Abstract

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

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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-ΔtGn and LPH-ΔtGn). 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-ΔtGn to the influenza hemagglutinin TMD-CT (LPH-ΔtGnHA).

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-ΔtGn vaccine candidate. Expression was successfully detected using the modified RVFV LPH-ΔtGn and LPH-ΔtGnHA 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-ΔtGn and LPH-ΔtGnHA vaccine candidates. They both elicited anti-Gn immune responses in vaccinated mice without the use of adjuvant. These result 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.

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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**

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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	*Bacillus thuringiensis israeliensis*
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	Hyptertranslatable
IFA	Immunofluorescence assay
IPTG	Isoprophylthio-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-morpholineethanesulforic 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

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