A study of the host-restricted lumpy skin disease virus as a vaccine vector using rabies virus as a model

Kate Aspden

Thesis presented for the degree of Doctor of Philosophy in the Division of Medical Virology, Department of Clinical Laboratory Science, Faculty of Health Sciences, University of Cape Town

February 2002

TABLE OF CONTENTS

ABSTRACT

Lumpy skin disease virus (LSDV) is a capripoxvirus that only replicates in ruminants. This means that transmission to non-ruminants is impossible and makes LSDV a safer alternative for use as a vaccine vector in non-ruminants than replication competent poxviruses. Furthermore, the large poxviral genome can accommodate the insertion of foreign DNA, thus making LSDV a suitable candidate vector for recombinant vaccines.

Rabies virus (RV) readily infects cattle and causes a fatal neurological disease. A stable vaccine, which does not require the maintenance of a cold chain and that is administered once to elicit lifelong immunity to rabies would be advantageous. The present study describes the construction of a live recombinant LSDV vaccine, expressing the RV glycoprotein gene (RG) under the control of a fowlpoxvirus early/late promoter (rLSDV-RG). The Neethling cattle vaccine strain of LSDV was used because of its safety in ruminants. This recombinant was used as a model to evaluate LSDV as both a replicating (in cattle) and non-replicating (in mice and rabbits) vaccine vector. Cattle inoculated with rLSDV-RG developed humoral immunity that was demonstrated in enzyme linked immunosorbent assays (ELISA) and neutralisation assays to RV. High titres of up to 1513 IU/ml of RV neutralising antibodies were induced. Furthermore, primed peripheral blood mononuclear cells displayed the ability to proliferate in response to stimulation with inactivated RV.

Promoter studies and DNA staining indicated that early and late viral gene expression occurs in ruminant and non-ruminant cells and that both types of cells support viral DNA replication in the cytoplasm of infected cells. Subsequently, it was found that the insertion of the RG gene into the ribonucleotide reductase gene of the LSDV genome did not alter the virus's ability to replicate, and that the rLSDV-RG replicated to maturity in both the bovine cell line (MBDK) and the primary ovine cells (LT) but not in the primate cell line, CV-1.

rLSDV-RG was assessed for its ability to generate both humoral and cellular immune responses against RG in rabbits and mice. In addition it was compared with two commercial rabies vaccines (Verorab and Rabisin, Pasteur Merieux, using the same prime/boost immunisation protocol). Rabbits inoculated intramuscularly with the rLSDV-RG produced RV neutralising antibodies as determined by ELISA (9 ± 2 IU/ml) and neutralisation assays (1.35 IU/ml). These antibody titres were comparable with titres elicited following Verorab immunisation and higher than those induced following Rabisin vaccination. BALB/c mice immunised with rLSDV-RG elicited comparable levels of RV-specific cellular immunity to that of mice immunised with Verorab and higher levels than those induced by Rabisin, as shown by T-cell proliferation to inactivated RV and intracellular cytokine staining. Compared with Verorab vaccination, rLSDV-RG vaccination was found to provide strong protection against an intracranial rabies virus challenge.

This study therefore indicates that lumpy skin disease virus could be used as a host-restricted vector in both ruminant and non-ruminant hosts for the production of live recombinant vaccines. In addition, rLSDV-RG shows potential to be used in ruminants as a cost-effective vaccine against both lumpy skin disease (LSD) and rabies.

ACKNOWLEDGEMENTS

1. I would like to thank my supervisor, Associate Professor Anna-Lise Williamson for all her patience, inspiration and assistance during the course of the project.
2. Thanks to all the people who made this project possible:
    - Noel Tordo from Pasteur Institute (Paris) for providing the RG gene
    - David Boyle from CSIRO (Geelong) for providing the plasmid pAF09
    - Anna-Lise Williamson from our department for providing the plasmid pLSDrr
    - State Vaccine Institute (Cape Town) for the use of their facilities during the rabies virus challenge experiment
    - Onderstepoort Biological Products (Pretoria) for providing the cattle, the horses and the LSDV (Neethling strain) vaccine
    - Albie van Dijk from Onderstepoort division of Biochemistry for all work in immunising cattle and horses as well as collecting samples
    - John Bingham from Onderstepoort rabies unit for the fluorescine labelled-nucleoprotein and rabies challenge virus strain
    - We appreciate the assistance given by the State Veterinary Surgeon, Dr Gary Burman, at Elsenburg (Western Cape, South Africa) in providing the control cattle sera.
    - Fritz Tiedt from our department for assistance with electron microscopic work
    - Jo-Ann Passmore from our department for immunology training
    - Vincent Sharp, Rodney Lucas, Noel Markgraaf and Hiram Arendse for assistance with animal work

- Nikki Johnston for diagram reproduction and moral support.
- James Maclean, Robin Thomas, Maureen Dennehy. Jo Van Harmelen and all the staff and students who supported an encouraged me along the way.
- Thanks to the National Research Foundation for funding the project and to Poliomyelitis Research Foundation, DR McIntosh Trust fund and National Research Foundation for bursaries. Margaret McFarlain for the scholarship that allowed me to attend the Business Planning course at the Graduate School of Business.

3. I'd like to extend a special thank-you to my husband, Scott Aspden, and my parents, Rod and Mary Turner-Smith for all the love and support you have given me over the years.

LIST OF ABBREVIATIONS

Bp	base pairs
β-gal	beta galactosidase
°C	degrees centigrade
CAM	chorioalantoic membrane
CDC	Centre for Disease Control
CEF	chick embryo fibroblast cells
CEV	cellular associate enveloped virions
cm	centimetres
CMI	cell mediated immunity
CPE	cytopathic effects
CVS	challenge virus standard
DMEM	Dulbeccos modified eagles medium
DNA	deoxyribonucleic acid
Dept	Department
ECOGPT	*E.coli gpt* gene
EEC	European Economic Community
EEV	extracellular enveloped virions
ELISA	enzyme linked immunosorbent assay
ER	endoplasmic reticulum
FCS	foetal calf serum
Fig	figure
ffu	focus forming units
FITC	fluorescein-isothiocyanate
g	grams
g	gravitational force
GFP	green fluorescent protein
GMP	guanylic acid
h	hours
HGPRT	hypoxanthine-guanine phosphoribosyl transferase
IC	intracranial/intracerebral
ID	intradermal
IEV	intracellular enveloped virions
IFN-γ	interferon gamma
IM	intramuscular
IMP	inosinate dehydrogenase
IMV	intracellular mature virions
IP	intraperitoneal
IU	international units
Kb	kilobases
Kda	kilodaltons
LD_{50}	50% lethal dose
LT	lamb testes
LSD	lumpy skin disease
LSDV	lumpy skin disease virus
LSDV-wt	lumpy skin disease virus – wild type (Neethling vaccine strain)
M	Molarity
Mag	magnification
mRNA	messenger RNA
μCi	micro curie
μg	micrograms

µl	microlitres
mM	millimoles
ml	millilitre
MVA	modified vaccinia Ankara
MPA	mycophenolic acid
N	normal (molarity)
NIH	National Institute of Health
nm	nanometers
OD	optical density
O.I.E.	Office des International Epizootics
ORF	open reading frame
PBMC	peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	polymerase chain reaction
pfu	plaque forming units
PS	penicillin streptomycin
PSF	penicillin streptomycin fungizone
RG	rabies virus glycoprotein
rLSDV-RG	recombinant lumpy skin disease virus expressing rabies virus glycoprotein gene
RNA	ribonucleic acid
RNP	ribonucleoprotein
rpm	revolutions per minute
RRV	recombinant rabies virus
RV	rabies virus
SC	subcutaneous
SD	standard deviation
SDS	sodium dodecyl sulphate
$TCID_{50}$	50% tissue culture infectious dose
TK	thymidine kinase
UCT	University of Cape Town
USA	United States of America
UK	United Kingdom
UV	ultra violet light
V	volts
VSV	vesicular stomatitis virus
VV	vaccinia virus
VV-RG	vaccinia virus – rabies glycoprotein recombinant
WHO	World Health Organisation
WR	Western Reserve
XGPRT	xanthine-guanine phosphoribosyl transferase
XMP	xanthosine monophosphate
YT	yeast tryptone

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1:

LITERATURE REVIEW

1.1. RABIES

1.1.1. Introduction

Rabies is a severe and fatal disease of the central nervous system (encephalomyelitis) of warm-blooded vertebrates, including man. Early symptoms of rabies in humans consist of fever, headache and malaise. As the disease progresses, neurological symptoms appear (Figure 1.1) that include insomnia, anxiety, confusion, paralysis, excitation, hallucinations, agitation, hypersalivation, difficulty swallowing and hydrophobia. Death usually occurs within days of the onset of symptoms (Dept of Health, 1996).

Figure 1.1. A dog showing advanced signs of rabies characterised by paralysis (http://.www.adam.com).

1.1.1.1. Rabies in southern Africa

The first confirmed outbreak of rabies in Africa, which resulted from the importation of an infected dog from England, occurred in 1892 in the eastern Cape Province (now Western Cape) of South Africa. The outbreak was controlled two years later (Hutcheon, 1894; cited by Swanepoel *et al.,* 1993). An epidemic of unconfirmed rabies in dogs was reported in Northern Rhodesia (now western Zambia) in 1901. During the following years the disease spread along the major trade routes and resulted in an outbreak in Southern Rhodesia (now Zimbabwe), which took twelve years to control (Edmonds, 1922; cited by Swanepoel *et al,* 1993). Rabies in viverrids (mongooses and genets) was detected in South Africa in 1928 and since then the disease has continued to occur predominantly on the plateau with spillover infections in cattle and other animals; the Yellow Mongoose (*Cynictis penicillata)* being the principle host of the virus (Swanepoel, 1996). From 1947, dog rabies spread from Northern Rhodesia (now Southern Zambia) into South West Africa (now Namibia), across northern and eastern Botswana into Zimbabwe and northern Transvaal (now Mpumalanga) by 1950, entered Moçambique (now Mozambique) in 1952, and spread into Swaziland in 1954. Dog rabies extended from southern Mozambique into Natal (now Kwazulu-Natal) in 1961 to cause a major epidemic that was finally brought under control in1968. The disease once again entered northern Kwazulu-Natal from Mozambique in 1976 and since then, dog rabies has been rife in the peri-urban settlements in the area. The disease spread to Lesotho in 1982 and into the Transkei in 1987. By 1990 it had reached Ciskei. The spread of the disease in dogs was followed by the emergence of rabies in jackals and cattle in central Namibia, northern Botswana, Zimbabwe and Mpumalanga. Furthermore, an unusual outbreak of rabies in kudu antelope occurred in central Namibia from 1977 to 1985, involving oral spread of the virus between individuals (cited by Swanepoel *et al.*, 1993). At present 600 to 700 rabies cases are being diagnosed each year in domestic and wild animals in South Africa. Table 1.1 shows most of the confirmed southern African rabies cases. The geographical distribution of the most important rabies virus vectors

is shown in Figure 1.2. Dogs, cattle and the yellow mongoose collectively constitute 85% of all animals in which the disease is confirmed. The 300-400 cases confirmed annually in dogs constitute approximately 90% of all cases of the disease diagnosed in KwaZulu-Natal, and 80% of all cases of the disease diagnosed in South Africa (Dept of Health, 1996).

Table 1.1. Confirmed cases of infection with rabies or rabies-related viruses in southern Africa (taken from Swanepoel, 1996).

VICTIMS		SOUTH AFRICA 1928-91	NAMIBIA 1938-91	BOTSWANA 1938-91	ZIMBABWE 1950-91
DOMESTIC ANIMALS					
Dogs		3322	611	611	3851
Cats		437	66	12	138
Cattle		2211	1395	706	1426
Goats		60	72	184	73
Horses and donkeys		53	25	30	73
Pigs		25	2	-	17
Water buffalo		-	1	-	-
Guinea pigs		1	-	-	-
Total domestic animals		6228	2228	1550	5658
WILD ANIMALS					
Yellow mongoose	*Cynictis penicillata*	2034	-	-	-
Unspecified mongooses		1272[a]	16	15	54[b]
Slender mongoose	*Galerella sanguinea*	16	1	-	39
Small grey mongoose	*G. purverulenta*	38	-	-	-
Large grey mongoose	*Herpestes ichneumon*	-	-	-	2
Banded mongoose	*Mungos mungo*	3	1	-	-
Water mongoose	*Atilax paludinosus*	11	-	-	1
Selous' mongoose	*Paracynictis selousi*	1	-	-	-
Dwarf mongoose	*Helogale parvula*	1	-	-	-
White-tailed mongoose	*Ichneumia albicauda*	2	-	-	4
Suricate	*Suricata suricatta*	112	4	-	-
Civet	*Civettictis civetta*	3	-	-	22
Small spotted genet	*Genetta genetta*	167	23	8	1
Honey badger	*Mellivora capensis*	18	24	5	38
Striped polecat	*Ictonyx striatus*	66[c]	2	-	5
Striped weasel	*Poecilogale albinucha*	1	-	-	-
Unspecified otter species		1	-	-	-
Lion	*Panthera leo*	-	4	-	-
Leopard	*P. pardus*	-	2	1	2
Cheetah	*Acinonyx jubatus*	-	4	-	-

Table 1.1. continued.

African wildcat	*Felis lybica*	13	15	-	3
Caracal	*F. caracal*	14	4	-	-
Serval	*F. serval*	-	-	1	2
Small-spotted cat	*F. nigripes*	3	-	-	-
Unspecified felids		168	46	1	-
Black-backed jackal	*Canis mesomelas*	206[d]	333	171	1449[e]
Side-striped jackal	*C. adustus*	-	-	-	166
Bat-eared fox	*Otocyon megalotis*	263	66	-	2
Wild dog	*Lycaon pictus*	-	1	-	3f
Cape fox	*Vulpes chama*	7	5	-	-
Aardwolf	*Proteles cristatus*	22	14	-	6
Brown hyaena	*Hyaena brunnea*	1	2	-	-
Spotted hyaena	*Crocuta crocuta*	1	-	4	5
Unspecified carnivores		-	-	20	-
Porcupine	*Hystrix africaeaustralis*	-	1	-	-
Ground squirrel	*Xerus inauris*	32	1	-	-
Greater canerat	*Thryonomys swinderianus*	2	-	-	-
Antbear	*Orycteropus afer*	-	-	-	-
Cape hyrax	*Procavia capensis*	8	-	-	-
Chacma baboon	*Papio ursinus*	1	-	-	-
Vervet monkey	*Cercopithecus aethiops*	-	1	-	2
Lesser bushbaby	*Galago moholi*	-	1	-	-
Warthog	*Phacochoerus aethiopicus*	1	-	-	-
Duiker	*Sylvicapra grimmia*	1 7	9	2	6
Steenbok	*Raphicerus campestris*	4	1	-	-
Kudu	*Tragelaphus strepsiceros*	3	385	-	2
Eland	*Taurotragus oryx*	-	17	-	3
Red hartebeest	*Alcelaphus buselaphus*	-	1	-	-
Blesbok	*Damaliscus dorcas phillipsi*	1	-	-	-
Reedbuck	*Redunca arundinum*	1	-	-	1
Springbok	*Antidorcus marsupialis*	3	1	-	-
Burchell's zebra	*Equus burchelli*	1	-	-	-
Unspecified herbivores		6	-	38	-
Epauletted fruit bat	*Epomophorus wahlbergi*	14[g]	-	-	-
Slit-faced bat	*Nycteris thebaica*	1	-	-	1
Long-fingered bat	*Miniopteris schreibersii*	1[h]	-	-	-
Scrub hare	*Lepus saxatilis*	1	-	-	-
Unspecified/unidentified		33	2	19	-
Total wild animals		4575	987	285	1825
Total		10803	3215	1835	7483
HUMANS		282	12	32	158

a Believed to be mainly *C. penicillata*

b Believed to be mainly *G. sanguinea*

c Probably includes some *P. albinucha*

d Possibly includes a few *C. adustus*

e Probably includes many *C. adustus*

f Captive animal/s

g Only two bats positively identified as *E. wahlbergi*

h Identification of species based on circumstantial evidence

Figure 1.2. The geographical distribution of the most important rabies vectors in South Africa (www.nbi-kzn.org.za/interest/fig1.htm). The area where the black-backed jackal (shaded in green) is the principal vector is in the northern regions of South Africa. The feral dog is responsible for the majority of infections in KwaZulu-Natal (shaded in red). In the central region of South Africa the yellow mongoose is the principle vector (shaded in yellow), while in the Western and Eastern Provinces the Bat-eared Fox is the main vector (striped shading).

1.1.1.2. Rabies epidemiology

Throughout the world, the incidence and epidemiology of human rabies is closely paralleled with the incidences of the disease in domestic animals (Baer *et al.*, 1996). It is of epidemiological importance that recent monoclonal antibody and nucleic acid studies have shown that strains of rabies virus that circulate in particular host species within given geographic regions, tend to undergo genetic adaptation, resulting in different biotypes. This results in changes in antigenicity and pathogenicity (Smith *et al.*, 1991). The biotypes seem to be adapted to circulate in specific animals, so that within an affected area the disease is present predominantly in a single host species. This host species appears to maintain the spread of the virus and disease in other animals represents spillover of infection resulting from contact with the major host species (Baer *et al.*, 1996). Infection with a lyssavirus that originated within a different reservoir population will generally lead to a fatal self-limiting rabies-like infection (or spillover), as in the case of humans, and only occasionally to a new stable enzootic infection (Blancou *et al.*, 1983,1991). A recent example of such a spillover was reported in Queensland, Australia, where a woman bitten by a flying fox (fruit bat) died after being diagnosed with Australian bat lyssavirus (Hanna *et al.*, 2000).

Countries that have been declared free of rabies are usually islands or peninsulas. They include Great Britain, Ireland, Iceland, Sweden, Norway, Denmark, Portugal, Spain, Gibraltar, Malta, Albania, Cyprus, Bahrain, Oman, Qatar, United Arab Emirates, Hong Kong, the Malaysian peninsula, Singapore, certain Indonesian and Philippine islands, Republic of Korea, Japan, Australia, New Zealand, Fiji, Hawaii, Libya, Cape Verde, Sao Tome, Comorres, Mauritius and Antarctica. Some of these countries have experienced reintroduction of the virus but have managed to control it. Several countries where rabies is absent in terrestrial vertebrates (lyssavirus 1), report the presence of bat-associated lyssavirus (Swanepoel, 1996; Hanna *et al.*, 2000), for example Australia.

Urban rabies in Europe, where the primary host is the red fox (*Vulpes vulpes*), became widespread only after the industrialisation of the eighteenth century. Dog rabies was still common in the 1940's and 50's in Western Europe but the introduction of rigorous vaccination campaigns decreased the rabies incidences markedly. Today, the residual cases in dogs, cats, domestic ruminants, deer, badgers, and martens are thought to be spillover infections from foxes. Human rabies has virtually disappeared from Western Europe with the control of dog rabies (Atanasiu *et al.*, 1968).

Rapid population growth and urbanisation in Asia have paved the way for the occurrence of rabies in that area. Thousands of cases of dog rabies are recorded each year in many Asian countries. India has the highest number of human deaths from rabies in the world; the number of deaths per annum ranging from 15000 to 25000 (Ahuja *et al.*, 1985). Despite the 700 000 rabies vaccines distributed throughout the country, the disease remains an important public health problem (John, 1997).

Dog rabies entered North America with the colonists from Europe and with the Eskimos from the Arctic. It was controlled by vaccination, and by 1988 there were no reported cases of human rabies. Skunks and foxes are presently the principal hosts of the virus. Infection of bats with rabies virus (lyssavirus 1) has been reported in the Americas (Beran, 1982). In South America there is a dual problem of urban rabies in dogs and sylvatic rabies in vampire bats (*Desmodus rotundus*). Sylvatic rabies is a common problem in the cattle in Mexico, Brazil and Venezuela where the virus is usually transmitted to cattle via a bite from a vampire bat or other wildlife reservoirs (De Mattos *et al.*, 1996), which makes herd vaccination the most viable method of disease prevention. Such vaccination programs are actively being implemented (De Mattos *et al.*, 1996; Ito *et al.*, 2001; Martinez-Burnes *et al.*, 1997; Rodregues da Silva *et al.*, 2000).

In North Africa rabies occurs in scattered urban foci, but is spread by the dogs of nomads. Occasionally it has been reported in hyaenas and jackals in that

region. In Sub-Saharan Africa rabies is more widely distributed than in the north. Because of the denser population, there is a tendency for the epidemics of dog rabies to spread over large areas and for the disease to be spread to domestic herbivores as well as wild vertebrates. Table 1.1 shows the confirmed cases of infection with rabies and rabies-related viruses recorded in four countries of southern Africa (Swanepoel, 1996).

1.1.1.3. Molecular epidemiology of rabies in southern Africa

In 1993, Nel and co-workers, using 3 regions of the rabies virus (RV) genome (the cytoplasmic domain of the glycoprotein gene, the G-L intergenic pseudogene and the antigenic domain II of the nucleoprotein gene), determined phylogenetic relationships between rabies viruses isolated from different canid hosts. The isolates differed in terms of nucleic acid sequence conservation, but were phylogenetially similar. These southern African isolates were distinctly different from other documented rabies virus groups. Furthermore, 4 mongoose (viverrid) rabies isolates were studied and found to be distinctly different from the canid isolates as well as from rabies isolates from other regions. Another group, von Teichman *et al.* (1995) studied the cytoplasmic domain and G-L intergenic pseudogene regions of the genomes of rabies viruses isolated from domestic dogs, jackals, bat-eared foxes (canids) and mongooses (viverrids) in southern Africa. High levels of sequence conservation and close phylogenetic relationships were found between the canid isolates; however, the viverrid isolates formed a distinct group that were distantly related to European and vaccine strains of RV. These two different groups of RV found in southern Africa have become known as the canid biotype and the viverrid biotype (Nel *et al.,* 1997). There is thought to be spillover between these two biotypes from time to time, however, little is known about the effect of such spillovers on the epidemiology of rabies in southern Africa.

1.1.2. Rabies virus morphology

Rabies is caused by an infection with rabies virus, the prototype virus (serotype 1) of the *Lyssavirus* genus which is a member of the *Rhabdoviridae* family (Rupprech *et al.,* 1990; van Reggenmortel *et* al., 2000 and Wagner, 1990). The virus contains a non-segmented negative strand RNA (Wagner, 1990) and five structural proteins (Madore and England, 1977). Rhabdoviruses have a distinctive morphology; they are rod-shaped particles with one end rounded and the other flattened giving them the appearance of bullets. Figure 1.3 is a diagrammatic representation of a rhabdovirus virion. The virions are approximately 180nm long and about 80nm in diameter (Wunner, 1991). The nucleocapsid (160-50nm) is surrounded by a lipid bilayer envelope that is derived from host cell membranes and through which flattened spikes or peplomers, each composed of three molecules of glycoprotein (RG), project over the entire surface of the virion.

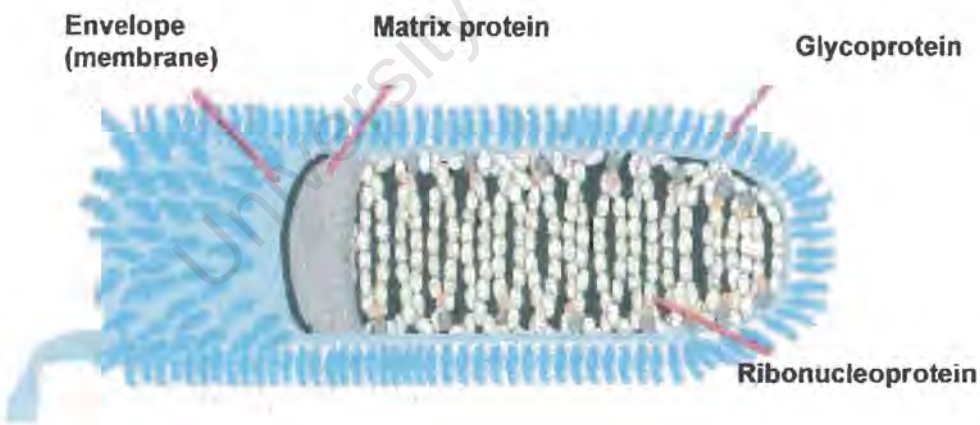


Figure 1.3A. A diagrammatic representation of a transverse section of the structure of rabies virus.

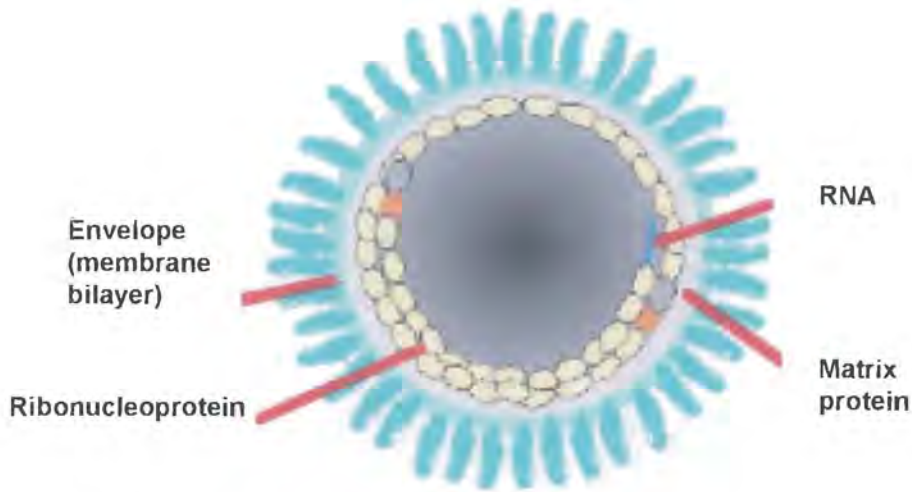


Figure 1.3B. A diagrammatic representation of the structure of rabies virus. (www.cdc.gov/ncidod/dvrd/rabies)

Of the five structural proteins comprising the rabies virion, only the glycoprotein (the nucleotide sequence of the RG gene is presented in figure 1.5) spans the viral lipid envelope. Beneath the lipid bilayer is a matrix protein, which binds the nucleocapsid to the envelope (Emerson, 1985). The helical nucleocapsid consists of an RNA genome (11932 nucleotides) that is bound to the phosphorylated nucleocapsid protein. An RNA-dependant RNA polymerase is also associated with the helix.

Figure 1.4. Negatively stained rhabdovirus as seen through an electron microscope. The bullet-like shape of the virus can be seen as well as the striations of the ribonucleoprotein (RNP) and the glycoprotein spikes in the outer membrane bilayer (http://www.virology.net/Big_Virology/Special/Rabies1/Rabies.htm; micrograph by Steven Vernon).

1.1.3. Rabies virus glycoprotein

The rabies virus glycoprotein (RG) is a trimeric type 1 transmembrane glycoprotein that is important in the pathogenisis of neurotropic rabies virus infection. It spans the viral envelope and is responsible for the recognition of receptor sites for the attachment of virus on the surface of susceptible cells and facilitates virus uptake by cells using low pH-induced membrane fusion (Gaudin *et al.,* 1992). The RG is responsible for inducing production of and binding to protective, virus-neutralising antibodies. It also stimulates and is a target for T-cell mediated immune response (Dietzschold *et al.*, 1990).

These RG spikes are 5-10nm long and 3nm in diameter. Mature monomeric RG protein has more than 500 amino acids making up 2-6 potential glycosylation sites, 12-16 highly conserved cysteine residues, 2-3 stretches of a-d hydrophobic heptad-repeats, an amino terminal hydrophobic signal peptide, a carboxy terminal hydrophobic transmembrane sequence and a carboxy terminal short hydrophylic cytoplasmic domain. The RG protein can assume three different states: the native state (N) detected at the surface of the virus, which is responsible for receptor binding; the activated hydrophobic state (A), which interacts with the target membrane as a first step in the fusion process; and the fusion-inactive conformation (I). These three states are structurally different, and are in a pH-dependant equilibrium (Gaudin *et al.*, 1999). This equilibrium is shifted toward the I state at low pH environments, such as the compartments of the Golgi. There is evidence that RG is shed in a soluble form from RV-infected cells (Morimoto *et al.,* 1993; Gaudin *et al.,* 1999), which, due to the inability of a pattern recognition receptor on the naïve T-helper cell to recognise a soluble protein, causes a typical Th-2 response (Jankovic *et al.,* 2001). Insoluble RG on the surface of the virus is recognised by pattern recognition receptors on the said naïve T-helper cell, which causes a typical Th-1 type immune response (Jankovic *et al.,* 2001). This dichotomy explains the th-1, and th-2 immune response that is known to be induced by RG. The soluble form of RG lacks the transmembrane and intracytoplasmic domains

(residues 440-505) and is secreted as a monomer regardless of the extracellular pH, however, it has the antigenic characteristics of the I state of RG. The transmembrane domain seems to be necessary for the correct folding of the ectodomain (Gaudin *et al.*, 1999). To investigate the importance of RG in protection against RV infection, Foley *et al.* (2000) constructed a recombinant RV (rRV) in which the ecto- and transmembrane domains were replaced with the corresponding regions of vesicular stomatitis virus (VSV) glycoprotein (another rhabdovirus). When mice were primed with either RV (non pathogenic) or rRV and subsequently challenged with a pathogenic strain of RV, it was evident that those primed with rRV were not protected. This confirms the crucial role of RG in RV vaccines.

```
ctgcaggggg gggggggggg aggaaagatg gttcctcagg ctctcctgtt tgtacccctt 60
ctggtttttc cattgtgttt tgggaaattc cctatttaca cgatactaga caagcttggt 120
ccctggagcc cgattgacat acatcacctc agctgcccaa acaatttggt agtggaggac 180
gaaggatgca ccaacctgtc agggttctcc tacatggaac ttaaagttgg atacatctta 240
gccataaaaa tgaacgggtt cacttgcaca ggcgttgtga cggaggctga aacctacact 300
aacttcgttg gttatgtcac aaccacgttc aaaagaaagc atttccgccc aacaccagat 360
gcatgtagag ccgcgtacaa ctggaagatg gccggtgacc ccagatatga agagtctcta 420
cacaatccgt accctgacta ccgctggctt cgaactgtaa aaaccaccaa ggagtctctc 480
gttatcatat ctccaagtgt agcagatttg gacccatatg acagatccct tcactggagg 540
gtcttcccta gcgggaagtg ctcaggagta gcggtgtctt ctacctactg ctccactaac 600
cacgattaca ccatttggat gcccgagaat ccgagactag ggatgtcttg tgacattttt 660
accaatagta gagggaagag agcatccaaa gggagtgaga cttgcggctt tgtagatgaa 720
agaggcctat ataagtcttt aaaaggagca tgcaaactca agttatgtgg agttctagga 780
cttagactta tggatggaac atgggtcgcg atgcaaacat caaatgaaac caaatggtgc 840
cctcccgatc agttggtgaa cctgcacgac tttcgctcag acgaaattga gcaccttgtt 900
gtagaggagt tggtcaggaa gagagaggag tgtctggatg cactagagtc catcatgaca 960
accaagtcag tgagtttcag acgtctcagt catttaagaa aacttgtccc tgggtttgga 1020
aaagcatata ccatattcaa caagaccttg atggaagccg atgctcacta caagtcagtc 1080
agaacttgga atgagatcct cccttcaaaa gggtgtttaa gagttggggg gaggtgtcat 1140
cctcatgtga acggggtgtt tttcaatggt ataatattag gacctgacgg caatgtctta 1200
atcccagaga tgcaatcatc cctcctccag caacatatgg agttgttgga atcctcggtt 1260
atccccccttg tgcacccccc ggcagacccg tctaccgttt tcaaggacgg tgacgaggct 1320
gaggattttg ttgaagttca ccttcccgat gtgcacaatc aggtctcagg agttgacttg 1380
ggtctcccga actgggggaa gtatgtatta ctgagtgcag gggccctgac tgccttgatg 1440
ttgataattt tcctgatgac atgttgtaga agagtcaatc gatcagaacc tacgcaacac 1500
aatctcagag ggacagggag ggaggtgtca gtcactcccc aaagcgggaa gatcatatct 1560
tcatgggaat cacacaagag tgggggtgag accagactgt gaggactggc cgtcctttca 1620
acgatccaag tcctgaagat cacctccccc tgggggggttc tttttaaaaa aaaaaaaaaa 1680
aaaaaaaaaa ccccccccc ccccctgctg
```

Figure 1.5. Rabies Virus Glycoprotein Nucleic Acid Sequence (1667bp) (Lathe *et al.*, 1985)

The mouse-adapted RV strain, CVS-24, has stable variants, CVS-B2c and CVS-N2c, which differ greatly in their pathogenicity for normal adult mice and in

their ability to infect non-neuronal cells. The RG, which has previously been implicated in RV pathogenicity, shows substantial structural differences between these variants. The possibility that pathogenicity is inversely related to RG expression levels is suggested by the finding that CVS-B2c, the less pathogenic variant, expresses at least fourfold-higher levels of RG in infected neurons than CVS-N2c. Although there is some difference in RG mRNA expression levels, the differential expression of RG appears to be largely determined by post-translational mechanisms that affect RG stability. Pulse-chase experiments have indicated that the RG of CVS-B2c is degraded more slowly than that of CVS-N2c, and the accumulation of RG correlated with the induction of programmed cell death in CVS-B2c-infected neurons. The extent of apoptosis was considerably lower in CVS-N2c, where RG expression was minimal. Thus down regulation of RG expression in neuronal cells evidently contributes to RV pathogenisis by preventing apoptosis (Morimoto *et al.*, 1999). Koprowski (1999) also suggests that expression of the RG in cells is related to apoptosis in those cells. RV-induced apoptosis is discussed in more detail in the next section.

1.1.4. Rabies virus pathogenesis

Transmission of rabies follows a bite by a rabid carnivore because RV is usually present in the saliva of the host from the time of the onset of the illness. The rate of transmission varies with the severity, location and multiplicity of bites. Nerve endings of the epithelial and subepithelial tissues of the skin and mucous membranes are the portals of virus entry into the recipient and thereafter centripetal spread of infection proceeds in the nerves to involve the central nervous system, and ultimately centrifugal spread to organs such as the salivary glands (Smith, 1996, www.avma.org)

The effect of the virus in the neurons has been shown to be cytocidal. Experimental introduction of virus followed by limb amputation in animals has shown that early viral replication usually occurs in muscle cells before invasion of the peripheral and central nervous system. However, other studies have

shown that early viral replication usually occurs in muscle cells before invasion of the peripheral and central nervous system. However, other studies have shown that the virus can enter the nervous system without prior amplification in tissues adjacent to the bite (Smith, 1996).

Another variable is the proximity of nerve endings to the bite area. Neurotropism is a characteristic feature of rabies virus infection and it is postulated that the virus recognises cholinergic binding sites (Smith, 1996).

The replication of RV is shown diagrammatically in figure 1.6. Infectious virions enter cells by receptor-mediated endocytosis. The low pH of the endosomal compartment induces a conformational change in the RG, which allows the viral envelope to fuse with the endosomal membrane, resulting in the release of the viral nucleocapsid into the cytoplasm (Gaudin *et al.*, 1999). After replication, progeny virions are released by budding through the distal surfaces of the cell.

The virus moves from the peripheral nerves to the central nervous system where it is transported by axonal flow. Hence, the initial spread of rabies virus is centripetal from the inoculation site to the brain, and once infection has become established in the central nervous system the virus is redistributed by centrifugal spread to a variety of organs, including sensory nerve end organs in nasal and oral cavities, retina and cornea. This ensures the transmission of the virus is to the salivary glands (Smith, 1996). Large amounts of virus are present in the saliva of infected hosts late in infection when chances of transmission are greatest.

The incubation period for rabies in humans varies between nine and sixty days, with a mean of thirty-four days. After the incubation period, patients develop fever, headache, malaise, sore throat, nausea, anorexia, diarrhoea, and fatigue. This is known as the prodromal phase. The wound site may become inflamed and cause discomfort at this stage of the illness. Anxiety, depression and insomnia are common. Patients then enter an acute, neurologic or agitated

phase. They lose the ability to swallow due to muscle spasms and exhibit hydrophobia as a result. This is known as the furious form of the disease. Death supervenes soon after patients enter the comatose stage of the illness. Some patients become paralytic during illness. This is known as the dumb form of the disease (Hatwick and Gregg, 1975).

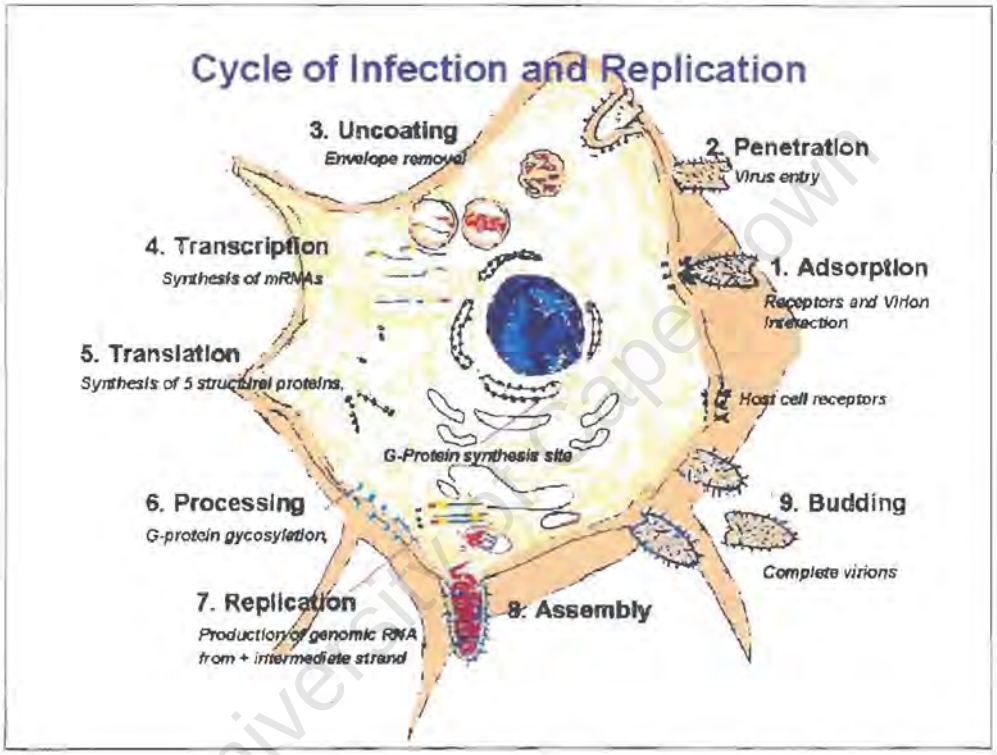


Figure 1.6. Diagramatic representation of the replication cycle of RV (www.cdc.gov/ncidod/dvrd/rabies)

A recent theory regarding the pathogenisis of RV was described by Galelli *et al.* (2000) who induced non-fatal paralysis in BALB/c mice by infecting them with the attenuated Pasteur strain of RV and characterised the spread and localisation of RV in the central nervous system. They suggested that three events were critically associated with the development of neurological symptoms. These were; the amount of virus in the central nervous system; the

infection. It is postulated that during RV infection, the levels of viral replication and primary degeneration of infected neurons by apoptosis are responsible for the infiltration of T lymphocytes capable of inducing secondary degeneration of neural cells. In addition, attenuated and highly neurolvirulent RV strains have distinct cellular tropisms. Highly neurovirulent strains such as the challenge virus standard (CVS) are neurotropic, whereas the attenuated strain ERA also infects non-neural cells. Thoulouze *et al.*(1997) report that both these strains of RV infect activated murine lymphocytes and the human lymphoblastiod Jurkat T-cell line *in vitro* and that both types of lymphocytes are more susceptible to infection with the ERA strain than the CVS strain. The ERA strain induces apoptosis of infected Jurkat T-cells and cell death is concomitant with RG expression – supporting the earlier suggestion of RG-induced apoptosis. Thoulouze *et al.* (1997) did report, however, that apoptosis did not hinder RV production *in vitro.* This finding is paradoxical in that RV infects lymphocytes and induces apoptosis, yet nevertheless induces a strong immune response. The suggestion is that apoptosis is a major function of the immune response to RV infection.

1.1.5. Rabies virus immunology and control

There is evidence that both antibodies and T-cells play a crucial role in immunity to RV (Celis *et al.*, 1989). Xiang *et al.* (1995), while exploring immune effector mechanisms required for protection to RV found that antibodies were important for protection. Mifune *et al.* (1981) showed that athymic nude mice were not protected from RV infection when vaccinated post-exposure with an inactivated vaccine. However, it was also found that the mice did not produce IgG. It was subsequently found that if the mice were given antirabies hyperimmune mouse ascites post-exposure, they were also not protected. This work demonstrates the need for both a humoral and cellular immune response for RV protection. Camelo *et al.* (2001) infected the eyes of BALB/c mice and athymic nude mice with CVS and discovered that the incidence and severity of the ocular disease were higher in the athymic nude mice than in BALB/c mice

athymic nude mice with CVS and discovered that the incidence and severity of the ocular disease were higher in the athymic nude mice than in BALB/c mice indicating that T lymphocytes are protective during RV ocular infection. They also observed that few T cells and neutrophils underwent apoptosis in RV-infected retina. This suggests that that T lymphocytes and neutrophils are able to enter the eye, escape immune privilege, and limit rabies ocular disease. Wunderli *et al.* (1991) showed that humoral neutralising antibodies play a protective role. They found that pre-exposure adoptive intravenous transfer of naïve or immune cells did not significantly protect naïve BALB/c mice from CVS challenge, however, immune serum and anti-rabies glycoprotein monoclonal antibodies (individually and in combination) did confer significant protection. Rabies glycoprotein has been further implicated as the major viral protein responsible for the induction of protection against rabies. Both humoral and cellular immune responses to rabies virus have been shown to be elicited by the RG on the surface of the virus. These proteins induce neutralising antibodies that are able to confer immunity against a lethal challenge of the virus. The ability of the protein to induce the production of neutralising antibodies is dependent upon the correct secondary and tertiary structure of the glycoprotein (Dietzschold *et al.,* 1990; Gaudin *et al.,* 1999; Koprowski, 1999).

Currently there are two strategies for controlling rabies in man. These comprise either post- exposure prophylaxis and treatment or pre-exposure prophylaxis for people who are at risk of becoming infected with the virus. Post-exposure prophylaxis involves eliminating the virus from the site of infection by both physical and chemical methods. After being bitten by an animal suspected of being rabid, the wound must be thoroughly cleansed. Secondly, it involves inducing immunity in the host before infection of the nervous system occurs. This is achieved by inoculation with rabies virus vaccine as well as with human anti-rabies immunoglobulin that is prepared by fractionation of pooled serum from immunised individuals. For high-risk people such as veterinarians, pre-exposure prophylaxis is administered by means of vaccination and regular booster vaccination with the inactivated rabies virus vaccine (Dept Health, 1996). In developing countries, particularly India, Semple's vaccine (sheep's

brain) is still used (John, 1997), however, the majority of rabies vaccines used for humans are inactivated RV. Data is not readily available on the cost of RV control in South Africa specifically, however, it must be noted that the RV control program in the United States of America was estimated to cost anything between $230 million and $1 billion per annum (Fishbein and Arcangeli, 1987), which today may be a conservative estimate. The result of this expenditure is that there are only one or two human deaths per year in the USA. Since the most cost-effective method of preventing the spread of rabies is to vaccinate the animal vectors, much work has gone into developing a diverse porfolio of commercial rabies vaccines for use in animals. Table 1.2 and 1.3 summarises the most readily available commercial veterinary rabies vaccines (www.avma.org).

Figure 1.7. Raccoon blood being drawn to assess RV immunity after consuming a bait containing vv-RG (http://www.co.fairfax.va.us/service/hd/rabiespic01.htm).

Table 1.2. Commercial inactivated RV vaccines.

Manufacturers	Product	Target	Booster
Fort Dodge	Trimune	Dogs/cats	*Yr1 then triennially
	Anumune	Dogs/cats	Annually
	RABVAC1	Dogs/cats	Annually
	RABVAC3	Dogs/cats/ horses	Annually
	ECLIPSE 3 1 FeLV/R	cats	Annually
	ECLIPSE 4 1 FeLV/R	cats	Annually
	Fel-O-Guard 3 1 FeLV/R	cats	Annually
	Fel-O-Guard 4 1 FeLV/R	cats	Annually
	Fel-O-Vax PCT-R	cats	*Yr1 then triennially
Merial	IMRAB 3	cats/dogs/sheep/cattle/ horses/ferrets	*Yr1 then triennially/annually
	IMRAB BOVINE PLUS	cattle/horses/sheep	Annually
	IMRAB 1	dogs/cats	Annually
	Feline 3 1 IMRAB	cats	*Yr1 then triennially
	Feline 4 1 IMRAB	cats	*Yr1 then triennially
	Rabies 1 LEUCAT	cats	Annually
	PUREVAX Feline 4/Rabies Rabies+LEUCAT	cats	Annually
	Rabisin	dogs/cats/cattle/horses	*Yr1 then triennially
Pfizer	RABDOMUN 1	dogs/cats	Annually
Intervet	PRORAB-1	dogs/cats/sheep	Annually
Bayer	PRORAB-3F	cats	*Yr1 then triennially
	MYSTIQUE II potomovac 1	horses	annually

* Yr1 means one vaccination at approximately 3 months of age.

Table 1.3. Licensed recombinant rabies vaccines

(from www.avma.org/)

Manufacturer	Product/description	Target	Booster/route
Merial	PUREVAX feline rabies Rabies glycoprotein, live canarypox vector	cats	Annually-sub cutaneous
	RABORAL V-RG Rabies glycoprotein, live vaccinia virus vector	Raccoons	As determined by local authorities - oral

The immunogenicity of the early nerve tissue vaccines (inactivated) was generally poor. In the 1950's production of a vaccine for dogs involved the use of the brains of suckling mice, while the vaccine for use in humans was prepared from embryonated ducks eggs, which induced allergies and was eventually discontinued. The use of attenuated live virus strains for vaccination did not find favour because of the danger that they could revert back to virulence. These vaccines have been used to vaccinate animals and have been shown to produce both a cell-mediated and humoral immune response (Lambot *et al.,* 2001). The vaccine currently in use in South Africa, and globally, consists of highly purified and inactivated virus propagated in human diploid cell cultures or Vero cell lines. It is considered to be both safe and thermostable (Barth *et al.,* 1985). Vaccination campaigns with this currently used vaccine have decreased the incidences of rabies in endemic areas such as KwaZulu-Natal, but the cost involved and the inaccessibility of many of the informal settlements to veterinarians have left many dog populations unvaccinated. Globally many other inactivated rabies vaccines are available for both human and animal use (Compendium of animal rabies control, 2000; Dept Health, 1996).

Table 1.4. A summary of the major rabies vaccines described in literature

Description	Target	Result	Rt/dose*	Reference
<u>Cell culture vaccines</u>				
Human diploid cell rabies vaccine (HDCV) – inactivated	Humans	Excellent – "gold standard"	IM: D0,D7, D28	Plotkin (1980)
Purified vero cell rabies vaccine (PVRV) Eg. Verorab	Humans	As good as HDCV	IM/ID: D0,D7, D28	Jaiiaroensup *et a.l* (1998)
Primary hamster kidney cell vaccine (PHKCV)	Humans	Good memory response after boosting	IM	Dietzschold and Hooper (1998)
Purified chick embryo cell culture rabies vaccine (PCECV) Eg. Rabipur	Humans, dogs	As good as HDCV	IM: D0, D7,D28 :	Zanetti *et a.l* (1998)
<u>Brain tissue vaccines</u>				
Suckling mouse brain (SMB) Semple's-	Humans	Failed in mice when challenged with wild RV	IP: D0 and D7	Yang and Zhang (1999)
Derived from RV – infected sheeps brain tissue	Humans, cattle, etc.	Semple induced autoimmune encephalomyelitis	IM	Piyasirisilp *et al.* (1999)
<u>Modified live-virus rabies vaccines</u>				
Street Alabama Dufferin (attenuated) (SAD)	Carnivores	Good in jackal, pathogenic in baboons and rodents	Oral	Bingham *et al.* (1992,1995)
SAD-Avirulent-Gif RG modified (SAG-2)	Mammals and birds	Protective and non-pathogenic	Oral	Bingham *et al.* 1997)

*IM denotes intramuscular route of inoculation; ID denotes intradermal route of inoculation; IP denotes intraperitoneal route of inoculation

1.1.5.3. Oral rabies vaccines (See table 1.4)

The concept of oral vaccination arose from the discovery that laboratory animals became immunised after oral infection with the rabies virus. This was demonstrated in the USA using live attenuated SAD (Street Alabama Dufferin) strain of virus. The study was successfully extended into Europe (Baer, 1988). This strategy is being tested for use in Africa to combat rabies in wildlife (Bingham *et al.*, 1995). When John Bingham tested the efficacy of the oral SAD (Berne) Rabies vaccine in 1995, he ascertained that the vaccine and the route of vaccination were both safe and effective in target (Black-backed and Side-striped Jackals) as well as in some non-target hosts. He had previously found that the SAD vaccine was pathogenic to Chacma baboons (Bingham *et al.*, 1992). In 1997, he tested the safety of SAG-2 oral rabies vaccine in various Zimbabwean wild non-target species, and he concluded that the vaccine was safe for use in the wild, even though one genet died after a month. He postulated that the inoculum probably would not be shed from the vaccinated animals and thus would not cause a problem in the wild. Field evaluation of two bait-delivery systems for the oral immunisation of dogs against rabies was performed in Tunisia (Matter *et al.*, 1998). These studies showed that fewer than half the dogs in the area consumed the baits. The project proved very costly and owner participation was minimal. Over the last decade or so, researchers have started to focus on the concept of live recombinant vaccines.

1.1.6.2. *Live recombinant rabies vaccines (See table 1.3 and 1.5)*

A recombinant vaccinia virus-rabies virus was constructed by Kieny *et al.*(1984), whereby the RG was inserted into the TK (thymidine kinase) gene of vaccinia virus (VV-RG) under the control of an early/late promoter. Inoculation of rabbits with VV-RG elicited high titres of RV-neutralising antibodies, confirming expression of the VV-RG in rabbit cells. Mice were also protected by immunisation with this recombinant following an intracerebral challenge with a lethal dose of live RV. However, Wiktor *et al.* (1984) demonstrated that if an amino acid change occurred within the RG gene, the altered gene did not

induce immunity in mice. Mice vaccinated intraperitoneally (IP) with 10^7 plaque forming units (pfu) of a VV-RG three weeks before challenge were protected against peripheral lethal infection. Also mice that were initially infected with rabies virus and subsequently vaccinated IP with the same recombinant virus were protected if vaccinated within twenty-four hours post infection (Fujii *et al.*, 1994). Similar VV-RG viruses were also tested in a variety of other hosts.

An effective oral rabies vaccine was developed and since 1987 field trials have been taking place in Europe where red foxes (*Vulpes vulpes*) are the principal wildlife reservoir. Foxes developed RV neutralising antibodies in response to a VV-RG and were resistant to challenge with street rabies after inoculation by subcutaneous, intradermal and oral routes (Blancou *et al.*, 1986). In another study, thirteen fox cubs were given a VV-RG orally. Twelve developed neutralising antibodies and eleven were protected when challenged. After a year, virus neutralising antibodies were not detected in the cubs but the cubs were protected when challenged. This shows that a single oral immunisation with VV-RG primed a potent memory response that was predominantly cellular in nature in the cubs. Residual pathogenicity of the vaccine vector was not observed and neither was there any horizontal transmission. Previous attempts to control the numbers and spread of the foxes, and thus the spread of RV, were ineffectual, and vaccination programs failed due to thermal instability of the attenuated rabies vaccine and its continued virulence in some wildlife species (Brochier *et al.*, 1991,1989).

Similar trials have been taking place in the USA where the raccoon and skunk are the most important wildlife hosts. Oral immunisation of raccoons and skunks with a VV-RG stimulated the production of RV-neutralising antibodies and protected against oral challenge as did intramuscular (IM) vaccination of dogs (Rupprecht *et al.*, 1986,1988; Tolson *et al.*, 1987). The candidate recombinant VV vaccine is safe in the target species but limited subclinical spread to, and seroconversion of contact animals has occurred (Rupprecht *et al.*, 1988). Studies of immunity and protection in other wild animals have

yielded similar results. Successful oral rabies vaccination with a recombinant raccoonpox virus expressing RG has been demonstrated (Esposito *et al.*, 1988,87). A raccoon poxvirus-rabies virus glycoprotein recombinant vaccine was tested in sheep with relative success (DeMartini *et al.*, 1993).

A fowlpoxvirus recombinant containing a RG gene was tested for its ability to elicit immunity and protect several non-avian species from challenge with live rabies virus (Taylor *et al.*, 1988). After inoculation with the fowlpoxvirus recombinant, both permissive (avian) and non-permissive (non-avian) cells expressed the RG as a membrane-associated antigen, despite the inability of the virus to replicate in non-permissive cells. Mice immunised with the fowlpox recombinant produced neutralising antibodies to RG and were protected when challenged with virulent rabies virus. Rabbits, rats, cats, dogs, and cattle were vaccinated with the fowlpoxvirus-rabies virus recombinant by one of intradermal (ID), subcutaneous (SC), or intramuscular (IM) inoculation routes, and each animal species developed significant antibody titres to rabies by day 14 after inoculation. Dogs and cats were protected when challenged. The anti-RG antibody titres in cattle increased after revaccination. When an inactivated fowlpoxvirus-rabies virus recombinant was injected into rabbits, no rabies antibodies were induced thus indicating that the *de novo* expression of the inserted gene is essential for effective immunisation.

Comparative efficacy studies have been performed in mice; dogs and cats of a canarypoxvirus-rabies virus recombinant (Taylor *et al.*, 1991), and results indicated that, whereas fowlpoxvirus recombinants are not as efficient as vaccinia virus recombinants, the canarypoxvirus recombinants are indeed comparable. The patented vector ALVAC (an avipoxvirus vector) has also been used to produce a RV recombinant virus (Cadoz *et al.*, 1992). Many different rabies vaccines are reported on in the literature with varying results. Table 1.5 summarises and compares these vaccines.

Table1.5. Summary of recombinant rabies vaccines and challenge results

Recom	Description	Inoc route +dose	Chal route+ dose	Result	Reference
RVac-G	Vaccinia virus expressing RG	10^{7} IP[a]	20MFPLD$_{50}$[f] mouse FP	100% survival in mice	Fujii *et al.*, 1994
RVac-N	Vaccinia virus expressing nucleoprotein of RV	10^{7} IP	20MFPLD$_{50}$ mouse FP	5-10% survival in mice	Fujii *et al.*, 1994
V-RN	Vaccinia virus expressing nucleoprotein of RV	$10^{8.5}$ ID[b]	$10^{6.3}$ MICLD$_{50}$[g] Dog IM	20-30% survival in dogs	Fekadu *et al.*, 1992
V-RG	Vaccinia virus expressing RG	$10^{8.5}$ ID	$10^{6.3}$ MICLD$_{50}$ Dog IM[h]	100% survival in dogs	Fekadu *et al.*, 1992
V-RG+N	Vaccinia virus expressing RG+nucleoprotein of RV	$10^{8.5}$ ID	$10^{6.3}$ MICLD$_{50}$ Dog IM	100% survival in dogs	Fekadu *et al.*, 1992
CPV-RG	Canarypoxvirus expressing RG	$10^{0.5-7.5}$ TCID$_{50}$[c] Mouse FP[d]	16 LD$_{50}$[j] mouse IC[i]	10-100% survival in mice	Taylor *et al.*, 1991
CPV-RG	Canarypoxvirus expressing RG	$10^{0.5-7.5}$ TCID$_{50}$ dog SC[e]	3.52 LD$_{50}$ dog IM	0-100% survival in dogs	Taylor *et al.*, 1991
CPV-RG	Canarypoxvirus expressing RG	$10^{0.5-7.5}$ TCID$_{50}$ cat SC	4.12 LD$_{50}$ cat IM	30-100% survival in cats	Taylor *et al.*, 1991
FPV-RG	Fowlpoxvirus expressing RG	$10^{1.5-7.5}$ TCID$_{50}$ Mouse FP	16 LD$_{50}$ mouse IC	0-70% survival in mice	Taylor *et al.*, 1991
VFP3	Fowlpoxvirus expressing RG	$10^{0.7-8.7}$ TCID$_{50}$ Mouse FP	10/12.5 LD$_{50}$ mouse IC	0-80%/0-50% survival in mice	Taylor *et al.*, 1988

a=intraperitoneal; b=intradermal; c=tissue culture 50% infectious dose d=via the footpad; e=subcutaneous; f=mouse footpad 50% lethal dose; g=Mouse intracerebral 50% lethal dose; h = intramuscular; i=intracerebral; j = 50% lethal dose.

1.2. LUMPY SKIN DISEASE VIRUS (LSDV)

1.2.1. Introduction

Lumpy skin disease (LSD) is caused by a capripoxvirus (of the family *Poxviridae*) of which the prototype strain, "Neethling", was first isolated in South Africa (Alexander *et al.*, 1957). The disease can vary between an acute, subacute or subclinical viral disease that primarily infects cattle. The acute form is characterised by fever and necrotic skin nodules. Nodules may also appear in the skeletal muscles and mucosae of the digestive and respiratory tracts. The peripheral lymph nodes are usually inflamed (Weiss, 1968). Clinically the skin lesions of LSD are similar to those caused by Allerton strain of bovid herpesvirus 2 (Alexander *et al.*, 1957). LSD has a mortality rate of up to 40% but is economically important because of the effects it has on cattle herds. Milk herds can stop producing milk as well as becoming debilitated; beef herds become emaciated and useless as beef producers; and bulls as well as cows can become infertile after contracting LSD (Henning, 1956 and Barnard *et al.*, 1994). In addition, the lesions cause permanent damage to the hides of infected cattle (see figure 1.8), which impacts heavily on the leather industry.

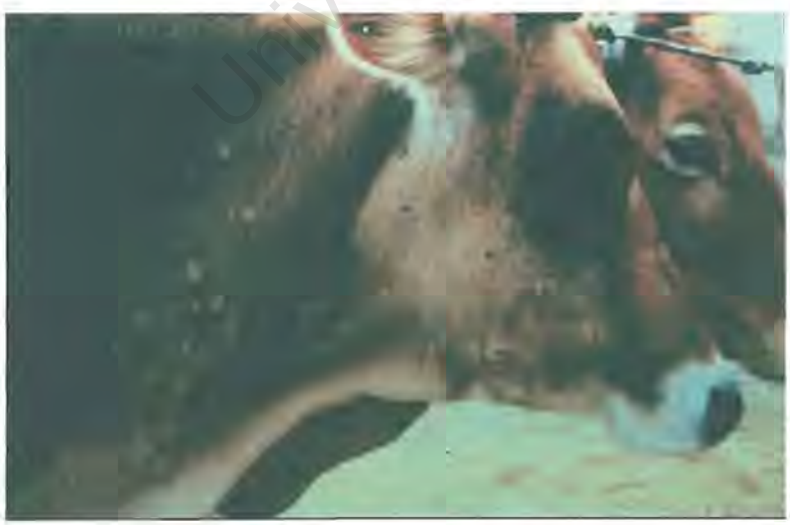


Figure 1.8. LSDV nodules on the neck of an infected animal (www.aphis.usda.gov/us/ep/fad_training/poxvol4index.htm).

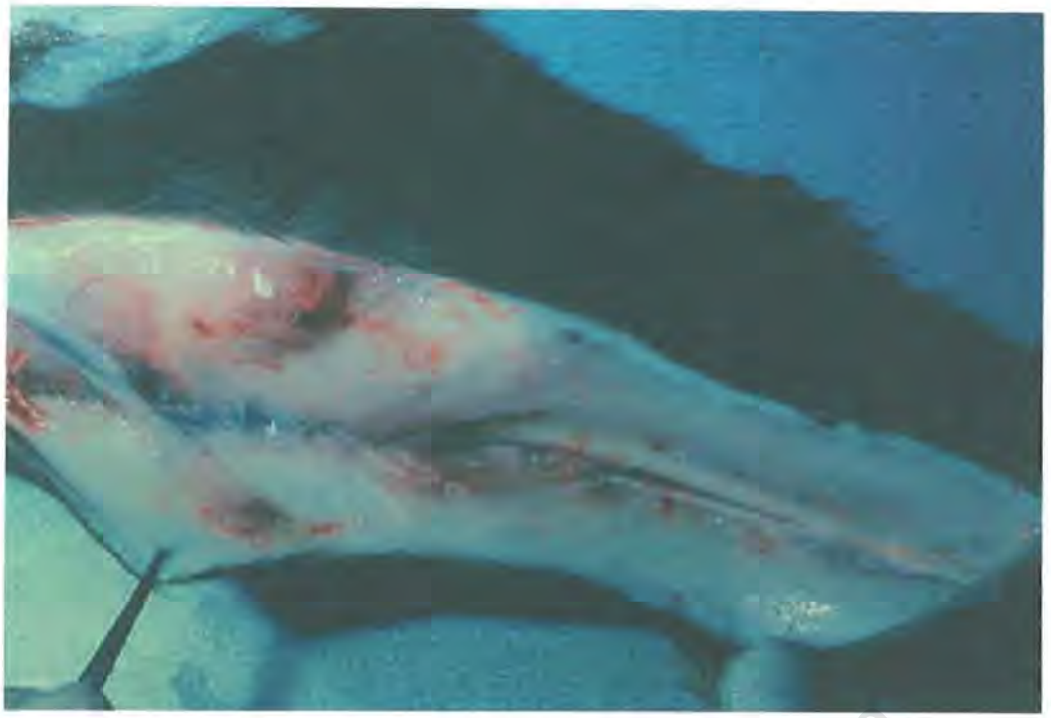


Figure 1.9. Cross-section through a LSDV nodule on the hock of an infected animal (www.aphis.usda.gov/us/ep/fad_training/poxvol4index.htm).

1.2.2. LSDV Pathogenesis

Subcutaneous or intradermal inoculation of cattle with LSDV results in a localised swelling at the site of inoculation after 4 to 7 days as well as enlargement of the regional lymph nodes. The skin nodules appear 7 to 19 days post inoculation (Weiss, 1968). Viraemia occurs after the initial febrile reaction and persists for 4 days. Epithelial, endothelial, pericytes, fibroblasts among other cells types become infected with LSDV. The virus is present in skin nodules, normal skin, lymph nodes, liver, kidneys, skeletal muscle, saliva and semen of infected animals. In experimentally infected cattle, LSDV was isolated from saliva for 11 days after the development of fever, in semen for 22 days and in skin nodules for 33 days but never in faeces or in urine. Pneumonia is a common and often fatal complication of LSD. Lesions may be found throughout the upper respiratory tract and focal or larger areas of grey consolidation may develop in the lungs together with larger areas of bronchopneumonia. Inhalation is a sequel of the necrotic lesions in the respiratory tract and it may prove fatal even months after the initial infection

when necrotic slough occurs from an old tracheal lesion (de Boom, 1948). The pneumonias seldom respond to treatment. Immunity after recovery from natural infection is lifelong in most cattle. Calves of immune cows receive maternal antibodies that allow the calves to resist clinical infection for up to six months of age (Weiss, 1968).

1.2.3. Diagnosis

Clinical symptoms provide a presumptive diagnosis of LSD, made on recognition of an epizootic disease of cattle, producing the characteristic skin nodules and systemic disease.

Animal inoculation may be used for diagnostic purposes, if quarantine facilities are available. The inoculations of calves with antibiotic-treated suspensions of tissue from the skin lesions may provide evidence for a viral aetiology and furnish material for virus isolation. LSDV will grow in embryonated hens' eggs, but this system is not sensitive enough for primary virus isolation (van Rooyen *et al.*, 1968).

Histopathology provides a means of distinguishing the lesions from a variety of other conditions with which LSD may be confused (Burden, 1959) such as streptothricosis, globidiosis, anaphylaxis and bovine herpes type 2. Skin biopsies should be taken under anaesthesia from recently formed skin nodules (including normal skin).

Virus Isolation may be atternpted in primary or secondary cultures from lamb or calf tissues. Lamb testis has been found to be very sensitive (Alexander *et al.*, 1957; Plowright and Witcomb, 1959). Clarified suspensions of ground biopsy tissue mixed with antibiotics can be used to infect such cell cultures. LSD-induced cytopathological changes become evident after 4-10 days in most cell cultures. These are focal initially, with groups of denser cells showing some

rounding and stranding. The foci gradually enlarge to include much of the monolayer.

Electron Microscopy of emulsified tissue fragments from skin biopsies may be stained to detect the brick-shaped poxvirus particles.

Serological methods, such as fluorescent antibody and serum neutralisation tests, may be used retrospectively to confirm recent infections.

1.2.4. Epidemiology

LSD was first described as a skin disease in northern Rhodesia (now Zambia) in 1929. The disease was referred to as pseudo-urticaria and was thought to be the result of an allergenic reaction to the bites of insects (MacDonald, 1931). By 1943 it had spread to Bechuanaland (now Botswana; von Backstrom, 1945) and by 1945 to Southern Rhodesia (now Zimbabwe; Husten, 1945) and to South Africa (Thomas and Mare, 1945; von Backstrom, 1945). In 1944 LSD was first reported in the Transvaal, South Africa (now Gauteng) from where it spread rapidly through South Africa (cited by Swanepoel, 1996). The disease continued to occur sporadically in other African countries. Although LSD was shown to be infectious and transmissible, the virus was only isolated in 1948 by Van Den Ende *et al.* However, they failed to show an aetiological relationship between this isolate and LSD (Haig, 1957; Alexander *et al.*, 1957), which indicates that the isolate may in fact have been Allerton virus (a herpesvirus often confused with LSDV). The panzootic in South Africa lasted until 1949 and affected approximately 8 000 000 cattle (Diesel, 1949). In 1959 lumpy skin disease virus (LSDV) was discovered to be the causal agent of lumpy skin disease (Neethling strain) by Prydie and Coackley, following isolation of the virus from skin lesions of diseased cattle and several passages in tissue culture.

LSD was identified in Kenya and in East Africa in 1957, in Sudan in 1972, and in West Africa in 1974. In 1983, it spread into Somalia (Davies, 1991). Many

years later, in 1988, LSD was discovered at a quarantine station in Egypt and from there it spread. In 1989 it was present in 22 of the 26 governorates in Egypt and subsequently 2 000 000 cattle were vaccinated with a sheepox vaccine (Ali *et al.,* 1990), the implication being that sheepoxvirus cross-reacts with LSDV and thus vaccination with a sheepox vaccine would confer protection from LSDV in cattle. Currently, the disease is found throughout sub-Saharan Africa, North Africa and the Middle East (Davies, 1991; Carn, 1993)

1.2.5. Host range and transmission

Cattle are the natural hosts of LSDV, in which all breeds and both sexes are susceptible (Weiss, 1968). The role of other animals in the epidemiology of LSD however, is questionable. Davies (1980) found that many buffalo showed a high titre of LSDV antibodies and thus concluded that they could act as a reservoir host. However, many buffalo do not show lesions (Hamblin *et al.*, 1990). Experimental infection of buffalo with LSDV also failed to generate the disease, whereas giraffes (*Giraffa cameloparladis*) and impala (*Aepyceros melampus*) died of LSD following experimental infection with the virus (Young *et al.*, 1970). Although there is no evidence indicating that sheep may act as carriers of LSDV, Barnard *et al.*, 1994, found LSDV was able to replicate and be recovered from sheep inoculated with varies field isolates of LSDV. Sheep that have been inoculated with LSDV reveal swelling at the site of inoculation, and regional lymph nodes become inflamed from which LSDV can be isolated, indicating that some replication of the virus can occur (Barnard *et al.*, 1994).

The mode of transmission was not established until recently but circumstantial evidence suggested that biting insects might play a role in spreading the infection. Weiss (1968) described the isolation of LSDV from the flies, *Stomoxys calcitrans* and *Biomyia fasciata*. Field observations of biting insects associated with cattle during epizootics of LSD suggested that mosquitoes, tabanids, *Culicoides* and *Glossina ssp.* also propagated the virus by mechanical

transmission. Chihota *et al* (2001) conclusively demonstrated that female *Aedes aegypti* are able to mechanically transmit LSDV from infected to susceptible cattle for a period of 2-6 days post infective feeding. Carn and Kitching (1995) also showed the transmission of LSDV to animals by intravenously feeding arthropod vectors. This is consistent with reports from field observations that outbreaks of LSDV remained contained in the absence of significant populations of biting flies. Furthermore, Carn and Kitching, 1995, showed that the transmission of LSDV was inefficient in the absence of arthropods as the uninfected cattle housed with infected cattle failed to seroconvert or show any signs of LSDV infection. The disease is more prevalent during the wet summer/autumn months, particularly in low-lying areas and along watercourses. However outbreaks have been known to occur during dry seasons. Deliberate attempts to infect susceptible animals by contact with an infected animal have been relatively unsuccessful, although infected saliva in communal drinking troughs has been implicated in transmission of LSDV. Henning, 1956, claimed that the disease could be transmitted to suckling calves through infected milk, although at this stage, there was some confusion as to the exact causal agent of LSD.

1.2.6. LSDV morphology, replication and stability

A mature LSDV virion measures approximately 200nm by 375nm and is enveloped (Munz and Owen, 1966). Within the outer membrane, tubular protein filaments are irregularly located on the surface. The virions are oval in profile and contain large lateral bodies.

Evidence suggests that there is only one immunological type of LSDV as all collected isolates cross-neutralise with antiserum from the prototype "Neethling" strain (Weiss, 1968). It is very closely related to sheepox and goatpox virus serologically and therefore was almost indistinguishable by routine laboratory tests (Burdin, 1959). Today, LSDV can be identified using a specific LSDV-neutralisation assay (personal communication, Dr Gerdes).

Gershon and Black, 1988, state that the genome size of capripoxviruses is between 143-147 kilobases (kb) in length, however, experiments done by Perlman (1993) indicated that the genome size of LSDV Neethling is in fact 152.61kb in length, 5-9kb larger than the size previously specified. Recently, Tulman *et al.* (2001) completely sequenced and reported the LSDV genome. They found that the genome was 151kb long and consists of a central coding region flanked by identical 2.4 kb inverted terminal repeats. They conclude that the genome consists of 156 putative genes. Previously, the genomic organisation of capripoxviruses was compared by cross-hybridisation of mapped genome fragments with those of other poxviruses. Gershon *et al.* (1989) found that capripoxviruses compare with poxviruses in that their sequences are more divergent at their termini than in the central region. Gershon and Black (1989) have also found that the centrally located 2.5kb genomic fragment from the Kenya sheep-1 strain contains three complete (one of which is the thymidine kinase ORF) and two incomplete open reading frames (ORFs). The additional ORF located directly downstream of the thymidine kinase gene is not present on the vaccinia and fowlpoxvirus genomes but does match a sequence immediately downstream of the Shope fibroma virus genome, of the genus leporipoxvirus (Gershon and Black, 1989). Genetically, LSDV is closely related to members of the *Poxviridae* family, more specifically the *Chordopoxvirinae,* but it contains a unique set of genes that are responsible for viral host range and consequently, virulence (Tulman *et al.*, 2001).

The virus replicates to high titres in cell culture although the development of cytopathic effects (CPE) may take up to eleven days during primary isolation. Replication is accompanied by the formation of intracytoplasmic inclusion bodies similar to those that occur in the skin lesions of infected cattle. The virus multiplies, albeit poorly, in chick embryos and on the choreoallantoic membrane (CAM) of embryonated hens' eggs.

LSDV is very stable and it was shown to survive for 33 days in necrotic dry skin lesions and for 6 months in infected tissue culture fluid at 4^0C (Tripathy *et al.*, 1981). Weiss (1968) also demonstrated that LSDV could remain viable for 18 days in scrapings from lesions in air-dried portions of the hide held at room temperature and for 10 years under dry ice refrigeration in tissue culture fluid. The virus is sensitive to ether and chloroform (unlike orthopoxviruses) and is inactivated by the detergent sodium-dodecyl-sulphate, suggesting that a lipid is incorporated in the structure of the virus (Plowright and Ferris, 1959). This finding implies that the envelope of LSDV is required for infection. LSDV is stable at varied pHs and is also heat stable.

1.2.7. Prevention and control

The European Economic Community (EEC) identified LSDV as a notifiable animal disease in 1989 and requested countries to report outbreaks of the disease within 24 hours to all other members of the Community. This measure was implemented to restrict and prevent LSDV infection in Europe. Capripox-free countries have imposed restrictions on the import of livestock and animal products from LSDV affected areas (Carn, 1993). Radical slaughter policies during outbreaks as well as radial vaccination policies have ensured the control of LSDV in most countries where LSDV is present (Carn, 1993). The Australian Veterinary Emergency Plan outlines an example of such a policy, which is a drastic disease strategy to prevent the introduction of LSDV into the commonwealth (not a LSDV endemic country) and to eradicate it should the situation arise (AUSVETPLAN, 1996).

Vaccination policies are essential as attempts to control the disease by quarantine and movement control is impractical because transmission of LSDV is due to arthropod vectors (Barnard *et al.*, 1994). In regions that have a high probability of LSD outbreak, vaccination of young stock before they enter the herd is generally followed.

The first work done on the development of a vaccine against LSD was carried out at Onderstepoort Veterinary Institute in Pretoria, South Africa, following severe outbreaks of LSD in the 1940s and 1950s. The Neethling strain was attenuated by passaging the virus in embryonated eggs (Van Rooyen *et al.*, 1959). The virus was further attenuated by Weiss (1968) after 60 passages in lamb kidney cells and 20 times on the chorio-allantoic membrane of hen's eggs. Today the attenuated virus is propagated in cell culture. The vaccine is administered subcutaneously with a minimum protective dosage of $10^{3.5}$ $TCID_{50}$ (Davies, 1991). One disadvantage of this vaccine is that it produces a localised lesion at the site of inoculation in approximately 50% of animals, but this usually regresses after a month (Weiss, 1968). There may also be a temporary decrease in milk production in dairy cattle. During the period 1993 to 31/05/2001, 211 322 LSDV vaccinations were administered in South African cattle. This was in response to 17 931 cases of the disease in the 1993 outbreaks in South Africa (Personal communication, Janet Meyer, Dept Agric, 25/07/2001). Hunter and Wallace (2001) also report that 200 000 doses of LSD vaccine are sold by Onderstepoort Biological products annually.

A similar attenuated vaccine was developed in Chad in the late 1960's by passaging a Madagascan strain of LSDV 101 times in rabbit kidney cells and 5 times in fetal calf kidney cells (Ramisse *et al.*, 1969). However, this vaccine is not widely used.

Subsequent vaccines have been developed using cross-reactive virus. The first was described by Capstick and Coackley (1961) who used the Kedong and Isiolo strains of a sheeppoxvirus that was grown in lamb testes and kidney cells and used to protect cattle against LSD. This vaccine was used extensively in Kenya between 1958 and 1959. However, this may cause infection in susceptible sheep. More recently, the Romanian and RM65 strains of sheeppox were used in Egypt and Israel respectively in an attempt to contain outbreaks of LSD in these regions (Davies, 1991). These sheeppox strains,

used as vaccines, were shown to be immunogenic in the field with no reported complications (Carn, 1993).

Attempts have also been made to produce subunit vaccines. Carn *et al.*, 1994, showed that goats inoculated with a fusion protein consisting of the capripoxvirus group specific antigen (KS-1 P32) linked to glutathione S-transferase (GST) could illicit a neutralising antibodies and reduced clinical symptoms on challenge. An advantage of using such a vaccine is that it can be used in countries where capripoxviruses are not endemic (Carn *et al.*, 1994).

1.3. PROJECT MOTIVATION

As the global population explodes, the necessity for infectious disease control becomes increasingly important. Currently vaccination is the most favourable method of control for viral diseases. Two types of vaccines exist, prophylactic and therapeutic vaccines, prophylactic being the more preferred. For decades, vaccinologists have been searching for improved systems to induce immunity. These systems include sub-unit vaccines, DNA vaccines, inactivated vaccines, recombinant vaccines and many others. More recently, recombinant vaccines, chimeric organisms that have been genetically modified to express genes from particular pathogens, are becoming a popular choice for inducing specific immunity in immunised individuals. Many recombinant vaccines (against rabies, measles and other infectious diseases) have been produced using VV as a vector. However as the incidence of HIV in the developing world is increasing, VV has become less desirable as a vaccine vector for use in humans because it can cause disseminated disease in immunocompromised individuals (Redfield *et al.*, 1987). Therefore, due to its transmissibility, VV cannot safely be used in animals. One solution to this problem lies in the use of replication deficient or host restricted poxvirus vaccine vectors.

Rabies virus readily infects cattle and causes a fatal neurological disease. A stable vaccine, which does not require the maintenance of a cold chain and that

is administered once to elicit lifelong immunity to rabies would be advantageous. The initial phase of this project concerns the production of a recombinant LSDV that expresses the glycoprotein of RV that could be used as a vaccine against rabies and lumpy skin disease in cattle.

The second phase will be the evaluation of LSDV as a vaccine vector in non-permissive hosts. Rabies virus has been chosen as the model for two reasons. Firstly, it is an incurable infectious disease that impacts heavily on the economies of many countries. Secondly, a well-developed challenge model exists with which to evaluate the candidate vaccine vector.

The activity of two VV promoters, P11 and P7.5, and a fowlpox early/late promoter will be evaluated in LSDV-infected lamb testes (LT), Madin Darby bovine kidney (MDBK) and primate kidney (CV-1) cells. Replication of LSDV DNA will be studied in the same cell lines. Electron microscopy will be used to study the morphogenisis of recombinant LSDV in the same cells lines. Cellular and humoral immune responses elicited by the candidate vaccine will then be evaluated in mice and rabbits. Finally, a live rabies virus challenge will be performed in order to assess the protective ability of the recombinant lumpy skin disease virus expressing the rabies virus glycoprotein gene.

CHAPTER 2:

PRODUCTION OF A RECOMBINANT LUMPY SKIN DISEASE VIRUS – RABIES VIRUS (rLSDV-RG) AND SUBSEQUENT EVALUATION OF IMMUNOGENICITY OF rLSDV-RG IN CATTLE

2.1. POXVIRUSES AS RECOMBINANT VACCINE VECTORS

A recombinant virus is a virus that has been genetically modified to express a foreign gene during its replication in various hosts. Such recombinant viruses completely or partially retain their ability to replicate and thus can potentially be used as recombinant vaccines to elicit humoral and cell-mediated immune responses to the immunogenic antigens that they were engineered to express. It is possible for recombinant viruses to elicit multivalent immunity if immunogens from multiple pathogens are inserted into the viral vector. Recombinant vaccines are thought to be safer than live attenuated vaccines, where the possibility of reverting back to the pathogenic strain exists.

Poxviruses infect both vertebrates and invertebrates (Moss, 1990). Table 2.1. lists the classification of the poxviruses that infect vertebrates (Fenner, 1992). As viruses, poxviruses are distinguished from other virus families by their size, complex morphology, large DNA genome, covalently closed termini (Baroudy *et al.,* 1982) and cytoplasmic site of replication (Moss, 1990). The mature virions are large enough to be seen under a light microscope but detailed structure can only be visualised by electron microscopy. The most well known member of the poxvirus family is the causative agent of smallpox, variola virus. And consequently, the vaccine developed to prevent smallpox was an orthopoxvirus that cross-reacts with variola virus. This poxvirus, vaccinia virus (VV), became the most thoroughly studied poxvirus of all time. For this reason the pioneering work on recombinant vaccines was done using VV and therefore much is known about the molecular biology, morphology and replication of this virus due to the experience gained from the development, production and use of the smallpox vaccine.

Table 2.1. Classification of poxviruses of vertebrates (from Fenner, 1992)

Family: *Poxviridae*	Subfamily: *Chordopoxviridae*
Genus	**Prototype virus**
Avipoxvirus	Fowlpox virus
Capripoxvirus	Sheepox virus
Orthopoxvirus	Vaccinia virus
Leporipoxvirus	Myxoma virus
Molluscipoxvirus	Molluscum contagiosum virus
Parapoxvirus	Pseudocowpox virus
Suipoxvirus	Swinepox virus
Yatapoxvirus	Yaba poxvirus

Poxvirus particles are characteristically brick-shaped with rounded edges (~ 300 x 230 x 180 nm, Moss, 1990). Extracellular virus particles have a membranous envelope derived from the Golgi vesicle membranes, which is labile and thus often lost during preparative procedures. The 'naked' vaccinia virion has three major structural entities, namely a central core, lateral bodies and an outer coat. The coat consists of an envelope (a 5 nm thick lipid bilayer) with surface tubules (best visualised by negative staining electron microscopy). The surface tubules are arranged over the virion's surface and are 50-100 nm long. The core is surrounded by a membrane (core envelope), on the outer surface on which there is a 'palisade' layer made up with a radial arrangement of rod-shaped molecules 5nm in diameter and 20nm long. Three coils, 250nm long and 50nm in diameter can be seen inside the core. Mature virions are biconcave in shape due to the presence two lateral bodies. The two lateral bodies in VV are ellipsoidal and are situated between the palisade layer of the core and the outer coat of the virion (Muller and Williamson, 1987).

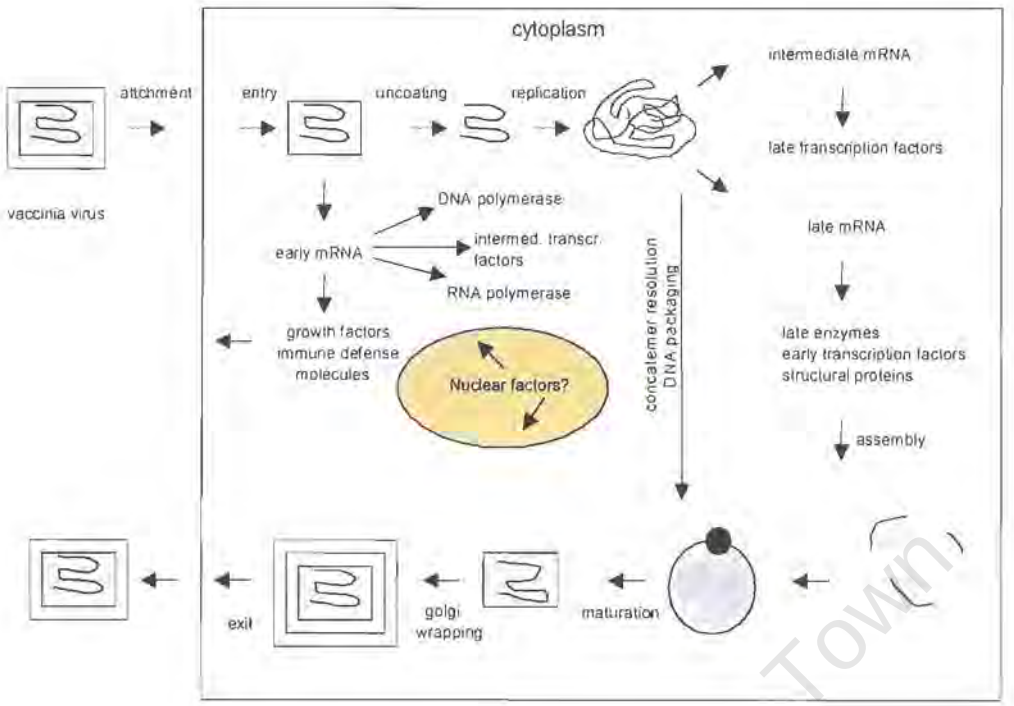


Figure 2.1. Replication cycle of vaccinia virus. Virions enter the cells by as yet unknown mechanism (see text for more detail), releasing cores into the cytoplasm. The cores synthesis early mRNAs that are translated into a variety of proteins. Uncoating occurs and the DNA replicated. Intermediate genes are transcribed and translated. Assembly begins once the late mRNAs have been translate. The concatemeric DNA intermediates are resolved and packaged in immature virions. Maturation takes place to finally produce the infectious virus. Taken from Moss, 1996 and modified by N.M.E. Johnston (2001).

Much of what is known about poxvirus replication is based on VV replication. Refer to figure 2.1 for a diagrammatic representation of the replication cycle of VV. It is generally accepted that poxviruses enter cells by means of fusion with the plasma membrane (Doms *et al.*, 1990) of the target cell, or alternatively, by endophagocytosis into the said cell (Dales and Kajioka, 1964). Experiments using lysomotropic agents (weak bases which can raise the pH of endosomes, thus blocking proteolysis) increased the infectivity of vaccinia virus (Janeczko *et al.*, 1987) and subcellular fractionation studies showed that virion proteins remained in plasma membrane-containing fractions and consequently, there was an absence of viral polypeptides in the fractions from endosomes

(Janeczko *et al.*, 1987; Mallon and Holowczak, 1985). This suggests that the plasma membrane is the most important site of entry. Doms *et al.* (1990) directly monitored cell fusion of VV intracellular mature virus (IMV), an infectious form of the virus, using fluorescence assays and hypothesized that a neutral pH, temperature-dependent process supported entry. Chung *et al.* (1998) showed that VV binds to cell surface heparan sulphate during infection and since entry is often a multiple stage process, it is possible that heparan sulfate-virus interaction could induce conformation rearrangements that may enhance subsequent fusion events. Chung *et al.* (1998) also found that one viral membrane protein, A27L, mediated virus attachment to the heparan sulfate moieties on cells. These findings suggests an attachment-fusion type entry, however, Pedersen *et al.* (2000), disputes this as they found the viral remnants attached to the plasma membrane, not fused with it. In summary, The virus gains entry into a cell when the viral envelope fuses with the cell membrane, releasing the naked particle into the cytoplasm of the cell, or when invagination of the cell membrane around the virion causes a vacuole to be incorporated in the cytoplasm (Muller and Williamson, 1987).

Once VV enters the cytoplasm, uncoating occurs. The virus undergoes at least two stages of disassembly (Pedley and Cooper, 1987). The first stage involves the loss of virion coat proteins and lipids, with the viral genome held in the core. This was demonstrated using the VV early transcription inhibitor actinomycin, to prevent core disassembly, indicating that early VV protein synthesis is required for core uncoating (Holowczak, 1972; Sarov and Joklik, 1972). During the second stage the genome becomes accessible to DNase for DNA replication.

Poxviruses differ from the majority of DNA viruses in that they replicate and transcribe their genome within the cytoplasm of infected cells and encode most of the proteins required for the synthesis of viral macromolecules (reviewed by Moss, 1996). Little is known about the replication of the DNA of poxviruses, but it is presumed to begin from a nick introduced near one or both ends of the genome. This nick exposes a 3'OH group that serves as a primer for strand-

displacement synthesis. This results in concatemer junctions as replication occurs through the hairpin. Concatemeric intermediates are resolved into unit-length genomes following the onset of late-stage transcription (Moss, 1996). Transcription of the early genes occurs immediately after infection and does not require *de novo* protein and DNA synthesis (Keck *et al.*, 1990). Transcription of the early genes are also regulated by A/T-rich promoters located upstream of the transcription initiation (Cochran *et al.*, 1985; Wei and Moss, 1987). Early genes that are transcribed include DNA polymerase and other proteins involved in transcriptional activity of late genes as well as immune modulators that diminish the host's immune response. Cellular protein synthesis is terminated during this process, probably due to viral protein(s) and/or the production of the polyadenylated nontranslated viral RNAs that effect ribosome function (Wei and Moss, 1975). The late genes encode major structural proteins and other components of viral particles and expression of these genes is dependent on DNA replication.

The basic pathway of virion assembly was established using electron microscopy (Muller and Williamson, 1987). Morphologically distinct regions of fibrillar material appear in the cytoplasm during DNA replication. These are presumed to be sites of virus replication. Crescent-shaped bilayer membranes become visible at the periphery of these viral 'factories'. Prominent spicules on one surface cause these membranes to curve until closed spherical particles filled with fibrillar material (DNA nucleoid) are formed (Muller and Williamson, 1987). A series of maturation steps occur, including proteolysis which gives rise to the intracellular mature virions (IMV) that represent the majority of infectious progeny (Moss and Rosenblum, 1973). Some IMVs become enveloped by two membranes derived from the Golgi complex (Schmelz *et al.*, 1994) or early endosomes (Tooze *et al.*, 1993) to form intracellular enveloped virus (IEV) particles. Recent studies by Hollinshead *et al.* (1999) indicated that a single lipid bilayer membrane surrounds VV IMVs. Hollinshead *et al.* (1999) stated that this membrane did not appear to be derived from host organelle membranes, and therefore questioned its origin. The IEV particles were seen to

induce the polymerisation of actin (Cudmore *et al.*, 1995) and migrate to the cell surface where the outer membrane fuses with the plasma membrane, forming extracellular enveloped virus (EEV). The EEVs can either be released from the cell or retained at the cell surface as cell-associated enveloped virus (CEV). CEV and EEV are thought to be responsible for cell-to-cell transmission and long-range spread (Blasco and Moss, 1992; Payne, 1980).

VV as a live vector is thought to be efficient, practical, economical and easy to administer. Production techniques have been developed, which make it attractive as a vaccine vector. VV is one of the best-characterised viruses to date as its entire genome of 191.6kb has been mapped as well as sequenced (Goebel *et al.*, 1990). A feature of the genome is the presence of large non-essential portions of DNA, which may be substituted with pieces of foreign DNA to form recombinant viruses. Isolated poxvirus DNA is not infectious because the virion contains enzymes, including RNA polymerase and other enzymes essential for the production of fully functional mRNA (Moss, 1996). Consequently, although the early stages of recombinant construction can take place *in vitro*, the final stages must involve replication of infectious virions and so must take place *in vivo*. VV has a broad host range that, in the past, has made it a useful vector. VV vectors are known to elicit good humoral and cellular immune responses in hosts and provide protective immunity in experimental models of disease.

Recombinant VV vaccines have been made against influenza, parainfluenza, measles, rinderpest, herpes simplex, pseudorabies, rabies, vesicular stomatitis, hepatitis B and Lassa fever viruses (reviewed by Moss *et al.*, 1984). Generally most animals immunised with these recombinant vaccines have produced high levels of neutralising antibody, but there are instances such as with hepatitis B, where protection was induced in the absence of significant antibody levels (Moss *et al.*, 1984) and others where neither antibody nor immunity was developed (Deubel *et al.*, 1988). Concern has been raised as to whether VV-specific immunity induced by a VV recombinant could reduce the efficiency of

subsequent VV-based vaccines in the same individual. Previous studies have showed that re-vaccination with the same recombinant can boost immunity and that subsequent vaccination with a heterologous VV recombinant can induce immunity to the second vaccine (Perkus *et al.*, 1985).

2.5. PRODUCTION OF RECOMBINANT POXVIRUSES

The most important consideration when constructing recombinant poxviruses is to ensure that the foreign gene (under the control of a poxvirus promoter) is inserted into a region of the poxvirus genome that is not essential for replication, and should contain its own translational start (ATG) and stop signals (TTTTTNT) to ensure that inserted genes are expressed correctly (Yuen and Moss, 1987). It is documented that in addition to the non-essential regions that can be replaced by foreign DNA, at least 25kb of extra DNA can be inserted into the genome of VV without any corresponding deletion (Smith and Moss, 1983). Thus different genes can be inserted to produce a polyvalent vaccine.

The simplest method of recombinant poxvirus production involves the construction of shuttle vectors or insertion plasmids. These plasmids are constructed to contain a poxvirus promoter that controls the expression of the foreign gene, a selectable marker such as TK- in VV (Moss, 1996) recombinants or ECOGPT (Brand, 1993) in other poxviruses, and a reporter gene such as green fluorescence protein or β-galactosidase. This "insertion cassette" has to be flanked by two halves of a non-essential region of poxvirus genome to facilitate homologous recombination.

Selectable markers are inserted into recombinant poxviruses in order to select recombinant viruses over wild-type viruses or alternatively, a common strategy, insertional inactivation, is used to select recombinant poxviruses. This involves the incorporation of the foreign gene into the thymidine kinase (TK) gene of the virus, thereby inactivating it. The thymidine kinase gene codes for an enzyme that is involved in the salvage pathway for the synthesis of thymidine

nucleotides. A number of TK^- cell lines have been isolated from different mammalian species and these cell lines are able to grow in medium that contains the thymidine analogue 5-bromodioxyuridine. Thus recombinants would be TK^- and can grow in TK^- cells in the presence of 5-bromodioxyuridine whereas non-recombinants are TK^+ and the presence of 5-bromodioxyuridine is lethal. An alternative positive selection method involves the use of mycophenolic acid (MPA), an inhibitor of purine metabolism. Cells are normally unable to synthesize guanylic acid (GMP) in a medium containing mycophenolic acid as this substance inhibits inosinate dehydrogenase (IMP) and so prevents the formation of xanthosine monophosphate (XMP). By the incorporation and expression of the *E.coli gpt* (ECOGPT) gene that codes for xanthine-guanine phosphoribosyl transferase (XGPRT) (Boyle and Coupar, 1988) an analogue of the mammalian enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT), this block can be overcome in the presence of xanthine and hypoxanthine. Hypoxanthine and guanine are substrates for HGPRT, while XGPRT can use xanthine and hypoxanthine for the synthesis of XMP. The pathway has been illustrated in figure 2.2.

Reporter genes that are included in the "insertion cassette" allow for easy tracking of the recombinant poxviruses as only recombinant poxviruses will be positive for reporter gene assays. Common reporter genes are green fluorescence protein (GFP) or β-galactosidase (β-gal). GFP fluoresces under UV light and β-gal can easily be assayed for (Appendix B3).

The production of recombinant LSDV was achieved using the simplified strategy outlined in figure 2.3 and subsequently 2.5.

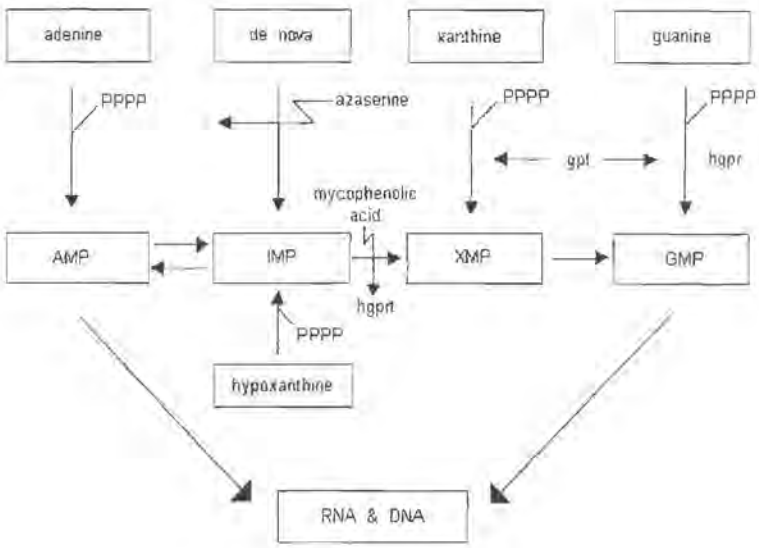


Figure 2.2. An illustration of the purine salvage pathway displaying enzymes and inhibitors of interconversions. Taken from Meuth and Harwood, 1991. Mycophenolic acid inhibits inosinate dehydrogenase (IMP). The E.coli gpt gene that encodes xanthine-guanine phosphoribosyltransferase overcomes this block. Xanthine is a poor substrate for HGPRT and is not normally used by animal cells as a precursor to guanine nucleotides.

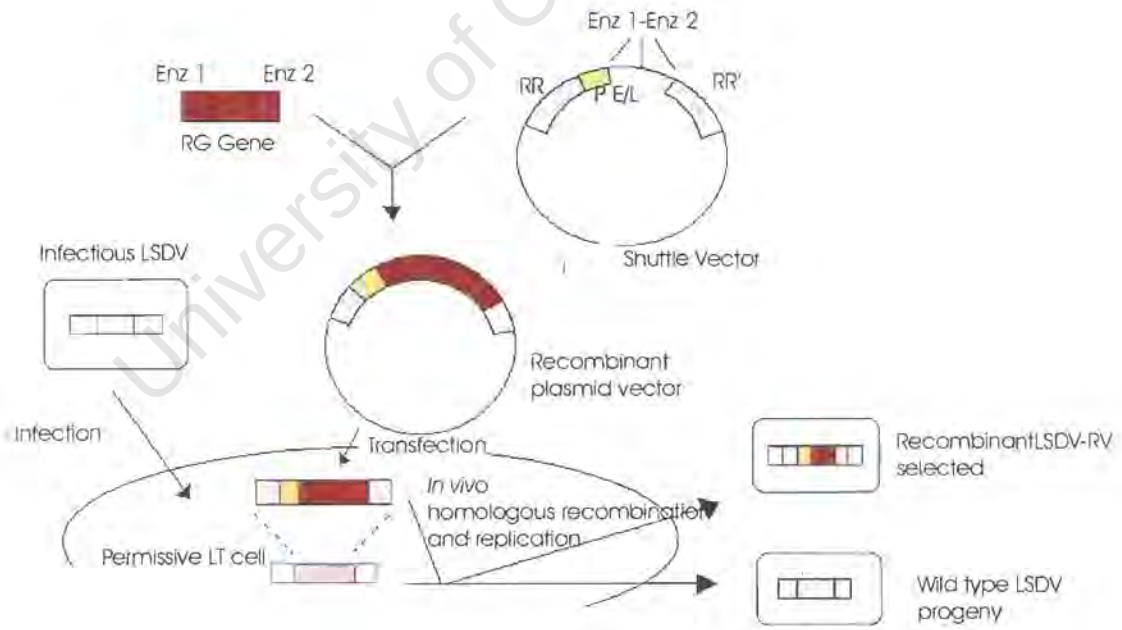


Figure 2.3. Production of recombinant LSDV. Enz = restriction sites, pE/L = fowlpoxvirus promoter, rr and rr' = two sections of a non-essential ribonucleotide reductase gene of LSDV. Adapted from Baxby, 1993 by N.M.E Johnston

2.3. RECOMBINANT CAPRIPOXVIRUSES

The LSDV vaccine (Neethling strain) has been shown to be a good recombinant vaccine candidate (Cohen *et al.*, 1997). Like vaccinia virus, a number of foreign genes can be inserted into the LSDV genome and furthermore, the correct post-translational modifications of viral glycoproteins is assumed to be generated. The LSDV vaccine strain is thermostable and LSDV has a host range limited to cattle, sheep and goats and thus reduces risk to humans, making it a relatively safe vaccine vector. In addition, the capripoxvirus vaccine is not transmitted horizontally from vaccinated to unvaccinated animals or vertically during pregnancy (Kitching *et al.*, 1986). The attenuated vaccine strain for LSDV was developed over thirty years ago (Weiss, 1968) and has proved safe and effective, adding to the benefits of selecting it as a candidate recombinant vaccine (Refer to section 1.2.7).

Capripoxviruses have been shown to be suitable candidates for the production of recombinant vaccines as demonstrated by Romero *et al.* (1994), who showed protection of cattle against virulent rinderpest and LSDV using a recombinant Kenya sheep-1 strain expressing the fusion (F) protein of rinderpest virus. Another Kenya sheep-1 recombinant vaccine, expressing the haemagglutinin (H) gene of the RBOK vaccine strain of rinderpest virus, was also developed by Romero *et al.* (1994). This recombinant produced significant amounts of the correctly folded H protein that was expressed on the membrane of infected cells. Immunised cattle were protected against virulent rinderpest virus challenge. Goats, vaccinated with recombinant capripoxvirus expressing either the fusion gene or the haemagglutinin gene of rinderpest virus or a mixture of the two, were protected from challenge with peste des petits ruminants virus (Romero *et al.*, 1995). Protection was also demonstrated in Kenyan cattle vaccinated with differing doses of an equal mixture of recombinant capripoxvirus expressing either the fusion gene or the haemagglutinin gene of rinderpest virus (Ngichabe *et al.*, 1997). The results of this trial indicated that cattle were fully protected against challenge with rinderpest virus and LSDV

(Ngichabe *et al.*, 1997). A recombinant capripoxvirus, expressing the major core structural protein of bluetongue virus, provided partial protection in sheep against challenge with virulent heterotypic virus (Wade-Evans *et al.*, 1996).

The attenuated LSDV vaccine strain is thus a good candidate for the production of new recombinant vaccines with the added advantage of not only immunising against the foreign gene product but also against virulent LSDV.

2.4. RABIES IN CATTLE

Cattle rabies is a problem, mainly in developing countries including South Africa (Swanepoel *et al,*1996), Namibia, Botswana (cited by Swanepoel *et al.*, 1996), Nigeria (Okoh, 1981) Zimbabwe (Kennedy, 1988 and Lauwrence, 1980), Mexico (Martinez-Burnes *et al.*, 1997), Venezuela (De Mattos *et al.*, 1996) and Brazil (Ito *et al.*, 2001 and Rodrigues da Silva *et al.*, 2000) Lithuania (Gylys *et al.*, 1998), Denmark (Westergaard, 1982), and Hungary (Voros *et al.*, 1999) have also reported cases of cattle rabies.

During the last century, the number of cattle rabies cases recorded was second only to the number of domestic dog rabies cases in South Africa and Zimbabwe. However, in Namibia and Botswana, cattle rabies was the most commonly reported (data collected and cited by Swanepoel *et al.*, 1996) rabies infection. The Department of Agriculture estimates that in the period 1993-2001/05/31, there have been 755 confirmed cases of cattle rabies in 528 outbreaks (Personal communication: Janet Meyer, Department of Agriculture). Canid rabies is commonly transmitted to cattle via the bite of carnivores such as jackals or dogs in Africa (Dept of Health, 2000). However, since so many vectors exist, in Africa, herd vaccination may be the most viable method of disease prevention in cattle. In support of this, 39 959 rabies vaccinations have been given to cattle in South Africa between 1993 and 2001, in order to prevent further rabies outbreaks (Personal communication. Janet Meyer. Dept of Agric).

It has been estimated that 30 000 cattle die annually in Brazil from RV infections (Rodrigues da Silva *et al.,* 2000), where sylvatic rabies is usually transmitted to cattle via a bite from a vampire bat (*Desmodus rotundus*) (De Mattos *et al.,* 1996). Voros *et al.* (1999) state that in Hungary, where cattle rabies is on the rise, cats, in addition to foxes, have been recognized as a major source of the infection. In Denmark, attempts to control the rabies epizootic in 1977-1980, where the number of cattle rabies cases was second only to that of foxes, included large-scale dog vaccination and fox population reduction by poisoning.

The physical manifestations of rabies virus infections in cattle are not unlike those seen in other species. The clinical signs include: excessive salivation, behavioral changes, muzzle tremors, bellowing, aggression, hyperaesthesia and/or hyper excitability, and pharyngeal paresis/paralysis (Hudson *et al.,* 1996). Rabies in cattle usually lasts 2-6 days depending on the severity of the infectious bite, however, the shorter the clinical course, the more prominent the nervous symptoms.

2.4.1. Prevention and Control

In many countries, eradication of the vector transmitting rabies has been the course of action taken in the control of the disease in cattle. This is often carried out using methods such as poisoning, which are detrimental to the environment. In addition, more than one vector is usually responsible for the transmission of the virus to cattle (outlined in section 2.4.), which makes cattle vaccination a viable method of control in this species. Table 1.2 shows the commercially available rabies vaccines and it is evident that those licensed for use in cattle are few and far between. Much research in the field of recombinant vaccine production is being undertaken to combat viral diseases, particularly for use in developing countries and in areas where the viral diseases are difficult to maintain. Cattle rabies is one of those diseases that could be controlled by the use of such a vaccine.

In addition, Kieny *et al.* (1984) reports that the very first recombinant vaccine trial took place in cattle. The recombinant vaccine being tested was a recombinant vaccinia virus expressing the rabies virus glycoprotein gene. However, there was much controversy over this trial that possibly caused a subsequent delay in the licensing of similar recombinant vaccines. The recombinant vaccine was developed in the USA and tested under the auspices of the Pan American Health Organisation in Argentina (Kieny *et al.*, 1984). The Argentinean authorities claimed that the vaccine was imported illegally and ordered the termination of the trial and for all test cattle to be slaughtered (reviewed by Baxby, 1993).

Table 2.2. Cattle rabies vaccine trials – an overview.

Vaccine	Booster	Results and comments	Refer
ERA (live modified RV)	Inactivated RV or ERA 2,4,8,12,16,weeks	Inactivated RV booster yielded higher levels of antibodies than did ERA, however, detectable antibody titres declined quickly.	Oliveira *et al.*,2000
Commercial French vaccine	16 months	Challenged IM with $10^{5.5}$MICLD$_{50}$ in month 55 with street RV. All controls died and test cattle survived.	Blancou *et al.*,1984
ERA	none	Antibody levels were highest at day 15 and lowered by day 30. From day 90 to month 15 the level remained at about 0.5 IU/ml.	Trani *et al.*, 1982
SMB (suckling mouse brain)	none	Circulating antibodies detected until day 90. Challenged after 1 year although some calves had no detectable antibodies and all were protected from a challenge strain that killed 75% of control animals.	Diaz and Lombardo, 1981.
Madiboven Rabdomun Rabisin	1 year	Before booster 42% of animals had antibody titres >0.5IU/ml. A year after booster, 96% of animals had antibody titres >0.5IU/ml. No significant difference was detected between the 3 inactivated vaccines. Thus booster is necessary.	Sihvonen *et al.*,1994; supported by Albas *et al.*,1998
		Ab titre (iu/ml) tested days p.i	
		10 15 20 30 40 50 95 120 150	
Vero cell culture (inactivated)	Day 3,7,14,28,90 Day 10,20,30,90	0.48 0.83 2.33 7.00 12.7 12.7 37.33 16.0 10.0 0.29 0.66 1.33 2.30 3.67 4.67 10.67 10.3 5.33	Basheer *et al.*, 1997.
Raksharab (inactivated)	Day 3,7,14,28,90 Day 10,20,30,90	0.37 0.75 1.26 2.83 5.00 6.67 16.00 9.33 4.67 0.25 0.54 0.75 1.67 2.67 2.67 8.00 3.33 1.67	Basheer *et al.*, 1997.
Semple's (sheeps' brain)	Day 1,2,3,4,5,6,7,8,9, 10	0.25 0.32 1.20 2.40 3.00 4.0 2.40 1.60 0.90	Basheer *et al.*, 1997.

Since rabies in cattle impacts heavily on the economies of affected countries, much work has been done to alleviate this problem. One approach is to vaccinate the animals that may transmit RV to cattle and the other is to vaccinate the cattle themselves. Table 2.2 briefly summarises rabies vaccine trials performed in cattle.

In this chapter, we assess the immunogenicity of a recombinant LSDV that expresses the glycoprotein gene of RV (rLSDV-RG) in cattle, compare it to those results shown in table 2.2 (see section 2.7), and explore the possibility of its use as a dual vaccine to protect cattle against LSD and rabies.

2.5. MATERIALS AND METHODS

2.5.1. Viruses and Cells.

LSDV (Neethling strain) was kindly supplied by Dr HG Jaeger of Onderstepoort Veterinary Institute (Onderstepoort, South Africa). The virus was passaged through primary lamb testis (LT) cells (Appendix B1) to obtain a titre of 3 X 10^6 focus forming units (ffu)/ml according to the method described by Brand (1993) (see Appendix B2), which was an adaptation of the method outlined for vaccinia virus propagation by Freshney (1987). LSDV and LSDV-recombinant viruses were prepared in primary passage LT cells in Dulbecco's Modified Essential Medium (DMEM; Highveld Biologicals, South Africa) supplemented with 10% foetal calf serum (FCS; Delta, South Africa).

2.5.2. Construction of the shuttle vector

A flow diagram of the cloning procedure is shown in Figure 2.4. The portion of the plasmid, pAF09 (kindly provided by David Boyle, of CSIRO, Australia), containing the ECOGPT selectable marker, under the fowl poxvirus early/late promoter (pAF), and the β-gal reporter gene, was excised using *Bgl*11 and *Sal*1

restriction endonucleases (Roche Pharmaceuticals, Germany, appendix A4). This fragment was then cloned into the cloning vector, pMTL25 (Chambers *et al.,* 1988) using the Rapid DNA ligation kit (Roche Pharmaceuticals, Germany, Appendix A5) to incorporate a multiple cloning site. pAF was excised from the resultant plasmid using *Pst*1, and inserted into the *Pst*1 site in pLSD-RR, a plasmid, developed in our laboratory, (Cohen *et al.,* 1997) by digesting LSDV DNA with *Hind* III and cloning into pECOR252 (Zappe, 1988). A clone (pLSD-RR) containing a 4.5kb fragment of LSDV DNA was sequenced and found to code for the ribonucleotide reductase gene (A-L Williamson; Cohen *et al.,* 1997). The ribonucleotide reductase gene was ascertained to be non-essential for LSDV replication by the creation of a LSDV gene library and experimental insertional inactivation. It was thus used as an insertion site, generating flanking sequences for homologous recombination into the LSDV genome. The resulting shuttle vector, pAFMCRR (Appendix C1) is a useful tool into which foreign genes can be inserted in order to construct recombinant LSDV.

2.5.3. Insertion of RG into LSDV shuttle vector.

The plasmid pT7G, containing the RG gene was kindly supplied by N Tordo (Pasteur Institute Paris, France). The RG gene is 1621 base pairs (bp) long (Anilionis *et al.,* 1981, Figure 1.5) and the gene product has a molecular weight of 60 kilodaltons (kDa). To clone the RG gene, primers [Forward primer: 5' AAG GGA TCC GGA AAG ATG GTT CCT CAG GC 3'; Reverse primer: 5' GTC GAC CGT TGA AAG GAC GGC CAG CTC 3'] based on gene sequences of RG in pT7G were designed and used to amplify the RG gene using the polymerase chain reaction (PCR). The PCR product was subcloned into the *Sma1* restriction endonuclease site of the cloning vector, pBluescript SK (Stratagene, USA). The RG gene was sequenced using an automated sequencer (Pharmacia Biotech, UK) by a commercial service (UCT) to ensure that no deletions or mutations had been introduced in the gene during PCR amplification. The RG gene was excised from pBluescript SK using *Sal*1 and *Bam*H1 restriction endonucleases (both supplied by Roche Pharmaceuticals,

Germany, Appendix A4) and cloned into the *Sal*1 and *Bam*H1 sites in the multiple cloning site (Appendix A5) of the shuttle vector (Figure 2.4), pAFMCRR (Appendix C1).

2.5.4. Production of rLSDV-RG and confirmation of RG expression.

A monolayer of 60% confluent LT cells grown in 6-well (35mm) plates Nunc, Denmark) were infected with LSDV at a multiplicity of infection of 0.1 ffu per cell for one hour and then transfected with the recombinant shuttle vector (pAFMCRRG) containing the RG gene mixed with the cationic liposome, DOTAP (Roche Pharmaceuticals, Germany) according to manufacturers instructions (Appendix B2). After 72 hours the transfection efficiency was assessed using the X-gal stain (Figure 2.7, appendix B3; Macket *et al.,* 1984). Romero *et al.* (1994) reports a method used for selecting recombinant virus using the β-galactosidase reporter gene (Appendix B3) and Brand (1993) describes the use of mycophenolic acid tolerance induced by expression of the ECOGPT gene in rLSDV-RG-infected cells as a method of selection (Figure 2.2). More specifically, individual blue foci were picked into 0.5ml growth medium (DMEM containing 10% FCS) from a culture of infected LT cells where the infecting rLSDV-RG had been immobilized using low melting temperature agarose containing selection medium (25μg/ml mycophenolic acid, 250μg/ml xanthine and 15μg/ml hypoxanthine in growth medium) and X-gal (see Appendix B4). Picking individual blue foci in order to further purify the recombinant virus constituted a further round of selection. The process was repeated five more times and finally, one representative focus was selected and propagated in LT cells grown in 175cm flasks in selection medium to prevent LSDV wild-type (LSDV-wt) break-through. After 8 passages through LT cells with selection, cells and growth medium were harvested and frozen and thawed three times, and centrifuged for 2 hours at 19 000g. The pellet containing the rLSDV-RG was resuspended in FCS-free DMEM and frozen at -70°C in aliquots. The recombinant virus was titrated by assaying for the *Lac Z* reporter

gene as described in Appendix B5.

Expression of the RG by LT cells infected with rLSDV-RG was confirmed by direct immunofluorescent staining (see Appendix B6). The rLSDV-RG LT cell layer was initially blocked using a 2% solution of ovalbumin in PBS, and subsequently stained with a 1:40 dilution of fluorescein-labeled rabies antibodies (Becton Dickinson, USA) in PBS.

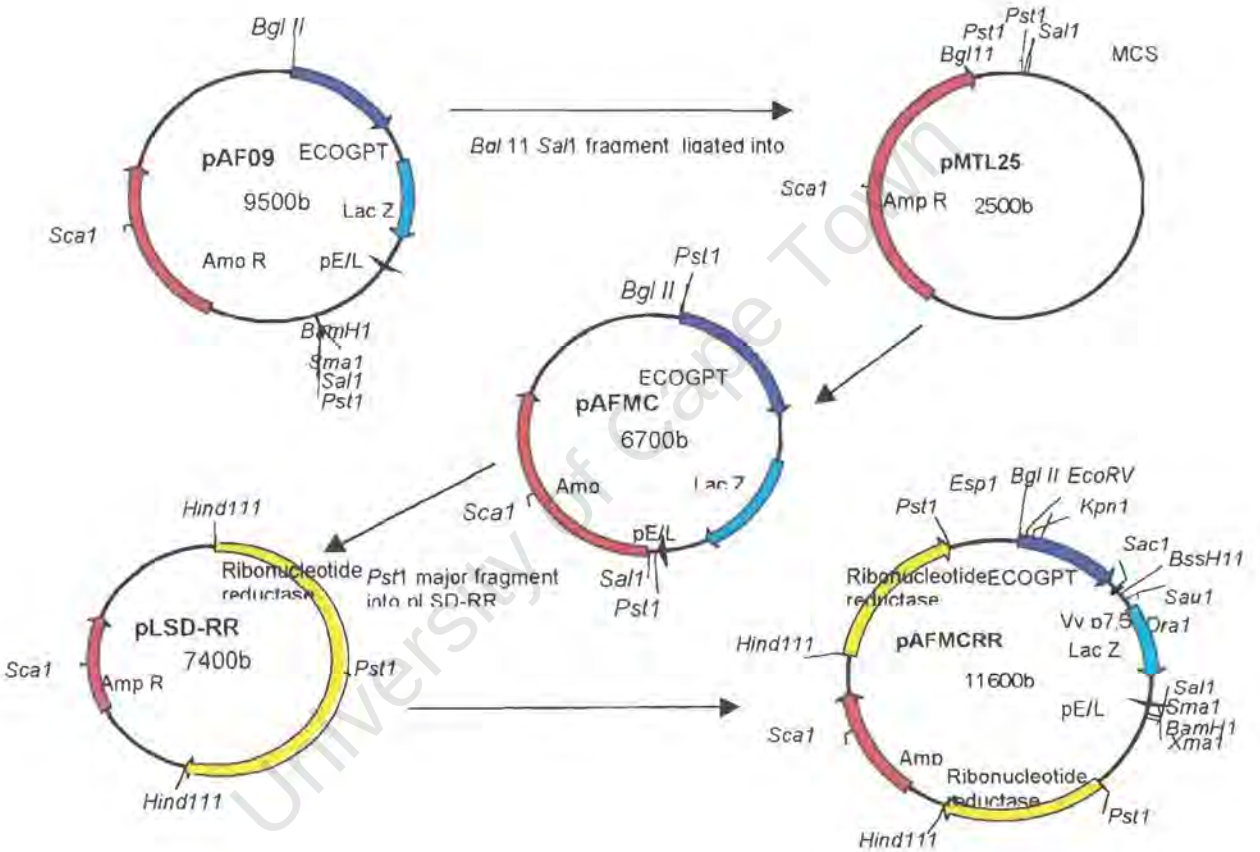


Figure. 2.4. Flow diagram (not to scale) of cloning strategy used to produce pAFMCRR (LSDV shuttle vector) and pAFMCRR. The *Bgl*11 – *Sal*1 fragment was excised from pAF09 and cloned into the cloning vector, pMTL 25. The major *Pst*1 fragment was then excised and cloned into pLSD-RR

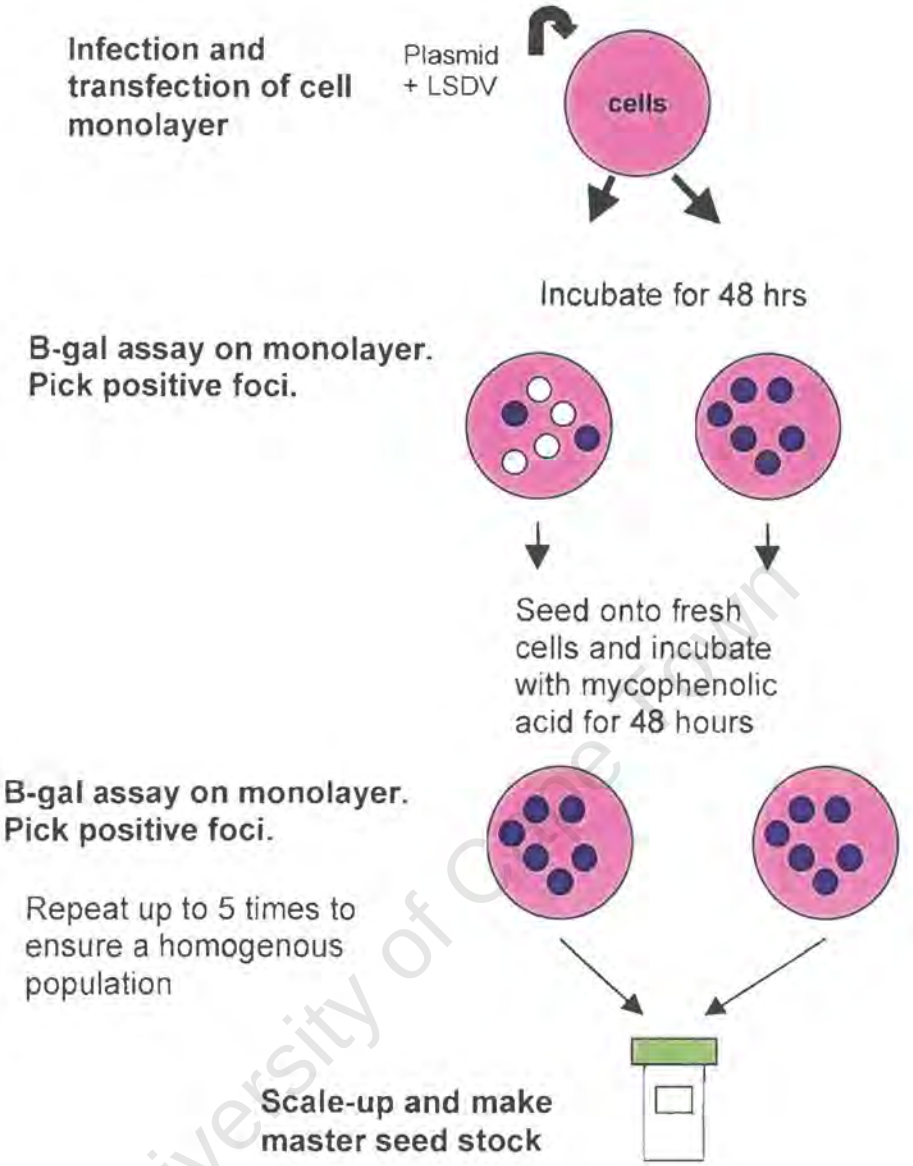


Figure 2.5. Production of recombinant LSDV (Adapted from Ring and Blair, 2001). In the production of rLSDV-RG, the positive foci were picked five times and subsequently passaged through LT cells eight times.

2.5.5. Southern Blot analysis to confirm presence of RG DNA in rLSDV-RG-infected cells

The DNA was extracted from the cells according to the method outlined in Appendix B7. The genomic DNA was then cut with *Bam*H1 and *Sal*1 endonucleases (both supplied by Roche Pharmaceuticals) according to the methods outlined in Appendix A4. The digested DNA from rLSDV-RG-infected cells, LSDV-wt-infected cells, and the plasmid pAFMCRRG was electrophoresed on a 0.8% agarose gel according to the method outlined in Appendix A3. The DNA from the gel was transferred to a nitrocellulose membrane according to method described in Appendix A6. The hybridization and detection was carried out according to the manufacturers' instructions for the Multiprime labeling system (Roche Pharmaceuticals). The method is outlined in Appendix A7 and A8.

2.5.6. Comparison of negatively stained LSDV-wt and rLSDV-RG

LT cells were grown in six well plates as previously described (Appendix B1) and subsequently infected with rLSDV-RG and LSDV-wt (Appendix B2) for three days. The supernatant was then removed from the cell layers and negatively stained with a 1% solution of phosphotungstic acid at pH6.2. The preparations were then viewed in a Hitach-600 electron microscope at 120 000 times magnification. For comparison purposes, a micrograph of similarly prepared LSDV-wt virions was kindly supplied by Linda Stannard.

2.5.7. Inoculation of cattle and horses with rLSDV-RG.

Four 12-15 month old male Friesland calves that had not previously been vaccinated against LSDV (from Onderstepoort Biological Products) were used in the study. Prior to the first inoculation (day 0), a blood sample was taken from each calf for serum preparation. As a first inoculation each calf received $5x10^5$ ffu given in 1ml in the left gluteal muscle (IM). After 28 days the calves

received booster inoculations of the same dose subcutaneously (SC) in the neck. On day 95, a second booster of the same dose was administered IM in the right gluteal muscle. On day 490, the cattle received a third booster inoculation (same dose) in the left gluteal muscle. Blood samples were collected on days 0, 14, 28, 42, 56, 70, 84, 95, 102, 112, 123, 143, 301, and 500 from all the calves and the serum prepared by centrifugation at 1000 g for 8 minutes at 4°C. One LSDV inoculated, year old bull was used as a control for the assessment of the cellular immune response. Two horses (non-permissive hosts in which LSDV does not replicate) were also inoculated IM with 5×10^5 ffu/ml rLSDV-RV in order to assess whether or not any immune responses detected in the cattle could be attributed to the subunit (cell debris protein) portion of the recombinant virus preparation. The horses also received IM booster inoculations of the same dose after 28 days and the serum was prepared and tested as described for the cattle samples. Four Friesland Heifers (four years old) that had been vaccinated with LSDV-wt at one year of age and subsequently boosted annually, were used as control animals to confirm that no cross-reaction occurred between LSDV-wt and RV during ELISAs. Blood was drawn and serum was prepared and tested as described in section 2.5.8.

2.5.8. Evaluation of the humoral immune response to RV.

Enzyme Linked Immunosorbent assays (ELISAs, Trousse Platelia Rage kit, Diagnostics Pasteur, France) were performed on the cattle and horse serum to measure the amount of anti-RG antibodies present in each sample. The serum was diluted 1:500 instead of the recommended 1:100 in order for the OD to be in the readable range. Since the kit did not provide a HRP conjugate suitable for use on cattle or horse serum, HRP anti-horse and anti-bovine conjugates (supplied by Dako, Denmark) were used at a dilution of 1:6000. The optical density (OD) of the ELISA plate was read at 492nm. The results were not corrected for pre-bleed results. Sera from four Friesland cows, that had been vaccinated against LSDV (Neethling vaccine strain) at one year of age and

subsequently boosted annually for three years, were used as controls in order to confirm that no cross reaction occurred in the ELISA between RV and LSDV antibodies raised as a product of vector-induced immunity. Highly specific rabies virus neutralisation assays were performed by John Bingham at Onderstepoort Veterinary Institute according to the FAVN test described by Cliquet *et al.* (1998) on the cattle serum samples to confirm the presence of RV neutralising antibodies and to quantify them. The animals were monitored for over a year to confirm the long-term immune response induced by rLSDV-RG vaccination

2.5.9. Lumpy Skin Disease Virus Neutralisation Assays.

LSDV neutralisation test was carried out according to the protocol set out by Office International Des Epizooties (O.I.E.) – World Health Organization for animal health by Dr Gerdes from Onderstepoort Veterinary Institute (South Africa). This test measures the ability of the antibodies generated in inoculated animals to neutralise LSDV in primary foetal calf testes cells. The test serum from cattle 9L34, 9L47, 9L48, and 9L49 was titrated against constant LSD virus [100 $TCID_{50}$ (50% tissue culture infective dose)] to calculate the neutralisation index. The sera were not prediluted prior to heat inactivation.

2.5.10. Lymphoproliferation assays for evaluation of cellular immune response.

Blood was collected from inoculated cattle on days 480 and 500 in a lithium heparin-coated blood collection tube (BD Vacutainer, UK). The tube was inverted 3-5 times to prevent coagulation of the red blood cells. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized whole blood by Ficoll-Hypaque density gradient centrifugation (Sigma, Germany). Freshly isolated PBMCs were seeded in quadruplicate into round-bottomed 96-well culture plates (2×10^5 cells.well^{-1}; Nunc, Denmark) and incubated in the presence of inactivated rabies virus (2 virus particles/cell; State Vaccine

Institute, Cape Town, South Africa) or unstimulated for 6 days at 37°C, 5% CO_2. [^{3}H]-thymidine (Sigma, Germany) (1 μCi per well) was added to each well for the last 18 h of the assay. The cells were harvested using an automated cell harvester (PHD, Cambridge Technology) and the radioactivity was measured by using a liquid scintillation counter (Tricard-4640). Student's t-test for paired samples was applied to the quadruplicate stimulation indices in order for "p" values to be calculated.

2.6. RESULTS

2.6.1. Construction of the shuttle vector

A suitable shuttle vector, pAFMCRR, was constructed to target foreign genes into the non-essential ribonucleotide reductase gene in the LSDV genome. The RG gene, once sequenced to confirm that no PCR errors had been introduced, was inserted into the pAFMCRR (Appendix C1). The resulting recombinant shuttle vector, pAFMCRRG (Figure 2.6) provided the necessary tool for the insertion of the RG gene into the ribonucleotide reductase gene of the LSDV genome.

2.6.2. Production of recombinant LSDV-RG

After a period of optimisation, where it was established that 10μg of DNA and 30μg of DOTAP liposomes were required to efficiently transfect $1x10^6$ LT cells, the transfection of the recombinant plasmid into LSDV-infected LT cells was successful. The reporter gene, *lac Z*, was transiently expressed in LSDV-infected LT cells transfected with pAFMCRRG and the positive foci can be seen in figure 2.7A. The shuttle vector was inserted by homologous recombination and the reporter gene stably expressed in the transfected cells. Recombinant foci, immobilised in agarose, were serially picked and passaged. Stable reporter gene expression was confirmed (see figure 2.7B,C, and D).

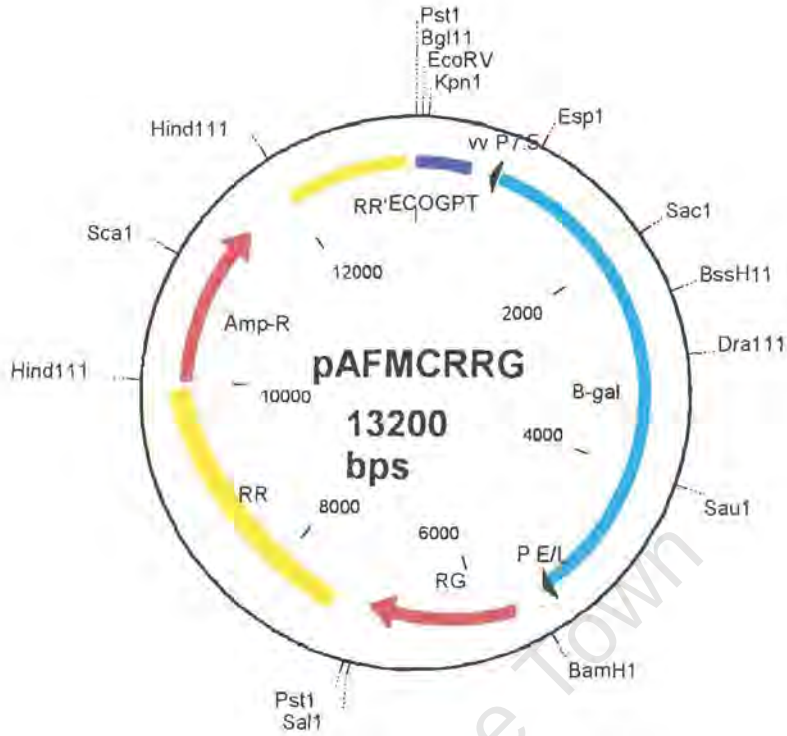


Figure 2.6. Recombinant shuttle Vector, pAFMCRRG, containing the rabies virus glycoprotein (RG) insert. Not to scale.

2.6.3. Immunofluorescence assay to confirm expression of RG by rLSDV-RG-infected LT cells

After 8 passages of rLSDV-RG through LT cells, expression of the RG was confirmed (figure 2.8) by immunofluorescence using FITC-labeled anti-rabies antibodies (Becton Dickinson, USA). Cells were counter-stained with Evan's Blue, which renders the nuclei of cells red when viewed under a fluorescent microscope. Cytoplasmic fluorescent staining was seen surrounding the nuclei of cells. These areas of fluorescence are likely to be viral factories, sites of rLSDV-RG replication.

2.6.4. Southern Blot confirming RG DNA in rLSDV-RG-infected cells

A Southern blot confirmed the presence of the RG gene in rLSDV-RG – infected LT cells. Extracted DNA from LSDV-wt – infected cells was used as a negative control. The RG fragment that was excised from pAFMCRRG and run in lane 3 was 1600bp according to the molecular weight marker (data not shown). Furthermore, a band of the same size is visible in lane 1 where the DNA extract from rLSDV-RG was analysed (see Figure 2.9). This confirmed the presence of RG DNA in rLSDV-RG-infected LT cells.

2.6.5. Comparison of negatively stained LSDV-wt and rLSDV-RG

Electron microscopy was done to determine whether or not the insertion of the RG gene into the ribonucleotide reductase gene of the LSDV genome altered the phenotypic appearance of LSDV. Negatively stained LSDV virions from naturally infected cattle are approximately 200 x 375 nm. Their surface is rope-like in appearance. This is caused by an irregular arrangement of tubular protein filaments. Virions can have either one, two or three membranes surrounding this tubular structure, however, the majority have two (ie. are enveloped). In figure 2.10C (LSDV-wt), the tubular structure is visible and figure 2.10A (rLSDV-RG) displays a similar phenotype. Figure 2.10D (LSDV-wt) and 2.10B (rLSDV-RG) have a similar double membrane arrangement and the size of the virions is similar. Figure 2.10 clearly shows that no major phenotypic alterations were introduced by the RG.

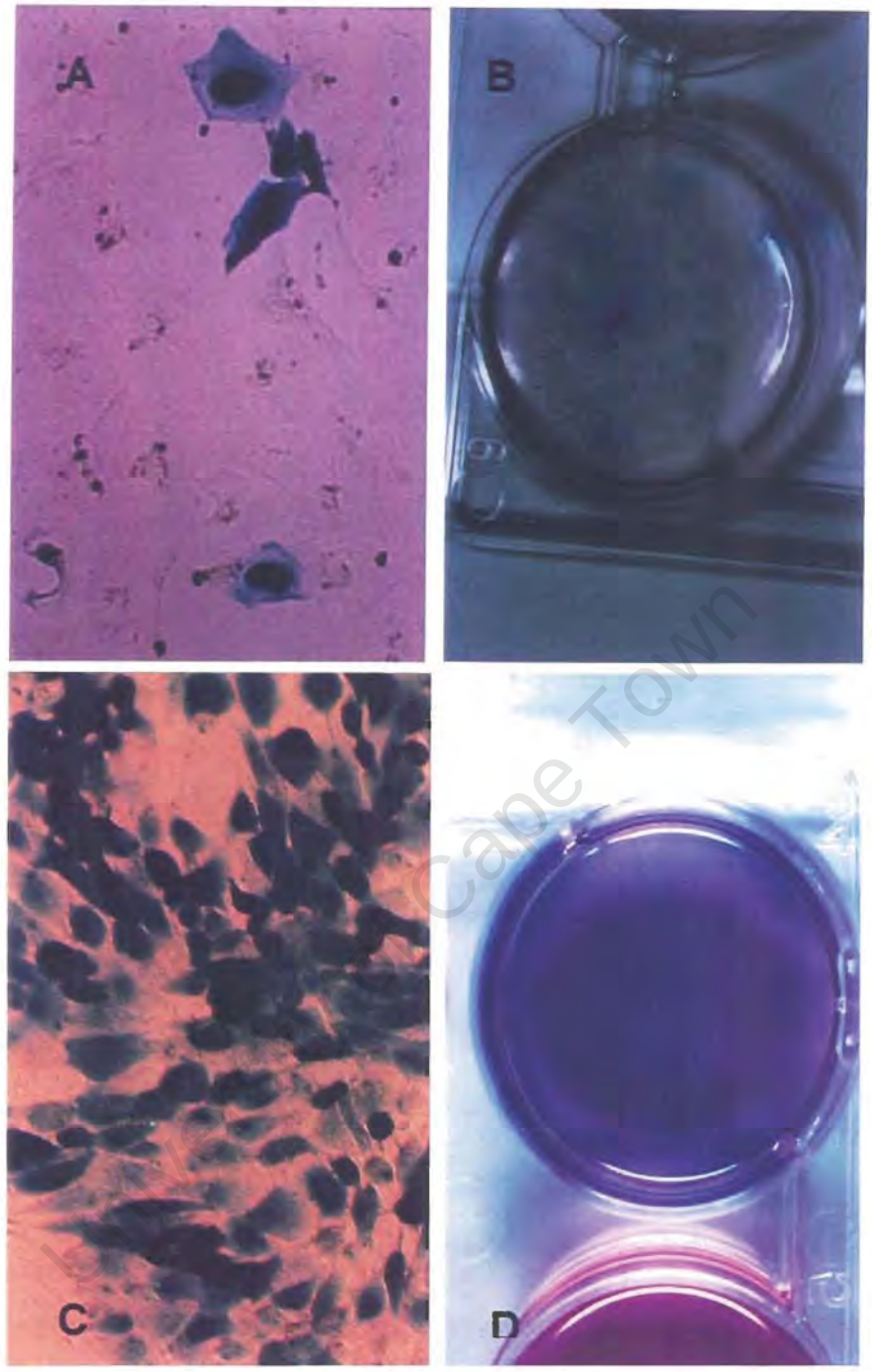


Figure 2.7. rLSDV-RG was generated according to the methods outlined by Macket *et al.* (1984). Transient expression of the lac Z reporter gene is confirmed using the X-gal stain. A: LSDV-infected LT cells transfected with pAFMCRRG and DOTAP, stained with X-gal, and photographed at 100x magnification using an Ilford tungsten film. An X-gal overlay was added to the cell layer to detect expression of the lac Z reporter gene. B: results of an X-gal overlay on cells infected with rLSDV-RG after a single passage at a 1x magnification. Notice the isolated blue foci. Positive foci were picked and passaged through LT cells 8 times until stable expression of the reporter gene was confirmed. C: X-gal stained LT cells infected with rLSDV-RG that had been picked 5 times and passaged 8 times, taken at 100x magnification with an Ilford tungsten film. D: an x-gal overlay on LT cells infected with rLSDV-RG that had been picked 5 times and passaged 8 times, taken at 1x magnification.

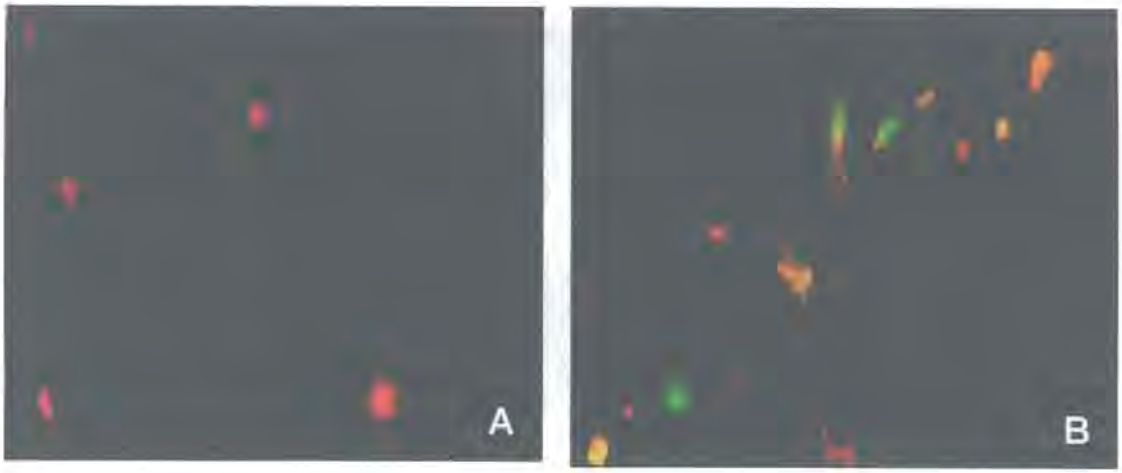


Figure 2.8. Direct immunofluorescent assays using FITC-labeled anti-RG antibodies (Becton, Dickinson, USA) at a 1:6000 dilution in PBS. LSDV-wt- infected LT cells at 100x magnification (A) display no immunofluorescence, while rLSDV-RG-infected LT cells at 100x magnification display immunofluorescence (B).

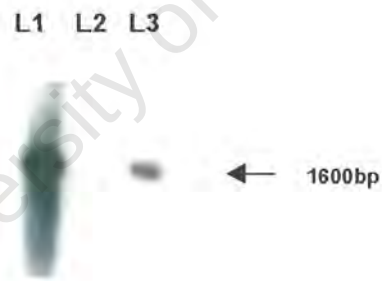


Figure 2.9. A 0.8% agarose gel was loaded with DNA extracted from rLSDV-RG – infected LT cells in lane 1 (L1); DNA extracted from LSDV-wt – infected LT cells in lane 2 (L2) and 200ng of RG excised from purified plasmid (pAFMCRRG; Figure 2.6) DNA in lane 3 (L3). The DNA extracts were digested with *Bam*H1 and *Sal*1restriction endonucleases. Similar amounts of DNA were loaded into lane 1 and 2. The resulting gel, after running for 2 hours was analysed by Southern blotting and probed with P^{32}-labelled purified RG DNA. The corresponding autoradiograph indicates the presence of the 1.6kb RG gene in rLSDV-RG-infected LT cells despite the occurrence of DNA degradation.

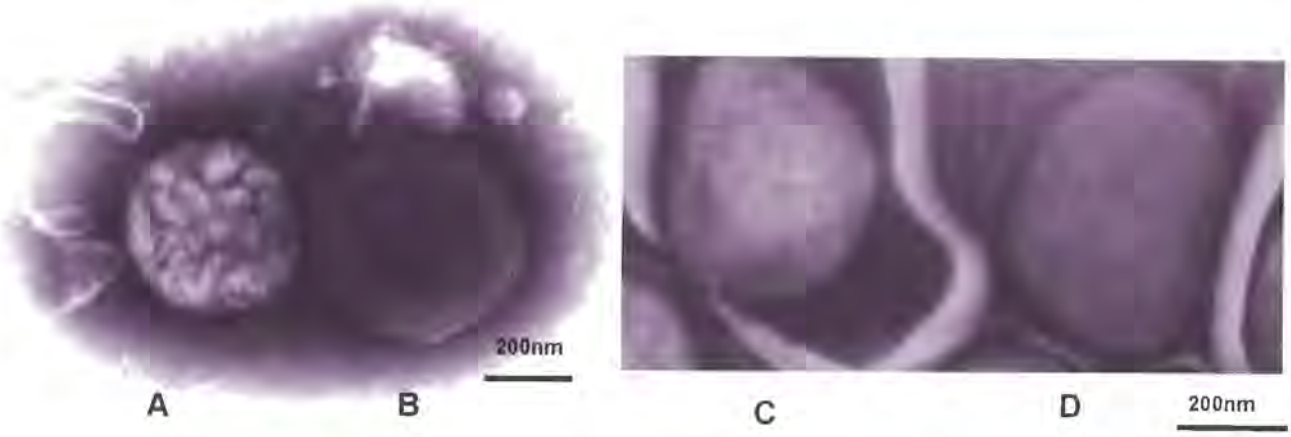


Figure 2.10. rLSDV-RG virions from infected LT cell supernatant, negatively stained with phosphotungstic acid and viewed in a Hitachi-600 electron microscope. The surface tubules can clearly be seen (A) as well as the internal core structure (B). LSDV-wt virions from infected LT cells negatively stained with phosphotungstic acid and viewed in a Hitachi-600 electron microscope (courtesy of L Stannard). Both external tubular (C) and internal structure (D) can be seen. There are no obvious phenotypic differences between the rLSDV-RG and the LSDV-wt virions.

2.6.6. Effect of immunisation with rLSDV-RG on cattle.

Following the primary IM inoculation of rLSDV-RG, no visible reaction was observed at the site of inoculation in the calves. After the first SC booster inoculation, all calves developed a lump 2cm in diameter at the site of injection, which is a typical LSDV vaccine reaction. In this experiment, this is a useful indication that the LSDV vector indeed replicates. After the second booster inoculation, calves 9L34 and 9L48 developed a small lump at the injection site, while the other two calves showed no reaction to the injection. After the third booster inoculation, 9L34 and 9L49 developed a 1cm sub-dermal lump, 9L47, a 0,5cm sub-dermal lump at the injection site, while 9L48 displayed no reaction.

2.6.7. Antibody response to vaccination with rLSDV-RG.

Figure 2.11 shows the rabies virus antibody response (represented by OD) of the four cattle, 9L37, 9L47, 9L48, and 9L49 inoculated with rLSDV-RG. Sera taken prior to the first inoculation (day 0) were negative with OD values of

taken prior to the first inoculation (day 0) were negative with OD values of 0.059, 0.052, 0.053 and 0.054 respectively. The test results were not corrected to negate the pre-bleed OD values.

All four test cattle seroconverted after the primary inoculation (day 0) and with OD values of 0.111, 0.943, 0.693 and 0.29. 9L49 had the lowest OD value after the first inoculation. It was speculated that the initial inoculation was not effective, however, since the OD values varied so notably, it may be a function of the individual animal's ability to respond to an immunogen. The four animals OD values varied because they were out-bred cattle and responded differently to the inoculations. For example, 9L47 did not appear to have a markedly increased OD reading after the first boost on day 28 where the other three cattle did. This may be because the antibody response after the first inoculation was still high and therefore no booster effect was seen. Alternatively, the second booster in this animal was not effective. After both the first and second booster inoculation, 9L34 appeared to have the best OD reading and thus the highest titres of RV antibodies. Both booster inoculations (at days 28 and 95) caused the antibody levels to rise sharply. All four cattle exhibited the highest OD values on days 42 and 95, a week after receiving a booster inoculation. Despite the differences in the individual OD values, all four cattle mounted a detectable anti-rabies antibody response after being immunised with rLSDV-RG.

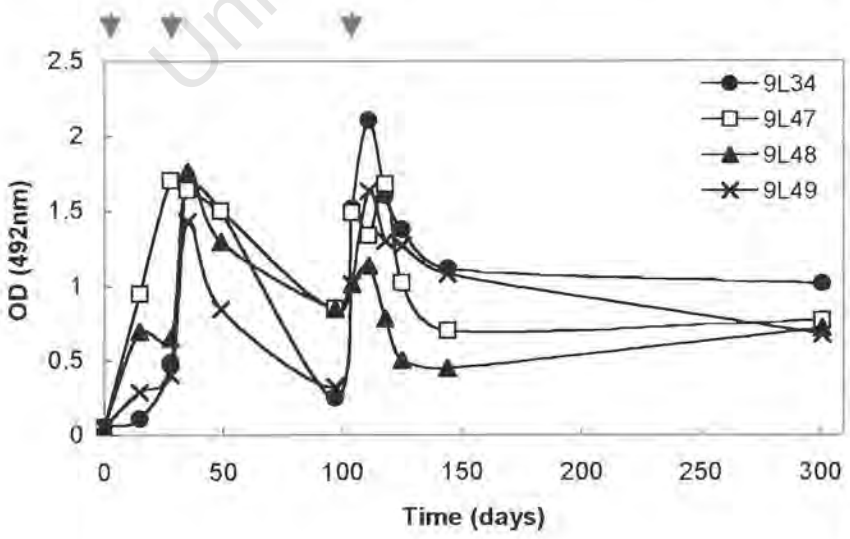


Figure 2.11. RG-specific antibody responses as measured by ELISA of cattle 9L34(•), 9L47(□), 9L48(▲), and 9L49(x) in response to an initial IM inoculation of rLSDV-RG ($1x10^6$ffu/ml), a SC booster inoculation of the same virus preparation ($1x10^6$ffu/ml) at day 28 and a second IM booster, also of the same virus preparation ($1x10^6$ffu/ml) at day 95. Each data point represents the mean OD (492nm) of duplicate determinations. The serum was diluted 1:500 in diluent. Arrows indicate the time of booster inoculations.

The sera from the four LSDV-wt-vaccinated cattle (controls) were negative for anti-RV antibodies using the ELISA with OD values of 0.084, 0.088, 0.053 and 0.065 respectively. This confirmed that the ELISA antibodies measured in the test cattle were specific to RV and not due to inoculation with the vector alone. Table 2.3 shows the results of the rabies virus neutralisation assays. For this test a value of 0.5 IU/ml was taken as the maximum level at which non-specific neutralisation can occur (Dutta *et al.,* 1992) and is considered to be the minimum antibody titre at which protection against rabies occurs (WHO). All four calves developed sustained rabies virus neutralising antibodies well above this level. Three of the calves, 9L34, 9L47 and 9L49, responded to the second booster vaccination, as indicated by a notable rise in the level of neutralising antibodies as well as in OD. The fourth animal, 9L48 had low neutralisation antibody titres and the lowest OD value seven days after the second boost. Once again, this can be attributed to the out-bred nature of the cattle or to an inefficient booster inoculation. Although the OD readings on day 28 and 95 were low, the neutralisation antibody titres were >14 IU/ml, which is twenty eight times higher than the minimum level of RV antibodies required for protection. On day 301, the neutralising antibody titres were low in 9L48 (although higher than the critical 0.5IU/ml) and the OD readings representing total RV antibody titres were stable, indicating that RV neutralising antibodies are not as long lasting as the other RV antibodies detected by ELISA. The neutralisation antibody titres of all four cattle on day 500 were well above that required for protection (0.5 IU/ml; Cliquet *et al.,* 1998) indicating that the immune response elicited by rLSDV-RG is long lasting. Furthermore, the OD reading on day 500 remains above those of the pre-bleed OD values in all four animals.

Table 2.3.

Rabies virus neutralising antibody levels (in IU/ml) in the cattle sera. A value of 0.5 IU/ml is taken as the maximum level at which non-specific neutralisation can occur (Dutta *et al.*, 1992). Animals were boosted on the days indicated in red.

Day	Calf 9L34	Calf 9L47	Calf 9L48	Calf 9L49
0 (Prebleed)	0.0	0.0	0.0	0.0
28	27.0	186.2	15.5	15.5
35 (7 days post boost 1)	501.0	165.9	55.0	95.5
48	1000.0	48.5	146.2	48.4
95	14.0	42.0	95.5	24.0
102 (7 days post boost 2)	1513.5	125.0	14.0	501.0
123	146.6	64.0	16.0	1000.0
143	166.0	42.0	24.0	166.0
301	27.0	81.3	2.9	20.4
500(14 days post boost 3)	9.2	5.3	7.0	27.9

None of the horse sera showed ELISA readings above the threshold of the assay, implying that there was no significant humoral response against the rLSDV-RG or other proteins in the vaccine preparation. This confirms that the immune responses detected in the cattle were produced against the live recombinant rLSDV-RG vaccine.

Table 2.4 shows the LSDV neutralisation results of the four test calves before inoculation and at various time points throughout the experiment. According to personal communication with Dr Gerdes (2001), who routinely performs the neutralisation assays to LSDV vaccinated animals at Onderstpoort Veterinary Institute, neutralisation results are not directly extrapolatable to protective ability and that the results obtained in this experiment are comparable with animals that have received the current LSDV vaccination. Only sera from animals that have been naturally infected in a LSDV outbreak have good LSDV neutralising ability using this test.

Table 2.4.

Lumpy skin disease virus neutralising antibodies (as a ratio to constant 100$TCID_{50}$ LSDV)

Day	Calf 9L34	Calf 9L47	Calf 9L48	Calf 9L49
0 (Prebleed)	1:6	<1:4	<1:4	1:4
48 (3 weeks post boost 1)	1:6	1:6	1:6	1:4
123 (3 weeks post boost 2)	1:12	1:24	1:12	1:6
301	1:4	1:12	1:6	1:4
500 (14 days post boost 3)	1:6	1:4	<1:4	1:4

2.6.8. Lymphoproliferation assays to evaluate cellular immune responses.

T cell proliferative activity to RG recall antigen was measured 391 days after the second boost (day 95) and 14 days after the third boost (day 486). Thirteen months following the last boost (day 95), the T cell responses of rLSDV-RG-immunised cattle, after *in vitro* restimulation with inactivated RV were significantly higher than the LSDV-wt immunised animal in ¼ rLSDV-RG-inoculated bulls (p=0.005; 9L47) and only weakly responsive in ¾ rLSDV-RG-inoculated bulls (9L34, 9L38 and 9L49; Figure 2.12.A). This RG-specific immunity was boosted following the third immunisation (day 486), with 3/4 rLSDV-RG immunised cattle (9L34, 9L48 and 9L49) displaying strong RV-specific T-cell proliferation responses to inactivated RV (Figure 2.12B; p=0.029, p=0.02 and p=0.01 respectively). The "p" values of 9L47 indicate that the stimulation index after the third boost (on day 486) was not significant, yet it was significant prior to the boost. This suggests that the booster inoculation (day 486) was not effective in 9L47.

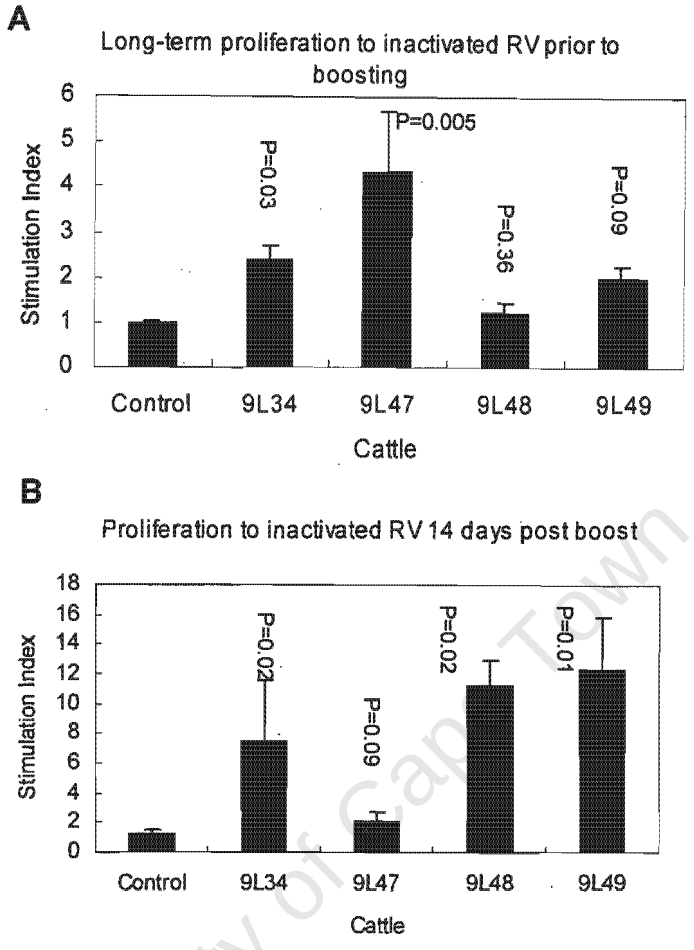


Figure 2.12. rLSDV-RV immunisation induces strong T cell proliferative responses to inactivated rabies virus following *in vitro* restimulation. (A) Proliferative response of PBMCs from cattle 391 days following second boost. (B) Proliferative response of PBMCs from the cattle 14 days after a third boost (day 486). *In vitro* PBMC proliferation to either no antigen (control) or inactivated rabies virus (2 particles/cell; test wells) determined by [^{3}H]-thymidine incorporation. Control animal had been vaccinated with LSDV-wt three months prior to testing. Results have been expressed as stimulation index and were calculated as follows: [cpm of test wells]÷[cpm of wells without recall antigen]. Each bar represents the mean stimulation index (± SD) of quadruplicate wells.

2.7. DISCUSSION

A shuttle vector (pAFMCRR) containing a reporter gene (*lac Z*), a poxvirus E/L promoter (p7.5), a selectable marker (ECOGPT) and a multiple cloning site was constructed that targets the inserted gene into the non-essential ribonucleotide

reductase gene of the LSDV genome. This shuttle vector is a tool that can be used to insert any other foreign DNA into the ribonucleotide reductase gene of LSDV. In this part of the study, the RG gene was then successfully inserted into pAFMCRR to construct a recombinant shuttle vector (pAFMCRRG) that was used to produce recombinant LSDV. The successful expression of the inserted RG supported work done in our laboratory (Cohen *et al.* 1999) showing that the ribonucleotide reductase gene is in fact not essential for LSDV replication. Bovine ephemeral fever virus glycoprotein gene was inserted into the ribonucleotide reductase gene of LSDV. Cohen *et al.* (1999) also inserted a gene from Rift Valley fever virus into an intergenic region in the genome. The thymidine kinase gene is also a suitable site for the insertion of foreign DNA into the LSDV genome (Romero *et al.*, 1994). LSDV now has three proven insertion sites which allows for additional genes to be inserted into recombinant LSDVs.

The RG was successfully amplified by PCR with no PCR-introduced errors. Care was taken to ensure that no poxvirus stop codons (TTTTTNT) were introduced prematurely thus ensuring that the gene is expressed in its entirety. The sequencing results and immunofluorescent assays using commercial rabies antibodies confirmed that the RG has the correct nucleotide sequence as well as being antigenically correct. The immunfluorescence primarily stained the areas around the nucleus. Because poxviruses are known to replicate in the cytoplasm of cells (reviewed by Moss. 1996), these fluorescing areas are likely to be viral factories in the cells.

Negatively stained rLSDV-RV from infected LT cells possess characteristic mature poxvirus morphology and can be described as eliptical with multiple membranes and condensed nuclear material inside them. In figure 2.10, it can be seen that virions superficially stained with phosphotungstic acid have distinct surface tubules, and that virions where the stain has permeated the envelope, display the layers of the core envelope and outer envelope. This finding is in keeping with the earliest documentation of the morphology of LSDV taken from a natural bovine infection (Weiss, 1968). Weiss documented that the two

"different forms of the virion" could be likened to the "M" and "C" forms of vaccinia virus and that the difference was attributed to a change in pH. However, this terminology is out of date and according to Muller and Williamson (1987), the "M" form is now referred to as "an intracellular mature virus (IMV)" and the "C" form is referred to as an "extracellular enveloped virus (EEV)". Furthermore, There do not appear to be any major phenotypic differences in the external morphology between LSDV-wt and rLSDV-RG when infecting LT cells. The virions are of similar size as well as structure. This finding confirms that the insertion of a foreign gene, the RG in particular, into the ribonucleotide reductase gene of the LSDV genome does not change LSDV phenotypically – a fact that further supports the finding that the ribonucleotide reductase gene is non-essential.

In this study immunogenicity of the rLSDV-RG recombinant virus was assessed in cattle, the natural hosts of LSDV, where the virus was expected to replicate productively and thus express sufficient quantities of the RG to induce an immune response. The cattle mounted good anti-RG antibody responses that in most animals increased substantially after booster inoculations, as determined by ELISA and RV neutralisation assays. The four LSDV-wt-inoculated cattle did not mount an immune response to RV, as determined by ELISA, which confirmed the specificity of the test. The ELISA measures all anti-rabies virus antibodies (in OD), while the neutralisation assay is a highly specific assay that measures only those antibodies that specifically neutralise live rabies virus in tissue culture (in IU/ml). This means that there may be differences between the two tests pertaining to ratios of neutralising antibodies to total antibody levels in serum samples at particular time points. However, all the neutralisation antibody titres are well above the critical 0.5IU/ml required for protection against rabies. This implies that the recombinant LSDV-RG replicated efficiently in cattle and adequately expressed the RG. In addition, the rLSDV-RG vaccine appears to produce a good rabies-specific T-cell proliferative response in rLSDV-RG-immunised cattle. The long-term cellular immunity to RV was boosted significantly in three out of the four cattle after the third booster.

a BHK-21 cell culture-inactivated vaccine; VSV (Pasteur Institute), a Vero cell culture adapted inactivated vaccine; and Semple's vaccine (King Institute), a 5% sheep's brain suspension inactivated with β-propiolactone] with various vaccination schedules (>5 vaccinations). They reported ELISA titres, in IU, of: 37.33 on day 95 induced by Raksharab inoculated on days 0,3,7,14,28 and 90; 16 on day 95 induced by VRV inoculated on days; 0,3,7,14,28 and 90; and 4 on day 50 induced by Semple's inoculated daily for 10 days from day 0. In our study, the neutralising antibody titres 7 days post boost (days 35, 109 and 500), compare well with those reported by Basheer *et al.* (1997), even though the number of vaccinations given in our study is 2 or 3 as compared to the 4,6 and 10 vaccinations of Basheer and co-workers (1997). Furthermore, the levels of neutralising antibodies detected in this study are likely to be significantly protective against rabies and the results warrant further trials to test for vaccine efficacy. It would also be valuable to perform LSDV ELISAs and/or challenges on such vaccinated cattle to test the dual vaccine aspect of the LSDV vector.

In conclusion, the LSDV-RG recombinant is a promising new candidate vaccine for protection against rabies in cattle. It has the potential to be developed as a cost-effective dual vaccine against both lumpy skin disease and rabies. Similar dual rLSDV vaccines have been successfully developed for other viral diseases, namely rinderpest and pestes des petits ruminants (Romero *et al.,* 1993, 1994a,1994b and 1995). This candidate recombinant vaccine would be convenient and cost-effective as both lumpy skin disease and rabies are serious diseases against which cattle in the developing world are regularly vaccinated.

A Detailed executive summary of a business plan to produce Caprirab, the trade name of this rLSDV-RG described in this chapter is presented in Appendix F. Further market research and due diligence is in progress to ascertain the viability of commercialising this product.

CHAPTER 3:
EVALUATION OF LSDV AS A HOST-RESTRICTED VACCINE VECTOR

3.1. INTRODUCTION TO HOST-RESTRICTED AND REPLICATION-DEFICIENT POXVIRUSES

Host-restricted poxviruses are safer as vaccine vectors than their counterpart vaccinia virus because their replication is limited and thus environmental spread is less likely to occur. The most well known host-restricted poxviruses to date are the avipoxviruses. They are able to enter mammalian cells and uncoat. However, they do not replicate productively. Their early genes are nevertheless expressed and so any gene under the control of an early promoter can potentially become an immunogen (see section 2.1). Previously NYVAC, a vaccinia virus-based vector was engineered as a host-restricted virus and more recently many more studies have focused on a modified vaccinia virus as a host-restricted vector for safety reasons. Currently modified vaccinia virus Ankara (MVA) is showing great potential as a viral vector.

3.1.1. NYVAC

NYVAC is a genetically modified version of VV. It has had 18 genes deleted and it has been found that these genes were associated with host range and pathogenesis (Tartaglia *et al.*, 1992). NYVAC has been patented and so widespread investigation into this possible vaccine vector has been limited.

3.1.2.Modified vaccinia virus Ankara

MVA was attenuated by more than 570 passages of vaccinia virus (strain Ankara) in chick embryo fibroblasts, during which multiple deletions and mutations occurred (Wyatt *et al.*, 1998). This resulted in the host range of the virus being severely restricted. Structural analysis revealed that the genome of MVA had suffered six major deletions resulting in the loss of 31 000 bp or 15% of its genetic information (Meyer *et al.*, 1991). Antoine *et al.* (1998) report that the MVA genome is 178kb as opposed to the 198kb of the VV copenhagen strain genome. MVA was found to be non-pathogenic in animals and

furthermore is replication deficient in mammalian cells. It was developed as a safe smallpox vaccine because unlike the prototype VV, it replicates very poorly in mammalian cells and is nonpathogenic even for immunocompromised animals (Hochsten-Mintzel *et al.*, 1972). After the eradication of smallpox, MVA was widely investigated as a safe and efficient expression vector (Sutter and Moss, 1992; Sutter *et al.*, 1995; Wyatt *et al.*, 1995). Efficient replication has only been described in primary chick embryo fibroblasts (CEF) and Syrian baby hamster kidney (BHK-21) cells (Carroll and Moss, 1997; Drexler *et al.*, 1998). The block in most non-permissive cells occurs at a late stage of the MVA replication cycle, thus allowing for the foreign protein to be expressed and an immune response to be elicited. Sutter and Moss (1992) demonstrated that the block in the replication of MVA in human cells occurs at a step in virion assembly, rather than at an early stage of infection as occurs with other poxvirus host range mutants. This results in recombinant gene expression being unimpaired. Furthermore, Sutter and Moss (1992) found that replication of MVA viral DNA appeared normal and that both early and late viral proteins were synthesised in human cells. However, proteolytic processing of viral structural proteins was inhibited and only immature virus particles were detected by electron microscopy.

Wyatt *et al.* (1998) discovered by performing marker rescue experiments on MVA that repair of multiple genes is required to restore the ability of MVA to replicate efficiently in human cells, thus the chances of MVA reverting to a virulent replicative strain are very low. In several instances, MVA appears to be more effective than a replication-competent vaccinia virus vaccine vector. This may be because MVA has deletions in the host immune evasion genes (Antoine *et al.*, 1998; Blanchard *et al.*, 1998).

Recombinant MVA is thus a promising human vaccine candidate due to its restricted host range, immunogenicity and avirulence in animal models, and has an excellent safety record as a smallpox vaccine.

3.1.3.Recombinant MVA

Insertion plasmids have been developed for construction of stable MVA expression vectors (Antoine *et al.*, 1996; Carroll and Moss, 1995; Scheiflinger *et al.*, 1996; Sutter and Moss, 1992; Wyatt *et al.*, 1995). Scheiflinger *et al.* (1996) discovered that co-integration of a functional fowlpox virus TK-gene allowed easy generation of recombinants, indicating that the genetically stable TK-gene region is a suitable insertion site, if the TK-gene activity is substituted.

Animal experiments have indicated that dedicated recombinant MVA viruses can provide protection against a wide variety of viral pathogens (Bender *et al.*, 1996; Sutter *et al.*, 1994; Hirsch *et al.*, 1996; Wyatt *et al.*, 1996). Sutter *et al.* (1994) and Bender *et al.* (1996) produced a recombinant MVA virus containing the influenza virus haemagglutinin and nucleoprotein that stimulated protective immunity against influenza challenge when inoculated into mice intramuscularly.

Wyatt *et al.* (1996) engineered MVA to express the fusion protein and haemagglutinin-neuraminidase glycoproteins (HN) of parainfluenza virus 3 (PIV3). Initial recombinant viruses, in which the HN gene was regulated by a very strong synthetic early/late promoter, replicated poorly in permissive chick embryo cells evidently due to toxic levels of the gene product. The promoter was replaced with a modified early/late promoter derived from the H5 gene of the vaccinia virus (weaker than the synthetic promoter but stronger than P7.5). Recombinant MVA employing the modified H5 promoter to regulate the fusion (F) or HN gene of PIV3 replicated to high titres in chick cells and expressed functional F or HN proteins as measured by syncitial formation upon dual infection of mammalian cells. Cotton rats inoculated with MVA expressing F or HN by intramuscular or intranasal routes produced high levels of antibodies to the F and HN proteins.

Belyakov *et al.* (1998) were able to induce a long-lasting cytotoxic T-lymphocyte response in mice by intrarectal immunisation with MVA expressing gp160 of

HIV-1. The response was comparable to that achieved using recombinant VV expressing the same gene. Hirsch *et al.* (1996) showed that viraemia in macaques infected with simian immunodeficiency virus (SIV) was less severe if the macaques had been immunised with MVA expressing the gag, pol and env genes of SIV prior to challenge with SIV. Seth et al. (1998) did similar work and demonstrated that rhesus monkeys vaccinated with MVA-SIVsm gag-pol developed a Gag epitope-specific CTL response. These results suggest that MVA may be useful in developing a recombinant HIV vaccine. Also, Hanke *et al.* (1998) compared routes of inoculation of MVA-based multi-CTL epitope vaccines for HIV-1 in mice and found that both intravenous (IV) and intramuscular (IM) inoculations induced CTL responses, although the IV response was moderately better than the IM response.

Genes encoding the glycosylated precursor of the membrane (prM) and envelope (E) proteins of a Korean strain of Japanese encephalitis virus (JEV) were inserted into the genome of MVA under the control of a strong synthetic or modified H5 vaccinia virus promoters. Mice were immunised IM or IP with the recombinants and were protected against challenge with JEV (Nam *et al.*, 1999). Allen *et al.* (2000), Hanke and McMicheal (1999) and Amara *et al.* (2001) have recently performed DNA/MVA prime/boost experiments in macaques, humans and mice with good results. The DNA and MVA recombinants used in these experiments were HIV-1 and SIV derived recombinants.

Where comparisons were made, the immunogenicity of MVA recombinants was equal to or better than that of vaccinia virus recombinants. In addition, MVA has been effective in a murine cancer immunotherapy model (Carroll *et al.*, 1997).

3.1 4. Avipoxviruses

Many of the hosts that members of the poxvirus family infect are avian hosts (Moss, 1996). As previously mentioned, however, poxvirus promoters (section 3.1.6.) seem to be conserved throughout the family and so the techniques and concepts developed for the construction of recombinant VV can be used for the construction of alternative poxvirus expression vectors. The avipoxviruses were initially investigated and developed as vaccine vectors for immunising birds against avian diseases, but fowlpoxvirus (Boyle and Coupar, 1988) and canarypoxvirus (Commercially known as ALVAC; Taylor *et al.,* 1988) have shown potential to be safe and efficient vaccine vectors for use in both humans and animals. Both fowlpoxvirus and canarypoxvirus can be grown in avian cells but late gene expression does not occur in mammalian cells (Tartaglia *et al.,* 1997; Somogyi *et al.,* 1993). Tartaglia *et al.* (1990) document that ALVAC displays an abortive phenotype that substantially reduces host protein expression by 6 hours post infection in cells of human origin.

3.1.5. Recombinant Avipoxviruses

Avipoxviruses such as fowlpox and canarypox are well known for their ability to only replicate in avian species. It was discovered that recombinant host-restricted viruses, when inoculated into mammalian hosts, could express their inserted gene and consequently confer protection (Taylor and Paoletti, 1988; Taylor *et al.,* 1988). Taylor *et al.* (1991) found that, for some unknown reason, the canarypox vector elicits a better immune response to the inserted antigen than does the fowlpox vector and one similar to that induced by VV.

Efficacy studies on a canarypox-rabies recombinant virus (Cadoz *et al.,* 1992 and Taylor *et al.,* 1991) demonstrated convincing protection and high levels of neutralising antibodies in mice, dogs and cats following challenge with live RV. Fowlpox virus - rabies virus recombinants proved not to be as effective as their

canarypoxvirus counterpart; however, protection from challenge in mice was achieved (Taylor *et al.*, 1991).

Canarypox recombinants expressing measles virus glycoproteins, equine influenza virus haemagglutinin protein, or feline leukemia virus *gag* have been quite successful on the veterinary front. Medically Canarypoxvirus vectors have been used to express HIV-1 and HIV-11 antigens and are showing great promise as cell-mediated immunity inducers (Paoletti, 1996).

3.1.6. Poxvirus promoters

The poxvirus RNA polymerase only recognises poxvirus promoters. These are regulatory sequences for initiation of transcription and they occur upstream of the RNA start sites. Detailed studies of promoters of poxviruses other than vaccinia virus have not yet been completed. However, avipoxvirus promoters apparently can function in vaccinia virus and vice versa (Kumar and Boyle, 1990; Boyle and Coupar, 1988; Taylor *et al.*, 1988). This suggests that promoter sequences amongst poxviruses are relatively conserved. Promoters function as early, late or early/late promoters, which allow gene products to be expressed at different times in the replication cycle depending on the controlling promoter. Most VV promoters are approximately 30bp in length and the most commonly used natural promoters, H5 and 7.5K (P7.5) consist of both early and late promoter elements. According to a review by Ring and Blair (2001), host restricted viruses demonstrate little or no late gene expression or DNA

replication in non-permissive cells, however, the majority do inhibit cell division and cause subsequent cell death.

In this part of the study the role of a late (P11) and an early/late (P7.5) poxvirus promoter was investigated in cells of ruminant and non-ruminant origin using transient expression assays. DNA replication was examined and a subsequent electron microscopic examination of those cells infected with rLSDV-RG provided information pertaining to the host restriction characteristic of LSDV. The humoral and cellular immune responses to rLSDV-RG were then examined in mice and the humoral immunity was evaluated in rabbits. Recombinant LSDV-RG was then evaluated as a rabies vaccine in comparison to a commercially used rabies vaccine in a live RV challenge experiment.

3.2. MATERIALS AND METHODS

3.2.1. Poxvirus promoter study

The ability of LSDV to express foreign genes under control of different promoters in infected permissive and non-permissive cells was assessed in a transient expression system (MacGregor and Caskey, 1989) using plasmids in which the reporter gene *lac Z* is under the transcriptional control of various VV promoters. The late promoter P11, on plasmid PAL1 (supplied by M.Mackett from Paterson Laboratories) and the early/late promoter P7.5, on plasmid PSC65, a VV shuttle vector, were transfected into LT, MDBK and CV-1 cells and their ability to express β-galactosidase was assayed using the X-gal stain (Appendix B3).

3.2.2. Stain for Cytoplasmic DNA replication

Hoechst's 332558 fluorescent stain, as described by Russel *et al.* (1975), (Appendix B8) was used to identify cytoplasmic DNA in infected cell monolayers at 24 hours post LSDV infection.

3.2.3. Preparation of Samples for Electron Microscopy.

Duplicate cultures of CV-1, MDBK, and LT cells were grown overnight to a confluency of 70% in 5 ml culture flasks before being infected with rLSDV-RG. Each culture flask was infected with 1 ffu/cell of recombinant virus and grown for another 48 hours. Cells were subsequently removed from the culture flasks using a rubber policeman, centrifuged at 250 g for 10 minutes to remove culture medium, and washed similarly with PBS. The pellets were stabilised in agarose and fixed in gluteraldehyde. Ultra-thin sections were prepared of rLSDV-RG-infected CV-1 cells, MBDK cell, and LT cells harvested 48 hrs post infection (p.i.). Cell cultures were rinsed three times with PBS, fixed in 2% gluteraldehyde for 2 hrs at 4°C, post-fixed in 1% OsO_4, then dehydrated and embedded in Spurr's resin by conventional methods. Ultra-thin sections were post-stained with uranyl acetate and lead citrate before examination with the electron microscope (Hitachi-600).

3.2.4. Evaluation of antibody responses in mice and rabbits.

(UCT Research Ethics Committee approval reference: 97/024)

Rabbits were inoculated intramuscularly (IM) or intradermally (ID) with $1x10^5$ ffu/ml of rLSDV-RG on week 0, week 4 and week 9. Rabbits inoculated with Verorab and Rabisin (Pasteur Merieux, France) were inoculated IM with 0.5 ml of inoculum (1 equivalent human/canine dose) at week 0, and week 4. Blood was taken every two weeks from all the rabbits and serum stored at -70°C until tested. Enzyme linked immunosorbent assays (ELISAs) were performed on serum to measure the amount of anti-rabies antibodies present in each serum sample. The Trousse Platelia Rage ELISA kit was used (Diagnostics Pasteur, France) according to the manufacturer's specifications. Neutralisation assays were performed at Onderstepoort Veterinary Institute (Onderstepoort, South Africa) on selected rabbit serum samples (according to the methods of Cliquet *et al.*(1998) to ascertain whether or not the antibodies neutralise live rabies

virus in a cell culture system. Controls included rabbits inoculated with tissue culture medium (DMEM); rabbits inoculated with LSDV (Neethling vaccine strain) wild type (LSDV-wt) and unvaccinated rabbits.

BALB/c mice (8-12 weeks old, Animal Unit, University of Cape Town, 3 mice/group) were inoculated ID on day 0 and day 28 (after four weeks) with rLSDV-RG ($5x10^4$ ffu/ml in 50 μl), Verorab (50 μl; 1/10 human equivalent dose) or Rabisin (50 μl; 1/10 canine equivalent dose). A group that was unvaccinated was monitored as a negative control. Blood samples were taken from the tail vein every two weeks. The blood was pooled within each group. The serum was prepared and analysed as was outlined for the rabbit serum above.

3.2.5. Lymphoproliferation assay in mice.

(UCT Research Ethics Committee approval reference: 97/024)

BALB/c mice (8-12 weeks old, Animal Unit, University of Cape Town, 3 mice/group) were inoculated ID on day 0 and day 28 with PBS (50 μl), rLSDV-RG ($5x10^4$ ffu/ml in 50 μl), LSDV-wt ($5x10^4$ ffu/ml in 50 μl), Verorab (50 μl; 1/10 human equivalent dose) or Rabisin (50 μl; 1/10 canine equivalent dose). On day 38, the mice were sacrificed and splenocytes were isolated by passage through a steel mesh (Sigma, Germany) to obtain a single cell suspension. Contaminating red blood cells were removed by centrifugation over Ficoll-Hypaque density gradients (Sigma, Germany). Freshly isolated splenocytes were seeded in quadruplicate into round-bottomed 96-well culture plates (2 x 10^5 cells/well; Nunc, Denmark) and incubated in the presence of inactivated rabies virus (2 virus particles/cell; State Vaccine Institute, Cape Town, South Africa) or unstimulated for 6 days at 37°C, 5% CO_2. [^{3}H]-thymidine (1 μCi per well, Sigma, Germany) was added to each well for the last 18 h of the assay. Cells were harvested using an automated cell harvester (PHD, Cambridge Technology) and the radioactivity was measured by using a liquid scintillation counter (Tricard-4640).

3.2.6. Immunophenotyping, intracellular cytokine staining and flow cytometry in mice. (Research Ethics Committee approval reference: 97/024)

BALB/c mice (8-12 weeks old, Animal Unit, University of Cape Town, 2 mice/group) were inoculated as described in section 3.2.5 with PBS, LSDV-wt, rLSDV-RV, Rabisin or Verorab and further boosted on day 56. Splenocytes were prepared as described and cells from each group were pooled. For immunophenotyping, the following panel of monoclonal antibodies (mAb) was used: CD3-APC, CD4-FITC, CD8-FITC, CD19-FITC, interferon (IFN)-γ-PE (all from Caltag, USA or Pharmingen, USA). Freshly isolated splenocytes (2-3 x 10^6/ml) were incubated with the mAb for 30 minutes on ice, washed with 10% fetal calf serum (FCS) PBS containing 0.01% NaN_3, and fixed with 1% paraformaldehyde in PBS. Isotypic control antibodies were used in all cases and all antibodies were used at the concentrations suggested by the manufacturer.

For intracellular cytokine staining, splenocytes were stimulated in 1 ml cultures (2-3 x 10^6 cell.ml^{-1}) in 24 well plates with either PMA/ionomycin (25 ng.ml^{-1}/1 μg.ml^{-1}, 4 hours), inactivated rabies virus (2 particles/cell, 20 hours, State Vaccine Institute, South Africa) or untreated at 37°C, 5% CO_2. Brefaldin A (10 μg.ml^{-1}; Sigma, Germany) was added after the first hour of incubation. Following stimulation, cells were washed once in 10% FCS PBS +0.01% NaN_3, stained with APC-labeled CD3 (Caltag, USA), and FITC-labeled CD4 or CD8 mAb (Caltag, USA) for 30 minutes on ice, washed once, and fixed for 10 minutes in 1% paraformaldehyde PBS. Cells were washed again with 10% FCS PBS +0.01% NaN_3 and then permeabilised using 0.1% Saponin (Fluka, South Africa) in PBS (containing 0.01% NaN_3). PE-conjugated anti-IFN-γ (Pharmingen, USA) was added immediately and cells were incubated on ice for 30 minutes. Finally, stained cells were washed once with 0.1% Saponin/PBS, once with PBS and fixed with 1% paraformaldehyde PBS. Cell fluorescence was measured using a FACSCalibar flow cytometer and data was analyzed

using CellQuest software (Becton Dickinson, USA).

3.2.7. Rabies virus potency test.

NMRI mice (3-4 weeks of age, South African Vaccine Producers, 4 mice/group) were held in an isolated animal unit at the State Vaccine Institute (Pinelands, Cape Town). All personnel handling the mice were rabies immune and prior approval from the ethics committee was granted (UCT Research Ethics Committee approval reference: 01/16). Care was taken that no material was removed from the holding unit until it had been incinerated. Wherever possible, disposable equipment was used. Equipment that was not incinerated was disinfected in an 80% iodine solution for 24 hours. Mice that succumbed to RV were incinerated after brain impressions had been taken and evaluated (refer to Appendix E).

Mice were inoculated intracranially (IC) or intramuscularly (IM) with various dilutions of CVS-11 (Rabies Unit, Onderstepoort Veterinary Institute, South Africa supplied at $4.8x10^1$ LD_{50}) as indicated in table 3.1.

The mice were monitored for 16 days post challenge to ascertain whether or not the IM route of inoculation would be potent enough to use in the rabies challenge experiment and which dose the intracranial challenge would give the most reproducible results. Mice that survived the full 16 days were euthanased and incinerated.

The dose used for the challenge experiment (section 3.2.8) was determined to be the most feasible using this potency test.

Table 3.1. Innoculation schedule of mice used to calculate challenge virus potency for rabies challenge experiment.

Group	Cage	Sex	Number	Route	Dose CVS-11 (in 30µl)
1a	1	male	4	IC*	$2.8x10^{1}$ LD_{50}***
1b	2	female	4	IC	$2.8x10^{1}$ LD_{50}
2a	1	male	4	IC	$1.8x10^{1}$ LD_{50}
2b	2	female	4	IC	$1.8x10^{1}$ LD_{50}
3a	1	male	4	IC	$8x10^{-1}$ LD_{50}
3b	2	female	4	IC	$8x10^{-1}$ LD_{50}
4a	1	male	4	IC	$8x10^{-2}$ LD_{50}
4b	2	female	4	IC	$8x10^{-2}$ LD_{50}
5a	1	male	4	IC	$8x10^{-3}$ LD_{50}
5b	2	female	4	IC	$8x10^{-3}$ LD_{50}
6a	1	male	4	IM**	$3.8x10^{1}$ LD_{50}
6b	2	female	4	IM	$3.8x10^{1}$ LD_{50}
7a	1	male	4	IM	$2.8x10^{1}$ LD_{50}
7b	2	female	4	IM	$2.8x10^{1}$ LD_{50}
8a	1	male	4	IM	$1.8x10^{1}$ LD_{50}
8b	2	female	4	IM	$1.8x10^{1}$ LD_{50}
9a	1	male	4	IM	$8x10^{-1}$ LD_{50}
9b	2	female	4	IM	$8x10^{-1}$ LD_{50}
10a	1	male	4	IM	$8x10^{-2}$ LD_{50}
10b	2	female	4	IM	$8x10^{-2}$ LD_{50}

$8x10^{-2}$ LD_{50} is interchangeable with $10^{0.08}LD_{50}$

* intracranial injection of live RV (CVS-11)

** intramuscular injection of live RV (CVS-11)

*** 50% lethal dose (ie. The dose where 50% of the mice would die from an intracranial challenge of live RV [CVS-11]).

3.2.8. Rabies virus challenge experiment.

(UCT Research Ethics Committee approval reference: 01/16)

NMRI mice (3-4 weeks old, South African Vaccine Producers; 30 mice/group) were held under conditions outlined in 3.2.7. Mice were inoculated IM on day 0 and day 14 with either PBS (50 µl), rLSDV-RG ($5x10^4$ ffu/ml in 50 µl), LSDV-wt ($5x10^4$ ffu/ml in 50 µl), or Verorab (1/10 human equivalent dose in 50 µl). On day 21, ten mice from each group were challenged intracranially (IC) with $8x10^{-1}$ LD_{50} LD_{50}, $8x10^{-2}$ LD_{50} or $8x10^{-3}$ LD_{50} dilution of live rabies virus (CVS-11; supplied by Onderstepoort Veterinary Institute's Rabies Unit, South Africa; refer to section 3.3.7). All mice that died within the first five days post-challenge were considered non-rabies related deaths (Seligman, 1973). Uncertain deaths were screened using a fluorescent antibody test of a brain impression (Dean *et al.*, 1996; Appendix E). Survival was monitored between days 5 and 14. During the monitoring period, mice that displayed advanced rabies symptoms were euthanased and considered to be rabies positive.

3.3. RESULTS

The following results evaluate LSDV as a vaccine vector in non-ruminant hosts. They are important in investigating certain properties of the vector such as its ability to express inserted genes in non-ruminant hosts, the level of replication attained in non-ruminant hosts as compared with that in ruminant hosts, and most importantly, its ability to illicit an immune response to the inserted gene in non-ruminant hosts. Having ascertained that a humoral and cellular immune response is elicited in cattle by rLSDV, we undertook to evaluate the immunity raised in non-ruminants to further assess the vector.

3.3.1. Promoter study

The aim of this study was to determine whether non-ruminant cells with either an early/late or a late promoter were able to express inserted genes and to

compare the results with the same experiment carried out in ruminant (permissive) cells. LT, MDBK and CV-1 cells were transfected with plasmids carrying the *lac Z* reporter gene under the control of either the late VV promoter P11 or the early/late VV promoter P7.5. The transfection medium was changed to standard tissue culture medium after eight hours and the cultures were assayed for their ability to express the *lac Z* reporter gene using the x-gal stain after twenty-eight hours. No endogenous β-gal activity was evident in the three cell types (uninfected controls; data not shown). Table 3.2 summarizes the results of the transient expression assay performed on LSDV-infected LT, MDBK and CV-1 cells.

Table 3.2. Transient expression assays

		Cell Type		
Plasmid	Promoter	**LT**	**MDBK**	**CV-1**
PAL1	**P11**	√	√	√
PSC65	**P7.5**	√	√	√

X-indicates that no β-gal expression was detected

√- Indicates that β-gal expression was detected

3.3.2. Stain for cytoplasmic DNA replication

The aim of this study was to determine whether or not viral DNA replication takes place in the cytoplasm of LSDV-infected non-permissive cells. Once again permissive cells were used as a comparison. Hoechst 33258 is a DNA fluorochrome, which specifically binds to the adenine-thymidine regions of DNA. Fixing the cells in cold acetone makes the membranes permeable and allows the stain to enter the cells and stain any available DNA in the cytoplasm of cells. Poxviruses are known to replicate in the cytoplasm and form virus factories.
Figure 3.1 is a collection of photographs showing the results obtained. DNA replication is evident in CV-1, MDBK and LT cells when infected with rLSDV-RG.

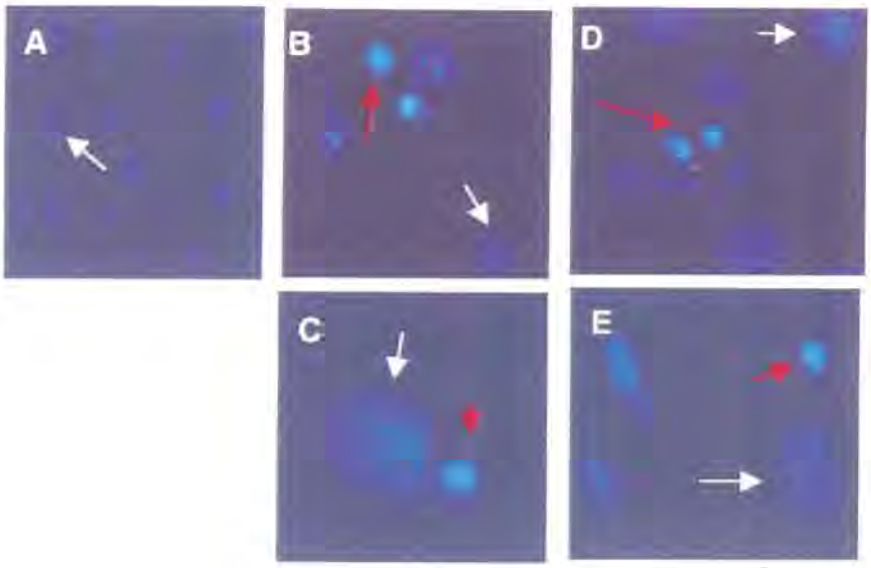


Figure 3.1. Hoechst stained cells to detect DNA. Areas of DNA replication are absent in uninfected CV-1 cells (A, 100x mag), evident in rLSDV-RG – infected CV-1 cells (B,100x mag), evident in rLSDV-RG – infected LT cells (C, 400x mag) and evident in rLSDV-RG – infected MDBK cells (D and E, both 100x mag) at 48 hrs post infection. White arrows indicate the nuclei of cells and red arrows indicate possible viral factories where DNA replication is taking place.

3.3.3. Electron microscopic examination of rLSDV-RG – infected permissive and non-permissive cells.

Having established that both DNA replication and late transcription of foreign genes occur in permissive and non-permissive cells infected with recombinant LSDV, we undertook to examine the ultrastructure of the same cell types infected with rLSDV-RG. Late transcription involves the translation of viral mRNAs into viral proteins, which implies that in an ultrastructural study, viral particles should be visible. The promoter study indicated that the P7.5 promoter used in the LSDV shuttle vector to express the inserted gene is functional in non-permissive cells. On the basis of these findings, we expected to find LSDV particles in the cytoplasm of all infected cells and that infection of these cells with rLSDV-RG would result in expression of the RG in these cells. Such expression of the RG should then induce an immune response to the RG in

ruminants as well as non-ruminants. This study was designed to visualize the rLSDV-RG in various cell types.

In all three cell-types, the replication of rLSDV-RG began with membrane crescent formation in electron dense areas adjacent to the nucleus. These areas are typically referred to as "viroplasms or viral factories" (Muller and Williamson, 1987; Prozesky and Barnard, 1982; Stannard *et al.,* 1998). In the permissive MBDK and LT cells, and the non-permissive CV-1 cells, replication of rLSDV-RG advanced rapidly and the crescents became filled with nucleic acid material. This material subsequently condensed as the crescents closed to form spherical virions. In permissive cells (LT and MDBK; figure 3.2A,B,C,D and E), the spherical virions condensed further and became oblong in shape. Some of these more mature intracellular virions acquired a second membrane by budding through intracellular vacuoles, others moved to the cell membrane and budded out to form extracellular virions with double membranes. Figures 3.2.A and B are representative of rLSDV-RG infections in ovine LT cells where the level of replication is quite advanced (viral particles are condensed and oblong in shape). Figures 3.2.C, D, and E are representative of rLSDV-RG infections in the permissive MDBK cells of bovine origin. These display evidence of advanced infection as demonstrated by intra- and extracellular virions, many of which have multiple membranes. rLSDV-RG replication in non-permissive, primate CV-1 cells proved not to be complete as virions were not oblong and did not appear to leave the cell (Figure 3.2F). A diagrammatic representation of the differences between reproduction levels observed in rLSDV-RG-infected CV-1 and MBDK cells, respectively is given in Figure 3.3.

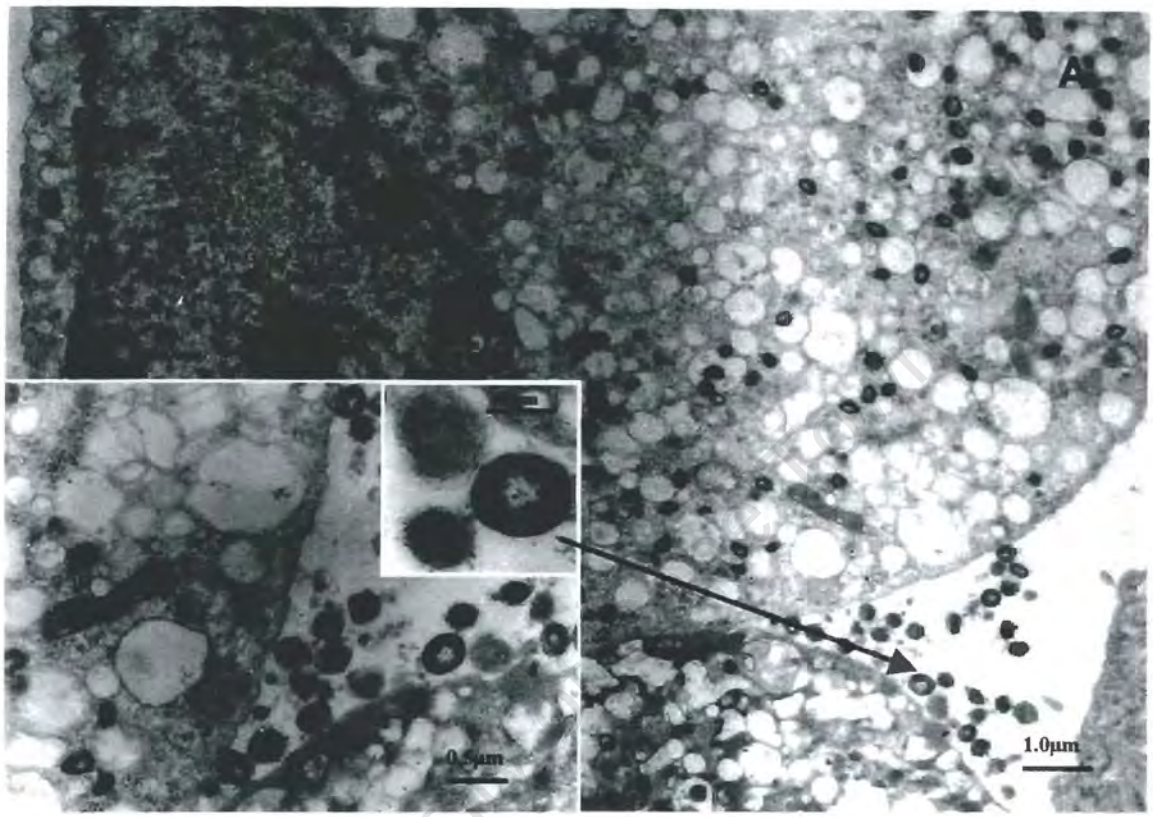


Figure 3.2A. Morphogenesis of recombinant LSDV within permissive cells. Ovine LT cells were infected with rLSDV-RG (pfu/cell, time, 6000x mag). Electron micrograph demonstrates mature virions inside and out side the cell. The nuclear material in the virion is condensed. The inserts (6600x and 60 000x mag) show high power virion structure where multiple membranes are visible.

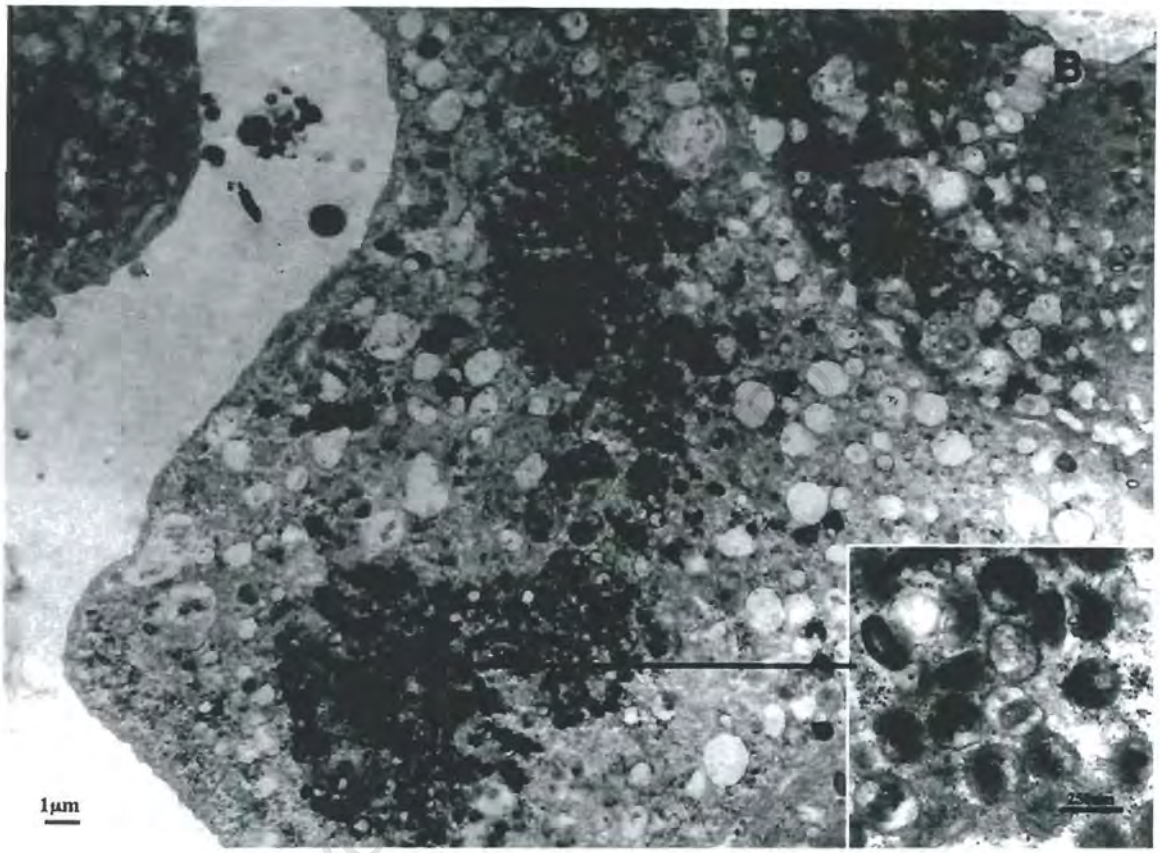


Figure 3.2B. Morphogenesis of recombinant LSDV within permissive ovine cells. LT cells infected with rLSDV-RG (6600x mag) showing evidence of a severely infected cell. Insert (26 000x mag) demonstrating virions at different stages of maturation present in "viral factories".

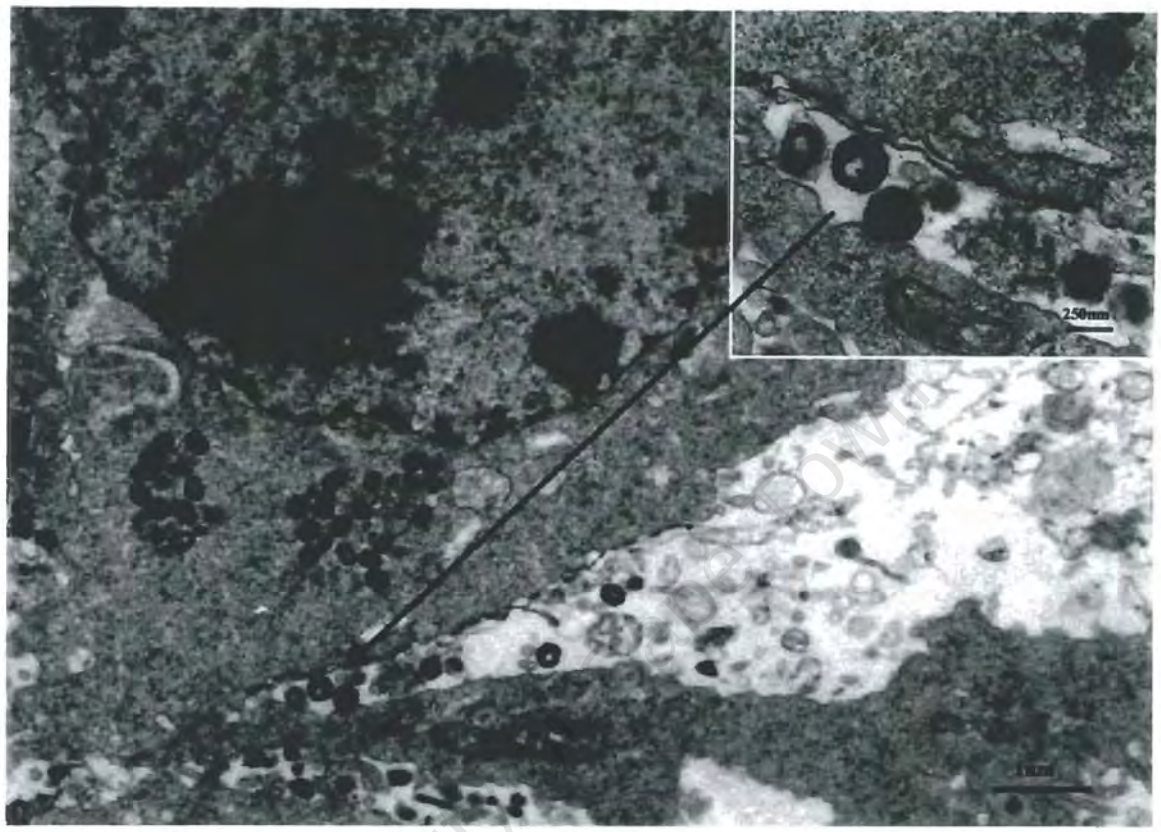


Figure 3.2C. Morphogenesis of recombinant LSDV within permissive bovine cells. rLSDV-RG-infected bovine MDBK cell (pfu/cell, time, 6600x mag) showing intracellular and extracellular virions in an advanced stage of maturation. (Insert 26 000 x mag). The mature virions are condensed and oblong in shape.

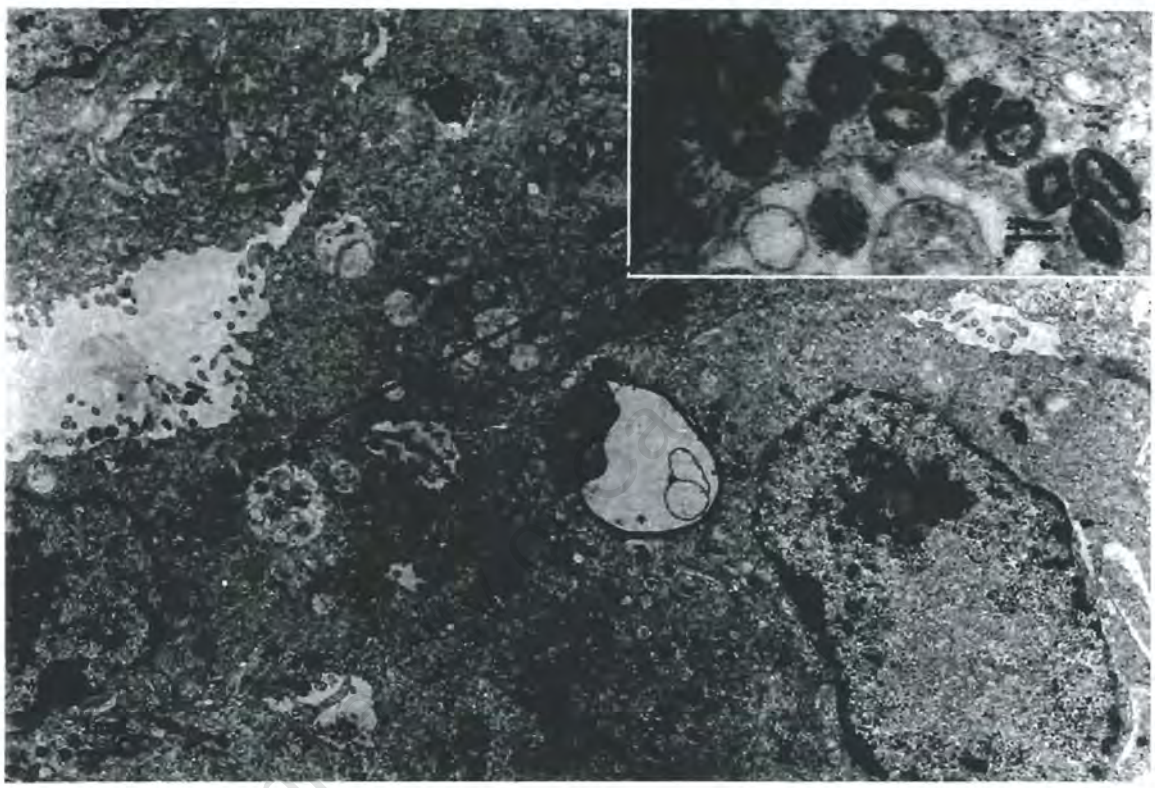


Figure 3.2D. Morphogenesis of recombinant LSDV within permissive bovine cells. rLSDV-RG-infected bovine MDBK cell (pfu/cell, time, 6600x mag) showing intracellular and extracellular virions in an advanced stage of maturation. (Insert 26 000 x mag). The mature virions have multiple membranes.

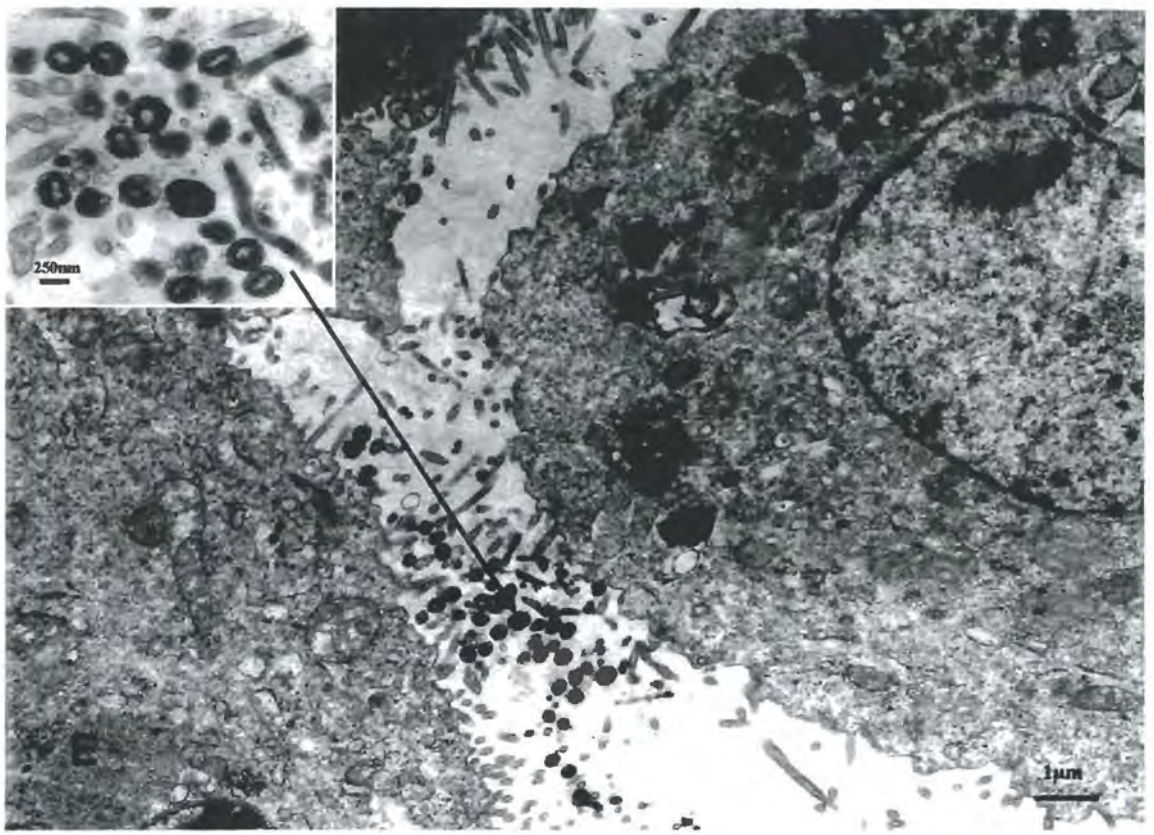


Figure 3.2E. Morphogenesis of recombinant LSDV within permissive bovine cells. rLSDV-RG-infected bovine MDBK cell (pfu/cell, time, 6600x mag) showing mainly extracellular virions in an advanced stage of maturation. (Insert 26 000 x mag).

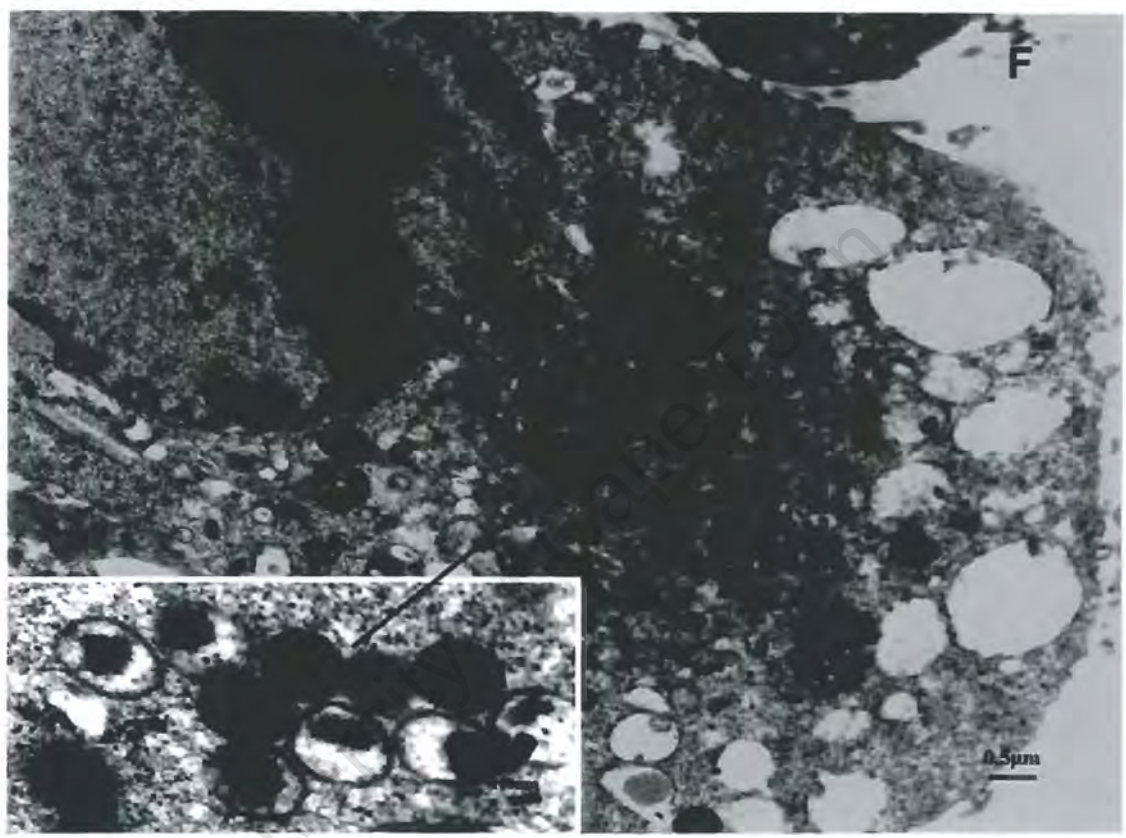


Figure 3.2F. Morphogenesis of recombinant LSDV within non-permissive (primate CV-1 cells) CV-1 cells were infected with rLSDV-RG (pfu/cell, time, 9000 x magnification). The insert is a 40 000 x magnification of the "viral factory". Early stages of viral maturation are evident here. The virions are circular with distinct areas of nuclear material. Very few virions reach the condensed stage and none were seen extracellularly. Only single membranes are visible in these virions.

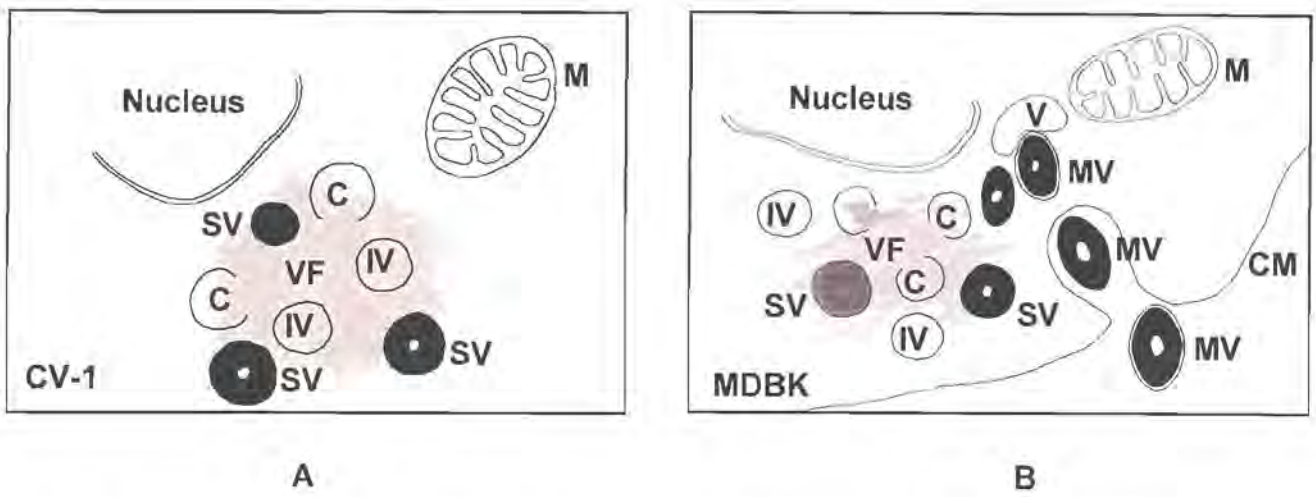


Figure 3.3. A diagrammatic representation of rLSDV-RG replication in (A) non-permissive CV-1 cells and (B) permissive MDBK cells. (M) indicates mitochondria, , which appear to have swollen cristae in both permissive and non-permissive infected cells, (C) indicates crescent-shaped membrane which precedes immature virion formation, (V) indicates vacuoles/intracellular membranous structure, (VF) indicates viral factory and electron dense areas where replication and maturation occurs, (IV) indicates immature virion, (MV) indicates mature virion, (CM) indicates cell membrane, and (SV) indicates semi-mature virion.

Although the morphology of rLSDV-RG is not identical to that of vaccinia virus, there seem to be similarities between the replication cycle of rLSDV-RG in "permissive cells" and vaccinia virus. In MBDK and LT cells, rLSDV-RG replicated to a more "mature" state than it did in CV-1 cells. This fact supports work done by Weiss (1968) and Young *et al.*(1977) stating that LSDV is species specific and that its replication is limited to ruminants. However, it can be seen in figures 3.2 and subsequently 3.3 that virions do enter non-ruminant cells and early replication does occur. This is the first electron microscopic examination of recombinant LSDV infections in cell culture, specifically in cells of non-ruminant origin. In this study, the degree of rLSDV-RG maturation within CV-1 cells, MDBK cell and LT cells was examined by thin-section electron microscopy and compared. In the bovine (MDBK) cells and LT cells, the replication cycle of rLSDV-RG appeared to be complete in that mature virions were observed inside and outside the cells (Figures 3.2. A,B,C,D,and E). These virions were more electron dense and less circular than those considered

"immature". In the case of rLSDV-RG-infected CV-1 cells, the virions did not reach the same level of maturity (Figure 3.2.F) in that they remained circular and were less electron dense. They were not observed outside the cells and the overall numbers of virions seen were smaller.

Table 3.3 summarizes the results obtained in this section.

Table 3.3.

Summary of assessment of LSDV as a vaccine vector in cells from permissive and non-permissive hosts.

Cell Type	Viral DNA replication	Expression from late pr**	Expression from E/L pr***	Expression of *lac Z**	Stage of viral Maturation
LT Permissive	yes	yes	yes	yes	Mature
MDBK Permissive	yes	yes	yes	yes	Mature
CV-1 Non-permissive	yes	yes	yes	yes	Virus particles with no maturation

*rLSDV-RG expression of *lac Z* reporter gene under control of P7.5 promoter in these cells.

** Vaccinia virus P11 late promoter.

*** Vaccinia virus P7.5 early/late promoter

3.3.4. Evaluation of RG-specific antibody responses in mice and rabbits.

Antibodies that react with RG antigen on ELISA were detectable in all of the rabbits immunised with rLSDV-RG, but not in unvaccinated rabbits following the first booster inoculation (Figure 3.4). The World Health Organization has defined the critical antibody level necessary for protection to be ≥0.5 IU/ml (unit equivalent to the international units defined by seroneutralisation; Cliquet *et al.* (1998); Dutta *et al.*, 1992). This level of RV-specific antibodies was attained in all rLSDV-RG-immunised rabbits tested after the first booster inoculation and increased substantially after the second booster inoculation. Similar levels of RV-specific antibodies were elicited in rabbits by rLSDV-RG irrespective of the

route of immunisation (IM *versus* ID). Rabbits immunised with the commercially available subunit vaccines (Verorab and Rabisin) generated similar levels of RV-specific antibodies as the rLSDV-RG–immunised rabbits. However, the responses to the commercial vaccines were detectable immediately following the primary immunisation whereas rabbits immunised with rLSDV-RV required a booster. None of the PBS, LSDV-wt, or tissue culture medium (DMEM)-inoculated control animals produced detectable antibodies to rabies antigen. Neutralising antibody titres were not determined in Verorab and Rabisin immunised rabbits.

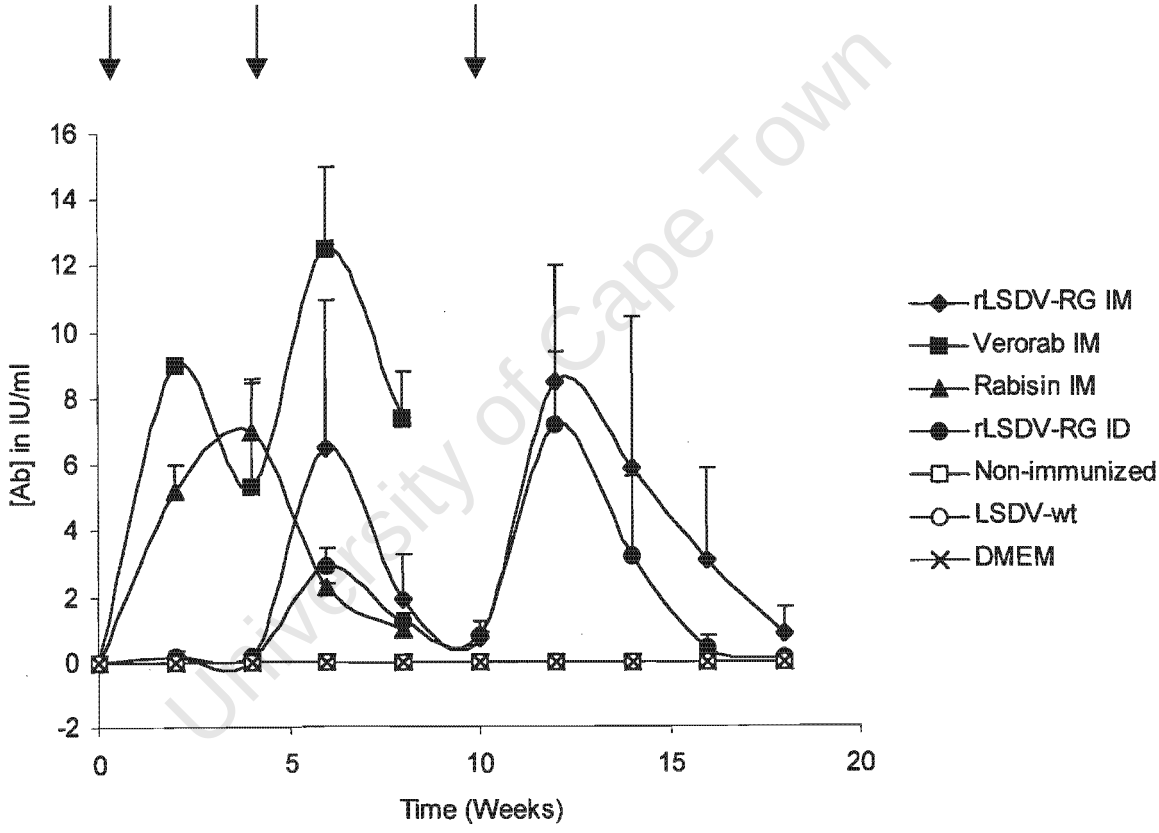


Figure 3.4. RV-specific antibody responses induced following intramuscularly (IM) and intradermal (ID) immunisation of rabbits with rLSDV-RG, Verorab and Rabisin. Rabbits were inoculated with $1x10^5$ffu/ml rLSDV-RG IM (♦) or ID (●), or with Verorab (■) and Rabisin (▲) (1 equivalent human or animal vaccination; IM). Arrows indicate times of inoculation.

The antibody responses of two of the rLSDV-RG animals (one IM and one ID) were tested for their ability to neutralise live RV (Table 3.4). By 12 weeks post-immunisation, both were found to have high titres of neutralising antibodies, whereas the control animals (PBS, LSDV-wt, or tissue culture medium (DMEM)-inoculated control animals) did not.

Table 3.4.

Results of Neutralisation Assay on Rabbit pre-immune and immune serum after immunisation with rLSDV-RG.

Route	Immunisation	Time (Weeks)	Dose *	Neutralisation antibody titre [IU/ml]
-	Unimmunised	0	-	0
		12	-	0
ID	rLSDV-RG	0	$1x10^5$ ffu/ml	0
		12	$1x10^5$ ffu/ml	1.35[a]
IM	rLSDV-RG	0	$1x10^5$ ffu/ml	0.17[a]
		12	$1x10^5$ ffu/ml	1.35[a]

[a] The 0.17 IU/ml titre is non-specific while those of 1.35 IU/ml are considered protective (Cliquet *et al.*, 1998).

* Test rabbits received one inoculation and two booster inoculations of rLSDV-RG while control rabbits remained unvaccinated.

BALB/c mice vaccinated with rLSDV-RG failed to produce measurable antibodies to rabies virus despite a booster inoculation. The commercial inactivated vaccines (Verorab and Rabisin) induced good humoral responses in mice; however, the 0,5IU/ml level was only attained after a booster inoculation (See figure 3.5).

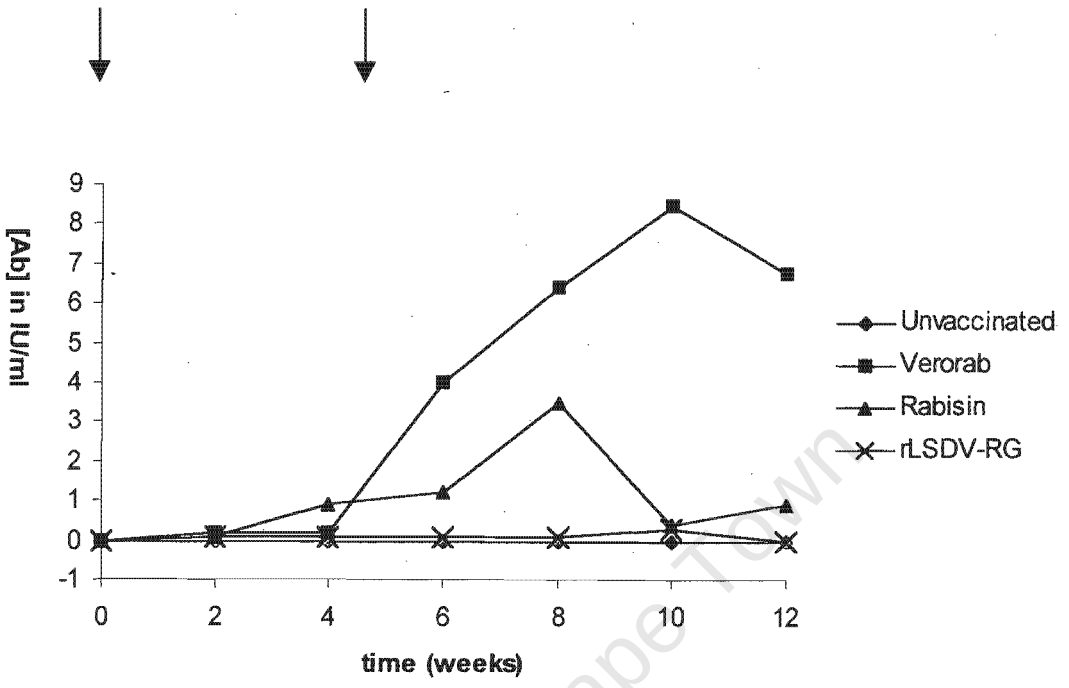


Figure 3.5. RV-specific antibody responses induced following intramuscularly (IM) immunisation of mice with rLSDV-RG, Verorab and Rabisin. Mice were inoculated with $5x10^4$ffu/ml rLSDV-RG IM (x), with Verorab (■), or with Rabisin (▲) (1/5 equivalent human or animal vaccination; IM). Arrows indicate times of inoculation for all groups of mice.

3.3.5. rLSDV-RG-immunisation induces RV-specific T cell proliferative activity in mice.

To investigate the ability of rLSDV-RG to induce cell-mediated immune (CMI) responses to rabies antigens, T-cell proliferative activity was investigated following immunisation. Splenocytes from mice immunised with rLSDV-RG showed good proliferation ($p=0.02$, when compared to LSDV-wt-inoculated

mice) to inactivated rabies virus that was comparable to levels induced by Verorab ($p=0.05$, when compared to LSDV-wt-inoculated mice; Figure 3.6). In comparison, splenocytes from Rabisin-immunised mice showed poor T-cell responsiveness to RV recall antigen that was similar to the level measured following immunisation with LSDV-wt ($p=0.2$). No RV proliferative response was found in PBS-immunised control mice. Phenotypic analysis of splenocytes isolated from immunised mice did not reveal any significant difference in the sub-populations present with $CD19^+$ B cells making up the dominant population (Table 3.5) with the exception of Verorab. Mice immunised with Verorab showed a 4-fold expansion of $CD4^+$ T cells in the spleen that was associated with a 5-fold reduction in $CD19^+$ B cells. Despite demonstrating a marked expansion of $CD4^+$ T cells, these animals did not generate T cell-specific proliferative responses following RV-stimulation that was significantly different to rLSDV-RG-immunised mice.

Table 3.5.

Immunophenotyping of mouse splenocyte populations. Ratios in relation to the numbers of "PBS" cells are shown in brackets.

Immunisation	CD4/CD3+ cells	CD8/CD3+ cells	CD19+ cells
PBS	19.69	6.98	66.68
LSDV-wt	22.10 (1.1)	6.68 (1.0)	57.18 (0.9)
rLSDV-RV	22.87 (1.2)	5.22 (0.7)	60.16 (0.9)
Rabisin	24.17 (1.2)	8.08 (1.2)	58.97 (0.9)
Verorab	79.02 (4.0)	1.98 (0.3)	11.92 (0.2)

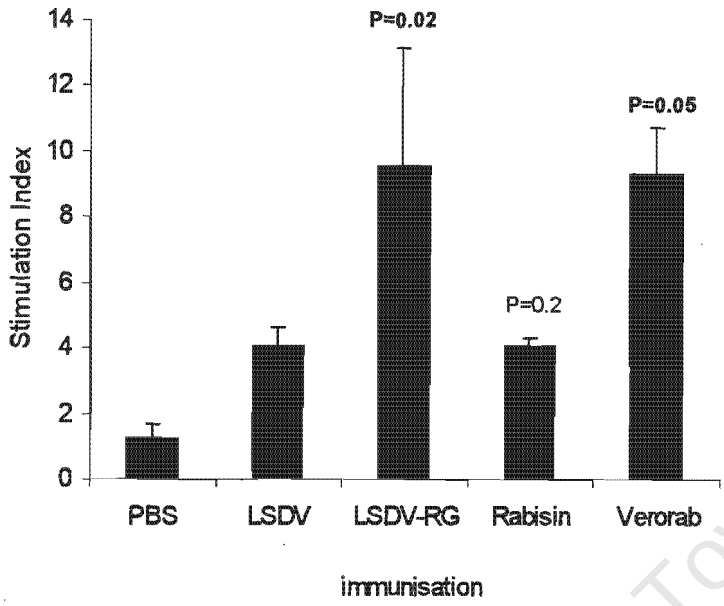


Figure 3.6. rLSDV-RV immunisation induces strong T cell proliferative responses to inactivated rabies virus following *in vitro* restimulation. BALB/c mice (8-12 weeks) were immunised twice (day 0 and 28) with either PBS, LSDV-wt, rLSDV-RV, Rabisin or Verorab and *in vitro* splenocyte proliferation to either no antigen (control) or inactivated rabies virus (2 particles/cell; test wells) determined by [^{3}H]-thymidine incorporation. Results have been expressed as stimulation index and were calculated as follows: [cpm of test wells]÷[cpm of control wells]. Each bar represents the mean stimulation index (± SD) of quadruplicate wells. Significant "p" values are shown in bold lettering.

3.3.6. Intracellular cytokine staining for IFN-γ following rLSDV-RG immunization in mice.

To examine *in vivo* cytokine expression in splenocytes isolated from rLSDV-RG-immunised BALB/c mice, we performed single cell analysis on either antigen (inactivated rabies virus)-stimulated cells or cells activated with PMA/ionomycin *ex vivo*. Figure 3.7A shows a typical intracellular cytokine staining result from a rLSDV-RV-immune spleen. PMA/ionomycin-induced levels of IFN-γ were high for both $CD4^+$ and $CD8^+$ T cells. Although antigen-specific rabies IFN-γ

responses were generally lower than levels measured following PMA/ionomycin stimulation, detectable amounts of IFN-γ were measured in the splenocytes from rLSDV-RV immune mice. $CD8^+$ T cells in particular (as well as $CD4^+$ T cells to a lesser extent) from rLSDV-RG-immune animals showed high levels of intracellular IFN-γ staining following *in vitro* stimulation with rabies antigen compared with Rabisin immune splenocytes and similar levels compared with Verorab splenocytes (Figure 3.7B). Splenocytes from PBS- or LSDV-wt immune animals, in contrast, showed only low levels of IFN-γ production following antigen-specific stimulation while maintaining strong responses to PMA/ionomycin activation.

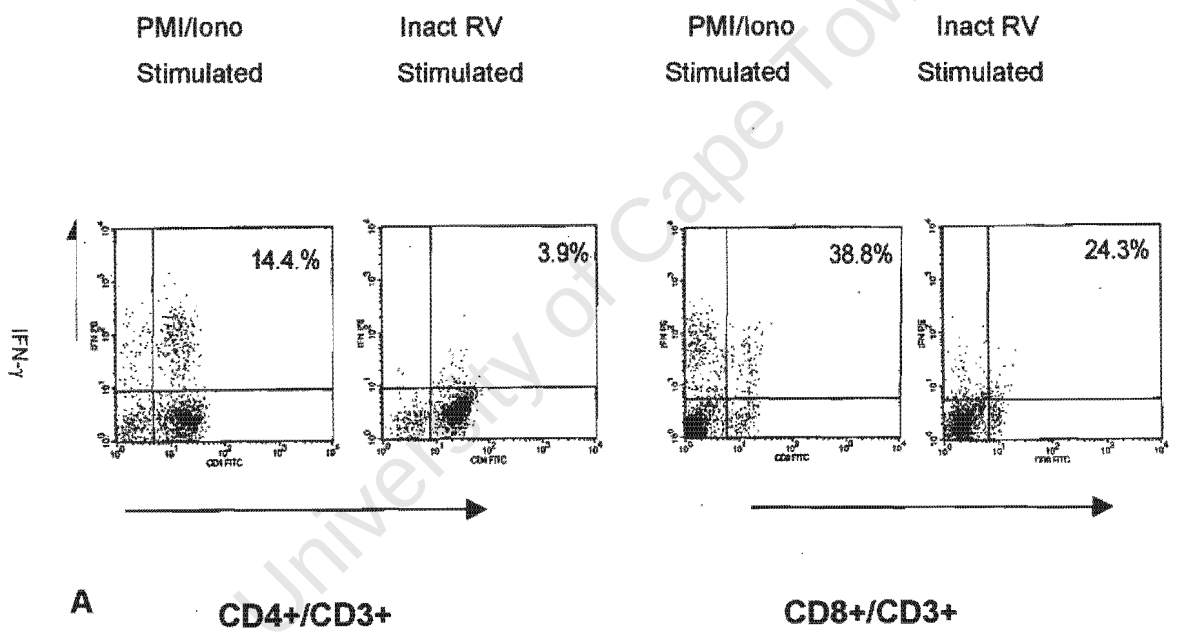


Figure 3.7A Intracellular cytokine staining for IFN-γ responsiveness following *in vitro* re-stimulation with inactivated rabies virus. BALB/c mice (8-12 weeks) were immunised twice (day 0 and 56) with either PBS, LSDV-wt, rLSDV-RG, Rabisin or Verorab and *in vitro* splenocyte cytokine responses to either no antigen (unstimulated; 4 or 20 hours), PMA/Ionomycin (25 $ng.ml^{-1}$/1 $\mu g.ml^{-1}$, 4 hours), inactivated rabies virus (20 hours, 2 virus particles/cell). Brefaldin A (10 $\mu g.ml^{-1}$) was added after the first hour of incubation. (A) Two parameter histogram analysis of intracellular IFN-γ production by CD4+ or CD8+ T cells isolated from LSDV-RV-immune mouse splenocytes. The values represented in the upper right quadrant of each histogram are the percentage $CD4^+$ or $CD8^+$ lymphocytes staining positive for intracellular IFN-γ.

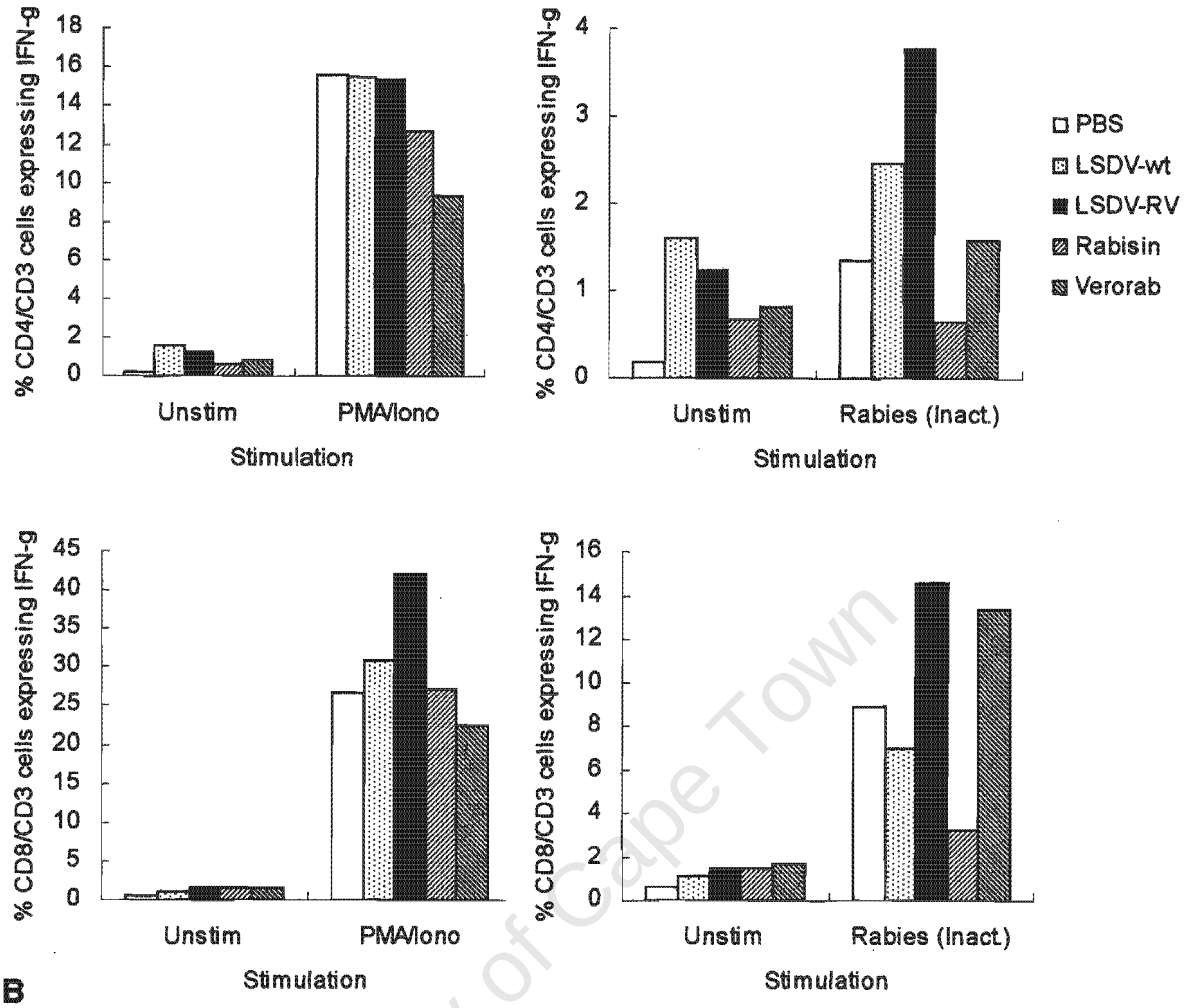


Figure 3.7B Intracellular cytokine staining for IFN-γ responsiveness following *in vitro* re-stimulation with inactivated rabies virus. BALB/c mice (8-12 weeks) were immunised twice (day 0 and 56) with either PBS, LSDV-wt, rLSDV-RG, Rabisin or Verorab and *in vitro* splenocyte cytokine responses were measured to either no antigen (unstimulated; 4 or 20 hours), PMA/Ionomycin (25 $ng.ml^{-1}$/1 $\mu g.ml^{-1}$, 4 hours), or inactivated rabies virus (20 hours, 2 virus particles/cell). Brefaldin A (10 $\mu g.ml^{-1}$) was added after the first hour of incubation. (B) Summary of intracellular cytokine responses following the various immunisation schedules. Each bar represents the mean percentage $CD4^+/CD3^+$ or $CD8^+/CD3^+$ lymphocytes expressing either IFN-γ.

3.3.7. Rabies virus potency test.

Before a live RV challenge could be performed, an evaluation of the potency of the RV (CVS-11) strain to be used in the challenge had to be carried out. The WHO describes a standard operating procedure for live rabies virus challenges in mice that involves the challenge virus being inoculated intracranially in to the

mice, but many references describe animals challenged with live RV peripherally in order to test candidate vaccine strains. We therefore undertook to compare the IC and IM routes of challenge with this particular RV strain. Table 3.6 shows the results of the potency test where it was discovered that in NMRI mice the IM route of inoculation, even at the highest dose of RV used, was fatal to only six out of eight mice. We therefore decided to give doses of $8x10^{-1}$, $8x10^{-2}$, and $8x10^{-3}LD_{50}$ of CVS-11 IC since the $8x10^{-1}$ challenge dose was fatal to all eight mice.

Table 3.6.

Innoculation schedule of mice used to calculate challenge virus potency for rabies challenge experiment and corresponding numbers of deaths due to rabies infection.

Group	Cage	Sex	Number	Route	Dose (in 30µl)	Deaths due to RV
1a	1	male	4	IC	$2,8x10^{1}$ LD_{50}	4
1b	2	female	4	IC	$2,8x10^{1}$ LD_{50}	4
2a	1	male	4	IC	$1,8x10^{1}$ LD_{50}	4
2b	2	female	4	IC	$1,8x10^{1}$ LD_{50}	4
3a	1	male	4	IC	$8x10^{-1}$ LD_{50}	4
3b	2	female	4	IC	$8x10^{-1}$ LD_{50}	4
4a	1	male	4	IC	$8x10^{-2}$ LD_{50}	1
4b	2	female	4	IC	$8x10^{-2}$ LD_{50}	2
5a	1	male	4	IC	$8x10^{-3}$ LD_{50}	1
5b	2	female	4	IC	$8x10^{-3}$ LD_{50}	1
6a	1	male	4	IM	$3,8x10^{1}$ LD_{50}	4
6b	2	female	4	IM	$3,8x10^{1}$ LD_{50}	2
7a	1	male	4	IM	$2,8x10^{1}$ LD_{50}	1
7b	2	female	4	IM	$2,8x10^{1}$ LD_{50}	1
8a	1	male	4	IM	$1,8x10^{1}$ LD_{50}	0
8b	2	female	4	IM	$1,8x10^{1}$ LD_{50}	0
9a	1	male	4	IM	$8x10^{-1}$ LD_{50}	0
9b	2	female	4	IM	$8x10^{-1}$ LD_{50}	0
10a	1	male	4	IM	$8x10^{-2}$ LD_{50}	0
10b	2	female	4	IM	$8x10^{-2}$ LD_{50}	0

The mice were monitored for 16 days post challenge. Any survivors were euthanased and brains were stained with FITC-labeled RV nucleoprotein

antibodies (prepared by Rabies Unit, Onderstepoort Veterinary Institute, South Africa) to ascertain whether or not mice in question were infected with RV (See Appendix E). All mice that had died by day 16 had positive brain impression stains while those that were euthanased on day 17 were negative. It was thus concluded that death up to day 16 was an accurate end-point to rabies infections in mice.

3.3.8. Rabies challenge experiment.

Once the RV challenge dose and route had been ascertained, we then aimed to determine the protective ability of rLSDV-RG in mice. We were careful to ensure that all parameters established in the previous test remained constant.

Mice were inoculated and boosted (fourteen days later) with PBS, rLSDV-RG, LSDV-wt or Verorab. Seven days after the booster inoculation the mice were challenged intracranially with live RV (CVS-11) at the doses determined by the rabies potency test. Mice were monitored for fourteen days after challenge. During the monitoring period, mice that displayed advanced rabies symptoms were euthanased and considered to be rabies positive. Figure 3.8A shows the survival curve of mice challenged with $8x10^{-1}$ LD_{50} of CVS-11. It can be seen that one mouse inoculated with Verorab died prior to challenge and one died post challenge as a result of rabies virus infection. Of the ten mice vaccinated with rLSDV-RG, one died and three were euthanased after displaying rabies-like symptoms. Seven of the PBS-inoculated mice died post-challenge and one was euthanased, while eight of the LSDV-inoculated mice died and one was euthanased. It is evident from figure 3.8A that in the rLSDV-RG and Verorab-vaccinated groups the onset of disease was later than in the control groups. In addition, the rLSDV-RG and Verorab-vaccinated groups were largely protected from high dose RV challenge. Figure 3.8B shows the survival curve of mice challenged with $8x10^{-2}$ LD_{50} of CVS-11. None of the rLSDV-RG and Verorab-vaccinated mice succumbed to disease post-challenge while between 50 and 60% of the inoculated mice in the negative control groups (PBS and LSDV)

were lost to rabies. $8x10^{-3}$ LD_{50} of CVS-11 is ineffectual in causing disease in this species. None of the mice displayed any rabies-like symptoms (data not shown). This experiment conclusively shows that protection against a live RV infection can be brought about by inoculation with rLSDV-RG and that the protective ability of this candidate vaccine is comparable with that of Verorab.

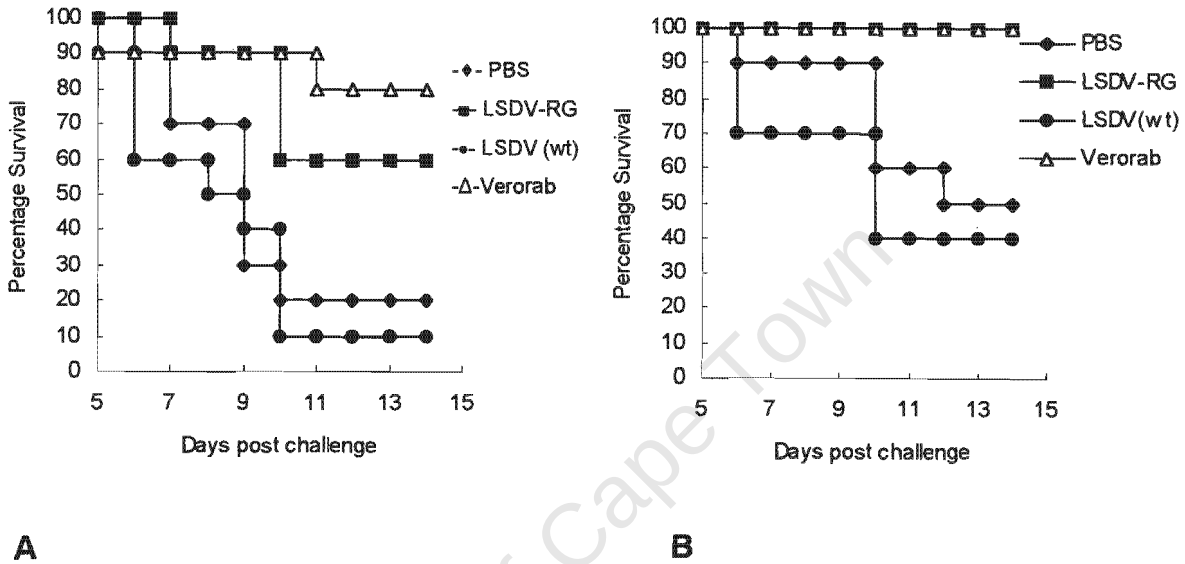


Figure 3.8. Survival curves of groups of ten NMRI mice inoculated on day 0 and 14 with PBS(♦),$5x10^4$ ffu/ml in 50µl rLSDV-RG(■),$5x10^4$ ffu/ml in 50µl LSDV-wt(●), or $1/10^{th}$ human dose of Verorab(Δ) and challenged with $LD_{50}=8x10^{-1}$ RV(CVS-11) (**A**), $LD_{50}=8x10^{-2}$ RV(CVS-11) (**B**), and $LD_{50}=8x10^{-3}$ RV(CVS-11) (Data not shown).

3.4. DISCUSSION

The primary purpose of this part of the study was to investigate LSDV as a possible vaccine vector for the production of recombinant vaccines, which will be safe and effective in non-ruminant hosts. Many studies are currently focusing on MVA as the recombinant vector of choice because of its safety in immunocompromised individuals (Barouche *et al.*, 2001). This vector, however, has a drawback; pre-existing cell mediated immunity (Baxby and Paoletti, 1992)

to cross-reactive viruses, such as vaccinia virus (VV), the vaccine used to immunise against smallpox. Many people still have VV-specific immunity due to prior vaccination against smallpox and a recombinant MVA vaccine would be less effective in such individuals.

Previous studies on fowlpoxvirus (Somogyi *et al.,* 1993) and canarypoxvirus (reviewed by Moss, 1996; Taylor *et al.,* 1993) have shown that these viruses undergo incomplete or abortive replication cycles in mammalian cells. This feature has become known as "host-restriction" and has subsequently been applied to other attenuated viral vectors such as MVA. Host-restricted vaccine vectors are safer than vaccine vectors with wide host ranges because the replication is restricted to a particular host species, which limits the threat of environmental spread. Weiss (1968) and Young *et al.*(1970) in separate investigations showed that LSDV is highly species-specific and that LSD is limited to ruminants, specifically cattle. In this study, we compared the replication cycle of recombinant LSDV in cells of bovine, ovine and primate origin in order to evaluate LSDV as an additional host-restricted vaccine vector.

Because the RG is inserted under the control of a poxvirus early/late promoter (pE/L), early expression of the foreign gene as well as late translation of protein occurs in rLSDV-RG-infected cells. This was confirmed in a transient expression assay, where it was ascertained that foreign genes are expressed by late (p11) and early/late (p7.5) poxvirus promoters in LSDV-infected LT, MDBK and CV-1 cells. Furthermore, using a Hoechst stain, it was confirmed that LSDV DNA replication occurs in all three types of cells. This result differs from the review compiled by Ring and Blair (2001), who state that host restricted viruses demonstrate little or no late gene expression or DNA replication in non-permissive cells. However, this may imply that LSDV replication is not as restricted to ruminants as is avipoxvirus replication to birds. The Hoechst-stained DNA was located in the cytoplasm of these cells as expected. A study performed by Prozesky and Barnard (1982) describes the pathogenesis of wild type LSDV in naturally infected cattle using electron

microscopy. Our study is the first ultrastructural comparison of the morphogenisis of rLSDV-RG in cultured cells of ruminant and non-ruminant origin. The bovine and ovine cells supported maturation of the infecting-virus in that the virions appeared to condense and extracellular virions were observed in rLSDV-RV-infected cell cultures. The ultrastructure of LSDV in bovine cells observed by Prozesky and Barnard (1982) suggests that the level of replication and maturity observed in our study in bovine and ovine cells represents complete LSDV replication. However, in primate cells, viral maturation was limited. Viral factories, which are electron dense areas consisting of nucleic acid material and proteinaceous membrane structures, were seen in these cells. This confirms that the areas stained with FITC-labeled anti-rabies antibodies in chapter two represented viral factories. Evidence of incomplete replication was found in the primate cells (CV-1) in that particles were seen in CV-1 cells but maturation in these particles was limited. This finding, with the result from the promoter study and Hoechst stain was extremely encouraging as it proved that non-permissive cells (cells not of ruminant origin) could support early stages of rLSDV-RG replication. This is important for vaccine considerations in that the cells of the vaccinated host (whether it be ruminant or not) need to support replication of the vector sufficiently for the antigenic protein to be expressed and an immune response to be elicited. Based on these results, we concluded that complete maturation of LSDV is host-restricted to cells of ruminant origin. However, gene expression of foreign DNA is expected to occur in both ruminant and non-ruminant cells. We subsequently undertook to evaluate the usefulness of such a virus as a host-restricted vaccine vector in non-ruminant hosts.

There is sufficient evidence that recombinant LSDV-RG expresses the inserted protein adequately in ruminant hosts so that immunity to that protein is elicited. In chapter two it was shown that, in cattle, rLSDV-RG does in fact induce cell mediated immunity to RV as well as neutralising antibody titres of up to 3000 times the threshold level of protection (0.5 IU/ml; Cliquet *et al.,* 1998) as prescribed by the WHO. This finding confirms that the RG is expressed by the bovine cells infected with rLSDV-RG in such a manner that RV-specific cell

mediated as well as humoral immunity is elicited in cattle. Furthermore, Romero *et al.* (1993,1994a,1994b) have demonstrated immunity against rinderpest in cattle vaccinated with a recombinant capripoxvirus expressing the fusion protein gene of rinderpest virus

In this chapter convincing evidence is presented on cell-mediated immunity in mice inoculated with rLSDV-RG. Rabbits similarly inoculated produced neutralising antibody titres that exceed the threshold of protection (0.5IU/ml; Cliquet *et al.,* 1998) by a factor of two. This confirms that despite the lack of productive replication of rLSDV-RG in non-ruminant cells, the RG is expressed adequately for a specific immune response to be elicited.

To further evaluate the efficacy of LSDV as a recombinant vaccine vector, we compared the immunogenicity of rLSDV-RG with two commercially available RV vaccines in two non-ruminant hosts (rabbits and mice). The rLSDV-RG recombinant vaccine was found to induce detectable anti-RG antibody responses in rabbits that increased dramatically after booster inoculations. This clearly demonstrated that the recombinant LSDV-RG adequately expressed the RG antigen in non-ruminant hosts. Compared with the commercial vaccines tested (Verorab and Rabisin), rLSDV-RG induced similar levels of RG-specific antibodies as Rabisin after the second rLSDV-RG boost but induced 2-fold lower antibody titres than Verorab. Following two immunisations, the rLSDV-RG vaccine induced RV neutralising antibody titres that exceeded those that have been found to provide protection from infection (Cliquet *et al.,*1998; Dutta *et al.,* 1992).

Rabbits immunised with 10^5 ffus of rLSDV-RG produced good RV antibodies as determined by ELISA. However, BALB/c mice immunised with $10^{4.5}$ ffus produced no detectable RV antibodies by the same test. It was speculated as to whether the difference in dose (500 000 virus particles) could have made such a difference in humoral immunity between the two species, or whether the fact that rabbits are phylogenetically related to ungulates (related to ruminants)

might have allowed rLSDV-RG to replicate more productively in rabbits than in mice.

In mice, immunisation with rLSDV-RG generated significant T cell proliferative ability to inactivated RV ($p=0.02$), indicating that a cell-mediated immune (CMI) response was induced. The T-cell proliferative ability of rLSDV-RG-immunised mice was comparable to mice immunised with Verorab ($p=0.05$) and better than mice immunised with Rabisin ($p=0.2$). Following rLSDV-RG immunisation, we also found evidence of a Th-1 type cytokine response in mice as determined by intracellular cytokine staining. rLSDV-RG induced more IFN-γ-producing CD4/CD3 and CD8/CD3 cells than did both commercial vaccines.

Most importantly, I have clearly shown that vaccination with rLSDV-RG protects mice against an aggressive intracranial challenge with live RV. Mice are not the natural hosts of LSDV and thus, according to our findings, rLSDV-RG is not expected to replicate productively, nevertheless, vaccination with rLSDV-RG induced protective immunity. As no antibodies to RG were detected in mice, it is assumed that this protection is cell mediated. Since intracranial challenge with live RV is the gold standard against which all rabies vaccines are compared, we can conclude that rLSDV-RG is comparable with other commercially used rabies vaccines in its ability to protect against RV infection. This finding demonstrates the efficiency of the LSDV vector in immunising against RV.

The dose of rLSDV-RG required to produce an immune response is low compared to other host restricted vaccine vectors. Mice were inoculated with $10^{6.7}$ $TCID_{50}$ of a fowlpoxvirus-RV recombinant virus (Taylor *et al.*, 1991), which protected 8/10 mice from an intracranial RV challenge. In this study, 10/10 mice inoculated with $10^{4.5}$ ffus of rLSDV-RG survived an intracranial RV challenge.

Mice inoculated with $10^{6.7}$ $TCID_{50}$ of a fowlpoxvirus-RV recombinant virus (Taylor *et al.*, 1991) produced RV-specific antibodies of 1.9 IU/ml. Dogs and cats

were given the same fowlpoxvirus-RV recombinant at doses of 10^{8} $TCID_{50}$ and they produced an average of 1.5 IU/ml RV-specific antibodies. Humans given a canarypoxvirus-RV recombinant (ALVAC-RG; Cadoz *et al.*,1992) in doses of $10^{3.5}$, $10^{4.5}$ and $10^{5.5}$ $TCID_{50}$ and boosted with the same doses after 4 weeks mounted RV-specific neutralising antibodies of <0.1, 0.58, and 1.9 IU/ml one week after the boost. The rabbits in this study produced up to 6 IU/ml of RV-specific antibodies as determined by ELISA and 1.35IU/ml as determined by RV neutralisation assays after being immunised with 10^{5} ffus of rLSDV-RG. From this data, it is clear that rLSDV-RG is effective at a dose that is far lower ($10^{4.5}$-10^{5} ffus) than that required for the other documented host-restricted vaccine vectors. This is a useful attribute as it translates to a lower cost per dose of the final vaccine.

For a vaccine to be protective against infection with rabies virus, previous studies have shown that neutralising antibodies are important as the primary line of defense (Dutta *et al.*, 1992; Xiang *et al.*, 1995) but that T-cells and the CMI response also plays a vital role (Mifune *et al.*, 1981). This study suggests that in non-ruminant hosts, rLSDV-RG elicits a predominantly cell-mediated immune response that is protective.

In conclusion, this study demonstrates that recombinant LSDV-RG shows potential as a vaccine against rabies in non-ruminant hosts. The advantages and efficacy of LSDV as a host-restricted vaccine vector that we report here provide strong evidence that this virus may be exploited as a safe, replication-restricted vaccine vector for a wide range of pathogens.

CHAPTER 4
DISCUSSION AND CONCLUSION

In this study, a recombinant LSDV expressing the rabies glycoprotein was produced. It was established that LSDV replicated in the cytoplasm in both ruminant and non-ruminant cells infected with rLSDV-RG. It was further established that in these cells, early and late gene expression takes place. The RG was expressed in rLSDV-RG-infected LT cells as determined by immunofluorescence and the cellular location of the fluorescence was restricted to viral factories adjacent to the nucleus of these cells. Electron microscopy confirmed the presence of such viral factories. The electron microscopic study of different cells infected with rLSDV-RG revealed that the recombinant virus partially replicates in non-permissive CV-1 cells but produces more mature virus particles in permissive bovine and ovine cells. We established that rLSDV-RG is a viable candidate vaccine for rabies in cattle and that both humoral and cellular immune responses are elicited in cattle. The RV antibody titres detected in rLSDV-RG-inoculated cattle are higher than the critical 0.5IU/ml titre established by the WHO for protection. On this basis, we conclude that rLSDV-RG is likely to protect cattle against rabies infections. Furthermore, preliminary market research indicates that such a vaccine would be useful in areas where cattle rabies is an economic problem. In the light of its success in cattle, LSDV was then evaluated as a vaccine vector in non-ruminants using RV as a model. It was found that rLSDV-RG elicited detectable RV neutralising antibody titres in rabbits that increased after booster inoculations. Furthermore, good cellular immunity was detected in rLSDV-RG inoculated BALB/c mice. Immunised mice were protected against an aggressive IC challenge with live RV despite the apparent lack of a RV-specific humoral immune response.

These results collectively mean that LSDV is a suitable vaccine vector for use in both ruminants and non-ruminants. In cattle, LSDV induces a good humoral as well as cellular immune response and in mice, the immune response appears to

be primarily cellular. In addition, rLSDV-RG has commercial applications as a rabies vaccine for cattle. This study is novel at a number of levels; namely, it contains the first ultrastructural examination of recombinant LSDV in cells of ruminant and non-ruminant origin; it has delivered a viable live recombinant LSDV vaccine against rabies for use in cattle; and it contains a substantial study of LSDV as a vaccine vector.

Because LSDV is endemic to Africa and the Middle East, recombinant LSDV vaccines have potential to be used in these areas. Rabies vaccines have market potential in Africa, India, South America and even parts of North America. Some of these regions have strict import regulations that may therefore limit the market size of a recombinant LSDV vaccine. However, Africa and India are good target markets for a recombinant LSDV-RV vaccine, such as the one produced in this study.

The first hurdle in the commercialisation of a vaccine presents as a product/development problem. Further trials and validation procedures require large-scale production of the vaccine in question, rLSDV-RG in this case. The doses need to be validated for registration as well as for comparison purposes. We have earmarked a facility capable of producing the required quantity and quality of rLSDV-RG for trials. An additional challenge is to produce the required doses at a cost that is low enough to suit the market. Since Africa and India consist primarily of developing economies, the cost of vaccines needs to be affordable. Furthermore there are ethical reasons for keeping healthcare product prices down. As a result, the scale-up procedure will be limited by the cost/dose of the vaccine.

In order for rLSDV-RG to be sold commercially, large-scale field trial needs to be performed. In this trial, an effective dose and vaccination schedule will have to be ascertained and a dose response curve produced, which may vary depending on the route of inoculation. It would be hugely beneficial for the vaccine to be

effective if given orally and therefore oral vaccination would be a focus of such a trial. The trial would have to be followed for the duration of an animal's lifespan to determine the duration of immunity elicited by rLSDV-RG at various doses. Ideally, a live RV challenge should be attempted to confirm protective capabilities of the candidate vaccine.

To complete the study of LSDV in non-ruminants, the trials described in the previous paragraph must be carried out in potential target species such as dogs and wildlife. In addition pathogenicity studies need to be carried out in nude mice to determine safety of the candidate vaccine. Non-human primate studies would have to be carried out to evaluate the potential of using LSDV as a vaccine vector in humans. As a proof-of-concept, the RG may be used as the inserted gene of choice because of the availability of models and tests for evaluation. However, in the future, other genes may be inserted in the LSDV genome in order to produce other novel recombinant vaccines.

In conclusion, this study has produced a novel vaccine vector with potential to be used to combat infectious diseases. Moreover, a rabies vaccine for cattle has been produced that has commercial implications and that could potentially impact favourably on the rabies problem in Africa and other developing nations.

APPENDIX A: MOLECULAR BIOLOGY TECHNIQUES

A1 Transformation of DNA into competent *E.coli* cells

$CaCl_2$ Shock procedure (Dagert and Ehrlich, 1979)

E.coli strain DHα was used for the production of competent cells. Five milliliters of DHα bacteria were grown overnight at 37°C in 2x YT broth, pH7.0 (per litre, 900ml deionised water, 16g bacto-tryptone, 10g bacto-yeast extract, 5g NaCl) in a McCartney bottle placed flat on a shaker for good aeration. The bacterial culture was then diluted 1/100 to 1/200 in 50 or 100ml 2xYT broth in a flask five to ten times the culture volume. The bacterial culture was grown to early log phase (OD_{600} 0.2-0.4) and the cells were harvested by centrifugation at 4000-5000rpm for 5 minutes at 4°C in the Beckman J2-21 centrifuge. The pellet was resuspended in1/2 the culture volume of 0.1M ice-cold $CaCl_2$ and then placed on ice for one to two hours. The cells were collected by centrifugation as before and then resuspended in 1/10 the original culture volume of 0.1M ice cold $CaCl_2$. The competent cells were then aliquoted in 100µl aliquots into ice-cold eppendorf tubes and 10% (v/v) ice-cold sterile glycerol was added. The cells were mixed and left on ice for 30 minutes before being frozen for storage at -70°C.

Transformation of $CaCl_2$ competent cells (Dagert and Ehrlich, 1979)

The relevant plasmid DNA (1-10ng) containing ampicillin resistance was mixed with 100µl of competent DHα Cells and left on ice for 30 minutes. The bacteria were then heat-shocked at 42°C for 2 minutes. 0.9ml of 37°C 2xYT broth was then added to the heat-shocked cells that were incubated at 37°C for 30-60 minutes. This is to allow for expression of the ampicillin and β-lactamase genes before plating the cells onto 2xYT plates containing Ampicilin and 0.5ml X-gal (5ml DMSO, 0.4g X-gal, 5ml distilled water) and 50µl IPTG (0.24g IPTG and

10ml distilled water) per 100ml agar. Colonies were grown overnight at 37°C and transformed bacteria identified.

If the marker gene, *lac Z*, was present on the plasmid transformed into the bacteria, the transformed bacteria appeared blue. The defective *lac Z* gene in the plasmid and the defective *lac Z* gene in the bacteria complement each other, allowing the expression of β-galactosidase, induced by the isopropyl-β-D-thio galactosidase (IPTG) in the agar. The β-galactosidase is able to metabolise 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-gal), a chromogenic substrate, to produce blue colonies. Selection of recombinant clones can occur if the multiple cloning site is within the *lac Z* gene of the plasmid is interrupted by the insertion of a foreign gene. The recombinant bacteria are therefore not able to express β-galactosidase and the colonies remain white.

A2 Plasmid Extraction

Small-scale plasmid preparation

The protocol was based on Sambrook *et al.* (1989). The selected recombinant bacteria were inoculated into 5ml of 2xYT broth containing 100µg/ml ampicillin and grown overnight at 37°C with shaking. Two milliliters of the culture was transferred to a 2ml eppendorf tube and centrifuged at 14 000rpm for two minutes in a microfuge. The supernatant was removed and the pellet re-suspended in 100µl of buffer 1 (50mM glucose, 10mM EDTA, 25mM Tris-Cl, pH8.0). After re-suspension, 400µl of buffer 2 (8.8ml distilled water, 0.2ml 10m NaOH, 1ml 10% SDS) was added and the tube placed on ice for 5 minutes after gentle mixing. The protein and genomic DNA was precipitated by the addition of 300µl of buffer 3 (3M K acetate and 1.3M formic acid) and placed on ice for 30 minutes. The flocculent was removed by centrifugation at 14 000rpm for 15 minutes in a microfuge and the clear supernatant transferred to a fresh eppendorf tube. The DNA was precipitated from the supernantant by the

addition of 1ml of isopropanol. The precipitated DNA was removed from the isopropanol by centrifugation at 14 000rpm in a microfuge for 15 minutes and then washed in 70% ethanol to remove salts and impurities. The DNA pellet was resuspended in the appropriate amount of TE (pH 7.6) or sterile, distilled water.

Large-scale plasmid preparation

For amplification of up to 100µg of plasmid, the Nucleobond®AX-PC-kit (Machery-Nagel, Germany) was used according to manufacturers' instructions. The AX-100 column is a silica-based, anion exchange column for purification of plasmid DNA from 10 to 100ml of broth. The desired transformed bacterial cells were grown in 50 ml of 2xYT broth containing 100µg/ml ampicillin overnight at 37°C with shaking. The bacterial cells were then pelleted at 5000rpm for 5 minutes at 4°C using the Beckmann J2-21 centrifuge. The supernatant was removed and the bacterial pellet carefully resuspended in 4ml of 4°C buffer S1 (50mM Tris/HCl, 10mM EDTA, 100 µg RNase A/ml, pH8.0). The resuspended bacteria were then mixed with 4ml of room temperature buffer S2 (200mM NaOH, 1% SDS) and incubated at room temperature for exactly 5 minutes. Four milliliters of 4°C buffer S3 (2.8M K acetate, pH5.2) was then added and the suspension gently mixed by inverting the tube. The mixture was incubated on ice for 5 to 10 minutes and then centrifuged in a Beckmann J2-21 centrifuge at 11 000rpm for 25 minutes at 4°C. The supernatant was removed and added to a AX-100 column, equilibrated with 2ml of buffer N2 (100mM Tris/H_3PO_4, 15% ethanol, 900mM KCl, pH6.3). Once the supernatant had run through the column, the column was washed with 2x 4ml of buffer N3 (100mM Tris/H_3PO_4, 15% ethanol, 1150mM KCl, pH 6.3). The washed plasmid DNA was then eluted from the column using 2ml of buffer N5 (100mM Tris/H_3PO_4, 15% ethanol, 1000mM KCl, pH 8.5). The eluted plasmid DNA was precipitated with 0.8 volumes of room temperature isopropanol and washed and resuspended as previously mentioned.

The resuspended DNA was diluted 1 in 100 in distilled water and scanned using the Beckmann DU-40 spectrophotometer from 310nm to 220nm wavelengths. The spectrophotometer was blanked against distilled water after which the samples were read. To determine the purity of the DNA, the 260nm peak (DNA) was divided by the 280nm (protein) value and if the ratio was between 1.7 and 2, the DNA was assumed to be pure. The DNA concentration was determined by multiplication of the A_{260} by 50 (the factor for double stranded DNA) multiplied by the dilution factor (100).

A3 Agarose gel electrophoresis (Sambrook *et al.*, 1992)

Agarose gel electrophoresis was performed using horizontal gel apparatus (Hoefer Scientific Instruments, San Francisco, USA) as described in Sambrook *et al.* (1989). The agarose powder was melted with frequent mixing to prevent clumping in 1x TAE (50x TAE/litre; 242g Tris base, 57.1ml glacial acetic acid, 100ml 0.5M EDTA, pH8.0) at concentrations of 0.8 to 2 %, depending on the sizes of the DNA fragments to be separated. Once melted, Ethidium Bromide (10mg/ml) dye was added to a final concentration of 1μg/100ml agarose. The agarose gel slabs were allowed to set at room temperature for 30 minutes after which the well combs were removed. DNA samples were mixed with 1/5 their volume of loading dye (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in deionised water) and added to the 1xTAE submerged wells. The agarose gel was electrophoresed at 5V/cm until the relevant fragments were separated. The DNA fragments were then visualized using a UVP transilluminator at 256nm wavelength and photographed either with a M645 1000S Polaroid camera or with a UVP computerised gel imager. Molecular weight markers were used to determine DNA fragment sizes; including marker VI (Roche Pharmaceuticals, Germany) and the 1kb ladder (Promega, Madison, WI,USA).

A4 Restriction endonuclease digestion (Sambrook *et al.*, 1992)

Restriction digests were carried out using the 10x buffer supplied by the manufacturer (Roche Pharmaceuticals, Germany). Two restriction enzymes were combined in a single digest if their buffers were compatible. If not, the digestion was done sequentially, and the DNA was purified to remove the first buffer and enzyme using ethanol precipitation, before the addition of the second enzyme and buffer. The restriction enzyme digests were performed as described in Sambrook *et al.*(1989). The majority of the restriction endonucleases are active at 37°C with the exception of *sma 1* which has an optimal temperature of 25 °C. For a single digestion reaction, the following reaction components were included in a 20μl reaction mix:

4μl 10x restriction endonuclease buffer

±1μg DNA

1μl 10 units/μl restriction endonuclease

Ethanol Precipitation

The reaction volume was made up to 90μl with TE pH8.0 (10mM Tris-Cl pH8.0; 1mM EDTA, pH8.0) in an eppendorf tube. One volume of phenol was added and the solution emulsified by shaking. The solution was then centrifuged in a microfuge at 14 000rpm for 2 minutes. The upper phase was transferred to a fresh eppendorf tube. This process was repeated and then one volume of chloroform/isoamyl alcohol (24:1) was added and the solution emulsified by shaking. The upper aqueous phase was recovered by centrifugation as before. 1/10 the volume of 3M sodium acetate pH4.8 and 2.5 volumes of absolute alcohol was added, the solution mixed and left on dry ice for 20 minutes. The DNA was collected by centrifugation at 14 000rpm for 20 minutes, washed with 70% ethanol and allowed to dry at room temperature for 5 minutes before being resuspended in the appropriate amount of TE buffer or distilled water.

A5 Ligation

The restriction endonuclease digested vectors were electrophoresed in 1% low melting temperature agarose to separate out the desired DNA fragments from the stuffer fragments as described by Sambrook *et al.*(1989). The relevant fragments were excised from the gel visualized under low intensity UV light. The Agarose was removed using a Cleanmix Kit (Talent, Italy) according to the manufacturers' recommendations. The vector and insert DNA was mixed together in a ratio of 1:3 and the Rapid Ligation Kit (Roche Pharmaceuticals, Germany) was used according to manufacturers' instructions to ligate the recombinant plasmids. The ligation mixture was incubated at 16°C overnight and the full 20µl was used to transform competent DHα *e.coli* cells (appendix A1). These transformed cells were then plated onto 2xYT agar containing 100µg/ml ampicillin and allowed to grow at 37°C overnight.

A6 DNA transfer to membrane (Sambrook *et al.*, 1992)

The gel containing DNA was placed in denaturing solution (0.5M NaOH and 1.5M NaCl) for 30 minutes. The gel was then rinsed in distilled water and placed in neutralising solution (20mM NaOH and 1M ammonium acetate) for 40 minutes. The gel was placed upside down on a sheet of glass in preparation for the transfer. Hybond N hybridization membrane was fitted to the gel and marked to ascertain correct orientation in future. The membrane was pre-wet in neutralising solution. Three pieces of Whatman 3MM chromatography paper, also soaked in neutralising solution were placed on top of the membrane. A 5cm thick layer of absorbent tissue paper was placed on top of the Whatman paper, along with a 2kg weight. The DNA was then transferred to the membrane by capillary action overnight. The DNA was fixed to the membrane by cross-linking with a UV cross-linker. The southern blot procedure was carried out using a Multiprime DNA labeling system (Roche Pharmaceuticals) according to the manufacturers instructions.

A7 Prehybridisation procedure (Sambrook *et al.*, 1992)

The membrane was prehybridised or blocked by soaking it in 25ml of prehybridisation solution (2xSSPE, 0.25% milk powder, 1% SDS) at 42°C for 5 hours or overnight.

A8 Labeling of probe and hybridisation
(Multiprime system, Roche Pharmaceuticals)

2-25 μg of linearised plasmid probe (RG gene) was denatured at 95-100°C for 2 minutes and placed on ice. The following reaction was set up on ice:

25 μg DNA	x
buffer (solution 1)	10μl
primer (solution 2)	5μl
dNTP	5μl
enzyme	2μl
water	to 50μl

The hybridization solution was incubated at room temperature overnight and then added to the prehybridisation solution, which continued to incubate at 42°C in a shaking incubator overnight.

The membrane was then soaked in washing solution (2x SSPE and 0.1% SDS) at room temperature to remove unhybridised probe. Three sequential 20 minute washes were then carried out in washing solution at 42°C. The membrane was then placed in plastic, adjacent to 2 Agfa RPI x-ray films, and placed in an x-ray cassette. The cassette was placed at -70°C. One x-ray was developed after 2 days and the other after 1 week.

APPENDIX B: TISSUE CULTURE TECHNIQUES

B1 Cell propagation

Primary Lamb Testes cells (LT; Wallace, 1994)

These cells were prepared according to the method outlined by Freshney (1987). The foetal lamb testes were obtained from the abattoir in Maitland, Cape Town. The testes were aseptically removed from the lambs and the cells were processed immediately. The testes were rinsed in physiological saline containing 0.5% penicillin, streptomycin and fungizone (PSF) (GibcoBRL,Paisely, UK). The fat and connective tissue was removed from the testes. The tissue that contains the rapidly dividing cells was washed again and cut into 2mm pieces. These pieces were thoroughly washed in crude trypsin (0.25%trypsin (Difco Laboratories,USA) in phosphate buffered saline) to remove excess blood. The pieces were then transferred to a sterile flat-bottomed flask containing crude trypsin. The mixture was stirred at 4°C overnight to allow the enzyme to penetrate the tissue. The trypsin was then removed by centrifugation at 1000rpm in a microfuge and the remaining cells were resuspended in Dulbecco's modified eagle's medium (DMEM) (Highveld Biological, South Africa) supplemented with 0.5% PS and 10% foetal calf serum (FCS). The cells were counted using a haemocytometer (as described below) and diluted to 10^7 cells per milliliter in DMEM containing 0.5%PSF and 10% FCS. 25ml polypropaline tissue culture flasks (Nunclon, nunc, Delta, Denmark) were seeded with approximately 200 000 cells per square centimeter. The cells were then incubated in a 37°C incubator with 5-10% CO_2 atmosphere for two to three days until the cells formed a confluent layer. Cells were split by adding activated trypsin (0.3% NaCl, 0.012% KH_2PO_4, 0.02% KCl, 0.091% Na_2HPO_4, 0.05% glucose, 0.02% EDTA, 0.25% PS, distilled water, pH7.8) for 5 minutes at 37°C and then diluting them 1/5 with fresh DMEM containing 10% FCS and 0.5% PSF.

Immortalised Cell Lines

CV-1 (African Green Monkey [*Cercopithecus aethiops*] kidney cell line), MDBK cells (Madin Darby bovine Kidney) were obtained from American Type Tissue Culture Collection (Rockville, MA, USA). Frozen stocks were thawed from liquid nitrogen storage by melting the cells in a 37°C water bath. The cells were pelleted by centrifugation at 1000rpm for 5 minutes and the storage medium containing 10% DMSO was removed. The cells were washed twice with DMEM containing 10%FCS and 0.5% PSF and then resuspended in DMEM containing 10%FCS and 0.5% PSF in a 25ml tissue culture flask as was used previously. These cells were maintained and split using the same procedure as was mentioned for LT cells.

To count the cells, 0.1ml of cell suspension was added to 0.15ml of physiological saline and 0.25ml of a trypan blue solution. This gave a dilution factor of 5. A small amount of the mixture was added to the chambers of a haemocytometer (Neubauer, Brand, Blaubrand, Germany) with the cover slip in place. Trypan blue enters cells that have a permeable membrane (i.e. non-viable) and stains them blue. The viable (clear) cells were counted in 1mm squares and an average taken. To calculate the viable cell concentration per milliliter, this average was multiplied with the dilution factor (5) and 10 000 (the total volume of the 1mm square with the coverslip in place is 0.1μl).

B2 Infection and transfection of cell-cultures (Adapted from Mahy (1985); Brand (1993) and Macket *et al.,* 1985)

Mammalian cells can take up DNA that is exogenously added and express the genes encoded by this DNA. A vector that replicates independently as an extrachromosomal element can be used to transform animal cells by infection or

transfection, for example, a virus genome that yields virions with recombinant genomes.

Cell monolayers were grown in Nunc 6-well plates (Delta, Denmark) until they were 60-80% confluent. Cells are usually infected with the virus at a titre of 0.1 plaque forming unit /cell (pfu/cell) or focus forming unit (ffu)/cell, which translates to1-2x10^5pfu/ml.

Viruses such as vaccinia virus (VV) cause a plaque in a cell monolayer as a characteristic cytopathic effect (CPE). However, viruses such as lumpy skin disease virus (LSDV) present foci as their CPE. This results from the cells in the monolayer piling up on each other in response to the infection. The titre of these two, host-restricted viruses is defined as focus forming units per ml (ffu/ml).

Cells were infected with LSDV (Neethling vaccine strain obtained from Onderstepoort Veterinary Institute); at a titre of 1x10^5 ffu/ml. The infected cells were then incubated at 37°C in a CO_2 incubator for an hour. If the cells were infected for the purpose of virus propagation, the infecting virus was removed from the cells at this stage and the DMEM with 10% FCS, 0.5% PSF and the specific selection medium containing mycophenolic acid was added to the cells. Cells were also transfected with DNA carrying the rabies virus glycoprotein (RG) (obtained from N Tordo, Pasteur Institute, Paris) gene at this point. A transfection-facilitating reagent called DOTAP (Roche Pharmaceuticals, Germany) was used to introduce the foreign DNA into the cells. DOTAP is a synthetic cationic lipid derived from oleic acid that forms unilamellar vesicles, liposomes, which bind negatively charged molecules such as DNA to their surface. The liposomes also adhere to anionic surfaces such as cell membranes and in this way mediate the DNA transfer into the cell. The hydrophobic and cationic portions of DOTAP are linked by ester bonds that are easily cleaved by esterases within the cell, promoting efficient degradation of the lipids once they have delivered the DNA (Leventis and Silvius, 1990).

Typically 10μg of DNA was used in the transfection of 10^6 cells. The DNA:DOTAP ration was optimized at 1:5. Since DOTAP is supplied at a concentration of 1μg/μl, 50μl of DOTAP was used in each well (containing 1 000 000 cells). The DNA and DOTAP was mixed together with 1M HBS (Hepes buffered saline) for 15 minutes before being added to the virus-infected cells. The DNA-DOTAP mixture was diluted in 2ml of DMEM containing 10%FCS and 0.5%PSF per well. This transfection medium was incubated on the cells at 37°C, 5-10% CO_2 overnight and then replaced by standard growth medium for two days under standard growth conditions. An X-gal stain was then performed on the cells to determine the transfection efficiency (appendix B3)

B3 X-Gal Stain (MacGregor *et al.*, 1991)

Growth medium (containing the recombinant virus) was removed from the transfected/infected cells and the cells were washed in PBS. The cells were then fixed in 4% paraformaldehyde for 5-10 minutes at room temperature. The fixative was removed and the cells once again washed in PBS. X-gal stain (working solution in 10 mls: 9.2ml PBS, 0.2ml 50mg/ml X-gal, 0.2ml 200mM k-ferricyanide, 0.2ml k-ferrocyanide 200mM, 0.2ml 200mM $MgCl_2$) was added to the cells and incubated under standard growth conditions. The blue cells (those expressing the *lac Z* reporter gene in the shuttle vector) were counted from 30 minutes after staining.

B4 Recombinant LSDV purification (Adapted from Romero *et al.*, 1994)

The growth medium that contains the recombinant virus was placed onto fresh cells at 60-80% confluency. The purification of the recombinant was performed in Nunc 6-well plates (Delta, Denmark). The plates were rocked every 30 minutes for 2 hours after which the inoculum was replaced with selection medium. The cells were incubated under standard growth conditions for two

days to allow for expression of the foreign DNA. The selection medium eliminates the presence of non-recombinant (wild-type) virus in the medium and so any virus present should be recombinant virus. The medium containing the 'selected' recombinant virus was removed and the cells washed in PBS. An overlay containing, 1% low melting temperature agarose, 0.2% 50mg/ml X-gal, made up to 10ml in selection medium was added to the cells and allowed to set. Once the overlay had hardened, the cells were incubated under standard growth conditions until blue plaques/foci were seen. These blue "colonies" were picked using a Pasteur pipette and dissolved in virus diluent (DMEM containing 100mM HBS and 0.5% PSF). The recombinant viruses were focus purified 4-8 more times in the same manner until all remaining wild type virus had been diluted out and the titre of the recombinant virus had increased sufficiently for large-scale virus growth.

B5 Growing viruses to high titres

VV and MVA are routinely grown up in fertilized hens' eggs, vv because it has no host restriction and eggs are convenient for this purpose and MVA because it is host restricted to chick cells. However, LSDV is host restricted to ruminant cells and does not grow to high titres in eggs, so this virus had to be grown up by multiple passages through LT cells. This was done by continuously infecting uninfected LTs as described in appendix B2. Once the total culture volume had reached 2 litres, the supernatant was centrifuged through a 36% sucrose cushion in a J2-21 Beckman rotor at 11 000 rpm for 2 hours. The supernatant was discarded and the pellet was resuspended in 50ml virus diluent (DMEM, 1M HBS, PSF). The concentrated rLSDV-RG was aliquoted into 0,5ml aliquots and frozen at -70°C for subsequent use.

Virus titration (Adapted from Paul, 1975)

The re-suspended virus diluted 10^{-3} to 10^{-9} was used to infect a 24 well plate (Nunc, Delta, Denmark) of 80% confluent LT cells. 200µl of diluted virus was added to each well, using three wells per dilution factor. The cells were incubated for 1 hour with occasional shaking at 37°C after which the virus was removed and 1ml of fresh DMEM with 10% FCS and PSF was added. The infected cells were incubated at 37°C with 5-10% CO_2 for two to three days until CPE were visible after which foci (ridges) were counted in the case of wild-type virus. In the case of rLSDV, an X-gal stain was performed as outlined in B3 and the blue foci were counted. The number of blue foci was multiplied by 5 to obtain a value per 1ml.

B6 Immunofluorescence (Adapted from Dean *et al.*, 1996)

Transfected or infected cells were grown on a glass cover slip in a 6 well plate for 2 to 3 days. The cells were fixed to the cover slip with acetone at 4°C for 10 minutes and the cover-slip air dried and washed with 1X PBS. 2% ovalbumin was added as blocking reagent for 20 minutes at RT with shaking and then the cover slips were washed twice with 1X PBS for 10 minutes each. A 1/300 dilution of the FITC-labeled antirabies glycoprotein antibodies (Becton Dickenson, USA) containing 1.5% BSA and 200µl of Evan's blue counter stain in 1X PBS was added and the cells incubated for 1 hour at 37°C. The cells were washed twice with 1X PBS for 10 minutes each, rinsed in distilled water and allowed to air dry. The cover slips were then mounted cells down on a slide for fluorescent microscopy using the SM-LUX (Leitz-Wetzlar, Germany) fluorescent

microscope at 450-490nm wavelengths and 40X magnification. Photographs were taken using Fugi® Sensia II IL400 film with a 2-3 minute exposure.

B7: Extraction of total DNA from infected cell cultures (Adapted from Bailey and Possee, 1991)

Lt cells, grown in tissue culture dishes (35 mm in diameter) were infected with rLSDV-RG when 70% confluent. After 2 hours of infection, the virus preparation was removed and the medium replaced with selection medium. Incubation was continued at 37°C for 3 days.

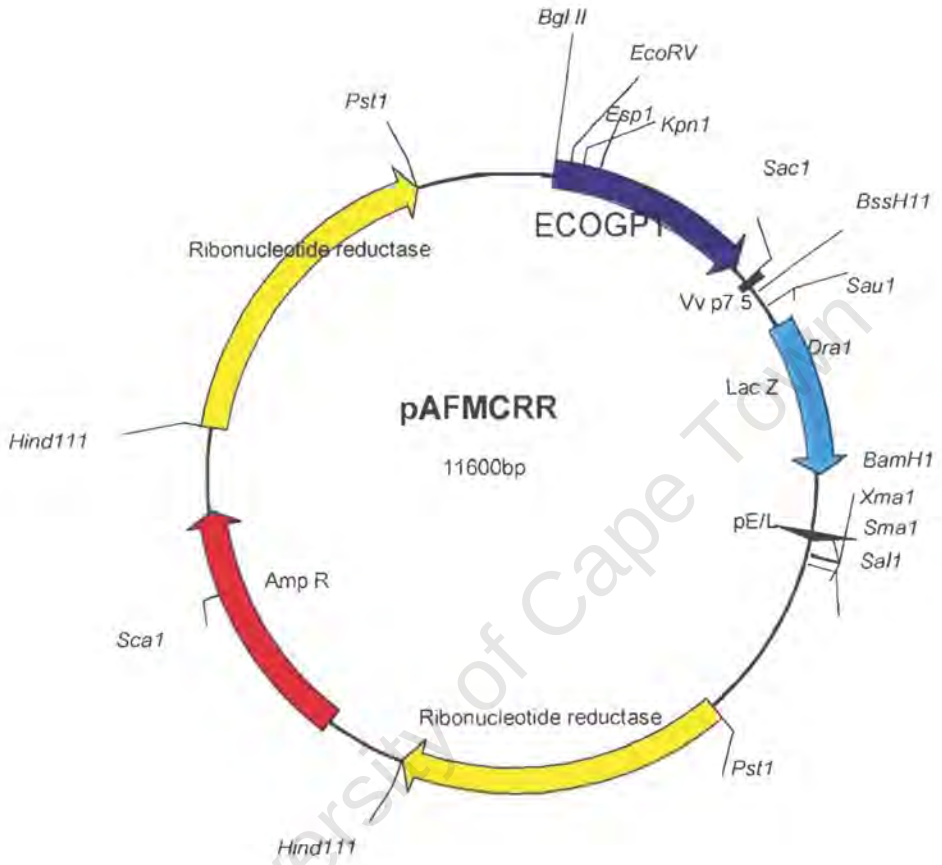
The medium was then removed from the cells and the cell layer was washed 3 times in PBS to remove inoculum from the cells. Lysis buffer (appendix D5), 500μl/dish was added along with Ribonuclease A to a final concentration of 40μg/ml and incubation was continued as before for 1 hour. The lysate was transferred to an eppendorf tube and Proteinase K was added to a final concentration of 100μg/ml. Incubation was carried out at 55°C for 2 hours. The lysate was extracted twice with pre-warmed phenol (37°C) and once with chloroform: isoamyl (24:1). The DNA was precipitated by the addition of sodium acetate (0.3M) and 2 volumes of absolute ethanol at -20°C overnight. The DNA was pelleted by centrifugation at 4°C for 15 minutes at 11 600g in a microfuge. The pellet was washed twice with 70% ethanol, dried and resuspended in ultra pure water overnight.

B8: Hoechst staining for cytoplasmic DNA replication (Russel *et al.*, 1975)

Hoechst 33258 (bisbenzimide; Sigma) is a DNA fluorochrome that specifically binds to the adenine- thymidine regions of DNA.

RLSDV-RG- infected LT cells (grown on coverslips) were fixed with Carnoys fixative (methanol:glacial acetic acid; 3:1) for 15 minutes. The cover slips were then immersed in a working solution of Hoechsts stain (0.25-0.5μg/ml of

bisbenzimide in dH_2O) and allowed to stand for 30 minutes. The cover slips were then rinsed twice in dH2O, mounted in mounting fluid and viewed using a fluorescent microscope with a 53/44 barrier filter and a BG-3 exciter filter.



C1: Shuttle vector for inserting foreign genes into LSDV (section 2.3.2.). Not to scale

D1: Activated Trypsin

10x Trypsin base	10ml
5% Trypsin stock solution	1ml
PS	0.5ml

Made up to 100ml with dH_20 and stored at 4°C.

D2: Dulbecco's Modified Eagle's Essential Medium (DMEM)/Hams F12

DMEM powder	67.75g
Hams F12 powder	53.55g
$NaHCO_3$	24.4g

Mixed with 10L dH_20 and the pH adjusted to 7.1 with 3M HCl. The solution was sterilized using a 0.2μm filter, with two 5ml reference samples taken at the start and end of the filtration process in order to establish sterility. The solution was aliquoted into 1L amounts and stored at 4°C.

D3: Hepes buffered saline (1M)

HEPES	23.8g
NaOH (0.3M)	80ml

The HEPES was dissolved in the NaOH and the pH adjusted to 7.2. The final volume of 100ml was made up with filtered dH_20. The buffer was stored at 4°C.

D4: Hypoxanthine

Hypoxanthine	0.1g
NaON (0.1N)	10ml

The solution was mixed and filter sterilized through a 0.2μm filter and stored at 4°C.

D5: Lysis buffer

100ml 1M Tris-HCl, pH 8
20μl 0.5M EDTA
1.5g sucrose
20mg lysozyme
2mg RNase
1mg BSA
distilled water to 10ml

D6: Mycophenolic acid (10mg/ml)

Mycophenolic acid	0.1g
NaOH (0.1N)	10ml

The solution was mixed and filter sterilized through a 0.2μm filter, aliquoted into 100μl aliquots and stored at 4°C in the dark.

D7: Paraformaldehyde (4%)

This solution was prepared in a fume hood and gloves and mask were worn. 4g of paraformaldehyde was dissolved in 80ml of PBS. The solution was heated to

60°C, 1N NaOH added drop-by-drop until the solution cleared and the pH was 9.8. The solution was then made up to 100ml and stored at 4°C.

D8: Penicillin-Streptomycin (PS)

Penicillin (Novo Nordisk)	$1x10^6$ units
Streptomycin (Novo Nordisk, 1g/3ml)	3ml

The mixture was made up to 20ml with physiological saline, filtered through a 0.2μm filter and store at -20°C in 2ml aliquots.

D9: Phenol

The phenol was melted at 68°C and hydroxyquinoline added to a final concentration of 0.1%. An equal volume of 0.5M Tris-HCl pH8 was added, mixed and the upper phase removed. This extraction was repeated until the pH of the phenol phase was greater than 7.8. An overlay of 0.2% β-mercaptoethanol was added and the solution was stored at -20°C.

D10: Phosphate buffered saline (PBS)

NaCl	8.0g
KCl	0.2g
KH_2PO_4	0.1g
$NaHPO_4$	0.91g

The ingredients were dissolved in 900ml of dH_2O and the pH adjusted to 7.5. The solution was made up to a final volume of 1000ml, autoclaved and stored at room temperature.

D11: Selection medium for rLSDV

MPA (stock solution)	25µl
Xanthine (stock solution)	250µl
Hypoxanthine (stock solution)	14µl
FCS	1ml
PS	0.05ml
F	0.1ml
DMEM/Hams F12	to 10ml

D12: Stop/loading buffer (6x)

Bromophenol blue	9.2ml
EDTA (0.5M, pH8)	0.4ml
Sucrose	4g
dh_20	to 10ml

D13: Substrate solution (x-gal stain)

PBS	9.2ml
x-gal (50mg/ml)	200µl
Potassium ferricyanide (50mM)	200µl
Potassium ferrocyanide (50mM)	200µl
$MgCl_2$	200µl

D14: Tissue culture medium

Foetal calf serum (FCS)	100ml
PS	5ml
F	10ml
DMEM/HamsF12	to 1000ml

D15: Trypan blue

Trypan blue powder	5g
Physiological saline	to 100ml

The mixture was filtered through Whatman number 1 paper and refiltered through a 0.2μm filter, and finally store at 4°C in 2ml aliquots.

D16: Trypsin base (10X)

NaCl	30g
KH_2PO_4	1.2g
KCl	2g
Na_2HPO_4	11.2g
Glucose	5g

The ingredients were dissolved in 700ml dH_2O. 2g of EDTA, dissolved in 100ml of dH_2O were added. 25ml of 4% phenol red (0.4g phenol red in 60ml 0.05N NaOH, made up to 100ml) was added and the pH adjusted to 7.8 with 1N NaOH. dH_2O was added to attain a final volume of 1000ml, the solution filtered through a 0.2μm filter and stored at -20°C in aliquots.

D17: Trypsin stock solution

10x trypsin base	10ml
Trypsin powder	5g

The trypsin base was added to 90ml of dH_2O and 0.5ml of PS. HCl (1M) was added dropwise until the solution turned yellow. The mixture was then heated to 37°C, the trypsin powder added and allowed to dissolve by letting it stand overnight at 4°C. The solution was filtered through a 0.2μm filter and stored at -20°C in aliquots.

D18: Virus diluent

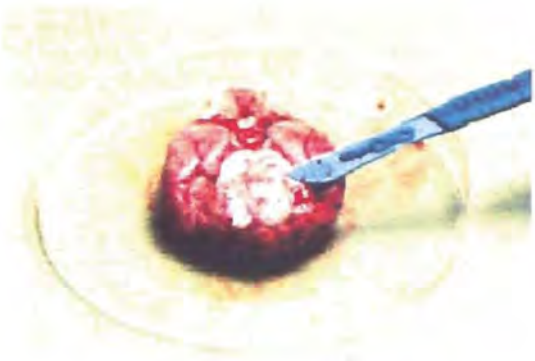
Hepes (1M)	1ml
PS	0.5ml
F	1ml
DMEM/HamsF12	to 100ml

D19: Xanthine (stock solution, 10mg/ml)

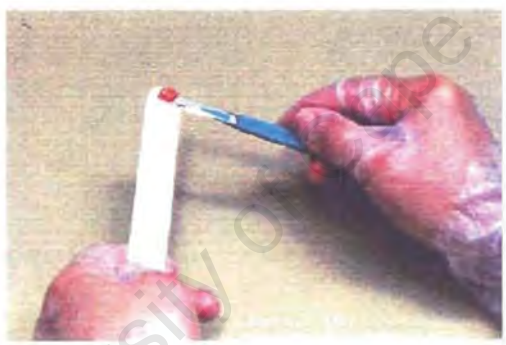
Xanthine	0.1g
NaOH (0.1N)	10ml

The solution was filtered through a 0.2μm filter and stored at -4°C.

APPENDIX E: FLUORESCENT ANTIBODY TEST FOR RABIES
(Adapted from www.cdc.gov/ncidod/dvrd/rabies)



The brain is removed from the animal, and the medulla oblongata identified. This is the white region at the lower base of the brain.



A piece of this tissue is placed on a tongue depressor or paper towel.

An impression slide is made from each tissue by carefully touching a clean microscope slide to the tissue. Excess tissue can be removed by blotting on paper towels or filter paper.

The slides are allowed to air dry. This usually takes about 30 minutes. After drying, the slides are fixed in acetone for 1-4 hours. Fixation allows the tissue to adhere to the slides and makes the cells permeable to the direct fluorescent antibody (dFA) reagents. This allows rabies virus nucleoprotein antigen present in the tissue to react with the labelled antibody. Rabies conjugate, anti-rabies nucleoprotein labeled with a fluorescent dye (fluorescein-isothiocyanate, FITC) is added to the fixed slides, and slides are incubated in a moist chamber for 30 minutes at 37°C to allow the reaction to occur. Slides are washed in phosphate- buffered saline (PBS) to remove unreacted conjugate, rinsed in water to remove any salt residue from the PBS, and mounted with coverslips, using a buffered mounting medium to hold the pH at approximately 8.5 before they are observed using a fluorescent microscope.

If the brain tissue is positive, rabies antigen will appear bright green under the fluorescent microscope.

APPENDIX F: EXECUTIVE SUMMARY OF BUSINESS PLAN FOR COMMERCIALISING CAPRIRAB

Executive Summary

Caprirab

A novel rabies vaccine developed by

NUVAX

Division of Medical Virology

Faculty of Health Sciences

University of Cape Town

Anzio Rd

Observatory

Cape Town 7925

South Africa

Kate Aspden

kturner@curie.uct.ac.za

082 681 6246

PhD Thesis: A Study of the host-restricted lumpy skin disease virus as a vaccine vector using rabies as a model.

Supervisor: Assoc Prof A-L Williamson (annalise@curie.uct.ac.za)

(021)406 6124

Introduction

Nuvax will be a company that specialises in the development and commercialisation of potential vaccines and vaccine-related products. We have identified a niche market between laboratories and the marketplace. Nuvax will develop innovations that arise from academic research teams to the point of commercialisation by ongoing consultation, facilitation, project management and potentially funding. Each innovation will be evaluated individually and a specific business plan and strategy is applied depending on the individual circumstances.

Mission: To provide a vehicle for successfully commercialising vaccines, reagents and other biotech products and in the process develop a centre of excellence in vaccine area, that creates world-class products and provides a career path for vaccine researchers. Nuvax does not anticipate turning into a marketing and sales organisation; however, we may take responsibility for manufacturing.

Furthermore, opportunities exist for contract research to be performed in various academic institutions for which facilitation is often required. Nuvax will have the capacity not only to perform certain contract research tasks but also to facilitate collaborative relationships.

The following Executive summary is specifically written for **Caprirab**, a candidate rabies vaccine developed by Kate Aspden as the subject of her PhD at the University of Cape Town's Faculty of Health Sciences. Nuvax will undertake to bring this potentially beneficial product to the point of commercialisation.

Table of contents

CAPRIRAB

1. Abstract

Recombinant vaccine* technology is an innovative method for producing novel vaccines for use in a wide variety of hosts. Vaccinia virus (the smallpox vaccine) has been used as a vector for producing recombinant vaccines with great success based on the knowledge gained through the smallpox epidemic. Unfortunately, with the advent of HIV, this vector is no longer safe as it can cause disseminated infections in immunocompromised individuals.

Kate Aspden developed a recombinant rabies vaccine using the lumpy skin disease virus vaccine as a vector during the course of her PhD at the University of Cape Town. This vector is safe because productive replication is restricted to cattle cells. The recombinant rabies vaccine (Caprirab) successfully protects mice against rabies infection. It produces long-term immunity to rabies in cattle that may potentially require only a single immunisation.

Work is in progress to increase the value of the vaccine by testing its ability to be administered orally (in baits) and by inserting genes from multiple disease-causing pathogens thereby making the vaccine multivalent

Since there are currently at least 4 international pharmaceutical companies supplying the world market with rabies vaccines and each company has manufacturing, marketing and distribution channels in place, we envisage licensing the production, marketing and sales to one of the above. Furthermore, since Caprirab does not require the level of processing that the existing rabies vaccines do, the lower production cost of the vaccine is an attractive advantage.

The Caprirab project will require approximately R1 500 000 for further animal trials and registration in order for the product to be licensed.

2.Product Description

Technology Description

The proposed vaccine is a live recombinant lumpy skin disease virus (Neethling vaccine strain) expressing the glycoprotein** of rabies virus in such a manner that rabies-specific immunity is induced in vaccinees. The vaccine is aimed at cattle and will confer dual immunity to both lumpy skin disease, a disfiguring disease of cattle, and rabies, a fatal neurological disease of mammals.

- The live nature of the vaccine results in a cell-mediated and antibody-mediated immune response against rabies in vaccinees.
- It has the ability of inducing immunity to both rabies and lumpy skin disease in cattle.
- It has the potential of being given orally in bait form (potential use in wildlife).
- It can elicit lifelong immunity after a single vaccination.
- It is stable, therefore maintenance of a cold chain is unnecessary.

Technical Definition

**Recombinant virus*: a virus that has been genetically modified to express a protein from another pathogen with the aim of inducing an immune response to the expressed protein and thus protection against the said pathogen.

***Glycoprotein:* coat protein that surrounds a virus. The primary protein against which immunity is conferred.

3. Stage of Development

Caprirab was produced in the laboratory and tested in cattle, mice and rabbits after performing convincingly in cell culture. The product is not yet ready for market in that a large-scale trial still needs to be performed in order to register the vaccine for use. This will take approximately 2

years (see appendix A) and cost in the region of R500 000. Registration in South Africa takes 6 months (Appendix F1) and costs R1600, however, registration for use internationally will be beneficial as the market in developing countries other than South Africa is large. We envisage the vaccine having major advantages in other developing countries such as Brazil, Mexico, and India where cattle rabies is an on-going problem. Further investigations are required with regards to registration of vaccines in the above countries.

At the current R/$ exchange rate, and laboratory capability production volumes, Caprirab will cost in the region of R10 per dose, however, if raw materials were purchased in $ and the production volume was scaled up, the cost price per dose could easily be reduced ten-fold. In addition, the vaccine does not require an inactivation step (which most other rabies vaccines do) and thus have a manufacturing cost advantage over inactivated rabies vaccines. The cost price per dose would thus competitive.

4. Market Analysis

The African market for lumpy skin disease vaccines for cattle is in the region of 200 000 doses per year. This market obviously fluctuates depending on whether or not outbreaks occur. The international market for lumpy skin disease vaccines is too small to be significant, however, the international markets for rabies vaccines in cattle are estimated to be in the region of 2 000 000 doses in South America, and 30 000 in India. These are conservative estimations. We expect the number of doses to increase with ease of administration and availability of an inexpensive effective vaccine. Rabies in cattle in Africa is becoming a problem, although very little reliable data is available.

Barriers to entry include: high development costs, the cost of clinical trials, regulatory approval requirements, competitors and public opinion.

Rabies vaccines currently sell for approximately R3 per dose for cattle and need to be given annually. This equates to R30 per animal (with a mean life-span of 10 years). We believe that the convenience of vaccinating a herd of cattle once will allow us to sell Caprirab at a premium price of approximately R15 ($2) per dose. Public acceptance will increase over time and thus so will the market share.

The market share that Caprirab can expect to corner would start in the region of 20% with the correct marketing. This equates to 400 000 doses in South America based on the current estimated figures. At R15 per dose, its market potential would result in a turn over of approximately R 6 000 000. Over time, both the market share and the size of the international market are expected to increase (see appendix F2).

5. Competitor Analysis

Large international pharmaceutical companies such as Bayer, Intervet, Meriel and Fort Dodge that all produce and supply rabies vaccines currently dominate the market. These companies have large manufacturing facilities that enable them to sell vaccines at a price that is affordable. They also have existing marketing and distribution channels that have effectively cornered the international rabies vaccine market.

The benefit of Caprirab is that in cattle, a single inoculation is required, as opposed to a stringent vaccination schedule required by other inactivated vaccines. It also has the potential of being given orally, which would save administration costs. It is a dual vaccine that potentially can become multivalent in cattle. Furthermore, it is safer than existing recombinant rabies vaccines.

6. Strategy

The most advantageous strategy is to conduct large-scale, phase three animal trials and register the vaccine for use in South Africa. Intervet currently has the tender for rabies vaccines in South Africa, and they would be our first choice as licensees. The prospect of increasing their market share by including prophylaxis to lumpy skin disease would be attractive to them. Subsequently Caprirab will be marketed to the big players in the international market for production and sales under a licensing agreement.

Licenses would be sold to companies on a regional basis depending on the regional market share that each potential licensee has captured. An upfront fee would be charged to the regional licensee to demonstrate intent to commercialise. A monthly royalty fee would be charged based on a percentage of sales. This fee would be subject to a minimum annual fee. Furthermore, Nuvax would exercise the right to audit the licensee and the agreement would contain a right of renewal. The licensee would benefit from regional exclusivity. Appendix F2 illustrates the cash flow model based on this strategy.

7. Sales and Marketing plan

Nuvax will focus on selling the Caprirab concept to Meriel, Fort Dodge, Bayer and Intervet with idea of licensing the vaccine to one of them. Caprirab would be the ideal candidate for a tender application due to its ability to protect after a single dose and the fact that it is safe. This strategy eliminates the need for developing specialised marketing and distribution channels. An expected revenue plan for 3-5 years based on the licensing model of Caprirab is shown in appendix F2.

8. Management and Staff (NUVAX)

Kate is completing her PhD in Medical Virology in the Faculty of Health Sciences at UCT. Her honours degree, completed in the same department, focused on creating a vector with which to produce recombinant viruses for vaccine use. She has commercial experience and intends to complete a MBA at UCT's Graduate School of Business.

Nuvax intends to source innovative vaccine concepts from research groups based at universities, colleges and technicons. In order to assess projects, a scientific advisory committee will be established. Networks with incubator companies and research groups as well as potential manufacturers will be established. Salaries will cost approximately R200 000 in the first year.

As Nuvax grows, we envisage employing additional consultants with expertise in vaccine development, commercialisation, and intellectual property management in order to commercialise multiple "vaccine related products" in tandem.

9. Intellectual Property

Currently, Caprirab is protected by a provisional patent (20013874). UCT shares the intellectual property rights with the inventors, Anna-Lise Williamson and Kate Aspden. The above parties will sign an agreement with the licensees and receive royalty payments.

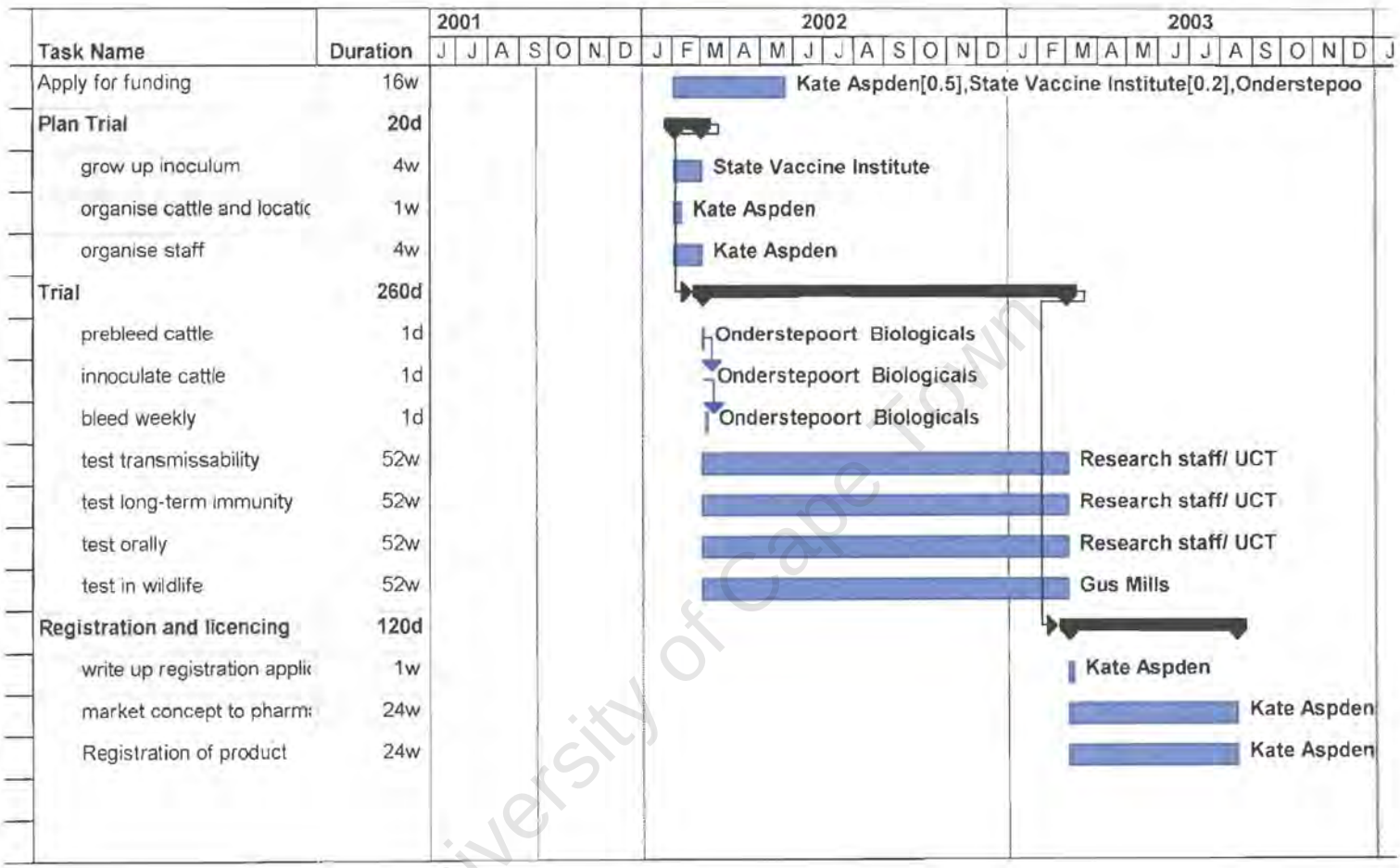
The above parties intend to take out a South African patent, and then submit a Patent Cooperation Treaty Application, which costs approximately R300 000. Nuvax then has 30 months in which to sell the licence to a major player in the pharmaceutical industry.

10. Funding Requirements for Caprirab project

The total investment for year 1 and 2 will be approximately R1 300 000, which is broken down as follows:

Cost of trial:	R500 000
Cost of PCT	R300 000
Cost of SA registration:	R1600
Salaries:	R200 000
Operating costs and sundries: Incl marketing and travel	R300 000
Total:	R1 301 600

Appendix F1



Appendix F2: Cash Flow ($)

Yr 3

	Africa	S America	India & East	Other	
Upfront Fee		10 000	10 000	10 000	10 000
Est annual doses		500 000	3 000 000	200 000	?
% market share		50	20	25	?
cost per dose		2	2	2	2
8% of sales		40 000	96 000	8000	?
Monthly income		4166.67	8833.33	750	?
Min annual fee		10 000	10 000	10 000	10 000

Est annual income in year 1 of marketing: $146 000

	Approx R1 293 560
Salaries	(300 000)
Sundries	(100 000)
Gross profit	R893 560
Tax@30%	(268068)
Net profit	R625 492

Yr 4

	Africa	S America	India & East	Other	
Upfront Fee					10 000
Est annual doses		500 000	3 000 000	200 000	100 000
% market share		60	40	35	20
cost per dose		2.1	2.1	2.1	2.1
8% of sales		50 400	201 600	11 760	3360
Monthly income		4200	16800	980	280
Min annual fee		10 000	10 000	10 000	5000

Est annual income in year 1 of marketing: $263 775

	Approx R2 373 975
Salaries	(320 000)
Sundries	(100 000)
Gross profit	R1 953 975
Tax@30%	(586 192.5)
Net profit	R1 367 782.5

REFERENCE LIST

ACHA, P.N., AND ARAMBULO III, P.V. 1985. Rabies in the tropics – history and current status. In Kuwert,E., Merieux, C., Koprowski, H and Bogel, K. (eds). *Rabies in the tropics.* Berlin:Springer-verlag.

AHUJA, S., TRIPATHY, K.K., SAHA, S.M. AND SAXENA, S.N. 1985. Epidemiology of rabies in India. In: Kuwert, E., Merieux, C., Koprowski, H and Bogel, K. (eds). *Rabies in the tropics.* Berlin:Springer-verlag.

ALBAS, A., PARDO, P.E., GOMES, A.A., BERNARDI, F., AND ITO, F.H. 1998. Effect of a booster-dose of rabies vaccine on the duratution of virus neutralising antibody titres in bovines. *Revista da Sociedade Brasileira de Medicina Tropical.* **31(4)**:367-371.

ALBERTS, B., BRAY, D.,LEWIS, J.,RAFF, M.,ROBERTS, K. AND WATSON, J.D. 1989. How are cells Studied. Ch 4..In *Molecular Biology of the Cell.* 2nd Edition. Garland Publishing Inc, New York, London: 135-201.

ALEXANDER, R.A., PLOWRIGHT, W., AND HAIG, D.A. 1957. Cytopathic agents associated with lumpy skin disease of cattle. *Bulletin of Epizootological Diseases in Africa.* **5**:489-492.

ALI, A.A., ESMET, M., ATTIA, H., SELIM, A., AND HAMID, Y.M. 1990. Clinical and pathological studies on lumpy skin disease in Egypt. *Veterinary Record.* **127**:549-550.

ALLEN, T.M., VOGEL, T.U., FULLER, D.H, MOTHE, B.R., STEFFEN, S., BOYSON, J.E., SHIPLEY, T., FULLER, J., HANKE, T., SETTE, A., ALTMAN, J.D., MOSS, B., MCMICHEAL, A.J., WATKINS, D.I. 2000. Induction of AIDS virus-specific CTL activity in fresh, unstimulated peripheral blood lymphocytes from rhesus macaques vaccinated with a DNA prime/modified vaccinia virus Ankara boost regime. *Journal of Immunology.* **164(9)**:4968-4978.

AMARA, R.R., VILLINGER, F., ALTMAN, J.D., LYDY, S.L., O'NIEL, S.P., STAPRANS, S.I., MONTEFIORI, D.C., XU, Y., HERNDON, J.G., WYATT, L.S., CANDIDO, M.A., KOZYR, N.L., EARL, P.L., SMITH, J.M., MA, H.L., GRIMM, B.D., HULSEY, M.L., MILLER,J., MCCLURE, H.M., MCNICHOLL, J.M., MOSS, B., ROBINSON, H.L. 2001. Control of a mucosal challenge and prevention of AIDS by a multiprotein DNA/MVA vaccine. *Science.***292(5514)**:69-74.

AMERICAN VETERINARY MEDICAL ASSOCIATION. 2000. Compendium of animal rabies control. www.avma.org/pubhlth/rabcont.asp.

ANILIONIS, A, WUNNER, W.H., AND CURTIS, P. 1981. Structure of the glycoprotein gene of rabies virus. *Nature.* **294**:275-278.

ANTOINE, G., SCHEIFLINGER, F., AND FALKNER, F.G. 1998. The complete genomic sequence of the modified vaccinia Ankara strain: comparison with other orthopoxviruses. *Virology.* **244(2)**:365-396.

ATANASIU, P., GAMET, A., GRAVIERE, P., LE GUILLOUX, M., GUILLON, J.C., AND VALLE, A. 1968. Reapparition de la rage en France. Premier cas chez un renard dans la Moselle. *Bulletin de l'Academie Veterinaire.***XLI:**161-163.

AUSUBEL, F.M., BRENT, R., KINGSTON, R.E., MOORE, D.D., SEIDMAN, J.G., SMITH, J.G., AND STRUHL, K. (eds) 1987. *Current protocols in molecular biology*. John Wiley and sons, NY, USA.

BACHMANN, M.F., KUNDIG, T.M., FREER, G., LI, Y., KANG, C.Y., BISHOP, D.H., HENGARTNER, H. AND ZINKERNAGEL, R.M. 1994. Induction of protective CTL with viral proteins. *European Journal of Immunology.***24:**2228-2236.

BAER, G.M. 1988. Oral rabies vaccination: An overview. *Reviews of Infectious Diseases.* **10**. :644-648.

BAER, G.M., BELLINI, W.J., AND FISHBEIN. D.B. 1996. Rhabdoviruses (Ch 32) In *Virology* (Third ed) Eds: Fields,B.N., Knipe,D.M., Howly,P.M. Lippincott-Raven Press, Ltd, New York :883-910.

BAILEY, M.J. AND POSSEE, R.D. 1991. Manipulation of baculovirus vectors. Chapter 13. In *Methods in Molecular Biology*, Volume 7: Gene Transfer and Expression Protocols. Murray, E.J. (ed). The Humana Press Inc. Clifton. N.J.

BALDICK, C.J.JR., AND MOSS, B. 1993. Characterisation and temporal regulation of mRNAs encoded by vaccinia virus intermediate stage genes. *Journal of Virology.* **67**:3515-3527.

BALL, L.A. 1987. High-frequency homologous recombination in vaccinia virus DNA. *Journal of Virology.* **61**:1788-1795.

BARNARD, B.J.H., MUNZ, E., DUMBELL, K., AND PROZESKY, L. 1994. *Infectious diseases of livestock.* Volume 1. eds Coetzer, J.A.W., Thomson,G.R., and Tustin,R.C. Oxford University Press:605-612.

BAROUCH DH, SANTRA S, KURODA MJ, SCHMITZ JE, PLISHKA R, BUCKLER-WHITE A, GAITAN AE, ZIN R, NAM JH, WYATT LS, LIFTON MA, NICKERSON CE, MOSS B, MONTEFIORI DC, HIRSCH VM, LETVIN NL. 2001. Reduction of simian-human immunodeficiency virus 89.6P viremia in rhesus monkeys by recombinant modified vaccinia virus Ankara vaccination. *Journal of Virology.* **75(11)**:5151-8

BAROUDY, B.M., VENTKATENSAN, S., AND MOSS, B. 1982. Incompletely bae-paired flip-flop terminal loops link the two DNA strands of the vaccinia virus genome into one uninterrupted polynucleotide chain. *Cell.* **28**: 315-324.

BARTH, R., BIJOK, U., GRUSCHKAU, H., JAEGER, O., AND WEINMANN, E. 1985. Purified chick embreyo cell (PCEC) rabies vaccine for human use- laboratory data. In Kuwert, E., Merieux, C., Koprowski, H., and Bogel, K. (eds) . *Rabies in the Tropics.* Springer-Verlag.

BASHEER, A.M., RAMAKRISHNA, J., AND MANICKAM, R. Evaluation of post-exposure vaccination against rabies in cattle. *New Microbioligica.* 1997.**20(3)**:289-94.

BAXBY, D. AND PAOLETTI, E. 1992. Potential use of non-replicating vectors as recombinant vaccines. *Vaccine.* **10**: 8-9.

BAXBY, D. 1993. Recombinant poxvirus vaccines. *Reviews in Medical Microbiology.* **4** :80-88.

BELYAKOV, I.M., WYATT, L.S., AHLERS, J.D., EARL, P., PENDLETON, C.D., KELSALL, B.L., STROBER, W.,MOSS, B., AND BERZOFSKY, J.A. 1998. Induction of a mucosal cytotoxic T-lymphocyte response by intrarectal immunization with a replication-deficient recombinant vaccinia virus expressing human immunodeficiency virus 89.6 envelope protein. *Journal of Virology.* **72(2)**:8264-8272.

BELYAKOV, I.M., DERBY, M.A., AHLERS, J.D., KELSALL, B.L., EARL, P., MOSS, B., STROBER, W. AND BERZOFSKY, J.A.1998. Mucosal immunization with HIV-1 peptide vaccine induces mucosal and systemic cytotoxic T lymphocytes and protective immunity in mice against intrarectal recombinant HIV-vaccinia challenge. *Proceedings of the National Academy of Sciences of the United States of America*.**95:**1709-1714.

BENDER, B.S., ROWE, C.A., TAYLOR, S.F., WYATT, L.S., MOSS, B., AND SMALL, P.A. 1996. Oral immunization with a replication-deficient recombinant vaccina virus protects mice against influenza. *Journal of Virology*. **12**:1032-1040.

BERAN, G.W. 1982. Ecology of dogs in the Central Philippines in relation to rabies control efforts. *Comparative Immunology, Microbiology and Infectious Diseases*. **5(1-3):**265-70.

BINGHAM, J., FOGGIN, C.M., GERBER, H., HILL,F. W.G., KAPPELER, A., KING, A.A., PERRY, B.D., AND WANDELER, A.I. 1992.Pathology of SAD rabies vaccine given orally in chacma baboons (*Papio ursius*) *The Veterinary Record*. **18**:55-56.

BINGHAM ,J., SCHUMACHER, C.L., AUBERT, M.F.A., HILLS, F.W.G., AND AUBERT, A. 1997. Innocuity studies of SAG-2 oral rabies vaccine in various Zimbabwean wild non-target species. *Vaccine*. **15(9)** :937-943.

BINGHAM, J., KAPPELAR, A., HILL,F. W.G., KING, A.A., PERRY, B.D., AND FOGGIN, M. 1995. Efficacy of SAD (Berne) rabies vaccine given by the oral route in two species of jackal (*Canis mesomelas* and *Canis adustus*). *Journal of Wildlife Diseases*. **31(3)** :416-419.

BINGHAM, J., JAVANGWE,S., SABETA,C.T., WANDELER, A.I., NEL, L.H. 2001. Report of unusual lyssaviruses (rabies and Mokola virus) identified respectively in Zimbabwe. *Journal of South African Veterinary Association*. **72(2):**92-94.

BLACK, D.N., HAMMOND, J.M., AND KITCHING, R.P. 1986. Genomic relationship between capripoxviruses. *Virus research*. **5**:277-292.

BLANCHARD, T.J., ALCAMI, A., ANDREA, P., AND SMITH, G.L. 1998. Modified vaccinia virus Ankara undergoes limited replication in human cells and lacks several immunomodulatory proteins: implications for use as a human vaccine. *Journal of General Virology*. **79**:1159-1167.

BLANCOU, J., ANDRAL, L., LAGRANGE, P.H., AND TSIANG, H. 1979. Kinetics of different specific immunological parameters after rabies vaccination in mice. *Infection and Immunity.* **24**(3):600-605.

BLANCOU, J., PEPIN, M. SOULEBOT, J.P., AND BRUN, A. 1984. The vaccination of cattle against rabies. Antibody kinetics, resistance to challenge three years after vaccination. *Annale Recherche.Veterinaire.* **15(4)**:543-547.

BLANCOU, J., AUBERT, M.F., AND ANDRAL, L. 1983. Studies on pathogenic, immunogenic, and protective efficiency of fox rabies virus before and after adaptation to cell culture: application to vaccination against rabies. *Canadian Journal of Microbiology.* **29(1)**:77-83.

BLANCOU, J., BALTAZAR, R.S., MOLLI, I., AND STOLTZ, J.F. 1991. Effective postexposure treatment of rabies-infected sheep with rabies immune globulin and vaccine. *Vaccine.* **9(6)**:432-7.

BLANCOU, J., KIENY, M.P., LATHE, R., LECOQUE, J.P., PASTORET, P.P.,SOULEBOT, J.P., AND DESMETTRE, P. 1986. Oral vaccination of the fox against rabies using a live recombinant vaccinia virus. *Nature.* **322**:373-375.

BLASCO, R., AND MOSS, B. 1992. Role of cell associated enveloped vaccinia virus in cell-to-cell spread. *Journal of Virology.* **66**:4170-4179.

BRAND, E.,G. 1993. Selectable markers for recombinant poxviruses. Masters Thesis. University of Cape Town, Faculty of Science.

BROCHIER, B., KIENY, M.P., COSTY, F., COPPENS, P., BAUDUIN, B., LECOCQ, J.P., LANGUET, B., CHAPPUIS, G., DESMETTRE, P., AFIADEMANYO, K. ET AL. 1991. Large-scale eradication of rabies using recombinant vaccinia-rabies vaccine. *Nature*; **354(6354)**:520-2.

BROCHIER, B., BLANCOU, J., THOMAS, I., LANGUET, B., ARTOIS, M., KIENY, M.P., LECOCQ, J.P., COSTY, F., DESMETTRE, P., CHAPPUIS, G. ET AL. 1989. Use of recombinant vaccinia-rabies glycoprotein virus for oral vaccination of wildlife against rabies: innocuity to several non-target bait consuming species.*Journal of Wildlife Diseases.***25(4)**:540-547.

BOYLE, D.B. 1992. Quantitative assessment of poxvirus promoters in fowlpox and vaccinia virus recombinants. *Virus Genes.* **6** :281-290.

BOYLE, D.B., AND COUPAR, B.E.H. 1988. A dominant selectable marker for the construction of recombinant poxviruses. *Gene.* **65**:123-128.

BURDIN, M.L. 1959. The use of histopathological examination of skin material for the diagnosis of lumpy skin disease in Kenya. *Bulletin of Epizootological Diseases in Africa.* **7**:27-36.

CADOZ, M., STRADY, A., MEIGNIER, B., TAYLOR, J., TARTAGLIA, J., PAOLETTI, E., AND PLOTKIN, S. 1992. Immunisation with canarypox virus expressing rabies glycoprotein. *The Lancet.* **339. 8807**. :1429-1449.

CAPSTICK, P.B., AND COACKLEY, W. 1961. Protection of cattle against lumpy skin disease. *Research in Veterinary .Sciences.* **2**:362-368.

CAMELO, S., CASTELLANOS, J., LAFAGE, M., AND LAFON, M. 2001. Rabies virus ocular disease: T-cell-dependent protection is under the control of signaling by the p55 tumor necrosis factor alpha receptor, p55TNFR. *Journal of Virology.* **75(7):**3427-3434.

CARN, V.M. 1993. Control of capripoxvirus infections. *Vaccine.* **11(13)**:1275-1279.

CARN, V.M. AND KITCHING, R.P. 1995. The clinical response of cattle experimentally infected with Lumpy Skin Disease (Neethling) virus. *Archives of Virology.* **140** :503-513.

CARN, V.M., AND KITCHING, R.P. 1995. An investigation of possible routes of transmission of lumpy skin disease virus (Neethling). *Epidermological Infections.* **114**:219-226.

CARN, V.M., TIMMS, C.P., CHAND, P., BLACK, D.N., AND KITCHING, R.P. 1994. Protection of goats against capripox using a subunit vaccine. *The Veterinary.Record.* **135**:434-436.

CARROL, M.W., AND MOSS, B. 1997. Host range and cytopathogenicity of the highly attenuated MVA strain of vaccinia virus: propagation and generation of recombinant viruses in a nonhuman mammalian cell line.*Virology.***238(2):**198-211.

CARROLL, M.W., OVERWIJK, W.W., CHAIMBERLAIN, R.S., ROSENBERG, S.A., MOSS, B., AND RESTIFO, N.P. 1997. Highly attenuated modified vaccinia virus Ankara (MVA) as an effective recombinant vector: a murine tumor model. *Vaccine.* **15(4)**:387-394.

CENTRE FOR DISEASE CONTROL website. www. Cdc.gov/ncidod/dvrd/rabies.

CELIS, E., OU, D., DIETZSCHOLD, B., OTVOS, J.R.L., AND KOPROWSKI, H. 1989. Rabies virus-specific T cell hybridomas: Identification of class II MHC-restricted T-cell epitopes using synthetic peptides. *Hybridoma.***3**:263-275.

CHAMBERS, S.P., PRIOR, S.E., BARSTOW, D.A., MINTON, N.P. 1988. The pMTL nic-cloning vectors. I. Improved pUC polylinker regions to facilitate the use of sonicated DNA for nucleotide sequencing. *Gene.* **68:**139-149.

CHIHOTA, C.M., RENNIE, L.F., KITCHING, R.P., AND MELLOR, P.S. 2001. Mechanical transmission of lumpy skin disease virus by Aedes aegypti (Diptera :Culicidae). *Epidemiology and .Infection.* **126(2)**:317-321.

CHOPPIN, P.W., AND SCHEID, A. 1980. The role of glycoproteins in adsorption, penetration and pathogenicity of viruses. *Reviews of.Infectious Diseases.* **2**:40-61.

CHUNG, C-S., HSIAO, J-C., AND CHANG, W. 1998. A27L protein mediates vaccinia virus interaction with cell surface heparan sulfate. *Journal of Virology.* **72(2)**:1577-1585.

CLIQUET, F., AUBERT, M., AND SAGNE, L. 1998. Development of F1 antibody virus neutralization test (FAVN) for the quantitation of rabies virus neutralizing antibodies. *Journal of immunological methods.* **212**:79-89.

COCHRAN, M.A., PUCKET, C., AND MOSS, B. 1985. In vitro mutagenisis of the promoter region for a vaccinia virus gene: evidence for tandem early and late regulatory signals. *Journal of Virology.* **54**:30-37.

COHEN, A., WILLIAMSON,A-L., PAWESKA,J., SWANEPOEL,R., BURT,F., JARDINE,J., AND VAN DIJK,A. 1999. Evaluation of the vaccine potential of a Rift Valley Fever-Lumpy skin disease virus recombinant in sheep. Abstract. Xith International congress of Virology:81.In Sydney, Australia.

COHEN, A., COX, D., VAN DIJK, A.A., KORBER, A., DUMBELL, K.R., AND WILLIAMSON, A-L. 1997. Lumpy skin disease virus as a vaccine vector for rift valley fever virus and bovine

ephemeral fever virus. *Proceedings of the 4th Congress of the European Society for Veterinary Virology, Edinbugh, Scotland, 24-17 August 1997.* :230-231.

COX J.H, DIETZSCHOLD B, AND SCHNEIDER LG. 1977. Rabies virus glycoprotein .II.Biological and serological characterization. *Infection and Immunity.* 16: 754-759.

COX, W.I., TARTAGLIA, J., AND PAOLETTI, E. 1993. Induction of cytotoxic T lymphocytes by recombinant canarypox (ALVAC) and attenuated vaccinia (NYVAC) virusexpressing the HIV-1 envelope glycoprotein. *Virology.* **195**:845-850.

CUDMORE, S., COSART, P., GRIFFITHS, G., AND WAY, M. 1995. Actin-based motility of vaccinia virus. *Nature.* **378(6557)**:636-638.

DAGERT, M. AND EHRLICH, S.D. 1979. Prolonged incubation in calcium chloride improves the competence of Escherichia coli cells. *Gene.* **6(1)**:23-8.

DALES, S., AND KAJIOKA, R. 1964. The cycle of vaccinia virus in Earle's strain L cells. 1. Uptake and penetration. *Virology.* **24**:278-294.

DAVIES, F.G. 1981. Lumpy Skin Disease. In *Virus diseases of food animals.* Gibbs,E.P.J.(eds) Academic Press Ltd.London :751-763.

DAVIES, F.G. 1991. Lumpy skin disease, an African capripox virus disease of cattle. *Bovine Veterinary Journal.* **147**:489-503.

DAVIES, F.G. 1991. Lumpy skin disease: a growing problem in Africa and the near East. *World Animal Review.* **68**:37-42.

DAVIES, R.E., AND MATTHEWS, C.K. 1993. Acidic-C terminus of vaccinia virus DNA-binding protein interacts with ribonucleotide reductase. *Proceedings of the .National.Acadamy of.Sciences,.USA.* **90**:745-749.

DEAN, D.J., ABELSETH, M.K., AND ATANASIU, P. 1996. The fluorescent antibody test. Chapter 7 in Laboratory Techniques in Rabies. Meslin F-X, Kaplan MM, Koprowski H. (ED.) World Health Organisation, Geneva. Fourth ed:88-95.

DE BOOM, H.P.A. 1948. Knopvelsiekte. *South African Scientific Bullitin.* **1**:44-46.

DEMARTINI, J.C., BICKLE, H.M., BRODIE, S.J., HE, B.X., AND ESPOSITO, J.J . 1993. Raccoon poxvirus rabies virus glycoprotein recombinant vaccine in sheep. *Archives of Virology*. **133**: 211-222.

DE MATTOS, C.A., DE MATTOS, C.C., SMITH, J.S., MILLER, E.T., PAPO, S., UTRERA, A., AND OSBURN, B.I. 1996. Genetic Characterization of rabies field isolates from Venezuela. *Journal of Clinical Microbiology*. **34(6):**1553-1558.

DEPARTMENT OF HEALTH. Guidelines for the medical management of Rabies in South Africa.2000.

DEUBEL, V., KINNEY, R.M., ESPOSITO, J.J., CROPP, C.B., VORNDAM, A.V., MONATH, T.P., AND TRENT, D.W. 1988. Dengue 2 virus envelope protein expressed by a recombinant vaccinia virus fails to protect monkeys against dengue.*Journal of General Virology*. **69 (8):**1921-1929.

DIAZ, A.M., AND LOMBARDO, R.A. 1981. Immunisation of calves with a rabies vaccine from suckling mouse brain. *Reviews of Argentinian Microbiology*. **13(2)**:45-48.

DIESEL, A.M. 1949. The epizootiology of lumpy skin disease in South Africa. *Proceedings of the 14th.International Veterinary Congress.* London. **2**:492-500.

DIETZSCHOLD, B., AND ERTL, H.C. 1991. New developments in the pre- and post-exposure treatment of rabies. Critical Reviews in Immunology. **10**(5):427-439.

DIETZSCHOLD, B., GORE, M., MARCHADIER, D., NIU, H.S., BUNSCHOTEN, H.M., OTVOS, L JR., WUNNER, W.H., ERTL, H.C., OSTERHAUS, A.D., AND KOPROWSKI, H. 1990. Structural and immunological characterisation of a linear virus-neutralising epitope of the rabies virus glycoprotein and its possible use in a synthetic vaccine. *Journal of Virology*. **64(8)**:3804-9.

DIETZSCHOLD, B., AND HOOPER, D.C. 1998. Human diploid cell culture rabies vaccine (HDCV) and purified chick embryo cell culture rabies vaccine (PCECV) both confer protective imunity against infection with the silver-haired bat rabies virus strain. *Vaccine*. **16(17)**:1656-1659.

DI TRANI, L., IRSARA, A., CIUCHINI, F., PESTALOZZA, S., AND ORFEI, Z. 1982. Antibody levels following ERA rabies vaccination in cattle. *Comparative Immunology, Microbiology and Infectious Diseases*. **5(1-3)**:199-203.

DOMS, R.W., BLUMENTHAL, R. AND MOSS, B. 1990. Fusion of intra- and extracellular forms of vaccinia virus with the cell membrane. *Journal of Virology.***64**:4884-4892.

DREESON, D.W. 1997. A global review of rabies vaccines for human use. *Vaccine.* **15(Supp)**:S2-6.

DREXLER, I., HELLER, K., WAHREN, B., ERFLE, V., AND SUTTER, G. 1998. Highly attenuated modified vaccinia virus Ankara replicates in baby hamster kidney cells, a potential host for virus propagation, but not in various human transformed and primary cells.*Journal of General Virology.*;**79 (2):**347-352.

DURBIN, A.P., CHO,C.J., ELKINS, W.R., WYATT, L.S., MOSS, B., AND MURPHEY, B.R. 1999. Comparison of the immunogenicity and efficacy of a replication-defective vaccinia virus expressing antigens of human parainfluenza virus type 3 (HPIV3) with those of a live attenuated HPIV3 vaccine candidate in rhesus monkeys passively immunized with PIV3 antibodies.*Journal of Infectious Diseases.* **179(6):**1345-1351.

DUTTA, J.K., PRADHAN, S.C., AND DUTTA, T.K. 1992. Rabies antibody titres in vacinees: protection, failure and prospects. *International Journal of Clinical Pharmacologogy Therapeutics and Toxicology.* **30(3)**:107-112.

EMERSON, S.U.1985. Rhabdoviruses. In : Fields, B.N.(ed) *Virology.* New York. Raven Press.

ESPOSITO, J.J., KNIGHT, J.C., SHADDOCK, J.N., NOVEMBRE, F.J., AND BAER, G.M. 1988. Successful oral rabies vaccination of raccoons with raccoon poxvirus recombinants expressing rabies virus glycoprotein. *Virology.* **165(1):**313-316.

ESPOSITO, J., BRECHLING, K., BAER, G., MOSS, B. 1987. Vaccinia virus recombinants expressing rabiesvirus glycoprotein protect against rabies. *Virus Genes.*;**1(1):**7-21.

EVANS, D.H., STUART, D. AND MCFADDEN, G. 1988. High levels of genetic recombination among co-transfected plasmid DNAs in poxvirus-infected mammalian cells. *Journal of Virology.* **62(2)** :367-375.

FALKNER, F.G., AND MOSS, B. 1988. *Escherichia coli gpt* gene provides dominant selection for vaccinia virus open reading frame expression vectors. *Journal of Virology.* **62(6)**:1849-1854.

FEKADU, M.,SUMNER, J.W.,SHADDOCK, J.H.,SANDERLIN, D.W.AND BAER, G.M. 1992. Sickness and recovery of dogs challenged with a street rabies virus after vaccination with a vaccinia virus recombinant expressing rabies virus N protein. *Journal of Virology*. **66(5)** :2601-2604.

FENNER, F., HENDERSON, D.A., ARITA, I., JEZEK, Z. AND LADNYI, I.D. 1988. Smallpox and its eradication. (WHO, Geneva):539-592.

FENNER, F. 1992. Chapter 1. Vaccinia virus as a vaccine, and poxvirus pathogenisis. In Recombinant Poxviruses. Binns, M.M. and Smith, G.L. (EDS). CRC Press. 1-45.

FISCHER, L., LE GROS, F.X., MASON, P.W., AND PAOLETTI, E. 1997. A recombinant canarypox virus protects rabbits against a lethal rabbit hemorrhagic disease virus (RHDV) challenge. *Vaccine* **15**:90-96.

FISHBEIN, D.B., AND ARCANGELI,S. 1987. Rabies prevention in primary care. A four-step approach. *Postgraduate Medicine*. **82(3)**:83-90, 93-5.

FRESHNEY, R.I. 1987.Dissagrigation of the tissue and primary culture. Ch 9. *In Culture of Animal Cells: A Manual of Basic Technique*. Alan R.Liss,Inc,New York:107-126.

FOLEY, H.D., MCGETTIGAN, J.P., SILER, C.A., DIETSCHOLD, B., AND SCHNELL, M.J. 2000. A recombinant rabies virus expressing vesicular stomatitis virus glycoprotein fails to protect against rabies virus infection. *Proceedings of the National.Acadamy of Sciences,.USA.*. **97(26)**:14680-14685.

FUJII, H., TAKITA-SONODA, Y., MIFUNE, K., HIRAI, K., NISHIZONO, A., AND MANNEN, K. 1994. Protective efficiency in mice of post exposure vaccination with vaccinia virus recombinant expressing either rabies virus glycoprotein or nucleoprotein. *Journal of General Virology*. **75**:1339-1344.

GALELLI, A., BALOUL, L., AND LAFON, M. 2000. Abortive rabies virus central nervous infection is controlled by T lymphocyte local recruitment and induction of apoptosis. *Journal of Neurovirology*. **6(5)**:359-372.

GAUDIN, Y., RUIGROCK, R.W., TUFFEREAU, C., KNOSSOW, M., AND FLAMAND, A. 1992. Rabies virus glycoprotein is a trimer. *Virology*. **187(2)**:627-632.

GAUDIN, Y., MOREIRA, S., BENEJEAN, J., BLONDEL, D., FLAMAND, A., AND TUFFEREAU, C. 1999. Soluble ectodomain of rabies virus glycoprotein expressed in eukaryotic cells folds in a monomeric conformation that is antigenically distinct from the native state of the complete membrane-anchored glycoprotein. *Journal of General Virology.* **80 (7):**1647-56.

GERSHON, P.D., ANSELL, D.M., AND BLACK, D.N. 1989. A comparison of the genome organization of capripoxvirus with that of the orthopoxviruses. *Journal of Virology* **.63(11):**4703-4708.

GERSHON, P.D. AND BLACK, D.N. 1988. A comparison of the genomes of capripoxvirus isolates of sheep, goats and cattle. *Virology.* **160**:473-476.

GERSHON, P.D. AND BLACK, D.N. 1987. Physical characterisation of the genome of a cattle isolate of capripoxvirus. *Virology.***160** :473-476.

GOEBEL, S.J., JOHNSON, G.P., PERKUS, M.E., DAVIS, S.W., WINSLOW, J.P., AND PAOLETTI, E. 1990. The complete DNA sequence of vaccinia virus. *Virology.*;**179(1):**247-66, 517-63.

GYLYS, L., CHOMEL, B.B., AND GARDNER, I.A. 1998. Epidemiological surveillance of rabies in Lithuania from 1986-1996. *Reviews in Science and Technology.* **17(3):**691-698.

HAIG, D.A. 1957. Lumpy skin disease. *Bulletin of Epizootological Diseases in Africa.* **5**:421-430.

HANKE, T., SCHNEIDER, J., GILBERT, S.C., HILL, A.V., AND MCMICHEAL, A. 1998. DNA multi-CTL epitope vaccines for HIV and Plasmodium falciparum: immunogenicity in mice. *Vaccine.*;**16(4):**426-35.

HANKE, T. AND MCMICHEAL, A. 1999. Pre-clinical development of a multi-CTL epitope-based DNA prime MVA boost vaccine for AIDS. *Immunology Letters.* **66(1-3):**177-181.

HANNA, J.N., CARNEY, I.K., SMITH, G.A., TANNENBERG, A.E., DEVERILL, J.E., BOTHA, J.A. SERAFIN, I.L., HARROWER, B.J., FITZPATRICK, P.F., SEARLE, J.W. 2000. Australian bat lyssavirus infection: a second human case, with a long incubation period. *Medical Journal of Australia.* **172(12):**597-599.

HARFORD, C.G., HAMLIN, A., AND RIDERS, E. 1966. Electron microscopic autoradiography of DNA synthesis in cells infected with vaccinia virus. *Experimental.Cell.Research.* **42**:50-57.

HARRIS, N., ROSALES, R., AND MOSS, B. 1993. Transcription initiation factor activity of vaccinia virus capping enzyme in independent of mRNA guanylylation. *Proceedings of the National.Acadamy of Sciences,.USA.* **90**:2860-2864.

HATTWICK, M.A.W., AND GREGG, M.B. 1975. The Disease (Rabies) in man. In Baer, G.M. (ed). The Natural History of Rabies. Vol 2. New York: Academic Press.

HENNING, M.W. 1956. Animal diseases in South Africa. Third ed. C.N.A. Cape Town: Central News Agency.

HIRSCH, V.M., FUERST, T.R., SUTTER, G., CARROLL, M.W., YANG, L.C., GOLDSTEIN, S., PIATAK, M.JR., ELKINS, W.R., ALVOID, W.G., MONTEFIORI, D.C., MOSS, B., AND LIFSON, J.D. 1996. Patterns of viral replication correlate with outcome in simian immunodeficiency virus (SIV)-infected macaques: effect of prior immunization with a trivalent SIV vaccine in modified vaccinia virus Ankara. *Journal of Virology.* **70(6):**3741-52.

HOSCHSTEN-MINSTEL, V., HUBER, H.C., AND STICKL, H. 1972. Virulenz und immunogenitat eines modifizierten vaccinia-virus (Stamm MVA). *Zeitschrift fur Immunologie und Forsch.* **144**:140-145.

HOLLINSHEAD, M., VANDERPLASSCHEN, A., SMITH, G.L., AND VAUX, D.J. 1999. Vaccinia virus intracellular mature virions contain only one lipd membrane. *Journal of Virology.* **73(2)**:1503-1517.

HOLOWCZAK, J.A. 1972. Uncoating of poxviruses. I. Detection and characterization of subviral particles in the uncoating process. *Virology.* **50**:216-232.

HUNTER, P., AND WALLACE, D. 2001. Lumpy skin disease in southern Africa: a review of the disease and aspects of control. *Tydskrif van.Suid.Afrikanse.veterinary.Versorging.* **72(2):**68-71.

HUSTON, P.D. 1945-1947. Report of the chief Veterinary Surgeon.

ICHIHASHI, Y., OIE, M., AND TSURUHARA, T. 1984. Location of DNA-binding proteins and disulfide-linked proteins in vaccinia virus structural elements. *Journal of Virology.* ;**50(3):**929-38.

ITO, M., ARAI, Y.T., ITAI, T., SAKAI, T., ITO, F.H., TAKASAKI, T., AND KURANE, I. 2001.Genetic characterization and geographic distribution of rabies virus isolates in Brazil: identification of two reservoirs, dogs and vampire bats. *Virology.* **5.284(2):**214-222.

JAIIAROENSUP, W., LANG, J., THIPKONG, P., WIMALARATNE, O., SAMRANWATAYA, P., SAIKASEM, A., CHAREONWAI, S., YENMUANG, W., PRAKONGSRI, S., SITPRIJA, V., AND WILDE, H. 1998. Safety and efficacy of purified vero cell rabies vaccine given intramuscularly and intradermally. (Results of a prospective randomized trial). *Vaccine.* **16(16)**:1559-1562.

JANECZKO, R.A., RODRIGUEZ, J.F., AND ESTABAN, M. 1987. Studies on the mechanism of entry of vaccinia virus in animal cells. *Archives of Virology.* **92**:135-150.

JANKOVIC,D., LIU, Z.,GAUSE, W.C. 2001. Th1- and Th2-cell commitment during infectious disease: asymmetry in divergent pathways. *Trends in Immunology.* **22(8):**450-457.

JOKLIK, W.K., PICKUP, D.J., PATEL, D.D., AND MOODY, M.D. 1988. Virulence genes of poxviruses and reoviruses. *Vaccine.* **6(2):**123-8. Review.

JOHN, T.J. 1997. An ethical dilemma in rabies immunization. *Vaccine.* **15(suppl:S):** 12-15.

KATES, J., AND BEESON, J. 1970. Ribonucleic acid synthesis in vaccinia virus. II. Synthesis of a poyriboadenylic acid. *Journal of Molecular Biology.* **50**:19-23.

KECK, J.K., BALDICK, C.J., AND MOSS, B. 1990. Role of DNA replication in vaccinia virus gene expression: a naked template is required for transcription of three late trans-activator genes. *Cell.* **61**:801-809.

KENNEDY, D.J. 1988. An outbreak of rabies in north-western Zimbabwe 1980-1983. *Veterinary Record.* **122(6):**129-133.

KIENY, M.P.,LATHE, R.,DVILLIEN, R.,SPEHNER, D.,SKORY, S.,SCHMITT, D.,WIKTOR, T. KOPROWSKI, H. AND LECOCQ, J.P. 1984. Expression of Rabies virus glycoprotein from a recombinant Vaccinia virus.*Nature*.**312**:163-166.

KITCHING, R.P. AND MELLOR, P.S. 1986. Insect Transmission of capripoxvirus. *Research in Veterinary Science*.**40**:255-258.

KITCHING, R.P.,BHAT, P.P.,. AND BLACK, D.N. 1989. The characterization of African strains of capripoxvirus. *Epidemiology and Infection*. **102**:335-343.

KITCHING, R.P.,HAMMOND, J.M. AND BLACK, D.N. 1986. Studies on the major common precipitating antigen of capripoxvirus. *Journal of General Virology*. **67**:139-148.

KITCHING, R.P.,HAMMOND, J.M. AND TAYLOR, W.P. 1987. A single vaccine for the control of capripox infection in sheep and goats. *Research in veterinary science*. **42**:53-60.

KITCHING, R.P., AND SMALE, C. 1986. Comparison of the external dimensions of capripoxvirus isolates. *Research in Veterinary Science*. **41**: 425-427.

KOPROWSKI, H. 1996. The mouse inoculation test. Chapter 6 in Laboratory Techniques in Rabies (4th ed) Eds: Meslin F-X, Kaplan MM and Koprowski H. World Health Organisation, Geneva. 80-87.

KOPROWSKI, H. 1999. *XI International Congress of Virology*. Plenary Session.

KRIJNSE LOCKER, J. KUEHN, A., SCHLEICH, S., RUTTER, G., HOHENBERG, H., WEPF, R., AND GRIFFITHS, G. 2000. Entry of the two infectious forms of vaccinia virus at the plasma membrane is signaling-dependant for IMV but not the EEV. *Molecular Biology of the Cell*. **11(7)**:2497-2511.

LAMBOT, M., BLASCO, E., BARRAT, J., CLIQUET, F., BROCHIER, B., RENDERS, C., KRAFFT, N., BAILLY, J., MUNIER, M., AUBERT, M.F., AND PASTORET,P.P . 2001. Humoral and cell-mediated immune responses of foxes (Vulpes vulpes) after experimental primary and secondary oral vaccination using SAG2 and V-RG vaccines. *Vaccine*. **19(13-14)**:1827-35.

LATHE,R., KIENY,M.P., LEMOINE,Y., LOISON,G., AIGLE,M. ANDLECOCQ,J.P.1985. Vectors for the expression of an antigenic rabies protein in eukaryotic cells, and their use in the preparation of a vaccine. *Patent: EP 0140762-A 1 08-MAY-1985;TRANSGENE S.A*

LAWRENCE, J.A., FOGGIN, C.M., AND NORVAL, R.A. 1980.The effects of war on the control of diseases of livestock in Rhodesia (Zimbabwe). *Veterinary Record*. **107(4)**:82-85.

LEVENTIS, R. AND SILVIUS, J.R. 1990. Interactions of Mammalian cells with lipid dispersions containing novel metabolizable cationic amphiphiles. *Biochemical and Biophysical Research Communications*. **1023**:124-132.

LITTAUA, R.A.,TAKEDA, A.,CRUZ, J. AND ENNIS, F.A. 1992. Vaccinia virus specific human CD4+ cytotoxic T-lymphocyte clones. *Journal of Virology*. **66(4)**:2274-2280.

LUO, D., AND SALTZMAN, W.M. 2000. Enhancement of transfection by physial concentration of DNA at the cell surface. *National.Biotechnology*. **18**:893-895.

MACDONALD, R.A.S. 1931. Pseudo-urticaria of cattle. Northern Rhodesia Department of Animal Health, Education and Welfare. Annual Report:20-21.

MACGREGOR, G.R., NOLAN, G.P., FIERING, S., ROEDERER, H. AND HERZENBERG, L.A. 1991. Use of *E.Coli* lacZ (B-galactosidase) as a reporter gene. In Methods in Molecular Biology. Volume 7:Gene Transfer and Expression Protocols. (eds) Murray,E.J. The Humana Press Inc, New Jersey:217-235.

MACKET, M., SMITH, G.L., AND MOSS, B. 1984. General method for production and selection of infectious vaccinia virus recombinants expressing foreign genes. *Journal of Virology*.**49(3)**:857-864.

MADORE, H.P., AND ENGLAND, J. M. 1977. Rabies virus protein synthesis in infected BHK-21 cells. *Journal of Virology*. **22**:102-112.

MAHY, B.W.J. ED. 1985. *Virology: A practical approach*. IRL Press Limited, Oxford, England

MALLON, V., AND HOLOWCZAK, J.A. 1885. Vaccinai virus antigens on the plasma membrane of infected cells. I. Viral antigens transferred from infecting virus particles and synthesized after infection. *Virology*. **141**:201-220.

MARAIS, D.; PASSMORE, J.A.; MACLEAN, J.; ROSE, R.; AND WILLIAMSON, A.L. 1999. A recombinant human papillomavirus (HPV) type 16 L1-vaccinia virus murine challenge model demonstrates cell-mediated immunity against HPV virus-like particles. Journal of General Virology. **80**(9):2471-2475.

MARTINEZ-BURNES, J., LOPEZ, A., MEDELLIN, J., HAINES, D., LOZA, E., AND MARTINEZ, M. 1997. An outbreak of vampire bat-transmitted rabies in cattle in northeastern Mexico. *Canadian Veterinary Journal.* **38(3):**175-177.

MATTER, H.C., SCHUMACHER. C.L, KHARMACHI. H., HAMMAMI. S., TLATLI. A., JEMLI. J., MRABET. L., MESLIN. F.X., AUBERT. M.F.A., NEUENSCHWANDER. B.E., AND HICHERI.K. E. 1998. Field evaluation of two bait delivery systems for the oral immunisation of dogs against rabies in Tunisia. *Vaccine.* **16(7)**:657-665.

MEYER, H., SUTTER, G., AND MAYR, A. 1991. Mapping of deletions in the genome of the highly attenuated vaccinia virus MVA and their influence on virulence. *Journal of General Virology.* **72 (5)**:1031-8.

MEYER, J., 2001. Department of Agriculture. Personal Communication.

MEUTH, M. AND HARWOOD, J. 1991. Selection of cells defective in pyramidine (TK-) and purine (APRT- and HPRT-) salvage. In *Methods in Molecular Biology. Volume 7 :Gene transfer and expression protocols.* (eds) Murray,E.J. The Humana Press Inc, New Jersey:339-359.

MIFUNE, K., TAKEUCHI, E., NAPIORKOWSKI, P.A., YAMADA, A., AND SAKAMOTO, K. 1981. Essential role of T-cells in the postexposure prophylaxis of rabies in mice. Microbiology and. Immunology. **25**(9):895-904.

MORIMOTO, K., HOOPER, D.C., SPITSIN, S., KOPROWSKI, H., AND DIETZSCHOLD, B. 1999. Pathogenicity of different rabies virus variants inversely correlates with apoptosis and rabies virus glycoprotein expression in infected primary neuron cultures. *Journal of Virology.* **73(1)**:510-8.

MORIMOTO, K., IWATANI, Y., AND KAWAI, A. 1993. Shedding of Gs protein (a soluble form of the viral glycoprotein) by the rabies virus-infected BHK-21 cells. *Virology.* **195(2):**541-9.

MOSS, B., AND ROSENBLUM, E.N. 1973. Protein cleavage and poxvirus morphogenisis: trypic peptide analysis of core precursors accumulated by blocking assembly with rifampicin. *Journal of Molecular Biology.* **81**:267-269.

MOSS, B., SMITH, G.L., GERIN, J.L., AND PURCELL, R.H. 1984. Live recombinant vaccinia virus protects chimpanzees against hepatitis B. *Nature.* **311(5981):**67-9.

MOSS, B. 1990. *Poxviridae and their replication.* In: Virology, second ed (Fields, B.N., Knipe, D.M., *et al* eds) Raven Press LTD. New York.

MOSS, B. 1996. Poxviridae: The viruses and their replication. (Ch 83) In *Virology* (3ed) Eds: Fields, B.N., Knipe, D.M., Howly, P.M. Lippincott-Raven Press, Ltd, New York:2637-2671.

MOSS, B. 1996. Genetically engineered poxviruses for recombinant gene expression, variation, and safety. *Proceedings of the National.Acadamy of Sciences,.USA.* 93:11341-11348.

MULLER, K.E., HOEK, A., RUTTEN, V.P., BERNADINA, W.E., AND WENTINK, G.H. Antigen-specific immune responses in cattle with inherited beta-2-integrin deficiency. *Vetrinary Immunopathology.* **58(1)**:39-53.

MULLER, G., AND WILLIAMSON, J.D. *Poxviridae.* Chapter 26 in Perspectives in Medical Virology (vol 3) Zuckerman, A.J. (ED).1987. 421-433.

MUNZ, F.K., AND OWEN, N.C. 1966. Electron microscopic studies on lumpy skin disease virus type "Neethling". *Onderstepoort Journal of Veterinary Research.* **33(1)**:3-8.

MURPHEY, B.R. AND CHANOCK, R.M. 1990. Immunisation against viruses. Ch 19. In *Virology,* Second Edition. Fields,B.N. , Knipe,D.M ., Chanock,R.M., Hirch,M.S., Melnick,J.L., Monath,T.P. and Roizman,B. (eds). Raven Press, LTD., New York :469-502.

NAGAFUCHI, S., KASHIWAGI, S., IMAYAMA, S., HAYASHI, J., AND NIHO, Y. 1998. Intradermal administration of Viral Vaccines. *Reviews in Medical Virology.* **8**: 97-111.

NAM, J-H., WYATT, L.S., CHAE, S-L., CHO, H-W., PARK, Y-K., AND MOSS, B. 1999. Protection against lethal Japanese encephalitis virus infection of mice by immunization with the highly attenuated MVA strain of vaccinia virus expressing JEV prm and E genes. *Vaccine.* **17**:261-268.

NEL, L.H., THOMSON, G.R., VON TEICHMAN, B.F. 1993. Molecular epidemiology of rabies in South Africa. *Onderstepoort Journal of Veterinary Research.* **(60)4**:301-306.

NEL, L.H., JACOBS, J., JAFTHA, J., MEREDITH, C. 1997. Natural spillover of a distinctly Canidae-associated biotype of rabies virus into an expanded wildlife host-ange in southern Africa. *Virus Genes.* **15(1)**:79-82.

NGICHABE, C.K., WAMWAYI, H.M., BARRET, T., NDUNGU, E.K., BLACK, D.N., AND BOSTOOK, C.J. 1997. Trial of a capripoxvirus-rinderpest recombinant vaccine in African cattle. *Epidemiolology and.Infection.* **118**:63-70.

OFFICE INTERNATIONAL DES EPIZOOTIES. Lumpy skin disease (Chapter 2.1.7). http://www.oie.int/eng/normes/mmanual/A_00024.htm.

OKOH, A.E. 1981.Rabies in farm livestock in Nigeria. *International Journal of Zoonoses.* **8(1):**51-56.

OLIVEIRA, A.N., ANDRADE, M.C., SILVA, M.V., MOURA, W.C., AND CORTEZ CONTREIRAS, E. 2000. immune response in cattle vaccinated against rabies. *Memorias do Instituto Oswaldo Cruz.***95(1)**:83-88.

OURMANOV I, BILSKA M, HIRSCH VM, MONTEFIORI DC. 2000. Recombinant modified vaccinia virus ankara expressing the surface gp120 of simian immunodeficiency virus (SIV) primes for a rapid neutralizing antibody response to SIV infection in macaques. *Journal of Virology.* **74(6):**2960-5

PAOLETTI, E. 1996. Applications of pox virus vectors to vaccination: An update. *Proceedings of the National.Acadamy of Sciences,.USA.***93**: 11349-11353.

PASTORET. P.P. AND BROCHIER.B. 1996. The development and use of a vaccinia-rabies recombinant oral vaccine for the control of wildlife rabies; a link between Jenner and Pasteur. *Epidemiological Infections.* **116**:235-240.

PASTORET, P.P., BROCHIER, B., BLANCOU, J., ARTOIS, M., AUBERT, M., KIENY, M.P., LECOCQ, J.P., LANGUET, B., CHAPPUIS, G., AND DESMETTRE, P. 1992. Development and deliberate release of a vaccinia – rabies recombinant virus for the oral vaccination of foxes against rabies. Chapter 5 in Recombinant Poxviruses. Binns, M.M. and Smith, G.L. eds. CRC Press. 163-206.

PAUL, J. 1975. *Tissue culture in biomedical research: II. Virology and host-parasite relationships.* Chapter 23. In Cell and Tissue Culture, 5th Edition. Longman Inc. N.Y.

PAYNE, L.G. 1980. Significance of extracellular virus in the in vitro and in vivo dissemination of vaccinia virus. *Journal of General Virology.* **31**:147-155.

PEDERSEN, K., SNIJDER, E.J., SCHLEICH, S., ROOS, N., GRIFFITHS, G., AND KRIJNSE LOCKER, J. 2000. Characterisation of vaccinia virus intracellular cores: implications for viral uncoating and core structure. *Journal of Virology.* **74(8)**:3525-3536.

PEDLEY, C.B., AND COOPER, R.J. 1987. The assay, purification and properties of vaccinia virus-induced uncoating protein. *Journal of General Virology.* **68**:1021-1028.

PENNINGTON, T.H., 1974. Vaccinia virus polypeptide synthesis: sequential appearance and stability of pre- and post-replicative polypeptides. *Journal of General Virology.* **25**:433-444.

PERKUS, M.E., GOEBEL, S.J., DAVIS, S.W., JOHNSON, G.P., LIMBACH, K., NORTON, E.K., AND PAOLETTI, E. 1990. Vaccinia virus host range genes. *Virology.*;**179(1)**:276-86.

PERKUS, M.E., PICCINI, A., LIMPISKAS, B.R., AND PAOLETTI, E. 1985. Recombinant vaccinia virus: immunization against multiple pathogens. *Science.***229**:981-984.

PERLMAN, L.J. 1993. Molecular characterization of the lumpy skin disease virus genome. A Masters Thesis. University of Cape Town. Souh Africa.

PIYASIRISILP, S., HEMACHUDHA, T., AND GRIFFIN, D.E. 1999. B-cell responses to myelin basic protein and its epitopes in autoimmune encephalomyelitis induced by Semple rabies vaccine. *Journal of Neuroimmunology.* **98(2)**:96-104.

PLOWRIGHT, W., AND FERRIS, R.D. 1959. Ether sensitivity of some mammalian poxviruses. *Virology.* **7**:357.

PLOWRIGHT, W., AND WITCOMB, M.A. 1959. The growth in tissue culture of a virus derived from lumpy skin disease of cattle. *Journal of Pathogenic Bacteria.* **78**:397.

PROSPERI, S., IRSARA, A., BATTELLI, G., AND SANGUINETTI, V. 1984. Vaccination of cattle with live and inactivated rabies vaccines: a study of antibody response. Veterinary *Research Communications.* **8(3)**:181-185.

PROZESKY, L., AND BARNARD, B.J.H. 1982. A study of the pathology of lumpy skin disease in cattle. *Onderstepoort Journal of Veterinary Research.* **49**: 167-175.

PRYDIE, J., AND COAKLEY, W. 1959. Lumpy skin disease: tissue culture studies. *Bulletin of Epizootological Diseases in Africa.* **7**:37-49.

RAMISSE, J., SERRES, H., AND RAKOTONDRAMARY, E. 1969. Adaptation aux cellules renales de lapin de virus associes a la dermatose nodulaire. *Revue d'Elevage et de Medecine Veterinaire des Pays Tropicaux* . **22(3)**:363-371.

REDFIELD, R.R., WRIGHT, D.C., JAMES, W.D., JONES, T.S., BROWN, C., AND BURKE, D.C. 1987. Disseminated vaccinia in military recruit with human immunodeficiency virus (HIV) disease. *New England Journal of Medicine*. **316(11)**:673-676.

RING, C.J.A. AND BLAIR, E.D. (EDS). 2001. Recombinant poxvirus expression vectors. In chapter 5, Genetically Engineered Viruses. Glaxo Wellcome Research and Development, Stevenage, UK. Bios Scientific Publishers Limited.

RODRIGUES DA SILVA, A.C., CAPORALE, G.M., GONCALVES, C.A., TARGETA, M.C., COMIN, F., ZANETI, C.R., AND KOTAIT, I. 2000.Antibody response in cattle after vaccination with inactivated and attenuated rabies vaccines. *Revista do Instituto de Medicina Tropical de Sao Paulo*. **42(2)**:95-98.

ROMERO, C.H.,BARRET, T.,EVANS, S.A.,KITCHING, R.P.,GERSHAN, P.D.,BOSTOCK, C. AND BLACK, D.N. 1993. Single capripox recombinant vaccine for the protection of cattle against rinderpest and lumpy skin disease. *Vaccine*. **11(7)**:737-742.

ROMERO, C.H.,BARRETT, T.,CHAMBERLAIN, R.W.,KITCHING, P.,FLEMMING, M.AND BLACK, D.N. 1994. Recombinant capripoxvirus expressing the hemagglutinin protein gene of rinderpest virus:Protection of cattle against rinderpest and lumpy skin disease virus. *Virology*. **204**:425-429.

ROMERO, C.H.,BARRETT, T.,KITCHING, R.P.,BOSTOCK, C. AND BLACK, D.N. 1995. Protection of goats against peste des petits ruminants with recombinants capripoxviruses expressing the fusion and haemagglutinin protein genes of a rinderpest virus. *Vaccine*. **13(1)**:36-40.

ROMERO, C.H.,BARRETT, T.,KITCHING, R.P., CARN, V.M. AND BLACK, D.N. 1994. Protection of cattle against rinderpest and lumpy skin disease with a recombinant capripoxvirus expressing the fusion protein gene of rinderpest virus. *The Veterinary Record* (August 13, 1994):152-154.

ROSALES, R., SUTTER, G., AND MOSS, B. 1994. A cellular factor required for transcription of vaccinia viral intermediate stage genes. *Proceedings of the National.Acadamy of Sciences,.USA.*. **91**:3794-3798.

RUPPRECHT, C.E., HAMIR, A.N., JOHNSTON, D.H., AND KOPROWSKI, H. 1988. Efficacy of a vaccinia-rabies glycoprotein recombinant virus vaccine in raccoons (Procyon lotor).*Reviews in Infectious Diseases*; **10 Suppl 4**: 803-9.

RUPPRECHT, C.E., WIKTOR, T.J., JOHNSTON, D.H., HAMIR, A.N., DIETSCHOLD, B., WUNNER, W.H., GLICKMAN, L.T., AND KOPROWSKI, H.1986. Oral immunization and protection of raccoons (Procyon lotor) with a vaccinia-rabies glycoprotein recombinant virus vaccine. *Proceedings of the National.Acadamy of Sciences,.USA.*;**83(20):**7947-50.

RUPPRECHT, C.E., BLASS, L., SMITH, K., ORCIARI, L.A., NIEZGODA, M., WHITFIELD, S.G., GIBBONS. R.V., GUERRA, M. AND HANLON, C.A. 2001. Human infection due to recombinant vaccinia-rabies glycoprotein virus. *New England Journal of Medicine.* **345(8):** 582-586.

RUSSEL, W.C., NEWMAN, C. AND WILLIAMSON, D.H. 1975. A simple cytochemical technique for demonstration of DNA in cells infected with mycoplasmas and viruses. *Nature.* **253**:461-462.

SAMBROOK, J., FRITSCH, E.F., AND MANIATIS, T. 1989. Molecular cloning: A laboratory manual, second ed. Cold Spring Harbour Lab. Press, NY.

SANZ, P., AND MOSS, B. 1999. Identification of a transcription factor, encoded by two vaccinia virus early genes, that regulate the intermediate stage of viral gene expression. *Proceedings of the National.Acadamy of Sciences,.USA.* **96(6)**:2692-2697.

SAROV, I., AND JOKLIK, W.K. 1972. Characterisation of intermediates in the uncoating of vaccinia virus DNA. *Virology.* **50**:593-602.

SCHEIFLINGER, F., FALKNER, F.G., AND DORNER, F. 1996. Evaluation of the thymidine kinase (tk) locus as an insertion site in the highly attenuated vaccinia MVA strain. *Archives of Virology.* **141**:663-669.

SCHMELTZ, M., SODEIK, B., ERICSSON, M., WOLFE, E.J., SHIDA, H,, HILLER, G., AND GRIFFITHS, G. 1994. Assembly of vaccinia virus: the second wrapping cisterna is derived from the trans Golgi network. *Journal of Virology.* **68**:130-147.

SCHNEIDER, J., GILBERT, S.C., BLANCHARD, T.J., HANKE, T., ROBSON, K.J., HANNAN, C.M., BECKER, M., SINDEN, R., SMITH, G.L., AND HILL, A.V. 1998. Enhanced immunogenicity for CD8+T cell induction and complete protective efficacy of malaria DNA vaccination by boosting with modified vaccinia Ankara. *Proceedings of the National.Acadamy of Sciences,.USA.* **4**:397-402.

SELIGMANN, E.B.J. 1973. The NIH test for potency. In Kapman MM and Koprowski H. (ED.) Laboratory techniques in rabies, Third ed:279.

SETH, A., OURMANOY, I., KURODA,M. J., SCHMITZ, J.E., CARROLL, M.W., WYATT, L.S., MOSS, B., FORMAN, M.A. HIRSCH, V.M., AND LETVIN, N.L. 1998. Recombinant modified vaccinia virus Ankara-simian immunodeficiency virus gag pol elicits cytotoxic T lymphocytes in rhesus monkeys detected by a major histocompatibility complex class I/peptide tetramer. *Proceedings of the National.Acadamy of Sciences,.USA.* **95(17):**10112-6.

SIHVONEN, L., KULONEN, K., AND NEUVONEN, E. 1994.Immunisation of cattle against rabies using inactivated cell culture vaccines. *Acta Vet Scand.* **35(4):**371-376.
Smith,G.L., and Moss,B. 1983. Infectious poxvirus vectors have capacity for at least 25,000 base pairs of foreign DNA. *Gene.* **25**:21-28.

SODEIK, B., DOMS, R.W., ERICSSON, M., HILLER, G., MACHAMER, C.E., VAN'T HOF, W., VAN MEER, G., MOSS, B. AND GRIFFITHS, G. 1993. Assembly of vaccinia virus: Role of the intermediate compartment between the endoplasmic reticulum and the golgi stacks. *Journal of Cell Biology.* **121(3):**521-541.

SMITH, J.S. 1996. New aspects of rabies with emphasis on epidemiology, diagnosis, and prevention of the disease in the United States. *Clinical Microbiology Reviews.* **9(2):**166-76. Review.

SMITH, J.S., FISHBEIN, D.B., RUPPRECHT, C.E., AND CLARK, K. 1991. Unexplained rabies in three immigrants in the United States. A virologic investigation.*New England Journal of Medicine.* **324(4):**205-11.

SMITH, G.L., AND MOSS, B. 1983. Infectious poxvirus vectors have capacity for at least 25 000 base pairs of foreign DNA. *Gene.* **25**:21-28.

SOMOGYI, P., FRAZIER, J., AND SKINNER, M.A. 1993. Fowlpox host range restriction: Gene expression, DNA replication and morphogenisis in non-permissive mammalian cells. *Virology.* **197**:439-444.

STANNARD, L.M., MARAIS, D., KOW, D., AND DUMBELL, K. 1998. Evidence for incomplete replication of penguin poxvirus in cells of mammalian origin. *Journal of General Virology.* **79**:1637-1646.

STEPHENSEN, C.B., WELTER, J., THAKER, S.R., TAYLOR, J., TARTAGLIA, J., AND PAOLETTI, E. 1997. Canine distemper virus (CDV) infection of ferrets as a model for testing morbillivirus vaccine strategies: NYVAC- and ALVAC- based CDV recombinants protect against symptomatic infection. *Journal of Virology.* **71**:1506-1513.

STERN, W. AND DALES, S. 1976. Biogenisis of vaccinia: Relationship of the envelope to virus assembly. *Virology.* **75**:242-255.

SUTTER, G., AND MOSS, B. Nonreplicating vaccinia vector efficiently expresses recombinant genes. 1992. *Proceedings of the National.Acadamy of Sciences,.USA.* **89**. 10847-10851.

SUTTER, G., WYATT, L.S., FOLEY, P.L., BENNINK, J.R., AND MOSS, B. 1994. A recombinant vector derived from the host-range restricted and highly attenuated MVA strains of vaccinia virus stimulates protective immunity in mice to influenza virus. *Vaccine.* **12**:1032-1040.

SUTTER, G., AND MOSS, B. 1995. Novel vaccinia vector derived from the host range restricted and highly attenuated MVA strain of vaccinia virus. Developments in Biological Standardization. **84**:195-200.

SWANEPOEL, R. 1996. Rabies. (ch 48) In *Infectious Diseases of Livestock.* Vol 1. Ed Coetzer.J.A., Thomson.G.R., and Kriek.N.P.J:494-540.

SWANEPOEL, R., BARNARD, B.J.H., MEREDITH, C.D., BISHOP, G.C., BRUCKNER, G.K., FOGGIN, C.M., AND HUBSCHLE, O.J.B. 1993. Rabies in southern Africa. *Onderstepoort Journal of Veterinary Research.* **60**:325-346.

TARTAGLIA, J., PINCUS, S., AND PAOLETTI, E. 1990. Poxvirus-based vectors as vaccine candidates. *Critical.Reviews in Immunology.* **10**:13.

TARTAGLIA J, PERKUS ME, TAYLOR J, NORTON EK, AUDONNET JC, COX WI, DAVIS SW, VAN DER HOEVEN J, MEIGNIER B, RIVIERE M, ET AL. 1992. NYVAC: a highly attenuated strain of vaccinia virus. *Virology.* **188(1)**:217-232

TAYLOR, J., TRIMARCHI, C., WEINBERG, R., LANGUET, B., GUILLEMIN. F.,DESMETTRE, P., AND PAOLETTI, E. 1991. Efficacy studies on a canarypox-rabies recombinant virus. *Vaccine.* **9**:190-193.

TAYLOR, J., WEINBERG,R., LANGUET, B.,DESMETTRE, P., AND PAOLETTI, E. 1988. Recombinant fowlpox virus inducing immunity in non-avian species. *Vaccine.* **6**:497-508.

TAYLOR J, PAOLETTI E. 1988. Fowlpox virus as a vector in non-avian species. *Vaccine.* **6**(6):466-8.

THOMAS, A.D., AND MARE, C.V.E. 1945. Knopvelsiekte. *Journal of South African Veterinary Medical Association.* **16**:36-43.

THOULOUZE, M.I., LAFAGE,., MONTANO-HIROSE, J.A., AND LAFON, M. 1997. Rabies virus infects mouse and human lymphocytes and induces apoptosis. *Journal of Virology.*;**71(10)**:7372-80.

TITOLI, F., PESTOLOZZA, S., IRSARA, A., PALLIOLA, E., FRESCURA, T., AND CIVARDI, A. 1982. Attenuated rabies virus, ERA strain, in cattle and dogs vaccinated with multiple doses. *Comparative Immunology, Microbiology and Infectious Diseases.* **5(1-3)**:193-197.

TOLSON, N.D., CHARLTON, K.M., STEWART, R.B., CAMPBELL, J.B., AND WIKTOR, T.J. 1987. Immune response in skunks to a vaccinia virus recombinant expressing the rabies virus glycoprotein. *Canadian Journal of Veterinary Research.* **51(3)**:363-6.

TOOZE, J., HOLLINSHEAD, M., REIS, B., RADSAK, K., AND KERN, H. 1993. Progeny vaccinia and human cytomegalovirus particles utilize early endosomal cisternae for their envelopes. *European.Journal of Cell.Biology.* **60**:163-178.

TRIPATHY, D.N., HANSON, L.E., AND CRANDELL, R.A. 1981. Poxviruses of Veterinary importance: Diagnosis of infections. Ch 6 in Comparative Diagnosis of Viral Diseases. Vol 3. Academic Press: 267-345.

TULMAN, E.R., ALFONSO, C.L., ZSAK, LU.L., KUTISH, G.F., AND ROCK, D.L. 2001. Genome of lumpy skin disease virus. *Journal of Virology.* **75(15)**:7122-7130.

VAN DEN ENDE, M., ALEXANDER, R.A., DON, P., AND KIPPS, A. 1948. Isolation is chick embryos of a filterable agent possibly related etiologically to lumpy skin disease of cattle. *Nature.* **161**:526.

VAN REGENMORTEL, M.H.V., FAUQUET, C.M., BISHOP, D.H.L., CARSTENS, E.B., ESTES, M.K., LEMON, S.M., MANILOFF, J., MAYO, M.A., MVGEOCH, D.J., PRINGLE, C.R., WICKNER, R.B. (Eds) 2000: Family Rhabdoviridae in Virus Taxonomy, Classifiation and Nomenclature of viruses. Seventh Report of the International Committee on Taxonomy of Viruses. Academic Press, San Diego, San Francisco, New York, Boston, London, Sydney, Tokyo. 563-583.

VAN ROOYEN, P.J., AND MUNZ, E.K. 1967-1968. Optimal conditions for the multiplication of "Neethling" type lumpy skin disease virus in embryonated eggs. *Onderstepoort Journal of Veterinary Research.*

VON BACKSTROM, U. 1945. Ngamiland cattle disease: preliminary report on a new disease. The aetiologcal agent being probably of an infectious nature. *Journal of South African Veterinary Medical Association.* **16**:29-35.

VON TEICHMAN, B.F., THOMSON, G.R., MEREDITH, C.D., NEL, L.H. 1995. Molecular epidemiology of rabies virus in South Africa: Evidence for two distinct virus groups. *Journal of General Virology.* **76(1):** 73-82.

VOROS, K., TANYI, J., AND KARSAI, F. 1999. Clinical experiences with rabies in cattle in hungary. *Deutsche Medizinische Wochenschrift.* **106(1):**46-48.

VOS, J.C., SASKER,M., AND STUNNENBERG,H.G. 1991. Promoter melting by a stage-specific vaccinia virus transcription factor is independent of the presence of RNA ploymerase. *Cell.* **65**:105-114.

VOS, J.C., AND STUNNENBERG, H.G. 1988. Derepression of a novel class of vaccinia virus genes upon DNA replication. *EMBO.Journal.* **7**:3487-3492.

glycoprotein gene. *Proceedings of the National.Acadamy of Sciences,.USA.*. **81(22):**7194-7198.

WUNDERLI, P.S., SHADDOCK, J.H., SCHMID, D.S., MILLER, T.J., AND BAER, G.M. 1991. The protective role of humoral neutralizing antibody in the NIH potency test for rabies vaccines. *Vaccine.* **9**(9):638-642.

WUNNER, W.H. 1991. The chemical composition and molecular structure of rabies viruses. In G.M. Baer (Ed) The natural history of rabies. Boca Roton,FL: CRC Press: 31-67.

WYATT, L.S., MOSS, B., AND ROZENBLATT, S. 1995. Replication-deficient vaccinia virus encoding bacteriophage T7 RNA polymerase for transient gene expression in mammalian cells. *Virology.*;**210(1):**202-5.

WYATT, L.S., CARROLL, M.W., CZERNY, C.P., MERCHLINSKY, M., SISLER, J.R., AND MOSS, B. 1998. Marker rescue of the host range restriction defects of modified vaccinia virus Ankara. *Virology.* ;**251(2):**334-42.

WYATT, L.S., SHORS, S.T., MURPHEY, B.R., AND MOSS, B. 1996. Development of a replication deficient recombinant vaccinia virus vaccine effective against parainfluenza virus 3 infection in an animal model. *Vaccine.* **14(15)**:1451-1458.

WYATT, L.S., WHITEHEAD, S.S., VENANZI, K.A., MURPHEY, B.R., AND MOSS, B. 2000. Priming and boosting immunity to respiratory syncytial virus by replication-deficient vaccinia virus MVA. *Vaccine.* **18**:392-397.

XIANG, Z.Q., KNOWLES, B.B., MCCARRICK, J.W., AND ERTL, H.C. 1995. Immune effector mechanisms required for protection to rabies virus. *Virology* .**214**(2):398-404.

YAMANOUCHI, K., BARRETT, T., AND KAI, C. 1998. New approaches to the development of virus vaccines for veterinary use. *Reviews in.Science and Technology. Office des.Internationale.Epizooties.* **17(3)**:642-653.

YANG, Y., AND ZHANG, J. 1999. An experimental study on the endurance of immunologic memory of intrdermal micro-injection with rabies vaccine and boosting immune effect. *Journal of Epidemiology.* **9**(4):209-215.

YOUNG, E., BASSON, P.A., AND WEISS, K.E. 1970. Experimental infection of game animals with lumpy skin disease virus (prototype strain Neethling). *Onderstepoort Journal of Veterinary Research*. **32**(2):79-88.

ZANETTI, C.R., DE FRANCO, M.T., VASSAO, R.C. PEREIRA, C.A., AND PEREIRA, O.A.C. 1998. Failure of protection induced by a Brazilian vaccine against Brazilian wild rabies viruses. *Archives of Virology*.**143**:1745-1756.

ZAPPE, H. 1988.The cloning and characterisation of an endogluconase and an endoxylanase from *Clostridium acetobutylicum* in *Eschericia Coli*. A PhD Thesis. University of Cape Town.

ZINKERNAGEL, R.M.; COOPER, S.; CHAMBERS, J.; LAZZARINI, R.A.; HENGARTNER, H.; AND ARNHEITER, H. 1990. Virus-induced autoantibody response to a transgenic viral antigen. *Nature*. **345**(6270):68-71.