THE PRODUCTION OF A CRIMEAN-CONGO HAEMORRHAGIC FEVER VIRUS DIAGNOSTIC ANTIGEN IN PLANTS

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Plagiarism declaration

I, Richard Atkinson, know the meaning of plagiarism and declare that all of the work in the dissertation, save for that which is properly acknowledged, is my own.

Full Name: _____ Signature: _____

Contents

Abbreviations

BeYDV . . . Bean-Yellow Dwarf Virus

CMV . . . . . Cauliflower Mosaic Virus

CCHF . . . . Crimean-Congo haemorrhagic fever

CCHFV . . . Crimean-Congo haemorrhagic fever virus

DIC . . . . . Disseminated Intravascular Coagulation

DNA . . . . . Deoxyribonucleic acid

ELISA . . . . Enzyme-linked immunosorbent assay

FPLC . . . . Fast protein liquid chromatography

GMP . . . . . Good Manufacturing Practice

LB . . . . . Lysogeny broth

LBA . . . . . Lysogeny broth agar

NP . . . . . Nucleocapsid protein

OD . . . . . Optical Density

PBS . . . . . Phosphate Buffered Saline

PCR . . . . . Polymerase chain reaction

RNA . . . . . Ribonucleic acid

TMV . . . . . Tobacco mosaic virus (TMV)

TMSV . . . . Tomato spotted wilt virus

TSP . . . . . Total soluble protein

USSR . . . . Union of Soviet Socialist Republics

VLP . . . . . Virus-like particle

Literature Review

1.1 Crimean-Congo haemorrhagic fever virus

1.1.1 Introduction

Crimean-Congo haemorrhagic fever (CCHF) is a highly infectious, life threatening disease caused by the Crimean-Congo haemorrhagic fever virus (CCHFV), a bunyavirus in the genus *Nairovirus*, family *Bunyaviridae*. The virus can be found across Asia, Africa and Europe; endemic regions include the Balkan Peninsula, Southern Russia, and most of sub-Saharan Africa (Leblebicioglu, 2010). The first reported cases of CCHFV infection occurred in 1944 amongst military workers, on the Crimean Peninsula of the then, Union of Soviet Socialist Republics (USSR) (Hoogstraal, 1979). It was not until 1967, when the virus was propagated in suckling white mice, that the morphological, antigenic and physiological characteristics were determined. This led to the discovery that Congo Haemorrhagic Fever (CHF) was antigenically identical to a virus isolated in what was then Zaire, now called the Democratic Republic of Congo, in 1956. The virus was then renamed to the now familiar Crimean-Congo Haemorrhagic Fever Virus (Casals, 1969).

1.1.2 Virus classification

The *Nairovirus* genus is named after Nairobi sheep disease virus (Crabtree et al., 2009). The genus contains 3 notable pathogens, in CCHFV, Dugbe virus and Nairobi sheep disease virus (Clerx et al., 1981).

CCHFV has a single stranded negative sense RNA genome, consisting of 3 strands: small (S), medium (M) and large (L). The S segment codes for a nucleocapsid protein (NP) that associates with the viral RNA to form a ribonucleocapsid. This protein is the most abundant viral protein produced by CCHFV and has been shown to produce a strong, specific humoral antibody response (Marriott and Nuttall, 1992). It is for this reason that indirect ELISA diagnostic tests use the N protein (Samudzi et al., 2012).

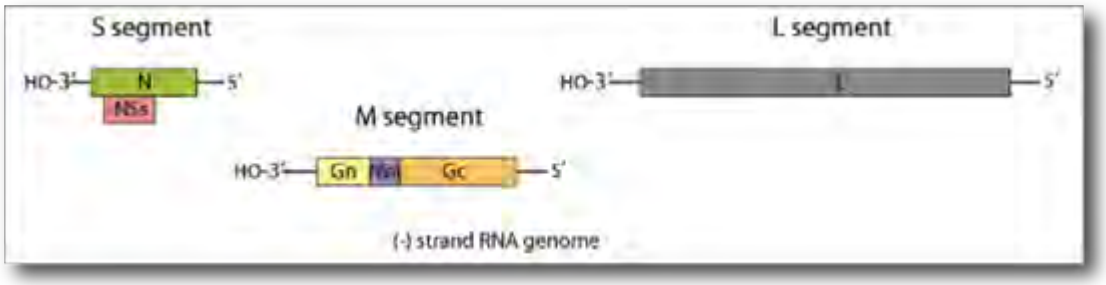


Figure 1.1: CCHFV genomic structure. Single stranded, negative sense RNA genome, consisting of 3 strands: small (S), medium (M) and large (L). Source:ViralZone:www.expasy.org/viralzone *SIB Swiss Institute of Bioinformatics*

The M segment encodes two glycoproteins, Gn and Gc, that are transcribed and translated as a single protein and modified post-translationally into two separate proteins; these glycoproteins play a role in both cell-type recognition and viral entry. The M segment also encodes for a small non-structural protein (Nsm) that is hypothesised to play a role in viral assembly. The L segment

codes for a RNA-dependent RNA polymerase that is responsible for viral RNA transcription (Figure 1.1).

All bunyaviruses have spherical virions of approximately 100 μm in diameter. The virion is enveloped, with Gn/Gc glycoprotein heterodimers extruding from the membrane across the surface of the virion (Figure 1.2).

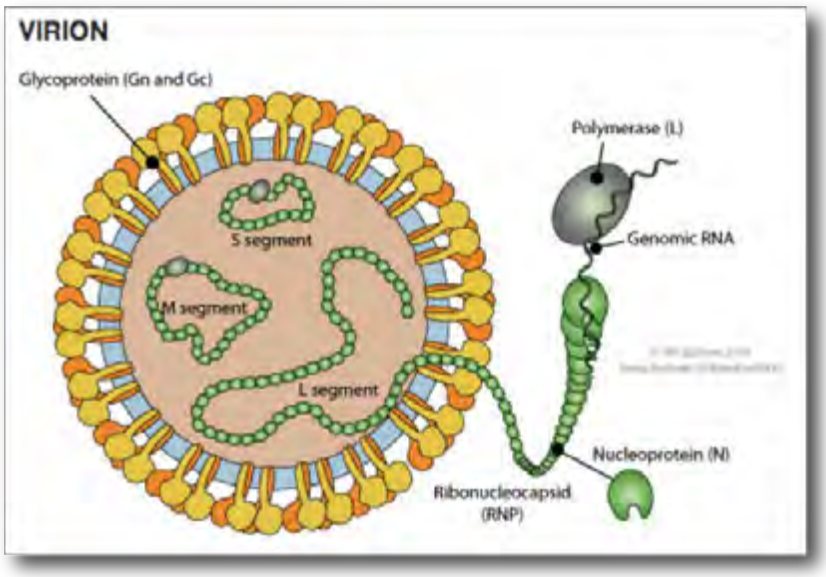


Figure 1.2: Structure of an enveloped, spherical CCHF virion which is approximately 100μm in diameter. Source:ViralZone:www.expasy.org/viralzone *SIB Swiss Institute of Bioinformatics*

1.1.3 CCHFV vectors and infection.

CCHFV has been isolated from a range of ticks, spanning 31 species and 7 genera. It has been found in both hard-bodied ixodid ticks (29 species) and soft-bodied argasid ticks (2 species) (Hoogstraal, 1979). The majority of these ticks, however, are not considered to be vectors for CCHFV and in the case of

argasid ticks, 4 different species have been independently shown to be replication incompetent hosts. There is a strong correlation between distribution of ticks from the *Hyalomma* genus and the distribution of CCHF infections. This association has led to the conclusion that ticks belonging to the genus *Hyalomma* are the primary infectious vectors for CCHFV (Hoogstraal, 1979).

Ixodid ticks follow a three-stage developmental pathway transforming from larvae into nymphs and finally maturing into adults. The larvae hatch from eggs laid by the female, and then attach to a host; this is normally a small vertebrate, such as a small mammal or bird. Once engorged on blood, they detach and molt into nymphs. The feeding and molting cycle is repeated again, after which they finally emerge as mature adults. The female adult ticks attach to large hosts to feed and lay eggs. The males have been observed to feed scarcely and their primary reason for attaching to large hosts is for mating purposes (Allan, 2001).

Transient viraemia has been demonstrated in a large percentage of small mammals in Africa, Asia and Europe. In some cases, they are able to transmit the virus to new ticks. The same is true of many large mammals, especially the preferred hosts of *Hyalomma* ticks, such as buffalo, zebra, rhinoceros and giraffe. The virus causes inapparent infection or mild fever and viraemia in its non-human hosts. Transmission occurs when a tick feeds on a host during viraemia and then feeds on an uninfected host during its subsequent life cycle stage. It is believed that the rate of transovarial transmission is too low to allow for the perpetuation of the virus. It is thus hypothesised that small mammals serve as a reservoir for the virus, infecting ticks during the nymph phase and allowing transmission to larger vertebrates (Hoogstraal, 1979).

Birds in general have been shown only to develop low levels of viraemia and it is believed that infection of ticks from birds does not occur (Shepherd et al., 1987). There is however, evidence of a phenomenon called nonviraemic transmission, where ticks carrying the virus pass it on to non-infected ticks

on the same host. Birds also play host to the nymph life stage of the ticks and thus could play a role in the dissemination of the virus within a specific region as well as along migratory pathways (Hoogstraal et al., 1961; Zeller et al., 1994).

As seen in Figure 1.3, *Hyalomma* ticks are present across all of Africa and a large portion of Europe and Asia. It has also been hypothesised that future climate change may increase the range of *Hyalomma* ticks, thus increasing the number of people who are at risk of CCHFV infection (Purnak et al., 2007).

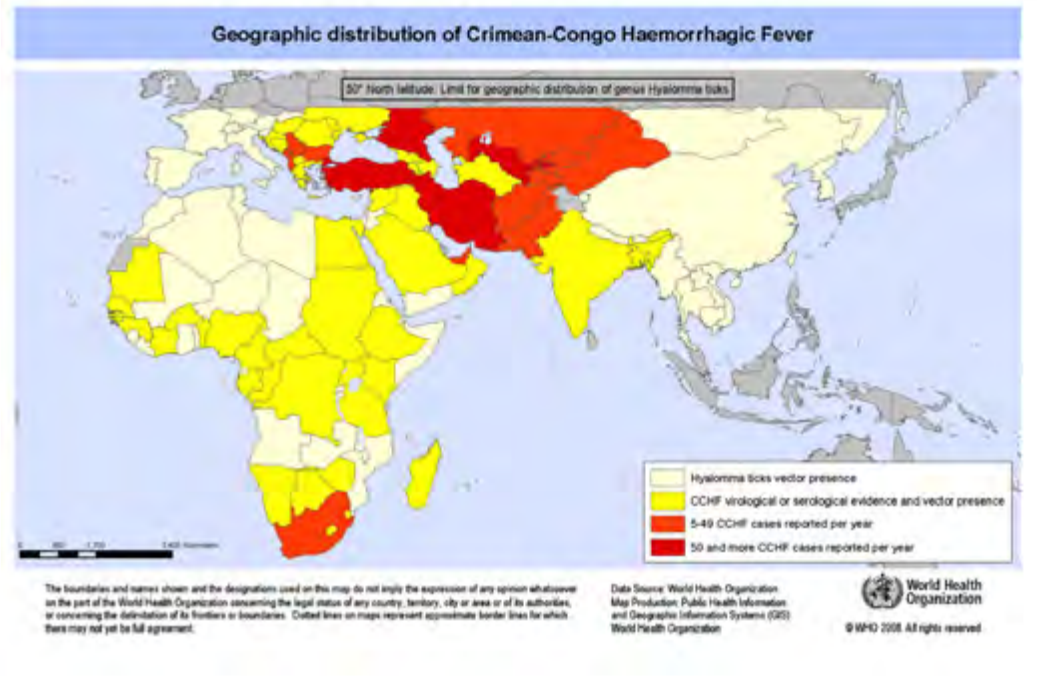


Figure 1.3: Geographic distribution of Crimean-Congo haemorrhagic fever virus.

1.1.4 Human infection

Human infection has a case fatality rate of up to 40 % (Bente et al., 2013), with infection most often occurs through the bite of a tick carrying the virus, or through contact with the tissue or blood of an infected patient or animal. For transmission to occur from infected blood, it must come into contact with an open wound or mucous membrane. The majority of CCHF cases in South Africa occur in the North West Province, Free State, and Northern and Western Cape (Horak et al., 2002). In a large portion of the cases, infection occurs in individuals who are involved in the livestock industry as well as people who visit rural areas (Swanepoel et al., 1989, 1983). An important fact to consider is that for humans to become infected, they do not have to be bitten by an infected tick; simply squashing the tick and coming into contact with their blood has been shown to cause infection. The virus can gain entry through small wounds on the hands or through mucous membranes such as the eyes (Hoogstraal, 1979).

Between 1981 and 2006, only 180 cases of CCHF have been diagnosed. In comparison to the serological evidence that the disease in livestock is widespread (Swanepoel, 1996), the prevalence of CCHF in human populations is low, even among high risk individuals. There are two possible explanations for this: first, that the majority of cases are asymptomatic and thus go undiagnosed. This, however, is unlikely as serological surveys of rural residents showed a low prevalence of CCHF antibodies in the population (Fisher-Hoch et al., 1992), discrediting this theory. The current explanations for low CCHF incidence in humans is that humans are not the preferred host for *Hyalomma* ticks, as well as the fact that the viraemia in livestock is comparatively low and short-lived when compared to other zoonotic viruses that are more readily acquired from infected tissue, such as Rift Valley fever virus (Swanepoel et al., 1987).

1.1.5 Clinical progression and diagnosis of human infection

As seen in Figure 1.4, the progression of CCHFV infection has 4 clinically distinct phases. These are the following:

1. The incubation phase, before the onset of symptoms, can last between 3 and 7 days.

2. The pre-haemorrhagic stage, classically characterised by a sudden onset of fever, headaches, myalgia and dizziness has been reported to last anything from 1 to 7 days. This large difference is possibly due to several factors, such as viral load and route of exposure. Diarrhoea, nausea and vomiting have also been reported in some cases.

3. The haemorrhagic stage is characterised by the onset of haemorrhagic symptoms. There is a large range in the severity of haemorrhagic symptoms, from mild petechial to large haematomas on the skin mucus membranes of the patient. There are also reports of bleeding from a range of other sites, including the nose, the intestines, the uterus and the urinary tract (Ergönül, 2006).

4. Patients that go on to recover generally show improvements by the ninth day of illness although symptoms such as light confusion, and amnesia can continue for a month or more (Swanepoel et al., 1989). Other symptoms of the convalescent stage include temporary loss of hair, difficulty in breathing and loss of both vision and hearing.

Diagnosis of CCHFV infection is dependent on the stage that the patient is in (Figure 1.4). During the initial stage of the infection, a PCR-based detection method is used, normally with primers targeting the S segment of the virus. The detection of antibodies against the virus (both IgM and IgG)

usually coincides with the onset of haemorrhagic symptoms (Vanhomwegen et al., 2012). The presence of antibodies is clinically significant as it has been shown to be an important marker for survivability of the infection. Patients who do not mount an antibody response to the infection by 7 days have a low survival rate (Swanepoel et al., 1989).

Antibody based detection of CCHFV at one stage required BSL4 containment (Vanhomwegen et al., 2012), as whole lysed virus-infected cells were the only source of antigen. This significantly increased the costs of the assay, as well as the time that it takes to perform. However, recently work has been done to produce recombinant CCHFV NP for use in ELISAs for diagnostics. (Samudzi et al., 2012).

The NP is an ideal diagnostic antigen, because it is the most abundant protein produced by the virus. As the protein is housed inside the viral particle, it is also a useful marker for viral clearing, as intact virus particle would not elicit a NP antibody response. The NP is also highly conserved across CCHFV virus strains (Deyde et al., 2006), allowing for improved detection of infection across a range of samples.

CCHFV NP has previously been expressed in insect (Zhou et al., 2011) and bacterial (Samudzi et al., 2012) cell culture. Insect cell culture has the drawback of high cost as well as the difficulty of producing and maintaining the required baculovirus clones (Saijo et al., 2002). Bacterial expression of CCHFV NP leads to insoluble protein being produced. This increased the downstream processing requirements, increasing the cost and time required to produce a protein ready for use in a diagnostic assay (Samudzi et al., 2012).

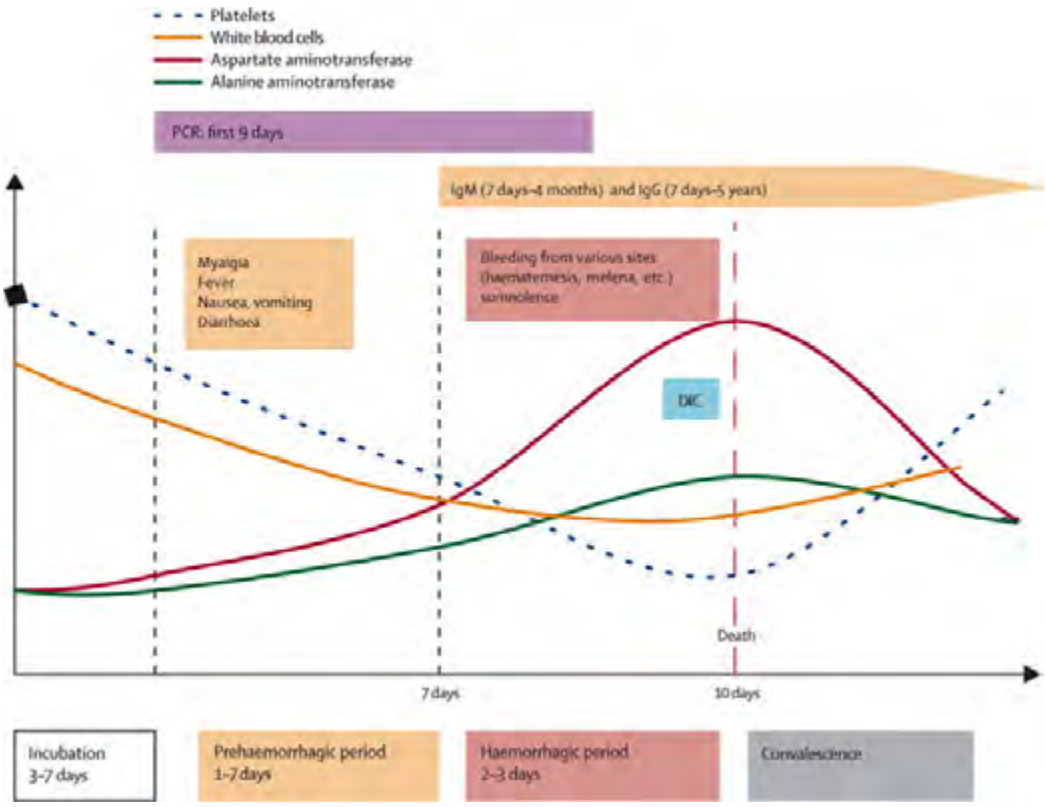


Figure 1.4: Clinical and laboratory, diagnostic course of a CCHFV infection (Bente et al., 2013).

1.1.6 CCHFV treatment

Treatment for CCHFV is mostly supportive, based on the symptoms of the patient. Fluid or whole blood replacement are common therapies to counteract the drop in blood pressure due to blood loss. In the past, early treatment with Ribavirin, an anti-vral drug, has been shown to be effective (Khan et al., 2003) although a more recent, blinded study has failed to show any therapeutic benefit when compared to supportive treatment (Koksal et al., 2010). Antibody based treatments have also been used, with limited documented

success. There is anecdotal evidence that treatment with hyperimmune serum from recovering CCHFV infected patients causes transient improvement of symptoms, however there has been no blinded clinical trial on the method (Bente et al., 2013).

1.1.7 Vaccination

In the 1970s, a formalin-inactivated, mouse brain-passaged CCHFV vaccine was developed and approved for use in the Soviet Union. A similar vaccine is currently in use in Bulgaria where it is given to high-risk individuals, such as soldiers and healthcare workers in endemic regions. There has however been no clinical trial conducted using this vaccine and serological studies of vaccinated individuals show a low level of neutralising antibodies produced. The only evidence for the vaccine's efficacy is based on the historical numbers of CCHFV infections before and after vaccination was introduced (1100 and 279, respectively). There has been little modern work on producing a CCHFV vaccine, mostly due to the lack of, until recently, an animal model to test the vaccine in. One group evaluated a multivalent DNA vaccine against the similar bunyavirus disease agents causing Rift Valley fever, tick-borne encephalitis, Hantaan River Virus and CCHFV, but it was shown to be only weakly antigenic in mice (Spik et al., 2006). Another study involved feeding mice transgenic tobacco plants expressing the CCHFV glycoproteins. The mice were shown to mount an antibody response to the glycoproteins, but no work was done to show neutralization (Ghiasi et al., 2011).

1.2 Plant-based protein expression

1.2.1 Introduction

Using plant cells as a platform for expressing recombinant proteins, either in cell culture or as part of a whole plant tissue, has been an area of interest since the late 1980s with the production of the first functional antibody in transgenic plants (Hiatt et al., 1989) and the early 1990s with the production of the first virus-like particle (VLP) in plants (Mason et al., 1992). Since then, there have been numerous changes and improvements in the field, from the movement away from stable transgenic plants to the use of deconstructed viral vectors for transient expression (Rybicki, 2014).

Proteins produced in plant-based expression systems have the advantages of being human pathogen-free as well as of containing post-translational modifications including glycosylation. Recent work has been performed on humanising the glycosylation patterns of proteins that *Nicotiana benthamiana* produces by transiently expressing the cellular machinery required for mammalian O-glycosylation and N-glycosylation (Yang et al., 2012).

1.2.2 Transgenic plants

Traditionally, expressing any protein in plants required the lengthy process of creating stable, nuclear- transformed, transgenic plant lines. Produced by using either *Agrobacterium*-mediated gene transfer (Deblaere et al., 1985) or by particle bombardment (Kikkert et al., 2005), these processes can take 2-4 months to produce recombinant proteins in *Nicotiana tabacum* and there is no guarantee that after that time the transgenic plant will express the recombinant protein. The other main limitation of nuclear transformation is the low yields of many proteins expressed using this method (Fischer et al., 2004).

To address the issue of low expression, research was done into transforming the chloroplast of the host plant. There are several advantages to this method: the full chloroplast genomes of many plant species have been sequenced, allowing for prediction of gene regulation once inserted; chloroplasts are maternally inherited, so containment of the foreign gene is easier; chloroplasts are also capable of accumulating large amounts of protein - up to 46 % TSP in stably transformed lines (Verma and Daniell, 2007). However, the long timescales required to generate transgenic plants are also present when performing chloroplast transformation. The desire for a system that can be evaluated in days, rather than months, lead researchers to focus their efforts on transient expression in plants. This focus has made transient plant expression a viable alternative to both nuclear and chloroplast transgenic plant expression (Rybicki, 2010).

1.2.3 Transient expression

Transient expression systems have allowed for rapid expression of proteins at higher levels of TSP than when nuclear transformation is performed (Daniell et al., 2009). Recombinant expression vectors can be introduced into the host cells using a process called agroinfiltration. This is achieved by vacuum infiltrating the host plant with a suspension of *Agrobacterium tumefaciens*, harbouring the recombinant expression vector of choice.

The expression vectors used are either simply *Agrobacterium*-derived plasmids, with the expression cassette inserted in the *Agrobacterium* T-DNA, or can include viral gene regulation elements, as well as functional elements, such as movement and replication proteins. The plant virus vectors that are currently in use are derived from Tobacco mosaic virus (TMV) (Marillonnet et al., 2004), Cauliflower Mosaic Virus (CMV) (Sainsbury and Thuenemann, 2009) and more recently Bean-Yellow Dwarf Virus (BeYDV) (Regnard et al.,

2010), among others.

Transiently expressed plant produced proteins are also not limited to the laboratory. Several proteins have been produced in GMP-certified facilities for use as human pharmaceuticals. Medicago Inc. have produced a pandemic influenza VLP vaccine (Vicente et al., 2011; D'Aoust et al., 2008) and the Fraunhofer USA Institute have produced an influenza vaccine based on the haemagglutinin subunit (Shoji et al., 2011). Both of these vaccines have completed phase I human clinical trials (Fischer et al., 2012). A plant-produced pharmaceutical that has held considerable public interest recently is ZMapp; this is a cocktail of three monoclonal antibodies, produced by LeafBio Inc., that is used as a treatment for Ebola virus disease. The antibodies are produced in *N. benthaniama* using both a TMV-based and PVX-based vector system and have recently been shown to induce full protection against a lethal viral challenge in non-human primates (Qiu et al., 2014).

1.3 Project aims and outline

Detection of CCHFV antibodies is currently expensive and hazardous, due to the need for live virus-infected tissue-culture and the associated BSL4 containment required for working with the cells and all resulting products. The aim of this work was to produce a CCHFV nucleocapsid protein (NP) for use in diagnostics, using a plant-based transient expression system to improve the safety as well as decrease the cost associated with using traditional diagnostic reagents. The approach included the following:

- Synthesis of the CCHFV *NP* gene and cloning into three different expression vectors in order to compare NP expression levels after *Agrobacterium*-mediated infiltration with the 3 different constructs. This included comparisons of NP expression when *Agrobacterium* constructs were co-infiltrated with a silencing suppressor as well as the comparison of NP expression levels after harvesting at different time periods post infiltration.

- Selection of the construct and conditions at which the highest levels of protein were expressed and the subsequent development of a purification protocol of NP in order to purify it.

- Testing of the ability of the plant-produced NP protein to be used as a diagnostic reagent to detect anti-CCHFV antibodies in serum from individuals previously infected with CCHFV.

Expression of Crimean-Congo haemorrhagic fever virus nucleocapsid protein in *Nicotiana benthamiana*

2.1 Introduction

The Crimean-Congo haemorrhagic fever virus (CCHFV) nucleocapsid protein (NP) is encoded for on the S segment of the virus genome and is an important antigen used for the diagnosis of CCHFV infection (Bente et al., 2013). Human and veterinary surveillance is an important part of monitoring and predicting potential outbreaks of the virus. Thus there is a great need for a specific, sensitive and cheap assay for infection that can be deployed across Africa, Asia and Russia.

Plant cells contain a defence system against viral infection, termed post-translational gene silencing. This can decrease yields of recombinant proteins expressed in plants. To counter this, a gene-silencing suppressor, such as the NSs protein from tomato spotted wilt virus (Prins et al., 1996), is often co-expressed with the protein of interest. This has been shown to increase the overall yield of recombinant proteins harvested from *N. benthaniama* leaves (Maclean et al., 2007).

To facilitate detection and purification of recombinant proteins, affinity tags are often added to the sequence of the protein of interest. The polyhistidine tag is often used in recombinant protein expression as it has several desirable properties: small size (0.84 kDa), low immunogenicity, minimal effect on protein function and the availability of high quality detection antibodies (Young et al., 2012). Combined with the ease and flexibility of purification that it allows, this makes the polyhistidine tag an ideal affinity tag.

In this chapter, I will discuss the expression optimisation of Crimean-Congo haemorrhagic fever virus nucleocapsid protein (NP) in *N. benthamiana*. Selecting the best combination of expression time and vector used as well as the inclusion of a RNA silencing suppressor resulted in a high level of expression, essential for further work on purification and characterisation.

The cloning and expression of CCHFV NP reported in this chapter lays the groundwork for further work in bulk expression and purification of NP as well as serology using the NP as a capture antigen.

2.2 Materials and methods

2.2.1 Codon optimisation of the *NP* gene

The coding sequence of the CCHFV isolate SPU 431/85 S segment encoding the *NP* gene (GenBank: DQ211648.1) was submitted to GenScript Inc. (USA) for *Nicotiana sp.* codon optimisation and gene synthesis. Codon optimisation was performed using OptimumGene^{TM} by Genscript Inc. The following parameters were optimised for *N. tabacum*:

- Codon usage bias
- GC content
- Unwanted restriction enzyme sites
- *Cis*-Acting Elements
- Repeat sequences

2.2.2 Gene synthesis

The CCHFV S segment (GenBank: DQ211648.1) was synthesised as a single gene by GenScript Inc. (USA) and cloned into pUC57-Kan (GenScript Inc. USA) (Figure 2.1) using the blunt cutting EcoRI site. This was designated pUC57-N.

2.2.3 Plant expression vectors

The plant expression vectors pTRAc-HT (provided by Rainer Fischer of the Fraunhofer Institute for Molecular Biology and Applied Ecology, IME, Germany), pRIC-3.0-HT (Regnard et al., 2010), and pEAQ-HT (Prof G Lomonos-

soff at the John Innes Centre, Norwich and Plant Biosciences Limited, Norwich) were used in this study.

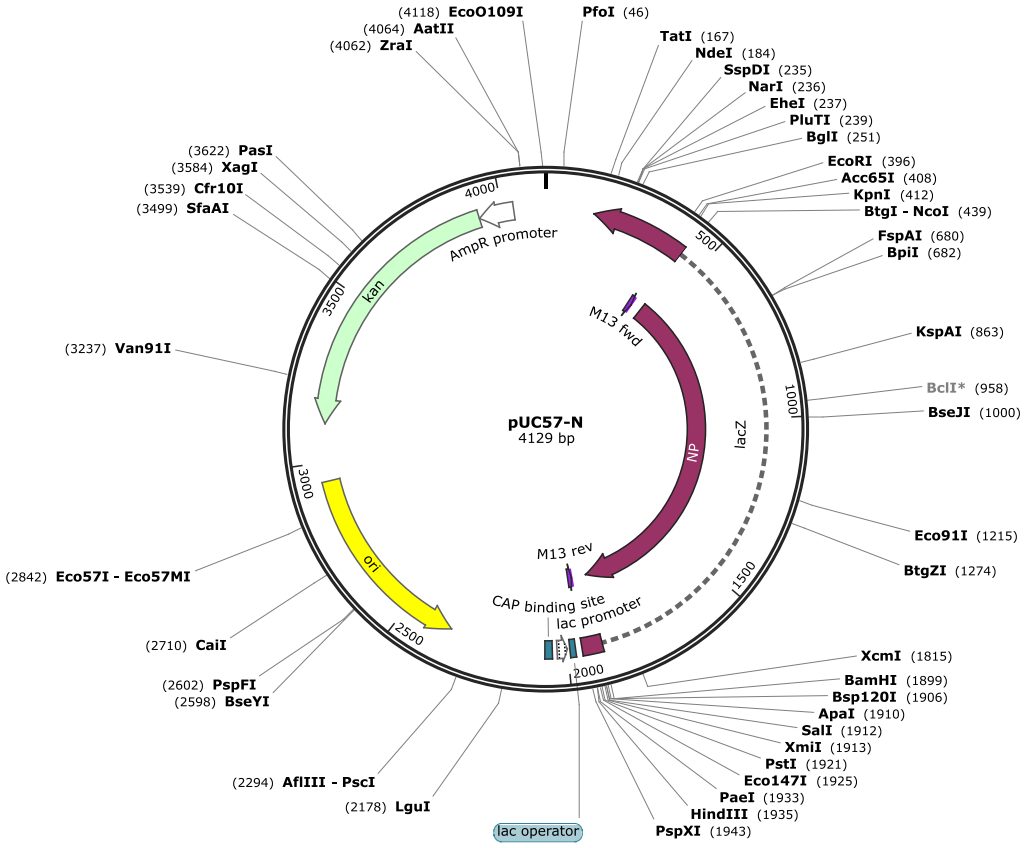


Figure 2.1: pUC57-N - The vector includes a *lacZ*-alpha site for blue/white cloning selection and an aminoglycoside phosphotransferase (*KanR*) gene to confer resistance to kanamycin. The *NP* gene is cloned into the single EcoRI site.

A summary of all of the vectors and their antibiotic resistance markers used in this study can be found below in Table 2.1.

Table 2.1: Plasmids used in this study

Plasmid	**Antibiotic and working concentration**
pUC57-N	Kanamycin, 50 µg/ml
pRIC 3.0-HT	Ampicillin, 100 µg/ml
pTRAc-HT	Ampicillin, 100 µg/ml
pEAQ-HT	Kanamycin, 50 µg/ml

2.2.4 Cloning strategy

The coding sequence for NP was cloned into pTRAc-HT, pRIC 3.0-HT and pEAQ-HT. For all of the expression vectors, a C-terminal polyhistidine tag was desirable and already present in the vector. The correct restriction enzyme sites were included on the 5' and 3' termini of the synthesised NP sequence to allow for direct cloning from pUC57-N into the various expression vectors. This process is shown in 2.2. Briefly, pTRAc-HT, pRIC 3.0-HT and pEAQ-HT and pUC57-N were isolated from single colonies grown on the appropriate selective media, using a QIAGEN Miniprep Kit (Qiagen, USA) as per the manufacturer's instructions. The plasmids were then digested using the appropriate restriction enzymes (see Figure 2.2) and ligated overnight at 4 °C using T4 ligase (Roche). The ligated DNA was transformed into chemically competent DH5α *E. coli* (*E. cloni*®*, Lucigen*) as per the manufacturers instructions and the resulting transformants were screened by colony PCR using either specific primers (Table 2.2) or restriction enzyme digests to confirm successful cloning.

Table 2.2: List of Primers used in this study.

Primer Name	**Sequence**	**Tm** (°C)	**Description**
pEAQ Forward	5' GACGAACTTGGAG AAAGATTGTTAAGC 3'	61,9	Vector-specific primer for use in PCR screening and sequencing.
pEAQ Reverse	5' GACCGCTCACCAA ACATAGAAATG 3'	61,6	Vector-specific primer for use in PCR screening and sequencing.
pTRAc Forward	5' CATTTCATTTGGA GAGGACACG 3'	61,3	Vector-specific primer for use in PCR screening and sequencing.
pTRAc Reverse	5' GAACTACTCACACAT TATTCTGG 3'	61,0	Vector-specific primer for use in PCR screening and sequencing.
pICH31070 Forward	5' CCTCACCCATCTTTT ATTAC 3'	58,4	Vector-specific primer for use in PCR screening and sequencing.
pICH31070 Reverse	5' ACACCGTAAGTC TATCTC 3'	57,1	Vector-specific primer for use in PCR screening and sequencing.

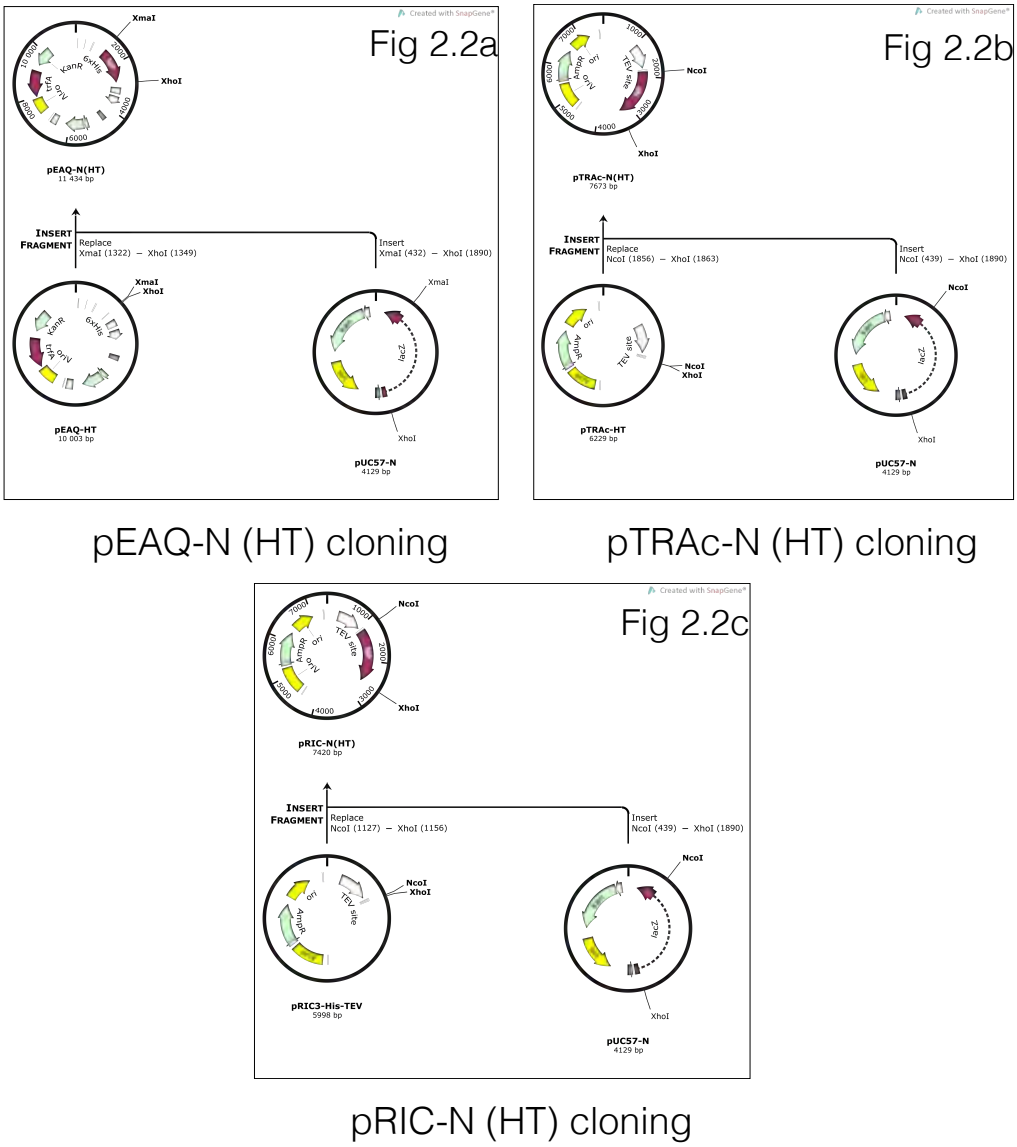


Figure 2.2: Cloning strategy for expression of NP. (a) XmaI and XhoI were used to clone *NP* into pEAQ- HT as well as including an in frame C-terminal his-tag. (b+c) NcoI and XhoI were used to clone *NP* into pTRAc-HT(b) and pRIC 3.0-HT(c) as well as including an in frame C-terminal his-tag.

2.2.5 *A. tumefaciens* transformation

A. tumefaciens transformation was performed by electroporation. The electrocompetent cells were prepared as previously described (Shen and Forde, 1989). The *A. tumefaciens* GV3101::pMP90RK was used for pRIC3.0-HT and pTRAc-HT constructs and the strain LBA4044 was used for pEAQ-HT constructs. Transformation was performed by mixing 100ul of electrocompetent cells with 500ng of plasmid DNA in a 1mm gap electroporation cuvette. Electroporation was performed at 1.8kV, 25mF, 200Ω with a Bio-Rad GenePulser (Bio-Rad Laboratories, USA). The cells were incubated in Lysogeny broth (LB) broth for 2 hours at 27 °C and then plated on the LB agar (LBA) plates containing the relevant selective antibiotics (Table 2.3). The plates were then incubated at 27 °C for 48h. Resulting single colonies were picked and grown in LB broth with the appropriate antibiotics for 48h. Plasmid was isolated using a QIAGEN Miniprep Kit (Qiagen, USA) as per the manufacturer's instructions and transformed back into competent DH5α *E. coli (E. cloni®, Lucigen)* to confirm successful transformation of the *A. tumefaciens* with the expression plasmid.

Table 2.3: *A. tumefaciens* strains used in this study

A. tumefaciens strain	Plasmids used	Antibiotic Selection	Notes
GV3101::pMP90RK	pRIC 3.0-HT	30 µg kanamycin	
	pTRAc-HT	50 µg carbenicillin	
		50 µg rifampicin	
LBA4044	pEAQ-HT	30 µg kanamycin	2 mM MgSO4 to prevent cell clumping
	pBIN-NSs	50 µg rifampicin	

2.2.6 Agroinfiltration of *N. benthamiana* leaves for small-scale transient protein expression trials

A single, freshly streaked colony of the appropriate *A. tumefaciens* strain was inoculated into 10ml of LB media containing the appropriate antibiotics (Table 2.3) and incubated with shaking at 150rpm at 27 °C for 24h. The resulting culture was then diluted into 50ml of induction medium (LB broth with 10 mM MES, 2 mM MgCl2, 1 mM acetosyringone, pH 5.6) to obtain an OD_{600} of 0.1 and then incubated with shaking at 150rpm at 27 °C for 24h. The culture was centrifuged for 10 minutes at 4000 x g at 4 °C to pellet the *A. tumefaciens* cells. The cells were then resuspended in infiltration medium (3 % (w/v) sucrose, 10 mM acetosyringone, 10 mM MES, 2 mM MgCl2, pH 5.6). The culture was diluted to the appropriate OD_{600} for infiltration (Table 2.4) and allowed to incubate at room temperature for 2 hours to induce the *vir* genes. Six to 8 week old *N. benthamiana* plants were infiltrated with the *A. tumefaciens* suspension by injection in to the abaxial airspaces of the ventral side of the leaves using a needleless syringe.

Table 2.4: Dilution of cultures for *A. tumefaciens* small-scale infiltration trials.

***A. tumefaciens* strain**	**Plasmid**	**OD_{600}**
GV3101::pMP90RK	pRIC-N (HT)	0,25; 0,5; 1,0
	pTRA-N(HT)	0,25; 0,5; 1,0
LBA4404	pEAQ-N(HT)	0,25; 0,5; 1,0
	pBIN-NSs	0,25

2.2.7 Small-scale protein extraction

Three leaf-disks were harvested from 3 different plants, using the cap of a 1.5ml microcentrifuge tube (each disk weighing approximately-10mg) and immediately flash-frozen and ground into a fine powder using liquid nitrogen in a mortal and pestle. The different plants were all of a similar age and efforts were made to ensure that similar age leaves were used for each data point. Three hundred µl of PBS was added to resuspend the leaf powder. The resulting extract was centrifuged at 13000 x g for 10 min at 4 °C to pellet the insoluble material. The extract was immediately prepared for SDS-Page by the addition of 5 x SDS sample buffer (25 % (v/v) glycerol, 0.5 M DTT, 5 % (w/v) SDS, 0.001 % (w/v) bromothymol blue) and incubation at 95 °C for 5 minutes. The protein sample was either used immediately or frozen at -20 °C.

2.2.8 Protein electrophoresis

Proteins in prepared samples were separated by SDS-Page (Sambrook and Russell, 2006). Ten-percent SDS-polyacrylamide gels were cast and run using the Biorad Mini-PROTEAN® Tetra Cell Systems (Bio-Rad Laboratories, USA) at a constant voltage of 125V as per the manufacturer's instructions. All gels were loaded with constant volumes of protein extract and the PageRuler pre-stained protein ladder SM6071 (Fermentas, Canada) was used as a molecular weight standard.

2.2.9 Western blotting

The electrophoresed protein was transferred to a nitrocellulose membrane (Hybond ECL, GE Healthcare, USA) using a Bio-Rad Trans-Blot® Semi-dry transfer blotter at 15V for 90 minutes. The membrane was then blocked using blocking buffer (5 % skim milk powder in PBST) for 30 minutes at room

temperature. Primary antibody (anti-6x His tag antibody, Abcam [HIS.H8]) was diluted as appropriate (1/2000) in blocking buffer and incubated at 4 °C for 16h, a NP-specific antibody was not available. The membrane was then washed 4 x 15 minutes in blocking buffer. Diluted secondary antibody conjugated to alkaline phosphatase was incubated on the membrane for 1 hour at 37 °C. The membrane was finally washed for 4 x 15 minutes in PBST lacking milk and the blot was developed using NBT/BCIP substrate (Sigma-Aldrich, USA).

2.3 Results

2.3.1 Gene optimisation

Codon optimisation of the *NP* for expression in *Nicotiana benthamiana* was successfully carried out. The Codon Adaptation Index (CAI) was improved from 0,73 to 0,87 (Figure 2.3a), Frequency of Optimal Codons (FOP) was shifted so that 64 % of the codons fell within the highest usage frequency of *N. benthamiana* (2.3b) and finally the GC content of the gene was reduced from 47.72 % down to 40.4 % to bring it in to line with the GC content of the *Nicotiana sp.* genome GC content (2.3c).

Several internal restriction enzyme sites that would have interfered with subcloning were also removed from the sequence, along with 3 different cis-acting elements (PolyA(AATGAA); PolyA(AATGGA) and PolyA(AAAAAAA)). The final gene sequence, aligned with the original sequence is presented in Figure 2.4.

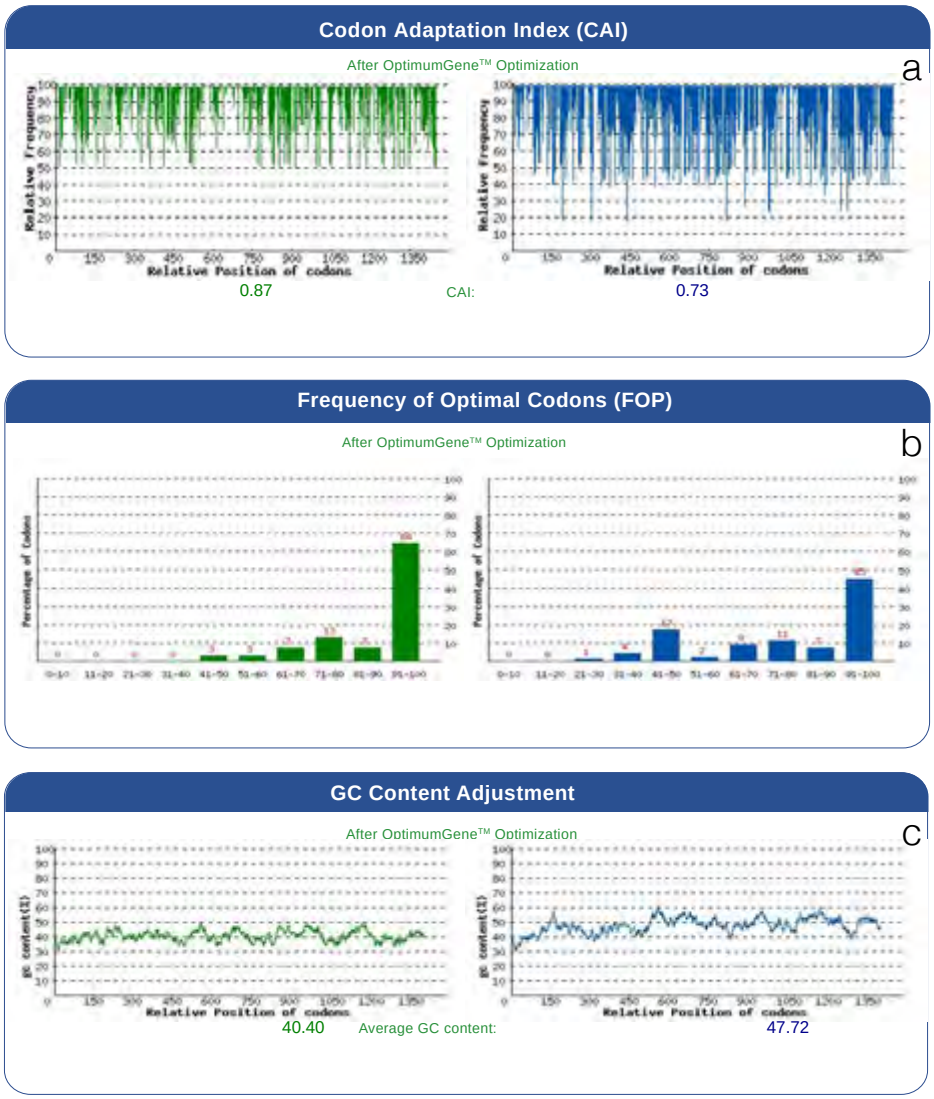


Figure 2.3: Codon and GC content optimisation of the *NP* gene. a) The distribution of codon usage frequency along the length of the gene sequence. A CAI of 1.0 is considered to be perfect in the desired expression organism, and a CAI of > 0.8 is regarded as good, in terms of high gene expression level. b) The percentage distribution of codons in computed codon quality groups. The value of 100 is set for the codon with the highest usage frequency for a given amino acid in the desired expression organism. c) The ideal percentage range of GC content lies between 30 % and 70 %. Peaks of GC content in a 60 base pair window have been removed.

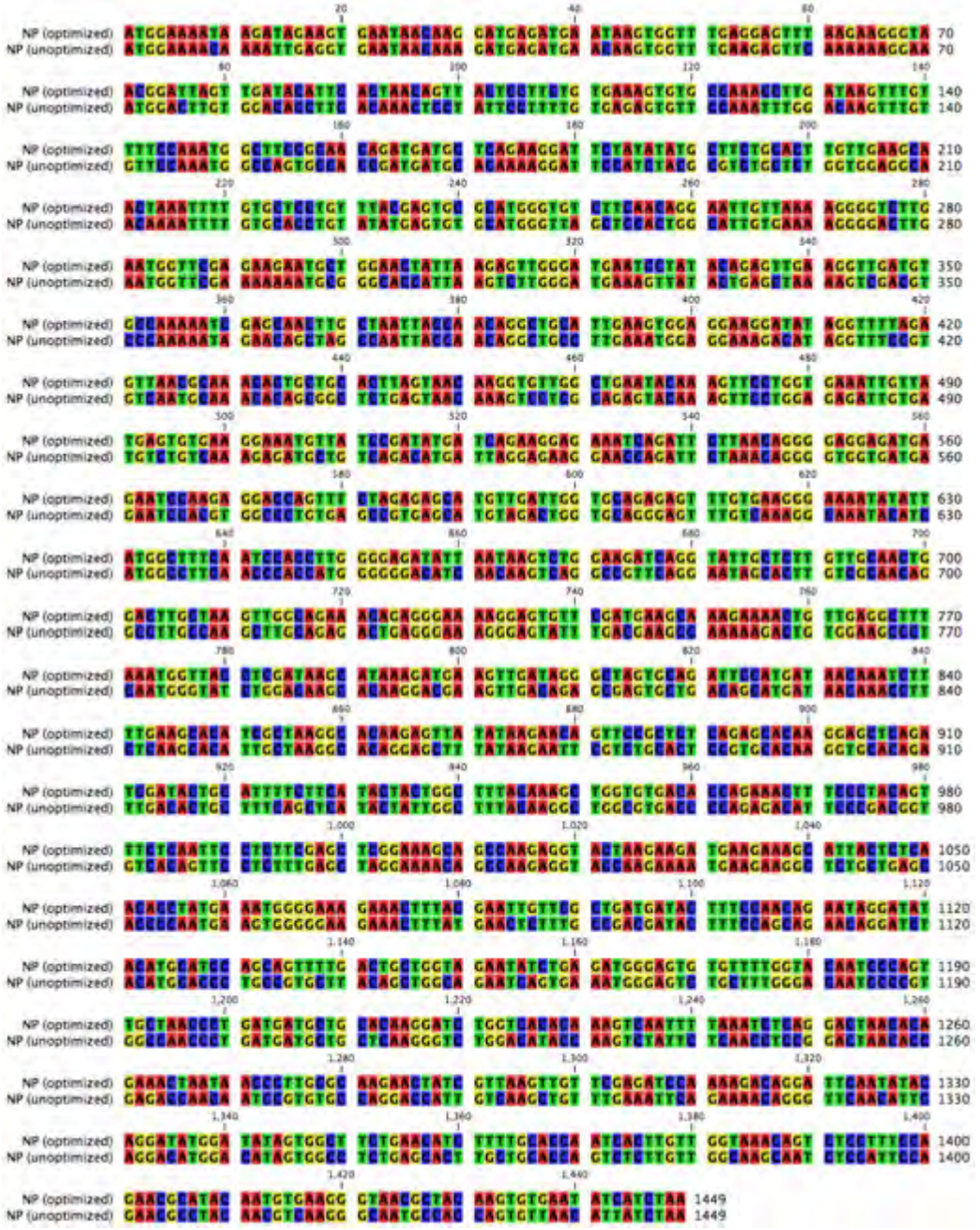


Figure 2.4: Alignment of codon optimised *NP* nucleotide sequence (NP (optimised)) with the wildtype (NP (unoptimised)) *NP* gene sequence (GenBank: DQ211648.1). The unoptimised gene is shown on the left and the optimised gene is shown on the right.

2.3.2 Cloning

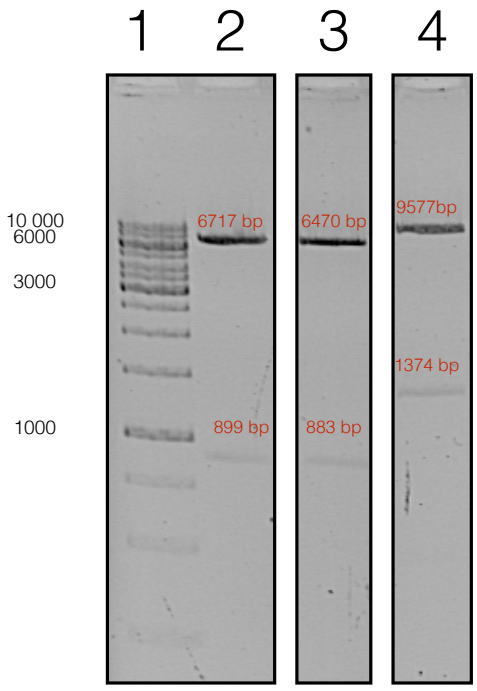


Figure 2.5: Restriction enzyme digests (XbaI) of recombinant expression constructs: (1) GeneRuler 1Kb DNA ladder (2) pTRAc-N(HT), (3) pRIC-N (HT) and (4) pEAQ-N(HT). Fragment sizes correspond to theoretical sizes calculated using SnapGene

2.3.3 Protein expression

Once the expression vectors were successfully transformed into *A. tumefaciens* and confirmed as described in the section 2.2.5, an expression trial was set up to compare the expression of NP over the course of 7 days, the effect of different concentrations of *Agrobacterium* (Table 2.4), as well as the effect of co-infiltration of a post translational silencing suppressor with the pRIC and pTRAc clones, in this case pBIN-NSs (Prins et al., 1996). The pEAQ-HT vector contains its own p19 silencing suppressor (Sainsbury and Thuenemann, 2009) gene, so co-infiltration with pBIN-NSs was not necessary when infiltrating with pEAQ-N(HT).

Initial trials showed that NP was expressed at its highest yield at an OD_{600} of 1.0 across all 3 vectors (data not shown). Subsequently all trials were done at an OD_{600} of 1.0.

Levels of NP in crude leaf extracts were assessed qualitatively by western blotting. NP was detected as a 55kDa sized protein using anti-6 x His tag antibody (Abcam, USA) (Figure 2.6 - lanes depicted as -). Leaves infiltrated with pRIC-N(HT) showed the expression of NP from day 1 through to day 7 post infiltration with the highest protein yield being observed on day 3 (Figure 2.6a). Leaves infiltrated with pTRAc-N(HT) showed peak expression of NP occurring from day 3 through to day 5 post infiltration (2.6b) Prior to day 3 and after day 5, expression yields decreased. Leaves infiltrated with only infiltration buffer showed no expression.

With regard to the barely discernible NP expression using pEAQ-N(HT), the culture used for infiltration was subsequently screened to determine whether the *NP* gene or the entire construct had perhaps been ejected from the *Agrobacterium* cells. However, it was confirmed that pEAQ-N(HT) was present in the *A. tumefaciens* used to infiltrate the plants, using back transformation and restriction enzyme digests (not shown) and therefore concluded

that the barely detectable presence of NP protein was due to low expression by the pEAQ-HT vector (Figure 2.6c). All further work was thus done using pRIC-N(HT) and pTRAc-N(HT).

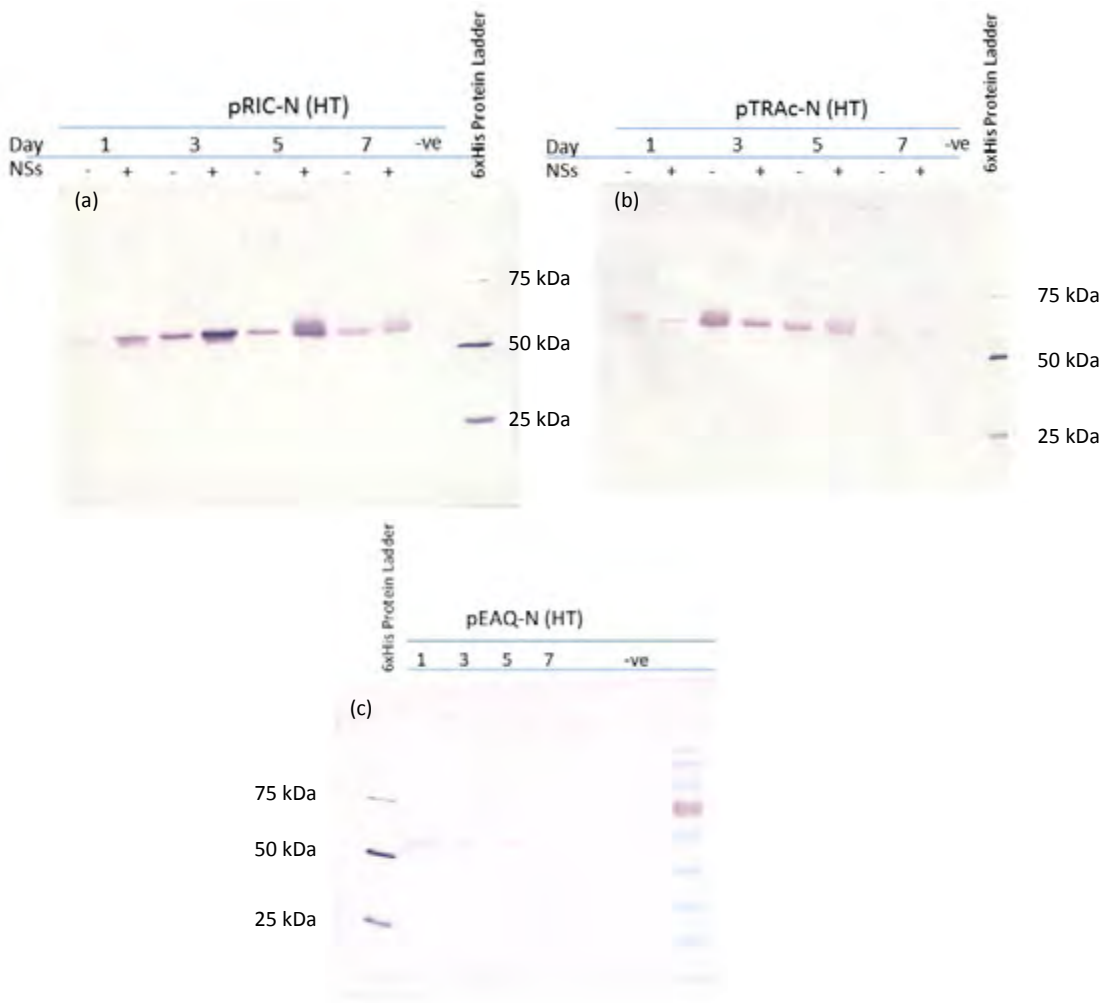


Figure 2.6: Expression time-trial of a) pRIC-N(HT), b) pTRAc-N (HT) and c) pEAQ-N (HT). (+) and (-) indicate samples infiltrated with and without NSs respectively. (-ve) represents samples infiltrated with infiltration buffer only.

Expression yields of NP in crude leaf extracts from leaves infiltrated with pRIC-N(HT) or pTRAc-N(HT) only (Figure 2.6 a and b, lanes depicted as -)

were compared when these were co-infiltrated with the pBIN-NSs silencing suppressor construct (Prins et al., 1996) (Figure 2.6 a and b, Lanes depicted as +).

Higher yields of NP were detected using pRIC-N(HT) when the construct was co-infiltrated with the silencing suppressor, particularly at days 3 and 5 post infiltration (Figure 2.7a). Co-infiltration of the silencing suppressor with pTRAc-N(HT) did not appear to qualitatively increase or decrease expression of NP (Figure 2.7b). In order to distinguish which of these two constructs to select for scaling up of NP production and subsequent purification, a further small-scale expression experiment incorporating parameters which resulted in the four highest levels was carried out to determine the optimum expression conditions. These were: infiltration of pTRAc-N(HT) at days 3 and 5 post infiltration with no silencing suppressor and pRIC-N(HT) at days 3 and 5 post infiltration with the silencing suppressor (Figure 2.6). Western blot results of this experiment are shown in Figure 2.7.

Crude leaf extracts pRIC-N(HT) co-infiltrated with the silencing suppressor and sampled at 3 days post infiltration produced the darkest bands on a western blot, suggesting that this combination of constructs and these conditions are optimal for recombinant NP expression. This construct was therefore selected for the scaled-up production and purification of NP.

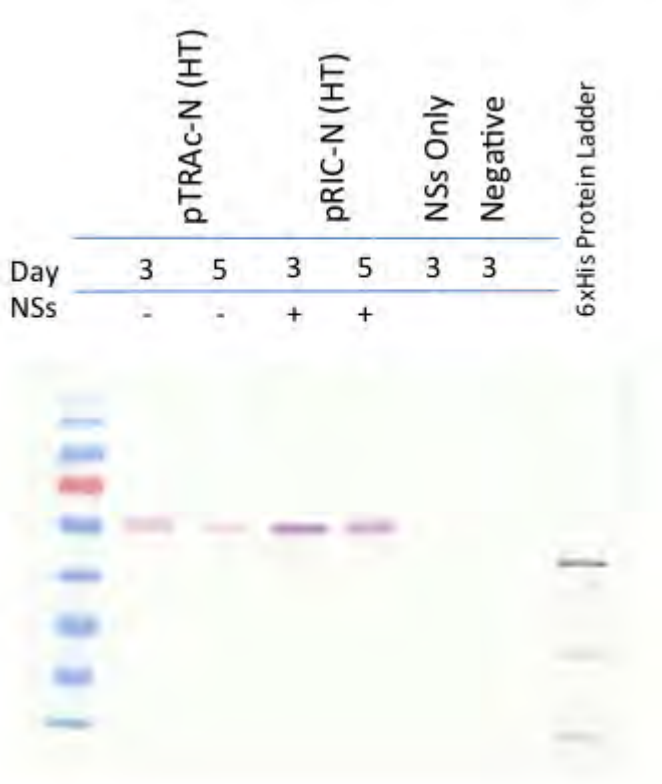


Figure 2.7: Comparison of expression between pRIC-N (HT) and pTRAc-N (HT) on day 3 and 5, with (+) and without (-) pBIN-NSs.

2.4 Discussion

A. tumefaciens mediated transient expression in *N. benthamiana* is an extremely fast and efficient method for evaluating the expression of novel proteins in plants. It has been shown to result in superior expression yields when compared to yields of recombinant protein expressed in transgenic plants (Rybicki et al., 2012).

Codon optimisation and synthesis of NP allowed for the gene to be rapidly cloned into the three expression vectors tested, cutting out the intermediate PCR steps that are required to add the correct restriction enzyme sites to a cDNA clone or any other natural DNA source. Coupled with the rapid, transient expression, this allowed us to rapidly determine the optimal expression conditions under which to conduct *Agrobacterium*-mediated infiltration in order to obtain maximal recombinant NP yields prior to continuing with scaling up production and purification and analysis of NP. Unfortunately, there was no reference standard available to quantify the amount of NP expressed in *N. benthamiana.* Comparisons of expressed NP yields by western blotting showed that while pRIC-N(HT) and pTRAc-N(HT) showed expression over a period of 1 to 7 days, pEAQ-N (HT) showed very low expression yields in comparison to pRIC-N(HT) and pTRAc-N(HT). Low protein expression is a complex problem, often driven by a variety of factors (Duong-Ly and Gabelli, 2014). As expression was achieved using the pRIC 3.0 and pTRAc vectors, optimisation of a pEAQ based expression system was abandoned. The expression vectors pTRAc-HT and pRIC 3.0-HT both make use of a modified Cauliflower mosaic virus (CaMV) 35S promoter to drive expression of the recombinant gene (Maclean et al., 2007). pRIC 3.0-HT has however been modified to include Bean yellow dwarf virus (BeYDV) replicating machinery *in cis*. This allows for many, replicating copies of the gene of interest to be produced, which also increases the mRNA levels of the gene present in the

cell (Regnard et al., 2010).

pBIN-NSs is derived from the S-segment of tomato spotted wilt virus (TMSV). pBIN-NSs acts by suppressing the gene silencing machinery of the host plant, in this case, *N. benthamiana* (Prins et al., 1996). In this study, we saw that inclusion of pBIN-NSs with pRIC-N(HT) allowed increased expression, but with pTRAc-N(HT) there appeared to be more expression when it was not included. This is most likely due to the host gene silencing machinery not being activated when pTRAc-N(HT) was used, due to the low copy number present in each cell, and it was likely detrimental to expression, because the cell's gene expression organelles, proteins and RNA had to be split between the expression of N and NSs. However, with pRIC-N (HT), the high levels of mRNA present in the cells may have induced a host gene silencing response, hence the increased expression yields seen when pBIN-NSs was included with pRIC-N(HT). The expression across both vectors, was however low, especially when compared to other proteins previously expressed using these vectors (Maclean et al., 2007; Regnard et al., 2010) In both cases, very little, to no signs of necrosis or physiological stress were observed on the leaves of infiltrated plants. At 3 days post infiltration, it was difficult to visually differentiate between plants infiltrated with the construct and plants infiltrated with only infiltration medium. This indicates that the protein is either does not effect the plans physiology, or is expressed in a concentration that is too low to effect the plants physiology.

In work done for this chapter, 3 different plant expression vectors were tested for their ability to express CCHFV NP with only two showing any meaningful levels of expression as detected by western blotting. Of the two that showed meaningful expression yield, there was a noticeable difference in the NP yields, showing that when expressing proteins in plants, it is best to test several different expression vectors when determining the optimal expression conditions. NP was successfully expressed and parameters established under

which to obtain the highest levels in *N. benthamiana* leaves as determined by comparative western blots. This enabled us to identify the recombinant constructs with which to continue working as pRIC-N(HT) and pBIN-NSs as well as to establish that harvesting of leaves at 3 days post infiltration was optimal, laying the way for further work on scaling up production and purification of NP.

Purification of Crimean-Congo haemorrhagic fever virus nucleocapsid protein

3.1 Introduction

Purification of a single protein from a complex host mix of proteins is often required for further characterisation and use downstream.

There are several strategies for purifying proteins, all of which take advantage of a certain biochemical characteristic of the protein itself, or a recombinant "affinity-purification tag" that has been added to the protein, to separate it from the complex mix. The general method for this separation is termed chromatography.

Using an affinity tag has several advantages, such as easy protein detection, improved solubility and stability, as well as several disadvantages, including interference with native protein structure and the inability to completely remove the tag if needed.

Affinity tag purification may also not produce a sufficiently pure protein. In cases like this, further biochemical methods, such as ion-exchange or gel filtration can be used to separate the remaining proteins.

The reliability and efficiency of chromatography can be improved by using a column-based, automated purification system. This allows for improved repeatability, as well as ease of scaling up if required.

For both time, cost and purification efficiency reasons, the protein of interest must be present in sufficiently high concentrations before continuing with the necessary chromatography. If this is not the case, a selective, concentrating step can be used. An example of this, ammonium sulphate precipitation, makes use of a proteins' varying solubility at varying concentrations of salt to separate these proteins. Ammonium sulphate precipitation can also be used to concentrate a protein sample after chromatography, such as gel filtration.

In this chapter, the purification of Crimean-Congo haemorrhagic fever virus nucleocapsid protein (NP) from a complex mix of host plant proteins, to a sufficient purity for further use and characterisation is described. Using a combination of precipitation and affinity tag purification coupled with an automated column chromatography system (ÄKTA Explorer, GE Healthcare), allowed for rapid, cost effective and scalable purification.

3.2 Materials and methods

3.2.1 Large-scale *Agroinfiltration* of *N. benthamiana*

Day 1: Pre-cultures of recombinant *A. tumefaciens* harbouring pRIC-N(HT) and pBIN-NSs were started by inoculating 5ml of YEB medium (per/L: beef extract; 5g; yeast extract: 1g; peptone: 5g sucrose: 5g; $MgSO_4$: 300 mg), containing the appropriate antibiotics (Table 2.3), from freshly streaked LBA plates of the respective *Agrobacterium* hosts. The cultures were incubated at 27 °C for 16h.

Days 2 and 3: The entire pre-cultures were inoculated into 250ml of YEB medium, containing the appropriate antibiotics but excluding rifampicin. These cultures were grown at 27 °C for 40h.

Day 4: The OD_{600} values of the cultures were determined and were subsequently diluted to an OD_{600} of 1.0 for pRIC-N (HT) and 0.25 for pBIN-NSs, using 2 x infiltration medium (per/L: sucrose: 100g; glucose: 3,6g; Ferty-2-Mega: 1g; pH 5.6). Acetosyringone was added to a final concentration of 200 µM.

Twenty 6 to 8 week old *N. benthamiana* plants were vacuum-agroinfiltrated by submerging whole plants in the infiltration medium, applying a vacuum of −90 Pa and quickly releasing the vacuum. The plants were then grown under conditions of a 16:8 hour light:dark cycle, a temperature of 24 °C and humidity of 55 % until ready for harvest (3 days post infiltration).

3.2.2 Protein extraction

Three days post infiltration the infiltrated leaves were harvested, weighed and 1 x PBS added at a volume of 3ml/g of leaf material. The mixture was then homogenised on ice until a uniform consistency was achieved and then incubated at 4 °C for 1h.

The extract was maintained at 4 °C for all subsequent steps.

The extract was clarified by first filtering through Miracloth^{TM} (22-24µm pore, Millipore, USA) and then centrifugation at 10 000 x g for 20 min was performed. This was repeated 3 times, retaining the supernatant at each step.

Finally, 0.02 % sodium azide was added to inhibit bacterial growth. The extract was either further purified immediately or stored at 4 °C until purification could be performed.

3.2.3 Ammonium sulphate precipitation

Precipitation was performed by adding solid ammonium sulphate ($(NH_4)_2SO_4$) to the protein extract prepared as describe in 3.2.2. The $(NH_4)_2SO_4$ was added slowly to the extract and kept at 4 °C with constant stirring. The amount of solid $(NH_4)_2SO_4$ required was calculated using the online tool available at `http://www.encorbio.com/protocols/AM-SO4.htm`. Once the $(NH_4)_2SO_4$ was added, the extract was left at 4 °C for 1 hour, with stirring, to equilibrate and for the protein to precipitate. Finally, the extract was centrifuged at 10 000 x g for 20 minutes, to pellet the extracted protein. The supernatant was removed and either discarded, or precipitated using a higher percentage of $(NH_4)_2SO_4$, as needed. The pellet was either stored at 4 °C, or dissolved in buffer and used immediately for downstream purification. For bulk precipitation, the 40-80 % fraction was used as it contained the majority of the target protein (Figure 3.1).

3.2.4 Chromatography

Chromatography was performed using an automated, fast protein liquid chromatography (FPLC) system (ÄKTAexplorer 100). The system has a multi-wavelength UV light absorption detector, as well as a conductivity meter, to aid the purification process.

FPLC systems have several advantages over manual processing, such as improved purity and consistency, as well as cost saving (Fichtali et al., 1992).

All samples were filtered through a 0.45 µm polyvinylidene fluoride (PVDF) syringe filter to ensure no particulate matter was present, which would interfere with or damage the chromatography matrix.

3.2.5 Nickel affinity chromatography

Nickel affinity chromatography was performed using a 1ml HisTrap HP pre-packed column (GE Healthcare, USA). The buffer flow rate throughout was set at 1ml/min. The column was equilibrated with 10 column volumes of 500mM NaCL PBS (standard 1 x PBS supplemented with NaCl to a final concentration of 500mM), supplemented with 20mM imidazole (Buffer A). Protein sample was injected directly onto the column using a 50ml SuperLoop and washed with 20 column volumes of buffer A. The protein was eluted using a linear gradient of imidazole (expressed as a percentage of Elution Buffer) from 20mM - 500mM. All eluting fractions were collected in 0.5mL aliquots and stored at 4 °C.

3.2.6 Protein quantification

Protein quantification was performed using gel densitometry. Serial dilutions of bovine serum albumin (BSA) were separated on a SDS polyacrylamide gel (see 2.2.8) alongside purified varying volumes of NP. The gel was then stained using Coomassie Blue. The BSA was used to construct a linear standard curve of the density, as measured by gel densitometry using a SynGene reader - GeneTools (version: 3.07.03). The NP sample concentration was calculated by determining the density of the protein band and then derived from the BSA standard curve.

3.3 Results

3.3.1 Ammonium sulphate precipitation

The crude extract was precipitated into 8 different fractions as described in 3.2.3, increasing the concentration of $(NH_4)_2SO_4$ by 10 % after each step. The majority of NP was found in the fractions ranging from 40 % to 80 % $(NH_4)_2SO_4$ as seen in Figure 3.1 by the presence of the 55 kDa NP band. Bulk $(NH_4)_2SO_4$ for further downstream processing was performed by precipitating the protein and discarding the precipitate obtained between 0 % and 40 % $(NH_4)_2SO_4$ and then retaining the protein precipitated using a 40 % and 80 % $(NH_4)_2SO_4$ solution.

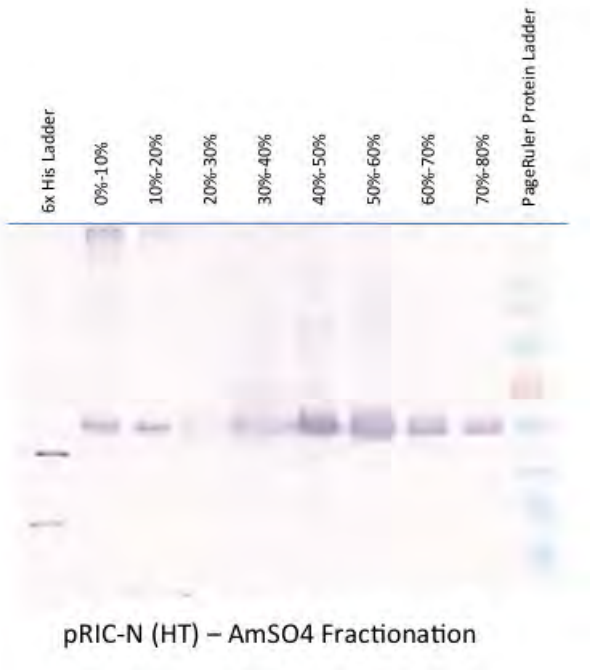
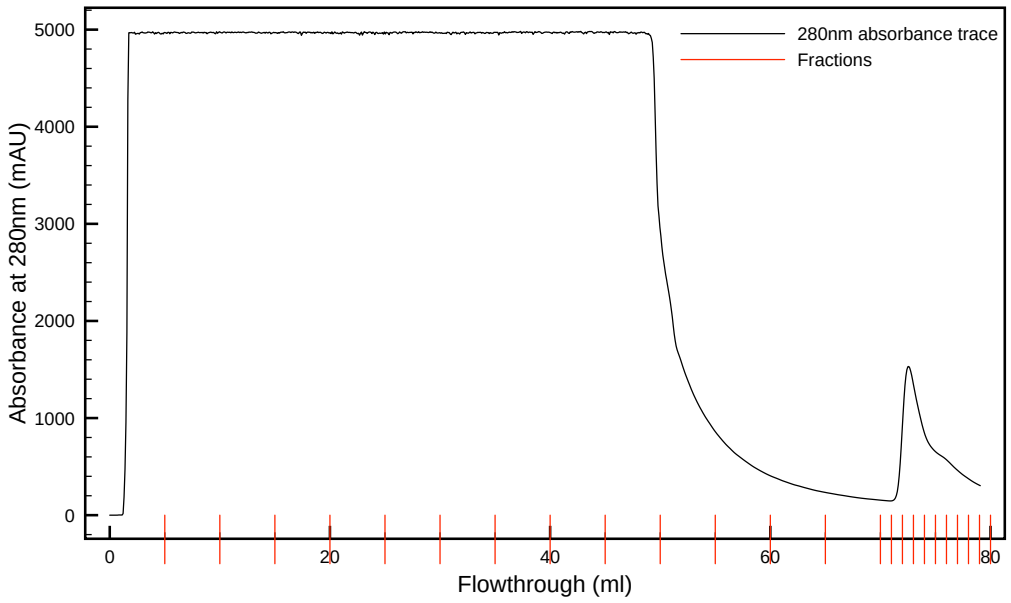


Figure 3.1: Western blot of equal volume of the 8 precipitated protein fractions ranging from 0 to 80 % $(NH_4)_2SO_4$ separated by SDS-Page. NP was detected using an anti-6 x his primary mouse antibody.

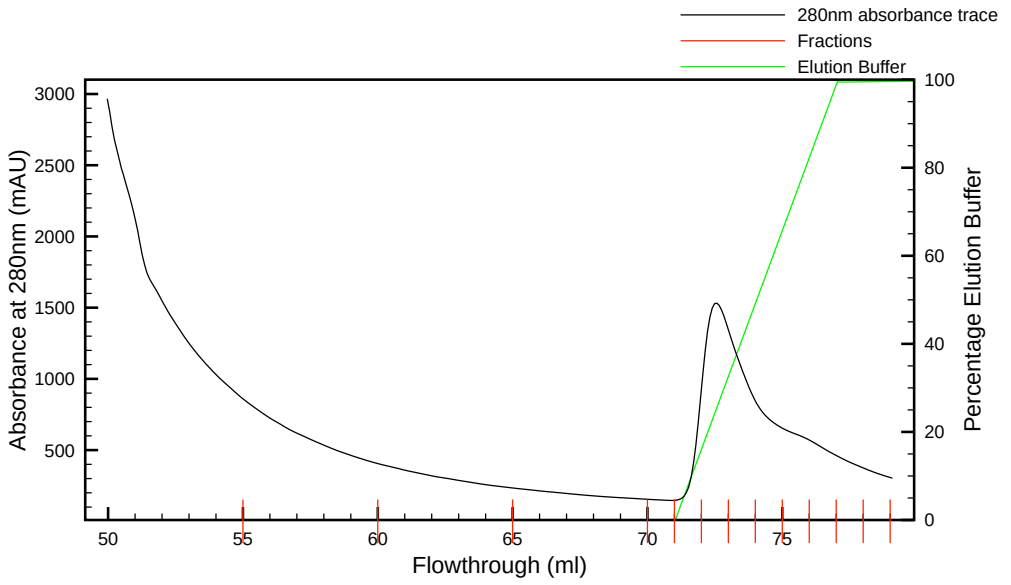
3.3.2 Nickel affinity

NP was purified successfully using a HisTrap HP pre-packed column. The elution profile shown in figure 3.2 shows a 280nm protein peak at an elution volume of 71ml-79ml. Selected fractions from an independent run, on a Coomassie-stained gel (figure 3.3a) and a cognate western blot probed with 6 x anti-his antibody (figure 3.3b) showed that negligible yields of NP were detected in the flow through (unbound) fraction (Flow through fraction 2), as well as in fractions 7 and 8, compared to the yields detected in the sample originally loaded onto the column (40 - 80 % $(NH_4)_2SO_4$ precipitation). The

Coomassie-stained gel (figure 3.3a) shows that the protein was successfully purified and concentrated to a point that it is the dominant band on the gel at the expected size (55kDa), with only minor contamination of other proteins present in the sample. There was also the added advantage of increasing the concentration of the protein - this can be seen in 3.3a where equal volumes of sample were added to each lane.

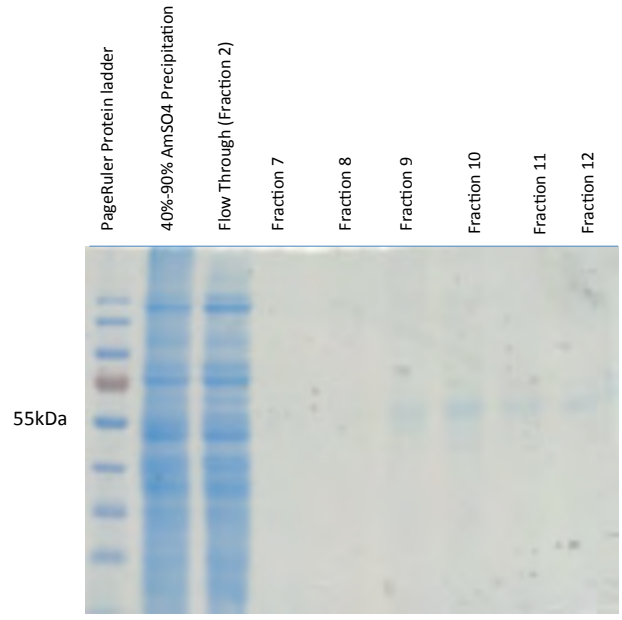


(a) Overview of NP histidine purification trace

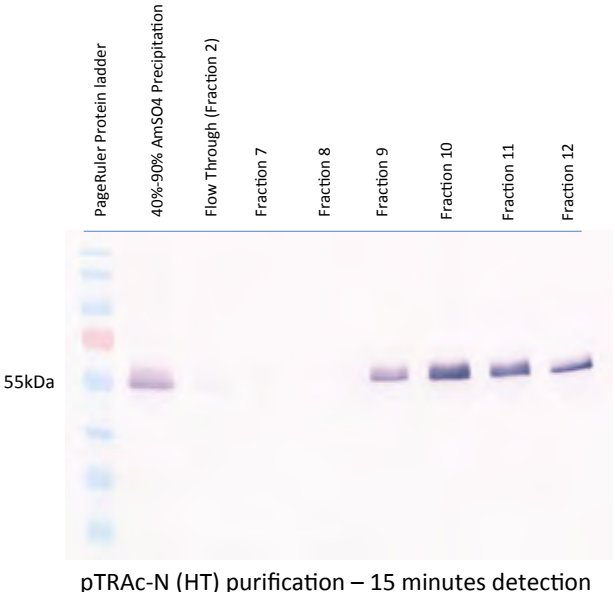


(b) Expanded view of elution portion of the NP histadine purification trace.

Figure 3.2: Purification traces of NP histidine purification. Produced using UNICORN 4.11 (GE Healthcare, USA).



(a) Coommassie Blue stain of the histidine affinity



(b) Western blot of histidine purification of NP. Detected using anti-6his primary mouse antibody.

Figure 3.3: Purification of NP using nickel affinity chromatography. a) Coommassie Blue stain and b) Western Blot of various fractions from the nickel affinity purification of NP.

3.3.3 Protein quantification

As the sample was not completely pure, testing the total soluble protein (Using the Bradford Method or similar), would not have given an accurate indication of the amount of NP present in the purified extract.

Thus, the concentration of NP was calculated from a linear curve generated by a range of BSA fraction V dilutions of known concentration as described in section 3.2.6. Once analysed, it was calculated that the final concentration of purified NP was 0.17mg/ml. This was calculated to be a final yield of 1.6mg of protein per kg of leaf material harvested. The calculations are summarised in Table 3.1.

Table 3.1: Calculation of NP yield.
Calculation = ((concentration*volume)/mass)*1000

Concentration (mg/ml)	Volume of Sample (ml)	Mass of Leaf Tissue Harvested (g)	**Yield per kg of leaf tissue (mg/kg)**
0.17	2.75	296	1.6

3.4 Discussion

Large-scale vacuum-infiltration is a required step for the *agroinfiltration*-based production of any plant-produced protein that requires milligram or greater quantities for downstream processing.

By incorporating ammonium sulphate precipitation as a first step of purification, a large number of contaminating proteins were easily and rapidly removed (data not shown). In addition, this step decreased the working volume of protein solution, thereby increasing the concentration of NP. This is important as the decreased volume requires less reagents for subsequent processing, thus reducing purification costs.

The second stage, nickel affinity chromatography performs a dual function of further removing a large number of unwanted, contaminating proteins, as well as concentrating the NP in the final protein solution to be further purified. The advantages of working with a concentrated protein include flexibility and ease of handling, as well as improved protein stability. Increased concentration of the 6 x his-tagged protein has been shown to improve the bind efficiency of nickel resin columns (Pers. Communication).

The use of an automated, column-based chromatography device, such as an ÄKTAexplorer, provided a rapid, reliable and most importantly, repeatable system for purification of CCHFV NP. An added advantage is that if required, the purification process can be scaled up by using larger volume chromatography columns.

In order to determine the feasibility of further scale-up and possibly commercial production of a plant produced protein, the yield of protein is required to be calculated. This is calculated in mg per kg of leaf material. As discussed in the previous chapter, this studies expression and subsequent purification level of 1.6mg/kg is low when compared to other proteins expressed and purified in plants (Maclean et al., 2007; Duvenage et al., 2013).

Steps for increasing this expression can include:

- Alternate codon optimisation.
- UTR modification.
- Use of expression vectors based on alternative viral genetics (e.g. Icon Genetics magnICON® system).

The purification methods used may also be inefficient. Further work is required to quantify the losses of expressed protein during purification and to examine alternate purification systems, such as ion-exchange chromatography, size-exclusion chromatography or a non-chromoatography based purification method, such as using elastin-like protein (ELP) as a fusion tag (Duvenage et al., 2013).

Analysis of the purification showed a significant decrease in the amount of contaminating protein, although not complete removal.

This purification process was repeated multiple times, across several batches of infiltrated leaves. Yield and purity, as estimated qualitatively by eye on both western blots and Coomassie stained gels, was consistent across batches. The method scaled consistently as the input material was increased (Data not shown). Potential further work could be to perform a detailed study on how well the purification scales as the input material increases to the levels required for commercial production of the protein.

Possibilities for further purification in order to remove the remaining contaminating proteins, if needed, include ion-exchange chromatography, which separates proteins based on their net surface charge; size-exclusion chromatography, to separate the proteins based on their globular, *in vitro* size. A final option, that would require molecular cloning, would be to add another affinity tag, such as glutathione S-transferase (GST), which can be purified using a glutathione-linked chromatography medium. An added advantage to using a

GST-tag is that it has been shown to increase the solubility and stability of the protein. The major disadvantage, and why it was not used in this study, is that the tag is large (220 amino acid residues) and can possibly interfere with other downstream processing such as further purification, antibody based detection and proper protein folding.

Detection of CCHFV antibodies in human serum using plant-produced NP

4.1 Introduction

Immunological detection of CCHFV-specific antibodies in human serum requires the use of BSL4 facilities, due to the need for using antigen produced from tissue-cultured cells harbouring live virus (Vanhomwegen et al., 2012). Efforts have been made to produce recombinant CCHFV NP in insect cells (Saijo et al., 2002) as well as bacteria (Samudzi et al., 2012). However, insect cell-produced proteins are expensive to make and require specialised facilities, and efforts to express NP in bacteria resulted in issues with low yields and insolubility.

In Chapter 3 I showed that plant-produced CCHFV NP can be expressed as a soluble protein with purified yields of up to 1.6 mg of protein per kg of leaf material, thus potentially providing a safe and cheap alternative to traditional methods. In this chapter, I describe the work done to test the immunological specificity of this plant-produced CCHFV NP using an indirect enzyme linked immunosorbent assay (ELISA) where the NP was used to detect

IgG antibodies in the serum samples of individuals previously infected with CCHFV.

4.2 Materials and methods

4.2.1 Serum samples

All samples were obtained with full informed consent of the donors. Ethics approval was obtained from the Ethics Committee at the Faculty of Health Sciences, University of the Free State (Ethics Number 152/06).

Negative panel

Serum samples were obtained from 13 individuals with no known history of CCHFV exposure. The serum was aliquoted into 2ml Cryotubes (Nalgene Nunc International, USA) and stored at 4 °C for immediate use, or -20 °C for long term storage.

Positive panel

Serum samples from 13 survivors of CCHFV infection collected between 5 and 13 years after illness were available for testing (Table 4.1). All samples had previously been tested positive for IgG antibody against CCHFV using indirect immunofluorescent antibody assays (EUROIMMUN) at the Department of Medical Microbiology and Virology, University of the Free State (Table 4.1).

Controls

Two known negative and 2 known positive serum samples, previously verified by Samudzi et al. (2012) were used as negative and positive assay controls, respectively.

Table 4.1: Details of 13 CCHFV positive samples used.

Laboratory Number	Patient Identifier	Date of Collection	Date of onset
2 13	TK	16-Jan-13	28-Nov-09
4 13	HC	24-Jan-13	23-Dec-08
5 13	AS	24-Jan-13	11-Feb-08
6 13	BS	24-Jan-13	07-Nov-00
7 13	JS	24-Jan-13	20-Dec-00
10 13	HD	14-Feb-13	10-Nov-10
13 13	SF	13-Mar-13	17-Mar-08
14 13	GC	13-Mar-13	27-Oct-08
15 13	JB	13-Mar-13	28-May-04
16 13	PB	13-Mar-13	25-Dec-10
19 13	NJ	18-Apr-13	31-Mar-09
20 13	JJ	18-Apr-13	unknown
21 13	WK	18-Apr-13	24-Mar-02

4.2.2 Preparation of mock antigen

Mock antigen was prepared by infiltration of *N. benthamiana* leaves with *Agrobacterium* containing an empty pRIC3.0-HT vector (as described in section 3.2.1) and harvesting of leaves after 3 dpi as for pRIC-N(HT)-infiltrated leaves. The crude extract was subsequently treated using the same NP purification protocol outlined in sections 3.2.2, 3.2.3 and 3.2.4, collecting the same fractions as those collected and pooled from the column.

4.2.3 Indirect Enzyme Linked Immunosorbent Assay (ELISA)

All sample, serum and detecting agent and substrate volumes used were 100 µl, incubations were performed at 37 °C and washes with 0.1 % Tween 20

in phosphate-buffered saline (TPBS) pH 7.0, three times, unless otherwise mentioned.

Varying dilutions of both antigen and secondary antibody were used to optimise the assay. All dilutions were prepared in glass vials to minimise possible interference from protein binding to plasticware.

Purified NP and a mock antigen were coated onto Nunc 96-well PolySorp plates (Nalge Nunc International, USA) in various dilutions depending on the application. Coated plates were incubated for 1 hour.

The plates were washed and then blocked and incubated for 1 hour with 10 % skim-milk powder 0.1 % TPBS for 1 hour at 37 °C and then washed as before.

Test serum was diluted 1/100 in 2 % skim-milk powder 0.1 % TPBS, added to the wells and then incubated for 1 hour at 37 °C, followed by a wash step.

Goat anti-human IgG HRPO conjugate (Zymed, USA) was diluted in 2 % skim-milk powder 0.1 % TPBS, added to the wells and then incubated for 1 hour, followed by a wash step.

ABTS substrate (KPL, USA) was added to each well and the reaction allowed to proceed for 30 min before absorbance was measured at 405nm and 650nm.

Final absorbance was measured as the difference between 405nm and 620nm to reduce any non-specific background absorbance.

The final value was calculated by subtracting the absorbance of the mock antigen from the absorbance of the NP to further reduce non-specific background.

In order to determine the optimum dilution of recombinant NP to coat the plates with, an antigen titration experiment was set up. Both NP and the mock antigen were serially diluted, doubling at each step from 1/50 to 1/3200. Two positive and two negative serum samples were used to account for potential variances in antibody titres between serum samples.

NP was subsequently used at a dilution of 1/400 for all further indirect ELISAs to test the remaining sera.

4.2.4 Data analysis

Normalisation of data

To account for background, cross reactivity, from unknown proteins in capture antigen, net OD is calculated by subtracting the OD reading of the mock antigen from the OD reading of the NP.

Net optical density (OD):

Net OD = OD in wells with NP minus OD in wells with mock antigen.

Net OD readings were converted to a percentage of a know high-positive, in house, control serum , termed Percentage Positivity (PP) as follows:

OD of test sample / OD of know high-positive) x 100 = PP.

Calculating cut-off values

The cut-off value used to separate positive results from negative is calculated using a panel of 13 known negative control serum samples. The control ELISA was repeated twice, resulting in 26 individual data points. Mean normalised absorbance and Standard Deviation (SD) were both calculated from the data. The cut-off value was calculated as the mean PP plus 2 standard deviations.

4.3 Results

4.3.1 Antigen titration

A titration of different dilutions of plant-produced NP and mock antigen were carried out using test positive and negative sera to determine which dilutions to use for the test ELISA. Results of the titration of the recombinant NP are shown in Table 4.2. The titration showed the expected results, with the positive serum samples showing a decreasing signal as the antigen dilution increased. The mock antigen shows a relatively stable signal across all dilutions. A dilution of 1/400 (0,0025) of recombinant NP was selected for further use in ELISAs as this concentration was the closest to the middle of the measurable dynamic range, allowing for the most flexibility when reading both strong and weak signals (Figure 4.1).

Table 4.2: Titration of recombinant NP and mock antigen to determine optimum dilution for use in ELISAs. Absorbance was measured as the difference between 405nm and 620nm. The reaction was visualised after 30 minutes. Red colouring represents the maximum value, yellow the median and green the minimum.

	Dilution	1/50	1/100	1/200	1/400	1/800	1/1600	1/3200	1/6400
NP Antigen	Positive a	1,5030	1,0940	0,8330	0,4350	0,2630	0,2100	0,1860	0,1650
	Positive b	1,5240	1,0040	0,8730	0,4790	0,2790	0,2030	0,1590	0,1570
	Negative a	0,2460	0,2040	0,1630	0,1440	0,1290	0,1010	0,1190	0,1110
	Negative b	0,2170	0,1960	0,1680	0,1480	0,1390	0,1430	0,1410	0,1190
Mock Antigen	Positive a	0,2120	0,2050	0,2140	0,1990	0,1950	0,1890	0,1850	0,1860
	Positive b	0,2110	0,1950	0,1610	0,1800	0,1640	0,1410	0,1650	0,1380
	Negative a	0,1350	0,1520	0,1240	0,1370	0,1230	0,1200	0,1220	0,1120
	Negative b	0,1680	0,1760	0,1720	0,1900	0,1810	0,1740	0,1740	0,1610

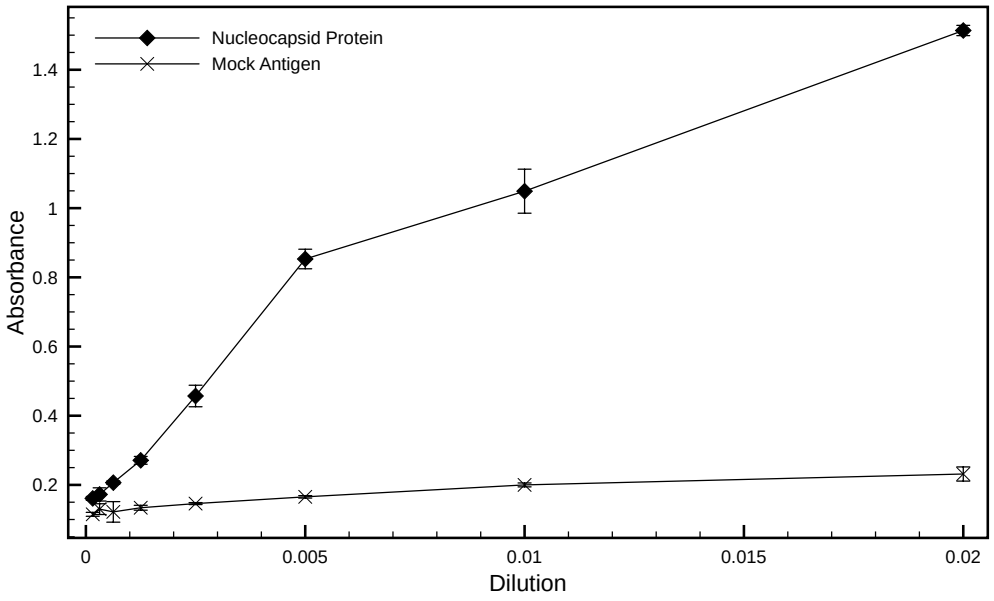


Figure 4.1: Graph of serial dilutions of both NP and mock antigen to determine the optimum dilution for further work ranging from 1/50 (0,02) to 1/6400 (0,00015625).

4.3.2 Detection of anti-CCHFV antibodies in human serum using plant produced NP.

To determine whether the plant-produced NP could accurately and reliably detect the presence of antibodies against CCHFV in human serum, an indirect ELISA was performed with 15 CCHFV positive (including 2, known highly reactive positive samples) and 15 known negative serum samples.

The absorbance values of the negative samples were used to calculate a cut-off value for determining a diagnostic positive reaction, as well as to account for any possible background. The cut-off value is calculated as the mean of the negative samples plus 2 standard deviations.

In Figure 4.2, negative serum samples are represented by numbers 1 to 15. The positive serum samples are represented by numbers 16 to 30. The ELISA was performed twice and each serum sample was measured in duplicate for each repeat.

There is a significant difference between all positive and all negative samples, illustrated as the mean fraction of the highest value to the measured sample, as well as the absence of any false positives.

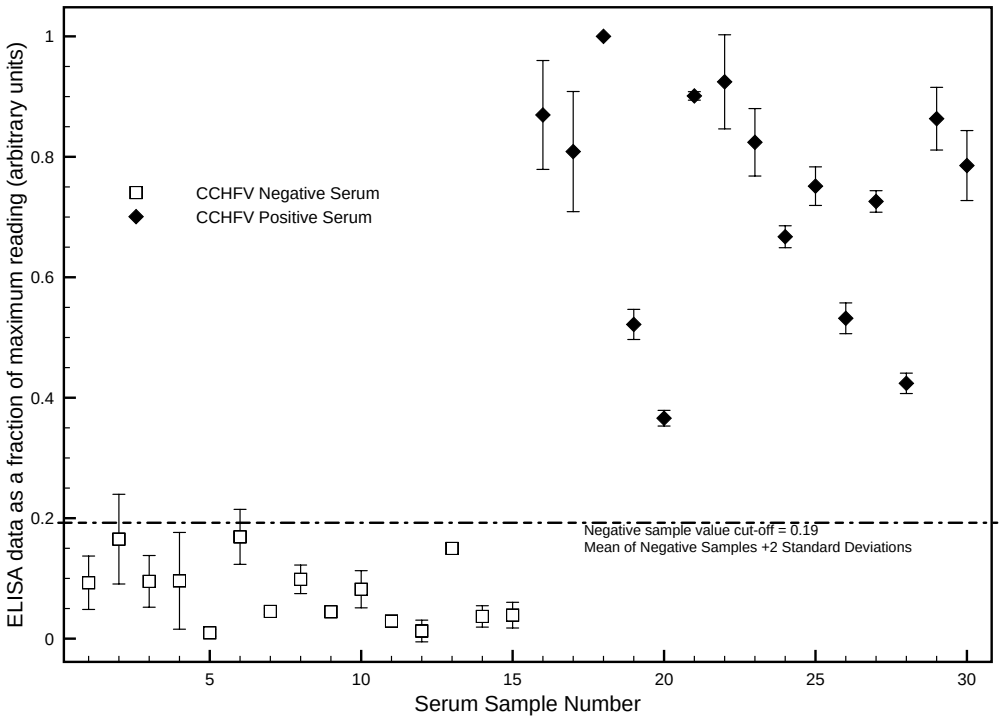


Figure 4.2: Detection of anti-CCHFV IgG in an indirect ELISA, using plant produced NP as a capture antigen. The error bars illustrate the variability between repeated ELISA experiments that were not accounted for by data processing.

4.4 Discussion

Traditional methods for diagnosis of CCHFV require high levels of bio-containment for production of antigen, making the process both costly, as well as potentially hazardous to the health of the individuals doing the testing and the production of the antigen.

The recombinant CCHFV NP antigen tested in this work represents a cheaper and safer alternative. It does not contain any live virus, eliminating exposure risk in the production phase and reducing the risk in the testing phase.

These results show that the antigen is capable of not only detecting CCHFV IgG antibodies in the serum of individuals previously infected with CCHFV, but it is also capable of distinguishing between the serum from a previously infected individual and that of an individual with no known history of CCHFV exposure.

The ability of the antigen to detect CCHFV IgG antibodies also shows us that the plant produced antibody is likely being folded and processed correctly by the plant hosts, leading to a protein conformation that is sufficiently similar to the native protein to bind to native CCHFV NP antibodies. We cannot however comment on the antigens antigenicity without further in-vivo immunological studies.

Previously, CCHFV NP has been expressed in *E. coli* (Samudzi et al., 2012), and showed similar results with regards to its ability to detect the presence of CCHFV IgG antibodies in the serum of individuals who have been previously infected by the virus as the plant produced NP used in this study. However, the *E. coli* expression of CCHFV NP had issues with protein solubility, leading to an increase in both cost and time required to purify the protein. The NP expressed in *N. benthamiana* is soluble in standard protein extraction buffers, simplifying the purification process.

Further work is required to fully qualify the antigen for use in routine diagnostics. This includes:

- Testing plant-produced NP against a larger range of negative samples to validate the antigen and ensure that there is a comparatively low level of false positives;
- Testing against a larger range of negative samples to ensure that there is a comparatively low level of false positives;
- Comparison of sensitivity, specificity and reproducibility, against other immunological diagnostic methods, such as virus neutralisation and immunofluorescence; and
- Stability assays to determine the antigens long term stability under different storage conditions. This is important as requirements such as refrigeration or lyophilization add both cost and difficulty of access to the diagnostic antigen.

A plant produced diagnostic antigen for CCHFV represents a cheap, safe alternative to traditional antigen based diagnosis. Plant produced proteins can be produced at large scale, but further work would be required to develop a commercial scale expression and purification process.

Ready access to a CCHFV diagnostic assay could lead to increased epidemiological surveillance, looking at viral prevalence in a range of hosts, including humans, animals and insects.

Conclusion

In order to study the true risk that a virus holds, it is necessary to know as much information as possible about its hosts, range, possible vectors, infectivity and case fatality. Traditionally this information can be obtained by performing large scale serological studies, looking for evidence of infection in possible vectors, as well as a range of hosts, including ones that are potentially asymptomatic, such as birds and livestock animals. Large studies of this kind have been difficult due to the lack of a safe, affordable serological assay for CCHFV.

To this end, the aim of this project was to express Crimean-Congo haeorrhagic fever virus nucleocapsid protein (NP) in *N. benthamiana* and to purify it sufficiently for it to be used in an indirect ELISA to detect antibodies in the serum of individuals previously exposed to CCHFV.

The gene encoding the NP was cloned into three different plant expression vectors, pRIC-3.0-HT, pTRAc-HT and pEAQ-HT. The resulting vectors were then used without and with co-expression of NSs, a TSWV protein that has shown to improve expression by reducing post-translational gene silencing. The replicating vector pRIC-3.0-HT used with NSs produced the highest expression levels of NP when comparing the intensities of the 55kDa sized NP band on a western blot. However, there was little or no change when NSs was used with pTRAc-HT: it is possible that the plants respond to

the high levels of foreign mRNA produced by the replicating pRIC-3.0-HT amplicon, by triggering RNA silencing, a tool in the plant's adaptive immune system. pTRAc-HT does not replicate so does not produce as much mRNA as pRIC-3.0-HT, so no silencing occurs, which negates the need for NSs.

To obtain sufficient NP for use in an ELISA, the expression of the protein needed to be scaled up. This was done by infiltrating 20 plants, using vacuum infiltration, resulting in 100-200 grams of leaf material after harvest.

In order to reduce the possibility of non-specific background in the ELISA due to human antibodies reacting with plant proteins, as well as to concentrate the protein, purification was required. A two-step approach was used: first, a bulk salting out using ammonium sulphate, to both reduce the volume of crude protein solution required for purification as well as to remove a large amount of contaminating protein, specifically RuBisCO which accounts for 50 % of soluble leaf protein. The second step made use of the 6 x-histidine tag fused to the N-terminus of the NP gene during cloning. The protein was bound to and eluted from a nickel affinity column, resulting in a more concentrated NP protein, as seen by the density of the 55kDa band on a Coomassie stained SDS gel. Additionally, the protein was much more pure, judged by the small number of extra bands present.

Purified plant-produced NP was successfully used as a capture antigen in an ELISA to detect CCHFV-specific IgG in the serum of individuals previously infected with the virus, as well as to distinguish between individuals with no history of infection and those previously infected. The plant produced protein compared favourably in its ability to detect CCHFV-specific IgG, with *E. coli* produced NP, returning no false-positive results and correctly identifying all of the CCHFV positive serum samples, as seen when using the *E. coli* produced NP as described by (Samudzi et al., 2012). However further work as described in Section 4.4, such as testing against larger positive and negative serum panels and studies on inter-batch variability, is required to

determine further how the plant produced protein compares to its *E. coli* produced counterpart.

To improve the utility of plant produced NP as a diagnostic reagent, further work could be done to determine the protein's ability to bind CCHFV-specific IgM antibodies, as these are sometimes present sooner during infection than IgG antibodies and would be a useful indicator in diagnosing CCHFV infection, sooner after exposure to the virus (**?**).

In order to qualify the protein for work in large scale serological studies, it needs to be tested on a larger range of tissue, such as animal serum and insect hemolymph (Vanhomwegen et al., 2012).

This study is the first report of a plant produced CCHFV NP. I hope that this work is used to improve monitoring and surveillance of CCHFV in animals populations as well as to improve the access to diagnostic services by both reducing the cost and improving the safety of the assay. Finally, I hope that others will build upon this work, by applying the lessons learnt in CCHFV NP production, to other *Bunyaviridae* species such as Rift-Valley Fever Virus and produce similar NP based, plant produced diagnostic antigens, further improving the availability and safety of diagnosis.

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