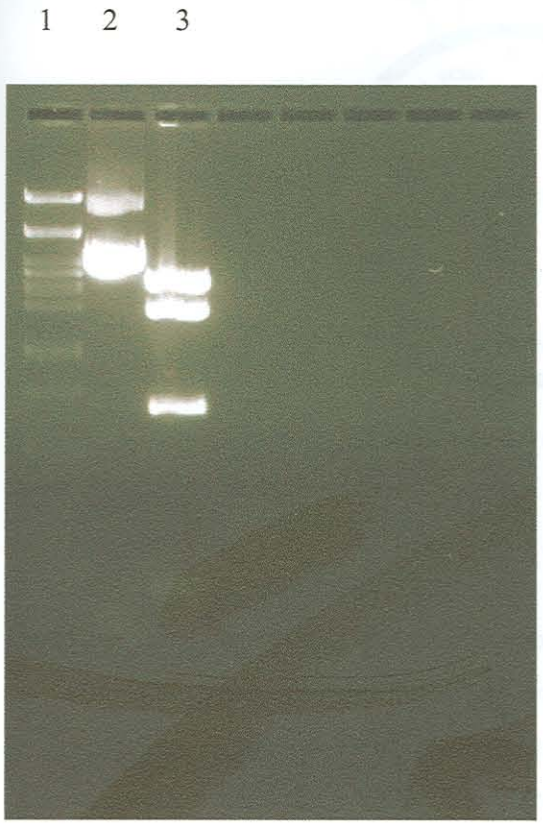


Figure. 8 Electrophoretic profile of the pEGFP-C1 plasmid upon *Mlu* I and subsequent *Nsi* I (Eco T22 I isoschizomer) restriction digestion (lane 3) on an ethidium bromide stained 0.8 % agarose gel. The 1.6 kb gelpurified fragment containing the EGFP gene was used in the subsequent cloning steps. Lane 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.

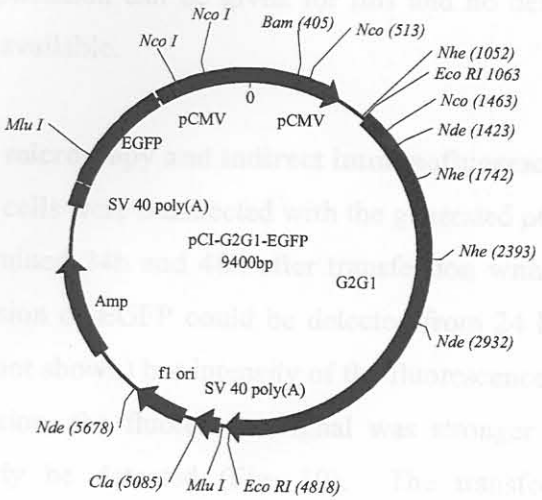


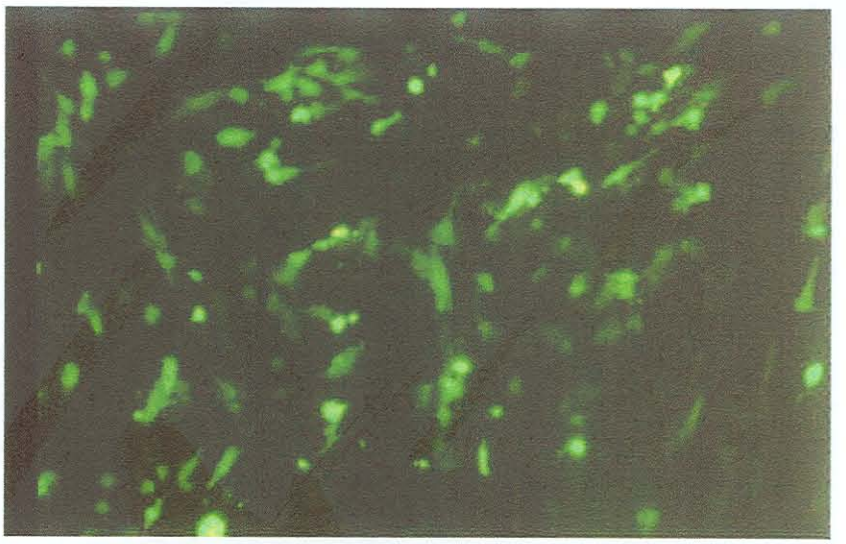
Figure 9 The pCI-G2G1-EGFP construct indicating the relevant restriction endonuclease recognition sites, fragments inserted and the orientations of the coding segments. For a diagrammatic summary of the cloning strategy and events, please see - Addendum 1 (p. 98).

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.

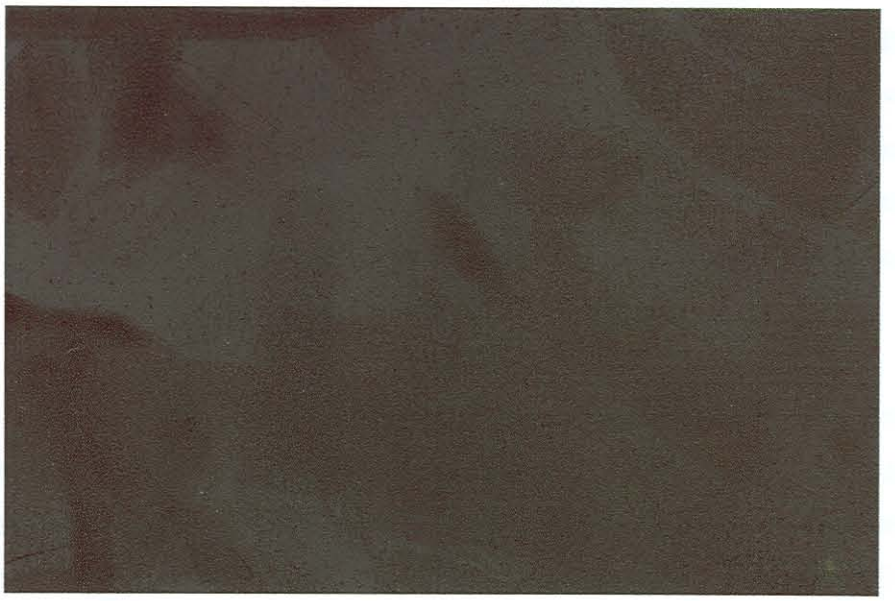
3.2 Fluorescent microscopy and indirect immunofluorescence

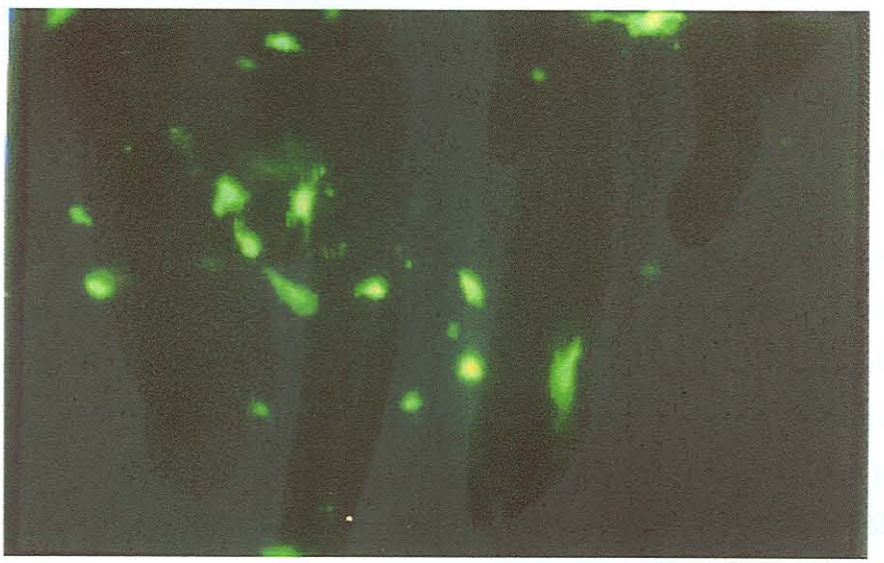
The BSC 40 cells were transfected with the generated pCI-G2G1-EGFP construct. The cells were examined 24h and 48h after transfection with plasmid DNA for EGFP expression. Expression of EGFP could be detected from 24 hours after transfection in this system (results not shown) but intensity of the fluorescence 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





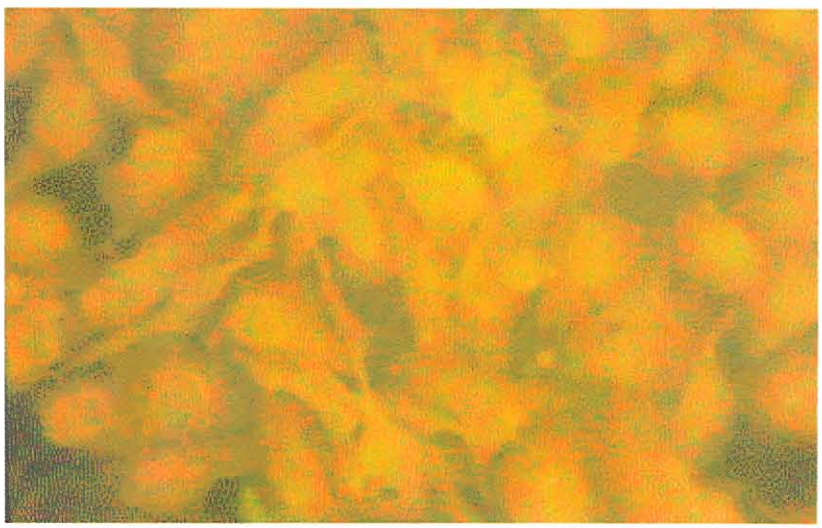
C

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.

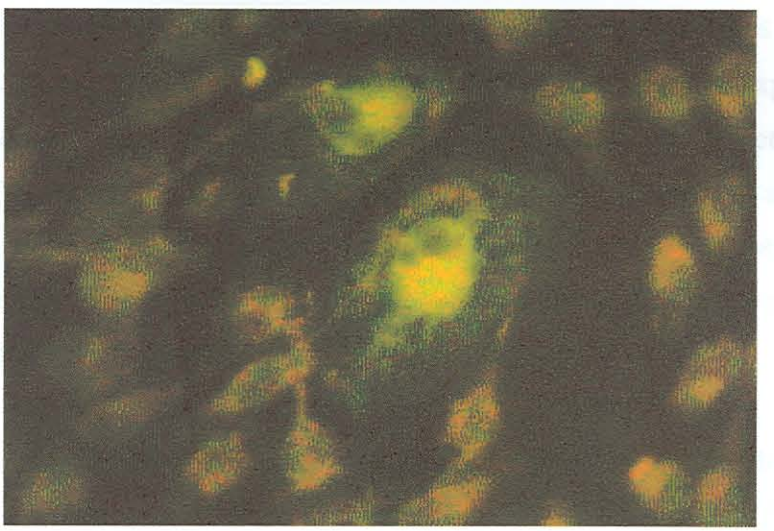
After detection of the EGFP in the transfected cells to confirm successful transfection and integrity of the subcloned EGFP gene cassette, indirect immunofluorescence was done to detect expressed glycoprotein. Both genes are in the same construct, therefore the amount of cells fluorescing due to expressed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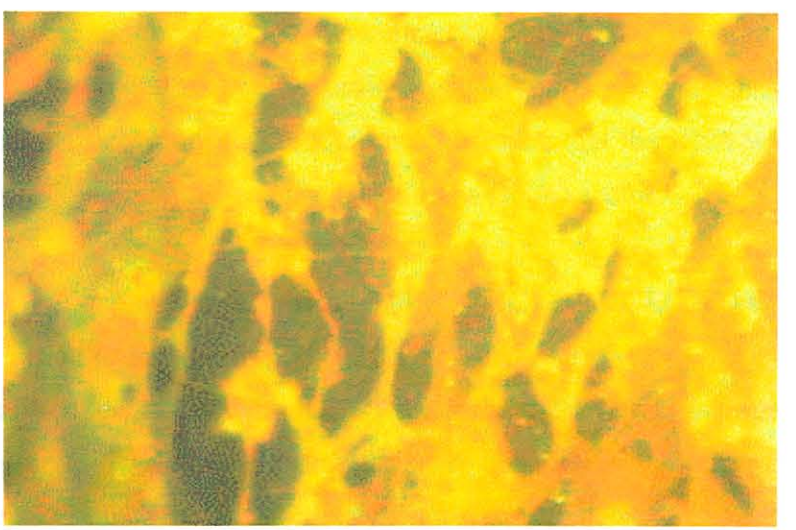


Figure. 11 - Transient expression of the RVFV glycoproteins after indirect immunofluorescent staining of BSC 40 cells with FITC conjugated secondary antibody (B, 7 seconds exposure time, 10 mm = 15 µm). Cells transfected with the pEGFP-C1 construct served as the negative control (A, 7 seconds exposure 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.

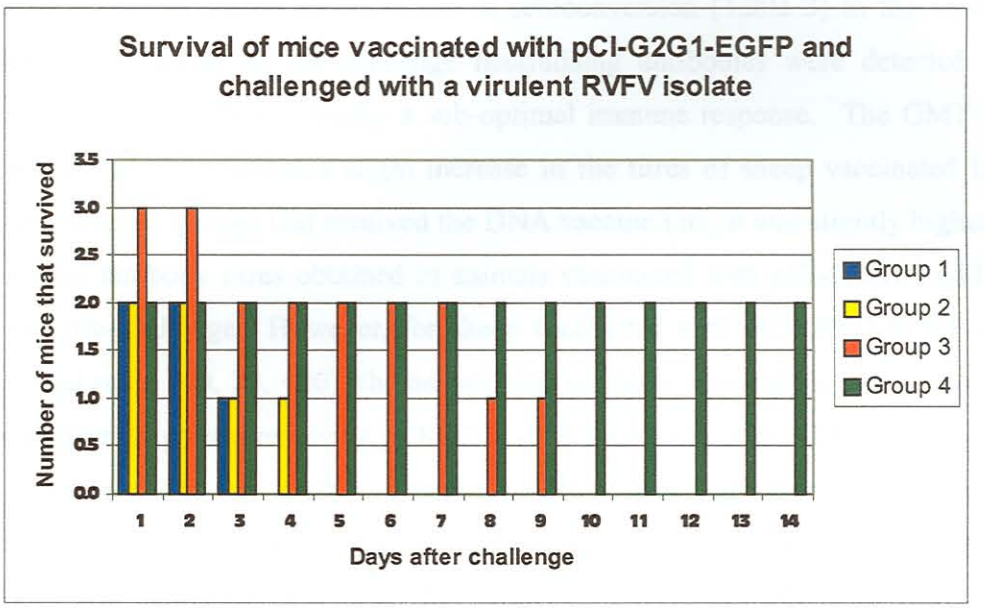


Figure 12 Survival of mice after immunisation with pCI-G2G1-EGFP and subsequent challenge with a virulent isolate of RVFV. The mice were observed for 14 days after injection with the virus. Group 1 – negative control, group 2 – pCI control, group 3 – DNA vaccinated mice, group 4 – positive control vaccinated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

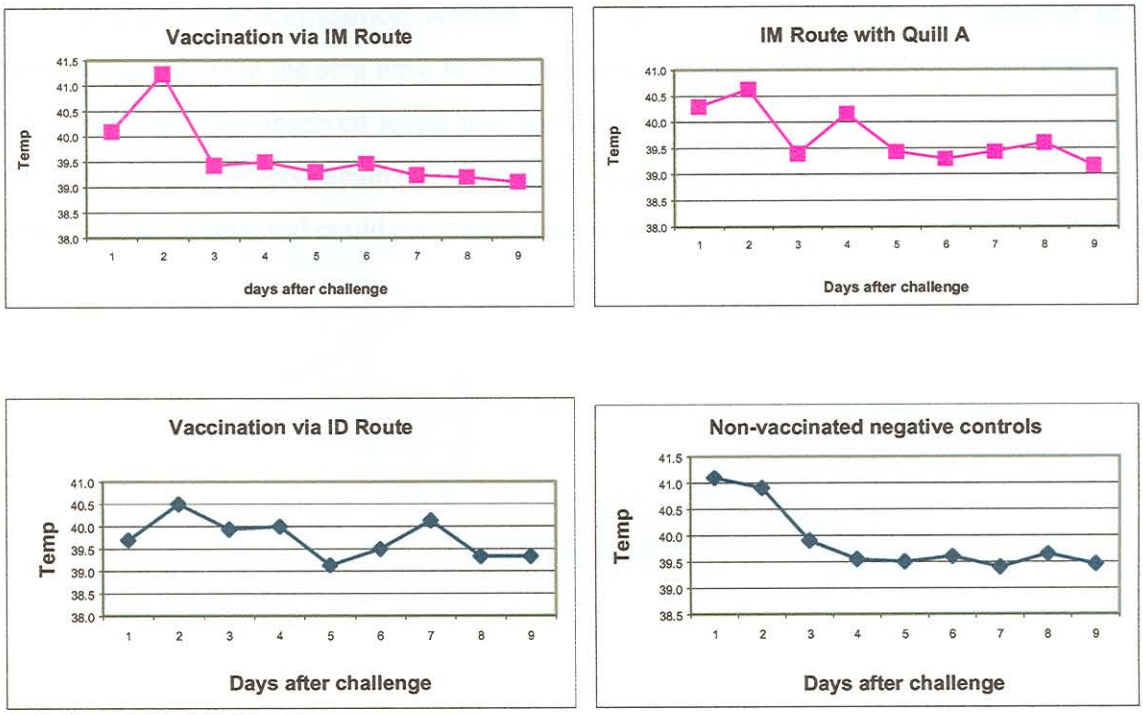


Figure. 13 - Average temperatures of vaccinated sheep taken daily post-challenge with a virulent RVFV strain (exact values shown in Addendum V). Different routes of inoculation were intradermally and intramuscularly (with or without Quil A used as adjuvant), (i.m –intramuscular, i.d – intradermal).

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.

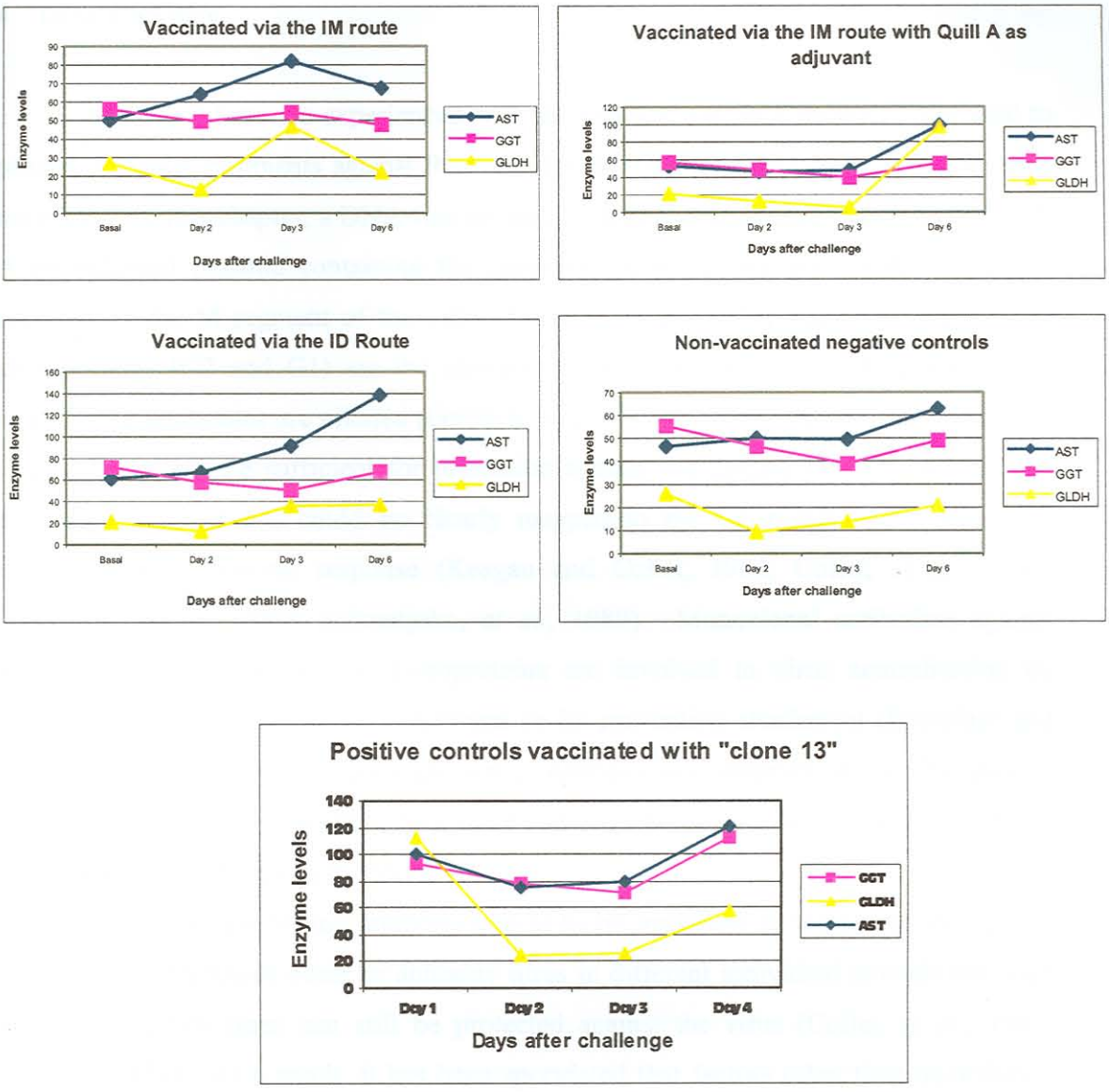


Figure. 14 - Liver enzyme levels of sheep over a six-day period post-challenge with a virulent strain of RVFV. Routes of inoculation were intramuscular (with or without Quil A as adjuvant) and intradermal. (AST – aspartate amino transferase, GGT – gamma-glutamyl transferase, GLDH – glutamate dehydrogenase).

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.

CHAPTER 3

CLONING AND EXPRESSION OF THE RIFT VALLEY FEVER VIRUS GLYCOPROTEIN GENES IN A BACTERIAL SYSTEM AND DEVELOPMENT OF A RT-PCR

1 INTRODUCTION

To identify the infectious agent in a suspected Rift Valley fever (RVF) case involve serological tests as well as culturing of the virus from infected samples collected during the febrile stage of the disease (OIE Manual, 1996). A disadvantage associated with the culturing of virus is that it is time consuming and virus-infected material has to be handled. Because RVF is zoonotic, handling of virus-infected material presents a health risk. Diagnosis based on serology include neutralisation tests ($PRNT_{80}$), Enzyme-linked Immunosorbent assay (ELISA), Heamagglutination inhibition (HI), Agar gel immunodiffusion (AGID), Immunofluorescence (IF), Radio-immuno assays (RIA) and complement fixation (CF). Although cross-reactions can occur while performing these tests (Swanepoel, *et al.*, 1986), such assays have the advantage of being performed using an inactivated antigen (OIE Manual, 1996; Swanepoel, *et al.*, 1986). Currently used ELISA assays can distinguish between very early infection and late infection as well as previous contraction or contact with the infectious agent by means of detecting circulating IgM or IgG antibodies using respectively a capture ELISA or a double sandwich ELISA (Niklasson, *et al.*, 1984). Disadvantages of these particular ELISA assays include the use of whole virus as antigen, low sensitivity, high cost and complexity because of the inclusion of several steps for detection of IgG or IgM.

In developing an alternative ELISA or alternative assay to assist in RVF diagnosis, use of a recombinant viral antigen, higher sensitivity, less complicated procedures and a more affordable assay would be preferred. A PCR diagnostic assay can assist in diagnosis and confirmation of the disease. It would have a high sensitivity and would allow differentiation between vaccinated and naturally infected animals in conjunction with serological techniques. PCR assays would have a quantitative function in evaluation of production of classical vaccines, in specific quality control thereof. Such

an assay could in time replace the ELISA based assays as main diagnostic test for diagnosis and confirmation of RVF and quality control of vaccine manufacturing procedures.

Alternative antigens for use as vaccines or to function in ELISA tests include subunits of the viral entity, whether recombinant or synthetic. Options in producing recombinant proteins include bacterial vector systems; baculovirus vector systems as well as Yeast vector systems (i.e. *Pichia pastoris*) for expression of the protein subunits. Because the bacterial expression system is simpler, expression can be regulated by cloning under the control of inducible promoters and expression levels of the antigen can be relatively higher than obtained in other systems, it may be used to produce recombinant antigen for use in an ELISA format assay. A disadvantage of this system is that downstream processing of the protein, including glycosylation, may not be correct or will not occur at all. The resulting gene product may be dysfunctional because of disruption or destroying of epitopes. Solubility of the antigen may also be low due to production of the antigen in the inclusion bodies in the bacterial cells. A system like the baculovirus expression system or the *Pichia pastoris* expression system can provide functions for post-translational modifications. Although the antigen will then be expressed in lower levels than that of the bacterial system, post-translational modification (i.e. glycosylation) would give a product similar to the natural protein. The expressed product might have a higher degree of solubility than the bacterial expressed protein.

In attempting to generate an alternative antigen to the currently used whole virus, the glycoprotein genes of RVFV were cloned into a bacterial expression system. The pPROEX-HTb bacterial expression vector was used to clone the glycoprotein encoding genes of the Rift Valley fever virus (RVFV) in. From this cloned segment, both glycoproteins can be expressed using the accompanying translational start sites. After induction of expression, protein products were examined by electrophoresis and subsequent western blotting using a RVFV specific antiserum.

A one-step RT-PCR assay was developed, optimised, evaluated and validated. Such an assay would have several useful applications as discussed including early and rapid diagnosis of the infectious agent in serum and whole blood. A one-step method was developed instead of a two-step method or a method where a nested reaction is

included. This means a more user-friendly procedure that eliminates unnecessary events where contamination is more likely to occur. Primers were designed to anneal in the intergenic region between the genes encoding the glycoproteins (G1 and G2) on the M segment of the RVFV genome. These docking sites allow the detection of the viral M segment. Additional docking sites would be on the S segment of the virus RNA encoding the nucleocapsid protein. However, since these primers were also used to detect the plasmid DNA vaccine during development thereof, the M segment proved to be the region of choice for primer docking sites. The specificity and sensitivity of the RT-PCR was determined and sera from animals that participated in a DNA vaccination trial and field samples (sheep sera) positive for RVFV obtained from Senegal was used to validate this test.

2 MATERIALS AND METHODS

2.1 Subcloning and expression of the Rift Valley fever virus glycoprotein genes

2.1.1 Subcloning of the RVFV glycoprotein genes into the pPROEX-HTb bacterial expression vector.

The *Nco* I/*Eco* RI fragment containing the glycoprotein genes was excised from the pSCRV-6 plasmid (see Chapter 2, Fig. 1) using enzymes supplied by New England Biolabs. The digestions were done using reaction conditions as indicated by the supplier. The subsequently gel-purified (Talent) fragment was directionally cloned into the pPROEX-HTb (Fig. 15) bacterial expression vector (Life Technologies) into the *Nco* I/*Eco* RI restriction sites, ensuring the inserted fragment to be in-frame with the translational start codon contained in the expression vector. An amount of 5U of the enzymes was used in the reactions in a final volume of 10 µl. The subsequent ligation reaction was performed in a final volume of 15 µl and 1.5 U T4 DNA ligase (Roche) was used with the appropriate amount of supplied buffer.

The pPROEX-HTb bacterial expression vector contains the sequence that codes for six histidine affinity tags to facilitate subsequent purification of the expressed protein. A Tobacco Etch virus (TEV) protease recognition site allows for removal of the histidine tags by protease cleavage from the expressed protein during purification (Fig. 15). The

histidine affinity tag would be located on the N-terminal of the expressed product. Expression of the protein from the genes cloned into the pPROEX-HT bacterial expression system is facilitated by isopropyl-β-D-thiogalactopyranoside (IPTG) induction (0.6 mM final concentration). Insertion of the gene fragment was verified by restriction digestion of the obtained recombinants. The confirmed correct recombinant plasmid was digested with *Mlu* I (AEC-Amersham) and analysed by electrophoresis on a 1% agarose gel stained with ethidium bromide.

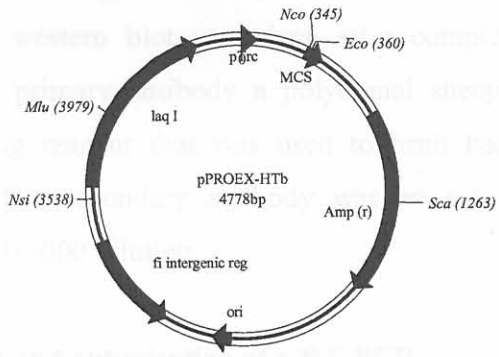


Figure. 15 - Diagrammatic representation of pPROEX-HTb bacterial expression vector indicating the multiple cloning site of the vector used to clone the glycoprotein gene fragment of RVFV excised from pSCRV-6.

2.1.2 Expression of the glycoprotein genes from the bacterial expression system

After verification of the suspected recombinants using restriction enzymes, a single colony of the stock culture streaked onto Luria-Bertani (LB) agar plate, was picked with a sterile toothpick. This was inoculated into 10 ml LB-broth and incubated at 37 °C. After the culture has reached a cell density of 0.5-1.0 at A_{590}, a 1 ml sample was removed and after pelleting the cells, it was resuspended in phosphate buffered saline (PBS). This sample served as the uninduced control. Isopropyl-β-D-galactopyranoside (IPTG) was subsequently added to the culture at a final concentration of 0.6 mM as indicated by the

accompanying plasmid product manual supplied by Life Technologies. Aliquots of 1ml of the induced cultures were removed at 3h post-induction, the samples were centrifuged and to the pellets were added PBS and an equal volume of sodium dodecyl sulphate (SDS) sample buffer (125 mM Tris-HCl (pH. 6.8), 4% (w/v) SDS, 20% (v/v) glycerol, 0.01% (w/v) bromophenol blue). Samples were taken in duplicate of which one received Dithiotrietol (DTT) (10 mM final concentration) added to the SDS sample buffer. The treated samples were stored at –70 °C until use. Samples were boiled and analysed by SDS-polyacrylamide gel electrophoresis on a 12% gel where 20 µl of the processed sample was loaded onto the gel. The supernatants as well as the pellets were treated in the same way. A western blot was done after completion of the electrophoresis procedure, using as primary antibody a polyclonal sheep-anti-RVF antibody diluted 1/100. The blocking reagent that was used to limit backgound was bovine serum albumine (BSA). The secondary antibody was an anti-sheep antibody coupled to peroxidase used at a 1/1000 dilution.

2.2 Development and optimisation of a RT-PCR

2.2.1 Designing of primers

Primers were designed to anneal at positions 1755-1775 and 2097-2117 for the forward (ARV1-F) and reverse (ARV2-R) primers respectively, on the M segment of the RVFV genome (Collet, *et al.*, 1985). Both primers were synthesized by Gibco BRL at an amount of 200 nmole each. The position where primer ARV1-F anneals, is at the 3' end of the G2 glycoprotein gene whereas ARV2-R anneals in the intergenic region between G2 and G1. The part that is being amplified is mainly the intergenic region. Sequences for ARV1-F and ARV2-R are 5' -tgt cac act gct ctc agt gcc–3' and for ARV2-R, 5' –gga gct tgc ctg aat ctg ttg-3' respectively. The amplicon size expected, after amplification of the template using these primers is 363 bp. Self-complementarity and alignments between the primers were checked as well as specificity for the template using the DNAMANSoftware (Version 2.6; Lynnon Biosoft).

2.2.2 Use of RVFV-specific primers in PCR reaction

A PCR was done using the plasmids pSCRV-6 and pCI-G2G1-EGFP as templates at an estimated 3ng dsDNA each to confirm the specificity of the primers for the template sequences. The pCI-G2G1-EGFP plasmid was also used at a 1:10 dilution of the plasmid. The composition of the reaction mixture was as follows:

Forward primer (ARV1-F)	(50 pmole/µl)	0.5 µl
Reverse primer (ARV2-R)	(50 pmole/µl)	0.5 µl
Takara ExTaq	5U/µl	1.0 µl
Supplied buffer (Takara)	10x	2.5 µl
dNTP's (Roche)	10 mM	1.0 µl
Template		3 ng
Water to a final volume of		25 µl

Magnesiumchloride ($MgCl_2$) was not added additionally to this reaction volume, thus the final concentration was 2.5 mM as included in the supplied buffer. The time-temperature profile of the reaction was 30 cycles of 94 °C for 30 sec; 61 °C for 30 sec; 72 °C for 30 sec.

2.2.3 Extraction methods for template RNA

Rift Valley fever virus (Smithburn strain used for live attenuated viral vaccine production) was obtained from Onderstepoort Biological Products and used to serve as template source during the optimisation of the RT-PCR. The virus has been passaged in mice 103 times, twice in Baby Hamster Kidney (BHK) cells and once in African Green Monkey kidney (VERO) cells to attenuate the virus. This attenuated virus stock that was obtained had a titre of 10^6 TCID/ml and was aliquotted and stored at –20 °C.

Two RNA extraction methods were evaluated. These were the QIAmp viral RNA extraction kit from Qiagen, and the method described by Chomczynski, *et al.*, (1987). Both methods were used according to the protocols described by either the manufacturer or the author respectively. Briefly, for the QIAmp viral RNA extraction kit, 140 µl of the naïve blood sample was used. To the sample was added the buffer containing carrier

RNA and absolute ethanol. This was loaded onto a column containing a silica-gel membrane supplied with the kit and centrifuged to retain the RNA in the column. The column was washed with the supplied buffers and the RNA eluted using the supplied elution solution. In the case of the method described by Chomczynski, *et al.,* (1987), 200 µl of the sample (serum or blood) was added to an equal volume of solution D (4M Guanidiumthiocyanate, 25 mM sodium citrate, 0.25 % Sarcosyl, 100 mM 2-mercapto ethanol) and 20 µl 2M NaAc. This was mixed and 500 µl phenol: chloroform (1:1) was added. After incubation on ice, the sample was centrifuged and the supernatant was added to 500 µl isopropanol before incubation at –20 °C to facilitate precipitation of the RNA. After centrifugation, the RNA pellet was washed with 70 % ethanol and then redissolved in 50 µl Diethylpirocarbonate (DEPC) treated water.

Blood was spiked with an estimated amount of 10^2 $TCID_{50}$ of the virus per extraction volume. The extractions were performed and the RNA obtained was stored for a short time at –20 °C until use. Naïve serum was also spiked with the virus to determine the possibility of extracting virus from blood and serum. A one-step RT-PCR method was performed with the following time-temperature profile. 37 °C for 30 min; 94 °C for 30 sec; **65 °C** for 30 sec; 72 °C for 30 sec in a 25 µl reaction volume. 30 cycles were completed. The annealing temperature was raised from 61 °C to 65 °C to increase specificity of the reaction. Reaction volumes had the following reagent composition :

Forward primer	(AVR1-F)	(50 pmole/µl)	0.5 µl
Reverse primer	(ARV2-R)	(50 pmole/µl)	0.5 µl
MMLV-RT (Promega)	200U/µl		0.1 µl
Ex Taq (Takara)	5U/µl		0.25 µl
Ex Taq buffer	10x		2.5 µl
dNTP's (Roche)	10mM		1.0 µl
HPRI (Promega)	110U/µl		0.01 µl
Template RNA			5 µl
Water to a final volume of			25 µl

Final concentration of $MgCl_2$ in this reaction was 2.0 mM included in the buffer.

2.2.4 Magnesiumchloride titration

A $MgCl_2$ titration was done to determine optimum $MgCl_2$ concentration to be used in the RT-PCR reaction. Final concentrations used in the titration assay were 0 mM; 1.5 mM; 2.5 mM; 5 mM; 7.5 mM and 10 mM. The titrations were performed in serum-extracted samples using the method of Chomczynski, *et al.*, (1987) after this method has been chosen as the preferred extraction method due to lower cost and ease of use. In this titration the annealing temperature was raised to 65 °C as was done for the reactions in which the extraction methods were compared. This would allow for higher specificity.

2.2.5 Sensitivity determination of RT-PCR

Sensitivity of the assay was determined by diluting the virus (titre of 10^6 $TCID_{50}$/ml) in naïve blood and serum samples. The total volume of sample for the extraction method needed to be 200 µl (Chomczynski, *et al.*, 1987). Dilutions of the virus were done in 100µl PBS and added to 100µl blood or serum. The amount of virus (Smithburn attenuated viral vaccine stock of 10^6 $TCID_{50}$/ml) added was 50µl, 25µl, 12.5µl, 6.25µl, 0µl respectively to an amount of PBS for a final volume of 100µl. This was then added to the 100 µl naïve serum or blood for a final volume of sample of 200 µl. Dilutions of virus and addition of virus to serum was also repeated after serially diluting the virus in PBS. The final dilution was 10^{-6} and the virus titre predicted for this dilution was 1 $TCID_{50}$. The RNA was extracted from these virus dilutions in serum or blood. The RT-PCR reaction was repeated as described for the magnesiumchloride titration.

2.3 Validation of the developed one-step RT-PCR.

The samples used in validation of this one-step RT-PCR assay were serum samples obtained from the sheep that have been injected with the DNA vaccine and challenged with a virulent strain of RVFV. The specific sera tested in this experiment were taken on day three post-challenge, since it is from day 2-5 that maximum virus titres

are expected for RVFV. RVF positive field samples obtained from Senegal were tested using the one-step RT-PCR protocol described here (results not shown). All the serum samples obtained from animals participating in the DNA vaccination trial were stored at –70 °C after preparation until used for RNA extraction using Chomczynski's method (Chomczynski, *et al.*, 1987). The one-step RT-PCR reaction was performed using the same conditions as for the sensitivity assay and magnesiumchloride titration previously described. The sheep were bled at day 3 and day 6 after challenge with the virulent viral strain and the serum samples were obtained from blood collected in this way.

3 RESULTS

3.1 Subcloning glycoprotein genes in the pPROEX-HTb bacterial expression vector

The glycoprotein genes were cloned directionally into the multiple cloning site of the pPROEX-HTb bacterial expression vector using the *Nco* I/ *Eco* RI restriction enzymes. Following cloning the gene fragment into the vector digested with these enzymes, the gene product should be expressed as a fusion protein with the six histidine tags at the N-terminal of the protein.

In doing small-scale plasmid DNA isolation of the recombinant plasmids, it was observed that the plasmid DNA yields obtained was very low in comparison to small-scale plasmid DNA isolations done from cultures containing the pPROEX-HTb vector alone. To be able to visualise the plasmid DNA extraction product on an ethidium bromide stained agarose gel, the amount needed to be loaded onto a gel was five times more than the amount needed for other constructs (Fig. 16) (see Chapter 2 as well). Insertion of the gene was verified by restriction digestion of the recombinant plasmid (7.7 kbp) with an enzyme having a unique restriction site in the inserted glycoprotein gene fragment and not in the 4.7 kbp vector (agarose gel not shown).

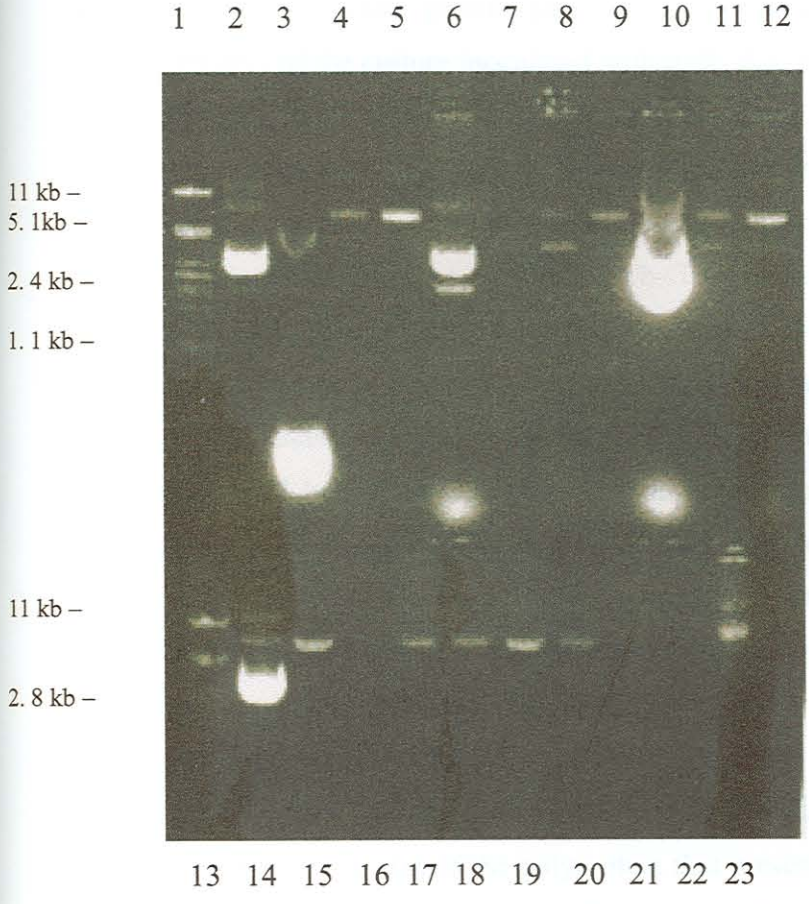


Figure. 16 Comparison of plasmid DNA yields obtained in culture containing recombinant pPROEX-HTb-G2G1 plasmid and the vector plasmid respectively. The first lanes (1 and 13) in the top and bottom row represent the lambda DNA digested with Pst I to serve as molecular weight marker. Next to the marker (lane 2 and 14) was loaded the vector control and in the remaining lanes the transformants picked from the solid medium and cultured overnight in 3 ml LB-broth (3 to 12 and 15 to 23).

3.2 Expression of the glycoprotein genes

After inoculation of the medium with pPROEX-HTb-G2G1 transformants, the cell density had to be between 0.5 and 1 AU at 590nm in order to ensure that the cells are in the logarithmic growth phase before inducing with IPTG. The absorbancy (cell density) of the culture inoculated with pPROEX-HTb-G2G1 increased much slower and reached the desired cell densities much later than the culture inoculated with pPROEX-HTb vector control. The vector control reached an absorbance of 0.5 after 4h30 min. A sample was taken from the vector control (non-induced vector control) after which the IPTG was added. The pPROEX-HTb-G2G1 culture reached an absorbancy of 0.5 after 6h30 min when the expression of the inserted glycoproteins could be induced. A non-induced control sample was taken from this culture before addition of IPTG. Two hours after induction the absorbance value of the test culture have not increased and even when incubated longer, no significant rise in absorbance values could be detected. Samples were taken to analyse for expression of the glycoprotein genes on an SDS-PAGE gel (Fig. 17). Electrophoresis profiles of the expression products on SDS-PAGE gels showed no detectable additional protein products at the expected size locations of the G2 and G1 glycoproteins to the vector background proteins after induction with IPTG. However, at the position of between 105 and 160 kDa, a faint band could be visualised in the lanes that represent the samples collected after induction and incubation of the induced sample overnight. The size of the polyprotein that presents the primary translation product was observed to be 130 kDa. In performing a western blot, it would be possible to detect if the protein represented by this band, is RVFV specific.

In an attempt to generate an alternative antigen for use in an ELISA based diagnostic assay, another inducible bacterial expression system has been used to examine expression of the glycoproteins from the RVFV M segment. The pRSET bacterial expression system (Invitrogen) is a phage-facilitated system that upon infection with the helper phage and induction with IPTG, drives expression of the genes cloned under the control of the inducible promoter present in the plasmid vector. The full-length fragment encoding the G2 and G1 glycoprotein genes as well as a truncated version of the G2 glycoprotein gene were cloned into the vector (Charlotte Ellis, Marie Hamman; Applied Biotechnology, Onderstepoort Veterinary Institute). From the clone containing the full-

length form of the M segment, no expression products could be obtained after induction (results not shown). However, when the truncated form of the G2 glycoprotein was expressed, a clear band at the expected molecular weight of 30 kDa could be detected. At this stage, this expressed antigen is being evaluated as alternative antigen in an ELISA based diagnostic assay against RVF.

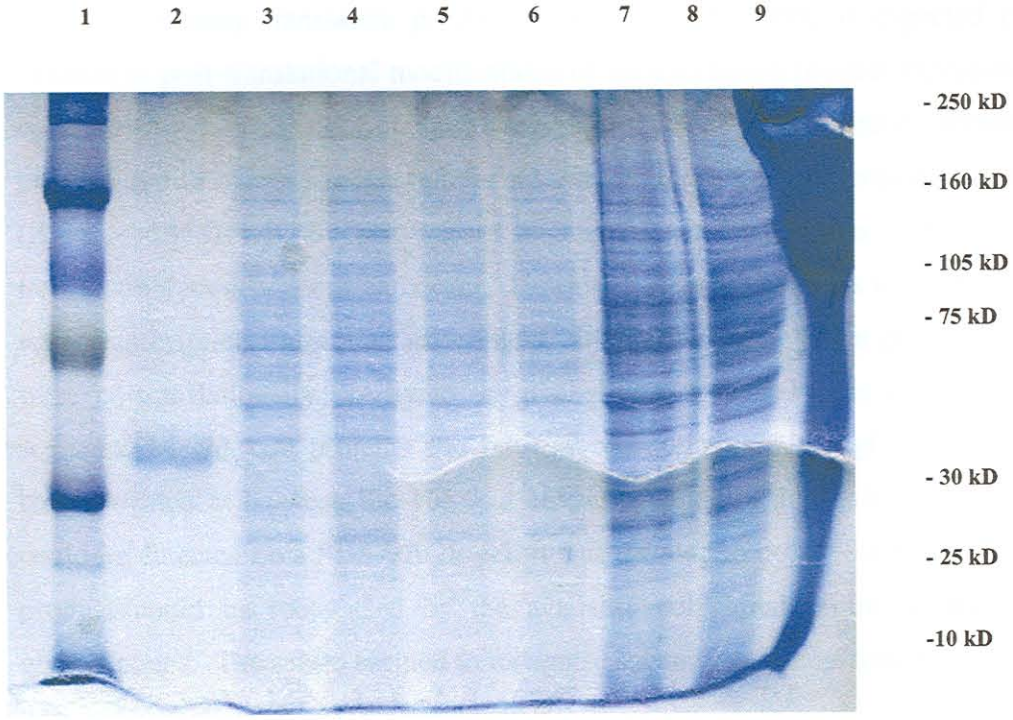


Figure. 17. SDS-PAGE gel with proteins expressed from pPROEX-HTb in *E. coli* liquid culture after induction with IPTG, separated on a 12 % SDS-polyacrylamide gel stained with Coomassie. The pellets were treated with PSB. The left lane (1) represents the molecular marker (kD) (Amersham). In lane 2 were loaded a truncated form of the G2 glycoprotein of RVFV expressed in the pRSET bacterial expression system. To the samples in lanes 3, 5 and 7 were added 15 mM DTT and the samples in lanes 4, 6 and 8 no additional DTT was added in the PSB. In lane 9 was loaded 20 µl of 10^6 pfu RVFV (Smithburn attenuated strain) in 200 µl PBS. Lanes 3 and 4 are the pre-induced culture, lanes 5 and 6 the 1hour post-induction samples and lanes 7 and 8 the overnight post-induction samples.

Sizes of the glycoproteins are respectively 65 and 56 kDa for G1 and G2 (Collet, *et al.*, 1987; Kakach, *et al.*, 1989). Because glycosylation is not expected to take place in the bacterial expression system, the glycan moieties would be absent causing the molecular weight of both proteins to be smaller, 53kDa and 57kDa respectively (Keegan and Collet, 1986), and the electrophoretic mobility of the proteins in a SDS-PAGE gel tends to increase (Kakach, L.T. *et al.*, 1989).

A primary translation product (Kakach, *et al.,* 1989) is expected due to the absence of post-translational modifications of the expression product expressed from the open reading frame (ORF) of the M segment of RVFV in a bacterial system. Such a product of 133 kDa was observed during a study of *in vitro* transcription and translation (Kakach, 1989). It did not appear in a eukaryotic expression system where post-translational modifications can occur. However, when microsomes were present in the *in vitro* transcription-translation experiment, this primary translation product of 133 kDa were co-translationally processed to give all the expected gene products of the M segment (14, 78 kDa proteins and both glycoproteins). Although only the additional bands located at between the 105 and 160 kDa bands in the molecular weight marker could be detected on a SDS-PAGE gel, it was decided to do a western blot to see if any product could be recognised by the available polyclonal serum against the RVFV glycoproteins. The serum seemed to recognise several bands non-specifically (Fig. 18).

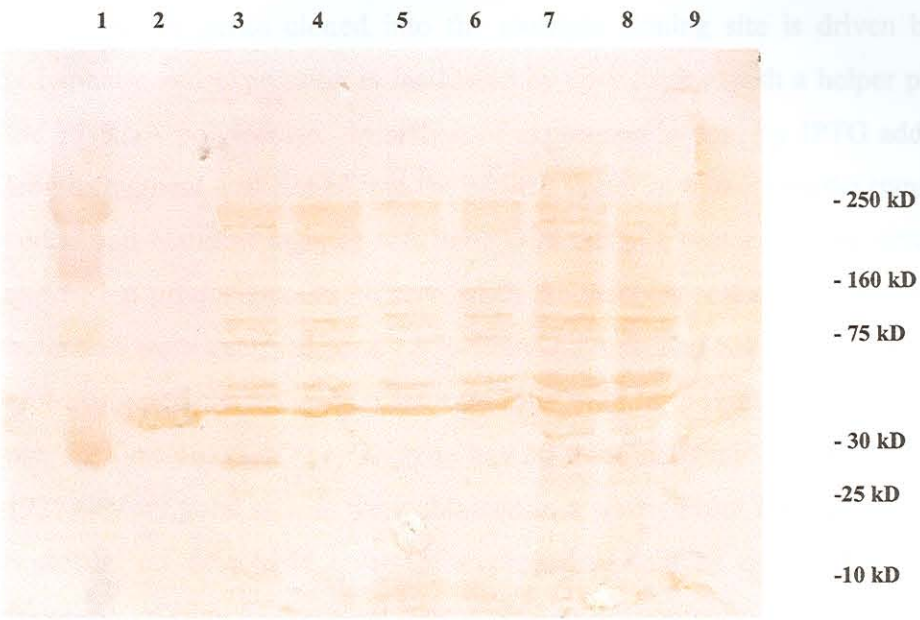


Figure. 18 Western blot of the proteins in pellets of *E. coli* cultures transformed with pPROEX-HTb pre-and post-induction with IPTG and treatment with DTT. The left lane (1) represents the molecular marker (kD) (Amersham). In lane 2 were loaded a truncated form of the G2 glycoprotein of RVFV expressed in the pRSET bacterial expression system (Invitrogen). To the samples in lanes 3, 5, and 7 were added 15 mM DTT and the samples in lanes 4, 6 and 8 no additional DTT was added in the PSB. In lane 9 was loaded 20 µl of 10^6 $TCID_{50}$ RVFV (Smithburn attenuated strain) in 200 µl PBS. Lanes 4 and 5 are the pre-induced culture, lanes 5 and 6 the 1hour post-induction samples and lanes 7 and 8 the overnight post-induction samples. The blot was blocked with 10 % albumine, developed with anti-RVFV serum (1/100) and the Protein G conjugated to peroxidase at a dilution of 1/1000 used as conjugate.

In the pRSET bacterial expression system, the sequence coding for six histidine molecules is 5' of the multiple cloning site in which the gene fragment had been cloned. Expression of genes cloned into the multiple cloning site is driven by the T7 RNA polymerase and expression is facilitated by co-infection with a helper phage expressing the T7 RNA polymerase. Induction of expression is done by IPTG addition. The full-length fragment was cloned into the pRSET vector in frame with the translation initiation codon and histidine tags, as was done with the pCI vector and the pPROEX-HTb. No expression products could be seen when the samples collected after time intervals after induction were analysed on a 12 % Coomassie stained SDS-PAGE gel or on a western blot using polyclonal serum (Charlotte Ellis and Marie Hamman). This encouraged us to use the first 43 % of the G2 gene having three antigenic determinants (Collet, *et al.*, 1987). Very good signals were obtained in a western blot for the truncated form of G2, however, no detectable levels of expression could be obtained with the full-length fragment of the M segment encoding the glycoprotein genes, whether in pRSET (Charlotte Ellis and Marie Hamman, results not shown) or pPROEX-HTb.

3.3 Development of a RT-PCR

The primer pair was found to be specific in annealing to the glycoprotein gene fragment. When using computer software to determine self-complementarity of the primer pair to itself, no self-complementary regions were found. Recognition of the primers to plasmid template at an annealing temperature of 61 °C were successful in giving an amplicon of the correct size of 363 bp (Fig. 19) and in the PCR reaction no non-specific amplification of background bands were observed.

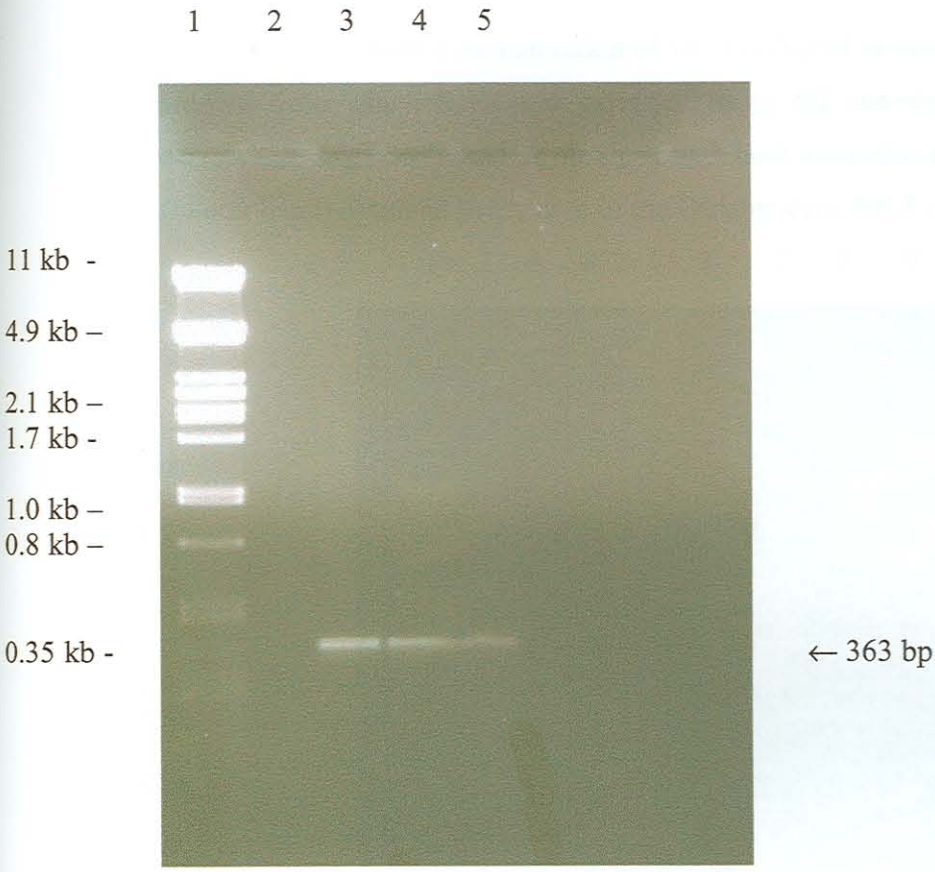


Figure. 19 The PCR amplicons obtained after using plasmid material as template to determine the specificity of the primers for the glycoprotein gene sequence. Lane 1 - molecular weight marker (λ DNA digested with Pst I), lane 2 - water control, lane 3 - pSCRV-6 as template, lane 4 - pCI-G2G1-EGFP as template, lane - 5 1:10 pCI-G2G1-EGFP. Amplicon size is 363 bp. A 1% agarose gel was used and the DNA was vizualised using ethidiumbromide.

Template RNA could be obtained by using extraction methods described by Chomczynski *et al.* (1987) and the Qiagen manufactured QIAmp Viral RNA extraction kit (Fig. 19). It was decided to use the one-step isolation method of Chomczynski for subsequent extraction procedures for economic reasons, availability of reagents, ease of use and quality of the obtained product. The samples used to compare the RNA extraction methods were naïve sheep blood spiked with 100 µl of the Smithburn

attenuated virus with a concentration of 10^6 $TCID_{50}$/ml or virus material only of the same amount from which RNA was extracted using the one-step extraction method. The correct size amplicon could be obtained with both extraction methods, with a less intense (fainter band) obtained in the case of the QIAmp Viral RNA extraction kit.

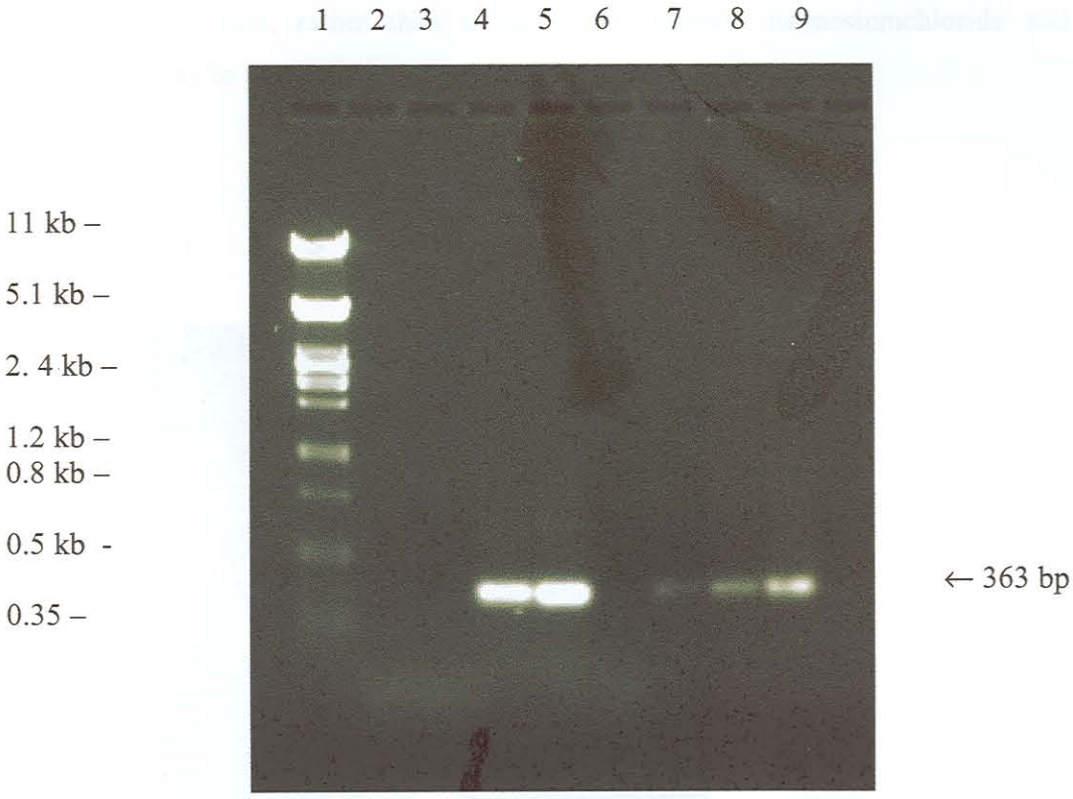


Figure.20 Comparison of extraction methods used to extract RNA from blood artificially spiked with the virus stock and virus stock (Smithburn attenuated virus strain) only. Lane 1-(λ DNA digested with Pst I), lane 2- water control; (lanes 3-5 with method described by Chomczynski, P. *et al.*, (1987) lane 3 - naïve blood; lane 4 - blood spiked with virus stock; lane 5 - virus stock; (lane 6 - 8 QIAmp viral RNA extraction kit) Lane 6 - naïve blood; lane 7 - spiked blood with virus stock; lane 8 – virus stock only (10^6 $TCID_{50}$/ml); lane 9 – plasmid DNA as positive control. Analysis done on a 1% agarose gel, stained using ethidiumbromide.

A titration was done to determine the optimal magnesiumchloride concentration in the RT-PCR. A concentration of 2 mM was found to be to be optimal in the RT-PCR reaction (Fig. 21). Since the concentration of magnesiumchloride present in the supplied enzyme buffer is 2.5 mM it was decided to keep the concentration at 2.5 mM in the reaction volume, rather than using buffer without magnesiumchloride and adding additionally to the reaction volume.

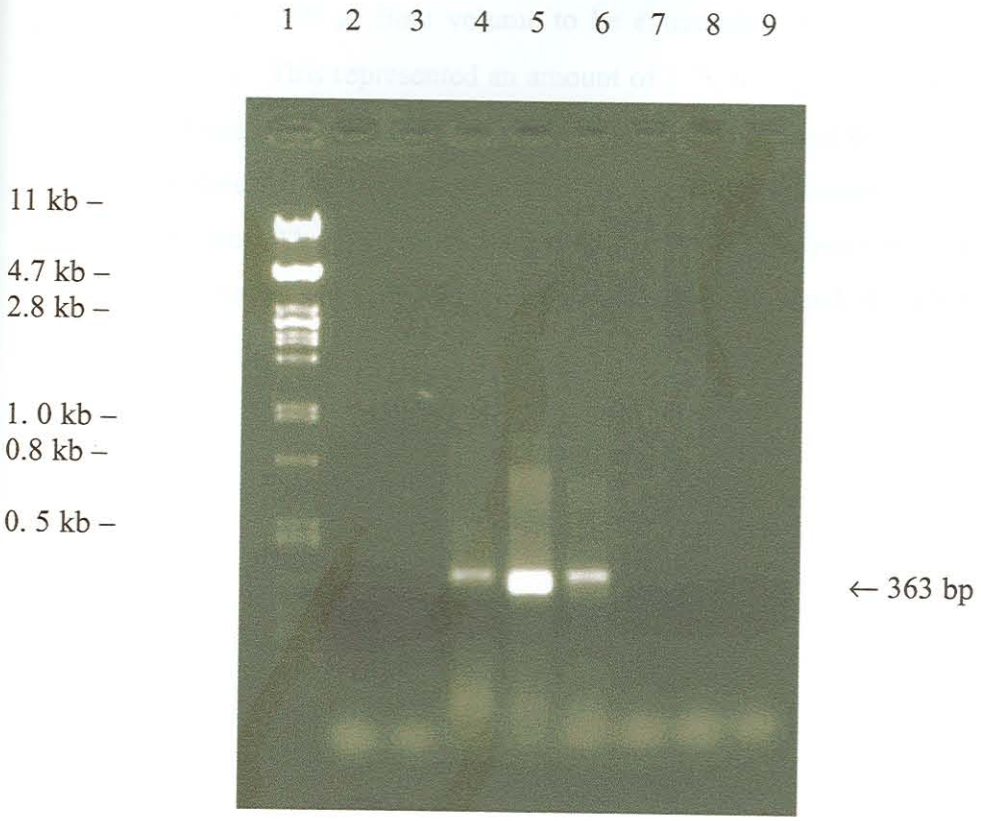


Figure 21 Titrations were done to determine the optimal concentration of magnesiumchloride to be used in the one-step RT-PCR reaction. Lane 1- Lamda DNA digested with Pst I as molecular weight marker. Lanes 2- Water control, 3- 0mM, 4- 1.5mM, 5- 2mM, 6- 2.5mM, 7- 5mM, 8- 7mM, 9- 10mM. Analysis was done on a 1% agarose gel, DNA visualised with ethidiumbromide.

A serial dilution of the Smithburn attenuated virus strain (10^6 $TCID_{50}$/ml) was done from 10^6 $TCID_{50}$ to 1 $TCID_{50}$ in serum and blood. At this stage no amplification product could be obtained from spiked blood. This compares to the results that were obtained in the comparison of extraction methods, where the products obtained were of a low yield when the QIAmp Viral RNA extraction kit was used to extract RNA from blood spiked with the virus. The absence of an amplicon is unclear but might be explained by the presence of a PCR inhibitor like haemoglobin. The faintest band of amplification product could be detected at a dilution of $5x10^{-4}$ (1 µl of the 10^{-3} virus dilution, 200 µl final volume to be extracted; 100 µl virus dilution in PBS + 100 µl serum). This represented an amount of 1 $TCID_{50}$ present in the volume extracted (20 µl volume of extracted material) of which 5 µl was used from the total of 20 µl, that could be detected in the one-step RT-PCR assay. The amount of viral RNA included in the one-step RT-PCR reaction amounts to 0.25 $TCID_{50}$/reaction. This gives an indication of non-viable virus present in the virus sample of which the RNA could still be detected (Fig.22).

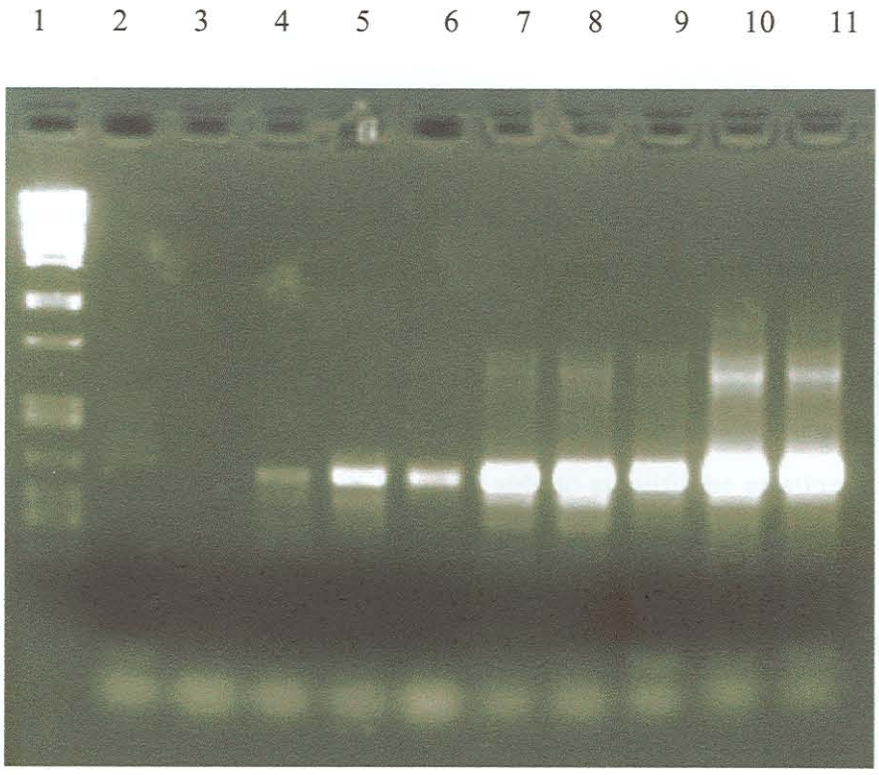


Figure.22 Titration of Smithburn attenuated virus material in serum to determine the relative sensitivity of the RT-PCR. Lane-1, Molecular weight marker; Lane-2, Water; Lane-3, Negative contol; Lane-4, 1/1000 dilution; Lane-5, 1/500 dilution; Lane-6, 1/100 dilution; Lane-7, 1/50 dilution; Lane-8, 1/10 dilution; Lane-9, 1 µl virus material diluted into 100 µl PBS and added to 100 µl serum; Lane-10, 6.25 µl virus material into 100 µl PBS added to 100 µl serum; Lane-11, only virus material (no further dilution into serum). DNA visualised on the 1% agarose gel using ethidiumbromide.

In order to validate the developed one-step RT-PCR procedure, sera were obtained from sheep participating in the DNA vaccination trial (for individual animal data see table 3, chapter 2). Amplicons could be obtained in all animal samples (Fig. 23). The result obtained in this case is an indication that virus template was detected in the vaccinated animals after challenge, indicated the presence of replicating virus. Since the positive controls in this experiment were vaccinated with an attenuated virus, replication of the viruses injected in the challenge dose in these animals is limited and thus much lower. However, the RT-PCR is still able to amplify the existing virus RNA. A further application of the RT-PCR might be the evaluation of a vaccine. To evaluate the DNA vaccine (Chapter 2) as well as other vaccines using the developed RT-PCR protocol, the aim would be to test sera obtained daily after challenge with a virulent strain and using this to monitor the virus clearance from the blood in this way. It would be expected that the vaccine eliciting higher levels of protection would lead to more rapid clearance of the virus from the blood.

These results indicate sufficient quantities of virus replicated in all animals for the RT-PCR to detect, whether vaccinated with the attenuated virus or the DNA vaccine. The intensities varied in some of the bands and this might be due to different levels of virus replication, even though differences in template amounts due to bias in the extraction procedure cannot be ruled out.

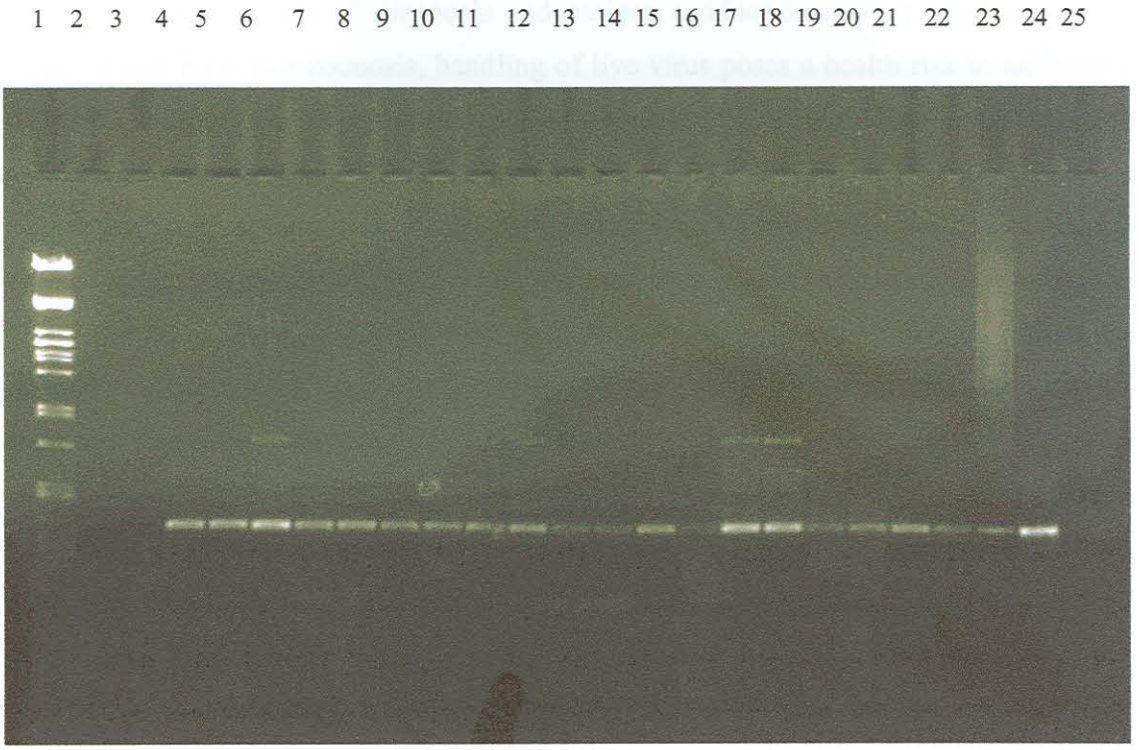


Figure.23 RT-PCR amplicon products obtained from serum samples of animals that participated in the DNA vaccine trial (injection with PCI-G2G1-EGFP) and subsequent challenge with a virulent strain of RVFV. The sera tested and shown in this figure were taken on day 3 post-challenge. The positive control used was viral spiked serum (virus titre of 10^3 $TCID_{50}$), indicated in lane 24. Lane 1 - molecular weight markers (Lambda DNA digested with Pst I), lane 2 the water control, lane 3 - negative serum, lane 4 - 3345, lane 5 - 3670, lane 6 - 3004, lane 7 - 3671, lane 8 - 3373, lane 9 - 3372, lane 10 - 3283, lane 11 - 3762, lane 12 - 3678, lane 13 - 3650, lane 14 - 3383, lane 15 - 3673, lane 16 - 3347, lane 17 - 3313, lane 18 - 3643, lane 19 - 3287, lane 20 - 3010, lane 21 - 3368, lane 22 - 3648, lane 23 - 3764 (Addendum V and VI list the animals that participated in the DNA vaccination trial, additional samples - sheep vaccinated with the "clone 13 " attenuated virus as positive controls).

4 DISCUSSION

Current methods of diagnosis and antigen production require handling of the virus. Since RVF is a zoonosis, handling of live virus poses a health risk to laboratory workers. A substitute for the whole virus as antigen would be recombinant viral proteins or viral protein subunits. The glycoproteins of RVFV is situated at the outside of the virus particle, it is antigenic and immunogenic. Since neutralising antibodies can be elicited against it, the glycoproteins should be useful in an ELISA-based diagnostic assay. The generation of such a recombinant viral protein can be done in different expression systems. These include eg bacterial expression systems, Baculovirus expression system and yeast expression systems.

The main advantage of bacterial expression systems is ease of use. On the other hand, the expressed proteins will not be glycosylated correctly due to the absence of post-translational modifications in prokaryotic cells. It was observed for the cloned genes of RVFV, that solubility of the expressed proteins is a problem due to expression of the proteins in the inclusion bodies found in the prokaryotic cell (Collet, *et al.,* 1987). Solubilising of the proteins using detergents may destroy epitopes on the protein or interfere with the subsequent downstream applications of the antigen.

In an attempt to generate recombinant viral antigen (glycoproteins) the glycoprotein gene fragment was subcloned in the pPROEX-HTb bacterial expression plasmid as well as in the pRSET phage-facilitated bacterial expression system (results not shown). No expression products could be detected after subcloning the RVFV glycoprotein gene fragment into the pPROEX-HTb. The cells were harvested and lysed 3 hours post-induction. Induction was done by adding 0.6 M IPTG. The supernatants (cell lysates) as well as the cell pellets were analysed on a 12% SDS-PAGE gel (Fig. 17). No additional protein bands representing the glycoproteins G2 and G1 were seen when compared to the negative vector control. In a western blot (Fig. 18), antibodies in RVFV-specific polyclonal sheep serum did not bind to proteins of sizes similar to G1 and G2 in either the cell pellet or the supernatant. However, it was observed that the *E. coli* culture transformed with pPROEX-HTb-G2G1 reached a cell density of 0.5 AU at 590 nm two hours after the *E.coli* culture transformed with pROEX-HTb after inoculation at the same

time. In addition, the plasmid DNA yield obtained of pROEX-HTb and pPROEX-HTb-G2G1 differed markedly (Fig. 16). These differences could be caused by an inhibiting effect of the subcloned gene on the replication of the plasmid, leading to lower yields in comparison to the vector alone. The expressed products might have a toxic effect on the cells, explaining the lower growth rate. However, it was seen in cloning the truncated version of the G2 glycoprotein, that the plasmid yield and growth rate of cells transformed with the resultant construct, compares to that of bacterial cells transformed with plasmid only.

Subcloning of the glycoprotein gene fragment into the Baculovirus pFASTBAC helper plasmid and subsequent expression of the gene product could result in a product more soluble (Schmaljohn, *et al.*, 1989) but a lower yield in comparison to the bacterial expression system. Although it is expected that post-translational modifications take place, it is not a given fact for each gene product. The bacterial system was chosen as first option, because it might have been possible that the expressed antigens have the capability to present the epitopes recognized by the RVFV specific antibodies in a way comparable to the natural situation (Keegan, *et al.,* 1986).

Subcloning the glycoprotein gene fragment into the pRSET expression plasmid, also did not result in detectable expression of G2 or G1 (see Chapter 3, results). However, the truncation of the M segment fragment lead to the expression of a truncated form of the G2 glycoprotein containing 43% of the N-terminal part of the G2 protein. Currently this product is being evaluated as recombinant RVFV antigen in an ELISA format assay. In preparation of this antigen, no effect on bacterial growth patterns could be detected as in the case of the full-length cloned fragment. The exact cause and level of influence of the full-length gene fragment or gene products of the full-length fragment in the bacterial system on the growth of the cells and copy number of the plasmid remains to be determined.

The one-step RT-PCR that was developed, evaluated and partially validated is sensitive enough to detect 0.25 $TCID_{50}$ in RVFV positive sera samples from which RNA has to be extracted (200 µl, equal thus to 1 TCID50/ml of infected sample). Maximum viremic titres of the virus observed in adult sheep were $10^{7,6}$ $MIPLD_{50}$/ml (Coetzer, J.A.W., *et al.,* 1994), indicating that diagnosis of the disease using this RT-PCR would be

possible. None of the related *Phleboviruses* causes disease in sheep that might be confused with RVF based on serology (Swanepoel, *et al.,* 1986). Thus in conjunction with serology, the diagnosis of RVF would have a high specificty. Since there was no outbreak in the area, field samples are not available to add to the validation data at this stage. RVFV positive samples obtained from Senegal were available to use in the one-step RT-PCR assay. Of these samples tested, some were found to be positive in the ELISA based diagnostic assay, were found to be negative when using the one-step RT-PCR. This could be explained by the fact that virus RNA was degraded in the samples at the time of RNA extraction since the samples were already repeatedly freeze-thawed. The one-step RT-PCR assay was also performed on the serum samples obtained from the sheep injected with pCI-G2G1-EGFP and challenged with a virulent RVFV. Amplicons of the expected size were obtained in all of the samples at varying intensities. This is an indication of RVFV present in sufficient levels to detect with the RT-PCR, even in vaccinated animals. However, a lower viral load than obtained in the negative control would give an indication of the ability of the vaccine to protect against the virus disease due to the immunological response that is being elicited.

The fully validated and evaluated test can be used as a diagnostic assay. Quantitative tests for quality control of classical vaccine production can be done using this RT-PCR assay. For discrimination between vaccinated and naturally infected animals, it would be necessary to design the primers according to the vaccine being tested for at that specific time. Diagnosis and differentiation would be done at this stage in conjunction with serology.

CHAPTER 4
CONCLUDING REMARKS

DNA vaccines offer numerous advantages over conventional vaccines for RVFV, although the conventional vaccines used to protect animals from the disease can lead to life-long protection (Smithburn, live, attenuated virus), or short-term protection for vaccinated cattle (inactivated, wild-type virus). For DNA vaccines, the advantages include sustained life-long presentation of the antigen in all vaccinees, activation of CD8+ CTL's, no reversion back to virulence, eliminating the use of the infectious agent in the vaccination, ease of production, lower cost, simpler quality control and elimination of the need for a cold chain. Because Rift Valley fever is a zoonotic disease, handling of the virus is not desirable. DNA vaccination offers a strategy that excludes handling of the infectious agent, thus it would be preferred above the currently used live, attenuated Smithburn (although the Smithburn strain in specific poses no real threat to human health) or inactivated vaccines.

It has to be remembered that when the efficiency of a DNA vaccine is evaluated in terms of neutralising antibodies after vaccination, the neutralising anti-RVFV antibodies elicited are monovalent polyclonal antibodies. When using monovalent polyclonal antibodies to detect whole virus, the test results (eg ELISA signal) obtained could be expected to be lower in comparison to antibodies detected when directed against the whole virus (polyvalent polyclonal antibodies). The low neutralising antibody titre could still be representative of a vacccine that confers significant levels of protection.

In this first study of a DNA vaccine for RVF, the construct has been developed and evaluated via a vaccination trial against virulent RVFV challenge. By employing an indirect immunofluorescence assay, it was observed that the RVFV glycoproteins were expressed from the DNA vaccine construct in mammalian cells, although at lower levels than what was expected. Subsequent vaccination of mice led to a delay of death after challenging of the mice with a virulent strain of RVFV. An indication that the target gene of the DNA vaccine was expressed and induced seroconversion in sheep, was obtained by testing the serum of the vaccinated sheep in a virus-neutralising antibody assay. The neutralising antibody levels were low compared to what is expected in a

situation where the live or inactivated vaccines were used. The group that received the vaccine via the intradermal route had the highest virus neutralising antibody titres. However, no appreciable levels of protection were observed. Under circumstances of controlled infection, it has been observed that sheep participating in vaccination trials did not show symptoms of the disease although they were challenged with virulent virus strains and even unvaccinated control sheep developed only mild infection (Swanepoel, *et al.*, 1986). In this study such an observation was also made. Even though the sheep were challenged with a virulent isolate and clinical signs did manifest in the animals, it was not an indication of a severe infection. It is not clear whether the weak performance of the DNA vaccine in the target animal is due to low level of antigen.

The DNA vaccination strategy needs further optimisation in terms of gene modifications (addition of leader signal sequence, truncation of any membrane insertion signals) to optimise expression and secretion of the target antigen to increase the levels of neutralising antibodies and to obtain protection levels against the disease in the target animals. Optimisation procedures also need to include route of administration, immunomodulation and immunostimulation and heterologous prime-boost strategies.

The currently used diagnostic ELISA format involves two assays that enable one to discriminate between early and late infection on the basis of IgM and IgG detection. In these assays, inactivated whole virus is used as the antigen. Binding of the virus to the microtitre plates is poor and therefore a double sandwich system capturing the RVFV specific IgM antibodies is used. A recombinant protein antigen could shorten the time it takes to complete such an assay by eliminating additional steps and eliminate handling and culturing of the virus, making the assay safer and less expensive.

In this study an attempt was made to express the glycoprotein antigen (G2G1) from the subcloned plasmid constructs in a bacterial expression system. Expression in this specific system proved to be unsuccessful in terms of yield when the full-length fragment encoding the glycoproteins from the RVFV M segment was used, but preliminary results using the pRSET phage-facilitated bacterial expression system using a truncated form of the gene is promising (data not relevant for this thesis). Sequences upstream of the translational start site were removed during the cloning of the truncated G2 gene fragment that only represents 43% of G2.

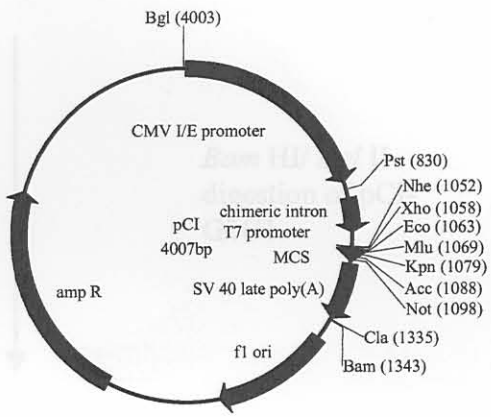
Future objectives regarding expression of the antigen include the use of a Baculovirus expression system or a yeast system i.e. *Pichia pastoris*. A major advantage of these systems is that glycosylation of gene products takes place in contrast to the case in bacteria. Although protein yield would be lower in comparison to what could be obtained in a bacterial system, post-translational modifications could be performed in these systems. Antigens resembling the naturally generated proteins could thus be produced.

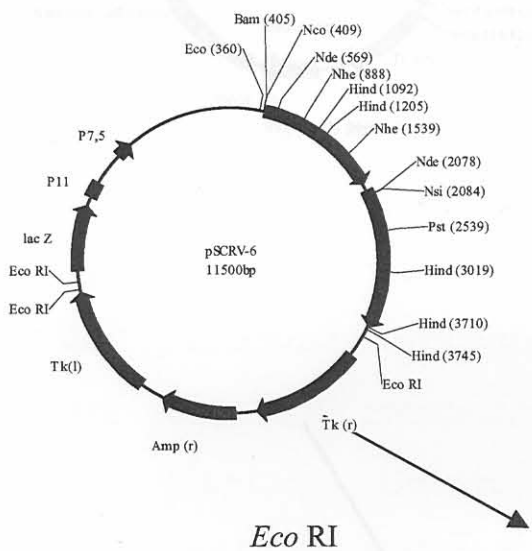
Currently used diagnostic assays detect either the antigen or the antigen specific antibodies. Again, because RVF is a zoonosis, it is not desirable to produce antigen (virus) for use in these assays and virus culturing is time consuming and expensive. Detection of early infection is possible with the ELISA assay detecting the IgM antibodies, but in some situations, it is not sensitive enough, since false negative results are obtained. A molecular based diagnostic tool such as a RT-PCR to detect the virus RNA genome can eliminate virus culturing and handling. Such an assay can be optimised to increase sensitivity. The RT-PCR assay that was developed in this study was shown to detect virus RNA in the serum samples from animals that participated in the DNA vaccine trial. Virus RNA could also be detected in blood spiked with virus. The level of sensitivity of the RT-PCR assay equals the detection of 0,25 $TCID_{50}$ in the reaction after extraction of RNA, indicating the ability to detect non-viable virus particles as well. This corresponds to a sensitivity level of 1 $TCID_{50}$/ml in the sample material to be evaluated. The RT-PCR has to be validated with respect to specificity (inclusion of unrelated but similar viruses as well as related viruses). A fully optimised, validated and evaluated RT-PCR assay can result in establishing a less time consuming and more user-friendly molecular diagnostic assay. Such a RT-PCR could furthermore be used as a tool in the production of RVFV vaccines since it could quantify the viral amounts and viral load. A PCR-based assay to discriminate between vaccinated and non-vaccinated individuals would be useful in monitoring outbreaks and would facilitate in the epidemiological study of the disease.

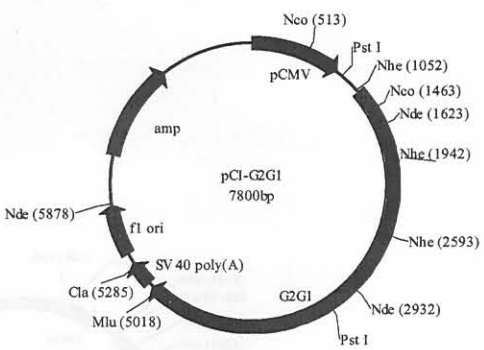
ADDENDUM I

Eco RI digestion of the pCI mammalian expression vector.

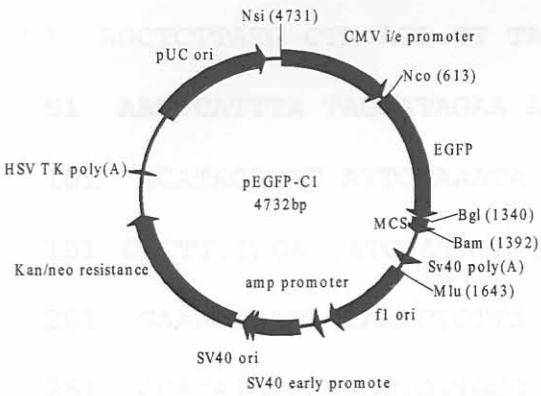
Eco RI excision of the 3.8 bp G2G1 gene fragment from the pSCRV-6 construct.







Bam HI/ *Bgl* II digestion of pCI-G2G1.



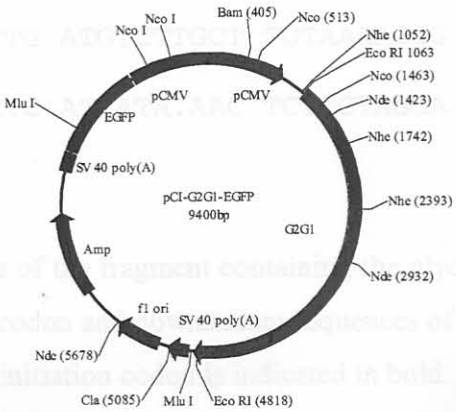
Bam HI/*Bgl* II digestion of pEGFP-C1 to remove MCS.

Religation

Nsi I/*Mlu* I digestion to excise the EGFP gene fragment

Subcloning of the EGFP gene fragment into pMTL 24 *Pst* I/*Mlu* I site

Bam HI digestion of pMTL24-EGFP



ADDENDUM II

```
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check: 875  from: 1  to: 710

 <rift2.00001------->

rift2_9.seq
Length: 710

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51  AATTCATTTA TAGCATAGAA AAAAACAAAA TGAAATTCTA CTATATTTTT

101  ACATACATAT ATTCTAAATA TGAAAGTGGT GATTGTGACT AGCGTAGCAT

151 CGCTT.TAGA CATCTATATA CTATATAGTA ATACCAATAC TCAAGACTAC

201  GAAACTGATA CAATCTCTTA TCATGTGGGT AATGTTCTCG ATGTCGATAG

251  CCATATGCCC GGT.GTTGCG ATATACATAA ACTGATCACT AATTCCAAAC

301  CCACCCGCTT TTTATAGTAA GTTTTTCACC CATAAATAAT AAATACAATA

351  ATTAATTTCT CGTAAAAGTA GAAAATATAT TCTAATTTAT TGCACGGTAA

401  GGAAGTAGAA TCATAAAGAA CAG.GACGGA TCCCCCATGG CAGGGATTGC

451  AATGACAGTC CTTCCAGCCT TA.CAGTTTT TGCTTTGGCA CCTGTTGTTT

501  TTGCTGAAGA CCCCCATCTC AGAAACAGAC CAGGGAAGGG GCACAACTAC

551  ATTGAC.GGA TGACTCATGA GGATGCCACA TGCAAACCTG TGACATATGC

601  TGGGGCATGT AGCAGTTTTG ATGTCTTGCT TGTAAAAGGG AAAATTTCCC

651  C.TTTTCCAG .CGTATGCTC AT.ATA.AAC TCTACTAGGA GGCAGGTTTA

701  CGACACCATT
```

DNA sequence of the insertion site of the fragment containing the glycoprotein genes of the RVFV as well as the initiation codon and downstream sequences of the G2 gene. The *Eco* RI insertion site as well as the initiation codon is indicated in bold. The insertion site was confirmed to be correct.

ADDENDUM III

```
>ref|NC_002044.1| Rift Valley fever virus M segment, complete sequence
           Length = 3884

 Score =  400 bits (202), Expect = e-109
 Identities = 264/274 (96%), Gaps = 8/274 (2%)
 Strand = Plus / Plus


429 ccatggcagggattgcaatgacagtccttccagcctta-cagtttttgctttggcacctg 487
    |||||||||||||||||||||||||||||||||||||| |||||||||||||||||||||
409 ccatggcagggattgcaatgacagtccttccagccttagcagtttttgctttggcacctg 468


488 ttgtttttgctgaagacccccatctcagaaacagaccagggaaggggcacaactacattg 547
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
469 ttgtttttgctgaagacccccatctcagaaacagaccagggaaggggcacaactacattg 528


548 ac-ggatgactcatgaggatgccacatgcaaacctgtgacatatgctggggcatgtagca 606
    || |||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
529 acgggatgactcaggaggatgccacatgcaaacctgtgacatatgctggggcatgtagca 588


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    |||||||||||||||||| |||||||||||||||||||||||| |||||||||||  ||
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665 a-aactctactaggaggcaggtttacgacaccat 697
    | |||||||||| |||||| ||| ||||||||||
648 agaactctacta-gaggca-gttcacgacaccat 679
```

Alignment of the sequences obtained with the RVFV confirming the presence of the RVFV glycoprotein genes in the fragment cloned from nucleotide 429, representing the initiation codon starting position 411 in the M segment. The initiation codon was found to be intact and in frame.

ADDENDUM IV

RVFV specific ELISA titres as OD values of a 1:10 dilution of mouse sera after vaccination of mice with a DNA construct containing the glycoprotein genes.

Pre-boost (week 3)		**Pre-challenge (week 9)**	
Sample	**OD**	**Sample**	**OD**
pCI 1	-0,082	pCI 1	-0,04
pCI 2	-0,114	pCI 2	0,033
pCI-G2G1-EGFP 1	-0,061	pCI-G2G1-EGFP 1	0,007
pCI-G2G1-EGFP 2	-0,047	pCI-G2G1-EGFP 2	0,012
pCI-G2G1-EGFP 3	0,043	pCI-G2G1-EGFP 3	0,014
		+ ve 1	-0,024
		+ ve 2	-0,078
		- ve 1	-0.012
		- ve 2	0,006
OD = (OD pos Ag)– (OD Neg Ag)			

ADDENDUM V

Temperature values taken daily for one week after challenge of the experimental animals with a virulent field strain of Rift Valley fever virus.

Days post challenge

DNA i.m.	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
3283	40.5	41.4	39.2	39	39.2	39.8	39.2	39.4	38.9
3345	39.8	41.6	39.6	40.2	39.8	39.4	39.4	39	39.4
3287	40	40.7	39.5	39.3	38.9	39.2	39.1	39.2	39
Avg	**40.1**	**41.2**	**39.4**	**39.5**	**39.3**	**39.5**	**39.2**	**39.2**	**39.1**
Sd	0.36	0.47	0.21	0.62	0.46	0.31	0.15	0.20	0.26
DNA with Quil A	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
3383	40.3	40.2	39.3	39.9	39.1	39.5	39.5	40	39.4
3368	40	39.7	39.4	41.3	39.4	39.5	39.4	39.3	39.1
3372	40.6	42	39.5	39.3	39.8	38.9	39.4	39.5	39
Avg	**40.3**	**40.6**	**39.4**	**40.2**	**39.4**	**39.3**	**39.4**	**39.6**	**39.2**
Sd	0.30	1.21	0.10	1.03	0.35	0.35	0.06	0.36	0.21
DNA i.d.	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
3313	39.3	39.8	39.7	39.2	38.7	39.2	39.6	39	39.5
3347	39.5	41.1	40	39.3	39.3	39.2	41.2	39.5	39
3373	40.3	40.6	40.1	41.5	39.4	40.1	39.6	39.5	39.5
Avg	**39.7**	**40.5**	**39.9**	**40.0**	**39.1**	**39.5**	**40.1**	**39.3**	**39.3**
Sd	0.53	0.66	0.21	1.30	0.38	0.52	0.92	0.29	0.29
Negative controls	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
3004-Control	41.5	41.2	39.9	39.8	39.6	39.8	39.5	39.9	39.3
3670-Control	40.7	40.6	39.9	39.3	39.4	39.4	39.3	39.4	39.6
Avg	**41.1**	**40.9**	**39.9**	**39.55**	**39.5**	**39.6**	**39.4**	**39.65**	**39.45**
Sd	0.57	0.42	0.00	0.35	0.14	0.28	0.14	0.35	0.21

ADDENDUM VI

Liver enzyme levels of trial animals after inoculation with the pCI-G2G1-EGFP DNA vaccine construct and challenge with a virulent Rift Valley fever virus strain.

DNA i.m.	Basal			Day 2			Day 3			Day 6		
	AST	GGT	GLDH	AST	GGT	GLDH	AST	GGT	GLDH	AST	GGT	GLDH
3283	47	51	19	58	40	7	70	69	51	52	37	12
3345	59	57	22	89	54	25	120	55	65	110	55	52
3287	44	60	40	45	54	7	56	39	24	40	51	3
Avg	**50**	**56**	**27**	**64**	**49**	**13**	**82**	**54**	**47**	**67**	**48**	**22**

DNA with Quil A	Basal			Day 2			Day 3			Day 6		
	AST	GGT	GLDH	AST	GGT	GLDH	AST	GGT	GLDH	AST	GGT	GLDH
3383	53	40	3	50	48	7	52	41	5	49	48	3
3368	46	64	34	40	51	27	31	45	8	179	72	271
3372	60	67	28	50	47	5	60	34	5	69	48	19
Avg	**53**	**57**	**22**	**47**	**49**	**13**	**48**	**40**	**6**	**99**	**56**	**98**

DNA i.d.	Basal			Day 2			Day 3			Day 6		
	AST	GGT	GLDH	AST	GGT	GLDH	AST	GGT	GLDH	AST	GGT	GLDH
3347	75	63	28	79	47	20	146	47	91	183	78	67
3373	48	68	5	72	59	11	79	49	13	145	59	21
3313	60	84	30	50	67	7	49	56	4	88	66	23
Avg	**61**	**72**	**21**	**67**	**58**	**13**	**91**	**51**	**36**	**139**	**68**	**37**

Negative g	Basal			Day 2			Day 3			Day 6		
	AST	GGT	GLDH	AST	GGT	GLDH	AST	GGT	GLDH	AST	GGT	GLDH
3010	51	49	35	52	39	10	52	37	13	58	45	29
3670	42	62	17	48	54	9	47	41	15	68	53	13
Avg	**47**	**56**	**26**	**50**	**47**	**10**	**50**	**39**	**14**	**63**	**49**	**21**

Positive	Basal			Day 2			Day 3			Day 6		
	AST	GGT	GLDH	AST	GGT	GLDH	AST	GGT	GLDH	AST	GGT	GLDH
3643	182	147	255	130	117	63	148	123	67	258	218	149
3648	66	69	47	51	59	9	54	42	11	67	63	12
3650	52	63	34	45	58	3	39	50	2	40	58	12
Avg	**100**	**93**	**112**	**75**	**78**	**25**	**80**	**71**	**26**	**121**	**113**	**57**

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PUBLICATIONS

Paper (in preparation):

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Development of a diagnostic one-step RT-PCR assay for the detection of Rift Valley fever in infected animals.