GENOMIC CHARACTERISATION OF SOUTHERN AFRICAN ISOLATES OF CAPRIPOX AND AVIPOX VIRUSES

BY

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Cape Town 1994

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dedicated to the Lord,
may His Will be done.

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ABSTRACT

A number of poxviruses, including members of the genera *Capripoxvirus* and *Avipoxvirus*, are being developed as recombinant vaccine vectors for use in veterinary medicine as they have restricted host ranges and have already been used as effective and safe vaccines in animals for many years. But, before recombinant vaccines can be used in the field, a thorough knowledge of the genetic diversity and stability of field isolates is necessary. The first part of this study was therefore initiated to compare the genomes of southern African vaccine strain and field isolates of the capripoxvirus, lumpy skin disease virus (LSDV). However, before this goal could be achieved, conditions for the growth and purification of LSDV virions and extraction of high quality viral DNA had to be optimised. It was found that propagation of LSDV at in lamb testes (LT) or foetal bovine testes (FBT) cells, using a combination of Dulbecco's modified Minimal Essential Medium (DMEM) and Ham's F-12 culture medium, resulted in a 2-3 fold increase in the yield of infectious virions when compared to "standard" culture conditions. Triton X-100 and β-mercaptoethanol, used to release virus particles from infected cells, and a Sarkosyl (sodium lauroyl sarcosinate) lysis buffer (including β-mercaptoethanol), used to lyse the virus, were found to yield the desired high quantity and quality of viral DNA.

Once optimal growth and purification conditions had been determined, DNA from the four LSDV field isolates and the vaccine strain was restricted with the endonucleases *Pst* I, *Eco* RI, *Hin* dIII and *Bam* HI. The resulting fragments were separated on agarose gels using field inversion gel electrophoresis (FIGE). Comparisons of the resulting electrophoretic patterns revealed a number of minor differences in the *Pst* I, *Hin* dIII, *Bam* HI and *Eco* RI restriction profiles. The terminal *Bam* HI and *Eco* RI fragments were identified by "snap-back" analysis. There were a number of distinct differences in the terminal regions of the isolates. On the basis of the lengths of the terminal fragments, the genomes of the virulent Neethling and Maputo isolates appeared to be similar, as were those of the vaccine strain and Haden, but Gaborone was found to be unique. The terminal regions of poxvirus genomes are known to be the least conserved genomic regions and it is thus of interest that these regions are not identical for different isolates of LSDV. This genomic polymorphism may be useful in the future for distinguishing between field isolates and recombinant vaccines.

Homologous recombination has been a standard technique used to introduce foreign genes into poxvirus genomes for the development of recombinant vector vaccines. An alternative technique makes use of *in vitro* ligation to clone genes into unique or rare restriction sites. The restriction sites of a number of rare restriction-site endonucleases were mapped within the genome of the LSDV vaccine strain. The restriction endonucleases *Sma* I, *Not* I, *Apa* I, *Nae* I, *Nar* I, *Ksp* I and *Bss* HII were tested. The genome lacked *Not* I, *Nar* I and *Ksp* I restriction sites, contained more than one *Nae* I and *Bss* HII site and single *Sma* I and *Apa* I sites. The unique *Sma* I and *Apa* I sites might prove useful for the development of a recombinant LSDV vaccine.

The second part of this study was the isolation of southern African field isolates of avipoxviruses from wild birds and the characterisation of these isolates using restriction digest analysis of their DNA. Avipoxviruses have been isolated from a large number of bird species. A number of vaccines have been available since the 1920s with commercial vaccines against fowlpox virus, pigeonpox virus and turkeypox virus being readily available. Poxvirus outbreaks in quails and psittacines have led to renewed interest in vaccine production, as well as in the development of avipoxvirus-based recombinant vector vaccines. For similar reasons as described for LSDV, a knowledge of the genetic diversity of field isolates of avipoxviruses is crucial before recombinant strains can be field tested. The DNA of an avipoxvirus isolated from a Rock pigeon, *Columba guinea*, was compared with the DNA from isolates from Turtle dove, Jackass penguin and parrot, as well as from a local vaccine strain (Jordaan) of pigeonpox virus. These restriction digest patterns were compared with strains of the well-characterised avipoxviruses, canarypox virus and fowlpox virus. The restriction patterns were found to be unique for avipoxviruses from each avian species and the Rock pigeon isolate was almost identical to the vaccine strain of pigeonpox virus.

ABBREVIATIONS USED IN TEXT:

A	adenosine
BEM	Basal Eagle's Medium
β gal	β-galactosidase
bp	base pair
C	cytosine
CAM	chorioallantoic membrane
°C	degrees celcius
CEF	chicken embryo fibroblast
CFK	calf foetal kidney
cm	centimeter
CO_2	carbon dioxide
CPE	cytopathic effect
D	Dalton
DAB	3,3'- diamino benzidine tetrahydrochloride
DMEM	Dulbecco's modified Minimal Essential Medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetra-acetic acid
EtBr	ethidium bromide
EtOH	ethanol
F	Forward Pulse (used during FIGE)
FBT	foetal bovine testes
FCS	Foetal Calf Serum
Fig.	Figure
FIGE	Field Inversion Gel Electrophoresis
g	gram or gravitational force
G	guanine
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic Acid
k	kilo
kbp	kilobase pair
kb	kilobase
KC	Kenya cattle
kDa	kilodalton
kg	kilogram

kPa	kilo Pascal
KS	Kenya sheep
l	litre
LSD	lumpy skin disease
LSDV	lumpy skin disease virus
LT	lamb testes
M	Molar
MDBK	Madin Darby Bovine Kidney
MEM	Minimal Essential Medium
mg	milligram
min	minute
µg	microgram
µl	microlitre
µm	micromolar
mA	milliamperes
ml	millilitre
mM	millimolar
mmol	millimoles
M.O.I.	Multiplicity of Infection
MW	molecular weight determined by gel electrophoresis
N	normal
NaCl	sodium Chloride
ng	nanograms
nm	nanometer
NaOH	sodium hydroxide
O/N	overnight
ORF	open reading frame
PBS	phosphate buffered saline
pfu	plaque forming units
PG	Penicillin and Gentamycin
pH	negative $\log_{10}$ of the hydrogen concentration
p.i.	post infection
PS	Penicillin and Streptomycin
r	ramp factor used in FIGE
R	Reverse pulse used in FIGE
RNA	ribonucleic acid

rpm	revolutions per minute
Sn	supernatant
SDS	Sodium dodecyl sulphate
T	thymine
TAE	Tris acetate EDTA
TE	Tris EDTA
TK	thymidine kinase
Tris	Tris-(hydroxymethyl)-aminomethane
UV	Ultra-violet
V	volts
v/v	volume per volume ratio
w/v	weight per volume ratio

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SECTION 1

LITERATURE REVIEW

CHAPTER 1

A GENERAL INTRODUCTION TO POXVIRUSES:

1.1 Introduction:

Poxviruses are large DNA-containing animal viruses which infect both vertebrate and invertebrate hosts and were the first viruses to be visualised by light microscopy (as reviewed by Moss, 1990). An important characteristic of poxviruses is their ability to replicate in the cytoplasm of infected cells. The family *Poxviridae* is divided into two subfamilies, namely the *Chordopoxvirinae* (vertebrate poxviruses) and the *Entemopoxvirinae* (insect poxviruses). These in turn consist of a number of genera (Table 1) (Matthews, 1982; Esposito, 1991). The *Orthopoxvirus* genus contains the two most well-known poxviruses; variola, which was the causative agent of smallpox and its benign relative vaccinia, which was used successfully as a vaccine against variola and played an important role in the global eradication of smallpox. Avipoxviruses have been isolated from many avian species and the type member, fowlpox virus, has caused considerable economic losses to the poultry industry (as cited by Ghildyal *et al.*, 1989). Capripoxviruses, which infect cattle, sheep and goats, are also economically important (as cited by Gershon and Black, 1988).

A number of members of the *Poxviridae* occur in southern Africa. Vaccinia virus was still being administered here as a vaccine against smallpox until a number of years ago, when it was confirmed that global eradication of the disease had been successful (Moss, 1990). Parapoxviruses and suipoxvirus occur worldwide (Fenner *et al.*, 1987). Lumpy skin disease virus (LSDV) is the only capripoxvirus found in South Africa, although it occurs throughout the rest of Africa along with the other members of the capripoxvirus genus, sheeppox virus and goatpox virus. Avipoxviruses have been isolated from a number of species of southern African birds (Kow, 1992).

The genome organisation and cell entry and exit mechanisms of vaccinia virus have been well studied, whereas this work is relatively new for the capripoxviruses and avipoxviruses. In view of this, the following sections on poxvirus structure and composition will mainly be discussed with relation to vaccinia virus.

TABLE 1.1 Family *Poxviridae*: Genera and Members

SUBFAMILIES	GENERA	SELECTED MEMBERS
CHORDOPOXVIRINAE (Vertebrate poxviruses)	*CAPRIPOXVIRUS*	lumpy skin disease virus, goatpox virus, sheeppox virus
	AVIPOXVIRUS	fowlpox virus, pigeonpox virus
	ORTHOPOXVIRUS	vaccinia virus, variola virus
	PARAPOXVIRUS	Orf virus
	LEPORIPOXVIRUS	Shope fibroma virus
	SUIPOXVIRUS	swinepox virus
	YATAPOXVIRUS	tanapox virus
	MOLLUSCIPOXVIRUS	*Molluscum contagiosum*
ENTEMOPOXVIRINAE (Insect poxviruses)	A	*Melontha melontha*
	B	*Amsacta moori*
	C	*Chironimus luridus*

(Adapted from Moss, 1990)

1.2 Virion structure:

Vaccinia virions appear by electron microscopy to be brick-shaped bodies approximately 300-400 x 170-260 nanometers (nm) (Westwood *et al.*, 1964). Early studies of electron micrographs of vaccinia virus revealed the presence of two types of particles; the one form, thought to be the complete form, consisted of a nucleoid with surrounding envelope material and the other, incomplete form, consisted of envelope material with or without nucleoid components (Nagington and Horne, 1962). Thin-sections of virions reveal a lipoprotein bilayer, called the outer membrane, surrounding a central core that encases the viral DNA. The core is surrounded by a palisade layer of rod-shaped molecules (Dales and Pogo, 1981) (Figure 1.1). In vertebrate poxviruses, the core appears biconcave with two structures, called lateral bodies, nestled in the concavities. The lateral bodies are attached to the outer membrane and are ellipsoidal in shape (Medzon and Bauer, 1970). The outer surface of the outer membrane is studded with randomly arranged surface tubule elements (MW 58,000) which give the virion its textured surface (Stern and Dales, 1976; Dales, 1963). An additional lipid-bilayer, called the envelope, is most often found surrounding mature particles. Its presence or absence seems to depend on the specificity of the virus isolate and of the host cell (Payne, 1979). The outer membrane and envelope contain a number of virus-encoded proteins, some of which are important in eliciting an immune response in infected hosts (Payne and Kristensson, 1985).

1.3 Genomic structure and organisation:

Poxvirus genomes consist of a linear, double-stranded DNA molecule which is covalently cross-linked at the terminals (Berns and Silverman, 1970; Gershelin and Berns, 1974; Black *et al.*, 1986). These terminal sequences have been shown to contain repeat sequences which are inverted with respect to one another (Wittek *et al.*, 1978). The inverted terminal repeats of the vaccinia virus genome are each 10 kilobases (kb) in length (Baroudy *et al.*, 1982). The termini are incompletely based-paired giving rise to 104 base pairs (bp) inverted loops which are adenine and thymine (A-T) rich. They also consist of two sets of 70 bp tandem direct repeats. The first set, consisting of 13 direct repeats, is disrupted by a 325 bp region of unique sequence, which is then followed by the second set of 18 direct repeats.

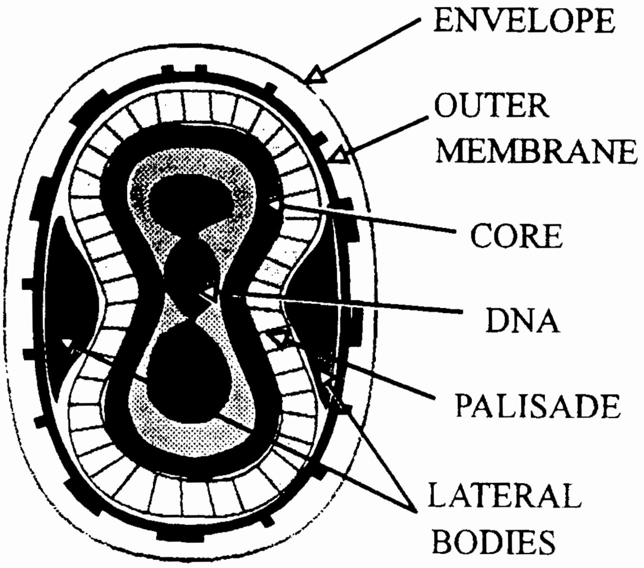

FIGURE 1.1
Schematic representation of a poxvirus virion. (Adapted from Dales and Pogo, 1981)

The genomes are large [the genome length of vaccinia virus (Copenhagen) is 191.6 kb], potentially coding for 263 proteins greater than 65 amino acids in length (review by Johnson *et al.*, 1993). At least 30 of these are structural proteins, and the majority are viral enzymes concerned with nucleic acid synthesis and processing, e.g. a multisubunit DNA-dependant RNA polymerase and a DNA polymerase (Broyles and Moss, 1986; Challberg and Englund, 1979).

Vaccinia virus genomes were found to be considerably more divergent towards their termini than in their central regions. "Host-range" genes have been mapped towards the left-hand termini of orthopoxvirus genomes (Gillard *et al.*, 1985), whereas genes coding for structural proteins and enzymes are placed more centrally.

1.4 Replication of poxviruses:

1.4.1 Virus cell entry:

Electron micrographs revealed attachment of enveloped and non-enveloped vaccinia virus to the plasma membrane at the surface of the cell (Dales and Kajioka, 1964). A number of mechanisms have been proposed for virus cell entry. The results of morphological studies suggest that vaccinia may obtain entry by endocytic mechanisms and by fusion of the viral envelope with the host plasma membrane. Experiments using methylamine and chloroquine [lysomotropic agents (weak bases which have the ability to raise the pH of endosomes, thus blocking proteolysis)] showed that these agents increased the infectivity of vaccinia virus (Janeczko *et al.*, 1987). Subcellular fractionation studies also revealed the absence of viral polypeptides from endosomes, whereas an abundance of these proteins co-migrated with the plasma membrane fraction. Since plaque formation is not inhibited by lysomotropic agents, pH-independent fusion with the plasma membrane may be the most important entry mechanism.

Once inside the cell, the virus undergoes at least two discrete stages of disassembly (Pedley and Cooper, 1987): The first stage is associated with the loss of virion coat proteins and lipids, although the genome is still protected within the core (Holowczak, 1972; Sarov and Joklik, 1972). During the second stage the genome becomes accessible to DNase. A putative, viral-encoded uncoating protein of 23 kilodaltons (kD) with trypsin-like activity is thought to be involved in uncoating (Pedley and Cooper, 1987).

1.4.2 Gene expression:

Almost immediately after entering the cell, and after uncoating, transcription takes place within the core particles producing functional and capped polyadenylated messenger RNA (mRNA) (Brakel and Kates, 1974). Adenosine triphosphate (ATP) is required for the extrusion of transcripts into the cytoplasm where they bind ribosomes for translation. RNA-DNA hybridisation studies revealed that about one-half of the genome is transcribed prior to DNA replication (review by Moss, 1990). Transcription factors have been identified which bind to, and initiate transcription from viral promoters (Li and Broyles, 1993). Although the large genomes are able to code for most of the enzymes required for gene expression, the host cell nucleus is required for the production of infectious virions (Yuwen *et al.*, 1993).

Vaccinia virus-encoded proteins have been located in the nucleus and one of these (E3L gene product) is able to bind double-stranded RNA (Yuwen *et al.*, 1993). Viral induction of the synthesis of host factors has been reported (Moss and Filler, 1970). These factors are thought to be required for the translation of viral mRNA. During the infection cycle, however, the virus eventually causes the complete inhibition of host protein synthesis.

Vaccinia virus gene expression is temporal, with classes of early, intermediate and late genes being identifiable (Moss, 1990). Early genes are expressed from 20-100 minutes post-infection, intermediate genes from 100-120 minutes post-infection and after the onset of DNA replication, whereas late genes are expressed only after replication (140 minutes post-infection) (Baldick and Moss, 1993). A number of regulatory mechanisms, controlling the switch from early to late gene expression, have been proposed (Moss, 1990). One of these suggests the involvement of regulatory factors which might suppress early gene transcription by binding to transcription factors or early gene promoters (McAuslan, 1963). In addition, early and intermediate gene transcripts have short half lives (30 minutes) providing the basis for rapid changes in gene expression (Baldick and Moss, 1993). Early protein synthesis is terminated once late gene products are being synthesised, unless the early genes contain late promoter sequences in addition to their early promoters (Cochran *et al.*, 1985). Late gene products consist mainly of structural proteins and enzymes to be packaged within the progeny virions.

The functional elements of promoter sequences are conserved between poxvirus genera; evidence of this is provided by recent studies demonstrating the ability of the thymidine kinase (TK) promoter of fowlpox virus to function in a recombinant vaccinia virus and of vaccinia virus promoters to function in a recombinant fowlpox virus (Coupar *et al.*, 1990; Prideaux *et al.*, 1990).

1.4.2.1 Early transcription:

A class of early promoters has been identified (Davison and Moss, 1989). They are A-T rich and contain a critical region for activity which corresponds to the downstream site of transcription initiation (AAAAATGAAAAAAAA). Probably the most important enzyme required for transcription is the vaccinia virus RNA polymerase and subunits (Broyles and Pennington, 1990). Another important gene is the DNA topoisomerase (Fogelsong and Bauer, 1984). Inhibition of the activity of this enzyme results in inhibition of transcription of viral genes from intact core particles. Vaccinia virus also codes for an early transcription factor (VETF) which consists of two subunits (70 and 83 kDa) and has ATPase activity required for early gene transcription (Broyles *et al.*, 1988; Broyles and Moss, 1988; Li and Broyles, 1993). An 85 kDa protein (called RAP94) has been identified which is tightly associated with the RNA polymerase, and along with the VETF is important for transcription of early genes.

The 5' ends of early mRNA transcripts are capped by a viral-encoded capping enzyme (Moss *et al.*, 1976) and the 3' ends are polyadenylated by a poly(A) polymerase (Nevins and Joklik, 1977). A transcription termination factor (VTF) was found associated with the capping enzyme (Shuman *et al.*, 1987). Termination signals (TTTTTNT) were discovered 20 to 50 bp upstream of the transcription termination sites (Yuen and Moss, 1987).

1.4.2.2 Intermediate gene transcription:

Expression of these genes is dependant upon DNA replication and requires transcription factors coded for by early genes (Wright and Moss, 1989). There appear to be two classes of intermediate genes, viz. early intermediate (consisting of at least two genes) (Vos and Stunnenberg, 1988) and true intermediate (consisting of three genes coding for late gene transcription factors; A1L, A2L and G8R) (Baldick and Moss, 1993). The promoter sequences of the true intermediate genes have been found to contain two critical regions for gene expression (AAATAA and TAAA) (Baldick *et al.*, 1992).

1.4.2.3 Late gene transcription:

Late gene expression is dependant on replication of the viral genome (Belle Isle *et al.*, 1981) and translation of the three late gene transcription factors. Most late genes occur in the central region of the genome and code for structural proteins and many of the enzymes destined for packaging within progeny virions. There are two classes of late genes; one which is expressed immediately after DNA replication and the other which is delayed. The promoter sequences of late genes consist of a critical region with the consensus sequence: TAAATG (Weir and Moss, 1984).

A major difference between early and late mRNA transcripts is that late transcripts do not have defined 3' ends and continue downstream through early genes, not recognising early gene termination sequences (Mahr and Roberts, 1984). The 5' capped ends of the late mRNAs also differ from those of early transcripts (Boone and Moss, 1977) and include poly(A) tracts which are thought to be important for ribosomal binding (Ahn and Moss, 1989). Vaccinia late transcription factor (VLTF-1), VETF and RNA polymerase have been identified in cell extracts purified late in the infection cycle (review by Moss, 1990a).

Proteolytic maturation of some vaccinia virus proteins (e.g. late gene core proteins 4a, 4b and 25K) occurs via cleavage of precursor polypeptides (Van Slyke *et al.*, 1991). A conserved "cleavage signal" sequence (Alanine-Glycine-Alanine) has been identified.

1.4.3 DNA replication:

The cytoplasm, as the site for replication, is unique to the poxviruses and African Swine Fever virus (Dales and Pogo, 1981; Harford *et al.*, 1966). Most, or all, of the genes required for DNA replication are encoded by the virus as it is still able to replicate in enucleated cells, although the nucleus is required for the production of infectious particles (Pennington and Follet, 1974; Yuwen *et al.*, 1993). Replication begins within the first three hours after infection (Harford *et al.*, 1966). Discrete "virus factories" have been identified which are electron dense regions in the cytoplasm where the virus replicates (Moss, 1990). No specific origins of replication have been found in the genome and it is speculated that replication occurs via nicking of the DNA at random points, followed by self-priming and replication giving rise to large concatemeric-branched structures. These might be resolved into unit genomes at a later stage. Concatemeric forms have been isolated when inhibitors of late protein synthesis are added to infected cells. Resolution occurs upon reversal, however, indicating that the concatemers are replicative intermediates.

A number of viral enzymes are required to facilitate the high levels of DNA synthesis. Two of these include the vaccinia virus single unit DNA polymerase (MW 110,000) (Charllberg and Englund, 1979; Traktman *et al.*, 1984) and a DNA ligase (Moss, 1990). Another enzyme involved in replication is a TK enzyme. The TK is important for nucleotide metabolism and is thus needed early in infection, especially in non-replicating infected cells with a low level of thymidylic acid. Although important, vaccinia virus can survive without it, making the TK gene a useful insertion site for recombinant vaccine development (refer to section 1.5). Other viral enzymes important for replication include a ribonucleotide reductase, which converts ribonucleotides into DNA precursors and a serine/threonine kinase (Banham and Smith, 1992).

Recombination of poxvirus DNA has been linked to replication and could explain the considerable amount of variation in the terminal regions of poxvirus genomes (as reviewed by Buller and Palumbo, 1991). Genetic recombination between orthopoxvirus genomes has been well documented (Ball, 1987; Sam and Dumbell, 1981) and it is thought that a member of the *Capripoxvirus* genus arose by genetic recombination between two other members (Gershon *et al.*, 1989 b). The ability of poxvirus genomes to recombine, and to replicate after regions of their genomes have been deleted and replaced with foreign DNA, has resulted in the development of recombinant vector vaccines (Taylor *et al.*, 1988; Cadoz *et al.*, 1992; Taylor *et al.*, 1992) (refer to section 1.5).

1.4.4 Virus assembly and release:

Many of the assembly proteins undergo proteolytic processing prior to assembly. Assembly is initiated in the cytoplasm and the first step is the appearance of crescent-shaped shells with a border of "spicules" on the convex surface and granular material adjacent to the concave surface (Dales and Pogo, 1981). Evidence exists to suggest that these crescent-shaped shells consist of a bilayer membrane derived from the intermediate compartment between the cellular endoplasmic reticulum and the Golgi stacks of the Golgi apparatus (Sordeik *et al.*, 1993). The spicules (MW 65,000) are thought to provide a scaffolding for the viral assembly (Essani *et al.*, 1982; Sodeik *et al.*, 1994). The immature envelope circularises and the nucleoproteins and DNA genome enter just before it is completely sealed (as reviewed by Buller and Palumbo, 1991). The immature virion then undergoes internal differentiation producing the core and lateral bodies. The surface spicules are replaced by surface tubular elements and the virion attains its brick-like morphological appearance. Mature virions are transported to the cell periphery either in vacuoles or bound in a double membrane (or envelope). There is much controversy over the origin of this second envelope as evidence exists to support an early endosomal origin (Tooze *et al.*, 1993) and an equal degree of evidence exists to support a trans Golgi network origin (Schmelz, 1994). A viral-encoded 42 kDa glycoprotein has been shown to be necessary for this second enveloping event (Wolffe *et al.*, 1993). Virions may also become associated with a proteinaceous A-type inclusion body which is thought to protect the virus from the external environment (Ichihashi *et al.*, 1971).

The virions are released from the cell either by fusion of the surrounding membrane with the plasma membrane or via budding from the cell surface, especially at sites on the microvilli.

1.5 Poxvirus recombinant vector vaccines:

The introduction of recombinant vaccinia virus in 1981 provided a new strategy for vaccine development (Sam and Dumbell, 1981). A recombinant vaccinia virus expressing the haemagglutinin of the swine influenza virus induced immune responses when inoculated into cattle, sheep and poultry (Boyle and Coupar, 1986). None of these animals displayed disease symptoms when inoculated with wild-type swine influenza virus and there was no detectable spread of either recombinant or wild-type virus from the inoculation sites or to uninoculated animals in close proximity.

The basic technologies used to construct vaccinia virus recombinants have been modified and extended to other members of the *Poxviridae* family, particularly members of the *Avipoxvirus* genus. Fowlpox and canarypox viruses have natural host ranges limited to avian species. As such, these viruses have been developed as species-specific recombinant viral vectors (Taylor *et al.*, 1988; Cadoz *et al.*, 1992; Taylor *et al.*, 1992). In April 1983, an avirulent avian influenza virus appeared in the chicken population in Pensylvania and in November that same year a highly virulent strain appeared in turkeys in Ireland. Conversion to virulence was obtained by inducing a single mutation in the hemagglutinin gene. This gene was cloned into a non-essential region of the fowlpox virus genome and recombinant virus was obtained expressing the virulent form of the hemagglutinin protein. When inoculated into chickens, birds survived challenge with homologous or heterologous influenza viruses. A recombinant pigeonpox virus has also been constructed expressing the fusion glycoprotein of Newcastle disease virus (Letellier *et al.*, 1991). The glycoprotein gene was inserted into the non-essential TK gene of the pigeonpox virus genome (Letellier, 1993). Chickens vaccinated with this construct via the follicular method induced high anti-fusion glycoprotein antibody titres, and good protection against challenge with virulent Newcastle disease virus was shown.

As avipoxviruses do not produce infectious particles in mammalian cells, it is surprising that fowlpox virus has been successfully used to vaccinate non-avian species. A recombinant fowlpox virus expressing the rabies glycoprotein, afforded protective immunity to live rabies challenge in mice, cats and dogs (Taylor *et al.*, 1988). Human trials of a recombinant canarypox virus also expressing the rabies glycoprotein resulted in induction of levels of neutralising antibody comparable with protection in animals (Cadoz *et al.*, 1992).

A number of pros and cons exist as to the use of vaccinia virus recombinant vaccines: Their molecular biology has been well studied and a number of selection techniques are available for the construction of recombinants (Moss, 1990; Merchlinsky and Moss, 1992): They stimulate all facets of the host's immune response (cellular and humoral) as the recombinant is able to replicate and present antigen to the cell surface as in a normal infection (Taylor *et al.*, 1992). The vaccines are safer than live, attenuated vaccines as convertance to virulence of the "protecting virus" is impossible as only a single immunogenic subunit of this virus is expressed. A recombinant vaccine has the potential to protect against a number of diseases at the same time if immunogens from more than one pathogen are cloned into its genome. Due to the large sizes of poxvirus genomes and their ability to package excess DNA, insertions of large fragments of foreign DNA is possible (Merchlinsky and Moss, 1992). Field trials of recombinant vaccines have already demonstrated their safety and effectiveness in controlling pathogens (Brochier *et al.*, 1991).

On the negative side, there are concerns as to the safety of vaccinia virus recombinants in immunosuppressed people, as vaccinia virus has been shown to cause complications in these patients, although the virus rarely causes problems in healthy subjects (as cited by Cadoz *et al.*, 1992). Another problem is the possibility that a recombinant vector might recombine with a more virulent strain in the field giving rise to a potentially pathogenic vector virus. Combined with this concern is the broad host-range of vaccinia virus, making it difficult to control virus spread, although avipoxvirus vector vaccines have an advantage over vaccinia virus in this respect, as they are host-specific and are unable to replicate in non-avian cells (Cadoz *et al.*, 1992).

The possibility of using members of the genus *Capripoxvirus* as host-restricted vaccine vectors for the expression of immunogens has been investigated (Gershon *et al.*, 1989a). Since then, a recombinant capripoxvirus, Kenya Sheep-1 (KS-1), expressing the hemagglutinin gene of the Rift Valley Fever virus, has been constructed (Romero, 1993). This recombinant is reported to protect cattle against challenge with virulent Rift Valley Fever virus.

The alternative development of avipoxvirus and capripoxvirus recombinant vaccines has necessitated the further molecular characterisation of these viruses in order to ascertain their potential safety and efficacy for use in the field. A thorough knowledge of the genetic diversity and stability of poxvirus field isolates might provide valuable information pertaining to the performance and stability of genetically engineered poxviruses once released into the field. This study was therefore initiated to compare the genomes of southern African vaccine and field isolates of the capripoxvirus, LSDV, and various avipoxviruses.

CHAPTER 2

LUMPY SKIN DISEASE VIRUS:

2.1 Introduction

Lumpy skin disease (LSD) was first described as a skin disease of cattle called "pseudo-urticaria" in Zambia in 1929 (as cited by Weiss, 1968). In subsequent years the infectious nature of the disease was recorded after its rapid spread throughout large parts of sub-Saharan Africa, including Madagascar. In 1957 a virus was isolated on numerous occasions from skin lesions of diseased cattle and, after passage in cell culture, was shown to be the cause of LSD. Morphological characterisation of LSDV (prototype Neethling) provided evidence of its membership in the *Poxviridae* family. LSDV is grouped with goatpox virus and sheeppox virus to constitute the *Capripoxvirus* genus (Matthews, 1982). The ultrastructural details seen on virions isolated in this department were very similar to those of vaccinia virus, but LSDV particles were somewhat less symmetrical in shape (Fig 2.1) (Weiss, 1968).

The name Capripox is derived from capri, Latin for goat, and pox, Old English for pustule or ulcer, and describes the symptoms exhibited by infected animals (Matthews, 1982; Weiss, 1968). For example, cattle infected with LSDV exhibit fever, swelling of the lymph nodes and raised nodules 5-50 mm in diameter on the skin covering the entire body (Erasmus *et al.*, 1986). The diseases induced by the capripoxviruses are a major cause of morbidity and mortality in sheep, goats and cattle in Africa and Asia (as cited by Gershon and Black, 1988). Lumpy skin disease (Neethling) was first diagnosed in South Africa in 1944 and affected eight million cattle with up to 75% mortality on some infected premises (Thomas and Mare, 1945; as cited by Weiss, 1968).

The capripoxviruses are economically the most important group of poxviruses (Kitching *et al.*, 1989). Their natural host range is restricted to sheep, goats and cattle and the central African and Asian isolates have been designated by Gershon and Black (1987) by their country and animal species of origin, e.g. Kenya cattle-1 was isolated from infected cattle during an outbreak of LSD in Londiani, Kenya.

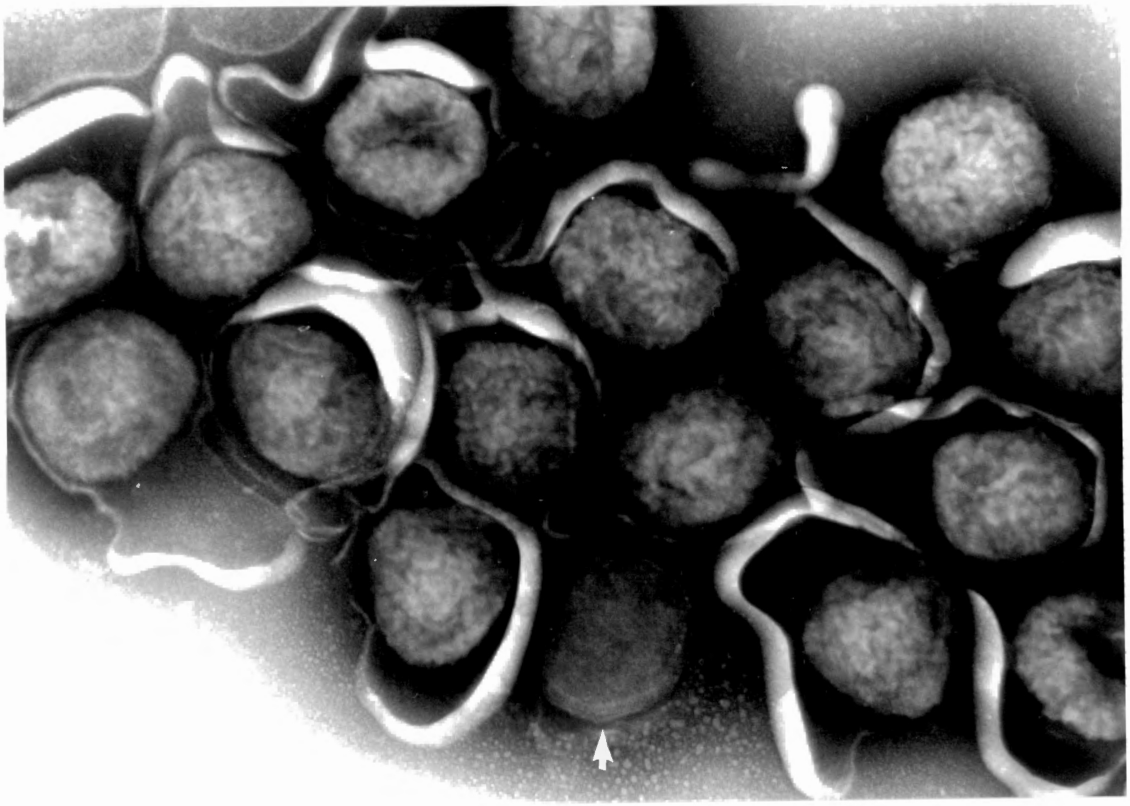

Figure 2.1
Electron micrograph of a cluster of LSDV particles purified from infected bovine kidney cells. The envelope around most of the virions has collapsed to reveal the surface tubular structure of the outer membrane. One particle (arrow) has been penetrated by stain and exposes the core outline and palisade layer (X 75 000). (Photograph kindly supplied by Dr L.M. Stannard, Medical School, UCT)

Although the majority of capripoxvirus strains examined show a preference for growth in only one of the three host species, some strains appear to be able to naturally infect both sheep and goats (Davies, 1981). However, in South Africa, capripoxvirus has only been isolated from cattle during outbreaks of LSD and has never been reported in sheep or goats (Thomas and Mare, 1945; as cited by Kitching *et al.*, 1989). Strains also vary in their pathogenicity in different host species and in different breeds of the same animal.

A number of successful live, attenuated vaccines have been developed to protect livestock from virulent capripoxvirus infections (Davies, 1976; Kitching and Taylor, 1985). A vaccine was developed from the Neethling strain of LSDV in South Africa and is used to protect cattle from LSD.

2.2 Genomic structure and organisation:

The original work on the elucidation of poxvirus genomic structures and gene organisation was performed on the vaccinia virus genome (for a summary refer to Chapter 1). More recently, however, similar studies have been performed on other poxvirus genomes, including those of capripoxviruses (Gershon and Black, 1987). Summation of the DNA restriction fragments generated by *Hin* dIII digestion of a cattle isolate from Kenya, Kenya cattle-1 (KC-1), yielded a genome length of 145.6 kbp (Gershon and Black, 1987). A later re-estimation of the size of this genome, including isolates from sheep (India sheep-1) and goats (Iraq goat-1), using pulse field gel electrophoresis yielded a genome length of 148 kbp for each isolate. These are much shorter than the genome lengths of orthopoxviruses and avipoxviruses, but are similar in lengths to the genomes of a number of parapoxviruses (Robinson *et al.*, 1987).

As observed for orthopoxvirus genomes, the termini of the KC-1 genome were shown to be covalently cross-linked (Gershon and Black, 1987). These terminal sequences have been shown to contain repeat sequences which are possibly inverted with respect to one another. The lengths of the terminal repeats of India sheep-1 DNA were determined to be between 2.25 and 3.40 kbp (Gershon and Black, 1988) and were shown to be inverted with respect to one another. Four tandemly repeated 81 - 82 bp elements were identified within these inverted terminal repeats (Gershon and Black, 1989).

A comparison of the whole genome organisation of a capripoxvirus [Kenya sheep-1(KS-1)] with an orthopoxvirus (vaccinia WR) using cross-hybridisation revealed a similar pattern of gene arrangement as observed within the *Orthopoxvirus* genus (Esposito and Knight, 1985; Gershon *et al.*, 1989). In general, poxvirus genomes are considerably more divergent toward their termini than in their central regions. "Host-range" genes have been mapped towards the left-hand termini of orthopoxvirus genomes (Gillard *et al.*, 1985) and it is thus possible that a similar set of genes are contained within the termini of capripoxvirus genomes. Thus, the central regions of KS-1 and vaccinia virus genomes are conserved, however, the terminal regions of vaccinia virus contains approximately 45 kb of DNA which is absent from the genome of KS-1. A number of open reading frames (ORFs), including one coding for a TK gene, are shared between KS-1 and vaccinia virus genomes, although the KS-1 genome contains one complete ORF in a centrally placed, conserved region, which is absent from the DNA of vaccinia virus and fowlpox virus, but is present downstream of the TK gene of Shope Fibroma virus DNA (Gershon and Black, 1989).

2.3. Characterisation of capripoxviruses

The agar gel immunodiffusion test (AGID) has been used to demonstrate the serological relationship between members of the orthopoxvirus group, the avipoxvirus group and the capripoxvirus group (as cited by Kitching *et al.*, 1986). Kitching *et al.* (1986) used [^{35}S]methionine-labelled viral antigen to improve the sensitivity of this test during their attempt to distinguish individual members of the *Capripoxvirus* genus. However, it was found that the AGID test could not be used to distinguish between the different members as isolates of sheeppox virus, goatpox virus, sheep-and-goatpox virus and LSD virus all share a common major precipitating antigen as revealed by polyacrylamide gel electrophoresis. It was also found that this antigen cross-reacted with sera taken from a lamb recovering from contagious pustular dermatitis virus (a parapoxvirus). These results indicated that the AGID test was not suitable as a diagnostic tool for capripoxvirus infection.

Restriction endonucleases have been successfully used to distinguish members of closely related orthopoxviruses and parapoxviruses (as cited by Black *et al.*, 1986). This technique has also been used on capripoxviruses (Black *et al.*, 1986; Gershon and Black, 1987; Gershon and Black, 1988). *Hin* dIII restriction digest patterns of a number of strains of capripoxvirus from sheeps, goats and cattle have confirmed the close degree of relatedness of this poxvirus group as evidenced from the AGID test (Kitching *et al.*, 1986; Gershon and Black, 1988).

Gershon and Black (1988) found that the capripoxvirus genomes differ from one another in length by not more than 2 kb with an average genome length of 148 kb (Black *et al.*, 1986; Gershon and Black, 1988; Gershon and Black, 1987). The South African vaccine strain of LSDV has, however, been accurately sized at 152.6 kb and differs from the Kenyan isolate in three *Hin* dIII fragments (Black *et al.*, 1986; Gershon and Black, 1988; Perlman, 1993). *Pst* I, *Sal* I and *Ava* I restriction site maps of a number of isolates of capripoxvirus have also been constructed (Fig. 2.2) (Gershon and Black, 1988; Perlman, 1993). These maps indicate that the South African LSDV vaccine strain shares a number of *Ava* I and *Pst* I restriction sites with KC-1, although the *Sal* I maps are quite different. It thus appears that at least these two isolates are genetically distinct and raises questions as to the evolution of LSD virus (or viruses) as it has only been reported in cattle over the last 65 years (as compared to sheep and goat pox which has been known for centuries) and appears to be maintained in wild buffalo populations (Davies, 1982; as cited by Kitching *et al*., 1989).

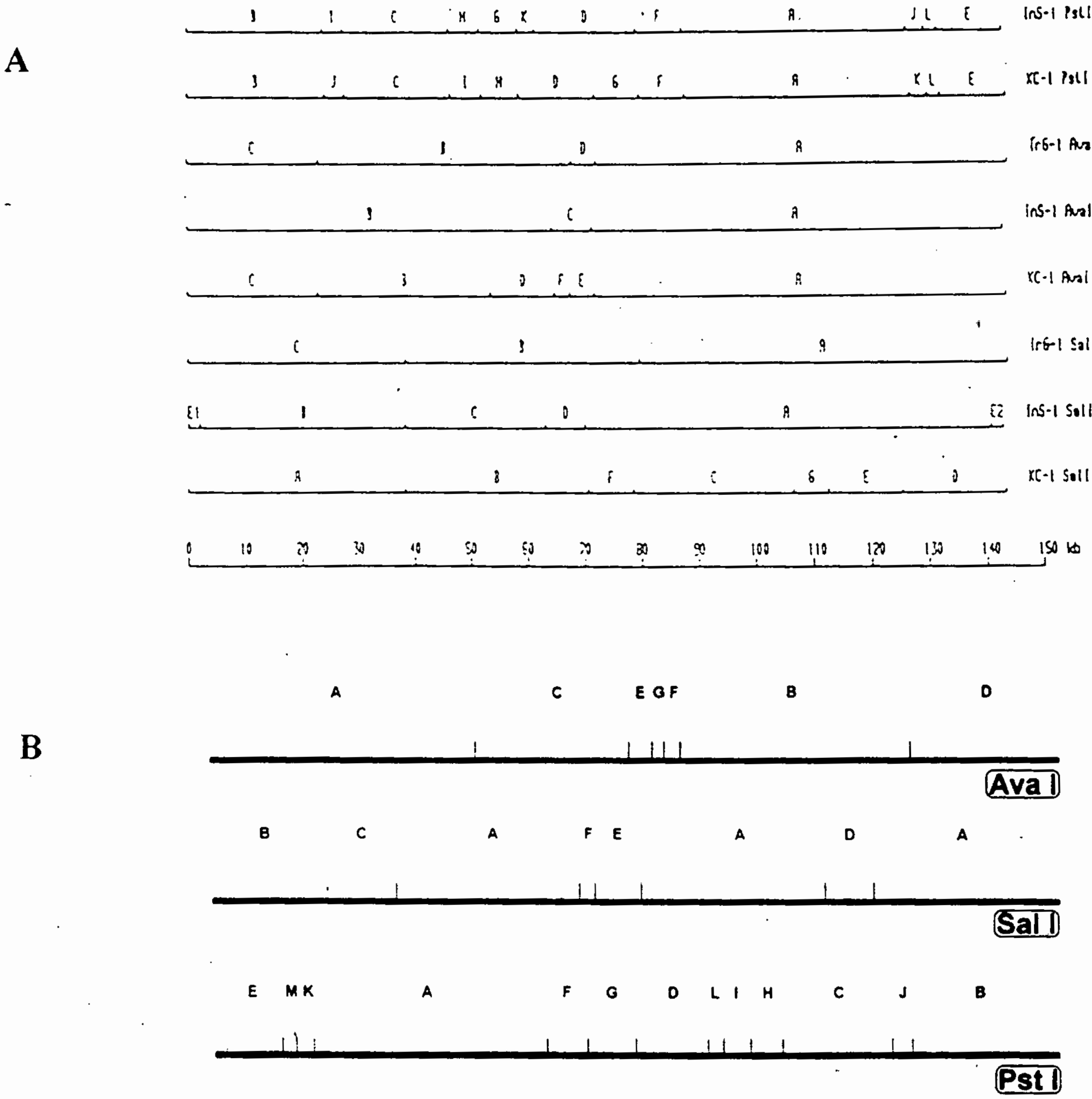

FIGURE 2.2
Genomic maps of capripoxvirus isolates. *Pst* I, *Ava* I and *Sal* I maps of India Sheep-1 (InS-1), Kenya Cattle-1 (KC-1), Iraqi Goat-1 (IrG-1) (A) and the South African vaccine strain of LSDV (B). (From Gershon and Black, 1988; Perlman, 1993). Note that the maps in A are reversed in respect to the maps in B.

Analysis of the *Hin* dIII restriction digest patterns of capripoxviruses have indicated that isolates from sheep and cattle are more closely related to one another than either are to goat isolates (Gershon and Black, 1988). These results in turn suggest that, if the capripoxviruses diverged from a common ancestor, the sheep and cattle isolates diverged from the goat isolates prior to diverging from one another.

Hin dIII restriction digest patterns of Kenya sheep isolates were found to be very similar, whereas when these patterns were compared with typical sheep isolates from other countries, e.g. Nigeria, India and Iraq, larger differences were observed (Gershon and Black, 1988). In fact, the Kenyan isolates were found to be more closely related to capripoxvirus isolates taken from Oman (sheep) and Yemen (goats) (Kitching *et al.*, 1989).

On the basis of these criteria, capripoxviruses can be divided into 5 groups: group 1 isolates are only isolated from sheep (e.g. India sheep-1), group 2 isolates are only isolated from goats (e.g. Iraq goat-1), group 3 isolates are isolated from sheep, goats and cattle, but are restricted to Africa (e.g. Kenya cattle-1), group 4 isolates are isolated from either sheep or goats in Africa or the Middle East (Gershon *et al.*, 1989 b) and group 5 isolates are isolated only from cattle in southern Africa [e.g. LSDV (Neethling)].

2.4 Epidemiology and Transmission:

LSD first appeared in Zambia in 1929 (as reviewed by Woods, 1988). Fourteen years later it appeared in Bechuanaland (Botswana) and soon spread throughout the country. LSD appeared in the Marico District of the Transvaal, South Africa in 1944 (Thomas and Mare, 1945; as cited by Weiss, 1968). Due to the extensive transport and communications network within South Africa, the disease had developed into an explosive epidemic within three years (as reviewed by Woods, 1988). It remained enzootic (potentially infectious, but stable in animal hosts). The disease then spread slowly north, probably through Mozambique and Tanganika (Tanzania), with an outbreak occurring in Kenya in 1957 with only 10 LSD associated deaths. By 1970 the disease had spread north into Sudan and then west into Nigeria in 1974, and Mauritania in 1977 (as cited by Kitching *et al.*, 1989). In 1981 a new epizootic of LSD was reported, which between 1981 and 1986 had affected Tanzania, Kenya, Zimbabwe, Somalia and the Cameroons. LSD was reported in Egypt in 1988 and was first reported outside of Africa in Kuwait in 1986, and in 1989 LSD was reported in Israel (Ali *et* *al*., 1990; as reviewed by Carn, 1993). Figure 2.3 shows the distribution of LSDV in Africa and Israel.

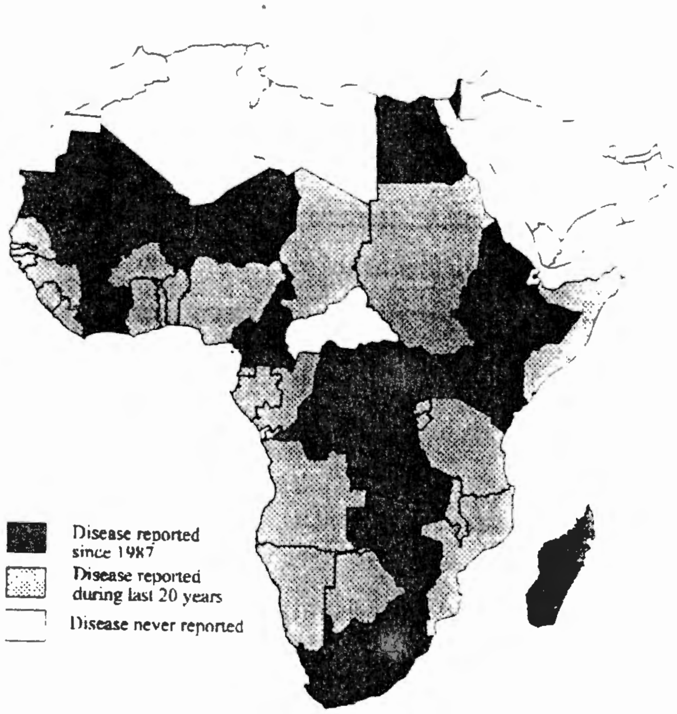

FIGURE 2.3
Distribution of LSDV throughout Africa (including Israel). (Adapted from Carn, 1993).

A vaccine was prepared from the South African Neethling isolate of LSDV and has been in use for many years (as cited by Weiss, 1968). Although sporadic outbreaks of LSD have occurred within the last fifty years in South Africa, a major outbreak was reported in 1990 (Dumbell, personal communication), suggesting that even though there is an effective vaccine, improper or inadequate use is made of the vaccine.

A characteristic of the epidemiology of LSD is the appearance of the disease in epizootics (major outbreaks of viral infection in animal hosts) which appear to spread north from southern Africa. Epidemic behaviour of LSD has been shown to be associated with certain climatic conditions, such as prolonged, or heavy rains which are considered to favour an increase in the insect population. Davies (1982) studied the distribution of LSD in Kenya and discovered that the highest incidence of the disease occurred in forests or at forest edges in a fairly high rainfall zone at 1000 - 2500 m above sea level. The virus is thought to be transmitted during the epidemics by biting insects, but their exact identity remains unknown. Weiss (1968) reported the isolation of LSDV from *Stomoxys calcitrans* and *Biomyia fasciata* and yet experimental transmission from these insects to cattle failed. Kitching and Mellor (1986) successfully transmitted Yemen-goat capripoxvirus between sheep using *Stomoxys calcitrans* as a vector. They were unable to achieve transmission using other Arthropod species, although they were able to isolate virus from sheep head flies (*Hydrotaea irritans*). Although cattle produce local reactions when inoculated with sheeppox or goatpox virus isolates, no natural transmission of these viruses to cattle has been reported, even when cattle were maintained in close contact with infected flocks in Kenya (Davies, 1981).

Antibodies to LSDV have been found in African buffalo, *Syncersus caffer*, and it is probable that they are the long-term maintenance hosts for the virus. Davies (1982) suggested that epizootics occur when the virus spreads from buffalo into a sufficiently large susceptible cattle population. Experimental transmission of LSDV (Neethling) to game animals, including giraffe, impala, buffalo and wildebeest, resulted in the deaths of the impala and giraffe (Young *et al.*, 1970). They exhibited typical symptoms and lesions associated with the disease and virus was successfully isolated from them. Neither the wildebeests or the buffaloes reacted clinically and they also failed to develop antibodies to LSDV.

2.5 Disease symptoms induced by LSDV:

Two viruses have been shown to cause lumpy skin disease-like symptoms in cattle (Weiss, 1968). Although the poxvirus, LSDV (Neethling), is generally associated with true lumpy skin disease, another virus, the Allerton herpesvirus, produces similar symptoms under field conditions (Prozesky and Barnard, 1982). Cattle infected experimentally with Allerton or

LSD (Neethling) viruses produced symptoms which, although similar, were distinguishable on the basis of lesion appearance and spread (Capstick, 1959). LSD is characterised by fever, swelling of the lymph nodes and large nodules in the skin over the whole body (Erasmus *et al.*, 1988). Soft yellow-grey nodules and ulcers also occur in the mucous membranes of the mouth, nose, respiratory tract and the reproductive organs and subcutaneous swellings of the legs are often seen (Figure 2.4 A). Seven to ten days after their first appearance, the nodules start to break away and form scabs which ultimately fall off. Early rejection of the skin lesions due to secondary bacterial infection often leaves purulent, raw ulcers and abscesses (Figure 2.4 B). The disease causes appreciable economic losses due to emaciation (excessive loss in weight), temporary and even permanent loss of milk production, sterility in bulls and damage to the hide (Green, 1959).

FIGURE 2.4. A

A cow infected with LSDV and displaying raised nodules covering the entire body. (Photo kindly supplied by Onderstepoort Veterinary Institute, Pretoria)

FIGURE 2.4 B.
A close-up view of a disrupted ulcer on the hide of a cow infected with LSDV.
(Photo kindly supplied by the Onderstepoort Veterinary Institute, Pretoria)

2.6 Control of LSDV:

As previously mentioned, certain strains of capripoxvirus infect more than one of the three natural hosts (Kitching and Taylor, 1985). Sheep, goats and cattle that have recovered from infection with capripoxvirus isolates from a heterologous host (i.e. one of the three hosts which is not a natural host for that isolate) are immune to challenge with virulent, homologous virus (Kitching, 1986a; Kitching, *et al.*, 1987). This characteristic of some capripoxvirus isolates has been successfully used in producing vaccines to control LSD, goatpox and sheeppox. Davies (1976) isolated capripoxvirus from a sheep (strain 0180) and from a goat (strain F53) in a group in which both sheep and goats were clinically infected with capripoxvirus (Kitching *et al.*, 1987). A second sheep isolate (0240) was collected later in the same outbreak. Davies attenuated the 0180 strain by 18 passages in bovine foetal muscle cells to produce a vaccine which is used in Kenya to protect sheep and goats against

Kenya sheeppox and goatpox, and cattle against LSD. The 0204 strain was passaged five times in lamb testes cells and inoculated intradermally into British sheep and goats. Only a mild local reaction resulted, without pyrexia, and the vaccine was shown to protect sheep and goats against challenge with virulent capripoxvirus isolates collected in Nigeria and India (Kitching and Taylor, 1985).

In South Africa, a LSD vaccine was developed at the Veterinary Research Institute, Onderstepoort. The vaccine was prepared in cell culture from a capripoxvirus strain (Neethling) that was attenuated through passage in embryonated eggs (as reviewed by Erasmus *et al.*, 1988). The live virus is freeze-dried and thoroughly tested to ensure it conforms to standards of safety and efficacy. The freeze-dried vaccine is stable and the efficacy is maintained for at least one year if stored at 4 °C. Calves acquire an immunity to LSD for up to six months of age from maternal colostrum of cows which have been previously immunised or which have recovered from the disease (Erasmus *et al.*, 1988). After six months, the calves are susceptible and must be vaccinated. The vaccine is administered as an injection and a single dose provides full protective immunity.

Under specific circumstances there could be considerable advantages to a dead vaccine, such as safety, stability in high ambient temperatures and its use in animals intended for export from capripoxvirus-free to capripoxvirus-enzootic areas (as cited by Kitching, 1983). However, numerous attempts to replace live capripoxvirus vaccines with dead preparations have been unsuccessful.

CHAPTER 3

AVIPOXVIRUSES

3.1 Introduction:

Avian poxvirus diseases have caused appreciable economic losses in poultry and in game birds such as quails (as cited by Winterfield and Reed, 1985). More recently, poxviruses have been identified in other bird species such as psittacines. As early as 1873, when Bollinger described the pathology of fowlpox, he mentioned the susceptibility of pheasants to the causative agent (as cited by Kirmse, 1969). Experimental transmission of avian poxviruses, especially fowlpox, to non-host wild bird species revealed differences in the degree of susceptibility of the non-host species to infection (Kirmse, 1969). This phenomenon has been used in the production of commercial vaccines such as a fowlpox virus vaccine being derived from a pigeonpox virus isolate (as cited by Ogawa *et al.*, 1993). Mammals inoculated with fowlpox virus showed no evidence of clinical disease and to date no mammalian cells have been able to support a productive infection of avian poxviruses (Taylor *et al.*, 1988). Thus certain avipoxviruses have been studied in great detail in recent years, both for their ability to cross-protect other avian species and for their development as potential recombinant vector vaccines (Winterfield and Reed, 1985; Taylor *et al.*, 1988).

3.2 Genomic structure and organisation:

As was the case with capripoxviruses, the genome structure and organisation of avipoxviruses have only recently been studied. The genome of fowlpox virus (the avipoxvirus type species) has been the most extensively studied and will therefore be reviewed as a representative of avipoxvirus genomes.

It has been shown that the fowlpox virus genome is composed of a single, double-stranded DNA molecule with covalently linked terminal hairpins. The genome is approximately 300 kb in length and consists of a 35 % guanine and cytocine (G+C) content (Szybalski *et al.*, 1963; Coupar *et al.*, 1990).

A number of genomic regions of fowlpox virus have been sequenced and have been compared with vaccinia virus. Regions have been identified containing ORFs similar to those found in vaccinia virus, as well as ORFs which appear to be unique to fowlpox virus (Tartaglia *et al.*, 1990; Coupar *et al.*, 1990). Analysis of the fowlpox virus 3.1 kbp and 6.1 kbp *Bam* HI fragments revealed extensive conservation between these fragments and the *Hin* dIII J fragment of vaccinia virus (Binns *et al.*, 1988). A TK gene, located on the *Hin* dIII J fragment of vaccinia virus, was however lacking. The fowlpox virus TK gene was mapped within the centrally placed *Pst* I D region of the fowlpox virus genome (Boyle *et al.*, 1987; Coupar *et al.*,.1990).

A 11.2 kbp *Bam* HI fragment of fowlpox virus was mapped towards one of the termini. This fragment was found to contain a number of ORFs which share homology with three predicted gene products of vaccinia virus, viz. the 42K early gene product, the 32.5K host range gene product and the 38K haemorrhagic gene product (Tomley *et al.*, 1988).

Tartaglia *et al.* (1990) sequenced and analysed the ORFs contained within a 10.5 kbp *Hin* dIII fragment of fowlpox virus and compared these ORFs with those found in the central portion of the vaccinia virus *Hin* dIII D region. Ten ORFs were identified, with eight of these being completely contained within the fragment. Five were found to share a high degree of sequence conservation at the amino acid level with vaccinia virus ORFs D4, D5, D6, D7 and D9 and the other five were found to be unique for fowlpox virus. Experiments have suggested that the shared vaccinia virus ORFs are essential for DNA replication. Putative promoter elements and early transcription termination sequences were identified within the 10.5 kbp fragment, suggesting that fowlpox virus genes are also temporally regulated.

As avipoxvirus genomes are much larger than those of other poxviruses, it is possible that they code for more proteins, or that a larger genome is required for other, as yet unknown, functions for expression in avian cells (Tartaglia *et al.*, 1990).

3.3 Characterisation of avipoxviruses:

Early attempts to differentiate avian poxviruses were made on the basis of host susceptibility, lesion characteristics on the dermal epithelium, growth on chorioallantoic membranes, cross-protection, cross-neutralisation, complement fixation, agar-gel precipitation and passive hemagglutination tests (as cited by Tripathy *et al.*, 1973). Antibody responses from the latter tests indicated that fowlpox virus was more closely related to turkeypox virus than to either pigeonpox or canarypox viruses (Tripathy *et al.*, 1973). Canarypox virus isolated from wild passerines (e.g. canaries and sparrows) proved to be highly virulent when inoculated into higher passerines and relatively inocuous in chickens, turkeys and pigeons (Giddens *et al.*, 1971). These results suggest that canarypox virus is quite distinct from fowlpox, turkeypox and pigeonpox viruses.

More recently, however, restriction enzyme analysis of viral DNA has been successfully used to distinguish avipoxvirus isolates. The genomes of fowlpox virus, pigeonpox virus, quailpox virus and other avipoxviruses, including canarypox virus have been characterised using *Bam* H1, *Eco* R1 and *Hind* III endonucleases (Ghildyal *et al.*, 1989; Schnitzlein *et al.*, 1988). Their results indicated that fowlpox virus, juncopox virus and pigeonpox virus are very closely related to each other with a probable sequence homology of greater than 80% (Ghildyal *et al.*, 1989). Quailpox virus, however, produced patterns distinct from the other species. When the immunogenic proteins were examined by immunoblotting, common as well as unique antigens were detected (Ghildyal *et al.*, 1989). As expected, the greatest disparity was between quailpox virus and the other three avipoxvirus species. These results are in agreement with cross-protection studies whereby pigeonpox virus has the ability to cross-immunise chickens against fowlpox virus, but not against quailpox or turkeypox viruses (Winterfield and Reed, 1985; as cited by Schnitzlein *et al.*, 1988).

3.4 Epidemiology and transmission

The avipoxviruses have been a successful genus in terms of their global distribution. They have been isolated from a vast number of avian species including most wild and domesticated birds (as cited by Kirmse, 1969; Giddens *et al.*, 1971; Winterfield and Reed, 1985). Although limited to replication in avian species, a number of avipoxviruses are able to naturally infect more than one avian host (Giddens *et al.*, 1971).

Mechanical, aerosol and insect transmission of poxviruses has been reported and insect transmission of avipoxviruses has been documented (Kitching and Mellor, 1986; as cited by Warner, 1968; Taylor *et al.*, 1988). The natural modes of transmission of avipoxviruses are, however, still unclear. Birds often preen themselves and each other and many sleep with their heads folded under their wings (personal observation). They also clean their beaks against branches and often share a favourite perching and roosting branch with other birds. These behavioural traits could result in mechanical and/or aerosol transmission of avipoxviruses and could explain the occurrence of pox lesions frequently associated with specific regions on birds (Figure 3.1). It is thus probable that all three modes of transmission are responsible for the spread of avipoxviruses.

3.5 Disease characteristics induced by avian poxviruses:

Fowlpox virus, pigeonpox virus and turkeypox virus usually cause a cutaneous disease of high morbidity, but of variable mortality (as cited by Giddens *et al.*, 1971). Large pocks with umbilicated centres form on the skin of fowlpox virus infected birds and are normally accompanied by lesions in the upper respiratory and digestive tract (as cited by Mockett *et al.*, 1987). The cutaneous lesions change from the initial papules to vesicles, then to pustules before finally forming a scab. The scab sloughs off releasing virus particles in intracellular inclusions which are available for transmission to other birds (as cited by Mockett *et al.*, 1987). Within the cutaneous lesions, which are generally confined to the epidermis, occur epithelial cells with pronounced hypertrophy and hyperplasia (Giddens *et al.*, 1971). The cytoplasm of these cells often contains Bollinger bodies, which are large eosinophilic inclusions composed of clusters of viral particles. Canarypox, however, produces a different intracellular disease scenario (Giddens *et al.*, 1971): The cytoplasmic inclusion bodies found in epithelial cells associated with canarypox are not as common or as distinct as the Bollinger bodies associated with fowlpox (Giddens *et al.*, 1971). This made early diagnosis of canarypox difficult.

During a study of wild birds naturally infected with canarypox virus, lesions were most commonly observed as a proliferative dermatitis around the eyes and/or base of the beak (Giddens *et al.*, 1971). The second most frequent location of lesions was in the skin of the axillary and upper pectoral regions beneath the folded wings. Often lesions of the breast were accompanied by multifocal papules on the legs. Post-mortem investigations of infected birds revealed lesion development in the respiratory tract. Complications associated with secondary bacterial or mycotic infection have been described when comparing the progression of natural poxvirus infection (Giddens *et al.*, 1971). Wild passerines, which were infected experimentally with canarypox virus, developed lesions five days afer inoculation (Giddens *et al.*, 1971). All infected birds were dead within 16 days post-inoculation.

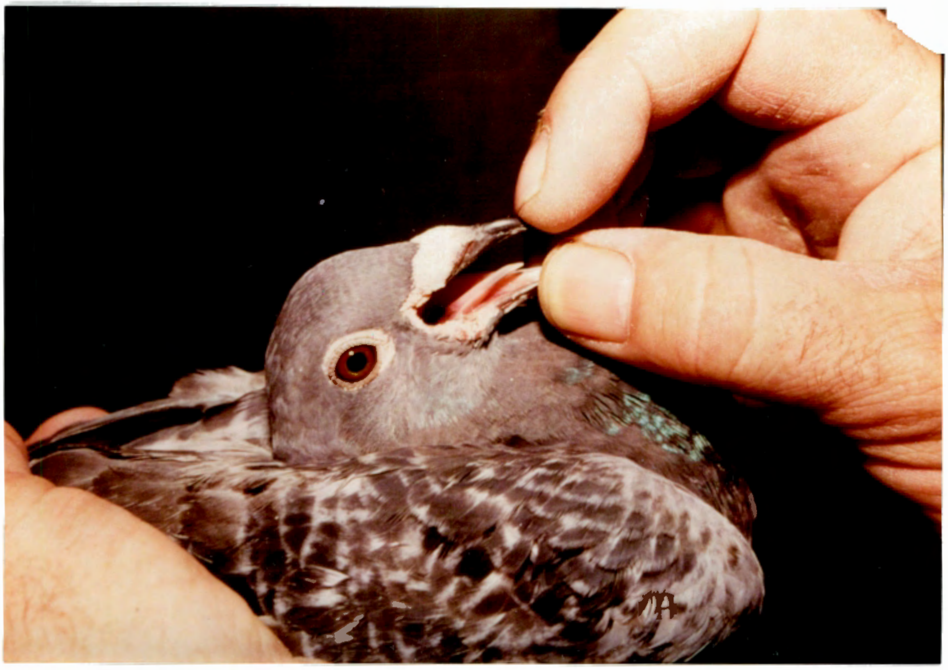

FIGURE 3.1 A
Lesions around the beak of a racing pigeon recovering from poxvirus infection.

FIGURE 3.1 B
Large pock covering the entire left eye of a juvenile Rock pigeon.

3.6 Control of avipoxviruses:

Fowlpox is one of the oldest known viral diseases affecting avian species and has been responsible for major economic losses to the poultry industry (as cited by Ghildyal *et al.*, 1989). Since the 1920's vaccination with a live attenuated fowlpox virus vaccine was found to be an effective method for control of this disease. Although fowlpox occurs world-wide, judicious farming practice, including vaccination and the use of insect-proof housing, has brought the disease under control. The vaccine is administered to day-old chicks as a web wing puncture.(as cited by Taylor and Paoletti, 1988). Older birds are re-vaccinated before laying commences. The vaccine usually consists of a mild naturally occurring strain or a strain attenuated by serial passage. In some countries attenuated strains of pigeonpox virus are used to vaccinate fowl against fowlpox (as cited by Taylor and Paoletti, 1988).

Commercial vaccines have also been available for the control of pigeonpox (as cited by Ghildyal *et al.*, 1989). In host-specificity studies, Kirmse (1969) found that pigeonpox virus failed to produce disease symptoms when inoculated into other wild and domestic birds, indicating a high degree of host specificity, although no attempts were made to infect other wild species of the *Columbidae*, which are known to be susceptible to avian poxviruses. Kirmse also noted partial immunity in pigeons infected and re-exposed to a highly virulent, monopathogenic pigeonpox strain (Kirmse, 1969). Some of the pigeons developed localised primary lesions when re-challenged three months after the initial lesions had disappeared. It was recorded in 1906 that pigeons recovered from pigeonpox were fully protected against re-infection, but that protection was of short duration (as reviewed by Kirmse, 1969). In some cases re-infection was possible after two months, but other pigeons were still immune after four to five months. A pigeonpox vaccine for use in racing pigeons in South Africa has been produced from a local pigeonpox virus strain (Jordaan) at the Onderstepoort Veterinary Research Institute (Erasmus *et al.*, 1988). As a result of the short term immunity afforded by pigeonpox, immunisation with the vaccine is recommended annually.

Winterfield and Reed (1985) tested a number of avipoxviruses in host and non-host species as potential vaccines. They found that quails, chickens and turkeys vaccinated with pigeonpox and fowlpox viruses were not protected against challenge with quailpox virus. When quails and chickens were vaccinated with quailpox virus and given pigeonpox and fowlpox challenge viruses, no protection was provided.

Clinical epornitics of pox have recently been diagnosed in psittacines (as cited by Winterfield and Reed, 1985). Psittacinepox virus applied as a vaccine in quail and chickens also failed to protect against quailpox virus challenge. However, quails, chickens and turkeys vaccinated with quailpox virus were protected against quailpox virus challenge. An isolate of psittacinepox virus, applied as a vaccine, protected chickens against challenge with the same virus isolate and also against challenge with two other psittacinepox virus isolates. When combined in a multivalent vaccine, quailpox, psittacinepox and fowlpox viruses induced excellent protection in chickens against challenge with the three respective viruses. From these studies it was concluded that quailpox virus had little, or no immunologic relationship to fowlpox, pigeonpox or psittacinepox viruses. Nor does it appear that psittacinepox virus has any antigenic relationship to fowlpox or pigeonpox viruses (as cited by Winterfield and Reed, 1985).

SECTION 2

CHARACTERISATION OF SOUTHERN AFRICAN ISOLATES OF LSDV

CHAPTER 4

DEVELOPMENT OF AN ACCURATE INFECTIVITY ASSAY FOR LSDV:

4.1 INTRODUCTION:

It was an important aim of this study to compare vaccine and field isolates of LSDV using restriction endonuclease digestion of viral DNA. LSDV does not reach the high titres experienced with other poxviruses, such as vaccinia virus when grown in cell culture (Esposito, 1981; Black, personal communication). Thus, before viral DNA could be isolated and subjected to molecular analysis, it was necessary to establish optimal conditions for the propagation and purification of LSDV. An accurate technique for determining the exact concentration of infectious virions had to be developed before optimal growth and purification conditions could be assessed. Infectivity assays were conducted in three different types of cells.

4.2 MATERIALS AND METHODS:

4.2.1 Cells used for infectivity assays:

A continuous cell line of bovine kidney cells [MDBK: American type culture collection (ATCC) catalogue, reference certified cell line (CCL) 22] were obtained from stocks maintained in liquid nitrogen (passage no. 186) in the Department of Medical Microbiology, UCT. DNA-fluorescence staining of these cells revealed the presence of mycoplasma. These were eliminated by six passages of the cells in the presence of a combination of antibiotics (tradename BM-Cyclin, Boehringer Mannheim, Germany).

Primary calf foetal kidney (CFK) and lamb testes (LT) cells were prepared as described below (4.2.3).

4.2.2. Sterile technique used for cell culture (Freshney, 1987):

A laminar flow hood (Laminaire, Model-4BH, Bino Instrumentation, Bellville, South Africa) was used at all times when dealing with culture cells or related chemicals (e.g. antibiotics). The following sterile technique was adopted:

The hood was kept free of extraneous items and the circulator fan was switched on at all times. The interior was irradiated with ultra-violet (UV) light for at least 10 minutes before the hood was to be used. The working surface inside the hood was cleaned thoroughly with 70% EtOH. All items to be used inside the hood were also cleaned with 70% ethanol (EtOH) before being returned to the hood. To ensure sterility, the operator's hands and arms were washed with soap and water and then with 70% EtOH. After work had been completed in the hood, all movable items were removed, the working surface was again washed with 70% EtOH and irradiated with UV light for at least 10 minutes.

4.2.3. Primary cell culture preparation:

Various techniques are available for producing primary cell cultures from animal tissue (Paul, 1972; Freshney, 1987) with the main variables being the source and type of animal tissue, the percentage of trypsin used and the temperature at which the tissue is trypsinised. A number of these techniques were tested and the following method was found to yield the most consistant and best cell growth:

Fresh foetal lamb or calf tissue was obtained from the State Abbotoir (Maitland, Cape Town) and placed in ice-cold phosphate buffered saline (PBS) (Appendix 1) with Penicillin-Streptomycin (PS) (2X) (Appendix 1). The kidneys or testes were washed in PBS (with 2X PS) in the laboratory and the outer connective tissue was removed using scissors and sterile technique. The tissue was then washed in PBS (with 2X PS) and cut up finely using two scapel blades. This tissue was placed in PBS (with 2X PS) and left on ice for 10 minutes (or longer if it contained a large quantity of blood). It was transferred to a small conical flask containing a magnetic bar and 1 % trypsin (Appendix 1) was added to just cover the tissue. This flask was heated to 30 °C and placed on a magnetic stirrer (Fried Electric, Haifa, Israel) to stir at 200 revolutions per minute (rpm) at room temperature for 30 minutes. This trypsin was discarded, replaced with fresh trypsin and the stirring was repeated as before. After 30 minutes the trypsin (containing digested tissue and single cells) was removed and fresh trypsin was added to the remaining tissue. The stirring was repeated and in the meantime the trypsinised tissue was diluted 1:1 in diluent, consisting of 10 mM N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and Dulbecco's modified Minimum Essential Medium (DMEM) (pH 7.4) (Appendix 1). The cells were pelleted in a Sigma 301K benchtop centrifuge (Sigma, Germany) at 1000 rpm (120g) for 10 minutes. The cell pellets were resuspended in DMEM supplemented with 10 % foetal calf serum (FCS) (Appendix 1) and were stored at 4 °C.

After 30 minutes of stirring, the freshly trypsinised tissue was removed from the flask and the cells were treated in a similar manner (as described above). The tissue remaining in the flask was subjected to two more cycles of trypsinisation. Cells recovered from each round of digestion were pooled and were then counted using a Neubauer haemocytometer. They were seeded in plastic culture flasks at a density of $1x10^5$ cells per cm^2. Fifty ml of culture medium (DMEM. 10% FCS), was added per 175 cm^2 flask (Nunclon, Nunc, Delta, Denmark). These were placed in a 5% CO_2 incubator (Queue, USA) at 37 °C for 48 hours before being examined for cell growth. Once the cells had formed a monolayer they were passaged as described below.

4.2.4. Passaging culture cells (Freshney, 1987):

The medium was removed from a culture flask which contained a confluent cell monolayer. The cell sheet was then washed with cold sterile PBS. The PBS was removed after one minute and Activated trypsin Versene (ATV) (Appendix 1) was added. The ATV was swirled over the cells and was then removed. Fresh ATV was then added. The flask was gently shaken and placed at 37 °C. Once the cells started lifting off the floor of the flask, it was removed from the incubator and the contents made up to a predetermined final volume with DMEM. 10% FCS. This volume depended on the split ratio of the cells (The split ratio of the cells refers to the number of confluent cell monolayers that can be obtained within 3-4 days after subculture of cells from one parental flask. The time taken for cells to become confluent depends on the specific cell line being used. For example, a cell line which grows quickly may be split 1:8, whereas a cell line which grows slowly may be split only 1:4. For a split ratio of 1:8, one flask of confluent cells are divided into eight flasks of the same size. If a different flask size is to be used, then the ratio must be adjusted accordingly. For example, four 150 cm^2 flasks are made from one 75 cm^2 flask for a split ratio of 1:8.). The cells were pipetted up and down in a pipette until no cell clumps were visible. The cell suspension was then divided evenly between the flasks and if necessary, extra medium was added to ensure that the cells were submerged to a depth of 4 mm. The cells were incubated at 37 °C in a 5% CO_2 incubator until confluent.

4.2.5. Source of LSDV:

The vaccine strain of LSDV (Neethling) was used for this study and all subsequent studies dealing with the culture and purification of LSDV. This isolate is derived from the original virulent LSDV (Neethling) isolate which was passaged in eggs and cell culture to produce an attenuated live vaccine at the Onderstepoort Veterinary Institute, Pretoria (as described by Weiss, 1968). The sample used in this study was kindly supplied by D. Kow (Department of Medical Microbiology, Medical School, UCT) after several passages in CFK cells.

4.2.6. Infecting culture cells with LSDV :

Virus stock was thawed at room temperature and diluted in diluent to the desired concentration. The medium was discarded from culture flasks containing a 90% confluent monolayer of cells, and enough diluent (containing virus) was added to each flask so that the diluent just covered the cells. The flasks were then incubated at 37 °C for 1 hour in a 5 % CO_2 incubator (Queue, U.S.A.) (they were rocked gently every 20 minutes). After the adsorption period, the virus was discarded and the cell sheet was covered with fresh diluent (37 °C). This diluent was replaced with DMEM. 4% FCS (or DMEM. 2% FCS for rapidly dividing cells). The flasks were then returned to the CO_2 incubator (37 °C) and the cells were monitored every day for cytopathic effects (CPE).

4.2.7 Infectivity assays:

4.2.7.1 Cell culture and virus titration:

MDBK, CFK and LT cells were each seeded into a 24-well titration plate (Nunclon, Delta, Nunc, Denmark) at a concentration of 1.1 x 10^5 cells per well (area of one well = 2.27 cm^2) in DMEM. 10 % FCS culture medium. The plates were then incubated in a 5 % CO_2 incubator at 37 °C. When the cells reached 90% confluency, the culture medium was removed. A 10-fold dilution series of a stock of LSDV was prepared in diluent. The first column (four wells per column) of each plate was used as a control (virus-free) and the remaining columns were used for consecutive dilutions. A 0.1 ml aliquot of each virus dilution was added per well. The plates were returned to the incubator. After a 60 minute adsorption period, the viral inoculum was removed, the cells were washed in PBS (37 °C) and the PBS was then replaced with 1 ml of DMEM. 4 % FCS. Incubation was continued for 3 days in a 5 % CO_2 incubator at 37 °C. The cells were viewed each day post-infection using an Olympus CK 2 inverted microscope (Olympus, Japan) for the appearance of CPE. The nature of the CPE was determined for each cell line by comparing the gross morphology of infected cells with that of non-infected cells. The results were photographed as described in Appendix 2.

4.2.7.2 Calculation of viral infectivity:

The number of foci of cells exhibiting CPE were counted in wells where between one and ten foci were present. The following techniques were then employed to obtain an accurate count of the number of CPE (foci) per well (each technique was performed on the same row of wells, in the order described below):

A. The foci were counted per well without removing the culture medium.

B. The culture medium was then removed and the cells were viewed for CPE.

C. The wells were divided into sections using a grid overlay marked on the lid of the titration plate. The use of an inverted microscope to view the cells resulted in the casting of a shadow-image of the grid over the cell sheet. The number of foci were then counted in each grid section, and these subtotals were added together to give the total number of foci per well.

D. The cells were stained with Ziehl Neelsen carbol fuchsin stain (Lennette and Schmidt, 1969).

Once the optimum conditions had been determined for counting the number of virus-infected cells per well, the concentration of the original virus stock could be accurately determined as follows:-

Assuming that one focus of infected cells (or plaque) in a cell sheet represents one infectious viral particle, then the virus concentration, quoted as plaque forming units per millilitre (p.f.u./ml), was calculated as follows:-

p.f.u./ml = X x Y x Z

where

X = the reciprocal of the dilution at which the number of foci averaged between one and ten per well

Y = the average number of foci per well inoculated with the dilution in X

Z = the reciprocal of the inoculum volume e.g. in this study 0,1 ml of each virus dilution was plated per well, therefore Z = 10

4.2.8 Techniques used to confirm the presence of LSDV in infected cells:

4.2.8.1 Indirect Immunoperoxidase staining technique:

In order to confirm whether the CPE were due to the presence of virus or due to other factors, the cells were stained using an indirect immunoperoxidase staining technique (Payne *et al.*, 1986).

MDBK cells were grown on glass coverslips in 6-well titration plates (Nunclon, Delta, Nunc, Denmark) (area per well = 5.3 cm^2). They were infected with virus as described (4.2.6.) and left in a CO_2 incubator at 37 °C for 1-3 days. The cells were then washed three times in PBS after the medium was removed. They were fixed in cold acetone for 5 minutes and were then air dried (after this stage they were always maintained in a moist environment). The fixed cells were washed in PBS and non-specific protein binding sites were blocked by immersion in 2 % ovalbumin (Sigma, U.S.A.), in PBS, for 20 minutes.

Thereafter cells were covered with hyperimmune bovine anti-LSDV serum [diluted 1:100 in 2% ovalbumin (in PBS)] for 1-2 hours at room temperature. After washing three times in PBS with 0.5% Triton X-100 (BDH, Poole, England), cells were covered with peroxidaseconjugated rabbit anti-bovine immunoglobulin-G (DAKO-immunoglobulins, Denmark) (diluted 1:400 in PBS) for 1 hour at room temperature. The Triton X-100 wash was repeated and the cells were exposed to substrate solution [(10 mg DAB (3,3'- diamino benzidine tetrahydrochloride) (Sigma, U.S.A.), 20 ml PBS and 40 μl 30 % hydrogen peroxide (BDH, Poole, England)] for 5 minutes.

After being washed gently in tap water, the cells were counter-stained with haemotoxylin (Gurr's, London) for 30 seconds and residual stain was removed by washing under running tap water. The cells were dehydrated by successive immersion in: 50 % ethanol - 70 % ethanol - 95 % ethanol - absolute ethanol - xylol (Merck, Germany) - xylol. Stained coverslips were finally mounted on glass slides using Entellan mounting fluid (Merck, Germany) and were viewed using a Leitz SM-LUX light microscope (Leitz and Wetzlar, Germany).

4.2.8.2 DNA-detection fluorescent staining:

Along with iridoviruses, poxviruses are unique amongst animal DNA viruses in their ability to replicate in the cytoplasm of infected cells (Moss, 1990). When poxvirus-infected cells are stained with a DNA-specific fluorescent stain and viewed using a fluorescence microscope, the cell nuclei fluoresce strongly, in addition to discrete areas in the cytoplasm. These cytoplasmic foci of fluorescence are due to sites of viral replication, called virus factories, containing large amounts of viral DNA.

The technique of Chen (1977) was used to stain infected and uninfected MDBK cells:

Chemical solutions:

Stock solution of Bisbenzamide fluorochrome stain: 5 mg of Hoechst no. 33258 powder (Hannover, Germany) was dissolved in 100 ml PBS. The solution was mixed thoroughly at 22-25 °C with a magnetic stirrer for 30 minutes and the bottle containing the stain was then wrapped in aluminium foil and stored in the dark at 2-8 °C.

Working solution of Hoechst no. 33258 stain: 1 ml of stock solution was dissolved in 100 ml of PBS (final concentration of 0.5 μg/ml). It was then stored in the dark at 2-8 °C after first being wrapped in aluminium foil. Before use, the solution was mixed thoroughly with a magnetic stirrer at 22-25 °C for 30 minutes.

Mounting fluid: 22,2 ml citric acid (0,1 M) (Merck, Germany), 27,8 ml disodium phosphate (0,2 M) and 50 ml glycerol were mixed. The pH was adjusted to 5,5 and the mounting fluid was stored at 2-8 °C.

Fixative: Glacial acetic acid - methanol (1:3)

Method:

Cells were grown on glass coverslips in 6-well titration plates (Nunclon, Delta, Nunc, Denmark) (area per well = 5.3 cm^2) and infected as described in 4.2.6. At 24 hour intervals the growth medium was removed from one well and the cells were fixed for 5 minutes with 3-4 ml of fixative. The fixative was removed and fixation was repeated for 10 minutes. The cells were air dried for 30 minutes, then covered with Hoechst stain for 30 minutes. The stain was then removed by washing the cells three times in deionised water. The coverslip was mounted on a glass slide in a drop of mounting fluid and the cells were viewed using a fluorescence microscope [Leitz SM-LUX microscope (Leitz and Wetzlar, Germany)] with UV light at a wavelength of 360 nm.

4.3 RESULTS:

4.3.1 Optimal conditions for virus titration:

4.3.1.1 Choice of cells:

Three different kinds of cells were evaluated in respect of the ease and reproducibility with which LSDV infection could be detected by criteria of CPE.

Comparisons of uninfected LT, CFK and MDBK cells with cells infected with LSDV are shown in Fig 4.1, A to F. Early plaque formation, characterised by foci of rounded cells, were observed in infected CFK (Fig. 4.1 B) and LT (Fig. 4.1 D) cells from three days post-infection. These foci eventually developed into irregular plaques, with the rounded cells lifting from the cell sheet, leaving a hole, by four to five days post-infection.

Foci, resembling raised ridges of cell growth, were observed from two days post-infection when MDBK cells were infected at 80 - 90 % confluency and three days post-infection when infected at 90 - 100 % confluency. Figure 4.1 F shows the presence of these foci as compared to the uninfected MBDK cells which lack them (Fig 4.1 E). These foci remained stable for a number of days, although they were most clearly discernible from the cell sheet until approximately four days post-infection by which time the rest of the cell monolayer had started producing a ridged appearance.

4.3.1.2 Calculating the concentration of infectious virions:

The foci produced in MDBK cells were much more easily recognisable than those produced in CFK and LT cells and developed more rapidly. The MDBK cells were also easier to maintain and grew more reliably than primary cells. For these reasons it was decided to use MDBK cells for virus titration studies.

A titration plate, consisting of a 90 % confluent monolayer of MDBK cells was infected with a 10-fold dilution series of a stock of LSDV as described (4.2.7.1), except that 6 wells were used for each dilution. The virus titre was calculated after 3 days.

The number of foci, in each of the 6 wells, at the 10^{-4} dilution, averaged between 1 and 10 per well. The optimal method for counting plaques was determined by comparing each of the four techniques described (the same row of six wells was used for each technique). The results are presented in Table 4.1:

Counting the foci without removing the cell culture medium or using a grid overlay (Technique A) gave rise to a low average count (3.0 p.f.u./well) with the greatest standard deviation (1.41). Removal of the cell culture medium and the use of a grid overlay (Technique C) produced the highest average count (6.3 p.f.u./well) with the lowest standard deviation (1.21). Removal of the cell culture medium without the use of a grid overlay (Technique B) resulted

TABLE 4.1: Comparisons of techniques used to titrate infectious LSDV.

TECHNIQUE [a]	NO. OF FOCI PER WELL AT 10^{-4} DILUTION OF LSDV.						AVERAGE NO. OF FOCI	STANDARD DEVIATION
	WELL NO.							
	1	2	3	4	5	6		
A	3	5	4	1	3	2	3.0	1.41
B	7	6	6	4	8	6	6.2	1.33
C	7	7	7	4	7	6	6.3	1.21
D	NR	NR	NR	NR	NR	NR	-	-

NR = not readable a A = foci counted in medium, B = foci counted with medium removed
C = foci counted with grid overlay, D = foci counted using stain

in a comparable average count (6.2 p.f.u./well), but with a greater standard deviation (1.33). The foci were uncountable when using the Ziehl Neelsen carbol fuchsin stain (Technique D) as the stain gave the entire cell monolayer a ridged appearance.

The data obtained using technique C was therefore used to determine the concentration of infectious virions in the original stock:

Infectious LSDV $= X \times Y \times Z$
$= 6{,}3 \times 10^4 \times 10$
$= 6{,}3 \times 10^5$ p.f.u./ml

4.3.2. Techniques used to confirm presence of LSDV in infected cells:

Confirmation of the viral nature of the foci produced by infected MDBK cells was obtained using two staining techniques; one which specifically detects the presence of viral antigen and the other which indicates the presence of DNA (both viral and cellular).

4.3.2.1 Immunoperoxidase staining of MDBK cells:

Fig. 4.2 B-D indicates that the raised ridges or foci seen on MDBK cells infected with LSDV are due to the presence of virus, and not other factors, as these ridges have been specifically labelled with anti-LSDV hyperimmune serum. Non-infected cells, and areas on infected cells which show no ridging, were not tagged with LSDV-specific antibody (as no brown stain is observable in these regions). Infected cells labelled with control, non-immune serum also failed to produce a brown stain (Fig. 4.2 A)

Viral antigen could be specifically labelled from as early as 24 hours post-infection in the cytoplasm of infected cells (Fig. 1.2 B). A progressive increase in the number of cells staining positive for viral antigen and a corresponding increase in the intensity of the peroxidase staining is shown in Fig. 4.2 C (48 hours post-infection) and Fig. 4.2 D (72 hours post-infection).

4.3.2.2 DNA-fluorescence staining of MDBK cells:

Staining of infected cells with a DNA-specific fluorescent stain (Hoechst no. 33258) revealed the presence of brightly fluorescing nuclei and discrete areas of fluorescence in the cytoplasm (Fig. 4.3 B) which were absent in non-infected control cells (Fig. 4.3 A). The possibility of mycoplasma contamination was ruled out as the cells and virus had been treated with BM-Cyclin (Boehringer Mannheim, Germany) for the eradication of mycoplasma. Electron microscopy also confirmed the absence of mycoplasma from virus stocks and infected cells. The cytoplasmic fluorescent "spots" were thus probably the factory sites associated with virion replication . These viral "factories" were present within 24-hours post-infection.

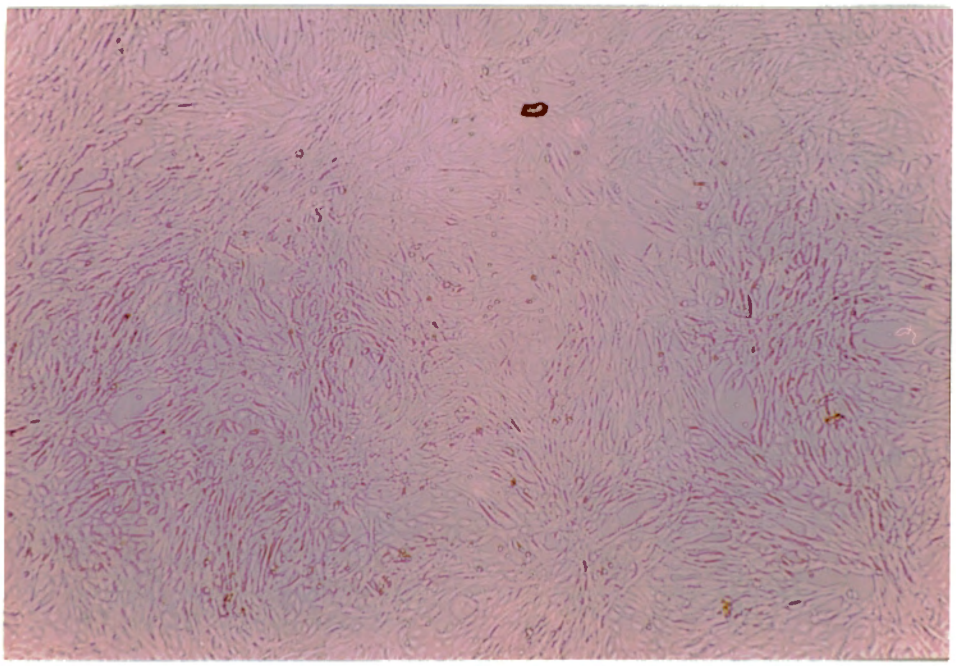

Figure 4.1 A.
Monolayer of uninfected CFK cells. (X 150)

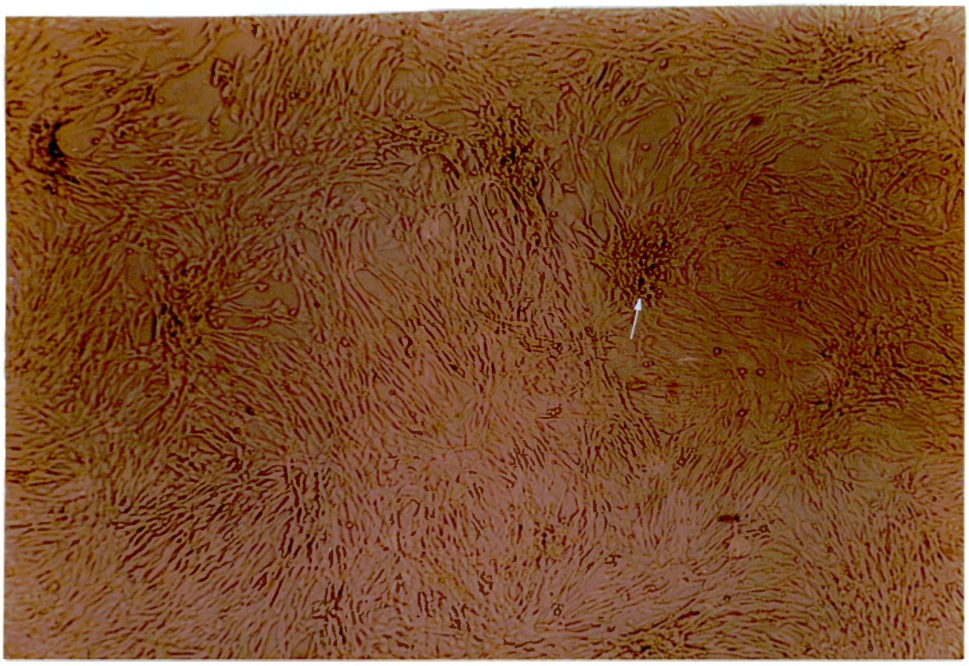

Figure 4.1 B.
Monolayer of CFK cells infected with LSDV (10^{-3} dilution). Three days post-infection. Note the early stage of diffuse plaque formation (arrow) characterised by foci of cells with atypical morphology. (X 150)

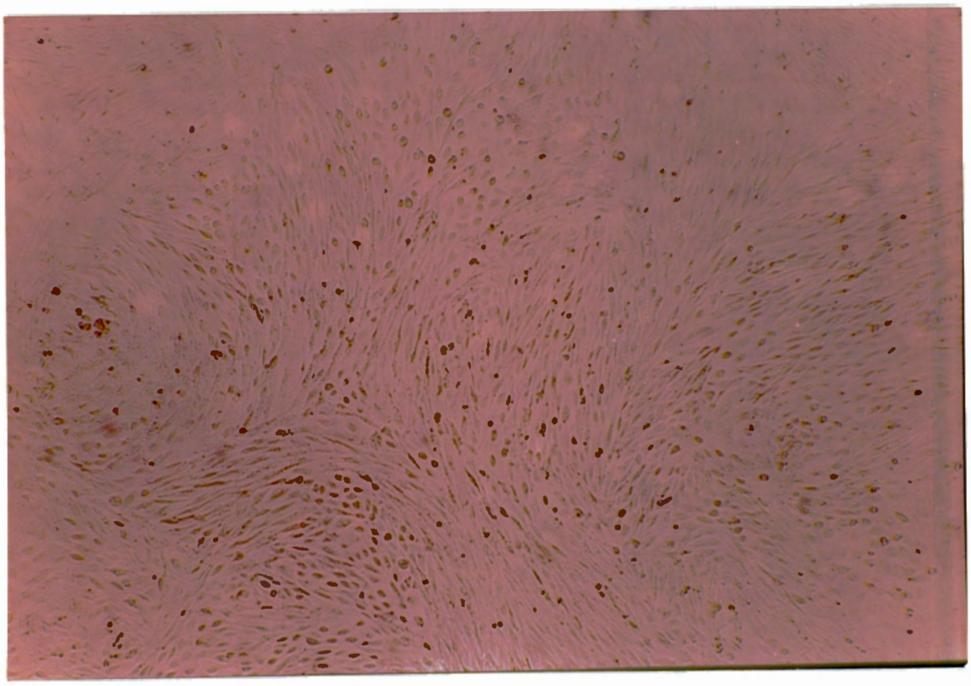

Figure 4.1 C.
Monolayer of uninfected LT cells. (X 150)

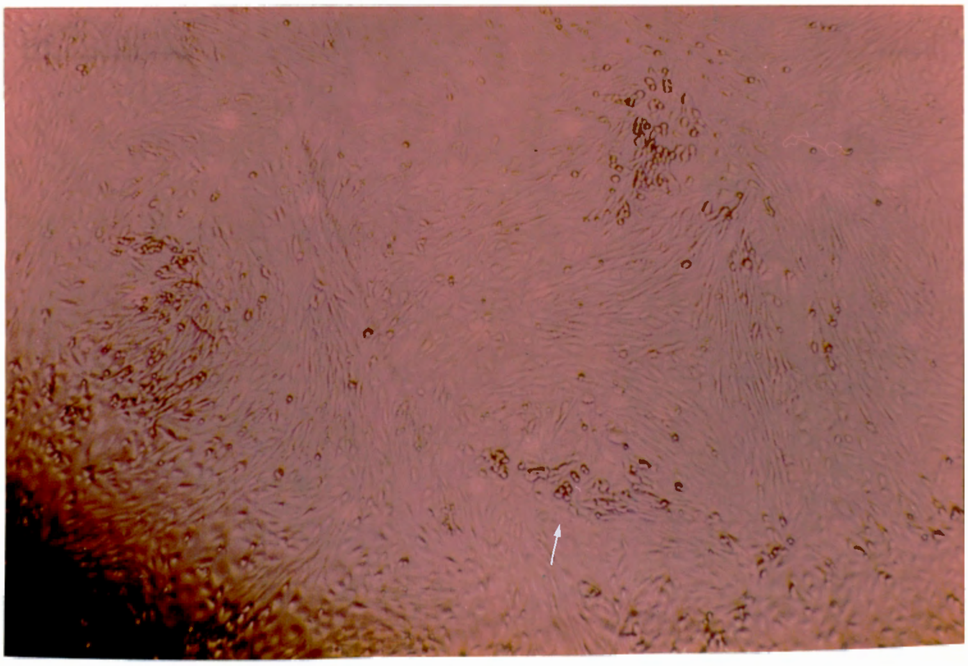

Figure 4.1 D.
Monolayer of LT cells infected with LSDV (10^{-3} dilution). Three days post-infection. Note the early stage of plaque formation (arrow) characterised by foci of rounded cells (X 150).

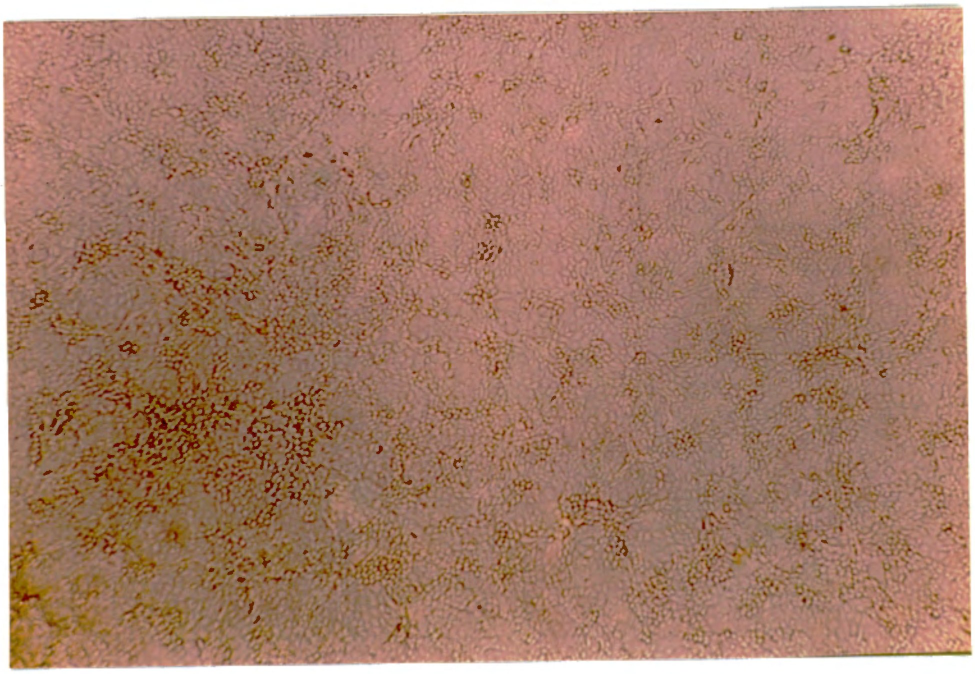

Figure 4.1 E.
Monolayer of uninfected MDBK cells. (X 150)

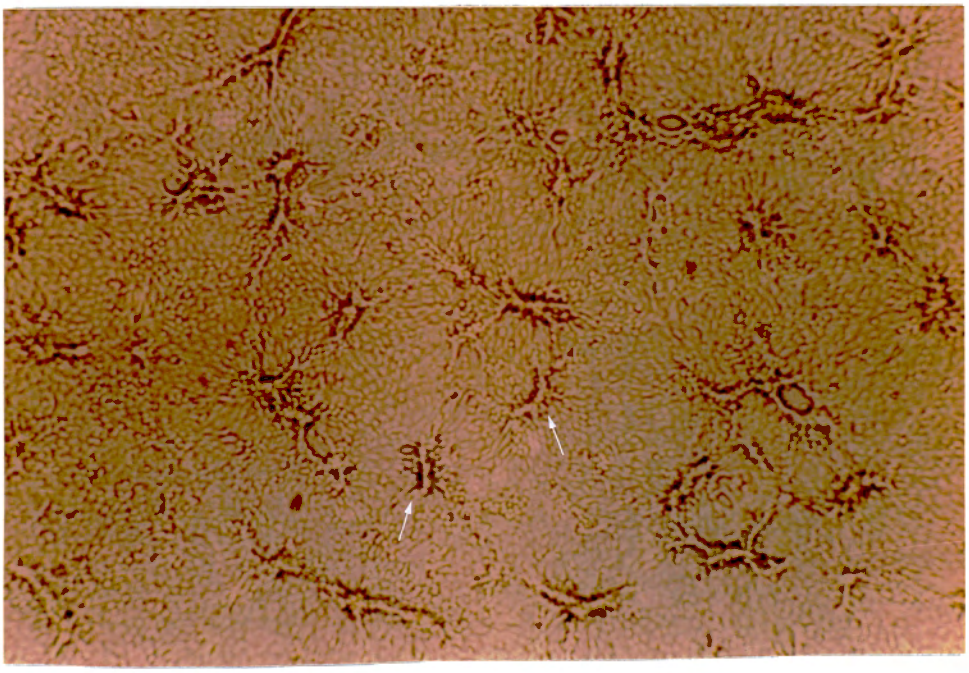

Figure 4.1 F.
Monolayer of MDBK cells infected with LSDV 3 days post-infection (10^{-2} dilution). Note the prominent foci (arrows). (X 150)

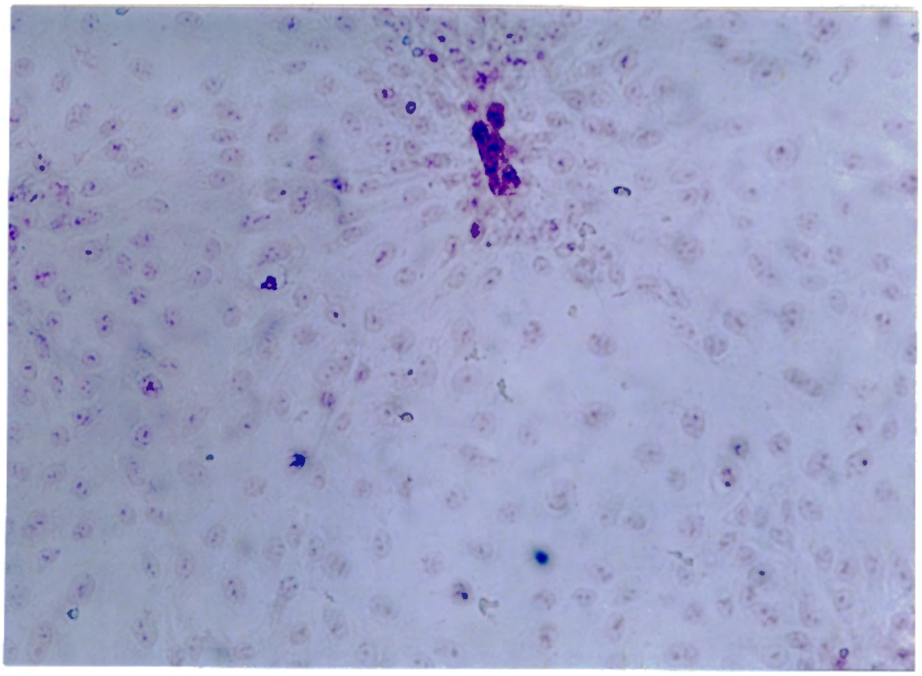

FIGURE 4.2. A

MDBK cells infected with LSDV (3 days post-infection) after incubation with serum from an uninfected cow (diluted 1/20). Cells were stained using an indirect immunoperoxidase technique. (X 300).

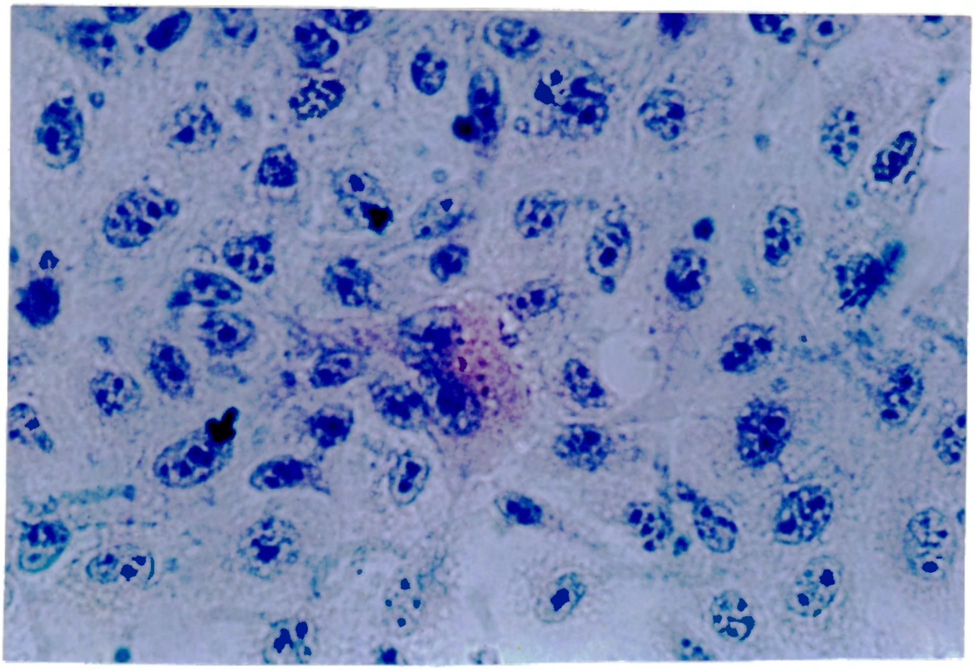

FIGURE 4.2. B

MDBK cells infected with LSDV (1 day post-infection) after incubation with immune serum from a cow infected with LSDV (diluted 1/100). Cells were stained using an indirect immunoperoxidase technique. (X 600).

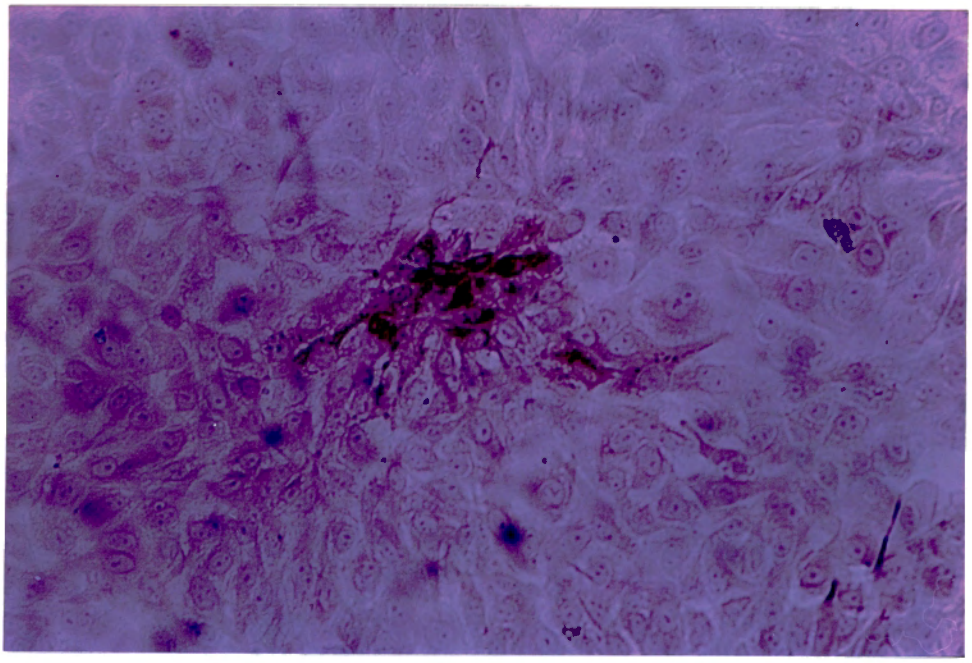

FIGURE 4.2. C
MDBK cells infected with LSDV (2 days post-infection) after incubation with immune serum from a cow infected with LSDV (diluted 1/100) and stained using an indirect immunoperoxidase technique. (X 300).

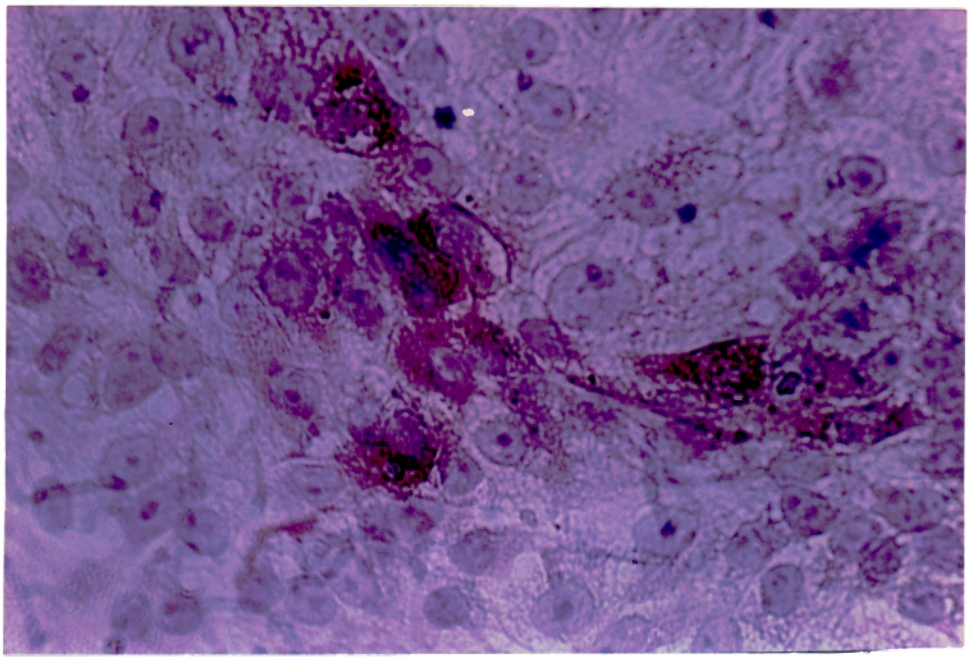

FIGURE 4.2. D
MDBK cells infected with LSDV (3 days post-infection). Cells were exposed to immune serum from a cow infected with LSDV (diluted 1/100) and were stained using an indirect immunoperoxidase technique (X 600).

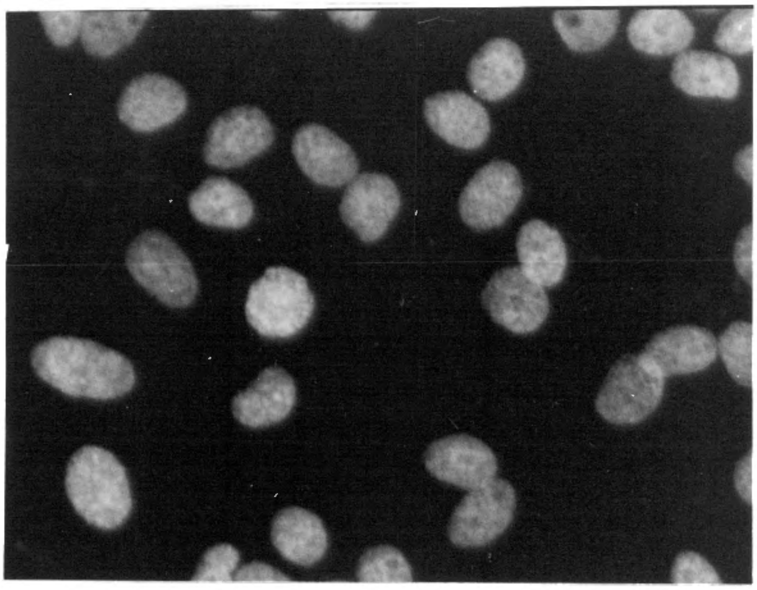

FIGURE 4.3. A
Uninfected MDBK cells stained for the presence of DNA using the Hoechst DNA-fluorescence stain. Only the cell nuclei fluoresce strongly. (X 600)

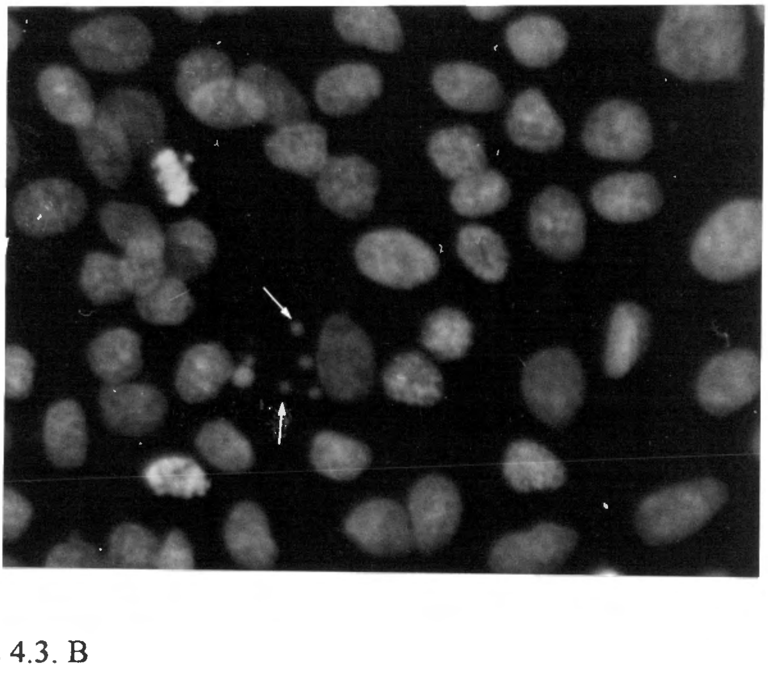

FIGURE 4.3. B
MDBK cells infected with LSDV (2 days post-infection) and stained for the presence of DNA using the Hoechst DNA-fluorescence stain. Arrows indicate discrete foci of DNA in the cytoplasm. (X 600)

4.4 DISCUSSION AND CONCLUSIONS:

It was found that LSDV could be propagated in three separate types of cells, viz. primary lamb testis (LT), primary foetal calf kidney (CFK) and a continuous line of bovine kidney cells (MDBK). A number of techniques were compared for the propagation of primary culture cells, as high yields of virus have been obtained from these cells (refer to Chapters 5 and 6). It was found that the use of foetal tissue yielded the best cultures, as these cells were the least differentiated and able to replicate for a number of generations in cell culture. A compromise was reached between the use of low temperatures for trypsinisation (4 °C) and high temperatures (37 °C), as neither of these techniques were found to yield cultures of a high viability. Tissue was heated to 30 °C and was left to cool to room temperature while being stirred with a magnetic stirrer. This method yielded the desired viability of cells with cultures attaining confluency within two days.

Evidence of LSDV infection was found in the appearance of distinctive CPE. After comparing the CPE produced by LSDV in the 3 cell types, it was decided that MDBK cells were the best suited for titration of LSDV. In MDBK cells, LSDV produces characteristic raised ridges or foci that appear within 2-3 days post-infection, whereas CPE were only detectable in infected CFK and LT cells from 3 days post-infection, and took 4-5 days to develop into discernible plaques. In addition, the foci produced by infected MDBK cells are more readily detectable than the CPE produced in LT and CFK cells, and the continuous cell line nature of the MDBK cells made them more readily available than primary cells. MDBK cells were therefore utilised for all virus titrations.

The optimal method for counting viral foci was determined using a number of different techniques. Conventional staining with the Ziehl Neelsen carbol fuchsin stain was found to mask the location of the viral foci as the stain gave the entire cell monolayer a ridged appearance. Thus foci were counted on unstained cells. Leaving the culture medium in the wells led to an inaccurate count as the medium produced a ring of shadow around the edge of the wells making it impossible to see foci falling within this ring. Removing the medium was found to eliminate this shadow and revealed foci previously hidden by the shadow. The use of a grid overlay yielded more consistent results as this divided the wells into smaller sections which could be scanned without the risk of counting the same foci twice or missing foci altogether. Removal of culture medium and division of the wells into sections using a grid overlay was thus found to be the most consistant and accurate technique for determining the exact number of foci per well.

A formula comprising the average number of foci on a number of wells, multiplied by both the reciprocal of the dilution, and the reciprocal of the inoculum volume, was used to determine the concentration of infectious virions in infectious units/ml.

Other factors exist which might have produced cytopathic effects in the MDBK cells, such as mycoplasma. However, mycoplasma contamination of cells and virus stocks was eliminated with the use of antibiotics. Confirmation of the localisation of viral antigen to the foci observed in infected MDBK cells was obtained using an indirect immunoperoxidase staining technique and LSDV-specific bovine hyperimmune serum. Viral antigen was detectable as early as 24 hours post-infection with copious amounts present in the cytoplasm of infected cells by 3 days post-infection. The DAB staining was restricted to foci, and control uninfected cells were not stained. This focus localisation of DAB also indicated a lack of the low level of endogenous peroxidase activity that is said to be associated with some cell lines.

Further evidence of LSDV infection in MDBK cells was obtained using the Hoechst DNA fluorescence stain. In addition to fluorescence of cellular DNA in cell nuclei, brightly fluorescing foci were observed in the cytoplasm of infected cells. Their appearance in the cytoplasm supports their viral nature as poxviruses are unique among DNA animal viruses (including iridoviruses) in their ability to replicate in the cytoplasm of infected cells (Moss, 1990). These DNA foci were very prominent by 24 hours post-infection which suggests that viral replication occurs in less than 24 hours. The absence of mycoplasma was confirmed by the fact that no DNA fluorescence was seen in the cytoplasm or at the cell surface of uninfected MDBK cells.

CHAPTER 5

YIELD OF INFECTIOUS VIRUS UNDER "STANDARD" CULTURE CONDITIONS:

5.1 INTRODUCTION:

Once an accurate technique had been determined for calculating the concentration of infectious virions, it was possible to study the growth characteristics and infectivity of LSDV. A number of factors, such as temperature and type of cell line, influence the yield of virions produced in infected cells. It was the aim of this study to optimise the conditions necessary to produce the maximum yield of virions in cell culture. However, before these conditions could be determined it was necessary to establish a set of "standard" growth conditions. These "standard" growth conditions could then be used as a guide in establishing whether altering one or more of the culture conditions had a negative or positive effect on the yield of LSDV virions.

The growth conditions were selected from reports on a number of techniques used to grow poxviruses, including capripoxviruses (Mackett et al., 1985; Esposito et al., 1981; Kitching et al., 1986). The "standard" conditions chosen were as follows: primary LT cells for the propagation of virions, a DMEM. 4 % FCS culture medium, the vaccine strain of LSDV (Neethling) as inoculating virus, a virus adsorption period of one hour and an incubation temperature of 37 °C.

The propagation of LSDV under these culture conditions was determined by calculating the change in titre of both intracellular and extracellular virions over a period of seven days and then plotting the total number of infectious units against time, on a graph.

5.2 MATERIALS AND METHODS:

Ten 25 cm^2 cell culture flasks (Nunclon, Nunc, Delta, Denmark) were seeded with primary LT cells in a DMEM. 10 % FCS culture medium (Appendix 1). The cells were incubated at 37 °C in a CO_2 incubator. Once 90 % confluent, the cells in each flask were infected with 1 x 10^6 p.f.u. from a freshly prepared stock of LSDV in 1 ml of diluent (Appendix 1). After a 60 minute adsorption period at 37°C, the virus inoculum was removed and the cells were washed with 1 ml of prewarmed diluent (37 °C). This diluent was replaced with 4 ml of DMEM. 4% FCS. At this stage one flask was removed and the contents titrated to establish the baseline (time = 0 hours post-infection) of infectious particles. The other flasks were returned to a CO_2 incubator for further incubation and one flask was removed at intervals of between 12 and 48 hours thereafter.

The presence of intracellular and extracellular infectious virus in each flask was assessed in the following way:

The medium was removed to a Universal bottle. The cells were washed in 2 ml of PBS (4°C). The PBS was discarded and the cells were washed in 2 ml trypsin (0,05%). This trypsin was discarded and was replaced with 1 ml trypsin (0,05%). The cells were incubated at 37°C until they detached from the flask. These cells were removed to a Bijou bottle (labelled CELLS) and 1 ml of DMEM. 4% FCS was added to the flask to wash off remaining cells. This wash was pooled with the cells in the Bijou bottle.

During this time, the medium in the Universal bottle was centrifuged at 1500 rpm for 10 min at 4°C in a Sigma 301K benchtop centrifuge, to sediment the cells. The supernatant fluid was then removed to a sterile bottle (labelled MEDIUM) and the cell pellet was resuspended in 1 ml of McIlvain's buffer (Appendix 1) and pooled with the rest of the cells. Both medium and cells were freeze-thawed three times between -20 °C and 4 °C to release virions from cells and the volume of each sample was adjusted to 6 ml with McIlvain's buffer.

An aliquot was removed from each sample for titration in MDBK cells (Chapter 4, 4.2.7.1) in 24-well titration plates (Nunclon). A 0,5 $\log_{10}$ dilution series was prepared of each sample (i.e. 1 ml of virus was diluted in 2.1 ml of diluent for each dilution step). Four wells were used per dilution and 0.1 ml of virus inoculum per well. A control row of wells (consisting of uninfected cells) was placed nearest the lowest virus dilution on each plate. The plates were incubated for 3 days at 37 °C and virus titre was calculated as follows:

Virus titre was determined by counting the number of foci in each of the 4 wells at the virus dilution that resulted in between 10 and 200 foci. The presence of intracellular and extracellular infectious virus was assessed in the following way:

A grid overlay was used (as described in 4.2.7.2) to obtain the most accurate count. The average number of foci per dilution was multiplied by the reciprocal of the dilution (termed the dilution factor) and the reciprocal of the inoculum volume. The resulting value (p.f.u./ml) was multiplied by the total volume of each virus sample (i.e. 6 ml) to give the total number of infectious particles per sample. These values were plotted against time (in hours) post-infection. Standard deviation values were also calculated for the distribution of foci in each set of 4 wells, in order to gauge the degree of variation in p.f.u. per dilution.

5.3 RESULTS:

A measure of the ability of LSDV to replicate in LT cells was obtained by infectivity titrations of both intracellular and extracellular virions, harvested at intervals over a seven day period. Results are presented in Tables 5.1 and 5.2 respectively, and are graphically illustrated in Fig. 5.1.

Each flask was inoculated with 1 x 10^6 p.f.u. of LSDV. Immediately after the viral inoculum was removed from the cells and they had been washed (0 hours post-infection), some infectious virus (9.8 x 10^3 p.f.u.) was recovered from the culture medium. This probably represents virus which had either loosely attached to the walls of the plastic flask, or was loosely bound to cells and became detached after the diluent wash and before the medium was removed from the flasks.

At the end of the adsorption period, 3.9 x 10^4 p.f.u. of infectious virions were recovered from the cellular sample. Uncoating of virions commences soon after they enter the cell, thus it is unlikely that internalised virus particles could be recovered from the cells and remain infectious. Therefore the infectious virus released from the cell sample at this stage probably represents virions that had attached to the outside of cells, but had not penetrated cell membranes.

Within the first 26 hours of infection, there was no significant change in the titre of extracellular virus (Table 5.1), but the amount of infectious virus recovered from the cells had increased approximately 35-fold (Table 5.2). These results suggest that viral replication had occurred within this time period, but that none of the progeny virions had been released from infected cells. The first indication that infectious virions were being released from the

TABLE. 5.1 Titre of infectious extracellular LSDV virions in relation to time after infection.

TIME POST INFECTION (HOURS)	NO. OF FOCI PER WELL				X. AVERAGE NO. OF FOCI PER WELL. [a]	Y. DILUTION FACTOR	TOTAL NO. OF FOCI. [b]
	WELL NO.						
	1	2	3	4			
0	23	14	14	17	17.0 (± 4.3)	9.61	9.80×10^3
26	10	6	7	5	7.0 (± 2.2)	9.61	4.04×10^3
52	23	25	16	12	19.0 (± 6.1)	29.8	3.40×10^4
68	36	35	36	34	35.3 (± 1.0)	92.4	1.96×10^5
75	19	43	37	38	34.3 (±10.5)	92.4	1.90×10^5
80	17	31	22	14	21.0 (± 7.4)	286	3.60×10^5
94	18	30	35	16	25.0 (± 9.2)	286	4.29×10^5
105	41	44	43	33	40.3 (± 5.0)	286	6.92×10^5
123	32	72	55	43	50.5 (±17.5)	286	8.67×10^5
168	57	65	34	37	48.3 (±15.2)	286	8.29×10^5

a = standard deviation values in brackets

b = X x Y x 6 x 10

TABLE 5.2 Titre of infectious intracellular LSDV virions in relation to time after infection..

TIME POST INFECTION (HOURS)	NO. OF FOCI PER WELL				X. AVERAGE NO. OF FOCI WELL WELL. [a]	Y. DILUTION FACTOR	TOTAL NO. OF FOCI. [b]
	WELL NO.						
	1	2	3	4			
0	16	22	20	30	22.0 (± 5.9)	29.8	3.93 x 10^4
26	118	69	82	64	83.3 (± 24.4)	286	1.43 x 10^6
52	29	24	34	34	30.3 (± 4.8)	887	1.61 x 10^6
68	35	29	25	32	30.3 (± 4.3)	887	1.61 x 10^6
75	69	44	36	40	47.3 (± 14.9)	887	2.58 x 10^6
80	60	74	80	50	66.0 (± 13.6)	2751	1.09 x 10^7
94	148	151	171	130	150.0 (± 16.8)	2751	2.48 x 10^7
105	78	89	104	86	89.3 (± 10.9)	2751	1.47 x 10^7
123	80	94	95	79	87.0 (± 8.7)	2751	1.44 x 10^7
168	36	53	61	47	49.3 (± 10.6)	2751	8.14 x 10^6

a = standard deviation values in brackets.

b = X x Y x 10 x 6

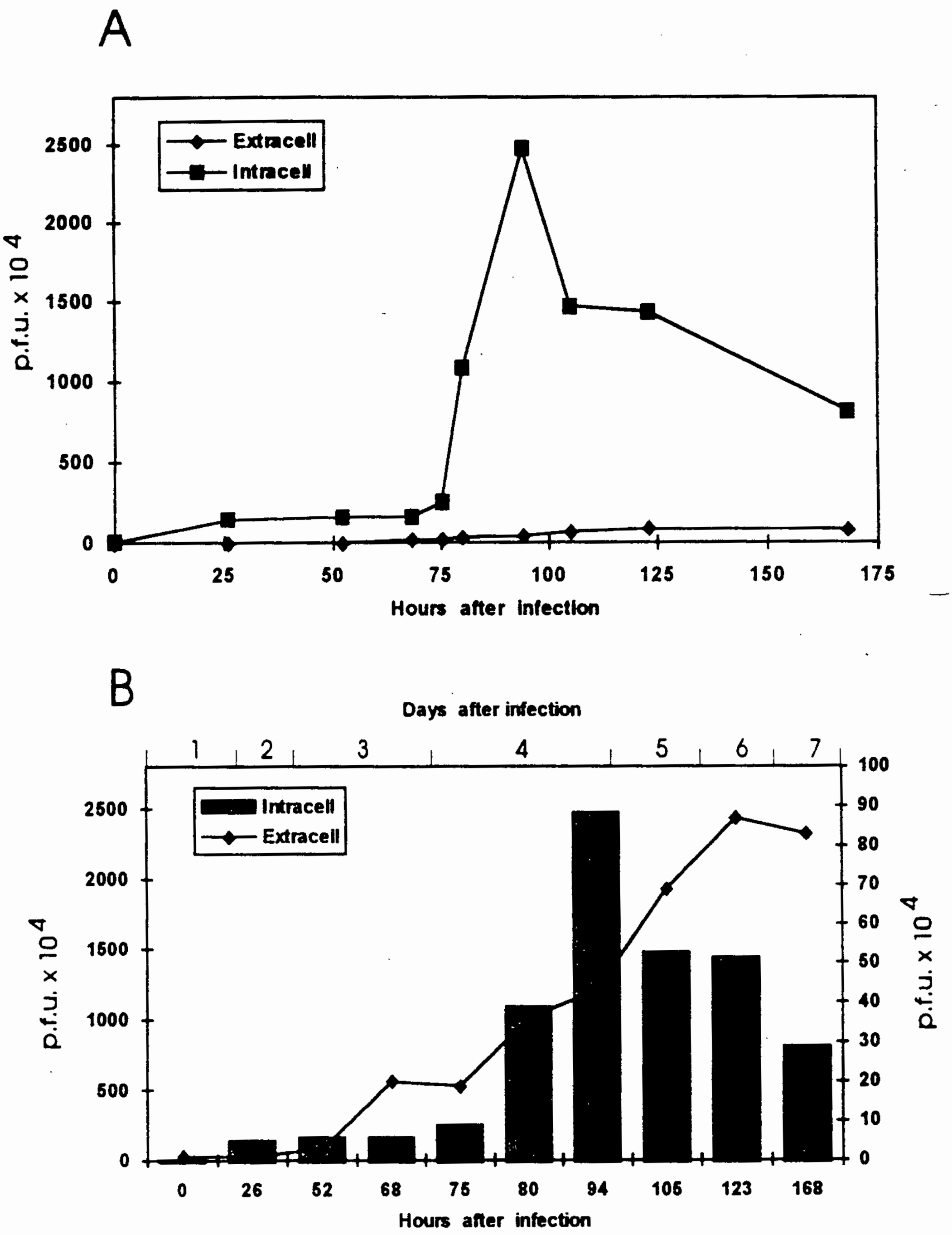

FIGURE . 5.1

Yields of infectious intracellular and extracellular LSDV particles (given as total p.f.u. x 10^4) measured over a 7 day period after infection. **A**; Presentation of data using the same scale to represent both intracellular and extracellular particles. **B**; Data presented on two scales to illustrate the smaller increases in titre of the extracellular particles (right-hand axis). Total yields of intracellular particles (left-hand axis) are represented as columns.

cells was seen at 52 hours after infection, and thereafter the titre of virus in the medium increased at a low but steady rate with approximately 2 x 10^5 p.f.u. of virus being added to the medium during each 24 hour period, up to day 6 (Fig. 5.1 B).

The number of infectious virions that could be recovered from the infected cells remained at a fairly constant level (about 1.6 x 10^6 p.f.u.) for the second and third days, but then increased rapidly to reach a peak of 2.4 x 10^7 p.f.u. by 94 hours after infection (Fig. 5.1 A). The large increase in intracellular virus was not reflected in the number of extracellular particles. Rather, as already stated, virions escaped from the cells at a constant rate, suggesting that cellular mechanisms or components dictate the rate of virion release. Almost 98% of the infectious virus recorded at 94 hours remained intracellular. From 5 days after infection, a gradual decrease in titre of intracellular virus was observed. A possible explanation is that late in infection, mature particles become trapped within intracytoplasmic inclusions, and upon lysis of the cell these particles remain clumped. This would effectively serve to reduce the number of infectious units in the cell lysate. Thus the total p.f.u. need not necessarily reflect the number of complete infectious virions.

5.4 DISCUSSION:

Although capripoxviruses have been propagated in culture by a number of other investigators (Weiss, 1968; Kitching *et al.*, 1986; Black *et al.*, 1986), no special attention has been given to the optimal culture conditions for LSDV. In this study, it was possible to establish baseline expectations for the propagation of LSDV in cell culture using a set of "standard" conditions. It was found that with an input virus titre of 1 x 10^6 p.f.u. per flask of primary LT cells, a maximum yield of 2.5 x 10^7 p.f.u. of infectious LSDV (pooled intracellular and extracellular virus) could be recovered at 94 hours post-infection. The majority (98%) of progeny virus remained intracellular, although a small proportion was recoverable from the medium. Esposito *et al.* (1981) made similar observations with vaccinia virus propagated in various cell cultures. The ultimate aim of the present thesis was to perform comparative analyses on the genomes of a number of different isolates of LSDV. This necessitates obtaining high concentrations of pure viral DNA. In view of the fact that very few virions are released from infected cells during the cycle of virus replication, it would be necessary to rupture the infected cells to release intact virus particles which would constitute the source of viral DNA. The manner in which this is achieved is discussed in Chapter 7. Over and above the cellular retention of large amounts of infectious virus, it is probable that the cellular fraction will contain a fair percentage of viral genomes which are incompletely packaged (and therefore uninfectious) but which could nonetheless serve as a source of viral DNA for molecular studies.

CHAPTER 6

ESTABLISHING OPTIMAL CULTURE CONDITIONS FOR LSDV

6.1 INTRODUCTION:

Once the propagation of the vaccine strain of LSDV had been established using a set of "standard" culture conditions (Chapter 5), it was possible to test the propagation of LSDV by changing one or more of these "standard" culture parameters and then comparing the resulting yield of virus with that obtained using "standard" conditions. Those altered parameters which resulted in an increased yield of infectious virions were considered favourable, and were then combined to form an optimum set of culture conditions.

6.2 MATERIALS AND METHODS:

6.2.1 Primary and continuous cell lines:

Primary LT, CFK and foetal bovine testes (FBT) cells were prepared as described in Chapter 4. Primary chick embryo fibroblast (CEF) cells were prepared in a similar manner, except that an entire 5-day old chicken embryo was used in the culture preparation.

Continuous lines of MDBK cells (described in Chapter 4), Vero (African green monkey kidney cells, ATCC Catalogue, reference CRL 1587), CV1 (African green monkey kidney cells, ATCC Catalogue, reference CCL 70), RK13 (5-week old rabbit kidney cells, ATCC Catalogue, reference CCL 37) and Don (Chinese hamster lung cells, ATCC Catalogue, reference CCL 16) were obtained from stocks maintained in liquid nitrogen in the Department of Medical Microbiology, UCT.

6.2.2 Egg inoculation:

6.2.2.1. Inoculation of CAMs with LSDV:

Embryonated hens' eggs (White Rock crossed with Cornish Game, Golden Grove Poultry Farm, Ottery, Cape Town) were candled and two pencil marks were made on the shell, one over the air sac and the other over an area of the chorioallantoic membrane (CAM) that was not directly above a blood vessel. The egg shell was then punctured at both positions using a punch "pen". Using the area of punctured shell as an entry point, the air sac was then pierced using a fountain pen nib. A drop of PBS was placed on top of the puncture hole over the CAM and this hole was then enlarged by rotating the fountain pen nib under the egg shell horizontally with the blunt end of the nib facing downwards, ensuring that the CAM was not pierced. The CAM was then dropped by removing the air from the air sac using a 5 ml rubber teat. The egg was allowed to stabilise for 60 min at 35 °C.

The virus stock was diluted in PBS to a concentration of 5×10^4 p.f.u./ml and 0,1 ml was placed on the CAM. Molten wax was spread over the hole above the CAM and the egg was gently swirled to spread the virus evenly. The egg was incubated at 35.5 °C for 5-7 days and then the virus was harvested.

6.2.2.2. Yolk sac inoculation:

The egg CAMs were dropped as described above and then a large hole was created in the egg shell above the dropped CAM. A drop of sterile paraffin oil was spread over the CAM, giving rise to a clear view of the yolk sac. A sterile needle was then inserted into the sac and the viral inoculum was injected. The egg was then wrapped in plastic cling-wrap and was placed at 35.5 °C for 5-7 days.

6.2.2.3. Harvesting of infected CAMs:

Gloves were worn at all times during the harvesting of the CAM's. The eggs were held in the left hand over a metal bucket containing Biocide 10 detergent (Lever Industrial, Durban, South Africa) while an incision was made starting at the air sac and continuing forwards and downwards towards the pointed end of the egg. The egg was then pried open, the blood vessels connecting the embryo and CAM were severed and the embryo was discarded into the Biocide. The CAMs were lifted away from the shell membranes and placed in physiological saline (Appendix 1) in a beaker. Only CAMs from live embryos were harvested.

After five CAMs had been harvested, they were washed in fresh physiological saline and transferred to a Universal bottle containing glass beads (0.3 mm diameter), to a depth of 1.5 cm. These bottles were kept on ice. Paper towel and saline were discarded into the Biocide and the scissors and forceps were boiled in water for 10 minutes in order to prevent contamination of other virus stocks. McIlvain's buffer was added to each Universal bottle (1 ml per CAM). The bottles were shaken for 2 minutes, were left on ice for 90 minutes and the disrupted membranes were then centrifuged at 1000 rpm (120g) at 4 °C for 10 min in a Sigma 301K benchtop centrifuge. The supernatant fluids were retained and were dispensed into Bijou bottles to be stored at -20 °C.

6.2.3. Establishment of optimal cell culture criteria for LSDV:

A number of experiments were performed to assess criteria for optimal LSDV infectivity and yield. The vaccine strain of LSDV (Neethling) was used for all experiments (as described in Chapter 4). For most of the experiments primary CFK cells were used. There is a seasonal availability of LT cells (used in the "standard" culture conditions), and CFK cells were more readily available than LT cells. They also divided more rapidly (than LT cells) and were therefore maintained using DMEM. 2 % FCS (as opposed to DMEM. 4 % FCS for LT cells). Unless otherwise stated, conditions used for the culture, harvesting and titration of virus were as follows:

Cell culture flasks (25 cm^2) (Nunclon, Delta, Nunc, Denmark) were each seeded with 6 x 10^5 primary CFK cells in 3 ml of DMEM. 10 % FCS (Appendix 1). The cells were incubated at 37 °C in a 5 % CO_2 incubator (Queue, U.S.A.) until they formed a 90 % confluent monolayer. The culture medium was then removed and replaced with 1 ml of 3 x 10^6 p.f.u. of LSDV in diluent. The flasks were returned to the incubator (37 °C). After an adsorption period of one hour, the virus inoculum was removed and the cells were washed with 1 ml of diluent (37 °C). The diluent was replaced with 3 ml of DMEM. 2 % FCS and the cells were incubated further at 37 °C in a CO_2 incubator.

The virus was released from the cells by adding 3 ml of McIlvain's buffer to the culture medium and then subjecting the cells to three cycles of freeze-thawing (between -20 °C and room temperature). A 0.1 ml aliquot of released virus was removed for titration on MDBK cells as described in Chapter 4 (4.2.7.1). The virus titre (p.f.u./ml) was converted to total virus yield (total p.f.u.) by multiplying the virus titre by the total volume of the virus suspension (i.e. 6 ml for most of the experiments). In those experiments where graphs were plotted, the total virus yield values were converted to $\log_{10}$ values and these were plotted against time post-infection.

6.2.3.1. LSDV growth at different temperatures.

The effects of temperature were assessed first in LT cells grown at 35 °C, 37 °C and 39 °C and virus yield was calculated after 24, 48, 72 and 96 hours post-infection. The method is as follows:

Thirteen culture flasks, containing 90 % confluent cell monolayers, were each inoculated with 1 x 10^6 p.f.u. of LSDV. Once the viral inoculum had been replaced with DMEM. 4 % FCS, the flasks were humidified in a CO_2 incubator at 37 °C for 30 minutes. One flask was then treated to release virus from infected cells (as described above) and 0.1 ml was removed for titration. The total yield of virus recovered from this flask was used as the time 0 post-infection value for the graph.

Twelve of the flasks were then placed in plastic containers (four flasks per container) and floated in waterbaths set at the relevant temperatures. One flask from each container was removed at 24, 48, 72 and 96 hours post-infection. Virus was released from infected cells and 0.1 ml aliquots were removed for titration. Graphs of the $\log_{10}$ total virus yield values (intracellular plus extracellular virus) versus time post-infection were plotted for the different incubation temperatures.

The experiment was repeated using CFK cells. An additional incubation temperature of 33°C was included. CFK cells incubated at 33 °C and 35 °C were also assessed for virus yield at 120 hours post-infection.

6.2.3.2 LSDV growth in different culture media.

Four different cell culture media were tested: Eagle's Minimal Essential Medium (MEM), DMEM, Basal Eagle's Medium (BEM) and DMEM combined 1:1 with HAM's F-12 medium (all media supplied by Highveld Biological Products, South Africa) (Appendix 1).

Four flasks of 90% confluent primary CFK cells were each infected with LSDV as described. The virus inoculum was removed and replaced with 3 ml of 2% FCS in either DMEM, MEM, BEM or DMEM + HAM's F-12 and the flasks were incubated at 37 °C for 3 days in a 5 % CO_2 incubator.

6.2.3.3 Pretreatment of viral stock with trypsin prior to infection.

Treatment of vaccinia virus with trypsin before infecting culture cells is reported to increase viral infectivity (Mackett et al., 1985). A similar approach was adapted for LSDV as follows:

Trypsin was added to a stock of LSDV (containing 3 x 10^6 p.f.u.) in diluent to give a final concentration of 0,25 mg/ml. This virus-trypsin mixture was incubated at 37 °C for 30 minutes. The trypsin was then inactivated by the addition of FCS to a final concentration of 0,1 %. The final volume of the treated virus was adjusted to 1 ml with diluent and was then used to infect one flask of primary CFK cells as described. Virus was harvested after 3 days and the titre was determined.

A control flask was infected in a similar manner, with 3 x 10^6 p.f.u. of stock virus (diluted in 1 ml of diluent) which was not pretreated with trypsin or FCS, although it was incubated at 37 °C for 30 minutes.

6.2.3.4 Changing the period of virus adsorption.

It is standard practice to infect cells with poxviruses for a period of 60 minutes at 37°C (Mackett *et al.*, 1985). It was the aim of this experiment to determine whether these conditions were relevant to LSDV infectivity, and whether changing the period of adsorption would have an effect on virus yield:

Each of two flasks was infected with LSDV as described. The virus inoculum was removed from one flask after an adsorption period of 60 minutes and from the other flask after 24 hours. Inocula were replaced with 3 ml DMEM. 2 % FCS. The cells were incubated for 3 days in total and the virus titres were then determined.

6.2.3.5 Splitting of infected cells:

Studies performed at Onderstepoort Veterinary Research Institute have shown that LSDV yield can be increased by infecting rapidly-dividing cells (Fick, personal communication). Those findings were tested in the following way:-

A flask containing a 50 % confluent monolayer of primary CFK cells was infected with 1.5 x 10^6 p.f.u. LSDV in 1 ml of diluent as described. The inoculum was removed after 60 minutes and was replaced with 3 ml DMEM.10 % FCS. The cells were incubated for 24 hours in a 5% CO_2 incubator at 37°C and were then split 1:2 (section 4.2.4). They were seeded into two new flasks and incubated for 3 days. After freezing and thawing, viral suspensions from both flasks were pooled, and a 0.1 ml aliquot was removed for titration on MDBK cells.

A control flask containing a 90 % confluent monolayer was infected with 3 x 10^6 p.f.u. of virus. The inoculum was removed after 60 minutes and was replaced with 3 ml DMEM.2 % FCS. The cells were incubated for 3 days in a 5 % CO_2 incubator at 37°C. After freezing and thawing,, a 0.1 ml aliquot was removed for titration on MDBK cells.

6.2.3.6 Infecting cells with different concentrations of LSDV:

Ten flasks of 90 % confluent CFK cells were infected with LSDV as follows: five of these were each infected with 1.5 x 10^7 p.f.u. and the other five were infected with 6 x 10^5 p.f.u. By means of cell counts (using a haemocytometer) it was calculated that each flask contained approximately 3 x 10^6 cells at the time of infection. Thus the multiplicity of infection (M.O.I.) was taken to be in the order of 5 p.f.u./cell and 0.2 p.f.u./cell for each set of cells respectively. After removal of the inoculum and washing with prewarmed diluent, 3 ml DMEM. 2 % FCS was added. At this stage, cells from one flask in each set of inoculum concentrations were disrupted, and 0.1 ml aliquots were removed for titration on MDBK cells (these titres were taken as the zero time post-infection values). The remaining flasks were incubated over a period of 4 days at 37 °C. Virus was harvested from one flask in each set at 24 hour intervals, and 0.1 ml of virus was used for titration on MDBK cells.

6.2.3.7 Growth in different cells.

The efficiency of replication of LSDV was tested in a number of different cells; viz. MDBK (both mycoplasma -ve and mycoplasma +ve), Vero, CFK, LT, CV1, RK13, Don and CEF cells.

Infected cells were incubated for 3 days, then the virus was harvested and titrated on MDBK cells.

A lower yield was obtained from Vero, Don, CV1 and RK13 cells. Therefore further studies were performed to assess whether LSDV could be passaged in these cell lines.

6.2.3.7.1 Growth of LSDV in Vero cells.

One flask containing a 90% confluent monolayer of Vero cells was infected with 3 x 10^6 p.f.u. of LSDV in 1 ml of diluent. The virus inoculum was removed after 60 minutes, the cells were washed with prewarmed diluent (37 °C) and this was then replaced with 3 ml of DMEM. 2% FCS. The flask was then placed at 37 °C in a 5 % CO_2 incubator. After a 3 day incubation period, virus was harvested and titrated on MDBK cells. A volume of 0,1 ml of the harvested virus was diluted in 0.9 ml of diluent which was then used to inoculate a new flask of 90 % confluent Vero cells. These cells were incubated for 3 days before virus was harvested. Virus was passaged three times in this manner.

6.2.3.7.2 Growth of LSDV in Don cells.

One flask containing a 90 % confluent monolayer of Don cells was infected with 3 x 10^6 p.f.u. of LSDV as described. After a 3 day incubation period, virus was harvested and titrated on MDBK cells. A volume of 0,1 ml of the harvested virus was added to 0.9 ml of diluent which was then used to inoculate a new flask of Don cells. LSDV was passaged five times in this manner. After the fifth passage, 0,1 ml of virus was inoculated onto Don cells growing on glass coverslips. The cells were incubated for a further 3 days and were then examined for the presence of LSDV antigen using the indirect immunoperoxidase staining technique described in Chapter 4 (4.2.8.1).

6.2.3.7.3 Growth of LSDV in CV1 cells:

One flask of 90 % confluent CV1 cells was infected with LSDV as described in 6.2.3.7.1 above. After 3 days the virus was harvested and passaged twice more in CV1 cells as described for Don cells above.

6.2.3.7.4 Growth of LSDV in RK13 cells:

One flask of 90 % confluent RK13 cells was infected as described in 6.2.3.7.1 above. After 3 days the virus was harvested and passaged twice more in RK13 cells as described for Don cells above.

6.2.4 Growth of LSDV in embryonated chicken eggs:

Van Rooyen *et al.* (1969) reported on the optimal conditions for the growth of LSDV on chicken egg CAMs. Using their recommendations, the growth of LSDV on CAMs was monitored by serial passage as follows:

Eight 7-day old embryonated hens' eggs were each infected with 5 x 10^3 p.f.u. of LSDV: Four were inoculated onto their CAMs (as described in section 6.2.2.1) and four were inoculated into their yolk sacs using a sterile needle (6.2.2.2). The infected eggs were placed in an incubator at 35,5 °C. Virus was harvested from the CAMs of four of the eggs (two from each inoculation route) after 5 days and from the CAMs of the other four eggs after 7 days. The virus titre was then determined by titration on MDBK cells.

A volume of 0,1 ml of virus that was recovered from the 7 day infection (CAM inoculation route) was used to inoculate the CAMs of another two 7-day old embryonated eggs. These were also incubated for 7 days at 35,5 °C before virus was harvested. The virus was passaged a further four times as described above, and the virus harvested after each passage was titrated on MDBK cells.

6.3 RESULTS:

6.3.1. LSDV growth at different temperatures:

The titres of LSDV obtained in LT cells at 35 °C, 37 °C and 39 °C are presented in Table 6.1 and Fig. 6.1 It can be seen that there is virtually no difference in the amounts of infectious virus recovered during the first three days from cultures grown at either 35 °C or 37 °C. However, by 96 hours post-infection, the virus titre at 37 °C had decreased slightly (about 0.5 log) whereas the virus titre at 35 °C had increased by a similar amount.

Replication of LSDV is apparently less efficient at 39 °C than at the other two temperatures. An initial increase in titre (from 6.0 x 10^4 p.f.u./ml at 24 hours to 6 x 10^6 p.f.u./ml at 36 hours) showed that the replication started at the same rate as reflected in cultures grown at either 35 °C or 37 °C, but thereafter the titre (at 39 °C) decreased and there was no extra gain in the recovery of infectious virus.

TABLE. 6.1: Effect of temperature on the growth of LSDV in LT cells

TIME POST INFECTION (HOURS)	TEMP (°C)	AVERAGE NO. OF FOCI PER WELL	DILUTION FACTOR	TOTAL NO. OF FOCI	LOG_{10} TOTAL NO. FOCI
0	-	8.0	10^2	$4.8 X 10^4$	4.7
24	35	3.0	10^3	$1.8 X 10^5$	5.3
	37	3.0		$1.8 X 10^5$	5.3
	39	1.0		$6.0 X 10^4$	4.8
48	35	16.0	10^4	$9.6 X 10^6$	7.0
	37	15.0		$9.0 X 10^6$	7.0
	39	10.0		$6.0 X 10^6$	6.8
72	35	8.0	10^4	$4.8 X 10^6$	6.7
	37	12.0		$7.2 X 10^6$	6.9
	39	2.0		$1.2 X 10^6$	6.1
96	35	28.0	10^4	$1.7 X 10^7$	7.2
	37	4.0		$2.4 X 10^6$	6.4
	39	3.0		$1.8 X 10^6$	6.3

The effect of temperature on the replication of LSDV was then investigated using CFK cells (in place of LT cells). Because of the apparent slight advantage seen at 35 °C at 96 hours post-infection in LT cells, the CFK experiments were designed to include titrations after an additional 24 hour period at 35 °C, and virus yields were also measured after replication at 33°C. The results are presented in Table 6.2 and Fig. 6.2. There was little difference (between LT and CFK cells) in the patterns of growth at either 35 °C or 37 °C. However, 39 °C was apparently even less favourable for LSDV replication in CFK cells than in LT cells, and no significant yield of infectious virus could be obtained at that temperature. The most favourable temperature for virus yield in CFK cells was 33 °C, where titres rose more rapidly and attained higher levels than those obtained from cells infected at higher temperatures. At both 33 °C and 35 °C, maximum titres were shown at 120 hours after infection, the main difference being that at 33 °C peak levels of infectious virus were reached and maintained from 48 hours after infection, but at 35 °C titres rose slowly and steadily throughout the 4-day period of infection.

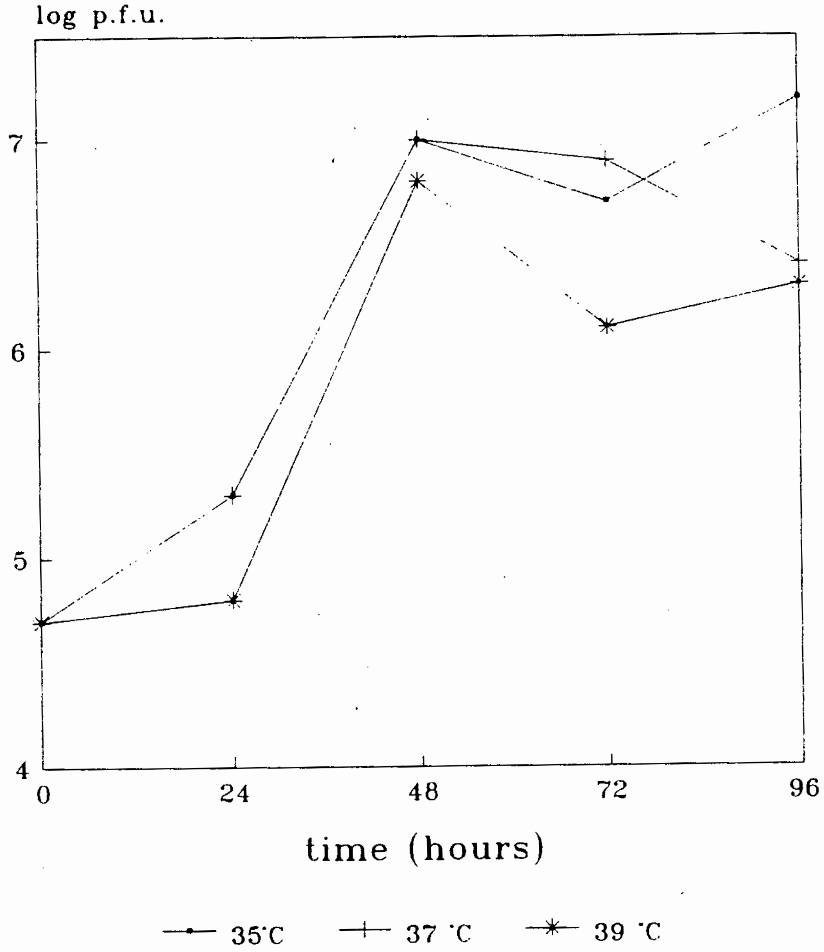

FIGURE 6.1.

Growth of LSDV at different temperatures in LT cells over a four day infection period. Log_{10} values of total p.f.u. are plotted against time.

TABLE 6.2: Effect of temperature on the growth of LSDV in CFK cells

TIME POST INFECTION (HOURS)	TEMP (°C)	AVERAGE NO. OF FOCI PER WELL	DILUTION FACTOR	TOTAL NO. OF FOCI	LOG$_{10}$ TOTAL NO. FOCI
0	-	8.5	10^3	5.1 X 10^5	5.71
24	33	4.7	10^4	2.8 X 10^6	6.50
	35	1.4	10^4	8.4 X 10^5	5.92
	37	2.0	10^4	1.2 X 10^6	6.08
	39	4.5	10^3	2.7 X 10^5	5.43
48	33	2.5	10^5	1.5 X 10^7	7.20
	35	5.0	10^4	3.0 X 10^6	6.48
	37	1.0	10^5	6.0 X 10^6	6.78
	39	6.5	10^3	3.9 X 10^5	5.59
72	33	1.5	10^5	9.0 X 10^6	6.95
	35	1.0	10^5	6.0 X 10^6	6.78
	37	3.6	10^4	2.2 X 10^6	6.34
	39	1.5	10^4	9.0 X 10^5	5.95
96	33	1.7	10^5	1.0 X 10^7	7.00
	35	1.3	10^5	7.8 X 10^6	6.89
	37	1.5	10^5	9.0 X 10^6	6.95
	39	2.5	10^3	1.5 X 10^5	5.18
120	33	3.0	10^5	1.8 X 10^7	7.30
	35	2.2	10^5	1.3 X 10^7	7.12

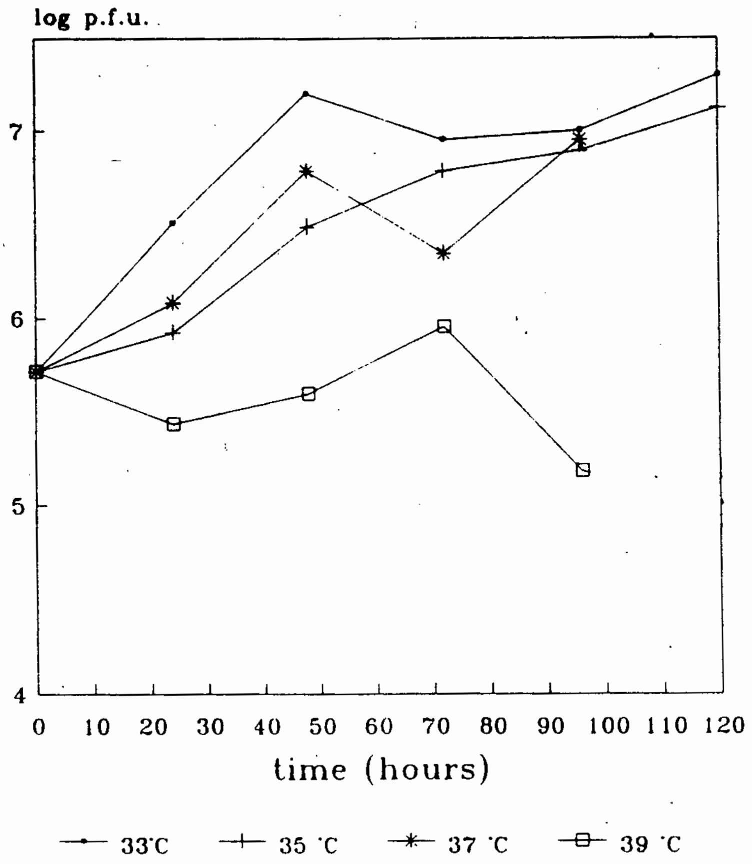

FIGURE 6.2.
Growth of LSDV at different temperatures in CFK cells over a four day infection period. Log_{10} values of total p.f.u. are plotted against time.

6.3.2. Growth in different cell culture media:

CFK cells infected with LSDV were grown in the presence of different culture media to assess whether the choice of medium might affect the ultimate yield of infectious virus. Titres are given in Table 6.3. Cells cultured in DMEM supplemented with Ham's F-12 nutrient medium, produced the highest yield of LSDV (4.2 x 10^7 p.f.u.). This titre was 2- to 3-fold higher than those obtained from cells grown in DMEM alone (1.2 X 10^7) or BEM (2.6 X 10^7), although more than 10-fold higher than when MEM was used (2.4 X 10^6).

6.3.3. Pretreatment of virus with trypsin:

Although it has been reported that pretreatment of vaccinia virus with trypsin increases its infectivity, the opposite effect was found for LSDV infectivity (Table 6.3). The titre obtained after 3 days with a LSDV stock which had been pretreated with trypsin (6.0 X 10^6 p.f.u.) was slightly lower than that obtained with virus which had not been treated (1.0 X 10^7 p.f.u.).

TABLE 6.3 : Growth of LSDV under various conditions

VARIABLE FACTOR	VARIABLE CONDITION	DAYS POST-INFECTION	TOTAL TITRE (P.F.U.)
GROWTH MEDIA	DMEM	3	1.2 X 10^7
	MEM	3	2.4 X 10^6
	BEM	3	2.6 X 10^7
	DMEM + F-12	3	4.2 X 10^7
TREATING VIRUS WITH TRYPSIN	WITH TRYPSIN	3	6.0 X 10^6
	NO TRYPSIN	3	1.0 X 10^7
PERIOD OF INFECTION	60 MINUTES	3	1.2 X 10^7
	24 HOURS	3	1.4 X 10^7
SPLITTING CELLS	SPLITTING CELLS	3	1.6 X 10^7
	CELLS NOT SPLIT	3	1.2 X 10^7
INFECTING CELLS AT DIFFERENT CONCENTRATIONS	6.0 X 10^5 p.f.u. PER FLASK	0	3.0 X 10^4
		1	1.0 X 10^5
		2	9.0 X 10^6
		3	1.2 X 10^7
		4	9.0 X 10^6
	1.5 X 10^7 p.f.u. PER FLASK	0	4.5 X 10^5
		1	1.4 X 10^6
		2	7.5 X 10^6
		3	1.7 X 10^7
		4	7.5 X 10^6

6.3.4. Changing the period of adsorption:

Leaving the virus inoculum in contact with the cells for a period of 24 hours, as opposed to one hour had no significant effect on the yield of LSDV recovered after 3 days ($1.4 X 10^7$ p.f.u. and $1.2 X 10^7$ p.f.u. respectively) (Table 6.3).

6.3.5. Splitting of infected cells:

The procedure of splitting the culture cells 1:2 at 24 hours after infection with LSDV, resulted in only a slight increase in the yield of LSDV recovered; $1.6 X 10^7$ p.f.u. as opposed to $1.2 X10^7$ p.f.u. for cells not split (Table 6.3). This difference was not considered to be significant.

6.3.6. Infecting cells with different concentrations of LSDV :

Titrations were performed to assess the extent to which the dose of infecting virus affected the ultimate yield of infectious particles. Two doses were compared in which inocula contained titres of either 5x less or 5x more than the standard inoculum dose. The results (Table 6.3) indicated that infection at an M.O.I. of 5 resulted in only a slight increase in the maximum yield of LSDV ($1.7 X 10^7$ p.f.u.) over that obtained with a starting M.O.I. of 0.2 ($1.2 X 10^7$ p.f.u.). Overall, however, the initial dose of infecting virus had a negliable effect on the growth of LSDV in CFK cells, or on the yield of LSDV virions (Table 6.3).

6.3.7. Growth in different cell lines:

Table 6.4 contains results of LSDV yields in different cell lines. Moderate titres (in the order of $3.0 x 10^6$) were obtained in MDBK cells, and the presence of mycoplasma had no adverse effect on LSDV growth. However, both primary LT and CFK cells resulted in a 7-fold increase in the yield of LSDV over that obtained in MDBK cells. No replication of LSDV was detectable in Vero, CV1 or RK13 cells and the virus yield consequently decreased rapidly with each passage. In Don cells, this decrease in LSDV yield occurred at a much slower rate and and virus was detectable until passage 5. Don cells infected at passage 6 failed to reveal the presence of viral antigen when stained using an indirect immunoperoxidase staining technique.

In CEF cells the first cycle of infection yielded titres equivalent to those obtained in MDBK cells. This titre was 10^3-fold lower on the second passage, but remained constant on passage three, indicating that CEF cells may well support the growth of LSDV to a limited extent.

TABLE 6.4 : Growth of LSDV in different types of cells.

CELL TYPE	VIRAL PASSAGE NO.	TOTAL TITRE (P.F.U.)
MDBK (mycoplasma -ve)	1	2.6×10^6
MDBK (mycoplasma +ve)	1	3×10^6
LT	1	2.1×10^7
CFK	1	1.6×10^7
VERO	1	1.5×10^4
	2	1.5×10^1
	3	$< 5 \times 10^1$
DON	1	5.7×10^5
	3	3×10^2
	5	$< 3 \times 10^2$
CV1	1	1.2×10^4
	2	$< 3 \times 10^3$
	3	1.5×10^1
RK	1	3.6×10^4
	2	1.5×10^3
	3	$< 1.5 \times 10^2$
CEF	1	3×10^6
	2	6.9×10^3
	3	6×10^3

6.3.8. Growth in embryonated chicken eggs:

Evidence was obtained that embryonated hens' eggs could support the replication of LSDV (Table 6.5). Inoculation into the yolk sac of 7-day old embryos resulted in premature death of the embryos despite the fact that only a low titre of viral inoculum (5 X 10^3 p.f.u.) was used. Although the yield of virus obtained after CAM inoculation was no higher than that obtained from inoculation of the yolk sac, only one embryo died when inoculated onto the CAM as opposed to three when inoculated into the yolk sac, showing that the former route of inoculation is safer. LSDV could be repeatedly passaged in eggs (via the CAM route of inoculation) and even after six passages, 1.5 X 10^5 p.f.u. per egg was recoverable (Table 6.6).

TABLE 6.5: Growth of LSDV in embryonated chicken eggs.

INOCULATION ROUTE	No. OF DAYS POST INFECTION	No. OF EGGS INOCULATED	No. OF EGGS SURVIVED	TITRE OF VIRUS RECOVERED PER EGG
YOLK	5	TWO	ONE	7 X 10^4
CAM	5	TWO	TWO	7.5 X 10^4
YOLK	7	TWO	NONE	-
CAM	7	TWO	ONE	2 X 10^5

TABLE 6.6: Passage of LSDV in embryonated chicken eggs.

PASSAGE NO.	INOCULUM PER EGG	No. EGGS INOCULATED	No. EGGS SURVIVED	TITRE PER EGG (P.F.U.)
2	2×10^4	ONE	ONE	7×10^5
3	2×10^3	FOUR	TWO	2.5×10^5
4	1×10^3	TEN	SIX	7.3×10^4
5	2×10^3	TEN	EIGHT	5×10^4
6	2×10^3	TEN	EIGHT	1.5×10^5

6.3.9. Optimal conditions for the culture of LSDV:

A comparison of the yields of infectious LSDV obtained by changing individual culture parameters indicates that certain conditions are more favourable for growth than others. The culture conditions, which produced the highest yields of infectious virus from each set of altered parameters, is presented in Table 6.7. Also shown is the good yields obtained using foetal bovine testes cells (FBT) grown in the presence of the most favourable culture medium.

Although none of the conditions tested produced a dramatic increase (> 10-fold) in the yield of infectious virus (when compared with the yield obtained using standard conditions), even an increase of two- to three-fold was considered as being significant.

TABLE 6.7 : Summary of optimal culture conditions for maximum yield of infectious LSDV.

PARAMETER	STANDARD CONDITION	OPTIMAL CONDITION
	37 °C, LT cells	35 °C
TEMPERATURE	9.0 x 10^6	1.7 x 10^7
	37 °C, CFK cells	33 °C
	9.0 x 10^6	1.8 x 10^7
CULTURE MEDIUM	DMEM	DMEM + HAMs F-12
	1.2 x 10^7	4.2 x 10^7
CELL TYPE	CFK	LT
	1.6 x 10^7	2.1 x 10^7
CELL TYPE and MEDIUM	LT, DMEM	FBT, DMEM + HAMs
	1.6 x 10^7	7 x 10^7

6.4 DISCUSSION:

Extensive tests were performed to establish optimal conditions of culture for maximum yields of infectious LSDV. Although few of the techniques tested had a profound influence on the established "standard" procedures (i.e. > 10-fold increase or decrease in viral yield), the combination of a number of changed parameters were found to be advantageous. Lowering the incubation temperature of infected cells to 33 °C, the combined use of DMEM and Ham's F-12 supplement as the culture medium, and the choice of primary bovine or ovine kidney or testes cells, all contributed to increasing the yield of infectious LSDV virions.

During these studies a number of interesting aspects concerning the *in vitro* culture of LSDV were observed.

LSDV has proved to be extremely host-specific with respect to natural transmission and infection as evidenced by the total absence of reports of capripoxvirus infections in sheep or goats in South Africa (cited by Kitching et al., 1989). In the present study, it was shown that cell cultures established from both ovine and bovine tissue were able to support the replication of the virus. Highest yields were obtained with primary cell cultures of either testes or kidney cells. A continuous cell line of bovine kidney cell origin (MDBK cells) was also permissive for propagation of LSDV, but a 5-fold increase in virus yield was obtained from primary CFK cells over that obtained in the MDBK cell line, even in the absence of mycoplasma.

CFK culture cells are routinely maintained at 37 °C. It is of interest that higher titres of LSDV could be obtained when cultures were grown at the lower temperatures of 33 °C and 35 °C. Enzymatic activity has strict temperature boundaries. Since poxviruses encode most of the enzymes required for their replication, it may be deduced that LSDV-encoded enzymes operate preferentially at 33 °C. It is possible that the advantage conferred by the lower temperature on the *in vitro* production of infectious virus may reflect the natural conditions of the site of virus replication *in vivo*.

The successful replication of LSDV *in vitro* is apparently dependent upon additional nutrient requirements supplied in the culture medium. Studies showed that standard MEM medium was suboptimal for good yields of infectious virions, and of the various culture media tested, best yields were obtained when cells were grown in DMEM with the addition of HAM's F-12 supplement. Media containing a broader spectrum of amino acids and vitamins, or higher concentrations of certain amino acids, in particular L-glutamine, were found to be best suited. The HAM's supplement contained, in addition, a number of critical trace-elements and organic factors required for cellular metabolism.

Receptors on the surface of the virion itself play a major role in virus infectivity. Chand and Black (personnal communication) have identified a 32 kDa major structural protein of a capripoxvirus (KS-1) which is involved in attachment of the virions to the surface of cultured lamb testis cells. The terminal portion of this protein is removed by trypsin, leaving a 22 kDa fragment associated with the virus surface, and reducing the virus infectivity by at least 10-fold. This may in part explain our observation that treatment of LSDV with trypsin prior to the inoculation of CFK cells resulted in reducing the final yield by almost half, despite the recommendation of Mackett *et al.* (1985) that pretreatment of vaccinia virus with trypsin results in increased infectivity.

Little is known about the viral receptors on susceptible cells, nor about the manner in which LSDV is able to enter each of the different types of cell. A constant observation during these studies was that a certain amount of infectious virus could be recovered from the cellular fraction at the end of the adsorption period. It was assumed that this represented virus particles which had adsorbed to the cell surfaces, but had not been internalised. The reason for their inability to penetrate the cells is unknown. The length of time for which the inoculum was left in contact with the cells did not result in any apparent increase in the efficiency of infection. In the case of herpesvirus infections it has been reported that multiple infections of a single cell are inhibited by herpesvirus-encoded proteins (Johnson and Spear, 1989). It is possible that similar mechanisms may exist for poxviruses, and might explain why a 25-fold increase in the M.O.I. of the inoculum did not result in an appreciable increase in the final yield of progeny virus particles.

In the present study, the yield of LSDV virions was measured only in terms of infectivity in MDBK cells. The majority of virions released from infected cells were shown by electron microscopy to be enveloped (unpublished data; see also Fig. 2.1). It is possible that procedures used to release intracellular virus (low ionic strength buffer, freezing and thawing, etc.) may damage or even completely remove the envelope. It is not known at this stage what effect this might have on virion infectivity. It has been reported that enveloped vaccinia virus particles are more rapidly adsorbed to the cell membrane (as cited by Sordeik *et al.*, 1993), but both enveloped and naked virions are infectious. The nature and prevalence of cell receptors on the various cell types will in part determine their degree of susceptibility to infection by the morphologically different forms of the virion.

The host-cell species specificity noted in natural infections of LSDV were reflected in the inability to achieve replication of LSDV virus in cells of rabbit, hamster or monkey origin (RK13, Don, CV-1 or Vero cells). Gershon and Black (1988) also reported failure in attempts to grow capripoxviruses in cells derived from hosts other than from goats, sheep or cattle, although Weiss et al. (1968) reported experimental induction of LSDV infection of a number of ovine and bovine cell lines as well as in foetal rabbit kidney and skin cells, chick embryo fibroblasts, adult vervet monkey kidney cells and baby hamster kidney cells.

Van Rooyen et al. (1969) reported growth and serial passage of LSDV in chicken chorioallantoic membranes which resulted in an attenuated virus, later developed for use as a vaccine against lumpy skin disease. As the vaccine strain of LSDV (Neethling) was used in all the present studies, it is probably not surprising that chick embryo cells were able to support LSDV replication and that the virus could also be repeatedly passaged in hens' eggs. However, only comparatively low yields of infectious virions could be recovered from the avian cells. It is known that host specificity in poxviruses is linked to the non-conserved terminal regions of their genomes which contain host-range genes (Gershon and Black, 1988). Serial passage of LSDV in CAMs, and even sheep cells, might have resulted in the loss or rearrangement of genes concerned with host-range specificity, thus endowing the vaccine strain with the ability to overcome the avian host-range restriction. DNA sequencing studies would be required to confirm this hypothesis.

CHAPTER 7

OPTIMISING PROCEDURES FOR PURIFICATION OF LSDV DNA:

7.1 INTRODUCTION:

Once an optimal set of conditions for the growth of LSDV had been established, the following step was to optimise procedures for the purification and recovery of viral DNA for restriction digestion analysis. In this respect a number of factors were considered as a balance had to be found between the maximum recovery of virions and obtaining virus that is free of contaminating cellular material. The lysis technique for recovering DNA from purified virus particles also required special attention as the DNA would have to be of a high quality and yield and sufficiently pure to ensure complete restriction with site-specific endonucleases.

A number of techniques are available for the purification and lysis of poxvirus virions (Mackett *et al.*, 1985; Black *et al.*, 1986). Most virion purification techniques involve the use of density gradient centrifugation, which inevitably result in the loss of an appreciable amount of virions. These losses are affordable when purifying virions from poxviruses which grow to high titres in cell culture, but some poxviruses, such as LSDV, do not, and it was thus necessary to consider alternative techniques for the purification of LSDV. Esposito *et al.* (1981) described the use of a non-ionic detergent and reducing agent for isolating orthopoxvirus DNA from the cytoplasm of infected cells. He found that incubation of infected cells in the presence of low concentrations of Triton X-100 and β-mercaptoethanol, and removal of cell nuclei by low speed centrifugation, resulted in a 5-fold increase in the yield of viral DNA over that recoverable from purified virions. This intracellular source of viral DNA was of a similar quality to DNA obtained from virions.

As has already been noted for LSDV growth in lamb testes cells, a high percentage of infectious virus remains intracellular (Chapter 5). Although techniques such as douncing and freeze-thawing are effective in releasing virus from infected cells, they appear to lyse cell nuclei resulting in cellular DNA contamination of viral preparations. A more gentle technique is thus necessary for recovering viral DNA from cells. It was thus the aim of this experiment to assess the viability of using Esposito's technique (Esposito *et al.*, 1981) for the recovery of LSDV from infected cells. In addition, a variety of virus lysis techniques were assessed in terms of obtaining the maximum yield and quality of viral DNA .

7.2 MATERIALS AND METHODS:

7.2.1. Pulse field gel electrophoresis:

Although conventional electrophoresis (non-pulsed) is sufficient for resolving small fragments (< 20 kbp), larger fragments migrate with a common mobility (Bostock, 1988). Alternating the direction of the electric field was found to enhance the separation of large DNA molecules by causing them to continually reorientate themselves as they migrate through the gel matrix (as reviewed by Lai *et al.*, 1989). Pulse Field Gel Electrophoresis [or Field Inversion Gel Electrophoresis (FIGE)] was one of the techniques developed to enhance the resolution of large DNA fragments. A forward to reverse pulse ratio of 3:1 is used, with long pulse times being required for the separation of large fragments and shorter pulse times for the separation of small fragments. Ramping the forward and reverse pulses has the added advantage of increasing the range of fragment sizes which can be separated during one electrophoretic run.

7.2.1.1 Preparation of gels and electrophoresis of DNA:

Agarose gels (0.8 %) were prepared by adding 0.8 g of agarose (Agarose MP, Boehringer Mannheim, Germany) per 100 ml of 0.5 x Tris-Borate-EDTA (TBE) buffer (Appendix 1). The agarose was dissolved by heating the solution to boiling temperature in a microwave oven (Sharp Carousel). After cooling to 56 °C, 250 ml of the 0.8 % agarose was cast in a large casting tray (25 cm x 19 cm x 0.75 cm). A 20-toothed comb was used and the gel was left to set for at least 2 hours at room temperature. Once the comb had been carefully removed, the gel was placed in a Hoefer Super Sub electrophoresis tank (Hoefer Scientific Instruments, San Fransisco, U.S.A.). Only 1.2 litres of 0.5 x TBE buffer was required for this tank as it employs a buffer recirculation system through a refrigerated waterbath (Haake D8-G, Germany). Internal buffer circulation was obtained using a magnetic stirrer linked to a Hoefer Multi-Spin Stir Controller. A constant temperature of 13-15 °C could thus be maintained for the duration of the run. After loading DNA samples, a non-pulsed prerun of 150 V (or as otherwise stated) for 15 minutes was initiated. The electrodes were then reconnected to a Hoefer PC 750 Pulse Controller and electrophoresis was continued using conditions as specified for each individual run.

NOTE: For each FIGE run, forward pulses (F) were approximately three times longer than reverse pulses (R) and the pulses were ramped by increasing the forward and reverse pulse times by an incremental rate (r) for the duration of the electrophoretic run.

7.2.1.2. Staining, viewing and photographing gels:

Following electrophoresis for the required time period, the power supply was disconnected and gels were stained in 500 ng/ml ethidium bromide (EtBr) (Merck, Germany) (diluted in 0.5 X TBE buffer) for 30 minutes. The fluorescing DNA bands were visualised on a UVP Transilluminator (model TM-40E, 302 nm wavelength) (UVP, U.S.A.). Gels were photographed as described (Appendix 2).

7.2.2 Virus purification for DNA isolation:

The method of Esposito *et al.* (1981) for the recovery of orthopoxvirus DNA from the cytoplasm of infected cells was adapted for the purification of LSDV. At each stage of the purification, an aliquot of virus was removed for titration in order to ascertain which fractions, produced during the purification procedure, contained LSDV virions.

One 175 cm^2 cell culture flask was seeded with primary LT cells. Once the cells had reached 90 % confluency, they were infected with 1×10^7 p.f.u. of LSDV vaccine as described (4.2.6). After 3 days, 90 % of the cells exhibited CPE. Intracellular and extracellular virus was then harvested as follows (Fig. 7.1):

The medium (20 ml), containing detached cells and extracellular virions, was harvested and the cells remaining attached to the floor of the flask were removed with mild trypsin digestion (4.2.4). These cells were pooled with the medium and an aliquot was removed for titration (labelled "Sample #1"). The rest of the pool was centrifuged at low speed in a Sigma 301K benchtop centrifuge (1500 rpm for 10 min at 4 °C). The supernatant fluid was removed and an aliquot was titrated ("Sample #2"). The cell pellet was resuspended in 4 ml of McIlvain's buffer, an aliquot was removed for titration ("Sample #3") and the volume was adjusted to 18 ml in McIlvain's buffer. This cell suspension was divided

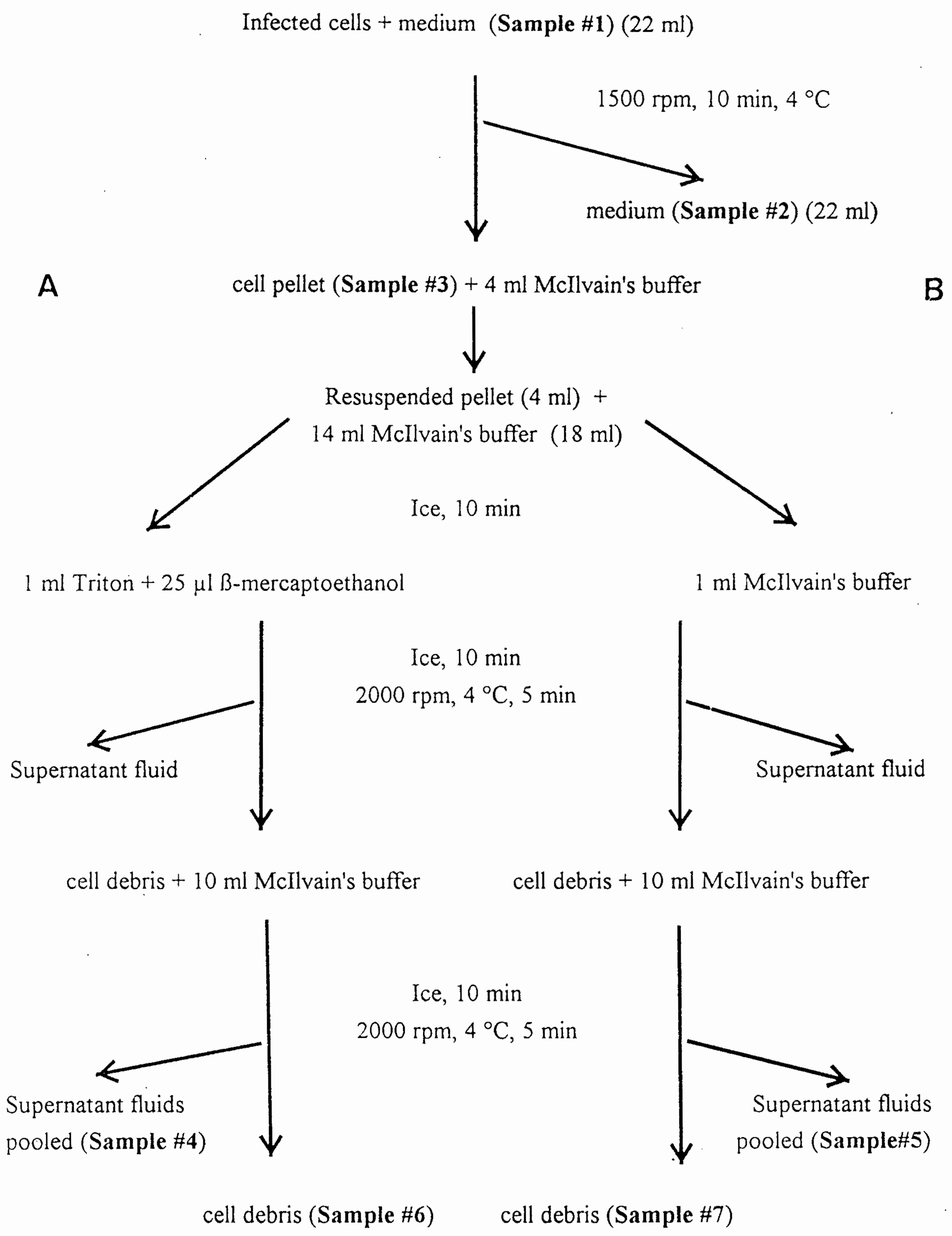

Figure 7.1. Flow diagram of virus purification from Triton-treated (A) and untreated (B) cells.

into two equal portions. These were placed on ice for 10 minutes and were then treated as follows:

1) One portion was treated to release intracellular virus by the addition of 1 ml of 10 % (v/v) Triton X-100 (diluted in McIlvain's buffer) and 25 µl ß-mercaptoethanol.

2) 1 ml of McIlvain's buffer was added to the other portion.

Triton-treated (1) and untreated (2) cells were processed in parallel in the following way:

After incubation for a further 10 minutes on ice, with occasional gentle shaking, the cell suspension (containing released virus) was centrifuged at 2000 rpm for 5 minutes. The supernatant fluid was collected. The cell debris was resuspended in McIlvain's buffer and was centrifuged at 2000 rpm for 5 minutes. This supernatant fluid was pooled with the first supernate and an aliquot was removed for titration (Sample #4 from Triton-treated cells and Sample #5 from untreated cells). An aliquot of the remaining cellular debris was also removed for titration (labelled "Sample #6" and Sample #7" for treated and untreated cells respectively).

The supernatant pool was then divided into three equal portions, and virus particles were concentrated by centrifugation at 11 000 rpm (19000g) for 60 minutes at 4 °C in a Beckman J2-21 centrifuge (JS 13-1 swing-out rotor).

7.2.3 Viral DNA purification:

The three virion pellets were each resuspended in 25 µl T.E. buffer (10 mM Tris-HCl, 1 mM EDTA, pH 9,0) and one of the following virion lysis buffers was added:-

1. 25 µl of lysis buffer A (1 % SDS, 20 mM EDTA, 20 mM ß-mercaptoethanol) (adapted from Mackett *et al.*, 1985).

2. 25 µl lysis buffer B (10mM Tris-HCl, pH 9; 20 mM NaCl; 100 mM EDTA; 2 % sodium lauroyl sarcosinate) (adapted from Esposito *et al.*, 1981).

3. 25 µl of lysis buffer C (2,5 µl 200 mM ß-mercaptoethanol; 100 mM Tris (pH 7,8) and 22,5 µl 54 % sucrose; 4 % sodium lauroyl sarcosinate) (adapted from Massung and Moyer, 1991).

Proteinase K (final concentration = 100 µg/ml) was added to each sample and they were incubated at 56 °C for two hours.

Each of the final 6 samples (three originating from Triton-treated cells and three from untreated cells) was assayed for DNA yield by field inversion gel electrophoresis (FIGE).

Parallel electrophoresis was performed on DNA extracted from the following samples:-

a) samples of cell debris (#6, #7) using lysis buffer C
b) uninfected MDBK cells using lysis buffer A
c) purified vaccinia virus (DIE), as a marker (Kindly supplied by Madelaine Richardson, previously of the Department of Medical Microbiology, UCT).
d) Triton-treated sample lysed with lysis buffer C, after phenol/chloroform clarification

Electrophoresis was performed as follows:

A 10 µl aliquot of each sample was diluted in 10 µl of T.E. buffer (pH 9,0) and 3 µl of Stop buffer (X6) (Appendix 1). These were loaded onto a 0,8 % agarose gel and subjected to FIGE using the following conditions:

Prerun: 200 V, 68 mA, 15 min at 14 °C,

Run: 200 V, 68 mA, 12 hours, Forward (F) = 1,0 sec, Reverse (R) = 0.3 sec and ramp (r) = 1,0 at 14 °C.

The gel was stained and visualised as described (7.2.1.2).

Samples #1 to #7 were titrated as described in Chapter 4 (4.2.7.1) after 3 cycles of freeze-thawing at -20 °C and room temperature. The titre of each sample (in p.f.u./ml) was multiplied by the respective volume (in ml) of the original sample to give the total number of infectious units.

7.3 RESULTS:

The number of infectious virus particles recovered at various stages of the extraction procedure is shown in Table 7.1. It was calculated that 3.9×10^7 p.f.u. could be obtained from one 175 cm^2 flask of primary LT cells (Sample #1) and 3.3×10^7 p.f.u. from the cell fraction alone (Sample #3). These estimates were drawn from titrations of infectious virions released from sample aliquots by freezing and thawing.

Alternative methods of achieving cell lysis and releasing intracellular virus particles were assessed. These included disruption of the cell membranes with a mixture of Triton X-100 and β-mercaptoethanol, or with the hypotonic McIlvain's buffer. The number of infectious particles released by each method were compared and this provided an indication of the minimal amount of intact viral DNA that could be obtained from the respective cell lysates. The use of Triton X-100 and β-mercaptoethanol allowed the recovery of 1.5×10^7 p.f.u. of infectious virus, which is slightly less than the titre estimated to be gained by freezing and thawing an equivalent volume of cells (50% of fraction #3) and slightly higher that obtained with McIlvain's buffer (1.2×10^7 p.f.u.). The small difference in yield of infectious virus recovered from the two experimental cell lysates was reflected in the relative amounts of infectious virus remaining in the cellular debris - 4.7×10^6 p.f.u. were recovered from Triton-treated cell debris, and 9.7×10^6 p.f.u. from the debris of cells lysed in McIlvain's buffer alone.

The quality and quantity of viral DNA that could be obtained from the respective cell lysates was assessed by subjecting virus particles released from infected cells (either with or without Triton X-100) to 3 different methods to release viral DNA. In order to assess the yield and quality of DNA, samples were electrophoresed using FIGE. This electrophoresis technique allows for separation of high molecular weight DNA (e.g. LSDV; 152 kb) from lower molecular weight cellular DNA (e.g. mitochondrial DNA; ≈ 20 kb).

TABLE 7.1 Distribution of LSDV virions during different stages of purification.

SAMPLE NO.	DESCRIPTION	TOTAL P.F.U. COUNTED
#1	POOLED MEDIUM AND CELLS	3.9 X 10^7
#2	MEDIUM	7.6 X 10^6
#3	CELL PELLET	3.3 X 10^7
#4	VIRUS RELEASED FROM CELLS TREATED WITH TRITON	1.5 X 10^7
#5	VIRUS RELEASED FROM UNTREATED CELLS (WITHOUT TRITON)	1.2 X 10^7
#6	CELL DEBRIS AFTER TRITON TREATMENT	4.7 X 10^6
#7	CELL DEBRIS WITHOUT TRITON TREATMENT	9.7 X 10^6

Vaccinia (DIE) DNA and DNA from uninfected cells (Fig. 7.2, lanes 11 and 10 respectively) were used as markers to help distinguish between viral and cellular DNA bands. Each lane in Fig. 7.2, consisting of DNA derived from cells infected with LSDV (lanes 1-8 and 13), contains a DNA band migrating slightly slower than the 145 kb marker fragment (lane 12). The relative position of this band is in agreement with the expected position of LSDV DNA (152 kb). As the band is absent from uninfected cells (lane 10), it can be assumed that it represents unit length genomes of LSDV DNA. Vaccinia virus DNA (lane 11) appears to co-migrate with the 194 kb marker fragment (lane 12), which is in agreement with the genome size of this isolate (190 kb).

Relative amounts of DNA were assessed by loading an equal proportion of each sample (1/5) per lane (Fig. 7.2, lanes 1-6). The use of lysis buffer B (lanes 1 and 4) resulted in a lower yield of DNA than when lysis buffer A (lanes 3 and 6) or C (lanes 2 and 5) were used.

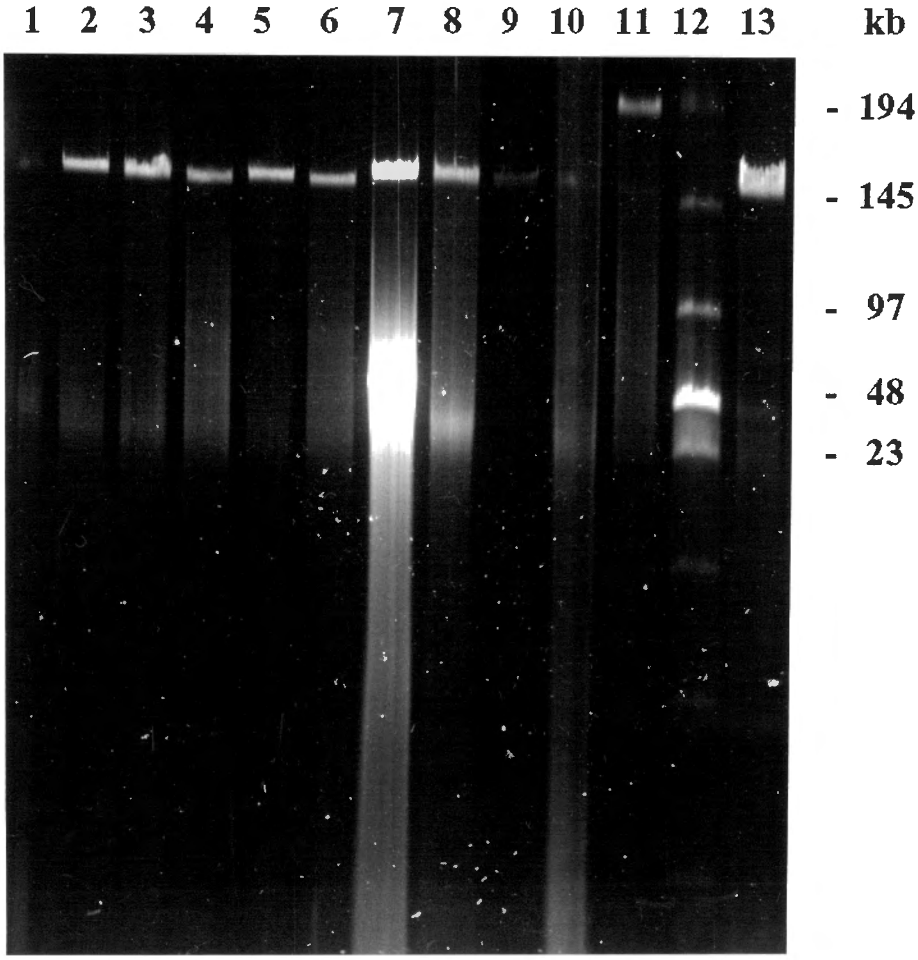

FIGURE 7.2

Agarose gel of LSDV (Neethling) vaccine DNA purified using various techniques. Lanes 1, 2 and 3 = untreated cells. Lanes 4, 5 and 6 = cells pretreated with Triton X-100. Virus particles lysed using lysis buffer B (lanes 1 and 4), or lysis buffer C (lanes 2 and 5) or lysis buffer A (lanes 3 and 6). Lane 7 = cell debris from untreated cells. Lane 8 = cell debris from cells pretreated with Triton X-100. Lane 9 = well leaked. Lane 10 = cell debris from uninfected cells treated with Triton X-100. Lane 11 = Vaccinia (DIE) DNA, uncut. Lane 12 = High Molecular Weight marker. Lane 13 = Phenol/Chloroform purified DNA (from lane 5). FIGE conditions: 200 V, 69 mA, 14°C, 12 hours. F = 1.0 sec, R = 0.3 sec, r = 1.0.

Cells lysed in standard lysis buffer (McIlvain's buffer only) (lanes 1,2,3) yielded a slightly higher quantity of viral DNA over that obtained from cells pretreated with Triton X-100 in conjunction with ß-mercaptoethanol (lanes 4,5,6), except for lanes 1 and 4 where the opposite result was obtained.

A comparison of lanes 7 and 8 shows that the amount of viral DNA remaining in the cell debris is proportional to the number of infectious virions in that less viral DNA appears to remain in cell debris after Triton treatment (lane 8) than in cell debris from untreated cells (lane 7). Although the viral bands in lanes 7 and 8 are intense, these DNA samples are three times as concentrated as the samples shown in lanes 1 to 6.

Although the viral DNA in lanes 1 to 6 appear to contain low levels of contaminating DNA only, they are probably also contaminated heavily with protein and lipid remnants of the lysed virus particles. Removal of these potential contaminants is necessary for restriction of viral DNA with endonucleases. Lane 13 contains DNA from the same sample as in lane 5 (but approximately three times more concentrated), which has been purified from these contaminants using phenol-chloroform extractions (8.2.2.2). The DNA was precipitated and resuspended in T.E. buffer. Not only does this treatment purify the DNA from protein and lipid contaminants, but it also appears to result in removal of low molecular weight DNA without degrading the viral DNA (lane 13). A small loss of DNA results during a phenol/chloroform extraction, but the recoverable DNA is pure, non-degraded and should thus restrict to completion with restriction endonucleases.

7.4. DISCUSSION:

A technique, which was first developed for the purification of orthopoxviruses from infected cells as an additional source of viral DNA besides extracellular virus (Esposito *et al.*, 1981), was evaluated for the purification of intracellular LSDV particles. Advantages are that the technique is rapid and alleviates the need for density gradient centrifugation. The use of a nonionic detergent (Triton X-100) and reducing agent (β-mercaptoethanol) to release viral particles from the cytoplasm of infected cells resulted in a good yield of infectious virions. It may be assumed that for virions to be infectious, they need to be intact. This result is in slight contrast to a report on the action of a non-ionic detergent (Nonidet P40) and reducing agent (β-mercaptoethanol), which were shown to free viral cores of vaccinia virions by solubilising the outer virion envelope (Easterbrook, 1966). The vaccinia virions were, however, subjected to much higher concentrations of chemicals at an elevated temperature and for a longer period than the LSDV infected cells. In addition, previous studies on the culture of LSDV (Chapter 5) have shown that intracellular particles are infectious and electron micrographs of intracellular LSDV particles have revealed the presence of membranes encapsulating groups of virus particles (Fig. 2.1) (Stannard, personal communication). It is thus possible that the mild chemical treatment of infected cells used in this study preferentially solubilized these cellular membranes to release individual virions, and that only a low percentage of the virion outer membranes were solubilized, leaving the majority of virus particles infectious.

A fair percentage of infectious virus particles were located in the cellular debris in both Triton-treated and untreated cells. It is possible that these particles were not released from infected cells, even after treatment with Triton, or that they were trapped beneath clumps of cellular material which caused them to co-precipitate during centrifugation. This loss of viral DNA was, however, considered negligible when compared to the high yield and purity of viral DNA recovered from virus particles in the supernatant fluid. The small loss of DNA resulting from phenol/chloroform purification, is also considered negligible, as, without this process, contaminating proteins and lipids would interfere with the restriction of viral DNA with endonucleases.

Various combinations of detergents and ß-mercaptoethanol were evaluated to find an optimal lysis buffer for release of DNA from intact virus particles. The lysis buffer with the highest concentration of ß-mercaptoethanol (lysis buffer C), in conjunction with Triton treatment of cells, proved to be the best for virion lysis, as almost no contaminating mitochondrial DNA co-precipitated with the viral DNA. Viral DNA preparations were also completely free of high molecular weight chromosomal DNA and restricted to completion with endonucleases (personal observation).

Thus, the combined use of Triton X-100 and ß-mercaptoethanol to release intracellular LSDV particles from infected cells, and a virus lysis buffer containing a high concentration of ß-mercaptoethanol, was found to produce a high yield and quality of viral DNA. In subsequent infections, involving the combined use of optimal culture and purification conditions and infection of ~1 x 10^8 cells, enough viral DNA was recovered on average for 150 restriction digests with restriction endonucleases such as *Pst* I and *Bam* HI (refer to Chapter 8).

CHAPTER 8:

RESTRICTION ENDONUCLEASE ANALYSIS OF LSDV ISOLATES:

8.1 INTRODUCTION:

Site-specific restriction endonucleases have been successfully used to distinguish different isolates of capripoxviruses (Black et al., 1986), whereas serological testing failed due to the presence of a common major antigen (Kitching *et al.*, 1986). *Hin* dIII restriction digest patterns of different capripoxviruses from Africa and Asia, including LSDV from South Africa, indicated a close degree of relatedness (Black *et al.*, 1986). Their results further showed that isolates from different hosts display specific differences in their restriction patterns, which appear to relate to the animal host. A very close relationship was shown between Kenya sheep-and-goatpox virus and different isolates of LSDV. The genome length of a Kenyan capripoxvirus, which is associated with outbreaks of LSD in Kenya (KC-1), has been estimated to be 145.6 kbp (Gershon and Black, 1987). This contrasts with the South African (Neethling) vaccine strain of LSDV which has recently been accurately mapped and sized to give a genome length of 152.61 kbp (Perlman, 1993). Recent molecular studies in Cape Town have identified LSDV homologues of the vaccinia virus intermediate class of transactivating genes, as well as a rifampicin gene (Cohen, personal communication). Early attempts to create a recombinant LSDV expressing a bluetongue virus antigen (virus protein 7), using heterologous recombination between the LSDV TK and vaccinia virus TK genes, were unsuccessful due to the heterologous nature of the LSDV and vaccinia virus TK genes (Brand, 1993). Parallel studies have identified LSDV genes which are strongly expressed during infection, and may contain promoters suitable for use in the development of a recombinant vaccine (Fick, 1992). No research has been published on analysis of virulent LSDV strains originating in southern Africa. As recombinant LSDV work is in progress, a comparison of restriction endonuclease patterns of southern African field isolates of LSDV would be of interest in determining whether these are closely related to the virulent South African (Neethling) strain.

Hin dIII, *Eco* RI, *Pst* I and *Bam* HI were used to cleave the DNA genomes of the four LSDV field isolates and the vaccine strain. Field inversion gel electrophoresis (FIGE) has been successfully used to separate and size poxvirus restriction digest fragments (Massung and Moyer, 1991; Bostock, 1988). FIGE was thus used to separate the LSDV DNA fragments and the resulting patterns were analysed.

8.2 MATERIALS AND METHODS:

8.2.1 Source of virus isolates:

Attenuated LSDV vaccine and virulent field isolates were originally obtained from the Virology Section, Onderstepoort Veterinary Institute, Pretoria as freeze-dried cell culture supernatant fluid. Virus particles were rehydrated in sterile, distilled water and were then passaged in cell culture as indicated.
(Refer to Figure 8.1 for the original locations of the LSDV isolates used in this study.):

1) LSDV (Neethling) - this isolate was isolated from infected cattle from the Marico District of the Western Transvaal, South Africa (Thomas and Mare, 1945). The virus was passaged 4 times in calf kidney cells and then 6 times in lamb kidney cells, before being freeze-dried. The rehydrated sample used in this study was passaged 11 times in MDBK cells and then twice in LT cells before viral and DNA extraction.

2) LSDV (Neethling) vaccine - this isolate is derived from the original virulent LSDV (Neethling) isolate which was passaged in eggs and cell culture to produce an attenuated live vaccine (as described by Weiss, 1968). The sample used in this study was kindly supplied by D. Kow (Department of Medical Microbiology, Medical School, UCT) after several passages in CFK cells. The virus was then passaged 12 times in MDBK cells. After 2 further passages in FBT cells, viral DNA was extracted from infected cells.

3) LSDV (Gaborone) - this virus isolate was also kindly supplied by D. Kow after several passages in CFK cells. It was then passaged 10 times in MDBK cells and viral DNA was extracted after two further passages in FBT cells. First described in 1959.

4) LSDV (Haden) - was rehydrated and passaged 13 times in MDBK cells. Viral DNA was extracted after two further passages in FBT cells. It was first described in 1954.

5) LSDV (Maputo) - This isolate was originally called "LSDV 74-8", but was changed to its present name for convenience in designating it by its source of origin. Rehydrated virus was passaged 7 times in MDBK cells and then twice in FBT cells for DNA extraction. First described in 1955.

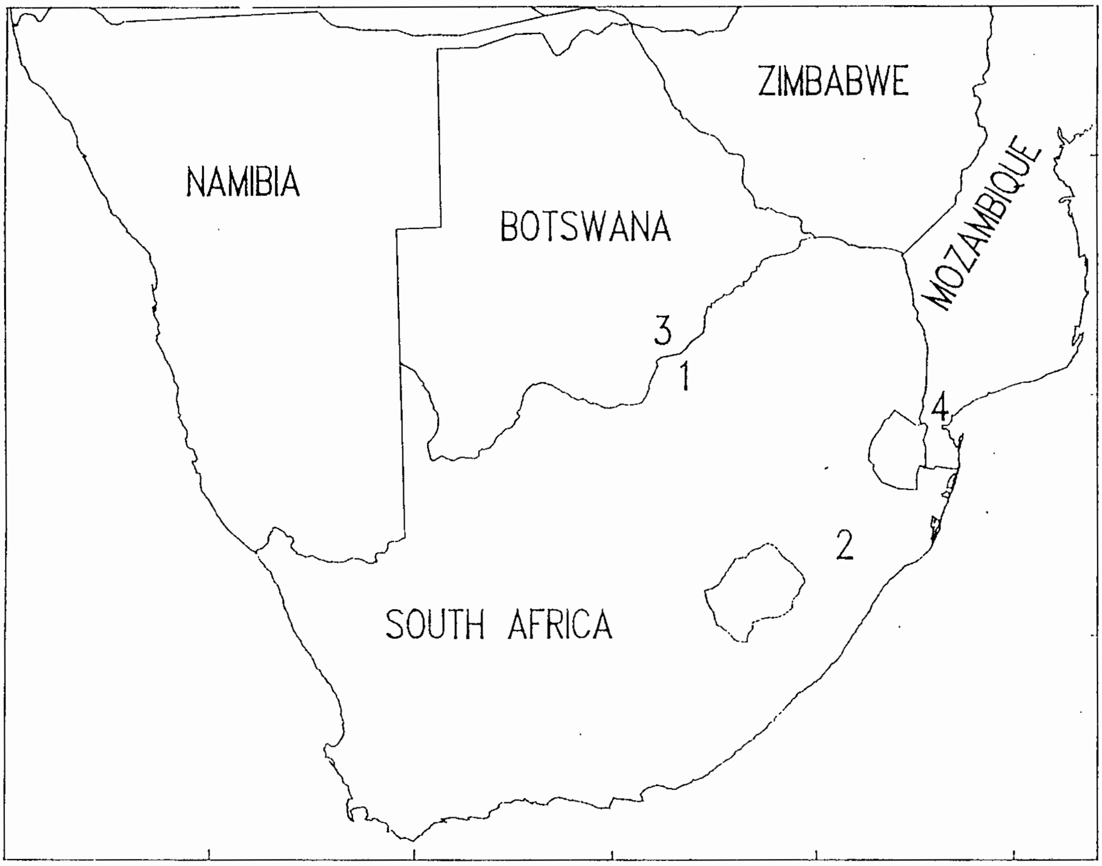

FIGURE 8.1.

A map of southern Africa indicating the locations of the LSDV isolates.
1 = LSDV (Neethling), 2 = LSDV (Haden), 3 = LSDV (Gaborone) and
4 = LSDV (Maputo).

8.2.2 Purification of LSDV:

8.2.2.1. Preparation of routine virus stocks :

The infected cells (LT or FBT) were harvested, either by shaking the flask vigorously to dislodge the cells (only possible when the cells were heavily infected) or by scraping the cells loose using a "rubber policeman" or by removing them gently using trypsin (4.2.4). The medium (DMEM and Ham's F12) containing the loose cells was dispensed into Universal bottles. These bottles were balanced and centrifuged at low speed in a Sigma 301K benchtop centrifuge at 1500 rpm (250*g*) for 10 min at 4°C. The cell pellets were lysed in 2 ml McIlvain's buffer and left on ice for 10 min. The lysed cells were centrifuged at 2000 rpm (450*g*) for 10 min and the supernatant fluid was collected. The cell debris was resuspended in McIlvain's buffer and was again centrifuged at 2000 rpm (450*g*) for 10 min. The supernatant fluid was collected and pooled with the first virus-containing supernatant fluid. An aliquot of the purified virus was removed and the titre of the virus was determined (Chapter 4, 4.2.7). The rest of the virus suspension was diluted in glycerol to produce a final concentration of 70 % glycerol and was dispensed into ampoules and frozen at -70°C.

8.2.2.2. Preparation of pure LSDV for DNA extraction (Esposito *et al.*, 1981):

The culture medium was removed from flasks containing infected cells exhibiting 90 % CPE. The infected cells were harvested by using trypsin to release them from the flasks (4.2.4). 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 (19000g), 60 min., 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 min. 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 min to disrupt the cells. The cell debris was removed by centrifugation at 2000 rpm (450g) for 5 min., 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 (2000 rpm (450g) for 5 min., 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 (19000g), 60 min., 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 (Appendix 1 and no. 3 from chapter 7) 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.

Viral DNA was purified as follows (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 min. 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 % EtOH and left O/N at -20 °C. The DNA was then concentrated by centrifugation at 14 000 rpm for 30 min. in an Eppendorf Centrifuge. The DNA pellet was washed in 70 % EtOH, resuspended in sterile distilled H_2O or TE buffer and stored at 4 °C.

8.2.3 Restriction endonuclease digestion and FIGE separation of LSDV isolates:

8.2.3.1. Endonuclease restriction:

DNA was purified from the four field isolates of LSDV and from the vaccine strain and was then restricted with the endonucleases *Hin* dIII, *Eco* RI, *Pst* I and *Bam* HI:

Digestion mixes were prepared in 1,5 ml Eppendorf tubes (Eppendorf, Germany): 10 µl DNA (the concentration of this DNA was variable and depended on the individual sample being digested), 2 µl 10X enzyme dilution buffer (the type of buffer depended on the specific enzyme being used), 1 µl restriction endonuclease (10 units) and 7 µl distilled H_2O to give a final volume of 20 µl. This mixture was incubated overnight in a waterbath at the manufacture's recommended temperature. The digestion was stopped with the addition of 3 µl 6X Stop Buffer (Appendix 1). The restricted DNA was then electrophoresed on an agarose gel.

8.2.3.2. FIGE separation of LSDV restricted DNA fragments:

Gels were prepared and samples were electrophoresed as described in Chapter 7 (7.2.1), except that a custom-made electrophoresis tank was used, containing 3,5 litres of pre-cooled 0.5 x TBE buffer (Appendix 1) (this large volume was required to act as a heat sink). No internal cooling system was employed, thus electrophoresis was performed in a cold room. Although this room was maintained at a constant temperature of 4 °C, a slight increase in temperature occurred over the entire course of each run (4 °C - 13 °C).

Digests, along with fragment size markers produced by either *Pst* I or *Hin* dIII digestion of phage Lambda DNA (Appendix 3), were loaded on 0,8 % agarose gels and the DNA restriction fragments were separated using the following FIGE conditions:

Prerun:	220 V, 70 mA, 4 °C, 15 min.
Run:	220 V, 4 °C, 16 hours
	F = 90 msec, R = 30 msec, r = 0,5

Separation of larger fragments (> 11 kb) was obtained by running the samples further using these conditions:

Run:	220 V, 4 °C, 9 hours
	F = 1.0 sec, R = 0.3 sec, r = 1.0

Gels were stained in EtBr (500 ng/ml), viewed under a UV Transilluminator and photographed with a Mamiya medium-format camera (7.2.1.2).

8.2.3.3 "Snap-back" analysis of LSDV DNA fragments:

The viral DNA was restricted with a restriction endonuclease. An ice bath was prepared by placing dry ice into absolute ethanol. A sample of the restricted DNA was denatured for 5 minutes in boiling water (100 °C) and was then plunged into the ice bath. It was removed after 2 minutes and centrifuged at 14000 rpm in an Eppendorf Centrifuge 5415 C

(Eppendorf, Germany) for 3 seconds to concentrate the DNA. The denatured DNA was then immediately loaded onto a 0.8 % agarose gel, alongside a non-denatured sample of the same restricted DNA.

8.3 RESULTS:

Although *Pst* I, *Ava* I and *Sal* I maps of the vaccine strain of LSDV are available (Perlman, 1993), *Ava* I and *Sal* I only restrict the genome in six and seven places respectively, producing a small number of relatively large fragments. In order to detect minor differences in the genomes of the isolates, it was thus necessary to restrict them with nucleases which have short, or frequently occurring recognition sequences and which would thus restrict the LSDV genome to produce a large number of relatively short fragments. *Pst* I, *Hin* dIII, *Eco* RI and *Bam* HI meet these criteria and were thus used in this study.

Comparisons of electrophoretic patterns of the DNA of all five isolates of LSDV restricted with *Pst* I, *Hin* dIII, *Eco* RI and *Bam* HI are illustrated in Figures 8.2, 8.3 and 8.4. Reference to a specific DNA band from a digest is made by refering to the largest fragment (that fragment which is nearset the top of the photograph) as "fragment A" and to the next largest fragment (one fragment below the top fragment) as "fragment B" etc. Approximate sizes are given for bands of interest using Lambda DNA restricted with *Pst* I and *Hin* dIII as reference markers. A more accurate sizing of bands would require the production of a ladder of Lambda fragments consisting of a large enough number of fragments so that each marker fragment co-migrates with each LSDV DNA fragment. This is due to the fact that fragments separated using conventional or pulse field gel electrophoresis do not migrate at a rate directly proportional to the size of fragment (Bostock, 1988).

As can be seen from Figure 8.2, all of the bands produced by *Pst* I restriction appear to have co-migrated, although it is possible that fragment E of the Maputo isolate (lane 6) has migrated slightly slower, and is thus larger, than the equivalent fragments of the other four isolates. The Maputo isolate contains a number of additional faint bands which may have arisen from partial digestion. Attempts were made to re-clarify the stock of Maputo DNA used for the *Pst* I digestion and a new stock of virus was prepared, from which DNA was extracted. However, re-digestion of both DNA stocks failed to resolve these extra bands (results not shown).

Hin dIII restriction patterns (Fig. 8.3), indicate that most of, but not all, the bands co-migrated. Due the large number of fragments produced using *Hin* dIII (> 30), it is difficult to distinguish between discrete viral bands and background levels of degraded DNA. This is especially true for fragments smaller than 2.5 kbp in size in lanes consisting of digests of the field isolates (lanes 4-6) . However, comparison of LSDV (Neethling) and the vaccine strain reveals the presence of a small 1.5 kbp band for LSDV (Neethling) (lane 3, arrow), which is absent in the vaccine strain (lane 2). Maputo (lane 6) appears to have produced an incomplete restriction pattern with *Hin* dIII, as was the case for *Pst* I restriction in Fig. 8.2.

Eco RI and *Bam* HI restriction digests revealed a number of interesting differences in the genomes of the LSDV isolates (Fig 8.4). Although most of the bands co-migrated, the H (≈5.9 kbp) and K (≈4.9 kbp) fragments of the *Eco* RI digests and the *Bam* HI fragments, G (≈9.0 kbp) and J (≈4.8 kbp), did not all co-migrate. These fragment length polymorphisms are summarised in Table 8.1. The positions of the fragments from the vaccine strain are compared relative to those from the virulent LSDV isolates. Except for Haden, which consists of co-migrating fragments (marked with a "="), the fragments from the virulent isolates are mostly larger than those of the vaccine strain (marked with a "+"). All fragments from the *Eco* RI and *Bam* HI digest patterns of the vaccine strain (Fig. 8.4, lanes 2 and 9) and LSDV (Haden) (lanes 4 and 11) appear to have co-migrated. LSDV (Neethling) and LSDV (Maputo) both seem to lack the *Eco* RI K fragment, although it is possible that it is co-migrating with another fragment to form a doublet. A closer inspection of the Neethling and Maputo *Eco* RI J fragments suggests that these have migrated as doublets (results not shown). Although it is less clear, it also appears that LSDV (Neethling) (lanes 3 and 10) and LSDV (Maputo) (lanes 6 and 13) produce identical *Eco* RI and *Bam* HI restriction patterns. LSDV (Gaborone) (lanes 5 and 12) is unique in the *Eco* RI and *Bam* HI restriction profiles it produces.

An analysis of the *Eco* RI and *Bam* HI digestion patterns was made using the "snap-back" method to identify the co-terminal fragments, as these are regions in poxvirus genomes where the sequences are generally non-conserved (Massung and Moyer, 1991; Gershon *et al.*, 1989). Terminal cross-linked restriction fragments have been shown to renature rapidly on release from denaturing conditions (as cited by Esposito *et al.*, 1981). These terminal fragments are thus able to co-migrate with their corresponding fragments from a non-denatured digest, when separated on an agarose gel, whereas the rest of the denatured fragments are unable to renature and are therefore not detectable as discrete bands.

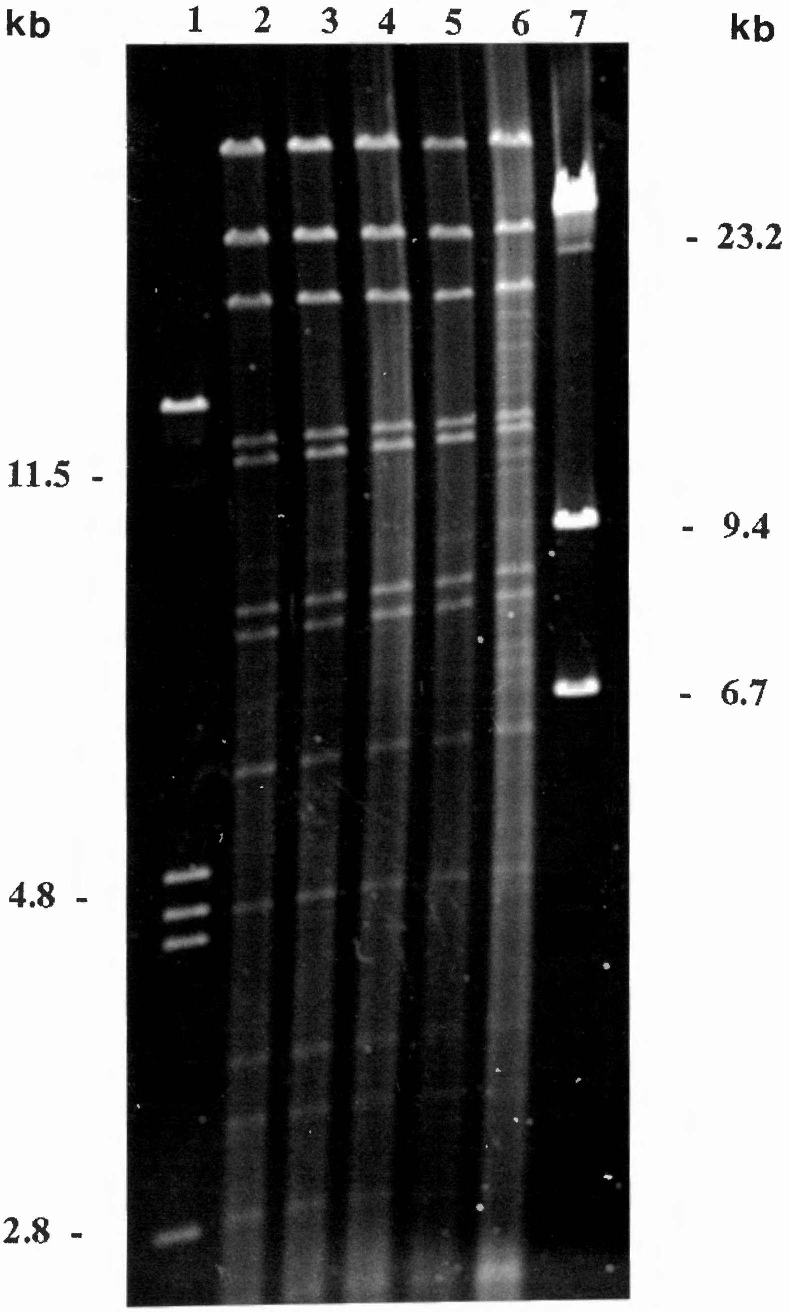

FIGURE 8.2
Agarose gel showing *Pst* I digests of the LSDV isolates separated using FIGE. Lane 1 = Lambda *Pst* I marker. Lane 2 = LSDV vaccine. Lane 3 = LSDV (Neethling). Lane 4 = LSDV (Haden). Lane 5 = LSDV (Gaborone). Lane 6 = LSDV (Maputo). Lane 7 = Lambda *Hin* dIII marker. FIGE conditions: Prerun: 220 V, 4 °C, 15 min. Run: 220 V, 4 °C, 16 hours, F = 90 msec, R = 30 msec, r = 0.5, then 220 V, 4 °C, 9 hours, F = 1.0, R = 0.3, r = 1.0.

Table 8.1. Comparisons of LSDV *Eco* RI and *Bam* HI fragment polymorphisms

ISOLATE	*Eco* RI		*Bam* HI	
	H	K	G	J
NEETHLING	+	absent	+	+
HADEN	=	=	=	=
GABORONE	+	+	+	+
MAPUTO	+	absent	+	+

\+ fragment larger than the vaccine strain
= fragment co-migrates with the vaccine strain
absent: fragment equivalent of the vaccine strain is not visible.

Denatured *Eco* RI and *Bam* HI digests of LSDV (vaccine) were prepared as described and were run (Fig. 8.4, lanes 1 and 8) next to the non-denatured *Eco* RI and *Bam* HI digests of LSDV (vaccine) (lanes 2 and 9). The presence of the co-terminal fragments are identifiable as these are the bands which have reannealed and co-migrated with corresponding bands from the non-denatured digests. The co-terminal fragments are thus the *Eco* RI H and K fragments and the *Bam* HI G and J fragments.

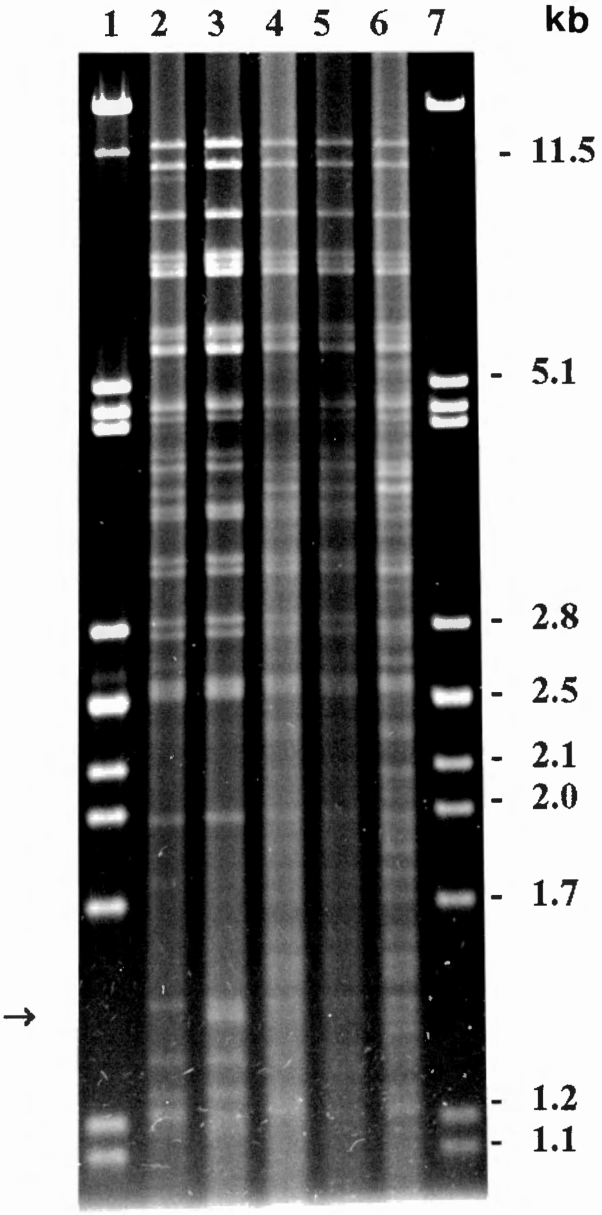

FIGURE 8.3
Agarose gel showing *Hin* dIII digests of the LSDV isolates separated using FIGE. Lane 1 = Lambda *Pst* I marker. Lane 2 = LSDV vaccine. Lane 3 = LSDV (Neethling). Lane 4 = LSDV (Haden). Lane 5 = LSDV (Gaborone). Lane 6 = LSDV (Maputo). Lane 7 = Lambda *Pst* I marker. FIGE conditions: Prerun: 220 V, 4 °C, 15 min. Run: 220 V, 4 °C, 16 hours, F = 90 msec, R = 30 msec, r = 0.5, then 220 V, 4 °C, 9 hours, F = 1.0, R = 0.3, r = 1.0. The arrow depicts the position of a 1.5 kbp fragment which is present in the Neethling digest only.

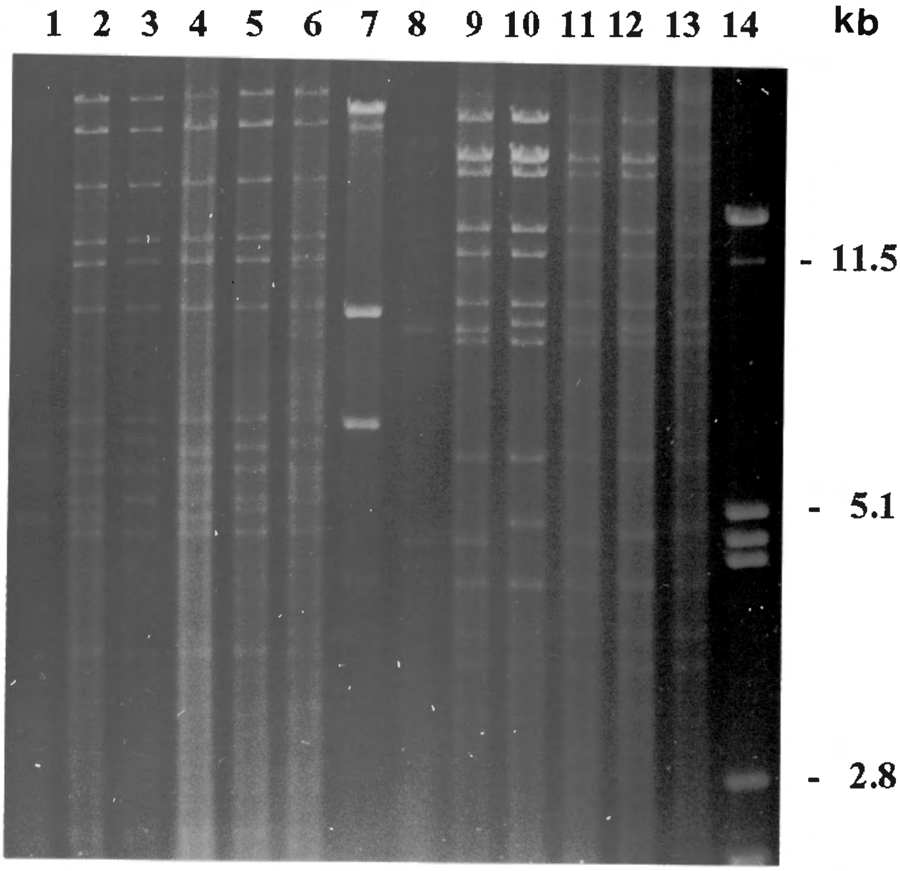

FIGURE 8.4
Agarose gel showing *Eco* RI (lanes 2 - 6) and *Bam* HI (lanes 9 - 13) digests of the LSDV isolates. Lane 1 = LSDV vaccine *Eco* RI (snap-back). Lane 2 = LSDV vaccine. Lane 3 = LSDV (Neethling). Lane 4 = LSDV (Haden). Lane 5 = LSDV (Gaborone). Lane 6 = LSDV (Maputo). Lane 7 = Lambda *Hin* dIII marker. Lane 8 = LSDV vaccine *Bam* HI (snap-back). Lane 9 = LSDV vaccine. Lane 10 = LSDV (Neethling). Lane 11 = LSDV (Haden). Lane 12 = LSDV (Gaborone). Lane 13 = LSDV (Maputo). Lane 14 = Lambda *Hin* dIII marker. FIGE conditions: Prerun: 220 V, 4 °C, 15 min. Run: 220 V, 4 °C, 16 hours, F = 90 msec, R = 30 msec, r = 0.5, then 220 V, 4 °C, 9 hours, F = 1.0, R = 0.3, r = 1.0.

8.4 DISCUSSION :

The restriction endonucleases *Pst* I, *Hin* dIII, *Eco* RI and *Bam* HI were used to cleave the DNA genomes of four southern African field isolates of LSDV and the vaccine strain. The genomic fragments were separated using FIGE. An analysis of the resulting patterns revealed a high degree of genomic conservation. These results are in agreement with Kitching *et al.* (1989) who described similar findings for comparisons of restriction pattern profiles of seven cattle isolates of capripoxvirus, including the LSDV (Neethling) isolate.

Comprehensive comparisons of the *Hin* dIII restriction digest patterns of the virulent LSDV isolates was not possible due to the large number of fragments generated using *Hin* dIII (>30) resulting in a low viral DNA to background DNA ratio. However, it did appear that the virulent Neethling strain contained a 1,5 kbp fragment which was absent from the vaccine *Hin* dIII digest.

Of major interest were the differences noted for the terminal fragments of the isolates. *Eco* RI and *Bam* HI digest patterns revealed slight differences in the terminal repeat regions of these isolates. Although less clear, it also appeared that the terminal E fragment (determined by Perlman, 1993) of the Maputo isolate was the only *Pst* I fragment which failed to co-migrate with corresponding fragments of the other isolates. Snap-back analysis was used to confirm the location of the *Eco* RI and *Bam* HI fragment polymorphisms in the terminal regions of the genome. The termini of poxviruses are generally non-conserved regions consisting of genes specific for host range (as cited by Gershon and Black, 1988). A comparison of the terminal polymorphisms of the vaccine strain and the field isolates indicated that the vaccine strain consisted of the shortest terminal regions. The loss of virulence of the vaccine, after a long passage history in eggs and culture cells, might thus be explained by the loss or rearrangement of genes in the terminal regions as compared to the virulent LSDV (Neethling) isolate from which it originated.

An overall analysis of the genomes of the five LSDV isolates compared in this study suggests that the Neethling and Maputo isolates are almost identical, the vaccine and Haden isolates are identical and the Gaborone isolate is the most diverse. It is interesting that the vaccine isolate is more similar to the Haden isolate than the Neethling isolate, from which it is meant to have originated. It would be difficult to explain why the terminal regions of certain isolates appear similar to each other (in respect to the conservation of restriction sites) and yet, different from other isolates, as factors such as history of passage in culture cells, recombination, distribution and date of isolation could have influenced the changes occurring in the genomes. It is also possible that the field isolates were not recent descendants of a common ancestor, thus allowing for a greater period of time for the genomes to diversify.

In lieu of the extremely narrow host-range exhibited by the vaccine strain of LSDV (Chapter 6) and the occurrence of deletions in the terminal regions of its genome, it would be of interest to determine whether the field isolates are able to replicate in cells other than those derived from sheep, cattle or chicken. This would seem unlikely, however, as the southern African isolates of LSDV have proved to be extremely host specific and in South Africa, LSDV has never been reported in hosts other than cattle (as cited by Kitching *et al.*, 1989). Experimental transmission to game animals was reported (Young *et al.*, 1970) and yet attempts to re-culture virus, claimed to have been recovered from infected animals displaying disease symptoms, failed in this laboratory.

CHAPTER 9

MAPPING OF RARE RESTRICTION SITES OF LSDV:

9.1 INTRODUCTION:

Poxviruses are being used as vectors for recombinant vector vaccines (Taylor *et al.*, 1988; Taylor *et al.*, 1992; review by Baxby and Paoletti, 1992). Homologous recombination *in vivo* has been extensively used for the construction of recombinant viruses (Falkner and Moss, 1990). A non-essential gene is used as an insertion site and this gene usually has the ability to be used as a selectable marker. A plasmid is constructed containing flanking sequences of the non-essential gene interrupted by a foreign gene. Cells infected with the wild-type virus are transfected with this plasmid. Double recombination between the virus and the plasmid results in the formation of a recombinant virus containing the foreign gene and displaying a negative phenotype for the non-essential gene. This altered phenotype can then be used to select only recombinant virus particles. This technique has its limitations due to rearrangements of DNA occurring during the bacterial cloning steps or loss of efficiency of homologous recombination with an increasing length of insert (as cited by Merchlinsky and Moss, 1992).

More recently, successful cloning of large fragments of DNA (up to 26 kbp) into the genome of vaccinia virus has been achieved with the use of *in vitro* ligation (Merchlinsky and Moss, 1992). Unique restriction sites found in non-essential regions of the genome were used, or were created, for use in the cloning strategy as insert DNA could then be ligated directly into whole genomic DNA. Propagation of recombinant viruses was made possible by first infecting mammalian cells with a conditionally lethal temperature-sensitive vaccinia virus mutant which provided the machinery necessary for replication and packaging, but which was unable to replicate itself. Purified vaccinia virus DNA, containing a foreign gene insert, was then transfected into these cells and incubation was performed at the non-permissive temperature of 40 °C for two days. Even without selection, up to 25 % of virions recovered from infected cells were found to contain inserts. Although the construction of recombinant capripoxviruses has been described using homologous recombination (Romero *et al.*, 1993), the use of *in vitro* ligation may provide a more efficient method for the cloning of very large inserts.

An aim of this thesis was the identification and mapping of rare restriction sites within the genome of LSDV vaccine strain. If these sites occur within non-essential regions of the genome, then they might eventually be used as cloning sites for the construction of a recombinant LSDV using *in vitro* ligation as described by Merchlinsky and Moss (1992) for the construction of a recombinant vaccinia virus.

9.2 METHODS:

Most poxvirus genomes contain a higher percentage of A-T bases than G-C bases (as reviewed by Moss, 1990) and therefore a number of restriction endonucleases were chosen which recognise long DNA sequences that are rich in guanine and cytosine. The enzymes chosen were: *Not* I, *Sma* I, *Nar* I, *Nae* I, *Ksp* I, *Apa* I and *Bss* HII (Boehringer-Mannheim, Germany).

Digests of vaccinia (DIE #7) virus DNA, using these enzymes, were set up in parallel as a control to ensure that the enzymes had retained their enzymatic activity. Purified DNA of LSDV (Neethling) vaccine and vaccinia virus was digested overnight as described (8.2.3.1). The digests were then run on a 0,8 % agarose gel using the following FIGE conditions (using the same gel tank and cooling apparatus as described in 7.2.1):

Prerun : 220 V, 70 mA, 4 °C, 15 min, no pulse
Run : 220 V, 70 mA, 4 °C, 12 hrs
F = 1,0 sec, R = 0,3 sec, r = 1,0

The gel was stained with EtBr, viewed and photographed as described (7.2.1.1).
In order to further separate the fragments, the gel was run for an extended period under the following conditions:

Run : 220 V, 70 mA, 4 °C, 22 hrs
F = 90 msec, R = 30 msec, r = 0,5

The gel was restained and photographed.

In order to map the precise locations of rare restriction sites, a number of double digests were prepared:

The first endonuclease restriction was prepared as described for single digests (8.2.3.1), except that no Stop Buffer was added. DNA was precipitated with ethanol as described (8.2.2.2) and resuspended in distilled H_2O. The second restriction enzyme and buffer was then added and restriction was continued overnight at the appropriate incubation temperature. The reaction was stopped with the addition of 3 µl 6X Stop Buffer and the sample was electrophoresed on an agarose gel.

LSDV (Neethling) vaccine DNA was restricted with *Pst* I, *Ava* I and *Sal* I [as the location of these sites has been mapped (Perlman,1993)]. The DNA was ethanol-precipitated, resuspended in distilled H_2O and then the second enzymes were added in their respective buffers in these combinations:

Pst I/*Sma* I, *Pst* I/*Apa* I, *Pst* I/*Bss* HII, *Ava* I/*Apa* I

Sal I/*Apa* I, *Sal* I/*Sma* I and *Ava* I/*Sma* I

The samples were incubated overnight, enzyme activity was stopped with Stop buffer (X6) and the digests were separated on a 0,8 % agarose gel using FIGE as follows:

Two sets of pulse conditions were used as *Ava* I and *Sal* I cleave LSDV DNA at fewer sites than *Pst* I, therefore producing larger fragments. Single digests of *Pst* I, *Ava* I and *Sal* I were also prepared for comparison with the double digests.

The *Pst* I single and double digests were run using these conditions:

Prerun : 220 V, 70 mA, 4 °C, 15 min.

Run : 220 V, 70 mA, 4 °C, 12 hrs
F = 1,0 sec, R = 0,3 sec r = 1,0

The *Ava* I and *Sal* I single and double digests were run using these conditions:

Prerun : 220 V, 70 mA, 4 °C, 15 min.

Run : 220 V, 70 mA, 4 °C, 9 hrs
F = 2 sec, R = 0,6 sec, r = 0

The electrophoretic conditions were then altered and the gel was run for a further 16 hours as follows:

Run : 220 V, 4 °C, F = 90 msec, R = 30 msec, r = 0,5

Gels were stained, viewed and photographed as described (refer to 7.2.1).

9.3 RESULTS:

LSDV vaccine DNA was restricted with a number of rare cutting restriction endonucleases. The fragments which were generated were separated on agarose gels using FIGE and the resulting patterns were analysed in order to determine which enzymes contained unique restriction sites within the LSDV vaccine strain genome. Fragments were sized to approximate values and the use of double restriction digestion, in conjunction with the accurate *Pst* I, *Ava* I and *Sal* I restriction maps of the LSDV vaccine genome elucidated by Perlman (1993) (Fig. 2.2 B), allowed relatively accurate determination of the location of unique sites.

The resulting single digest restriction profiles are illustrated in Figures 9.1 and 9.2 (Fig. 9.2 is the same gel as in Fig. 9.1, but after extended electrophoresis), and an estimation of the number and size of the fragments generated are summarised in Table 9.1. It can clearly be seen that LSDV (Neethling) vaccine lacks *Not* I, *Ksp* I and *Nar* I restriction sites (lanes 9, 12 and 13) as the single bands in these lanes have co-migrated with the uncut LSDV DNA (lane 7). The enzyme activities of the three endonucleases were active as evidenced by their ability to cleave vaccinia virus (Die #7) DNA (lanes 3, 4 and 5).

Sma I and *Apa* I (lanes 8 and 10) cleaved LSDV DNA once producing fragments of approximately 85 and 67 kbp with *Sma* I, and 30 and 122 kbp with *Apa* I. *Bss* HII (lane 14) cleaved the genome twice producing three closely sized fragments (ranging in size between 42 and 60 kbp). *Nae* I (lane 11) restriction produced five fragments which add up to a total genome size greater than 152.6 kbp. It can therefore be assumed that at least one of these sites restricted partially. Further attempts to resolve the partial digestion failed.

The LSDV vaccine genome thus contains unique *Sma* I and *Apa* I restriction sites. As it only contains two *Bss* HII sites, determining the location of these sites was also of interest.

TABLE 9.1. Sizes and number of the restriction fragments generated by single digestion of the LSDV vaccine DNA with rare restriction endonucleases.

FRAGMENT No.	SIZE OF FRAGMENT (kbp)							
	uncut	*Sma* I	*Not* I	*Apa* I	*Nae* I	*Nar* I	*Ksp* I	*Bss*HII
A	152.6	85.6	152.6	122.6	*	152.6	152.6	60.6
B		67.0		30.0	70.6			50.0
C					55.0			42.0
D					27.0			
E					*			

* partial digest fragments

In order to determine the exact location of the restriction sites of *Sma* I, *Apa* I and *Bss* HII, double digests of these enzymes, and *Pst* I, *Ava* I or *Sal* I, were prepared.. Single digestion sizes of *Pst* I, *Ava* I or *Sal* I fragments are presented in Table 9.2. These results can be seen in Figs. 9.3, 9.4 and 9.5 and are summarised in Table 9.3. Tables 9.2 and 9.3 represent the fragment number and sizes obtained from single and double digestion of LSDV DNA with *Pst* I, *Ava* I and *Sal* I.

Sma I cut the *Pst* I D fragment (12,3 kbp) to produce two fragments of 9,1 and 3,2 kbp (smaller band not visible on the photograph) (Fig. 9.3, lane 3).

Apa I cleaved the *Pst* I J fragment (3,6 kbp) to produce two fragments of 3,4 kbp and 0,2 kbp (smaller band not visible on the photograph) (Fig. 9.3, lane 4).

Bss HII cut the *Pst* I A (42,0 kbp) and C (19,76 kbp) fragments producing three bands of approximately 35, 8,2 and 7 kbp (Fig. 9.3, lane 5). These fragments do not add up to the expected total of 61,76 kbp (sum of the *Pst* I A and C fragments) and it is thus possible that at least one band is co-migrating with another *Pst* I band (No further attempts were made to map the location of the *Bss* HII restriction sites as unique restriction sites were considered more important).

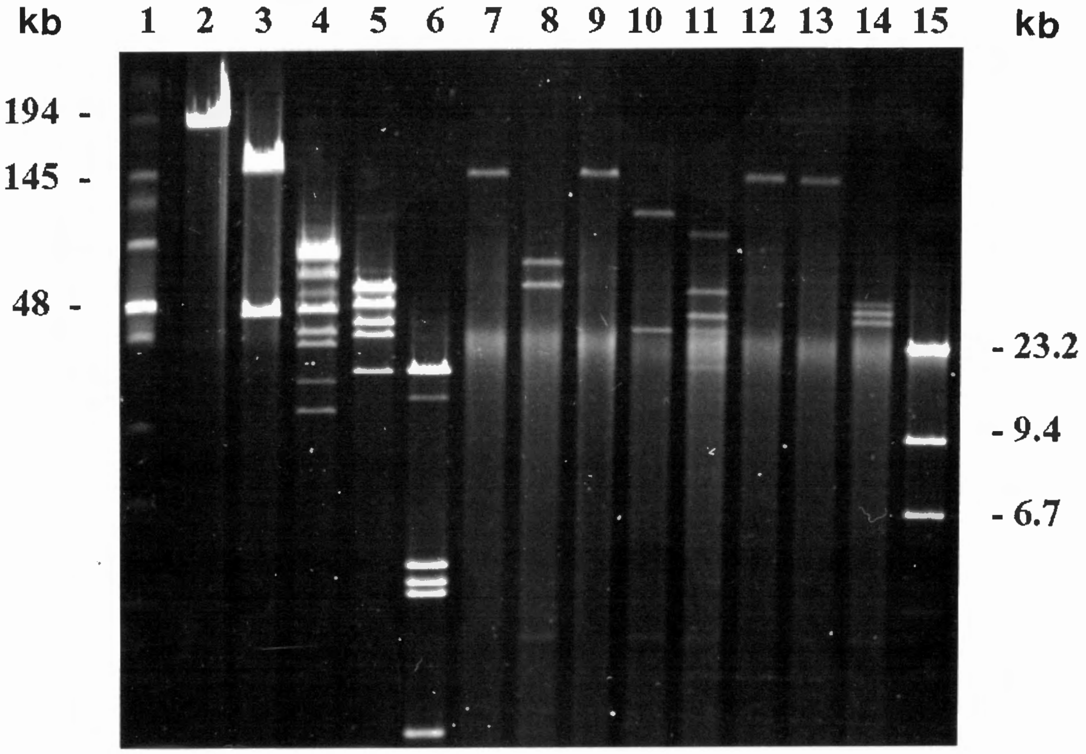

FIGURE 9.1

Agarose gel of LSDV and vaccinia (DIE #7) DNA restricted with rare restriction site endonucleases: 1st run. Lane 1 = High Molecular Weight marker. Lane 2 = Vaccinia uncut. Lane 3 = Vaccinia *Not* I. Lane 4 = Vaccinia *Nar* I. Lane 5 = Vaccinia *Ksp* I. Lane 6 = Lambda *Pst* I marker. Lane 7 = LSDV uncut. Lane 8 = LSDV *Sma* I. Lane 9 = LSDV *Not* I. Lane 10 = LSDV *Apa* I. Lane 11 = LSDV *Nae* I. Lane 12 = LSDV *Nar* I. Lane 13 = LSDV *Ksp* I. Lane 14 = LSDV *Bss* HII. Lane 15 = Lambda *Hin* dIII marker. FIGE conditions: 150 V, 54 mA, 14 °C, 12 hours, F = 1.0 sec, R = 0.3 sec, r = 1.0.

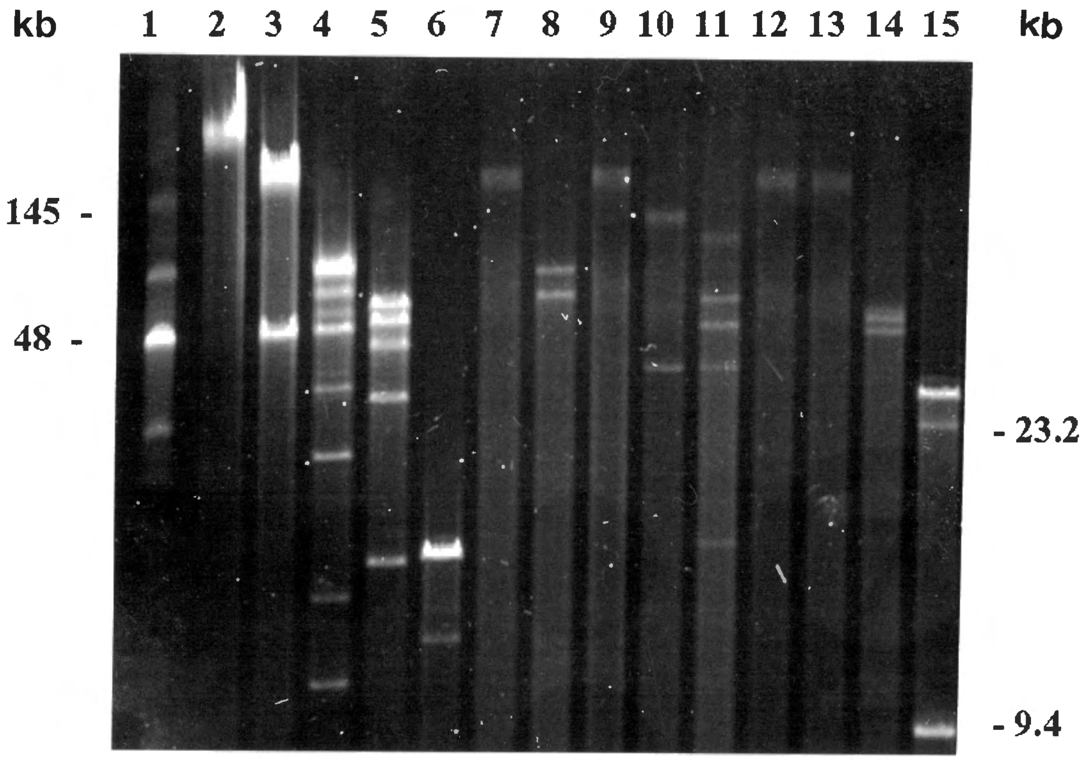

FIGURE 9.2

Agarose gel of LSDV and vaccinia (DIE #7) DNA restricted with rare restriction site endonucleases; 2nd run: Lane 1 = High Molecular Weight marker. Lane 2 = Vaccinia uncut. Lane 3 = Vaccinia *Not* I. Lane 4 = Vaccinia *Nar* I. Lane 5 = Vaccinia *Ksp* I. Lane 6 = Lambda Pst I marker. Lane 7 = LSDV uncut. Lane 8 = LSDV *Sma* I. Lane 9 = LSDV *Not* I. Lane 10 = LSDV *Apa* I. Lane 11 = LSDV *Nae* I. Lane 12 = LSDV *Nar* I. Lane 13 = LSDV *Ksp* I. Lane 14 = LSDV *Bss* HII. Lane 15 = Lambda *Hin* dIII marker. FIGE conditions: 150 V, 65 mA, 14 °C, 22 hours, F = 90 msec, R = 30 msec, r = 0.5.

From Fig. 9.4 it can be seen that the 41,62 kbp *Ava* I B fragment (lane 2) was cleaved by *Apa* I to produce two fragments of 37 and 4,62 kbp (smaller band not visible on the photograph). *Apa* I cleaved one of the three *Sal* I A fragments (33,15 kbp; lane 4) to produce two bands of 30 and 3.2 kbp (smaller band not visible on the photograph) (lane 5).

From Fig. 9.5, it is unclear whether any one of the *Ava* I fragments is cleaved by *Sma* I as there is no noticeable change in the migration of any of the *Ava* I fragments when comparing the *Ava* I single (lane 2) and double (lane 3) digest patterns.

TABLE 9.2. Sizes of the restriction fragments generated by double and single digestions of LSDV vaccine DNA with *Pst* I, *Ava* I and *Sal* I.

Ava I	Ava I/Sal I	Sal I	Sal I/Pst I	Pst I	Pst I/Ava I
A ^{T}47.35	A' " 26.16	A' " ''' ^{T}33.15	A' " 26.5	A 42.0	A 30.1
B 41.62	B 20.7	B ^{T}20.7	B 12.5	B ^{T}26.5	B 26.4
C 28.2	C 19.05	C 12.5	C 12.1	C 19.76	C 19.76
D ^{T}26.16	D 14.1	D 8.71	D 11.2	D 12.3	D 12.1
E 4.54	E 12.5	E 8.33	E 8.71	E ^{T}12.1	E 12.00
F 2.92	F 8.71	F 2.8	F 8.2	F 8.5	F 8.5
G 2.1	G' " 6.75		G 7.05	G 8.2	G 8.2
	H 2.96		H 6.95	H 6.0	H 6.0
	I 2.92		I 6.0	I 4.7	I 4.7
	J 2.8		J 4.7	J 3.6	J 4.3
	K 2.1		K 3.6	K 3.25	K 3.6
	L 1.58		L 3.25	L 2.82	L' " 3.25
			M 3.0	M 2.4	M 2.92
			N 2.85		N 2.82
			O 2.82		O 2.4
			P 2.4		P 2.1
			Q' " 1.44		Q 0.20
			R 1.35		R 0.15
152.89	**153.24**	**152.49**	**152.56**	**152.13**	**152.85**

T = terminal fragment, $^{\prime\ \prime\prime\ \prime\prime\prime}$ = no. of co-migrating fragments.

(Perlman, 1993)

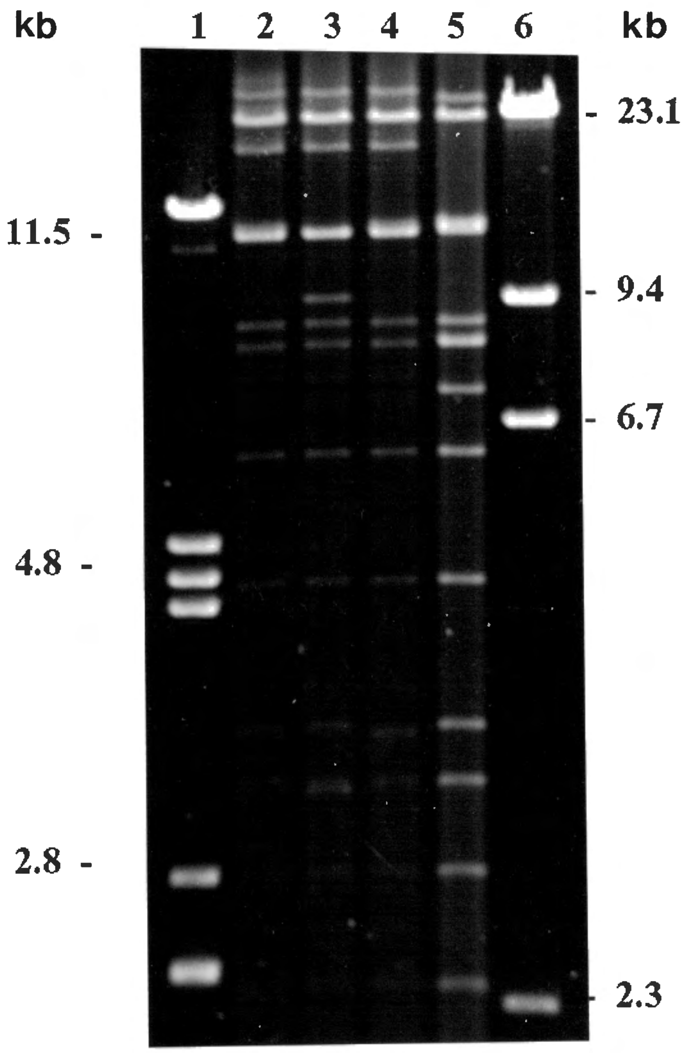

FIGURE 9.3

Agarose gel of LSDV vaccine DNA *Pst* I double digests of rare restriction endonucleases. Lane 1 = Lambda *Pst* 1 marker. Lane 2 = *Pst* I. Lane 3 = *Pst* I/ *Sma* I. Lane 4 = *Pst* I/ *Apa* I. Lane 5 = *Pst* I/ *Bss* HII. Lane 6 = Lambda *Hin* dIII marker. FIGE conditions : 100 V, 14 °C, 12 hours, F = 1.0, R = 0.3, r = 1.0

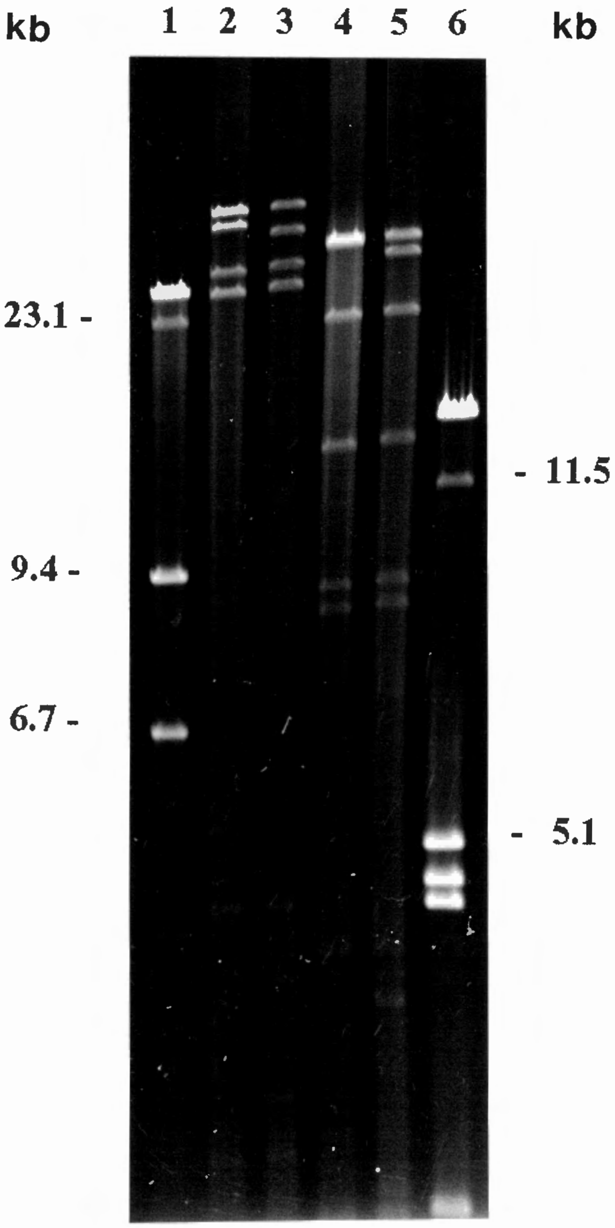

FIGURE 9.4

Agarose gel of LSDV (vaccine) DNA double digests. Lane 1 = Lambda *Hin*d III marker. Lane 2 = LSDV *Ava* I. Lane 3 = LSDV *Ava* I/*Apa* I. Lane 4 = LSDV *Sal* I. Lane 5 = LSDV *Sal* I/*Apa* I. Lane 6 = LSDV *Pst* I marker. FIGE conditions: Prerun: 220 V, 4 °C, 15 min. Run: 220 V, 4 °C, 16 hours, F = 90 msec, R = 30 msec, r = 0.5, then 220 V, 4 °C, 9 hours, F = 1.0 sec, R = 0.3 sec, r = 1.0

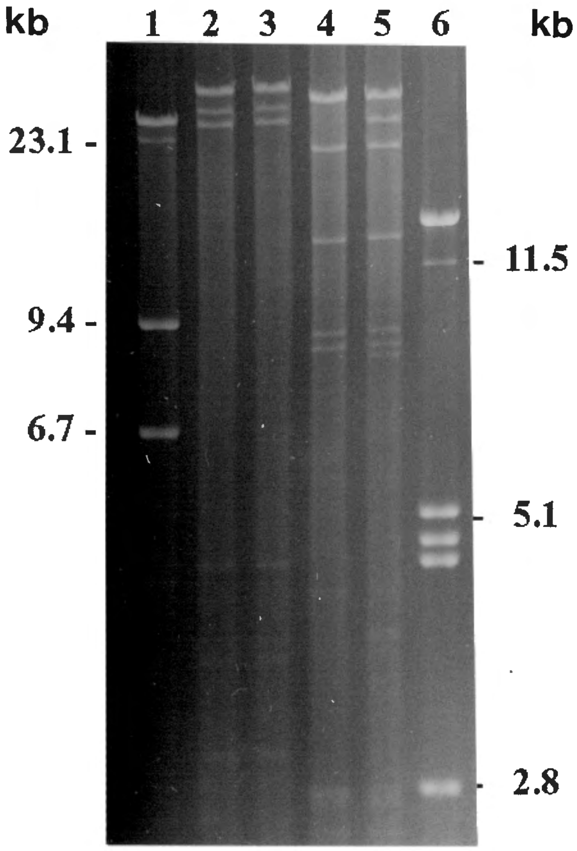

FIGURE 9.5

Agarose gel of LSDV (vaccine) DNA double digests. Lane 1 = Lambda *Hin*d III marker. Lane 2 = LSDV *Ava* I. Lane 3 = LSDV *Ava* I/*Sma* I. Lane 4 = LSDV *Sal* I. Lane 5 = LSDV *Sal* I/*Sma* I. Lane 6 = LSDV *Pst* I marker. FIGE conditions: Prerun: 220 V, 4 °C, 15 min. Run: 220 V, 4 °C, 16 hours, F = 90 msec, R = 30 msec, r = 0.5, then 220 V, 4 °C, 9 hours, F = 1.0 sec, R = 0.3 sec, r = 1.0

A clear distinction between LSDV DNA cleaved with *Sal* I only (Fig. 9.5, lane 4) and *Sal* I/*Sma* I (lane 5) is possible as *Sma* I cleaves one of the *Sal* I A fragment triplets (33.15 kbp) to produce two fragments of 25.0 kbp and 8.1 kbp (lane 5).

TABLE 9.3. Sizes of the restriction fragments generated by double and single digestions of LSDV vaccine DNA.

	FRAGMENT SIZES * (kbp)											
	Pst I	Pst I SmaI	Pst I Apa I	Pst I BssH	Ava I	Ava I Apa I	Sal I	Sal I Apa I	Ava I	Ava I SmaI	Sal I	Sal I SmaI
A	42.0	42.0	42.0	35.0	47.4	47.4	33.2	33.2	47.4	47.4	33.2	33.2
B	26.5	26.5	26.5	26.5	41.6	37.0	20.7	30.0	41.6	41.6	20.7	25.0
C	19.8	19.8	19.8	12.3	28.2	28.2	12.5	20.7	28.2	28.2	12.5	12.5
D	12.3	12.1	12.3	12.1	26.2	26.2	8.7	12.5	26.2	26.2	8.7	8.7
E	12.1	9.1	12.1	8.5	4.5	4.6	8.3	8.7	4.5	4.5	8.3	8.3
F	8.5	8.5	8.5	8.2	2.9	4.5	2.8	8.3	2.9	2.9	2.8	8.1
G	8.2	8.2	8.2	7.0	2.1	2.9		3.2	2.1	2.1		2.8
H	6.0	6.0	6.0	6.0		2.1		2.8				
I	4.7	4.7	4.7	4.7								
J	3.6	3.6	3.4	3.6								
K	3.3	3.3	3.3	3.3								
L	2.8	3.2	2.8	2.8								
M	2.4	2.8	2.4	2.4								
N		2.4	0.2									

* shaded areas represent sizes of fragments of interest

The exact location of the unique *Apa* I and *Sma* I restriction sites could then be determined using the results described above and the *Ava* I, *Sal* I and *Pst* I restriction map (Fig. 9.6): The *Apa* I site is located either 0.2 kbp from the left- or right-hand end of the *Pst* I J fragment (Fig. 9.3). As double restriction of *Apa* I with *Ava* I results in restriction of the *Ava* I B fragment to produce two fragments of 37.0 and 4.62 kbp, and as the *Pst I* site (separating the *Pst I* B and J fragments) and *Ava* I site (separating the *Ava* I D and B fragments) are located close to each other, it is apparent that the *Apa* I site is located 0.2 kbp from the left-hand end of the *Pst* I J fragment. If it was located nearer the right-hand end, then double restriction with *Ava* I would have failed to result in a noticeable shortening of the *Ava* I B fragment.

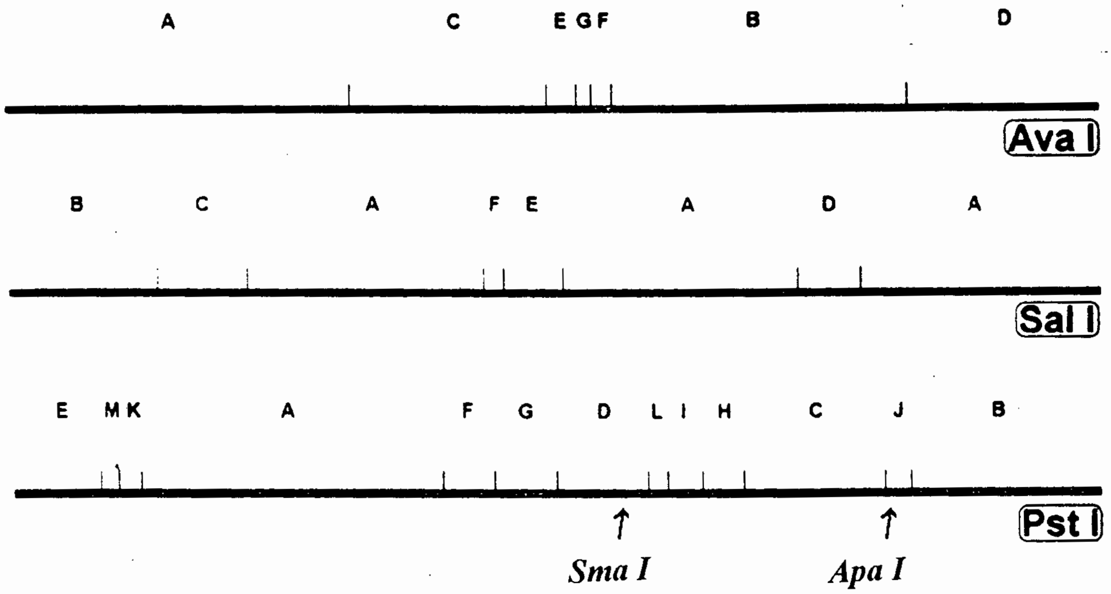

FIGURE 9.6
Genomic map of LSDV (Neethling) vaccine showing the location of *Ava* I, *Pst* I and *Sal* I restriction sites. The location of the unique *Apa* I and *Sma* I sites have also been indicated. (Reproduced with kind permission of the author, Perlman, 1993).

The *Sma* I site is located either 3.0 kbp from the left- or right-hand end of the *Pst* I D fragment (Fig. 9.3). Although it is unclear which *Ava* I fragment is restricted by *Sma* I, as double restriction of *Sma* I with *Sal* I results in restriction of the central *Sal* I A triplet fragment (as this is the only *Sal* I A fragment which overlaps with the *Pst* I D fragment) to produce two fragments of 25.0 and 8.1 kbp. As the *Pst I* site (separating the *Pst I* G and D fragments) is located close to the *Ava* I site (separating the *Ava* I D and B fragments), it is apparent that the *Sma* I site is located 3.0 kbp from the right-hand end of the *Pst* I D fragment. If it was located nearer the left-hand end, then double restriction with *Sal* I would have resulted in the *Sal* I A fragment being shortened by only 3.0 kbp.

9.4 DISCUSSION:

The construction of recombinant poxviruses using *in vitro* ligation requires the creation or identification of unique restriction endonuclease sites (Merchlinsky and Moss, 1992). Seven rare cutting endonucleases were tested on LSDV (Neethling) vaccine DNA. *Not* I, *Ksp* I and *Nar* I failed to restrict, even though the enzymes had retained their full enzymatic activity as evidenced by their ability to restrict vaccinia virus (DIE #7) DNA. Using site-directed mutagenesis, it would be possible to create *Not* I, *Ksp* I or *Nar* I restriction sites within any non-essential region in the LSDV genome for use as a cloning site for foreign DNA.

The LSDV genome was shown to contain two *Bss* HII restriction sites. Attempts to map the exact locations of these sites however, failed as co-migrating fragments were generated when using double digests of *Pst* I and *Bss* HII. Mapping the *Bss* HII sites using *Ava* I and *Sal* I was not attempted as only unique restriction sites were of interest.

Two of the enzymes tested, *Sma* I and *Apa* I, restricted the genome once producing fragments of almost exactly 87 and 65 kbp, and 122 and 30 kbp respectively. If the *Sma* I and/or *Apa* I restriction sites occur in non-essential regions, then they may be used as cloning sites without the need to use site-directed mutagenesis. In order to test this possibility, it would be necessary to attempt to clone a reporter gene into the *Sma* I and *Apa* I sites. A helper virus, such as an avipoxvirus, could then be used to recover recombinant LSDV virions expressing the reporter gene. If either of these cloning sites proved successful, then the reporter gene could be replaced with an antigen from a pathogen, which would eventually result in the development of a recombinant vaccine. The identification of unique restriction sites is thus a useful first step in creating a recombinant virus for use as a recombinant vector vaccine (Merchlinsky and Moss, 1992).

SECTION 3

AVIPOXVIRUSES

CHAPTER 10

ISOLATION AND CHARACTERISATION OF AVIPOXVIRUSES:

10.1 INTRODUCTION:

Avipoxviruses have been isolated from many bird species and are probably also able to infect those species from which they have not yet been isolated (Kirmse, 1969). It was as recent as 1969 when the first cases were diagnosed in Norway in wild terrestrial birds (as cited by Holt and Krogsrud, 1973). The introduction of avian poxviruses to the Hawaiian Islands has been attributed to the extinction of much of its avian fauna (as reviewed by Warner, 1968).

Early attempts to sample the diversity of the avian poxviruses met with limited success due to inherent limitations of the tests applied (as cited by Mockett, 1987). However, the ability of site-specific restriction endonucleases to cleave DNA at sequence-dependant recognition sites opened the way for the characterisation of viruses at the molecular level. Analysis of the patterns produced by restriction cleavage of poxviral DNA, and electrophoretic separation, indicated that members of the *Orthopoxvirus* genus possess a considerable degree of homology between the internal regions of their genomes, while the corresponding terminal regions can be quite variable (as cited by Schnitzlein *et al.*, 1988; as cited by Gershon and Black, 1988). In contrast, members of the *Parapoxvirus* genus contain DNA molecules with extensive heterogeneity as reflected in the variability of their DNA restriction patterns (as cited by Schnitzlein *et al.*, 1988). Members of the *Capripoxvirus* genus have also been successfully distinguished using restriction endonuclease pattern analysis of their DNA (Gershon and Black, 1988). Recent comparisons of avipoxviruses have indicated that isolates from different bird species possess a high degree of sequence diversity (Coupar, personal communication; Kow, 1992). Their findings contradict those of Schnitzlein *et al.* (1988) who reported that the restriction endonuclease profiles of fowlpox, juncopox and pigeonpox viruses were very similar implying greater than 80% homology between the isolates.

Although fowlpox and pigeonpox vaccines are commercially available, previously vaccinated birds are still susceptible to poxvirus infections (as cited by Ghildyal *et al.*, 1989). These infections are probably a reflection of the host range of antigenically distinct avipoxviruses. In this regard, commercial poultry and pigeons may be susceptible to various members of the *Avipoxvirus* genus. Thus a thorough knowledge of field and vaccine strains is important in distinguishing poxvirus re-infections of vaccinated birds. Poxviruses have been isolated from a number of southern African bird species and it is in the interests of vaccine safety and efficacy to characterise these in relation to one another and to other well-characterised avipoxviruses such as fowlpox and canarypox virus.

This thesis contains the first reported study of the isolation and characterisation of an avipoxvirus isolated from a juvenile Rock pigeon. The pigeon was discovered in the Rondebosch suburb of Cape Town and died within 24 hours of developing macroscopic lesions on its head and feet. The virus isolate was compared with other local isolates and strains of fowlpox virus, canarypox virus and pigeonpox virus.

10.2 MATERIALS AND METHODS:

10.2.1 Source of avipoxvirus isolates:

a. Fowlpox and canarypox reference isolates were obtained from stocks originally supplied to the Medical Microbiology Department of the University of Cape Town by Professor A. Mayr from the University of Munich, Germany.

b. Penguinpox virus was isolated on two separate occasions from Jackass Penguins at SANCOB, Tableview, Cape Town.

c. Parrotpox virus was obtained from imported parrots displaying pocks held in quarrantine at the Onderstepoort Veterinary Institute, Pretoria.

d. The Turtle dovepox virus was isolated from lesions on a Turtle dove found in Cape Town.

All the isolates were passaged in embryonated chicken CAMs by D. Kow (1992). She prepared crude stocks of semi-purified virus (refer below) and stored them at - 20 ° C. These crude stocks were then inoculated onto 20-40 CAMs in order to obtain high titres of virus for the extraction of DNA.

10.2.2 Inoculating egg CAMs with avipoxviruses:

Egg CAMs were inoculated as described for LSDV (6.2.2.1), except that eggs were only incubated for 3-4 days.

10.2.3. Harvesting of infected CAMs:

CAMs were harvested as described for LSDV (6.2.2.3).

10.2.4. Preparation of pure virus for DNA extraction.

Once all the CAMs had been harvested, 3.75 ml of McIlvain's buffer and 1.25 ml of Arklone-X (1,1,2-trichloro-1,2,2-trifluroethane) (AECI, South Africa) was added per Universal bottle. These were shaken vigorously for 1.5 minutes and were then centrifuged at 1000 rpm (120*g*) at 4 °C for 10 min in a low speed Sigma 301K benchtop centrifuge. The supernatant fluids were transferred into fresh Universal bottles and the precipitates were resuspended in another 3.75 ml of McIlvain's buffer and 1.25 ml of Arklone-X. The shaking was repeated for 1.5 min and the resuspended precipitates were centrifuged at 1000 rpm (120g) at 4 °C for 10 min. The supernatant fluids were pooled and this time the precipitates were resuspended in 5 ml of McIlvain's buffer . Shaking and centrifugation was repeated as described above. All the supernatant fluids were pooled and left on ice for 90 min. They were then centrifuged at 3000 rpm at 4 °C for 15 min and the supernatant fluids were placed into polycarbonate centrifuge tubes. A sucrose cushion was formed by pipetting 0.5 ml of 36% sucrose (in TE buffer) into the bottom of each tube. The balanced tubes were centrifuged in a pre-cooled Beckman JS13-1 (Beckman Instruments, C.A., U.S.A.) rotor (the buckets also having been pre-cooled) at 11 000 rpm (19000g) at 4 °C for 60 min in a Beckman J2-21 (Beckman Instruments, C.A., U.S.A.) centrifuge. The virus pellets were resuspended in a small volume of TE buffer. A biphasic gradient was then prepared in a number of centrifuge tubes using a 2:1 ratio of 10 % T_{10} dextran (Sigma, USA) (in TE buffer) and 36 % sucrose (in TE buffer) by first layering the dextran and then the sucrose into the bottom of each tube. The virus suspensions were layered on top of the biphasic gradients and centrifuged at 11 000 rpm (19000g) at 4 °C for 60 min in a Beckman J2-21 centrifuge in a JS13-1 rotor. The pellets, containing purified virus, were either :

(i) resuspended in a small volume of McIlvain's buffer, dispensed into Bijou bottles and stored at - 20 °C or

(ii) resuspended in 1 ml TE buffer and lysis buffer (Appendix 1) for DNA extraction (as described in Chapter 8, 8.2.2.2).

10.2.5. Preparation of routine viral stock.

Egg CAMs were harvested (as described above) and 0,75 ml of 90% glycerol [diluted in McIlvain's buffer (pH 7.4)] and 0,25 ml of Arklone-X was added per membrane. The membranes were shaken in Universal bottles containing glass beads for 2 min and were left on ice for 90 min. The disrupted membranes were then centrifuged at 1000 rpm (120g) at 4°C for 10 min in a Sigma 301K benchtop centrifuge. The supernatant fluids were retained and were dispensed into Bijou bottles to be stored at -20 °C.

10.2.6. Agarose gel electrophoresis:

a) Conventional electrophoresis using medium-sized gels: These gels were cast in relatively small casting trays (22 cm x 12.5 cm x 0.75 cm) (as described, 7.2.1) and electrophoresis was performed overnight at 40 V in electrophoresis tanks containing 1300 ml of 1 x TAE. 14- or 20-toothed combs were used. No pulsing was used.

b) FIGE: Gels were prepared and electrophoresis was performed as described in Chapter 7 (7.2.1.1). The same electrophoresis apparatus was used, including the cooling system.

10.2.7 Isolation of a virus from a Rock pigeon:

Lesions, from the head and feet of a dead, juvenile Rock pigeon (also called a Speckled pigeon) (*Columba guinea*), were removed and placed in a solution of McIlvain's buffer, PS (X5) and Fungizone (2,5 µg/ml) (Appendix 1). The lesions were then ground finely in a mortar and placed at 4 °C overnight. A 10-fold dilution series of this mixture was prepared in McIlvain's buffer and PS (X1). The CAMs of six embryonated chicken eggs were inoculated with different dilutions of the lesion mixture. The eggs were incubated at 37 °C for three days. The CAMs were removed and examined for the presence of pocks and bacterial and fungal infections. CAMs which displayed pocks, and appeared free of bacterial and fungal infections, were ground in Universal bottles containing glass beads (4mm diameter) and a solution of 90 % (v/v) glycerol (diluted in McIlvain's buffer). A crude virus stock of the infected CAMs was prepared as described (10.2.5) and was stored at - 20 °C. An aliquot of this sample was later removed and inoculated onto a number of CAMs. The virus was passaged four times in this manner.

10.2.8 Characterisation of the virus from a Rock pigeon as a poxvirus:

Chicken CAMs which displayed pocks were fixed in a 2% (v/v) solution of gluteraldehyde (diluted in PBS). These were then photographed as described (Appendix 2).

Crude stocks of the virus were inoculated onto 40 chicken CAMs, and after a 3 day incubation period the CAMs were harvested and virus was purified from them as described (10.2.4). After centrifugation through a dextran-sucrose gradient, virion pellets were prepared for electron microscopy by Dr L.M. Stannard, Department of Medical Microbiology, UCT. Particles in a drop of distilled water were placed on a formvar-coated grid, negatively stained with 2% phosphotungstic acid (pH 6.2), and examined in a Hitachi 600 transmission electron microscope operating at 75 kV.

10.2.9. Restriction endonuclease analysis of avipoxvirus DNA:

DNA was extracted from the purified Rock pigeon virus as described (10.2.4) and, along with DNA from the well-characterised fowlpox and canarypox viruses (kindly supplied by Mrs D. Marais, Department of Medical Microbiology, UCT), was digested with the restriction endonucleases *Eco* RI and *Bam* HI (Chapter 8, 8.2.3.1). Restriction was terminated with the addition of Stop buffer (X6). The digests were then run on a 0,8 % agarose gel in a Hoefer Super Sub electrophoresis tank,. using the following FIGE conditions:

Prerun: 100 V, 34 mA, 10 min, 14 °C
Run : 100 V, 19 hours, 14 °C, 34 mA
F = 450 msec, R = 150 msec, r = 0,1

The gel was then stained, viewed and photographed as described (7.2.1.2).

DNA was also prepared from an avipoxvirus isolated from a Turtle dove (*Streptopelia capicola*). *Pst* I was used to digest this DNA, as well as DNA from a local poxvirus isolate from a Jackass penguin (*Spheniscus demersus*), the Rock pigeon isolate, fowlpox virus, canarypox virus and parrotpox virus (DNA from the penguin and parrot poxviruses was also kindly supplied by Mrs D. Marais, Department of Medical Microbiology, UCT). The digests were run on a 0,8 % agarose gel in a Hoefer Super Sub electrophoresis tank using the following FIGE parameters:

Prerun : 130 V, 12 °C, 15 min.
Run : 130 V, 12 °C, 12 hours
F = 1.0 sec, R = 0.3 sec, r = 1.0

The gel was stained, viewed and photographed as described (7.2.1.2).

A pigeonpox virus vaccine, produced locally from a naturally occuring strain (Jordaan), was obtained from Onderstepoort Veterinary Institute (Erasmus *et al.*, 1988). DNA was extracted from purified virions and was digested with *Eco* RI. This digest was then run on a medium-sized 0,8 % agarose gel under non-pulsed electrophoretic conditions, along with an *Eco* RI digest of the Rock pigeon isolate. The gel was run in 1X Tris-acetate-EDTA buffer as follows:

Run : 40 V, 34 mA, 16 hours, 25 °C.

The gel was stained, viewed and photographed as described.

10.3 RESULTS:

10.3.1 Characterisation of the virus isolated from a Rock pigeon:

Chicken CAMs infected with the Rock pigeon virus produced macroscopic pocks within three days post-infection (Fig 10.1). The white appearance of the pocks suggests that they are not haemorrhagic.

Electron microphotographs of the Rock pigeon virus purified from the CAMs of embryonated chicken eggs (Fig. 10.2, A to C) revealed the presence of brick-shaped particles with characteristic poxvirus morphology. A number of these particles are apparent in Fig. 10.2 C. The particles are separated by large quantities of collagen, probably derived from the CAMs. Higher magnification reveals finer ultrastructural detail (Fig. 10.2 A and B). The outer surface of the particles displays ridges which resemble surface tubular elements.

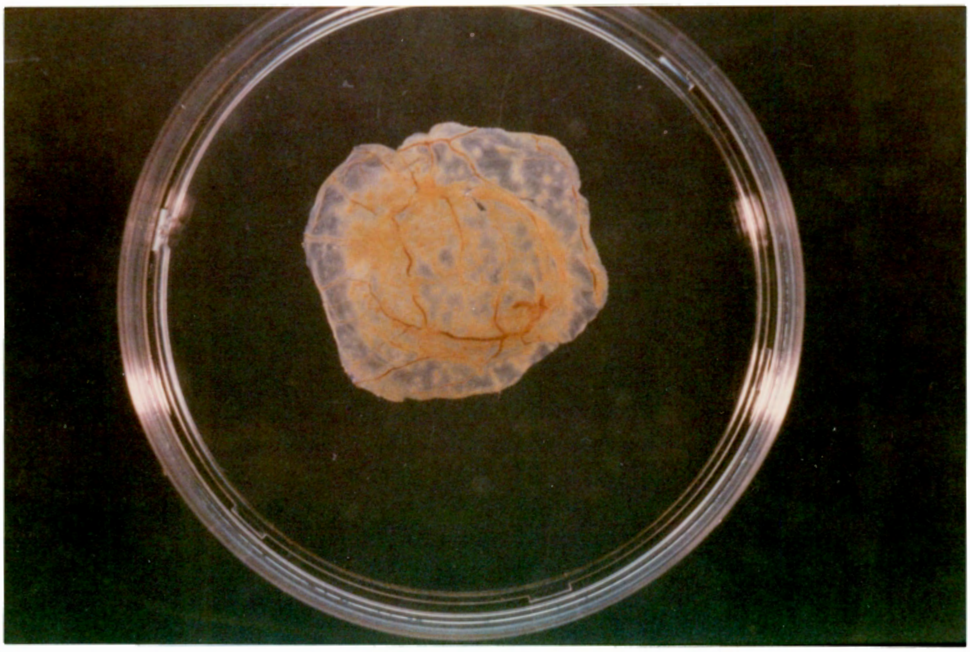

FIGURE 10.1
Macroscopic pocks produced on the CAMs of embryonated chicken eggs infected with the Rock pigeon virus.

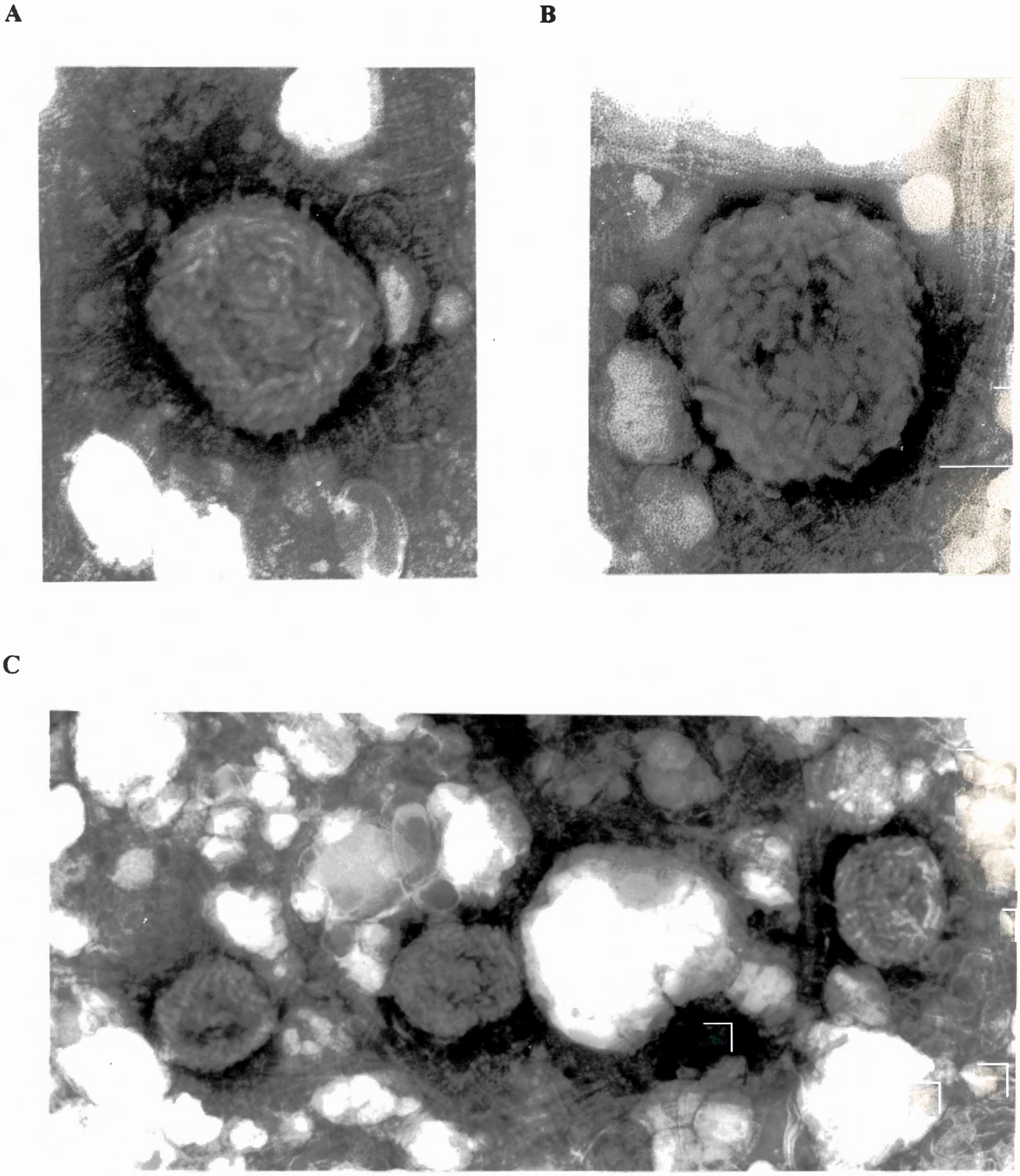

FIGURE 10.2
Electron micrographs of the poxvirus-like particles isolated from CAMs infected with the Rock pigeon virus. (A and B X 150 000, and C X 35 000). (Photographs kindly supplied by Dr L.M. Stannard, Department of Medical Microbiology, UCT)

10.3.2 Restriction endonuclease analysis of avipoxviruses:

Eco RI and *Bam* HI restriction endonuclease digests of fowlpox, canarypox and the Rock pigeon virus DNA were separated on an agarose gel using FIGE (Fig 10.3). Both *Bam* HI and *Eco* RI digests of Rock pigeon virus DNA (lanes 1 and 5 respectively) revealed a distinctly different number and distribution of bands to *Bam* HI and *Eco* RI digests of either fowlpox virus (lanes 2 and 6) or canarypox virus (lanes 3 and 7) DNA.

In contrast, when *Eco* RI digests of the local vaccine strain (Jordaan) of pigeonpox virus and the Rock pigeon virus, were separated on an agarose gel using conventional electrophoresis, most, if not all, the fragments co-migrated (Fig 10.4).

Pst I restriction digests of the DNA from well-characterised avipoxviruses (fowlpox virus and canarypox virus) were compared with digested DNA from local isolates (penguinpox virus, dovepox virus and Rock pigeon virus) and from imported parrotpox virus. The use of FIGE to separate the restriction fragments (Fig. 10.5) showed that the Rock pigeon viral DNA (lane 2) differed from poxviruses isolated not only unrelated avian species [such as penguin (lane 4), fowl (lane 5), canary (lane 6) and parrot (lane7)] but also from more closely related species such as dove (lane 3).

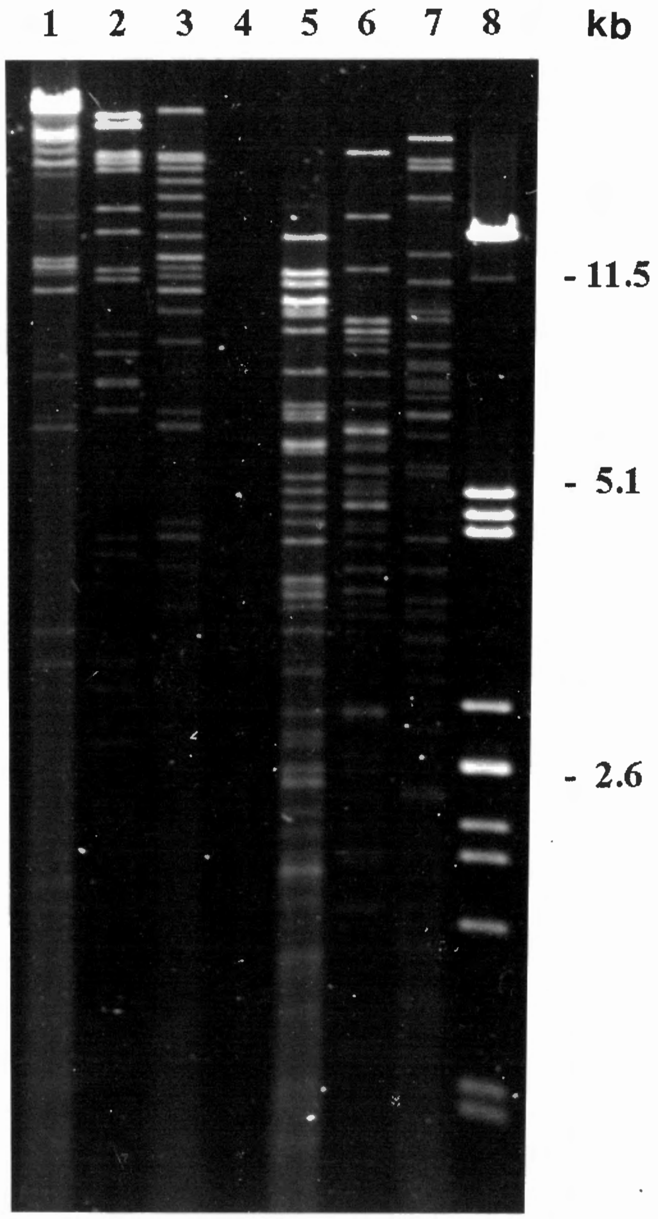

FIGURE 10.3

Agarose gel of restriction digests of Rock pigeon virus (lanes 1 and 5), fowlpox virus (lanes 2 and 6) and canarypox virus (lanes 3 and 7) DNA. Lane 1 = Rock pigeon *Bam* HI. Lane 2 = fowlpox *Bam* HI. Lane 3 = canarypox *Bam* HI. Lane 4 = empty. Lane 5 = Rock pigeon *Eco* RI. Lane 6 = fowlpox *Eco* RI. Lane 7 = canarypox *Eco* RI. Lane 8 = Lambda *Pst* I marker. FIGE conditions: 100 V, 19 hours, 14 °C, F = 450 msec, R = 150 msec, r = 0.1.

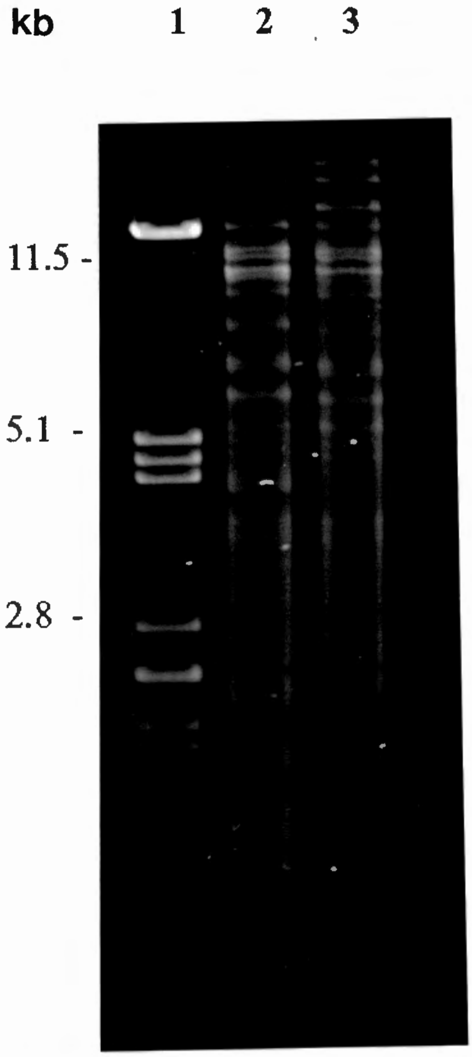

FIGURE 10.4.

Agorose gel showing *Eco* RI digests of DNA from the Rock pigeon isolate and pigeonpox vaccine. Lane 1 = Lambda *Pst* 1 marker. Lane 2 = Rock pigeon, Lane 3 = pigeonpox vaccine. Non-pulsed gel electrophoresis on medium sized gel: 40 V, 30 mA, 16 hours, 24 °C.

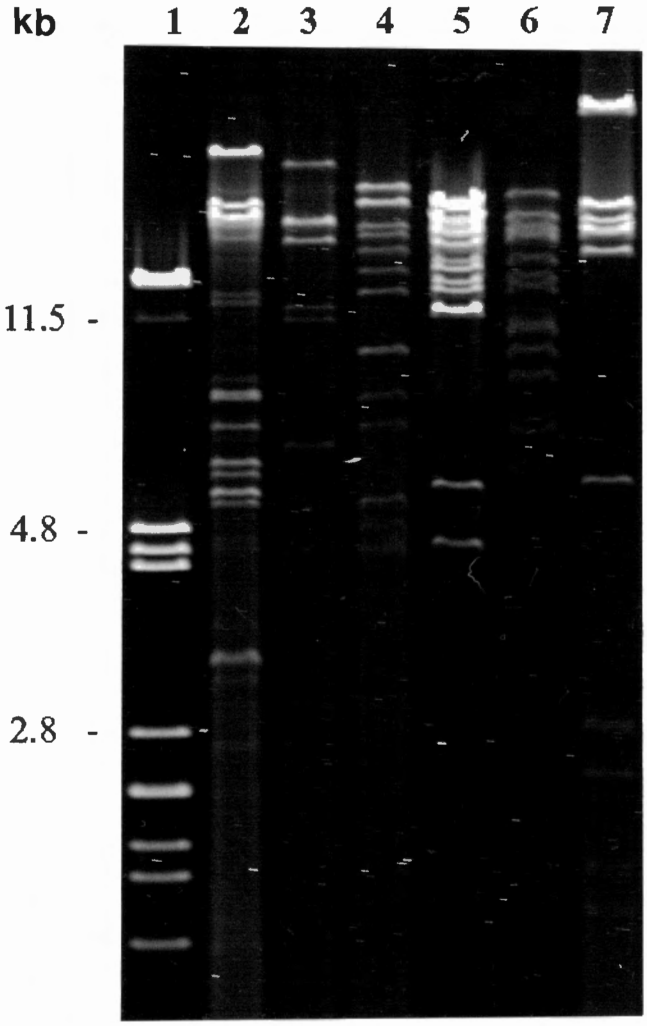

FIGURE 10.5

Agarose gel showing *Pst* I digests of DNA from various avipoxvirus isolates. Lane 1 = Lambda *Pst* I marker. Lane 2 = Rock pigeon. Lane 3 = Turtle dovepox. Lane 4 = penguinpox. Lane 5 = fowlpox. Lane 6 = canarypox. Lane 7 = parrotpox. FIGE conditions: 130 V, 12 °C, 12 hours, F = 1.0 sec, R = 0.3 sec, r = 1.0.

10.4 DISCUSSION:

A pathogen extracted from lesions on the head and feet of a dead juvenile Rock pigeon (*Columba guinea*) was identified as a poxvirus by its ability to form characteristic pocks on the CAMs of infected eggs. The pocks resembled those produced by fowlpox virus and a vaccine strain (Jordaan) of pigeonpox virus (Kow, 1992; personal observation). Electron microscopy confirmed that the virus particles extracted from the CAMs had typical poxvirus morphology. They appeared as brick-shaped particles with tubular structures on their outer surface, and were similar in size and shape to other avipoxviruses (Holt and Krogsrud, 1973).

Analysis of the DNA extracted from these particles after restriction with site-specific endonucleases, showed that the restriction pattern profiles were distinct from patterns produced by other well-characterised avipoxviruses and also from local isolates of poxviruses from Jackass penguin and Turtle dove. Yet they were similar, if not identical, to patterns produced by DNA extracted from a local vaccine strain (Jordaan) of pigeonpox virus.

These results provide conclusive evidence that the Rock pigeon pathogen is a member of the *Avipoxvirus* genus of the *Poxviridae* family and is closely related to a local vaccine strain of pigeonpox virus. Although the use of FIGE to compare the restriction patterns of the DNA of the pigeonpox virus isolates, might have yielded a more accurate comparison than conventional electrophoresis, time constraints prevented the preparation of more viral DNA. The origin of the Rock pigeon isolate is unclear. It is possible that pigeonpox virus is endemic in Rock pigeons and other southern African members of the *Columbidae*, but on the other hand its appearance in these indigenous species may be fairly recent due to introduction of the virus to southern Africa through birds imported from Europe and America. Another possibility is that the vaccine strain (Jordaan) of pigeonpox virus was transmitted to Rock pigeons and, although protective in racing pigeons (for which the vaccine was specifically developed), has proved to be pathogenic for Rock pigeons. The pathogenic nature of this virus is of concern as it has the potential to pose a serious threat to the populations of indigenous pigeons found throughout southern Africa.

Of further interest is the degree of genomic diversity exhibited by avipoxviruses as evidenced by restriction pattern polymorphism of their genomic DNA. Even restriction patterns of closely related members of the same family (Rock pigeon and Turtle dove) were distinct. Although some avipoxviruses (such as fowlpox virus and pigeonpox virus) are able to cross-protect non-host avian species, other members are unable to do so, such as quailpox virus and parrotpox virus (as cited by Winterfield and Reed, 1985). These observations may relate to evolutionary ties between different avipoxviruses or to selective constraints imposed on the viruses by their avian hosts. Sequence analysis of shared genomic regions may prove a more productive method in providing answers to these questions.

DISCUSSION

CHAPTER 11

DISCUSSION:

11.1 LSDV:

LSDV is a member of the *Capripoxvirus* genus of the *Poxviridae* family (Matthews, 1982). This genus consists of two other members, namely goatpox virus and sheeppox virus, and collectively they are economically the most important group of the poxviruses (Kitching *et al.*, 1989). They are responsible for causing severe stock losses of cattle, sheep and goats throughout Africa and parts of Asia. The use of restriction endonucleases to compare the genomes of capripoxviruses has revealed that members of this genus fall into one of five groups depending on their degree of host-specificity and geographic distribution (Gershon and Black, 1988). It has also been shown that capripoxviruses causing LSD in central Africa are genetically distinct from isolates occurring in southern Africa (Gershon and Black, 1988; Perlman, 1993). In addition, the southern African isolates of LSDV are extremely host-specific as they have only been isolated from cattle, whereas Kenyan isolates appear to be able to infect sheep and possibly even goats (Weiss, 1968; Davies, 1981).

Although sporadic outbreaks of capripoxvirus infections occur, effective and safe attenuated, live vaccines have been in use for a number of decades (Kitching, 1986 b). These vaccines have been useful in limiting the epidemic spread of capripoxviruses and yet their total eradication has been impossible due to their various modes of transmission, including insect transmission, and their probable long term maintenance in wild ungulates (Kitching and Mellor, 1986; Davies, 1982). With the recent advent of recombinant vaccines and the use of poxviruses as vector vehicles for these vaccines, capripoxviruses have been targeted for development as potential vectors as they are host-range restricted to natural infection in only sheep, goats or cattle, and the vaccine strains have proven to be effective and safe (Gershon *et al.*, 1989a; Erasmus, 1988). A recombinant capripoxvirus, KS-1, has already been developed and is reported to protect cattle against challenge with virulent Rift Valley fever virus (Romero, 1993). Present studies are concentrating on the development of a recombinant vector vaccine from the southern African vaccine strain of LSDV due to its greater degree of host-range restrictiveness.

There is evidence to suggest that genetic recombination between the genomes of two capripoxviruses led to the formation of a third virus (Gershon *et al.*, 1989 b). It would be dangerous to release a vaccine virus into the field if there was a chance that recombination could occur between the vaccine and a virulent virus strain. The result of such an event could be the formation of an altered vaccine displaying virulent properties. There is possibly an even greater risk of this event occurring with a recombinant vector vaccine which might have been made more unstable due to the insertion of foreign genes into its genome. Thus before a recombinant LSDV vaccine can be released into the field, it is necessary to obtain an indication of the degree of stability of the LSDV genome. This aim was achieved by comparing the genomes of virulent southern African field isolates, collected almost forty years ago, with the presently used vaccine strain of LSDV. These results would also give an indication of the mutational rate of the genomes and might provide a useful means for distinguishing virulent field isolates from the vaccine strain. However, the limiting levels of replication experienced with LSDV cultured under "standard" conditions (25 infectious particles per cell) necessitated the optimisation of virus culture and purification conditions before the genomes could be compared.

It was realised at the outset of the project that an accurate method for titrating infectious LSDV particles was unavailable. It was thus necessary to develop such a technique before commencing studies on viral replication and growth in cell culture. Evidence of viral replication was obtained by infecting a number of different types of cell with the vaccine strain of LSDV and then observing the cells for cytopathic effects. A continuous line of bovine kidney cells (MDBK) was found to produce the most easily recognisable cytopathic effects. The nature of the CPE in MDBK cells was quite distinct from infected primary bovine kidney cells. It is possible that the transformation of the MDBK cells to produce a continuous line results in major changes in the manner in which the cells react to viral infection stemming from possible genetic changes. An indirect immunoperoxidase staining technique was used to confirm that the foci seen in infected MDBK cells were associated with virus particles. Although the use of this technique allowed the detection of viral antigen within 24 hours post-infection, it was not considered for use in titrating infectious LSDV as it was extremely time consuming. A 10-fold dilution of a stock of LSDV, plated onto MDBK cells and left to incubate at 37 °C for two to three days, yielded discrete foci which could then be counted. Optimal titration conditions were obtained with the use of a grid overlay and the culture medium removed to expose the entire cell sheet.

The vaccine strain of LSDV was found to replicate more efficiently in primary cultures of foetal bovine or ovine tissue than in any other cell type tested. These results are in agreement with the host-range restrictiveness of southern African isolates of LSDV (Weiss, 1968). Low levels of replication were, however, obtained in chicken embryo fibroblasts and on the CAMs of embryonated hens' eggs. Although a 2 log increase in virus yield was obtained per egg CAM inoculated, increasing the concentration of viral inoculum failed to result in an appreciable increase in the amount of recoverable virus (results not shown). It is thus possible that the CAMs are only able to support a limited amount of LSDV replication and that once this limit is obtained, further replication is inhibited via the embryo's immune system. The ability of LSDV to replicate in avian cells and not in most mammalian cells raises interesting questions as to the evolution of this virus. Vaccinia virus is also able to replicate in avian cells and is less host-range restrictive than LSDV (Joklik, 1962). The genome of vaccinia virus is much larger than LSDV and it is possible that the larger vaccinia virus genome contains genes necessary for replication in a wider range of animal cells, as well as genes responsible for eluding the host's immune system.

Temperature was also found to affect LSDV replication. Lower temperatures than the standard 37 °C used to incubate culture cells were found to favour LSDV replication. LSDV infection is most commonly associated with the formation of nodules and ulcers on the skin, surface areas of respiratory tracts and the reproductive organs and it is thus probable that the average temperature of these organs is lower than the rest of the body (Weiss, 1968). It would be of interest to determine whether the LSDV enzymes associated with replication, transcription and translation have an optimal activity below 37 °C.

Different types of culture media affected LSDV replication. The optimal culture medium, DMEM mixed 1:1 with Ham's F-12, contained more amino acids, vitamins, trace elements and organic chemicals than any of the other media tested, suggesting that the virus requires a wide range of organic and inorganic chemicals for optimal replication in cell culture.

The best culture conditions for LSDV replication were obtained when foetal bovine testes cells were infected, and the DMEM plus Ham's F-12 culture medium was used. Under these conditions, 70 infectious LSDV particles were recoverable per cell. The recovery of viral DNA was optimised when the intracellular source was harvested as it was shown under "standard" culture conditions that almost 98% of infectious particles remained intracellular. A non-ionic detergent (Triton X-100) and reducing agent (ß-mercaptoethanol) proved instrumental in releasing this additional source of viral DNA from infected cells. A fair percentage of viral DNA was lost in the cellular debris fraction and it is possible that the development of better purification techniques might facilitate the recovery of this DNA. Of the viral lysis buffers tested, the buffer containing the highest percentage ß-mercaptoethanol proved the most effective as the use of this buffer resulted in the recovery of viral DNA practically free of contaminating cellular DNA.

Once conditions for the culture of LSDV and recovery of a high quality and quantity of DNA had been improved, it was possible to prepare large amounts of DNA required for restriction endonuclease analysis of the five LSDV isolates. The genomes of the four field isolates and vaccine strain were restricted with *Pst* I, *Hin* dIII, *Bam* HI and *Eco* RI. The restriction patterns produced using FIGE were practically the same for all the isolates, except for some of their terminal regions. The terminal fragment polymorphisms allowed the isolates to be divided into three groups: the first group comprising the Maputo and Neethling isolates, the second group the Haden and vaccine isolates and finally the third group comprising just the Gaborone isolate. No feasible explanation can be offered as to why the vaccine and Haden isolates are more similar to each other than either is to the Neethling isolate from which the vaccine was derived. It is possible that during the development of the vaccine, the Haden isolate, and not the Neethling isolate, was used for the production of the vaccine.

It thus appears that the southern African isolates of LSDV used in this study are extremely stable, and share a high degree of genomic conservation, as they yield almost identical restriction pattern profiles. Furthermore, they do not appear to recombine with other isolates or within their own genomes as evidenced by the fact that none of the isolates contained a mixture of different sized terminal fragments. These results are important for prospects on the development of genetically engineered LSDV recombinants as it suggests that it would be relatively safe to release these into the field without the risk of reversion to virulence.

Although homologous recombination has been used to create recombinant vector vaccines, *in vitro* ligation has been shown to yield a higher percentage of recombinants (Merchlinsky and Moss, 1992). This technique makes use of rare or unique restriction sites for the insertion of foreign genes. Two such sites were mapped within the LSDV vaccine genome. The *Sma* I site has been mapped within the essential ribonucleotide reductase gene, whereas the *Apa* I site is located near one of the terminal regions. As the terminal regions generally contain "host-range" genes, it is possible that the *Apa* I site might prove useful as a cloning site (as cited by Gershon and Black, 1988). The vaccine strain also lacked *Not* I, *Nar* I and *Ksp* I restriction sites and, if necessary, it would be possible to create any one of these sites in a non-essential region of the genome using site-directed mutagenesis.

In summary, it was possible to improve the culture conditions of southern African field isolates and the vaccine strain of LSDV once its growth, using standard culture conditions and an accurate infectivity assay, had been established. Restriction digestion analysis of the viral genomes indicated that they were highly conserved and extremely stable making the vaccine strain an excellent candidate for development as a recombinant vector vaccine. This prospect was enhanced by the identification and mapping of unique restriction sites, one of which might prove useful for the insertion of foreign genes.

11.2 Avipoxviruses:

Avipoxviruses are also economically important and are known to cause diseases in a large number of avian species (as cited by Winterfield and Reed, 1985). They have been targeted for use as recombinant vector vaccines due to their ability to express early gene products in mammalian cells, even though they are restricted to avian cells for complete replication and production of progeny virions (Taylor *et al.*, 1988). They are also more host-specific than viruses such as vaccinia virus, making them attractive candidates as recombinant vaccines.

A knowledge of the genomic diversity and stability of avipoxviruses is also important for similar reasons to those described for LSDV. Comparisons were made of southern African isolates of avipoxviruses with well-characterised isolates from canaries and fowl. The isolation and characterisation of an avipoxvirus from a Rock pigeon presented an almost identical restriction pattern profile to that of a local vaccine strain of pigeonpox virus. However, when compared with other avipoxviruses, even with a closely related Turtle dovepox virus isolate, the restriction profiles produced were extremely different, indicating a low degree of sequence homology. These results indicate that avian poxviruses have evolved independently from each other and have shared a long and close evolutionary tie with their avian hosts. Although there are reports of close serological ties between various avipoxviruses, they are also reported to be extremely host-specific (Tripathy *et al.*, 1973; Kirmse, 1969). These reports of host-specificity are supported by the high degree of genome diversity as evidenced by the widely different restriction profiles.

The isolation and characterisation of avipoxviruses from a number of birds of the same species, such as Rock pigeons, would prove instrumental in determining the exact extent to which the virus is related to its host and would give an indication of the degree of sequence diversity of viruses isolated from the same host species. These results might then prove useful for determining the likelihood of natural recombination occurring between different avipoxviruses. Even though avipoxviruses undergo an abortive infection in mammalian cells, and there are no reports yet of any avipoxviruses able to replicate in mammalian cells, it is impossible to be certain, that under the right conditions, a productive infection cannot still occur.

APPENDICES

APPENDIX 1 : CHEMICAL SOLUTIONS

All chemicals, unless otherwise indicated were obtained from BDH (Poole, England).

A. CHEMICAL SOLUTIONS USED FOR CELL CULTURE:

These solutions were either prepared according to the manufacture's instructions or were adapted from recipes provided in Paul (1972).

CELL CULTURE MEDIA :

Foetal calf serum (FCS) was first heat inactivated before being added to culture media:

A bottle of FCS (Delta Bioproducts, South Africa) was thawed at room temperature and placed at 56 °C for precisely 30 min. The FCS was dispensed into 50 ml and 20 ml amounts after filtering through a 0,2 µm filter and was then refrozen at -20°C.

DMEM .10 % FCS :

400 ml Dulbecco's Minimum Essential Medium (DMEM) (Highveld Biological, S.A.)
50 ml FCS
2.5 ml PG or PS solution
32 ml of 5 % bicarbonate of soda.

A 10 % FCS concentration was used for early growth of cells in culture.

DMEM .4 % FCS:

450 ml DMEM
20 ml FCS
2.5 ml PG or PS
32 ml of 5 % bicarbonate of soda.

A 4 % FCS concentration was used for the maintenance of most types of culture cells.

DMEM. 2 % FCS:

450 ml DMEM
10 ml FCS
2.5 ml PG or PS
32 ml of 5 % bicarbonate of soda.

A 2 % FCS concentration was used for the maintenance of rapidly dividing culture cells.

The following media were prepared in similar concentrations of FCS and antibiotic to those described above :

Eagle's Minimum Essential Medium (MEM) (consisting of essential amino acids and vitamins), Basal Eagle's Medium (BEM) (consisting of lower concentrations of amino acids than MEM) and a 1:1 combination of DMEM (consisting of higher concentrations of, and additional, amino acids and vitamins than MEM) and Ham's F12 medium (HAM's F12) (consisting of higher concentrations of, and additional, amino acids and vitamins than MEM, as well as the addition of inorganic salts and cellular organic biochemicals)

NOTE : Bicarbonate of soda was added to the above media according to the manufacture's instructions.

PHOSPHATE BUFFERED SALINE :

NaCl	8,00 g
KCl	0,20 g
KH_2PO_4	0,12 g
Na_2HPO_4 (anhydrous)	0,91 g
or $Na_2HPO_4.2H_2O$	1,14 g
or $Na_2HPO_4.12H_2O$	2,28 g

Chemicals were dissolved in 800 ml of distilled water and made up to 1 l with distilled water. The pH was adjusted to 7,5. The solution was filter sterilized and dispensed into 100 ml and 500 ml aliquots.

PHYSIOLOGICAL SALINE

NaCl (8.5g) was dissolved in 800 ml of distilled water. The solution was made up to 1 l with distilled water, dispensed in 100 ml amounts and autoclaved at 104 kPa at 121 °C for 15 min to sterilize.

0,4% PHENOL RED :

Phenol Red (Gurr's, London) powder (0.4g) was dissolved in 60 ml of 0,05 N NaOH with the use of gentle heating. Once cooled to room temperature, the solution was made up to 100 ml in distilled water. It was then filter sterilized, dispensed in 20 ml amounts and stored at 4°C.

PENICILLIN - STREPTOMYCIN (PS)

Penicillin (Novopen, Novo Nordisk (PTY) LTD, South Africa) (5 x 10^6 units) was dissolved in 20 ml of physiological saline. Streptomycin (Novostrep, Novo Nordisk (PTY) LTD, South Africa) (5.0g) was added and the volume was adjusted to 100 ml with physiological saline. 2,0 ml of 1 M HEPES (Sigma, USA) buffer (pH 7,3) and 0,6 ml of 0,4% phenol red were added and the pH was adjusted to 7,3 with 1 N NaOH. The final volume was made up to 125 ml with physiological saline. The antibiotic solution was filter sterilized, dispensed in 2,5 ml amounts and stored at -20 °C. For use, 0.5 ml was added to 100 ml of culture medium (i.e. at a concentration of 100 µg/ml).

PENICILLIN - GENTAMYCIN (PG)

Penicillin (Novopen, Novo Nordisk (PTY) LTD, South Africa) (1 X 10^6 units) was dissolved in 25 ml of Gentamycin (Fermentmycin, Lennon LTD, South Africa). The solution was filter sterilized, dispensed in 2,5 ml amounts and stored at -20 °C. For use, 0.5 ml was added to 100 ml of culture medium (i.e. at a concentration of 100 µg/ml).

NOTE: Fermentmycin was found to be toxic for all primary and secondary culture cells such as LT and CFK cells.

FUNGIZONE SOLUTION:

One vial of Fungizone (50 mg) (Fungizone Intravenous, Squibb Laboratories (PTY) Limited, Isando, South Africa) was reconstituted in 1 ml of distilled H_2O. The solution was then removed from the vial and adjusted to a final volume of 200 ml with distilled H_2O. The Fungizone was filtered through a 0.2 µm filter, dispensed in 5 ml amounts and stored at -20°C. The Fungizone was used at a final concentration of 2.5 µg/ml.

TRYPSIN BASE (10X)

NaCl	30.0 g
KH_2PO_4	1.20 g
KCl	2.00 g
Na_2HPO_4	9,10 g
or $Na_2HPO_4.2H_2O$	11.2 g
glucose	5.00 g

The above chemicals were dissolved in 700 mls of distilled water. EDTA (2.0g), dissolved in 100 ml of distilled water, was added to the above solution. Phenol red (25 ml of a 0.4% stock) was then added and the solution was stirred, while adjusting the pH to 7.8 with 1 N NaOH, until a clear red colouration was obtained. The final volume was adjusted to 1 l with distilled water. The trypsin base was filtered through a 0.2 µm filter and was dispensed in 20 ml aliquots. It was stored at -20°C.

5% TRYPSIN STOCK SOLUTION :

Trypsin base (10 ml) and 0.5 ml of PS or PG was added to 90 ml of distilled H_20. HCl (1.0 N) was added dropwise until the solution turned bright yellow. This solution was then heated to 37°C for a few minutes in a beaker. Trypsin (Difco Laboratories, U.S.A.) powder (5.0g) was carefully sprinkled onto the surface of the yellow solution making sure that no lumps were formed. This was done slowly and without stirring. Once all the trypsin had been added, it was left to dissolve by either letting it stand at room temperature for a few hours or by leaving it overnight at 4°C. Once most of the trypsin had dissolved, it was filtered through a 0.2 µm filter, dispensed in 1 ml amounts into Bijou bottles and stored at -20°C.

ACTIVATED TRYPSIN VERSENE (ATV):

A 0.05 % solution of trypsin was used for mild digestion of cells in order to remove them from culture flasks for cell passage or for virus isolation. The working solution of trypsin was prepared using sterile technique as follows:

10 ml of trypsin base was thawed and was added to 90 ml of sterile distilled H_2O. PG or PS (0.5 ml) and 1 ml of 5 % trypsin stock was added and the solution was stored at 4 °C.

5% BICARBONATE OF SODA :

$NaHCO_3$ (100g) was dissolved in 1.5 l of distilled H_2O. Phenol red (2 ml of 0.4 % stock) was added and the volume was made up to 2 l using distilled H_2O. Dry ice was then added to a 2 l conical flask. The neck of the flask was partially closed using a rubber stopper which had a silicon tube running through it. The other end of the tube was placed in the bicarbonate solution and the CO_2 released by the dry ice was allowed to bubble through the solution until it became a pale pink colour. The solution was then filtered through a 0.2 μm filter and was dispensed into Bijou or Universal bottles filled to the brim. The bottles were stored at 4 °C.

B. GENERAL CHEMICAL SOLUTIONS:

McILVAIN'S BUFFER :

Solution A : 0.1 M citric acid (Merck, Germany)

Solution B : 0.2 M $Na_2HPO_4.12H_2O$

Solution A (1.83 ml) and 18,17 ml of solution B was added to 800 ml of distilled H_2O (Lennette and Schmidt, 1969). The pH of the mixture was adjusted to 7.4 using solutions A and B (A = acid and B = base). The buffer was made up to a final volume of 1 l using distilled H_2O. It was then autoclaved at 104 kPa at 121 °C for 10 min and stored at 4 °C.

DILUENT:

HEPES buffer (Sigma, USA) (1 ml of a 1 M stock) was added per 100 ml of DMEM and the pH was adjusted to 7.4 with 1 N NaOH (Williamson, 1968). The solution was then filtered through a 0.2 µm filter and stored at 4°C.

VIRUS LYSIS BUFFER C:

Solution A : 4 % Na-n-laurylsarcosinate (Sarkosyl)
54 % sucrose
Solution B : 200 mM ß-mercaptoethanol (Merck, Germany) (wrapped in aluminium-foil)

Both solution A and B were made in 100 mM Tris (pH 7.8) (Massung and Moyer, 1991). Solution A was autoclaved at 69 kPa at 115 °C for 10 min and both solutions were stored at 4°C.

STOP/LOADING BUFFER (X6)

0.25 % Bromophenol Blue (Merck, Germany)
0.25 % Xylene Cyanol (Merck, Germany)
40 % (w/v) sucrose (dissolved in distilled H_2O) (Sambrook *et al.*, 1989)

TRIS - EDTA BUFFER (TE, pH 9.0)

EDTA (Boehringer Mannheim, Germany) (93.05g) was added to 400 ml of distilled H_2O (Sambrook *et al.*, 1989). The EDTA was dissolved in NaOH (Univar, Saarchem, South Africa) and the pH was adjusted to 9.0. The final volume was made up to 500 ml with distilled H_2O. Tris (Boehringer Mannheim, Germany) (12.01g) was then dissolved in 80 ml distilled H_2O. The pH was adjusted to 9.0 using HCl and the final volume was made up to 100 ml with distilled H_2O. The 0.5 M EDTA and 1 M Tris-Cl solutions were diluted together in distilled H_2O (1:500 and 1:100 respectively) to give a solution containing 1 mM EDTA and 10 mM Tris-Cl (pH 9.0).

ELECTROPHORESIS BUFFERS:

10X Tris-Borate-EDTA buffer (TBE):

108 g Tris (Boehringer Mannheim, Germany)
55.0 g Boric acid (Merck, Germany)
9.3 g EDTA

These chemicals were mixed and made up to a final volume of 1 litre in distilled H_2O (Sambrook *et al.*, 1989).

50X Tris-Acetate-EDTA buffer (TAE):

242 g Tris
57.1 g Glacial acetic acid
100 ml EDTA (0.5 M, pH 8.0)

This solution was made up to 1 litre in distilled H_2O (Sambrook *et al.*, 1989).

APPENDIX 2: PHOTOGRAPHY:

a) Photographic images for laboratory records were made using a Video Copy Processor (Mitsubishi, Japan) linked to a Gel Band Amplifier (UVP Gel Band Amplifier 5000, UVP, U.S.A.). Gels were viewed on a Sony SSM-320CE Black and White Monitor (Sony, Japan) and images were recorded onto thermal paper (Mitsubishi, Japan). Gels which were illustrated in this thesis (or for publication) were photographed with bottom illumination using a Mamiya M645 1000 S medium format camera (Mamiya, Japan). This camera was fitted with a Haze UV filter (Korea), CPC Red filter (Japan) and a Kenko Close-up no. 1 ring (Japan). The camera was permanentley set at F5.6. Film used was Ilford FP4 or Ilford FP4 Plus (Ilford LTD, England) with exposure times of 60 seconds and 30 seconds respectively.

b) Fluorescence microscopy: These samples were illuminated with UV light at a wavelength of 360 nm and photographed with a Wild MPS 51S SPOT camera linked to an electronic exposure meter (Wild PHOTOAUTOMAT MPS 45, Wild, Switzerland) mounted on a Leitz SM - LUX (Leitz and Wetzlar, Germany) light microscope. Black and white 35 mm Ilford HP5 (400 ASA) film was used and the camera's "Normal field" light metering system with 100 second exposure times.

c) Photomicroscopy of stained cells: The same camera and microscope were used as for the fluorescence microscopy (above). Kodak Ektachrome 160 tungsten EPT 135-36 (Kodak, Eastman Kodak Company, U.S.A.) film was used and the camera's "Dark field" light metering system with the camera set for "automatic exposure".

d) Cells in culture dishes: Cells were photographed using Kodak Ektachrome 160 tungsten EPT 135-36 film and an Olympus OM 101 camera mounted on an Olympus CK2 inverted microscope (Olympus, Japan). The camera's automatic exposure setting was used.

e) Pocks on chicken CAM's were photographed with Kodak Ektachrome 160 tungsten EPT 135-36 film at F5.6 and a 1/60 second exposure time. A blue background was used with side and bottom illumination. The sample was raised 15 cm above the base platform.

NOTE: Black and white negatives were developed using Ilford ID-11 developer according to the manufacture's instructions. Prints were made on Ilford Multigrade Paper using Filter no. 4.5. Colour slides were converted to prints by the Medical Graphics Department, Groote Schuur Hospital, Cape Town.

APPENDIX 3 : DNA MARKERS

Three DNA molecular weight markers were used for calculating the sizes of LSDV and avipoxvirus DNA fragments. The first two contained either *Hin* dIII or *Pst* I restriction fragments of phage Lambda CI 857 Sam 7 (Boehringer Mannheim, Germany), and the other one contained a mixture of *Hin* dIII restriction fragments and concatemers of Lambda DNA [(Low Range PFG Marker (BioLabs, New England, U.S.A.)]. The sizes of the DNA markers are indicated in Tables A, B and C below. The Lambda *Pst* I and *Hin* dIII markers each contain an extra band that coincides with the top bands in each case. This is due to the presence of Lambda *cos* sites which link two of the restriction fragments causing them to migrate slower than single fragments. Only sizes for single fragments are indicated below:

TABLE A: Sizes of Lambda DNA *Pst* I fragments

Fragment no.	Size (kbp)	Fragment no.	Size (kbp)	Fragment no.	Size (kbp)
1	11.5	11	1.7	21	0.22
2	5.1	12	1.2	22	0.21
3	4.8	13	1.1	23	0.20
4	4.5	14	0.8	24	0.16
5	2.8	15	0.5	25	0.15
6	2.6	16	0.47	26	0.09
7	2.5	17	0.45	27	0.09
8	2.4	18	0.34	28	0.07
9	2.1	19	0.27	29	0.02
10	2.0	20	0.25		

TABLE B: Sizes of Lambda *Hin* dIII fragments

Fragment no.	Size (kbp)
1	23.1
2	9.4
3	6.7
4	4.4
5	2.3
6	2.0
7	0.56

TABLE C: Sizes of Low Range PFG marker DNA.

Fragment no.	Size (kbp)	Fragment no.	Size (kbp)
1	194.0	7	6.5
2	145.5	8	4.3
3	97.0	9	2.3
4	48.5	10	2.0
5	23.1	11	0.5
6	9.4	12	0.1

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