Department of Biological Sciences

Heterologous expression of LSDV immunogenic epitopes as TMV Coat Protein fusions in *Nicotiana benthamiana* plants.

By

Melitah Motlhale

(ID 200500988)

Supervisors: Dr S.W. Mpoloka

Dr T.K Sebunya

Submitted to the School of Graduate Studies in partial fulfilment of the requirements for the degree of Masters of Science in Applied Microbiology at the University of Botswana, 2015.

APPROVAL PAGE

This dissertation has been approved as meeting the required standards of scholarship for partial fulfilment for the degree of Masters of Science (Applied Microbiology), University of Botswana.

_____ _____

Supervisor: Dr S.W. Mpoloka Date

_____ _____

Co-Supervisor: Dr T.K. Sebunya Date

DECLARATION

This dissertation is my original work except where due reference is made. It has not been and shall not be submitted for the award of any degree in any other University.

Melitah Motlhale

_____ _____

Student signature Date

ACKNOWLEDGEMENTS

I would like to start by extending my gratitude to the Department of Biological Sciences staff for giving me the opportunity of a lifetime to undertake this programme. I would like to thank my project supervisors Dr S.W. Mpoloka and Dr T.K. Sebunya for constantly supporting, directing and encouraging me throughout the project. Their assistance and guidance is greatly appreciated. I wish to thank them for providing detailed comments on my dissertation and for always availing themselves for consultations in their hectic schedule.

Heartfelt thanks go to Dr Larry Grill and the Pitzer College Plant Vaccine Group for giving me an opportunity to do some of the laboratory work in their laboratory, and for supplying me with materials, plasmids, cultures and *Nicotiana benthamiana* seeds used in this project. Thank you to Prof E. Rybicki UCT for the *Agrobacterium tumefaciens* strains. I would like to thank Dr L. Grill, Ms Nina Timberlake and Ms Caitie Tanaka for the technical support provided; I will forever be grateful and cherish the friendship developed during my studies. I am also grateful to Pitzer College and the University of Botswana for funding this project.

I wish to thank the University of Botswana (Biological Sciences), Botswana National Veterinary Laboratory staff and the Department of Veterinary Services in the Ministry of Agriculture for the assistance granted to me in regard to the statistical information and maps on the LSD outbreaks in Botswana. Special thanks to Mrs O. Tagwa, Dr Kobedi Segale and Dr Joseph Hyera.

Special thanks to my loved ones, Kelvin Phiri, Aku N. Ntumy, Bakang Baloi, Mthandazo Dube and Bonolo Kambai for their support, appreciation, humour and the generosity they granted me both in trying and in good times.

To all those who have contributed to this project but not specifically mentioned, thank you so much.

DEDICATION

This is dedicated to my parents, Mr Nonofo Motlhale and Mrs Banyana Motlhale, for their support and encouragement throughout my graduate studies. I could not have made it this far without their love and support.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Ab.....Antibody

AP.....Alkaline phosphatise

APHIS.....Animal and Plant Health Inspection Service

APS.....Ammonium per sulphate

ASM.....American Society for Microbiology

BEDIA.....Botswana Export Development Investment Authority

BSA.....Bovine serum albumin

CaPVs.....Capripoxviruses

ChPV.....Chordopoxvirus

CP.....Coat protein

cDNA.....Complementary deoxyribonucleic acid

DEFRA.....Department of Environment Food and Rural Affairs

DMSO.....Dimethyl sulphoxide

DNA.....Deoxyribonucleic acid

EDTA.....Ethylenediaminetetraacetic acid

ELISA.....Enzyme linked immunosorbent assay

EtBr.....Ethidium bromide

EtOH.....Ethanol

FAO.....Food Agriculture Organization

FMD.....Foot and mouth disease

Gent.....Gentamicin

GFP.....Green fluorescent protein

GPV.....Goat pox virus

HEPES.....*N*-2-Hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid

HPV.....Human papilloma virus

HRP.....Horse radish peroxidase

ITR .....Inverted terminal repeats

Kan.....Kanamycin

LB.....Luria-Bertani

LSD.....Lumpy skin disease

LSDV.....Lumpy skin disease virus

Mab.....Monoclonal antibody

Min.....minute

NS.....Non structural

OD.....Optical density

OIE.....Office of International Des Epizooties

O/N.....Overnight

ORF.....Open reading frame

PAGE.....Poly acrylamide gel electrophoresis

PBS.....Phosphate buffered saline

PCR.....Polymerase chain reaction

ShPV.....Sheep pox virus

Rif.....Rifampicin

RNA.....Ribonucleic acid

rTMV.....Recombinant tobacco mosaic virus

rtPCR.....Reverse transcriptase polymerase chain reaction.

SDS.....Sodium dodecyl sulphate

ss(+)RNA.....single stranded positive sense ribonucleic acid

TAE.....Tris-acetate EDTA buffer

TBS.....Tris-buffered saline

TMV.....Tobacco mosaic virus

UTR.....Untranslated region

UV.....Ultraviolet light

VIPR.....Virus Pathogen Resource

VLP.....Virus-like particle

wtTMV.....Wild-type Tobacco mosaic virus

ABSTRACT

Lumpy skin disease (LSD) is an economically important disease in Botswana. Currently the disease has no cure. In Botswana this disease is endemic; it has serious economic effects on beef export and the tourism industry. The only way of controlling LSD is by vaccination, separation of healthy animals from the diseased and killing or eliminating the diseased animals. Current strategies in vaccine production use intact or inactivated pathogen strains to induce immunity, as well as subunit vaccines which are commercially produced in yeast or mammalian cell cultures. Vaccine production is expensive. This study aimed at production of a candidate vaccine for lumpy skin disease using tobacco mosaic virus (TMV) as a vector for coat protein fusion production. The major immunodominant region in the lumpy skin disease virus (LSDV) is the P32 protein; two epitopes (LSDV-A and LSDV-B) from this protein were selected and used as antigens for the vaccine production. The epitopes were fused to the CP of TMV then cloned into a TMV based *Agrobacterium* compatible binary vector (pJL TRBO). Six weeks old *Nicotiana benthamiana* plants were agroinfiltrated with the recombinant TMV (rTMV). The rTMV was extracted from the plants three weeks post infection using polyethylene glycol (PEG) precipitation method. Reverse transcription polymerase chain reaction (rtPCR) of raw material from infected leaves showed that the rTMV was not expressed in the plants. The virus extracts showed that the LSDV epitopes were not successfully expressed in the plants; the epitopes were not attached to TMVCP. Western blots and ELISA showed that the rTMV was not able to elicit an immune response by reacting to anti-LSDV bovine sera. The study indicated the potential to develop a system to locally generate cheap and effective vaccines against LSDV even though there are some limitations that are to be eliminated.

Chapter 1

1.0 INTRODUCTION

1.1 Lumpy skin disease

Lumpy skin disease is an acute cattle disease caused by the lumpy skin disease virus. The virus is classified in the genus *Capripoxvirus* (CaPVs) which represents one of eight genera within the chordopoxvirus (ChPV) subfamily of the *Poxviridae* (Buller *et al*., 2005; Diallo and Viljoen, 2007; Woods, 1988). The capripoxvirus genus is currently comprised of lumpy skin disease virus (LSDV), Sheeppox virus (ShPV), and Goatpox Virus (GPV).

These viruses are responsible for some of the most economically significant diseases of domestic ruminants in Africa and Asia (Fenner, 1996). Capripoxvirus infections are generally host specific and have specific geographic distributions (Coetzer *et al*., 1994; Davies, 1991). However, CaPVs are serologically indistinguishable from each other. They are able to induce heterologous cross-protection and in some instances, to experimentally cross-infect (Capstick, 1959; Tulman *et al*., 2001).

Restriction fragment analysis and limited DNA sequence data support a close relationship between the CaPVs (Bhanuprakasha *et al*., 2006; Black *et al*., 1986; Kara *et al*., 2003; Kitching *et al*., 1989; Stram *et al*., 2008). The molecular basis of CaPV host range restriction and virulence remains to be elucidated. The disease is found in Southern and Eastern Africa but in recent years it extended Northwest through the continent into sub Saharan West Africa and into the Middle East (Babiuk *et al*., 2008; House *et al*., 1990; Tuppurainen and Oura, 2012). LSD strain of *Capripoxvirus* is antigenically indistinguishable from strains causing sheep pox and

goat pox (Buller *et al*., 2005). However, LSD has a different geographical distribution to ShPV and GPV (**Figure 2**), suggesting that cattle strains of *Capripoxvirus* do not infect and transmit between sheep and goats (Babiuk *et al*., 2008; Patel and Heldens, 2009).

Lumpy skin disease is an infectious, eruptive, occasionally fatal disease of cattle of high importance (Coetzer *et al*., 1994; Davies, 1991; Fenner, 1996). LSD is characterised by fever, enlarged lymph nodes, firm, circumscribed nodules on the skin and ulcerative lesion of the mucous membrane of the mouth and sometimes death (Richmond, 1998). Transient viremias have also been described for cattle infected with LSDV (Carn and Kitching, 1995a; Tuppurainen *et al*., 2005). Internal lesions are often seen at necropsy, especially in the lungs. Lesions in the trachea, rumen, tongue, kidney, nasal turbinates, and reproductive organs have also been reported (Afshar *et al*., 1986; Gulbahar *et al*., 2000; Kitching, 1999). Frequently, the centre of the lesion dies, becomes dry, forms a deep scab, and falls out, leaving a raw area that becomes infected (APHIS, 2003).

LSD has morbidity averaging 10% and mortality averaging 1% in affected herds although mortality rate over 75% have been recorded (Diesel, 1949). Although LSD does not usually have a high mortality rate (<10%), it causes severe economic losses due to reduced productivity in animals; particularly reduced milk production. In dairy herds it causes a sharp drop in milk production, often leading to secondary bacterial mastitis (Ayelet *et al*., 2014). It also causes damage to hides, abortions in pregnant cows and temporary or permanent sterility in males, **Figure 1** (DEFRA, 2008). The deleterious effect that the disease has in affected bulls' ability to mate and to produce fertile sperm is under appreciated. However it has important economic

implications, especially under subsistence farming and in farming systems where a single sire is used (Coetzer, 2004).

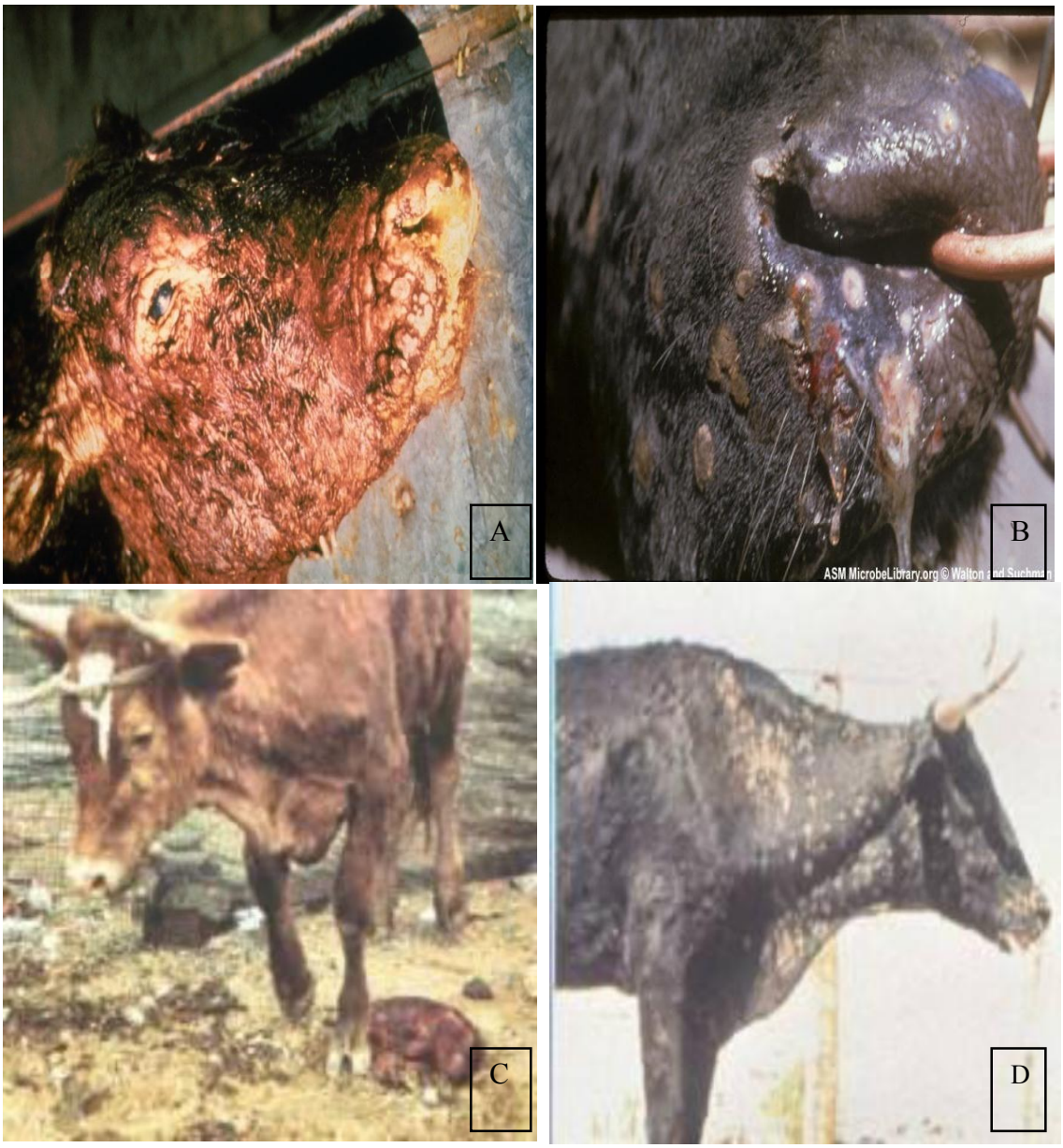


Figure 1: The typical clinical symptoms of LSD. Pictures **A** and **D** show nodules on the skin, **C** shows severe weight loss and a foetus carcase on its side as a result of abortion (**DEFRA**, 2003). **B** shows a closer magnification of the nodules and increased production of mucus (**ASM**, 2011).

LSD causes significant economic losses and consequently it is identified as a high priority disease for control. LSD is an Office of International Des Epizooties (OIE) 'List-A' disease, which identifies diseases with the potential for rapid spread and severe economic losses (Irons *et al*., 2005). This disease has a major influence on international trade requiring immediate notification of animal disease. It must be reported to all other member states and the commissioner within 24 hours of an outbreak (OIE, 2008).

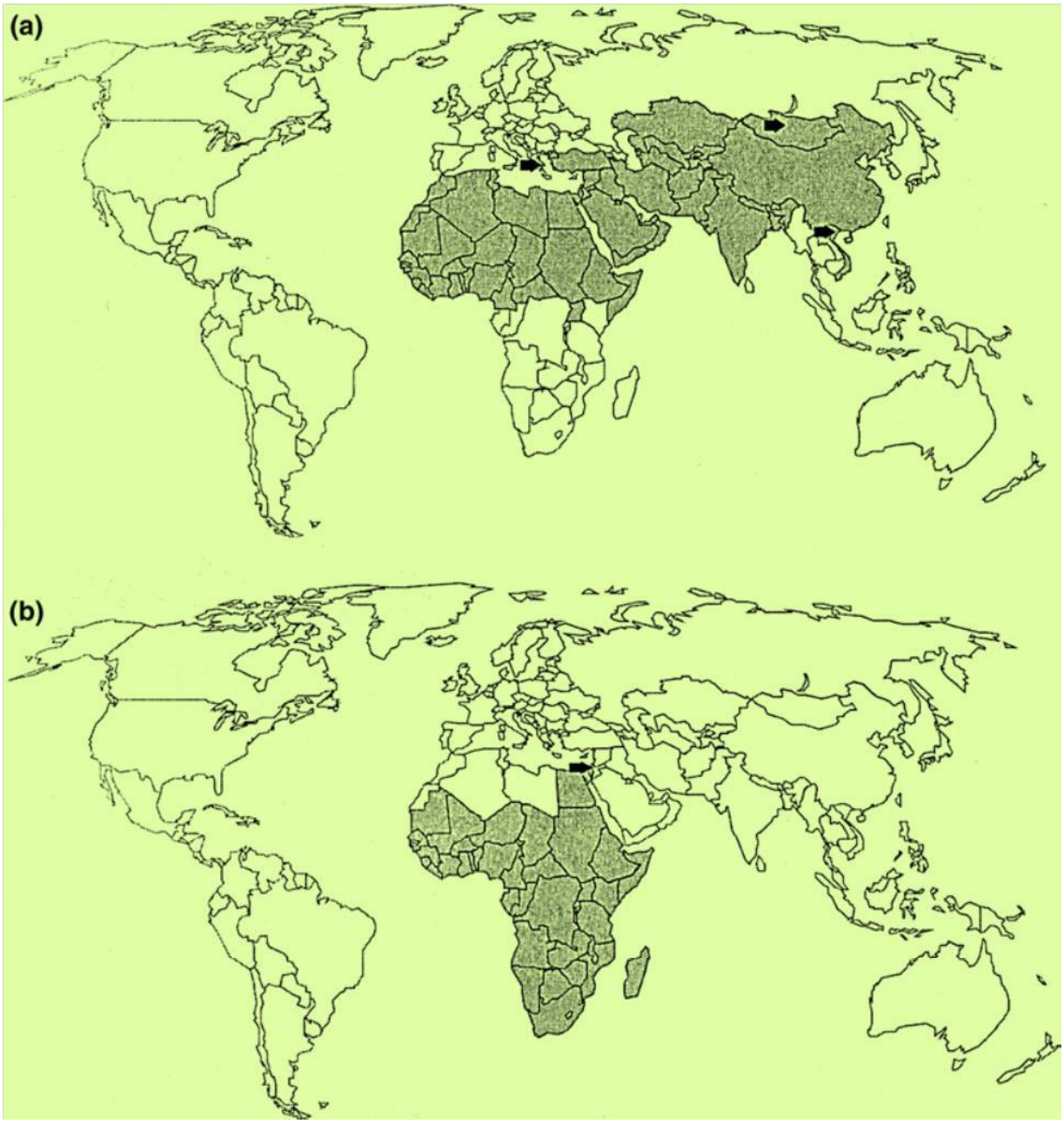


Figure 2: Map showing likely global distribution of sheeppox and goatpox (**a**) and LSD (**b**) viruses. Recent outbreaks marked with arrows (Babiuk *et al*., 2008).

1.2 Transmission of LSDV

Transmission of the LSD virus is thought to be predominantly by insects, natural contact transmission in the absence of insect vectors has been shown to be inefficient (Carn and Kitching, 1995b; OIE, 2002). Until 1988, the occurrence of LSD was confined to sub-Saharan Africa, but has since spread into Egypt. Two laboratory-confirmed outbreaks of LSD outside Africa have been documented, one in Egypt and one in Israel in 1989. LSDV is not transmissible to humans (Ireland and Binepal, 1998).

LSDV has been isolated from flies *Stomoxys calcitrans* and from *Biomyia fasciata* (Weiss, 1968). Transmission may occur *via* infected saliva in the absence of an insect vector. Natural capripox infections occur throughout endemic areas as a result of contact between diseased and susceptible animals (Davies, 1976). Though no specific vector has been identified to date, transmission studies suggest that the most prevalent method for the spread of LSD is mechanical transmission by insects such as *S. calcitrans* (Kitching and Mellor, 1986: Mellor *et al*., 1987). Mosquitoes like *Culex mirificens* and *Aedes natrionus* (Chihota *et al*., 2001), and other flies could also play a major role in LSDV transmission (Santi *et al*., 2006). Field observations and supporting evidence indicate that the disease is transmitted by biting flies (Kitching and Mellor, 1986).

Mosquitoes have been shown to be efficient mechanical vectors of LSDV and are able to transmit the virus to a susceptible bovine over a period of 2-6 days post infective feeding. LSDV has been proven to be transmissible to calves through infected milk. LSDV may be excreted for long periods after obvious clinical signs are no longer seen (Irons *et al*., 2005). For clinical

diagnosis, incubation period is approximately 12 days (OIE, 2008). Wildlife does not seem to play any significant role in the clinical epidemiology of the disease although suspect clinical disease has been described in species such as Asian water buffalo, springbok and oryx (antelope species) (Kitching and Mellor, 1986; Young *et al*., 1970).

There are distinct differences between the geographical distribution of sheeppox, goatpox and lumpy skin disease virus. In the last 50 years the geographic range of sheep pox and goatpox (**Figure 2a**) has been restricted to mainly Asia and Africa, extending from Africa North of the Equator (Kitching *et al*., 1989).

1.3 History and epidemiology of LSD

Lumpy skin disease is generally confined to Africa (**Figure 2b**) with a potential of spreading to some Asian countries, (Babiuk *et al*., 2008; House *et al*., 1990). Until the 1980s, this disease was only found south of the Sahara desert and in Madagascar, but in 1988, it spread into Egypt (Woods, 1988). It also occurs in other Middle Eastern countries. In 1989, an outbreak in Israel was eradicated by slaughter and vaccination (OIE, 2008). The economic significance of the disease was demonstrated in the first large-scale outbreaks in South Africa in the late 1940s; over 8 million cattle were affected with a mortality rate of over 75% (Diesel, 1949, Von Backsrom, 1945) **Table 1**.

Periodic epidemics occur in most African countries. The sub-Saharan region is most commonly affected with morbidity rates generally varying between 1% and 20%. Cows in 1% to 7% of cases may abort (OIE, 2012). In Africa periodic epidemics of LSD outbreaks occur. Outbreaks

were recorded in Ethiopia from January 2007- December 2011 (Ayelet *et al*., 2014). The disease has a severe impact on the socio-economic status of the continent as the cattle industry is of great importance. There is a real risk of LSDV establishing itself in the Middle East and spreading into Asia and Europe (Kitching and Carn, 2004). Eradicating this disease remains a challenge hence the need for research on possible effective vaccine candidates.

Table 1: The earliest LSD outbreaks recorded in Africa and some parts of The Middle East.

Year	Location	Reference
1929	Zambia	(MacDonald, 1931; Morries,1931; Coetzer, 2004)
1943	Botswana	(Von Backsrom, 1945)
1945	Zimbabwe, Mozambique,	(Houston, 1945)
1945	South Africa	(Diesel, 1949;Thomas and Mare, 1945; Von Backsrom, 1945)
1947	Lesotho	(De Sousa Dias and Limpo Serra, 1956; Diesel, 1949)
1954	Madagascar	(Ordner and Lefevre, 1987)
1957	Kenya, Uganda, Somalia, Tanzania (East Africa)	(MacOwan, 1959; Burdin, 1959)
Early 1970s	Sudan	(Ali and Obeid, 1977; OIE, 1996)
1970s -1985	Almost all the sub Saharan countries, most central and west African countries. For examples Chad and Niger 1973, Nigeria, 1974, Ivory coast 1976 and Somalia, 1983	(Davies, 1991; Nawathe *et al*., 1978; Ordner and Lefevre, 1987)
1988	Egypt	(Ali *et al*., 1990; Hafez *et al*., 1992)
1989	Israel and Saudi Arabia	Abraham and Zissman, 1991; Greth *et al*., 1992; Yeruham *et al*., 1995)

1.3.1 Epidemiology of LSD in Botswana

LSD is endemic and widespread countrywide. **Table 2** shows the annual outbreaks of LSD in Botswana. Outbreaks usually take place during the rainy season. Therefore, farmers are advised

to vaccinate their cattle before the rainy season as a prevention strategy (Quinn *et al.*, 2002). In Botswana, farmers are advised to use tetracycline- or sulphonamide-based drugs on infected animals to treat for opportunistic bacterial infections. The LSD live-attenuated vaccine being used currently in Botswana is imported from South Africa (News, 2011). Botswana Ministry of Agriculture Annual reports on LSD outbreaks indicate that the disease caused severe symptoms and in some cases cattle death (**Table 2**).

Table 2: LSD outbreaks in Botswana from the year 2000-2011 (Ministry of Agriculture, 2000-2011).

Year	No. Dead animals	No. Affected animals	Number of risk
2000	44	343	8846
2001	15	77	2667
2002	4	0	6
2003	0	7	3165
2004	0	68	364
2005	253	62	1097
2006	34	272	5635
2007	9	168	2032
2008	12	113	2044
2009	1	19	742
2010	8	126	2692
2011	26	103	1704

1.4 The lumpy skin disease virus

1.4.1 The LSDV genome

The poxvirus genome consists of a linear, double stranded DNA (dsDNA) molecule which is covalently cross linked at the terminal repeats (Gershelin and Berns, 1974; Black *et al.*, 1986). These terminal sequences have been shown to contain repeated sequences that are inverted with respect to one another (Wittek *et al.*, 1978).

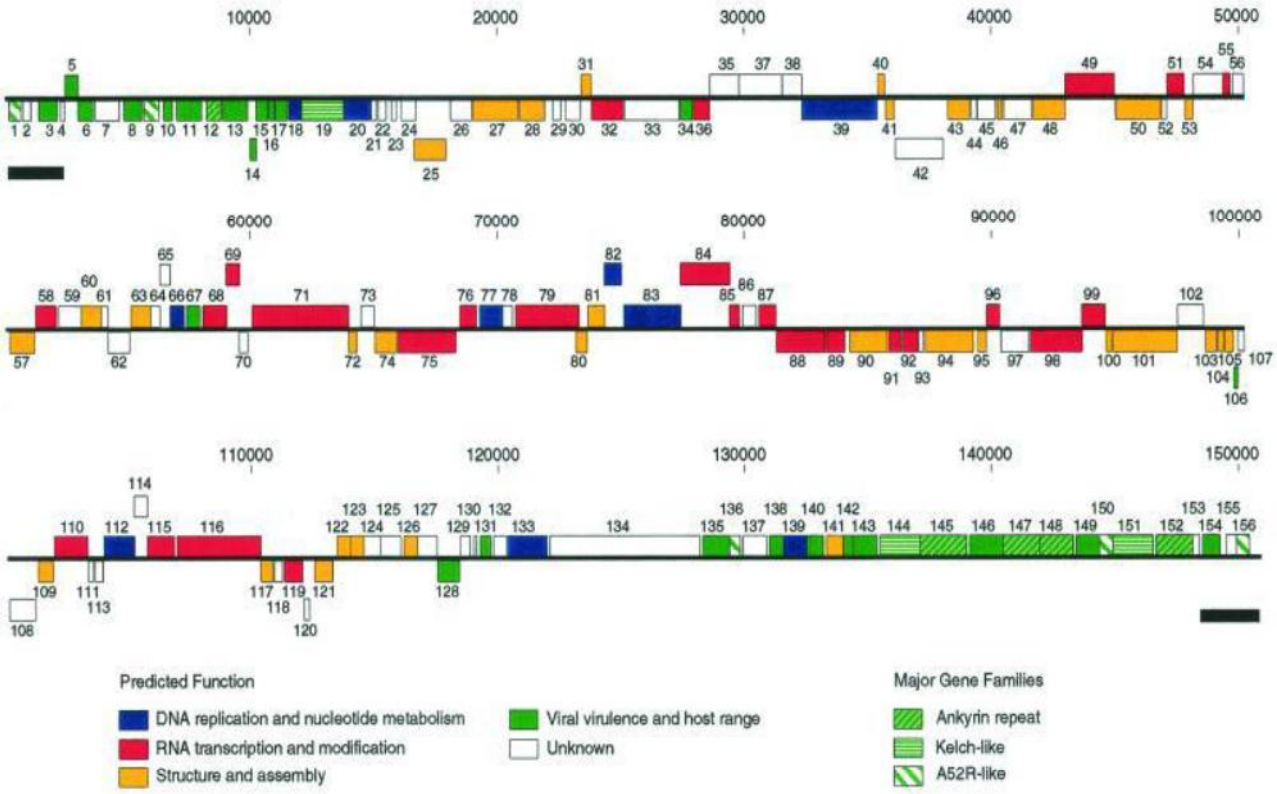


Figure 3: Linear map of the LSDV genome. ORFs are numbered from left to right based on the position of the methionine initiation codon. ORFs transcribed to the right are located above the horizontal line; ORFs transcribed to the left are below. Genes with similar functions and members of gene families are colored according to the figure key. ITRs are represented as black bars below the ORF map (Tulman *et al*., 2001).

The 151-kbp LSDV genome contains 156 putative genes and consists of a central coding region bounded by identical 2.4 Kbp-inverted terminal repeats (Gershon and Black, 1989) (**Figure 3**). Comparison of LSDV with chordopoxviruses of other genera reveals 146 conserved genes which encode proteins involved in transcription and mRNA biogenesis, nucleotide metabolism, DNA replication, protein processing, virion structure and assembly, viral virulence and host range. In the central genomic region, LSDV genes share a high degree of colinearity and amino acid identity (average of 65%) with genes of other known mammalian poxviruses, particularly

suipoxvirus, yatapoxvirus, and leporipoxviruses. In the terminal regions, colinearity is disrupted and poxvirus homologues are either absent or share a lower percentage of amino acid identity (average of about 43%).

The 156 putative genes in the LSDV genome represent a 95% coding density and encode proteins 53 to 2025 amino acids. LSDV contains the majority of conserved pox viral genes involved in basic replicative mechanisms. These include at least 26 genes encoding RNA polymerase subunits, mRNA transcription initiation, elongation and terminal factors. Enzymes directing post transcriptional processing of viral mRNA are also included (Tulman *et al*., 2001). LSDV proteins potentially involved in nucleotide metabolism include homologues of thymidine kinase, dUTP pyrophosphatase and the small subunit of ribonucleotide reductase. It encodes at least 30 homologues of pox viral proteins known to be structural or involved in virion morphogenesis and assembly (Davies, 1991).

1.4.2 Virion structure of LSDV

LSD appears by electron microscopy to be oval- or brick-shaped, **Figure 4** (Westwood *et al*., 1964). Early electron microphotographs of the capripoxvirus (**Figure 5**) revealed the presence of two types of particles; one form was thought to be the complete form, consisting of a nucleoid with surrounding envelope material, and the other incomplete form consisting of an envelope with or without nucleoid components (Nagington and Hornes, 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 (Dale and Pogo, 1981).

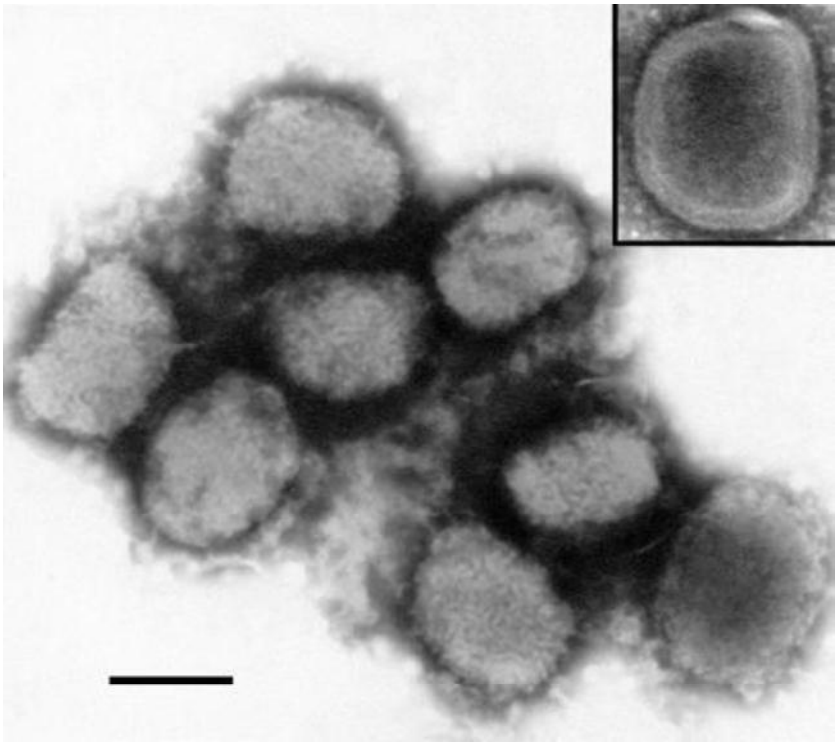


Figure 4: LSDV particles caption; Electron micrograph of virus particles (scale bar = 200nm) (Davies, 2012).

The virion contains numerous antigens, most of which are shared by all members of the genus (Fenner *et al*., 1987). DNA analysis using restriction endonucleases on both field samples and South African Neethling strain showed 80% homology between strains of the capripoxvirus (Black *et al*., 1986). The Neethling strain is the wild type strain of LSDV (Kitching, 1986). The viriods are ovoid and classified as "C" (capsule or "C" form) or "M" if surrounded by a membrane or covered in cordlike elements (mulberry or "M" form) (Kitching and Smale, 1986; Munz and Owen, 1966).

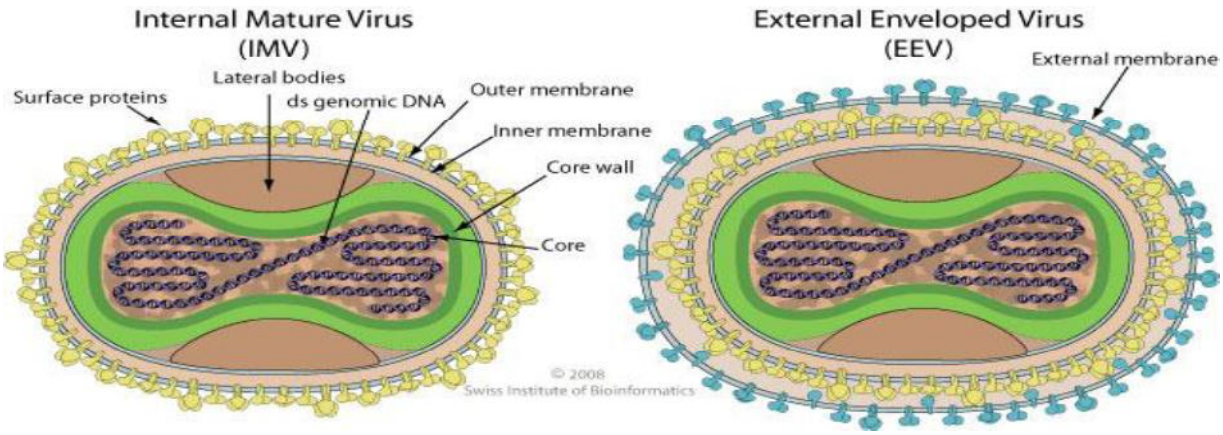


Figure 5: Two distinct infectious virus particles exist; the intracellular mature virus (IMV) and the extracellular enveloped virus (EEV). The virus is enveloped and brick-shaped with dimensions; 300 x 270 x 200nm. The surface membrane displays surface tubules or surface filaments (Swiss Institute of Bioinformatics, 2008).

1.4.2.1 P32 antigen

The immunodominant region of the LSDV is the P32 protein. The P32 antigen is a structural protein present in all capripoxvirus isolates and contains a major antigenic determinant (Chand, 1992; Chand *et al*., 1994). Thus P32 is important in pathogenicity, diagnosis, prevention and control of capripoxvirus (Tian *et al*., 2010). Most of the differences between the different Capripoxvirus strains involve genes and gene families with likely functions involving viral virulence and host range (Tian *et al*., 2010).

Although LSDV resembles leporipoxviruses in gene content and organization, it also contains homologues of interleukin-10 (IL-10), IL-1 binding proteins, G protein-coupled CC chemokine receptor, and epidermal growth factor-like protein which are found in other poxvirus genera (Tulman *et al*., 2001). These data show that although LSDV is closely related to other members

of the *Chordopoxvirinae*, it contains a unique complement of genes responsible for viral host range and virulence.

1.4.2.2 Capripoxvirus life cycle

The *Poxviridae* are enveloped unsegmented dsDNA viruses; unlike many dsDNA viruses that replicate in the host nucleus poxviruses encode their own replication machinery and therefore replicate in the cytoplasm. Viral genes are expressed in a bi-phasic manner with early-expressed genes encoding non-proteins involved in a genome replication and late-expressed genes encoding the viral structural proteins (OIE, 2008)Poxviruses assemble large virions that can be seen under the microscope (Chen *et al*., 2003).

Attachment of the viral protein to host glycosaminoglycans mediates endocytosis of the virus into the host cell. The viral proteins fuse with the plasma membrane to release the core into the cytoplasm. During the early phase early genes are transcribed in the cytoplasm by viral RNA polymerase. Early expression begins minutes post infection, as early expression ends the core is completely uncoated as early expression ends, the genome is now free in the cytoplasm. During the intermediate phase intermediate genes are expressed, triggering genomic DNA to replicate. The late phase constitutes late genes expression producing all structural genes.

Assembly of progeny virion starts in the cytoplasmic viral factories producing a spherical immature particle. This virus particle matures into brick shaped intracellular mature virion (IMV). IMV-virion can be releases upon cell lysis, it can also acquire a second double membrane

from trans-golgi and bud as external enveloped virion (EEV) (OIE, 2010; Moss, 2006; Moss, 2001).

1.4.2.3 LSDV serotypes and subtypes

Available evidence from studies on the South African Onderstepoort Neethling strain suggests that there is only one immunological type of LSDV and only one serotype. Virus isolates collected over an extended period from a large number of natural cases originating from outbreaks of the disease in Kenya, Botswana and Malawi showed complete, cross neutralization with the Neethling strain (OIE, 2010; Voster and Mapham, 2008; Fauquet *et al*., 2005).

1.4.2.4 Immune response to LSDV

Immunity to capripoxvirus infection is predominantly cell mediated and therefore requires a replicating agent to be stimulated (Carn, 1993). Most progeny viruses remain inside cells with the exception of enveloped viruses which are released into the blood (Boulter and Appleyard, 1973). By spreading from cell to cell, the virus is inaccessible to circulating antibodies. Circulating antibodies against capripoxvirus limit the spread of the virus in experimental sheep, but this does not prevent replication of the virus at the site of infection.

All the viruses in the *Capripox* genus share a common major antigen for neutralizing antibodies; thus, animals recovered from infection by one virus are protected from infections with the other. This is demonstrated by serological evidence (Davies and Otema, 1978), cross infection and cross protection experiments (Capstick, 1959; Kitching and Taylor, 1985).

Plant-derived vaccines can be administered orally to elicit a mucosal immune response. These vaccines possess dual advantage of preventing antigens from being degraded as they pass through the gastrointestinal tract, simultaneously delivering an antigen into the mucosal immune system (Hefferon, 2012).

1.4.2.5 Control of LSD

In endemic countries, vaccination against LSD is the most effective method for controlling the disease, thus far control of LSD has been only partially successful (OIE, 2012). Where LSD is not present the most important method is restriction of the importation of animals and those animal products that may contain live virus from endemic countries (Carn, 1993). In Botswana there are veterinary cordon fences used mostly during outbreaks but currently more focused on control of foot and mouth disease.

In case of outbreaks, isolation and prohibition of animal movements, slaughtering of infected and in contact animals, and ring vaccination in a radius of 20-50 km should usually be sufficient to eradicate the disease (Carn, 1993; APHIS, 2003). Proper disposal of dead animals, for example incineration, is also beneficial. Other control measures include disinfection of premises, and vector control in premises and on animals. Intensive insect control, including widespread use of insecticides can be used (OIE, 2002).

1.4.2.5.1 Vaccination

1.4.2.5.1.1 Current vaccines

Many poxviruses (including LSDV) have been attenuated by serial passage in either cell cultures or on the chorioallantoic membranes of embryonated chicken eggs to serve as live attenuated vaccines (Van Rooyen *et al*., 1969; Kirnse, 1969; Taylor and Paoletti, 1988; Winterfield and Reed, 1985). The highly attenuated strain has proven to be safe and effective and to provide long-term immunity although over time the effectiveness has been challenged and more frequent vaccinations are now recommended (Hunter and Wallace, 2001; OIE 2010; Weiss, 1968).

Different vaccines have been widely and successfully used for the prevention of LSD in cattle populations in Africa. In Southern Africa, the Onderstepoort LSD freeze dried G 0110 (Act 36/1947) live attenuated vaccine is used. The *Neethling* strain of LSD was passaged 50 times in tissue cultures of lamb kidney cells and then 20 times in embroyonated eggs (OIE, 2008). The strain proved to be immunogenic for cattle, although local reactions do occur in a high proportion of animals at the vaccination site. It is produced in tissue culture and issued as a freeze-dried product (Weiss, 1968). Immunity conferred lasts up to three years.

The second type is the sheep goat pox virus (SGPV) vaccine for cattle, (Capstick and Coackley, 1961). The use of this vaccine has been restricted to countries where sheep and goat pox viruses are endemic. The heterologous attenuated virus vaccine include sheep goat poxvirus, but may cause severe reaction, therefore this option is not advised in countries free from goat poxvirus (OIE, 2002). In Kenya, an effective vaccine has been produced from a local sheep and goat pox virus. This was shown to immunize cattle against LSD (Capstick and Coackley, 1961; Kitching, 2003).

The SGPV was passaged 16 times in pre-pubertal lamb testis or foetal muscle cell cultures and used for vaccination at this level. Local reactions have not been observed, but some *Bos taurus* breeds have shown lymphadenitis with signs of mild, generalized LSD-like lesions following vaccination (approximately 0.02%). Neutralizing antibodies to LSDV persist for at least two to three years after vaccination. In other cases, some animals have antibody levels which are too low to be demonstrated but they nevertheless are still resistant to challenge (Carn and Kitching, 1995b; Weiss, 1968).

Control of LSDV in Egypt depends on prophylactic vaccination programs using live attenuated cell cultures adapted sheep pox vaccine (SPV) (Brenner *et al*., 2006; Michael *et al*., 1994). Two other strains of SPV have recently been used as a prophylaxis against LSD. The Romanian strain, prepared in the skin of lambs for use against sheep pox, was used in several million cattle in Egypt and was able to illicit an immune response. Another sheep pox strain, the Yugoslavian RM 65 prepared in tissue culture, was used in Israel. No complications have followed the use of these strains in cattle (FAO, 1991). Immunity lasts at least two years.

The use of the live attenuated LSD/SPGV vaccine has been successful in reducing LSD outbreaks (Hunter and Wallace, 2001; Weiss, 1968). However there are several concerns and limitations. These include the need for high containment facility for production of the vaccine making the cost of the vaccine to be high. There is need for improved vaccines which produce rapid protection and can be cheaply produced.

1.4.2.5.2 Plant based vaccines

1.4.2.5.2.1 Simple virus-based vectors

Plants are now gaining widespread acceptance as a general platform for large scale production of recombinant proteins. The first recombinant pharmaceutical proteins are reaching the final stages of clinical evaluation and many more are in the development pipeline (Cummings *et al*., 2014; Desai *et al*., 2010; Fischer *et al*., 2004; Grimsley *et al*., 1986; Hefferon, 2012; Lindbo, 2007a; Lindbo, 2007b; Mortimer *et al*., 2015; Paul and Ma, 2011; Penney *et al*., 2011; Rybicki, 2014). Viral vectors have been used for some time for the expression of foreign proteins or for chimaeric coat proteins in plants (Gleba *et al*., 2005; Steinmetz *et al*., 2009; Yusibov *et al*., 2006).

Economic losses in agriculture caused by viral infections led to the development of genetic systems that allow manipulation of the virus to manage plant diseases; these genetic systems have also led to the development of viruses as beneficial molecular tools (Yusibov *et al*., 2002). The utility, variety and application of these genetic systems have expanded greatly since the early use of cDNA-derived *in vitro*-synthesised RNA to infect plants with recombinant tobacco mosaic virus (rTMV) (Yusibov *et al*., 1999). Examples of some of the vaccine epitopes which have been expressed on TMV include antigens from human papillomavirus, foot and mouth disease virus, HIV-1 and rotavirus (Noris *et al*., 2011; Turpen *et al*., 1995; Wigdorovitz *et al*., 1999; Rybicki, 2009; Yusibov *et al*., 2011).

One of the first plant-based vaccines described was rTMV with malarial epitopes expressed on the virion surface (Turpen *et al*., 1995; Koo *et al*., 1999). Large Scale Biology Corp had the

recombinant TMV technology (Geneware^{TM}) as one of their main products and it was successfully used to express patient-specific non-Hodgkin lymphoma tumor-derived single-chain vaccine for FDA approved clinical trial (McCormick and Palmer, 2008; McCormick *et al*., 1999, 2006). Cottontail rabbit papillomavirus (CRPV) major protein L1 was also produced either transgenesis or via rTMV expression and protected rabbits against CRPV challenge (Kohl *et al*., 2006). In animals rTMV expressing surface located epitope from CRPV and ROPV (rabbit oral papillomavirus) L2 minor capsid was also used as an injectable vaccine (Palmer *et al*., 2006).

High-level expression of the introduced genes and the rapid accumulation of appropriate products that are easily purified from the host plant are the primary characteristics that make plant viruses well suited as transient expression vectors. Moreover, plant viruses generally have a wide host range that allows expression of a gene in different plant species using the same vector construct (Yusibov *et al*., 2002).

The vast majority of viruses that infect plants are single-stranded RNA viruses that replicate in the cytoplasm. Many of these viruses replicate to very high levels in plants and a few have been used to express foreign genes (Scholthof *et al*., 1996). One of such viruses is TMV. Other plant viruses that have been used successfully for either peptide expression or for whole antigens include the Bamboo mosaic virus (BaMV), Cowpea mosaic virus (CPMV), Papaya mosaic virus (PapMV) and Cucumber mosaic virus (CMV) among others.

There are challenges associated with expression of whole genes using replicating plant virus vectors constructs which spread via natural means in plants. In many cases there is loss of the

transgene during the expression period, especially when expression gets depressed overtime. A cottontail rabbit papillomavirus (CRPV)-L1 protein gene expression in *N. benthamiana* via rTMV was lost progressively as infected plants developed, leading to lower yields of proteins than were obtained in transgenic tobacco (Kohl *et al*., 2006). The elegant means of getting around the problem and completely redesigning plant virus involves the use of *A. tumefaciens* for systemic delivery of viral genomes (Rybicki, 2010).

1.4.2.5.2.2 Agroinfiltration of viral vectors

The most widely used method of generating transgenic plants is the *A. tumefaciens*-mediated transformation. This technically simple plant transient expression system takes advantage of *A. tumefaciens'* molecular machinery to transfer DNA into plant cells (Gleba, *et al*., 2007; Grimsley *et al*., 1986; Maclean *et al*., 2007; Rybicki 2009, 2010, 2014). *A. tumefaciens* is a soil bacterium that has evolved a natural genetic engineering system; it contains a segment of DNA that is transferred from the bacterium to the plant cells (Snustad and Simmons, 2006)Achieving an *A. tumefaciens*-mediated transient expression involves infiltration of intracellular spaces in plant leaves with a suspension of the bacterium, using a syringe or vacuum. This should result in mobilization of the T-DNA into the nuclei of a large portion of the cells. Infiltrating more leaves or the whole plant would improve viral recovery.

Because it is effective, easy and inexpensive to use, this system has become the most commonly used for production of proteins in plants. However the approach sometimes expresses very low T-DNA, thus low levels of proteins of interest (Lindbo, 2007a). An agro-infiltration delivered

TMV based vector has also been used to produce many antigens, among others, H5N1 influenza virus haemagglutinin (Hahn *et al*., 2014; Paul and Ma, 2011; Rybicki, 2010). Several vectors have a host range problem, with amplified expression being limited mainly to *N. benthamiana*. Tobacco among the leafy plants has been widely used as a host because it is easy to transform and regulation of transgene expression is well understood. Being a non-food, non-feed crop, tobacco reduces the risk of contaminating the food/feed chain (Chakauya *et al*., 2006; Lee and Yang, 2006).

1.5 Tobacco mosaic virus (TMV)

1.5.1 TMV genome

TMV is a single stranded positive sense RNA, rod shaped virus. RNA is encapsulated with viral coat proteins. It is approximately 6400 nucleotides long (Grill, 1993; Mims *et al*., 2004), with dimensions of 300nm x 15-18nm. The TMV has only four open reading frames (Goelet *et al*., 1982). The 126 kDa protein and 183 kDa read through proteins are translated from the 5' end of the genomic RNA. The movement protein (MP) (30 kDa) and 17.5 kDa capsid protein are translated from the 3'-coterminal subgenomic mRNAs produced during replication of the viral RNA, **Figure 6** (Hunter *et al*., 1976; Beachy and Zaintlin, 1977).

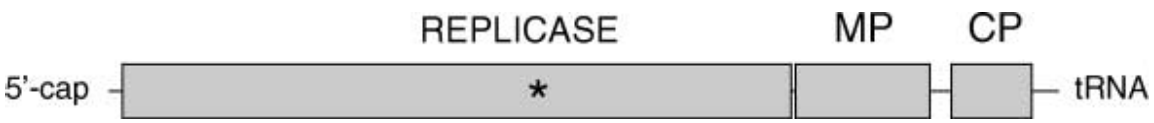


Figure 6: Genetic map of Tobacco mosaic virus (TMV). The approximately 6,400 nucleotide TMV RNA has a 5'-cap and the 3'-tRNA-like structure. The open reading frames are indicated by boxes. The replicase consists of a 126 kDa protein and, by read-through of an amber stop codon (asterisk), the 183 kDa protein. The replicase proteins are translated from the genomic

RNA. The 30 kDa movement protein (MP) and the 17.5 kDa capsid protein (CP) are expressed from separate subgenomic RNAs (not shown). (Scholthof, 2004).

The MP is necessary for the virus to pass through the plasmodesmatal opening between the cells to support cell-to-cell movement. The capsid protein is the single structural unit protecting the RNA in virions and is required for long-distance movement throughout the plant (Yusibov *et al*., 2002). The Coat Protein (CP) of TMV is one of the most accumulated proteins in infected plants. The CP can reach up to 10% of leaf dry weight (Copeman *et al*., 1969; Siegel *et al*., 1978; Fraser, 1987). The structure of the CP is known (Namba *et al*., 1989; Butler *et al*., 1992). Fusion of the viral CP and a foreign protein resulted in one of the first hybrid constructs (Takamatsu *et al*., 1987; Dawson *et al*., 1988).

The TMV virions are stable for decades and the virions can also survive heating. A broad range of crop species and weeds mostly *Solanaceae*, which includes tobacco, tomato, pepper plants, can be infected experimentally (Awram *et al*., 2002; Knapp *et al*., 2001).

1.5.2 TMV as a vector expressing rTMV proteins

There are several different methods to genetically alter plants to produce foreign proteins. Generally, these methods introduce foreign DNA sequences into plant cell DNA, which requires time-consuming manipulations. Naturally occurring plant viruses are capable of altering plant genomes such that the plant produces virally encoded products within a matter of days or weeks (Grill, 1993). A recombinant TMV (rTMV), once introduced into a leaf of a tobacco plant is able to replicate and express the foreign gene in the plant within a short time.

Tobacco serves as a good host for foreign protein expression due to the fact that it is one of the highest biomass producing cash-crop in the world. Tobacco under optimal conditions can produce up to 2000 Kg per acre per year (Turpen *et al*., 1995). However, tobacco plants contain numerous toxic alkaloids and phenolic compounds and this makes purification of heterologously produced recombinant proteins a difficult process (Awram *et al*., 2002).

N. benthamiana is recommended as the best tobacco species to use for expression of foreign proteins because it does not contain high levels of toxic phenolics and alkaloids that are associated with other tobacco species. TMV's utilization as an expression vector has already been well established and has been used to produce many different kinds of proteins including allergens, antibodies and vaccine candidates (Gleba *et al*., 2005; Grill, 1993; Grill *et al*., 2005; Lindbo, 2007a; Smith *et al*., 2006).

This vector has in turn allowed for the development of economical methods for the expression of foreign products. The amount of foreign product produced depends on different factors. These include the protein being expressed, choice of the expression system and the host plant used. Depending on the TMV based expression vectors used, the infection may result in systemic spread causing high expression of recombinant proteins. Active replication of recombinant virus is characterized by many symptoms; these include leaf curling, mottling, chlorosis, necrosis and growth stunting. The symptoms are not lethal to the plant (Culver *et al*., 2002).

Fusion of foreign amino acid sequences to the TMV CP has been shown to induce severe chlorosis and necrosis in *N. benthamiana* plants (Turpen *et al*., 1993; Bendahmane *et al*., 1999),

even though this is not always the case. The symptoms of infection are not necessarily an indication of production of rTMV, but are instead due to TMV assembly. The CP enables systemic infection; deletion of the TMV CP limits the viral ability to spread systemically throughout the plant. Without the CP the virus is still able to move locally from cell to cell but loses for systemic infection ability (Santi *et al*., 2006).

1.5.3 TMV transient expression vectors

Genetically modified plant viruses are powerful tools with a variety of applications. Accumulation of specific recombinant proteins in whole plants provides a cost-effective source of therapeutic reagents for human and animal diseases and this application has immediate potential in health care application (Yusibov *et al*., 2002). TMV and its tobamovirus relatives have advantages for consideration as transient expression vectors.

One advantage is their long history of experimentation. TMV was the first virus to be recognized and purified, and the first virus for which virion structure and the coat protein sequence were determined (Yusibov *et al*., 2006). TMV represents a reduced biohazard as a recombinant virus in the field, it is only spread mechanically. TMV virions can easily be purified in large quantities by relatively simple procedures and equipment (Yusibov *et al*., 2002).

The CP is immunogenic in mammals due to its ability to activate T cells (Loor, 1967). This has made this virus a potential vector for expressing foreign epitopes fused to the CP for development and application of novel vaccines (Jiang *et al*., 2006; Dawson and Lehto, 1990; Lacomme *et al*., 1998). The CP subunits in the TMV particles are assembled in a highly ordered

fashion, with the N- and C-termini both located on the surface of the TMV particle (Namba *et al*., 1989). TMV CP fusion would result in a TMV particle displaying the epitope (red) on the outside of the TMV virion (**Figure 7**). The epitope would be covalently linked to the TMV CP (brown), but otherwise the TMV virion would assemble as normal with the TMV RNA (green) enclosed in the virion.

Thus foreign protein fused to the termini of CP could be displayed outside the virus particle if the fused CP subunits are still able to encapsidate the recombinant viral genome in proper order (Turpen *et al*., 1995; Sugiyama *et al*., 1995). However, the limited capacity of typical TMV-based vectors like pJL TRBO, for expressing longer foreign peptides (more than 20 residues), has hobbled its applications to the purpose that required longer peptides (Beachy *et al*., 1996; Jiang *et al*., 2006; Wu *et al*., 2003).

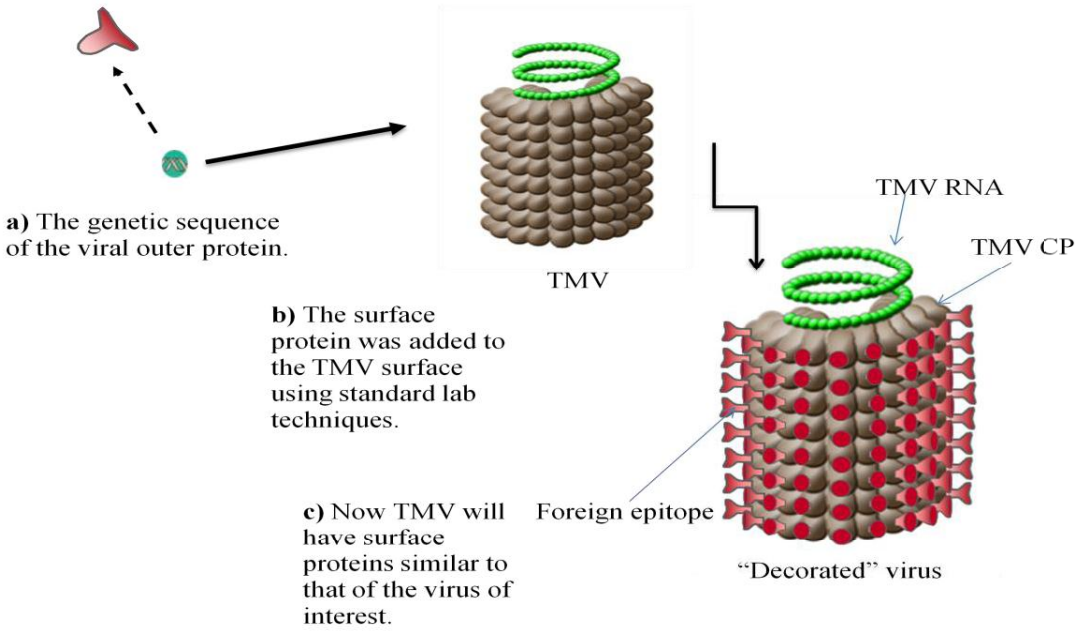


Figure 7: Diagram of "decorated" TMV virion expressing a foreign epitope (red).

1.6 Statement of the problem

LSD is a viral disease. LSD clinical disease has been described in species such as Asian water buffalo, springbok and oryx (antelope species) but it mainly affects cattle (VIPR, 2012). The rate at which outbreaks take place in Botswana and in Africa as a whole is a major cause of concern (OIE, 2012). The disease is viral in nature and currently the only means of controlling it is by vaccination and separating healthy animals from the affected ones.

LSD is endemic in Botswana. Its socio-economic effects do not only affect the beef industry (beef export for the Botswana government), but also affects individual farmers who are dependent on animal husbandry for their livelihoods. Cattle population in Botswana fluctuates between 2.5 and 3 million (BEDIA, 2012). Individual farmers have to buy the current vaccine for their cattle. The cost of production of the currently used vaccine is high. Several millions of Botswana Pula are mainly attributed to manufacturing, research and development (BEDIA, 2011).

In Botswana, currently a chemically attenuated live vaccine is the primary option in use. Although live attenuated virus vaccines are generally considered safe, reported cases of reversion to virulence have highlighted the inherent safety problems of live attenuated vaccines (Cizman *et al*., 1989; Christensen *et al*., 1992; Minor, 1993; Xie *et al*., 1998; Huang *et al*., 1999; Nielsen *et al*., 2001). Mutations can occur when the vaccine virus replicates within the animal and cause a virulent strain.

There is no guarantee under the right conditions the live attenuated live vaccine might not be able to revert to virulence. The need for cold chain storage for these vaccines makes them costly, especially for farmers in rural areas. The use of eggs or tissue culture for the conventional methods of vaccine production also causes a need for alternative methods, since the vaccine may harbour contaminants like endotoxins or pathogens (Fischer and Emans, 2000; Giddings *et al*., 2000). All these reasons justify the need for development of a locally produced, cost effective vaccine.

1.7 Objectives of the study

1.7.1 Aim

The main aim was to clone two LSDV epitopes into the TMV binary vector (pJL TRBO) and to investigate the ability of the rTMV to retain the cloned epitopes and cause an infection in *N. benthamiana* plants using *Agrobacterium tumefaciens*. Basically, the aim was to develop a method for production of TMV based subunit vaccines against LSDV using a plant based production system.

1.7.2 Specific objectives

- ✓ To identify two immunodominant regions (LSDV A and LSDV B) of the LSDV genome.
- ✓ To clone the two epitopes into the pJL TRBO plasmid.
- ✓ To transform *A. tumefaciens* with a TMV based binary vector (pJL TRBO).
- ✓ To agroinfect *N. benthamiana* plants with the transformed *A. tumefaciens*.
- ✓ To purify rTMV from infected *N. benthamiana* leaf material.

- ✓ To determine immunogenicity of rTMV by ELISA and western blotting.

1.8 Significance of the study

The study pioneers the production of safe, efficient and cost-effective vaccines. The plant-based vaccine production method could be adapted for any disease. This study could potentially equip Botswana with a substantial vaccine production industry and minimise the country's reliance on imported vaccines.

Currently the Onderstepoort lumpy skin disease vaccine for cattle is being used in Botswana. This vaccine is imported from South Africa, it is to be refrigerated before use, calves from vaccinated cows should be vaccinated at six months of age and an annual booster vaccination should be given. The yearly outbreaks of this disease in Botswana are of major concern, currently the effectiveness and efficacy of the vaccine being used is still questionable. Not all farmers afford to purchase this vaccine and keep it under the right conditions before use.

This would have major implications in Africa, even world-wide especially in countries that have a huge beef and tourism industry. In 2003/04, agriculture contributed 2.3% of Botswana's gross domestic product (GDP) out of which about 70% to 80% was attributed to the cattle industry (BEDIA, 2012).

1.9 Rationale

Vaccination is useful for eradication of infectious diseases in animals and human beings. The ultimate goal of vaccination is to protect vaccinated animals against re-infection by the same pathogen and provide sterile immunity. Current strategies in vaccine production use intact or inactivated pathogen strains to induce immunity. Subunit vaccines which are commercially produced in yeast or genetically engineered mammalian cell cultures are also used (Andersson, 2000; Turpen, 1999).

Subunit vaccines are composed of one or more subunits of an antigenic protein from a disease causing organism. They are usually produced in bioreactors or fermenters and also tend to require extensive purification prior to use (Korban *et al*., 2002). Some vaccines, especially parenteral vaccines (vaccines administered by injection), contain preservatives such as formaldehyde, thiomersal (a mercury based compound) and aluminium sulphate. These commercially produced vaccines tend to be ineffective and are costly (Hunter and Wallace, 2001).

The beef industry contributes significantly to the economy. Livestock plays a critical role in terms of income, savings, food security and employment. In Botswana, outbreaks of diseases such as LSD, rabies and foot and mouth disease (FMD) are of great concern since these are endemic in southern Africa. Development and improvement of vaccines is a suitable way to combat infectious endemic diseases in both wild and domestic animals.

Plants offer advantages in terms of production scale, economy, safety, ease of storage and distribution (Canizares *et al*., 2005; Gidding *et al*., 2000; Neimer *et al*., 2014). Use of plants with comparison to the traditional methods eliminates the need for fermentation facilities and overcomes the possible risks of contamination of subunit vaccines by mammalian pathogens (Santi *et al*., 2006). Pathogens that infect plants do not infect animals whereas mammalian pathogens can infect the animal population (Gidding *et al*., 2000).

The production of subunit vaccines has been validated using different plant heterologous expression approaches (Grill, 1993; Yusibov *et al*., 1999; Babiuk, 2002; Kang *et al*., 2004; Jiang *et al*., 2006). Plants can express a large variety of proteins and perform the post translational modifications required for proper biological function (Hofbauer and Stoger, 2013; Mason *et al*., 2004; Sabalza *et al*., 2014). Hence they offer great potential as production systems for recombinant proteins (Lindbo, 2007a). Recombinant protein expression can reach very high levels (range) of expression in a relatively short time, ranging from 3-14 days post infection, depending on the system used (Canizares *et al*., 2005; Turpen, 1999). The success of viral vector infection depends on the synthesized construct being stable within the plant (Chakauya *et al*., 2006).

Several expression vectors have been developed using different types of plant viruses: the most commonly used plant virus is the tobacco mosaic virus (Mason *et al*., 2004). Plant viral vectors offer several advantages. TMV-based vectors express the highest amounts of foreign proteins in plants (Wagner *et al*., 2004). The TMV vector produced between 600 and 1200 micrograms of recombinant proteins per gram of infiltrated tissue by 6 days post infiltration (DPI) (Lindbo,

2007a). TMV vectors can be used to produce many different kinds of proteins in plants including allergens, antibodies, or antibody fragments and vaccine candidates (Lindbo, 2007a). Plant virus based expression vectors can be used for production of vaccines for infectious diseases such as LSD as they allow for rapid, effective expression of proteins in the whole plant (Lindbo, 2007a).

In this study TMV was used as an expression vector for the immune dominant region of the LSDV genome in *N. benthamiana* plants geared towards the production of a LSD vaccine.

Chapter 2

2.0 MATERIALS AND METHODS

2.1 Experimental design

The experimental work was done in three different stages. Stage one involved, identification and cloning of the LSDV epitopes into pJL TRBO, a TMV based vector (from the Grill Vaccine lab, Ptitzer College). Cloning was done using a series of PCR steps to insert the epitopes into the TMV coat protein (CP). Initially the wild-type CP (wtCP) was amplified from pJL 36 plasmid followed by restriction digestions and ligations to insert the recombinant TMV CP into pJL TRBO. The plasmid carrying the LSDV epitope and the coat protein was transformed into *E. coli* (DH5α), to bulk up the constructs.

For the second stage, the Agro-infiltration of *N. benthamiana* with the recombinant pJL TRBO (pJL TRBO containing LSD epitopes) was done as well as extraction of the rTMV from plants. *A. tumefaciens* was transformed with rpJL TRBO. Agro-induction was done, and Agro infiltration was performed on six weeks old *N. benthamiana* plants. The success of the infiltration was tracked by monitoring disease symptoms, after viral expression the virus particles were extracted from the plant leaf material.

The third stage involved testing of the plant extracts to verify the expression of the LSDV epitopes. A reverse transcription PCR (rtPCR) was carried out using rTMV extract from infected *N. benthamiana* leaves. This was to show that the LSDV epitopes were still associated with the TMV coat protein in viral RNA. The extracts were then subjected to SDS PAGE analysis,

western blotting, and ELISAs to confirm the presence of TMV and that the rTMV still had the LSDV antigens capable of eliciting an immune response in animals. **Figure 8** shows a simplified flow diagram of the sequence of events followed during the study.

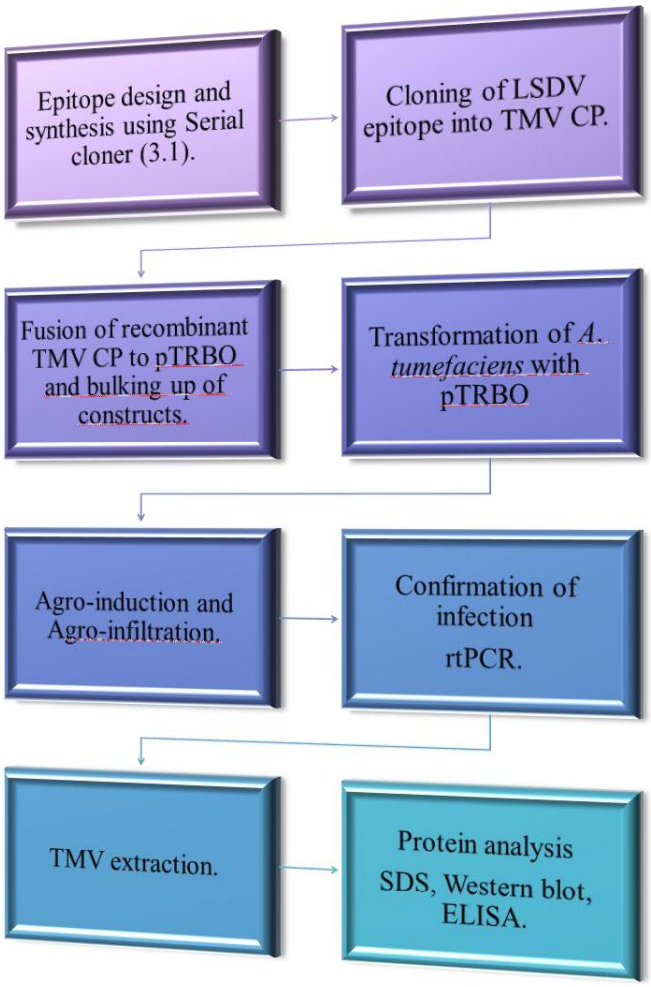


Figure 8: Flow diagram showing the sequence of events in the study.

2.2 Stage I

2.2.1 Epitope identification

The major LSDV antigenic sites are located on the P32 structural protein. The protein is found in all *Capripoxviruses*, and contains the major immunogenic determinants. Two particular amino-acid sequences; 92-118 LSDV A (27 amino acids (a.a) and 156-175 LSDV B (20 a.a) (Tian *et al*., 2010) were identified (see **Table 3**). The consensus sequences for LSDV A and LSDV B are the epitope sequences used in the study. The LSDV A was reduced to 20 amino acids since the plasmid used can only accommodate about 20 amino acids while the LSDV B was used as it is. The LSDV DNA analysis was carried out online using a programme called Serial Cloner (version 3.1). Primer synthesis was done at Inqaba Biotechnological Industries in Pretoria, South Africa.

Table 3: Sequences of the synthesized LSDV epitopes.

LSDV A				
Sequence	**Amino acid sequence**	**Site**	**Length (a.a)**	**Reference**
Original LSDV A	EAKSSIAKHFSLWKSYADADIKNSENK	92-118	27 a.a	(Tian *et al*., 2010)
Consensus LSDV A	SSIAKHFSLWKSYADADIKN	95-114	20 a.a	(Tian *et al*., 2010)
Consensus Sequence	**AGC AGC ATT GCG AAA CAT TTT AGC CTG TGG AAA AGC TAT GCG GAT GCG GAT AAA AAC**			
LSDV B				
Sequence	**Amino acid sequence**	**Site**	**Length (a.a)**	**Reference**
Original LSDV B	FHNSNSRILFNQENNNFMYS	156-175	20.a.a	(Tian *et al*., 2010)
Consensus LSDV B	FHNSNSRILFNQENNNFMYS	156-175	20 a.a	(Tian *et al*., 2010)
Consensus Sequence	**TTT CAT AAC AGC AAC AGC CGC ATT CTG TTT AAC CAG GAA AAC AAC AAC TTT ATG TAT AGC**			

2.2.2 Cloning of LSDV epitopes into the Coat Protein by PCR

Primers specific to the sequences generated were designed and the LSDV epitopes were inserted into the TMV CP via three rounds of PCR. The primers used in this study were designed with the method of inserting the epitopes of interest in the CP (see **Table 4**). The method used to achieve this was adopted from Wu *et al*., (2003). This method used three rounds of PCR to enable the removal of wtTMV CP from the pJL 36 plasmid, then inserting the epitopes into the cloning site within the TMV CP.

2.2.2.1 Round 1 PCR

The first round of PCR was done to amplify the coat protein from the pJL 36 plasmid and to that end primers were designed to amplify the entire 480bp TMV CP. Primers specific to the TMV CP were used to amplify the TMV CP from pJL 36 and introduce the restriction sites *NotI* and *AvrII*. The PCR 1 product was run on an agarose gel and the 480bp amplicon was excised, gel purified and used as the template for the round 2 PCR.

2.2.2.2 Round 2 PCR

In the second round of PCR, the TMV CP forward primer was used. The reverse primer constituted a sequence complementary to the TMV CP just downstream of the insertion site, with the LSDV epitopes extending from the end. This round binds the CP downstream of the insertion sites. The final product constitutes the TMV CP with insert attached but missing the CP sequence

upstream of the insertion sites. The round 2 PCR product was run on an agarose gel and the approximately 500bp amplicon was excised, gel purified and used as the template for the PCR 3.

2.2.2.3 Round 3 PCR

In the third round of PCR the reverse primer consisted of a sequence complementary to the end of the LSDV epitopes and this time the remainder of the TMV CP (**Table 4**) (sequence after the insertion site) was hanging from the end. The TMV CP forward primer was used as the forward primer. The amplicon for PCR 3 is about 600bp in size, it was excised and purified from the agarose gel.

The reaction mix for all the PCRs (**Table 5**), first round PCR and the second round PCR used the same reaction mix (**Table 5**) and same parameters. Due to the length and GC content of the reverse primers used in this round, the original PCR programme was no longer effective. Gradient PCR was done in order to find the optimal annealing temperature for the Round 3 primers, which was finally determined to be 70.4°C (**Table 6**). All other parameters in the PCR programme remained unchanged.

Table 4: Properties and sequences of primers used to clone the LSDV epitopes into the TMV CP.

Round 1	
Forward Primer **TMV Forward** 5'gggcctaggATGCCTTATACAATCAACTCT-3'* Primer length: 30 Bases Melting Temperature (Tm) = 67.37°C	**Reverse Primer** **TMV Reverse** 5'cccgcggccgcCTAAGTAGCCCGGAGTTGT-3'** Primer length: 30 Bases Melting Temperature (Tm) = 76.93°C
Round 2	
LSDV A	**LSDV B**
Forward Primer **TMV Forward** 5'gggcctaggATGCCTTATACAATCAACTCT-3' Primer length: 30 Bases Melting Temperature (Tm) = 67.37°C	**Forward Primer** **TMV Forward** 5'gggcctaggATGCCTTATACAATCAACTCT-3' Primer length: 30 Bases Melting Temperature (Tm) = 67.37°C
Reverse Primer **LSDV A Reverse 1** 5'GTTTTTAATATCCGCATCCGCATAGCTTTTCCACAGGCTAAAATGTTTCGCAATGCTGCTggtccagacaagtccact-3' Primer length:78 Bases Melting Temperature (Tm) = 76.89°C	**Reverse Primer** **LSDV B Reverse 1** 5'GCTATACATAAAGTTGTTGTTTTCCTGGTTAAACAGAATGCGGCTGTTGCTGTTATGAAAggtccagacaagtccact-3' Primer length: 78 Bases Melting Temperature (Tm) = 75.31°C
Round 3	
LSDV A	**LSDV B**
Forward Primer **TMV Forward** 5'gggcctaggATGCCTTATACAATCAACTCT-3 Primer length: 30 Bases Melting Temperature (Tm) = 67.37°C	**Forward Primer** **TMV Forward** 5'gggcctaggATGCCTTATACAATCAACTCT-3' Primer length: 30 Bases Melting Temperature (Tm) = 67.37°C
Reverse Primer **LSDV A Reverse 2** 5'gggggcggccgcctaagtagccggagttgtGTTTTTAATATCCGCATC-3' Primer length: 48 Bases Melting Temperature (Tm) = 75.55°C	**Reverse Primer** **LSDVB Reverse 2** 5'gggggcggccgcctaagtagccggagttgtGCTATACATAAAGTTGTT-3' Primer length: 48 Bases Melting Temperature (Tm) = 76.69°C

* Lower case letters indicate the *AvrII* site.
** Lower case is *NotI* site.

Table 5: PCR reaction mix.

Reagent	Volume (50µL reaction)
Pfu DNA polymerase (Fermentas)	2 units
dNTP Mix (2mM each) (Fermentas)	5µL
Forward Primer (Inqaba)	1 µM
Reverse Primer(Inqaba)	1 µM
Template DNA*	1 µL
10X Pfu buffer with $MgSO_4$ (Fermentas)	5 µL
Water	35µL
Total	**50 µL**

* For colony PCR the template DNA is replaced with a bit of the colony sample as the template.

Table 6: The temperature regimes used in the PCR.

Step	Cycle	Temperature	Time
1	Denaturation	94°C	3 min
2	Denaturation	94°C	15 sec
3	Annealing*	56°C	30 sec
4	Elongation	72°C	45 min
5	Number of Cycles	Repeat 2-4 30 times	
6	Elongation	72°C	5min
7	Hold	Hold at 4°C	

*Annealing temperature for PCR 3 was changed to 70.4ºC

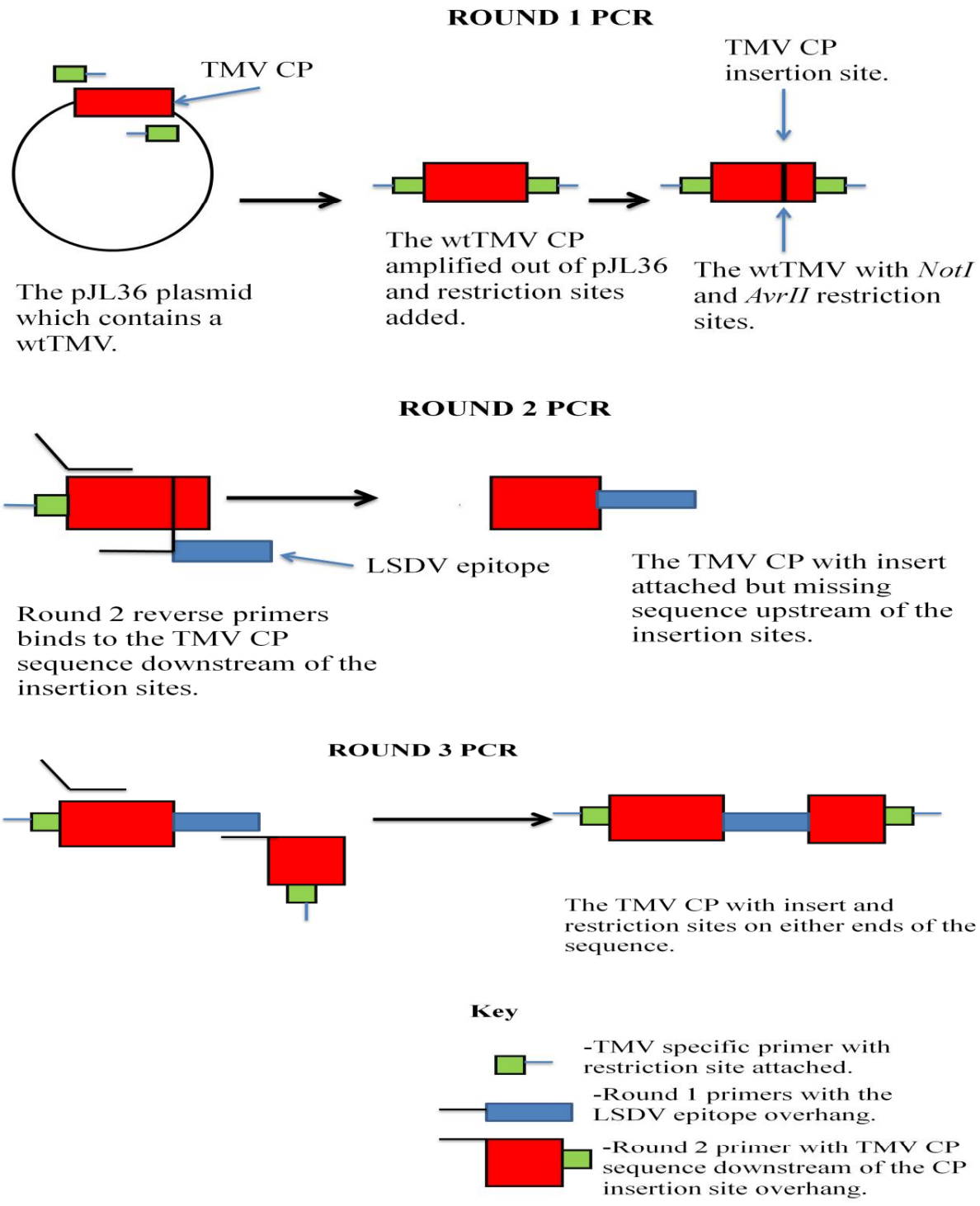


Figure 9: A flow diagram of the cloning process.

2.2.3 Gel electrophoresis

Successful amplification of all PCRs, as shown in **Figure 9**, was confirmed by agarose gel electrophoresis. DNA samples, along with appropriate molecular weight markers, 5μL of ThermoScientific O' Gene Ruler 100bp DNA ladder ready to use (Thermo Scientific) ; 1kb DNA ladder (Promega) or Quick-Load 1kb Extent DNA ladder (Biolabs), were loaded into the wells and ran at 80 volts/cm for approximately 60 minutes in TAE buffer. Bands were visualised using a UV light box and the Gene Snap (Gene Genius Bio Imaging System).

2.2.4 Gel purification

After each round of PCR required for the cloning of the LSDV epitopes into the TMV CP, and after each restriction digest, the DNA was subjected to gel purification. The amplicons and digested materials were run on a 1% (w/v) agarose gel. Long wavelength UV light was used to visualise the bands on the gel and the bands were excised using a sterile razor blade and transferred to a sterile 2mL microcentrifuge tube. The TMV CP amplicon was identified as a 480bp band by comparison with a 100bp DNA ladder (Thermo Scientific). The amplified DNA was purified from the gel using the Zyppy Zymoclean™ Gel DNA Recovery kit (Zymo, California, USA), according to the manufacturer's instructions. DNA was eluted in 10μL sterile distilled water and quantified using a nanodrop. All DNA gel purifications in this study were performed in this manner.

2.2.5 Plasmid bulking up

2.2.5.1 Preparation of *E. coli* competent cells (DH5α)

The competent cells were made using a method modified from the $CaCl_2$ method, (Dagert and Ehrlich, 1979; Sambrook and Russell, 2000). A colony from an overnight culture was inoculated into 10mL of 2xYT broth and incubated overnight on a shaker at 37°C. 5mL of the overnight culture was then inoculated into 100mL of fresh, pre-warmed 2x YT broth and incubated at 37°C with shaking till the culture reached an optical density of 1 (OD_{600}). The cells were then pelleted by centrifugation at 5000rpm for 5 minutes under refrigerated conditions (4°C).

The supernatant was discarded and the cell pellets were re-suspended in 30mL ice cold 100mM $MgCl_2$ after which the suspension was allowed to sit on ice for 10 minutes. The cells were re-pelleted under the same conditions as before and re-suspended in the same volume of ice cold 100mM $CaCl_2$. The suspension was then incubated on ice for 30 minutes after which the cells were pelleted out again under the same conditions and re-suspended in 10mL ice cold 100mM $CaCl_2$ plus 20% glycerol. The 200µL aliquots were then placed into sterile microcentrifuge tubes and stored at -80ºC. The level of competence was determined by transforming the cells with the pUC19 plasmid using the transformation method described above, with kanamycin (50mg/L) being replaced with ampicillin (100mg/L).

2.2.5.2 Preparation of *A. tumefaciens* competent cells (GV3101)

A 10mL overnight culture of *A. tumefaciens* (strain GV3101) in 2x YT broth containing rifampicin and gentamicin was prepared from a single colony. 4mL of the overnight (O/N)

culture was used to inoculate 100mL of 2x YT containing rifampicin (20µg/mL) and gentamicin (50µg/mL) and then incubated with shaking at 30°C. When the culture reached an OD_{600} of between 0.5 and 1.0 it was removed and placed on ice.

The cells were then pelleted by centrifugation at 5000rpm for 5 minutes under refrigerated conditions (4°C) and the supernatant discarded. The cell pellet was re-suspended in 2mL of ice cold 20mM $CaCl_2$. Then 0.1mL aliquots of the suspension were dispensed into pre-chilled microcentrifuge tubes and stored at -80°C. To determine the level of competence the cells were transformed with the pUC19 plasmid using the transformation method described in **Section 2.3.2**, with kanamycin being replaced with ampicillin as the pUC19 plasmid carries an ampicillin resistance gene instead of a kanamycin resistance gene.

2.2.6 Preparation of recombinant plasmids

2.2.6.1 Restriction digest

To clone the three TMV CP constructs, restriction enzyme digestion was carried out on the purified pJL TRBO plasmid and the purified recombinant TMV CP amplicons carrying the LSDV A and LSDV B epitopes. pJL TRBO plasmid was digested with *AvrII* and *NotI* simultaneously according to the manufacturer's instructions. Samples were then mixed with an appropriate volume of 6X gel loading buffer and analysed by 1.2% (w/v) agarose gel electrophoresis. The linearized plasmid was excised from the gel and purified using the gel purification method, as described previously (**Section 2.2.4**).

2.2.6.2 Ligation

For this study three different TMV constructs were generated: TMV containing either of the LSDV epitopes (named LSDV-A and LSDV- B), and wild type TMV were made by ligating the appropriate epitope TMV CP fusion to the linearized pJL TRBO. T4 ligase (Fermentas) and ligation buffer were also added to the mixture. The ligation mix was then incubated at room temperature overnight. The ligation mix was used to transform DH5α competent cells and colony PCR was used to select for transformants carrying the correct plasmid. The selected transformants were then used to inoculate LB containing antibiotics and plasmid extractions followed. The final plasmids generated are listed in **Table 7**.

Table 7: Names assigned to the plasmids generated and their composition.

Plasmid Name	Composition
pLSDV-A	pJL TRBO containing the LSDV-A epitope
pLSDV-B	pJL TRBO containing the LSDV-B epitope
pTMV-CP	pJL TRBO containing the wtTMV CP from pJL 36

The constructs were introduced into competent *E. coli* cells by transformation.

2.2.7 Transformation into DH5α *E. coli* cells

Transformation of competent *E. coli*: DH5α cells was carried out for DNA amplification of the various plasmids used in the study. To transform the plasmids into competent *E. coli* cells, the standard heat-shock method was used (Sambrook and Russell, 2000). Frozen competent cells

were removed from the -80°C freezer and thawed on ice, then 100ng of plasmid DNA was added and the mixture was incubated on ice for 30 minutes. A heat shocked was performed, for the competent cells to take up the DNA, by placing the reaction mixture in a water bath at 42°C for 45 seconds and immediately back on ice for 5 minutes. Approximately 1mL of warmed 2x YT broth (37°C) was subsequently added and the resulting culture was incubated at 37°C for 3 hours with shaking (200 rpm) to allow for expression.

The cells were then spread on LB agar plates with kanamycin (50μg/L). Plates were then incubated overnight at 37°C and the colonies obtained were inoculated in LB broth containing kanamycin for plasmid (Plasmid containing the construct) isolation from the *E. coli* culture, using the Zyppy plasmid miniprep kit. The isolated plasmids were visualised by agarose gel electrophoresis. Furthermore, as another measure of verification, colony PCR of the isolated plasmid was done, using the Round 3 parameters as outlined in **section 2.3.2.1**.

2.2.8 Plasmid isolation

To isolate pJL TRBO, DH5α: pJL TRBO was cultured in Luria-Bertani (LB) broth with kanamycin (50μg/mL) in order to isolate pJL TRBO from the bacterial cells. Plasmid isolation was done using the Zyppy Plasmid MiniPrep kit (Zymo Research). Isolation of the plasmid of interest was confirmed by agarose gel (1.5%) electrophoresis against a 1kb DNA ladder (Promega) and a Quick-Load 1kb Extent DNA ladder (Biolabs). The concentration of the isolate was determined using a NanoDrop spectrometer (A_{260}).

2.3 Stage II

2.3.1 Growth conditions for the plants

The *N. benthamiana* seeds were planted in individual Styrofoam cups containing organic compost. The plants were grown in a greenhouse with an 18 hour photoperiod at 25-27°C. After 2-3 weeks, seedlings were transplanted into individual cups (1 seedling per cup). The plants were housed in the University of Botswana greenhouse during germination and growth, and were watered daily as required. The plants were fertilized once a week using a commercial hydroponic fertilizer (Nutrifeed starke Ayres- Garden care).

2.3.2 Transformation into *A. tumefaciens* (GV3101).

The *A. tumefaciens* GV3101 was transformed with plasmids purified from *E. coli* using the freeze thaw method (Jyothishwaran *et al*., 2007). 100µL of *A. tumefaciens* cells were removed from the -80°C freezer and thawed on ice. A bottle of LB media was removed from the fridge and allowed to warm to room temperature. Plasmid DNA (100ng) was added to the thawed cells and the micro-centrifuge tubes were tapped gently several times to mix. The tubes were then placed in liquid nitrogen for 5 minutes. Immediately the tubes were transferred to the 37ºC water bath for 5 minutes. About 1mL of room temperature LB broth was added to each tube. The tubes were capped and inverted 6 times to mix.

The tubes were incubated at room temperature for 4 hours. Then the cells were centrifuged at 7000xg for 7 minutes to pellet the cells. Then 0.9mL of the culture was removed and the cell

pellets were resuspended in the remaining media using a P1000 pipette. The entire suspensions were plated on LB containing 50µg/mL kanamycin, 25µg/mL gentamicin and 10ug/mL rifampicin using glass rods. The plates were incubated overnight at 25ºC. *A. tumefaciens* was transformed with pTMV, pLSDV-A, and pLSDV-B for use in agro-infection. Colony PCR was done to check that the transformation was successful and that the insert was still present within the vector as indicated below.

2.3.2.1 Colony screening

Confirmation of successful transformation was carried out by colony PCR using a method adapted from Mirhendi *et al*., (2007). A colony was picked using a sterile micropipette tip (0.5µL) and mixed into the same PCR mix used in normal PCR reactions (**Table 5**). The same thermocycler conditions (see **Table 6**), and PCR round 1 primers were used (see **Table 5**). This method was applied to each recombinant TMV CP transformation in DH5α and *A. tumefaciens* GV3101.

2.3.3 Agro-induction

Individual colonies of *A. tumefaciens* transformed with Ptmv, pLSDV-A and LSDV-B plasmid were inoculated into LB broth (containing rifampicin, gentamicin and kanamycin) and grown to an OD_{600} of 1.0 in L-MES media at 25ºC overnight. The cells were collected by centrifugation at 10Krpm for 5 minutes and re-suspended in agro-induction media (Refer to the appendix for

media/ reagent composition), and then the cells were allowed to sit at room temperature overnight.

2.3.4 Agro-infection

Six week old *N. benthamiana* plants were infected by infiltrating the agro-induced cells into the abaxial surface of 3 leaves using a 1-3mL needleless syringe. Pressure was applied from the top side of the leaf with a gloved finger, and the syringe was depressed slowly and evenly to inject the liquid culture into the leaf.

The plants were infected with *A. tumefaciens* carrying the pTMV, pLSDV-A, pLSDV-B plasmids; *A. tumefaciens* with no plasmid and water (**Table 8**). Expression was visible within 2-3 days. Symptoms of TMV infection include, light and dark mottled areas on leaves, infected leaves are often curled and puckered. Virus was extracted within 3 weeks.

Table 8: Composition and function of materials used to infect *N. benthamiana* plants.

Plants infected with	Purpose
pTMV	This plasmid constituted the wtTMV CP amplified out of pJL 36 and inserted into pJL TRBO with no LSDV epitope fused to the TMV CP. The virus produced was also used as wtTMV in the ELISAs and western blots
pLSDV-A	pJL TRBO containing the LSDV-A epitope.
pLSDV-B	pJL TRBO containing the LSDV-B epitope
Water	Served as a negative control

2.3.5 rtPCR

Confirmation of successful infection of the *N. benthamiana* plants was by reverse transcription PCR (rtPCR) of the TMV coat protein. Crude extract from leaves were obtained seven days post-inoculation by grinding the leaves in liquid nitrogen and re-suspending in TE buffer with DNase added. rtPCR reactions were performed using an rtPCR kit (First Strand cDNA Synthesis Kit, Fermentas), as described by the supplier, using TMV CP specific primers. The product of the rtPCR was used as the template for the standard PCR using the conditions already described above.

2.3.6 Virus extraction

All virus assays were carried out on semi-pure extracts from infected *N. benthamiana* plants, 3 weeks post-infection; 20g (recorded as fresh weight-A) of systemically infected leaves were harvested from the plants infected with pLSDV-A, pLSDV-B, pTMV, water and *A.tumefaciens* with no plasmid. The leaves were ground using a pestle and mortar until homogenized. The grinding was done in 4 x weight-A volume of virion extraction buffer, which was added in small amounts until the leaves were well macerated. The homogenate was filtered through two layers of cheesecloth into 50mL polypropylene centrifuge tubes, making sure that as much of the liquid was collected while preventing any of the particles from entering the filtrate. The liquid was heated for 5 minutes at 50°C.

The tubes were centrifuged at 12K rpm for 25 minutes to pellet out the green plant material. The supernatant was transferred into a clean 50mL centrifuge tube and the volume was recorded

(volume-B). The green pellet was re-suspended in about 1mL phosphate buffer and saved for analysis. Then volume-B x 0.12ml of 40% PEG and volume-B x 0.17ml of 5M NaCl were added to the supernatant to precipitate the virus. The supernatant was left over night in a fridge. The following day the supernatant was centrifuged at 15K rpm for 25 minutes at 4°C. After centrifugation a white pellet was visible and the supernatant was poured off. The pellet was re-suspended in 1/10 volume-A of 50mM phosphate buffer (pH 5.7). The virus was stored in the fridge until analysis was completed.

2.4 Stage III

2.4.1 Virus analysis

The extracted virus was subjected to SDS PAGE, western blot, and ELISA analysis to confirm the presence of TMV, to check if the LSDV epitopes are covalently bonded to the TMV virion and to check if the LSDV/TMV display particle was capable of eliciting an immune response in animals.

2.4.1.1 SDS-PAGE

Equal volumes of viral extract of each treatment were combined with SDS-PAGE loading dye. The samples were loaded on a 12% separating gel with a 4% stacking gel (Laemmli, 1970). The PROTEAN® II xi 2-D (BIO-RAD) system was used to prepare and run the gel according to the manufacturer's instructions.

2.4.1.2 Western blotting

One piece of nitrocellulose membrane and 20 sheets of filter paper, the size of the gel, were cut and soaked in transfer buffer for 20 min. After the electrophoresis, the membrane was blotted using a semi-dry electro-blotter (Fisher Scientific). 10 sheets of filter paper were placed on the anode followed by the nitrocellulose membrane, gel and the remaining filter paper. The cathode was then placed on top. A constant voltage of 30V was run through the setup for 45 minutes to effect blotting of the SDS gel onto the membrane.

Upon completion of the transfer, the membrane was washed twice in Tris-buffered saline (TBS) to remove the methanol from the transfer buffer then it was placed in freshly made blocking buffer with 5% milk TBS-T (Tris-buffered saline- Tween/Triton), and shaken for an hour at room temperature. The membrane was then washed twice with TBS-Tween/Triton, then twice with TBS at room temperature, 15 min each. The membrane was then probed with either goat anti-TMV (polyclonal anti TMV, Dr I Becker, University of Cape Town, South Africa) or bovine anti LSDV (polyclonal anti-LSDV, OVI, South Africa), diluted in 3% BSA (in TBS) for 4 hours at room temperature with shaking.

After the four hours had elapsed the membrane was washed as before then probed with the appropriate secondary antibody (anti-Goat/Sheep monoclonal IgG, alkaline phosphatase conjugate, Sigma; or anti-Bovine monoclonal IgG alkaline phosphatase conjugate, Santa-Cruz Scientific) diluted in 3% BSA for 4 hours at room temperature with shaking. The membrane was then washed four times with TBS-Tween/Triton for 15 minutes each at room temperature. To develop the membrane a tablet of NBT/BCIP (Sigma) was diluted in 200mL of sterile water and added to the membrane and then allowed to sit in the dark till sufficiently dark bands appeared.

The reaction was stopped by washing away the NBT/BCIP with sterile distilled water (QIAGEN, 2002).

2.4.1.3 ELISA

A 96-well plate (number of wells used varied depending on the number of samples available) was coated with 50µl of capture antibody in carbonate buffer overnight at 4°C. For LSDV-TMV sandwich ELISAs, anti LSDV antibodies (bovine anti LSDV polyclonal sera obtained from the OVI, South Africa) were used as the capture antibody while for the TMV ELISA, anti-TMV antibody (goat anti TMV polyclonal sera, Dr I Becker, University of Cape Town) was used. The plate was covered with cling wrap to prevent drying.

The wells were then emptied and washed 5 times with phosphate-buffered saline (PBS) and then dried by tapping gently on paper towel to remove all the liquid in the wells. 200µl ELISA blocking buffer was then added and the plate was incubated for 1 hour at 37ºC. To each well 50µl of purified TMV extracted from agro-infected plants in glycerol was added and the plate was incubated for 30 minutes at room temperature.

The sample was then removed and the plate washed 3 times with 200µl PBS and dried as before, after which 50µl of the sandwich antibody (goat anti TMV polyclonal sera, Dr I Becker, University of Cape Town), diluted 1:10000 in blocking buffer, was added to each well. The plate was covered and incubated for 2 hours at room temperature. The antibody was removed and the plate washed 4 times with PBS and dried as before. 50µl secondary antibody; alkaline phosphatase conjugated monoclonal anti goat/ sheep IgG (Sigma); diluted in blocking buffer to

the appropriate concentration was added to each well and the plate covered and incubated for 1 hour at room temperature. The antibody was removed and washed off 4 times with PBS after which 100µl NBT/BCIP solution was added to each well and colour allowed to develop for 30 minutes then the plate was photographed. **Figure 10** shows the flow diagram for the ELISA design.

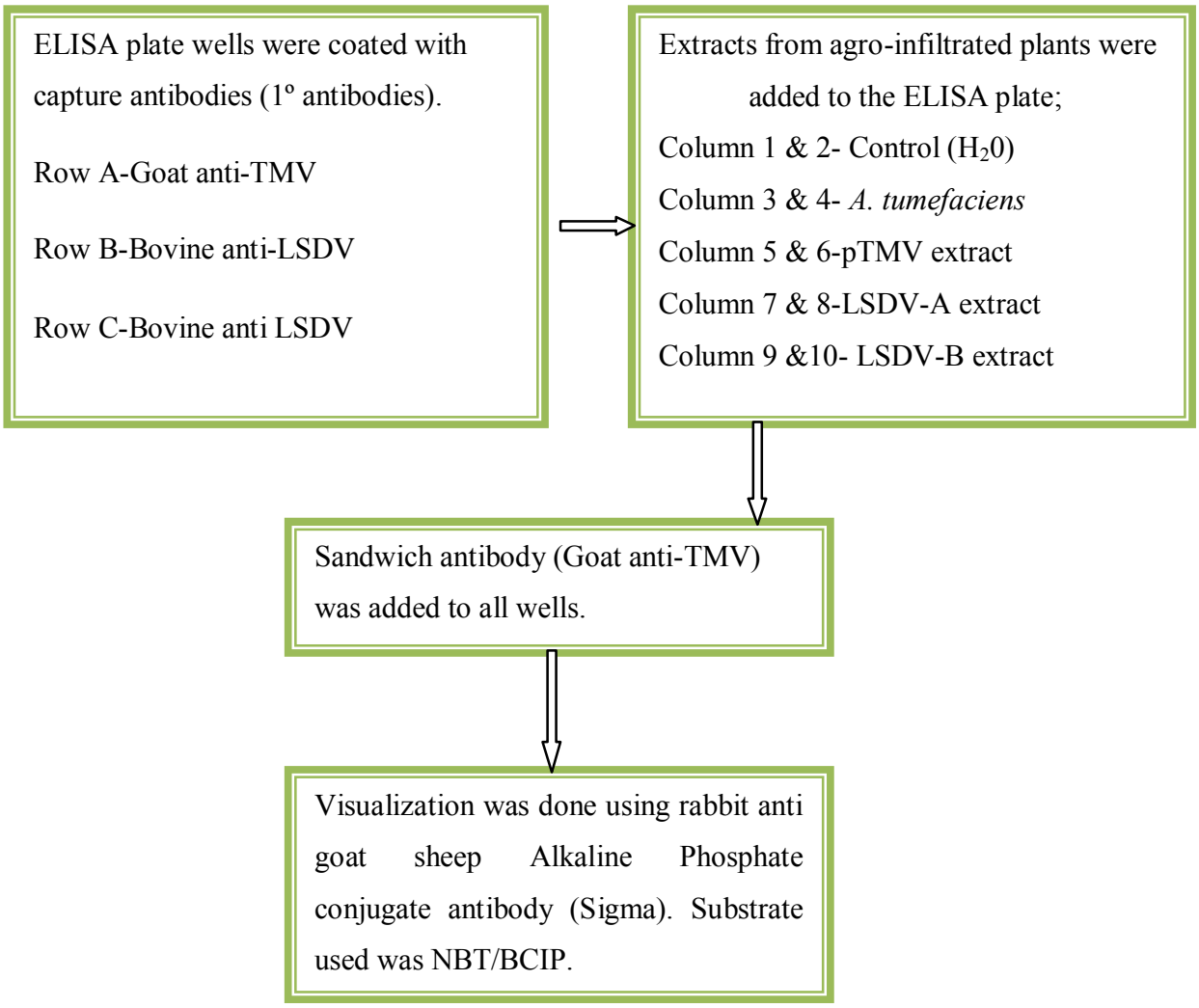


Figure 10: The ELISA design.

Chapter 3

3.0 RESULTS

3.1 Plasmids bulking up

3.1.1 Transformation efficiency of *E. coli* (DH5α)

The competent cells were prepared successfully. This was seen as the newly prepared competent cells were able to take up the different plasmid and grow on nutrient agar containing ampicillin (for pUC19) and rifampicin, kanamycin and gentamycin. The transformation of the plasmids into DH5α was also successful. However, the transformation efficiency of the competent cells seemed to drop when the TMV based plasmids (pJL TRBO and pJL 36) were used to transform the cells.

3.1.2 Transformation efficiency of *A. tumefaciens* (GV3101)

The *A. tumefaciens* competent cells were able to take up the different plasmids and grow on nutrient agar containing with specific antibiotics.

3.1.3 Plasmid isolation

The isolation of pJL 36 and pJL TRBO plasmids from DH5α cells was successful as shown by bands electrophoresing between the 9413bp and 23130bp marker. This corresponds to the expected 11578bp for pJL 36 and 10606bp for pJL TRBO (**Figure 11**). Two bands are visible on the gel photograph which is not uncommon with plasmids. One band represents the super coiled

form of the plasmid while the other is the normal plasmid. The super coiling did not interfere with downstream processes in the study.

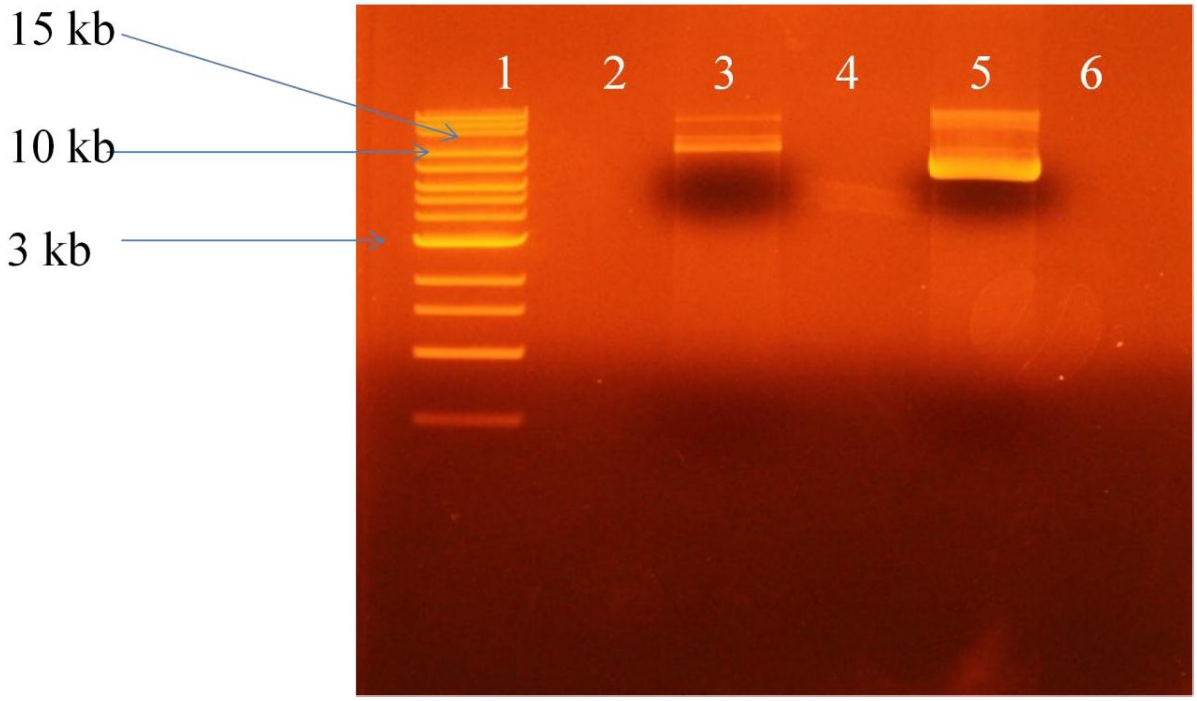


Figure 11: Agarose gel electrophoresis of plasmid extracted from *E. coli*. Lane 1: Quick load 1Kb Extend ladder (New England BioLABS). Lane 2: Blank. Lane 3: pJL36. Lane 4: Blank. Lane 5: pJL TRBO. Lane 6: Blank.

3.2 Cloning epitopes into pJL TRBO

3.2.1 PCR

The three rounds of PCR used to introduce the epitope into the TMV CP were successful. In the first round of PCR the TMV CP was amplified out of pJL36 to give a band 480 bases long (**Figure 12a** Lane 2). The second round resulted in a construct of 540 bases long (**Figure 12a** Lane 3 and 5 for LSD A and LSD B respectively) while the final round resulted in an amplicon 560 base pairs long (**Figure 12 a** Lane 4 and 6 for LSD A and LSD B respectively).

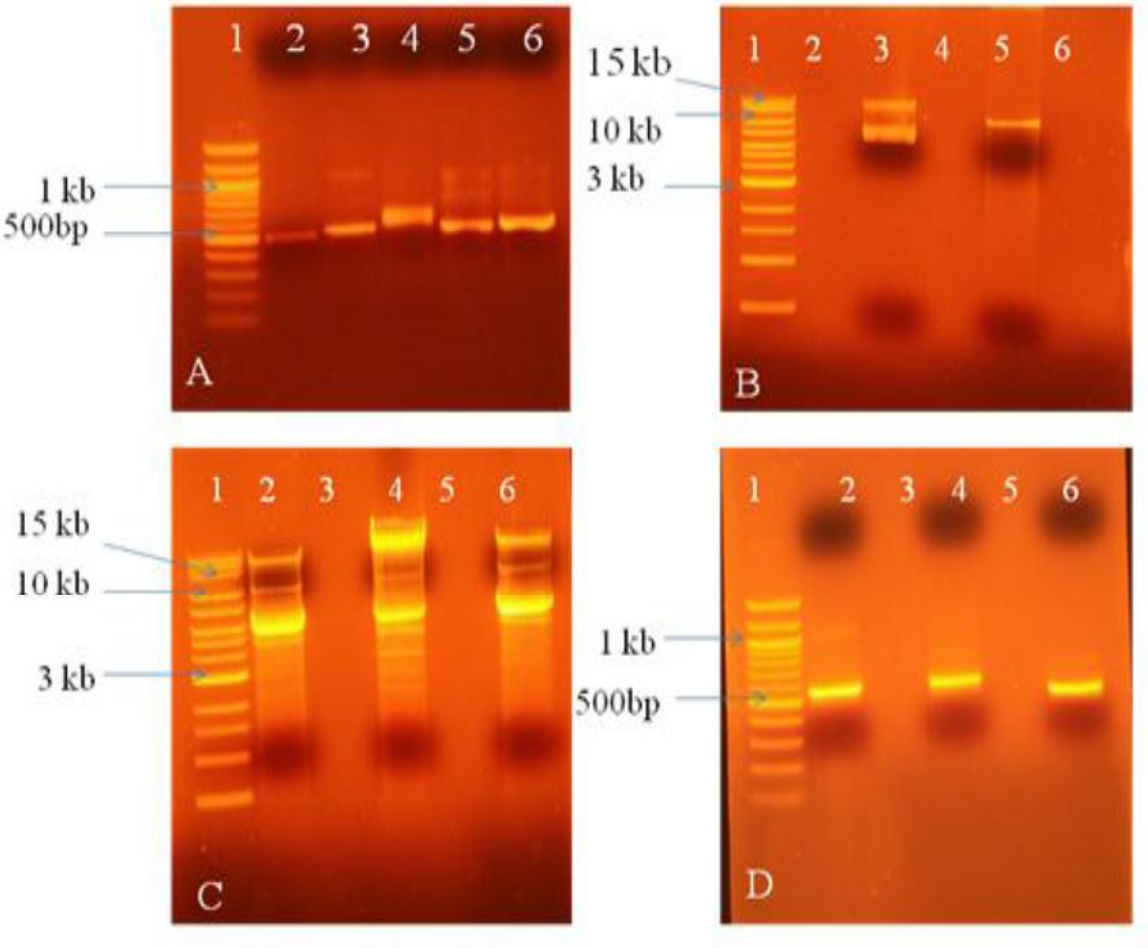


Figure 12: a) Agarose gel electrophoresis of products of three rounds of PCR. Lane 1: Quick load DNA Ladder. Lane 2: TMV CP (PCR 1). Lane 3: 2nd round PCR product of LSD A. Lane 4: 3RD round of PCR product for LSD A. Lane 5: 2nd round PCR product of LSD B. Lane 6: 3rd round PCR product for LSD B. **b)** Restriction enzyme digests of pJL TRBO. Lane 1: Quick load 1Kb Extend Ladder. Lane 2: Blank. Lane 3: pJL TRBO uncut. Lane 4: Blank. Lane 5: pJL TRBO cut using *NotI* and *AvrII*. Lane 6: Blank. **c)** Plasmids isolated from *E. coli* after ligation of pJL TRBO and TMV CP constructs. Lane 1: Quick load 1 Kb Extend Ladder. Lane 2: pLSDV-A. Lane 3: Blank. Lane 4: pLSDV-B. Lane 5: Blank. Lane 6: pTMV. **d)** Colony PCR results for *E. coli* colonies carrying the TMV CP. Lane 1: Quick load DNA Ladder. Lane 2: pLSDV-A. Lane 3: Negative control (water). Lane 4: pLSDV-B. Lane 5: Blank. Lane 6: pTMV.

3.2.2 Restriction digest

In order to insert the recombinant TMV coat protein constructs, pJL TRBO (**Figure 12b**) and the TMV CP constructs (**Figure 12a**), were subjected to restriction endonuclease activity (*NotI* and *AvrII*). Since only a few bases are removed due to the digestion, the TMV CP constructs appear to be the same length on the agarose gel (**Figure 12b**). There is only a slight difference between the linearised pJL TRBO and the circular form of the plasmid (**Figure 12a)**. In lane 3 (**Figure 12a)**, the pJL TRBO has been linearised by digestion with both *NotI* and *AvrII* and is shown to be electrophoresing slightly lower than the circular and super coiled forms of the plasmid.

3.2.3 Ligation

The linearised pJL TRBO was ligated to the different coat protein constructs to form plasmids named pTMV, LSDV-A and LSDV-B, depending on the epitope fused to the TMV coat protein (**Table 5**). The electrophoresis of these plasmids showed bands running between the 9413bp and 15000bp markers as expected from the plasmids that are approximately 11150bp in length (**Figure 12b**). The pJL TRBO (**Figure 12b** lane 2) plasmid was used as a control to compare the newly constructed plasmids and it shows that the plasmids are of the expected size based on rate of electrophoresis.

3.2.4 Screening of the TMV CP transformants in *E. coli.*

A colony PCR was carried out on the colonies that grew on LB agar containing kanamycin. These colonies were carrying the plasmid containing kanamycin resistance. Colony PCR was carried out to further confirm the presence of the plasmid which would also be carrying the TMV CP as well as the epitopes of interest. Gel electrophoresis of the DNA product from the colony PCR showed successful amplification of the DNA falling between the 500bp and the 600bp band. The TMV CP is expected to be about 490bp in length, thus the pTMV was about the expected size (**Figure 12d**: Lane 6); whereas fragments obtained for the LSDV-A and LSDV-B (**Figure 12d** lane 2 and 4 respectively) were slightly bigger than the pTMV as expected. There was no product for the negative control where water was used as the template (**Figure 12d**: lane3).

3.3 Screening of TMV CP transformants of *A. tumefaciens.*

Colonies that grew on LB containing kanamycin, rifampicin and gentamicin were assumed to be carrying the plasmid containing kanamycin resistance since the *A. tumefaciens* (GV3101) already contains resistance to gentamycin and rifampacin. A colony PCR was done to confirm the presence of the plasmid which would also contain the TMV CP and the epitopes of interests. Gel electrophoresis of the amplicons from the colony PCR revealed a successful amplification of the DNA fragment falling between the 500bp and the 600bp. For the negative control there was no amplicon (**Figure 13**: lane 3). The pTMV (**Figure13**: lane 6) was slightly smaller in size as compared to LSD A (**Figure 13**: lane 2) and LSD B (**Figure 13**: lane 4).

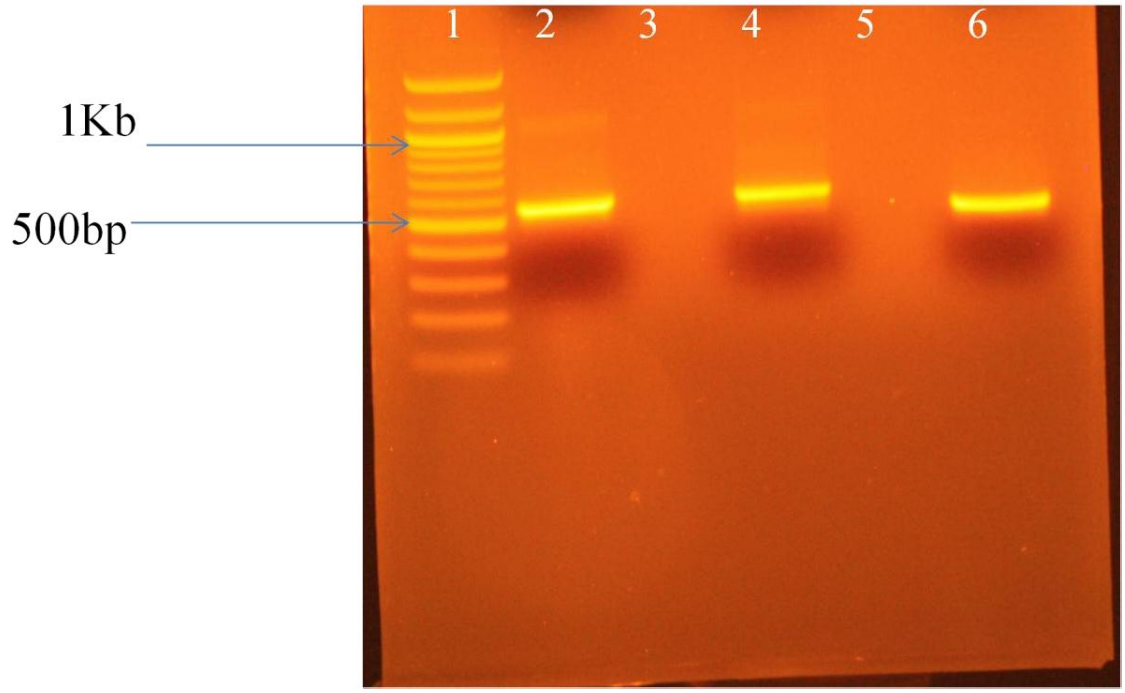


Figure 13: Colony PCR results for *A. tumefaciens* colonies carrying the TMV CP. Lane 1: 100bp Quick load DNA Ladder (New England BioLabs). Lane 2: pLSD A. Lane 3: Negative control (water). Lane 4: pLSD B. Lane 5: Blank. Lane 6: pTMV.

3.4 Confirmation of infection of *N. benthamiana* plants.

3.4.1 Visual confirmation

Two weeks post infiltration typical symptoms of TMV infection were not observed these include curling, mottling and chlorosis of leaves (**Figure 14b**). Leaf necrosis was clearly visibly in all infiltrated leaves **Figure 14c and d**.

Figure 14: Comparison of a healthy and Agro-infected *N. benthamiana* plants. **a**) A healthy *N. benthamiana* plant. **b**) Typical symptoms of TMV infected *N. benthamiana* plant, arrows showing curling and molting leaves. **c**) and **d**) Leaf necrosis indicated by arrows was visible in the agro infiltrated leaves.

3.4.2 rtPCR of Agro-infiltrated plants.

The raw plant material was used to carry out rtPCR using the TMV CP specific primers to amplify the DNA fragments. The negative controls being plants infiltrated with pure water and plants infiltrated with clean *A. tumefaciens* (with no plasmid) are blank as TMV CP was not present in the plant material. The pLSDV A and pLSD B did not have amplicons, the pTMV did show an amplicon between the 500bp and 600bp as expected.

3.5 Virus extraction and analysis

The nature of the extracted virus was determined by subjecting the extracts to SDS analysis, western blotting and ELISA.

3.5.1 SDS PAGE and western blot

The TMV CP has a molecular weight of 17.6 kDa. The pTMV ran between the 15k Da and 20 kDa markers in the SDS PAGE gel. The pLSDV-A and pLSDV-B could not be detected on the SDS PAGE gel. The positive control being the pJL 24 was detected running at about 17.6 kDa. The negative controls (water and *A. tumefaciens* without plasmid) did not show any bands.

The western blots of gels loaded with the same material and same order as the SDS PAGE gels showed that only pTMV was detected with goat anti- TMV antibodies. Refer to **Appendix 1**: **Figure 15** for the SDS PAGE and the western blot. The pLSDV-A and pLSDV-B were not detected as expected using bovine anti-LSDV antibodies. The negative controls did not show any bands in the western blots (data not shown due to poor picture quality).

3.5.2 ELISA

To test the potential antigenicity of the viral extract a sandwich ELISA was used. The negative controls; water *A. tumefaciens* with no plasmid pTMV, pLSDV-A and pLSDV-B were used as samples for the ELISA. The plates were coated with goat anti-TMV antibodies (Row A) and anti-LSDV sera (Row B). The samples were then captured on the plates and the anti-TMV

antibodies were used as secondary antibodies. AP conjugated rabbit anti goat antibody was used in conjunction with NBT/BCIP to visualize the reaction. The ELISA showed that the viral extract pTMV reacted with the anti-TMV antibody, refer to **Appendix 1**: **Figure 16**. LSDV constructs did not react with the anti-LSDV sera. The pTMV did not react with the LSDV sera since it was not carrying an LSDV epitope. The negative controls from plants infiltrated with water and *A. tumefaciens* without plasmid tested negative for the LSDV (data not shown).

Chapter 4

4.0 DISCUSSION

Administration of vaccines is by far the most humane and cost effective method of combating the spread of diseases (Streatfield, 2005). Several systems have been used to produce vaccines including chemical synthesis, fermentation and development of transgenic plants (Wigdorovitz *et al.*, 2004). Production costs, bulk up capacity and stable storage still remain a great challenge in vaccine production.

Plant based vaccines offer potential remedies to several disadvantages of conventional vaccines (Fischer *et al*., 2012; Streatfield and Howard, 2003; Rybicki, 2014; Tiwari *et al*., 2009; Tremblay *et al*., 2010). In this study the possibility of using a TMV-based vector to produce epitopes of LSDV as TMV CP fusions in *N. benthamiana* was explored, in order to develop a candidate vaccine for LSDV. The P32 antigen is important in pathogenicity, diagnosis, prevention and control of capripoxvirus (Tian *et al*., 2010). The study mainly focused on the P32 structural protein, and attempted to establish a cost effective method of vaccine production for LSDV in a country that is heavily reliable on the cattle industry.

4.1 Epitope design

The epitopes selected and used in this study, had to be suitable to elicit effective and lasting protection against LSDV thus the use of part of the structural protein 32 (P32). P32 was used because it is the immunodominant region of LSDV. The consensus sequences generated were

derived from the sequences studied before (Tian *et al*., 2010); these included the amino acids 95-114 and 156-175 of the structural protein P32.

The choice of epitopes was limited, because there is a size limit associated with the CP fusions (Mortimer *et al*., 2015). A maximum of 25 amino acids may be inserted into the TMV CP without disrupting the proper folding of the TMV CP. Thus the epitopes used were 20 amino acids in length to fit into the acceptable range for TMV CP fusions. The epitopes used were synthesized as opposed to being amplified out of the LSDV genome, and this ensured that the epitopes used were of the right sequence as the ones generated from the consensus sequence.

The coat protein of several plant viruses, including TMV (Hamamoto *et al*., 1993), cow pea mosaic virus (Porta *et al*., 1994; Usha *et al*., 1993), tomato bushy stunt virus and alfalfa mosaic virus have been used successfully as carrier molecules. For this study TMV CP was used. Plant virus CP fused to a foreign peptide can self assemble into particles. It is increasingly being used in the production of antigenic peptides in plants.

In addition to the safe and inexpensive production environment provided by plants, in-frame fusion of peptides to plant virus CP provides a means of easy and low cost purification, an important aspect of large scale peptide manufacturing (Belanger *et al*., 2000; Fahad *et al*., 2015; Verma and Daniell, 2007).

4.2 Cloning of LSDV epitopes into pJL TRBO

The LSDV epitopes were successfully inserted into the TMV CP using three rounds of PCR. Due to the size of reverse primers used in PCR 2 and 3, respectively large sizes of products were formed. Pure amplicons of the right sizes were produced by gel purifying the product using the Zyppy Zymoclean^{TM} Gel Recovery kit.

From PCR 1 to the 3rd PCR the product size increased gradually as expected (**Figure 14**). This was due to the LSDV epitope sequence being added to the second round of PCR and only a small piece of the TMV CP being excised. In the 3rd round PCR, the excised part of TMV CP is re-introduced therefore increasing the length of the TMV CP sequence. The primers also added the restriction sites (*NotI* and *AvrII*) corresponding to the enzymes found in the pJL TRBO multi-cloning site to the end of the TMV CP. This aids easy insertion of the constructs (the modified TMV CP) into the pJL TRBO.

The coat proteins carrying the different constructs were cloned successfully into the binary vector pJL TRBO. The vector was designed without the TMV CP, for high protein expression in plants (Lindbo, 2007a). The CP is not essential for cell to cell transport but is for efficient systemic movement in *N. benthamiana* and other host species (Dawson *et al*., 1988; Saito *et al*., 1990; Spitsin *et al*., 1999). The use of recombinant TMV virion as a vaccine has advantages over the whole virus or peptides alone (McCormick *et al*., 2006; McCormick and Palmer, 2008).

The pJL TRBO containing the different constructs was successfully transformed into *E. coli* (DH5α). The plasmid carrying the constructs was then extracted and purified from *E. coli* and

used to transform *A. tumefaciens* (GV3101). The transformation was successful as shown by colony PCR. Due to time constrains the amplicons from the colony PCR were not sequenced. The colonies that carried the constructs were used for Agro-infiltration.

4.3 Agro-infiltration of *N. benthamiana*

Bacterial expression systems have the advantage of speed and abundant production. However, they are limited in many instances by their inability to produce properly folded proteins. In this study, Agro-infection was not successful as the typical TMV infection symptoms were not observed in *N. benthamiana* plants post infiltration. However, the leaves showed signs of necrosis. Different TMV CP substitutions, deletions and peptide additions have also been shown to induce necrotic response in plants independent of any known host resistance response (Bendahmane *et al*., 1999; Culver, 2002; Dawson *et al*., 1988; Lu *et al*., 1998; Saito *et al*., 1989).

Although the native CP was expressed the recombinants were not expressed and this could be due to a number of factors. In some cases, epitopes expressed on the surface of plant viruses interfere with the normal virus infection cycle. Such results were observed with TMV and CPMV where the insertion of certain foreign sequences resulted in interference of viral assembly (Bendahmane *et al*., 1999). Those foreign epitopes aided loss of systemic infection, and or induction of a necrotic response in the host (Takamatsu *et al*., 1990; Portal *et al*., 1994; Beachy *et al*., 1996). Such effects limit the use of viruses for production of peptides, including their use as vaccines (Bendahmane *et al*., 1999).

The CP units in the TMV particles are assembled in a highly ordered fashion, with the N- and C-termini both located on the surface of the TMV particles (Namba *et al*., 1989; Durham *et al*., 1971). The epitopes fused to the CP could only be displayed outside the virus particles if the fused CP subunits are still able to encapsidate the recombinant viral genome properly (Turpen *et al*., 1995; Sugiyama *et al*., 1995). In this study, there were problems when it comes to the assembly of virus-like particle in the plant cells. This resulted in unsuccessful expression of the epitopes of interest.

4.4 Virus extraction and analysis

4.4.1 Virus extraction

In this study the virus expressing the epitopes of interest was not obtained in the extraction made from the infiltrated leaf material. Agro-infiltration was repeated on several occasions and the expression was still unsuccessful. The generated constructs were not stable in the plants. However, unsuccessful expressions of certain short epitopes are often experienced, the corresponding rTMV genomes become less infectious (Takamatsu *et al*., 1990).

It has been reported that a group of rTMV genomes encoding short peptides with transmembrane domain induce necrotic lesions (Li *et al*., 2006). The display of foreign peptides on the surface of TMV has been proven to be an effective way to produce valuable peptide medicines using tobacco. However, some foreign peptides have been severely affected by the viral infectivity, viral particle formation and even symptom appearance on susceptible hosts (Bendahmane *et al*., 1999; Portal *et al*., 1994; Takumatsu *et al*., 1990; Li *et al*., 2006).

It has been suggested that various criteria such as the amount of the amino acid residues (Wu *et al*., 2003), pI/charge value of foreign peptides (Bendahmane *et al*., 1999) and fusion sides (Fischen *et al*., 1995; Hamamoto *et al*., 1993; Sugiyama *et al*., 1995; Takamatsu *et al*., 1990; Turpen *et al*., 1995) in the TMV CP correlate with the infectivity and stability of the rTMV in tobacco plants. Unfortunately, there is no single criterion that could correlate the host response to the virus infection (Li *et al*., 2006).

Therefore, for successful epitope expression in *N. benthamiana* plants there are several factors to consider. These include; stability of the constructs after being cloned into the vector and the presence of transmembrane domain encoded by the constructs (Bendahmane *et al*., 1999). Any possible transmembrane sequence in foreign peptide must be avoided in the mass expression of foreign peptides in tobacco plants using a TMV-based vector (Li *et al*., 2006).

4.4.2 Serology

To enable the rTMV to elicit an immune response when used as a vaccine, the epitopes fused to the TMV CP would have to be expressed on the surface of TMV virion. In this study the epitopes were not successfully expressed on the TMV surface as CP fusions since could not be detected using ELISAs and western blots. The TMV CP used in this study was approximately 480bp (160a.a long). Estimation of the molecular weight of the pTMV is 17.6 kDa so the expectation was that the pLSDV constructs would run between 15 and 20kDa. The bands were not visualized on SDS and even on western blot.

The use of the anti-LSDV sera also showed that the LSDV antigens were not synthesised in the plants. The negative controls (water and *A. tumefaciens* with no plasmid) did not show any reaction to sera indicating that the antibodies were not reacting non-specifically to unknown plant derived antigens. The wtTMV CP did not react to the anti- LSDV antibodies. This clearly indicated that anti-LSDV antibodies were not reacting to TMV CP. The covalent association between the TMV CP and LSDV epitopes used in this study was not achieved. This was further confirmed with a sandwich ELISA, for both pLSDV-A and pLSDV-B constructs were not attached to the TMV CP. The controls tested negative when probed with anti-LSDV antibodies.

4.5 Limitations

The TMV CP can only be fused with a maximum of 24 amino acids. Larger epitopes compromise the folding of the CP and result in unsuccessful TMV virion formation (Wu *et al*., 2003; Beachy *et al*., 1996). As a result the sizes of the epitopes used in this study were limited. The size also limits the number of epitopes that can be fused to the TMV CP simultaneously. Only linear epitopes can be fused to the TMV CP but in some cases the non-linear epitopes are the major immunodominant regions. Instability of the epitopes in the viral genome can also present some problems (Scholthof *et al*., 1996)

4.6 Conclusion

Previous studies showed that subunit vaccines can be generated using TMV CP fusions in *N. benthamiana* plants. However, for this study the epitopes of interest were not successfully

expressed in *N. benthamiana* plants. This clearly indicates that epitope design should be thoroughly investigated to successfully express the epitopes in plants. It was noted that not all epitopes can be expressed on the surface of TMV. The agro infiltration method is quick and cost effective. It takes 3 weeks from infiltration to harvest as compared to the traditional methods which takes at least 6 months. This method can be optimized and used in plant vaccine production systems. The success of this method would result in better, more specific and cheap vaccine production.

4.7 Recommendations

In order to improve this study the following recommendations were made:

- The use of an alternative cloning method for insertion of duplex epitopes into modified pJL TRBO vectors like linker, helical linker or CP154. The method is appropriate for insertion of epitopes that are more than 20 amino acids in length. These vectors are already being tested by the Grill lab in Pitzer College Plant Vaccine Group (unpublished data).
- The PCR amplification of the TMV genome outside of the CP gene to determine whether it was in fact present where the peptide and or the CP was not.
- The use of transformed plants expressing native TMV CP. The use of plants that already express the native TMV CP should help stabilize TMV virions with longer epitopes, since each virion should be a mixture of native CP subunit and CP subunits with an epitope attached.

- The use of multiple epitopes and selecting the best expressed epitopes to use for vaccine production. Concoction or a mixture of epitopes should be tested to see which would give the best and long lasting protection against LSDV.
- Sequencing and analysis of the amplicons after the colony PCR from *A. tumafaciens* and after the rtPCR of the plant extracts can also be done to confirm if the LSDV epitopes were still attached to the TMV CP.
- The use of longer epitopes using a different vector can also be investigated to compare how short epitopes fare compared to longer epitopes.

Chapter 5

5.0 REFERENCES

Abraham, A. and Zissman, A. (1991). Isolation of lumpy skin disease virus from cattle in Israel. *Israel Journal of Veterinary Medicine*, volume 46, pp. 20-23.

Afshar, A., Bundza, A., Myers, D.J., Dulac, G.C. and Thomas, F.C. (1986). Sheep pox: experimental studies with the West African isolate. *Canadian Veterinary Journal,* volume 27, pp. 301-306.

Ali, A.A., Esmat, M., Selim, A. and Abdel-Hamid, Y.M. (1990). Clinical and Pathological studies on lumpy skin disease in Egypt. *Veterinary Record*, volume 127, pp. 549-550.

Ali, B.H. and Obeid, H.M. (1977). Investigation of the first outbreaks of lumpy skin disease in Sudan. *British Veterinary Journal*, volume 133, pp. 184-189.

Andersson, C. (2000). *Production and delivery of recombinant subunit vaccines*. Stockholm, Royal Institute of Technology.

APHIS, (2003). *Animal and Plant Health Inspection Service; Factsheet*. California, United States Department of Agriculture.

ASM, (2011). ASM MicrobeLibrary.org [online] Available at: www.ASMMicrobeLibrary.org [Accessed 06 August 2014].

Awram, P., Gardner, R.C., Foster, R.L. and Bellamy, A.R. (2002). The potential of plant viral vectors and transgenic plants for subunit vaccine production. *Advances in Virus Research*, volume 58, pp. 81-124.

Ayelet, G., Haftu, R., Jemberie, S., Belay, A., Gelaye, E., Sibhat, B., Skjerve, E. and Asmare, K. (2014). Lumpy skin disease in cattle in central Ethiopia: Outbreak investigation and isolation and molecular detection of lumpy skin disease virus. *Revue Scientifique et technique (International Office of Epizootics)*, volume 33, no. 3, pp. 1-23.

Babiuk, L.A. (2002). Vaccination: A Management Tool in Veterinary Medicine. *The Veterinary Journal*, volume 164, pp. 188-201.

Babiuk, S., Bowden, T.R., Boyle, D.B., Wallace, D.B. and Kitching, R.P. (2008). Capripoxviruses: An Emerging Worldwide Threat to Sheep, Goats and Cattle. *Transboundary Emerging Diseases*, volume 55, no. 7, pp. 263-272.

Bachrach, H.L., Moore, D.M., McKercher, P.D. and Polatnick, J. (1975). Immune and antibody responses to an isolated capsid protein of foot-and-mouth disease virus. *Journal of Immunology*, volume, 115, pp.1636-1641.

Bhanuprakasha, V., Indranib, B.K., Hosamania, M. And Singha, R.K. (2006). The current status of sheep pox disease. *Comparative Immunology, Microbiology and infectious Diseases*, volume 29, pp. 27-60.

Beachy, R.N., Firhchen, J.N. and Hein, M.B. (1996). Use of plant viruses for the delivery of vaccine epitopes. *Annals of the New York Academy of Science*, volume 792, pp.43-49.

Beachy, R.N. and Zaitlin, M. (1977). Characterisation and *in vitro* translation of the RNAs from less than full-length virus related nucleoprotein rods present in tobacco mosaic virus preparation. *Virology*, volume 81, pp. 160-169.

BEDIA, (2011). *Botswana Export Development Investment Authority, Report.* Available at www.bedia.co.bw [Accessed 24 March 2014].

BEDIA, (2012). *Botswana Export Development Investment Authority, Report.* Available at www.bedia.co.bw [Accessed 12 October 2013].

Belanger, H., Fleysh, N., Cox, S., Bartman, G., Deka, D., Trudel, M., Koprowski, H. and Yusibov V. (2000). Human respiratory syncytial virus vaccine antigen produced in plants. *The FASEB Journal*, volume 14, pp. 2323-2328.

Bendahmane, M., Koo, M., Karrier, E. and Beachy, R.N. (1999). Display of epitopes on the surface of TMV; Impact of charge and isoelectric point of the epitope on virus host interactions. *Journal of Molecular Biology*, volume 290, pp.9-20.

Black, D.N., Hammond, J.M. and Kitching, R.P. (1986). Genomic relationship between capripoxviruses. *Virus Resources*, volume 5, pp. 227-292.

Boulter, E.A. and Appleyard, G. (1973). Differences between extracellular and intracellular forms of poxvirus and their implications. *Progress in Medical Virology*, volume 16, pp. 86-108.

Brenner, J., Haimovitz, M., Oron, E., Stram, Y., Fridgut, O., Bumbarov, V., Kuznetzora, L., Oved, Z., Waserman, A., Garazzi, S., Perl, S., Lahav, D., Edery, N. and Yudin, H. (2006). Lumpy skin disease (LSD) in a large herd in Israel. *Israel Journal of Veterinary Medicine*, volume 61, pp.73-77.

Brooksby, J.B. (1982). Portraits of viruses: foot-and-mouth disease virus. *Intervirology*, volume 18, pp. 1-23.

Buller, R.M., Arif, B.M., Black, D.N., Dumbell, K.R., Esposito, J.J., Lefkowitz, E.J., McFadden, G., Moss, B., Mercer, A.A., Moyer, R.W., Skinner, M.A. and Tripathy, D.N. (2005). *Poxviridae; Virus Taxonomy; Eighth Report of the International Committee on the Taxonomy of Viruses*. Oxford: Elsevier Academic Press.

Butler, P.J.G., Bloomer, A.C. and Finch, J.T. (1992). Direct visualization of the structure of the "20s" aggregate of coat protein of tobacco mosaic virus. The "disk" is the major structure at pH 7.0 and the photo helix at lower pH. *Journal of Molecular Biology*, volume 224, pp. 381-394.

Canizares, M.C., Nicholson, L. and Lomonossoff, G.P. (2005). Use of viral vectors for vaccine production in plants. *Immunology and Cell Biology*, volume 83 no 3, pp. 263-270.

Capstick, P.B. (1959). Lumpy skin disease-experimental infection. *Bulletin of Epizootic Diseases of Africa*, volume 7, pp. 51-62.

Capstick, P.B. and Coackley, W. (1961). Protection of cattle against lumpy skin disease. Trials with a vaccine against Neethling type infection. *Research in Veterinary Science*, volume 2, pp. 362-368.

Carn, V. (1993). Control of Capripox infections. *Vaccine*, volume 11 no. 13, pp. 1275-1279.

Carn, V.M. and Kitching, R.P. (1995a). An investigation of possible route of transmission of lumpy skin disease virus (Neethling). *Epidemiology and Infections*, volume 114 no. 1, pp. 219-226.

Carn, V.M. and Kitching, R.P. (1995b). The clinical response of cattle experimentally infected with lumpy skin disease (Neethling) virus. *Archive Virology*, volume 140, pp. 503-513.

Chakauya, E., Chikwamba, R. and Rybicki, E.P. (2006). Riding the tide of biopharming in Africa; consideration for risk assessment. *South African Journal of Science*, volume 102, pp. 284-288.

Chand, P. (1992). *Molecular and Immunological characterization of a major envelope protein of capripoxvirus*. University of Surrey. Guildford, England.

Chand, P., Kitching, R. and Black, D.N. (1994). Western blot analysis of virus-specific antibody responses for capripox and contagious pustular dermatitis viral infections in sheep. *Epidemiology and Infections*, volume 133, pp. 377-385.

Chen, N., Danila, M.I., Feng, Z., Buller, R.M.L., Wang, C., Han, X., Lefkowitz, E.J. and Upton, C. (2003). The genomic sequence of ectromelia virus, the causative agent of mousepox. *Virology*, volume 317, pp.165-186.

Chihota, C.M., Rennie, L.F., Kitching, R.P. and Mellor, P.S. (2001). Mechanical transmission of lumpy skin disease virus by *Aedes aegypti* (Diptera: Culicidae). *Epidemiology and Infections*, volume 126, pp.317-321.

Christensen, L.S., Medveczky, I., Strandbygaard, B.S. and Pejsak, Z. (1992). Characterization of field isolates of suid herpesvirus 1 (Aujeszky's disease virus) as derivatives of attenuated vaccine strains. *Archives of Virology*, volume 124, pp. 225-234.

Cizman, M., Mozetic, M., Radescek-Rakar, R., Pleterski-Rigler, D. And Susec-Michieli, M. (1989). Aseptic meningitis after vaccination against measles and mumps. *Pediatric Infectious Disease Journal*, volume 8, pp. 302-308.

Coetzer, J.A.W. (2004). *Infectious Diseases of Livestock*. 2nd Edition. Cape Town: Oxford University Press.

Coetzer, J.A.W., Thomson, G. R. and Tuskin R.C. (1994). *Infectious Diseases of Livestock with Special Reference to Southern Africa*. Cape Town: Oxford University Express.

Copeman, R. J., Hartman, J. R. and Watterson, J.C. (1969). Tobacco mosaic virus in inoculated and systematically infected tobacco leaves. *Phytopathology*, volume 59, pp. 1012-1013.

Culver, J.N. (2002). Tobacco mosaic virus Assembly and Disassembly: Determinants in Pathogenicity and Resistance. *Annual Review of Phytopathology*, volume 40, pp. 287-308.

Culver, J.N., Figueira, A.R., Golem, S. and Goregaoker, S.P. (2002). A nuclear localization signal and membrane associated domain contribute to the cellular localization of the tobacco mosaic virus, 126-kDa replicase protein. *Virology*, volume 301, pp. 81-89.

Cummings, J.F., Guerrero, M.L., Moon J.E., Waterman, P., Nielsen, R.K., Jefferson, S., Gross, F.L., Hancock, K., Katz, J.M. and Yusibov, V. (2014). Safety and immunogenicity of a plant-produced recombinant monomer hemagglutinin-based influenza vaccine derived from influenza A (H1N1) pdm09 virus: A phase 1 dose-escalation study in healthy adults. *Vaccine*, volume 32, pp. 2251-2259.

Dagert, M. and Ehrlich, S.D. (1979). Prolonged incubation in calcium chloride improves the competence of *Escherichia coli* cells. *Gene*, volume 6 no.1, pp. 23-28.

Dales, S. and Pogo, B.G.T. (1981). *The virus particles-elementary bodies; Biology of Poxviruses.* New York: Springer-Verlag.

Davies, F.G. (1976). Characteristics of a virus causing Pox disease in sheep and goats in Kenya, with observations on the epidemiology and control. *Journal of Hygiene.* volume76, pp. 163-171.

Davies, F.G. (1991). Lumpy skin disease, an African Capripox virus disease of cattle. *Britain Veterinary Journal*, volume 147, pp. 489-503.

Davies, F.G. (2012). *LSDV virus particles caption Electron microgram of virus particles* (scale bar=200nm). CAB International.

Davies, F.G. and Otema, C. (1978). The antibody response in sheep infected with a Kenyan sheep and goat poxvirus. *Journal of Comparative Pathology*, volume 88, pp. 206-210.

Dawson, W.O., Bubrick, P. and Grantham, G.L. (1988). Modifications of tobacco mosaic coat protein gene affecting replication, movement and symptomology. *Phytopathology*, volume 78, pp.783-789.

Dawson, W.O. and Lehto, K.M. (1990). Regulation of tobamovirus gene expression. *Advances in Virus Research*, volume 38, pp. 307-342.

De Sousa Dias, A. and Limpo Serra, J. (1956). La dermatose nodulaire au Mozambique. *Bulletin de I'Office International Des Epizooties*, volume 46, pp. 612.

DEFRA, (2003). *Department for Environment Food and Rural Affairs Newsletter*. Pretoria.

DEFRA, (2008). *Department for Environment Food and Rural Affairs Newsletter*. Pretoria.

Desai, P.N., Shrivastava N. and Padh H. (2010). Production of heterologous proteins in plants; strategies for optimal expression. *Biotechnology Advances*, volume 28 no.4, pp. 427-435.

Diallo, A. and Viljoen, G. J. (2007). Genus *Capripoxvirus*. In: Mercer, A.A., Schmidt, A. and Weber O. (Editors). *Poxviruses*, pp. 167-181. Birkhauser, Basel, Switzerland.

Diesel, A.M. (1949). The epizootiology of lumpy skin disease in South Africa. *Proceedings of the 14th International Veterinary Congress, London*, volume 2, pp. 492-500.

Durham, A.C.H., Finch, J.T. and Klug, A. (1971). States of aggregation of tobacco mosaic virus protein. *Nature*, volume 229, pp.37-42.

Fahad, S., Khan, F.A., Pandupuspitasari, N.S., Ahmed, M.M., Liao, Y.C., Waheed, M.T., Sameeullah, M., Darkhshan, Hussain, S., Saud, S., Hassan, S., Jan, A., Jan, M.T., Wu, C., Chun, M.X. and Huang, J. (2015). Recent developments in therapeutic protein expression technologies in plants. *Biotechnology Letters*, volume 37, no. 2 pp. 265-279.

FAO, (1991). Food and Agriculture Organization of the United Nations. Corporate Document Depository.

Fauquet, C., Fauquet, M. and Mayo, M.A. (2005). *Virus Taxonomy: VIII Report of the International Committee on Taxonomy of Viruses*. New York: Academic Press.

Fenner, F. (1996). *Fields virology; Poxviruses*. Philadelphia: Lippincott-Raven Publishers.

Fenner, F., Bachmann, P.A., Gibbs, E.P.J., Murphy, F.A., Studdert, M.J. and White, D.O. (1987). *Poxviridae, Veterinary Virology*. New York: Academic Press.

Fichen, J.H., Beachy, R.N. and Hein, M.B. (1995). Plant virus expressing hybrid coat protein with added murine epitope elicits autoantibody response. *Vaccine,* volume 13, pp. 1051-1057.

Fischer, R. and Emans, N. (2000). Molecular farming of pharmaceutical proteins. *Transgenic Research*, volume 9, pp. 279-299.

Fischer, R., Schillberg, S., Hellwig, S., Twyman, R.M. and Drossard, J. (2012). GMP issues for recombinant plant derived pharmaceutical proteins. *Biotechnology Advances*, volume 30, pp. 434-439.

Fischer, R., Stoger, E., Schillberg, S., Christou, P. and Twyman, R.M. (2004). Plant-based production of biopharmaceuticals. *Current Opinion in Plant Biology*, volume 7, pp. 152-158.

Fraser, R.S.S. (1987). *Biochemistry of virus-infected plants*. Letchworth: Research studies.

Gershelin, P. and Berns, K.I. (1974). Characterisation and localisation of the naturally occurring cross-links in Vaccinia virus DNA. *Journal of Molecular Biology*, volume 88, pp. 785-796.

Gershon, P.D. and Black, D.N. (1989). A Capripoxvirus pseudogene whose only intact homologs are in other poxvirus genomes. *Virology*, volume 172, pp.350-354.

Gidding, G., Allison G., Brooks D. and Carter, A. (2000). Transgenic plants as factories for biopharmaceuticals. *Nature Biotechnology*, volume 18, pp.1151-1155.

Gleba, Y., Klimyuk, V. and Marillonnet, S. (2007). Viral vectors for the expression of proteins in plants. *Current Opinion in Biotechnology*, volume 18, no.2, pp 134-141.

Gleba, Y., Klimyuk, V. and Marillonnet, S. (2005). Magnifection-a new platform for expressing recombinant vaccines in plants. *Vaccine*, volume 23, no 17-18, pp. 2042-2048.

Goelet, P., Lomonossoff, G.P., Butler, P.J.G., Akam, M.E., Gait, M.J. and Karn, J. (1982). Nucleotide sequence of tobacco mosaic virus RNA. *Proceedings of the National Academy of Science of the U.S.A*, volume 79, pp. 5818-5822.

Greth, A., Gourreau, J.M., Vassart, M., Nguyen-Ba-Vy., Wyers, M. and Lefevre, P.C. (1992) Capripoxvirus disease in an Arabian Oryx (*Oryx leucoryx*) from Saudi Arabia. *Journal of Wildlife Diseases*, volume 28 no. 2, pp. 295-300.

Grill, L.K. (1993). Tobacco mosaic virus as a gene expression vector. *Biosource Genetic Corporation*, volume 333, pp. 20-23.

Grill, L.K., Palmer, K.E. and Pogue, G.P. (2005). The use of plant viruses for production of plant-derived vaccines. *Critical Review in Plant Sciences*, volume 24, pp. 309-323.

Grimsley, N., Hohn, B., Hohn, T. and Walden, R. (1986). "Agroinfection" an alternative route for viral infection of plants by using the Ti plasmid. *Proceedings of the National Academy of Science of the United States of America*, volume 83, pp. 3282-3286.

Gulbahar, M.Y., Cabalar, M., Gul, Y. and Icen, H. (2000). Immunohistochemical detection of antigen in lamb tissues naturally infected with sheeppox virus. *Journal of Veterinary Medicine*, volume 47, pp. 79-80.

Hafez, M.A.M., Tawfik, A.M., Maysa Shaker, H.M. and El-Danaf, N.A. (1992). Clinical and pathological studies on lumpy skin disease first recorded in Egypt. *Bulletin of Animal Production in Africa*, volume 40, pp. 225-233.

Hahn, S., Giritch, A., Bartels, D., Bortesi, L. and Gleba, Y. (2014). A novel and fully scalable *Agrobacterium* spray-based process for manufacturing cellulose and other cost-sensitive proteins in plants. *Plant Biotechnology Journal*, volume 1, pp.1-9.

Hamamoto, H., Sugiyama, Y., Nakagawa, N., Hashida, E., Matsunaga, Y., Takemoto, S., Waranabe, Y. and Okada Y. (1993). A new tobacco mosaic virus vector and its use for the systemic production of angiotensin-I-converting enzyme inhibitor in transgenic tobacco and tomato. *Biotechnology*, volume 11, pp. 930-932.

Hefferon, K.L. (2012). Plant virus expression vectors set the stage for biopharmaceutical proteins. *Virology*, volume 433, no. 1, pp. 1-6.

Hofbauer, A. and Stoger, E. (2013). Subcellular accumulation and modification of pharmaceutical proteins in different plant tissues. *Current Pharmaceutical Design*, volume 19, pp.5495-5502.

House, J.A., Wilson, T.M., El Nakashly, S., Karim, I.A., Ismail, I., El Danaf, N., Moussa, A.M. and Ayoub, N.N. (1990). The isolation of lumpy skin disease virus and bovine herpes virus-4 from cattle in Egypt. *Journal of Veterinary Diagnostic Investigation*, volume 2 pp. 111-115.

Houston, P.D. (1945). *Report of the chief Surgeon*. Southern Rhodesia.

Huang, W., Hussey, M. and Michel, F. (1999). Transmission of varicella to gravid via close contacts immunized with varicella-zoster vaccine: a case report. *Journal of Reproductive Medicine*, volume 44, pp. 905-907.

Hunter, P. and Wallace, D. (2001). Lumpy skin disease in Southern Africa. A review of the disease and aspects of control. *Journal of South African Veterinary Association*, volume72, pp. 68-71.

Hunter, T., Hunt, T., Knowland, J. and Zimmern, D. (1976). Messenger bRNA for the coat protein of tobacco mosaic virus. *Nature*, volume 260, pp. 759-764.

Ireland, D.C. and Binepal, Y.S. (1998). Improved detection of Capripoxvirus in biopsy samples by PCR. *Journal of Virology Methods*, volume 74, pp. 1-7.

Irons, P.C., Tuppurainen, E.S.M. and Venter, E.H. (2005). Excretion of lumpy skin disease virus in bull semen. *Theriogenology*, volume 63, pp. 1290-1297.

Jiang, L., Li, M., Zhou, Z., Wu, L., Fan, J., Zhang, Q., Zhu, H. and Xu, Z. (2006). A modified TMV- based vector facilitates the expression of longer epitopes in tobacco. *Vaccine*, volume 24, no. 2, pp. 109-115.

Jyothishwaran, G., Kotresha, D., Selvaraj, T.M., Srideshikan, S., Rajvanshi, P.K. and Jayabaskaran, C. (2007). A modified freeze-thaw method for efficient transformation of *Agrobacterium tumefaciens*. *Current Science*, volume 93 no. 6, pp.770-772.

Kang, T., Kang, K., Kim, J., Kwon, T., Jang, Y. and Yang, M. (2004). High-level expression of the neutralizing epitope of porcine epidemic diarrhoea virus by tobacco mosaic virus-based vector. *Protein Expression and Purification*, volume 38 no 1, pp. 129-135.

Kara, P.D., Afonso, C.L., Wallace, D.B., Kutish, G.F., Abolnik, C., Lu, Z., Vreede, F.T., Taljaard, L.C.F., Zsak, A., Viljoen, G.J. and Rock, D.L. (2003). Comparative sequence analysis

of the South African vaccine strain and two virulent field isolates of the lumpy skin disease virus. *Archives of Virology*, volume 148, pp. 1335-1356.

Kirnse, P. (1969). Host specificity and pathologenicity of poxviruses from wild birds. *Bulletin of the Wildlife Disease Association,* volume 5, pp. 376-386.

Kitching, R. (1986). Passive protection of sheep against capripoxvirus. *Research in Veterinary Science*, volume 41, pp. 247-250.

Kitching, R. (1999). *Encyclopedia of Virology 3rd Edition; Capripoxviruses*. San Diego, California: Academic Press.

Kitching, R. P. (2003). Vaccines for lumpy skin disease, sheep pox and goat pox. In vaccines for OIE list A and emerging animal diseases (Brown F and Roth J.A Editors). Proceedings of an International Symposium 16-18 September 2002, Ames Iowa. *Developments in Biology (Basel)*, volume 114, pp. 161-167.

Kitching, R.P., Bhat, P.P. and Black, D.N. (1989). The characterization of African Strains of Capripoxvirus. *Epidemiology and Infections*, volume 102, pp. 335-343.

Kitching, R.P and Carn, V.M (2004). Sheep pox and goat pox. *Office International des Epizooties Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (mammals, birds and bees)*. OIE, Paris.

Kitching, R.P. and Mellor, P.S. (1986). Insect transmission of Capripox virus. *Research in Veterinary Science*, volume 40, pp. 255-258.

Kitching, R.P. and Smale, C. (1986). Insect transmission of Capripox virus. *Research in Veterinary Science*, volume 41, pp.425-427.

Kitching, R.P. and Taylor, W. (1985). Transmission of Capripoxvirus. *Research in Veterinary Science*, volume 39, pp. 196-199.

Knapp, E., Dawson, W.O. and Lewandowski, D.J. (2001). Conundrum of the lack of defective RNAs (dRNA) associated with tobamovirus infections: dRNAs that can move are not replicated by the wild type virus; dRNAs that are replicated by the wild type virus do not move. *Journal of Virology*, volume 75, pp. 5518-5525.

Kohl, T., Hitzeroth, I.I., Steward, D., Varsani, A., Govan, V.A., Christiansen, N.D., Williamson, A.L. and Rybicki, E.P. (2006). Plant-produced cottontail rabbit papillomavirus L1 protein protects against tumor challenges: a proof of concept study. *Clinical Vaccine Immunology*, volume 13, pp. 845-853.

Koo, M., Bendahmane, M. and Lettieri, G.A. (1999). Protective immunity against murine hepatitis virus (MHV) induced by intranasal or subcutaneous administration of hybrids of tobacco mosaic virus that carries an MHV epitope. *Proclamations of National Academy of Science USA,* volume 96, pp.7774-7779.

Korban, S.S., Krasnyansk, S.F. and Buetow, D.E. (2002). Foods as production and delivery vehicles for human vaccines. *Journal of American College of Nutrition*, volume 21 no. 3, pp. 212-217.

Lacomme, C., Smolenska, L. and Wilson, T.M.A. (1998). Genetic engineering and the expression of foreign peptides or proteins with plant virus-based vectors. *Genetic Engineering*, volume 20, pp. 225-237.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, volume 227, pp. 680-685.

Lee, M.W. and Yang, Y. (2006). Transient expression assay by agro-infiltration of leaves. *Methods in Molecular Biology*, volume 323, pp. 225-229.

Li, Q., Li, M., Jiang, L., Zhang, Q., Song R. and Xu, Z. (2006). TMV recombinants encoding fused foreign transmembrane domains to the CP subunit caused local necrotic response on susceptible tobacco. *Virology*, volume 348, pp.253-259.

Lindbo, J.A. (2007a). TRBO: A high-efficiency tobacco mosaic virus RNA-based over expression vector. *Plant Physiology*, volume 145, pp. 1232-1240.

Lindbo, J.A. (2007b). High-efficiency protein expression in plants from agroinfection-compatible Tobacco mosaic virus expression vectors. *BMC Biotechnology*, volume 7, pp. 52.

Loor, F. (1967). Comparative immunogenicities of tobacco mosaic virus, protein subunits and reaggregated protein subunits. *Virology*, volume 33, no. 2, pp. 215-220.

Lu, B., Taraporewala, Z.F., Stubbs, G. and Culver J.N. (1998). Intersubunit interactions allowing a carboxylate mutant coat protein to inhibit tobamovirus disassembly. *Virology*, volume 244, pp. 13-19.

MacDonald, R.A.S. (1931). *Pseudo-urticaria* of cattle. In: Weiss K.E, editor, *Annual Report*. Northern Rhodesia Department of Animal Health, pp 20-21.

Maclean, J., Koekemser, M, Oliver, A.J., Stewart, D., Hitzeroth, I.I., Rademacher, T., Fischer R., Williamson, A.L. and Rybicki, E.P. (2007). Optimization of human papillomavirus type 16 (HPV-16) L1 expression in plants: comparison of the suitability of different HPV-16 L1 gene

variants and different cell compartment localization. *Journal of General Virology*, volume 88, pp. 1460-1469.

MacOwan, R.S.D. (1959). Observation on the Epizootiology of lumpy skin disease during the first year of its occurrence in Kenya. *Bulletin of Epizootic Diseases of Africa*, volume 7, pp.7-20.

Mason, H.S., Chikwamba, R. and Santi, L. (2004). *Mucosal Immunity*. 3rd Edition. New York: Elsevier.

McCormick, A.A., Corbo, T. A., Wykoff-Clary, S., Nguyen, L.V., Smith, M.L., Palmer, K.E. and Pogue, G.P. (2006). TMV-peptide fusion vaccines induced cell-meditated immune responses and tumor protection in two mice murine models. *Vaccines*, volume 24, pp. 6414-6423.

McCormick, A.A., Kumagai, M.H., Hanley, K., Turpen, T.H., Hakim, I., Grill, L.K., Tuse, D., Levy, S. and Levy, R. (1999). Rapid production of specific vaccines for lymphoma by expression of tumor-derived single-chain Fv epitopes in tobacco plants. *Proceedings of National Academy of Science of the United States of America*, volume 96, pp.703-708.

McCormick, A.A. and Palmer, K.E. (2008). Genetically engineered tobacco mosaic virus as nanoparticle vaccines. *Expect Reviews of Vaccines*, volume 7, pp.33-41.

Mellor, P.S., Kitching, R.P. and Wilkinson, P.J. (1987). Mechanical transmission of Capripoxvirus and African swine fever virus by *Stomoxys calcitrans*. *Research in Veterinary Science*, volume 43, pp. 109-112.

Michael, A., Soliman, S.M., Saber, M.S., Fayed, A. and Moussa, A.A. (1994). Vaccination of cattle against LSD with tissue culture sheeppox vaccine. *Journal of Veterinary Medicine, Assiut, Egypt*, volume 1, pp. 536-541.

Minor, P.D. (1993). Attenuation and reversion of the Sabin vaccine strain of poliovirus. *Developments in Biological Standardization*, volume 78, pp.17-26

Mims, C., Dockrell, H.M., Goering, R.V., Roitt, I., Wakelin, D. and Zuckerman, M. (2004). *Medical Microbiology*. *3rd Edition*. New York: Elsevier Limited.

Ministry of Agriculture, Botswana. (2000-2011). *Annual Reports*. Gaborone: Ministry of Agriculture.

Mirhendi, H., Diba, K., Rezaei, A., Jalalizand, N., Hosseinpur, L. and Khodadadi, H. (2007). Colony- PCR is a rapid and sensitive method for DNA amplification in Yeasts. *Iranian Journal Publications in Health*, volume 36, no. 1, pp. 40-44.

Morris, J.P.A. (1931). *Pseudo Urticaria* of cattle. In: Annual Report. Department of Animal Health, p.20.

Mortimer, C.L., Dugdale, B. and Dale, J.L. (2015). Updates in inducible transgene expression using viral vectors: From transient to stable expression. *Current Opinion in Biotechnology*, volume 32, pp.85-92.

Moss, B. (2006). Poxvirus entry and membrane fusion. *Virology*, volume 344, pp. 48-54.

Moss, B. (2001). *Poxviridae*: the viruses and their replication. In *Field Virology* 4th Edition, pp. 2849-2883. Edited by Knipe D.M. and Howley P.M. Lippioncott Williams and Wilkins. Philadelphia.

Munz, E.K. and Owen, N.C. (1966). Electron microscopic studies on lumpy skin disease virus type "Neethling". *Onderstepoort Journal of Veterinary Research*, volume 33, pp. 3-8.

Nagington, J. and Hornes, R.W. (1962). Morphological studies of the ORF and Vaccinia vaccines. *Virology*, volume 16, pp. 248-260.

Namba, K., Pattanayek, R. and Stubbs, G. (1989) Visualization of protein-nucleic acid interactions in virus: refined structure of intact tobacco mosaic virus at 2.9 Å resolution by fiber diffraction. *Journal of Molecular Biology*, volume 208, no. 2, pp. 307-325.

Nawathe, D.R., Gibbs, E.P.J., Asagba, M.O. and Lawman, M.J.P. (1978). Lumpy skin disease in Nigeria. *Tropical Animal Health and Production*, volume 10, pp.49-54.

News, Botswana Daily newspaper (2011). [Accessed January 28, 2011]. Gaborone, Botswana.

Nielsen, H.S., Oleksiewicz, M.B., Forsberg, R., Stadejek, T., Bøtner, A. and Storgaard, T. (2001). Reversion of a live porcine reproductive and respiratory syndrome virus vaccine investigated by parallel mutations. *Journal of General Virology*, volume 82, pp. 1263-1272.

Niemer, M., Mehofer U., Acosta, J.A.T., Verdianz, M., Henkel, T., Loos, A., Strasser, R., Maresch, D., Rademacher, T., Steinkellner, H. and Mach, L. (2014). The human anti-HIV antibodies 2F5, 2G12 and PG9 differ in their susceptibility to proteolytic degradation: Down-regulation of endogeneous serine and cysteine proteinase activities could improve antibody production in plant-based expression platforms. *Biotechnology Journal*, volume 9, pp. 493-500.

Noris, E., Poli, A., Cojoca, R., Ritta, M., Cavallo, F., Viglio, S., Matic, S. and Landolfo, S. (2011). A human papillomavirus 8 E7 protein produced in plants is able to trigger the mouse immune system and delay the development of skin lesions. *Archives of Virology*, volume 156(4), pp. 587-595.

OIE, (1996). *Manual of Diagnostic Tests and Vaccines for Terrestrial animals*. Paris: World Organisation for Animal Health.

OIE, (2002). *Manual of Diagnostic Tests and Vaccines for Terrestrial animals*. Paris: World Organisation for Animal Health.

OIE, (2008). *Manual of Diagnostic Tests and Vaccines for Terrestrial animals*. Paris: World Organisation for Animal Health.

OIE, (2010). *Manual of Diagnostic Tests and Vaccines for Terrestrial animals*. Paris: World Organisation for Animal Health.

OIE, (2012). *Manual of Diagnostic Tests and Vaccines for Terrestrial animals*. Paris: World Organisation for Animal Health.

Ordner, G. and Lefevre P.C. (1987). *La dematose nodulaire contagieuse des bovins.* Paris: Colection Etudes et Synthesede l'IEMVT.

Palmer, K.E., Benko, A, Doucette, S.A., Cameron, T.I., Foster, T., Hanley, K.M., McCormick, A.A., McCulloch, M., Pogue, G.P., Smith, M.L. and Christensen, N.D. (2006). Protection of rabbits against cutaneous papillomavirus infection using recombinant tobacco mosaic virus containing L2 capsid epitopes. *Vaccine*, volume 24, pp. 5516-5525.

Patel, J.R. and Heldens, G.M.J. (2009). Review; Immuno-prophylaxis against important virus disease of horses, farm animals and birds. *Vaccine*, volume 27, pp. 1797-1810.

Paul, M. and Ma, J.K.C. (2011). Plant-made pharmaceuticals: leading products and production platforms. *Biotechnology and Applied Biochemistry*, volume 58, no. 1, pp. 58-67.

Penney, C.A., Thomas, D.R., Deen, S.S. and Walmsley, A.M. (2011). Plant-made vaccines in support of the Millennium Development Goals. *Plant Cell Reports*, volume 30, no.5, pp. 789-798.

Porta, C., Spall, V.E., Loveland, J., Johnson, J.E., Barker, P.J. and Lomonossoff, G.P. (1994). Development of cowpea virus as a high-yielding system for the presentation of foreign peptides. *Virology*, volume 202, pp. 949-955.

OIAGEN, (2002). *QIAexpress Detection and Assay Handbook*. 3rd Edition.

Quinn, P.J., Markey, B.K., Leonard, F.C., Hartigan, P., Fanning, S. and FtzPatrick, E.S. (2002). *Veterinary Microbiology and Microbial Diseases.* New York: John Wiley and Sons, Inc.

Richmond, V.A. (1998). *Foreign animal diseases*. New York: United States Animal Health Association.

Rybicki, E.P. (2014). Plant based vaccines against viruses. *Virology Journal*, volume 11, no 1, pp.1-20.

Rybicki, E.P. (2010). Plant-made vaccines for humans and animals. *Plant Biotechnology Journal,* volume 8, pp. 620-637.

Rybicki, E.P. (2009). Plant-produced vaccines: Promise and reality. *Drug Discovery Today*, volume 14, no.1-2, pp.16-24.

Sabalza, M., Christou, P. and Capell, T. (2014). Recombinant plant-derived pharmaceutical proteins: current technical and economic bottleneck. *Biotechnology Letters*, volume 36, pp. 2367-2379.

Saito, T., Yamanaka, K. and Okada, Y. (1990). Long-distance movement and viral assembly of *tobacco mosaic virus* mutants. *Virology*, volume 176, pp. 329-336.

Saito, T., Yamanaka, K., Watanabe, Y., Takamatsu, N., Meshi, T. and Okada, Y. (1989). Mutational analysis of the coat protein gene of tobacco mosaic virus in relation to hypersensitive response in tobacco plants with the *N′* gene. *Virology*, volume 173, pp. 11-20.

Sambrook, J. and Russell, D. (2000). *Molecular cloning: A Laboratory manual*. *3rd Edition*. Cold Spring: Harbor Laboratory Press.

Santi, L., Giritch, A. and Roy, C.T. (2006). Protection conferred by recombinant *Yersinia pestis* antigens produced by a highly scalable plant expression system. *Proceedings of the National Academy of Science of the United States of America*, volume 103, no. 4, pp.861-866.

Scholthof, H.B., Scholthof, K.B.G. and Jackson, A.O. (1996). Plant virus gene vectors for transient expression of foreign proteins in plants. *Annual Review of Phytopathology*, volume 34, pp. 299-323.

Scholthof, K.B.G. (2004). Tobacco mosaic virus; a model for Plant Biology. *Annual Review of Phytopathology*, volume 42, pp. 13-34.

Siegel, A., Hari, V. and Kolaez, K. (1978). The effect of tobacco mosaic virus infection and virus-specific protein synthesis in protoplasts. *Virology*, volume 85, pp. 494.

Smith, M.L., Lindbo, J.A., Dillard-Telm, S., Brosio, P.M., Lasnik, A.B., MacCormick, A.A., Nguyen, L.V and Palmer, K.E. (2006). Modified tobacco mosaic virus particles as scaffolds for display of protein antigen for vaccine application. *Virology*, volume 348, no. 2, pp. 475-488.

Snustad, D.P. and Simmons, M.J. (2006). *Principles of Genetics*. *4th Edition*. New Jersey: John Wiley and Sons, Inc.

Spitsin, S., Steplewski, K., Fleysh, N., Belanger, H., Mikheeva, T., Shivprasad, S., Dawson, W, Koprowiski, H. and Yusibov, V. (1999). Expression of alfalfa mosaic coat protein in *tobacco mosaic virus* (TMV) deficient in the production of its native coat protein supports long distance movement of a chimeric TMV. *Proceedings of the National Academy of Science of the United States of America*. Volume 96, pp. 2549-2553.

Steinmetz, N.F., Lin, T., Lamonossoff, G.P. and Johnson J.E. (2009). Structure-based engineering of icosahedral virus for nanomedicine and nanotechnology. *Current Topics in Microbial Immunology,* volume 327, pp. 23-58.

Stram, Y., Kuznetzova, L.., Friedgut, O., Gelman, B., Yadin, H. and Rubinstein-Guini, M. (2008). The use of lumpy skin disease virus genome termini for detection and phylogenetic analysis. *Journal of Virolo gical Methods*, volume 151, pp. 225-229.

Streadfield, S.J. (2005). Plant-based vaccines for animal health. *Revue Scientifique et technique (International Office of Epizootics)*, volume 24, no. 1, pp. 189-199.

Streadfield, S.J. and Howard, J.A (2003). Plant production systems for vaccines. *Expert Review of Vaccines*, volume 2, no. 2, pp. 763-775.

Sugiyama, Y., Hamamoto, H., Takemoto, S., Watanabe, Y. and Okada, Y. (1995). Systemic production of foreign peptides on the particles surface of tobacco mosaic virus. *FEBS Letters*, volume 359, pp. 247- 250.

Swiss Institute of Bioinformatics. (2008). Report.

Takamatsu, N., Ishikawa, N., Meshi, T. and Okada, Y. (1987). Expression of bacterial chloramphenicol acetyl-transferase gene in tobacco plants mediated by TMV-RNA. *EMBO Journal*, volume 6, pp. 307.

Takamatsu, N., Watanabe, Y., Yanagi, H., Meshi, T., Shiba, T. and Okada, Y. (1990). Production of enkephalin in tobacco protoplasts using tobacco mosaic virus RNA vector. *FEBS Letters*, volume 269, pp. 73-76.

Taylor, J. and Paoletti, E. (1988). Fowlpox virus as a vector in non-avian species. *Vaccine*, volume 6, pp. 466-467.

Thomas, A.D. and Mare, C.V.E. (1945). "Knopvelsiekte." *Journal of the South African Veterinary Medical Association*, volume 16, pp. 36-43.

Tian, H., Chen, Y., Wu, J., Shang, Y. and Liu, X. (2010). Serodiagnosis of sheeppox and goatpox using an indirect ELISA based on synthetic peptide targeting for the major antigen P32. *Virology Journal*, volume 7, pp. 245.

Tiwari, S., Mishra, D.K., Roy, S., Singh, A., Singh, P.K. and Tuli, R. (2009). High level expression of a functionally active Cholera toxin B: rabies glycoprotein fusion protein in tobacco seeds. *Plant Cell Reports*, volume 28, pp.1827-1836.

Tremblay, R., Wang, D., Jervnikar, A.M. and Ma, S.W. (2010). Tobacco; a highly efficient green bioreactor for production of therapeutic proteins. *Biotechnology Advances*, volume 28, pp. 214-221.

Tulman, E.R., Afonso, C.L., Lu, Z., Zsak, L., Kutish G.F. and Rock, D.L. (2001). Genome of lumpy skin disease virus. *Journal of Virology*, volume 75, pp. 7122-7130.

Tuppurainen, E.S., Venter, E.H. and Coetzer, J.A. (2005). The detection of lumpy skin disease virus in samples of experimentally infected cattle using different diagnostic techniques. *Onderstepoort Journal of Veterinary Research*, volume 72, pp.153-164.

Tuppurainen, E.S. M. and Oura, C.A.L. (2012). Review: Lumpy Skin Disease: An Emerging Threat to Europe, the Middle East and Asia. *Transboundary and Emerging Diseases*, volume 59, pp. 40-48.

Turpen, T.H. (1999). Tobacco mosaic virus and the virescence of biotechnology. *Biosource Technologies Inc*, volume 354, pp. 664-673.

Turpen, T.H., Reinl, S.J., Charoenvit, Y., Hoffman, S.L., Fallarme, V. and Grill, L.K. (1995). Malarial epitopes expressed on the recombinant tobacco mosaic virus. *Nature Biotechnology*, volume 13, no. 1, pp. 53-57.

Turpen, T.H., Turpen, T.A.M., Weinzettle, N., Kumagai, M.H. and Dawson, W.O. (1993). Transfection of whole plants from wounds inoculated with *Agrobacterium tumefaciens* containing cDNA of TMV. *Journal of Virology Methods*, volume 42, no. 2-3, pp. 227-239.

Usha, R., Rohll, J.B., Spall, V.E., Shanks, M., Maule, A.J., Johnson, J.E. and Lomonosoff, G.P. (1993). Expression of an animal virus antigenic sites on the surface of a plant virus particle. *Virology*, volume 197, pp. 366-374.

Van Rooyen, P.J., Munz, K.E. and Weiss, K.E. (1969). The optional condition for the multiplication of the Neethling type lumpy skin disease virus in embryonated eggs. *Onderstepoort Journal of Veterinary Research*, volume 36, pp. 165-174.

Verma, D., and Daniell, H. (2007). Chloroplast vector system for biotechnology applications. *Plant Physiology*, volume 145, pp.1129-1143.

VIPR, (2012). *Virus Pathogen Resource*. Report.

Von Backsrom, U. (1945). Ngamiland cattle disease. Preliminary report on a new disease, the etiology agent probably being of an infectious nature. *Journal of the South African Veterinary Medicine Association*, volume 16, pp. 29-35.

Voster, J.H. and Mapham, P.H. (2008). Lumpy skin disease. *Livestock Health and Production Review*, volume 1, pp. 16-21.

Wagner, B., Fuchs, H., Adhami, F., Ma, Y., Scheiner, O. and Breiteneder, H. (2004). Plant expression systems for transient production of recombinant allergens in *Nicotiana benthamiana*. *Methods*, volume 32, no. 3, pp. 227-234.

Weiss, K. (1968). Lumpy skin disease virus. *Virology Monographs*, volume 3, pp. 111-131.

Westwood, J.C.N., Harris, W.J., Zwartouw, H.T., Titmus, D.H.T. and Appelyard, G. (1964). Studies on the structure of Vaccinia virus. *Journal of General Microbiology*, volume 34, pp. 67-78.

Wigdorovitz, A., Carrillo, C., Dus Santos, M.J., Trono, K., Peralta, A., Gomez, M.C., Rios, R.D., Franzone, P.M., Sadir, A.M., Escribano, J.M. and Borca, M.V. (1999). Induction of a protective antibody response to foot and mouth disease virus in mice following oral or parenteral immunization with alfalfa transgenic plant expressing the viral structural protein VP1. *Virology*, volume 255, no. 2, pp. 347-353.

Wigdorovitz, A., Mozgovoj, M., Santos, M.J., Parreno, V., Gonez, C., Perez-Filgueira, D.M., Trono, K.G., Rios, R.D., Franzone, P.M., Fernandez, F., Carrillo, C., Babiuk, L.A., Escribano, J.M. and Borca, M.V. (2004). Protective lactogenic immunity conferred by an edible peptide vaccine to bovine rotavirus produced in transgenic plants. *Journal of General Virology*, volume 85, pp. 1825-1832.

Winterfield, R.W. and Reed, W. (1985). Avianpox: Infection and immunity with quail, psittacine, fowl and pigeon poxvirus. *Poultry Science*, volume 64, pp. 65-70.

Wittek, R., Menna, A., Muller, K., Schumperli, D., Bosley, P.G. and Wyler, R. (1978). Inverted terminal repeats in rabbit poxvirus and vaccinia virus DNA. *Journal of Virology*, volume 28, pp. 171-181.

Woods, J.A (1988). Lumpy skin disease-a review. *Tropical Animal Health and Production*, volume 20, pp. 11-17.

Wu, L.G., Jiang, L.B., Zhou, Z.A., Fan, J.H., Zhang, Q.Q., Zhu, H.H., Han, Q. and Xu, Z.K. (2003). Expression of foot-and-mouth disease virus epitope by tobacco mosaic virus-based vector. *Vaccine*, volume 21, pp. 4390-4398.

Xie, H., Cass, A.R. and Barrett, A.D. (1998). Yellow Fever 17D vaccine virus isolated from healthy vaccines accumulates very few mutations. *Virus Research*, volume 55, pp. 93-99.

Yeruham, I., Nir, O., Braverman, Y., Davidson, M., Grinstein, H., Haymovitch, M. and Zamir, O. (1995). Spread of lumpy skin disease in Israel dairy herds. *The Veterinary Record*, volume 137, pp. 91-93.

Young, E., Basson, P.A. and Weiss, K.E. (1970). Experimental infection of game animals with lumpy skin disease virus (prototype strain Neethling). *Onderstepoort Journal of Veterinary Research*, volume 37, pp. 79-87.

Yusibov, V., Hooper, D.C. and Spitsin, S. (2002). Expression in plants and immunogenicity of plant virus based experimental Rabies vaccine. *Vaccine*, volume 20, pp. 3155-3164.

Yusibov, V., Rabindran, S., Commandeur, U., Twyman, R.M. and Fischer, R. (2006). The potential of plant virus vector for vaccine production. *Drugs R.D*, volume 7, pp. 203-217.

Yusibov, V., Shivprasad, S., Turpen, T.H., Dawson, W. and Koprowski, H. (1999). Plant viral vectors based on tobamoviruses. *Current Topics in Microbiology and Immunology*, volume 240, pp. 81-94.

Chapter 6

6.0 APPENDICES

6.1 Appendix 1: Virus extraction and analysis

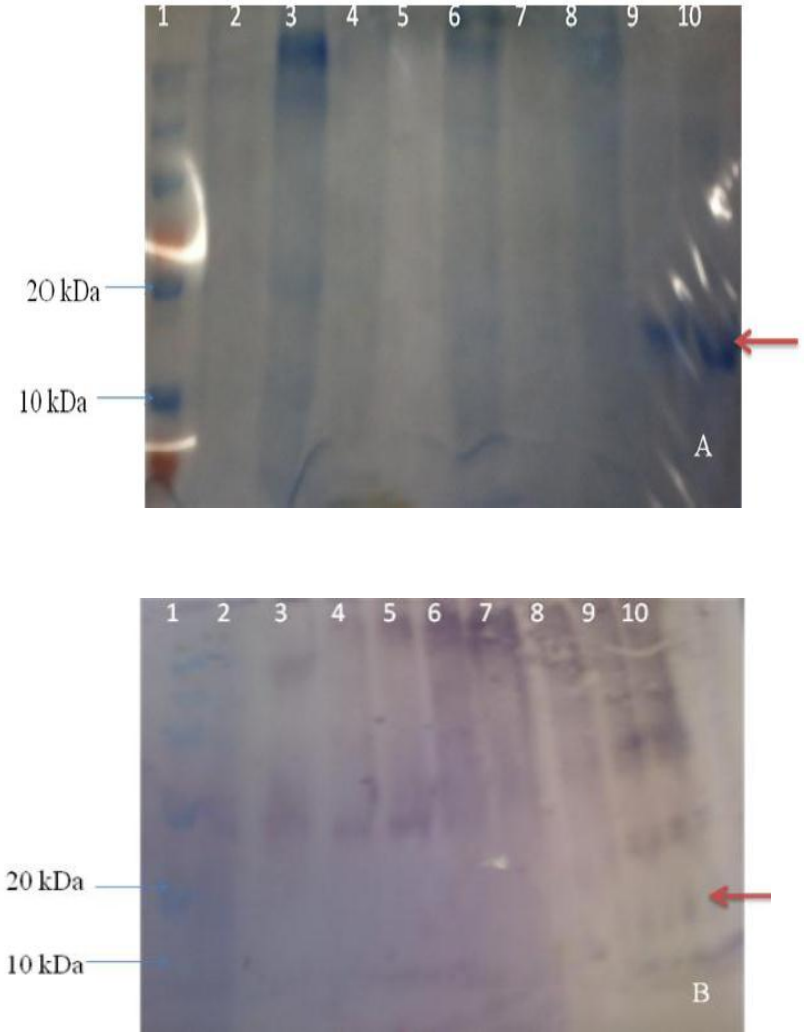


Figure 15: SDS PAGE and western blot analysis of viral extracts. **a**) Coomassie stained SDS gel **b**) The western blot was carried out using anti TMV. The gel and the blot were loaded as follows; lane 1: the pre-stained marker (biorad). Lane 2: water. Lane 3: pLSDV-A. Lane 4: water. Lane 5: *A. tumefaciens* without plasmid. Lane 6: pLSDV-B. Lane 7: water. Lane 8: pLSDV-B. Lane 9 pTMV. Lane 10: pJL 24**.**

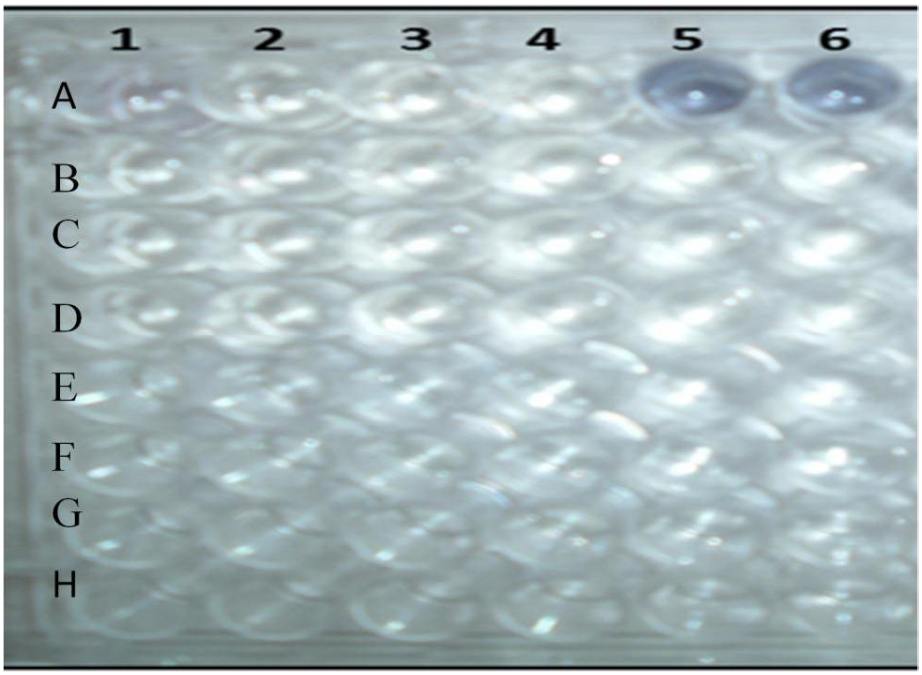


Figure 16 : Sandwhich ELISA of the extracted TMV constructs. Row A: coated with anti-TMV antibodies, Row B: anti-LSDV sera. Column A: 1 and 2: coated with water, Column 3 and 4: coated with *A. tumefaciences* without plasmid. Column 5 and 6: coated with pTMV.

6.2 Appendix 2: Plasmid Maps

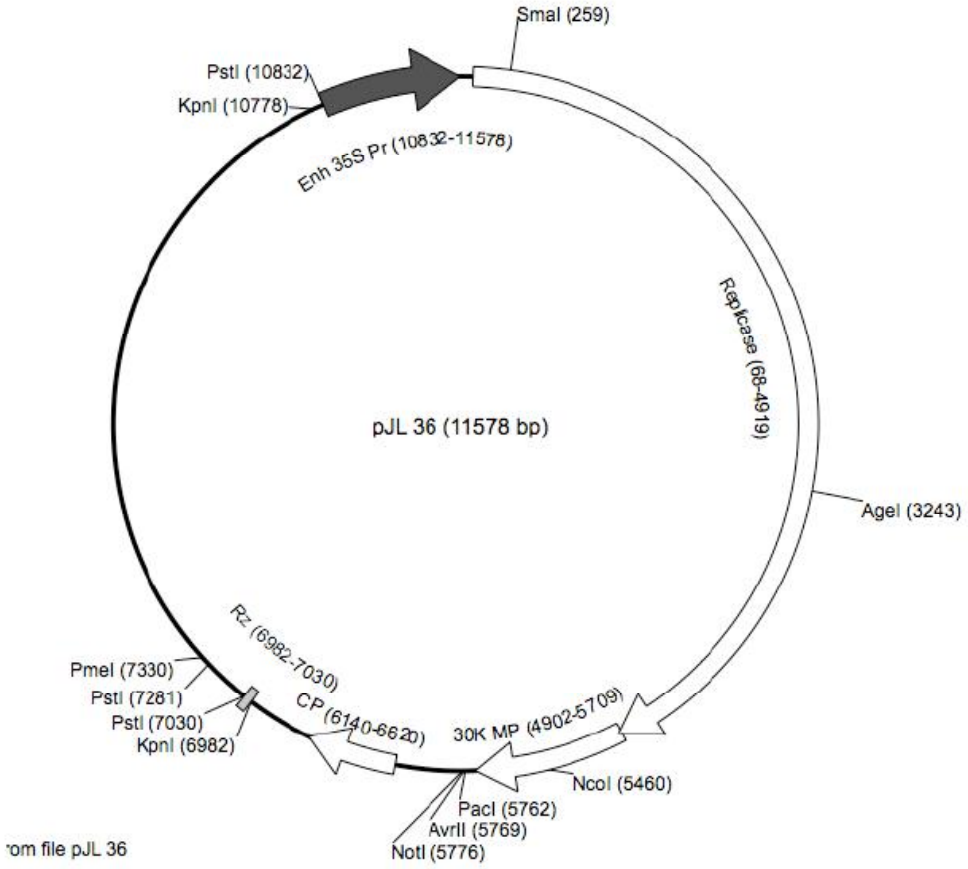
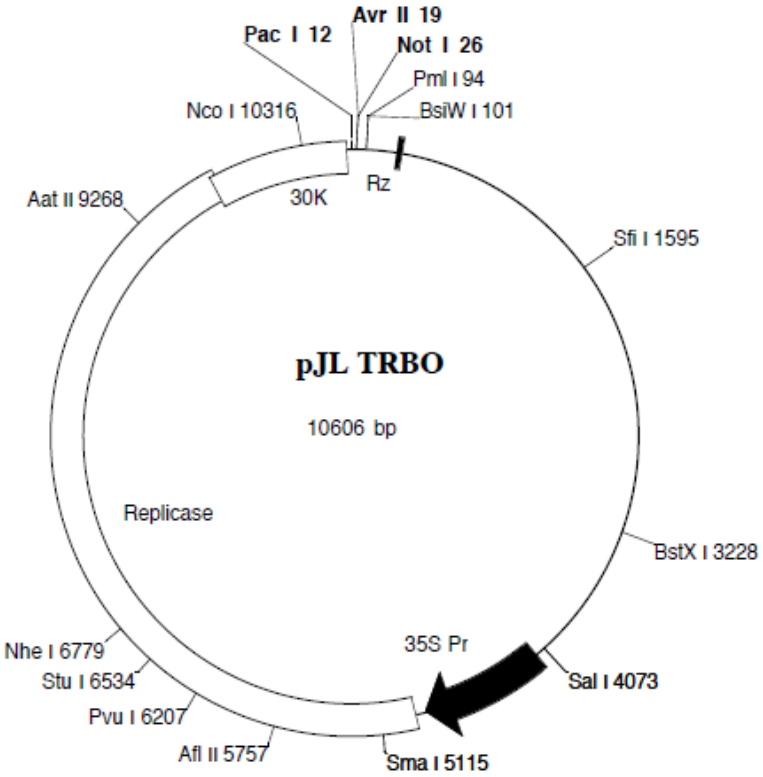


Figure 17: pJL 36 vector map.

Binary vector, Kanamycin resistance marker. T-DNA borders not shown in map. Clone gene of interest into *PacI-AvrII-NotI* multiple cloning site. (Lindbo, 2007a)



Multiple cloning Site (MCS)

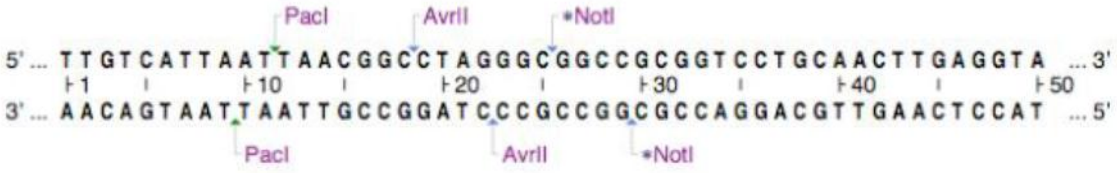


Figure 18: pJL TRBO

The binary plasmid, replicates in *E. coli* or *A. tumefaciens*. It has Kan resistance selection (50µg/mL). The T-DNA borders are not shown in map. This TMV based replicon is used for high level gene expression in plants. The gene of interest is cloned into *PacI-AvrII-NotI* polylinker [ttaattaa, cctagg, gcggccgc] (see MSC sequence map). This replicon does NOT have coat protein gene, so the replicon will NOT move systemically, and will not form virion particles (Lindbo, 2007b).

6.3 Appendix 3: Buffers and solutions

30% Acrylamide

29g Acrylamide

1g N,N'methylbisacrylamide

Dissolve in 60mL of dH_20

Adjust volume to 100mL with dH_20

Filter sterilise (0.45 micron pore size)

Store at 4°C

100mg/mL Ampicillin

1g Ampicillin sodium salt

Add dH_20 up to 10mL

Filter sterilise (0.22 micron pore size)

Aliquot 500µL into microcentrifuge tubes

Store at -20°C

50mg/mL Kanamycin

1g Kanamycin sulphate

2mL H_20

Filter sterilise

Store in 500µL aliquots at -20°C

25mg/mL Gentamicin

1g Gentamicin

4mL H_20

Filter sterilise

Store in 500µL aliquots at -20°C

50mg/mL Rifampicin

1g Rifampicin

2mL DMSO

Filter sterilise

Store in 500µL aliquots at -20°C

10% APS

1g Ammonium per sulphate (MW 228.2)

Dissolve in 10mL dH_20

Store in 200µL aliquots at -20°C

10X PBS

80g NaCl

2g KCl

11.5g Na_2HPO_4

2g KH_2PO_4

Make up to 1L. Autoclave.

1% Agarose

1g Agarose

Make up to 100mL with 1X TAE

0.5µg/mL (200mL) Ethidium Bromide

Add 10µl of 10mg/mL Ethidium Bromide (BioRad) to 200ml ddH_20. Wrap in foil and store at 4°C

6X Gel loading dye

25mg Bromophenol Blue

3mL Glycerol

10mL dH_20

Coomassie stain

250mL Isopropanol

100mL Glacial acetic acid

1g Coomassie Blue

650mL dH_20

Destain

250mL Isopropanol

100mL Glacial acetic acid

650mL Water

Transfer buffer (pH 9.2) (Semi-dry)

5.82g Tris Base

2.93g Glycine

200mL Methanol

Make up to 1L with dH_20

LB broth (1L)

10g NaCl

10g Triptone

5g Yeast extract

Autoclave

LB agar (1L)

10g NaCl

10g Triptone

5g Yeast extract

18g Agar

Boil then autoclave

50X TAE (1L)

242g Tris base (MW=121.1)

57.1 mL Glacial acetic acid

100ML 0.5M EDTA

Mix the Tris with about 600mL ddH_20. Add EDTA and acetic acid. Bring final volume to 1 L with ddH_20. Store at room temperature.

Induction Media

2mL 0.5M MES pH5.7

2mL 0.5M $MgCl_2$

200µL 0.1M Acetosyringone

Make up to 100mL with sterile d H_20

L-MES media

50mL LB broth

1mL 0.5M MES pH5.7

10µl Acetosyringone

50µl Kan, 25µl Gent and 10µl Rif

TBS (500mL)

50mL 1M Tris-Cl

4.5g NaCl

pH to 7.8

TBS-Tween/Triton

50mL 1M TRIS-CL

14.6g NaCl

0.25mL Tween 20

1mL Triton-X 100

pH to 7.8

Make up to 500mL with d H_20

80% Glycerol v/v

80mL Glycerol

20mL H_20

Autoclave

3% BSA (Blocking buffer)

3g BSA

100mL TBS

NBT/BCIP solution

1 tablet in 20mL dH_20

(Sigma Aldrich)

2X YT broth (1L)

10g Yeast extract powder

16g Triptone

5g NaCl

Autoclave

100mM $MgCl_2$

50mL 1M MgCl2

450mL dH_2O

Autoclave

100mM $CaCl_2$

50mL 1M CaCl2

450mL dH_2O

Autoclave

100mM $CaCl_2$ and 20% Glycerol

50mL 1M CaCl2

100mL Glycerol

350mL dH_2O

Autoclave

20mM $MgCl_2$

10mL 1M MgCl2

490mL dH_2O

Autoclave

20% PEG (8000)

20g PEG 8000

100mL sterile dH_20

Filter sterilise

5M NaCl

146g NaCl

Make up to 1L with dH_20

20% SDS

20g SDS

100mL dH_20

0.5M MES pH5.7

19.52g MES

60mL sterile dH_20

Adjust pH to 5.7 with 10M NaOH

Make up to 100mL with sterile dH_20

Filter sterilize into sterile bottle

Sample treatment buffer (10mL)

2.5mL 4x Stacking Buffer

4mL 10%SDS

4% PEG (8000)

4g PEG 8000

100mL sterile dH_20

Filter sterilise

2mL glycerol

0.2mL β-mercaptoethanol (1M)

0.2mg bromophenol blue

Store as 500µL aliquotes at -20°C.

Carbonate buffer (50mM, pH 9.6)

Make a stock solution of 1M $NaHCO_3$ (84,01g/L of water)

Dilute 1:20 before use and adjust pH to 9.6 with NaOH.

Store at 4°C.

4x Resolving buffer (200mL)

36.3g Tris-base

pH 8.8, adjusted with HCl.

Autoclave.

4x Stacking buffer

3g Tris-base

Make up to 50mL with dH_20

pH 6.8, adjusted with HCl.

Autoclave.

20% SDS

10g SDS

Make up to 50mL with dH_20

Autoclave.

0.01% beta mercaptoethanol βMe (50 μL)

0.5M $MgCl_2$

10.16g $MgCl_2.6\ H_20$

Make up to 100mL with sterile distilled water

Filter sterilize into sterile bottle

10x Running buffer

30.3g Tris-base

144.2g glycine

10g SDS

Water to 1L

pH 8.5, adjusted with HCl.

12% SDS PAGE gel

10.2 mL H_2O

7.5 Ml 4X Resolving Buffer

0.15 mL 20% SDS

12.0 mL 30% Acrylamide
0.15 mL 10% APS
0.02 TEMED

Extraction buffer

50Mm NaAcetate pH 5.0 (2.05g)

Adjust pH to 5.0

0.1% Na metabisulfite