Determination and analysis of the complete genome sequences of a vaccine strain and field isolate of Lumpy Skin Disease Virus (LSDV)

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by

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Preface

I wish to acknowledge the following, without whom these few pages would not be:

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AUM

Jai Shree Ganesha Namah

CONTENTS

Summary

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by

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In this study, the genomes of both the attenuated South African lumpy skin disease virus (LSDV) Neethling vaccine strain (LW) and a virulent field isolate from a recent outbreak namely the South African lumpy skin disease virus (LSDV) Neethling Warmbaths isolate (LD) have been cloned, sequenced and analysed. The genomic sequences of the South African LSDV Neethling Warmbaths isolate (LD) and the South African LSDV Neethling vaccine strain (LW), were compared to each other. The virulent South African isolate, LD was also compared to the previously sequenced virulent LSDV Neethling strain 2490 (LK), to determine molecular differences.

The LSDV genome is approximately 150 kbp in size and consists of 156 putative genes. Of the 156 potential encoded proteins of the virulent LSDV field isolates, the South African LSDV Neethling Warmbaths isolate (LD) and the LSDV Kenyan Neethling strain 2490 (LK), 120 were identical, 21 showed differences of a single amino acid, 7 showed two amino acid differences, while only one showed three amino acid differences. These were mostly found in the variable terminal regions. The

LSDV Kenyan Neethling strain 2490 (LK) was isolated in Kenya in 1958 and than re-isolated in 1987 from lesions of an experimentally infected cow (Tulman *et al.* 2001). The South African LSDV Neethling Warmbaths isolate (LD) was isolated from lesions of a severely infected calf in the Northern Province of the Republic of South Africa, on the farm Bothasvlei in 2001 (David Wallace, Biotechnology Division, Onderstepoort Veterinary Institute, Republic of South Africa; Personal communication, 2001). Considering the geographically distant African regions of the isolates, namely South Africa (LD) and Kenya (LK) as well as the time when these viruses were isolated, minimal genetic variation was observed thereby suggesting that lumpy skin disease virus is genetically stable.

When the attenuated vaccine strain (LW) was compared with the South African field isolate LD, a total of 480 amino acid differences were observed in 121 of the 156 potential encoded proteins. These were again mainly in genes of the terminal regions and a number of these led to frameshifts that caused truncated open reading frames (ORFs) as well as deletions of up to nine amino acids and insertions of up to 42 amino acids. These modified open reading frames (ORFs) encode proteins that are involved in various aspects, such as the regulation of host immune responses [a soluble interferon (IFN)-gamma receptor, and an interleukin-1 (IL-1) receptor-like protein], gene expression (*mut*T motif proteins), DNA repair (superoxide dismutase), host-range specificity (ankyrin-repeat protein, kelch-like proteins) including proteins with unassigned functions. These differences could lead to a reduction in immuno evasive mechanisms and virulence factors present in attenuated LSDV strains.

At this stage, it is not possible to define which amino acid differences in particular are responsible for dramatic alterations in viral virulence. A good indication, however are differences occurring in functional domains. A mutation in a transmembrane region, for example, could alter the levels of secretion of a protein involved in the regulation of the host immune response. We conclude that the attenuated effect is likely to be the sum of the altered phenotypes of the expressed proteins, although it is also likely that a few specific proteins carry more weight. Further studies to determine the functions of the relevant encoded gene products will hopefully confirm this. The molecular design of an effective vaccine is likely to be based on the strategic manipulation of such genes.

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

A	-	adenine
Å	-	angstrom
aa	-	amino acid
ANK	-	ankyrin
ATP	-	adenosine triphosphate
ATPase	-	adenosine triphosphatase
bp	-	base pair
BTB	-	Broad-complex, Tramtrack and Bric-a-Brac
° C	-	degrees celcius
C	-	cytosine
CAM	-	chorio-allantoic membrane
CCD	-	charge-coupled device
CCP	-	complement control protein
ChPV	-	chordopoxvirus
CIP	-	calf intestinal phosphatase
c.p.e	-	cytopathic effects
CPV	-	cowpox virus
CsCl	-	cesium chloride
Da	-	dalton
dH_2O	-	distilled water
DNA	-	deoxyribonucleic acid
dATP	-	2'-deoxyadenosine-5'-triphosphate
dNTP	-	2'-deoxynucleoside-5'-triphosphate
dUMP	-	deoxyuridine monophosphate
dUTP	-	deoxyuridine triphosphate
dUTPase	-	deoxyuridine triphosphatase
EBV	-	epstein-barr virus

EEV	-	extracellular enveloped virus
ELISA	-	enzyme-linked immunosorbent assay
ER	-	endoplasmic reticulum
EtBr	-	ethidium bromide
EV	-	ectromelia virus
FBT	-	foetal bovine testes
FPV	-	fowlpox virus
G	-	guanine
GPCR	-	G-protein-coupled receptors
GPV	-	goatpox virus
GTP	-	guanosine triphosphate
IEV	-	intracellular enveloped virus
IFN	-	interferon
Ig	-	immunoglobin
IL	-	interleukin
IMV	-	intracellular mature virus
IPTG	-	isopropyl-β-$_D$-thiogalactopyranoside
ITR	-	inverted terminal repeats
IV	-	immature virion
kbp	-	kilobase pair
kDa	-	kiloDaltons
LB	-	Luria-Bertani
LD	-	South African LSDV Neethling Warmbaths isolate
LFT	-	lamb foetal testes
LK	-	LSDV Kenyan Neethling strain 2490
LSD	-	lumpy skin disease
LSDV	-	lumpy skin disease virus
LW	-	South African LSDV Neethling vaccine

		strain
MCS	-	multiple cloning site
MCV	-	molluscum contagiosum virus
MDBK	-	madin-darby bovine kidney
mg	-	milligram
ml	-	millilitre
mm	-	millimeter
mM	-	millimolar
mRNA	-	messenger ribonucleic acid
MTV	-	monkey tumor virus
MYX	-	myxoma virus
ng	-	nanogram
NTP	-	nucleoside triphosphate
NPH	-	nucleoside triphosphate phosphohydrolase
OIE	-	Office International des Epizooties
ORF	-	open reading frame
OVI	-	Onderstepoort veterinary institute
PAP	-	poly(A) polymerase
PCR	-	polymerase chain reaction
PLD	-	phospholipase D
POZ	-	poxvirus and zinc finger
RFV	-	rabbit fibroma virus
RNA	-	ribonucleic acid
rpm	-	revolutions per minute
RNR	-	ribonucleotide reductase
SCRs	-	short consensus repeats
SERPIN	-	serine proteinase inhibitors

SFV	-	shope fibroma virus
ShPV	-	Sheeppox virus
SMART	-	Simple Modular Architecture Research Tool
SOD	-	superoxide dismutase
SODC	-	copper/zinc superoxide dismutase
SPV	-	swinepox virus
T	-	thymine
TF	-	transcription factors
UDP	-	uridine diphosphate
µg	-	microgram
µl	-	microlitre
UNG	-	uracil DNA glycosylase
UV	-	ultraviolet
VETF	-	viral early transcription factor
VV	-	vaccinia virus
X-gal	-	5-bromo-4-chloro-3-indolyl-β-$_D$-galactopyranoside
YLDV	-	yaba-like disease virus

CHAPTER I

Review of the Literature

1.1 INTRODUCTION TO POXVIRUSES

1.1.1 Poxviridae

The *Poxviridae* comprise a large family of complex DNA viruses that replicate in the cytoplasm of vertebrate and invertebrate cells (Moss, 1996). The general properties of *Poxviridae* include (a) a large complex virion containing enzymes that synthesise mRNA, (b) a genome composed of a single linear double-stranded DNA molecule of 130-300 kilobase pairs (kbp) with a hairpin loop at each end, and (c) a cytoplasmic site of replication (Moss, 1996). Most poxvirus-induced diseases in animals and humans involve characteristic pock-like lesions of the skin (Levy *et al.* 1994).

1.1.2 Classification of poxviruses

The family *Poxviridae* is divided into two subfamilies, *Chordopoxvirinae* (poxviruses of vetebrates) and *Entemopoxvirinae* (poxviruses of insects) (Table 1, Table 2). The subfamily *Chordopoxvirinae*, comprises eight genera, and a few unclassified species (Table 1) (Fenner, 1996).

Table 1. Host range and geographic distribution of genera and unclassified members of the subfamily *Chordopoxvirinae*

Genus and species	Reservoir host	Geographic Distribution	Other naturally infected hosts
Orthopoxvirus			
Camelpox virus	Camels	Africa, Asia	Nil
Cowpox virus	Rodents	Europe, western Asia	Cats, cows, humans, zoo animals
Ectromelia virus	Rodents	Europe	Silver fox, blue fox, mink
Monkeypox virus	Squirrels	Western and Central Africa	Monkeys, humans
Raccoon poxvirus	Raccoons	Eastern United States	Nil
Skunk poxvirus	Skunks	United States of America	Nil
Tatera poxvirus	Gerbils	Western Africa	Nil
Uasin Gishu virus	Unknown	Eastern Africa	Horses
Vaccinia virus (prototype)	Unknown	Worldwide	Humans, cows, buffaloes
Variola virus	Humans	Worldwide (now extinct)	Nil
Volepox virus	Voles	Western USA	Nil

Parapoxvirus			
Ausdyk virus	Camels	Africa, Asia	Nil
Bovine papular			
stomatitis virus	Cattle	Worldwide	Humans
Orf virus (prototype)	Sheep	Worldwide	Other ruminants and humans
Pseudocowpox virus	Cattle	Worldwide	Humans
Red deer poxvirus	Red deer	New Zealand	Nil
Seal parapoxvirus	Seals	Worldwide	Humans
Capripoxvirus			
Sheeppox virus (prototype)	Sheep	Asia, Africa	Nil
Goatpox virus	Goats	Asia, Africa	Nil
Lumpy skin disease virus	Cattle	Africa	Nil
	Buffalo	Africa	Nil
Suipoxirus			
Swinepox virus (prototype)	Swine	Worldwide	Nil
Leporipoxvirus			
Myxoma virus (prototype)	*Sylvilagus brasiliensis*	South America	*Oryctolagus*, other leporids
	Sylvilagus bachmani	Western United States	*Oryctolagus*, other leporids
Fibroma virus	*Sylvilagus floridansis*	Eastern United States	*Oryctolagus*
Hare fibroma virus	*Lepus capensis*	Europe	Nil
Squirrel fibroma virus	*Sciurus carolinensis*	Eastern United States	? Woodchuck
Western Squirrel fibroma	*Sciurus griseus*	California	Nil
Avipoxvirus			
Many species	Birds	Worldwide	Humans, as vaccine vectors
Yatapoxvirus			
Tanapox virus	? Rodents	Eastern and central Africa	Humans
Yabapox virus	? Monkeys	Western Africa	Humans (rare)
Molluscipoxvirus			
Molluscum contagiosum			
virus (prototype)	Humans	Worldwide	Nil
Unclassified			
Macropod poxvirus	Kangaroos	Australia	Nil
	Quokkas		
Crocodilian poxvirus	Crocodiles	Australia	Nil
		Southern Africa	
	Caimans	Florida	

(Fenner 1996)

Table 2. *Entomopoxvirinae* genera and member viruses

Subfamily	Genera	Member viruses
Entomopoxvirinae	Entomopoxvirus A	*Melolontha melolontha* (prototype)
	Entomopoxvirus B	*Amsacta moorei* (prototype)
	Entomopoxvirus C	*Chironimus luridus* (prototype)

(Moss 1996)

1.1.3 Smallpox

Smallpox, caused by variola virus, was a disease of major importance, due to the disfigurements and deaths it caused during recurring epidemics that affected world

history (Moss, 1996; Levy *et al.* 1994). Already endemic in India 2 000 years ago, smallpox had spread to China and Japan in the East and Europe and northern Africa in the West by 700 AD. The spread resulted from European voyages of discovery and colonisation. It was than introduced to the Caribbean by African slaves in 1518, to Mexico in 1520, to Peru in 1524, and independently from Africa to Brazil in 1555. Repeated introductions from Europe and to a lesser extent Africa into North America occurred from 1617 onward (Fenner, 1996). In 1967 a Smallpox Eradication Unit was established at the World Health Organisation (WHO) headquarters, with a goal of global eradication within a decade (Fenner, 1996). The WHO program achieved its target in October 1977 with the world being certified free of endemic smallpox in 1979 (Fenner, 1996).

1.1.4 Poxvirus infections of veterinary importance

With the exception of molluscum contagiosum and smallpox, all poxvirus infections of humans are zoonoses. The causative viruses of several of these diseases cause significant diseases of livestock while others are transmitted to humans from wildlife sources. In addition, several poxviruses that do not affect humans are important pathogens of livestock (Fenner, 1996).

Orthopoxvirus infections occurring in animals include camelpox and mousepox. Camelpox, caused by camelpox virus, is a severe generalised disease of camels. The disease naturally affect only old world camels, namely *Camelus dromedarius* and *Camelus bactrianus* (Fenner, 1996; Munz and Dumbell, 1994(c); Pfeffer *et al.* 1996). Camelpox virus has a narrow host range and can be differentiated from other orthopoxviruses by its biological characters and its genomic restriction endonuclease map (Fenner, 1996). The disease may on occasion cause serious loss due to mortality. (Munz and Dumbell, 1994(c)). Ectromelia virus (EV), the causative agent of mousepox, is a natural pathogen of mice that has also been reported to occur in silverfox, bluefox and mink (Fenner, 1996; Turner *et al.* 2000). The infection causes inapparent disease or acute death with extensive necrosis of the liver and spleen is dependent on the genotype of the mouse (Fenner, 1996). Mice that survive the acute disease often develop a generalised pustular rash (Fenner, 1996).

Suipoxvirus, leporipoxvirus, and avipoxvirus infections in animals include swinepox, myxomatosis and fowlpox respectively. The genus *Suipoxvirus* has only one member namely swinepox virus (SPV) which causes swinepox (Fenner, 1996; Massung and Moyer, 1991; Massung *et al.* 1993). The virus is worldwide in distribution and endemic in many swine populations in both developed and underdeveloped countries (Massung *et al.* 1993). Infections are restricted to porcine species and are relatively mild and self-limiting (Massung *et al.* 1993; Munz and Dumbell, 1994(b)). Swinepox is most commonly mechanically transmitted between pigs by the bite of the pig louse, *Hematopinus suis*, which is common in many herds; flies may also be mechanical vectors (Fenner, 1996; Munz and Dumbell, 1994(b)). The genus *Leporipoxvirus* contains five species of which myxoma virus is the only one of any veterinary importance (Cameron *et al.* 1999; Fenner, 1996). Myxoma virus causes a mild, benign infection in its evolutionary host, the North American bush rabbit (*Sylvilagus californicus*) or the South American tapeti (*Sylvilagus brasiliensis*), but it causes a rapid systemic and lethal infection known as myxomatosis in European rabbits (*Oryctolagus cuniculus*) with mortality rates of up to 100% (Cameron *et al.* 1999; Fenner, 1996). Transmission is usually due to mechanical transfer of virus by biting arthropods, most commonly mosquitoes or rabbit fleas (Fenner, 1996). The members of the genus *Avipoxvirus* within the *Chordopoxvirinae* subfamily only infect non-mammalian hosts (Afonso *et al.* 2000). Avipoxviruses are a large family of cytoplasmic DNA viruses infecting more than 60 species of wild birds (Afonso *et al.* 2000). Fowlpox virus (FPV), the prototypal member of the *Avipoxvirus*, is a serious disease of poultry that has occurred world-wide for centuries (Afonso *et al.* 2000; Fenner, 1996). Two forms of disease are associated with different routes of infection. The most common form, occurs following infection by biting arthropods that serve as mechanical vectors for viral transmission; and the second being the diphtheric form, involves droplet infection of the mucous membranes of the mouth, the pharynx, the larynx, and sometimes the trachea (Afonso *et al.* 2000; Fenner, 1996). Poxviruses diseases of poultry and other domestic birds have significant economic impact.

Poxviruses infections in domestic animals are, with three important exceptions, generally of limited economic importance (Coetzer *et al.* 1994). The exceptions are lumpy skin disease (LSD), which has caused infrequent but severe epidemics in cattle in Africa, particularly southern Africa, since its first appearance in the 1940's; Sheep-

and goatpox, which has caused serious losses in a number of regions in Africa but which have not yet occurred in southern Africa; and orf, which is a significant problem in sheep and goats world-wide (Coetzer *et al.* 1994). Sheeppox (ShPV) and goatpox (GPV) are acute or sub-acute contagious and often fatal diseases of sheep and goats, causing high mortality in young animals and significant economic loss, although mild and inapparent infections can also occur (Fenner, 1996; Munz and Dumbell, 1994(a)). They occur as enzootic infections in southwestern Asia, the Indian subcontinent, and most parts of Africa except southern Africa but the possibility of their importation necessitates constant vigilance (Fenner, 1996; Munz and Dumbell, 1994(a)). The pathogenesis of sheep- and goatpox is similar to that of other generalised pox diseases (Munz and Dumbell, 1994(a)). During an outbreak, the virus is probably transmitted between sheep by aerosol, and mechanical transmission of virus by biting arthropods such as stable flies may also occur (Fenner, 1996). Sheeppox (ShPV), goatpox (GPV) and lumpy skin disease virus (LSDV) are all capripoxviruses.

Lumpy skin disease (LSD), the subject of this thesis will be discussed in further detail.

1.2 LUMPY SKIN DISEASE

Lumpy skin disease (LSD), caused by lumpy skin disease virus (LSDV), is an important disease of cattle in Africa (Fenner, 1996; Weiss, 1963). It is characterised by fever, multiple firm, circumscribed skin nodules, necrotic plaques in the mucous membranes (chiefly of the respiratory tract and oral cavity) and swelling of the peripheral lymph nodes (Figure 1 and Figure 2) (Barnard *et al.* 1994; Fenner, 1996; Hunter and Wallace, 2001; OIE manual 2000; Tulman *et al.* 2001; Weiss, 1963; Weiss, 1968).

Figure 1. LSD – A calf in Egypt affected with LSD; note the large skin nodules.
(http://www.vet.uga.edu/vpp/gray_book/FAD/lsd.htm)

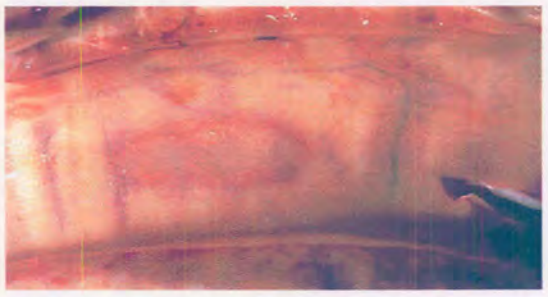

Figure 2. LSD - An LSD lesion in the tracheal mucosa.
(http://www.vet.uga.edu/vpp/gray_book/FAD/lsd.htm)

1.2.1 History of Lumpy skin disease

In 1929, a skin disease called pseudo-urticaria was noticed in the territory then known as Northern Rhodesia (Zambia) (Morris, 1931). At the time the lesions were thought to be caused by the bites of insects, but it is likely that the disease was LSD (Barnard *et al.* 1994; Weiss, 1968). Von Backström was the first to recognise lumpy skin disease as being an infectious malady when an outbreak occurred in Ngamiland during 1943 (Von Backström, 1945). It was reported for the first time in the Marico District of Western Transvaal (now the North West Province) in 1944 and then spread rapidly throughout South Africa during the ensuing years, despite enforced control measures (Henning, 1949; Thomas and Mare, 1945; Weiss, 1968). During this period it is estimated that 8 million cattle were affected (Diesel, 1949). By 1947 the disease had become firmly established and enzootic in South Africa and had also been reported from Swaziland (Weiss, 1968). Sporadic outbreaks continued to occur in South Africa throughout the ensuing years with the exception of 1953/54, 1957, 1962 and 1967, when the disease assumed epizootic proportions (Weiss, 1968). The disease

appeared to be confined to southern Africa until 1956 when it was encountered in Central and East Africa and since then has spread northward, westward and to Madagascar (Barnard *et al.* 1994). In 1989, the disease was recorded for the first time outside Africa, in southern Israel (Barnard *et al.* 1994).

1.2.2 Etiology

Early attempts to isolate and characterise the etiological agent of LSD indicated that a virus was the causative agent (Thomas and Mare, 1945). MacDonald in 1931 was unable to cultivate the causal agent of LSD on ordinary laboratory media, and he also failed to transmit it by means of blood inoculations. From field observations, Von Backström (1945) concluded that lumpy skin disease was infectious, but it was not until Thomas, Robinson and Alexander (1945) succeeded in demonstrating the transmissibility of the infectious agent by means of inoculation of susceptible animals with emulsified nodules.

With the development of tissue culture techniques, Alexander *et al.* in 1957 succeeded in isolating three distinct groups of cytopathogenic agents associated with lumpy skin disease (Weiss, 1963). Group I includes an orphan virus, bovine herpesvirus-4, Group II contains Allerton virus, bovine herpes mammilitis, or bovine herpesvirus-2, and Group III contains a virus which resembles vaccinia virus (Munz and Owen, 1966; Weiss, 1963; Weiss, 1968).

Orphan agents which produce morphological changes similar to group I viruses appear to be widespread among cattle in Africa (Weiss, 1963). The orphan virus was found to be non-pathogenic and in most cases resulted from mixed infections with the Group III virus (Hunter and Wallace, 2001). Allerton virus was found to be widespread in cattle throughout South Africa and was shown to be pathogenic for cattle by intradermal, intravenous and subcutaneous routes of inoculation, and its clinical signs were often confused with those of true LSD (Thomas and Mare, 1945; Weiss, 1963).

One of the first purified Group III virus isolates was a South African isolate named the Neethling-isolate. Agents belonging to group III, have been shown to be

responsible for producing skin lesions in cattle and corresponding with the original histopathological description of lesions of lumpy skin disease by Thomas and Maré in 1945. From the observations and experimental work carried out in Africa up to 1959 in South Africa and Kenya, there is no doubt that the Neethling type virus is the cause of true lumpy skin disease (Prydie and Coackley, 1959; van Rooyen *et al.* 1959; Weiss, 1963).

1.2.3 Epizootiology

The mode of transmission of LSD has not been proven conclusively, although circumstantial evidence suggested that biting insects played a major role in the dissemination of infection (Barnard *et al.* 1994). It is more prevalent during the wet summer and autumn months, particularly in low-lying areas and along water courses but outbreaks may occur during the dry season (Ali and Obeid, 1977; Diesel, 1949; Haig, 1957; Henning, 1956; Nawathe *et al.* 1982; von Backström, 1945; Weiss, 1968). Attempts in the 1960's to isolate LSD from various species of insects, including *Culex* and *Aedes* species of mosquitoes, *Culicoides* species, various species of ticks and *Muscidae* proved unsuccessful (Barnard *et al.* 1994; Weiss, 1968). However the virus was recovered from *Stomoxys calcitrans* and *Biomyia fasciata*, although attempts at transmitting the infection experimentally with these insects were unsuccessful (Barnard *et al.* 1994; Hunter and Wallace, 2001; Weiss, 1968). Experimental transmission of LSD was first achieved in 1987, with the fly *Stomoxys calcitrans* (Mellor *et al.* 1987).

In 1995 Carn and Kitching concluded that naturally occurring cases of generalised LSD may follow spread by intravenously feeding arthropods. It has also been shown that the female mosquitoes *Aedes aegypti* (Figure 3) are capable of mechanical transmission of LSDV from infected to susceptible cattle (Chihota *et al.* 2001). These findings are the first to demonstrate unequivocally that LSDV is insect-transmitted, and that mosquito species are competent vectors (Chihota *et al.* 2001).

Figure 3. *Aedes aegypti* (http://klab.agsci.colostate.edu/aegypti/aegypti.html)

Lumpy skin disease may spread in the absence of insects but direct contact between animals is inefficient (Barnard *et al.* 1994; Haig, 1957; Henning, 1956; Weiss, 1968). Deliberate attempts to infect susceptible animals, through handling infected animals first and thereafter immediately handling susceptible animals, proved unsuccessful (Barnard *et al.* 1994; Weiss, 1968). Transmission did however occur when common drinking troughs were used indicating that infected saliva might play a role in the dissemination of the disease (Barnard *et al.* 1994; Haig, 1957; Henning, 1956; Weiss, 1968). The disease is transmissible to suckling calves through infected milk (Barnard *et al.* 1994; Henning, 1956; Weiss, 1968). The spread of the LSD appears to be facilitated through movement of clinically sick or inapparently infected animals along the main roads or by rail (Barnard *et al.* 1994; MacOwen, 1959; Weiss, 1968).

It has been suggested that sheep might possibly act as carriers of lumpy skin disease but these observations have not been confirmed (Barnard *et al.* 1994; Capstick, 1959; Weiss, 1968).

1.2.4 Cultivation

Lumpy skin disease virus (LSDV) can be propagated in a large variety of animal cells grown in tissue culture (Weiss, 1968). Besides lamb and calf kidney tissue cultures, the virus multiplies and produces cytopathic changes in cultures of lamb and calf testes, the sheep kidney cell strain of MADIN and DARBY, lamb and calf adrenal and thyroid cultures, foetal bovine muscle, foetal lamb and calf muscle, sheep embryonic kidney and lung, rabbit foetal kidney and skin, chicken embryo fibroblasts, in a line of adult vervet monkey kidney cells and in lines of baby hamster kidney cells

(Alexander and Weiss, 1959; Binepal *et al.* 2001; Prydie and Coackley, 1959; Weiss, 1968; Weiss and Geyer, 1959).

1.2.5 Characterisation of capripoxvirus isolates

Epidemiological investigation of endemic virus diseases relies heavily on the ability to distinguish between closely related strains of virus (Kitching *et al.* 1989). Up to the mid-1980's it had not been possible to distinguish between strains of capripoxvirus using standard laboratory tests (Kitching and Taylor, 1985; Kitching *et al.* 1989). Capripoxviruses isolates also cannot be distinguished by analysis of their proteins, and cannot be distinguished on the basis of their immunogenicity since isolates from the *capripoxvirus* genus induce antibody to a common major antigen (Gershon and Black, 1988; Kitching *et al.* 1986). The common immunogenic properties of capripoxviruses are also evidenced by the ability of specific attenuated or low-virulence capripoxvirus isolates to protect all three host species from capripoxvirus infection (Gershon and Black, 1988; Kitching and Taylor, 1985).

In 1986 Black, Hammond and Kitching compared DNA fragments generated by *Hind*III restriction endonuclease digestion of field and vaccine strains of capripoxvirus and were able to distinguish between them (Black *et al.* 1986).

1.2.6 Symptoms

The severity of clinical signs of LSD depends on the strain of capripoxvirus and the breed of host (OIE manual 2000). *Bos taurus* is more susceptible to clinical disease than *Bos indicus* (OIE manual 2000). However, even among groups of cattle of the same breed kept together under the same conditions, there is a large variation in the clinical signs presented, ranging from subclinical infection to death (OIE manual 2000). There may be failure of the virus to infect the whole group, depending on vector prevalence (OIE manual 2000).

The incubation period after an artificial infection with virulent blood or fresh nodule emulsion varies from 4 to 14 days (Henning, 1949). Under natural conditions, the first symptom commonly appears two to five weeks after exposure to infection (Henning,

1949, Weiss, 1963; Weiss, 1968). Natural cases of LSD are manifested initially by lacrimation, fever, loss of appetite, and disinclination to move (Hunter and Wallace, 2001). Skin nodules, the characteristic feature of the disease, appears later at roughly four to 10 days after the initial temperature reaction (Figure 4 and Figure 5) (Barnard *et al.* 1994; Hunter and Wallace, 2001; Weiss 1968). It is randomly distributed ranging in diameter from 5 to 50 mm, and involves both the skin and subcutaneous tissues and sometimes even the underlying musculature (Barnard *et al.* 1994; Hunter and Wallace, 2001; Weiss, 1968). The number of nodules may range from a few to several hundred in severely infected animals (Barnard *et al.* 1994). Soft, yellowish-grey nodules may also occur in the mucous membranes of the mouth, nose, reproductive organs as well as the respiratory tract (Weiss, 1963; Weiss, 1968). During the course of the disease, induration of the skin nodules may occur and these indurated lesions may persist for years. The skin nodules usually undergo complete necrosis (Weiss, 1963; Weiss, 1968). If extensive necrosis occurs in the upper respiratory tract, pneumonia may result from inhalation of secondarily infected necrotic tissue (Barnard *et al.* 1994).

Figure 4. Generalised LSD infection

Figure 5. Severe LSD skin lesions

http://www.fao.org/WAICENT/FAOINFO/AGRICULT/AGa/AGAP/WAR/Warall/u4900b/u4900b0d.htm

1.2.7 Diagnostic techniques

A presumptive diagnosis of LSD can be confirmed by electron microscopic demonstration of virus in negatively-stained preparations of biopsy specimens taken from affected skin or mucous membranes. Virus isolation from skin lesions is also possible. Electron microscopic identification of LSD virus particles can be achieved within hours of receipt of the specimen, but virus identification in cell cultures by

fluorescent antibody techniques requires at least 48 hours (Barnard *et al.* 1994; Davies *et al.* 1971).

The most specific serological test for LSD is the virus neutralisation test. Because immunity to LSD is predominantly cell-mediated, the test is not sufficiently sensitive to identify animals that have had contact with LSD virus and developed only low levels of neutralising antibody. Other tests include the agar gel immunodiffusion test and the indirect immunofluorescent antibody test. Both these tests are less specific due to cross-reactions with antibody to other poxviruses. Western blotting using the reaction between the P32 antigen of LSD virus with test sera is both sensitive and specific, but is difficult and expensive to carry out. The use of this antigen, expressed by a suitable vector, in an enzyme-linked immunosorbent assay (ELISA) offers the prospect of an acceptable and standardised serological test (OIE manual 2000).

An antigen detection ELISA has been developed, using a polyclonal detection serum raised against a recombinant immunodominant antigen of capripoxvirus (OIE manual 2000).

1.3 MORPHOLOGY OF POXVIRUSES

The virions of poxviruses are larger than those of other animal viruses and are just discernible by light microscopy (Moss, 1996). Poxviruses are typically described as brick-shaped, while "ovoid" describes their shape better (Figure 6), (Levy *et al.* 1994). They are roughly 400 x 240 x 200 nm particles of the order of 4 x 10^9 Da in mass. The internal structure as revealed by thin sections is a biconcave nucleoid core covered by a 9 –nm membrane. The dumbbell-like shape of the core is due to its being located between two "lateral bodies' (Figure 7), (Levy *et al.* 1994; Moss, 1996).

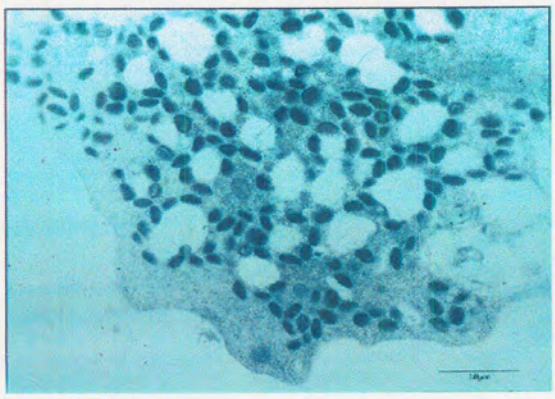

Figure 6. Thin section of a poxvirus lesion.
(http://www.aphis.usda.gov/vs/ep/fad_training/Poxvol4/page41_4.htm)

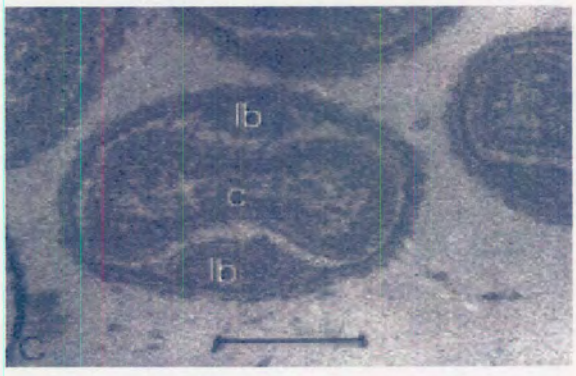

Figure 7. Micrograph of cowpoxvirus thin section (Dr. F. Fenner, Australian National University, 1997). lb- lateral bodies, c- core. The bar represents 100 nm.
(http://www.ncbi.nlm.nih.gov/ICTVdb/Images/Fenner/poxc.htm)

In 1966, Munz and Owen observed virus particles morphologically similar to the M-forms of vaccinia virus (VV) described by Westwood *et al.* (1964). The term M-forms refers to those particles in vaccinia virus which appeared to have a beaded surface like a mulberry (Westwood *et al.* 1964). These lumpy skin disease virus particles depicted in Figures 8, stained at pH 6.5, consisted of a complex interwoven network of strands each with an approximate width of 70 to 90 Å and presenting an irregular surface structure (Weiss, 1968).

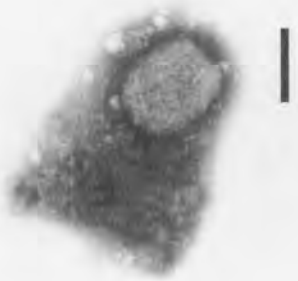

Figure 8. Electron micrograph of lumpy skin disease virus particles stained with phosphotungstic acid (PTA) at a pH of 6.5. The bar represents 200nm. (Picture courtesy of Dr. G.H. Gerdes and Mr. J. F. Putterill, Onderstepoort Veterinary Institute)

At pH 8.5, negatively stained preparations showed a predominance of particles with multilayered capsules. These particles did not show the show the thread-like surface structure but consisted of a homogeneous granular mass enveloped by a capsule approximately 280 Å thick. The capsule has an irregular outer membrane, a thicker central part and a distinct inner membrane (Weiss, 1968). These particles resembled the C-forms of VV as described by Westwood *et al.* (1964). The term C-forms refer to those vaccinia virus particles that appear as larger more electron-dense bodies with a capsule of complex structure (Westwood *et al.* 1964). In preparations stained at pH 7.0, both the C- and M-forms were observed. The virus particles measured approximately 3500 Å in width, with an axis ratio in the region of 1.2 (Munz and Owen, 1966).

1.4 GENOME OF POXVIRUSES

Poxviruses are linear double-stranded DNA genomes that vary from about 130 kilobase pairs (kbp) in parapoxviruses to about 300 kbp in avipoxviruses (Levy *et al.* 1994; Moss, 1996). According to Tulman *et al.* (2001) lumpy skin disease virus (LSDV) is 151 kpb. Poxvirus genomes contain a central coding region flanked by inverted terminal repeat (ITR) regions (Moss, 1996). The ITRs are identical but oppositely orientated sequences at the two ends of the genome and are found in all poxviruses examined (Figure 9), (Antoine *et al.* 1998; Afonso *et al.* 1999; Afonso *et al.* 2000; Cameron *et al.* 1999; Esposito and Knight, 1985; Garon *et al.* 1978; Goebel

et al. 1990; Knight *et al.* 1992; Lee *et al.* 2001; Massung *et al.* 1994; Senkevich *et al.* 1997; Shchelkunov *et al.* 1998; Tulman *et al.* 2001; Willer *et al.* 1999(b); Wittek *et al.* 1978). The lengths of the ITRs are variable even within a genus (Massung *et al.* 1994; Moss, 1996). The ITRs may include coding regions, where some genes are present at both ends of the genome (Moss, 1996). This is evident in LSDV (Tulman *et al.* 2001). The LSDV genome is flanked by identical 2,4 kbp-inverted terminal repeats (Tulman *et al.* 2001). The most terminal 241 nucleotides of the assembled LSDV Neethling type strain 2490 sequence as sequenced by Tulman *et al.* (2001) contain 7.5 copies of a 24 base pair (bp) imperfect tandem repeat and are similar to those described in ShPV (Gershon *et al.* 1989). Terminal regions involve genes and gene families with likely functions in viral virulence and host range (Tulman *et al.* 2001).

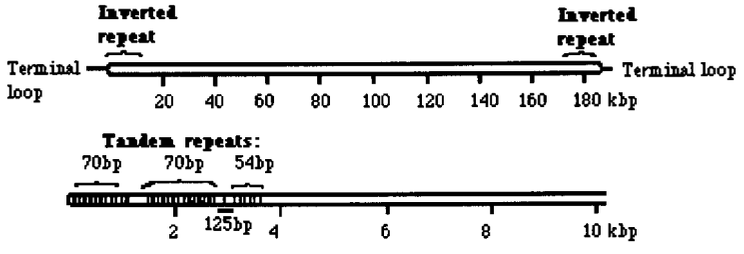

Figure 9. A representation of the entire linear double stranded VV DNA genome and an expansion of the 10,000 base pair (bp) inverted left terminal repetition are shown. (Moss 1996).

Poxvirus DNA differs from most other viral DNAs in that the two strands of virus DNA are connected by hairpin loops to form a covalently continuous polynucleotide chain as shown in Figure 9 (Baroudy *et al.* 1982; Levy *et al.* 1994; Moss, 1996). The loops are A+T-rich, cannot form a completely base-paired structure, and exist in two forms that are inverted and complementary in sequence (Baroudy *et al.* 1982; Moss, 1996). The nucleotide composition of most poxvirus genomes sequenced thus far are highly A+T-rich, ranging from between 55% and 82% with the exception of molluscum contagiosum virus (MCV) having an A+T content of 36% (Afonso *et al.* 1999; Afonso *et al.* 2000; Antoine *et al.* 1998; Cameron *et al.* 1999; Goebel *et al.* 1990; Lee *et al.* 2001; Senkevich *et al.* 1997; Shchelkunov *et al.* 2000; Tulman *et al.* 2001; Tulman *et al.* 2002; Willer *et al.* 1999). The nucleotide composition of the LSDV genome is approximately 73% A+T and is uniformly distributed (Tulman *et al.* 2001). The large, conserved central region predominantly contains genes essential for

virus structure and replication (Buller and Palumbo, 1991; Esposito and Knight, 1985; Fenner *et al.* 1989).

1.5 CONTROL OF LUMPY SKIN DISEASE

Since arthropods have been shown to be important in the transmission of LSD, control by quarantine and controlling of the movement of cattle is generally ineffective (Barnard *et al.* 1994; Chihota *et al.* 2001). Control is therefore essentially confined to immunoprophylaxis (Barnard *et al.* 1994).

In 1961, Capstick and Coackley demonstrated that 3 strains of sheeppox were able to protect cattle against experimental LSD infection with the '*Kedong*' strain being the choice vaccinating agent. The reason for the choice of the '*Kedong*' strain was as follows: the strain was isolated before LSD broke out in the Colony, and had been handled in a laboratory in which Neethling-type virus was not being used (Capstick and Coackley, 1961). Thus little chance existed that accidental contamination of the strain with Neethling-type virus had occurred either in the field or in the laboratory as such contamination would have been difficult to detect as both viruses behave similarly in tissue culture and serologically (Capstick and Coackley, 1961). This vaccine was used extensively with good results as local reactions were not seen in cattle, although some *Bos taurus* breeds exhibited mild generalised symptoms with care being taken as the strain was still virulent to sheep (Capstick and Coackley, 1961; Carn, 1993).

According to the OIE Manual of standard Diagnostic tests and Vaccines (OIE 1996, OIE 2000), two live attenuated strains of capripoxvirus have been used as vaccines specifically for the control of LSD. These were a strain of Kenya sheep and goat pox virus (Capstick and Coackley, 1961; Carn, 1993; OIE 1996; OIE 2000). All strains of capripoxvirus examined thus far, share a major neutralising site, so that animals recovered from infection with one strain are resistant to infection with any other strain (OIE 1996; OIE 2000).

In LSD-endemic areas such as South Africa, vaccination is the only viable means of control (Hunter and Wallace, 2001). The South African vaccine was developed by

attenuation of a field isolate in tissue culture and on the chorioallantoic membranes of embryonated hen's eggs (Hunter and Wallace, 2001; Weiss, 1968). The attenuated South African vaccine strain has been shown to protect against clinical disease (Weiss, 1968), but outbreaks during 1990/1991 and subsequent years have challenged the assertion that immunity is life-long, with more frequent vaccination now being recommended (Hunter and Wallace, 2001; Kitching, 1996). Some of the reasons for vaccine failure are:

1. 50 per cent of the cattle develop a swelling at the point of inoculation and this may be accompanied by a reduction in milk yield,
2. Vaccination of animals already incubating the disease,
3. Confusion with '*pseudo lumpy skin*' disease caused by Allerton virus,
4. Vaccinated cows that develop an antibody response will confer maternal immunity to LSD lasting 6 months whereafter calves may develop LSD if not vaccinated timeously,
5. Mishandling of the vaccine through exposure to sunlight and high temperatures or storage after reconstitution.

(Barnard *et al.* 1994; Carn, 1993; Hunter and Wallace, 2001).

A new generation of capripox vaccines that uses the capripoxvirus genome as a vector for the genes of other ruminant pathogens, for instance genes of rinderpest and pestes des petits ruminant viruses are being developed (OIE 1996; OIE 2000). The recombinant vaccine may thus provide protection against LSD and rinderpest in a single vaccination (OIE 1996; OIE 2000; Romero *et al.* 1993; Romero *et al.* 1994).

1.6 AIMS OF THIS STUDY

Lumpy skin disease (LSD) in an important, insect-borne infectious disease of cattle, occurring epidemically or sporadically in southern and eastern Africa. The etiological agent is a capripoxvirus related to sheeppox and goatpox viruses. The Neethling strain poxvirus is the prototype strain of lumpy skin disease virus (LSDV). Vaccination with an attenuated strain of lumpy skin disease virus (LSDV) is the only viable means of LSD control in endemic areas such as South Africa. The South African vaccine was developed by passage of a field isolate in tissue culture and on the chorioallantoic membranes of embryonated chicken eggs (Weiss 1968).

Although the local vaccine has been proven safe and effective (Weiss, 1968), problems were encountered during the 1990-1991 LSD outbreak as well as subsequent outbreaks (Hunter and Wallace, 2001). Outbreaks of LSD are frequent with increased incidence during high rainfall seasons (Dr. G.H. Gerdes, Virology Division, Onderstepoort Veterinary Institute, Republic of South Africa; Personal communication, 2001). 587 cases of LSD in South Africa was reported in 2000 with 228 cases being reported in 2001 (Odendaal 2002). The problems encountered may be due to the climatic conditions favouring the reproduction and spread of insect vectors (Hunter and Wallace, 2001). Low levels of vaccination during this period and in subsequent years, may also contribute to the spread of LSD (Hunter and Wallace, 2001). Frequent vaccinations are now recommended. The molecular characterisation of the virus is therefore an important step in the process of developing more effective diagnostic reagents and vaccines, as well as in the understanding of mechanisms of viral pathogenesis and epidemiology. A suitably tailored vaccine could also allow for the rapid determination of vaccine status and virus presence in cattle during LSD outbreaks. This would be of considerable importance in the timely control of disease outbreaks.

The first aim of this study was to clone and sequence the LSDV genome. This was achieved using the "shotgun" cloning and automated sequencing approach. Oligonucleotides designed from LSDV sequences was then used to primer-walk gaps in the DNA in certain regions.

The second aim after determining the nucleotide sequence of the clones, was to assemble the fragments into a continuous LSDV DNA sequence. Computer analysis was used to assemble the sequences into the correct orders and orientations.

The third aim of this investigation was to use computer analysis, to translate assembled LSDV nucleotide sequence into amino acid sequences. Open reading frames were identified and possible functions were assigned to the putative LSDV proteins.

The fourth aim of this investigation was to identify functional domains occurring in the putative LSDV proteins of the South African lumpy skin disease virus (LSDV) Neethling vaccine strain (LW), the South African lumpy skin disease virus (LSDV) Neethling Warmbaths isolate (LD) and the LSDV Neethling strain 2490 (LK). Those putative LSDV proteins containing amino acid differences between the virulent South African isolate LD and the South African vaccine strain LW, as well as having functional domains were compared to determine whether these amino acid differences occurred within these domains. The virulent Kenyan isolate LK and the South African isolate LD, were also compared to each other as above to determine whether the amino acid differences occurred within these functional domains.

The first and second aims were performed at Plum Island Animal Disease Centre (PIADC), the third aim at both PIADC and Onderstepoort Veterinary Institute (OVI), and the fourth aim only at OVI.

Parts of this thesis have been accepted for publication:
Kara, P.D., Afonso, C.L., Wallace, D.B., Kutish, G.F., Abolnik, C., Lu, Z., Vreede, F.T., Taljaard, L.C.F., Zsak, A., Viljoen, G.J., and Rock, D.L. Comparative Sequence Analysis of the South African Vaccine Strain and Two Virulent Field Isolates of Lumpy Skin Disease Virus. *Archives of Virology*

Parts of this thesis have been presented as a poster at the XIVth International Poxvirus and Iridovirus Workshop – Lake Placid, New York, 20-25 September 2002:
Kara, P.D., Afonso, C.L., Wallace, D.B., Kutish, G.F., Abolnik, C., Lu, Z., Vreede, F.T., Taljaard, L.C.F., Zsak, A., Viljoen, G.J., and Rock, D.L. Molecular

Characterisation of the South African Vaccine Strain and Two Field Isolates of Lumpy Skin Disease Virus.

CHAPTER II

Materials and Methods

2.1 INTRODUCTION

Sequencing of large clones or genomes is done by the shotgun approach (Figure 10). Briefly:

(1) Genomic DNA is sheared or enzyme restricted to yield random fragments of the required size.

(2) The fragments are cloned in a universal vector.

(3) Sequencing reactions are performed with a universal primer on a random selection of the clones in the shotgun library. These sequencing reads are assembled into contigs, identifying gaps (where there is no sequence available) and single-stranded regions (where there is sequence for only one strand). A contig is a series of two or more individual DNA sequence determinations that overlap.

(4) The gaps and single-stranded regions are then targeted for sequencing to produce the full sequenced molecule.

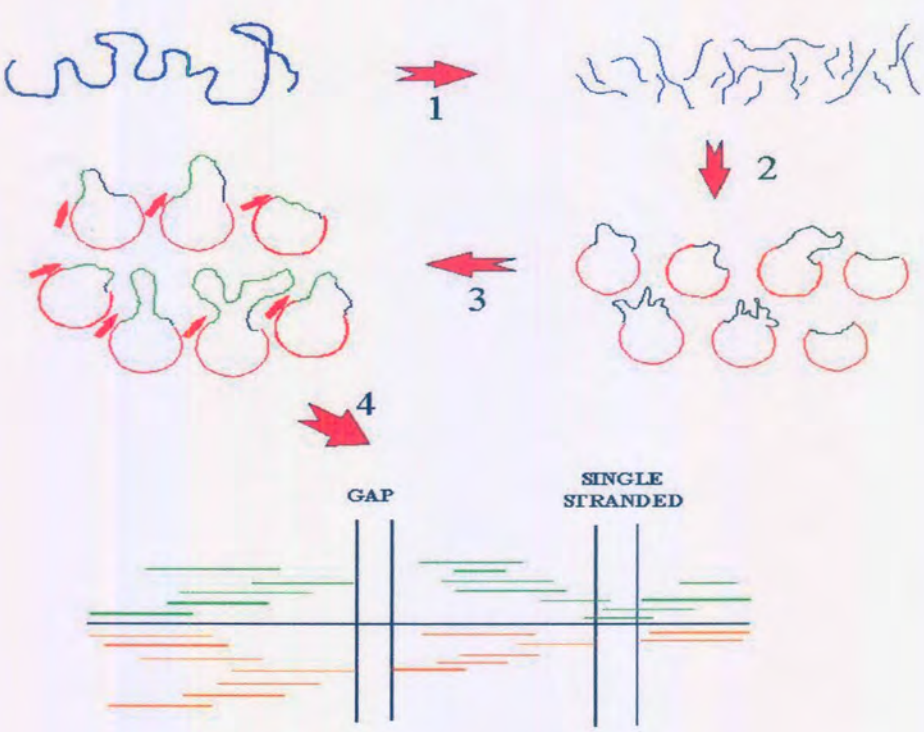

Figure 10. The shotgun approach to genomic sequencing.

(1) Genomic DNA is sheared or enzyme restricted to yield random fragments of the required size.

(2) The fragments are cloned in a universal vector.

(3) Sequencing reactions are performed with a universal primer on a random selection of the clones in the shotgun library. These sequencing reads are assembled in to contigs, identifying gaps (where there is no sequence available) and single-stranded regions (where there is sequence for only one strand).

(4) The gaps and single-stranded regions are then targeted for sequencing to produce the full sequenced molecule.

Genome DNA composition, structure, repeats and restriction enzyme patterns were analysed. Open reading frames (ORFs) were evaluated for coding potential with minor ORFs being excluded. Gene families are than analysed and annotated.

The approach to the cloning, sequencing and analysis of both the South African LSDV Neethling vaccine strain (LW) and the LSDV Neethling Warmbaths isolate (LD) is outlined in figure 11.

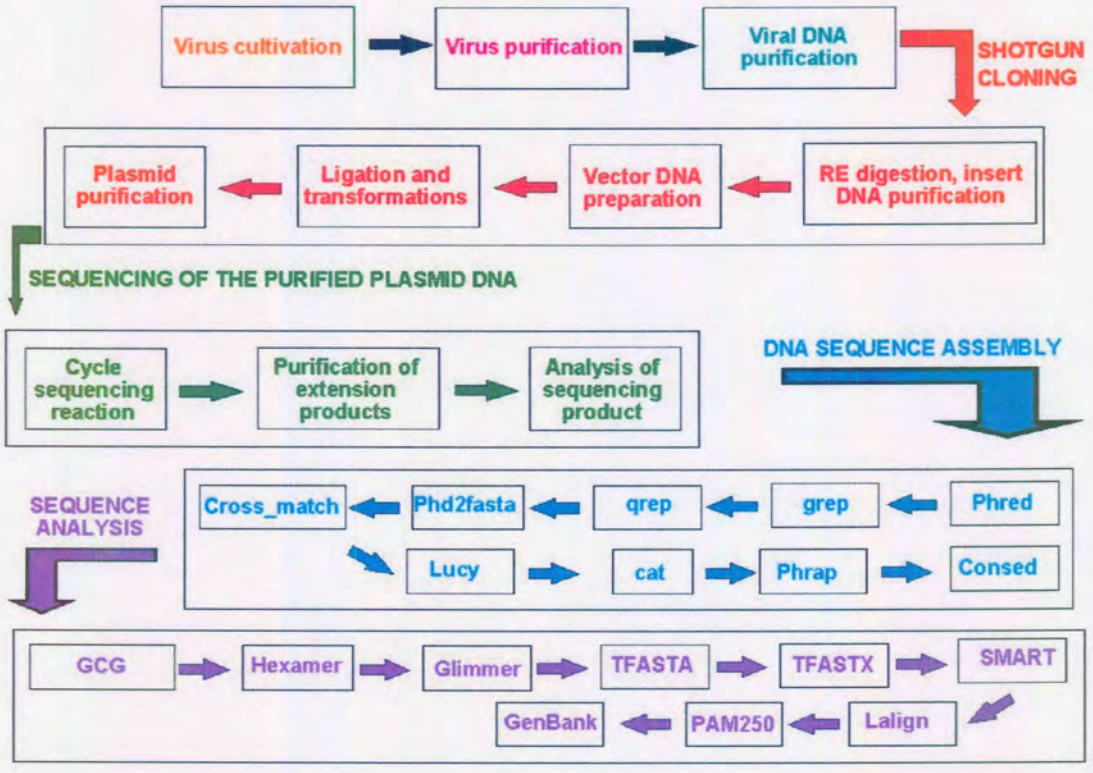

Figure 11. Schematic diagram of the procedure for the cloning, sequencing and analysis of LSDV

2.2 MATERIALS AND METHODS

2.2.1 Virus cultivation.

2.2.1.1 History of the viruses

The LSDV Neethling strain 2490 (LK) was originally isolated in Kenya in 1958, passed 16 times in lamb testes (LT) cells, and was subsequently reisolated in 1987 from lesions of an experimentally infected cow (Tulman *et al.* 2001). The South African LSDV Neethling vaccine strain was cultivated in monolayers of lamb kidney tissue cultures through 61 serial passages followed by 20 passages in the chorio-allantoic membrane (CAM) of embryonated chicken eggs. It was then passaged thrice in lamb kidney monolayers, ten times in Madin-Darby bovine kidney (MDBK) cells and finally five times in foetal bovine testes (FBT) cells (van Rooyen *et al.* 1969). The LSDV Neethling Warmbaths isolate (LD) was isolated from lesions of a severely infected calf in the Northern Province of the Republic of South Africa, on the farm Bothasvlei in 2001 (David Wallace, Biotechnology Division, Onderstepoort Veterinary Institute, Republic of South Africa; Personal communication, 2001). The isolate was passaged twice in Madin-Darby bovine kidney (MDBK) cells and twice in lamb foetal testes (LFT) cells (David Wallace, Biotechnology Division, Onderstepoort Veterinary Institute, Republic of South Africa; Personal communication, 2001). The difference in the passages of the vaccine strain and the virulent isolate of LSD was to keep the virulent isolate as close to a wild-type virus as possible.

2.2.2 Virus purification.

Purification of LSDV from cell cultures was based primarily on the method described by Esposito (Esposito *et al.* 1981). The culture medium was removed from flasks containing infected cells exhibiting 90% C.P.E. The infected cells were harvested by using trypsin to release them from the flasks. The cells were pooled with the medium, dispensed into centrifuge tubes and 0.5 ml of 36% sucrose was layered at the bottom of each tube. The virus/cell suspension was centrifuged at 11 000 rpm (19000 g) for

60 minutes at 4° C in a Beckman J2-21 (Beckman Instruments, CA, U.S.A.) high speed centrifuge in a JS13-1 (Beckman Instruments, C.A., U.S.A.) rotor. The pellet was resuspended in 9 ml of McIlvain's buffer (4 mM) and left on ice for 10 minutes. ß-mercaptoethanol (26 µl) and 1 ml of 10% Triton X-100 (in McIlvain's buffer) were then added and the suspension was further incubated on ice for 10 minutes to disrupt the cells. The cell debris was removed by centrifugation at 2000 rpm (450 g) for 5 minutes at 4° C in a Sigma 301K benchtop centrifuge (Sigma, Germany) and the supernatant fluid was collected. The cell debris was resuspended in McIlvain's buffer and the centrifugation was repeated at 2000 rpm (450 g) for 5 minutes at 4° C in a Sigma 301K benchtop centrifuge). The supernatant fluid was collected and pooled with the first virus-containing supernatant fluid. This fluid was transferred to a centrifuge tube and 0.5 ml of 36% sucrose [prepared in TE (10 mM Tris, 1 mM EDTA, pH 9.0)] was layered beneath. Virus was then pelleted [centrifuged at 11 000 rpm (19000 g) for 60 minutes at 4° C in a Beckman J2-21 high speed centrifuge in a JS13-1 rotor]. The pellet was resuspended in 0.5 ml of TE buffer. Lysis buffer and 26 µl proteinase K (Boehringer Mannheim) (final concentration = 100 µg/ml) were added and the virus mixture was incubated for 3 hours at 56° C (David Wallace, Biotechnology Division, Onderstepoort Veterinary Institute, Republic of South Africa; Personal communication, 2001).

2.2.3 Purification of viral DNA.

Viral DNA was obtained according to a standard and routine procedure in use in the Biotechnology Division (Onderstepoort Veterinary Institute) based on the DNA purification procedures described by Sambrook *et al.* 1989. To remove extraneous proteins, one volume of phenol (Merck, Germany) was added to the viral extract and the mixture was inverted gently a number of times. It was then centrifuged in an Eppendorf Centrifuge 5415 C (Eppendorf, Germany) at 14 000 rpm for 5 minutes. The aqueous phase was carefully removed using a truncated sterile pipette tip and the viral DNA was further purified using 0.5 volumes each of phenol and chloroform (24:1 chloroform:isoamylalcohol), in the manner described above. This was then followed by a final purification step using one volume of chloroform. The DNA was precipitated by the addition of sodium acetate (NaAc) (final concentration = 0.3 M) (Lasechem, LASEC, South Africa) and two volumes ice cold 100% ethanol and left

overnight at -20° C. The DNA was then concentrated by centrifugation at 14 000 rpm for 30 minutes in an Eppendorf Centrifuge. The DNA pellet was washed in 70% ethanol, resuspended in sterile distilled water (dH_20) or TE buffer and stored at 4° C (David Wallace, Biotechnology Division, Onderstepoort Veterinary Institute, Republic of South Africa; Personal communication, 2001).

2.2.4 Cloning

2.2.4.1 Restriction endonuclease digestion and purification of insert DNA.

Random DNA fragments were generated by incomplete enzymatic digestion of the LW and LD LSDV strains with *Tsp*509 I endonuclease (New England Biolabs, Beverly, MA). The LSDV genome is highly A+T rich with an A+T nucleotide content of 74% that is uniformly distributed throughout the genome (Tulman *et al.* 2001). *Tsp*509 I endonuclease is a 4 base pair cutter that is highly AT preferential, therefore *Tsp*509 I endonuclease was used. Briefly, 5 µg of DNA were digested with 1 unit of *Tsp*509 I for 5 minutes at 65° C in a heating block, to obtain fragments with an average size of 2-4 kbp. DNA fragments smaller than 1 kbp were removed by centrifugation through Clontech chroma-spin columns according to the manufacturer's instructions.

2.2.4.2 Preparation of vector DNA.

The vector used for the cloning of all insert DNA was the pUC19 cloning vector (Roche). The vector contains commonly used six-base restriction endonuclease sites in its multiple cloning site (MCS). pUC19-plasmid containing *E. coli* cells were inoculated into LB medium containing ampicillin, and grown with shaking overnight in a 37° C incubator. The vector plasmid was isolated from the pelleted bacterial cells by the modified alkaline lysis method for large–scale preparations of plasmid DNA and purified by equilibrium centrifugation in (cesium chloride) CsCl-ethidium bromide gradients as described in Maniatis (Birnboim and Doly, 1979; Ish-Horowicz and Burke, 1981; Maniatis *et al.*, 1982; Sambrook *et al.*, 1989) and resuspended in dH_2O. Vector plasmid DNA was linearised with *Eco*R I (Roche) in 25 µl reaction

volumes: ~500 ng of plasmid DNA, 10X buffer supplied with the restriction enzyme, 0.5 U of restriction enzyme and distilled water (dH_2O) to make up the volume. The reaction was incubated at 37° C for 60 minutes and loaded onto an ethidium bromide stained 1% agarose gel, alongside undigested plasmid DNA and the λ-*Pst* I marker. This was electrophoresed at 90-100 V for 45 minutes to confirm the digestion. Once digested the DNA was cleaned with phenol, 2x chloroform, followed by ethanol precipitation (Sambrook *et al.*, 1989). The linear vector DNA was resuspended in distilled water (dH_2O) and the concentration determined by spectrophotometry.

The ends of the linearised vector were dephosphorylated to prevent self-ligation. 0.5U of Calf-Intestinal Phosphatase (CIP) (Roche) was added to the total volume of purified linear DNA, together with the CIP 10X buffer, and dH_2O. The dephosphorylation reaction was incubated in a water bath at 37° C for 20 minutes. The reaction volume was raised to 200 µl by the addition of distilled water (dH_2O), and a 3X volume (600 µl) of Phenol/Chloroform/Isoamyl alcohol was added to purify the vector DNA. After vortexing, centrifugation (13 000 g's) and removal of the aqueous DNA-containing phase, DNA was ethanol precipitated and washed before being resuspended in distilled water (dH_2O). The DNA concentration was determined by spectrophotometry.

2.2.4.3 Ligation and transformations.

The ligation reaction was carried out using the rapid ligation kit according to manufacturer's instruction (Roche). The larger inserts were cloned into the dephosphorylated *Eco*RI site of pUC19 vector plasmid, which were then cultured in *E. coli* DH10β cells according to the manufacturer's instructions (GibcoBRL, MD). Approximately 500 ng of insert was ligated with 250 ng of vector. Cells were plated on LB plates containing ampicillin, X-gal (5-bromo-4-chloro-3-indolyl-β-$_D$-galactopyranoside) and IPTG (isopropyl-β-$_D$-thiogalactopyranoside) and grown overnight at 37° C. DNA inserted into the multiple cloning site (MCS) inactivates the *lac*Z' gene providing blue/white selection of recombinants on X-gal media. *E. coli* cells that carry the recombinant plasmids are identified as ampicillin resistant, white colonies.

2.2.4.4 Plasmid purification.

Individual bacterial colonies were picked from agar plates and inoculated into 96-well culture plates containing 1.5 ml of TB medium with carbenicillin (Sigma) per well. Cells were grown overnight at 37° C, with shaking. The plates were spun at 3000 rpm for 10 minutes and the liquid dumped. Double stranded recombinant pUC19 plasmids were then purified using alkaline lysis according to the manufacturer's instructions (Eppendorf 5 Prime, Boulder, CO). The purified recombinant plasmid DNA was now ready for sequencing.

2.2.5 Sequencing

2.2.5.1 Sequencing of the inserted fragments from the purified recombinant plasmid DNA:

2.2.5.1.1 The automated DNA sequencing reaction.

The ABI PRISM® BigDye™ Primer *v*3.0 Ready Reaction Cycle Sequencing Kit For –21 M13 and M13 REV primers (PE Biosystems, Foster City, CA) was used according to the manufacturer's instructions. The protocol was modified as described in the following sections. DNA templates were sequenced from either end with M13 forward and reverse primers (Invitrogen) using dideoxy chain terminator sequencing chemistries (Sanger *et al.* 1977) (Table 3).

Table 3. Commercial primers for the sequencing of bacterial vector plasmids (Invitrogen).

Primer	DNA sequence	MW (Da)	% G+C	Tm
M13 forward	5'- TGT AAA ACG ACG GCC AGT -3'	5529	50	48
M13 reverse	5'- CAG GAA ACA GCT ATG ACC -3'	5499	50	48

a) The cycle sequencing reaction

- A typical sequencing reaction was assembled in 96-well reaction plates, on ice, as follows:

2.5 µl	plasmid DNA template [final concentration = 0.5 µg]
7.5 µl	diluted reaction mix (1:2)
10 µl	total volume

The 1:2 dilution of the reaction mix is made up as follows:

400 µl	Ready Mix (ABI)
200 µl	5X Sequencing buffer
20 µl	Primer (M13 forward or reverse) (~4 µg) [final concentration =0.2 µg/µl]
880 µl	dH_2O
1500 µl	(200X reactions)

Cycle sequencing was performed on a GeneAmp PCR System 9600 thermal cycler™ (Perkin Elmer) under the following conditions:
Denaturation: 96° C for 10 seconds/ Annealing: 50° C for 30 seconds/ Elongation: 60° C for 4 minutes (40 cycles).

- The sequencing of full length genomic DNA for the closure of gaps as well as regions of low sequence reads, the sequencing reaction was assembled in 96-well reaction plates as follows:

2 µg	genomic DNA
7 µl	Reaction mix
1 µl	Primer (M13 forward or reverse) (20 ng)

- The Reaction mix is made up as follows (7µl):

400 µl	Ready Mix (ABI)
200 µl	5X Sequencing buffer
150 µl	dH_2O
750 µl	

The first step of cycle sequencing was performed on a GeneAmp PCR System 9600 thermal cycler™ (Perkin Elmer) under the following conditions:
Initial Denaturation: 98° C for 3 minutes/ Denaturation: 98° C for 10 seconds/ Annealing: 50° C for 30 seconds/ Elongation: 60° C for 4 minutes (99 cycles).

- The sequencing of PCR products for regions of low sequence reads, the sequencing reaction was assembled in 96-well reaction plates as follows:

2.5 µl	DNA [final concentration = 0.5 µg]
7 µl	diluted reaction mix (1:2)
1 µl	Primer (M13 forward or reverse) (20 ng)

Cycle sequencing was performed on a GeneAmp PCR System 9600 thermal cycler™ (Perkin Elmer).

Gap closure was achieved by primer-walking and gap-spanning clones. Certain areas of the sequence where regions of low sequence reads were obtained were re-sequenced from the original clones as well as from PCR products. The primers used for the vaccine strain (LW) are as follows:

Table 4. The primers for LW used for the closure of gaps as well as sequencing areas with low sequence reads (All primers were manufactured by Invitrogen)

Primer	Position	DNA sequence	MW (Da)	% G+C	Tm
lwy1f.log	13110	5'- CCACTTAATTATAAAATTAAGAACAAAATC -3'	9158	20	51
lwy1r.log	13553	5'- AATTGGAGGTATATCACAAACAAGG -3'	7742	36	53
lwy2f.log	135872	5'- TGTATCATATTACAATGGCTTAATAATGAG -3'	9239	27	53
lwy2r.log	136295	5'- TTTGGAAAACTGTTCCATGATTTAG -3'	7682	32	51
lwy3f.log	80803	5'- GAAACGAGGAAGACGTTATTAAATGT -3'	8085	35	53
lwy3r.log	81369	5'- ATTGATATGAATGCGAAAACAAAAG -3'	7751	28	49

The primers used for the wild-type strain (LD) are as follows:

Table 5. The primers for LD used for the closure of gaps as well as sequencing areas with low sequence reads (All primers were manufactured by Invitrogen)

Primer	Position	DNA sequence	MW (Da)	% G+C	Tm
ld1f	11266	5'- CAA CGA TAT AGA AGC TAA TAT TAC TGA AGG -3'	9266	33	56
ld1r	11770	5'- GAT ATA GGA GGA GGA GTT ATA GAT GAA GA -3'	9133	38	57
ld2f	14237	5'- AGT TCG TTT GAA AAT GTT AAT CCT G -3'	7682	32	51
ld2r	15263	5'- CCG TTA GTA GTG AGG ATA ATG ATA CTA AAT -3'	9287	33	56
ld3f	35398	5'- GAA GAG CTA TTT GGA CAG TGA TTT T -3'	7745	36	53
ld3r	36121	5'- GAA ATA AAC GAA ATC GTT ATA AAC GG -3'	8040	31	52
ld5f	39970	5'- GAT GTC ATT TTC GAT GAT AAT GCT T -3'	7673	32	51
ld5r	40858	5'- GAA GAA CCT AAA TCA GTT ATT CAG ACG -3'	8296	37	55
ld6f	68878	5'- CAC TGA AGA AAA TGA AAA TGA CGA T -3'	7736	32	51
ld6r	69348	5'- GTT CAT AAA CAA TAA CAT CAG TTA AGT GG -3'	8929	31	54
ld7f	108166	5'- GGA CTC ACT CAC ATT ATT CTT GGA T -3'	7604	40	54
ld7r	108704	5'- GTC ATA TCA TCT ATT TTA AAT TCG AGT TCT -3'	9134	27	53
ld8f	109259	5'- AGA AAG AGT TAA ATC TAC AGC TTA CTC AAA -3'	9212	30	55
ld8r	109950	5'- GAA CTG TTG CCT TAT CCT GAG TTA G -3'	7658	44	56

Id9f	119677	5'- GGC AAA GGA TAT AAC TTT AAA AGT TTC ATA -3'	9257	27	53
Id9r	120515	5'- GAT TTG TAA ATC GTT TAA ATA ATA AAC CC -3'	8881	24	52
Id10f	122907	5'- GAT AAT TGT ACC TTA ACA CTT CCT GTT ACT -3'	9104	33	56
Id10r	123545	5'- GAT ACA TAT GCA AAC AGA ATG GAT CTA -3'	8320	33	54
Id11f	130926	5'- GTT AAC AAA ACA AGA TAG CAT ATC TGG TA -3'	8938	31	54
Id11r	131789	5'- CTA CAA ATA ATG GTC CAT TGC TCT T -3'	7589	36	53
Id12f	133514	5'- GCG AGG TGT TAG CTA TAC TTT AGT AGG A -3'	8691	43	58
Id12r	134200	5'- CAA CGA CAA CTT CAT AGA TTT GAT TAT -3'	8239	30	52
Id13f	134196	5'- TGA TAT AAT CAA ATC TAT GAA GTT GTC G -3'	8622	29	53
Id13r	134890	5'- GCA ATC TAT TGC CAT ATC TAA TTT TGT -3'	8212	30	52
Id21f	69742	5'- GAT AAG GTT CCG CAT ACC TTT ATA GT -3'	7956	38	55
Id21r	70424	5'- GCT ATC ATC GAT GAA TAT TCA TTG TAT TT -3'	8869	28	53
Id22f	72874	5'- GAG TAG TGA AAA TTA TAT GTC CGT TGA A -3'	8685	32	54
Id22r	73609	5'- TGG AAT CTG AAA TTA AAA GAT TAA CAA ATA -3'	9275	20	51
Id23f	8611	5'- ATT TAT ATC GGA TCC ATT TTC TAT GAG -3'	8251	30	52
Id23r	9316	5'- GGA GTA TAT TTA TGT TCC ATA ACT ACG GAT -3'	9230	33	56
Id24f	12786	5'- AAA ACT TAA AAT TTT CAT CTA TTA TGT TGC -3'	9152	20	51
Id24r	13357	5'- GAT GTG TAC GAT GTT GCA GAT AAA TA -3'	8067	35	53
Id25f	18152	5'- GTT TCA TCA TCG TCG ATC TTG TAC T -3'	7586	40	54
Id25r	18841	5'- GAT GAT TTA TAT TCA GTT AAT GGT GGG -3'	8392	33	54

b) Purification of extension products

Following the sequencing reaction the DNA was precipitated with 75 µl of 70% ethanol (EtOH) at room temperature for 15 minutes. The 96-well reaction plates were then spun at 2800 rpm for 20 minutes at 20° C. The supernatant was then dumped and the pellet washed with 75 µl of 70% EtOH. The 96-well reaction plates were spun at 2800 rpm for 10 minutes and dried for 20 minutes. The DNA was resuspended in 25 µl dH_2O.

c) Analysis of sequencing reaction

Reaction products were run on an ABI PRISM® 3700 DNA Analyzer (PE Biosystems, Foster City, CA). The ABI PRISM® 3700 DNA Analyzer combines capillary electrophoresis, with computer controlled instrument operation and data analysis.

2.2.6 DNA sequence assembly

2.2.6.1 Hardware

ABI sequence software (version 3.0) was used for lane tracking and trace extraction. The following was used: Sun Sparc computers (unix) running the operating system Solaris 5.6; a PC with an AMD 333 chip running unix (Solaris X86 7); a PC with an AMD 333 chip running Linux 2.2 (Caldera 2.3) a unix system; and apple mac power PC with mac os8.

2.2.6.2 DNA sequence assembly

Chromatogram traces were base-called with Phred (Ewing *et al.* 1998b) which also produced a quality file containing a predicted probability of error at each base position. The sequences were assembled with Phrap (Ewing *et al.* 1998a), using the quality files and default setting to produce a consensus sequence with some subsequent manual editing using the Consed sequence editor (Gordon *et al.* 1998).

a) Extracting the trace files from the ABI sequencer using the program Phred:
Phred is a base calling program for DNA sequence traces that was developed by Phil Green and Brent Ewing from the University of Washington and can be obtained from http://www.phrap.org. [Command line: *phred -id prep -pd prepd*]. Phred extracts the ABI chromatogram (trace) files in the directory prep putting the resulting phd files in the directory prepd. These phd files have the called base and a quality value related to a probability that the base called is correct.

b) Pattern searching program:
grep is a unix pattern matching program that searches one or more input files for lines containing a match to a specified pattern, the pattern in the above case being TRIM. grep can be obtained from http://www.gnu.org/software/grep/grep.html. [Command line: *grep TRIM prepd/* > trim.list*]. This command looks for the occurrence of the word TRIM in the phd files located in the directory prepd and directs the output to a file called trim.list. A value labelled TRIM is found in the phd file indicating the position where the called bases have a probability of greater than 0.05 of being correct. Therefore you can see short reads as well as traces where the reaction did not work.

c) Summary of the quality of the gel file:

qrep is a program that summarises the quality of a gel file. [Command line: *qrep -pd prepd -cd prep > std.out*]. The program qrep finds the phd files in the directory prepd and the chromatogram files in the directory prep and directs the output to a file called std.out. The output has a summary of the average read length, the average length and standard deviation of the bases called. These are statistics on how good the run was namely, the overall quality of the run.

d) phd2fasta program makes FASTA files from the phd files:
phd2fasta is a program by Phil Green and Brent Ewing and can be obtained from the University of Washington. [Command line: *phd2fasta -id prepd -os new.fasta -oq new.fasta.qual*]. When the phd2fasta program runs, it reads the phd files from the directory prepd thereby making sequence and quality value FASTA files from these phd files, which the programs phrap and cross_match need as input. In addition, phd2fasta ignores those phd files that containing no sequence, which phred automatically creates from chromatograms that lack traces.

e) cross_match is a general purpose screening and alignment program:
Cross_match is a program by Phil Green from the University of Washington, for rapid protein and nucleic acid sequence comparison and database search. [Command line: *cross_match – new.fasta screen.seq –screen –minscore 20 –minmatch 12 > sc.out*]. The cross_match alignment program is used to compare each read in a file namely new.fasta to a database of cloning and sequencing vectors. The –screen option in the above command tells cross_match to produce another fasta file, nearly identical to new.fasta, except that recognised vector sequences are replaced by x. If there were pox sequence mixed in with something else you could screen out the pox sequence. The poxvirus database is a list of all poxviral sequences in genbank. Clones being sequenced from the one direction using the M13 forward primer are screened here. Those clones containing poxvirus sequence are identified and these clones are than sequenced in the opposite direction using the M13 reverse primer.

f) Preparation of raw sequence fragments for sequence assembly:
Lucy is a program from The Institute of Genomic Research (TIGR) and is available at www.tigr.org. [Command line: *lucy –v PUC19 PUC19splice new.fasta new.fasta.qual –debug > lu.out*]. It screens vector etc. in the same manner as cross_match. It gives

some useful statistics, such as the number of sequences with no insert, all vector sequences, bad quality sequences, as well as short sequences. It prepares raw DNA sequence fragments for sequence assembly.

g) Concatonate one file onto the end of another file:
cat is the unix command to concatonate one file onto the end of another file.
[Command line: *cat new.fasta.screen >> ld.fasta.screen*
*cat new.fasta.qual >> ld.*een.qual*].

h) Assembly of sequences using the program Phrap:
Phrap is the University of Washington's sequence assembly program for assembling shotgun sequencing projects. [Command line: *phrap ld.fasta.screen -ace -forcelevel 3 > phrap.out &*]. The command assembles the sequence in a file namely, ld.fasta.screen and makes a file called ld.fasta.screen.ace, which can be read into the consed editor. Forcelevel 3 defines the merging of contigs where 0 is the default value, with 0 being very stringent and 3 less so. The value varies from 0 to 12.

2.2.6.3 Consed 10.0

Consed is the editor portion of the phred/phrap/consed package, for viewing and editing assemblies assembled with the phrap assembly program (Gordon *et al.* 1998). The PRIMER-PICKING program available with the consed program was used to design the primers listed in Tables 4 and 5.

2.2.6.4 Sequence analysis

Genome DNA composition, structure and restriction enzyme patterns were analysed with the Wisconsin Genetics Computer Group (GCG) programs (Devereux *et al.* 1984, Afonso *et al.* 1999). Open reading frames (ORFs) longer than 30 amino acids with a methionine start codon (Staden et al. 1982, Staden 1982) were evaluated for coding potential using the Hexamer (ftp.sanger.ac.uk/pub/rd) and GLIMMER (Salzberg *et al.* 1998, Delcher *et al.* 1999) computer programs. Hexamer is a program that scans a piece of DNA for likely coding regions, while GLIMMER is a system for finding genes in microbial DNA and is the microbial gene finder at The Institute of

Genomic Research (TIGR). The Kenyan LSDV Neethling strain 2490 (LK) (Tulman *et al.* 2001), the South African LSDV Neethling Warmbaths isolate (LD) and South African LSDV Neethling vaccine strain (LW) strains were compared using the TFASTA and TFASTX program which does a Pearson and Lipman search for similarity between a query peptide sequence and any group of nucleotide sequences taking frameshifts into account (Pearson and Lipman 1988). Gene families were analysed and annotated as previously described (Afonso *et al.* 1999).

SMART (a Simple Modular Architecture Research Tool) allows for the identification and annotation of genetically mobile domains and the analysis of domain architecture (http://coot.embl-heidelberg.de/SMART) (Letunic *et al.* 2002, Schultz *et al.* 1998, Schultz *et al.* 2000). SMART is able to detect more than 600 domain families found in nuclear, signalling and extra-cellular proteins (Letunic *et al.* 2002). These domains have been extensively annotated with respect to functional class, sub-cellular localisation, phyletic distribution and tertiary structure (Letunic *et al.* 2002, Schultz *et al.* 1998, Schultz *et al.* 2000). Information on more than 600 domain types in more than 54 000 different proteins is stored in SMART using a relational database management system (Letunic *et al.* 2002, Schultz *et al.* 2000). Each domain's hit borders, raw bit score, and Expect (*E*) value are recorded, together with protein accession code, description and species names structure (Letunic *et al.* 2002, Schultz *et al.* 1998, Schultz *et al.* 2000). For each protein in the relational database, features such as transmembrane regions, coiled-coils, signal peptides and internal repeats are also included (Letunic *et al.* 2002, Schultz *et al.* 2000). SMART consists of a library of Hidden Markov Models (HMMs), which provide statistically sound *E*-values, giving a robust estimate of the significance of a domain hit (Letunic *et al.* 2002, Schultz *et al.* 2000).

Once domains were identified in an ORF, the region was then aligned with William Pearson's *lalign* programme (http://www.ch.embnet.org/software/LALIGN_form.html) to identify amino acid differences occurring within such domains. The *lalign* program compares two sequences looking for local sequence similarities. This programme implements the algorithm of Huang and Miller (1991). The algorithm of Huang and Miller is a local alignment algorithm, which locates matching segments within two sequences, while global alignment methods align entire sequences, including

unconserved regions (Huang and Miller, 1991). Local alignment methods are appropriate when sequences may show isolated regions of similarity, like conserved domains. Local alignment algorithms directly discover alignments with both substitutions and gaps (Buhler, 2001).

The PAM250 matrix (Gribskov *et al.* 1986) was used to identify whether amino acid differences occurring within the functional domains as identified by SMART (Table 9, Table 10, Table 11, Table 12, Table 13 and Table 14) were conservative or non-conservative using a cut-off value of 0.4.

2.2.7 Nucleotide sequence accession number. The LD and LW genome sequences have been deposited in GenBank under accession nos. AF409137 and AF409138 respectively.

CHAPTER III

Results

3.1 RESULTS

The genomes of the South African LSDV Neethling vaccine strain (LW) and the virulent South African LSDV Neethling Warmbaths isolate (LD) were sequenced. The LD and LW genomes were then assembled into contiguous sequences of 150,793 base pairs (bp) and 150,509 bp respectively. The complete sequences of LD and LW can be found in GenBank under accession numbers AF409137 and AF409138 respectively. By comparison, the genome sequence of the virulent LSDV Kenyan Neethling strain 2490, described here as LK, was assembled into a contiguous sequence of 150,773 bp by Tulman *et al.* (2001). The terminal hairpin loops were not sequenced and therefore the leftmost nucleotide of each assembled genome was arbitrarily designated as base 1. The nucleotide composition of both the LD and LW genomes are 74% A+T and is uniformly distributed. The final DNA consensus sequences for the LD and LW genome represented on average a 6- and 9-fold redundancy at each base position respectively.

LW and LD each contains 156 open reading frames (ORFs) that have been annotated here as putative genes (Figure 12). The same number of ORFs were also present in the LSDV Kenyan Neethling strain 2490 (LK) (Tulman *et al.* 2001). These genes encode proteins of 53 to 2,025 amino acids.

In addition to these amino acid differences, six deletion and eight insertion sites occurred. The genes involved included:

- ORF067 – a single amino acid deletion in LD - encoding a putative host range protein
- ORF141 – a single amino acid deletion in LD - encoding a putative host range protein
- ORF011 – a four amino acid deletion in LD - encoding a chemokine receptor-like protein
- ORF119 - a single amino acid insertion in the LD - encoding the RNA polymerase sub-unit RPO35
- ORF128 - a single amino acid insertion in the LD - encoding a CD47-like protein
- ORF151 - a single amino acid insertion in the LD - encoding a kelch-like protein
- ORF022 - a two amino acid insertion in LD - encoding a hypothetical protein of unknown nature
- ORF144 - a three amino acid insertion in LD - encoding a kelch-like protein

A total of three frameshifts occurred in LD. Two of the LD ORFs are truncated due to such frameshifts. These are ORF019, encoding a kelch-like protein and ORF026, encoding a hypothetical protein of unknown nature. The other frameshift occurred in ORF013, encoding an interleukin-1 receptor-like protein, with the C-terminus encoding-end producing a stop codon where the last 14 amino acids are out of frame. ORFs containing frameshifts were labeled a (e.g. LD013a), while those ORFs with frameshifts resulting in a truncation were labeled a and b (e.g. LD026a and LD026b) in the Table 6.

Table 6. Comparison between the Kenyan LSDV Neethling Strain 2490 (LK) and the South African LSDV Neethling Warmbaths isolate (LD).

LK (a)		**LD** (b)		**Frameshift(#)**	**Total**	
new	**Length**	**new**	**Length**	**Insertions (+)**	**aa** (c)	
orfs	**aa** (c)	**orfs**	**aa** (c)	**Deletions (-)**	**changes**	**Predicted structure and/or function** (d)
LK011	381	LD011	377	4-		CC chemokine receptor-like protein
LK012	211	LD012	211		2	Ankyrin repeat protein
LK013	341	LD013a (e)	328	#	1	Interleukin-1 receptor-like protein
LK017	176	LD017	176		1	putative integral membrane protein, apoptosis regulator
LK019	569	LD019a (e)	290	#		Kelch-like protein
LK022	112	LD022	114	2+	1	hypothetical protein

LK026	302	LD026a (e)	153	#		hypothetical protein
		LD026b (e)	107			
LK027	638	LD027	638		1	putative EEV maturation
LK033	735	LD033	735		2	hypothetical protein
LK035	402	LD035	402		2	hypothetical protein
LK036	201	LD036	201		1	RNA polymerase subunit RPO30
LK056	174	LD056	174		1	hypothetical protein
LK059	336	LD059	336		1	putative myristylated protein
LK061	92	LD061	92		1	hypothetical protein
LK067	198	LD067	197	1-		putative host range protein
LK073	190	LD073	190		1	hypothetical protein
LK075	798	LD075	798		1	RNA polymerase-associated protein RAP94
LK087	253	LD087	253		1	*mut*T motif, putative gene expression regulator
LK089	287	LD089	287		1	mRNA capping enzyme, small subunit; VITF
LK103	190	LD103	190		1	putative virion core protein
LK110	480	LD110	480		3	putative DNA helicase transcriptional elongation factor
LK117	148	LD117	148		1	putative fusion protein
LK119	302	LD119	303	1+		RNA polymerase subunit RPO35
LK127	273	LD127	273		1	hypothetical protein
LK128	300	LD128	301	1+		CD47-like protein
LK129	123	LD129	123		1	hypothetical protein
LK133	559	LD133	559		1	DNA ligase-like protein
LK134	2025	LD134	2025		1	similar to variola virus B22R
LK140	240	LD140	240		1	putative RING finger host range protein, N1R
LK141	225	LD141	224	1-		putative EEV host range protein
LK143	302	LD143	302		2	Tyrosine protein kinase-like protein
LK144	547	LD144	550	3+	2	Kelch-like protein
LK145	634	LD145	634		2	Ankyrin repeat protein
LK149	337	LD149	337		1	Serpin-like protein
LK151	550	LD151	551	1+	2	Kelch-like protein
LK152	489	LD152	489		1	Ankyrin repeat protein

(a) LK, Kenyan LSDV Neethling Strain 2490

(b) LD, South African LSDV Neethling Warmbaths isolate.

(c) aa, amino acids.

(d) Function was deduced either from the degree of similarity to known genes or from the presence of Prosite signatures.

(e) ORFs containing either an amino-terminal or carboxy-terminal frameshift were labelled [a] (e.g. LD013a), while those ORFs with frameshifts resulting in a truncation were labelled [a] and [b] (e.g. LD026a and LD026b).

3.1.2 Comparison between the virulent South African LSDV Neethling Warmbaths isolate (LD) and the South African LSDV Neethling vaccine strain (LW)

Major amino acid differences were observed in the comparison between the highly cell-attenuated SA vaccine strain (LW) and the virulent Warmbaths strain (LD). 118 of the 156 genes (76%) common to both viruses were found to contain between one to 42 amino acid differences (Table 7). The effects of the amino acid differences as well as the frameshifts between the cell-attenuated vaccine strain and the virulent isolate remains speculative until they have been further studied and confirmed.

A total of 30 amino acid insertions and 21 amino acid deletions occur, ranging from a total of one to 11 amino acids encoded by 12 genes and a total of one to nine amino acids encoded by 10 genes respectively.

ORFs containing either an amino-terminal or carboxy-terminal frameshift were labelled a (e.g. LD013a), while those ORFs with frameshifts resulting in a truncation were labelled a and b or a, b and c (e.g. LW019a, LW019b, and LW019c) [Table 7]. A total of nine frameshifts occurred between the LD Warmbaths isolate and the LW vaccine strain. Of the nine frameshifts, four of the frameshifts have resulted in a truncation. These four genes and their putative functions are:

- LW019 and LD019 - kelch-like protein
- LD026 - hypothetical protein of unknown nature
- LW134 - similar to variola virus B22R
- LW144 - kelch-like protein.

The other five frameshifts occured in:

- LD013 encoding an interleukin-1 receptor-like protein which has a C-terminal frameshift resulting in the last 14 amino acids being out of frame.
- LW035 encoding a hypothetical protein of unknown nature which has a N-terminal frameshift where the first 26 amino acids are out of frame.
- LW086 encoding a protein with a *mut*T motif which has a C-terminal frameshift where the last 7 amino acids are out of frame.

- LW087 encoding a *mut*T motif - putative gene expression regulator which has two frameshifts at the C-terminal end resulting in the last 53 amino acids being out of frame.
- LW131 encoding a superoxide dismutase-like protein which has a frameshift where the last 77 amino acids are out of frame.

Table 7. Comparison between the South African LSDV Neethling Warmbaths isolate (LD) and the South African LSDV Neethling vaccine strain (LW).

LD (a)		**LW** (b)		**Frameshift (#)**	**Total**	
new	**Length**	**new**	**Length**	**Insertions (+)**	**aa** (c)	
orfs	**aa** (c)	**orfs**	**aa** (c)	**Deletions (-)**	**changes**	**Predicted structure and/or function** (d)
LD001 (f)	159	LW001	159		2	hypothetical protein
LD003 (f)	240	LW003	240		2	putative ER-localized apoptosis regulator
LD005	170	LW005	171	1+	2	Interleukin-10-like protein
LD006	231	LW006	230	1-	3	Interleukin-1 receptor-like protein
LD007	355	LW007	355		3	hypothetical protein
LD008	275	LW008	275		19	putative soluble Interferon gamma receptor
LD009	230	LW009	230		13	alpha amanitin-sensitive protein
LD010	162	LW010	162		6	LAP/PHD-finger protein
LD011	377	LW011	381	4+	4	CC chemokine receptor-like protein
LD012	211	LW012	211		3	Ankyrin repeat protein
LD013a (e)	328	LW013	341	#	6	Interleukin-1 receptor-like protein
LD017	176	LW017	176		8	putative integral membrane protein, apoptosis regulator
LD018	146	LW018	146		2	dUTPase
LD019a (e)	290	LW019a (e)	290	#		Kelch-like protein
LD019b (e)	270	LW019b (e)	125			
		LW019c (e)	100			
LD020	321	LW020	321		3	Ribonucleotide reductase, small subunit
LD021	86	LW021	86		3	hypothetical protein
LD022	114	LW022	112	2-	3	hypothetical protein
LD024	216	LW024	216		1	hypothetical protein
LD026a (e)	153	LW026	302	#		hypothetical protein
LD026b (e)	107					
LD027	638	LW027	639	1+	5	putative EEV maturation
LD028	370	LW028	370		2	putative palmitylated virion envelope protein
LD029	145	LW029	145		1	hypothetical protein
LD030	219	LW030	219		1	hypothetical protein
LD032	474	LW032	474		2	Poly(A) polymerase large subunit PAP_L
LD033	735	LW033	735		12	hypothetical protein
LD034	177	LW034	177		1	putative PKR inhibitor
LD035	402	LW035a (e)	377	#	7	hypothetical protein
LD036	201	LW036	201		1	RNA polymerase subunit RPO30
LD037	566	LW037	566		3	hypothetical protein
LD039	1010	LW039	1010		8	DNA polymerase
LD040	95	LW040	95		1	putative redox protein
LD041	130	LW041	130		1	putative virion core protein
LD042	684	LW042	684		4	hypothetical protein

LD043	314	LW043	314		2	putative DNA-binding virion core protein
LD045	276	LW045	276		2	putative DNA-binding phosphoprotein
LD046	78	LW046	78		1	hypothetical protein
LD047	394	LW047	394		2	hypothetical protein
LD048	433	LW048	433		1	putative virion core protein
LD049	676	LW049	676		5	putative RNA helicase NPH-II
LD050	596	LW050	595	1-	0	putative metalloprotease
LD052	110	LW052	110		1	hypothetical protein
LD054	437	LW054	437		3	hypothetical protein
LD056	174	LW056	174		2	hypothetical protein
LD057	373	LW057	373		2	putative virion core protein
LD059	336	LW059	336		2	putative myristylated protein
LD061	92	LW061	92		3	hypothetical protein
LD062	318	LW062	318		1	hypothetical protein
LD064	131	LW064	131		4	Putative membrane protein
LD065	147	LW065	148	1+	0	hypothetical protein
LD067	197	LW067	197	1+,1-	2	putative host range protein
LD068	333	LW068	333		1	Poly(A) polymerase small subunit, PAP_S
LD071	1285	LW071	1285		4	RNA polymerase subunit RPO147
LD073	190)	LW073	190		2	hypothetical protein
LD074	322	LW074	322		2	putative IMV envelope protein p35
LD075	798	LW075	798		5	RNA polymerase-associated protein RAP94
LD076	223	LW076	226	3+	2	putative late transcription factor VLTF-4
LD079	842	LW079	842		3	mRNA capping enzyme, large subunit
LD080	155	LW080	155		1	putative virion protein
LD081	245	LW081	245		2	putative virion protein
LD082	218	LW082	218		1	Uracil DNA glycosylase
LD083	786	LW083	786		7	putative NTPase
LD084	635	LW084	635		2	putative early transcription factor, small subunit $VETF_S$
LD085	163	LW085	163		1	RNA polymerase subunit RPO18
LD086	213	LW086a (e)	210	#	2	*mut*T motif
LD087	253	LW087a (e)	200	#	3	*mut*T motif, putative gene expression regulator
LD088	635	LW088	635		1	putative transcription termination factor NPH-I
LD089	287	LW089	287		2	mRNA capping enzyme, small subunit; VITF
LD090	549	LW090	549		1	putative rifampicin resistance protein
LD093	75	LW093	75		1	hypothetical protein
LD094	661	LW094	661		2	putative virion core protein P4b
LD095	161	LW095	161		2	putative virion core protein
LD096	170	LW096	170		2	RNA polymerase subunit RPO19
LD097	375	LW097	375		2	hypothetical protein
LD098	714	LW098	714		6	Early transcription factor $VETF_L$
LD101	904	LW101	904		1	Virion core protein P4a
LD102	317	LW102	317		3	hypothetical protein
LD103	190	LW103	190		2	putative virion core protein
LD107	95	LW107	95		1	hypothetical protein
LD108	377	LW108	377		3	putative myristylated membrane protein
LD109	196	LW109	196		1	putative phosphorylated IMV membrane protein
LD110	480	LW110	480		7	putative DNA helicase transcriptional elongation factor
LD112	430	LW112	430		3	putative DNA polymerase processivity factor
LD113	115	LW113	117	2+	1	hypothetical protein
LD114	168	LW114	179	11+	0	hypothetical protein

LD115	385	LW115	385		2	putative intermediate transcription factor VITF-3
LD116	1156	LW116	1156		3	RNA polymerase subunit RPO132
LD118	140	LW118	140		1	hypothetical protein
LD119	303	LW119	303		1	RNA polymerase subunit RPO35
LD122	196	LW122	196		2	putative EEV glycoprotein
LD123	171	LW123	171		1	putative EEV protein
LD125	288	LW125	288		1	hypothetical protein
LD126	181	LW126	172	9-	1	putative EEV glycoprotein
LD127	273	LW127	273		3	hypothetical protein
LD128	301	LW128	300	1-	9	CD47-like protein
LD129	123	LW129	123		5	hypothetical protein
LD130	81	LW130	81		4	hypothetical protein
LD131	161	LW131a (e)	108	#, 1-, 2+	19	Superoxide dismutase-like protein
LD132	176	LW132	177	1+	4	hypothetical protein
LD133	559	LW133	559		8	DNA ligase-like protein
LD134	2025	LW134a (e)	721	#		similar to variola virus B22R
		LW134b (e)	1243			
LD135	360	LW135	360		7	putative IFN-alpha/beta binding protein
LD136	153	LW136	153		3	hypothetical protein
LD137	335	LW137	335		4	hypothetical protein
LD138	186	LW138	186		6	Ig domain, OX-2-like protein
LD139	305	LW139	305		4	putative Ser/Thr protein kinase
LD140	240	LW140	240		10	putative RING finger host range protein, N1R
LD141	224	LW141	225	1+	3	putative EEV host range protein
LD142	134	LW142	132	2-	2	putative secreted virulence factor
LD143	302	LW143	302		5	Tyrosine protein kinase-like protein
LD144	550	LW144a (e)	269	#		Kelch-like protein
		LW144b (e)	281			
LD145	634	LW145	636	2+	42	Ankyrin repeat protein
LD146	413	LW146	412	1-	9	Phospholipase D-like protein
LD147	498	LW147	498		1	Ankyrin repeat protein
LD148	447	LW148	447		12	Ankyrin repeat protein
LD149	337	LW149	337		2	Serpin-like protein
LD150	161	LW150	161		1	hypothetical protein
LD151	551	LW151	549	2-	4	Kelch-like protein
LD152	489	LW152	489		10	Ankyrin repeat protein
LD153 (f)	91	LW153	91		1	hypothetical protein
LD154 (f)	240	LW154	240		2	putative ER-localized apoptosis regulator
LD156 (f)	159	LW156	159		2	hypothetical protein

(a) LD, South African LSDV Neethling Warmbaths isolate.

(b) LW, South African LSDV Neethling vaccine strain.

(c) aa, amino acids.

(d) Function was deduced from either the degree of similarity to known genes or from the presence of Prosite signatures.

(e) ORFs containing either an amino-terminal or carboxy-terminal frameshift were labelled [a] (e.g. LD013a), while those ORFs with frameshifts resulting in a truncation were labelled [a] and [b] or [a], [b] and [c] (LW019a, LW019b and LW019c).

(f) ORF 001, ORF 002, ORF 003 and ORF 004 are identical to ORF156, ORF155, ORF154 and

ORF153 respectively. 51 amino acids encoded by ORF004 situated in the inverted terminal repeats.

For clarity, the amino acid differences occurring in an open reading frame (ORF) will be presented in this section in a systematic way (Table 8).

Table 8. Systematic representation of open reading frames (ORFs) containing amino acid differences between LD and LW.

ORFs (a)	Predicted structure and/or function
ORFs containing a single amino acid difference	
ORF024	hypothetical protein
ORF029	hypothetical protein
ORF030	hypothetical protein
ORF034	putative PKR inhibitor
ORF036	hypothetical protein
ORF040	putative redox protein
ORF041	putative virion core protein
ORF046	hypothetical protein
ORF048	putative virion core protein
ORF052	hypothetical protein
ORF062	hypothetical protein
ORF068	poly(A) polymerase small subunit, PAP_S
ORF080	putative virion protein
ORF082	uracil DNA glycosylase
ORF085	RNA polymerase subunit RPO18
ORF088	putative transcription termination factor NPH-I
ORF090	putative rifampicin resistance protein
ORF093	hypothetical protein
ORF101	virion core protein P4a
ORF107	hypothetical protein
ORF109	putative phosphorylated IMV membrane protein
ORF113	hypothetical protein
ORF118	hypothetical protein
ORF119	RNA polymerase subunit RPO35
ORF123	putative EEV protein
ORF125	hypothetical protein
ORF126	putative EEV glycoprotein
ORF147	ankyrin repeat protein
ORF150	hypothetical protein
ORF153	hypothetical protein
ORFs containing two amino acid differences	
ORF001	hypothetical protein
ORF003	putative ER-localized apoptosis regulator
ORF005	interleukin-10-like protein
ORF018	dUTPase
ORF028	putative palmitylated virion envelope protein
ORF032	poly(A) polymerase large subunit PAP_L

ORF043	putative DNA-binding virion core protein
ORF045	putative DNA-binding phosphoprotein
ORF047	hypothetical protein
ORF056	hypothetical protein
ORF057	putative virion core protein
ORF059	putative myristylated protein
ORF067	putative host range protein
ORF073	hypothetical protein
ORF074	putative IMV envelope protein p35
ORF076	putative late transcription factor VLTF-4
ORF081	putative virion protein
ORF084	putative early transcription factor, small subunit $VETF_S$
ORF086	*mut*T motif
ORF089	mRNA capping enzyme, small subunit; VITF
ORF094	putative virion core protein P4b
ORF095	putative virion core protein
ORF096	RNA polymerase subunit RPO19
ORF097	hypothetical protein
ORF103	putative virion core protein
ORF115	putative intermediate transcription factor VITF-3
ORF122	putative EEV glycoprotein
ORF142	putative secreted virulence factor
ORF149	serpin-like protein
ORF154	putative ER-localized apoptosis regulator
ORF156	hypothetical protein
ORFs containing three amino acid differences	
ORF006	interleukin-1 receptor-like protein
ORF007	hypothetical protein
ORF012	ankyrin repeat protein
ORF020	ribonucleotide reductase, small subunit
ORF021	hypothetical protein
ORF022	hypothetical protein
ORF037	hypothetical protein
ORF054	hypothetical protein
ORF061	hypothetical protein
ORF079	mRNA capping enzyme, large subunit
ORF087	*mut*T motif, putative gene expression regulator
ORF102	hypothetical protein
ORF108	putative myristylated membrane protein
ORF112	putative DNA polymerase processivity factor
ORF116	RNA polymerase subunit RPO132
ORF127	hypothetical protein
ORF136	hypothetical protein
ORF141	putative EEV host range protein
ORFs containing four amino acid differences	
ORF011	CC chemokine receptor-like protein
ORF042	hypothetical protein
ORF064	putative membrane protein
ORF071	RNA polymerase subunit RPO147
ORF130	hypothetical protein
ORF132	hypothetical protein

ORF137	hypothetical protein
ORF139	putative Ser/Thr protein kinase
ORF151	kelch-like protein
ORFs containing five amino acid differences	
ORF027	putative EEV maturation
ORF049	putative RNA helicase NPH-II
ORF075	RNA polymerase-associated protein RAP94
ORF129	hypothetical protein
ORF143	tyrosine protein kinase-like protein
ORFs containing six amino acid differences	
ORF010	LAP/PHD-finger protein
ORF013	interleukin-1 receptor-like protein
ORF098	early transcription factor $VETF_L$
ORF138	OX-2-like protein
ORFs containing seven amino acid differences	
ORF035	hypothetical protein
ORF083	putative NTPase
ORF110	putative DNA helicase transcriptional elongation factor
ORF135	putative IFN-alpha/beta binding protein
ORFs containing eight amino acid differences	
ORF017	putative integral membrane protein, apoptosis regulator
ORF039	DNA polymerase
ORF133	DNA ligase-like protein
ORFs containing nine amino acid differences	
ORF128	CD47-like protein
ORF146	phospholipase D-like protein
ORFs containing ten amino acid differences	
ORF140	putative RING finger host range protein, N1R
ORF152	ankyrin repeat protein
ORFs containing twelve amino acid differences	
ORF033	hypothetical protein
ORF148	ankyrin repeat protein
ORFs containing thirteen amino acid differences	
ORF009	alpha amanitin-sensitive protein
ORFs containing nineteen amino acid differences	
ORF008	putative soluble Interferon gamma receptor
ORF131	superoxide dismutase-like protein
ORFs containing forty two amino acid differences	
ORF145	ankyrin repeat protein

(a) Open reading frames

CHAPTER IV

Discussion

4.1 DISCUSSION

The genome of the sequenced virulent field isolate (LD) was compared to the previously sequenced genome of the virulent LSDV Kenyan Neethling strain 2490 (described here as LK), and was found to have minimal differences. Only 10 of the 156 putative genes, containing functional domains were found to have amino acid differences between them. The genomes of a vaccine strain (LW) and a virulent field isolate (LD) of LSDV was sequenced and compared to each other. Of the 156 putative genes, only 51 containing functional domains were found to have amino acid differences occurring between them. Only these will be discussed further.

4.1.1 Transcription and mRNA biogenesis.

The genomes of both LD and LW were found to contain at least 26 putative genes encoding proteins involved in poxviral transcriptional processes (not shown). These include RNA polymerase subunits, mRNA transcription initiation, elongation, and termination factors, and the enzymes that direct post-transcriptional processing of viral mRNA (Moss 1996). RNA polymerase subunits include homologues of the VV RPO147 (ORF071), RPO132 (ORF116), RAP94 (ORF075), RPO35 (ORF119), RPO30 (ORF036), RPO22 (ORF069), RPO19 (ORF096), RPO18 (ORF085), and RPO7 (ORF055) (Tulman *et al.* 2001). Orthologues of all the above RNA polymerase subunits are present in the genomes of both sheeppox and goatpox virus sharing a 96% to 100% amino acid (aa) identity (Tulman *et al.* 2002). Three RNA polymerase subunits namely RPO30 (ORF036), RAP94 (ORF075) and RPO35 (ORF119), have amino acid differences occurring in the two wild types LD and LK, although none of these involve functional regions (Simple Modular Architecture Research Tool [SMART] analysis).

Table 9. SMART analysis of ORFs involved in transcription and mRNA biogenesis.

ORF (a)	Functional Domain	LK (b)		LD (c)		LW (d)		no. of aa Changes	Type of aa changes in functional domains between LK and LD	no. of aa Changes	Type of aa changes in functional domains between LD and LW
		Begin	End	Begin	End	Begin	End				
ORF049	DEAD-like helicases superfamily domain (DEXDc3)	0		159	362	159	362			0	
	Helicase superfamily c-terminal domain (HELICc3)			410	503	410	503			0	
ORF071	RNA polymerase alpha subunit domain			186	787	186	787			1	M->T*
	RNA polymerase A / beta' / A" subunit domain			891	1256	891	1256			1	Y->H*
ORF079	mRNA capping enzyme, large subunit			4	842	4	842			4	I->T*, D->N, T->P*, K->R
ORF084	DEAD-like helicases superfamily domain (DEXDc3)			15	200	15	200			0	
	Transmembrane segment			41	63	41	63			0	
	Helicase conserved C-terminal domain			366	458	366	458			0	
ORF086	mutT domain			26	212	26	207			2	E->D, L->F
ORF087	mutT-like domain	47	231	47	231	47	200	1	E->K*	1	K->E*
ORF088	DEAD-like helicases superfamily domain (DEXDc3)			28	222	28	222			0	
	Helicase superfamily c-terminal domain (HELICc3)			396	482	396	482			0	
ORF089	Poxvirus mRNA capping enzyme, small subunit	1	287	1	287	1	287	1	A->V*	2	V->I, V->A*
ORF110	DEAD-like helicases superfamily domain (DEXDc3)	77	264	77	264	77	264	1	P>S	3	S->P, R->L*, K->R
	Helicase superfamily c-terminal domain (HELICc3)	338	416	338	416	334	416	0		1	L->F
ORF116	RNA polymerase beta subunit domain			70	1075	70	1075			2	A->V*, C->S

(a) ORF, Open reading frame.

(b) LK, Kenyan LSDV Neethling Strain 2490.

(c) LD, South African LSDV Neethling Warmbaths isolate.

(d) LW, South African LSDV Neethling vaccine strain.

* amino acid differences that are non-conservative using the PAM250 matrix (Gribskov and Burgess. 1986) where 0.4 is the cut-off value.

Between LD and LW however, seven of the nine RNA polymerase subunits have amino acid (aa) differences, with only ORF's 071 (RPO147) and 116 (RPO132) having non-conservative amino acid differences (Table 9). Non-conservative amino acid (aa) differences occurred in both the functional domains of ORF071 (RPO147) while ORF116 (RPO132) with a single functional domain had a single non-conservative difference therein. RPO147 and RPO132 are homologous to the corresponding subunits of cellular RNA polymerases and more closely resemble those of eukaryotes and archaebacteria than those of eubacteria (Moss 1996, Patel and Pickup 1989).

Differences have been noted in several of the transcription factors (TF) [early (E), intermediate (I) and late (L)] between LD and LW. Since these viruses are fully functional in their host, these changes are considered conservative. They may however play a role in non transcription activities. Orthologues are also present in the genomes of both sheeppox and goatpox virus sharing an 86% to 100% amino acid identity (Tulman *et al.* 2002). Functional domains only occurred in ORF084 (VETF-1) with no amino acid differences present therein (Table 9).

ORF 051 (VV G2R) and ORF110 (VV A18R) present in both LD and LW encodes an elongation factor for late transcription in which amino acid differences occurred only in ORF110 between both the virulent LK and LD as well LD and LW. A single non-conservative aa difference between LD and LW occurred in one of the functional domains of ORF110, namely the DEAD-like helicases superfamily (DEXDc) domain (Table 9). DexH/D proteins are essential to all aspects of cellular RNA metabolism and the replication of many viruses. Their functions include the hydrolyses of nucleoside triphosphates (NTPs) and the unwinding of RNA (Jankowsky *et al.* 2001). Orthologue of ORF110 is present in the genomes of both sheeppox and goatpox virus sharing a 97% amino acid identity (Tulman *et al.* 2002). ORF110 is a homologue of VV A18R protein. ORF110 like VV A18R contains conserved motifs belonging to the DEXH family of DNA and RNA helicases (Gorbalenya *et al.* 1989; Koonin and Senkevich. 1992; Simpson and Condit. 1995). ORF110 may therefore function during the early and late phases of viral transcription (Simpson and Condit. 1995).

Vaccinia virus (VV) nucleoside triphosphate phosphohydrolase I (NPH-I) and NPH-II were the first DexH box NTPases to be purified and characterised (Martins *et al.* 1999; Paoletti and Moss, 1974; Paoletti *et al.* 1974). The genomes of LD and LW were both found to contain the transcriptional terminator NPH-I (ORF088) and the RNA helicase NPH-II (ORF049). Orthologues of NPH-I and NPH-II were present in both sheeppox and goatpox virus sharing a 97% to 99% amino acid identity (Tulman *et al.* 2002). VV NPH-I catalyses the hydrolysis of ATP and dATP and serves as a transcription termination factor during the synthesis of viral early mRNAs (Martins *et al.* 1999; Deng and Shuman, 1996; Deng and Shuman 1998). VV NPH-II is an NTP-dependant helicase that catalyses the unidirectional unwinding of 3'-tailed duplex RNAs, a process that is critical for vaccinia virus replication (Gross and Shuman, 1996; Gross and Shuman, 1998). ORF049 and ORF088 may well play a role in virus replication and the synthesis of early viral mRNAs respectively. Functional domains occurred in both ORF049 and ORF088 with no aa differences present therein, while no aa differences were present between the virulent LK and LD (Table 9).

Both mRNA capping enzyme subunits encoded by ORFs 079 and 089 are present in the genomes of LD and LW. Orthologues of both these capping enzyme subunits are present in the genomes of both sheeppox and goatpox virus sharing a 97% to 99% amino acid identity (Tulman *et al.* 2002). Non-conservative aa differences have occurred in both ORF079 and ORF089 between LD and LW (Table 9). ORF089 contains a non-conservative aa difference between the virulent LK and LD strains. mRNA capping occurs by a series of three sequential enzymatic reactions in which the 5' triphosphate-terminated primary transcript is converted to a diphosphate-terminated RNA by RNA triphosphate, capped with GMP by RNA guanylyltransferase and than methylated by RNA (guanine-7-) methyltransferase (Cong and Shuman, 1995; Higman *et al.* 1992; Myette and Niles, 1996; Wang *et al.* 1997). In VV, the three steps in cap formation are catalysed by a heterodimeric protein encoded by VV D1 and D12 genes (Cong and Shuman, 1995). ORF079 and ORF089 are homologues of VV D1R and D12L respectively. Five sequence motifs within the VV D1 protein are arranged in the same order and with similar spacing in LSD (ORF079) as well as in the capping enzymes of

other DNA viruses (Cong and Shuman, 1995; Yu and Shuman, 1996). The motifs are motif I: KxTG at aa position 260 to 263, motif II: xYx at aa position 288, motif III: GexV at aa position 302 to305, motif IV: EGVIL at aa position 375 to 376 and motif V: KxxxxxTxD at aa position 392 to 400 [Underlined amino acids are important residues (Cong and Shuman, 1995; Yu and Shuman, 1996)]. The capping enzyme of ORF079 and ORF089 may therefore play a pivotal role in regulating viral gene expression at both the mRNA transcription and processing level (Higman *et al.* 1992; Myette and Niles, 1996). The non-conservative amino acid differences that do occur in both ORF079 and ORF089 are outside motifs I to V and may therefore not have an effect on the protein.

LD and LW encoded the proteins ORF086 and ORF087 containing *mut*T-like motifs similar to VV D9R and D10R, which is a negative regulator of viral transcription (Koonin 1993; Shors *et al.* 1999). Schors *et al.* (1999) indicates that the D10R protein might bind to or hydrolyse cap structures thereby affecting the stability and translatability of mRNAs. Orthologues of the above ORFs are present in both the genomes of sheeppox and goatpox virus sharing a 96% to 98% amino acid identity (Tulman *et al.* 2002). Functional domains occurred in both ORF086 and ORF087 with non-conservative amino acid differences present only in ORF087 (Table 9). A C-terminal frameshift occurs in both ORF086 and ORF087 leading to the absence of the last five and last 29 respective encoded amino acids of the mutT-like domain. The MutT proteins or Nudix hydrolases are a family of versatile, and widely distributed "house-cleaning" genes with the function of cleansing the cell of potentially deleterious metabolites and to modulate the accumulation of intermediates in biochemical pathways (Bessman *et al.* 1996; Koonin. 1993; Shors *et al.* 1999).

4.1.2 Nucleotide metabolism.

The genomes of LD and LW contain several homologues of poxviral genes involved in nucleotide metabolism. These include a dUTP pyrophosphatase (ORF018), the small subunit of ribonucleotide reductase (RR) (ORF020) and thymidine kinase (ORF066) (Moss. 1996; Roseman *et al.* 1996). Orthologues of the above genes are found in both the

Table 10. SMART analysis of ORFs involved in nucleotide metabolism.

ORF (a)	Functional Domain	LK (b)		LD (c)		LW (d)		no. of aa Changes	Type of aa changes in functional domains between LK and LD	no. of aa Changes	Type of aa changes in functional domains between LD and LW
		Begin	End	Begin	End	Begin	End				
ORF018	dUTPase domain			15	144	15	144			1	G->C*
ORF020	ribonucleotide reductase domain			34	301	34	301			3	S->N*, E->D, D->V*
ORF039	DNA polymerase type-B family domain			249	766	249	766			4	T->I*, E->D, N->D, V->I
ORF133	DNA ligase domain	208	405	208	405	208	405	0		4	S->N*, L->S*, I->T*, D->N

(a) ORF, Open reading frame.

(b) LK, Kenyan LSDV Neethling Strain 2490.

(c) LD, South African LSDV Neethling Warmbaths isolate.

(d) LW, South African LSDV Neethling vaccine strain.

* amino acid differences that are non-conservative using the PAM250 matrix (Gribskov and Burgess. 1986) where 0.4 is the cut-off value.

genomes of sheeppox and goatpox virus sharing a 97% to 98% amino acid identity (Tulman *et al.* 2002). Non conservative amino acid differences only occurred in ORF018 and ORF020 (Table 10).

High levels of dUTP have been shown to be lethal to cells. dUTPase removes dUTP from the dNTP pool thereby generating dUMP, and is involved in maintaining fidelity of DNA replication (Roseman *et al.* 1996). It is advantageous that LSDV encodes both a dUTPase (ORF018) to reduce the dUTP pool and a uracil DNA glycosylase (ORF082) that acts by excision repair to remove dUTP that has been misincorporated into the replicating DNA (Aravind and Koonin, 2000; Ellison *et al.* 1996; Krokan *et al.* 1997; Stuart *et al.* 1993; Tomilin and Aprelikova, 1989). The same complement of nucleotide metabolism genes found in leporipoxviruses are present in LSDV, with the exception of the large subunit of ribonucleotide reductase. Ribonucleotide reductase (RNR) provides the precursors for DNA synthesis by the reduction of ribonucleotides to their respective deoxyribonucleotides by presumably acting in conjunction with a hydrogen donor. Ribonucleotide reductase (RNR) accepts UDP as a precursor and ultimately contributes to the intracellular dUTP pool (Hendricks and Mathews, 1998; Rajagopal *et al.* 1995; Roseman *et al.* 1996).

LD and LW contain seven homologues of ChPV genes necessary for, or potentially involved in, DNA replication. The genes involved include those encoding DNA polymerase (ORF039), DNA processivity factor (ORF112), DNA topoisomerase (ORF077), uracil DNA glycosylase (ORF082), ATP-GTP binding protein (ORF083), DNA ligase (ORF133), and the replication essential protein kinase (ORF139).

ORF039 is a homologue of VV E9L, a DNA polymerase with an orthologue present in both sheeppox and goatpox virus. Poxviral polymerases can promote strand annealing and strand-transfer reactions that are an integral step in most recombination schemes (Willer *et al.* 1999a). A single non-conservative difference between LD and LW occur within the functional domain (Table 10).

ORF082 (D4R) of LD and LW encodes a uracil- DNA glycosylase protein (UNG). A single conservative amino acid difference occurred between LD and LW. Uracil DNA glycosylases (UDGs) are major repair enzymes protecting DNA from mutational damage caused by uracil incorporation (Aravind and Koonin, 2000; Ellison *et al.* 1996; Krokan *et al.* 1997). The uracil DNA glycosylase is essential for virus viability and functions in the initial phase of the base excision repair pathway of uracil in DNA by cleaving the glycosidic bond, resulting in an apyrimidinic (AP) site (Aravind and Koonin, 2000; Ellison *et al.* 1996; Krokan *et al.* 1997, Stuart *et al.* 1993). The LSDV uracil DNA glycosylase may therefore function in a similar manner. Ellison *et al.* (1996) showed that mutations at three active-site amino acids of vaccinia virus resulted in proteins being defective in uracil excision but still retaining their ability to bind DNA. The three active-site amino acids namely Asp_{68}, Asn_{120} and His_{181} are conserved between LD and LW. It is therefore unclear what effect the single conservative amino acid difference may have.

ORF133 of LD and LW encodes a homologue of VV A50R, a DNA ligase protein. An orthologue is also present in sheeppox and goatpox virus sharing a 96% to 97% amino acid identity (Tulman *et al.* 2002). ATP-dependant DNA ligases are encoded by yeasts, mammalian cells, prokaryotic DNA viruses and two classes of eukaryotic DNA viruses namely poxviruses and African swine fever virus (Barker *et al.* 1985; Barker *et al.* 1987; Barnes *et al.* 1990; Colinas *et al.* 1990; Hammond *et al.* 1992; Kerr and Smith, 1989; Parks *et al.* 1994; Parks *et al.* 1998; Sekiguchi and Shuman, 1997; Shuman and Ru, 1995, Skinner *et al.* 1994; Tomkinson *et al.* 1991; Wang *et al.* 1994). Poxviral ligases share a high degree of homology with DNA ligase III, an enzyme involved in meiosis and DNA repair (Parks *et al.* 1998). The ATP-dependant DNA ligases are strand-joining enzymes that catalyse the joining of the 5' phosphate terminated donor strands to the 3' hydroxyl-terminated acceptor strands via three sequential nucleotidyl transfer reactions (Lehman. 1974; Lindahl and Barnes, 1992; Shuman and Ru, 1995; Sekiguchi and Shuman, 1997). A functional domain occurring in ORF133 has three non-conservative aa differences between LD and LW (Table 10). Mutant ligase poxviruses show a replicative deficiency seen when the viruses are cultured on particular cell lines. The deficiency results in a generalised reduction in the rate of DNA synthesis, although ligase-deficient poxviruses

do eventually reach titres comparable to wild-type virus, suggesting that ligase deficiencies do not create an absolute impediment to viral replication (Colinas *et al.* 1990; Kerr and Smith, 1991; Park *et al.* 1998). ORF133 may therefore exhibit a reduction in viral synthesis.

4.1.3 Protein modification.

The genomes of LD and LW contain at least five genes encoding putative protein modification functions: two serine/threonine protein kinases (PK), ORF025 and ORF139; a metalloprotease (ORF050); a tyrosine/serine protein phosphatase (ORF072) and one tyrosine PK-like protein (ORF143). Orthologues of the above five genes are present in both the sheeppox and goatpox virus sharing a 97% to 99% amino acid identity (Tulman *et al.* 2002). ORF050 is a homologue of VV G1L. No functional domain is observed in ORF050. ORF050 may function in viral protein processing and virion morphogenesis (Banham *et al.* 1992; Whitehead and Hruby, 1994). Both ORF139 and ORF143 encode a protein with a single functional region (Table 11).

A single non-conservative difference in the functional domain occurred between both LD and LW in ORF143. ORF139 encodes a homologue of VV B1R, which is expressed early during infection. It localises in cytoplasmic factories and is packaged into virions. The protein functions directly or indirectly in the replication of VV DNA, and appears to be essential for the transcription of intermediate but not late viral genes (Banham *et al.* 1992; Chen and Broyles, 1992; Kovacs *et al.* 2001; Rempel and Traktman, 1992). ORF139 may therefore function in a similar manner. The B1R gene is conserved among many but not all poxviruses (Kovacs *et al.* 2001).

Table 11. SMART analysis of ORFs involved in protein modification.

ORF (a)	Functional Domain	LK (b)		LD (c)		LW (d)		no. of aa Changes	Type of aa changes in functional domains between LK and LD	no. of aa Changes	Type of aa changes in functional domains between LD and LW
		Begin	End	Begin	End	Begin	End				
ORF139	Protein kinase domain	0		23	303	23	303			3	C->Y, G->V*, S->P
ORF143	Protein kinase domain	56	295	56	295	56	295	2	R>K, N>K	3	K->R, K->N, T->S*

(a) ORF, Open reading frame.

(b) LK, Kenyan LSDV Neethling Strain 2490.

(c) LD, South African LSDV Neethling Warmbaths isolate.

(d) LW, South African LSDV Neethling vaccine strain.

* amino acid differences that are non-conservative using the PAM250 matrix (Gribskov and Burgess. 1986) where 0.4 is the cut-off value.

4.1.4 Virion structure and assembly.

At least 26 known VV structural proteins for LSDV that have been identified, and the majority of these are associated with the intracellular mature virus particle (IMV) (Tulman *et al.* 2000). Homologues of VV core proteins include F17R (ORF031), E11L (ORF041), I1L (ORF043), G7L (ORF057), L4R (ORF063), I7L (ORF066), D2L (ORF080), D3R (ORF081), A3L (ORF094), A10L (ORF101), and A12L (ORF103). Orthologues of the core proteins are present in the genomes of both sheeppox and goatpox virus sharing a 93% to 99% amino acid (aa) identity (Tulman *et al.* 2002). Of the core proteins mentioned, the only proteins with functional domains (SMART) are those encoded by ORF057 and ORF094 with non-conservative aa differences not present therein (Table 12).

ORF046 (I5L), ORF060 (L1R), ORF074 (H3L), ORF090 (D13L), ORF100 (A9L), ORF104 (A13L), ORF105 (A14L), ORF109 (A17L), and ORF117 (A27L) are genes of LD and LW homologues of VV IMV membrane associated proteins. Orthologues of the above proteins are present in the genomes of both sheeppox and goatpox virus sharing a 93% to 100% amino acid (aa) identity (Tulman *et al.* 2002). Only ORFs 046, 074 and 109 contain functional domains. However, no non-conservative aa differences occurred therein. The C-terminal membrane anchor protein encoded by VV H3L (ORF074) is located on the surfaces of intracellular mature virions with H3L deletion and repression mutants resulting in decreased virus particle production ($_{Da}$ Fonseca *et al.* 2000). ORF109 may function similarly to VV A17L in being essential for the formation of viral progeny, and acting at an early stage in the virion assembly process (Betakova and Moss, 2000; Rodríguez *et al.* 1995). ORF117 (VV A27L) may play a role in virus-cell attachment, virus-cell fusion, and virus release from cells (Chung *et al.* 1998; Sanderson *et al.* 2000; Vázquez and Esteban, 1999). Vázquez and Esteban (1999) showed that the amino-terminal of VV A27L contains a heparin binding domain, a fusion domain and a domain responsible for interaction with either proteins or lipids in the Golgi stacks for EEV formation and virus spread.

Table 12. SMART analysis of ORFs involved in virion structure and assembly.

ORF (a)	Functional Domain	LK (b)		LD (c)		LW (d)		no. of aa Changes	Type of aa changes in functional domains between LK and LD	no. of aa Changes	Type of aa changes in functional domains between LD and LW
		Begin	End	Begin	End	Begin	End				
ORF057	Transmembrane segment			106	128	106	128			0	
ORF094	Signal peptide			1	48	1	48			0	
	Poxvirus P4b major core protein			1	661	1	661			2	D->N, F->L
ORF141	Signal peptide	1	18	1	18	1	18	0		0	
	(CCP) complement control protein domain	25	84	25	84	25	84	0		1	E->K*
	(CCP) complement control protein domain	89	145	89	145	89	145	0		0	
	Transmembrane segment	189	211	189	211	189	211	0		0	

(a) ORF, Open reading frame.

(b) LK, Kenyan LSDV Neethling Strain 2490.

(c) LD, South African LSDV Neethling Warmbaths isolate.

(d) LW, South African LSDV Neethling vaccine strain.

* amino acid differences that are non-conservative using the PAM250 matrix (Gribskov and Burgess. 1986) where 0.4 is the cut-off value.

ORF040 present in LD and LW encodes a homologue of VV E10R. No functional domains are present in ORF040 (SMART). The E10R protein is conserved in all poxviruses, and is a member of the ERV1/ALR (Essential for Respiration and Vegetative growth protein / Augmenter of Liver Regeneration protein) family which appears to be represented in all eukaryotes (Senkevich *et al.* 2000b) where these proteins are involved in growth and metabolism. ORF040 encodes a protein involved in virion morphogenesis and disulphide bond formation (Senkevich *et al.* 2000a, Senkevich *et al.* 2000b). ORF040 also encodes a protein similar to the African swine fever virus 9gI protein which affects virion morphogenesis, replication in vitro, and virulence in vivo (Lewis *et al.* 2000).

A vaccinia virus protein designated p39, which was shown to be expressed late in infection mapped to VV WR A5L (Western Reserve) corresponding to VV A4L Copenhagen (Demkowicz *et al.* 1992; Maa and Esteban. 1987; Williams *et al.* 1999). ORF095 is similar to VV A4L (Copenhagen), VV A5L (WR), MCV MC107L, FPV 168 core protein genes as well as to the myxoma virus (MYX) M093L, rabbit fibroma virus (RFV) S093L, and the Yaba monkey tumor virus (MTV) B11L (Tulman *et al.* 2001, Williams *et al.* 1999) encoding genes. No functional domains are present in ORF095. ORF095 like VV A5L (WR) may be essential for virus growth, synthesised late in the viral life cycle and may be required for the progression of immature virion (IV) particles to infectious intracellular mature virion (IMV) particles (Williams *et al.* 1999).

Four genes of LSDV potentially encode proteins present in the extracellular enveloped virus (EEV) outer envelope which are involved in or associated with the release of EEV, and affecting virus infectivity, while 2 genes, ORF126 (VV A36R) and ORF141 (VV B5R), resembling VV EEV proteins are also present (Blasco *et al.* 1991; McIntosh, *et al.* 1996; Moss. 1996; Roper *et al* 1996). VV EEV is important for cell-to-cell and long-range virus spread (Lorenzo *et al.* 1998). The four genes are ORF027, ORF028, ORF122 and ORF123, which encode homologues of VV F12L, F13L, A33R and A34R respectively. Orthologues of the above proteins are present in both sheeppox and goatpox virus sharing a 91% to 99% amino acid (aa) identity (Tulman *et al.* 2002).

No functional domains were detected in ORF 027 but two phosholipase D (PLD) active site motifs at positions 114 to 141 and 307 to 334 were identified in ORF 028 (SMART). Non-conservative amino acid differences were not present within these domains. The phospholipase D (PLD) superfamily is a diverse group of proteins which includes enzymes involved in phospholipid metabolism, a bacterial toxin, poxvirus envelope proteins and bacterial nucleases (Bárcena *et al.* 2000; Borrego *et al.* 1999; Interthal *et al.* 2001; Koonin. 1996; Sung *et al.* 1997). A HxKxxxxD (HKD) motif conserved in all members of the PLD superfamily are critical for PLD biochemical activity (Sung *et al.* 1997). ORF028, like the VV P37 (F13L) gene contains only one HKD motif which is encoded as NKD [NxKxxxxD] (Koonin. 1996; Sung *et al.* 1997). Sung *et al.* has shown that VV P37 requires this (H)KD site in order to be functional. VV P37 is the major constituent of the envelope and is located in the inner surface of the extracellular enveloped virus (EEV) envelope, mediates the intracellular mature virus (IMV)-membrane interaction required for the wrapping process. ORF028 like VV P37 may have a central role in virus envelopment. (Bárcena *et al.* 2000; Borrego *et al.* 1999; Koonin. 1996; Sung *et al.* 1997).

ORF122 is similar to the VV A33R gene, which is highly conserved in all orthopoxviruses with a homologue also present in the distantly related *Molluscum contagiosum* virus, suggesting that it has an important role (Roper *et al.* 1996; Roper *et al.* 1998). An orthologue of ORF122 is present in both sheeppox and goatpox virus sharing a 95% amino acid identity (Tulman *et al.* 2002). ORF122 encodes a transmembrane segment at position 45 to 67 (SMART). A single conservative aa difference occurred therein. In the case of VV, a severe reduction in cell-to-cell spread was attributed to a defect in the formation of actin tails and specialised virus-tipped microvilli, for which this A33R protein is required (Roper *et al.* 1998). Wolffe *et al.* showed also that the A33R protein guides the A36R protein to the IEV membrane, where it becomes tyrosine phosphorylated as a signal for actin tail formation (Wolffe *et al.* 2001).

ORF123 encodes a protein with an amino-terminal signal peptide, an overlapping transmembrane segment and a C-type lectin (CTL) or carbohydrate-recognition domain (SMART). The differences that occurred in ORF123 between LD and LW occurred outside these regions. ORF123 is similar to the VV A34R gene product and may have multiple functions in the virus life cycle and may be essential for the normal infectivity of EEV: it has an important role in virus plaque formation, EEV release, EEV infectivity and virus virulence (McIntosh and Smith, 1996).

ORF126 is a homologue of VV A36R, an envelope glycoprotein found only on IEV and is required for actin-based motility and viral virulence (Parkinson and Smith, 1994; Sanderson *et al.* 1998; van Eijl *et al.* 2000; Wolffe *et al.* 1998). A transmembrane segment with no aa differences were present in ORF126. The F12L (ORF027) protein is required for actin tail formation which is essential to efficient cell-to-cell spread, normal plaque size and virulence (Zhang *et al.* 2000).

Four functional domains have been identified in ORF141 (Table 12). A single non-conservative aa difference was observed between LD and LW in the first complement control protein (CCP) domain. The CCP modules contain approximately 60 amino acid residues and have been identified in several proteins of the complement system. Given the similarity of the domain to complement control proteins, the ORF141 protein may be involved in viral evasion from host immune responses. The protein encoded by ORF141, may have a function similar, by amino acid similarity, to B5R which has developed as a multifunctional protein with a role in virus morphogenesis, production of EEV and, potentially, in regulation of host complement activation (Engelstad and Smith, 1993; Herrera *et al.* 1998; Hollinshead *et al.* 2001; Lorenzo *et al.* 1998; Mathew *et al.* 1998; Rodger and Smith, 2002; Wolffe *et al.* 1993).

ORF028 (F13L) together with ORF141 (B5R) and ORF123 (A34R) may function in a similar manner to their VV homologues and may be needed for the wrapping of IMV particles since the intracellular enveloped virus (IEV) is reduced or abolished in their absence (Zhang *et al.* 2000).

There are homologues of five genes present in both LD and LW, representing two conserved poxviral gene families with putative structural encoded functions: ORF024 and ORF060 are the homologues of VV F9L and L1R genes respectively, and comprise one gene family (Senkevich *et al.* 1997). ORF059, ORF070, and ORF108 are homologues of VV G9R, J5L and A16L genes respectively and comprise a second gene family. Only ORFs 024, 059 and 108 contain functional domains, with non-conservative aa differences not present. The functions of the A16L and G9R myristylated proteins have not yet been determined, but are possibly targeted to membranes (Grosenbach and Hruby, 1998). ORF024, a hypothetical protein of unknown function has been shown to encode a protein of the L1L / F9 / C19 poxvirus orf family (SMART). The function of the L1L / F9 / C19 poxvirus orf family is unknown. Both ORF059 and ORF108 encode a DUF230 domain as well as a transmembrane segment (SMART). The DUF230 domain is a poxvirus protein of unknown function.

4.1.5 Immune evasion functions.

Viral homologues of cellular interleukin-10 (IL-10) have been shown to possess immunosuppressive and immunostimulatory activities (Lockridge *et al.* 2000; Moore *et al.* 1990; Rode *et al.* 1993; de Waal Malefyt *et al.* 1991; Hsu *et al.* 1990; Muller *et al.* 1998; Vieira *et al.* 1991; Fleming *et al.* 1997; Gesser *et al.* 1997). Several herpesviruses including Epstein-Barr virus (EBV), equine herpesvirus 2, and several primate cytomegaloviruses encode IL-10 homologues (Lockridge *et al.* 2000; Moore *et al.* 1990; Rode *et al.* 1993). The genome of LD and LW (ORF005) was found to encodes a homologue of IL-10. Functional domains are present within ORF005 (Table 13). Non-conservative aa differences between LD and LW occurred in both the amino-terminal signal peptide domain as well as the overlapping transmembrane segment. Signal peptides target proteins for secretion in both prokaryotes and eukaryotes, taking part in an array of protein-protein and protein-lipid interactions resulting in initiation of protein translocation through a proteinaceous channel in the endoplasmic reticulum (ER) of eukaryotic cells (Jain *et al.* 1994; von Heijne, 1998). Changes within the signal peptide

Table 13. SMART **analysis** of ORFs involved in immune evasive functions.

ORF (a)	Functional Domain	LK (b)		LD (c)		LW (d)		no. of aa Changes	Type of aa changes in functional domains between LK and LD	no. of aa Changes	Type of aa changes in functional domains between LD and LW
		Begin	End	Begin	End	Begin	End				
ORF005	Signal peptide			1	26	1	26			2	A->V*, I->V,
	Transmembrane segment			7	27	7	27			2	A->V*, I->V,
	Interleukin-10 domain			35	168	35	168			0	
ORF006	Signal peptide			1	19	1	19			1	F->L
	Immunoglobulin domain			29	137	29	136			2	S->L*, I->S*
	Immunoglobulin domain			142	231	141	230			1	S->N*
ORF008	Signal peptide			1	18	1	18			1	S>Y*
	Interferon - gamma receptor domain			16	213	19	213			17	N->K, G->E, I->T*,
											D->V*, N->S*, S->D*,
											K->R, T->I*, Q->K,
											N->D, S->T*, I->V,
											K->T*, K->N, I->A*,
											V->I, E->A*
ORF011	7 Transmembrane receptor			104	351	104	351			3	M->I, T->I*, M->T*
ORF013	Transmembrane segment	7	29	7	29	7	29	0		1	C->Y
	Immunoglobulin domain	30	126	30	126	30	126	0		2	K->E*, V->I
	Immunoglobulin-like domain	103	198	103	198	103	198	0		1	V->I
	Immunoglobulin domain	128	233	128	233	128	233	0		1	D->A*
ORF128	Signal peptide	1	26	1	26	1	26	0		0	
	Transmembrane segment	122	144	123	145	122	144	0		3	T->A, M->L, I->L
	Transmembrane segment	157	179	160	182	159	181	0		0	
	Transmembrane segment	189	211	189	211	188	210	0		0	
	Transmembrane segment	218	240	226	248	225	247	0		0	
	Transmembrane segment	255	277	255	277	254	276	0		0	
	Immunoglobulin like domain	39	95	39	95	39	94	0		5	V->I, N->D, E->G,
											K->T*, N->D
ORF135	Signal peptide			1	15	1	15			0	
	Immunoglobulin domain			62	160	62	160			3	T->K*, A->T, R->Q
	Immunoglobulin C-2 type domain			171	233	171	233			1	I->L
	Immunoglobulin domain			261	359	261	359			1	R->Y*
ORF138	Immunoglobulin domain			27	126	27	126			3	S->N*, V->I, Q->K
	Transmembrane segment			10	32	10	32			2	G->S, S->T*
	Transmembrane segment			160	179	160	179			1	F->L

(a) ORF, Open reading frame.

(b) LK, Kenyan LSDV Neethling Strain 2490.

(c) LD, South African LSDV Neethling Warmbaths isolate.

(d) LW, South African LSDV Neethling vaccine strain.

* amino acid differences that are non-conservative using the PAM250 matrix (Gribskov and Burgess. 1986) where 0.4 is the cut-off value.

domain can affect protein secretion, while mutations in transmembrane domains can partially or completely disrupt the receptor surface expression and function.

Viral interleukin-1 binding proteins bind and inactivate host IL-1, preferentially IL-1β, which affects viral virulence (Spriggs *et al.* 1992; Alcami *et al.* 1992). ORF135, ORF006 and ORF013 are similar to cellular and viral IL-1R orthologues (IL-1R) but differ from each other other (Tulman *et al.* 2001). Functional domains are present in all three ORFs (Table 13). ORF135 encodes a protein with a signal peptide domain as well as two immunoglobulin (Ig) domains and an Ig C-2 type domain. Non-conservative differences occur in both the Ig domains. The protein encoded by ORF135 is most similar to VV B18R (strain Western reserve [WR]). It is an interferon (IFN) -α/β binding protein which is both soluble and present on the cell surface where it binds to and inhibits the cellular binding and antiviral activity of mammalian α, β, and ω type I IFN (Alcami *et al.* 2000; Colamonici *et al.* 1995; Symons *et al.* 1995). ORF135 likely serves a similar function. ORF006 and ORF013, like other poxviral orthologues of VV IL-1 binding protein are most similar to mammalian IL-1R, particularly to type II IL-1R (IL-1R II) (Smith and Chan, 1991). ORF006 encodes a protein with a signal peptide domain as well as two Ig domains. Non-conservative differences occur within both the Ig domains. ORF013 encodes a protein with a transmembrane segment, two Ig domains and an Ig-like domain. Non-conservative differences occur within both the Ig domains. The frameshift occurs outside the domains identified. ORF006 encodes a protein similar to the VV (strain Wyeth) B18R protein, (which binds interferon less efficiently than B18R from other VV strains) lacks a third Ig domain in the carboxyl-terminus and may perform a different immunomodulatory function (Alcami *et al.* 2000). ORF013 is disrupted in the genomes of both the sheeppox and goatpox viruses (Tulman *et al.* 2002).

The IFN-γ R occurring in myxoma virus was the first poxvirus IFN-γ R described (Upton *et al.* 1992). It has been shown that an IFN-γ R is highly conserved among members of the poxvirus family (Alcami and Smith, 1995; Blanchard *et al.* 1998; Mossman, *et al.* 1995). ORF008 encodes a homologue of the soluble VV B8R and myxoma virus (MYX) M007 IFN-γ receptors (IFN-γ R), that bind IFN-γ and influence viral virulence (Alcami *et*

al. 1995; Mossman *et al.* 1995; Tulman *et al.* 2001; Upton *et al.* 1992; Verardi *et al.* 2001). An orthologue of ORF008 is also present in the genomes of sheeppox and goatpox virus (Tulman *et al.* 2002). Functional domains are present in ORF008 (Table 13). A single as well as nine non-conservative aa differences occurred in the signal peptide as well as the complex of interferon-gamma receptor domain respectively. The VV B8R protein is secreted, binds soluble IFN-γ and prevents binding to cellular receptors (Alcami and Smith, 1995). Poxviruses are therefore able to inhibit both the antiviral and immune functions of IFN-γ (Alcami and Smith, 1996). ORF008 may similarly inhibit the antiviral and immune functions of IFN-γ. Šroller *et al.* (2001) showed that in experiments on rabbits, VV (western reserve) lacking the IFN-γ R was attenuated as skin lesions induced by the deletion mutants tended to disappear earlier than those caused by the wild-type virus. Verardi *et al.*, (2001) found that recombinant VV with a deleted B8R gene are attenuated for normal and nude mice without having a concomitant reduction in immunogenicity. Deletion of the VV B8R gene thus leads to attenuation of the virus in a mouse model as demonstrated above. The only vaccinia virus strain lacking IFN-γ R is the highly attenuated MVA (modified vaccinia virus Ankara) [Blanchard *et al.* 1998]. Thus far deletion mutants where a part or whole of the B8R protein was deleted has been studied. It is therefore unclear whether the non-conservative amino acid differences that have occurred have an effect on the attenuation of LSDV. The effects of these differences will have to be further studied. The amino acid differences observed in ORF008 between LD and LW, may influence viral virulence while changes within the signal peptide domain may affect protein secretion.

LD and LW (ORF011) encodes a homologue of a CC chemokine receptor. Chemokine receptors are integral membrane proteins that transduce extracellular signals to the intracellular environment through heterotrimeric guanine nucleotide-binding (G) proteins (Palczewski *et al.* 2000; Schoneberg *et al.* 1999). Heterotrimeric guanine nucleotide-binding protein (G-protein)-coupled receptors (GPCRs) share a conserved transmembrane structure comprising seven α helices Palczewski *et al.* 2000). Certain mutations in GPCR impair stability of the receptor on the membrane; therefore these mutant receptors are unable to translocate to cell surface (Ling *et al.* 1999). It has been

reported that chemokine receptor CCR5 containing certain point mutations express poorly on the cell surface (Farzan *et al.* 1998). ORF011 encodes a transmembrane-domain receptor belonging to the rhodopsin family (Table 13), with 3 amino acid differences occurring within this region between LD and LW with 2 of the 3 differences being non-conservative. The role of viral G-protein chemokine receptor homologues during poxviral infection possibly involves modification of the host anti-viral immune responses, and have been shown to affect host range for leporipoxviruses (Lalani *et al.* 1999).

ORF010 encodes a protein with two transmembrane segments, as well as a ring finger and an overlapping zinc finger domain. Conservative aa differences between LD and LW occur in all of these regions. An orthologue of ORF010 is present in the genomes of both the sheeppox and goatpox virus sharing a 94% to 95% amino acid (aa) identity (Tulman *et al.* 2002). ORF010 encodes a herpesvirus similar protein known to affect virus-mediated inhibition of class I major histocompatibility antigen (MHC-I) and NK-mediated cytotoxicity receptor expression (Ishido *et al.* 2000; Stevenson *et al.* 2000).

Cellular CD47, an integrin-associated protein, acts as a marker for immune cell recognition by possibly affecting adhesion-dependant cell functions through its association with the cytoskeleton (Oldenborg *et al.* 2000; Wu. *et al.* 1999), but also affects common intracellular signal transduction mechanisms (Drbal *et al.* 2000; Frazier *et al.* 1999; Shahan *et al.* 2000; Wang *et al.* 1999). Other viral integrin-associated proteins such as VV A38L to which LSDV ORF128 shows homology, affect granulocyte, B-cell, smooth muscle and tumour cell chemotaxis, antigen presentation and cell maturation. They also affect the production of pro-inflammatory cytokines such as IL-12, TNF-α, and INF-γ, T-cell development and activation as well as lymphocyte cell death (Armant *et al.* 1999; Avice. *et al.* 2000; Demeure *et al.* 2000; Hermann *et al.* 1999; Mateo *et al.* 1999; Pettersen *et al.* 1999; Vallejo *et al.* 2000; Yoshida *et al.* 2000; Cameron *et al.* 1999; Parkinson *et al.* 1995). Functional domains are present in ORF128 (Table 13). A single non-conservative aa difference is present in the immunoglobulin-like

domain between LD and LW. The encoded product of ORF128 is therefore likely to influence host immune responses and/or intracellular signalling mechanisms.

ORF138 encodes a homologue of MYX M141R protein, and is similar to other viral and cellular OX-2-like proteins which are dendritic cell surface antigens involved in co-regulation of T-cell stimulation and development of type 1/ type 2 cytokine (Tulman *et al.* 2000; Cameron *et al.* 1999; Gorczynski *et al.* 1999; Gorczynski *et al.* 2000; Russo *et al.* 1996). An orthologue is present in the genomes of both the sheeppox and goatpox virus sharing a 78% to 80% amino acid (aa) identity (Tulman *et al.* 2002). Viral OX-2 like proteins may interfere with development of effective antiviral cellular immune responses. Functional domains are present in ORF138 (Table 13).

4.1.6 Other virulence, host range, and cellular functions.

Poxviruses are unique among viruses in encoding members of the serine proteinase inhibitor (serpin) superfamily. Multiple serpin genes are present in orthopoxviruses, leporipoxviruses, and fowlpox virus (FPV), with ORF149 in LD and LW being similar to MYX M151R (Serp 2) and the single serpin in SPV (Massung *et al.* 1993). An orthologue is present in the genomes of both the sheeppox and goatpox virus sharing a 94% to 95% amino acid (aa) identity (Tulman *et al.* 2002). Poxviral serpins perform host-range functions involving efficient viral gene expression, membrane fusion, anti-inflammatory activity, and/or regulation of cellular apoptosis through inhibition of a number of caspases including IL-1 β-converting enzyme (ICE), cathepsin G, the CTL-derived protease granzyme B and others within the apoptosis regulatory cascade (Ali *et al.* 1994; Brooks *et al.* 1995; Kettle *et al.* 1997; Moon *et al.* 1999; Messud-Petit *et al.* 1998; Shisler. *et al.* 1999; Turner *et al.* 1998; Turner *et al.* 2000). Only conservative aa differences occurred in the serine proteinase inhibitor domain between both LK and LD and LD and LW.

Table 14. SMART analysis of ORFs involved in other virulence, host range and cellular functions.

ORF (a)	Functional Domain	LK (b)		LD (c)		LW (d)		no. of aa Changes	Type of aa changes in functional domains between LK and LD	no. of aa Changes	Type of aa changes in functional domains between LD and LW
		Begin	End	Begin	End	Begin	End				
ORF012	Ankyrin repeats domain	21	50	21	50	21	50	0		0	
	Ankyrin repeats domain	51	82	51	82	51	82	1	K>N	0	
	Ankyrin repeats domain	87	119	87	119	87	119	1	S>F*	2	F->S*, I->V
	Ankyrin repeats domain	123	154	123	154	123	154	0		0	
	Ankyrin repeats domain	158	188	158	188	158	188	0		1	M->I
ORF131	Copper / zinc superoxide			12	161	12	83			19	F->L, N->I*, E->M*, H->G*,
	dismutase domain (SODC)										G->L*, D->E, L->I,
											G->W*, N->E, Y->F, S->I*,
											N->Q*, K->T*, Y->N*, G->M*,
											I->E*, S->Y*, Y->L*, L->Y*
ORF145	Ankyrin repeats domain	111	143	111	143	111	143	0		2	S->G, I->V
	Ankyrin repeats domain	147	179	147	179	147	179	0		0	
	Ankyrin repeats domain	184	216	184	216	184	216	0		4	G->N, S->R*, N->D, T->S*
	Ankyrin repeats domain	255	287	255	287	255	287	0		4	M->V, V->I, L->I, N->S*
	Ankyrin repeats domain	292	323	292	323	292	323	0		1	S->P
	Ankyrin repeats domain	327	360	327	360	327	360	0		6	V->I, I->V, E->D,
											H->N, D->E, Y->H*
	Ankyrin repeats domain	405	434	405	434	405	434	0		1	V->A*
ORF147	Ankyrin repeats domain			1	31	1	31			0	
	Ankyrin repeats domain			35	66	35	66			0	
	Ankyrin repeats domain			69	104	69	104			0	
	Ankyrin repeats domain			146	177	146	177			0	
	Ankyrin repeats domain			181	214	181	214			0	
	Ankyrin repeats domain			256	285	256	285			0	
	Ankyrin repeats domain			289	318	289	318			0	
ORF148	Ankyrin repeats domain			50	78	41	78			0	
	Ankyrin repeats domain			82	114	82	114			1	I->L
	Ankyrin repeats domain			117	148	117	148			0	
	Ankyrin repeats domain			152	185	152	185			2	N->D, E->D
	Ankyrin repeats domain			227	256	227	256			0	
ORF152	Ankyrin repeats domain	31	62	31	62	31	62	1	N>I*	2	V->I, I->N*
	Ankyrin repeats domain	66	100	66	100	66	100	0		0	
	Ankyrin repeats domain	104	137	104	137	104	137	0		1	A->V*
	Ankyrin repeats domain	141	174	141	174	141	174	0		1	E->G
	Ankyrin repeats domain	179	212	179	212	179	212	0		0	
	Ankyrin repeats domain	255	284	255	284	255	284	0		0	
	Ankyrin repeats domain	288	317	288	317	288	317	0		0	

(a) ORF, Open reading frame.

(b) LK, Kenyan LSDV Neethling Strain 2490.

(c) LD, South African LSDV Neethling Warmbaths isolate.

(d) LW, South African LSDV Neethling vaccine strain.

* amino acid differences that are non-conservative using the PAM250 matrix (Gribskov and Burgess. 1986) where 0.4 is the cut-off value.

A homologue of VV C7L encoded by ORF067 present in both LD and LW, is involved in host range specificity. VV C7L is required for replication of VV in specific cell types, and it can functionally replace the VV K1L ankyrin repeat protein in conferring host range specificity (Oguiura *et al.* 1993; Perkus *et al.* 1990). The mechanism by which it does this is not known. VV C7L homologues are also present in leporipoxviruses (Cameron, C. *et al.* 1999). LSDV like the Yaba-like disease virus (YLDV) lacks a counterpart of K1L (Lee, H-J. *et al.* 2001). ORF067 has a Poxvirus / C7 / F8A domain and has a high degree of similarity to the CF8a protein of the sheep pox virus (isolate Kenya sheep-1, KS-1) (BLAST) (Gershon and Black. 1989).

Proteins encoded by ORF003, ORF154 and ORF017, are homologues of MYX which prevent apoptosis in infected cells (Tulman *et al.* 2001). Orthologues of the above genes are present in the genomes of both the sheeppox and goatpox virus sharing a 94% to 99% amino acid (aa) identity (Tulman *et al.* 2002). Proteins encoded by ORF003 and ORF154 are homologues of MYX M004, which is an endoplasmic reticulum-localized virulence factor required for productive infection of lymphocytes (Barry *et al.* 1997). No amino acid differences were found in the functional domains of ORFs 003, 017 and 154. ORF017 encodes is a homologue of MYX M011L which is a membrane-bound virulence factor that localises to the mitochondria by means of a 25 amino acid COOH-terminal targeting motif, where it inhibits induction of apoptosis by preventing transition of mitochondrial permeability (Everett *et al.* 2000; Macen. *et al.* 1996; Opgenorth *et al.* 1992). M011L is specifically required for inhibition of apoptosis in MYX-infected monocytes/macrophages and it functions in suppression of host inflammatory responses (Everett *et al.* 2000; Opgenorth *et al.* 1992).

ORF140 encodes a homologue to the shope fibroma virus (SFV) N1R, rabbit fibroma virus (RFV) N1R, ectromelia virus (EV) p28, and is similar to the the N1R/p28 gene family of FPV, and the *Heliothis amerigera* entomopoxvirus 17K ORF (Tulman, E.R. *et al.* 2001; Afonso. *et al.* 2000; Senkevich. *et al.* 1994; Senkevich. *et al.* 1995; Brick *et al.* 1998; Upton *et al.* 1994). An orthologue is present in the genomes of both the sheeppox and goatpox virus sharing a 94% to 95% amino acid (aa) identity (Tulman *et al.* 2002).

RING fingers are known to mediate protein-protein interactions and help direct protein ubiquitination, tagging proteins for destruction (Borden 2000; Joazeiro *et al.* 2000). Ectromelia p28 is a host range factor required for viral replication in mouse macrophages and for viral virulence in mice. In addition, p28 and N1R are able to inhibit UV light-induced apoptosis (Brick. *et al.* 2000) SFV N1R protein binds double- and single-stranded DNA, reduces apoptosis at late times and thus it may serve to increase the spread of the virus infection in an infected animal host (Brick *et al.* 2000). A number of other proteins possessing RING motifs have been shown to be involved in the regulation of apoptosis. (Brick *et al.* 2000; Crook *et al.* 1993). ORF140 is therefore likely to encode proteins with virulence and host range functions. Conservative aa differences occurred in the functional domain.

ORF034, a putative PKR inhibitor encodes an amino-terminal Z-DNA binding domain and a carboxy-terminal double-stranded RNA binding motif (SMART). An orthologue is present in the genomes of both the sheeppox and goatpox virus sharing a 97% amino acid (aa) identity. A single conservative amino acid difference occurred in the Z-DNA binding domain. ORF034 is the homologue of VV E3L protein, a protein which confers resistance to the antiviral effects of interferon (IFN) (Brandt and Jacobs, 2001; Chang *et al.* 1992). E3L is a member of a family of double-stranded RNA (dsRNA)-binding proteins which function to specifically bind dsRNA is a sequence –independent manner (Bass *et al.* 1994; Brandt and Jacobs, 2001; Chang and Jacobs, 1993). Brandt and Jacobs showed that while the amino terminus is dispensable for supporting replication in cells in culture, both the carboxy-terminal double-stranded RNA binding protein and the amino-terminal of E3L are required for full viral pathogenesis (Brandt and Jacobs, 2001). The presence of ORF034 in LSDV indicates the presence of IFN-resistance mechanisms similar to those found in orthopoxviruses and leporipoxviruses.

Ankyrin (ANK) repeat proteins have been found in a wide variety of organisms ranging from humans to viruses and are present in the nucleus, cytoplasm and extracellular milieu (Michealy and Bennett. 1995; Sedgwick and Smerdon. 1999; Shchelkunov *et al.* 1993). ANK-repeat proteins carry out a wide range of biological functions with the number of

repeats in any one protein being variable (Sedgwick and Smerdon. 1999). Poxviral ANK-repeat genes encode proteins that have been associated with host range functions in MYX, cowpox virus (CPV), and VV, and proteins which possibly inhibit virally induced apoptosis (Gillard. *et al.* 1986; Ink *et al.* 1995; Mossman *et al.* 1996; Perkus *et al.* 1990; Spehner *et al.* 1988; Sutter *et al.* 1994). In CPV, which has a relatively broad host range, at least 16 ANK-repeat genes have been identified (Shchelkunov *et al.* 1998). Loss or disruption of many of these genes in other orthopoxviruses with a more restricted host range, has suggested that loss of ANK genes may be associated with a narrowing of host range (Antoine *et al.* 1998; Shchelkunov. *et al.* 1998). While the mode of action of these proteins is unknown, ANK-repeat motifs of other proteins are clearly involved in mediating protein-protein interactions (Lin *et al.* 1999; Sedgwick and Smerdon. 1999). Orthologues of the ankyrin repeat proteins are present in the genomes of both the sheeppox and goatpox virus sharing a 94% to 97% amino acid (aa) identity (Tulman *et al.* 2002). ORF012, ORF145, ORF147, ORF148, and ORF152 encode proteins in LD and LW containing ANK-repeat motifs (Table 14). Non-conservative aa differences were found in one of the five ANK-repeat domains between LK and LD and LD and LW in ORF012; in four of the seven ANK-repeat domains between LD and LW in ORF145; in one of the seven ANK-repeat domains in ORF152 between LK and LD; and two of the seven ANK-repeat domains in ORF152 between LD and LW.

The ORF146 encoded protein is similar to the VV K4L protein and eukaryotic phospholipase D (PLD) (Tulman *et al.* 2001; Koonin 1996; Ponting and Kerr, 1996). ORF146 encodes a protein with two phospholipase D (PLD) domains at positions 115 to 142 and 322 to 348 (SMART), with an amino acid difference between LD and LW occurring within the first PLD domain. Furthermore, ORF146 is similar to ORF028, a homologue of the VV F13L IMV membrane protein that also contains a PLD superfamily motif with phospholipase activities (Baek *et al.* 1997). ORF146, like VV K4 contains two HKD motifs. VV P37 (F13L) appears to be more important than K4 as vaccinia virus mutants lacking K4, replicate and spread as efficiently as the wild-type virus (Blasco and Moss, 1991; Sung *et al.* 1997). An orthologues is present in the genomes of both the

sheeppox and goatpox virus sharing a 95% to 96% amino acid (aa) identity (Tulman *et al.* 2002).

Three main forms of superoxide dismutase (SOD) occur and contain either Mn, Fe, or both Cu and Zn. MnSODs and FeSODs are found in prokaryotes with the latter also occurring in plants, while Cu-Zn SODs are found in eukaryotes and few species of bacteria (Almazán *et al.* 2001). ORF131 encodes a protein with a copper / zinc superoxide dismutase (SODC) domain. ORF131 (VV A45R) is similar to cellular Cu-Zn superoxide dismutase (SODC) and the SOD-like genes found in leporipoxviruses and orthopoxviruses (Almazán *et al.* 2001; Bawden *et al.* 2000; Cameron *et al.* 1999; Senkevich *et al.* 1996; Tulman *et al.* 2001; Tomalski *et al.* 1991; Willer *et al.* 1999b). LSDV, like all other poxviruses except *Amsacta moorei* virus lacks residues in the SOD protein that would predict enzymatic activity (Almazán *et al.* 2001; Tulman *et al.* 2001). Poxvirus SODs associate with virion structural proteins (Almazán *et al.* 2001; McCraith *et al.* 2000). Almazán *et al.* (2001) showed that a VV A45R mutant lacking the majority of the A45R gene replicated normally in vitro and had unaltered virulence in mice and rabbits. However, in a murine intranasal model, infection with the deletion mutant of VV A45R resulted in the early onset of signs of illness, suggesting a possible function in the progression of the infection (Almazán *et al.* 2001). A functional domain present in ORF131 (Table 14). A total of fourteen non-conservative aa differences occurred within the SODC domain. The C-terminal frameshift in LW results in a truncated protein in which last 54 amino acids of the SODC domain is absent. ORF131 like the deletion mutant of VV A45R may play a role in the progression of infection.

LSDV encodes several homologues of proteins in the terminal genomic regions with unknown functions that are found in other poxvirus genera (Tulman *et al.* 2000). These are ORF002, ORF004, ORF007, ORF021, ORF129, ORF130, ORF134, ORF137, ORF153 and ORF155.

ORF007 encodes a homologue of the VV C4L/C10L family of genes present in orthopoxviruses and FPV, but absent in leporipoxviruses and MCV. The function of these

proteins are unknown but may involve host range given the terminal genomic location of the encoding genes, their dispensable nature for VV replication in cell culture, and the interaction of VV C10L with a known VV host range protein namely VV K1L (Kotwal *et al.* 1988; McCraith *al.* 2000; Perkus. *et al.* 1991). ORF134 encodes a product similar to the VAR B22R putative membrane protein and its homologues (Afonso *et al.* 2000; Massung *et al.* 1994; Tulman *et al.* 2000). Yaba MTV E11R is the only known gene product similar to that of ORF130.

LSDV encodes two putative proteins namely ORF022 and ORF132, which lack homology to any other known protein (Tulman *et al.* 2001, Table 6 and Table7). No functional domain is encoded by ORF022 (SMART). ORF132 encodes a protein with an amino-terminal signal peptide as well as an overlapping transmembrane segment at position 4 to 21 (SMART). An amino acid difference between LD and LW in ORF132 occurred within the transmembrane segment.

4.1.7 Gene families of unknown function.

Three proteins encoded by LD and LW are similar to the *Drosophila* kelch protein (Tulman *et al.* 2001; Bork *et al.* 1994): encoded by ORF019, ORF144 and ORF151. All contain a Broad-complex, Tramtrack and Bric-a-Brac (BTB) domain also known as POZ (poxvirus and zinc finger) domain as well as Kelch-like domains (SMART). Four kelch domains are present in both ORF019 and ORF144 encoded proteins while three kelch domains are found in that of ORF151. The BTB (for BR-C, ttk and bab) or POZ (for pox virus and zinc finger) domain is present near the N-terminus of a fraction of zinc finger (zf-C2H2) proteins and in proteins that contain the Kelch motif such as Kelch and a family of poxvirus proteins (Bardwell *et al.* 1994; Read *et al.* 2000; Zollman *et al.* 1994). The BTB/POZ domain mediates homomeric dimerisation and in some instances heteromeric dimerisation (Bardwell *et al.* 1994). ORF151 has a non-conservative aa difference in the encoded BTB domain as well as an amino acid difference in one of the kelch domains at position 400 to 445 between LD and LW. A non-conservative aa difference occurred only in the kelch domain at position 352 to 398 between LK and LD.

A frameshift occurred in ORF144 between LD and LW, resulting in a truncation of the protein. The encoded BTB region occurs in one half of the ORF namely LW144a, and the four kelch domains occurred in the other half namely LW144b. Furthermore, ORF019 encodes a protein that is truncated between both LK and LD and LD and LW. The BTB domain occurs in the one half with the four kelch domains occurring in the other half. Although the function of poxviral kelch-like proteins is unknown, they are non-essential for replication of VV in cell culture (Kotwal *et al.* 1988; Perkus *et al.* 1991). Kelch-repeat motifs are found in functionally diverse proteins and they are involved or associated with protein-protein interactions (Adams *et al.* 2000; Bork *et al.* 1994; Hughes. *et al.* 1999; Scarr *et al.* 2000; Schmid *et al.* 1994).

The lumpy skin disease virus genes, ORF001, ORF009, ORF136, ORF150 and ORF156 are similar to the Family 5 poxviral genes which includes VV A52R (Smith *et al.* 1991). Although the function of most of these genes are unknown, many are not required for growth in cell culture and VV N2L has been associated with resistance to α-amanitin (Kotwal *et al.* 1988; Perkus *et al.* 1991; Tamin *et al.* 1991). VV A52R functions as an antagonist for host cell IL-1 and Toll-like receptor-mediated intracellular signaling including IL-1R, Toll-like receptor 4 and IL-18R-mediated induction of NFκ-B activation (Bowie *et al.* 2000). The potential for IL-1/Toll-like receptor inhibition by a family of poxvirus proteins is significant, considering the role of IL-1/Toll-like receptor signalling in innate immune responses and inflammation (Aderem and Ulevitch, 2000; Fitzgerald and O'Neill, 2000). ORF001, ORF156 both encode an amino-terminal signal peptide and an overlapping poxvirus B15-like protein at position 3 to 144 (SMART). This is a family of poxvirus proteins including B15, C6, and T3A. Members of this family are approximately 150 residues long and have no known function. Substitutions occur within these domains of ORF001 and ORF156 between LD and LW.

CHAPTER V

Conclusion

The passage of a pathogenic virus (LSDV) through cell culture has reduced its virulence for cattle. This phenotypic attenuation resulted from small genetic changes in the genotype of the genome of the virus. By seeking out the molecular differences between a virulent field strain such as LK or LD, and an attenuated vaccine strain such as LW, an attempt was made to analyse the different isolate's genomes and to identify the genes responsible for virus virulence and host range specificity.

The LSDV Neethling Warmbaths isolate (LD) was obtained from a severely infected calf and was amplified in cell culture. DNA from both this isolate (LD) and the South African LSDV Neethling vaccine strain was purified. Both the genomes of LD and LW were cloned, sequenced and assembled into a contiguous sequence. The sequences were than analysed and finally compared to each other, with the LSDV Neethling Warmbaths isolate (LD) also being compared to the previously sequenced virulent LSDV Kenyan Neethling strain 2490 (LK).

In LSDV alone, 156 putative genes have been identified. Between the two virulent field isolates, namely LSDV Neethling strain 2490 (LK) and LSDV Warmbaths isolate (LD), 29 (18%) of the 156 genes common to both viruses were found to contain between one to three amino acid differences, affecting genes mostly but not exclusively of the variable terminal regions. The ORF with the greatest variation between LK and LD was found to be ORF110 encoding a putative DNA helicase transcriptional elongation factor (3 aa differences). In addition to these amino acid differences, six deletion and eight insertion sites occurred. Three frameshifts were also observed between LK and LD with two of the frameshifts resulting in truncations of the protein. The two ORFs are ORF019, a kelch-like protein and ORF026 a hypothetical protein with unknown nature. The remaining frameshift occurred in ORF013, an IL-1 receptor (IL-1R)-like protein. Despite the geographic distance and time separation between the origins of the isolates (South Africa

[LD] and Kenya [LK]), minimal genetic variation was observed thereby suggesting that lumpy skin disease is genetically stable.

In contrast to the comparison between the two virulent strains, major differences were observed in the comparison between the highly cell-attenuated South African vaccine strain (LW) and the virulent Warmbaths isolate (LD) where 121 genes (77%) were found to have amino acid differences.

Variations in amino acid differences vary from single residues up to 42. Those ORFs with the greatest variation between LD and LW were found to be ORF152, encoding an ANK repeat protein (10 aa differences), ORF009 encoding an α amanitin-sensitive protein (13 aa differences), ORF144, encoding a kelch-like protein (15 aa differences), ORF008, encoding a putative soluble IFN-γR (19 aa differences) and ORF145, an ANK-repeat protein having 42 amino acid differences. Further differences are due to 32 amino acid insertions and 22 amino acid deletions across the genome, where insertions of up to 11 amino acids and deletions of up to nine amino acids were observed. Nine frameshifts occurred between the LD Warmbaths isolate and the LW vaccine strain, with four of these frameshifts resulting in the truncation of these proteins. The remaining frameshifts occur in ORF013, an IL-1R-like protein, LW035 a protein of unknown function which has a N-terminal frameshift where the first 26 amino acids are out of frame, LW086 encoding a protein with a *mut*T, LW087 encoding a a *mut*T motif - putative gene expression regulator having two frameshifts at the C-terminal end resulting in the last 53 amino acids being out of frame and LW131 encoding a SOD-like protein with a frameshift resulting in the last 77 amino acids being out of frame.

Variations are not limited to the terminal regions of the LSDV genome, and many of the affected genes from the central region are involved in viral DNA replication. The impediment of viral DNA replication has been shown to play a key role in the attenuation of a pathogenic virus (Kiyotani *et al.* 2001). Several virulence factors occurring in both field and vaccine isolates have also undergone amino acid changes, as have several proteins, such as the interleukin-like proteins which are secreted and play an important

role in suppressing the host immune response as well as the IFN-γ R which bind IFN-γ and influence viral virulence. A target gene for further studies is the IFN-γ R which may be one of the key virulence factors since a VV mutant lacking the IFN-γ R was shown to induce skin lesions in rabbits that tended to disappear earlier than those of the wild-type, while in a mouse model, a VV recombinant with a deleted IFN-γ R was shown to be attenuated.

It is not known which particular amino acid difference or differences are responsible for significant alterations in viral virulence. Attenuation is therefore likely to be due to the sum of several altered protein phenotypes. It is however also likely that a few specific proteins have greater significance. Further studies are required to identify those amino acid residues, which specifically influence viral virulence and host specificity such as those that form parts of epitopes or enzyme active centres. We have, however, attempted to narrow the search by highlighting the amino acid differences occurring in recognised functional domains. Mutations in the transmembrane regions of proteins could alter the levels of secretion. This could play an important role in virus virulence if, for example, the protein was involved in host immune regulation like ORF005 (the IL-10 homologue), ORF138, (the OX-2 like protein) and most notably ORF017, a membrane-bound virulence factor. Virulence or more likely host specificity may be affected by functionally active domain copy numbers for example the differences observed in ANK, kelch and BTB/POZ domains between the attenuated vaccine and wild-type strain. Viral pathogenicity could also be potentiated by interference with apoptosis, with which RING finger proteins such as ORF140 are associated. The results of this comparison should at least narrow the search for the most important determinants of LSDV virulence to its bovine host, and consequently aid in the development of and safer vaccines.

CHAPTER VI

Appendices and References

6.1 Appendix I

Table 15. SMART analysis of the South African LSDV Neethling Warmbaths isolate (LD) and the South African LSDV Neethling vaccine strain (LW) open reading frames (ORF's).

LD (a)		**LW** (b)			
new	**Position**	**new**	**Position**		**SMART Analysis** (d)
orfs	**(length, codons)**	**orfs**	**(length , codons)**	**Predicted structure and/or function** (c)	**Functional Domain**
LD001 (e)	717-241(159)	LW001 (e)	592-116(159)	hypothetical protein	signal peptide
					B15-like protein
LD002 (e)	1183-791(131)	LW002 (e)	1058-666(131)	hypothetical protein	transmembrane segment
LD003 (e)	2155-1436(240)	LW003 (e)	2029-1310(240)	putative ER-localized apoptosis regulator	signal peptide
LD004 (e)	2398-2228(57)	LW004 (e)	2272-2102(57)	hypothetical protein	no domains
LD005	2450-2959(170)	LW005	2324-2836(171)	Interleukin-10-like protein	signal peptide (overlapping with transmembrane segment)
					transmembrane segment
					Interleukin-10 domain
LD006	3668-2976(231)	LW006	3544-2855(230)	Interleukin-1 receptor-like protein	signal peptide
					immunoglobulin domain
					immunoglobulin domain

LD007	4757-3693(355)	LW007	4633-3569(355)	hypothetical protein	no domains
LD008	5668-4844(275)	LW008	5544-4720(275)	putative soluble Interferon gamma receptor	signal peptide
					complex of interferon -gamma receptor domain
LD009	6393-5704(230)	LW009	6271-5582(230)	alpha amanitin-sensitive protein	no domains
LD010	6933-6448(162)	LW010	6810-6325(162)	LAP/PHD-finger protein	transmembrane segment
					transmembrane segment
					Ring finger domain
					Zinc finger domain
LD011	8111-6981(377)	LW011	7999-6857(381)	CC chemokine receptor-like protein	7 transmembrane receptor (rhodopsin family)
LD012	8853-8221(211)	LW012	8742-8110(211)	Ankyrin repeat protein	ankyrin repeats
					ankyrin repeats
					ankyrin repeats
					ankyrin repeats
					ankyrin repeats
LD013a (f)	9918-8935(328)	LW013	9809-8787(341)	Interleukin-1 receptor-like protein	transmembrane segment
					immunoglobulin domain
					immunoglobulin-like domain
					immunoglobulin domain
LD014	10247-9981(89)	LW014	10139-9873(89)	putative eIF2 alpha-like PKR inhibitor	Ribosomal protein S1-like RNA-binding domain
LD015	10719-10237(161)	LW015	10611-10129(161)	putative Interleukin-18 binding protein	Immunoglobulin domain
LD016	11026-10760(89)	LW016	10916-10650(89)	EGF-like growth factor	Epidermal growth factor-like domain
LD017	11547-11020(176)	LW017	11437-10910(176)	putative integral membrane protein, apoptosis regulator	transmembrane segment
LD018	12026-11589(146)	LW018	11919-11482(146)	dUTPase	dUTPase domain
LD019a (f)	12945-12076(290)	LW019a (f)	290	Kelch-like protein	BTB domain

LD019b (f)	13784-12975(270)	LW019b (f)	125		Kelch domain
		LW019c (f)	100		Kelch domain
					Kelch domain
					Kelch domain
LD020	14814-13852(321)	LW020	14699-13737(321)	Ribonucleotide reductase, small subunit	ribonucleotide reductase domain
LD021	15115-14858(86)	LW021	14999-14742(86)	hypothetical protein	signal peptide
LD022	15500-15159(114)	LW022	15378-15043(112)	hypothetical protein	no domains
LD023	15949-15734(72)	LW023	15826-15611(72)	hypothetical protein	no domains
LD024	16677-16030(216)	LW024	16552-15905(216)	hypothetical protein	L1L / F9 / C19 poxvirus orf family
LD025	17998-16658(447)	LW025	17873-16533(447)	putative Ser/Thr protein kinase	Protein kinase-like domain
LD026a (f)	18495-18037(153)	LW026	18817-17912(302)	hypothetical protein	no domains
LD026b (f)	18942-18622(107)				
LD027	20867-18954(638)	LW027	20745-18829(639)	putative EEV maturation	no domains
LD028	21986-20877(370)	LW028	21864-20755(370)	putative palmitylated virion envelope protein	Phospholipase D domain
					Phospholipase D domain
LD029	22625-22191(145)	LW029	22503-22069(145)	hypothetical protein	no domains
LD030	23361-22705(219)	LW030	23237-22581(219)	hypothetical protein	no domains
LD031	23435-23746(104)	LW031	23308-23619(104)	putative DNA-binding virion core phosphoprotein	no domains
LD032	25177-23756(474)	LW032	25050-23629(474)	Poly(A) polymerase large subunit PAPL	no domains
LD033	27381-25177(735)	LW033	27254-25050(735)	hypothetical protein	no domains
LD034	27926-27396(177)	LW034	27800-27270(177)	putative PKR inhibitor	Z-DNA-binding domain
					Double-stranded RNA binding motif domain
LD035	28592-29797(402)	LW035a (f)	28541-29671(377)	hypothetical protein	E5R poxvirus protein family
LD036	28593-27991(201)	LW036	28466-27864(201)	RNA polymerase subunit RPO30	C2C2 Zinc finger domain

LD037	29809-31506(566)	LW037	29684-31381(566)	hypothetical protein	no domains
LD038	31516-32313(266)	LW038	31391-32188(266)	hypothetical protein	Poxvirus E8 protein
					transmembrane segment
LD039	35345-32316(1010)	LW039	35220-32191(1010)	DNA polymerase	DNA polymerase type-B family domain
LD040	35379-35663(95)	LW040	35254-35538(95)	putative redox protein	no domains
LD041	36055-35666(130)	LW041	35930-35541(130)	putative virion core protein	no domains
LD042	38096-36045(684)	LW042	37971-35920(684)	hypothetical protein	transmembrane segment
LD043	39146-38205(314)	LW043	39021-38080(314)	putative DNA-binding virion core protein	no domains
LD044	39371-39156(72)	LW044	39246-39031(72)	hypothetical protein	transmembrane segment
LD045	40202-39375(276)	LW045	40077-39250(276)	putative DNA-binding phosphoprotein	no domains
LD046	40483-40250(78)	LW046	40359-40126(78)	hypothetical protein	signal peptide
					transmembrane segment
					transmembrane segment
LD047	41685-40504(394)	LW047	41561-40380(394)	hypothetical protein	transmembrane segment
LD048	42979-41681(433)	LW048	42855-41557(433)	putative virion core protein	no domains
LD049	42985-45012(676)	LW049	42861-44888(676)	putative RNA helicase NPH-II	(DEXDc3) DEAD-like helicases superfamily domain
					(HELICc3) helicase superfamily c-terminal domain
LD050	46802-45015(596)	LW050	46675-44891(595)	putative metalloprotease	no domains
LD051	47125-47790(222)	LW051	46998-47663(222)	putative transcriptional elongation factor	no domains
LD052	47131-46802(110)	LW052	47004-46675(110)	hypothetical protein	transmembrane segment
LD053	48137-47760(126)	LW053	48010-47633(126)	putative glutaredoxin	signal peptide
LD054	48140-49450(437)	LW054	48013-49323(437)	hypothetical protein	coiled coil domain
LD055	49454-49642(63)	LW055	49327-49515(63)	RNA polymerase subunit RPO7	RNA polymerases N / 8 kDa subunit
LD056	49645-50166(174)	LW056	49518-50039(174)	hypothetical protein	no domains

LD057	51304-50186(373)	LW057	51177-50059(373)	putative virion core protein	transmembrane segment
LD058	51334-52113(260)	LW058	51207-51986(260)	putative late transcription factor VLTF-1	Viral Trans-Activator Protein
					transmembrane segment
LD059	52143-53150(336)	LW059	52016-53023(336)	putative myristylated protein	transmembrane segment
LD060	53154-53888(245)	LW060	53027-53761(245)	putative myristylated IMV envelope protein	L1L/F9/C19 pox virus orf family
					transmembrane segment
LD061	53929-54204(92)	LW061	53802-54077(92)	hypothetical protein	transmembrane segment
LD062	55173-54220(318)	LW062	55046-54093(318)	hypothetical protein	no domains
LD063	55198-55956(253)	LW063	55071-55829(253)	putative DNA-binding virion core protein	Alpha-macroglobulin receptor domain
LD064	55975-56367(131)	LW064	55848-56240(131)	Putative membrane protein	transmembrane segment
LD065	56327-56767(147)	LW065	56200-56643(148)	hypothetical protein	no domains
LD066	56798-57328(177)	LW066	56668-57198(177)	Thymidine kinase	Thymidine kinase
LD067	57404-57994(197)	LW067	57273-57863(197)	putative host range protein	Pox C7 F8A domain
LD068	58056-59054(333)	LW068	57923-58921(333)	Poly(A) polymerase small subunit, PAPS	Poly A polymerase regulatory subunit domain
					VV Protein Vp39 in complex with S-Adenosylhomocysteine
LD069	58972-59526(185)	LW069	58839-59393(185)	RNA polymerase subunit RPO22	no domains
LD070	59936-59538(133)	LW070	59803-59405(133)	hypothetical protein	(DUF230) Poxvirus proteins of unknown function
					transmembrane segment
LD071	60022-63876(1285)	LW071	59889-63743(1285)	RNA polymerase subunit RPO147	RNA polymerase alpha subunit domain
					RNA polymerase A / beta' / A" subunit domain
LD072	64399-63887(171)	LW072	64266-63754(171)	putative protein-tyrosine phosphatase	Dual specificity phosphatase, catalytic domain
LD073	64415-64984(190)	LW073	64282-64851(190)	hypothetical protein	transmembrane segment
LD074	65952-64987(322)	LW074	65819-64854(322)	putative IMV envelope protein p35	transmembrane segment
LD075	68378-65985(798)	LW075	68244-65851(798)	RNA polymerase-associated protein RAP94	no domains

LD076	68522-69190(223)	LW076	68386-69063(226)	putative late transcription factor VLTF-4	no domains
LD077	69235-70185(317)	LW077	69107-70057(317)	DNA topoisomerase	Eukaryotic DNA topoisomerase I, catalytic core
LD078	70208-70648(147)	LW078	70080-70520(147)	hypothetical protein	no domains
LD079	70682-73207(842)	LW079	70554-73079(842)	mRNA capping enzyme, large subunit	mRNA capping enzyme, large subunit
LD080	73639-73175(155)	LW080	73511-73047(155)	putative virion protein	no domains
LD081	73641-74375(245)	LW081	73513-74247(245)	putative virion protein	no domains
LD082	74375-75028(218)	LW082	74247-74900(218)	Uracil DNA glycosylase	Uracil-DNA glycosylase domain
LD083	75074-77431(786)	LW083	74946-77303(786)	putative NTPase	no domains
LD084	77431-79335(635)	LW084	77303-79207(635)	putative early transcription factor, small subunit VETFS	(DEXDc3) DEAD-like helicases superfamily domain
					Helicase conserved C-terminal domain
LD085	79363-79851(163)	LW085	79235-79723(163)	RNA polymerase subunit RPO18	no domains
LD086	79895-80533(213)	LW086a (f)	79767-80396(210)	*mut*T motif	mutT domain
LD087	80536-81294(253)	LW087a (f)	80409-81008(200)	*mut*T motif, putative gene expression regulator	mutT-like domain
LD088	83210-81306(635)	LW088	83082-81178(635)	putative transcription termination factor NPH-I	(DEXDc3) DEAD-like helicases superfamily domain
					(HELICc3) helicase superfamily c-terminal domain
LD089	84100-83240(287)	LW089	83972-83112(287)	mRNA capping enzyme, small subunit	Poxvirus mRNA capping enzyme, small subunit
LD090	85789-84143(549)	LW090	85661-84015(549)	putative rifampicin resistance protein	no domains
LD091	86268-85819(150)	LW091	86140-85691(150)	putative late transcription factor VLTF-2	Poxvirus trans-activator protein A1
LD092	86996-86301(232)	LW092	86868-86173(232)	utative late transcription factor VLTF-3	no domains
LD093	87220-86996(75)	LW093	87092-86868(75)	hypothetical protein	no domains
LD094	89214-87232(661)	LW094	89086-87104(661)	putative virion core protein P4b	signal peptide
					Poxvirus P4b major core protein
LD095	89824-89342(161)	LW095	89647-89165(161)	putative virion core protein	no domains
LD096	89865-90374(170)	LW096	89688-90197(170)	RNA polymerase subunit RPO19	no domains

LD097	91501-90377(375)	LW097	91324-90200(375)	hypothetical protein	no domains
LD098	93666-91525(714)	LW098	93489-91348(714)	Early transcription factor VETFL	no domains
LD099	93723-94592(290)	LW099	93546-94415(290)	putative intermediate transcription factor subunit VITF-3	no domains
LD100	94855-94622(78)	LW100	94678-94445(78)	putative IMV membrane protein	signal peptide
					transmembrane segment
					transmembrane segment
LD101	97570-94859(904)	LW101	97393-94682(904)	Virion core protein P4a	no domains
LD102	97585-98535(317)	LW102	97408-98358(317)	hypothetical protein	coiled coil domain
					transmembrane segment
					transmembrane segment
LD103	99107-98538(190)	LW103	98930-98361(190)	putative virion core protein	no domains
LD104	99375-99175(67)	LW104	99200-99000(67)	putative IMV membrane protein	transmembrane segment
LD105	99744-99460(95)	LW105	99567-99283(95)	putative IMV membrane protein	signal peptide
					transmembrane segment
					transmembrane segment
LD106	99922-99764(53)	LW106	99745-99587(53)	putative virulence factor	transmembrane segment
					transmembrane segment
LD107	100199-99915(95)	LW107	100022-99738(95)	hypothetical protein	no domains
LD108	101316-100186(377)	LW108	101139-100009(377)	putative myristylated membrane protein	transmembrane segment
					(DUF230) Poxvirus proteins of unknown function
LD109	101922-101335(196)	LW109	101745-101158(196)	putative phosphorylated IMV membrane protein	transmembrane segment
					transmembrane segment
					transmembrane segment
LD110	101937-103376(480)	LW110	101760-103199(480)	putative DNA helicase transcriptional elongation factor	(DEXDc3) DEAD-like helicases superfamily domain

					(HELICc3) helicase superfamily c-terminal domain
LD111	103584-103363(74)	LW111	103407-103186(74)	hypothetical protein	no domains
LD112	103931-105220(430)	LW112	103760-105049(430)	putative DNA polymerase processivity factor	no domains
LD113	103932-103588(115)	LW113	103761-103411(117)	hypothetical protein	transmembrane segment
LD114	105192-105695(168)	LW114	105021-105557(179)	hypothetical protein	no domains
LD115	105723-106877(385)	LW115	105553-106707(385)	putative intermediate transcription factor VITF-3	no domains
LD116	106911-110378(1156)	LW116	106741-110208(1156)	RNA polymerase subunit RPO132	RNA polymerase beta subunit domain
LD117	110841-110398(148)	LW117	110672-110229(148)	putative fusion protein	Chordopoxvirus fusion protein
LD118	111264-110845(140)	LW118	111095-110676(140)	hypothetical protein	transmembrane segment
LD119	112176-111268(303)	LW119	112007-111099(303)	RNA polymerase subunit RPO35	no domains
LD120	112369-112148(74)	LW120	112200-111979(74)	hypothetical protein	no domains
LD121	113312-112551(254)	LW121	113143-112382(254)	putative DNA packaging protein	no domains
LD122	113444-114031(196)	LW122	113275-113862(196)	putative EEV glycoprotein	transmembrane segment
LD123	114067-114579(171)	LW123	113897-114409(171)	putative EEV protein	signal peptide
					transmembrane segment
					C-type lectin or carbohydrate-recognition domain
LD124	114610-115182(191)	LW124	114440-115012(191)	hypothetical protein	no domains
LD125	115222-116085(288)	LW125	115052-115915(288)	hypothetical protein	no domains
LD126	116147-116689(181)	LW126	115977-116492(172)	putative EEV glycoprotein	transmembrane segment
LD127	116703-117521(273)	LW127	116506-117324(273)	hypothetical protein	no domains
LD128	118433-117531(301)	LW128	118233-117334(300)	CD47-like protein	signal peptide
					transmembrane segment
					transmembrane segment
					transmembrane segment

					transmembrane segment
					transmembrane segment
					Immunoglobulin domain
LD129	118531-118899(123)	LW129	118331-118699(123)	hypothetical protein	no domains
LD130	118971-119213(81)	LW130	118770-119012(81)	hypothetical protein	no domains
LD131	119272-119754(161)	LW131a (f)	119076-119399(108)	Superoxide dismutase-like protein	Copper / zinc superoxide dismutase domain (SODC)
LD132	119792-120319(176)	LW132	119593-120123(177)	hypothetical protein	signal peptide
					transmembrane segment
LD133	120352-122028(559)	LW133	120156-121832(559)	DNA ligase-like protein	DNA ligase domain
LD134	122187-128261(2025)	LW134a (f)	121991-124153(721)	similar to variola virus B22R	signal peptide
		LW134b (f)	124335-128063(1243)		transmembrane segment
LD135	128334-129413(360)	LW135	128133-129212(360)	putative IFN-alpha/beta binding protein	signal peptide
					immunoglobulin domain
					immunoglobulin C-2 type domain
					immunoglobulin domain
LD136	129464-129925(153)	LW136	129263-129721(153)	hypothetical protein	no domains
LD137	129990-130994(335)	LW137	129790-130794(335)	hypothetical protein	no domains
LD138	131027-131584(186)	LW138	130827-131384(186)	Ig domain, OX-2-like protein	Immunoglobulin domain
					transmembrane segment
					transmembrane segment
LD139	131626-132540(305)	LW139	131426-132340(305)	putative Ser/Thr protein kinase	Protein kinase domain
LD140	132575-133294(240)	LW140	132374-133093(240)	putative RING finger host range protein, N1R	Ring finger domain
LD141	133346-134017(224)	LW141	133145-133819(225)	putative EEV host range protein	signal peptide
					(CCP) complement control protein domain

					(CCP) complement control protein domain
					transmembrane segment
LD142	134022-134423(134)	LW142	133824-134219(132)	putative secreted virulence factor	no domains
LD143	134463-135368(302)	LW143	134258-135163(302)	Tyrosine protein kinase-like protein	Protein kinase domain
LD144	135540-137189(550)	LW144a (f)	135320-136126(269)	Kelch-like protein	BTB domain
		LW144b (f)	136120-136962(281)		Kelch domain
					Kelch domain
					Kelch domain
					Kelch domain
LD145	137230-139131(634)	LW145	137011-138918(636)	Ankyrin repeat protein	ankyrin repeats domain
					ankyrin repeats domain
					ankyrin repeats domain
					ankyrin repeats domain
					ankyrin repeats domain
					ankyrin repeats domain
					ankyrin repeats domain
LD146	139263-140501(413)	LW146	139120-140355(412)	Phospholipase D-like protein	Phospholipase D domain
					Phospholipase D domain
LD147	140571-142064(498)	LW147	140418-141911(498)	Ankyrin repeat protein	ankyrin repeats domain
					ankyrin repeats domain
					ankyrin repeats domain
					ankyrin repeats domain
					ankyrin repeats domain
					ankyrin repeats domain

					ankyrin repeats domain
LD148	142115-143455(447)	LW148	141962-143302(447)	Ankyrin repeat protein	ankyrin repeats domain
					ankyrin repeats domain
					ankyrin repeats domain
					ankyrin repeats domain
					ankyrin repeats domain
LD149	143479-144489(337)	LW149	143327-144337(337)	Serpin-like protein	signal peptide
					Serine proteinase inhibitors (SERPIN)
LD150	144531-145013(161)	LW150	144379-144861(161)	hypothetical protein	no domains
LD151	145058-146710(551)	LW151	144907-146553(549)	Kelch-like protein	BTB domain
					Kelch domain
					Kelch domain
					Kelch domain
LD152	146779-148245(489)	LW152	146622-148088(489)	Ankyrin repeat protein	ankyrin repeats domain
					ankyrin repeats domain
					ankyrin repeats domain
					ankyrin repeats domain
					ankyrin repeats domain
					ankyrin repeats domain
					ankyrin repeats domain
LD153 (e)	148293-148565(91)	LW153 (e)	148136-148408(91)	hypothetical protein	signal peptide
					transmembrane segment
LD154 (e)	148638-149357(240)	LW154 (e)	148481-149200(240)	putative ER-localized apoptosis regulator	signal peptide
LD155 (e)	149610-150002(131)	LW155 (e)	149452-149844(131)	hypothetical protein	transmembrane segment

LD156 (e)	150076-150552(159)	LW156 (e)	149918-150394(159)	hypothetical protein	signal peptide
					B15-like protein

(a) LD, South African LSDV Neethling Warmbaths isolate.

(b) LW, South African LSDV Neethling vaccine strain.

(c) Function was deduced from either the degree of similarity to known genes or from the presence of Prosite signatures.

(d) SMART: Simple Modular Architecture Research Tool

(e) ORF 001, ORF 002, ORF 003 and ORF 004 are identical to ORF156, ORF155, ORF154 and ORF153 respectively. 51 amino acids encoded by ORF004 is situated in the inverted terminal repeats.

(f) ORFs containing frameshifts were labelled [a] (e.g. LD013a), while those ORFs with frameshifts resulting in truncations were labelled [a] and [b] or [a], [b] and [c] (e.g. LW019a, LW019b and LW019c).

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