

Abstract

Development of Rift Valley fever virus candidate vaccines and reagents produced in *Nicotiana benthamiana*

By

Sandiswa Mbewana

Biopharming Research Unit

Department of Molecular and Cell Biology, Faculty of Science

University of Cape Town, South Africa

Rift Valley fever (RVF) is a haemorrhagic fever agent caused by an infection with an enveloped negative-stranded RNA Rift Valley fever virus (RVFV). It belongs to the genus *Phlebovirus* in the family *Bunyaviridae*. The virus is spread by infected mosquitoes and affects ruminants and humans, causing high numbers of neonatal fatalities in animals and occasional fatalities in humans. It is endemic to parts of Africa and the Arabian Peninsula, but is described as an emerging virus due to the wide range of mosquitoes that could spread the disease into non-endemic areas, posing serious health and agricultural problems. The disease can be prevented by vaccination, but there is currently no Food and Drug Administration-approved RVFV vaccine that can be used outside endemic areas, while there are two live attenuated vaccines available for use in endemic areas. These vaccines have the potential for reversion, and are therefore not recommended for use in countries where RVFV is not endemic. This indicates the need for more RVFV vaccine research and development. This work focused on the development of a RVFV vaccine candidate that would allow for differentiation between infected and vaccinated animals as well as humans.

A readily available wild type Egyptian ZH548 RVFV *GnGc* polyprotein-encoding gene sequence was initially used for developing the candidate vaccines. The *GnGc* genes were cloned into various plant expression vectors with and without a histidine tag. Initial strategies and efforts made to express *GnGc* protein resulted in a lack of recombinant protein detection. Thus, a different *GnGc* DNA sequence representing a South African isolate M35/74 was selected to make putative vaccine candidates; this gene was *Nicotiana* sp.- and human-codon optimised and both were synthesised. Both genes were modified for various cloning strategies into different plant expression vectors. Gene expression was conducted by transient expression of recombinant constructs in *Nicotiana benthamiana* via *Agrobacterium tumefaciens*-mediated gene transfer. Protein expression analysis was verified by western blotting of crude leaf extracts separated using SDS-PAGE. Protein expression could not be detected from the full length *GnGc* glycoprotein gene.

The lack of recombinant protein expression from the full length *GnGc* lead to the RVFV *Gn* gene being used for further studies. *Gn* was modified by truncation of its transmembrane domain and cytoplasmic tail (TMD-CT) and substituting its native signal peptide with two heterologous signal peptides, namely protein disulphide isomerase (PDI) and murine mAB24 heavy chain (LPH), to make a soluble recombinant protein vaccine candidates (PDI- Δ tGn and LPH- Δ tGn). Virus-like particles are known to be more immunogenic than recombinant proteins alone because they display authentic conformation of the virus, thus displaying their immunogenic epitopes. In addition to the recombinant protein vaccine, a second type of vaccine candidate was designed to form chimaeric VLPs by fusing the LPH- Δ tGn to the influenza hemagglutinin TMD-CT (LPH- Δ tGnHA).

Gene expression was conducted by transient expression of recombinant constructs in *N. benthamiana* via *A. tumefaciens*-mediated gene transfer. Protein expression analysis was verified by western blotting of crude leaf extracts separated using SDS-PAGE. Unfortunately, no recombinant protein expression was detected using the PDI- Δ tGn vaccine candidate. Expression was successfully detected using the modified RVFV LPH- Δ tGn and LPH- Δ tGnHA vaccine candidate crude leaf extracts. Production

of these recombinant vaccine candidates was scaled up and protocols for their purification were developed. The soluble LPH-ΔtGn was initially partially purified by ammonium sulphate precipitation and then by affinity chromatography. The protein yields were calculated to be ~ 56.81 mg/kg fresh weight. The chimaeric particulate LPH-ΔtGnHA candidate was purified by differential centrifugation. The protein yields were calculated to be ~ 59 mg/kg fresh weight. Protein was characterised by transmission electron microscopy (TEM). TEM showed chimaeric Gn particles of ~49 – 60 nm. Preliminary immunogenicity studies of both modified RVFV Gn candidate vaccines was carried out in BALB/c mice. The vaccine candidates were found to be immunogenic in mice.

The second focus of this research was on the development of a diagnostic reagent. Diagnosis of RVFV is carried out by various methods including ELISA, using the nucleocapsid protein (N-protein) as a diagnostic agent. N-protein is the most abundant non-structural protein in the virion. It has been used as a diagnostic reagent for other viruses in the *Bunyaviridae* family. N-protein is currently produced from live virus preparations which involve high levels of biosafety, high production costs and have restricted use outside of RVF endemic areas due to potential escape of the virus in non-endemic areas. This work describes the production of a cost-effective and safer diagnostic N-protein antigen using plants as an expression system.

The South African isolate M35/74 RVF N-protein was human-codon optimised and synthesised. Recombinant protein expression was successfully detected in plants when fused with a histidine tag. Purification by affinity chromatography yielded high amounts of protein ranging from 500 - 558 mg/kg fresh weight. TEM of purified preparations showed that the protein forms ring shaped structures of ~10 nm. Preliminary data carried out using purified protein showed that the N-protein is stable after at least 8 months when stored at both 4 and - 80 °C. Preliminary ELISAs carried out revealed that plant-produced N-protein was functional as it could successfully differentiate between serum isolated from infected and non-infected animals, thus indicating it has potential use as a diagnostic reagent.

In conclusion, this research demonstrates the potential of LPH- Δ tGn and LPH- Δ tGnHA vaccine candidates. They both elicited anti-Gn immune responses in vaccinated mice without the use of adjuvant. These results show that these vaccine candidates have potential as RVFV candidate vaccines and their development should be further investigated. The plant-produced N-protein detected IgG antibodies against RVFV-infected sheep serum.

Acknowledgements

While my name maybe alone on the front cover of this thesis. I am by no means its sole contributor. There are several people behind this piece of work, who deserve to be both acknowledged and thanked here:

The Almighty, for the uncommon favour and blessings.

My family, this journey would not have been possible without the support of my family, an inspiring mother, and my sisters, who supported me emotionally and otherwise.

Dr Ann Meyers, for acting as co-supervisor and advice and critical discussion.

Dr Inga Hitzeroth, for acting as my mentor and helping shape my career as a researcher.

Prof Edward Rybicki, for acting as supervisor and an opportunity to advance my career.

My dear friends and lab colleagues, particularly Dr Albertha Van Zyl, Dr Siyabulela Ntutela and Dr Mbulelo Maneli for your continued support, encouragement, advice and support. Nathi Tshabalala, Ondella Stungu, Siphhelele Ngcai, Nobathembu Mbombela, and Nobesuthu Tom for the prayers, the good and bad times, the laughter and tears through, the bitter and sweet moments.

AmaMpondo, Thahla, Ndayeni, Ngqungqushe and amaNtande, ooDlomo, for your favour and protection.

Brandon Webber for the assistance with analytical chromatography, **Mohammed Jaffer** for assistance with the transmission electron microscopy, which were accompanied by intense conversation of national importance.

Maryke Ferreira, Vuyokazi Mareledwane, Felix Mjiwa from the Agricultural Research Council-Onderstepoort Veterinary Institute for the testing of the N-protein antigen.

National Research Foundation, Poliomyelitis Research Foundation, UCT Science Faculty, Carnegie Co-operation for financial support.

PREFACE

This thesis is presented as a compilation of nine chapters. Each chapter is introduced separately and is written to the style of the **Frontiers-Science Journal**

CHAPTER 1 GENERAL INTRODUCTION AND PROJECT AIMS

CHAPTER 2 LITERATURE REVIEW

CHAPTER 3 RESEARCH RESULTS

Expression strategies for RVFV *GnGc* genes in *N. benthamiana* to identify suitable candidates for vaccine or reagent development

CHAPTER 4 RESEARCH RESULTS

The modification and expression of RVFV Gn as a candidate subunit vaccine

CHAPTER 5 RESEARCH RESULTS

The modification and expression of a chimaeric RVFV virus-like particle candidate vaccine

CHAPTER 6 RESEARCH RESULTS

Production of a potential Rift Valley fever N-protein in plants for use as potential diagnostic antigen

CHAPTER 7 CONCLUSION

CHAPTER 8 APPENDIX

CHAPTER 9 REFERENCES

List of abbreviations

Abbreviations

5FU	5-fluorouracil
6xhis	Histamine
aa	Amino acids
ACT2	Arabidopsis actin 2
ARC-OVI	Agricultural Research Council – Onderstepoort Veterinary Institute
BeYDV	Bean yellow dwarf virus
bp	Base pair
BRU	Biopharming Research Unit
BSL	Biosafety level
BTI	<i>Bacillus thuringiensis israeliensis</i>
CaMV	Cauliflower mosaic virus
Carb	Carbenicillin
CDC	Centre for Diseases Control
CPMV	Cowpea mosaic virus
CTL	Cytotoxic T lymphocyte
DIVA	Differentiate Infected and Vaccinated Animals
dNTP	deoxy-ribosenucleoside triphosphates (dATP, dCTP, tTTP and dGTP)
dpi	Days post infiltration
DTT	DL-dithiothreitol

EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
Env	Envelope protein
ER	Endoplasmic reticulum
FDA	Food and Drug Administration
FMD	Food and Mouth Disease
FMDV	Foot and mouth disease virus
FRhL-2	Entebbe strain in diploid cells
FW	Fresh weight
Gc	Carboxy-terminal glycoproteins
GFP	Green fluorescent protein
Gn	Amino-terminal glycoproteins
HA	Hemagglutination assay
HI	Hemagglutination inhibition
HT	Hypertranslatable
IFA	Immunofluorescence assay
IPTG	Isopropylthio- β -D-galactoside
Kan	Kanamycin
L	Large

LA	Luria agar
LAMP	Loop mediated isothermal amplification
LB	Left border/ Luria-broth
LC-MS	Liquid chromatography – mass spectrometry
LIR	Long intergenic region
LPH	Murine mAb24 heavy chain
LSDV	Lumpy skin disease virus
M	Medium/molar
M1	Matrix protein
MCS	Multiple cloning site
MES	2-morpholineethanesulfonic acid
MP	Movement protein
MRC5	Human diploid lung cells
N	Nucleocapsid protein
nAbs	Neutralising antibodies
NBT/BCIP	Nitro blue tetrazolium chloride/5-bromo-4chloro-3-indolyl phosphate
NP	Nucleoprotein
NSR	Nonspreading RVFV
O/N	Overnight
OIE	Office for International des Epizooties
Ori	Origin of replication

PAGE	Polyacrylamide gel electrophoresis
PB(s)	Protein bodies
PDI	Protein disulphide isomerase
PTGS	Post-translational gene silencing
R/Rev	Reverse
RB	Right border
Rif	Rifampicin
RNP	Ribonucleoprotein
RT	Room temperature
RVF	Rift valley fever
RVFV	Rift Valley Fever virus
SANS	South African National Accreditation System
SIR	Short intergenic region
Sp.	Species
TBSV	Tomato bushy stunt virus
T-DNA	Transfer-DNA
TE	Transient expression
Ti	Tumour-inducing
TMV	Tobacco mosaic virus
Tris	Tris(hydroxymethyl)aminomethane
TSP	Total soluble protein
TSWV	Tomato spotted wilt virus
U	Unit

USDA	U.S Department of Agriculture
UTR	Untranslated region
VEEV	Venezuelan equine encephalitis virus
VLP	Virus-like particles
VN	Virus neutralization
wt	weight
WT	Wild type
x-gal	5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside

Contents

Chapter 1: General Introduction and project aims	1
1.1 General Introduction	1
1.2 Project Aims	3
Chapter 2: Literature review	5
2.1 Rift Valley Fever	5
2.2 Viral structure	5
2.3 Viral entry and transcription.....	7
2.4 RVFV outbreaks	9
2.5 Clinical Symptoms.....	11
2.5.1 RVFV in animals	11
2.5.2 RVFV in humans.....	11
2.6 Bioterrorism concerns	12
2.7 Diagnosis	13
2.8 Control and prevention	14
2.9 RVFV Vaccines	14
2.9.1 Live attenuated vaccines	15
2.9.2 Inactivated vaccines	18
2.9.3 Recombinant viral vaccines	19
2.9.4 Virus-like particles as vaccines	19
2.10 Plant production systems	21
2.11 Plant expression vectors	23
2.11.1 Plant organelle targeting vectors	24
2.11.2 Geminivirus-based expression vector	26
2.11.3 Comovirus-based expression vector	27
2.11.4 Tobamovirus-based expression vector	28
2.12 Codon optimisation.....	29
2.13 Signal peptide fusions	30
2.14 Final Remarks	31
Chapter 3: Expression strategies for RVFV GnGc genes in N. benthamiana to identify suitable candidates for vaccines or reagent development	32
3.1 Introduction	32
3.2 Materials and Methods	34
3.2.1 Rift Valley fever virus (RVFV) GnGc genes	34

3.2.2 Plant expression vectors.....	34
3.2.3 Bacterial and plant growth conditions	35
3.2.4 DNA manipulation and analysis	36
3.2.5 Gene amplification by PCR.....	37
3.2.6 Sub-cloning of RVFV glycoprotein genes for sequencing.....	37
3.2.7 Cloning of glycoprotein genes into plant expression vectors	38
3.2.8 Cloning of WT strain RVFV GnGc glycoprotein gene	38
3.2.9 Cloning of human and Nicotiana sp. codon optimised RVFV GnGc	40
3.2.10 Agrobacterium transformation.....	41
3.2.11 Transient expression of recombinant proteins	42
3.2.12 Small scale protein extraction	42
3.2.13 Protein expression analysis	43
3.3 Result	44
3.3.1 Cloning of WT Gn and GnGc	44
3.3.2 Expression of WT Gn and GnGc in <i>N. benthamiana</i>	45
3.3.3 Cloning of human (h) and <i>N. benthamiana</i> (p) codon optimised Gn and GnGc	45
3.4 Discussion	49
Chapter 4: The modification and expression of RVFV Gn as a candidate subunit vaccine	54
4.1 Introduction	54
4.2 Materials and Methods	56
4.2.1 Cloning and sequencing of constructs	56
4.2.2 Modification of <i>N. benthamiana</i> codon optimised pGn and construction of plasmids	56
4.2.3 Modification of human-codon optimised Gn (hGn) and construction of plasmids	60
4.2.4 Agrobacterium transformation and recombinant protein expression.....	60
4.2.5 Optimal buffer composition	61
4.2.6 Large scale protein expression	61
4.2.7 Protein purification of pEAQ-HT-LPH- Δ ptGn	61
4.2.8 Protein expression analysis	63
4.2.9 Liquid chromatography - mass spectrometry (LC-MS)	63
4.2.10 Immunisation of mice	63
4.2.11 Indirect ELISA detection of anti-Gn antibodies in mouse sera.....	64
4.3 Results	65

4.3.1 Cloning of the recombinant modified Gn constructs into plant expression vectors	65
4.3.2 Expression and detection of recombinant protein in crude leaf extracts ...	68
4.3.3 Purification of pEAQ-HT-LPH- Δ tGn product	73
4.3.4 Total soluble protein (TSP) quantification	74
4.3.5 Immunisation of mice with LPH- Δ tGn and animal serum analysis	74
4.4 Discussion	77
Chapter 5: The modification and expression of a chimaeric RVFV virus-like particle candidate vaccine.....	82
5.1 Introduction	82
5.2 Materials and Methods	85
5.2.1 Genes	85
5.2.2 Fusion of <i>Nicotiana</i> sp. codon optimised glycoprotein (Δ tGn and tGn) with avian influenza transmembrane domain and cytosolic tail of HA.....	85
5.2.3 Cloning of Δ tGnHA and tGnHA into pTRAc and pTRAc-ERH.....	86
5.2.4 Cloning of LPH- Δ tGnHA into pEAQ-HT plant expression vector.....	86
5.2.5 <i>Agrobacterium</i> transformation and recombinant protein expression.....	86
5.2.6 Large scale expression of pEAQ-HT-LPH- Δ tGnHA	87
5.2.7 Staggered infiltration with M1 and N-protein.....	87
5.2.8 Purification of pEAQ-HT-LPH- Δ tGnHA chimaeric particles.....	87
5.2.9 Protein expression analysis	87
5.2.10 Transmission electron microscopy (TEM).....	88
5.2.11 Immunisation of mice.....	88
5.2.12 Indirect ELISA detection of anti-Gn antibodies in mouse sera.....	88
5.3 Results	88
5.3.1 PCR fusion of <i>Nicotiana</i> sp. codon optimised glycoprotein (Δ tGn and tGn) with avian influenza TMD/CT of HA.....	88
5.3.2 Transient expression of recombinant proteins in <i>Nicotiana benthamiana</i> .	90
5.3.3 Protein purification and characterisation of LPH- Δ tGnHA.....	91
5.3.4 Staggered infiltration of LPH- Δ tGnHA with M1 and N-protein.....	92
5.3.5 Large-scale production of chimaeric particles.....	98
5.3.6 Immunisation and animal serum analysis	100
5.4 Discussion	102
Chapter 6: Production of Rift Valley fever virus N-protein in plants for use as a potential diagnostic antigen.....	106
6.1 Introduction	106

6.2 Materials and Methods	108
6.2.1 Growth conditions and DNA manipulation	108
6.2.2 Cloning of N-protein into pEAQ-HT	108
6.2.3 Agrobacterium transformation and recombinant protein expression analysis	108
6.2.4 Large scale protein expression	109
6.2.5 Protein purification	109
6.2.6 Protein expression analysis	110
6.2.7 Liquid chromatography - mass spectrometry (LC-MS)	110
6.2.8 Transmission electron microscopy (TEM)	110
6.2.9 IgG ELISA assays	110
6.2.10 Protein stability assays	111
6.3 Results	112
6.3.1 Cloning of Rift Valley fever nucleocapsid (N) protein gene	112
6.3.2 Transient expression and detection of recombinant N-protein in crude leaf extracts	113
6.3.3 Purification of recombinant protein	114
6.3.4 IgG capture ELISA	117
6.3.5 Protein stability	119
6.4 Discussion	120
Chapter 7: Conclusion	125
7.1 Conclusion	125
7.2 Future work	128
Chapter 8: Appendix	129
Appendix A: Gene alignment between wild type, plant- and human-codon optimised sequences	129
Appendix B: Polymerase chain reaction primer sequences	134
Chapter 9: References	137
9.1 Journal Articles	137
9.2 Web page	154