PLANT-EXPRESSED DIAGNOSTIC PROTEINS AND THEIR USE FOR THE IDENTIFICATION AND DIFFERENTIATION OF INFECTED AND VACCINATED ANIMALS WITH FOOT-AND-MOUTH DISEASE VIRUS

By

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DECLARATION

Name: Scott de Beer

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Declaration

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"If I have seen further than others, it is by standing upon the shoulders of giants"

—Isaac Newton

ABSTRACT

The Foot-and-mouth disease virus (FMDV) affects cloven-hoofed animals and is endemic in most parts of Africa, South America and southern Asia. South Africa is considered a FMDV-free zone but the virus is maintained within the wildlife in the Kruger National Park (KNP), making mitigation of outbreaks a high priority. Diagnostic methods are usually costly due to the high production cost of the reagents used, meaning that regular monitoring and diagnosis of animals around the KNP for FMDV is expensive due to the large amounts of serum continuously being tested. I propose an alternative plant expression platform for the local production of more cost effective diagnostic reagents capable of distinguishing between infected and vaccinated animals (DIVA). I selected the non-structural 3ABC polyprotein of FMDV to express, as it is a suitable candidate as a coating antigen in a competitive enzyme linked immunosorbent assay (C-ELISA) for the detection of neutralizing antibodies in livestock sera. I also chose other variations of the full polyprotein (3AB, $3AB_1$ and 3B) for expression as they have previously been shown to be effective in FMDV diagnosis. I also selected a second reagent to be expressed: this was the CRAb-FM27 single chain variable fragment (scFv), which binds a 3B epitope on the 3ABC polyprotein and has previously shown to be effective as a competing antibody in a C-ELISA. The 3B antigen and the scFv were successfully expressed and purified from *N. benthamiana*, which to my knowledge is the first time either has been shown. The plant produced scFv successfully bound the 3B antigen in an Indirect-ELISA (I-ELISA). Separately, the plant produced 3B antigen could be used to successfully differentiate FMDV infected and vaccinated guinea pig serum in an I-ELISA. However, testing of these reagents in tandem within a C-ELISA to DIVA sera was inconclusive, and further research is required to optimise C-ELISA conditions.

ABBREVIATIONS

ARC	Agricultural Research Council
BEI	Binary ethyleneimine
bp	Base pairs
BSA	bovine serum albumin
C-ELISA	competitive-ELISA
CF	complement fixation
CFT	complement fixation test
CPE	cytopathic effect
CRAb-FM	chicken recombinant antibody-foot-and-mouth
CV	column volumes
DIVA	Distinguishing vaccinated from infected animals
DNA	Deoxyribonucleic acid
dNTP	deoxy-ribonucleoside triphosphates (dATP, dCTP, dTTP and dGTP)
dpi	days post infiltration
EDD-OVI	Exotic Disease Division at the Onderstepoort Veterinary Institute
EDTA	ethylenediaminetetra-acetic acid
eIF-4A	RNA helicase
eIF-4G	eukaryotic initiation factor
EITB	enzyme-linked immunoelectrotransfer blots
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FMDV	Foot-and-mouth disease virus
FPLC	Fast protein liquid chromatography
g	gram(s)
GMO	genetically modified organisms
h	Hour(s)
I-ELISA	indirect-ELISA
Igs	Immunoglobulins
IMAC	immobilized metal-ion affinity chromatography
INTA	National Agricultural Technology Institute
IPTG	isopropylthio-β-D-galactoside
IRES	internal ribosome entry site

kb	kilobase(s)
kDa	kilodalton(s)
KNP	Kruger National Park
L	litre(s)
LB	Luria-Bertani
LPBE	liquid-phase blocking-ELISA
LPH	lactase-phlorizin hydrolase
L^{pro}	L protease
M	molar
mAbs	monoclonal antibodies
MES	2-morpholinoethanesulfonic acid
mg	milligram(s)
min	minute(s)
mL	millilitre(s)
mM	millimolar
ng	nanogram(s)
NSP	Non-structural protein
O/N	overnight
OD	optical density
OIE	Oficina Internacional de Epizootias
OP	oesophageal-pharyngeal
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
RE	restriction enzyme
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
RT-LAMP	reverse transcription loop-mediated isothermal amplification
RT-PCR	reverse transcriptase-PCR
scFv	single chain variable fragment
SDM	site-directed mutagenesis
SDS	sodium dodecyl sulphate
sec	second(s)
SP	Structural protein
SPCE	solid-phase competition-ELISA
TBE	Tris-borate-EDTA buffer
T-DNA	transferred-DNA
Ti	tumour inducing
Tris	Tris(hydroxymethyl)aminomethane

TSP	total soluble protein
V	Volts

Symbols

µg	microgram(s)
µL	microlitre(s)
%	percentage
°C	degrees Celsius

CHAPTER 1

LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

The Foot-and-mouth disease virus (FMDV) causes a highly contagious acute infection that affects cloven-hoofed animals (order: *Artiodactyla*). The virus is often linked to livestock infection – that is, cattle, sheep, goats (ruminants) and pigs - but is also known to infect over 70 species of wildlife (Alexandersen and Mowat, 2005). The virus causes Foot-and-mouth disease (FMD) which is categorised as a list A disease, that has the ability to spread rapidly and potentially cause serious economic and agricultural loss (Stear, 2005).The strain put on a country during an outbreak can be devastating, even with the virus usually only having a short-term effect on the animal. The visible production losses include reduction in milk by about 80% (Bayissa *et al.*, 2011) and young animal mortality rates of 2-3% (Rufael *et al.*, 2008). The invisible losses due to FMD are abortions, where farmers must decide to keep the animal for up to a year without production or cull it. As a direct impact, nations without FMD-free status cannot trade live animals with FMD-free countries, and restrictions are made with regards to livestock products. This cut-off to a lucrative market that some countries depend heavily upon, can have compounding negative effects on their economies (Knight-Jones and Rushton, 2013).

The aggressive nature of the virus is exemplified by its ability to spread quickly through direct and indirect methods. The most common form of transmission is through direct contact with infected animals. The virus is known to have been recovered in most bodily excretions (milk, saliva, semen, urine and faeces) and the primary mechanism of spread is through the inhalation of aerosolised droplets into the respiratory tract. Indirect contact with contaminated personal, surfaces and foods have also shown to be responsible for transmission (Alexandersen and Mowat, 2005; Klein, 2009; Admassu *et al*., 2015).

The incubation period of the virus can vary depending on viral dose, route of transmission and the animal species being infected (Alexandersen, Zhang, *et al*, 2003). This ranges from 2-

14 days and at the end of this time frame, ample amounts of virus can be shed and disseminated. The early clinical signs include fever, extensive salivation, depression and lameness, which are then followed by the formation of vesicular lesions around the mouth, muzzle, udder and feet. The inability to walk and eat is a result of the lesions and contributes to weight loss (Alexandersen, Quan, *et al.*, 2003; Alexandersen, Zhang, *et al.*, 2003; Alexandersen and Mowat, 2005; Admassu *et al.*, 2015).

The disease is controlled by restricting trade of livestock and animal products from known FMD endemic countries. Countries are classed into FMDV-free with or without vaccination, depending on their current prevention procedures. During an emergency outbreak within a FMDV-free country, "stamping-out" procedures are deployed. The world organization for animal health (Oficina Internacional de Epizootias, OIE) defines it as, the slaughter and disposal of all infected and in-contact animals, the disinfection and cleaning of all equipment, and restricting movement of animals and personal (OIE, 2016). In contrast, countries where FMD is endemic, prophylactic vaccinations and limitation of animal movement are employed to protect high yielding livestock (Admassu *et al.*, 2015). The highly infectious nature of the virus and the ease with which it can spread amongst a population of livestock and wildlife animals, shows the importance of control and prevention. The first line of defence in this case is to have a well-established, rapid and accurate method of diagnosis. The combination of continuous systematic inspection of clinical signs and virological and serological diagnosis are currently used and have been shown to be highly successful in combatting outbreaks.

1.2 VIRAL CLASSIFICATION AND GEOGRAPHICAL DISTRIBUTION

FMDV is classified within the *Aphthovirus* genus as a member of the *Picornaviridae* family (Belsham, 1993) of small non-enveloped isometric ssRNA-containing viruses. There are seven serotypes of the virus, each consisting of varying subtypes distributed around the world. The O and A types are the most widely distributed, appearing in many parts of Africa, South America, southern Asia and the Far East. Type C occurs in the Indian sub-continent and the Asia 1 serotype in southern Asia, to where these serotypes have become confined. The SAT serotypes (SAT1, SAT2 and SAT3) are usually restricted to sub-Saharan Africa. Central and

North America, Europe and Australasia are amongst the few that are FMDV-free. (Knowles and Samuel, 2003). Figure 1.1 shows the distribution of the seven serotypes.

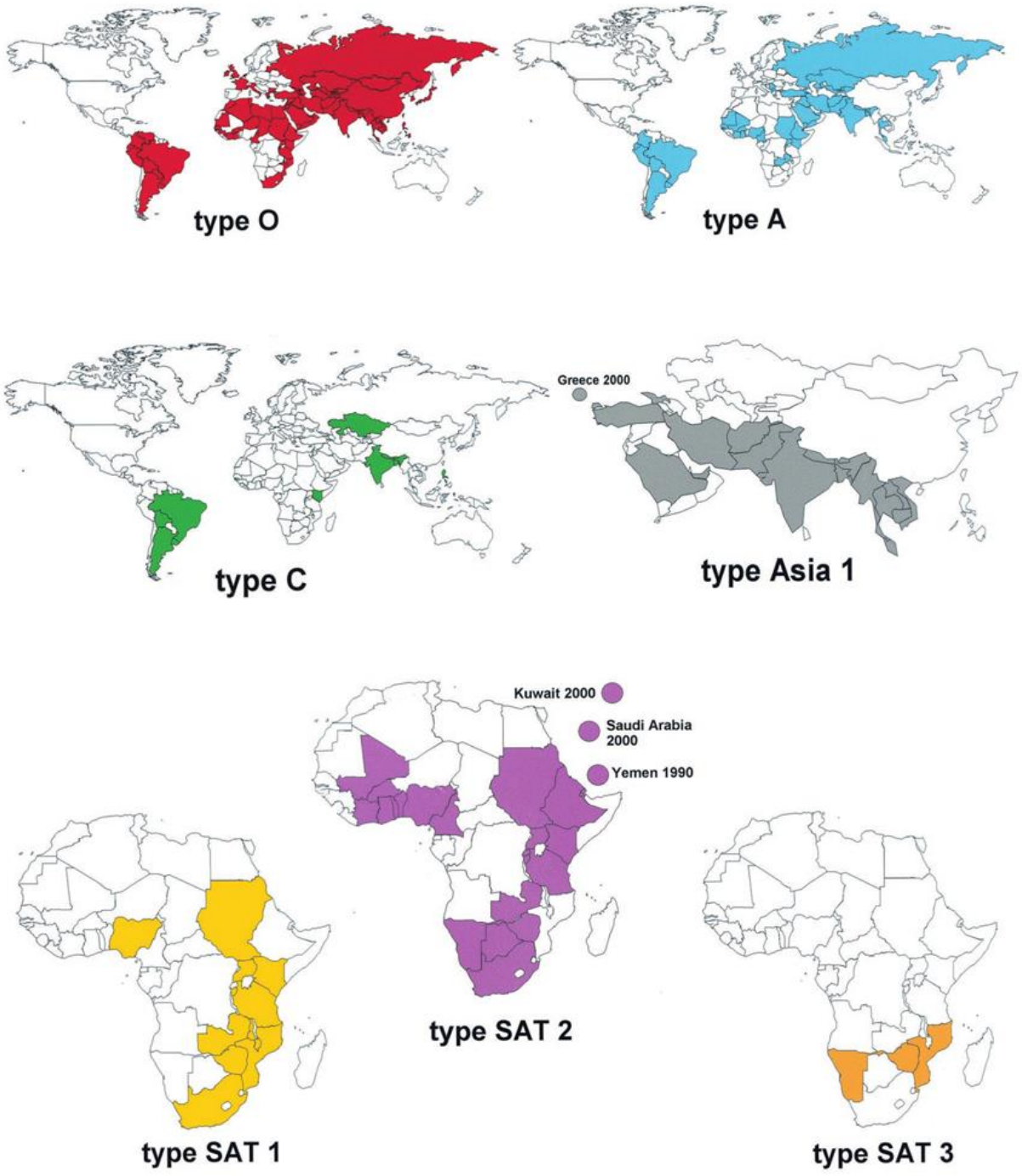

Figure 1.1. Distribution of the seven serotypes globally.

Reported to the OIE from 1990-2002. Taken and adapted from Grubman and Baxt (2004).

1.3 VIRAL COMPONENTS

The FMDV genome consists of single-stranded plus-sense RNA, which is approximately 8500 nucleotides in length, enclosed by four structural proteins to form a non-enveloped icosahedral capsid (Crowther, 1986; Sobrino *et al.*, 2001; Grubman and Baxt, 2004). The genome is flanked by 5' and 3' untranslated regions (UTRs), which do not encode viral proteins but show complex secondary structure involved in viron replication and translation. A single open reading frame (ORF) is encoded between the UTRs that translates into a single polyprotein, which undergoes multiple post-translational proteolytic cleavages to produce both intermediate and mature structural and non-structural proteins (NSPs) (Mason, Grubman and Baxt, 2003; Grubman and Baxt, 2004). A representation of the viral genome is shown in Figure 1.2.

1.3.1 THE 5' UNTRANSLATED REGION

The 5' UTR is about 1300 bases in length and is divided into five functional elements that are involved in virus translation and RNA replication. These are the S fragment, the poly(C) tract, the pseudoknots, the cis-acting replication element, and the internal ribosome entry site (IRES). The 5' terminus is covalently bound to a genome-linked protein (VPg), encoded by the 3B region, which is important for initiation of RNA synthesis and viral RNA encapsulation (Belsham, 1993; Mason, Grubman and Baxt, 2003). The 5' UTR elements are well described by Mason et al. (2003).

1.3.2 THE POLYPROTEIN

The long polyprotein encoded between the 5' and 3' UTRs is divided into four protein regions (Figure 1.2). These are the L protease (L^{pro}), P1 (precursor of the structural capsid proteins), P2 and P3 which produce a range of non-structural proteins involved in viral replication. Processing of these four regions is achieved through the action of viral encoded proteases and autocatalytic cleavage which gives rise to the structural and non-structural proteins.

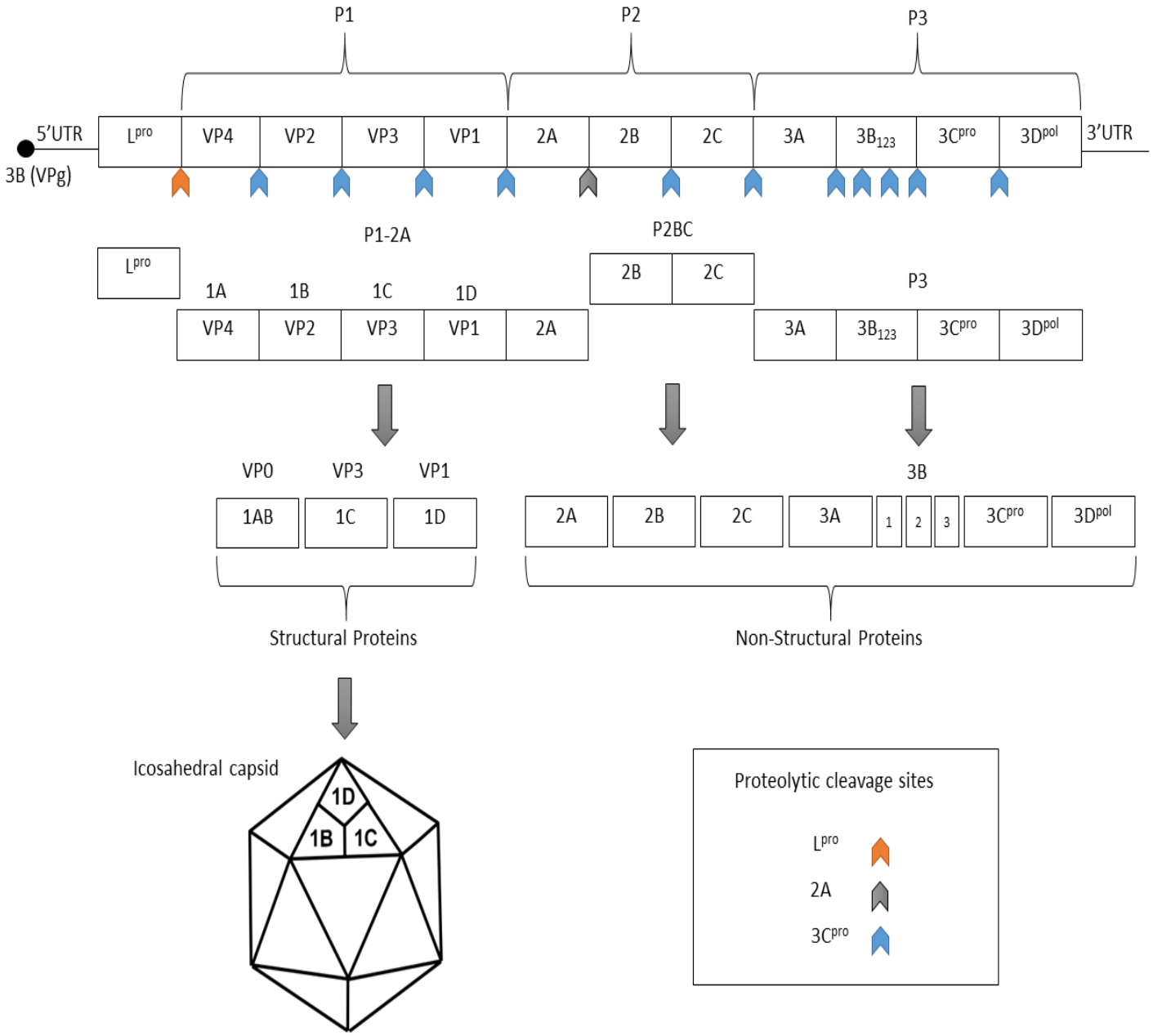

Figure 1.2. Schematic diagram of the FMDV genome.

The protein-encoding **region** or ORF is indicated by the open boxes and the lines either side indicate the RNA UTR's. The proteins are named according to the L434 convention with the nomenclature of Rueckert and Wimmer (1984)

1.3.2.1 Proteases

The 5' terminus of the ORF contains two in-frame start codons (AUG), which encode the Lab (major protein) and Lb leader proteases (L^{pro}) (Robertson *et al.*, 1985; Sangar *et al.*, 1987). The L^{pro} cleaves itself from the C-terminus of the polypeptide chain and is responsible for the cleavage of the host cell protein, eukaryotic initiation factor (eIF-4G). This results in the shut-down of the host cap-dependent mRNA translation and subsequent ceasing of host protein

synthesis. As the FMDV RNA initiates translation in a cap-independent manner via its IRES, viral protein synthesis is uninhibited (Devaney *et al.*, 1988).

One of the most versatile viral encoded proteases is the 3C protease ($3C^{pro}$), as it is responsible for the majority of polyprotein processing by proteolytic cleavage (Vakharia *et al.*, 1987; Clarke and Sangar, 1988). The $3C^{pro}$ has also been shown to cleave eIF-4G but at different locations to that of L^{pro}. It also cleaves the host RNA helicase (eIF-4A), a component of the cap-binding complex (Belsham, McInerney and Ross-Smith, 2000). It has also shown proteolytic activity towards host H3 histone (Falk *et al.*, 1990) and therefore not only inhibits host translation, but plays an important role in host transcription shut-down.

The P2 region of the polyprotein encodes the 2A peptide which is involved in the autocatalytic cleavage between the 2A-2B junction. This 'autoprotease', which is 18 amino acids in length, separates itself from the 2BC-P3 polyprotein, forming the P1-2A precursor. (Donnelly *et al.*, 2001).

1.3.2.2 Structural proteins

The P1 region of the viral genome encodes for the structural proteins (Figure 1.2), with the P1-2A precursor being catalytically cleaved by $3C^{pro}$ into the VP0, VP3 and VP1 proteins that assemble into single protomers. The icosahedral capsid (Figure 1.2) consists of 60 protomers, five of which assemble into a pentamer and twelve pentamers into the full 28-30nm icosahedral capsid (Sobrino *et al.*, 2001).

1.3.2.3 Non-structural proteins

The non-structural proteins arise from the P2 and P3 region of the viral polyprotein (Figure 1.2). They are involved in genome replication and participate in structural protein folding and assembly. The P2 polyprotein is autocatalytically processed by 2A, resulting in the 2BC polyprotein, which is further processed by $3C^{pro}$ into mature 2B and 2C proteins. These proteins have been known to associate with cellular membranes as well as interfering with the secretary pathway and preventing delivery of proteins to the cell surface (Moffat *et al.*, 2005).

The P3 polyprotein is also processed by $3C^{pro}$, giving rise to the 3A, 3B, 3C and 3D proteins (Mason, Grubman and Baxt, 2003). The 3A protein has been shown to associate with intracellular membranes, and is thought to anchor the replication complex of picornavirus to the endoplasmic reticulum (ER) membrane (Weber *et al.*, 1996; Suhy, Giddings and Kirkegaard, 2000; O'Donnell *et al.*, 2001). The 3B protein consists of three highly conserved non-identical proteins that are involved in linking to the viral RNA at the 5' UTR, by phosphodiester bonds (Forss and Schaller, 1982). These viral-genome linked proteins are involved in RNA synthesis and encapsulation, and their copy-number has been linked to host range and pathogenic potential of FMDV (Falk, Sobrino and Beck, 1992; Pacheco *et al.*, 2003). The $3C^{pro}$ is a serine protease and is largely involved in polyprotein processing as mentioned before. The 3D protein is a RNA-dependent RNA polymerase ($3D^{pol}$) (Newman *et al.*, 1979) and is responsible for positive and negative-strand RNA synthesis. The $3D^{pol}$ is a major component of the virus infection-associated antigen (VIAA), and has shown as an indicator of infection (Cowan and Graves, 1966) as its presence would indicate viral replication.

1.3.3 THE 3' UNTRANSLATED REGION

The FMDV genome contains a poly(A) tract at the 3'-end which is thought to be involved in processes required for polyadenylated mRNAs, as well as circularization of the RNA genome as a mechanism for positive strand replication (Herold and Andino, 2001; Mason, Grubman and Baxt, 2003). The 3' UTR is thought to have *cis*-acting sequences required for the initiation of genome replication, but it is still poorly understood (Mason, Grubman and Baxt, 2003).

1.4 VACCINES AND NON-STRUCTURAL PROTEINS

The production of vaccines has been explored in depth, from the classic killed whole virion preparations, to the more modern alternatives of using genetically engineered attenuated strains. To then expressing the VP1 protein and its fragments as synthetic peptides or in microorganisms (Küpper, 1984; Brown, 1988), to the use of adenoviruses to express P1 for cattle protection (Sanz-Parra *et al.*, 1999). The use of genetically engineered attenuated strains of the virus have been shown to protect cattle (McKenna *et al.*, 1995) and progress is being made in making recombinant empty viral capsids (Lewis, Morgan and Grubman, 1991).

Conventional vaccines against FMDV consist of different serotypes of chemically inactivated virus. Binary ethyleneimine (BEI) is used to neutralise the virus, which is then formulated along with an adjuvant before it can be used. Aluminium hydroxide gel plus saponin, is used for ruminants. Whereas oil-based adjuvants are used for swine (Doel, 1999). The preparation of these vaccines requires the growth of the virus in tissue culture in high-containment facilities, as it is non-attenuated and still virulent. Purification of the inactivated virus using polyethylene glycol (PEG) and ultracentrifugation has been shown to be successful. Depending on the procedure used by the manufacturer, variable levels of non-structural proteins will be present as a result of the growth cycle of the virus in tissue culture (Wagner, Card and Cowan, 1970; Lubroth *et al.*, 1996; Clavijo, Wright and Kitching, 2004). The virus infection-associated antigen protein which includes the $3D^{pol}$, in particular, has been shown to be present in vaccine preparations as it is incorporated within the virion. Therefore, animals vaccinated using inactivated FMDV have antibodies directed towards VIAA after multiple doses (O'Donnell *et al.*, 1997).

1.5 DIAGNOSIS OF FMDV

The implementation of a well-established diagnostic procedure is important for early detection and to mitigate the spread during FMDV outbreaks. The combination of clinical analysis of symptoms, and virological and serological diagnosis has been shown to be most effective in FMDV control. This is particularly important as swine vesicular disease, vesivirus, vesicular stomatitis and vesicular exanthema symptoms cannot be discriminated from those due to FMDV infections (Alexandersen, Zhang, *et al.*, 2003). A range of nucleic acid, antibody and viral antigen detection techniques have been developed in order to optimise swift and effective identification of the virus. Distinguishing vaccinated from infected animals (DIVA) is of great importance, as countries that use vaccination outbreak mitigation would need to differentiate their livestock to prevent any unnecessary culling.

1.5.1 CLINICAL DIAGNOSIS

Clinical signs of the virus follow after an incubation period of between 2-14 days, these include fever, lameness and lesions on the mucous membranes in and around the mouth as well as

on the feet and around the mammary glands (Admassu *et al.*, 2015). These debilitating symptoms have an effect on milk production, weight loss and although the mortality rate is low in adults, the virus affects the hearts of developing young which leads to increased mortality due to necrotizing myocarditis (Grubman and Baxt, 2004). In sheep, goats and deer, these lesions are not as noticeable, therefore making it difficult to identify infected animals and making them a dangerous source of infection. The diagnosis from clinical signs become complex as there are a range of other viral disease that display the same symptoms, including swine vesicular disease and vesicular stomatitis that cause identical lesions to FMDV (Rémond, Kaiser and Lebreton, 2002). The severity of acute clinical signs can vary and is dependent on viral dose, route of transmission and the animal species being infected (Alexandersen, Zhang, *et al.*, 2003). Therefore suspected infection requires laboratory confirmation, by the recovery of virus from epithelial tissue, blood, vesicular fluid and oesophageal secretions (Rémond, Kaiser and Lebreton, 2002). Recent work by Gloster et al. (2011), have investigated the use of thermal imaging for the early detection of FMDV by measuring the temperature around the hoof and eye areas. Success with this was limited however, as the ambient temperature and animal's movement prior to measurement affects the results. Recent work has also been done on pre-clinical detection of the virus with use of an air filter sampling system, which has shown promise as a non-invasive screening method (Waters *et al.*, 2014; Pacheco *et al.*, 2017). Early clinical diagnosis along with virological diagnostics could prove to be more effective than waiting for clinical signs to appear.

1.5.2 VIROLOGICAL DIAGNOSIS

Identification of the viral agent involves virus isolation or reverse transcriptase-PCR (RT-PCR) on samples of the oesophageal-pharyngeal (OP), epithelial tissue, vesicular fluid, milk or blood. The use of enzyme-linked immunosorbent assay (ELISA) and complement fixation (CF) testing is also used in combination as an immunological diagnosis for capturing structural components of the virus.

1.5.2.1 Viral isolation

Viral isolation and characterisation has been considered to be the 'gold standard' for FMDV diagnosis. It involves the inoculation of primary cell lines (calf thyroid or pig, calf, or lamb

kidney) or established cell lines (BHK-21) with suspected sample suspensions. The observation of a cytopathic effect (CPE) indicates viral replication. This is a time consuming and expensive method which requires maintenance of cell lines and a facility that meets the requirements for containment of group 4 pathogens. The established cell lines are known to exhibit inconsistency in comparison to the primary cell lines and attempts at immortalising primary cells to reduce costs, has been shown to display less sensitivity (Ferris *et al.*, 2002; Rémond, Kaiser and Lebreton, 2002).

1.5.2.2 Immunological methods

The complement fixation test (CFT) has been used for many years as a means of detecting capsid proteins of the virus from epithelial samples. This test requires serotype-specific antisera and is used to distinguish between different strains of FMDV (Gritsenko, Sobko and Chepurkina, 1975; Ferris *et al.*, 1984). However, there are disadvantages to the CFT: interpretation becomes difficult due to pro- and anti-complementary activity of the samples and it requires propagation of the virus in cell culture if the viral titres are too low, which is time consuming (Ferris and Dawson, 1988; Rémond, Kaiser and Lebreton, 2002). These complications have given rise to the development of an antigen capture ELISA, which is now the preferred method of FMD viral antigen detection and identification of serotype (Ferris and Donaldson, 1992; OIE, 2012). Recent developments in viral antigen detection have recently been made by Yang et al. (2015), with the use of a multiplex lateral flow strip test for a more rapid means of detection. This test has been shown to be comparable to the double antibody sandwich ELISA and is showing promise as a valuable tool for rapid detection and serotyping (Yang *et al.*, 2015).

1.5.2.3 Nucleic acid recognition

The development of RT-PCR methods of FMDV diagnostics has been achieved by use of universal primers that are able to differentiate between serotypes (Callens and De Clercq, 1997). The serotype-specific primers used, generally correspond to the VP1 genome coding region which is responsible for the antigenic diversity between groups of the virus. Primers were also designed to differentiate FMDV from other known enteroviruses and other viruses of vesicular diseases. These primers are based on the highly conserved $3D^{pol}$ coding region of

the FMDV genome (Meyer *et al.*, 1991; Rodríguez *et al.*, 1992; Callens and De Clercq, 1997). Recent developments have been made in order to better diagnose clinical samples of FMDV using RT-PCR and sequencing (McKillen *et al.*, 2011; Le *et al.*, 2012; Xu *et al.*, 2013). A novel reverse transcription loop-mediated isothermal amplification (RT-LAMP) test has been developed, which has been shown to be significantly more sensitive and quicker than RT-PCR (Chen *et al.*, 2011; Madhanmohan *et al.*, 2013; Kasanga *et al.*, 2014), which is paramount in diagnosing a highly infectious disease. In addition, advancements in next-generation sequencing have allowed for a universal protocol to be developed, which uses whole genome sequencing of FMDV for better diagnosis and eliminates the need for genome-specific PCR amplification (Logan *et al.*, 2014).

1.5.3 SEROLOGICAL DIAGNOSIS

There are four main reasons for serological testing of FMDV, as stated in the OIE terrestrial manual of 2012. These are 1) to confirm suspected cases of FMDV; 2) to validate the absence of infection; 3) to prove efficacy of vaccination and; 4) to endorse animals for export or import (OIE, 2012). Different tests and interpretations are based on whether the animal population has been vaccinated or not during an outbreak, or as a means of an ongoing vaccination programme for mitigation. There are two types of serological testing done: those that identify antibodies binding to structural proteins (SP) and those that detect antibodies binding to non-structural proteins (NSP) of the virus.

1.5.3.1 Structural protein testing

The advantage of using SPs as diagnostics is that they are able to detect antibodies elicited from different serotypes and are therefore very serotype-specific. The disadvantage is that the detection of antibodies to SPs does not distinguish between infected and vaccinated animals. Some of these tests include the virus neutralisation test (VNT) (Golding, Hedger and Talbot, 1976), the solid-phase competition-ELISA (SPCE) (MacKay *et al.*, 2001; Chénard *et al.*, 2003; Paiba *et al.*, 2004) and the liquid-phase blocking-ELISA (LPBE) (Hamblin, Barnett and Hedger, 1986; Hamblin *et al.*, 1987). These are the OIE-prescribed tests for trade, and are used to confirm ongoing or previous infections in non-vaccinated animals. They are serotype-specific but sensitivity is dependent on the virus being used in the tests matching those strains

in the field. The VNT is performed in tissue-culture microtitre plates and requires the use of BHK-21, lamb or pig kidney cells as well as live virus. Although this test is sensitive, it is also laborious, time consuming and needs biosafety level 3 facilities. On the other hand, the ELISA based tests require serotype-specific monoclonal or polyclonal antibodies, do not depend on tissue culture and are much quicker and correlate well with the VNT (OIE, 2012). It has been shown that both the SPCE and LPBE are able to detect lower levels of antibodies compared to the VNT but the specificity of the SPCE was higher than that of the LPBE but equivalent to that of the VNT (MacKay *et al.*, 2001).

1.5.3.2 Non-structural protein testing

The advantage of using NSPs over SPs as a diagnostic antigen is that the presence of antibodies to NSPs can be used to distinguish infected from vaccinated animals. NSPs can detect antibodies elicited by live replicating virus but not by inactivated virus used for vaccinations, with the exception of the VIAA. The disadvantage is that these tests are still not serotype-specific and would only indicate infection of FMDV or not. Therefore these diagnostics are usually used for certifying animals for trade and between regions that are FMDV-free with vaccination (OIE, 2012). The prescribed tests are usually based on a combination of different formats of ELISAs and immunoblotting (Bergmann *et al.*, 2000).

The advancement of recombinant techniques using a range of *in-vitro* expression systems has allowed for different NSPs of the virus to be examined and for the most immunogenic to be identified. A variety of recombinant NSPs have been tested from both the P2 and P3 regions of the viral genome. The work done by Rodríguez et al. (1994) assessed the FMDV Leader, 2C, 3A, 3B, 3C, 3AB, 3ABC and 3D NSPs for their immunogenicity in swine to determine if anti-FMDV antibodies against these proteins were detected in infected and vaccinated animals. The results indicated that the 3ABC polyprotein was the most antigenic and was able to successfully distinguish infected from vaccinated animals (Rodríguez *et al.*, 1994). Work done by Mackay et al. (1998), examined serum from infected and vaccinated cattle and confirmed that the 3ABC polyprotein was the most immunogenic of all the NSPs. It was also noted that the 3D protein gave false positive readings of infection from post-vaccinated serum of cattle that received less than five vaccinations, whereas the 3ABC polyprotein would not. It was only

after ten or more vaccinations that the other NSPs could not differentiate between infection and vaccination (Mackay *et al.*, 1998). This positive correlation between increased number of vaccinations and the inability to distinguish infected from vaccinated animals using NSPs is linked to the impurity of the vaccines used, as mentioned in section 1.4.

With the 3ABC polyprotein showing promise as a diagnostic reagent for DIVA, much work has been done to show its effectiveness in a range of different diagnostic tests. The 3ABC polyprotein has been shown to function in indirect-ELISAs (I-ELISA) (Sørensen *et al.*, 1998; Srisombundit *et al.*, 2013) and competitive-ELISAs (C-ELISA) (Sharma *et al.*, 2012) with great sensitivity and specificity. With comparisons being made with other NSPs, it has again been confirmed that the 3ABC polyprotein is the most immunogenic. The full 3ABC polypeptide has also been truncated into the smaller individual NSPs (Figure 1.2) and tested in a range of ELISAs. The $3AB_1$ polypeptide has also been shown to successfully distinguish infected from vaccinated animals in an I-ELISA (Nanni *et al.*, 2005; Jaworski *et al.*, 2011). Use of the 3AB polypeptide has been explored in I-ELISAs (Chung *et al.*, 2002) and shown to be just as effective as the 3ABC polyprotein (Sørensen *et al.*, 1998). Much work has also been done using the 3B (all three copies $B_1B_2B_3$) protein in an I-ELISA (Mohapatra *et al.*, 2014), and results in an I-ELISA have shown comparability to three commercial diagnostic kits (Gao *et al.*, 2012). The 3B protein has also been shown to be effective as a synthetic peptide in an I-ELISA (Shen *et al.*, 1999).

The OIE has adopted an I-ELISA based on the *E. coli*-produced 3ABC polyprotein (NSP) as its index screening method, developed by PANAFTOSA (Stear, 2005). This is being used to help discriminate between infected and vaccinated animals in countries that used emergency FMDV vaccines to regain their FMDV-free status. This has helped reduce the need for unnecessary mass slaughter of vaccinated animals (vaccinate-to-live) (Brocchi *et al.*, 2006). This method of diagnosis has been used as a reference to validate other NSP diagnostic tests. The OIE manual of diagnostic tests and vaccines for terrestrial animals indicate the index screening method for PANAFTOSA to be I-ELISAs and enzyme-linked immunoelectrotransfer blots (EITB). It also states that C-ELISAs have equivalent diagnostic performance (Stear, 2005).

Investigation into the comparison between the different NSP diagnostic test kits was done, in order to determine which might have better DIVA capabilities. Two studies looked at comparing commercial kits; CHEKIT-FMD-3ABC bo-ov (CHEKIT) ELISA (Bommeli Diagnostics/Intervet) and Ceditest ® FMDV-NS (Cedi Diagnostics B.V.) a 3ABC C-ELISA as well as the PANAFTOSA I-ELISA. The studies investigated the sensitivity (proportion of true positives) and the specificity (proportion of true negatives) identified by each of the tests. The results indicated that sensitivity and specificity of the C-ELISA and PANAFTOSA I-ELISA were high, whereas the CHEKIT ELISA had very low sensitivity but a high specificity (Bronsvoort *et al.*, 2006). The CHEKIT ELISA showed similar results as before but the C-ELISA had a high sensitivity but a low specificity when compared to the VNT used as the 'gold standard' for serological testing (Bronsvoort *et al.*, 2004). The authors state that the use of the VNT as a 'gold standard' is questionable due to the different antibody responses in cattle giving unreliable results. Therefore, there might be justification in only comparing NSP tests to each other, without assuming the status of the animals using the VNT. The indirect- and competition-ELISAs have been shown to be highly effective but due to high variability in specificity, false positives might overwhelm sero-surveillance of large populations and the use of EITB in combination might be necessary.

1.6 COMPETITIVE-ELISA

Competitive-ELISAs have been shown to be highly sensitive and specific in the detection of binding antibodies towards NSPs of FMDV (Bronsvoort *et al.*, 2006). This method of diagnosis has an advantage over the commonly used I-ELISA for FMDV detection. With the virus being able to infect multiple species, diagnosis using an I-ELISA would require a range of host specific secondary-conjugated antibodies for detection. This makes screening livestock an expensive and complex process, whereas the workings of a C-ELISA would only require one detection antibody irrespective of the origin of the serum. In an I-ELISA, NSPs are used as the capture antigens in these tests and it is the detection of bound antibodies from animal serum to these antigens that would confirm infection. In a C-ELISA the same NSPs are coated but when the suspected animal serum is added, a known antibody is added which competes for the binding of the antigen (hence termed competitive-ELISA) (Figure 1.4). Therefore, to

determine infection, the detection of the known antibody through the use of a specific secondary conjugate can be achieved. The detection signal would therefore be inversely proportional to the infection status.

1.6.1 ANTIBODY PRODUCTION

Antibodies are used in many applications including research, therapeutics and diagnostics. Polyclonal antibodies have been useful for the detection of multiple antigens, widely used in research and diagnostics, but the sera contain a diverse number of different antibodies which have unknown specificities. Therefore, monoclonal antibodies (mAbs) are greatly sought after due to their specific binding capabilities. The breakthrough that was hybridoma technology gave rise to the continuous production of specific mAbs (Köhler and Milstein, 1975).

The production of a mAb initially starts by immunising an animal with the desired target protein/antigen, then extracting the immune cell from the spleen and fusing these cells to myeloma cells to create immortalised hybridoma cell lines that continuously produce mAb (Ward *et al.*, 1999). Antibody phage display and other technologies have improved the screening and generation of monospecific binders but effective production systems are limiting (Schirrmann *et al.*, 2011). Immunoglobulins (Igs) consist of heavy and light chains (HC and LC) that require sophisticated folding to generate the intramolecular disulphide bonds (SS) for stabilisation (Figure 1.3). Many commonly used host expression systems (e.g. bacteria) do not facilitate the mechanisms required for complex folding and therefore smaller antibody fragments were designed (Boss *et al.*, 1984). These fragments consist of only the variable region of the Ig molecule and therefore still have the binding capacity of the full Ig but a reduced complexity. These variable fragments consist of the heavy and light variable (V_H and V_L) regions which can be linked together for stabilisation, to create a single chain variable fragment (scFv) (Figure 1.3) (Frenzel, Hust and Schirrmann, 2013). Improvements in recombinant DNA technology and antibody engineering have now allowed for antibody fragments or scFv genes to be cloned and expressed in a magnitude of systems; bacteria (Skerra and Plückthun, 1988), yeast (Gram and Ridder, 2001), mammalian (Ho, Nagata and Pastan, 2006), plant (Galeffi *et al.*, 2006) and insect cells (Choo *et al.*, 2002).

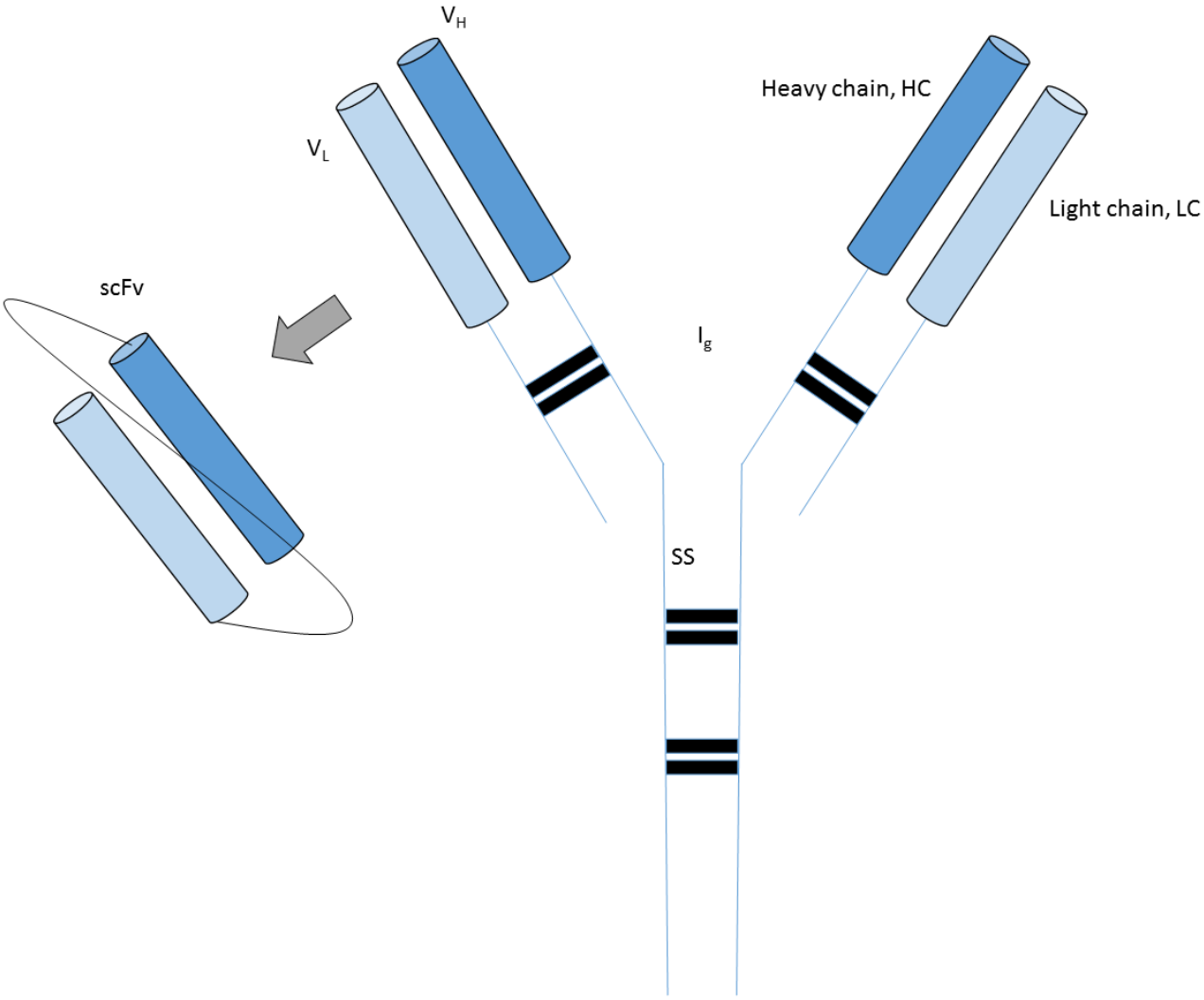

Figure 1.3. Diagram showing full immunoglobulin (Ig) and scFv.

The full Ig molecule consisting of the heavy and light chains (HC and LC), the heavy and light variable (VH and VL) regions and with disulphide bonds (SS) joining the two chains. Showing the variable regions linked together for a recombinantly made scFv.

1.6.2 COMPETITIVE-ELISA REAGENTS FOR FMDV DIAGNOSIS

It has been mentioned previously that NSPs have successfully been shown to DIVA when used in ELISAs. In a C-ELISA the 3ABC polyprotein has again been shown to function well as the coating antigen to detect infection. In many cases, the 3ABC antigen is expressed within *E. coli* and once purified, used to coat the wells of the ELISA plate. The competing antibody however, has been used from different sources. The work done by Clavijo et al. (2004) used purified *E. coli*-expressed 3ABC antigen to infect guinea pigs and the serum (polyclonal) was successfully used as the competing antibody (Clavijo *et al.*, 2004). Similarly, work done by

Sharma et al. (2012), expressed the 3ABC polyprotein in *E. coli* and once purified, monoclonal antibodies were developed by hybridoma cell lines (Sharma *et al.*, 2012). These studies were successful in distinguishing infected from vaccinated animal serum but the use of animals and hybridoma culture is a lengthy, complex and expensive approach for continual antibody production. In order to circumvent this problem a group decided to use antibody phage display technology in order to generate a phage display library of recombinant scFvs which could be used as the competing binding antibody in a C-ELISA to diagnose FMDV. Foord et al. (2007) used recombinant *E. coli*-expressed and purified 3ABC polyprotein (NSP) to immunise chickens. A chicken phage display library was made and biopanning used to isolate the 3ABC-specific phage antibody clones, which when sequenced revealed three distinctive genetic sequences.

The three specific scFv genes were expressed in an *E. coli* system, and when tested showed binding capabilities to the 3ABC polyprotein, specifically to epitopes on the 3B region. The three-chicken recombinant antibody-foot-and-mouth (CRAb-FM) 26, -27 and -29 clones were

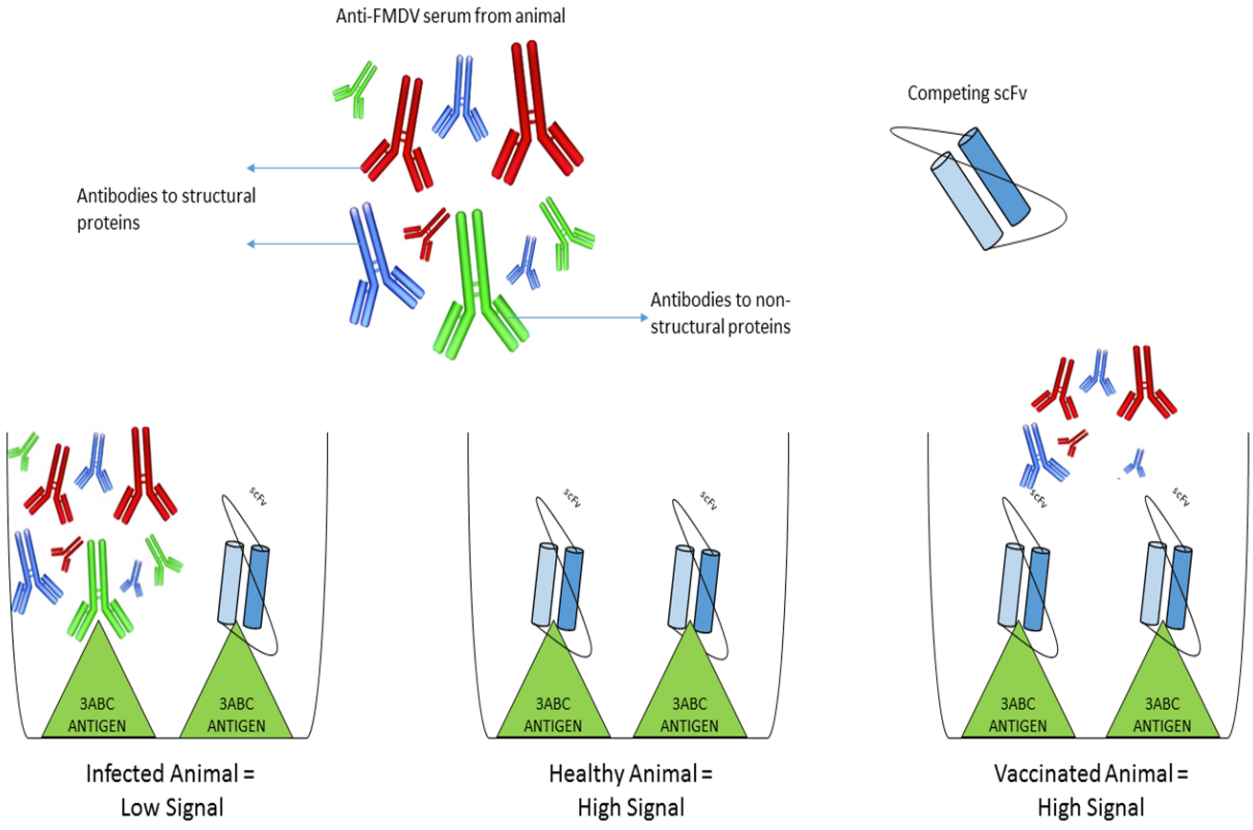

Figure 1.4. Diagram of a competitive-ELISA showing how differentiation of infected and healthy or vaccinated animals would be performed with a scFv as the competing agent.

evaluated in a C-ELISA. The CRAb-FM27 clone was best for differentiating sera from pigs, cattle and sheep that were from naïve, FMDV-infected and FMDV-vaccinated samples (Foord *et al.*, 2007). This is the first serological diagnostic assay which successfully derived both antigen and detecting scFv from a bacterial expression system for FMDV diagnosis. This safe and economic production of diagnostic reagents without the need for live virus and the costly use of continuous cell culture maintenance is most desirable.

1.7 THE SOUTH AFRICAN DILEMMA

South Africa (SA) is considered a FMDV-free without vaccination country but with the Kruger National Park (KNP) being recognised as the only infected area within the country. The epidemiology of the disease has shown that all three SAT serotypes of the virus are maintained within the African buffalo (Condy *et al.*, 1985; Di Nardo *et al.*, 2015) and that more than 70 other species of wildlife can be infected (Grubman and Baxt, 2004). The prevalence of the virus within these animals has created a reservoir for the virus, making the KNP a lingering threat to South Africa. Recommendations by the OIE were to isolate the KNP from the rest of the country using game-proof fencing and to set up a buffer zone adjacent to the park and a surveillance zone just outside of that, for livestock to be continuously monitored (Figure 1.5). In the buffer zone, animals are vaccinated twice a year with a trivalent SAT 1, 2 and 3 vaccine. There is a seven-day inspection of livestock and strict movement of animals and animal products into the surveillance and free zones for outbreak mitigation. There are 14-day inspections of livestock within the surveillance zone, along with ongoing serological testing of serum of animals translocating into the free zone. There is also continuous monitoring taking place within the KNP and other private game reserves holding wildlife, specifically within the African buffalo (*Syncerus caffer*) population (Brückner *et al.*, 2002).

With vaccinations taking place in and around the park, distinguishing infected from vaccinated animals is of great importance. Complications have arisen in the past, during emergency outbreaks, where animals had come into contact with infected wildlife and DIVA recognition of livestock sera was required. The large number of livestock and wildlife sera obtained from this continuous monitoring requires the need for level 3 bio-containment facilities. South

Africa's Exotic Disease Division at the Onderstepoort Veterinary Institute (EDD-OVI) in Pretoria was founded to handle highly contagious diseases such as FMDV and African swine fever. This facility routinely manages between 20-30 thousand serological tests per year, which can increase to around 75 000 during an outbreak (Brückner *et al.*, 2002).

Outbreaks are known to put tremendous strain on a nation's economy from the direct and indirect impact the virus has on animals. In September 2000 to February 2001 SA experienced outbreaks in Mpumalanga, KwaZulu-Natal and Mhala. The total operational costs to contain the problem including having over 900 personnel to maintain roadblocks, set up animal quarantine camps, dispose of culled animals, control bio-security cordons and perform continuous diagnosis, was estimated at ZAR90 million (Brückner *et al.*, 2002). The monitoring of animals around the KNP may be challenging and expensive but controlling the spread and returning a region to a FMDV-free status is even more so and adds to the stress put on the economy. This illustrates the importance of having a fast, accurate diagnostic protocol as well as a more cost-effective one to limit the enormous costs of containment.

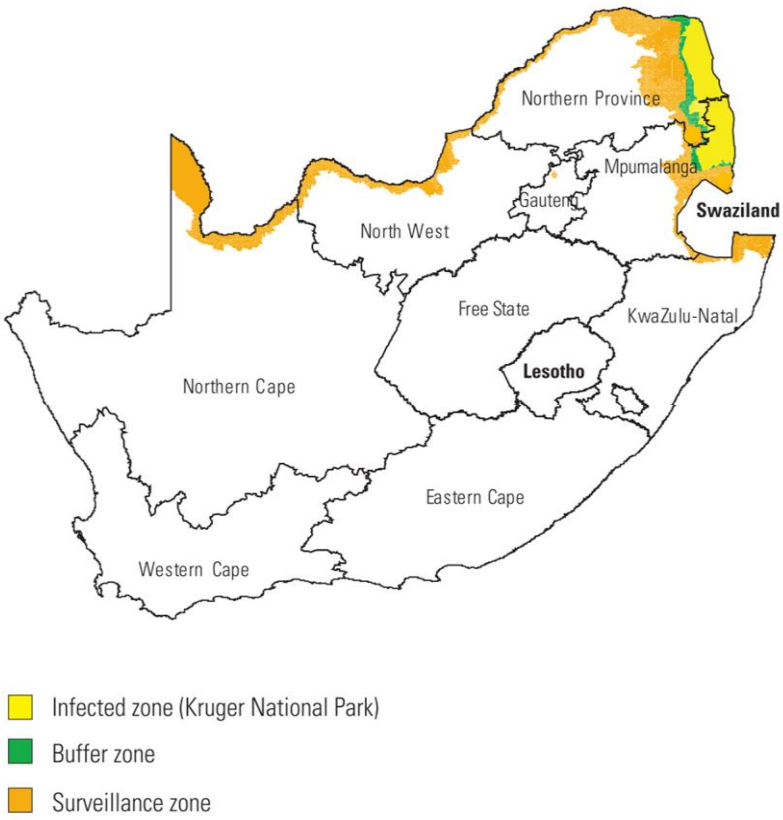

Figure 1.5. Indicating the areas of South Africa where FMDV is controlled.

The control zones located next to the Kruger National park and neighbouring countries and the white zone indicating the FMDV-free zone. Figure taken and adapted from Brückner et al. (2002).

1.8 PLANT EXPRESSION AS A SOLUTION

The advancement over the last few decades in recombinant DNA technology has given rise to a multitude of different protein expression systems. Recombinant proteins used for research, diagnostics and therapeutics have been expressed in both prokaryotic and eukaryotic cell systems, each of which systems having different advantages. The shared objective in the ongoing research is to identify a single system that has the collective advantages of these. The model expression system would offer high levels of functionally active proteins which are safe, production of which would be scalable, and could be produced at a reduced cost. The expression of recombinant DNA in mammalian cells has produced biologically active molecules but, scalability of this system is costly as cell culture maintenance is expensive. This system is also susceptible to human or other mammalian pathogen contamination which can present a considerable threat if used as a therapeutic. Microorganisms have been modified to accommodate high levels of production, however, these products are also limited due to the considerable difference between the natural protein and the bacterially-expressed ones. An example of this is the lack of post-translational modifications often required for correct conformation and folding through disulphide bridges and glycosylation, which is seen in mammalian but not bacterial systems (Egelkrout, Rajan and Howard, 2012).

The use of plants as a recombinant protein expression system in recent years has shown potential for covering many of these advantages, and has been proposed as an effective alternative expression system (Hefferon, 2014). Firstly, plants are a more economically viable method of production in comparison to mammalian cell culture, which has been shown in antibody production (Nandi *et al.*, 2016). Secondly there is no risk of containing retroviruses or other pathogens which can occur in cell cultures. Thirdly, plants contain a secretory pathway and endomembrane system similar to that of mammalian cells, which allows for post-translational modification of recombinant proteins which is not observed in bacterial production of complex proteins (Chen *et al.*, 2013).

The transformation of plants with foreign DNA can be done by a physical or biological means. Physically a particle gun, electroporation or PEG-mediated transformation can be used to

transform plant cells with recombinant DNA for transgenic or transient expression (Rao *et al.*, 2009). The advantage with physical transformation is that it is simple but, complications can arise when the physical toll on the host tissue causes cell damage during transformation, reducing successful integration (Egelkrout, Rajan and Howard, 2012). The biological means of transformation involves the natural ability of *Agrobacterium* to incorporate its transferred-DNA (T-DNA), located on the tumour inducing (Ti) plasmid, into the plant cell. The left and right borders (LB and RB) located on either side of the T-DNA are used to harbour recombinant DNA which would then be transferred and transcribed within the plant cell (Tzfira and Citovsky, 2006). This can facilitate episomal transient expression, using either plasmid or replicating plant virus-derived vectors, or stable transformation using non-viral vectors (Egelkrout, Rajan and Howard, 2012).

Transgenic expression of recombinant proteins is often highly successful, and once the gene has been integrated into the plant genome, it is stable over many generations (Li *et al.*, 2006). However, this approach is often time consuming as several months are required for transformation, which limits high throughput investigation of highly expressed recombinant proteins. Once generated, the stable transgenic lines are considered genetically modified organisms (GMO) and precautions would need to be taken to prevent exposure to the environment, which again adds to the cost of production (Rybicki, 2009).

The advantages of using transient expression is that a larger variety of proteins can be screened for possible expression in a plant system in a shorter period of time, hence improving high throughput testing. The yields have been shown to be competitive with transgenic and other expression systems (Gleba, Klimyuk and Marillonnet, 2007). Moreover, containment is not an issue as the genes are somatically expressed and are not heritable. Simple transient expression is achieved using modified Agrobacterium-derived plasmid vectors, which do not replicate in plant cells. The virus-derived vectors which do replicate consist of two types: these are first generation and second-generation vectors. The first generation of viral vectors (gene substitution and gene insertion vectors) involved the use of fully functional RNA plant viruses, which contained the coding sequence of the gene of interest. The gene of interest is usually expressed from a strong viral promoter like the coat

protein promoter. The limitations of the full-virus approach are that large inserts of >1Kb are not usually expressed and only short peptides could be expressed fused to the viral coat protein. The infection process is usually slow and asynchronous leading to not all parts of the plant being infected, and the vectors are generally unstable which leads to issues of low yields of protein. These limitations prompted the development of the second generation of vectors (deconstructed and modular viral vectors, both RNA and DNA), which involve the use of deconstructed virus vectors containing only the elements necessary for expression. These second-generation viral vectors combined the best features of viruses for their speed and high level of expression and the efficiency of transformation using *Agrobacterium*. The deconstructed RNA viral vectors gave rise to a new expression strategy in plants, called magnifection (Gleba, Klimyuk and Marillonnet, 2007).

Magnifection in essence uses deconstructed viral vectors that have been integrated between the LB and RB into a single modified Ti plasmids of *Agrobacterium* for the transformation and recombinant protein expression in plant tissue (Gelvin, 2003; Gleba, Klimyuk and Marillonnet, 2005, 2007; Lico, Chen and Santi, 2008).

The modular vector system utilises binary vectors which contain the essential components of the viral vectors, along with the gene of interest between the LB and RB on a separate T-DNA plasmid. The Ti plasmid, would have the virulence genes acting in *trans* to transfer the T-DNA. This modified Ti plasmid is referred to as the helper plasmid (Lee and Gelvin, 2007; Shah, Almaghrabi and Bohlmann, 2013). The two systems of second generation vectors uses the process of *Agrobacterium*-mediated transfer to introduce the bacteria into the plant by vacuum or syringe infiltration (Chen *et al.*, 2013). This process addresses the limitations of the earlier expression based technologies in plants by having: increased levels of protein expression and high yields, faster and cheaper research and development, and reduced costs in biosafety as well as upstream processing (De Martinis *et al.*, 2016).

1.9 AIMS AND OBJECTIVES

The Foot-and-mouth disease virus has been shown to be a lingering threat to South Africa as the wildlife in the KNP and other wildlife reserves act as a viral reservoir. FMD is one of the most devastating diseases for any country's economy, where billions of dollars have been spent to mitigate outbreaks and eradicate it. The nations which have successfully done so, have gained the FMDV-free status from the OIE, which allows them to maintain animal and animal product trading with other FMDV-free countries. Once declared as FMDV-free, maintaining this status requires strict monitoring of livestock through various diagnostic methods prescribed by the OIE. These are used to prevent or detect possible outbreaks as soon as possible and to reduce the direct and indirect impact of the virus. This monitoring of animals is generally done during their import and export but SA also has the viral reservoir to consider. This additional burden requires continuous inspection of animals around susceptible areas, which adds to the huge cost associated with outbreak mitigation. The cost of diagnosis varies depending on the inquiry and method required to answer it. The Agricultural Research Council (ARC) at the Onderstepoort Veterinary Institute supply a price list for each test. The range of these costs per animal are; ZAR229 (NSP-ELISA test to DIVA), ZAR367 (VNT), ZAR555 (virus isolation), ZAR1963 (RT-PCR) and over ZAR2000 for sequencing (ARC, 2017).

Vaccines are used around the buffer and surveillance zones of the KNP and therefore regular inspection requires rapid diagnoses that involves distinguishing infected from vaccinated animals. The NSP-ELISA is used for this as it is the quickest of the tests (4 days compared to 5-8 days for VNT or virus isolation), can distinguish infected from vaccinated animals and is the cheapest (ARC, 2017).

The focus of this project, therefore, was to determine if diagnostic reagents for DIVA ELISA tests could be made in a plant expression system, and to test whether they were functional, and whether they could be made less costly. The reagents investigated were the highly immunogenic 3ABC NSP of the virus and a scFv (CRAb-FM27) found previously in another laboratory to recognise the 3ABC polyprotein. Expression and functionality of truncated

versions of the 3ABC polyprotein were also tested ($3AB_1$, 3AB and 3B). The expression and functionality of a plant-produced CRAb-FM27 scFv, previously shown to be functional in a C-ELISA to DIVA sera (section 1.6.2) was also investigated.

The objectives of the project were:

- To transiently express these FMDV NSPs in a plant system and purify them using immobilized metal-ion affinity chromatography (IMAC). The plant expression vectors used are the autonomously replicating geminivirus-derived pRIC3.0 (Regnard *et al.*, 2010) and the non-replicating pTRAKc. The same NSPs were to be expressed and purified in an *E. coli* system to serve as positive controls and a point of reference for comparison to plant expression.
- To transiently express the CRAb-FM27 scFv in a plant system using the pTRAKc vector and to purify it using IMAC. *E. coli*-expression of the scFv was to be done again to serve as a positive control during plant expression and to compare its functionality to the plant made one.
- To test these two reagents for functionality using an indirect-ELISA and again in tandem in a competitive–ELISA to determine if they are able to differentiate between infected and vaccinated animal serum.

CHAPTER 2

EXPRESSION AND PURIFICATION OF FMDV 3ABC VARIANT ANTIGENS IN *E. COLI* AND *N. BENTHAMIANA*

2.1 INTRODUCTION

The types of diagnostic reagents required for use in FMDV antibody detection differ depending on whether the FMDV serotype is to be identified or if the animal's status needs to be distinguished from infected or vaccinated. In an FMDV-free country that does not require vaccination of animals, animal serum would only need to be tested for the presence of antibodies as a result of infection and therefore structural proteins (SPs) could be used in a serotype-specific ELISA test to determine this. Countries that have FMDV-free with vaccination status, would need to differentiate between the animals that have been vaccinated and those that are infected. Serological tests using ELISAs with non-structural proteins (NSPs) of the virus are employed to do so, as only binding antibodies from an infected animal would be detected.

Buffalo and other animals in the Kruger National Park in South Africa act as a reservoir for the virus, and vaccination of cattle is therefore used in the buffer zone surrounding the park to prevent spread. Accordingly, it is of great importance that the animals translocating from controlled zones into FMDV-free areas of the country are distinguishable from infected ones. This becomes an expensive process, and with a range of serological, immunological and nucleic acid tests being used (e.g. VNT, CFT and RT-PCR), the NSP-ELISA is the quickest and cheapest option.

A variety of non-structural proteins exists as part of the virus's replicating machinery and much research has been done to determine the most immunogenic ones. Research has shown that the 3ABC polypeptide has worked well in an ELISA as a NSP diagnostic reagent for the detection of anti-FMDV antibodies. Variations or truncated regions of the full 3ABC polypeptide ($3AB_1$, 3AB and $3B_{123}$) have also been shown to be as effective in an ELISA to

distinguish infected from vaccinated animal serum. The polyprotein consists of three main parts: region 3A helps anchor the viral transcripts to the ER; the 3B region consists of three non-identical segments B_1, B_2 and B_3 each of which encode a viral genome-linked protein involved in the initiation of RNA synthesis and encapsulation of viral RNA; and, the 3C region is a protease ($3C^{pro}$) that is involved in cleavage of the 3ABC polyprotein into its individual components.

The important characteristic of an antigen used as a diagnostic reagent is its ability to distinguish infected from vaccinated animals (DIVA). The main focus of this project was to explore the expression of these known NSPs in plants, which could lead to an increased level of protein containing the necessary post-translational modifications and which could potentially be used as an alternative expression platform for more cost-effective diagnostic reagent production. The testing of FMDV 3ABC expression and several variants thereof in the plant *Nicotiana benthamiana* was accordingly carried out in five studies. The first two studies included using the whole polyprotein of two different serotypes: these were serotype O (3ABC-O) and serotype A (3ABC-A). These have previously been shown to be successfully expressed in an *E. coli* system and were used as reagents in ELISAs for FMDV detection. The third study used a truncated version of the 3ABC-O protein which lacks the $3C^{pro}$ region, yielding the 3AB variant (3AB-O). The fourth involved the removal of the 3A and $3C^{pro}$ regions from 3ABC serotype O, giving rise to the $3B_{123}$ region (3B-O). The final variant was the $3AB_1$ protein, which is used at the National Agricultural Technology Institute (INTA) in Argentina ($3AB_1$-Arg). Expanding the range of antigen types by having multiple variants of 3ABC was thought to broaden the chances of achieving successful expression in plants.

Genes encoding the protein variants described above were cloned into two plant expression vectors, pRIC3.0-HT and pTRAKc in order to compare expression levels of recombinant proteins. Transient expression of these proteins in *N. benthamiana* was done using two different binary vectors. The pRIC3.0-HT vector was selected as it functions to increase expression by autonomous replication. The expression for each protein using pRIC3.0-HT vector was compared to the pTRAKc vector, which is non-replicating.

In addition, the same genes were cloned into the *E. coli* expression vector pProEX-HTb in order to produce recombinant protein of the cognate variants for positive controls as well as to be compared to the functionality of the plant produced antigens during testing of the proteins against animal serum (described in Chapter 4).

2.2 MATERIALS AND METHODS

2.2.1 PLASMID AMPLIFICATION AND ISOLATION.

The plant and *E. coli* expression vectors were maintained in *E. cloni®* DH5-α competent cells (Lucigen, USA). These vectors included the pProEX-HTb (Thermo Fisher Scientific) *E. coli* expression vector and the pRIC3.0-HT (Biopharming Research Unit (BRU) in the department of Molecular and Cell Biology (MCB) at the University of Cape Town) and pTRAKc-ERH (supplied by Rainer Fischer, Fraunhofer Institute, Aachen) plant expression vectors. Cultures were streaked out on Luria-Bertani (LB) (1.0% Tryptone, 0.5% Yeast Extract, 1.0% NaCl, 1.5% Bacterial Agar) plates containing the appropriate antibiotics for selection (Table 2.1) and incubated overnight at 37°C. Colonies for each clone were inoculated in 10ml LB broth with the relevant antibiotics and incubated overnight at 37°C. Plasmid DNA was extracted from overnight cultures using the QIAprep Spin Miniprep kit (QIAGEN, USA) and DNA concentrations were measured using a UV-VIS NanoDrop spectrophotometer (NanoDrop technologies, USA) at a wavelength of 260nm.

Table 2.1. Antibiotics used for bacterial selectivity.

Vector	Bacterial strain	Selective antibiotic
pProEX-HTb	*E. coli* DH5α	Ampicillin (100µg/ml)
pJET2.1/blunt		
pUC57		
pRIC3.0-HT		
pTRAKc		
pRIC3.0-HT	*A. tumefaciens* GV3101::pMP90RK	Rifampicin (50µg/ml) Kanamycin (30µg/ml) Carbenicillin (50µg/ml)
pTRAKc		

2.2.2 CONSTRUCT DESIGN AND SYNTHESIS.

Three variants of the antigen gene were synthesised: the first was the full 3ABC polypeptide from FMDV serotype O (*3ABC-O*) (GenBank, Accession number AY614501) which was wild type and had no modifications. The second was the full polypeptide gene from serotype A (GenBank, Accession number AFE84732), which contained mutations (*mu3ABC-A*). The third was only the 3B region of the polypeptide from serotype O (*3B-O*) and the fourth was the 3AB1 region of the polypeptide from serotype A (*3AB1-Arg*, sequence obtained from INTA). Synthesis of the antigen genes was done by GenScript (China): these were codon optimised for *Nicotiana benthamiana* and cloned in pUC57. All genes contained a protein disulphide isomerase (PDI) signal sequence (GenBank, Accession number Z11499) fused to the 5' terminus. A histidine tag (6Xhis tag), was fused to the 5'end of *mu3ABC-A, 3B-O* and *3AB1-Arg* genes.

2.2.3 SITE DIRECTED MUTAGENESIS.

It is hypothesised that the $3C^{pro}$ region of the polyprotein could possibly be responsible for a reduction of recombinant 3ABC expression due to it causing proteolysis of host cell proteins. Therefore site-directed mutagenesis (SDM) was employed to disrupt the active site of the $3C^{pro}$ region. The *3ABC-O* gene was used as the template for SDM of the $3C^{pro}$ region of the polypeptide as first described by Ho et al. (1989) and resulted in the *mu3ABC-O* variant. Two mutations (Cys 142 Ser and Cys 163 Gly) were added by substitution of single base pairs using sets of primers (Table 2.2) and PCR. This was done using flanking primers (For-A and Rev-D) to bind either end of the target sequence along with internal primers (For- C1+*TaqI* and Rev-B1+*TaqI*) containing mismatched bases to bind the region where the mutation occurred. The first round of PCR created the AB and CD fragments which were run on an agarose gel and purified. These fragments were mixed together along with the flanking primers for the second round of PCR. The B and C region of the fragments were complementary to each other, containing the changed sequence, and therefore hybridised and in turn created the new internal primers resulting in the final A to D sequence containing the first Cys142Ser mutation. The complete mutated fragment was run on an agarose gel, purified and ligated into the pJET1.2/blunt cloning vector (Thermo Scientific, CloneJET PCR Cloning Kit) as per the manufacturer's instructions. Mini-prepped plasmids of the first mutation were then subjected

to the same procedure for introduction of the second mutation using the same flanking primers but different internal ones (For-C2+*MnlI* and Rev-B2+*MnlI*). All PCRs were performed using a high fidelity DNA polymerase (Accuzyme™, Bioline) as per the manufacturer's instructions, to reduce misreading and for blunt end formation of products.

Confirmation of the added mutations was done using the RE digest of the final sequence. The mutations were created such that the sequence change at the nucleotide level created a synonymous mutation to include restriction enzyme sites. The Cys142Ser mutation created a *TaqI* restriction site and the Cys163Gly mutation created a *MnI* restriction site. Digestion of the mutated gene with the respective enzymes was performed and run on a 2.5% agarose gel. The banding pattern observed would determine if the mutation was made or not.

Table 2.2. Primers and conditions used for site-directed mutagenesis. Red nucleotides indicating base change for substitution mutations and underlined regions indicating RE sites.

Round	Primers	Conditions
Mutation 1 (Cys142ser) *TaqI* RE site	For-A 5'- GACCATGGCGAATGT-3' Rev-B1+*TaqI* 5'- CCATCCATCGATACAACA-3'	Denatured at 95°C for 30 sec Annealing at 42°C for 30 sec Extension at 72°C for 1 min 30 sec Final Extension at 72°C for 2 min 30 cycles
	For- C1+*TaqI* 5'- TGTTGTATCGATGGATGG-3' Rev-D 5'- ATCTCGAGTCATTCGTGGTG-3'	
Mutation 2 (Cys163Gly) *MnlI* RE site	For-A 5'- GACCATGGCGAATGT-3' Rev-B2+*MnlI* 5'-GCTCCACCTCCGTAACCAG-3'	Denatured at 95°C for 30 sec Annealing at 45°C for 30 sec Extension at 72°C for 1 min 30 sec Final Extension at 72°C for 2 min 30 cycles
	For-C2+*MnlI* 5'- CTGGTTACGGAGGTGGAGC-3' Rev-D 5'- ATCTCGAGTCATTCGTGGTG-3'	

2.2.4 TRUNCATION OF *3ABC-O*.

The pUC57-*3ABC-O* plasmid was subjected to PCR in order to truncate and remove the $3C^{pro}$ region of the polypeptide gene. This was done using a high fidelity DNA polymerase along with specific primers and cycling conditions (Table 2.3). Amplification of the *3AB* region would result in the loss of the *3C* region and the *3AB-O* gene was ligated into the pJET1.2/blunt cloning vector as per manufacturer's instructions. The variations of the *3ABC* gene, its elements and its different serotypes can be seen in Figure 2.1

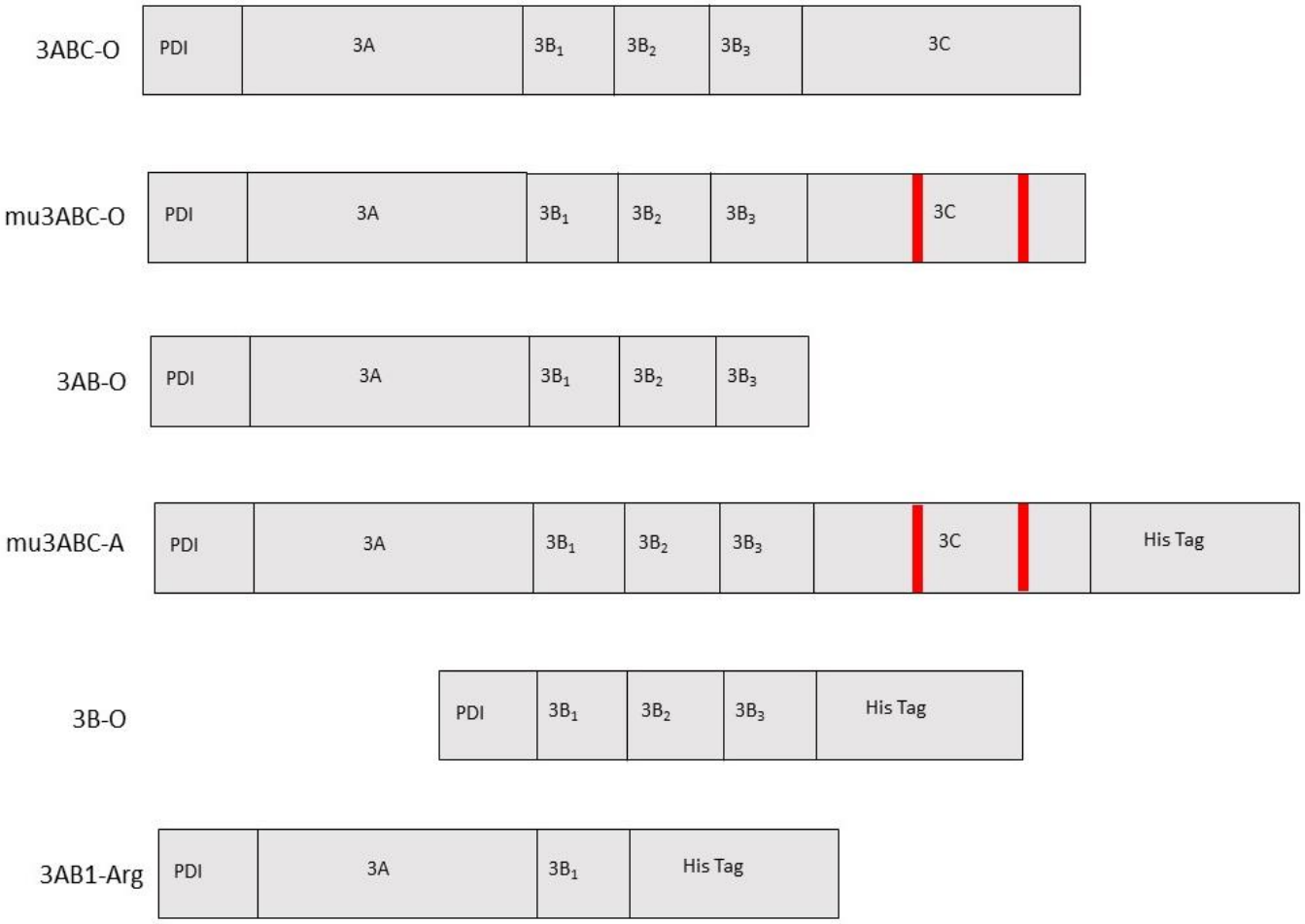

Figure 2.1. Schematic of the five *3ABC* NSP gene variants.

Mu3ABC-A, 3B-O and *3AB1-Arg* have a 3' 6Xhis tag, whereas the *mu3ABC-O* and *3AB-O* genes do not. They each contain a 5' PDI signal for targeting to the endoplasmic reticulum. Red bars indicate mutation sites in the $3C^{pro}$ region.

Table 2.3. Primers and conditions used for 3ABC-O truncation.

Template	Primers	Conditions
pUC57-3ABC-O	Forward 5'-TACCATGGCGAAGAATGTTGCTATT-3' Revers 5'-TACTCGAGTTATTCTGTTACAATCAAATTC-3'	Denatured at 95°C for 30 sec. Annealing at 50°C for 30 sec. Extension at 72°C for 1 min 30 sec. Final Extension at 72°C for 2 min 30 cycles

2.2.5 CLONING STRATEGY AND SUBCLONING.

The cloning strategy for the *3ABC-O, mu3ABC-O* and *3AB-O* genes into the plant expression vectors was as follows: cloning of the genes into pRIC3.0-HT was done by restriction enzyme (RE) digest using the 5' *NcoI* and 3' *XhoI* sites. These sites allowed for the retention of the 6Xhis tag within the vector at the 5' end. Cloning of these same genes into the pTRAKc-ERH vector used the same restriction enzymes. This resulted in the retention of the 5' lactase-phlorizin hydrolase (LPH) signal sequence (Maclean et al. 2007), which is used as an ER targeting signal, but the removal of the 6Xhis tag and SEKDEL signal (ER retention signal) within the vector.

The cloning strategy for the *mu3ABC-A, 3B-O* and *3AB1-Arg* genes into the two plant expression vectors was as follows: The pRIC3.0-HT vector was digested at the *AflIII* and *XhoI* sites and the genes digested using the *NcoI* and *XhoI* sites. Ligation of the gene into the vector abolished the *NcoI* site and removed the native 6Xhis tag within the vector. The same genes were cloned into the pTRAKc-ERH expression vector using *EcoRI* and *XhoI*. This resulted in the removal of the LPH signal, 6Xhis tag and SEKDEL signal from the vector.

The cloning strategy for *3ABC-O, mu3ABC-O, 3AB-O, mu3ABC-A, 3B-O* and *3AB1-Arg* genes into the *E. coli* expression vector (pProEX-HTb) was done using the *NcoI* and *XhoI* RE sites. The use of these sites resulted in the retention of the 5' 6Xhis tag in the expression vector. The *3ABC-O, mu3ABC-O* and *3AB-O* genes do not contain a 6Xhis tag and therefore would only contain one 5' 6Xhis tag from the vector. The *mu3ABC-A, 3B-O* and *3AB1-Arg* genes already had a 6Xhis tag fused to the 3' terminus and therefore once cloned into pProEX-HTb would contain both a 5'and 3'his tag. See Table 2.4 for final constructs.

All plasmids (100ng) were digested using appropriate restriction enzymes (Thermo Fisher Scientific) incubating for 1 hour at 37°C and fragments resolved by electrophoresis at 120 Volts (V) for 1 hour on a 1% agarose gel (0.5 µg/ml)0.04 µl/ml ethidium bromide). O'GeneRuler 1kb DNA ladder (Fermentas, Canada) was used to determine the size of the DNA

fragments. A QIAquick gel extraction kit (Qiagen, USA) was used to purify the inserts and the expression vector fragments from the gel, as per manufacturer's instructions. A ligation reaction of 1:3 (vector: insert) molar ratio was used and T4 ligase (Fermentas, Canada) was added and incubated overnight at 4°C for ligation. Transformation of recombinant plasmids into *E. cloni®* DH5α competent cells was performed as per manufacturer's instructions.

Five 3ABC NSP gene variants	***E. coli* expression vector**	**Plant expression vectors**	
	pProEX-HTb	**pRIC3.0-HT**	**pTRAKc**
3ABC-O	pProEX-HTb-*3ABC-O*	pRIC3.0-HT-*3ABC-O*	pTRAKc-*3ABC-O*
Mu3ABC-O	pProEX-HTb-*Mu3ABC-O*	pRIC3.0-HT-*Mu3ABC-O*	pTRAKc-*Mu3ABC-O*
Mu3ABC-A	pProEX-HTb-*Mu3ABC-A*	pRIC3.0-HT-*Mu3ABC-A*	pTRAKc-*Mu3ABC-A*
3AB-O	pProEX-HTb-*3AB-O*	pRIC3.0-HT-*3AB-O*	pTRAKc-*3AB-O*
$3AB_1$-Arg	pProEX-HTb-*$3AB_1$-Arg*	pRIC3.0-HT-*$3AB_1$-Arg*	pTRAKc-*$3AB_1$-Arg*
3B-O	pProEX-HTb-*3B-O*	pRIC3.0-HT-*3B-O*	pTRAKc-*3B-O*

Table 2.4. Showing final cloned antigen genes into *E. coli* and plant expression vectors.

2.2.6 RECOMBINANT PLASMID CONFIRMATION.

In order to confirm successful transformation of recombinant expression vectors into either *E. coli* or *A. tumefaciens*, colonies were screened using polymerase chain reaction (PCR). All PCR reactions were done using the Bio-Rad MyCycler (Bio-Rad Laboratories, USA) and KAPATaq ready mix (KAPABiosystems, USA, KK1024). PCR reactions were performed per the manufacturer's instructions in 20µl reactions. PCR products were resolved on an agarose gel, along with a 1kb DNA ladder as described in section 2.2.5. Primers and cycling conditions for each set of reactions are shown in Table 2.5. Primers were synthesised by the MCB Oligonucleotide Synthesis Service, UCT.

Confirmation of recombinant expression vectors was also achieved using restriction enzyme analysis. Each recombinant plasmid was mini-prepped and digested using the same REs as used in the cloning steps in section 2.2.5. The fragments were resolved on an agarose gel to observe the banding pattern. Confirmation of recombinant vectors was also achieved via sequencing (Macrogen). Theoretical subcloning of the various genes into the expression vectors was initially done using CLC Genomics Workbench (Qiagen).

Table 2.5. Vector specific primers and conditions used for colony PCR.

Vector	Primers	Conditions
pProEX-HTb	Forward 5'-CGTACTACCATCACCATCAC-3' Revers 5'-TGATTTAATCTGTATCAGGCTG-3'	Initial denaturing 95°C for 5 min Denatured at 95°C for 30 sec Annealing at 55°C for 30 sec Extension at 72°C for 1 min 30 sec Final Extension at 72°C for 2 min 30 cycles
pRIC3.0-HT	Forward 5'-ATCCTTCGCAAGACCCTTCCTCT-3' Reverse 5'-AGAGAGAGATAGATTTGTAGAGA-3'	Initial denaturing 95°C for 5 min Denatured at 95°C for 30 sec Annealing at 54°C for 30 sec Extension at 72°C for 1 min 30 sec Final Extension at 72°C for 2 min 30 cycles
pTRAKc		

2.2.7 *E. COLI* EXPRESSION OF RECOMBINANT PROTEIN.

The recombinant pProEX-HTb antigen constructs and empty pProEX-HTb (negative control) were maintained in *E. coli* glycerol stocks (25% glycerol) and inoculated initially in 10ml LB broth with appropriate antibiotics (Table 2.1). These were incubated at 37^{o}C with shaking overnight (16-18 hours). The starter cultures were used to inoculate a 50ml LB broth with appropriate antibiotics and again incubated overnight at 37°C. The pProEX™ HT Prokayotic Expression System (Thermo Fisher Scientific) protocol was followed as per manufacturer's instructions. The culture was induced using isopropyl β-D-1-thiogalactopyranoside (IPTG), at a final concentration of 0.6mM, when the optical density (OD_{600}) reached 0.6-0.1 (exponential growth phase). The culture was again incubated at 37^{o}C. As a small-scale time trial, 1ml samples were taken every hour for 3 hours after induction and analysed for optimisation purposes. Large scale expression was done using the optimised time of harvest determined in the small-scale trial. The cultures were centrifuged (10 000xg) for 10 minutes after the incubation period and the pelleted cells' wet weight was determined. The cell pellets were kept for protein extraction.

2.2.8 RECOMBINANT *E. COLI* PROTEIN EXTRACTION.

The extraction of recombinant protein from the *E. coli* cell pellet from section 2.2.7 was achieved using the BugBuster™ protein extraction reagent (Novagen). The empty vector negative control cells were treated in the same way during extraction. The protocol was followed for soluble protein extraction, as per the manufacturer's instructions. The pellet, collected after isolation of the soluble fraction, was kept and resuspended in 2ml extraction buffer (1X phosphate-buffered saline (PBS), 8M urea, pH8) (same buffer as the equilibration buffer for batch binding). This was kept shaking at room temperature (22-24^{o}C) for 1 hour to allow for disruption of inclusion bodies and solubilisation of proteins. Centrifugation at 10 000xg for 20 minutes was done to isolate the supernatant, which was kept at -20^{o}C for later analysis.

2.2.9 *AGROBACTERIUM TUMEFACIENS* TRANSFORMATION.

A. tumefaciens strain GV3101::pMP90RK was obtained from culture collection of the BRU. The cells were made electrocompetent using the method described by Wen-Jun and Forde (1989). A 100µl volume of thawed electrocompetent cells were added to a chilled 0.1cm electroporation cuvette (Bio-Rad, USA) and 400ng of recombinant plasmid DNA was added and mixed. Cells were electroporated using a GenePulser (Bio-Rad, USA) set at 1.8kV, 25µF and 200Ω. Transformed cells were then combined with 900µl LB broth and incubated for 2 h at 27°C. Cells were plated onto LB plates with appropriate antibiotics (Table 2.1) and incubated for 2 days at 27°C. Colony PCR was used to screen transformed colonies as described before in section 2.2.6. Selected colonies were inoculated into 10ml LB broth with the respective antibiotics and incubated overnight at 27°C.

2.2.10 *A. TUMEFACIENS*-MEDIATED TRANSIENT EXPRESSION.

Starter cultures of *A. tumefaciens* containing recombinant pRIC3.0-HT or pTRAKc constructs (Table 2.4) were grown in LB broth with the appropriate antibiotics (Table 2.1) at 27^{o}C overnight (Maclean *et al.*, 2007). The empty pRIC3.0-HT and pTRAKc vectors in *A. tumefaciens* cells were grown in the same way to be used as negative controls.

Starter cultures were used to inoculate LB Broth base (LBB) (Tryptone 2.5g/L, Yeast extract 12.5g/L, NaCl 5g/L, 10 mM 2-morpholinoethanesulfonic acid [MES], 20 µM acetosyringone in water, pH 5.6) with the appropriate antibiotics (Table 2.1) and incubated at 27^{o}C overnight with agitation.

Cultures were diluted to the required optical density (OD_{600}) in a resuspension solution (10 mM magnesium chloride ($MgCl_2$), 10 mM MES, and 200 µM acetosyringone in water, pH 5.6).

Each of the constructs were infiltrated into six-week-old *N. benthamiana* plants as a small-scale time trial at varying OD_{600} of 0.5 and 1.0. Each construct consisting of a different OD_{600} suspension was injected into the abaxial side of the leaves of one plant, using a blunt-end syringe (Maclean *et al.*, 2007).

Larger scale vacuum infiltration of the plants was achieved by submerging the leaves from 12 plants into the bacterial suspension and applying a negative pressure (-100KPa) under vacuum. The plants were again left to grow under constant conditions, 16 hours of light and 8 hours of dark, at 24°C.

2.2.11 RECOMBINANT PLANT PROTEIN EXTRACTION.

The small-scale time trial of each of the expressed constructs involved harvesting equal-size disks (using the lid of an Eppendorf vial as a cutter) from leaves. The leaf disks were sampled at 1, 3 and 5 days post infiltration (dpi), and two optical densities (OD_{600} 0.5 and 1.0) were investigated. A soluble (1XPBS, pH7.4) and insoluble (1XPBS, 8M urea, pH8) extraction buffer was investigated and used to extract protein separately on each sample. To each, 150µl of extraction buffer was added and the clippings homogenised within 1.5ml Eppendorf tubes using micro-pestles. The samples were then centrifuged at 6000xg for 8 minutes and the supernatant from each collected and stored at -20^{o}C.

The optimal conditions for large scale expression were determined by the small-scale time trial, which determined at which day whole leaves should be harvested, along with the optimised OD_{600} and extraction buffer to use. Extraction buffer (1XPBS, 8M urea, pH8) was

added at a 1:3 weight (grams) to volume (millilitres) ratio. The leaves were then homogenised using a blender (Hamilton Beach Commercial, USA) and passed through Miracloth™ (Millipore, USA) to remove cell debris. Centrifugation at 13 000xg for 20 minutes isolated the supernatant which was again passed through Miracloth™ and stored at -20^{o}C.

2.2.12 BRADFORD ASSAY.

Total protein concentrations from all the samples were determined using a Bradford assay (DC Protein Assay, Bio-Rad); the microplate assay protocol was followed as per manufacturer's instructions. A standard curve using a range of bovine serum albumin (BSA, Thermo Scientific) at concentrations of 0.2, 0.5, 0.75, 1.0 and 1.5 mg/ml was made. Dilutions were made with the same extraction buffer contained in the sample. The absorbance was measured at 750nm on a BIO-TEK® Powerwave XS microtitre plate reader.

2.2.13 SDS-PAGE.

The volumes of protein samples to be tested were varied based on their total protein concentration in order to load equal amounts into each well. The samples (50µg total protein) were treated with sample application buffer (SAB) (10% SDS, 1M TrisCl pH7.5, 100mM EDTA, glycerol, dH2O, mercaptoethanol, bromophenol blue) and boiled for 10 minutes. Samples resolved at 120 V on either a 10% or 15% polyacrylamide gel by SDS-PAGE and protein sizes were compared against 5µl PageRuler™ prestained protein ladder (Fermentas) or Color Prestained protein standard, broad range (New England Biolabs).

2.2.14 COOMASSIE BLUE STAINING.

Visualisation of total protein on acrylamide gels was performed using Coomassie Brilliant Blue G250 stain (1% Coomassie blue, 48% methanol, 15% acetic acid, 36% dH2O) overnight with agitation. Destaining solution (30% methanol, 10% acetic acid, 60% dH2O) was added and agitation took place for 1 hour. Further destaining was done using 7% acetic acid for 1 -2 hours at room temperature.

2.2.15 WESTERN BLOTTING.

Once samples had been separated by SDS-PAGE, proteins were transferred to a nitrocellulose membrane using a Bio-Rad Trans-Blot® Semi-Dry transfer cell (Bio-Rad Laboratories, USA) set to 15 V and run for 1 hour. The membranes were blocked for 30 minutes using blocking buffer (1XPBS pH 7.4, 5% milk powder, 10% Tween 20) at room temperature with agitation. Diluted primary antibody (mouse anti-his (1/2000) (Bio-Rad, USA, #MCA1396)) was added to the membrane and left overnight at 4°C with agitation. The membrane was washed using blocking buffer four times and the secondary conjugate antibody (goat Anti-mouse Alkaline phosphatase (1/10000) (Sigma-Aldrich, USA, A3562)) was added. This was incubated at 37°C for 90 minutes with agitation. The membrane was then washed four times with wash buffer (1XPBS pH 7.4, 10% tween 20) at room temperature with agitation for 15 minutes. Once washed, NBT/BCIP substrate (Roche, USA) was added (2ml per blot) and the blot left in the dark for up to an hour for the developed bands to be visualised.

2.2.16 BATCH BINDING PURIFICATION OF RECOMBINANT *E. COLI* ANTIGEN.

Purification of the recombinant *E. coli*-expressed proteins was done using immobilized metal-ion affinity chromatography (IMAC) through use of the batch binding method. Binding-resin (His-select Nickel Affinity Gel, Sigma-Aldrich) was used to purify the insoluble protein (see section 2.2.8) and was done under denaturing conditions as per the manufacturer's instructions. This involved using 8M urea to solubilise the proteins and low pH to elute proteins from the resin. The equilibration buffer (1XPBS, 0.5M Nacl, 8M urea, pH8) and wash buffer (1XPBS, 0.5M Nacl, 8M urea, pH6.3) was used at a set pH, whereas the elution buffer (1XPBS, 0.5M Nacl, 8M urea) had a range of pH conditions for elution optimisation (pH6, pH5 and pH4.5). The same procedure was followed for the negative control (empty *E. coli* vector).

2.2.17 LARGE-SCALE AFFINITY PURIFICATION OF RECOMBINANT PLANT ANTIGEN.

Purification of the recombinant *N. benthamiana* expressed antigen proteins was done using IMAC through use of a 5ml prepacked Nickel Sepharose™ High Performance column (HisTrap™ HP, GE Healthcare). The column was equilibrated with 5 column volumes (CV) of equilibration buffer (1XPBS, 0.5M NaCl, 8M urea, pH8). The column was then loaded with the

plant extract using a 150ml SuperLoop; this was followed by 10 CV washes using wash buffer (1XPBS, 0.5M NaCl, 8M urea, pH8). Elution of the recombinant protein was done over 20 CV of a linear gradient of elution buffer (1XPBS, 0.5M NaCl, 8M urea, 0.5M imidazole, pH8) from 0%-100%, with the final elution step being 5 CV of 100% elution buffer. The ÄKTA Explorer™ Fast protein liquid chromatography (FPLC) system and UNICORN 4.11 software (GE Healthcare) were used. The fractions were stored at -20^{o}C for later analysis.

2.2.18 QUANTIFICATION OF AFFINITY-PURIFIED PROTEIN.

Purified *E. coli* protein was quantified using the Bradford assay (as described in section 2.2.12.) using a BSA standard. Purified plant samples were quantified via gel densitometry (Syngene GeneTools image analysis software (version 3.07.03)) of western blots (section 2.2.15) using purified plant produced scFv (CRAb-FM27) used as a standard curve (as described in Chapter 3).

2.2.19 LYOPHILISATION.

Purified plant produced 3B-O protein was concentrated by lyophilisation, where 1ml aliquots of the samples were placed in Eppendorf tubes and frozen at -80^{o}C overnight. The tubes were then opened and covered with Parafilm (Parafilm M®, Bemis) in which pinholes were made. The samples were then placed into holding vessels and freeze dried overnight. The freeze-dried contents of the tubes were stored at ambient temperature or reconstituted in 200µl of dH_2O and stored at -20°C for later analysis.

2.3 RESULTS

2.3.1 INITIAL EXPRESSION OF *3ABC-O*

Initially the full length *3ABC-O* gene was synthesised, cloned into the *E. coli* expression vector pProEX-HTb, and into the plant expression vectors pRIC3.0-HT and pTRAKc and tested for its ability to express recombinant 3ABC-O in *N. benthamiana* (by syringe infiltration) or in *E. coli* (by induction with IPTG). Expression of 3ABC-O was tested for by screening both leaf samples

and partially purified *E. coli* extract samples by western blot using an anti-his antibody (not shown). However, no expression was observed, even after multiple attempts.

2.3.2 GENE MODIFICATION

It was hypothesised that the $3C^{pro}$ region of the polyprotein was possibly responsible for the lack of recombinant 3ABC-O expression due to it causing proteolysis of host cell proteins. It was therefore decided that modifications of the *3ABC-O* gene were necessary to encourage expression. The first modification was to mutate and thereby abolish the active site of the $3C^{pro}$. The second was to remove the $3C^{pro}$ region by truncation of the polypeptide gene to yield *3AB* and the third was to explore and test three new variants of *3ABC*.

2.3.2.1 Site-directed mutagenesis of *3ABC-O*

Substitution mutations were introduced into the $3C^{pro}$ region of the *3ABC-O* gene with use of the primers and conditions mentioned in Table 2.2. The two mutations Cys142Ser and Cys163Gly are known to improve solubility and disrupt the active site, respectively. The mutations were designed such that they would include synonymous mutations for the addition of restriction sites *TaqI* and *MnlI,* respectively. These added RE sites were used to confirm successful site-directed mutagenesis mutation by restriction enzyme digest and analysis of the banding pattern by gel electrophoresis. Confirmation of a successful Cys142Ser substitution mutation would show the absence of a 431 bp fragment, whereas the presence of this band would indicate a lack of mutation (Figure 2.2 A). Confirmation of the Cys163Gly substitution mutation would show the absence of a 629 bp fragment and the presence of a 462 bp fragment (Figure 2.2 B). The mutations in the gene were then further confirmed by sequencing (not shown).

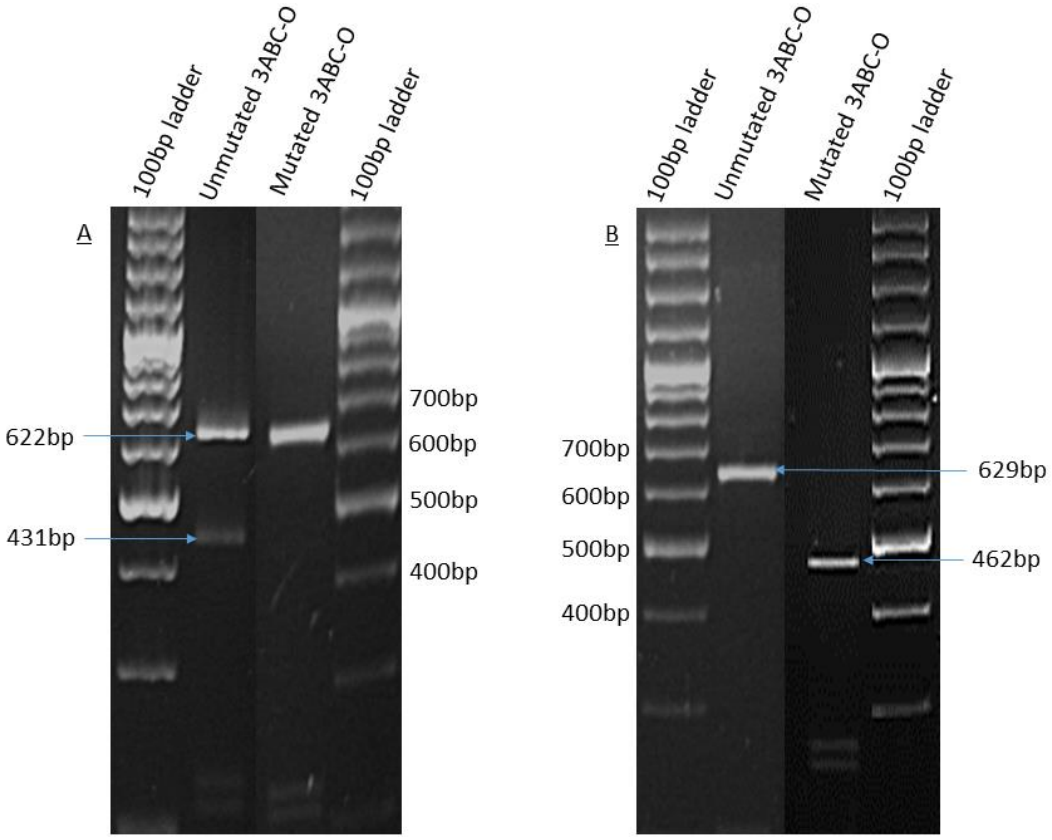

Figure 2.2. 2.5% agarose gel displaying banding patterns of digested *3ABC-O* gene to confirm mutations.

(**A**) Banding pattern after *TaqI* digest to identify if Cys142Ser mutation is present (mutated *3ABC-O*) and not present (unmutated *3ABC-O*), if the 431bp band is visible. (**B**) Banding pattern after *MnlI* digest to identify if Cys163Gly mutation is present (mutated *3ABC-O*) with the loss of the 431bp band or not present (unmutated *3ABC-O*) with the 629bp band seen.

2.3.2.2 Truncation of *3ABC-O*

Truncation of the *3ABC-O* gene was achieved using PCR. Amplification of *3ABC-O* with primers and conditions in Table 2.3 resulted in the *3AB-O* variant, seen as a 728 bp fragment during colony PCR (Figure 2.3).

2.3.2.3 Synthesis of new variants

The three new genes (*mu3ABC-A, 3B-O and 3AB1-Arg*) were successfully synthesised by GenScript.

2.3.3 CONSTRUCT CONFIRMATION

The five variations of the *3ABC* gene were cloned into both the *E. coli* expression vector (pProEX-HTb) and plant expression vectors (pRIC3.0-HT and pTRAKc) (Table 2.4). Colony PCR was used to confirm successful cloning.

2.3.3.1 Construct confirmation in *E. coli* expression vector

The variant antigen genes were successfully cloned into the pProEX-HTb *E. coli* expression vector. The following fragment sizes were observed, confirming successful cloning: *3ABC-O* (1371 bp), *mu3ABC-O* (1371 bp), *3AB-O* (728 bp), *3AB1-Arg*, (809 bp) *3B-O* (500 bp) and *mu3ABC-A* (1592bp) (Figure 2.3). Positive colonies were selected and RE digest and sequencing was used to further confirm successful cloning (not shown).

2.3.3.2 Construct confirmation in plant expression vectors

The five variants of the *3ABC* polyprotein gene were subcloned into two plant expression vectors pRIC3.0-HT and pTRAKc-ERH. Confirmation of successful cloning and transformation into *A. tumefaciens* was achieved using colony PCR, with the use of vector specific primers (Table 2.5). Results from the screening of these colonies is shown in Figure 2.4, which shows appropriate band sizes for each gene in the different vectors: pRIC3.0-HT-*mu3ABC-O*

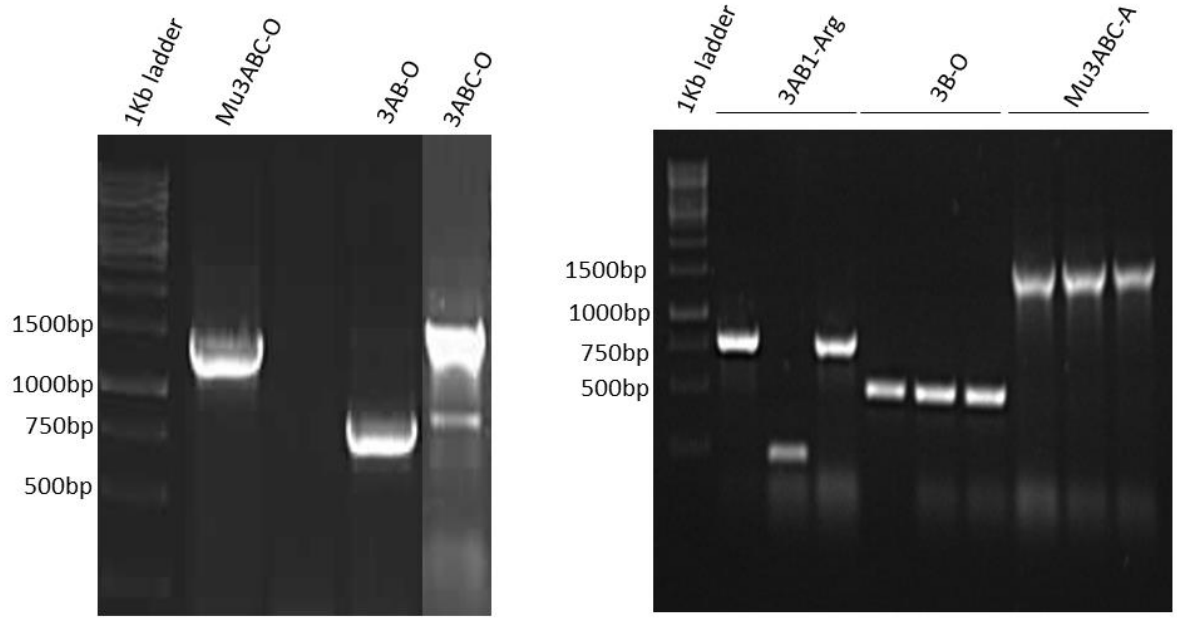

Figure 2.3. 1% agarose gel displaying *E. coli* colony PCR confirming pProEX-HTb vector with antigen gene inserts.
PCR products; *mu3ABC-O* and *3ABC-O* (1371bp), variant *3AB-O* (728bp), *3AB1-Arg* (809bp), *3B-O* (500bp) and *mu3ABC-A* (1592bp).

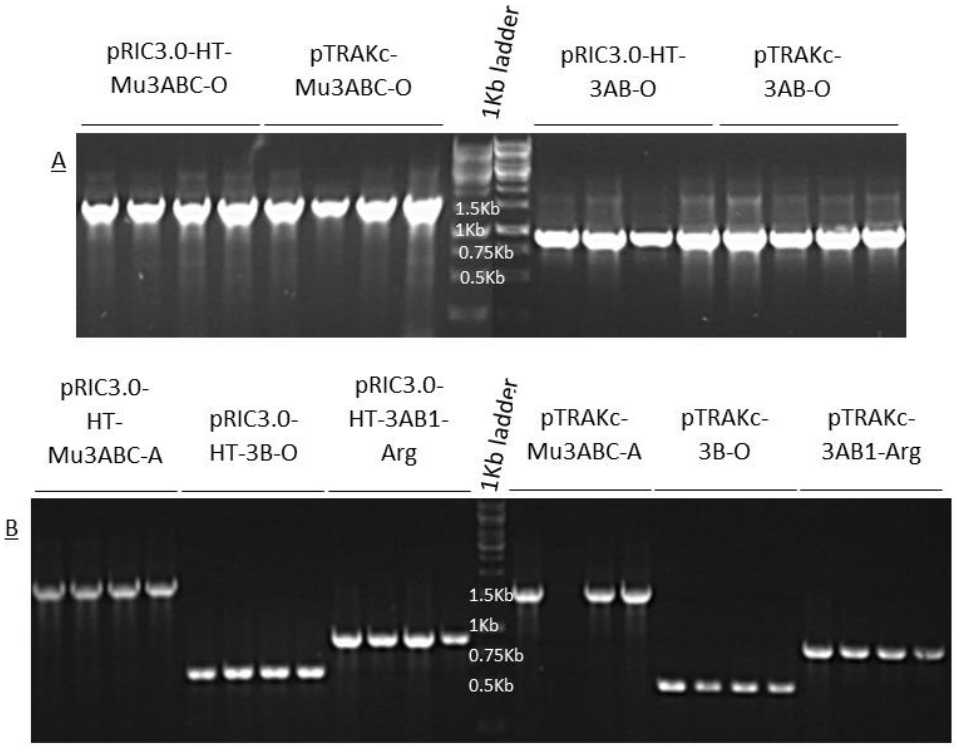

Figure 2.4. 1% agarose gel displaying *Agrobacterium* colony PCR confirming plant vectors with antigen gene inserts.
A) *mu3ABC-O* and *3AB-O* both within pRIC3.0-HT (1528bp and 889bp respectively) and pTRAKc (1593bp and 954bp respectively). B) *mu3ABC-A, 3B-O* and *3AB1-Arg* within pRIC3.0-HT (1618bp, 526bp and 835bp respectively) and pTRAKc (1586bp, 494bp and 803bp respectively).

(1528bp), pRIC3.0-HT-*mu3ABC-A* (1618bp), pTRAKc-*mu3ABC-O* (1593bp), pTRAKc- *mu3ABC-A* (1586bp), pRIC3.0-HT-*3AB-O* (889bp), pRIC3.0-HT-*3B-O* (526bp), pRIC3.0-HT-*3AB1-Arg* (835bp), pTRAKc-*3AB-O* (954bp), pTRAKc-*3B-O* (494bp) and pTRAKc-*3AB1-Arg* (803bp). Positive clones were confirmed by RE digest using the same RE sites used for cloning (not shown).

2.3.4 ANALYSIS OF ANTIGEN EXPRESSION

Once positively transformed colonies with the five variants had been identified in the *E. coli* and plant expression vectors, expression was performed on a small scale. This was done in order to determine whether recombinant vectors were successfully expressing the antigen, and to the optimal conditions for large scale expression and extraction.

2.3.4.1 Analysis of *E. coli* expressed antigens

Detection of expressed recombinant protein was analysed by anti-his western blotting of protein extracts sampled at 1, 2 and 3 hours post induction with IPTG. Expression of 3ABC-O

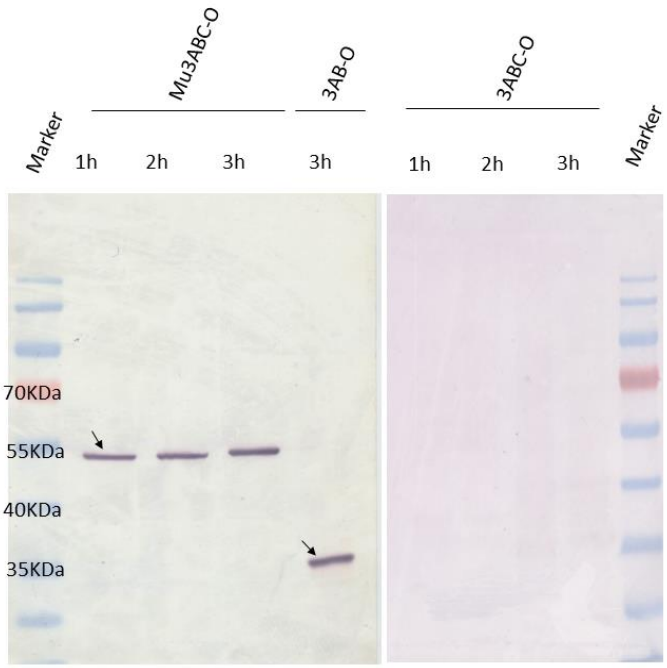

Figure 2.5. Western blot of *E. coli* expressed mutated and truncated 3ABC antigen displayed as an hourly time trial.

Mutated antigen (Mu3ABC-O) and Non-mutated antigen (3ABC-O) expression shown over hours (h) 1, 2 & 3. Truncated variant (3AB-O) expression shown at the third hour. Marker (PageRuler™). Black arrows showing mu3ABC-O at 53KDa and 3AB-O at 35KDa. Anti-his 1/2000.

was not detected over 3 hours post induction. However, the *mu3ABC-O* gene which contained both mutations (Cys142Ser and Cys163Gly), was expressed in consistent amounts over 3 hours as visualised by a 53KDa protein band (Figure 2.5). The 3AB-O variant was expressed and sampled over three hours (not shown) of which protein from the third hour after induction is shown in Figure 2.5 as a 35KDa band.

Initially the mu3ABC-A, 3B-O and 3AB1-Arg *E. coli* constructs were successfully expressed and samples taken every hour for three hours after induction. Each of the constructs showed increasing amounts of protein over three hours (not shown). Figure 2.6 shows protein sampled 2 hours after induction for the three new variants, visualised in an anti-his western blot. The mu3ABC-A polyprotein is seen at 53KDa protein band in Figure 2.6, along with multiple lower bands. Expression of 3B-O protein is expected at 13KDa as indicated by the black arrow in Figure 2.6 but a second higher molecular weight band is seen at around 20KDa indicated by the blue arrow. The 3AB1-Arg protein was expected at around 24KDa but with a faint band seen at this position (Figure 2.6, black arrow). There are multiple bands seen in the 3AB1-Arg expression lane, with the darkest band at around 30KDa. Figure 2.6 also shows the

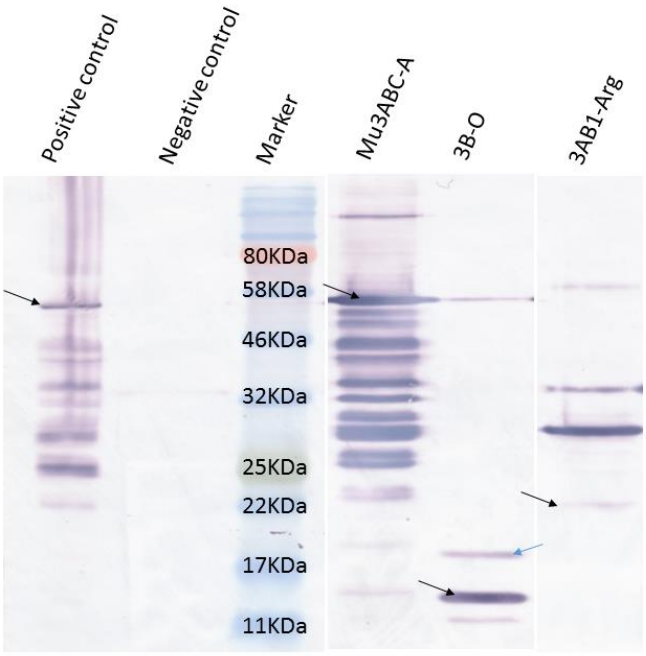

Figure 2.6. Western blot of *E. coli* expressed antigens (three new variants).

Positive control (*E. coli*-expressed mu3ABC-O), Negative control (crude *E. coli* extract containing empty pProEX-HTb vector), Marker (Color Prestained protein standard), mutated 3ABC-A (53KDa), truncated 3B-O (13KDa) and 3AB1-Arg (24KDa) expression at 2 hours. Black arrows showing position of proteins and blue arrow indicating ≈20KDa upper band for 3B-O protein. Anti-his 1/2000

lack of bands in the negative control lane, indicating no non-specific antibody binding. The multiple bands seen in Figure 2.6 are not observed in Figure 2.5, and is suspected to be due to the 6Xhis tag being located at both terminals of the mu3ABC-A, 3B-O and 3AB1-Arg proteins, which could influence expression, or if degraded would give a number of bands.

2.3.4.2 Analysis of plant expressed antigens

Transient expression of all constructs in plants on a small scale was repeated 3 times. Each of the pRIC3.0-HT and pTRAKc constructs were syringe-infiltrated at an OD_{600} of 0.5 or 1.0 into three plants. Leaf disks were sampled on days 1, 3 and 5 dpi and homogenised with both a denaturing (1XPBS + 8M urea) and native (1XPBS) buffer, separately. The samples were analysed by an anti-his western blot, using a crude *E. coli* sample of the cognate protein as the positive control and a crude leaf sample infiltrated with an empty pRIC3.0-HT or pTRAKc vector used as a negative control.

Analysis of crude leaf extracts by western blot was done in order to determine the optimal day of harvest, optimal bacterial OD_{600} at which to infiltrate cultures and best extraction

buffer to use. To ensure a true comparison between the samples, equal amounts of total protein were loaded into each well. A Bradford assay was used to determine total protein in each of the samples.

Analysis of expression of mu3ABC-O, 3AB-O and 3AB1-Arg from both pTRAKc and pRIC3.0HT vectors showed no expression (not shown). This was observed for each biological repeat experiment.

Expression of mu3ABC-A and 3B-O, however, was successful. The small-scale time trials showed mu3ABC-A expression was highest with the pTRAKc expression vector at 3 days post infiltration at an OD_{600} 1.0 as the most dense 53KDa band is seen in that lane (Figure 2.7 B). Multiple bands were seen for the pTRAKc-mu3ABC-A construct expression (Figure 2.7 B, orange, blue and red arrows), which are suspected to be due to proteolytic cleavage by native plant proteases. No expression was seen with the pRIC3.0-HT vector (Figure 2.7 A).

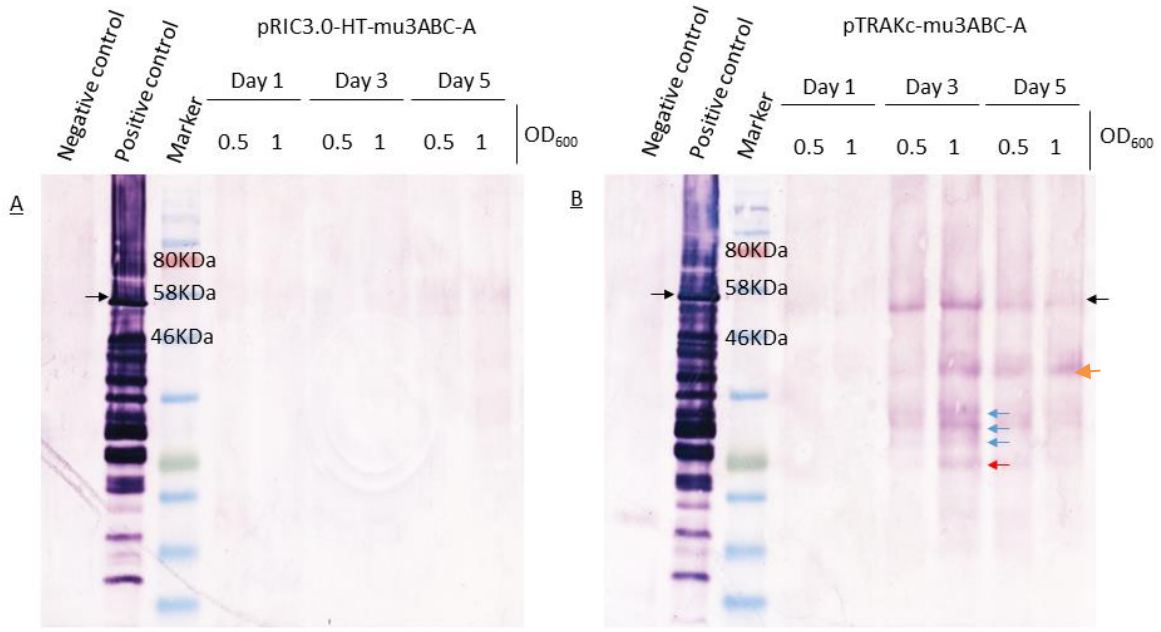

Figure 2.7. Western blot displaying small scale time trial of plant-expressed mu3ABC-A.

(**A**) pRIC3.0-HT-mu3ABC-A and (**B**) pTRAKc-mu3ABC-A construct sampled. Negative control (crude leaf extract infiltrated with empty pRIC3.0-HT or pTRAKc vector), Positive control (Crude *E. coli* expressed mu3ABC-A), Marker (Color Prestained protein standard). Days 1, 3 and 5 post-infiltration, each with an OD_{600} of 0.5 and 1 tested. Black arrows indicating position of mu3ABC-A protein (53KDa), orange arrow indicates possible degradation within the 3A region of the polyprotein (35KDa), blue arrows indicating processed polyprotein being cleaved within the 3B region of mu3ABC-A (B_3C= 26, B_2B_3C= 28 and $B_1B_2B_3C$= 30 KDa) and red arrow indicating $3C^{pro}$ alone (≈24KDa).

The small-scale time trials conducted for 3B-O expression showed the highest levels of expression when using the pRIC3.0-HT vector, with no protein bands observed when using the pTRAKc vector (Figure 2.8 A and B respectively). The highest expression levels of 3B-O were observed at 5 days post infiltration (dpi) using an infiltration culture OD_{600} of 1.0, as the protein bands were more dense in those particular lanes (Figure 2.8 A). The 3B-O protein is expected to be 13KDa, which is seen as the lower band in Figure 2.8 A, but again a second band is also seen at a higher molecular weight at around 20KDa. This upper band is also seen in the *E. coli* positive control and is suspected to be an alternative product due to post translational modifications. The plant-produced 3B-O protein has greater upper band density than the lower, which might indicate that plant expression might favour the higher molecular weight modification.

Extraction of both the mu3ABC-A and 3B-O proteins was only successful using a denaturing extraction buffer (1XPBS + 8M urea), as no protein was seen when using 1XPBS only (not shown). Therefore, the NSPs appear to be insoluble

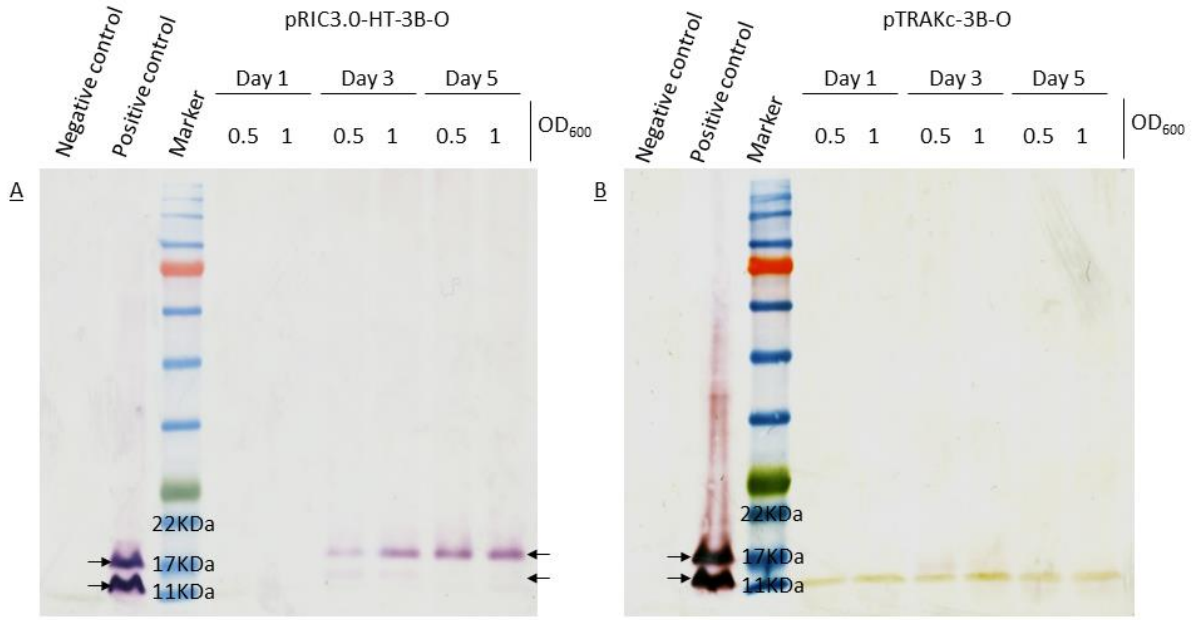

Figure 2.8. Western blot displaying small scale time trial of plant-expressed 3B-O.

(**A**) pRIC3.0-HT-*3B-O* and (**B**) pTRAKc-*3B-O* constructs sampled. Negative control (crude leaf extract infiltrated with empty pRIC3.0-HT or pTRAKc vector), Positive control (Crude *E. coli*-expressed 3B-O), Marker (Color Prestained protein standard). Days 1, 3 and 5 post-infiltration, each with an OD_{600} of 0.5 and 1 tested. Black arrows indicating position of 3B-O double band (lower band 13KDa, upper band ≈20KDa).

2.3.5 LARGE SCALE AFFINITY PURIFICATION

Expression of all five variants of the 3ABC polypeptide was successful in *E. coli,* but only the 3B-O and mu3ABC-A variants were expressed in plants. The plant expressed 3B-O protein showed higher expression than the mu3ABC-A protein and with less degradation, and therefore it was decided that the 3B-O protein would be continued with. Large scale expression of 3B-O was carried out using *E. coli* and *N. benthamiana*, using the optimal conditions determined in section 2.3.4, so that optimal purification conditions could be determined and 3B-O could be purified.

2.3.5.1 Purification of *E. coli* expressed 3B-O

The pProEX-HTb-*3B-O* glycerol stock was grown up, induced and cells harvested 3 hours post induction. Purification of the *E. coli*-expressed antigen was achieved using the batch binding method, to generate sufficient recombinant protein for functionality testing. The fractions collected during purification were analysed on an anti-his western blot as well as a Coomassie

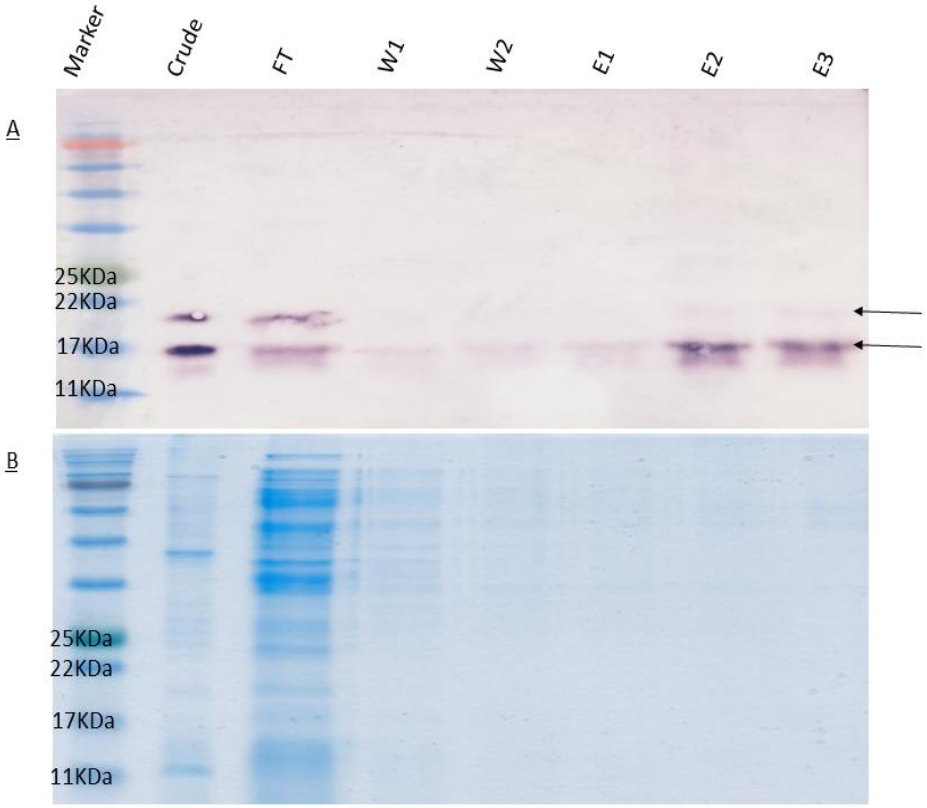

Figure 2.9. Batch binding purification of *E. coli*-expressed 3B-O, fractions analysed.

(**A**) Anti-his 1/2000 western blot and (**B**) Coomassie stained SDS-PAGE. Marker (Color Prestained protein standard), crude (5ul cell culture pre-purification), FT (Flow Through), W1 (Wash 1), W2 (Wash2), E1 (Elution 1, pH6), E2 (Elution 2, pH 5), E3 (Elution 3, pH 4.5). Elution of 2ml was done and 30µl loaded into each lane. The black arrows indicate the upper (≈20KDa)

stained gel (Figure 2.9 A&B respectively). The western blot showed the 3B-O protein present in the flowthrough (FT), as the common double band is observed, but very little seen in the wash fractions (W), with only the lower band being faintly seen. Decreasing the pH of the buffers was used to elute the protein from the resin. To determine the optimal pH for elution a range of pH elution buffers were tested. The E1 sample showed very little 3B-O elution, seen by the faint band in Figure 2.9 A, which had a pH of 6. The E2 sample showed a dark band at 13KDa and a very faint band at ≈20KDa, with a similar result observed for the E3 sample. This indicated that the elution of 3B-O was highest at a pH 5 (E2) and pH 4.5 (E3). The same samples were analysed by Coomassie-staining (Figure 2.9 B), which showed a decrease in total protein between the crude and eluted fractions, indicating purification had been successful. The western blot also showed that once the protein had been eluted from the resin, the lower band seemed denser compared to the upper band.

2.3.5.2 Purification of plant expressed 3B-O

Purification of *N. benthamiana*-produced 3B-O protein using the pRIC3.0-HT-*3B-O* construct was done using the ÄKTA system. In order to obtain a substantial working volume for purification, a large amount of protein was required. Therefore, the construct was vacuum infiltrated into 12 plants at the optimal conditions determined during the small-scale testing (see 2.3.4.2.).

The ÄKTA system displays absorbance readings at 280nm on a chromatogram. The chromatogram for the purification of 3B-O is shown in Figure 2.10, which indicated a high protein absorbance in the flowthrough fractions (fractions 1-18) and then a considerable drop during the wash phase (fractions 19-27). The initial absorbance was due to the high concentration of plant proteins present in the sample. The appearance of a second high absorbance peak was observed in the elution fractions (fractions 29-32), which smoothed out into the shoulder (fractions 33-35). The increasing imidazole gradient, during elution, continued up to fraction 48 and then plateaued from fraction 49-53 with no other peaks of absorbance seen.

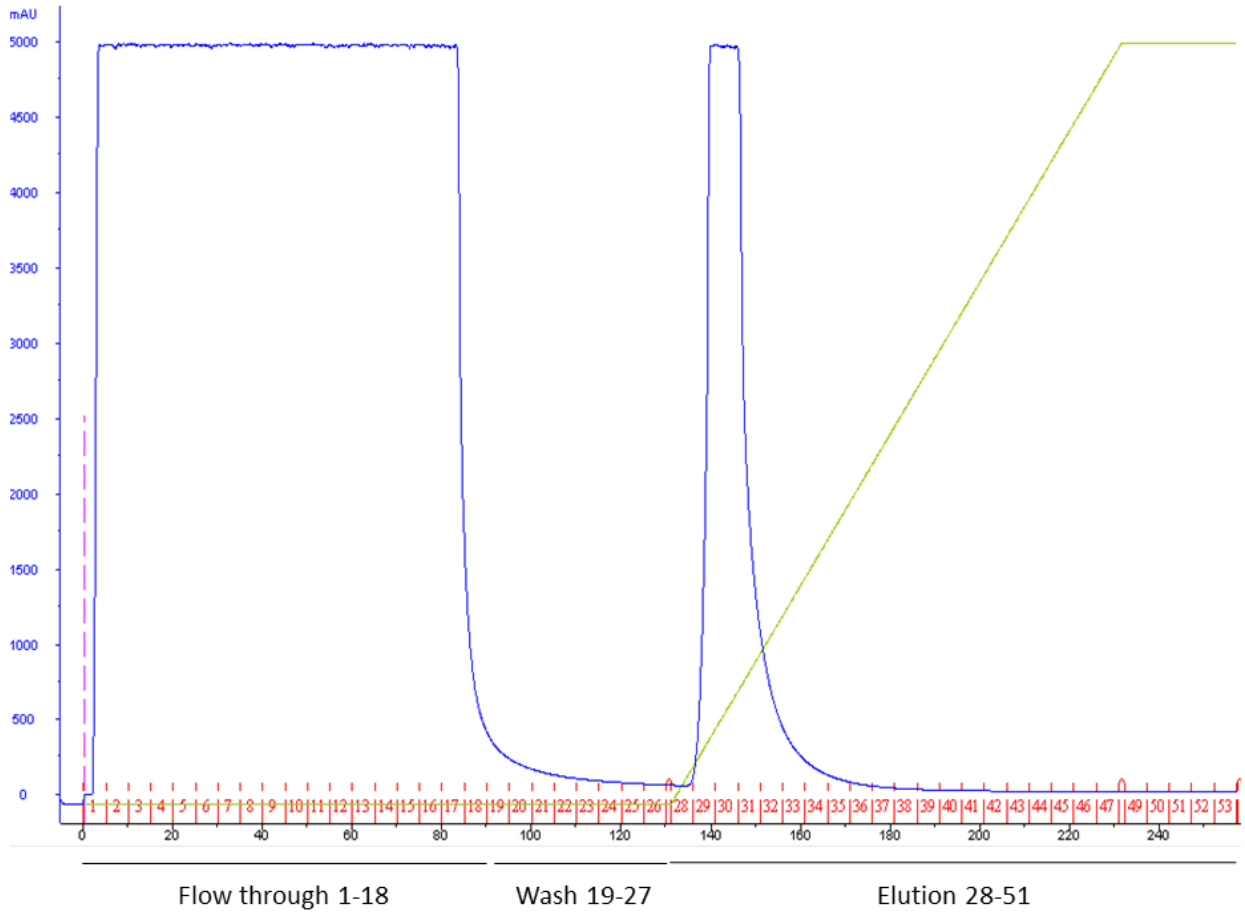

Figure 2.10. Chromatogram trace of ÄKTA purification of *N. benthamiana*-produced 3B-O.

Blue line indicating Absorbance at 280nm (Y axis), green line showing gradient increase of elution buffer as a percentage of 0.5M Imidazole. X axis indicating fractions of Flow through (1-18), Wash (19-27) and Elution (28-51).

The successful purification of the 3B-O protein was confirmed by analysing the ÄKTA fractions on an anti-his western blot and Coomassie stained gel (Figure 2.11 A & B respectively). The western blot showed that minimal levels of 3B-O protein was captured in the flowthrough and none was present in the wash fractions. Analysis of the eluted fractions within the second absorbance peak (E29-35), indicated that the 3B-O protein was present in that peak as well as within the shoulder of it (Figure 2.11 A, black arrows). The darker bands are seen in fractions E31 and E32, indicating that this was where the majority of the protein was being eluted. The common double band is seen within the elution fractions, with the upper (≈20KDa) band appearing to be the darkest and most prominent.

The Coomassie stained gel (Figure 2.11 B) shows the total protein within the fractions and gives an estimate of how pure the samples were. The flowthrough fraction (FT8-10) showed a smear suggesting a large amount of various proteins were present. This correlates well with

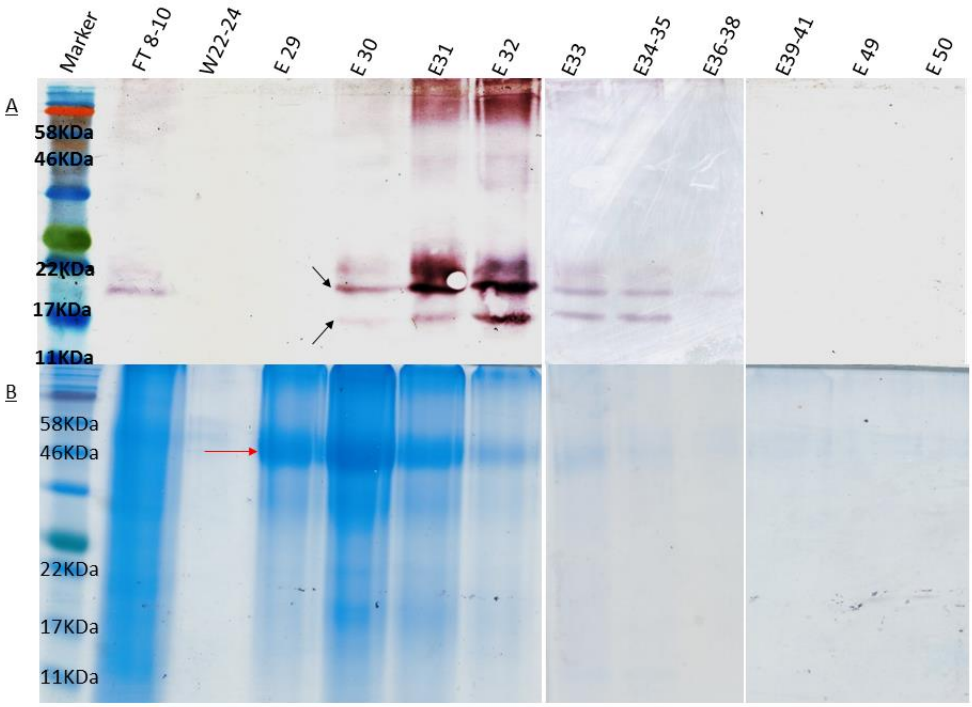

Figure 2.11. Fractions from the ÄKTA purification of *N. benthamiana*-produced 3B-O.

(A) Western blot Anti-his 1/2000 and B) SDS-PAGE coomassie stained. Marker (Color Prestained protein standard), Flow Through fractions (FT 8-10), Wash fractions (W 22-24), Elution fractions (E 29-50). Black arrows showing position of purified double 3B-O protein (upper ≈20KDa and lower 13KDa bands) and the red arrow indicates high molecular weight contaminant (≈55KDa).

the initial large peak seen in the chromatogram in the same fractions. The eluted fractions (E30-32), show a substantial amount of other high molecular weight plant proteins being present (Figure 2.11 B, red arrow). Therefore, the high absorbance observed in the second peak is probably due to the presence of native plant proteins and does not represent the level of 3B-O protein alone. The Coomassie stained gel indicates that the eluted fractions collected across the second peak contain co-eluting contaminating proteins and therefore show that these fractions do not consist purely of the 3B-O protein. However, a reduction of the contaminant in the E33-35 fractions (shoulder of second peak) was also seen, indicating that these might be the purest of the fractions.

2.3.6 Quantification

Once purification of the 3B-O protein was completed, the eluted (semi-pure) fractions were quantified. The *E. coli* expressed 3B-O was quantified using a Bradford assay. The plant purified 3B-O preparation contained levels of native plant proteins in the elution fractions

and therefore total protein via a Bradford assay was considered to be inaccurate. Therefore, a western blot was run and densitometry was used for quantification of plant 3B-O.

2.3.6.1 Quantification of *E. coli* purified 3B-O

The purified *E. coli* 3B-O protein was quantified by Bradford assay, which gave a concentration range of 0.095µg/µl to 0.102µg/µl (not shown) at a volume of 2ml.

2.3.6.2 Lyophilisation and quantification of plant purified 3B-O

The elution fractions E30-35 contained the 3B-O protein but at very low levels, as seen in the western blot (Figure 2.11 A). Accordingly, the fractions were subjected to lyophilisation (freeze drying) in order to concentrate the protein once reconstituted in a $1/5^{th}$ of the starting volume (not shown). The fractions were then quantified using densitometry by western blot, which indicated that fraction E31 gave the highest concentration of 0.0057µg/µl at a volume of 5ml.

2.4 DISCUSSION

As most studies of FMDV non-structural protein expression have been done in an *E. coli* system, I did the same, both so as to have a positive control, and to have proteins for comparison to the plant made proteins. However, preliminary work done to express FMDV 3ABC-O in both *N. benthamiana* and *E. coli* was not successful as seen by a complete absence of protein bands of 53kDa in size on western blots. This was surprising, as the FMDV 3ABC polyprotein has previously been expressed in *E. coli* (Clavijo *et al.*, 2004; Nanni *et al.*, 2005; Jaworski *et al.*, 2011; Gao *et al.*, 2012; Sharma *et al.*, 2012; Mohapatra *et al.*, 2014) and insect cells (Sørensen *et al.*, 1998; Chung *et al.*, 2002; Srisombundit *et al.*, 2013).

The *3C* region of the FMDV genome encodes a serine protease ($3C^{pro}$), which is responsible for proteolytic cleavage of the viral encoded polyprotein into its individual structural and non-structural proteins (Vakharia *et al.*, 1987; Clarke and Sangar, 1988). It has been shown that $3C^{pro}$ is responsible for cleavage of host cell eIF-4A (RNA helicase) as well as the histone H3, which is responsible for shutting down of host transcription (Falk *et al.*, 1990; Belsham,

McInerney and Ross-Smith, 2000). It is therefore possible that in my case, transient expression of the 3ABC polyprotein could have resulted in inhibition of host cell transcription and inhibition of recombinant expression in *N. benthamiana* and *E. coli* as a result of the activity of $3C^{pro}$.

It was thus decided to manipulate the *3ABC* gene and mutate/remove the *3C* coding region in order to encourage expression of this protein. Sariya et al. (2011) showed that disrupting the active site of the $3C^{pro}$ enhances expression of the full 3ABC polypeptide in *E. coli*. Thus, in this work, I introduced two substitution mutations (Cys142Ser & Cys163Gly) into *3ABC* to encourage recombinant expression (Sariya *et al.*, 2011; Srisombundit *et al.*, 2013). The addition of the first mutation (Cys142Ser) alone did not enhance expression (not shown). It was only after the addition of the second mutation (Cys163Gly), which is responsible for disrupting the active site of the 3C protease, that expression was observed (Figure 2.5).

In addition to mutating *3ABC*, the *3C* coding region was removed to create a truncated version (*3AB-O*). The expression of the truncated polyprotein was successful (Figure 2.5, lower band), and confirmed the 3C protease involvement in reducing expression.

In addition to the above variant genes tested for expression of recombinant protein, three new genes were synthesised: these were *mu3ABC-A, 3B-O and 3AB1-Arg*. This was done to give a greater variety, to explore the possibilities of a better antigen option, as well as to increase the chances of successful expression. Investigation into the expression of both serotype O and A non-structural proteins was done as infected animal serum from these two serotypes had been obtained for testing purposes. The selection of the non-structural proteins to be tested was also influenced by the competing scFv to be used, as the CRAb-FM27 scFv (discussed in Chapter 3) is known to bind epitopes found within the 3B region of the polypeptide (Foord *et al.*, 2007). Therefore, the NSPs to be investigated would have to contain the 3B region. The 3ABC full length polyprotein had previously been shown as the best NSP for a FMDV diagnostic reagent (Mackay *et al.*, 1998). It was for this reason that the *3ABC-O* (serotype O) and *3ABC-A* (serotype A) were chosen. The truncated *3AB-O* gene was also a candidate for study, as a difference in expression between the mu3ABC-O might be

seen as a result of the removal of the 3C protease portion. The 3AB protein has also been used to successfully distinguish infected from vaccinated animal serum in an I-ELISA (Sørensen *et al.*, 1998; Chung *et al.*, 2002). The *3B-O* gene had been selected as it has previously been shown to differentiate infected from vaccinated serum just as effectively as the full length polyprotein (Gao *et al.*, 2012). The $3AB_1$ protein has also been successfully used as a DIVA reagent (Nanni *et al.*, 2005; Jaworski *et al.*, 2011) and therefore was also chosen.

Constructs encoding each of the 5 genes (*mu3ABC-O, 3AB-O*, *mu3ABC-A, 3B-O and 3AB1-Arg)* resulted in the appropriate recombinant protein expression using an *E. coli* expression system (Figures 2.5 and 2.6). It was noted, however, that additional bands were visualised in the lanes containing proteins encoded by *mu3ABC-A, 3B-O and 3AB1-Arg*. It is unclear as to why this is, but is hypothesise that it could be because *mu3ABC-A, 3B-O* and *3AB1-Arg* are cloned in such a way that the resulting recombinant protein has both an N and C-terminal 6Xhis-tag. It is thought that with recombinant proteins having a histidine tag at either end, cleavage or degradation anywhere in the centre of the protein would lead to twice as many bands compared to proteins with just one 6Xhis-tag, and shown at different sizes. This is seen in the anti-his western blot in Figure 2.6, where the positive control (mu3ABC-O) which contains only one 6Xhis-tag shows 8 distinct bands, whereas the mu3ABC-A which has a 6Xhis-tag at both ends of the protein has 16 bands. The multiple bands observed in Figure 2.6, thought to be from protein degradation, could be a result of storing the samples at -20°C and not analysing it fresh, as done for the samples in figure 2.5.

The same 5 genes cloned into plant expression vectors pTRAKc and pRIC3.0-HT and infiltrated into *N. benthamiana* did not all result in transient expression of recombinant protein. Only the mu3ABC-A and 3B-O proteins were successfully expressed (Figures 2.7 and 2.8 respectively, black arrows). It was interesting to see that the vector which had the highest expression levels of 3B-O based on the density of the protein bands in a western blot, was pRIC3.0-HT - whereas it was the pTRAKc vector for mu3ABC-A. The *N. benthamiana*-produced mu3ABC-A protein showed multiple bands in the anti-his western blot (Figure 2.7 B, orange, blue and red arrows). It is suspected that the mu3ABC-A protein could have undergone proteolysis during expression, resulting in multiple bands. Naturally, it is the viral $3C^{pro}$ (serine

protease) that cleaves the polyprotein into individual mature proteins during processing at various sites to produce 3A, $3B_1$, $3B_2$, $3B_3$ and 3C proteins (Figure 1.2). With the $3C^{pro}$ region of the polyprotein being deactivated by mutation, it has been hypothesised that native plant serine proteases (Pillay *et al.*, 2013) might be processing the polyprotein in a similar way. If this were the case and cleavage were to take place at the same sites of the polyprotein as it would naturally, multiple proteins would be observed on an anti-his western blot. The 6Xhis tag is fused to the C-terminus of the recombinant mu3ABC-A protein, and thus only a select few proteins would be observed when cleaved. The $3C^{pro}$ protein, if cleaved from the polyprotein alone, would be seen at ≈24KDa as indicated by the red arrow in Figure 2.7 B. The 3B region is made up of three segments (B_1, B_2 and B_3) and depending on where proteolysis takes place, it would either give the $B_1B_2B_3C$ (≈30KDa), B_2B_3C (≈28KDa) or B_3C (≈26KDa) indicated by the blue arrows in Figure 2.7 B. Proteolysis within the 3A region of the polyprotein could explain the presence of a ≈35KDa band (Figure 2.7 B, orange arrow) appearing below the full mu3ABC-A polyprotein but above the $B_1B_2B_3C$ band. If the hypothesis of native plant serine proteases processing the non-structural proteins of the virus is true, this might explain why expression for the mu3ABC-O, 3AB-O and $3AB_1$-Arg proteins are not observed - if these proteins were more susceptible to proteolysis than the mu3ABC-A or 3B-O proteins. If the same processing, but to a lesser degree, is taking place for the 3B-O protein individual proteins (B_1, B_2 and B_3) would not have been seen as they could have migrated off the SDS-PAGE due to their low molecular weight. The expression of the NSPs did not allow for the retention of the recombinant proteins in the ER, which has shown to protect against proteolysis, and which could have improved the expression levels and quality of these proteins. However, this was not explored due to time constraints. The extraction of these NSPs was achieved using 8M urea, which suggests that these proteins are insoluble. It is known that the 3A protein associates with intracellular membranes, and which is thought to anchor the replication complex of picornavirus to the ER membrane (Weber *et al.*, 1996; Suhy, Giddings and Kirkegaard, 2000; O'Donnell *et al.*, 2001), therefore, it is suspected that the NSP variants which included the 3A region would be insoluble and difficult to extract. Extraction using 8M urea showed to effectively solubilise the proteins and hence no other detergents were investigated.

The 3B-O protein was chosen to continue with as it showed dense protein bands in a western blot (Figure 2.8 A), indicating good expression and possibly less degradation, in comparison to the mu3ABC-A protein (Figure 2.7 B). The pRIC3.0-HT-*3B-O* construct encoding the *3B-O* gene was expressed on a larger scale in plants in order to develop a purification protocol for producing sufficient amounts to test in an ELISA. This was done by nickel affinity chromatography using the ÄKTA fast protein liquid chromatography system. 3B-O was successfully purified to a certain degree although analysis of fractions collected indicated the presence of a co-eluting contaminant. It is suspected that this high molecular weight contaminant (≈55KDa) is RuBisCO, the most abundant protein found in plants (Portis Jr, 2001). The addition of imidazole in the binding buffer is known to reduce non-specific binding of contaminants to the nickel resin during purification. However, due to the 3B-O protein eluting very early on at a low concentration of imidazole (0.05M), imidazole in the binding buffer might have resulted in the loss of 3B-O. This makes it difficult to isolate the 3B-O protein from other native plant proteins, which share a similar affinity towards the nickel resin. It is possible that RuBisCO has a low affinity for the nickel resin similar to that of the his-tag of the 3B-O protein, resulting in both proteins eluting at a similar imidazole concentration. The lowering of the imidazole gradient or making it a more gradual increase of imidazole during elution is known to help separate proteins with slightly similar affinity towards the resin. This technique separates protein peaks that are close together but it seems even when this was done, the contaminant again co-eluted even within the later elution fractions (E33-E35). This indicated less contaminating protein but also less 3B-O protein (Figure 2.11 A & B). Therefore, it is hypothesised that there might be a more direct interaction taking place between RuBisCO and the 3B-O protein, therefore resulting in co-elution of the two proteins. However, possible pre-purification techniques such as ammonium sulphate precipitation, pH precipitation or protamine sulphate precipitation (Kim *et al.*, 2013) could be employed to assist in the removal of RuBisCO and other native plant proteins prior to IMAC. However, these techniques were not explored due to time constraints as well as, if there is an association or an affinity between RuBisCO and 3B-O (as is suspected during the elution of 3B-O from the nickel column due to RuBisCO co-eluting along with it (figure 2.11)), RuBisCO might draw the 3B-O protein out of solution as it precipitated.

3B-O protein in the eluted fractions was quantified via western blot densitometry as the concentrations were too low for quantification using gel densitometry. An attempt to concentrate these fractions was made by freeze drying and resuspending in 1/5th of the initial volume. Samples analysed on a western blot indicated that freeze drying did not have a degrading effect on the protein as only the two bands were seen as previously shown (not shown). The concentration of the 3B-O protein was still not sufficient to detect the protein by Coomassie and therefore densitometry was done as a western blot. The standard used for quantification was the plant expressed scFv (CRAb-FM27 described in Chapter 3) as this was expressed in high enough quantities to be well quantified by densitometry using a Coomassie-stained gel. The scFv was also chosen as the standard as it had the same single C-terminal 6Xhis-tag as the 3B-O protein and therefore was more comparable. Yields of 3B-O protein from the large-scale purification were calculated to be around 6.13µg per gram of infiltrated leaf material and a concentration range between 0.0042 µg/µl to 0.0057µg/µl after purification. The total 3B-O protein within the fraction was compared to the total 3B-O protein within the crude extract (crude plant extract taken before purification) and showed a 58% recovery of the 3B-O protein after purification.

The production of 3B-O protein in *E. coli* was also scaled up which was then purified in order to have a comparison for functionality of plant-produced 3B-O in ELISAs. The *E. coli*-produced 3B-O was purified by batch binding, which showed less co-eluting native proteins than that seen in the plant purified 3B-O, when comparing the Coomassie staining in Figure 2.9 B and Figure 2.11 B respectively. It was for this reason that quantification of *E. coli* 3B-O was done using the Bradford assay, which gave a concentration range of 0.095µg/µl to 0.102µg/µl. The *E. coli*-expressed and purified 3B-O protein was not high enough to perform densitometry in the form of a Coomassie-stained gel due to the bands not being visible. The same was not possible for densitometry as a western blot as the protein contained two his-tags, which would not be comparable to a standard that only contained one.

The *N. benthamiana* and *E. coli*-produced 3B-O is seen as a double band in an anti-his western blot and it was first thought to be due to dimer formation. This was not the case as a dimer of the 13KDa 3B-O protein would result in a 26KDa band, but the upper band was around

20KDa. It is possible that the two bands might be different post-translational forms of the same protein. It has been shown that both prokaryotes and eukaryotes have the ability to post-translationally modify proteins (Dell *et al.*, 2010; Grangeasse, Stülke and Mijakovic, 2015). Glycosylation of proteins can increase the molecular weight and therefore show increased sizes of proteins on SDS-polyacrylamide gels. It is possible that the higher molecular weight band represents a more glycosylated form of the protein. However, when the 3B-O sequence was run through an *in-silico* prediction server for N-Glycosylation sites (Gupta, Jung and Brunak, 2004) no glycosylation sites were identified. Lysine acetylation is one of the major post-translational modifications in chromatin remodelling, activation of transcription factors and metabolic enzymes. It has also recently been found that lysine acetylation takes place within other non-histone proteins within both prokaryotic and eukaryotic systems (Ouidir, Kentache and Hardouin, 2016), particularly in *E. coli* and plants (Nallamilli *et al.*, 2014). When the 3B-O sequence was run through a lysine acetylation prediction site (Xue, Li and Yao, 2006), it showed many internal lysine sites that are susceptible to acetylation. It has also been shown that acetylation of proteins has an effect on their size and increases their apparent molecular weight (Guan *et al.*, 2010). It is possible that in this case the 3B-O protein has undergone internal lysine acetylation, which has caused an increase in its size and the appearance of two bands. It has also been noted that the *N. benthamiana*-produced 3B-O protein gave a darker upper (≈20KDa) band when compared to the *E. coli* expressed 3B-O protein, which gave a darker lower (13KDa) band (Figure 2.8 A). This might be due to the extensive post-translational modifications or the high level of acetylation that take place in plants which is not seen to the same extent in bacteria.

In summary, the expression and purification of the 3B-O protein was successful in both the *N. benthamiana* and *E. coli* systems. This antigen was then tested for its ability to distinguish infected from vaccinated animal serum in an I-ELISA (Chapter 4).

CHAPTER 3

EXPRESSION AND PURIFICATION OF A SINGLE-CHAIN VARIABLE FRAGMENT MONOCLONAL ANTIBODY IN *E. COLI* AND *N. BENTHAMIANA*

3.1 INTRODUCTION

The diagnosis of FMDV, for which there is a continual requirement worldwide, is often done using serological analyses. These methods use structural and non-structural proteins (SP and NSP) purified from the virus in an ELISA format in order to either detect serotype-specific antibodies, or to distinguish serum between infected and vaccinated animals. The use of a competitive-ELISA (C-ELISA) with these viral proteins allows for a more sensitive analysis that can be performed on a wide range of animal species. This is due to specific competing antibodies being raised towards the viral proteins which are used in the ELISA, which therefore only requires detection of a single antibody during analysis. The competing antibodies can be whole immunoglobulin (Ig) molecules or recombinant single chain variable fragments (scFvs). The disadvantage of antibody production in hybridoma cell lines is that it is often a lengthy and expensive process that requires the use of animals. The advances in recombinant expression and phage display for expression of scFvs, has significantly reduced both the time and cost necessary for production of monoclonal antibodies. The expression of recombinant full length antibodies (IgG and IgA) and scFvs in a plant system has been shown to be highly successful and an economically viable method of antibody production when compared to mammalian cell culture (Daniell, Streatfield and Wycoff, 2001; Nandi *et al.*, 2016). The use of plants for the expression of immunoglobulins has shown a significant reduction in the cost of production from USD1000/gram for cell culture (hybridoma), to USD50/gram in plants (Daniell, Streatfield and Wycoff, 2001).

Foord *et al.* (2007) identified a scFv isolated from a chicken phage display library that is able to bind to the 3ABC NSP of FMDV, strain O1K. The chicken recombinant antibody foot-and-mouth clone 27 (CRAb-FM27) scFv was identified as having specificity for 3ABC and was

shown to bind epitopes found on the 3B region of the polypeptide. Recombinant CRAb-FM27 was subsequently produced in an *E. coli* expression system from which it was purified and shown to work effectively as a competing agent in a C-ELISA to differentiate between FMDV infected and vaccinated animals (Foord *et al.*, 2007).

This Chapter describes the transient expression of CRAb-FM27 in *N. benthamiana* as a possible reagent for use in a C-ELISA for FMDV diagnosis, and as a cheaper alternative to *E. coli* expression. CRAb-FM27 was synthesised and cloned into the pTRAKc-ERH non-replicating plant expression vector in 2 different ways: one, so as to direct the scFv into and retain it in the endoplasmic reticulum (ER); the second, to direct it into the ER and subsequently allow it to be secreted into the apoplastic space of the plant cell. Previous work has shown that ER retention has improved the yields of scFv expression (Schouten *et al.*, 1996), as has secretion into the apoplast (Fischer *et al.*, 1999). It was also thought that extraction of the scFv from the apoplastic space would be easier for purification purposes due to the reduced number of contaminating proteins in this compartment.

Expression of CRAb-FM27 has previously been achieved only in an *E. coli* system (Foord *et al.*, 2007), and its expression in a plant system would be novel. This Chapter also describes the expression of the same scFv in *E. coli,* which was done in parallel in order to generate a positive control for the plant expression analysis experiments, and to be used as a comparison for functionality testing in an ELISA of the plant expressed scFv, described in Chapter 4.

3.2 MATERIALS AND METHODS

3.2.1 PLASMID AMPLIFICATION AND ISOLATION.

This was carried out as described in section 2.2.1.

3.2.2 CONSTRUCT DESIGN AND SYNTHESIS.

The *CRAb-FM27* gene sequence was obtained from Foord et al. (2007) and was codon optimised for *Nicotiana benthamiana,* synthesised and cloned into pUC57 by GenScript

(China). It was designed to have an *NcoI* restriction site at the 5' end and then two subsequent *NotI* and *XhoI* sites at the 3'end, with a 6xhis tag located between them. An *XbaI* site was placed at the extreme 3' end after the *XhoI* site.

3.2.3 CLONING STRATEGY AND SUBCLONING.

The cloning strategy for the *CRAb-FM27* gene was designed so as to generate two different plant constructs. The gene was digested and ligated into the pTRAKc-ERH expression vector using the *NcoI* and *NotI* sites in order to excise the 6xhis tag from the 3' end of the gene but to retain the 6xhis tag and SEKDEL signal in the vector. This generated the pTRAKc-ERH-*CRAb-FM27* construct which encoded a 3' 6xhis tagged protein, which was intended to be retained within the endoplasmic reticulum (ER) as a result of the presence of the SEKDEL signal. The second construct involved digesting the *CRAb-FM27* using *NcoI* and *XbaI* restriction sites and ligation into the pTRAKc-ERH expression vector. This resulted in the removal of the 6xhis tag and SEKDEL signal from the vector but the retention of the 3' 6x his tag within the gene. This generated the pTRAKc-A-*CRAb-FM27* construct which encoded a 3' 6xhis tagged protein that would not be retained within the ER.

CRAb-FM27 was also cloned into the *E. coli* pProEX-HTb expression vector. This was achieved using the *NcoI* and *XhoI* RE sites which resulted in the gene having a 6xhis tag at both the 5' end (retained within vector) and 3' end (retained within gene).

The cloning protocol was carried out as described in section 2.2.5.

3.2.4 RECOMBINANT PLASMID CONFIRMATION.

This was carried out as described in section 2.2.6. Table 2.5 indicates the primers and cycling conditions used in colony PCR.

3.2.5 *E. COLI* EXPRESSION OF RECOMBINANT PROTEIN.

The pProEX-HTb-*CRAb-FM27* construct was expressed using the protocol described in section 2.2.7.

3.2.6 RECOMBINANT *E. COLI* PROTEIN EXTRACTION.

The BugBuster™ protein extraction protocol was carried out as described in section 2.2.8 but only the soluble fraction was kept.

3.2.7 *A. TUMEFACIENS* TRANSFORMATION.

The pTRAKc-ERH-*CRAb-FM27* and pTRAKc-A-*CRAb-FM27* constructs were transformed into *A. tumefaciens* using the same protocol as described in section 2.2.9.

3.2.8 *A. TUMEFACIENS*-MEDIATED TRANSIENT EXPRESSION.

The pTRAKc-ERH-*CRAb-FM27* and pTRAKc-A-*CRAb-FM27* constructs were transiently expressed on a small scale and large scale using the protocols described in section 2.2.10.

3.2.9 RECOMBINANT PLANT PROTEIN EXTRACTION.

Extraction of proteins from leaves infiltrated with pTRAKc-ERH-*CRAb-FM27* and pTRAKc-A-*CRAb-FM27* was done as described in section 2.2.11, with the exception of only extracting the soluble fraction with extraction buffer (1XPBS, pH7.4).

Apoplast extraction of the CRAb-FM27 protein was done from leaves infiltrated with both constructs. Once the two constructs had been vacuum infiltrated into three plants each, the whole leaves were harvested on day 3 post infiltration. Equal amounts of leaf biomass from each construct was weighed out and submerged in extraction buffer (1XPBS). The extraction buffer was infiltrated into the apoplastic space using a vacuum as described in section 2.2.10. The leaves were dabbed dry, rolled up and placed into 20ml syringe barrels (without plungers). Handling of the leaves was carefully done so as to limit damage and rupturing of the cells within the leaf. These leaf filled syringes were placed into 50ml Falcon tubes which facilitated a gap between the end of the syringe and the bottom of the tube. The removal of the extraction buffer from the apoplastic space was achieved by centrifugation (300xg) for 30 minutes. This was repeated until the leaves appeared to be dry and the apoplastic extract was collected at the bottom of the tubes. Extracts were stored at -20^{o}C for later analysis.

3.2.10 BRADFORD ASSAY.

This was performed as described in section 2.2.12.

3.2.11 SDS-PAGE.

This was performed as described in section 2.2.13.

3.2.12 COOMASSIE BLUE STAINING.

This was performed as described in section 2.2.14.

3.2.13 WESTERN BLOTTING.

The same procedure was carried out as in section 2.2.15. Western blots carried out for the detection of the scFv were done using two different primary antibodies. 1) The anti-his antibody (Mouse anti-his (1/2000) (Bio-Rad, USA, #MCA1396)) along with the secondary conjugate antibody (Goat Anti-mouse Alkaline phosphatase (1/10000) (Sigma-Aldrich, USA, A3562)) was used for the detection of the 6Xhis tag on the recombinant protein. 2) The anti-chicken alkaline phosphatase conjugate (Anti-chicken IgY (IgG) (whole molecule)-Alkaline Phosphatase, Sigma-aldrich) was used for the direct detection of the chicken recombinant antibody Foot and mouth 27 (CRAb-FM27) scFv being expressed.

3.2.14 BATCH BINDING PURIFICATION OF RECOMBINANT *E. COLI* SCFV.

This was performed as described in section 2.2.16. The soluble fraction from section 3.2.6 was subjected to purification and was done so under native conditions as per the manufacturer's instructions. The equilibration and wash buffers were the same and consisted of 1XPBS, 0.5M NaCl at a pH7.4. The elution buffer used consisted of 1XPBS, 0.5M NaCl, pH7.4 with four different imidazole concentrations, which were used to identify optimal elution buffer conditions. The range of imidazole concentrations were 0.01M, 0.1M, 0.25M and 0.5M for each elution buffer used. The elution process started with the elution buffer with the lowest imidazole concentration and then sequentially increased. The fractions collected were stored at -20^{o}C for later analysis.

3.2.15 LARGE-SCALE AFFINITY PURIFICATION OF RECOMBINANT PLANT-PRODUCED SCFV.

The procedure was followed as described in section 2.2.17. The buffers used were the same but lacked 8M urea.

3.2.16 QUANTIFICATION OF AFFINITY-PURIFIED PROTEIN.

The quantification of the purified *E. coli* protein was quantified using the Bradford assay (as described in section 2.2.12.) using a BSA standard.

The purified plant protein samples were quantified via gel densitometry using Syngene GeneTools image analysis software (version 3.07.03). The samples were separated by SDS-PAGE and stained using Coomassie blue and quantified against a BSA standard curve (0.125, 0.062, 0.031 and 0.015 mg/ml).

3.2.17 GLYCO-PROFILING OF PLANT PRODUCED SCFV.

The purified plant scFv was lyophilised as described in section 2.2.19 and sent to the Department for Chemistry, Division of Biochemistry at the University of Natural Resources and Life Sciences, Vienna (BOKU) for glycoanalysis. Analysis by liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) of reduced glycans using PNGase A treatment was performed.

3.3 RESULTS

3.3.1 *E. coli*-EXPRESSION AND PURIFICATION OF CRAb-FM27 scFv

3.3.1.1 Construct confirmation

The *CRAb-FM27* gene was successfully digested from the pUC57 vector and ligated into *E. coli* expression vector pProEX-HTb to generate pProEX-HTb-*CRAb-FM27*. Positive clones were confirmed by colony PCR. Figure 3.1 shows colony PCR products of three *E. coli* transformants, each containing the amplified DNA band of the expected size (932bp). Further confirmation was achieved by RE digestion (not shown).

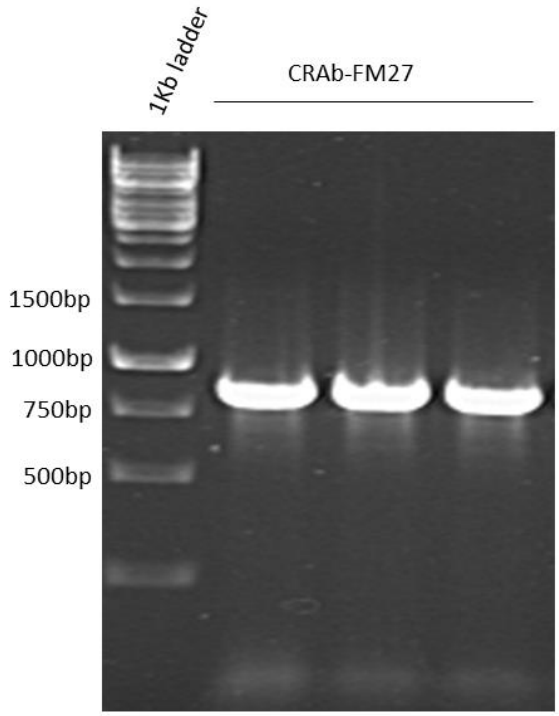

Figure 3.1. 1% agarose gel displaying colony PCR products confirming pProEX-HTb-*CRAb-FM27* transformation into *E. coli*. 1Kb ladder (1Kb DNA ladder), CRAb-FM27 (PCR products of three *E. coli* colonies with DNA fragments confirming *CRAb-FM27* gene ligation into pProEX-HTb seen as 932bp fragments).

3.3.1.2 *E. coli* expression analysis

Expression of CRAb-FM27 in *E. coli* was carried out and induced cultures of the pProEX-HTb-*CRAb-FM27* were sampled every hour for three hours. The samples were analysed by anti-his western blot to determine initial expression, and at which time after induction the highest levels of expression were visualised. Figure 3.2 shows a western blot of scFv protein expression up to three hours after induction. Bands of the expected size (approximately

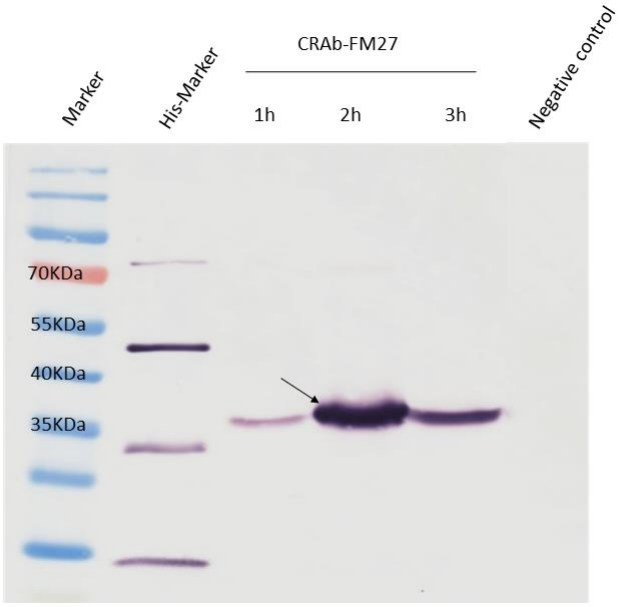

Figure 3.2. Western blot displaying hourly small scale time trial of *E. coli* expressed scFv.
Marker (PageRuler™), His-marker (histidine ladder, positive control), *E. coli*-expressed CRAb-FM27 shown over hours (h) 1, 2 & 3 post-induction. Negative control (crude extract of *E. coli* containing an empty pProEX-HTb vector). Black arrow indicating position of scFv at 35KDa. Anti-his 1/2000.

35KDa) were seen as indicated by the arrow in Figure 3.2. The highest level of expression was observed 2 hours after induction as visualised by the darkest band. A histidine marker was used as a positive control and a crude preparation of *E. coli* hosting pProEX-HTb only was used as the negative control, which showed no non-specific binding (background) of the primary antibody.

3.3.1.3 Purification of *E. coli*-produced CRAb-FM27

Purification of the CRAb-FM27 scFv produced in *E. coli* was done using the batch binding method. The binding and wash buffers contained no imidazole but the elution buffers ranged from 10mM-500mM imidazole to determine at which concentration the protein would be eluted. The fractions collected during purification were analysed on an anti-his western blot and Coomassie stained gel (Figure 3.3 A & B respectively). The western blot shows some scFv present in the flow through (FT) and none detected in the two wash fractions (W1&W2). The elution fractions (E1-E4) show that most of the protein was eluted at 0.01M imidazole (E1) as it gave the darkest band on the western blot. The subsequent elutions (E2-E4) contained less of the scFv, with a steep reduction in band intensity observed between them. The Coomassie gel (Figure 3.3 B) shows a reduction of contaminating proteins from the crude sample and enrichment in fractions of the scFv. The flow through and wash fractions show the presence

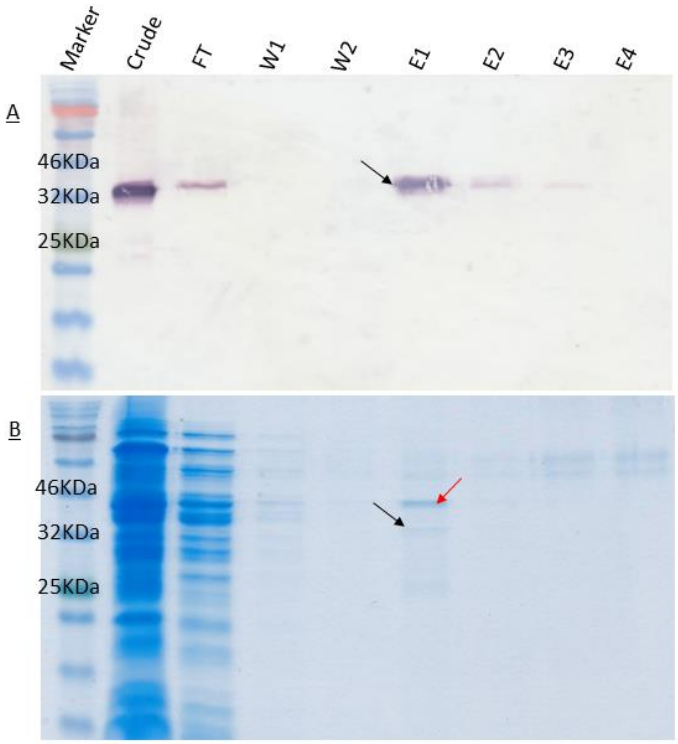

Figure 3.3. Batch binding purification of *E. coli*-expressed CRAb-FM27 fraction analysis.
(**A**) Anti-his 1/2000 western blot and (**B**) Coomassie stained SDS-PAGE. Marker (Color Prestained protein standard), crude (5ul cell culture pre-purification), FT (Flow Through), W1 (Wash 1), W2 (Wash2), E1 (Elution 1, Imidazole 0.01M), E2 (Elution 2, Imidazole 0.1M), E3 (Elution 3, Imidazole 0.25M), E4 (Elution 4, Imidazole 0.5M). Black arrows indicating CRAb-FM27 at ≈35KDa and red arrow indicating contaminating protein.

of other proteins as seen by the bands in the Coomassie. The reduction of band intensity between the FT and wash fractions indicates successful removal of unbound native *E. coli* proteins. The elution fractions in the Coomassie-stained gel show the presence of possible native *E. coli* proteins (red arrow) that co-eluted with the scFv (black arrow), shown in lane E1. Quantification of the protein was carried out by Bradford assay, as using gel densitometry was not possible (not shown). This yielded 0.104µg/µl, for a final yield of 520µg.

3.3.2 *N. BENTHAMIANA*-EXPRESSION AND PURIFICATION OF CRAB-FM27 SCFV.

3.3.2.1 Cloning of CRAb-FM27 into plant expression vectors and construct confirmation

The *CRAb-FM27* gene was successfully digested from the pUC57 vector and ligated into the pTRAKc-ERH plant expression vector. Subcloning using different RE sites allowed for the generation of two variations of the same construct; pTRAKc-ERH-*CRAb-FM27* and pTRAKc-A-*CRAb-FM27*. Positive clones were confirmed by colony PCR. This was carried out on four colonies per construct and products run on a 1% agarose gel (Figure 3.4). The pTRAKc-ERH-*CRAb-FM27* showed all four colonies contained the construct as a 1041 bp product was seen

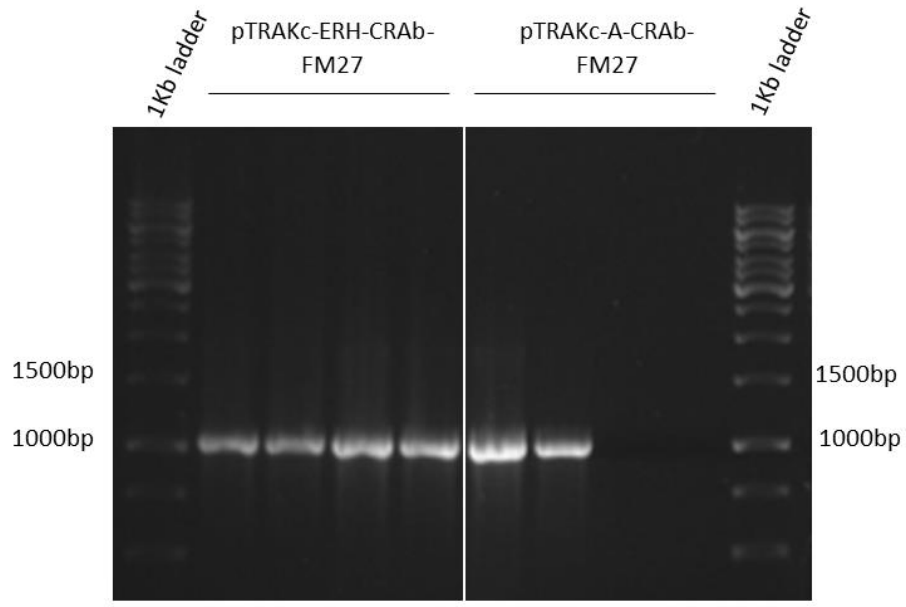

Figure 3.4. 1% agarose gel displaying *Aarobacterium* colony PCR confirming plant vectors with *CRAb-FM27* gene insert. 1Kb ladder (DNA ladder), pTRAKc-ERH-*CRAb-FM27* (1041bp) and pTRAKc-A-*CRAb-FM27* (1023bp) indicating four colonies per construct and DNA fragments indicating successful construct transformation into *Agrobacterium.*

in each lane. However, only 2 of the four colonies showed positive 1023 bp PCR products for the pTRAKc-A-*CRAb-FM27* construct.

3.3.2.2 Small-scale plant expression of CRAb-FM27

Initially the two constructs (pTRAKc-ERH-*CRAb-FM27* and pTRAKc-A-*CRAb-FM27*) were syringe-infiltrated into three plants each at bacterial OD_{600} values of 0.5 and 1.0. Crude leaf extracts were screened after 1, 3 and 5 days post infiltration (dpi) and analysed for recombinant scFv expression by anti-his western blot. This was done to determine the optimal day of harvest as well as the optimal bacterial OD_{600} to use for large scale expression (Figure 3.5). The positive control used was the crude sample of *E. coli*-expressed CRAb-FM27 and the negative control was extracted crude leaf sample infiltrated with *A. tumefaciens* hosting pTRAKc-ERH only. Total protein for each sample was measured by Bradford assay, for equal amounts to be loaded into each lane. This gives a more reliable and accurate comparison between the differences seen in expression of the two variations of recombinant constructs.

Crude extracts from leaves infiltrated with pTRAKc-ERH-*CRAb-FM27* (Figure 3.5 A) showed the highest expression level of scFv on day 3 after infiltration at an OD_{600} of 1.0, as the band in this lane is darker than those seen on day 1 (which barely showed any expression) and day 5 (which is very similar to day 3). However, crude extracts from leaves infiltrated with the

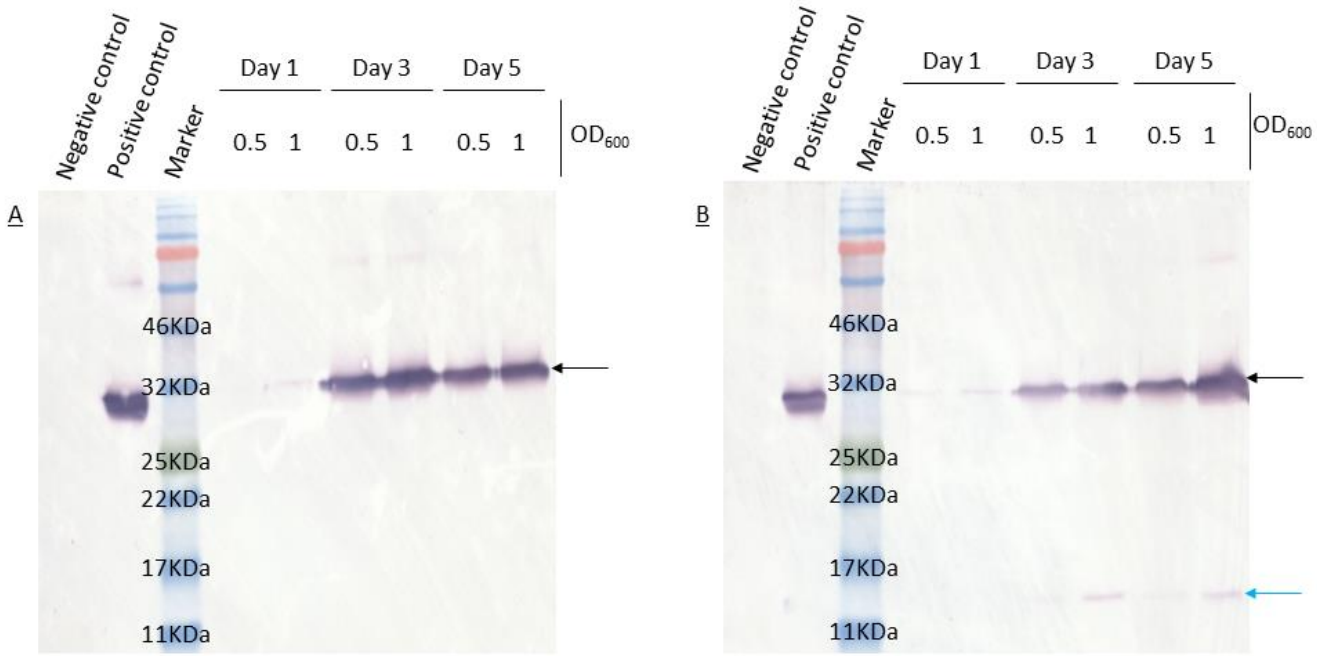

Figure 3.5. Western blot displaying small scale time trial of *N. benthamiana*-expressed CRAb-FM27 scFv.

(**A**) pTRAKc-ERH-CRAb-FM27 and (**B**) pTRAKc-A-CRAb-FM27 constructs sampled. Negative control (crude leaf extract infiltrated with empty pTRAKc vector), Positive control (Crude *E. coli*-expressed CRAb-FM27), Marker (Color Prestained protein standard). Days 1, 3 and 5 post infiltration, each with an OD600 of 0.5 and 1 tested. 50µg of total soluble protein loaded into each lane. Black arrow indicating position of CRAb-FM27 scFv (≈35KDa) and blue arrow indicating possible degraded scFv (15KDa). Anti-his 1/2000.

pTRAKc-A-*CRAb-FM27* construct showed the highest expression on day 5 after infiltration with a culture OD_{600} of 1.0 (Figure 3.5 B). Additional faint bands of a lower molecular weight (≈15KDa) were seen in the leaf extracts from the pTRAKc-A-*CRAb-FM27* constructs (Figure 3.5 B), indicated by the blue arrow. These were repeatedly observed for three biological experiments when infiltrated with pTRAKc-A-*CRAb-FM27* but not observed in samples infiltrated with pTRAKc-ERH-*CRAb-FM27*.

In order to further confirm successful expression in plants of the CRAb-FM27 scFv the same samples were run on a western blot for each construct as before. The blot was then probed using an anti-chicken alkaline phosphatase conjugate antibody for the direct detection of the scFv (not shown). The same protein bands of the same molecular weight (≈35KDa) as in Figure 3.5 were observed. This showed that the specific chicken scFv was being expressed as it could be detected using an anti-chicken antibody.

3.3.2.3 Apoplast extract comparison between constructs

Apoplast extraction was carried out to determine if extraction using this method would be more effective than homogenising whole leaves. There is substantially less protein within the apoplastic space in comparison to within the cells and so it was thought that secretion of recombinant proteins into this space would consist of less contaminating proteins within the sample extracts. This would reduce the need for extensive purification and hence simplify recombinant protein extraction.

Three plants were vacuum infiltrated with pTRAKc-ERH-*CRAb-FM27* and another three with pTRAKc-A-*CRAb-FM27* at an OD_{600} of 1.0. Three days post infiltration the same weight of leaf material from each plant and for each construct was subjected to apoplast extraction. The samples were analysed by an anti-his western blot and a comparison of all three biological repeats (B1, B2 and B3) was done between both constructs (Figure 3.6). The results showed successful extraction of the scFv from the apoplast in all samples (Figure 3.6, black arrow), with the majority seen from the pTRAKc-A-*CRAb-FM27* extract as expected when compared to that of the pTRAKc-ERH-*CRAb-FM27* extract. This blot also showed that the scFv extracted from the pTRAKc-A-*CRAb-FM27* construct alone, had a lower molecular weight band of

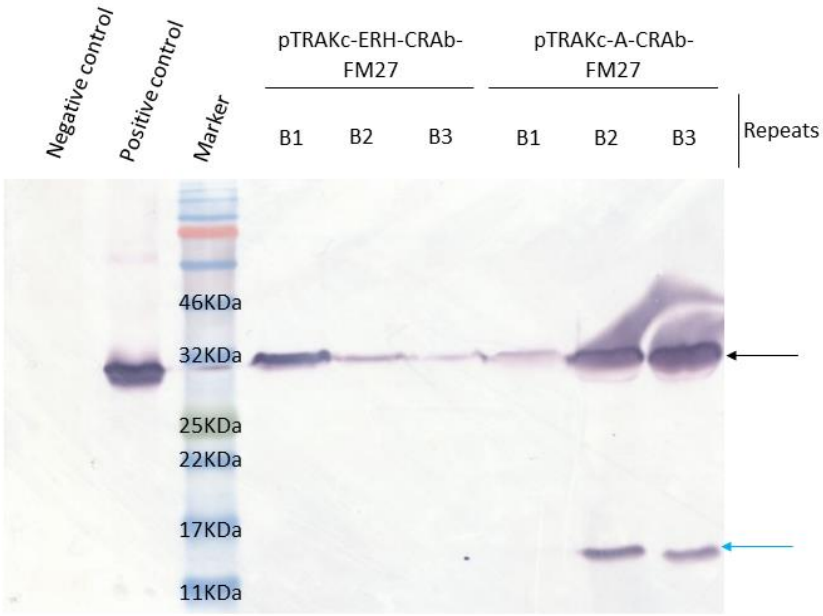

Figure 3.6. Western blot displaying *N. benthamiana* leaf apoplastic extracts containing CRAb-FM27.

Negative control (crude leaf extract infiltrated with empty pTRAKc vector), Positive control (crude *E. coli* expressed CRAb-FM27), Marker (Color Prestained protein standard), Biological repeats (B1, B2 and B3) of apoplastic extracts taken on day 3 post infiltration at OD_{600} 1.0 of both constructs. Black arrow indicating position of CRAb-FM27 scFv (≈35KDa) and blue arrow indicating possible degraded scFv (15KDa). Anti-his 1/2000.

approximately 15KDa (Figure 3.6), indicated by the blue arrows, and as seen before in Figure 3.5 B.

3.3.2.4 Large-scale affinity purification of recombinant plant-produced scFv

The pTRAKc-ERH-*CRAb-FM27* construct was chosen to continue purification with, as it showed the highest yields of protein the soonest after infiltration and also showed no signs of degradation, i.e. the lower molecular weight bands visualised from samples infiltrated with pTRAKc-A-*CRAb-FM27* (Figure 3.5). To achieve a suitable working volume for purification using IMAC (nickel resin) and the ÄKTA FPLC system, a large-scale infiltration was done. The pTRAKc-ERH-*CRAb-FM27* construct was vacuum infiltrated into 12 plants, using the optimal parameters established for the small-scale expression (see section 3.3.2.2.). The homogenised plant extract was then purified using the ÄKTA system. The flowthrough, wash and elution fractions are shown in a chromatogram depicting protein absorbance readings at 280nm (Figure 3.7). The flowthrough region early on in the chromatogram shows a large absorbance peak spanning the fractions (1-15). This peak is thought to contain mostly the native plant proteins which have not bound to the resin during the loading of the plants extract onto the column. This initial peak decreases rapidly during the wash phase (fractions 16-20). The elution phase consisted of an increasing imidazole concentration gradient across fractions 21-40 and then an increase to 0.5M imidazole across fractions 41-46 (Figure 3.7, green line as a percentage of 0.5M imidazole). A second peak (increase in protein absorbance) is seen from fraction 23 to fraction 25. A third peak of absorbance across fractions 27-28 is also seen. Subsequent to the third peak, the absorbance at 280nm gradually increases across fractions 31-42 which then peaks again and plateaus (43-46). The absorbance seen across these fractions seems to follow the increase of imidazole being added. It is thought to be the impurities within the imidazole, which tend to absorb light at 280nm, as it was not of a pure grade.

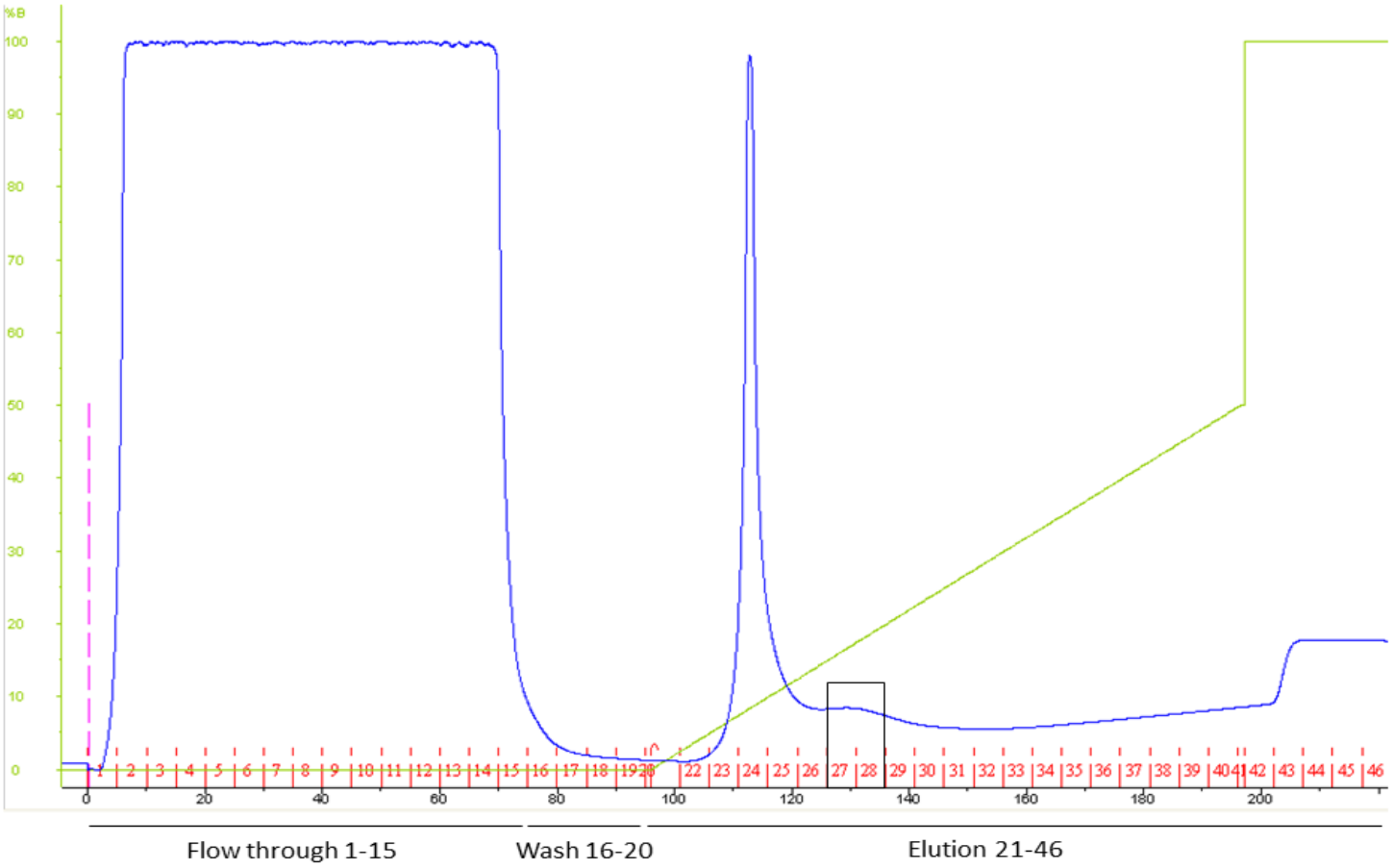

Figure 3.7. Chromatogram trace of ÄKTA purification of *N. benthamiana*-produced CRAb-FM27.
The X axis indicating fractions of the Flow through (1-15), Wash (16-20) and Elution (21-46). Y axis showing % of elution buffer. Blue line indicating absorbance at 280nm, green line showing increasing gradient as a percentage of elution buffer containing 0.5M Imidazole. Black box indicating were best purity and quantity of scFv was found.

The fractions from the ÄKTA purification were analysed by an anti-his western blot and Coomassie staining, in order to confirm which of the protein peaks contained the CRAb-FM27 protein (Figure 3.8 A & B respectively). The flow through (FT) fractions 5-7 contained in the first large absorbance peak, contained very little scFv as only a faint band was seen on the western blot. The Coomassie-stained gel showed many protein bands and a smear of protein in the FT 5-7 sample, suggesting that the high absorbance in these fractions largely consists of contaminating plant proteins. The wash fractions (W 17-19) in the western blot show no scFv protein bands indicating that it was successfully bound to the column. There were no protein bands seen in the W17-19 samples on the Coomassie gel either (Figure 3.8 B). Eluted fractions E23-E25 across the second protein peak showed the presence of scFv as visualised by a ≈35kDa band. However, the Coomassie-stained gel of the same fractions showed a range

of other proteins bands of different sizes and very little scFv. However, the eluted fractions from the third peak across E27 and E28 showed successful enrichment of the scFv (Figure 3.7, black box) as seen by the darkest band on the western blot and a single protein band on the Coomassie-stained gel with little contaminating proteins observed. No protein bands were visualised in fractions E40-42 indicating that all the scFv was eluted. There were no protein bands in these fractions on the Coomassie-stained gel either, suggesting that the peak visualised on the chromatogram may have been due to absorbance of impurities in the imidazole. Quantification of fraction E27-28 was done by SDS gel densitometry using Coomassie staining and a BSA standard curve (not shown). This yielded 0.098µg/µl of the scFv which was eluted and pooled to give a total of 10ml and therefore giving a total of 980µg of scFv.

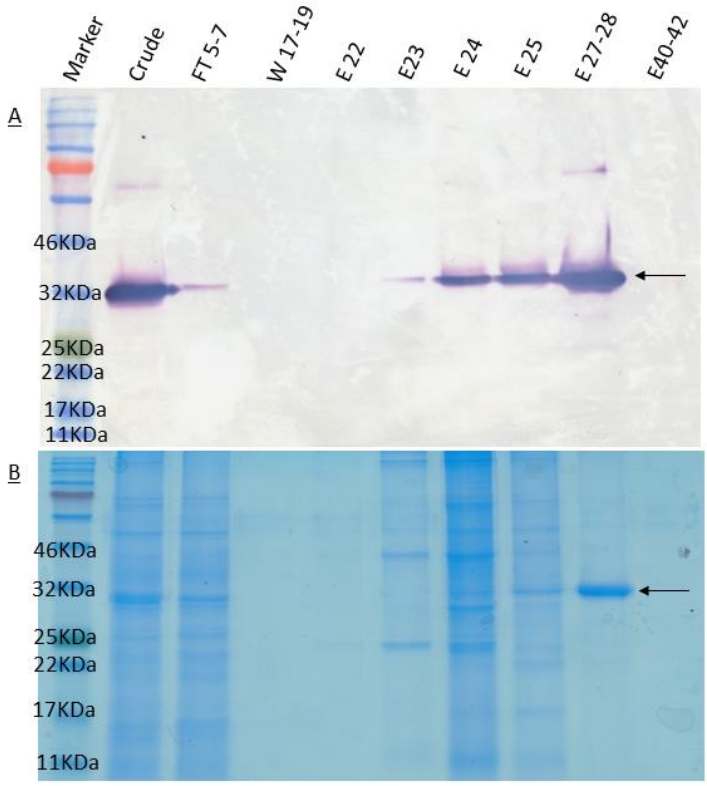

Figure 3.8. Analysis of ÄKTA fractions for purification of *N. benthamiana*-produced CRAb-FM27 scFv.

(**A**) Western blot Anti-his 1/2000 and (**B**) SDS-PAGE Coomassie stained. Marker (Color Prestained protein standard), Crude (pre-purification crude leaf extract), Flow Through fractions (FT 5-7), Wash fractions (W 17-19), Elution fractions (E 22-42). Black arrows showing position of CRAb-FM27 scFv (≈35KDa).

3.3.3 GLYCO-PROFILING OF PLANT PRODUCED CRAB-FM27

A lyophilised sample of purified plant made scFv was analysed for N-glycosylation. Results are shown as a percentage of the different glycoforms in Table 3.1. The predominant glycoforms are Man8, Man9 and MMXF and GnGnXF3 which show to make up over 75% of the glycoforms within the scFv protein. The other glycoforms screened for included Man5, Man6, Man9+Hex, MMX, MUX, Man4XF3, Man4X, GnGnF, AGnF6, AAF6, GnM, GnGn and AA, which were not present.

Table 3.1. The relative proportion of the different glycoforms are depicted as a percentage.

Glycan species	Relative abundance (%)
Man7	11.44
Man8	15.57
Man9	16.99
MMXF3	21.89
GnGnXF3	22.53
GnMXF3	7.24
MUXF3	4.34

3.4 DISCUSSION

The CRAb-FM27 scFv was identified by Foord et al. (2007) and cloned and expressed in *E. coli* to be used as a competing reagent in a C-ELISA, and was successful in differentiating between FMDV-infected and -vaccinated animal serum. This work describes the expression of the scFv in *N. benthamiana,* as expression of recombinant proteins in a plant system is potentially a cheaper method of production. It was decided that expression of the scFv in an *E. coli* system would be conducted in parallel to show reproducibility of expression and to generate positive control proteins to verify the plant expression work.

The small-scale expression of the scFv in plants was used for optimisation of parameters (time of harvest and culture OD_{600}) to be used for large scale production. Two variations of recombinant pTRAKc vectors expressing the scFv were tested to determine whether targeting to different subcellular organelles would influence the level of protein expression. The first

was termed pTRAKc-ERH-*CRAb-FM27*, which had a SEKDEL signal to retain the scFv in the endoplasmic reticulum (ER) (Denecke, De Rycke and Botterman, 1992). Retention of recombinant proteins within the ER is thought to protect proteins by shielding them from proteases found within the cytoplasm, and therefore help increase yields (Pillay *et al.*, 2013). The second variant was the pTRAKc-A-*CRAb-FM27* construct which lacks the SEKDEL signal, therefore allowing the scFv to move through the secretory pathway into the apoplastic space. Previous work has shown that targeting and extracting proteins from the apoplastic space yields higher levels of more pure scFvs (Fischer *et al.*, 1999). This is due to the limited amount of native plant proteins present in the apoplast compared to within the cell.

The optimisation time trials were used to determine optimal OD_{600} for infiltration and time of harvest for both the pTRAKc-ERH-*CRAb-FM27* and pTRAKc-A-*CRAb-FM27* constructs, using leaf clippings (Figures 3.5 A &B). The results showed that both constructs yielded successful expression of the scFv. However, the pTRAKc-ERH-*CRAb-FM27* vector showed higher levels of expression earlier (at 3 dpi) compared to pTRAKc-A-*CRAb-FM27* (at 5 dpi). The pTRAKc-A-*CRAb-FM27* samples showed expression of the scFv as a ≈35KDa protein band but a smaller-sized band of approximately 15KDa (Figure 3.5 B, blue arrow) was also visualised. This lower molecular weight band could be degradation of the scFv due to the lack of ER retention and therefore exposure to plant proteases (Pillay et al, 2013).

The molecular weight of CRAb-FM27 protein is not disclosed in the Foord et al. (2007) paper, but the theoretical size calculated *in silico* using the ExPASy server tool (Gasteiger *et al.*, 2005) was calculated to be 27.5KDa. However, this work shows that the successfully expressed scFv in *E. coli* and *N. benthamiana* was approximately 35KDa in size (Figure 3.2 and Figure 3.5 respectively). It is possible that this perceived increase in molecular weight may be due to post-translationally modified amino acids. Post-translational modification of proteins (e.g. *N*-glycosylation) has been known to increase the apparent molecular weight (MW) of a protein on a SDS-PAGE (gel-shift) (Lu *et al.*, 1997; Selcuk Unal *et al.*, 2008).

N-glycosylation is one of the major post-translational modifications in eukaryotic systems and is largely similar between mammals and plants. The initial processing of proteins in all

eukaryotes, starts in the ER and mainly consists of the oligo-mannosidic N-glycans; $Glc_3Man_7GlcNAc_2$ (Man7), $Glc_3Man_8GlcNAc_2$ (Man8) and $Glc_3Man_9GlcNAc_2$ (Man9). In plants, the Man8 glycoform is usually moved down the secretory pathway, through the *cis*, *medial* and *trans*-Golgi compartments to form complex *N*-glycans (e.g. GnGnXF3). The GnGnXF3 glycoform is then sorted into either the vacuole or apoplast for further processing into the MMXF3 complex *N*-glycan (Bosch *et al.*, 2013). Glycosylation is a complex process and consists of many precursor glycan forms and therefore, to determine the extent of *N*-glycosylation, the plant purified CRAb-FM27 protein was analysed for its N-glycan profile. The Man7, Man8 and Man9 glycoforms made up around 44% of the total *N*-glycosylation, which was to be expected as the protein was targeted and retained within the ER. The majority of the other glycoforms detected consisted of the complex GnGnXF3 and MMXF3 *N*-glycans, which again made up around 44% of the total glycosylation. This was an unexpected result as these glycoforms are usually processed in the Golgi and vacuole/apoplast, which the scFv should not have been exposed to. This is due to the scFv containing the SEKDEL signal for retention in the ER. However, incomplete ER retention of recombinant proteins possessing a SEKDEL signal has been identified as a common trait for recombinantly expressed proteins in seeds (Arcalis *et al.*, 2004; Petruccelli *et al.*, 2006; He *et al.*, 2012). Even though the extracts analysed were from the leaves and not from the seeds, these findings might suggest that the retention in the ER of the recombinant scFv using the SEKDEL signal, might not be entirely efficient. With the scFv consisting of complex *N*-glycoforms (Table 3.1) it could explain why the scFv is seen as a band having a higher molecular weight on the western blot (≈35KDa) than at the calculated 27.5KDa. There is also a slight difference in size between the *E. coli*-produced CRAb-FM27 (slightly lower MW) and the *N. benthamiana*-produced CRAb-FM27 (slightly higher MW) (Figure 3.5). This difference, estimated to be about 2-3KDa, could be due to the extensive *N*-glycosylation, or possibly the high levels of acetylation that take place in plants which is not seen to the same extent in bacteria (Dell *et al.*, 2010). However, the latter possibility was not tested. Moreover, when the scFv protein sequence was run through an *in-silico* prediction server for N-Glycosylation sites (Gupta, Jung and Brunak, 2004) no glycosylation sites were identified.

In order to further confirm that the plant and *E. coli*-produced CRAb-FM27 protein was derived from a chicken antibody, a western blot with CRAb-FM27 protein was probed with an

anti-chicken IgY alkaline phosphatase conjugate antibody (not shown). The western blot showed the same sized band (≈35KDa) as observed with the anti-his blot (not shown), indicating that the recombinant scFv was folded correctly for binding of the secondary antibody. This also confirmed that the anti-chicken conjugate antibody could be used in future work to prove the functionality of the scFv in ELISAs and western blots (Chapter 4).

The distribution of recombinant CRAb-FM27 in the apoplast was surveyed in extracts from leaves infiltrated with pTRAKc-ERH-*CRAb-FM27* and pTRAKc-A-*CRAb-FM27* and harvested at 3 dpi. It was expected that the pTRAKc-A-*CRAb-FM27* (lacking the SEKDEL ER retention signal) would direct the recombinant protein into the apoplast and that the pTRAKc-ERH-*CRAb-FM27* (having the ER retention signal) would result in the retention of the recombinant protein in the ER. When infiltrated plant leaves were subjected to an apoplast extract protocol, a faint band was detected indicating low levels of scFv from the pTRAKc-ERH-*CRAb-FM27* expression (Figure 3.6). One would have expected no band, but it is possible that the apoplast extraction method may have caused some physical damage to the leaves resulting in 'leakage' of the recombinant protein into the preparation. It has also been hypothesised that the retention of the scFv within the ER is not completely efficient, allowing for secretion into the apoplastic space, as the *N*-glycoforms detected in the scFv shows processing of the protein in the Golgi and apoplast (as explained above). Higher levels of the scFv from the pTRAKc-A-*CRAb-FM27* construct were observed, which was expected. However, there appears to be an increase in the presence of degraded proteins, as seen by the lower band (Figure 3.6, blue arrow). This could possibly be due to proteolysis caused by the abundant levels of proteases in the apoplast. The advantage of using an apoplastic extract is the low abundance of native plant proteins present compared to a homogenised extract, reducing the need for complex purification. This low abundance of proteins in the apoplastic fluid compared to the whole leaf homogenate was observed and confirmed when running the samples on a Coomassie (not shown). However, an increase in recombinant scFv proteolysis, possibly resulting from the known high abundance of proteases within the apoplast (Delannoy *et al.*, 2008; Pillay *et al.*, 2013), led to the conclusion that apoplast secretion was an inefficient method of production.

The pTRAKc-ERH-*CRAb-FM27* construct was therefore selected for further experimentation as infiltration resulted in greater levels of recombinant protein being produced earlier, and retention of the scFv within the ER appeared to limit degradation of the protein. With the optimised conditions for scFv expression in both *N. benthamiana* and *E. coli* having been established, a large-scale expression for both was done in order to optimise purification. Purification of *E. coli* made scFv was successful using the batch binding method. The scFv was eluted at a very low imidazole concentration (0.01M) which suggested that the binding buffer should not contain imidazole during purification of plant produced scFv, when using the ÄKTA system. The lack of imidazole in the binding buffer resulted in increased non-specific binding of proteins to the resin but would reduce the risk of losing the scFv in the flow through and wash fractions. In order to limit co-elution of contaminating native plant proteins, a shallow imidazole gradient was used to better separate the scFv and obtain a more pure fraction. The plant-produced scFv was successfully eluted with the majority of the protein collecting in fractions E27-28 and containing very few contaminating proteins (Figure 3.8).

The purified samples of CRAb-FM27 from both *E. coli* (batch binding) and from *N. benthamiana* (ÄKTA) were then quantified. *N. benthamiana* production gave a range between 0.047µg/µl and 0.098µg/µl, with a best recovery of 85% of the total scFv from the crude extract, after purification. Quantification of the *E. coli* expressed scFv could not be achieved using gel densitometry, as the concentration of the protein was too low to be seen on a Coomassie-stained gel. A western blot could not be used for densitometry as the *E. coli* expressed scFv had two 6xhis tags, whereas the standard would only have one and so would not be comparable. Therefore, the *E. coli*-expressed scFv was quantified using a Bradford assay. The concentration of the protein ranged from 0.094µg/µl to 0.104µg/µl; however, this was the total protein concentration which included some contaminating proteins.

The CRAb-FM27 scFv was successfully expressed and purified from both *E. coli* and *N. benthamiana* systems and its functionality was tested in an ELISA (Chapter 4), to see if they could bind the 3B-O antigen (produced in work reported in Chapter 2).

CHAPTER 4

TESTING THE FUNCTIONALITY OF *N. BENTHAMIANA* AND *E. COLI* PRODUCED REAGENTS

4.1 INTRODUCTION

A competitive-ELISA (C-ELISA) in the field of FMDV diagnostics could reduce the cost of testing, as the reagents required for testing will not be confined to just one species of animal sera being investigated. This is because the antigen is coated into the wells of the ELISA plate, and animal serum to be analysed for infection is added, along with the competing antibody. The detection of the bound competing antibody to the antigen is done using a secondary antibody, which will determine the infection status of the animal. Therefore, a secondary antibody to detect specific antibodies of the animal under investigation is not needed. In the context of the Kruger National Park (KNP) in South Africa and the surrounding buffer and FMDV surveillance areas, this is important when screening animals in these regions. The reason for this is that livestock, which is translocated to and from the zones of the KNP, consists of other animal species as well as cattle.

The desired reagents for an FMDV C-ELISA would consist of a NSP with which to coat the wells of the plate, and an antibody to compete with antibodies in the animal serum for the binding of the antigen. In Chapter 2, I showed that of the candidates expressed in *N. benthamiana*, the FMDV-NSP 3B-O was the most favourable to use as a coating antigen as high levels could be expressed in plants and successfully purified. The same 3B-O protein was also successfully expressed and purified using an *E. coli* expression system, to be used for comparative functionality testing. In Chapter 3 it was shown that the CRAb-FM27 scFv could be successfully expressed and purified from both *N. benthamiana* and *E. coli*. Thus, it was decided to use this as the competing antibody in a C-ELISA together with the plant and *E. coli*-produced coating antigen 3B-O. *E. coli*-expressed CRAb-FM27 scFv was tested for comparative functionality.

It is vital that the CRAb-FM27 scFv is able to bind the 3B-O antigen. To determine which of the systems produced functional proteins, combinations of *E. coli* and plant produced antigen

and scFv were tested together. The combinations tested, were to determine if the scFv could bind the antigen and were as follows: plant-produced scFv tested against plant and *E. coli*-produced 3B-O antigen, and *E. coli*-produced scFv tested against plant and *E. coli*-produced 3B-O antigen. The initial testing of the scFv was done via western blotting, and then subsequently by an I-ELISA, to show functionality and binding capability to the antigen in this format.

It is also imperative that antibodies in infected but not in vaccinated animal serum can bind and recognise the 3B-O antigen. Therefore, the recombinant *N. benthamiana* and *E. coli*-produced 3B-O antigen was initially tested in an I-ELISA, with infected animal serum to show whether it could demonstrate its ability to differentiate between serum from infected and vaccinated animals. Having established this, both the recombinant 3B-O antigen and CRAb-FM27 scFv proteins were used in combination in a C-ELISA to identify their potential as functional diagnostic reagents in this format.

4.2 MATERIALS AND METHODS.

4.2.1 FUNCTIONALITY TESTING OF CRAB-FM27 ON A WESTERN BLOT.

Plant-produced and *E. coli*-produced CRAb-FM27 antibodies were tested for their ability to bind to plant-produced or *E. coli*-produced 3B-O. The 3B-O antigen purified from both plant and *E. coli* expression systems (approximately 300ng) were run on separate SDS polyacrylamide gels and blotted onto nitrocellulose. Purified plant and *E. coli*-produced CRAb-FM27 scFvs were diluted (1:3, approximately 300µg) in blocking buffer and used to separately probe both plant-produced and *E. coli*-produced 3B-O blots. Alkaline phosphatase-conjugated anti-chicken antibody was used as the secondary antibody (Anti-chicken IgY (IgG) (whole molecule)-Alkaline Phosphatase, Sigma-Aldrich) in a 1:5000 dilution. The procedure was carried out as discussed in section 2.2.15.

4.2.2 FUNCTIONALITY OF RECOMBINANT CRAB-FM27 IN INDIRECT-ELISA (I-ELISA).

The combination for testing the binding of the scFv to the antigen in an I-ELISA was similar to that described in section 4.2.1. Purified plant-produced 3B-O and *E. coli*-produced 3B-O (100 µl undiluted, 1:2 and 1:10 diluted in coating buffer (1XPBS, pH7.4)) were used to coat the wells of separate 96-well Maxisorp™ microtitre plates (Nunc-Immuno™, Sigma-Aldrich). The controls included coating buffer only and 1:100 dilutions of either *E. coli* or plant negative crude extract (extracted from *E. coli* transformed with empty pProEX-HTb vector or from plants infiltrated with *Agrobacterium* transformed with empty pRIC3.0-HT vector, as negative controls) for background normalisation. The *E. coli* and plant-produced scFv was diluted (1:3) and coated in three of the wells per plate that were to be probed using the same scFv. This was a maximum absorbance control to confirm detectability of the scFv using the secondary antibody (positive control). The plates were incubated overnight at 4^{o}C with shaking. The plates were blocked with blocking buffer (5% non-fat dry milk in 1XPBS) using 200µl per well and incubated at 37^{o}C for 1 hour with shaking. Wells were rinsed with 200µl of 1XPBS before the recombinant purified scFv expressed in *E. coli* or *N. benthamiana* was added. The scFv was diluted in blocking buffer at a 1:3 dilution and 100µl added per well. The plates were incubated at 37^{o}C for 1 hour and again rinsed with 1XPBS. The plates were washed (1XPBS, 0.05% tween 20 - PBST) three times with 200µl. The anti-chicken alkaline phosphatase conjugated IgY (Sigma-Aldrich) was added to each well at a 1:5000 dilution in blocking buffer at 100µl per well. The plate was incubated at 37^{o}C for 1 hour and then washed four times in PBST. After washing, 200µL SIGMAFAST™ p-Nitrophenyl phosphate substrate (pNPP, Sigma) was added per well and plates incubated in the dark for 30 minutes. The absorbance was detected at 405 nm on a BIO-TEK® Powerwave XS microtitre plate reader. This experiment was carried out in quadruplicate.

4.2.3 FUNCTIONALITY OF RECOMBINANT 3B-O ANTIGEN USING I-ELISA TO DETECT ANTI-FMDV ANTIBODIES IN ANIMAL SERUM.

The same methodology was followed as described in section 4.2.2, with the following changes. 107ng of 3B-O antigen from *E. coli* and *N. benthamiana* was separately coated across a 96-well plate. Either *E. coli* or leaf crude extract from an empty vector (negative control, 107ng total protein coated) was used for background normalisation. *E. coli* expressed and

purified $3AB_1$ protein obtained from collaborators at INTA (Buenos Aires, Argentina), which has previously been shown to detect infected animal serum, was used as a positive control. The same amount of $3AB_1$ protein was diluted in coating buffer and added to the wells to be used as a positive control. Each row of wells on the plate was coated with the test antigen (3B-O from *E. coli* or *N. benthamiana*), a background control (*E. coli* or plant negative), and a positive control ($3AB_1$). All serum samples tested but one were from guinea pigs which had previously been infected with guinea pig-adapted FMDV; the remaining serum sample was from a vaccinated guinea pig (FMDV-inactivated virus, serotype C85) (Table 4.1). Serum samples were taken 15-20 days post-infection or vaccination. These serum samples were diluted to 1:10 with blocking buffer and 100µl added per well. Each serum sample was added to a separate row of wells. Anti-Guinea pig alkaline phosphatase-conjugated (Anti-Guinea Pig IgG (whole molecule) −Alkaline Phosphatase, Sigma-Aldrich) secondary antibody was used at 1:5000. The absorbance readings were read at 405nm and experiments were performed in quadruplicate.

Table 4.1. FMDV-serum from infected and vaccinated Guinea pig of different serotypes and subtypes.

Serotype	Subtype	Status
C	C85	Infected
C	CC59	Infected
A	A24	Infected
A	A87	Infected
O	O1	Infected
O	O1C	Infected
C	C85	Vaccinated

4.2.4 Competitive-ELISA to test functionality of recombinant antigen and scFv in the presence of FMDV anti-serum.

The *E. coli* and *N. benthamiana*-produced 3B-O antigen and the *N. benthamiana*-produced scFv were tested together to determine if they could differentiate infected from vaccinated Guinea pig serum in a competitive-ELISA. The *E. coli* and *N. benthamiana*-produced 3B-O antigen was diluted (107ng) in coating buffer and coated in separate 96-well Maxisorp™ microtitre plate overnight at 4°C. The background control included either *E. coli* or leaf material (extracted from an empty vector, negative; 107ng total protein) for background

normalisation. The plate was blocked as previously described and the serum from FMDV-infected Guinea pigs added at a range of dilutions (1:10, 1:100, 1:1000 and 1:10 000). This was incubated at 37^{o}C for 1 hour and thereafter, without discarding the diluted serum, the plant-produced scFv diluted to 1:3 in coating buffer (1XPBS, pH7.4) was added. The maximum absorbance control included the addition of the scFv to the antigen coated well, in the absence of the animal serum. This would give the maximum absorbance and therefore the binding of the scFv to the antigen without the competition of the serum. The plate was re-incubated at 37^{o}C for 1 hour, followed by three wash steps as before. The anti-chicken alkaline phosphatase-conjugated secondary antibody (diluted at 1:5000) was added and incubated for 1 hour at 37^{o}C. The plate was washed four times and the pNPP substrate added and incubated as described previously. The absorbance was read at 405nm and experiments performed in triplicate.

4.3 RESULTS

4.3.1 Functionality of recombinant CRAb-FM27 scFv on a Western blot.

Having previously described the successful expression and purification of both the 3B-O antigen and CRAb-FM27 scFv in *N. benthamiana* and *E. coli,* the functionality of the recombinant CRAb-FM27 was tested for its ability to bind to recombinant 3B-O in a western blot. The 3B-O antigen produced in *N. benthamiana* and *E. coli* were separately probed with either the *N. benthamiana* or *E. coli*-expressed scFv. In all the western blots tested, a positive control blot was run with the same samples but probed using the anti-his antibody, which was used to confirm the presence of the antigen. The negative control was either crude *N. benthamiana* leaf extract infiltrated with *Agrobacterium* containing an empty pRIC3.0-HT vector or crude *E. coli* extract transformed with empty pProEX-HTb vector, depending on the system the antigen was being expressed in.

The 3B-O *E. coli* produced antigen was successfully detected as a 13 KDa sized protein with the *N. benthamiana*-produced scFv (Figure 4.1 A, black arrow). The positive control western blot (Figure 4.1 B) showed the same 13KDa band when probing with the anti-his primary

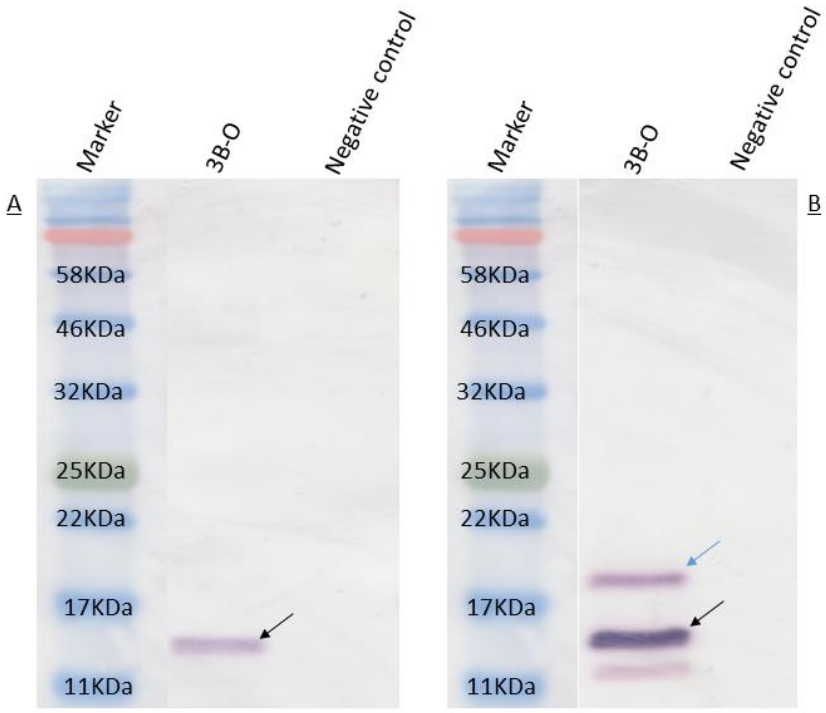

Figure 4.1. Western blot to detect 3B-O antigen using plant made scFv.

(**A**) Probed with purified plant scFv (1:3 dilution) as primary antibody and conjugate anti-chicken as secondary antibody (1:2000). (**B**) Probed with anti-his antibody (1:2000) and conjugate anti-mouse secondary antibody (1:10 000). Marker (Color Prestained protein standard), 3B-O (*E. coli*-produced antigen), negative control (crude *E. coli* extract containing empty pProEX-HTb vector). Black arrows indicating lower 13KDa protein band and blue arrow indicating upper ≈20KDa protein band.

antibody. However, in addition a second ≈20 KDa size protein band was seen (Figure 4.1 B, blue arrow). In both blots the negative controls showed no protein bands.

The *N. benthamiana* expressed 3B-O antigen was also run on a western blot and probed in the same way as the *E. coli* produced 3B-O. The *N. benthamiana*-expressed scFv only showed detection of the *N. benthamiana*-expressed 3B-O protein as extremely faint bands at the lower (13KDa) position (data not shown). Whereas, the anti-his probed blot again showed the upper and lower bands as previously seen for the 3B-O antigen.

Western blots with *N. benthamiana* or *E. coli*-produced 3B-O were probed with the *E. coli*-produced scFv; however, these showed no protein bands in either blot (data not shown). However, the positive anti-his blots again showed the upper and lower bands as seen before in Figure 4.1 B, indicating that the 3B-O antigen was present.

4.3.2 FUNCTIONALITY OF RECOMBINANT CRAB-FM27 IN I-ELISA

The detection of the *E. coli*-produced 3B-O by the *N. benthamiana*-expressed scFv was successful and was clearly demonstrated in a western blot (Figure 4.1). Detection of *N. benthamiana*-expressed 3B-O using the same scFv was not definitive as the bands seen on a western blot were extremely faint. The lack of detection of the 3B-O from both expression systems using the *E. coli*-expressed scFv on a western blot could be a result of the lack of sensitivity of the assay. The requirement of the 3B-O antigen to present conformationally appropriate epitopes for scFv binding, might be the reason for the limited detection observed. Therefore, an I-ELISA was performed using these samples as it is a more sensitive assay which allows for conformational dependence.

The 3B-O antigen produced from both *E. coli* and *N. benthamiana* was coated onto the wells of an ELISA plate at varying dilutions. It was assumed that due to there being a low concentration of antigen in the samples, that an undiluted, 1/2 dilution and a 1/10 dilution would allow for a better range for a signal to be observed. The scFv was added and allowed to bind before addition of the secondary anti-chicken conjugate. These readings were then compared to a background control, of either crude leaf extract or crude *E. coli* extract from empty vector samples, probed for by the scFv in the same way. It was though that due to the 3B-O antigen being partially purified and therefore, having a very low concentration of native plant or *E. coli* proteins in the sample, it was decided that a 1/100 dilution of crude negative extract was to be used as a background control as it might consist of native proteins at a similar concentration of that of the partially purified antigen. It was also thought that a high concentration of negative crude plant or *E. coli* extract would give too much background binding, and therefore could interfere with the absorbance reading. The scFv was coated alone in the well as a maximum absorbance control or as a positive control to show that the secondary antibody was detecting the scFv. This control would give a maximum absorbance in the assay and therefore, no other signal should exceed this and if so could be considered an outlier. The combinations of 3B-O antigen and scFv to be tested are the same as mentioned in the western blot testing. The I-ELISAs for each of these combinations were displayed graphically in Figure 4.2.

The first panel (Figure 4.2 A) shows the *N. benthamiana*-produced 3B-O antigen coated at different dilutions, and probed for using the *N. benthamiana*-produced scFv. The readings of the diluted 3B-O samples detected by the scFv were compared to the plant background control (1:100 crude leaf extract). A significant difference was only observed in the 1:2 dilution of the antigen, signifying that the undiluted and the 1:10 dilution samples were not suitable for detection. The last column indicates a maximum absorbance of the coated plant produced scFv, and indicates a significant difference to the background.

The same *N. benthamiana*-produced scFv was used to probe an ELISA plate coated with the *E. coli*-produced 3B-O antigen at different dilutions (Figure 4.2 B). The scFv was able to significantly detect the antigen at both an undiluted and 1:2 dilution, but with the latter having a higher reading. The higher reading observed in the 1:2 dilution is also seen in Figure 4.2A, further suggesting that this is the optimal dilution of the antigen. The 1:10 dilution gave a low reading similar to that of the background control. The maximum absorbance of the scFv again showed a significant difference to the background, as seen in the last column.

The *E. coli*-produced scFv was also used to probe for the *N. benthamiana*-produced 3B-O antigen (Figure 4.2 C) as well as the *E. coli*-produced 3B-O antigen (Figure 4.2 D). The readings for all the antigen dilutions were not significantly different to that of the background control. However, the maximum scFv reading, shown in the last column, is significantly different to the background, indicating that the scFv is present, even though detected at very low levels in comparison to the *N. benthamiana*-expressed scFv maximum absorbance positive controls in Figure 4.2 A and B.

A constant 1:3 dilution of the recombinant scFv from both *E. coli* and *N. benthamiana* was used in each of the ELISAs. It was suspected that the difference in activity between the differently expressed scFvs might be due to keeping the dilution of them the same and not using a constant concentration. To determine if the scFv from both *E. coli* and *N. benthamiana* varied in activity due to a concentration issue, a checker board titration was carried out. Whereby, varying dilutions of both expression systems of the 3B-O antigen were coated on an ELISA, and probed with varying concentrations of both expression systems of the scFv. It

was surmised that this would give an indication of the best ratio of antigen to scFv to be used. It was seen that the *N. benthamiana*-produced scFv was best used at a 1:3 dilution when detecting either plant or *E. coli* antigen at 107ng. The *E. coli*-produced scFv however did not give significant readings, even when an undiluted sample was used, in detecting either of the 3B-O antigens (not shown). These results coincided with those seen in the I-ELISA in Figure 4.2 and the western blots indicated that the *E. coli*-produced scFv is not effectively binding the antigen. Therefore the *N. benthamiana*-expressed CRAb-FM27 scFv was taken forward for the subsequent experiments.

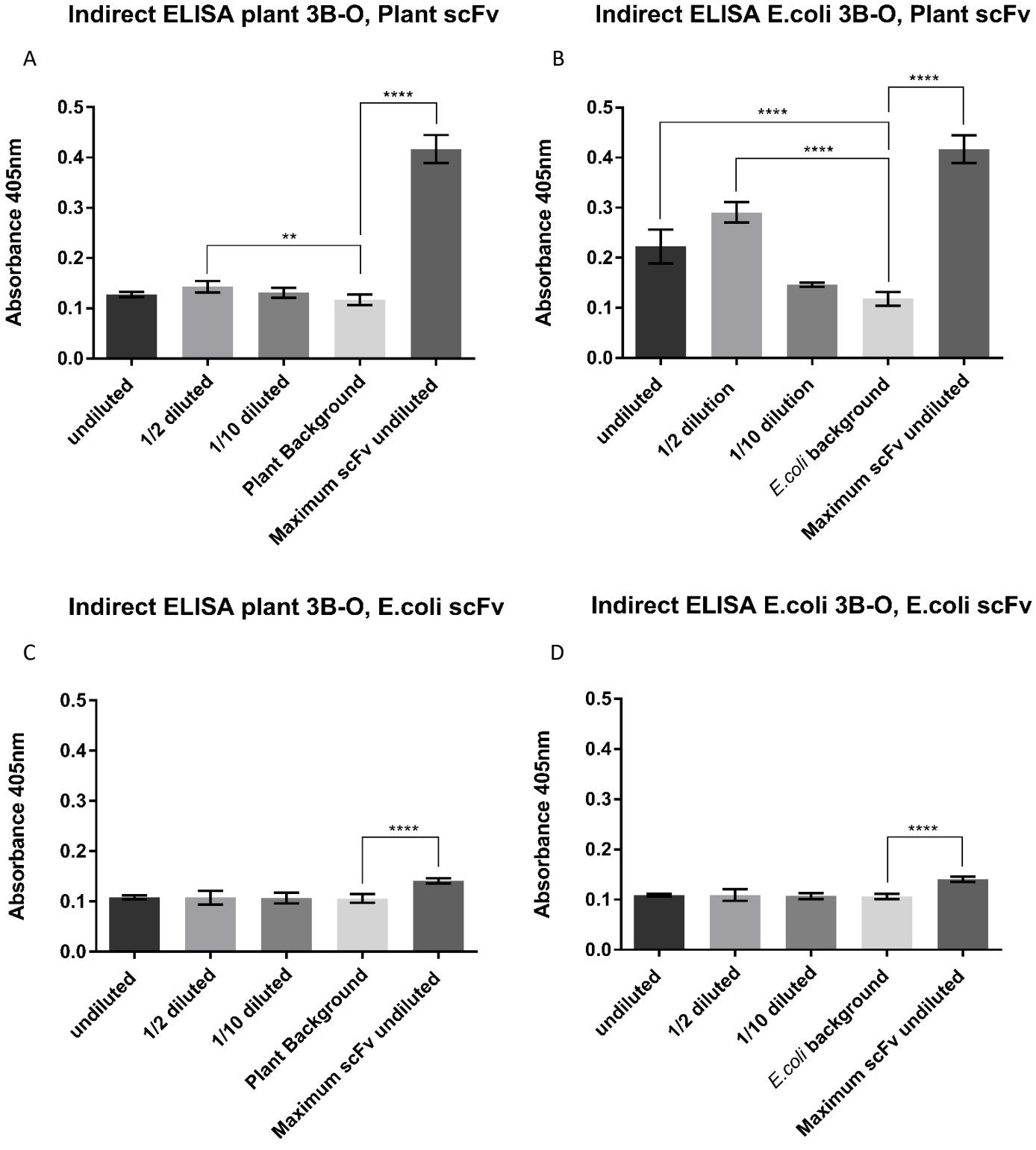

Figure 4.2. Indirect-ELISA of coated 3B-O antigen probed with CRAb-FM27 scFv.

(**A**) *N. benthamiana*-produced 3B-O coated and probed with *N. benthamiana*-produced scFv. (**B**) *E. coli*-produced 3B-O coated and probed with *N. benthamiana*-produced scFv. (**C**) *N. benthamiana*-produced 3B-O coated and probed with *E. coli*-produced scFv. (**D**) *E. coli*-produced 3B-O coated and probed with *E. coli*-produced scFv. Antigen coated as undiluted, 1:2 and 1:10 dilutions. Background controls included 1:100 dilutions of either *N. benthamiana* or *E. coli* empty vector crude extracts. Maximum scFv undiluted samples were coated and used as positive controls for presence of scFv. ScFv used at 1:3 dilution to probe for antigen and secondary anti-chicken alkaline phosphatase conjugate (1:5000) was used. The error bars indicate standard deviation with 95% confidence level and double asterisks (**) indicates P < 0.01 and quadruple asterisks (****) indicates P<0.0001.

4.3.3 I-ELISA TO TEST FOR THE PRESENCE OF ANTI-FMDV ANTIBODIES TOWARDS RECOMBINANT 3B-O.

It is vital that antibodies from infected animal serum can recognise recombinantly produced 3B-O antigen, for it to be considered a diagnostic candidate. Furthermore, for it to be considered as a possible DIVA diagnostic reagent, it would have to show that antibodies in animal serum from a vaccinated animal would not be able to be detected.

To test the functionality of *E. coli*-produced and *N. benthamiana*-produced 3B-O antigens, an I-ELISA was carried out to determine whether the recombinant antigens could detect antibodies in serum from 6 different samples of FMDV-infected animals. In addition, the recombinant antigens were tested to determine whether they could differentiate this serum from that of vaccinated animal serum.

Purified recombinant 3B-O antigen, either *N. benthamiana* or *E. coli*-produced, was coated into microtiter plate wells. The 6 different guinea pig serum samples containing antibodies representing 6 different FMDV serotypes and two subtypes (Table 4.1) were added to wells of the coated plates. Each row of the plate was designated one serum sample and each row consisted of a test sample (3B-O antigen from either *N. benthamiana* or *E. coli*), a background control (*E. coli* or *N. benthamiana* crude sample from an empty vector) and a positive control ($3AB_1$ sample from INTA). The ability of the 3B-O protein to distinguish infected and vaccinated serum is critical, and therefore to one of the rows serum from a vaccinated animal was added (Table 4.1). The statistical analysis of the data was done using an ordinary one-way ANOVA test, which did a multiple comparison between the test samples and the backgrounds.

Results from these experiments are shown in Figure 4.3. Absorbance values of background controls and test samples were compared and plotted next to each other for each serum sample tested. The *E. coli*-produced 3B-O tests showed to have significantly higher absorbance values to that of the background controls for each of the 6 infected sera tested (Figure 4.3A). Serum sample O1 showed a lower absorbance value than the other 5 samples tested. The *E. coli*-produced 3B-O showed similar absorbance values to that of the

background control for the C85 serum sample from a vaccinated animal, indicating that it did not bind to antibodies in the serum.

The *N. benthamiana*-produced 3B-O tests showed significantly higher absorbance in comparison to the background controls of 5 of the 6 infected serum samples (Figure 4.3 B). The O1 serum sample showed a higher absorbance value for the test when compared to the background control but was not significant. The C85 vaccinated animal serum showed similar absorbance values between the *N. benthamiana*-produced 3B-O test and the plant background control, suggesting that antibodies in the serum do not recognise the 3B-O protein.

For the *E. coli*-produced $3AB_1$ (Figure 4.3 C) there was a significant increase in the absorbance of the 6 serum samples between the $3AB_1$ test and *E. coli* background control. The O1 sample again had a lower reading than the other 5 samples but this was still significantly higher than the background. There was no significant difference in absorbance between the $3AB_1$ protein and the background control when testing the C85 vaccinated animal serum. These results confirm that infected and vaccinated serum was distinguishable with 95% confidence when using the recombinant *E. coli* and *N. benthamiana*-produced 3B-O antigens.

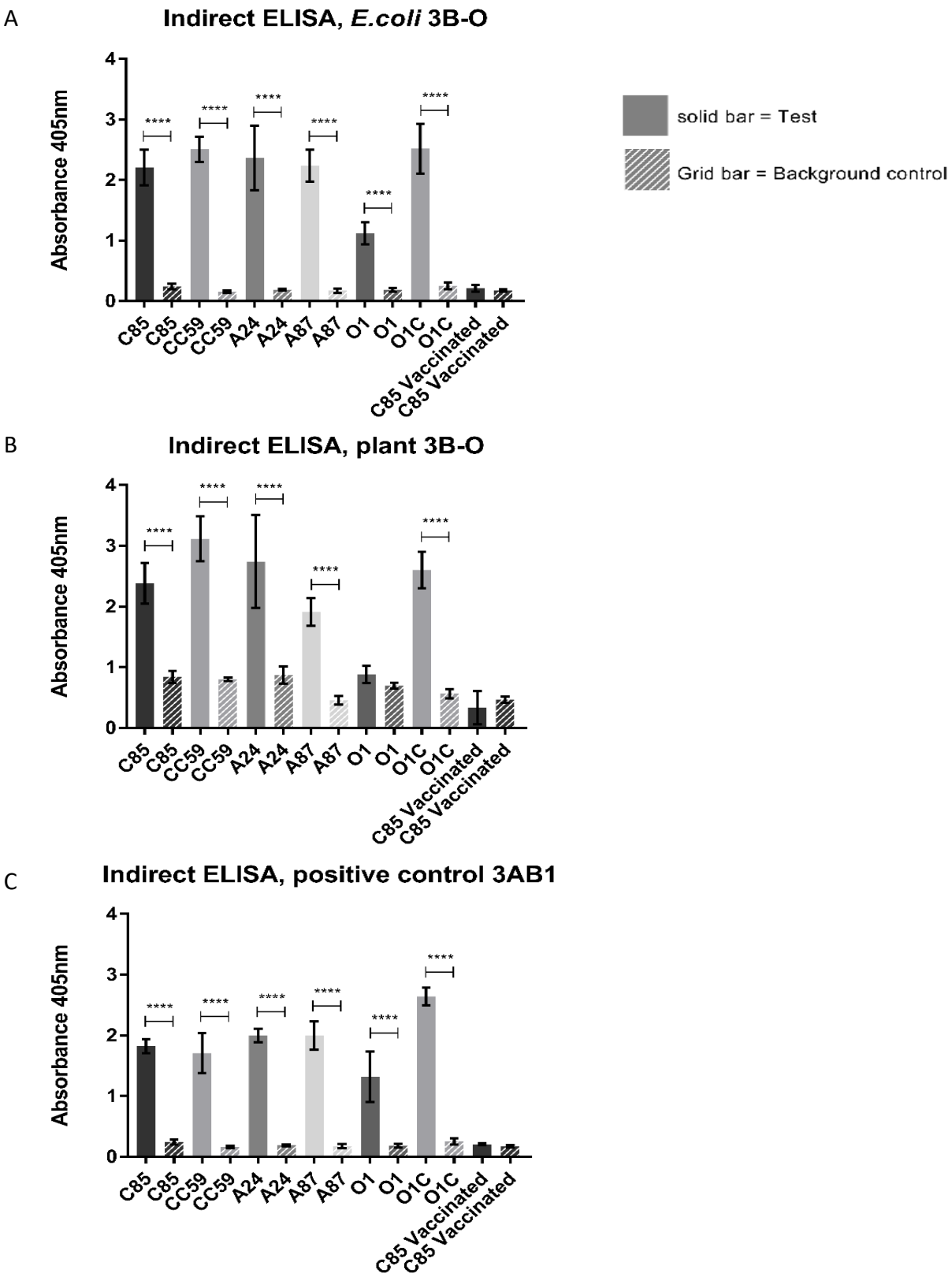

Figure 4.3. Indirect-ELISAs coated with recombinant antigen and probed with infected guinea pig serum.

(A) *E. coli*-produced 3B-O coated. (**B**) *N. benthamiana*-produced 3B-O coated. (**C**) $3AB_1$ protein coated as positive control. Solid bars indicate antigen being tested and gridded bar indicates background control, for each of the serum. Guinea pig serum used at 1:10 dilution and anti-Guinea pig alkaline phosphatase conjugate used at 1:10 000. The error bars indicate standard deviation with 95% confidence level and quadruple asterisks (****) indicate P<0.0001.

4.3.4 COMPETITIVE-ELISA TO TEST FUNCTIONALITY OF RECOMBINANT ANTIGEN AND SCFV IN THE PRESENCE OF FMDV ANTI-SERUM.

The scFv produced in *N. benthamiana* could bind the 3B-O antigen (section 4.3.2), and the 3B-O antigen produced in both *E. coli* and *N. benthamiana* was able to distinguish between serum from FMDV infected and vaccinated animals (section 4.3.3). With these results, an ELISA was carried out to determine whether these two reagents could be used together in a C-ELISA to differentiate between animal sera.

The 3B-O antigen from both *N. benthamiana* and *E. coli* was coated in the wells of an ELISA plate and different dilutions of serum from infected guinea pigs added. *N. benthamiana*-produced scFv was added to each of the wells at a final dilution of 1:3 and given a chance to compete with the antibodies for the binding of the 3B-O antigen, with further incubation. The *E. coli* crude samples containing the empty pProEX-HTb expression vector and *N. benthamiana* crude samples from leaves infiltrated with *Agrobacterium* containing plant expression vectors lacking the gene of interest, were again used as the background controls. *E. coli* and *N. benthamiana* –produced 3B-O antigen was coated as before, but with only the scFv added (without animal serum), to be used as a maximum absorbance control. The maximum absorbance reading was used to calculate the percentage of inhibition of the scFv in the test samples.

The results for both the *E. coli* and *N. benthamiana*-produced 3B-O C-ELISAs were inconclusive, as all the samples tested gave similar readings to the background control. The absorbance values were very low and similar across the different dilutions of the 6 infected animal sera, which was the same seen for the vaccinated animal serum. The expected results, which would have shown a successful C-ELISA test, would have shown an increase of absorbance as the dilutions of the infected animal serum was increased (i.e. decreasing concentration of antibodies). It would also have shown a consistent high absorbance reading across the vaccinated serum dilutions. The maximum absorbance control showed a similar low absorbance reading to that of the background and other test samples. This control was used as a positive control to show maximum binding of scFv to the antigen to determine the percentage of inhibition when comparing absorbance readings against the test sample

readings. Therefore, it should have given a very high absorbance reading. The C-ELISA for both *E. coli* and *N. benthamiana*-produced 3B-O using the *N. benthamiana*-produced scFv was repeated in triplicate but each experiment gave similar results (not shown).

4.4 DISCUSSION

Production and purification of both the 3B-O antigen and CRAb-FM27 scFv was successful in both *N. benthamiana* and *E. coli*. The objective of the project was to produce functional proteins which could be used in combination with each other to distinguish between serum from FMDV-infected and vaccinated animals in a C-ELISA.

In order for these proteins to be used for diagnostic purposes, they would each have to display specific characteristics. Firstly, the scFv would need to show specific antigen binding. This is of great importance, as the scFv must act as a competing antibody when used in a C-ELISA. The *E. coli* produced CRAb-FM27 scFv has been shown, by another research group, to bind the full length non-structural 3ABC polypeptide and more specifically the epitopes found on the 3B region (Foord *et al.*, 2007). I wanted to test whether the plant produced CRAb-FM27 scFv would behave similarly, and to determine if it could act as a possible diagnostic candidate. The initial testing of this interaction was done via a western blot, where the 3B-O antigen was probed using the recombinant scFv as an antibody, in order to give some idea of the binding capabilities of the scFv for the antigen.

The results indicated that *N. benthamiana*-produced scFv was capable of detecting the *E. coli*-produced 3B-O antigen as seen by a defined band on the western blot (Figure 4.1A). In addition, the *E. coli* 3B-O antigen was shown to be present and recognised by the anti-his antibody used as a positive control in a western blot (Figure 4.1 B). When comparing the western blots of the 3B-O protein either probed with the plant produced scFv or anti-his antibody, I saw the expected lower band (13KDa) in both, but only the higher band (≈20KDa) emerging in the anti-his blot. This double band was observed throughout the expression of 3B-O in both *E. coli* and *N. benthamiana* (see results section 2.3.4.1 and 2.3.4.2) when analysed using an anti-his western blot. The disappearance of the upper (≈20KDa) band was

observed when using the *N. benthamiana*-produced scFv to probe the blot, and it was only the lower 13KDa band that was detected (Figure 4.1 A). It was surmised that the scFv was only able to detect the lower band and not the upper band of the 3B-O protein as the smaller molecular weight form of the protein is conformationally correct to allow for binding of the scFv. It has been hypothesised that the double band seen in the western blot of the 3B-O protein is due to one or more of the many types of post-translational modifications (PTMs) that take place within the cell (discussed in Chapter 2). The N-acetylation of lysine residues in proteins takes place within both prokaryotic and eukaryotic systems (Ouidir, Kentache and Hardouin, 2016) and is thought to influence the expression of the 3B-O protein such that two different conformations results in a change in sizes (Guan *et al.*, 2010). The lower band (13KDa) is surmised to be the least post-translationally modified form of the protein as it is closest to the predicted size of the protein than the upper band (≈20KDa). This might explain why only the lower band is detected by the scFv, as the epitopes to which the scFv would bind, might be hidden by the extensive acetylation or other PTMs that are found in the larger (upper band) form of the protein.

The detection of the *N. benthamiana*-produced 3B-O protein using the same *N. benthamiana*-produced scFv was not as clear as in the *E. coli* western blot, as very faint protein bands were seen. The faint band seen was of the lower molecular weight protein. It has been hypothesised that due to the plant system (eukaryote) having an extensive secretary pathway, increased post-translational modification would occur in comparison to a bacterial system (prokaryote) (Dell *et al.*, 2010). It was also noted that the plant expressed 3B-O produced a darker, more dense upper band than the lower one (Figure 2.8), suggesting increased concentrations which is possibly due to the increase in PTMs taking place. This might explain why the lower band of the plant made 3B-O antigen, shows such a faint band when probed with the scFv on a western blot, as there is very low concentrations of the lower form being present.

Western blots probed with the *E. coli*-produced scFv did not show any binding to either the *E. coli* or *N. benthamiana*-produced 3B-O antigen. It is possible that the *E. coli*-produced scFv may therefore be non-functional in recognising the 3B-O antigen, as a result of less PTMs

taking place during *E. coli* expression (Frenzel, Hust and Schirrmann, 2013) compared to plant expression and hence, influencing and possibly reducing the activity of the scFv. This contradicts the evidence published by Foord et al. (2007), who showed that the *E. coli*-expressed CRAb-FM27 scFv was able to detect the *E. coli*-produced antigens 3B and 3ABC on a western blot.

Since results from the western blot were inconsistent with expectations, an ELISA was employed using combinations of these reagents. It was shown in an I-ELISA that the *N. benthamiana*-produced scFv could significantly detect the 3B-O antigen made from both *N. benthamiana* and *E. coli* (Figure 4.2 A&B respectively). However, the *E. coli* made scFv could not (Figure 4.3 C&D), which confirmed the non-functionality of the *E. coli*-produced scFv. Consequently, only the plant-produced scFv was employed for further work.

The second characteristic to determine suitability of the reagents for diagnostic use, was to verify whether the 3B-O antigen could be recognised by antibodies from infected animal serum, but not antibodies from vaccinated animal serum. It has previously been shown that FMDV 3B can be used instead of the full length 3ABC polyprotein as a coating reagent in an ELISA in order to successfully distinguish infected from vaccinated animal serum (Shen *et al.*, 1999; Gao *et al.*, 2012; Mohapatra *et al.*, 2014).

An indirect-ELISA was employed to distinguish infected and vaccinated guinea pig serum from three serotypes, each with two subtypes: serotype C (subtypes C85 and CC59), serotype A (subtype A24 and A87) and serotype O (subtype O1 and O1C) (Table 4.1). In order to show distinguishability of sera, serum from an animal sample vaccinated with FMDV C85 was used. Results showed that the 3B-O antigen produced in *E. coli* was detectable by antibodies from infected guinea pig serum for all serotypes and subtypes tested. The plant-produced 3B-O antigen showed similar results. Interestingly, the absorbance readings from each of the sera tested with plant-made 3B-O varied in comparison to the *E. coli* antigen tests as they were slightly higher, along with increased error bars. The difference in absorbance could be a result of antibody accessibility to the differently expressed antigens. It could also be due to higher titres of antibodies being present in the serum which recognise native plant proteins, which

have been co-purified with the plant-produced 3B-O, than there are antibodies towards the native *E. coli* proteins. There was a significant difference in absorbance readings between the plant background and plant produced 3B-O samples for each of the tested sera, except for the O1 subtype. This general trend of a lower absorbance reading for the O1 subtype sample seen in the plant made antigen test, the *E. coli* antigen test and as well as the positive control, could be due to the serum being old and therefore the antibodies losing binding activity (Correia, 2010). It could also mean that there was a lower titre of antibodies in that particular sample, which before gave a lower reading.

Overall, I have shown that the *N. benthamiana* and *E. coli*-produced 3B-O antigen is significantly recognised by antibodies in serum from FMDV-infected samples, but not by antibodies in the serum sample from a vaccinated animal. This indicates the potential of the 3B-O antigen, expressed from both systems, to be used as a diagnostic reagent for DIVA capacity. A limitation of this particular experiment is that there was only one vaccinated sample representing one subtype tested. This experiment should be repeated with more sera isolated from vaccinated as well as from healthy unvaccinated animals in order to calculate significance.

The *N. benthamiana* and *E. coli*-produced 3B-O was subsequently used in a C-ELISA, along with the *N. benthamiana*-produced CRAb-FM27 scFv to determine whether they were able to differentiate infected from vaccinated serum. The advantage of a C-ELISA is that only two common reagents are needed in order to test a range of samples that might come from different animal species. In this case, to determine which of the guinea pig serum has been infected or not, an ELISA plate is coated with the 3B-O antigen and the serum under investigation is added. To this, the plant made scFv would be added in order to compete with antibodies in the serum for the binding of the antigen. To determine which of the animals is infected, only a single secondary conjugate antibody (anti-chicken in this case) is needed, which would detect the scFv and determine the infectious status of the animal. This alleviates the need for specific or multiple secondary antibodies towards antibodies of the animal suspected to be infected (i.e. anti-guinea pig conjugate). The lack in scFv detection (i.e. low absorbance readings) would indicate an infected sample, as the scFv has been outcompeted

by antibodies, raised against live replicating virus. Whereas detection of the scFv (i.e. high absorbance readings) would indicate a lack of binding antibodies and that the animal has been vaccinated or is healthy.

C-ELISAs were conducted using either the plant or *E. coli* made 3B-O antigen as a coating antigen to select for specific NSP antibodies (i.e. those found in sera from infected animals), and plant-produced scFv as the competing antibody. Unfortunately, the results were inconclusive for both, as the infected serum samples gave very low absorbance readings in all three replicates (not shown). The low absorbance readings in the infected samples was to be expected; however, the vaccinated sample should have shown a high absorbance reading if the C-ELISA had worked, but had given similar readings to the infected samples. The positive control also showed low absorbance readings, when it should have shown maximum absorbance readings. The very low level of absorbance observed in the positive control suggests that there is an underlying problem with the interaction between the scFv and the 3B-O antigen, as the lack of raised absorbance indicates no or very little binding between these two reagents. This is contradictory to results obtained with the I-ELISAs which showed that the scFv was able to bind to 3B-O. The only differences between these experiments was 1) that the scFv showed binding to the antigen in the absence of the serum but a lack of binding in its presence. 2) The scFv used in the C-ELISA was purified from a new batch of plants, but processed in the same way. An explanation to why the C-ELISA was unsuccessful could be due to the scFv being inhibited by an unknown molecule found within the serum, or that the new batch of scFv was non-functional due to it not reacting to the antigen alone in the maximum absorbance control.

Optimisation of the C-ELISA could not be fully explored due to time constraints. There are still many avenues to be explored in order to certify these proteins as diagnostic reagents. Therefore, future work would include a complete repeat of antigen and scFv expression in the successful expression systems. Once sufficient quantities of each protein are obtained, testing using multiple experimental conditions could be done. There are many types of coating, blocking, and wash buffers for ELISAs that would need to be tested. There are a range of different ELISAs that could be tested to determine which might be most effective (e.g.

capture-ELISA to better expose the antigen). Titrations of the antigens and scFv to find the optimal working concentration of each, and a possible titration of the animal serum in order to determine the best dilution to be used in a C-ELISA could also be done.

CHAPTER 5

CONCLUSION

The diagnosis of FMDV disease employs three different approaches: these are clinical, virological and serological testing. The clinical approach relies largely on symptomatic inspection of the animals. This is often challenging as other viruses and other disease agents are known to cause similar or even identical symptoms. Consequently, by the time the animal has been identified as FMDV-infected by symptomatic criteria, the virus has probably already spread. Thus, clinical analysis is often combined with a more thorough or more specific approach of diagnosis. This includes virological and serological testing of animal tissue or fluids. The virological method of diagnosis includes virus isolation and/or direct detection of viral structural proteins or viral nucleic acid. This manner of diagnosis is usually expensive and time consuming and requires high levels of biosafety for live virus handling. Serological methods include identification of binding and/or antibodies in animal serum using both the structural (SP) and non-structural (NSP) viral proteins. These methods generally use ELISA-based techniques which can distinguish between different serotype infections using binding of antibodies from animal sera to structural proteins such as the VP1, or determine if the animal has been infected or vaccinated by use of non-structural proteins such as 3B or 3ABC polyproteins. The use of ELISAs, with either SPs or NSPs, is found to be the most inexpensive and generally the most rapid form of diagnosis.

A country or region that is considered FMDV-free without the use of vaccinations, would use SP-based ELISA testing in order to detect infection. Complications arise when vaccinations are used as a form of protection and for mitigation of outbreaks. Countries where FMDV is endemic often use prophylactic vaccines to protect high value animals, whereas countries that are FMDV-free often use the same vaccines for outbreak control. If SP-ELISAs are used for diagnosis in this case, vaccinated animals can be mis-identified as being infected (false

positives), resulting in unnecessary culling. In order to prevent false positives, NSP-ELISAs are employed which have the ability to distinguish between infected and vaccinated animals.

Although South Africa is currently classified as a FMDV-free zone, the Kruger National Park (KNP) is considered an infected zone within the country. To retain FMDV-free status, South Africa has isolated the KNP to prevent the spread of the virus from wild animals to livestock near its borders. Control zones have been implemented around the park in order to monitor the livestock in close proximity to the infected zone. Monitoring of livestock involves the utilisation of a range of diagnostics; however, due to vaccines being used to mitigate outbreaks, NSP-ELISAs are largely utilised. The continuous screening of large amounts of animal serum samples becomes expensive, and therefore the development of cheaper reagents to be used in these ELISA kits could reduce this financial burden, and potentially widen the scale of testing.

This project's focus was using transient plant expression for the production of FMDV diagnostic reagents. Plant expression has been shown to be an effective, safer and cheaper alternative to many other recombinant expression systems for the production of recombinant proteins. The use of a competitive-ELISA could reduce the cost of diagnostic testing as fewer reagents are needed in order to screen samples from multiple species. This work looked at the expression of plant-produced non-structural FMDV proteins and their functionality when used in combination with a plant-produced scFv antibody in a C-ELISA.

The NSPs investigated were the full length FMDV 3ABC polypeptide from both serotype O and serotype A of the virus. The 3AB polyprotein from serotype O was also investigated, along with the $3AB_1$ polyprotein from serotype A and the 3B region from serotype O. These proteins are highly conserved amongst FMDV serotypes, which makes them excellent candidates for a broad range diagnostic system that can distinguish infected from vaccinated animals, across a wide range of serotypes.

The expression of these NSPs was explored in a plant system as recombinant proteins produced in this manner are potentially easier and more cost effective to produce than using

the conventional bacterial or insect cells expression systems. Amongst the five variations of the non-structural polypeptides tested, the 3B-O protein was successfully expressed and purified from *N. benthamiana* leaves. To our knowledge this is the first time that a non-structural portion of the FMDV viral genome has been expressed in plants. In order to have a point of reference for comparison of plant expression, the 3B-O protein was also successfully expressed and purified in an *E. coli* system. Both *N. benthamiana* and E*. coli*-produced 3B-O proteins were then tested in an indirect-ELISA in order to determine if they could differentiate between antiserum from FMDV-infected and -vaccinated guinea pigs. Results indicated that the 3B-O proteins produced from both expression systems were able to distinguish between serum from vaccinated and infected animals; this was from guinea pigs infected with a range of FMDV serotypes – namely, A, O and C. These results confirm that recombinant 3B-O protein produced in plants has the potential to be a highly suitable diagnostic candidate for ELISA.

The second diagnostic reagent needed for C-ELISAs is a competing antibody that will bind the FMDV-NSP (3B-O) used to coat the ELISA plate. Foord et al. (2007) reported the identification of a single chain variable fragment (scFv), termed chicken recombinant antibody-Foot-and-Mouth 27 (CRAb-FM27), which recognised epitopes on the 3B region of the FMDV 3ABC polypeptide. They successfully expressed both the 3ABC antigen and the scFv in an *E. coli* system and showed that these two reagents worked in combination with each other in a C-ELISA to distinguish FMDV-infected serum from vaccinated serum.

In this project I tested the expression of the CRAb-FM27 scFv in a plant system to further reduce the cost of production. The scFv was both successfully expressed and purified from *N. benthamiana*. This is the first report of expression of this specific scFv (CRAb-FM27) in plants. The scFv was also concurrently successfully expressed and purified in an *E. coli* system, to serve as a control and a reference for future experiments. The functionality of the scFv was tested in order to see if it could bind its target protein. The plant-produced scFv showed binding activity towards both the *N. benthamiana* and *E. coli*-produced 3B-O protein in both a western blot and in an I-ELISA. However, the *E. coli*-produced scFv did not bind to the plant or the *E. coli*-expressed 3B-O protein, which is contrary to the findings of Foord et al. (2007).

The combined use of the two plant-produced reagents (3B-O and CRAb-FM27) was tested in a C-ELISA in order to determine if they could distinguish between sera from FMDV-infected and vaccinated guinea pigs. Despite the experiment being repeated three times, the C-ELISA was unsuccessful in that CRAb-FM27 did not bind to 3B-O. Furthermore, the positive control (CRAb-FM27 added to 3B-O with no test serum) was negative. This indicates that there is an underlying issue with the scFv not effectively binding the 3B-O protein, which is contrary to what was shown when testing the interaction between the scFv and 3B-O antigen in an I-ELISA. This disparity in results suggests that further work is needed in optimising C-ELISA conditions. Titrations for both the scFv and animal serum need to be optimised to improve the binding capabilities of the scFv to the antigen. The application of a capture-ELISA might be useful in presenting the antigen more effectively, and hence further exposing the epitopes for the scFv to bind. Unfortunately, these optimisation steps were not explored due to time constraints.

Future work could include additional experimentation of the NSP expression with different plant vectors (e.g. pEAQ-HT, deconstructed viral vectors). This would be to determine if the other NSPs (3ABC-O, 3AB-O and $3AB_1$-Arg), which were not successfully expressed using the pRIC3.0-HT or pTRAKc vectors, could be produced in plants. In addition, these NSPs could be targeted and retained within other plant subcellular compartments, for example the ER or chloroplast, in order to reduced proteolysis and increase protein levels. The expression of many recombinant proteins in plants is limited by host gene silencing. The use of a gene silencing suppressor, for example P19, could be explored to enhance the expression of the NSPs. Screening for an alternative antibody or scFv for antigen detection could be explored using phage display scFv libraries newly available in the BRU, where a range of candidates could be generated from a library using the plant-produced NSP for screening purposes. This would help possibly identify more sensitive and specific scFvs, which could be used in a C-ELISA, for the competitive binding towards the plant produced antigens. If other competing antibodies or scFvs were to be identified for binding to the NSPs, they could be recombinantly expressed along with an enzyme (e.g. alkaline phosphatase or horseradish peroxidase) fused to it – technology that is also available in the BRU. This could generate a competing reagent that would function independently without the need for a secondary antibody for detection. Subsequently, a secondary conjugated antibody would not be necessary to detect the primary

antibody or competing agent in the ELISA. Therefore, the recombinant competing antibody or scFv would be directly detectable using the appropriate substrate, further making this a more affordable method of diagnosis.

In conclusion, the individual reagents for a C-ELISA were successfully expressed in plants and showed appropriate functionality in an I-ELISA, although not in a C-ELISA. This provides proof of concept that both functional antigens and their cognate scFvs can be made in plants, thereby providing a more cost-effective platform for production of candidate diagnostic FMDV reagents.

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