

**Novel expression and production of
Foot-and-mouth disease virus
vaccine candidates in
*Nicotiana benthamiana***

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"A scientist in his laboratory is not a mere technician: he is also a child confronting natural phenomena that impress him as though they were fairy tales."

~ Marie Curie ~

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ABSTRACT

Foot-and-mouth disease, also known as FMD, is caused by the aphthovirus Foot-and-mouth disease virus (FMDV), which is a highly contagious disease of cloven-hoofed animals. It is endemic in Africa, parts of South America and southern Asia. In South Africa, the disease is controlled essentially through prophylactic vaccination. Current vaccines on the market are chemically inactivated virus strains. However, these are not considered ideal due to the possibly insufficient inactivation which could fail to render the virus harmless. Research on recombinant vaccines which obviate the need for high biosafety requirements for vaccine preparation has shown that recombinant FMDV virus-like particles (VLPs), devoid of viral genetic material, are an ideal vaccine candidate as they are as immunogenic as the virions themselves when administered to animals. These VLPs are formed by the assembly of the FMDV capsid proteins VP0, VP1 and VP3, which are generated upon the proteolytic cleavage of the capsid precursor protein P1-2A, by the FMDV 3C-protease.

The expression platforms used to co-express and produce the component capsid proteins and the protease are usually mammalian, insect or *E. coli* cells. The use of these expression systems requires extensive bioreactor infrastructure and sterile conditions for vaccine preparation which are costly. In addition, some studies have shown how the co-expression of the 3C-protease can prove to be deleterious when expressed at a high concentration in expression systems. In order to circumvent this, and encourage more efficient production of the capsid proteins and subsequent VLP assembly, researchers have shown that the levels of the 3C-protease can be down-regulated by introducing mutations in the 3C gene or a ribosomal frameshift in the gene sequence which subsequently reduce its deleterious effect.

Our laboratory has previously shown that similar FMDV VLPs can be assembled by the expression of FMDV P1-2A (referred to as oP1-2A, in this study), in the absence of the 3C-protease in the plant *Nicotiana benthamiana*, albeit in low amounts. This platform does not require high

biocontainment facilities for the production of recombinant proteins and VLPs and the process is easily scalable.

This study centers mainly on optimisation of the FMDV capsid protein expression in *N. benthamiana* in order to increase VLP yields. I first used codon-optimisation as an approach to improve expression of the capsid proteins and compared expression in the presence and absence of the 3C-protease, using mP1-2A-3C (a new codon-optimised construct), mP1-2A and oP1-2A. Electron microscopy (EM) showed that VLPs resulting from the expression of both mP1-2A-3C and mP1-2A were very low in yield, and irregular in shape and size compared to those produced using oP1-2A. The stability of the plant-produced VLPs was assessed by counting numbers of VLPs, when it was seen that expression of mP1-2A-3C compared to oP1-2A produced an average of 1 VLP per field of view versus 3 VLPs per view, for the same magnification. Furthermore, maturation trials at room temperature was performed on the oP1-2A VLPs, whereby a time-point between 30 to 45 minutes was considered ideal to produce stable VLPs.

It is also known that FMDV, unlike the other members of the *Picornaviridae* family, is acid and heat-labile. The second aim of this study was to promote the stability, and hence encourage the amount of the VLPs produced, by engineering acid and heat-resistant mutants, namely, VP1 N17D, VP2 H93C and VP1 N17D/VP4 S73N using site-directed mutagenesis. A fourth mutant, VP3 A118V which is acid sensitive was used as a control in downstream experiments. The mutants were subjected to a lower than normal pH and a higher than normal temperature. Expression of oP1-2A from the pH and heat assays was assessed to be better than its mutants. The optimum VLP count of 3 VLPs per field of view, was achieved from expression of oP1-2A, after treatment at pH 6.2, compared to 2 VLPs or 1 VLP per field of view for the other mutants tested under all the different conditions.

The final aim of this study was to test the immunogenicity of the VLPs from expression of oP1-2A in Balb/C mice. Due to the low yields of VLPs obtained from purification through a continuous gradient, a partial purification method was adopted. Two experimental groups of animals were either vaccinated with P1-2A VLPs or with adjuvanted P1-2A VLPs. A control group was administered with partially-purified plant extract, previously infiltrated with pEAQ-*HT*. The two

experimental groups elicited a marginal increase in humoral immune response at 41 days post vaccination (dpv), which increased significantly at 58 dpv.

To my knowledge, this is the first study showing that VLPs produced from expression of FMDV P1-2A only, in tobacco plants, can withstand otherwise degradative acidic and heat conditions. This characteristic has potential for extending the shelf-life of such a candidate vaccine. I also implemented maturation steps to further promote the stability of such VLPs. Finally, the partially purified VLPs showed that they stimulate a significant FMDV P1-2A-specific immune response, particularly in combination with the adjuvant Montanide suggesting that it has potential as a candidate FMDV vaccine.

ABBREVIATIONS

A	Alanine
Ad5	Adenovirus serotype 5
AEC	Animal Research Ethics Committee
ANOVA	One-way analysis of variance
BCIP	4-chloro-3-indoxyl-phosphate
bp	base pairs
BSA	Bovine Serum Albumin
BSL 2	Biosafety Level 2
C	Cysteine
CPMV	Cowpea mosaic virus
<i>cre</i>	cis-acting replicative element
CysPs	cysteine proteinases
D	Aspartic acid
DIVA	Differentiating Infected from Vaccinated Animals
DNA	deoxyribonucleic acid
dpi	days post infiltration
dpv	days post vaccination
EDTA	Ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EM	Electron Microscopy
FMD	Foot-and-mouth disease
FMDV	Foot-and-mouth disease virus
g	grams
GFP	green-fluorescent protein
H	Histidine
h	hour
HBcAg	hepatitis B virus core antigen gene
His	histidines
I-ELISA	Indirect ELISA
HS	heparan sulfate
iFMDV	inactivated virus
IPTG	isopropyl β -D-1-thiagalactopyranoside
IRES	internal ribosome entry site
kb	kilobase

kDa	kilo-Dalton
KNP	Kruger National Park
LB	Luria broth
M	Molar
MES	2-morpholinoethanesulfonic acid
mg	miligram
min	minutes
ml	mililitres
MM	Molecular Marker
N	asparagine
NBT	nitroblue tetrazolium
NFDM	non-fat dairy milk
nm	nanometres
NS	non-structural
NTE	Sodium chloride, Tris, EDTA buffer
OD	Optical Density
OIE	Office International des Epizooties
ORF	open reading frame
PBS	phosphate-buffered saline
PCR	Polymerase Chain Reaction
PKs	pseudoknots
pNPP	p-Nitrophenyl phosphate
PTGS	posttranscriptional gene silencing
RF	replicative form
RGD	Arg-Gly-Asp
RI	replicative intermediate
RNA	Ribonucleic acid
RT-PCR	RNA using reverse- transcription PCR
S	Serine
S	Svedberg units
SAT	South African Territories
SAVP	South African Veterinary Products
sec	seconds
siRNA	small interfering RNAs
TBS	Tris-Buffered Saline
TEM	Transmission Electron Microscopy
TSP	Total Soluble Protein
TST	TBS-Tween
UTR	Untranslated region
UV	ultraviolet

V

V

VLPs

VPg

VPs

WT

μg

μl

Valine

Volts

virus-like particles

virus protein, genome linked

Viral Proteins

wild-type

micrograms

Microliter

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CHAPTER 1

LITERATURE REVIEW

Foot-and-mouth disease vaccines: The past and the present

1.1. GENERAL INTRODUCTION

Foot-and-mouth disease (FMD), which is caused by the Foot-and-mouth disease virus (FMDV), is a highly contagious disease affecting domestic and wild-cloven hoofed animals. Both young and old adult animals are affected by the disease; in the former, the mortality rate is higher due to myocardial dysfunctions (Brooksby, 1982). The disease does not generally cause high mortality in adult animals but can be very debilitating, causing decreases in milk and meat production, while many countries face significant economic losses of domestically important livestock (Guo et al., 2013). Animals such as cattle, sheep, goat and buffaloes are carriers (thus asymptomatic) of FMDV and can harbour the virus for 2 to 3 years (Brooksby, 1982). FMDV is known to be endemic in African buffalo (*Syncerus caffer*) residing in the Kruger National Park (KNP) and in game parks in the vicinity. The magnitude of the disease impact depends on factors such as the time of detection of the incursion, the scale of the original infection, density of the livestock and herd, and the effectiveness of control measures (Gibbens et al., 2001; Tomassen et al., 2002). Nonetheless, outbreaks are sometimes sporadic: for instance, South America, Asian countries and the Middle East are still affected by incidences. FMDV is on the Office International des Epizooties (OIE) list of notifiable animal diseases, and they are authorized to recognize countries and geographical zones according to their FMDV-free status for trade purposes. These are categorized into those that are either free from FMD with vaccination, or free from FMDV without vaccination or suspended of the FMD free status without vaccination (Bruckner et al., 2002).

The mechanisms of FMDV transmission have long been scrutinized, and it is understood that the virus is spread mostly via the airborne route - either in the form of dust particles or as aerosols (Hyslop, 1973). Animals are also likely to be infected if they are exposed to lesions on the skin and from contact with mucous membranes of sick animals. However, this route of transmission

is less likely and requires about 10 000 times more virus. Virus is also secreted in the milk, semen and faeces, and the initial zones of viral replication are commonly located in the organs of the respiratory tract (Pega et al., 2013). The fact that the disease could be asymptomatic should not be disregarded: it has been shown that live FMDV can be isolated from esophageal-pharyngeal fluids of animals during convalescence; furthermore, African buffalo can be carriers for up to 3 years (Bruckner et al., 2002; Condy et al., 1985). As the virus disseminates to other areas of the body, the hallmarks of the disease such as vesicular lesions on the feet and on the mouth, are observed, followed by fever and smacking of the tongue (Alexandersen et al., 2002; Salt et al., 1996). Vaccinated animals are also prone to the acute disease if persistently exposed to the infectious virus leading to an active replication phase for up to 72 hours post-infection (Pega et al., 2013). Eventually, these acute phases often contribute to complications during disease outbreaks (Alexandersen et al., 2002).

Only countries which have demonstrated that animals are not showing any signs of viral infection are declared FMD-free (Rodriguez and Grubman, 2009), and this is established by the constant monitoring of animals. Usually, the 'hotspots' are developing countries which lack the adequate facilities to contain or to eradicate the disease. There are some methods which help to distinguish infected from naïve animals, such as the antigen capture enzyme-linked immunosorbent assay (ELISA) and virus nucleic acid detection. The latter is carried out by amplification of viral genome RNA using reverse- transcription PCR (RT-PCR). The presence of FMDV infection using this method should further be confirmed via inoculation of samples into sensitive cell cultures. Positive testing for infection should include implementing a strategy to control the outbreak within a feasible time-frame (Callens and De Clercq, 1997; Rweyemamu et al., 2008). There are also tests to differentiate infected from vaccinated animals (Differentiating Infected from Vaccinated Animals or DIVA) which is based on detection of antibodies to non-structural proteins (Bergmann et al., 1993). Vaccination, although it would not offer an enzootic country FMDV-free status, helps to control the spread of the virus and has long been developed for that purpose. Prophylactic FMD vaccines currently in use, help to differentiate between infected and vaccinated animals, as these are all inactivated virion preparations made using chemically-treated live virus, and consequently do not make non-capsid proteins in vaccinated hosts.

Candidate vaccines not yet on the market include subunit antigen, live attenuated virus, peptide, DNA, and empty capsid or virus-like particle vaccines. The inactivation of live virulent virus poses risks such as accidental release of the live virus, and therefore require high biocontainment facilities; live attenuated vaccines engender issues such as reversion to virulence; therefore, the other above-mentioned vaccines (peptide, recombinant protein and empty capsid vaccines) are preferred for future development (Lombard et al., 2007; Rodriguez and Grubman, 2009).

1.2. FMDV SEROTYPES AND GEOGRAPHICAL DISTRIBUTION

Seven serological FMDV serotypes (O, A, C, Asia 1, also referred to as the Euroasiatic types and South African Territories [SAT] 1, 2 and 3) have been identified to date; within each serotype there are several subtypes and variants. These serotypes can be distinguished by the variability of their respective VP1 sequences, and have been assigned their names depending on which country the first outbreak occurred (Jamal et al., 2011). Asian and north African countries are mostly affected by serotype O, while South America harbors serotypes O, A and C. Type Asia 1 is confined within the south Asian countries and the rest of Africa is mostly affected by SAT 1, 2 and 3 (Figure 1.1) (Bachrach et al., 1975; Pattnaik et al., 2012).

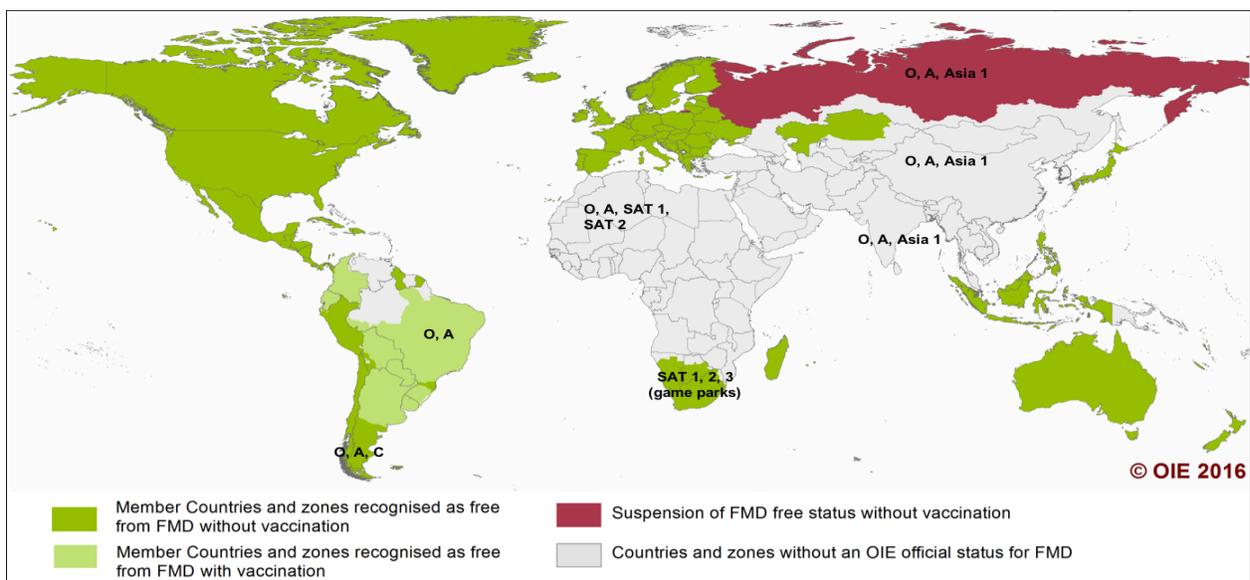


Figure 1.1: World-wide distribution of FMD as stipulated by the OIE 2016. Map taken and adapted from http://www.oie.int/fileadmin/Home/js/js2/images/eng/FMD_world_ENG.png.

Countries exporting animal products usually aim at achieving the FMD-free status, as the effect of the disease often impacts heavily on the economy, with the slowing down of the trade of livestock and meat products. The OIE is an international committee which assigns countries the FMD-free status based on certain stringent rules. The FMD committee and other Epizootics Commission meet twice a year, and after evaluation of certain submitted documentation and other information collected from a delegate, it is then proposed that a member country be recognised as free of the disease. The OIE member countries are also given a 60 day consultation period (Bruckner et al., 2002; Vosloo et al., 2002).

Western European countries have implemented vaccination programs which resulted in the cessation of disease outbreaks after the 1990s. South America followed in those footsteps, and together with animal culling prevented the spread of the ailment and by the end of the 20th century, FMD was brought under control in many countries on the African and Asian continents; Australia has always been spared from FMDV infections since the very beginning (Correa Melo et al., 2002; Rweyemamu et al., 2008).

South Africa lost its FMD-free status in 2000 when a piggery in KwaZulu-Natal was diagnosed with a case of serotype O, while a few months later FMDV SAT 1 was isolated from a feedlot within the vicinity of the KNP whose fence separating it from the nearby game park was damaged by floods. Currently, the buffalo populations in the park and cattle just outside harbour all three of the SAT serotypes (Figure 1.1) (Bruckner et al., 2002). As a result of proper containment and vaccination programs, North American countries and those in the Middle East recently acquired the FMD-free status by the OIE without vaccination (Figure 1.1).

1.3. CLASSIFICATION AND STRUCTURE OF FMDV

FMDV is a prototypic member of the genus *Aphthovirus* within the family *Picornaviridae* (Rueckert and Wimmer, 1984). The acronym 'aphtho' is a derivative of a Greek word referring to lesions on the mouth which pertains to the symptoms of the FMD (Thomson et al., 2003). While the equine rhinitis A virus is the only other virus in this genus, FMDV is structurally similar to polioviruses, possibly the best-known members of the same family (Li et al., 1996).

1.3.1. Genomic structure of the virus

The FMDV genome is a plus-sense, single-stranded RNA molecule of approximately 8500 bases, which encodes both structural and non-structural proteins (Rueckert and Wimmer, 1984). Figure 1.2 shows a schematic diagram of the viral RNA: this is translated as a long single open reading frame (ORF) into a polyprotein. This polyprotein is in turn post-translationally cleaved by the FMDV-encoded proteases which autocatalytically cleave themselves from the polyprotein. As a result, both intermediate, and mature structural and non-structural (NS) proteins are produced (Robertson et al., 1985; Rueckert and Wimmer, 1984).

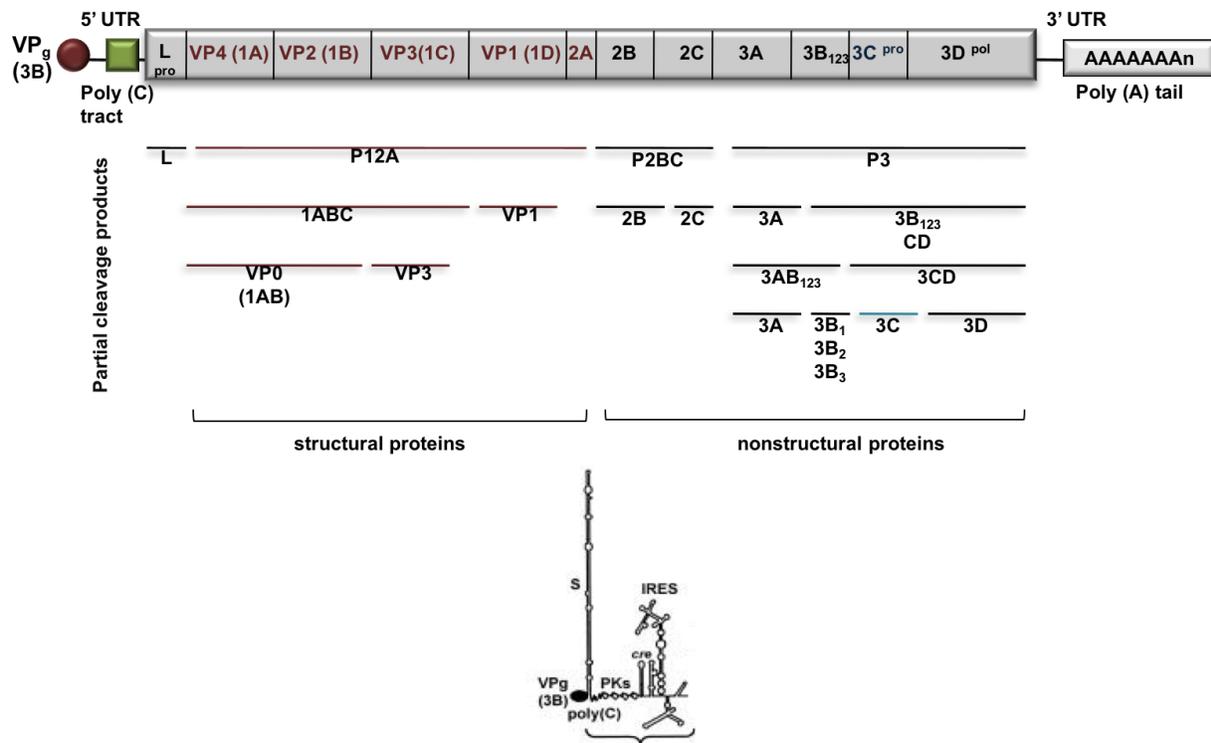


Figure 1.2: Schematic map of the genomic structure of the FMDV. The ORF is represented in the boxed area with the viral proteins labeled per the nomenclature set by Rueckert and Wimmer (1984). An expanded view of the 5' UTR is presented under the ORF. Adapted from Grubman and Baxt (2004).

Based on initial cleavage products, the ORF is distinguished as having four regions, namely L, P1, P2 and P3 (Strebel and Beck, 1986). The genome itself is covalently linked to a protein called 3B (VP_g [virus protein, genome linked] for designation of the protein) (Grubman, 1980). Upstream of the 5'-UTR, an S element of 360 bases folds into a stem-loop structure. Its main function is to

maintain genome stability in infected cells (Costa Giomi et al., 1984). The poly(C) tract of 100 bases, consisting of 90% C residues, is located downstream of the S-segment (Escarmis et al., 1992). The function of the poly(C) tract is discussed in the description of the infectious cycle in section 1.4. Following this component, there is a series of RNA pseudoknots (PKs) whose function has not yet been established. Located further 3' of these pseudoknots is a short hairpin-loop structure which has been identified as a cis-acting replicative element (*cre*) that is vital for RNA genome replication (Mason et al., 2002). A highly conserved 450 base stem-loop structure, the internal ribosome entry site (IRES), is located between the *cre* and the ORF (Belsham and Brangwyn, 1990). This is the entry site for the ribosomes to enter the genome during translation, which is further discussed in the infectious cycle section 1.4 (Jang et al., 1988). In juxtaposition to the 5'-UTR, the L-protein ORF, also known as the 'Leader' protein, encodes a papain-like protease which is autocatalytically cleaved at its C-terminus. It is referred to as L and not P0 to avoid confusion with the naming of the coat protein VP0. The protease plays a major role in limiting host protein synthesis and contributes to virulence (Piccone et al., 1995; Strebel and Beck, 1986).

Downstream of the L region is the P1, which encodes the capsid polyprotein precursor containing the proteins VP0, VP1 and VP3. This is followed by P2, encoding the NS proteins 2A, 2B and 2C. Although 2A forms part of the P2 region, it is cleaved as a P1-2A precursor. It acts as a protease whose mode of action relies on auto-proteolytic cleavage and participates indirectly in the maturation cleavage of the capsid proteins. Following the P2 region, the P3 region encodes the NS proteins 3A, 3B, 3C and 3D. The 3C component is known as a protease (Klump et al., 1984) which cleaves P1-2A polyprotein into the VPs. Its mode of action depends on its highly conserved active site consisting of the triad Cys163-His46-Asp84, and it cleaves proteins at the Gln-Gly, Glu-Gly or Glu-Ser sites (Palmenberg, 1990; Robertson et al., 1985). The 3D NS acts as the viral RNA-dependent RNA polymerase (Newman et al., 1994). The functions of these proteins will further be discussed in section 1.4.

The stop codon of the polyprotein ORF is followed by another UTR, which also contains elements that contribute to viral replication. At the extremity, the poly (A) tract of variable length plays a

role in the FMDV RNA replication as discussed in the infectious cycle section 1.4 (Melchers et al., 1997; Pilipenko et al., 1996).

1.3.2. Structure of the virus

Picornaviruses have a characteristic isometric particle encapsidating the viral genome. Electron microscopy shows that the FMD virion has a smooth outer surface and the virion has a diameter of 30 nm. From crystallography studies, the structure of the virion is distinct in that it resembles a ball of pentamers. The outer surface of the virion consists of 60 copies of a protomer: every protomer consists of one copy of each of the viral capsid proteins VP1, VP2, VP3, and VP4 which is located internally within the virion structure. Thus, VP1, VP2 and VP3 confer the antigenic properties of the virion. VPs 1 to 3 associate together into an eight-stranded wedge-shaped barrel (Jackson et al., 2003) and these strands are connected by loops to form the outer coat of the virion (Figures 3A and 3B). Five protomers (Figure 1.3B) form a pentamer (Figure 1.3C), 12 copies of which assemble to form a complete virion. Together with the encapsidated RNA, the virion has a sedimentation rate of 140 Svedberg units (S). The different intermediates of assembly and disassembly of the virus or the particle have different sedimentation coefficients, with the protomer and pentamer having coefficients of 5S and 12S, respectively as illustrated by Figure 1.3 (Sobrino et al., 2001).

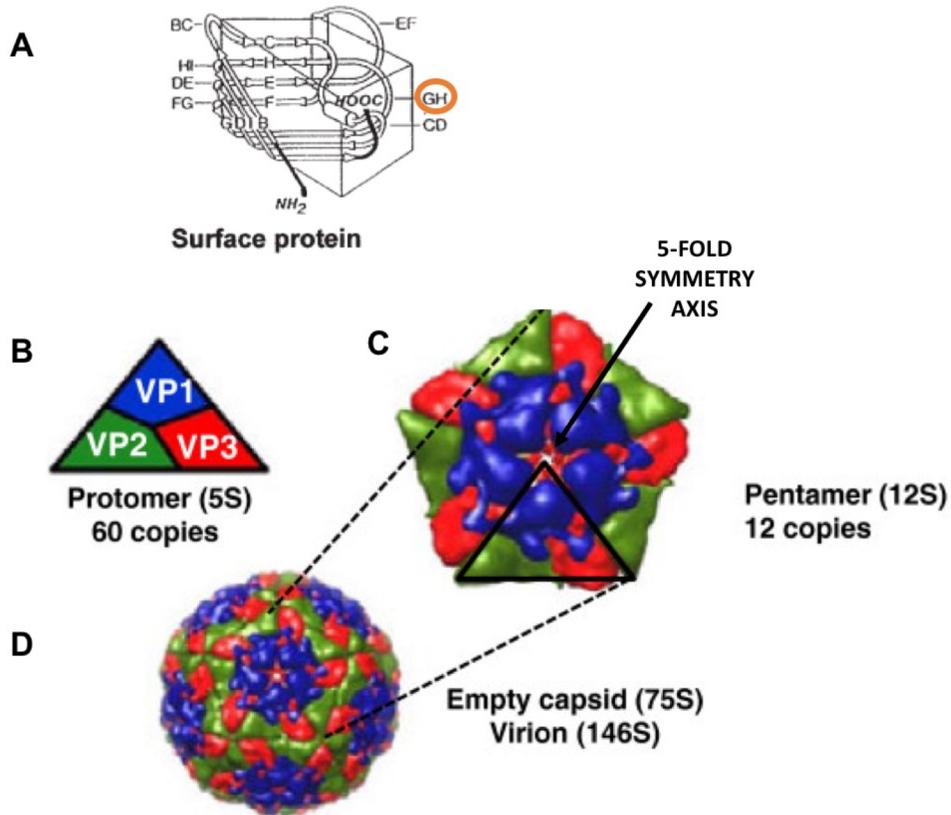


Figure 1.3: Schematic image of the structure of the FMDV capsid proteins and the intermediates of assembly and disassembly. (A) The tertiary structure of VP1, VP2 and VP3. (B) The arrangement of VP1, VP2 and VP3 as a protomer. (C) The arrangement of five protomers into a pentamer. (D) Structure of an empty capsid/ virion. Adapted from Sobrino et al. (2001) and Jamal and Belsham (2013).

The FMD virion is acid and heat-labile compared to the other Picornaviruses. Its capsid dissociates into pentamers at pH values below 6.5 (Caridi et al., 2015; Carrillo et al., 1985; Vazquez-Calvo et al., 2012). The acid sensitivity is thought to be related to the mechanism of viral uncoating and genome penetration from the endosomes (Caridi et al., 2015). Disassembly of the virion is promoted by the protonation of the histidines (His) residues at the interpentameric interfaces (Martín-Acebes et al., 2010). The poor thermostability of the FMDV particles is also correlated to these His residues where there are electrostatic repulsions between the pentamers which mediates sensitivity of the virion to thermal dissociation (Mateo et al., 2008).

1.4. INFECTIOUS CYCLE OF THE VIRUS

The FMDV infectious cycle is similar to the other members of the *Picornaviridae* family and is relatively short, as illustrated in Figure 1.4 (De Jesus, 2007; Rueckert and Wimmer, 1984). The virus is known to be cytocidal and infected cells usually show cytopathic effects (Alexandersen et al., 2003; Arzt et al., 2010).

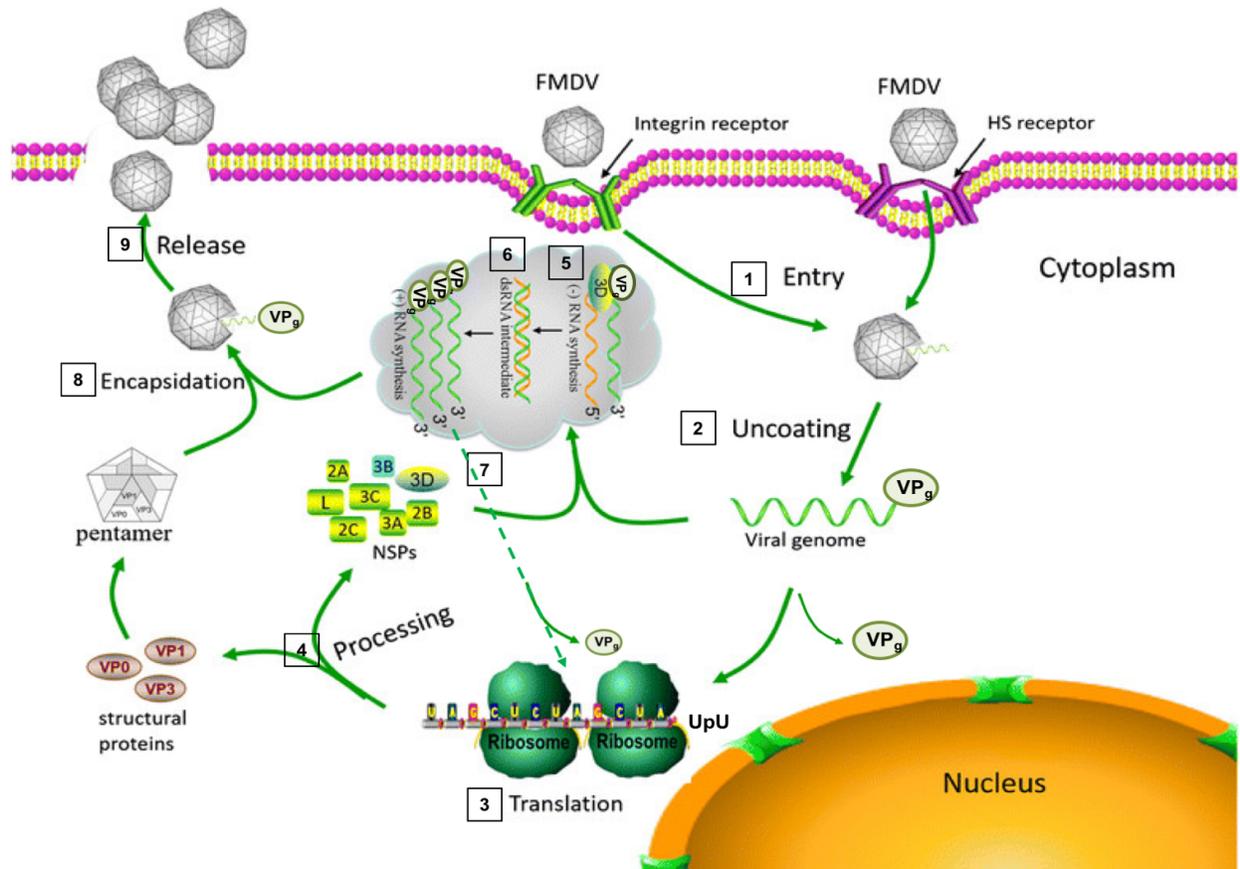


Figure 1.4: The infectious cycle of FMDV. HS: Heparan sulfate. NSPs: Non-Structural Proteins. VP_g: virus protein, genome-linked. Adapted from Gao et al. (2016).

1.4.1. Adsorption and uncoating

Infection starts in the epithelial cells of the mouth and in the tongue. The early interactions of FMDV with the host cell start at the surface membranes, with entry via receptor-mediated (integrin and heparan sulfate [HS] receptors) endocytosis (Figure 1.4, step 1). Though many receptors are involved, Robertson and his co-workers demonstrated that G-H loops (Figure 1.3A)

of the Arg144 in the VP1 of the FMDV capsid are involved in docking the virus to a host's membrane, and a series of events follow involving biochemical alterations and redistribution of internal cellular membranes (Mason et al., 1994; Robertson et al., 1985). Furthermore, studies conducted by Pierschbacher and Ruoslahti, earlier in 1984, showed there was a highly-conserved tripeptide sequence Arg-Gly-Asp (RGD) in VP1. This sequence is responsible for cell recognition and binding, similar to what is found for fibronectin receptors (Pierschbacher and Ruoslahti, 1984). Through the adsorption process, structural alterations in the virus capsid result in the virus particle injecting its RNA into the cell (Figure 1.4, step 2), and an uncoating process occurs where the 140S virion breaks down into 12S pentamers (Baxt, 1987; Baxt and Bachrach, 1982).

1.4.2. Viral translation

Following adsorption and internalization of the viral genome is the onset of translation. The genome-linked protein VP_g is first cleaved off by cellular enzymes before the translation of incoming RNA, and then associates with ribosomes in the host cell and participates in a translation initiation complex (Figure 1.4, step 3) (Ambros and Baltimore, 1980; Ambros et al., 1978). Protein synthesis begins from the IRES located upstream of the ORF. The poly(C) found in the 5'UTR (Figure 1.2) contributes to translation and binding of the genome to the initiation complex. The 3'UTR is known to also play a role in this vital process as some studies show that the abolishment of the poly (A) tail lowers translation efficiency *in vitro* (López de Quinto et al., 2002). As a result, the genome is translated into a long single polypeptide which is co- and post-translationally cleaved by the FMDV proteases (Figure 1.4, step 4) as mentioned in the genomic structure section 1.3.1. The occurrence of autocatalytic cleavage of 2A from the P2 polypeptide is quite debatable, as it has been argued many a time that the process relies on a translational machinery modification (Donnelly et al., 2001). This in turn allows the P1-2A complex to be released from the ribosome, and other downstream maturation of proteins occurs mainly from the action of the 3C-protease on the precursor polypeptides (Palmenberg, 1990) .

1.4.3. Viral transcription and replication

The processes of transcription and replication are as intricate as that of translation. For replication to occur, the synthesis of a minus-strand RNA in the smooth endoplasmic reticulum

is necessary (Figure 1.4, step 5): this will then act as a template for further synthesis (Gamarnik and Andino, 1998). The replication machinery (Figure 1.4, step 6) is quite complex and has still not been fully elucidated. It is thought that translation of the plus strand should be completed before the synthesis of the minus-strand begins. It is also observed that the 3CD complex breaks down into the 3C^{pro} and 3D^{pol} components during replication. In picornavirus-infected cells, the 5'-ends of both the plus and minus-strand are attached to VPgs, and a small protein-linked dinucleotide UpU acts as a primer for the RNA polymerase. In this way, the polymerase can distinguish between viral RNA and cellular RNA in order to proceed with translation of the cellular RNA to produce the plus-strand viral RNA. The nascent strands elongate (Figure 1.4, step 5), resulting in double-stranded molecules - namely, the replicative form (Osorio et al.) (Agol et al., 1999) - after which a new plus-strand can now be synthesized. For the process to occur, the RF has to unwind. FMDV 2C has helicase properties which are suggested to participate in the unwinding mechanism (Dmitrieva et al., 1991; Gorbalenya et al., 1989). The 3D^{pol} takes over during elongation and makes use of the replicative intermediate (RI) to give rise to new plus-strand viral RNA (Figure 1.4, step 6). As the new RNAs are made, some will either form complexes with the ribosomes to produce more viral RNA, where the VPg is cleaved off (Figure 1.4, step 7), or will form the genome of new virus particles to form the capsid, where the VPg remains covalently-linked to the genomic RNA (Figure 1.4, step 8) (Goodfellow, 2011).

1.4.4. Encapsidation of viral RNA and maturation

The replication cycle culminates in the encapsidation of the plus-strand FMDV RNA (Figure 1.4, step 8), upon which there is maturation cleavage of VP0 to VP2 and VP4, giving rise to the mature virion (Arnold et al., 2008; Hogle et al., 1985; Jacobson and Baltimore, 1968). As described in the structure section 1.3.2, the capsid precursor is cleaved by 3C^{pro} to yield the capsid proteins which assemble resulting in the formation of the virion (Newman et al., 1994). Ultimately, cell lysis occurs, and there is release of the new viral progeny particles (Figure 1.4, step 9) (Traylen et al., 2011).

1.5. VACCINES AGAINST FMDV

Control of FMD has been quite a challenge for the last hundred years, and the manufacture and use of vaccines has long been a priority. Vaccine development, however, also brings other challenges, with the mutation rates of the virus and the number of subtypes and topotypes on the different continents which did not make it easy to derive safe and stable vaccines against each serotype. Countries which are disease-free have adopted a culling strategy supplemented with other sanitary measures, and have furthermore implemented rapid vaccination in their mitigation regime to limit the spread of the infection (Correa Melo et al., 2002; Paton and Taylor, 2011).

1.5.1. Inactivated vaccines

Currently, inactivated whole-virus vaccines are commercially available to address FMDV outbreaks. The origins of this vaccine date back to the early nineteen forties, in Germany, where Waldmann and his coworkers discovered that virus isolated from cattle could be inactivated using formaldehyde. This inactivated form when later injected in cattle would result in FMDV immunity in the animals. Eventually, it was shown that the inactivated virus adsorbed onto aluminium hydroxide could potentially cause disease, even though it was conferring some protection when injected. They then first adsorbed the virus, and then used formaldehyde to inactivate it (Waldmann et al., 1941). The method used for vaccine production was cattle being injected in the dorsal epithelium of the tongues to produce the live virus. Vaccinated animals were culled and the tongues were processed to produce 200 doses of vaccine per animal. This strategy indeed helped to reduce the outbreaks in Europe but the production was lengthy, and tedious. Therefore, Waldmann's vaccine became a stepping stone to develop the same vaccine, but this time *in vitro*, with Frenkel and his researchers finding that the virus would replicate in bovine tongue epithelium in 1950. The epithelium would be collected from the slaughterhouse and incubated with a particular strain of virus (Barteling and Vreeswijk, 1991; Frenkel, 1951; Lombard et al., 2007). Countries such as the Netherlands and France quickly adopted the FMDV inactivated virus (iFMDV) vaccination strategy; finally, it was discontinued in 1991 in Europe where there was no need for vaccination anymore, as the European countries were disease-free. The inactivated vaccines are still in use nowadays and recently, Quattrocchi and his coworkers

showed how vaccine formulated with Montanide adjuvant provided early protection in cattle. In this case, the archaic aluminium hydroxide was replaced by an oil-based adjuvant which is still currently used (Mohan et al., 2013). It was demonstrated that the iFMDV provided 100% protection against viral challenge at 4 and 7 days post-vaccination (dpv), which required only a single dose of the iFMDV with the adjuvant (Quattrocchi et al., 2014). Other forms of production include virus production in cell culture, such as in baby hamster kidney cells (BHK-21), which is inactivated by binary ethyleneimine. However, there are disadvantages to using inactivated vaccines which include the high cost of cell culture, insufficient inactivation, and the risk of release of the wild-type virulent virus from production facilities.

1.5.2. Live attenuated vaccines

In the early nineteen sixties, researchers in countries such as Britain, France, Germany and Uruguay became heavily engaged in the development of an attenuated FMD vaccine (Sutmoller et al., 2003). This was regarded as a desirable alternative to the inactivated vaccines, whose quality would often be undesirable due to poor quality control. In parallel, the attenuated polio vaccine was successfully produced to combat poliomyelitis, and developing countries could easily produce this in their laboratories as it was cheaper and safer to make than the inactivated one. The common method for production of attenuated vaccines at that time involved the serial passage of the virus in foreign hosts such as baby rabbits (Cunha et al., 1964), embryonated chicken eggs or one-day old chicks (Gillepsie, 1954). The 'adapted' virus would then be tested in cattle and further tested on a larger scale when pathogenicity was reduced and the cattle survived after viral challenge (Polacino et al., 1985). Sutmoller and his researchers at that time, after vaccination of some 5000 cattle, noticed that vaccinated animals would often remain asymptomatic; however, there was a high mortality of calves which were vaccinated with rabbit-adapted attenuated FMDV. After that, countries which usually imported meat from countries using these vaccines were reluctant to do so anymore, and unsurprisingly, these vaccines were no longer used (Sutmoller et al., 2003).

The technique behind the live attenuated vaccine design was improved by genetic engineering (Doel, 2003). Nevertheless, the live vaccine helped to eradicate the disease in many parts of Europe. Nonetheless although they proved to be very good elicitors of the immune response,

there was always the possibility that infected animals which have a weak immune system might not respond favourably to the vaccine doses. Furthermore, live attenuated vaccine had to be continually kept on ice especially if it had to be shipped over long distances and may have perished upon arrival at its destination. Another shortcoming of this particular type of vaccine, is that there is a lack of ability to discriminate between naturally infected and vaccinated animals (Doel, 2003; Lombard et al., 2007; Sobrino et al., 2001).

1.5.3. Peptide vaccines

The antigenic properties of FMDV virions (refer to structure of the virus section 1.3.2) have been analysed extensively, and this allowed another class of candidate FMD vaccines in the form of synthetic peptides. These were developed to circumvent the issues faced with vaccines made up of the whole virus (Grubman and Baxt, 2004). There has been engineering of peptides which correspond to B cell epitopes in the G-H loops of the viral capsid (Sobrino et al., 2001). DiMarchi et al. (1986) showed that cattle were protected when they were immunized with a peptide encoding the G-H loop and the C-terminal (residues 200-213) of VP1. Francis and his group in 1990 demonstrated that synthetic peptides elicited neutralizing antibodies to all the seven known serotypes of FMDV in guinea-pigs (Francis et al., 1990) while Pfaff and coworkers also demonstrated similar results much earlier using mice as an animal model (Pfaff et al., 1982). Taboga et al., (1996) tested four different types of peptides derived from FMDV C3 Argentina 85 type which included site A: the G-H loop of VP1, AT: where a T-cell epitope was added to site A, AC: site A combined with the C-terminal of VP1 and ACT: where the former mentioned motifs were represented collinearly. Large-scale vaccination of 138 cattle was carried out. Unfortunately, it was concluded that none of the peptides afforded protection above 40%, with protection showing limited correlation with serum neutralization activity. It was also noticed that there was an escape by the challenge virus to another mutant form *in vivo* (Taboga et al., 1997). More recently, however, it has been shown how a dendrimeric peptide vaccine raised titres of FMDV-neutralizing antibodies as well as activating an FMDV-specific T cell response in pigs (Cubillos et al., 2008). There are many drawbacks associated with this strategy to provide a suitable vaccine to combat FMDV, such as the limited number of antigenic sites which are recognized by the immune system, the disperse antigenic regions on VP1 or the quasispecies

nature of FMDV (Domingo et al., 1990). As a result, there is the generation of many antigenic variants which gives rise to outbreaks in already vaccinated animals. Therefore, there is a need to provide peptide vaccines which would provide complete protection (Zhang et al., 2011), and no such vaccines are commercially available.

1.5.4. Recombinant DNA and protein vaccines

Recombinant proteins of FMDV have also been extensively studied, and have also been investigated as candidate FMDV vaccines. An effective immune response is achieved either by having a series of epitopes on a single polypeptide chain or by having B and T cell polypeptide proteins (McCullough et al., 1992). Bae et al., in 2009, used the 3D sequence which is highly conserved and immunogenic, along with VP1 which has both B and T cell epitopes. The genes were codon-optimised and cloned into mammalian cell expression vectors. Downstream experiments demonstrated that BALB/c mice co-immunized with the 3D and VP1 DNA or proteins showed humoral and cellular immune responses (Bae et al., 2009). Moreover, in 2011, Andrianova et al. produced a recombinant protein comprising of viral epitopes. They used the B-cell epitopes of proteins VP1 and VP4 and the T-cell epitopes of proteins 2C and 3D. The polypeptide was expressed in both bacterial cells (*E. coli*) and in tobacco plants (*Nicotiana benthamiana*) using a phytovirus expression system. The proteins were purified and an efficient immune response was recorded from inoculated guinea-pigs (Andrianova et al., 2011). From the success of these two studies, vaccines in the form of recombinant polypeptide viral proteins could therefore be implemented on the market to control FMD.

1.5.5. Live vector vaccines

Live vector vaccines became the next promising FMD vaccine technology developed, using a replication-defective human adenovirus serotype 5 (Ad5) which encodes FMDV transgenes (Sanz-Parra et al., 1999). Mayr et al. initially constructed two Ad5-FMDV vectors with the sequence encoding the capsid and a wild-type (WT) or an inactive mutant form FMDV 3C-protease from a FMDV laboratory strain Ad5-A12 (Mayr et al., 1999; Mayr et al., 2001). They found that the 3C-protease was important for processing of the capsid precursor, and the Ad5-A12 3C WT form which was inoculated in mice, elicited a neutralizing antibody response. Swine were administered

two doses of either the wild-type or the mutant vaccine and were challenged 2 weeks after the boosts. The sera of the animals which were inoculated with the wild-type form showed the presence of FMDV-specific neutralizing antibodies (Mayr et al., 2001) and all the animals from the same group were protected from clinical disease, compared to the naïve animals which showed signs of disease. The same group also developed another construct that includes the capsid region of serotype A24, which was used as a vaccine in Brazil for a while. For strict control of FMD, a single dose of the vaccine should protect animals; the same group tested one dose of Ad5-A24 in swine and showed there was only a partially protective response within 5 days. They further vaccinated cattle with the same Ad5-A24 and used two doses at first, with the second boost administered after 9 weeks. The results showed complete protection after viral challenge which implies that this type of vaccine could be used for semi-annual or annual vaccinations in enzootic countries (Mayr et al., 2001).

1.5.6. DNA vaccines

DNA vaccines were considered one of the most versatile methods to induce humoral and cellular immune responses in the 1990s, using immunization with naked DNA. In 1997, Ward et al. were among researchers who successfully produced a candidate DNA vaccine to combat FMDV. They transfected BHK cells with a prototype plasmid pWRMH containing the cDNA of the A12 FMDV capsid precursor. As the level of transfection of the cells was low, they added a cDNA encoding the hepatitis delta virus ribozyme to the 3' end of the FMDV cDNA. They also created another plasmid (pWRMHX) with a gene engineered to abolish the cell binding site from VP1. Suckling mice were inoculated and pWRMHX was shown to successfully produce empty capsids without the worry of the virus going through a secondary infectious cycle. Swine were also administered pWRMHX and showed protection against FMD and produced neutralizing antibodies, while only 20% of the vaccinated animals with pWRMHX were protected after challenge (Ward et al., 1997). Moreover, the potential of FMDV DNA vaccines could be significantly improved by the co-expression of immunogens and cytokines relevant to the induction of protective immunity (Gurunathan et al., 2000). However, the efficiency of uptake of the nucleic acid is low and the mechanism of action is poorly understood.

1.5.7. Empty capsid vaccines

As described in section 1.5.6, empty FMDV capsids can be produced from the co-expression of the capsid precursor P1-2A and the 3C^{pro} to produce the capsid proteins VP0, VP1 and VP3. The self-assembly of capsid proteins into empty capsid particles which lack the viral genome, also known as virus-like particles (VLPs), can occur (Figure 1.3) (Bachrach et al., 1975; Jamal and Belsham, 2013). VLPs have a sedimentation coefficient of 75S, allowing for centrifugation-based purification for vaccine production. Production of VLPs relies on the cloning of these genome encoding regions into appropriate expression vectors using various expression platforms. These structures confer similar antigenicity in animals to that of infectious FMD virions (Grubman et al., 1985; Rowlands et al., 1975; Rweyemamu et al., 1979). Previous work by Lewis and co-workers has shown that bacterial cells could be an appropriate vehicle for effective and safe production of empty capsids to be used as FMDV vaccines. Biochemical assays supported the fact that portions of the processed P1 fragments assembled into capsid intermediate complexes which induced a satisfactory neutralizing antibody response in guinea pigs using a full P1 construct, while a truncated P1 construct without co-expression with 3C did not yield any such results (Lewis et al., 1991). Production of recombinant vaccines in *E. coli* proved to be feasible but there were certain negative issues, such as an inability to perform post and co-translational modifications, glycosylation, phosphorylation and processing.

Researchers also tested the production of FMDV VLPs in insect cells using recombinant baculoviruses: Roosien et al. (1990) produced empty FMDV capsid proteins via cDNA clones using combinations of cassettes with P1-2A, including and excluding the sequences encoding L and 3C proteases. Expression of the cassettes resulted in assembly of FMDV proteins, however, very low expression levels of the FMDV proteins were detected and no capsid particles were visible under the electron microscope. While this experiment was partly successful and required optimisation, Porta et al. (2013b) validated this experiment. They used a different expression vector and varied the 3C-protease activity to avoid the deleterious effects of the protease which, when fused to the C-terminus of the P1-2A capsid precursor, abolished expression. The techniques involved the site-directed mutagenesis of 3C and an HIV-1 ribosomal frameshift site between the P1-2A and 3C sequences. The experiment was monitored via the production of VP1 protein and

implementation of 3C mutants generated the most VP1 as confirmed by western blotting. Sucrose density gradient ultracentrifugation was carried out and the fractions were analysed using the electron microscope, which revealed the presence of VLPs in the extracts of infected insect cells. The experiment proved to be successful for FMDV A, O and SAT2 serotypes (Porta et al., 2013b).

The production of recombinant vaccines using bacteria or insect cells carries some drawbacks, including the fact that large volumes of culture are needed for scale-up, and using insect cells requires expensive culture media. The use of silkworm larvae could help to overcome such issues, as these have a higher recombinant protein production capacity per unit biomass than insect cells and *E. coli*. In 2008, Li et al. developed a recombinant silkworm baculovirus construct Bm-P12A3C bearing the complete P1-2A and 3C protease coding regions of FMDV serotype Asia1. The polyprotein was expressed in the silkworm larvae, and cattle were vaccinated: 4 out of 5 of the animals were protected with their sera showing a relatively high titre of FMDV-specific neutralizing antibodies (Li et al., 2008). Li et al. (2012) came up with a better approach, using the pupae instead of the larvae. This method facilitated large-scale production and the pupae did not require feeding at regular intervals. Furthermore, the silkworm pupae have a prolonged survival at 2 to 8°C compared to silkworm larvae, and also do not require mulberry leaves or an artificial diet during their growth. Therefore, the silkworm pupae have been used as a bioreactor to express proteins of interest at any time of the year. Cattle were vaccinated with extracts from pupae infected with the recombinant baculovirus. Contrary to the use of the larvae, all the cattle in this experiment developed antibody responses after 14 days. The same methods were used and produced better results with higher antibody titres in the vaccinated animals. Thus, a better alternative to the silkworm larvae, requiring the constant supply of mulberry leaves, would be the pupae which conferred other advantages such as purification of the product directly from the system (Li et al., 2012).

In 2013, Mignaqui et al. produced FMDV empty capsids in mammalian cells. They made use of serum-free mammalian cells, the vector pTT5, the genes P1-2A and 3C producing the structural proteins VP0, VP1 and VP3. They achieved relatively high expression levels of P1-2A and 3C, using pTT5-P12A3C vector for transfection. The P1-2A fragment was successfully cleaved by 3C into

VP0, VP1 and VP3. 3C had a deleterious effect on expression, especially when present in a high amount, but there was still a higher expression level than when the individual structural proteins were expressed. However, the expression of P1-2A in the absence of 3C did not result in the detection of the individual capsid proteins. Overall, the system allowed for scalability but there were many issues such as the high cost for the biocontainment manufacturing facilities, coupled with the expense of cell culture media (Mignaqui et al., 2013).

1.5.8. Plants as biofactories

Plants could help circumvent many of the negative issues discussed above; namely, the requirement for biocontainment facilities, the limited time-frame for the efficient production of recombinant vaccines, and the lack of post-translational modifications faced with the use of bacterial cells. In addition, plant production is affordable, and can be easily be scaled up without fear of gene leakage in the environment. (Rybicki, 2009; Shah et al., 2013).

1.5.8.1. Transgenic vaccines in plants

In 1996, the Arntzen lab was the first to discuss the possibility of producing vaccines in plants, and specifically in transgenic tobacco and in potatoes (Mason et al., 1996). Production of recombinant vaccines in transgenic plants became the new method for circumventing several issues posed by expression systems as previously stated. This type of genetic transformation refers to the insertion of the gene of interest into the plant genome, so that these plants act as bioreactors for the mass production of recombinant vaccines against disease agents such as FMDV, influenza or human papillomaviruses, as examples (Dus Santos and Wigdorovitz, 2005; Gleba et al., 2007; Rybicki, 2009).

The first case study of expressing an FMDV antigen in transgenic plants was performed by Carrillo et al. in 1998. Transgenic *Arabidopsis thaliana* plants expressing the complete FMDV VP1 via the pROK1 binary plasmid were produced and the plant extracts were used to inoculate mice, whose sera were collected after ten days and were analysed via ELISA. The results demonstrated that the mice developed a strong immune response to FMD. The immunized mice were further challenged with 50% lethal doses of FMDV and all animals were shown to be effectively protected. This was the first milestone towards FMDV immunization using the expression of an

antigen in transgenic plants (Carrillo et al., 1998). In 2005, Dus Santos and Wigdorovitz developed a similar strategy where they fused *gus A* (a reporter gene) with VP1 under the control of the CaMV35S promoter (Dus Santos and Wigdorovitz, 2005). Mice showed complete protection after viral challenge and their paper depicts the utilization of P1 and 3C-protease as effective experimental immunogens.

Transgenic Alfalfa plants provided another alternative to generate recombinant vaccines, and Dus Santos et al. (2005) proved that these bioreactors were very efficient and reliable. Here, the genome fragment spanning the P1, 2B and 3C encoding regions was cloned as cDNA into a plant expression vector, and agroinfiltrated into alfalfa plants. Empty capsids were then assessed using scanning electron microscopy. Mice were subsequently intraperitoneally injected with the plant extracts, and further boosted at 15 days intervals where the experimental animals developed anti-FMDV antibodies at a high titre (Dus Santos et al., 2005). Wigdorovitz et al. in 1999, also made use of transgenic alfalfa plants to express only the structural protein VP1. After confirmation of the presence of the transgenes in the said plants, mice were either immunized via needle or fed with leaf extracts. In both cases, they developed a virus-specific immune response as speculated. Furthermore, the mice were protected from challenge which brought to the scientific world for the first time, an edible vaccine which could provide immunity (Wigdorovitz et al., 1999a).

The experiments and strategies illustrated above, while touting transgenic plants as an effective tool to produce recombinant vaccines against FMDV, however, proved to be disadvantageous in several ways. The experiments explicitly showed that the yield of recombinant antigen stimulated from the transgenic plants was relatively low and would thus inevitably require further purification and concentration processes. This could lead to high costs of production. Moreover, the generation of the transgenic plants is quite a lengthy and laborious process (Gleba et al., 2007; Rybicki, 2009).

1.5.8.2. Production of vaccines in plants via transient expression

Transient expression in plants can be achieved by using recombinant *Agrobacterium*-mediated gene delivery. Agroinfiltration is a common method for uniformly introducing recombinant

bacteria with the gene of interest in whole leaves of plants, and it is scalable. The process has been shown to be a highly convenient method while alleviating many factors such as cost and time compared to the use of transgenic plants, whose regeneration is lengthy (Rybicki, 2009). Moreover, advances in the transient expression technology have demonstrated that significant increases in quantities of proteins can be produced compared to transgenic plants, whether by introducing bacterial binary vectors or use of recombinant plant viral vectors (Thuenemann et al., 2013; Yusibov and Rabindran, 2008).

Recently, researchers have produced a number of bovine candidate vaccines transiently in plants, mainly to take advantage of the technology's affordability and scalability (Ruiz et al., 2015). For FMDV, the transient expression system demonstrated its usefulness recently when Habibi et al. (2014) conducted a study investigating the transient expression of the coat protein VP1 in spinach leaves. The plants leaves were agroinfiltrated with the chimaeric gene consisting of the two G-H loops of VP1 and its presence and expression were confirmed using protein dot-blot and ELISAs. The high levels of expression showcased the potential of this method for the production of a recombinant subunit vaccine on a large scale without any major drawbacks (Habibi et al., 2014). Earlier, as mentioned in section 1.5.4, Andrianova et al. (2011) showed that the production of a FMDV recombinant vaccine consisting of VP1, VP4 and the T-cell epitopes of 2C and 3D, via transient expression in *N. benthamiana*, successfully provided protective immunity when administered in guinea-pigs (Andrianova et al., 2011).

1.6. AIMS OF THIS STUDY

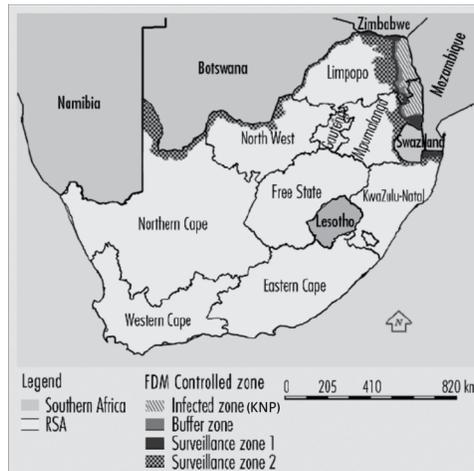


Figure 1.5: Map indicating the Foot-and-mouth disease control areas in South Africa. The Kruger National Park (KNP) is referred as the infected zone. Adapted from Grant et al. (2008).

Since 1957, South Africa has successfully managed to contain FMDV, and the OIE provided the country with disease-free status without vaccination in 1995. However, this status was lost as of September 2000 when the first case of serotype O was diagnosed in a piggery in KwaZulu-Natal after illegal feeding of untreated swill sourced from ships. Furthermore, till this date the Kruger National Park (KNP) (Figure 1.5) is treated as the infected zone in the region, with SAT1, SAT2 and SAT3 being maintained in the African buffalo population. The viruses are also present in around other 70 wildlife species in the vicinity (Bruckner et al., 2002; Suttmoller et al., 2003; Thomson et al., 2003). In that instance, KNP was isolated from the rest of the country with a fence built around the game park, thus erecting a buffer zone (Figure 1.5, light-grey area). Farm animals in the area are vaccinated yearly, while those in the surveillance zones (Figure 1.5, dark-grey area) around the buffer zone, are monitored regularly (Bruckner et al., 2002).

Current vaccines against FMDV are chemically inactivated virus strains, and although effective, there is a risk of incomplete inactivation which might lead to accelerated release of the wildtype virus (Knight-Jones et al., 2016; Lombard et al., 2007). The vaccines are produced in large bioreactors which require high biocontainment facilities. However, recent research on recombinant vaccines, which help to circumvent the need for high biosafety requirements, have shown that recombinant FMDV VLPs composed of the FMDV P1-2A capsid proteins are

immunogenic (Mignaqui et al., 2013; Porta et al., 2013b). The manufacture of such vaccines relies on the co-expression of the FMDV capsid precursor P1-2A with the 3C-protease, which often has deleterious effects when expressed in bacterial or insect cells as discussed before. Thus, researchers have mainly truncated the 3C-protease to produce satisfactory levels of empty capsids (Lewis et al., 1991; Porta et al., 2013b).

However, these are produced in mammalian or insect cells, which still represent a biosafety hazard from possible contamination with agents which could infect target animals. Plants such as *N. benthamiana*, on the other hand, provide a safer host for the production of recombinant proteins and VLPs, as their use considerably reduces the risk of gene leakage into the environment because of the lack of cross-infecting disease agents (Shah et al., 2013). Plant production platforms can further be classified into either transgenic or transient expression systems. While the transgenic method deals with the insertion of the gene of interest into the plant genome, for the transient system the gene is not integrated into the genome and expression is accomplished by *Agrobacterium*-mediated infiltration. The transient system is a much quicker method to produce recombinant protein (days vs. months) as it does not rely on the long process required to select for genes of interest that have integrated into the host chromosome (Gleba et al., 2007; Rybicki, 2009).

Gene transfer relies on the use of expression vectors with the gene of interest under the control of a promoter. Sainsbury et al. (2009) engineered a plant expression vector, pEAQ-*HT*, having a modified 5'-UTR and the 3'-UTR from the Cowpea mosaic virus (CPMV) RNA-2 (CPMV-*HT*) within a binary vector pBINPLUS. This has been shown to enhance the level of transiently expressed recombinant proteins in plants. Recently, Shah et al. (2013) compared transient expression of the green-fluorescent protein (GFP) using different plant expression vectors, including the pEAQ-*HT*. They observed that transient expression with the pEAQ-*HT* vector resulted in the highest expression levels of GFP, which the authors postulated was a result of a P19 expression cassette being integrated into the T-DNA of the vector. P19 sometimes play an important role for expression of recombinant proteins in plant expression systems as it is known that posttranscriptional gene silencing (PTGS) has a negative impact on expression of proteins. PTGS is a defense mechanism against foreign RNAs in eukaryotes, whereby the plant produces

homologous small interfering RNAs (siRNAs) in response to foreign molecules. Thus, the presence of a silencing suppressor such as the P19, would counteract the host's PTGS by binding to the siRNAs (Bartel, 2004; Shah et al., 2013). As a result, P19 being on the same T-DNA as genes of interest would lead to the expression of both P19 and the gene of interest from the same cells. In this study, the pEAQ-*HT* vector was selected for the expression of selected FMDV viral genes in *N. benthamiana*.

Therefore, the specific objectives of this study were:

- (i) It has been shown by Veerapen et al., 2017 (manuscript submitted for publication) that infiltration of *N. benthamiana* leaves with the P1-2A construct in the absence of 3C resulted in the expression of VLPs, albeit at low levels. Thus, the first objective of my study consisted of the comparison of transiently expressed *N. benthamiana* codon-optimised FMDV Type A P1-2A-3C constructs. Expression of the capsid precursor was also monitored in the absence of the 3C-protease. FMDV Type A constructs were used for expression as a proof of concept, and similarly, FMDV Type SAT 1, 2 and 3 P1-2A can also be expressed for future studies.
- (ii) Furthermore, FMDV VLPs are known to be acid-labile which are also prone to disassembly at extreme temperatures. Unlike enteroviruses which release their RNA by receptor-mediated uncoating (Porta et al., 2013a), the FMDV capsids must dissociate to release their genome which partially explains the weak electrostatic interactions at low pH and at elevated temperatures. Vaccines which are made up of these empty capsids should therefore be able to withstand such conditions to promote a longer shelf-life. Towards the long-term aim of developing a vaccine which will alleviate the constraints posed by the current methods of production and to promote shelf-life of such a vaccine, the second objective was to transiently express derived-FMDV acid and heat-resistant mutants' proteins to produce highly stable VLPs, in the absence of the 3C-protease.
- (iii) The third objective was to test the immunogenicity of the candidate vaccine produced from the expression of the P1-2A capsid precursor only, in the presence and in the absence of an adjuvant.

CHAPTER 2

Transient expression of different codon-optimised FMDV constructs *in planta* and expression of FMDV capsid precursor in *E. coli*

2.1. INTRODUCTION

FMDV vaccines currently available on the market consist of purified, inactivated whole virus preparations (Robinson et al., 2016). However, there are drawbacks to manufacturing these, including the high costs of biocontainment as well as the risk of failure of complete inactivation and subsequent viral escape, and the regulatory limitation of some countries such as the US that means they cannot produce vaccines using live virus. These factors have led to the development of candidate recombinant vaccines. One of the strategies has been the production of virus-like particles (VLPs) as they lack the infectious viral genetic material which negates the risk of infection (Kushnir et al., 2012). It has been shown that co-expression of the FMDV ORF *P1-2A* encoding the three capsid proteins (Grubman et al., 1985), and the non-structural *3C* protease which cleaves *P1-2A* into the component capsid proteins, results in the assembly of the capsid proteins into VLPs. These have been produced in various host cell expression systems including silkworm larvae (Li et al., 2012), *E. coli* (Lewis et al., 1991; Xiao et al., 2016), insect cells (Cao et al., 2009; Porta et al., 2013b; Roosien et al., 1990), Vaccinia (Abrams et al., 1995), mammalian cells (Mignaqui et al., 2013), transgenic alfalfa and tomato (Dus Santos et al., 2005; Dus Santos and Wigdorovitz, 2005). In most cases, the VLPs have been shown to be immunogenic when injected in animals (Porta et al., 2013a; Rodriguez and Grubman, 2009).

Nevertheless, these above-mentioned systems relied on the expression of a mutated form of the *3C*-protease as its presence in cells has been proven to be detrimental. In a previous study (Veerapen et al., 2017, manuscript submitted for publication), our group demonstrated that the *P1-2A* capsid precursor can be proteolytically cleaved inside plant cells in the absence of *3C*. We had transiently co-expressed *P1-2A* with different concentrations (OD_{600} of 0.25 and 0.5) of the protease in *N. benthamiana* as well as in the absence of *3C*. Surprisingly, expression of the capsid proteins VP0, VP1 and VP3 was observed by western blot analysis and VLPs of ~30 nm, consistent

in shape and regularity, were observed both from the co-expression of P1-2A with 3C and from expression of P1-2A only. However, the number of VLPs per field of view was low.

Messenger RNA secondary structures such as hairpin loops can cause interference with the translation of a protein. Given a DNA or RNA sequence, the secondary structure can be predicted and therefore the translation efficiency of a protein can also be predicted, making it necessary to test whether codon optimisation of the protein gene to be expressed, can give rise to unwanted structures (Malys and McCarthy, 2011; Malys and Nivinskas, 2009). The TripleT Biosciences company (Wageningen) has developed an algorithm that would combine the use of optimal codons to build an ideal mRNA structure for any given gene. Their expertise relied on analysing how comparison between overall codon use in highly expressed genes differs between monocots and dicots. These conserved expression codons, which help increase expression in the plants, were used to optimise the codon optimisation of three genes. This further showed how the protein yield was increased upon stable and transient expression in plants. They extended this study into analysing whether the expression-linked codon use was applicable across different kingdoms, namely Bacteria, Fungi and Animalia, where they concluded that a common codon use bias prevails over all species. Moreover, from computational analyses of different mRNA characteristics, it was revealed that a similar selection pressure across kingdoms exists, which increases both stability and translatability. It was also shown that from the combination of gene expression data with available protein abundance, that an increase in the number of stem-loop transitions together with a reduction of stem size would result in an increase in translation efficiency. Considering all these analyses, an algorithm was thus developed and tested successfully in plants, showing an increase in protein production of the same three genes tested before (Westerhof, 2016).

One of the ways of increasing protein and therefore VLP production would be to increase translation efficiency of the RNA of interest. Therefore, one of the aims of this study was to optimise the expression of FMDV VLPs in *N. benthamiana* by firstly, investigating the translation efficiency of differently codon-optimised P1-2A-3C constructs in order to try and increase VLP yields. We tested the expression of P1-2A-3C which was codon-optimised using the TripleT biosciences algorithm, and compared this with the original P1-2A-3C construct previously used

by Veerapen et al., (2017) (manuscript submitted for publication). In addition, the effect of maturation time on VLP formation after crude extraction from infiltrated leaves was investigated. As the detection of expressed protein by western blotting required an antibody, since there is no commercially available antibody against P1-2A, this chapter also describes the production of a polyclonal antibody against the capsid precursor P1-2A in *E. coli*.

2.2. MATERIALS AND METHODS

2.2.1. Genes

FMDV Type A P1-2A and 3C (GenBank accession numbers: M7287 and FJ265680, respectively) were codon-optimised for *Nicotiana* species and synthesized by GenScript (USA). FMDV Type A P1-2A-3C was codon-optimised for *Nicotiana species* using a different algorithm by Triplet Biosciences (Wageningen) and synthesized by GenScript.

2.2.2. Codon-optimised oligonucleotides

The original P1-2A-3C construct (provided by Dr. Vanesa Ruiz, INTA, Argentina) which was used to produce the second *N. benthamiana* codon-optimised construct will be referred to as oP1-2A-3C and mP1-2A-3C respectively. Their RNA structures are as illustrated in Figure 2.1 below. The structure of oP1-2A-3C was illustrated as this was used as a template to generate the codon-optimised construct mP1-2A-3C. Furthermore, oP1-2A-3C was further used as a template in downstream experiments to generate the construct oP1-2A via PCR.

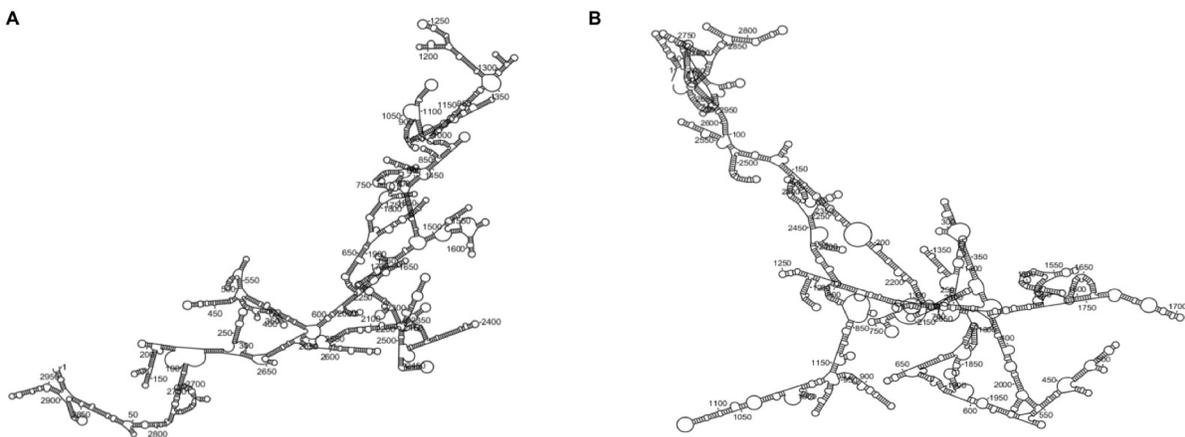


Figure 2.1: The RNA structures of oP1-2A-3C and mP1-2A-3C as generated by CLC Workbench 6.0 (QIAGEN Bioinformatics). (A) oP1-2A-3C, $\Delta G = -873.0$ kcal/mol. (B) mP1-2A-3C, $\Delta G = -955.1$ kcal/mol.

Codon optimised genes were received from GenScript (USA) in the pUC57 vector. These constructs were used as template DNA for Polymerase Chain Reaction (PCR) amplification of the mP1-2A-3C, mP1-2A and oP1-2A genes.

Sense and anti-sense oligonucleotides (Table 1) were designed to include *AgeI* and *XhoI* restriction enzyme sites to facilitate cloning of the amplicons into plant expression vectors. For expression, a start codon was inserted into the oP1-2A sense oligonucleotide and a stop codon was inserted into oP1-2A and mP1-2A anti-sense oligonucleotides.

Table 2.1: Oligonucleotides used for amplification of mP1-2A-3C, mP1-2A and oP1-2A.

Primer	Restriction site	Oligonucleotide sequence (5' to 3')	Length (bp)	Tm (°C)
mP1-2A-3C forward	<i>AgeI</i>	AT <u>ACCGGT</u> CCCATGGGTGCT	20	62.1
mP1-2A-3C reverse	<i>XhoI</i>	AT <u>CTCGAGT</u> TACATGACGTGGACG	24	62.2
mP1-2A forward	<i>AgeI</i>	AT <u>ACCGGT</u> CCCATGGGTGCT	20	62.1
mP1-2A reverse	<i>XhoI</i>	AT <u>CTCGAGT</u> CA ACCAGGGTTAGAC	24	62.1
oP1-2A forward	<i>AgeI</i>	AT <u>ACCGGT</u> AT GGGAGCAGGTC	21	56.2
oP1-2A reverse	<i>XhoI</i>	AT <u>CTCGAGT</u> CA ACCTGGGTTAGA	23	55.2

¹The restriction sites are underlined. The start and stop codons are in bold.

2.2.3. PCR amplification of mP1-2A-3C, mP1-2A and oP1-2A

Polymerase Chain Reaction (PCR) was performed for the specific amplification of the genes of interest using the primers listed in Table 2.1. PCR reactions consisted of 60 ng of template DNA, 1x Phusion® HF buffer (New England Biolabs), 200 μ M dNTPs (KAPA), 0.5 μ M forward primer, 0.5 μ M reverse primer, 2 mM MgCl₂ and 1.0 U Phusion® High-Fidelity DNA polymerase (New England Biolabs). Alternatively, the KAPA HiFi HotStart ReadyMix PCR Kit was also used for gene

amplification and consisted of template DNA, 1x KAPA HiFi HotStart ReadyMix, 0.3 μ M forward primer, 0.3 μ M reverse primer. Control reactions contained all reagents except the DNA template, or forward primer only or reverse primer only. Thermocycling conditions were as follows: initial denaturation at 98°C for 30 sec, followed by 30 cycles of denaturation at 98°C for 10 sec, annealing at 65.5°C for 30 sec and extension at 72°C for 30 sec. A final extension step of 72°C for 5 min was included.

PCR products were analysed on 1% w/v TBE (89 mM Tris base, 89 mM boric acid and 2 mM EDTA [pH 8]) agarose gels, containing 2.5 mg/ml ethidium bromide and visualized under short wavelength ultraviolet (UV) illumination. The O'GeneRuler™ 1 kb marker (Thermo Scientific) was used to size the DNA fragments.

2.2.4. Cloning

2.2.4.1. Restriction enzyme digest

Restriction enzyme digest, with *AgeI* (Thermo Scientific) and *XhoI* (Thermo Scientific), were carried out on miniprep mP1-2A-3C, amplified mP1-2A and oP1-2A PCR fragments as well as the pEAQ-*HT* vector to facilitate directional cloning into the vector. Restriction digests consisted of 500 ng DNA, 1 U of each of the restriction enzymes and a 1x final concentration of Buffer O (Thermo Scientific). Digests were carried out for 2 hours at 37°C. Digested fragments were analysed on a 0.8% agarose gels with 2.5 mg/ml ethidium bromide.

2.2.4.2. Gel extraction

The appropriate bands for mP1-2A-3C, mP1-2A, oP1-2A and pEAQ-*HT* fragments were visualized under long UV wavelength and purified from agarose gels using QIAquick® gel extraction kit (Qiagen) according to the manufacturer's instructions.

2.2.4.3. Ligation

Purified mP1-2A-3C, mP1-2A and oP1-2A fragments were ligated into pEAQ-*HT*, using a vector to DNA ratio of 1:5, 1:7 and 1:7 respectively. The ligation reaction consisted of the pEAQ-*HT* linearized vector, relevant DNA fragments, 1 U of T4 DNA ligase (Thermo Scientific) and supplied 1x T4 buffer (Thermo Scientific). Ligations were incubated at 4°C overnight.

2.2.5. Transformation of competent *E. cloni*TM DH5 α cells

Competent *E. cloni* cells (*E. cloni*TM, Lucigen) were transformed via a ‘heat-shock’ step with the appropriate recombinant plasmid DNA as described in a protocol by Sambrook et al., (1989). The pEAQ-*HT* plasmid contains a kanamycin resistance gene, which allows for the selection of transformed cells. Therefore, 100 μ l of the transformed cells were plated onto Luria Bertani (LB) agar containing kanamycin (50 μ g/ml) and incubated at 37°C overnight.

2.2.6. Confirmation of recombinant clones

Recombinant clones were selected and inoculated into 10 ml of LB broth containing kanamycin (50 μ g/ml) and incubated overnight at 37°C in a shaking incubator. The cells were pelleted and plasmid extraction was performed using QIAprep[®] Spin Miniprep kit (Qiagen) according to the manufacturer’s instructions. Plasmid DNA was digested with *Age*I and *Xho*I as described in section 2.2.3.1 to confirm the presence of the genes of interest.

2.2.7. Nucleotide sequencing

To further confirm the presence of the gene of interest in the vector, 100 ng/ μ l plasmid DNA was sequenced (Macrogen, Netherlands) using pEAQ-*HT* primers (Table 2.2). The sequences were analysed using CLC Workbench 6.0 (QIAGEN, Bioinformatics).

Table 2.2: Oligonucleotides used for sequencing of pEAQ-*HT*-mP1-2A-3C, pEAQ-*HT*-mP1-2A and pEAQ-*HT*-oP1-2A.

Primer	Oligonucleotide sequence (5’ to 3’)	Length (bp)	Tm (°C)
pEAQ- <i>HT</i> forward	TTCTTCTTCTTGCTGATTGG	20	46.3
pEAQ- <i>HT</i> reverse	CACAGAAAACCGCTCACC	18	49.3

2.2.8. Electroporation of competent *A. tumefaciens* AGL-I

Cells of the *Agrobacterium sp.* AGL-I (now known as *Rhizobium radiobacter* AGL-1 [ATCC BAA-101TM]) were made electrocompetent using a method adapted from Shen and Forde (1989). One hundred microliters of electrocompetent cells was transferred to a chilled 0.1 cm electroporation cuvette (Bio-Rad) and 100 to 500 ng of recombinant plasmid DNA was added. The electroporation was performed as described by Maclean et al. (2007). The cells were plated on LB agar containing

kanamycin (50 µg/ml) and carbenicillin (25 µg/ml) to select for recombinant AGL-I, and incubated at 27°C for two days. Putative positive clones were screened by PCR as described in section 2.2.2.

2.2.9. *Agrobacterium* infiltration

Each of the recombinant *A. tumefaciens* were inoculated in 10 ml of LBB broth (0.25 % tryptone, 1.25 % yeast extract, 0.5 % NaCl, 10 mM 2-(N-Morpholino) ethanesulfonic acid [MES], pH 5.6) infiltration medium containing the appropriate antibiotics. The cultures were incubated overnight at 27°C with agitation at 200 rpm. The pre-inoculum was transferred to a 50 ml LBB medium, with the respective antibiotics and incubated at 27°C overnight with agitation. The resuspension medium used to dilute the cultures to the desired OD₆₀₀ contained 10 mM ethanesulfonic acid (MES), 20 µM acetosyringone, and appropriate antibiotics.

Cell suspensions of OD₆₀₀ 0.25 to 1.0 were used for the infiltration of each strain into *N. benthamiana* leaves. Small-scale infiltration was performed using a 1 ml syringe filled with the *Agrobacterium* strains which was forced into the air spaces of leaves. The plants were kept at 22°C for 3 to 7 days before harvest. For large-scale infiltration 500 ml LBB infiltration medium was adjusted to the required OD₆₀₀ and plants were vacuum infiltrated using a vacuum pump with the appropriate recombinant construct.

2.2.10. Protein extraction and analysis

Six leaf discs were harvested per construct by cutting out leaf sections using the lid of an Eppendorf vial, ground up in liquid nitrogen and protein extracted using 200 µl of 1x phosphate-buffered saline (PBS) (137 mM sodium chloride [NaCl], 10 mM disodium hydrogen phosphate [Na₂HPO₄], 2.7 mM potassium chloride [KCl], 2 mM potassium dihydrogen phosphate [KH₂PO₄]), pH 7.4.

2.2.10.1. SDS-PAGE

Protein extracts were treated with 5x sample application buffer and denatured at 95°C for 10 min. Proteins were separated on 15% SDS-acrylamide gels. Colour Prestained Protein Standard (New England Biolabs) was used as a molecular weight marker.

2.2.10.2. Western blot

After electrophoresis, proteins were transferred onto nitrocellulose membranes at 15 volts (V) for 90 min using a Trans-blot® semi-dry transfer cell (Bio-Rad). Membranes were then blocked for 1 hour at room temperature using blocking buffer (1x PBS containing 10% v/v Tween-20 [PBS-T], 5% non-fat dairy milk [NFDM]). Membranes were incubated overnight at 4°C with the primary antibodies used to detect the capsid proteins which were either polyclonal anti-serum from guinea-pigs having been infected with FMDV, serotype A1 (diluted 1: 100 in blocking buffer) or mouse anti-His antibody (Sigma-Aldrich) (diluted 1: 2000). Membranes were washed four times for 15 min each with blocking buffer and afterward incubated with either 1: 10 000 alkaline phosphatase conjugated anti-guinea pig (Sigma-Aldrich) or alkaline phosphatase conjugated anti-mouse antibody for the polyclonal serum or anti-His antibody respectively for 1 hour at 37°C. A 1: 5000 alkaline phosphatase conjugated anti-rabbit antibody (Sigma-Aldrich) was used as secondary antibody if the polyclonal anti-P1-2A rabbit sera was used. The incubation was followed by four washes for 15 min with 1x PBS-T and the proteins were detected with 5-bromo-4-chloro-3-indoxyl-phosphate (BCIP) and nitroblue tetrazolium (NBT) phosphate substrate (BCIP/NBT 1-component, KPL).

2.2.10.3. Dot blot

Two microliter of sample was placed onto a piece of nitrocellulose membrane, previously divided into 1 cm² squares. The membrane with the sample was left to air-dry and was then treated as described in the western blot section 2.2.10.2.

2.2.10.3. Coomassie staining

Fifteen percent SDS-PAGE gels were stained using Coomassie Brilliant Blue R250 stain (1% Coomassie blue, 48% methanol, 15% acetic acid, 36% deionised water) and destained in destaining solution (30% methanol, 10% acetic acid, 60% deionized water).

2.2.11. Extraction and purification of empty capsids

Large scale protein extraction was performed by homogenizing plant material in 2x NTE buffer pH 8.0 (0.1 M NaCl, 0.05 M Tris, 0.004 EDTA-disodium dehydrate). The extract was filtered

through Miracloth™ (Merck) and centrifuged at 9600 x *g* for 10 min. Clarified sap was loaded onto a 30% sucrose cushion and centrifuged at 175 000 x *g* for 2 h 45 min in a SW 32 Ti rotor (Beckman). Maturation was carried out by resuspending the pellet in NTE buffer pH 8.0 with 3500 U Benzonase (Sigma-Aldrich) for 30 min. The matured sample was loaded onto a 5 to 20% continuous sucrose gradient (w/v in NTE) and the gradient was developed at 175 000 x *g* for 2 h 30 min in a SW 32 Ti rotor, at 12°C (Beckman). The tube was then fractionated from the bottom into 1.5 ml fractions.

2.2.12. Transmission Electron Microscopy (TEM)

TEM analysis was performed on purified fractions. Three mm carbon coated copper grids (mesh size 200) were made hydrophilic by discharging at 25 mA for 30 sec using a Model 900 SmartSet Cold Stage Controller (Electron Microscopy Sciences). Grids were floated on 20 µl of sample for 4 min, after which grids were washed twice in water. The grids were negatively stained with 2% w/v uranyl acetate for 1 min using a Tecnai F20 electron microscope.

2.2.13. *E. coli* expression of P1-2A

The pProEx-Hta-P1-2A recombinant construct (provided by the Biopharming Research Unit) was expressed in *E. coli*. Ten milliliters of LB broth was inoculated with the recombinant *E. coli* construct and grown overnight at 37°C with agitation. The pre-inoculum was used to inoculate 50 ml LB which was incubated overnight at 37°C with agitation. The culture was induced with isopropyl β-D-1-thiogalactopyranoside IPTG (0.6 mM) when the optical density reached a cell density of OD₆₀₀ of 0.6. P1-2A was partially purified using the BugBuster® Protein Extraction Reagent (Novagen) according to the protocol provided for inclusion body purification with additional washes using 1x PBS to solubilize the protein.

2.2.14. Polyclonal P1-2A antibody production in rabbits

Female New Zealand white rabbits sourced from South African Veterinary Products (SAVP, Johannesburg) were housed under Biosafety Level 2 (BSL 2) conditions in the Research Animal Facility at the Health Sciences Faculty, University of Cape Town. Approval for this study was granted by the Animal Research Ethics Committee at the University of Cape Town (AEC no. 016-006). Three-month-old rabbits were inoculated with 100 µg *E. coli* produced P1-2A as described

in section 2.2.12, followed by four inoculation boosts, within a two-week interval for the first boost and one week interval for subsequent boosts. The sera were collected and tested on western blots via serial dilutions.

2.3. RESULTS

2.3.1. Generation of mP1-2A-3C, mP1-2A and oP1-2A constructs

The *mP1-2A-3C* gene was digested from the pUC57-mP1-2A-3C construct using *AgeI* and *XhoI* for subsequent cloning into the pEAQ-*HT* vector for downstream experiments. A product size of ~2.9 kb was gel excised as illustrated in Figure 2.2A.

The 2.2 kb amplicons of mP1-2A and oP1-2A were successfully amplified (Figures 2.2B and 2.2C respectively) and modified at the 5' and 3' termini for cloning into pEAQ-*HT*. PCR controls included using reactions containing reverse or forward primer only, and no DNA template. Fragments were excised from the agarose gel and purified, after which they were successfully ligated into the pEAQ-*HT* vector.

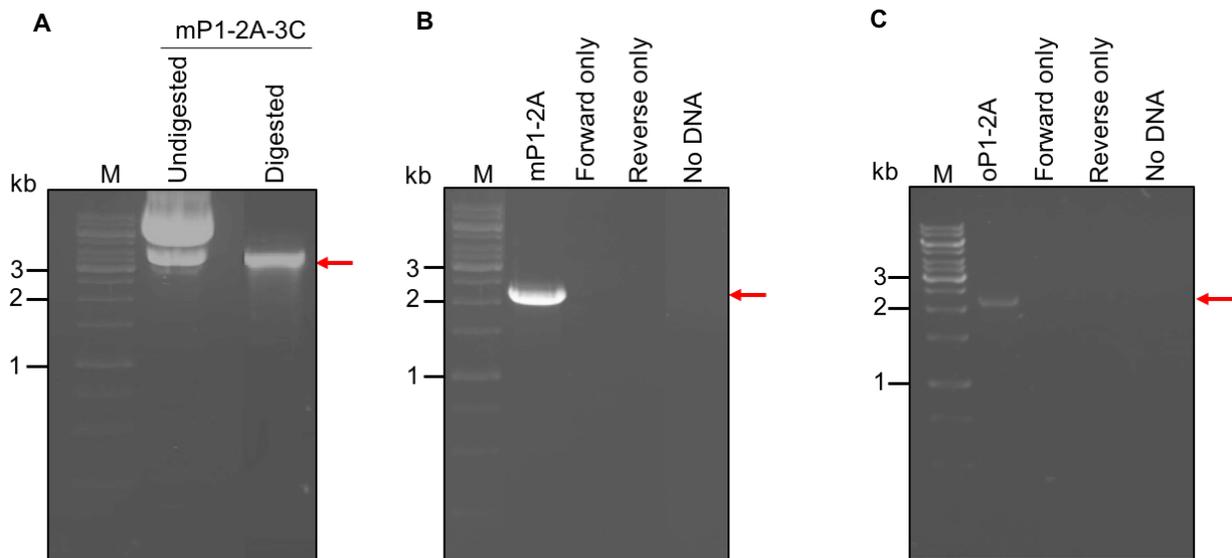


Figure 2.2: Generation of constructs mP1-2A-3C and mP1-2A from pUC57-mP1-2A-3C and oP1-2A from pUC57-oP1-2A-3C. (A) Restriction enzyme digest of mP1-2A-3C from pUC57-mP1-2A-3C with *AgeI* and *XhoI* to generate a product of ~2.9 kb, the undigested pUC57-mP1-2A-3C. (B) mP1-2A PCR product amplified from pUC57-mP1-2A-3C of ~2.2

kb. (C) oP1-2A PCR product from pUC57-oP1-2A-3C of ~2.2 kb. Controls for B and C: forward primer only, reverse primer only and no DNA template. (M) O'GeneRuler™ 1 kb (Thermo Scientific).

Ligated constructs pEAQ-HT-mP1-2A-3C, pEAQ-HT-mP1-2A and pEAQ-HT-oP1-2A were transformed into *E. coli* and the recombinant clones were digested to confirm the presence of the respective inserts. Figure 2.3 shows some of the putative recombinant clones which had the inserts after restriction digest. The pEAQ-HT-mP1-2A-3C recombinant clones showed the expected banding pattern of ~10 kb and ~2.9 kb (Figure 2.3A), while restriction enzyme digests of selected clones of pEAQ-HT-mP1-2A and pEAQ-HT-oP1-2A gave the expected products of ~10 kb and ~2.2 kb (Figures 2.3B and 2.3C respectively).

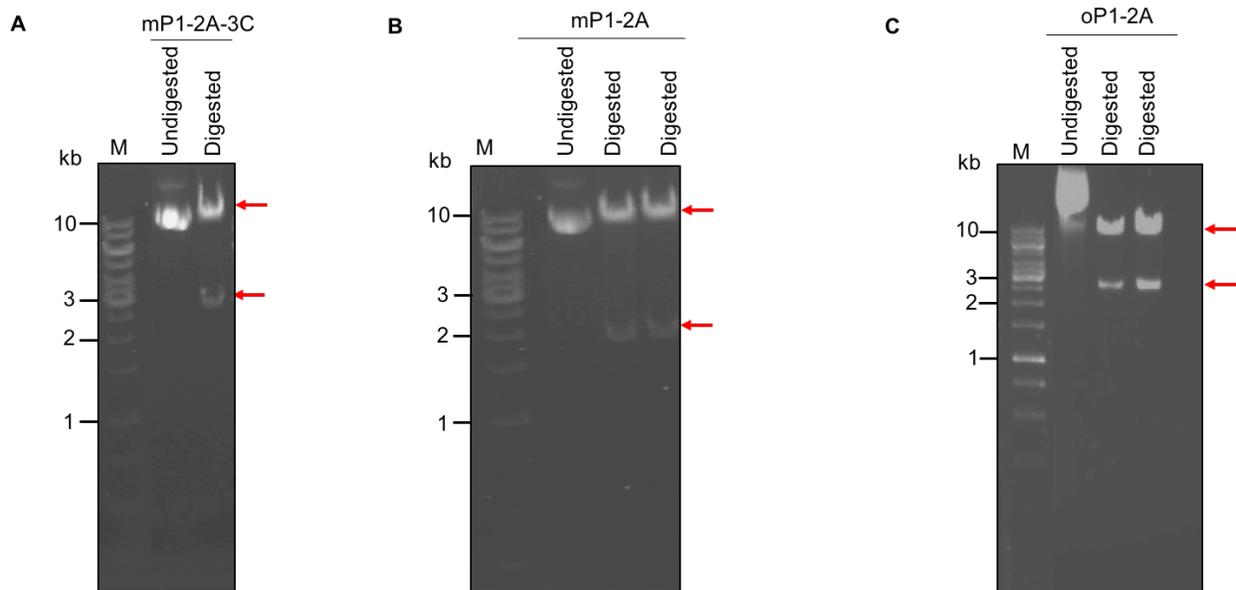


Figure 2.3: Restriction enzyme digestion of pEAQ-HT-mP1-2A-3C, pEAQ-HT-mP1-2A and pEAQ-HT-oP1-2A. The clones were verified by restriction enzymes *AgeI* and *XhoI*. (A) Digested pEAQ-HT-mP1-2A-3C giving products of bands of sizes ~10 kb (pEAQ-HT) and ~2.9 kb (mP1-2A-3C). (B) and (C) Digested pEAQ-HT-mP1-2A and pEAQ-HT-oP1-2A respectively, giving bands of sizes of ~10 kb (pEAQ-HT) and ~2.2 kb (mP1-2A/oP1-2A). (M) O'GeneRuler™ 1 kb (Thermo Scientific).

Recombinant constructs confirmed by restriction enzyme digests and sequencing were used for transformation of *Agrobacterium* AGL-I clones.

2.3.2. Transient expression of FMDV capsid proteins in *N. benthamiana*

Recombinant pEAQ-*HT*-mP1-2A-3C, pEAQ-*HT*-mP1-2A and pEAQ-*HT*-oP1-2A *Agrobacterium* constructs were infiltrated into *N. benthamiana* and protein accumulation was analysed after 3, 5 and 7 dpi. Western blot analysis of FMDV oP1-2A showed that VP1 and VP3 (~27 kDa) were expressed at all dpi for the different OD₆₀₀. However, VP0 (~34 kDa) was detectable only at 5 dpi at OD₆₀₀ of 0.25 and 0.5 (Figure 2.4). The highest accumulation of the capsid proteins was determined to be therefore at 5 dpi, either at OD₆₀₀ of 0.25 or 0.5.

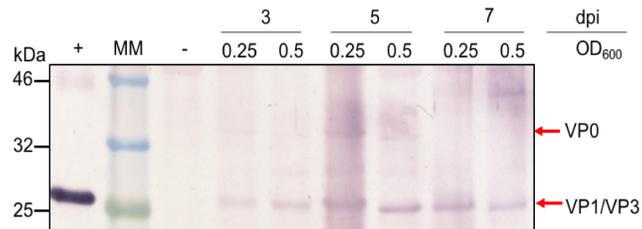


Figure 2.4: Western blot analysis of the time trials of the expression of FMDV oP1-2A *A. tumefaciens* AGL-I with VP0 (~34 kDa) and VP1/VP3 (~27 kDa). The OD₆₀₀ of pEAQ-*HT*-oP1-2A was varied at 0.25 and 0.5. (+) inactivated FMDV. (-) crude plant material infiltrated with pEAQ-*HT* without any insert. (MM) Colour Prestained Protein Standard (New England Biolabs).

From western blot analysis of mP1-2A-3C and mP1-2A expression, bands representing VP0 were barely visible at 5 dpi at OD₆₀₀ of 0.25 and 0.5 in both cases with VP1 and VP3 not detected in the crude extracts (data not shown). Necrotic phenotypes were also observed on leaves at 5 and 7 dpi (data not shown).

2.3.3. Purification of plant-produced VLPs

It has been shown that cleavage of the FMDV P1-2A precursor protein into mature structural proteins leads to assembly of empty capsids (Cao et al., 2009). To optimise the production of VLPs, plants were infiltrated with either recombinant pEAQ-*HT*-mP1-2A-3C, pEAQ-*HT*-mP1-2A or pEAQ-*HT*-oP1-2A at OD₆₀₀ of 0.5, and harvested at 5 dpi, as this was determined to be the best conditions for optimum expression using pEAQ-*HT*-oP1-2A (Figure 2.4). Forty grams of leaves of each construct was harvested at 5 dpi and extracted in 2x NTE buffer (pH 8.0). Putative VLPs were pelleted through a 30% sucrose cushion to remove as many contaminants as possible. Subsequently, the pellet was resuspended in NTE buffer and matured in the presence of

Benzonase, to improve maturation in the absence of nucleic acids, after which it was then centrifuged through a linear gradient to purify VLPs. All the fractions were first analysed on dot-blots (data not shown) and selected fractions were consequently analysed by western blot.

The western blot analysis of purified pEAQ-*HT*-mP1-2A-3C shows the presence of the capsid proteins VP0 (~34 kDa) with VP1 and VP3 being approximately the same size (~27 kDa) in fractions 8 and 9, though the yields were low as evidenced by the observation of faint bands (Figure 2.5B), while western blot analysis of purified pEAQ-*HT*-mP1-2A shows the presence of only VP0 in fractions 1 to 9 (Figure 2.5C). Figure 2.5D depicts purified pEAQ-*HT*-oP1-2A with the western blot showing the presence of all three capsid proteins in fractions 4 to 7.

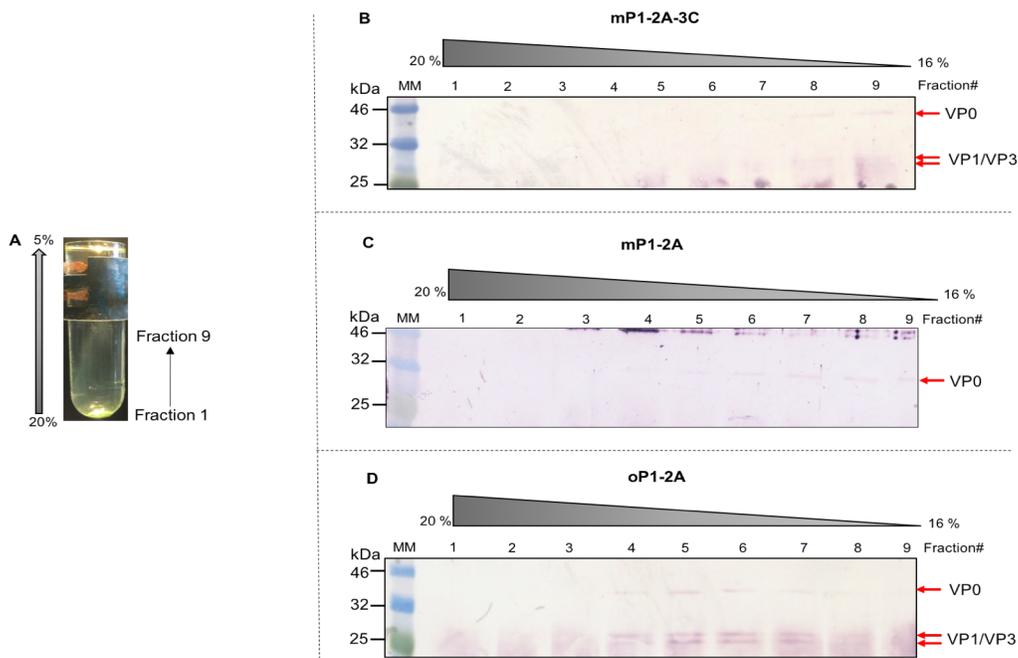
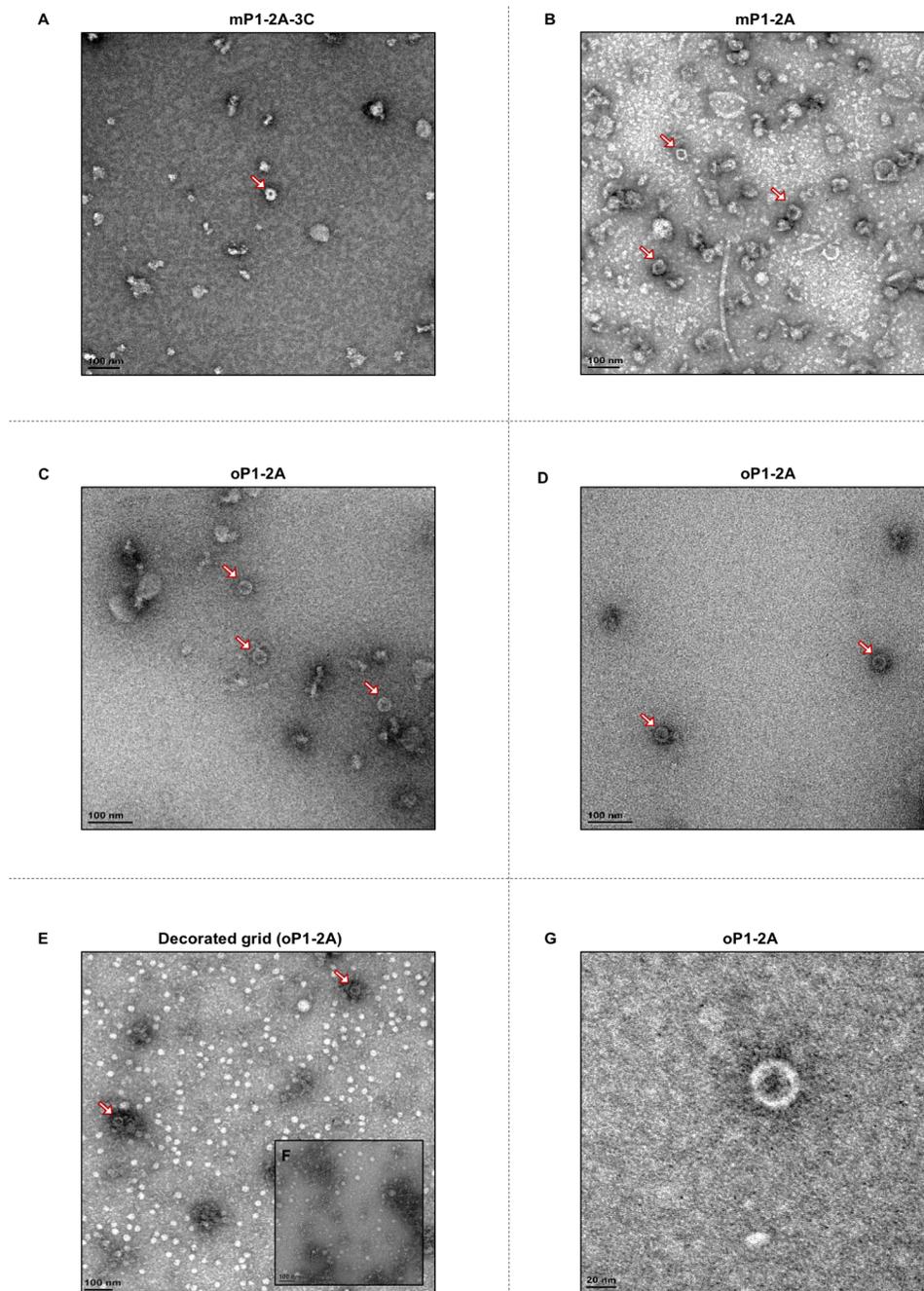


Figure 2.5: Western blot and EM analyses of purified fractions from large scale expression of the three different constructs with the western blot showing the expression of the capsid proteins VP0 (~34 kDa), VP1 and VP3 (~27 kDa). (A) Tube showing positions of fractions. (B) expression of pEAQ-*HT*-mP1-2A-3C. (C) expression of pEAQ-*HT*-mP1-2A. (D) expression of pEAQ-*HT*-oP1-2A. (MM) Colour Prestained Protein Standard (New England Biolabs).

Transmission electron micrograph of selected purified fractions of mP1-2A-3C revealed the presence of one VLP per field of view, and the presence of VLPs of variable sizes (30 to 35 nm) from mP1-2A fractions. EM analysis of oP1-2A (Figures 2.6C and 2.6D) with the red arrows

indicate the most stably sized VLPs of ~30 nm, with a count of 3 VLPs per field of view for 5 fields of view. VLPs were decorated with polyclonal anti-FMDV A1 from guinea-pigs to confirm their



identity, which indeed showed a dark halo around the VLPs compared to the non-decorated grids (Figure 2.6E), with an empty grid decorated with the same antibody used as a negative control. Figure 2.6G illustrates an expanded view of a oP1-2A VLP.

Figure 2.6: EM analyses of purified fractions from large scale expression of the three different constructs. (A) mP1-2A-3C, magnification: 56 000x. (B) mP1-2A, magnification: 56 000x. (C) and (D) oP1-2A., magnification: 64 000x (E) Decorated grid of oP1-2A VLPs with the polyclonal anti-FMDV A1 anti-guinea pig, magnification: 56 000x. (F) Decorated grid with polyclonal anti-FMDV A1 anti-guinea pig, magnification: 56 000x. (G) Expanded view of oP1-2A VLP, magnification: 128 000x.

2.3.4. Maturation trials of putative VLPs from expression of pEAQ-HT-oP1-2A

As seen from the results in section 2.3.3 above, expression of oP1-2A resulted in the best expression from the large-scale infiltration, and therefore this particular construct was chosen for the maturation trial experiments using the optimum conditions determined in 2.3.3.

VLPs are formed from capsid proteins which self-assemble to form the particle. Usually, this formation is dependent on many external factors such as temperature and pH of the environment. Grubman and Baxt (2004) described how FMDV VLPs are stable at pH above neutrality, and we thus investigated the effect that different maturation times would have on capsid assembly while maintaining a pH of 8.0. Maturation was carried out on resuspended pellets in the presence of Benzonase at room temperature for 30, 45 and 60 min before loading the matured material onto continuous gradients. Figure 2.7 shows the western blot and EM analyses of purified fractions collected from the continuous gradients. Figure 2.7A (i) shows the presence of the different VPs in purified fractions 2 to 8 after 30 minutes of maturation. Concentric VLPs measuring ~30 nm were visualized under TEM (Figure 2.7A (ii)). Similar results were obtained after maturation for 45 minutes where the bands representing the VPs were visible in fractions 1 to 9 with fractions 6 to 9 (Figure 2.7B (i)) showing the presence of all three VPs that are required for the formation of the particles (Figure 2.7B (ii)) (Porta et al., 2013b). However, after 60 minutes of maturation, the particles appeared to be prone to disassembly as illustrated by the EM image (Figure 2.7C (ii)) with detection of the capsid proteins in only fractions 2 to 4 from the western blot (Figure 2.7C (i)). Based on these results, maturation in further experiments was carried out at room temperature for 30 to 45 minutes.

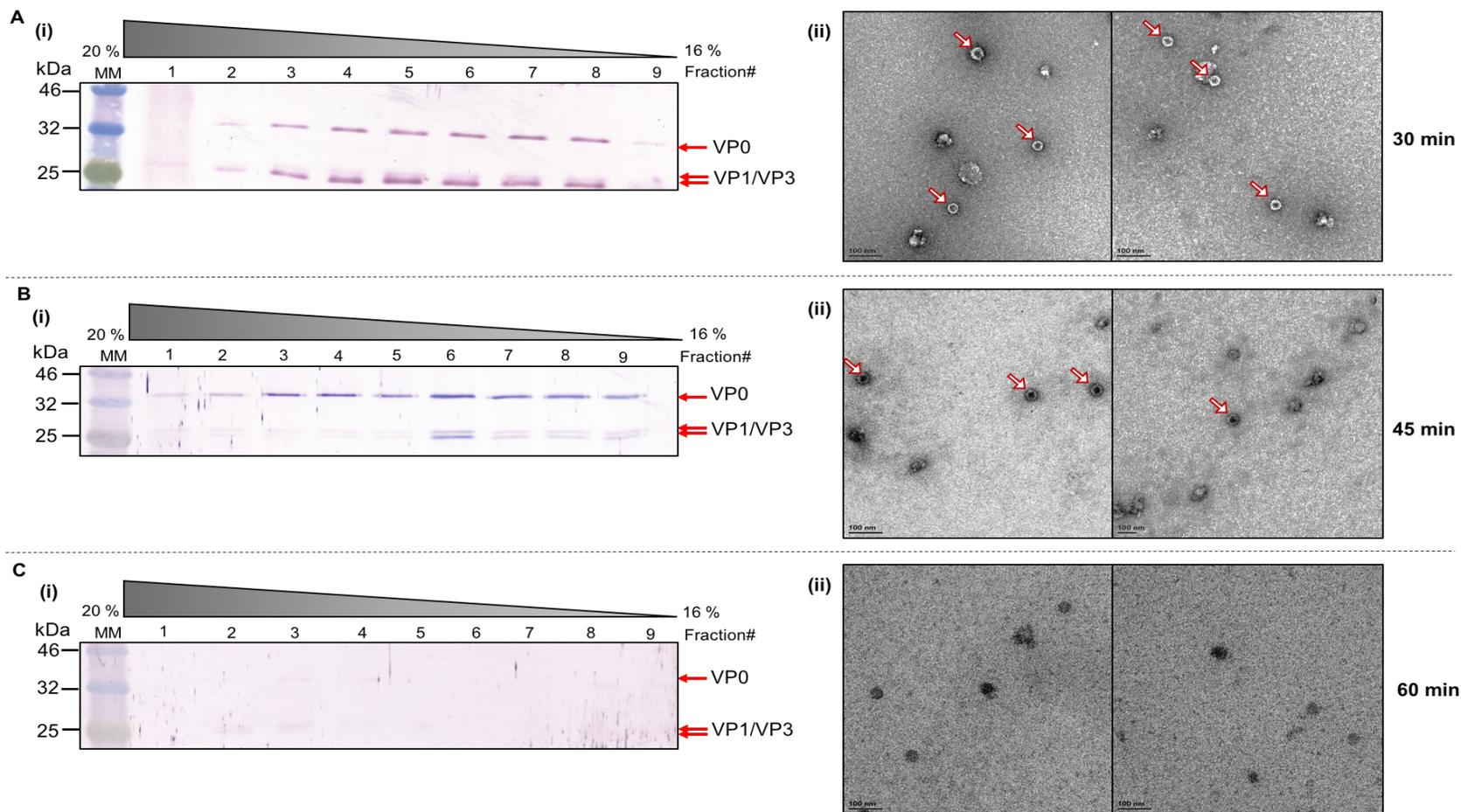


Figure 2.7: Western blot and EM analyses of expression of oP1-2A after maturation at room temperature from different time points showing expression of VP0 (~34 kDa), VP1 and VP3 (~27 kDa) from a continuous gradient and VLPs of ~30 nm. Maturation after (A) 30 min, (B) 45 min, (C) 60 min. Magnification: 64 000x. (MM) Colour Prestained Protein Standard (New England Biolabs).

2.3.5. Expression of oP1-2A capsid precursor in *E. coli*

The P1-2A capsid precursor gene was previously cloned into pProEx-HTa and was used for expression in *E. coli*. A time-trial after induction with IPTG was conducted which showed that best protein expression was obtained after 2 hours at an IPTG concentration of 0.5 mM (data not shown). These parameters were used for large-scale expression of P1-2A. Partial purification of inclusion bodies allowed the visualization of a band of ~83 kDa representing P1-2A on both western blot and on Coomassie-stained gel after 12 washes in 1x PBS (Figure 2.8). T₀ represents the reference time point prior to induction, while W1 to W12 are representative of multiple washes performed after protein extraction using BugBuster[®]. Western blot analysis showed no P1-2A present in wash fractions 1 and 2 but from wash 3 to wash 12, the protein was detected, with the highest levels in wash 12 (Figure 2.8).

The P1-2A band sized ~83 kDa was quantified by gel densitometry using a BSA standard curve. The purification process was repeated until 800 µg of protein was obtained for antibody production in rabbits.

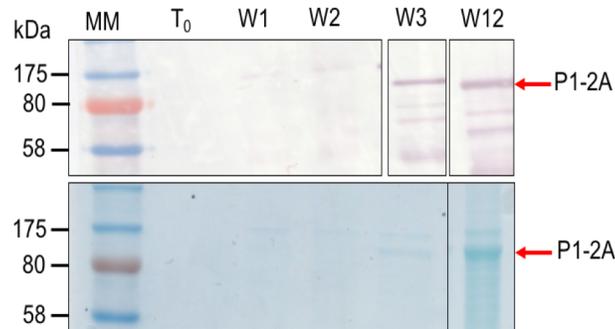


Figure 2.8: Western blot and Coomassie-stained gel analyses of the expression of the capsid precursor P1-2A in *E. coli*. The washes are represented by 'W' and uncleaved P1-2A is represented by a band size of ~83 kDa. (MM) Colour Prestained Protein Standard (New England Biolabs).

Sera from rabbits were collected and analysed for the presence of P1-2A antibodies. Western blots of crude oP1-2A plant extract was probed with a serial dilution (data not shown) of the sera to determine the optimal dilution at which to use the polyclonal antibody. The optimum titer for detection of P1-2A was determined to be 1:100, from bleeds collected from the third and fourth

inoculation boosts, as shown in Figure 2.9 where bands representing VP0 (~34 kDa), VP1 and VP3 (~27 kDa) are successfully detected with the rabbit-raised serum. The positive control used for comparison with the rabbit-raised serum consisted of using the polyclonal anti-FMDV obtained from guinea-pigs serum at 1:100 as shown in Figure 2.4.

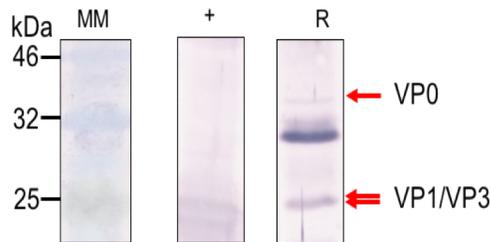


Figure 2.9: Western blot analysis of oP1-2A crude plant extract harvested at 5 dpi. (+) primary antibody, polyclonal anti-FMDV A1 anti-guinea pig (1:100). (R) primary antibody, anti-P1-2A, anti-rabbit (1:100). (MM) Colour Prestained Protein Standard (New England Biolabs).

The rabbit raised serum was used for subsequent western blots in this study (Chapter 3) and for ELISA (Chapter 4).

2.4. DISCUSSION

Foot-and-mouth disease (FMD) is prevalent in many regions around the world and is responsible for significant economic losses. The disease is endemic in Africa, affecting buffalo populations in the Kruger National Park, posing a threat to other livestock in the region (Bruckner et al., 2002). Therefore, there is an urgent need to screen and vaccinate the buffalo herds regularly and rapid response vaccines should be available when an outbreak occurs. The current vaccine available in South Africa to combat FMD consists of inactivated whole virus preparations which can be a biosafety hazard both due to the risk of release of the virus and incomplete inactivation. The expression of FMDV VLPs using recombinant technology constitutes a feasible strategy to overcome these issues. These empty viral capsids emulate the conformation of native virus particles and have been shown to be as immunogenic as the virions when injected in animals (Porta et al., 2013a).

In this study, expression of FMDV capsid proteins was optimised by investigating expression of P1-2A-3C and P1-2A constructs which were plant (*N. benthamiana*) codon-optimised to determine whether VLP yields could be increased. Proteolytic processing of P1-2A by the 3C-protease is important in the production of proteins VP0, VP1 and VP3, which self-assemble to form the viral capsid (Grubman and Baxt, 2004; Li et al., 2012). In this study, oP1-2A was expressed in the absence of the 3C-protease as our group has shown previously that proteolytic cleaving into VP0, VP1 and VP3 can occur in plants in the absence of 3C, and it was further used for expression comparison purposes. The two other codon-optimised constructs which were made to compare the expression of the FMDV capsid proteins were mP1-2A-3C and mP1-2A.

Small-scale infiltration with recombinant pEAQ-HT-oP1-2A *A. tumefaciens* AGL-I maintained at OD₆₀₀ of 0.5 resulted in successful expression of the capsid proteins VP0, VP1 and VP3 (Figure 2.4). The decrease in the accumulation of the capsid proteins at 7 dpi correlated with development of necrotic leaves, which could have been the result of a Hypersensitive Response (HR) generated by the plants (Hao et al., 2006). In similar studies carried out using alternative expression systems such as mammalian (Mignacqui et al., 2013) and insect cells (Roosien et al., 1990), P1-2A recombinant protein was detected as an uncleaved polyprotein of 83 kDa. The FMDV 3C-protease is a highly conserved viral enzyme and adopts a chymotrypsin-like fold. Its active site consists of the catalytic triad Cys¹⁶³-His⁴⁶-Asp⁸⁴, in a similar conformation to the Ser-His-Asp triad conserved in almost all serine proteases. The picornavirus 3C is thus classified as a chymotrypsin-like cysteine protease and the cleavage sites contain glutamine followed by small residues such as Gly, Ser and Ala (Birtley et al., 2005). The tobacco plant *N. benthamiana* is known to harbor cysteine proteases which might be involved in many processes such as proprotein processing, programmed cell-death and especially in protein turnover. While the tobacco genome encodes at least sixty putative cysteine proteinases (CysPs), they are poorly characterized (Duwadi et al., 2015; Hao et al., 2006). In this study, cleavage of the FMDV capsid precursor into VP0, VP1 and VP3 after expression in plants could be attributed to the presence of a large variety of cysteine proteases that have been shown to be present in plants.

It was determined by western blot analysis that the highest accumulation of FMDV VPs occurred at 3 and 5 dpi, after which VP0 was not visible at 7 dpi (Figure 2.4). On the other hand, expression

of mP1-2A-3C and mP1-2A resulted in low level accumulation of the capsid proteins despite having been codon-optimised to increase expression of the VPs. This could be due to the low ΔG of -955.1 kcal/mol of the mP1-2A-3C secondary structure (Figure 2.1), which is much lower than of the oP1-2A-3C construct having a ΔG of -873.0 kcal/mol. This means that even though the RNA structure of mP1-2A-3C is more open and 'expanded', its low ΔG would likely make it less stable than the RNA structure of oP1-2A-3C, resulting in lower levels of protein expression.

To determine whether the transiently expressed capsid proteins assembled into VLPs, production was scaled up by vacuum infiltrating twenty *N. benthamiana* plants at an OD₆₀₀ of 0.5 for all three constructs. Fifty grams of leaves were harvested at 5 dpi and the crude extract was filtered and centrifuged at 9600 *xg* for 10 minutes. Clarified supernatant was loaded onto 30 % sucrose cushion to pellet the capsid proteins, which was then loaded onto linear sucrose gradients of 5 to 20 % to purify VLPs; purified fractions were analysed by western blot (Figure 2.5). For mP1-2A-3C, VP0, VP1 and VP3 were observed in fractions 8 and 9 (Figure 2.5A (i)) and the EM analysis showed very few VLPs (1 VLP per field of view for 5 views) were purified (Figure 2.6A). Figure 2.5B illustrates the very low levels of VP0 with barely any detection of the other two VPs for mP1-2A produced VLPs. EM analysis demonstrates variable sizes of VLPs which could pertain to the low levels of VP1 and VP3 and the absence of VP0 resulting in non-uniform particles (Figure 2.6B). Figure 2.5C shows successful co-purification of all three VPs using the oP1-2A construct. TEM analysis of fraction 7 showed the presence of VLPs of approximately 30 nm in size (4 VLPs per field of view for 5 views) (Figures 2.6C and 2.6D) which is slightly more than obtained from the expression of mP1-2A-3C and mP1-2A.

A maturation process is a common method used to provide an ambient environment to enable VLP formation from the assembly of VP0, VP1 and VP3 present in the pelleted sample (Veesler and Johnson, 2012). Figure 2.7 while providing a close assessment of the structure of the VLPs formed from the expression of oP1-2A, helps to determine the optimum time-point at which the most structurally intact VLPs can be obtained. Thus, it was determined that the optimum time for maturation was 30 to 45 minutes (Figures 2.7A(ii) and 2.7B(ii)) after which the particles were shown to disassemble, possibly due to the ambient temperature to which they are exposed as was pointed out in the literature (Mateo et al., 2008). Pentameric subunits containing VP1 and

VP3 are intermediates of FMDV VLP assembly and disassembly, and it has been found that a high density of Histidine (His) residues which promote noncovalent interactions between the subunits, is located close to the interpentameric interface which tend to dissociate at extreme conditions (Liang et al., 2014; Martin-Acebes et al., 2010). The literature also points out that mild heating or prolonged exposure of the empty capsids at a slightly higher temperature than 37°C could cause the irreversible dissociation of particles into stable pentamers (Mateo et al., 2008). This could therefore be a plausible explanation for the presence of pentamers (~7-8 nm) (Oem et al., 2007) as shown after maturation of 60 minutes (Figure 2.6C (ii)).

The capsid precursor P1-2A was also expressed in *E. coli* as a reagent, for immunisation of rabbits whose sera would be used in further western blot and ELISA analyses. The partial purification of the P1-2A from inclusion bodies included numerous washes in both BugBuster[®] and in PBS, to remove other contaminating *E. coli* proteins which were shielding the detection of P1-2A as illustrated by Figure 2.8. Washes 3 and 12 revealed the presence of a band representing P1-2A (~83 kDa) from both Western blot and Coomassie Blue analyses.

To conclude, it was shown that the P1-2A capsid precursor can be expressed in the absence of 3C-protease, and that expression of oP1-2A resulted in the highest accumulation of the capsid proteins VP0, VP1 and VP3 needed for VLP assembly. It was further shown that maturation of 30 to 45 minutes resulted in the purification of stable and regularly shaped VLPs. Therefore, oP1-2A was selected for larger scale expression to analyse the immunogenicity of the VLPs in mice.

CHAPTER 3

Transient expression of heat and acid resistant FMD VLPs *in planta*

3.1. INTRODUCTION

Picornaviruses have a viral capsid which protects them from environmental factors and which contributes to viral dissemination and infection (Caridi et al., 2015). However, aphthoviruses, which are not acid resistant, lose their infectivity and immunogenicity due to their disassembly in culture environments below a neutral pH (Liang et al., 2014). In the case of FMDV, this phenomenon results in the disintegration of its capsid into 12S pentameric subunits containing five copies each of VP1 and VP3. In the literature, it has been hypothesized that acid sensitivity is associated with the uptake of FMDV virions in endosomes, where the pH is very low, thus triggering viral uncoating and genome release. Therefore, although acid sensitivity is detrimental to capsid stability, the process allows for the release of viral RNA into the host cell in order to establish infection (Carrillo et al., 1985; van Vlijmen et al., 1998). An environment conferring neutral pH will improve the stability of the virus and is thus thought it will also improve stability of VLPs. Nonetheless, though a 'neutral' environment will address the issue of acid sensitivity, the capsids are moreover thermolabile as stated by Doel and Baccarini (1981) and Curry et al. (1995). Thus, FMDV is both acid- and thermo- labile.

The propensity of the capsids to dissociate into pentamers at low pH and at moderate temperatures, is relevant to this study. It is possible that the generation of more stable FMDV VLPs could result in a higher yield. Work carried out by researchers has shown that site-directed mutations in the amino acid sequence of the capsid precursor, P1-2A, can enhance acid resistance by lowering the pH required for uncoating (Martin-Acebes et al., 2010; Vazquez-Calvo et al., 2014); this method also provides a higher thermostability, relaxing the requirements for a faultless cold chain without the need to modify production procedures (Mateo et al., 2008; Park, 2013).

Acid and heat lability of FMDV has been shown to be particularly modulated by amino acids located at the pentamer interface or in the N terminus of VP1 (Figure 3.1). Caridi et al. (2015) and

Liang et al. (2014) demonstrated how a mutation in the VP1 capsid protein from asparagine to aspartic acid (N17D) conferred acid resistance to the virion in the presence of the lysosomotropic weak base ammonium chloride (NH_4Cl). The latter acts as a proton sink within endosomes. Liang et al. (2014) further showed how the double mutant VP1 N17D/VP4 S73N could also be a potential acid resistant mutant. The FMDV capsid has a cluster of Histidine (His) residues with a pK_a of 6.8 close to the pentamer interface. In an acidic environment, these residues become protonated leading to the disassembly of the capsid via electrostatic repulsions between capsid subunits (Acharya et al., 1989; Curry et al., 1995). While the empty capsid seems to be more acid resistant than the virion itself (Curry et al., 1995), it is still not thermostable (Curry et al., 1997; Doel and Baccarini, 1981) due to the presence of these His-residues. In order to address this issue, it is important to target these His-covalent mediated interactions between pentamers and to replace the His with other amino acid residues such as cysteine as shown by Porta et al. (2013a) and Kotecha et al. (2015). They engineered a mutation in VP2 resulting in the mutant VP2 H93C, whereby mutated FMDV empty capsids were more stable when treated at extreme pH and temperature. Otherwise, as shown by Caridi et al. (2015) and Martin-Acebes et al. (2010), other mutations such as the replacement of Alanine to Valine in VP3 (A118V), which is close to the pentamer interface, would give rise to increased acid sensitivity. Figure 3.1 illustrates the location of the mutations with respect to pentamer-pentamer interactions.

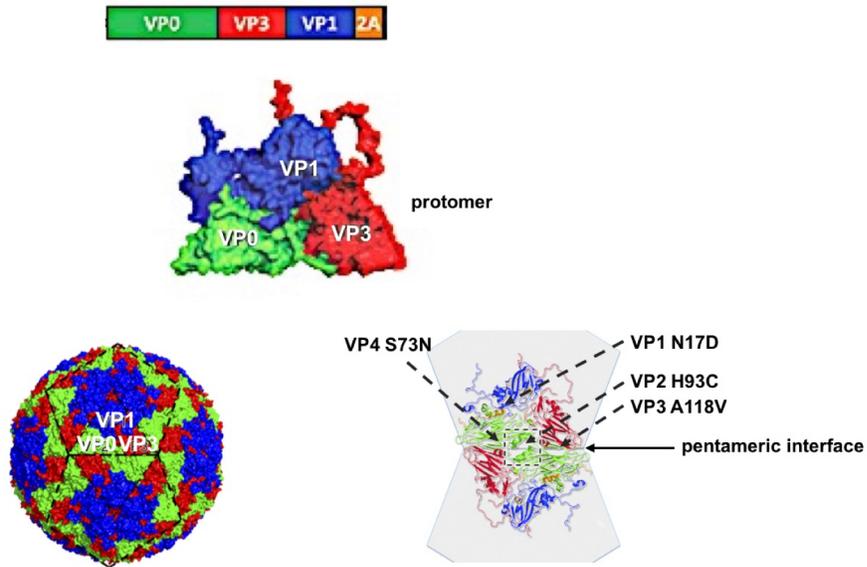


Figure 3.1: Illustration of the FMDV capsid proteins which form the empty capsid, with an expanded form of the interactions at the pentamer interface. Adapted from Kotecha et al. (2015).

My aim was to engineer the same mutations described (Table 3.1) in the P1-2A capsid precursor to address the acid and thermolability issues, and to determine whether this would influence the number of FMDV VLPs assembled in the transient plant expression system, as the previous studies focused on the use of insect cells as expression platform. Both the mutants and the wild-type oP1-2A were expressed in the absence of the 3C-protease, compared if expression of the mutants would be better than oP1-2A, which should result in higher levels of component VP proteins and subsequently a higher amount of assembled VLPs.

Table 3.1: Mutations with their respective properties and references.

Mutation	Properties	References
VP1 N17D	Acid resistant	(Caridi et al., 2015; Liang et al., 2014)
VP1 N17D/ VP4 S73N	Acid resistant	(Liang et al., 2014)
VP2 H93C	Heat and acid resistant	(Kotecha et al., 2015; Porta et al., 2013a)
VP3 A118V	Acid sensitive	(Caridi et al., 2015; Martin-Acebes et al., 2010)

3.2. MATERIALS AND METHODS

3.2.1. Site-directed mutagenesis

Site-directed mutagenesis was performed on the *oP1-2A* sequence to generate the sequences encoding for the acid resistant mutants VP1 N17D and VP1 N17D/VP4 S73N, acid and heat mutant VP2 H93C, and the acid sensitive mutant VP3 A118V. Figure 3.2 below illustrates the positions of the mutations in the *oP1-2A* gene. Generation of the mutants were achieved via PCR using the primers listed in Table 3.2. The first round of PCR consisted of using the forward oP1-2A primer and the mutant's reverse primer in the same reaction mix, while a second reaction mix contained the forward mutant's primer along with the oP1-2A reverse primer, yielding two half products. Subsequently, the second round of PCR with the oP1-2A forward and reverse primers involved using the half products obtained from the two previous PCR reactions as DNA templates (used in a 1:1 ratio). Mutants were confirmed by DNA sequencing (Macrogen, USA).

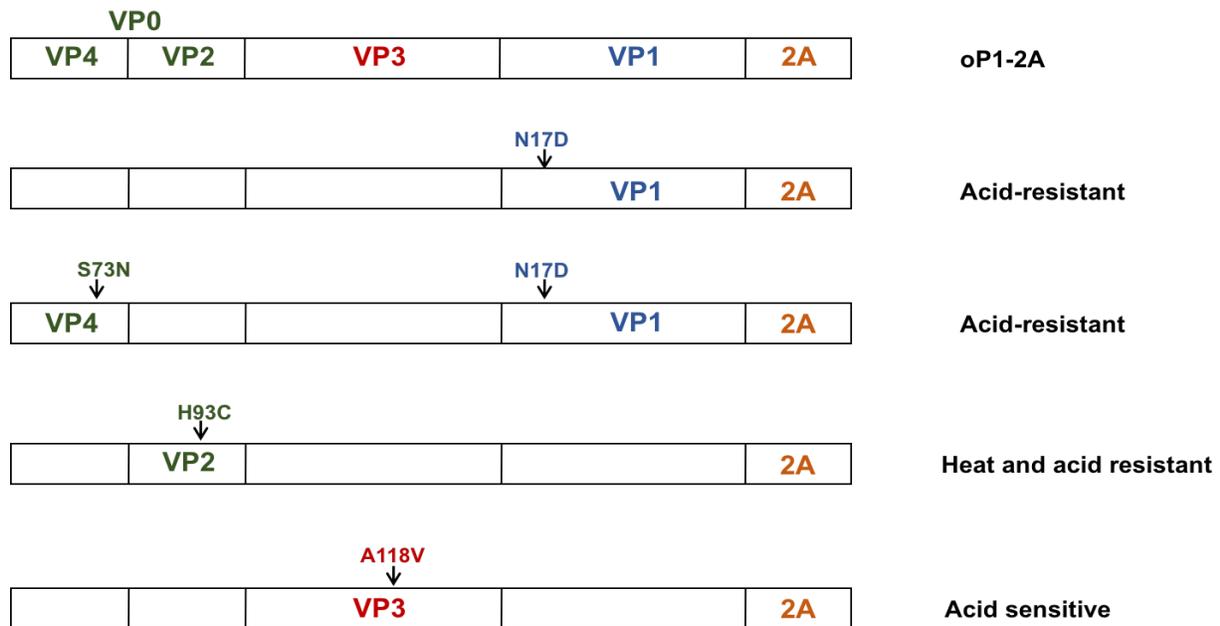


Figure 3.2: Schematic representation of the mutations generated in the *oP1-2A* gene.

Table 3.2 below shows all the primers which were synthesized to target specific nucleotides changes (underlined).

Table 3.2: Oligonucleotides used for site-directed mutagenesis.

Primer	Oligonucleotide sequence (5' to 3')	Length (bp)	Tm (°C)
VP1 N17D forward	GTTACTACAACCGTGGAA <u>G</u> ATTATGGTGGAGAGACCC	37	74.0
VP1 N17D reverse	GGGTCTCTCCACCATAAT <u>C</u> TTCCACGGTTGTAGTAAC	37	74.0
VP2 H93C forward	ACGGTGTTTTTGGAT <u>G</u> CCTTGTGGATAGTT	30	71.0
VP2 H93C reverse	AACTATCCACAAGGC <u>A</u> TCCAAAAACACCGT	30	71.7
VP4 S73N forward	GGTTTTCAAGATTAGCTA <u>A</u> TTAGCTTTTTTCTGGTCTCT	39	76
VP4 S73N reverse	AGAGACCAGAAAAAGCTGA <u>A</u> TTAGCTAATCTTGAAAACC	39	71
VP3 A118V forward	GGTTCAACTGATAGTAAGGU <u>C</u> AGAUUAUGGUUGCAUACA	40	70.2
VP3 A118V reverse	TGTATGCAACCATATACT <u>G</u> ACCTTACTATCAGTTGAACC	40	70.7

¹Nucleotides which were changed from the original sequence *oP1-2A* are underlined.

3.2.2. PCR amplification of VP1 N17D, VP2 H93C, VP4 S73N and VP3 A118V

PCR was performed as described in section 2.2.3, using the KAPA HiFi HotStart ReadyMix PCR Kit, and the primers listed in Table 3.2.

3.2.3. Cloning

Restriction enzyme digests, gel extractions, ligations and transformation of competent *E. coli*TM were performed as described in sections 2.2.4.1, 2.2.4.2, 2.2.4.3 and 2.2.5, respectively. Ligation ratios for VP1 N17D, VP2 H93C, VP4 S73N and VP3 A118V were 1: 5, 1:9, 1:9 and 1:5, respectively.

3.2.4. *Agrobacterium* infiltration

Agrobacterium infiltration and time-trials were carried out as described in section 2.2.8. The conditions for the large-scale infiltration and harvest for the mutants are summarized in Table 3.3.

Table 3.3: Optimum conditions determined from time-trials of expression of the mutants.

Mutant	Days post infiltration (dpi)	OD ₆₀₀
VP1 N17D	5	0.25
VP2 H93C	5	0.5
VP1 N17D/VP4 S73N	5	1.0
VP3 A118V	5	1.0

3.2.5 Protein extraction and analysis

SDS-PAGE, western blotting and dot-blotting of mutants were performed as described in sections 2.2.10.1, 2.2.10.2 and 2.2.10.3, respectively. Total soluble protein (TSP) concentrations of crude extracts were determined with the DC Protein Assay (Bio-Rad), using serial dilutions of bovine serum albumin (BSA, Sigma-Aldrich) as a protein standard. The absorbance was read at A₇₅₀ using a PowerWave XS™ (BioTek®) microplate spectrophotometer. Data were analysed using Microsoft® Excel. Forty micrograms of TSP were loaded into each well of the polyacrylamide gels for time-trial analysis.

3.2.6. Sedimentation and purification of empty capsids

Large-scale protein extraction was performed by homogenizing plant material in 2x NTE buffer pH 8.0 (0.1 M NaCl, 0.05M Tris, 0.004 EDTA-disodium dehydrate) and clarified sap was loaded onto a 30% sucrose cushion as described in section 2.2.11. Maturation was also performed as described in section 2.2.11, after which matured samples were loaded onto 15 to 45% continuous Optiprep™ gradients (v/v in NTE). Gradients were centrifuged at 175 000 *xg* for 2 h 30 min in a SW 32 Ti rotor (Beckman), at 12°C, after which tubes were fractionated from the bottom and analysed by western blotting.

3.2.7. Thermo-stability/pH stability assays

Acid and thermostability assays were carried out as described by Porta et al. (2013a) with some modifications. A 400 μ l aliquot that contained the capsid proteins which was analysed by EM and confirmed to contain empty capsids, was diluted either (a) with 133 μ l NTE buffer pH 8.0 and incubated in a water bath at 56°C for 1 hour or (b)(i) 50 mM sodium acetate buffer pH 4.6 to give a final pH of 5.2 or (ii) 50 mM sodium acetate buffer pH 4.6 to give a final pH of 6.2 and incubated for 15 min at room temperature before neutralization using 2 M NaOH. Treated samples were loaded onto 5 ml continuous Optiprep™ gradient ranging from 15 to 45% and the gradient was developed at 175 000 xg (SW 55Ti, 35 min, 12°C). One ml fractions were collected from the bottom of the tubes. Five hundred microliters of each fraction was precipitated with an equal volume of saturated ammonium sulphate overnight at 4°C. Precipitates were collected by centrifugation at 16 000 xg for 15 min and analysed by western blot.

3.2.8. Transmission Electron Microscopy (TEM)

TEM analysis was performed as described in section 2.2.12.

3.3. RESULTS

3.3.1. Generation of coat protein mutants

VP1 N17D, VP2 H93C, VP1 N17D/VP4 S73N and VP3 A118V mutants were successfully generated by site-directed mutagenesis using the primers in Table 3.2. Point mutations were incorporated into the oP1-2A amplicon, thus replacing the original nucleotides, resulting in the VP1 N17D, VP2 H93C, VP1 N17D/VP4 S73N (consisting of two base substitutions) and VP3 A118V mutants. The VP1 N17D/VP4 S73N mutant was engineered by using the mutant VP1 N17D as a template. As illustrated in Figure 3.3, the partial mutant products (product A and product B) were first amplified and subsequently used as template DNA to produce final mutant products. Figure 3.3 shows the partial and final PCR products of the mutant VP1 N17D with Figures 3.3A and 3.3B illustrating products A (~1.645 kb) and B (~5.55 kb), respectively. Figure 3.3C represents the PCR mutated product VP1 N17D as indicated by a fragment size of ~2.2 kb.

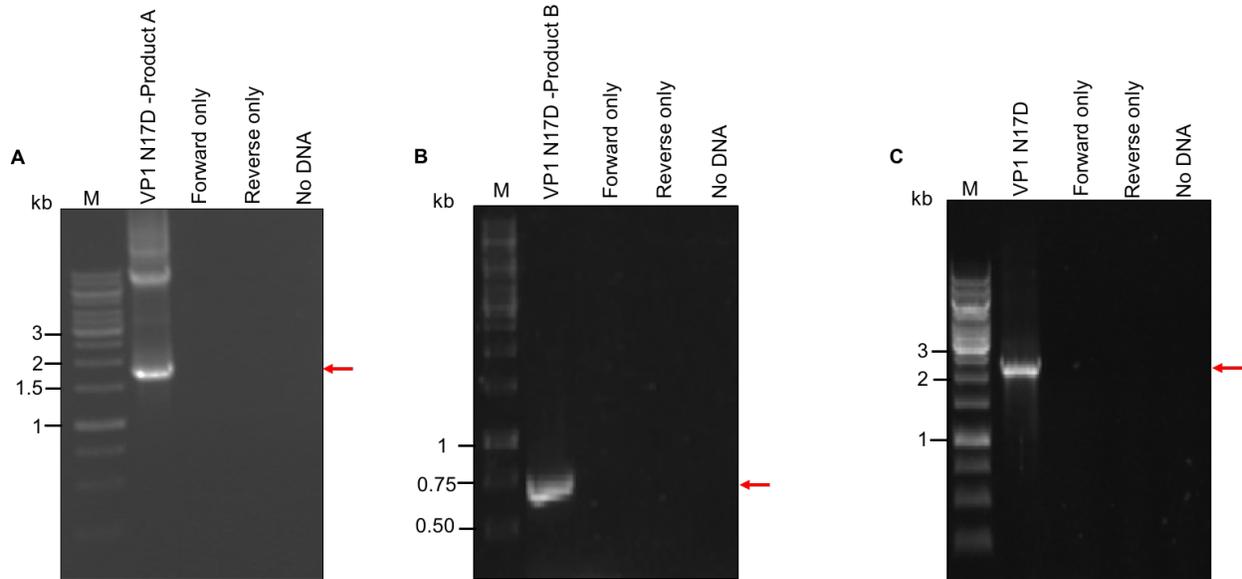


Figure 3.3: Generation of partial products of mutant VP1 N17D to give the final mutant product as shown by the red arrows. (A) PCR product A (~1.645 kb) using oP1-2A forward and VP1 N17D reverse primers. (B) PCR product B (~5.55 kb) using VP1 N17D forward and oP1-2A reverse primers. (C) PCR product VP1 N17D (~2.2 kb) using oP1-2A forward and oP1-2A reverse primers. (M) O'GeneRuler™ 1 kb (Thermo Scientific).

Mutants VP2 H93C, VP1 N17D/VP4 S73N and VP3 A118V were made in a similar way to VP1 N17D. Partial PCR products for VP1 N17D were ~1.6 kb and ~0.6 kb for products A and B, respectively (data not shown), while fragment sizes of ~1.65 kb and ~5.5 kb were identified as partial products of mutant VP1 N17D/VP4 S73N with ~1.2 kb and at ~1.0 kb fragments obtained for mutant VP3 A118V (data not shown). All the bands identified were gel excised and used as templates in the downstream PCRs to yield the final mutants of product size ~2.2 kb (data not shown).

Colony PCR was performed to screen for putative positive colonies which were further confirmed by an enzyme restriction digest with *AgeI* and *XhoI* (Figure 3.4). Restriction analysis confirmed positive clones with bands of ~10.0 kb and ~2.2 kb identified to be the pEAQ-*HT* vector and the mutants, respectively. Sequencing analysis further confirmed these mutations.

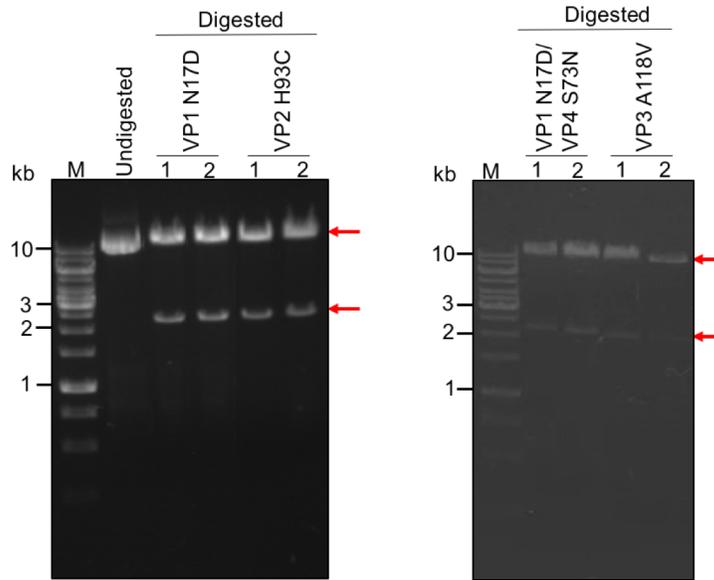


Figure 3.4: Restriction enzyme digestion of pEAQ-*HT*-VP1 N17D, pEAQ-*HT*-VP2 H93C, pEAQ-*HT*-VP1 N17D/VP4 S73N and pEAQ-*HT*-VP3 A118V clones were verified by restriction enzymes *AgeI* and *XhoI* giving products of band sizes ~10 kb and ~2.2 kb. (M) O'GeneRuler™ 1 kb (Thermo Scientific).

Recombinant constructs confirmed by sequencing were used for transformation into *Agrobacterium* AGL-I. Positive clones were identified by colony PCR (data not shown).

3.3.2. Transient expression of derived mutant capsid proteins *in planta*

Recombinant pEAQ-*HT*-VP1 N17D, pEAQ-*HT*-VP2 H93C, pEAQ-*HT*-VP1 N17D/VP4 S73N and pEAQ-*HT*-VP3 A118V *Agrobacterium* constructs were infiltrated at OD₆₀₀ of 0.25, 0.5 and 1.0 in *N. benthamiana* and capsid protein accumulation was monitored at 3 dpi, 5 dpi and 7 dpi by western blot (Figure 3.5).

In order to determine at which conditions (dpi and OD₆₀₀) the mutants were best expressed via assessment of the highest accumulation level of the capsid proteins, equal amounts of TSP (40 µg) of time-trial samples collected were loaded onto polyacrylamide gels (Figure 3.5). Analysis of the crude extracts of the mutants show that in some cases VP0 (~34 kDa) is cleaved into VP2 (~28 kDa) and VP4 (~6 kDa), where the latter was not expected to be observed on western blots due to its small size. As pointed out by literature, aberrant cleavage of VP0 into VP2 and VP4, is a commonly observed occurrence upon expression of mutant capsids. It has been stipulated that

it could be due to an attempt of the expressed mutant VLPs to encapsulate viral RNA (Curry et al., 1995; Curry et al., 1997; Porta et al., 2013a), as a simulation of the native viral RNA to undergo such cleavage of VP0, upon encapsidation of RNA by the virion. Similarly, in this study, this phenomenon was also observed from the expression of the mutant capsid proteins in the absence of the 3C-protease. Some form of degradation product was also noticeable (Figure 3.5, illustrated by (*)) for all the mutants tested.

Expression of the VP1 N17D mutant (Figure 3.5A) allowed the observation of the VPs 1, 2 and 3 at 3 and 5 dpi with some form of protein degradation at 5 dpi for the different OD₆₀₀ tested. Interestingly, at 7 dpi, bands representing VP2 were undetectable, but expression of VPs 0, 1 and 3 were observed at OD₆₀₀ of 0.5 and 1.0. The highest accumulation of the VPs, according to the intensity of bands on western blots, were determined to be at 5 dpi, OD₆₀₀ of 0.25.

Analysis of expression of the heat and acid resistant mutant, VP2 H93C (Figure 3.5B) showed that VPs 1 and 3 were noticeable throughout the time-trials at all 3 dpi and ODs tested, while VP2 was only detectable at 3 dpi, OD₆₀₀ of 0.25 and at 5 dpi OD₆₀₀ of 0.5 and 1.0. It was also noted how the degradation product (*) was visible at all the dpi and ODs.

Figure 3.5C illustrating expression of the second acid-resistant mutant VP1 N17D/VP4 S73N at 3, 5 and 7 dpi, further supports the expression of VP2 (at 5 dpi, OD₆₀₀ of 1.0) and of the degradation product (*) (3 dpi at OD₆₀₀ of 0.25, 5 dpi at OD₆₀₀ of 0.25 and 1.0). VP1 and VP3 were nonetheless present at 3 dpi, at ODs of 0.25 and 1.0, and at all the ODs for 5 dpi. Expression of all the VPs seemed to have decreased by 7 dpi with the absence of bands from western blotting. However, bands representing the degradation product (*) were visible at all the ODs tested.

Although acid sensitivity would not affect expression of the respective capsid proteins, it could potentially lead to conformational instability of the VLPs at extreme conditions. Expression of the capsid proteins was observed up to 7 dpi with the acid sensitive mutant VP3 A118V (Figure 3.5D). Expression of VP2 expression was detected as from 5 dpi, with an increase in OD₆₀₀ from 0.25 to 1.0. The capsid protein was only noticeable at OD₆₀₀ of 0.5 after 7 days. VPs 1 and 3, on the other side, were present as from 3 dpi till 5 dpi for all the ODs and were only detected at

OD₆₀₀ of 0.5 at 7 dpi. The degradation product (*) was also observed at 3 and 5dpi using ODs of 0.25, 0.5 and 1.0, and at 7 dpi using an OD₆₀₀ of 0.5.

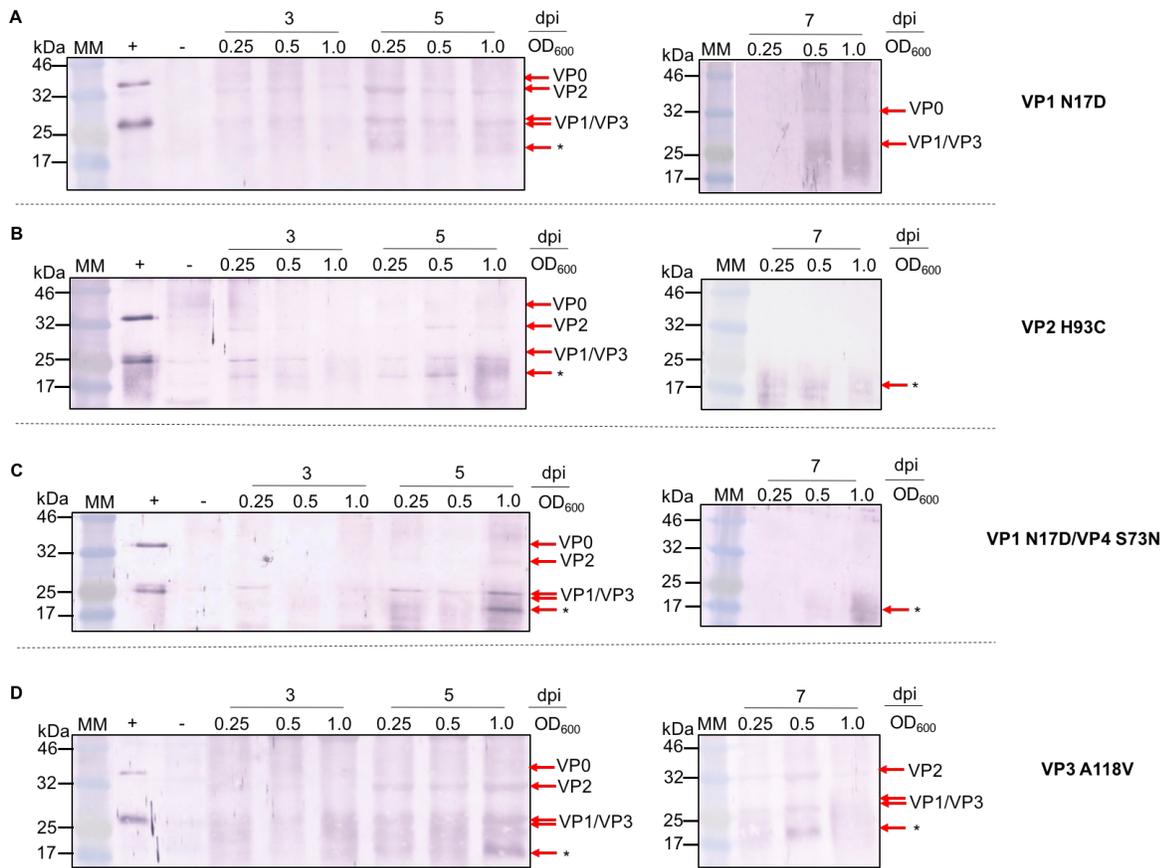


Figure 3.5: Western blot analysis of the time trial of the expression of FMD mutants. (A) VP1 N17D. (B) VP2 H93C. (C) VP1 N17/VP2 S73N. (D) VP3 A118V *A. tumefaciens* AGL-I. The OD₆₀₀ of recombinant pEAQ-HT-VP1 N17D, pEAQ-HT-VP2 H93C, pEAQ-HT-VP1 N17D/VP4 S73N and pEAQ-HT-VP3 A118V were varied at 0.25, 0.5 and 1.0. (+) Purified oP1-2A fraction from a sucrose gradient. (-) crude plant material infiltrated with pEAQ-HT without any insert. (MM) Colour Prestained Protein Standard (New England Biolabs).

Based on the results observed in Figure 3.5, the conditions selected for downstream experiments using the mutants are summarized in Table 3.3.

3.3.3. Production of VLPs

To optimise production of mutant VLPs and to assess particle formation at pH 8.0, plants were infiltrated with either pEAQ-HT-VP1 N17D, pEAQ-HT-VP2 H93C, pEAQ-HT-VP1 N17D/VP4 S73N, or with pEAQ-HT-VP3 A118V using the optimum conditions shown in Table 3.3. The recombinant

pEAQ-*HT*-oP1-2A was infiltrated at an OD₆₀₀ of 0.5 and harvested at 5 dpi, as determined previously in section 2.3.2. As a control, plants were alongside infiltrated with pEAQ-*HT* (without any insert).

Sixty grams of biomass expressing each construct was used in this study and extracted in 2x NTE buffer (pH 8.0), after which putative VLPs were pelleted as described in section 2.2.11 and resuspended in NTE buffer (pH 8.0). The resuspended pellets were matured at room temperature for 30 minutes before loading onto a continuous gradient to purify putative VLPs. Figure 3.6A shows a representative 15 – 45% continuous OptiprepTM gradient after final centrifugation for 2 h 30 min in a SW 32Ti rotor at 175 000 *xg*. The whole tube was fractionated from the bottom and fractions were analysed on dot-blot prior to western blotting.

Western blot analysis after purification of the VP1 N17D and VP1 N17D/VP4 S73N constructs, showed the presence of only VP2 (~28 kDa) in fractions 21 to 25 for VP1 N17D (Figure 3.6B) and in fractions 18 to 25 for VP1 N17D/VP4 S73N (Figure 3.6D), illustrating that some cleavage of VP0 had occurred even though the other VPs were not detected. The presence of the capsid proteins in top fractions however, suggest these could be soluble proteins which did not assemble into VLPs. However, VP4 was not detected which could be due to its small molecular weight and the probability of it running off the gel or it was not detected by the antibody. Cleavage of the capsid proteins is a common phenomenon as described by Porta et al. (2013a).

On the other hand, VPs 0, 1 and 3 were detected after purification of both VP2 H93C and oP1-2A in fraction 18 (Figure 3.6C) and in fractions 17 to 19 respectively (Figure 3.6F), with the highest expression being detected in fraction 17 (oP1-2A, OptiprepTM concentration = 19%) (Figure 3.6F).

Western blot analysis of the purified acid-sensitive mutant, VP3 A118V, showed the presence of VP1 (~27 kDa), VP2 (~28 kDa), VP3 (~27 kDa) and a product of around 15 kDa which could be a degradation product (Figure 3.6E(i)).

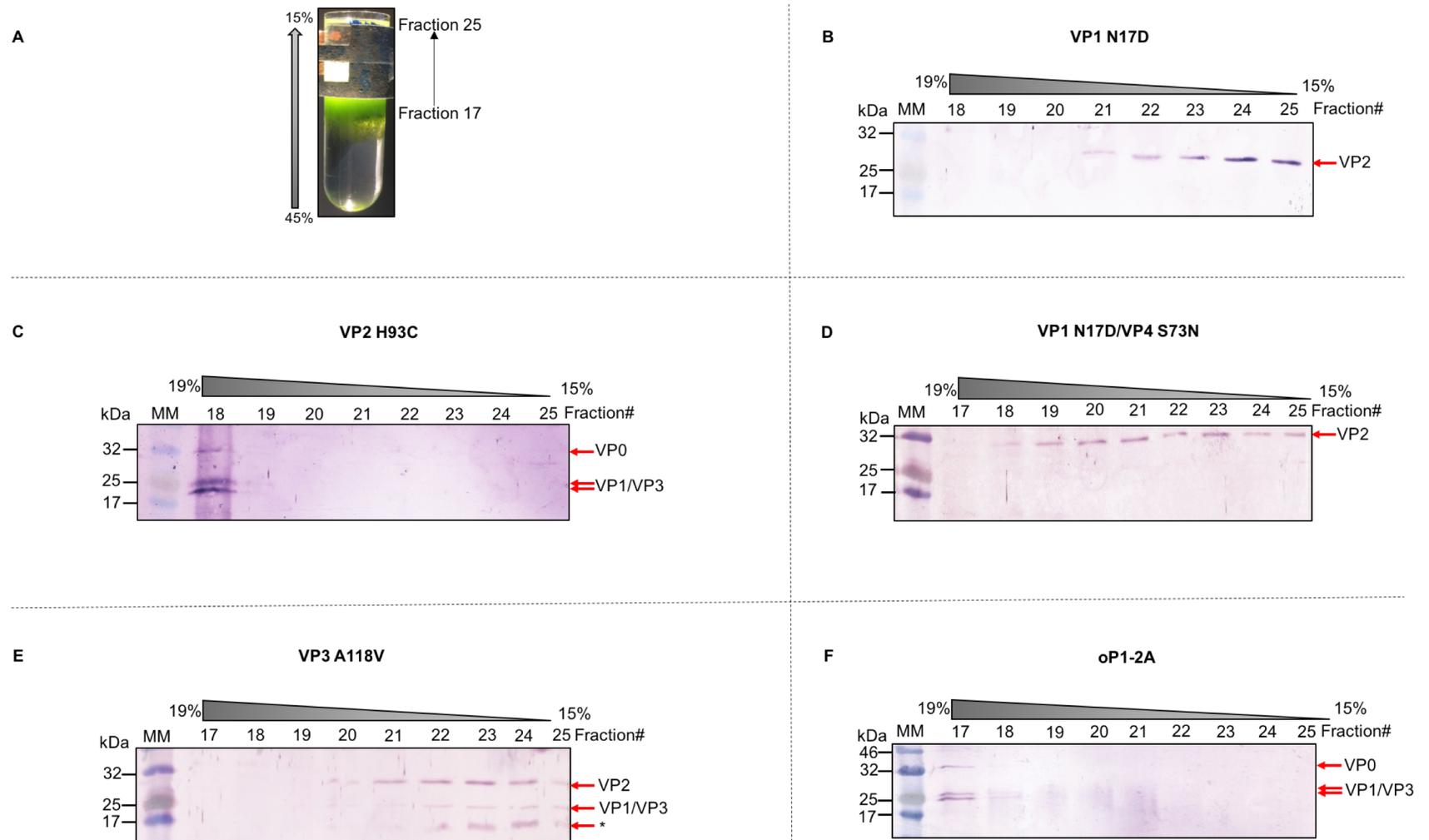


Figure 3.6: Optiprep™ gradient purification and western blots of plant-produced mutants' VLPs compared to the production of non-mutant oP1-2A VLPs. (A) Linear Optiprep™ gradient. (B), (C), (D), (E) and (F) VP1 N17D, VP2 H93C, VP1 N17D/VP4 S73N, VP3 A118V and oP1-2A respectively. (MM) Colour Prestained Protein Standard (New England Biolabs).

EM analyses of selected positive fractions as determined by western blots were performed (Figure 3.7). A purified fraction of pEAQ-*HT* was also analysed under the EM and no VLPs were observed (Figure 3.7A). Purified fractions of VP1 N17D and , VP2 H93C in both instances (Figures 3.7B and 3.7C, respectively) show putative VLPs of ~30 nm (red arrows), with a count of 2 VLPs per field of view and 1 VLP per field of view for five fields of view for VP1 N17D and VP2 H93C, respectively. The presence of other protein aggregates was also noted, which could be some unassembled particles and contaminating host cell proteins. Similarly, the presence of VLPs measuring ~30 nm in diameter, which is consistent with the size of empty FMD capsids (Grubman and Baxt, 2004), were confirmed by EM analysis (red arrows) (Figure 3.7D) in a positive fraction of VP1 N17D/VP4 S73N. The VLP count resulted in 1 VLP per field of view for five fields of view.

On the other hand, no assembled VLPs were observed from EM analysis of VP3 A188V (Figure 3.7E); however, protein aggregates were visible in all the fields of view analysed. It was also noted of the presence of regularly shaped VLPs from expression of oP1-2A (Figure 3.7F) with a count of 3 VLPs per field of view for five fields of view.

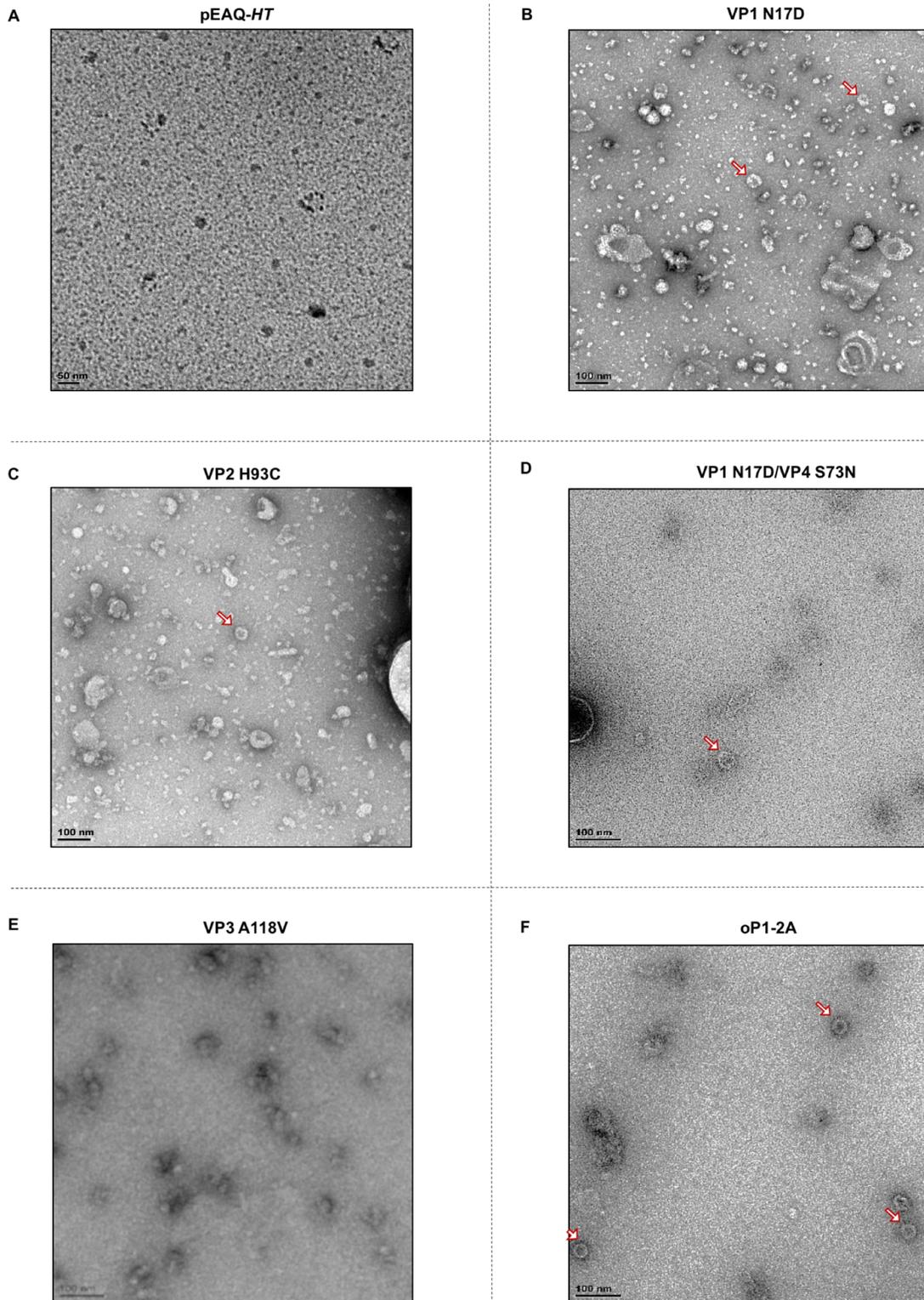


Figure 3.7: EM images of selected purified fractions after purification of VLPs at pH 8.0. (A), (B), (C), (D), (E) and (F) pEAQ-*HT*, VP1 N17D, VP2 H93C, VP1 N17D/VP4 S73N, VP3 A118V and oP1-2A respectively. Magnification of (A): 25 500x. Magnification of (B), (C), (D), (E) and (F): 53 000x. Red arrows point out VLPs of ~30 nm.

To further ascertain if the aberrant cleavage of VP0 into VP2 and VP4 (Curry et al., 1997; Porta et al., 2013a) could have been triggered by encapsidation of plant nucleic acids during VLP formation, the absorbance of selected purified fractions of the mutants were measured at 260 nm (Figure 3.8). The absorbance values at 280 nm were also recorded to correlate protein levels present in the fractions to RNA levels (Figure 3.8).

As previously pointed out by a study performed by Mignaqui et al. (2013), fractions harboring empty capsids would give a value close to zero for absorbance readings recorded at 260 nm due to the lack of nucleic acids. In the present study, attention is drawn to the fact that all the selected fractions showed a reading above zero indicating the presence of nucleic acids in the samples (Figure 3.8). Graphs illustrating the absorbance values for mutants VP1 N17D, VP2 H93C, VP1 N17D/VP4 S73N, VP3 A118V and oP1-2A have a similar trend with A_{260} readings ranging from 103.7 to 123.9 (Figure 3.8A), 61.116 to 121.949 (Figure 3.8B), 114.46 to 126.24 (Figure 3.8C), 118.53 to 123.24 (Figure 3.8D), and 118.6 to 123.7 (Figure 3.8E) respectively. Graphs representing VP1 N17D (Figure 3.8A), VP2 H93C (Figure 3.8B) and VP1 N17D/VP4 S73N (Figure 3.8C) depict the relationship between the decrease in the absorbance recorded at 260 nm to that at 280 nm. Although the absorbance recorded at A_{260} could potentially relate to free nucleic acids lying in the purified samples, the fact that capsid formation was relying on the encapsidation of heterogeneous nucleic acids should not be disregarded. The RNA levels remained constant from fractions 16 to 25 for both the acid sensitive mutant VP3 A118V (Figure 3.8D) and the non-mutated oP1-2A (Figure 3.8E). A peak (Fraction 22) and a trough (Fraction 21) were observed in the A_{280} readings of oP1-2A (Figure 3.8E). Overall, it is clear that some mechanism during empty capsid formation was triggering the interaction between the capsid proteins, thereby inducing the packaging of single-stranded RNA that is not of viral origin (Saunders and Lomonosoff, 2015).

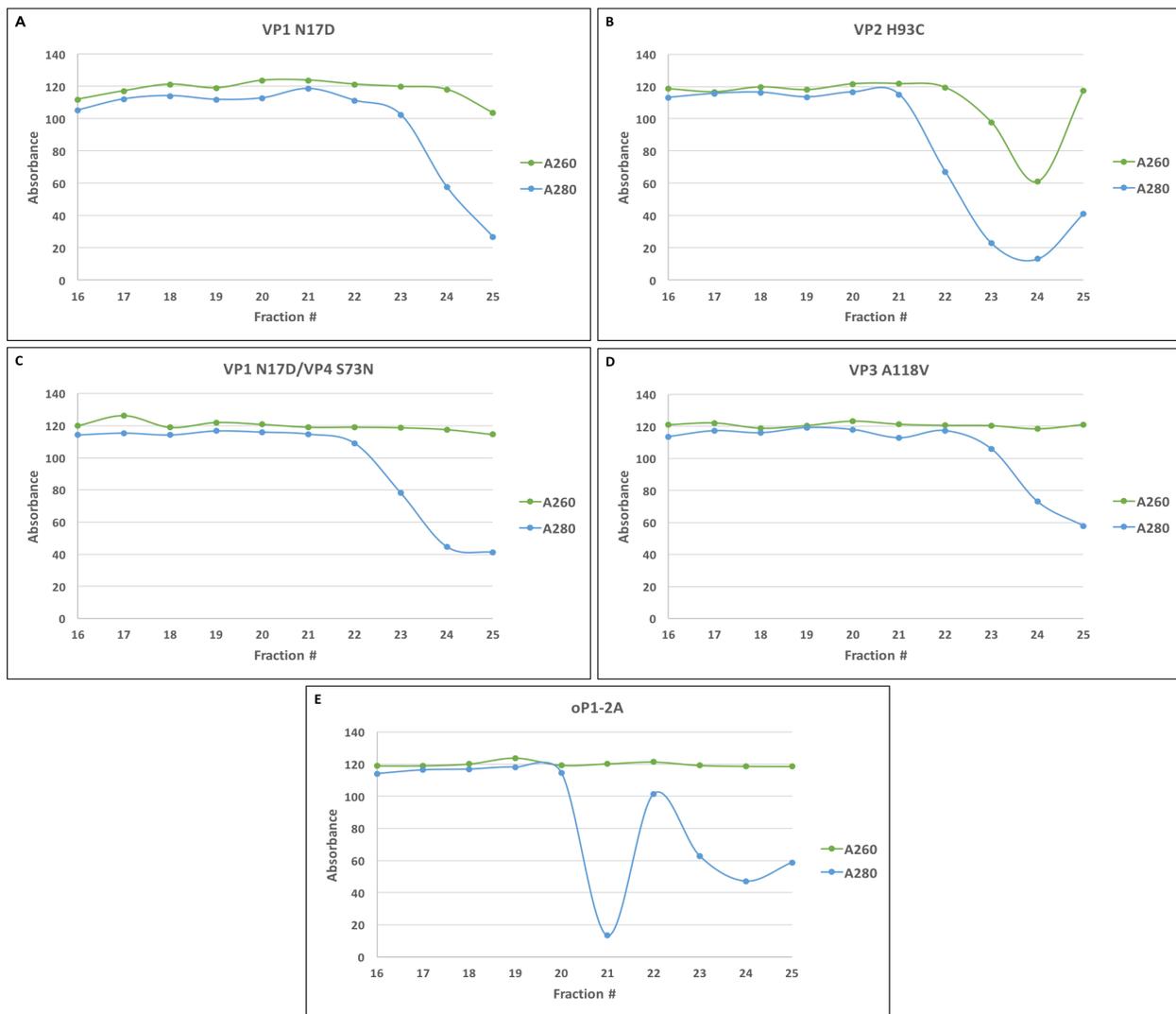


Figure 3.8: Detection of presumptive RNA and protein levels from A₂₆₀ and A₂₈₀ readings of purified fractions after purification. (A) VP1 N17D. (B) VP2 H93C. (C) VP1 N17D/VP4 S73N. (D) VP3 A118V. (E) oP1-2A.

3.3.4. Thermostability/ pH stability assays of mutants' and oP1-2A VLPs

To investigate the thermo- and pH stability of the recombinant particles, aliquots of selected positive fractions (VP1 N17D: fraction 22 (Figure 3.6B); VP2 H93C: fraction 18 (Figure 3.6C); VP1 N17D/VP4 S73N: fraction 19 (Figure 3.6D); VP3 A118V: fraction 22 (Figure 3.6E); oP1-2A: fraction 17 (Figure 3.6F) as determined by western blot and EM analyses (Figure 3.7), were subjected to either acidification for 15 min at room temperature (pH 5.2 and pH 6.2) or heating to 56°C for 1 hour before repeat sedimentation on a continuous 15 to 45% Optiprep™ gradient. Fractions collected after centrifugation were analysed on dot blots. In some cases, an opaque band was

observed after centrifugation. The bands were collected separately after which the rest of the tube was fractionated from the bottom as per usual (Figure 3.9A).

Acidification was carried out by adjusting the pH from 8.0 to either pH 5.2 or pH 6.2 with sodium acetate buffer. Samples were incubated for 15 min at room temperature, after which VLPs were sedimented on Optiprep™ gradients. Analysis of gradient fractions by dot-blot showed that VP1 N17D derived proteins treated at pH 5.2 were present in fractions 4, 5 and in the band, and in all the fractions after treatment at pH 6.2 (Figure 3.9B(i)). The strongest signal as illustrated by the darkest dot from the dot-blot was detected in fraction 4, which implies that empty capsids were stable and were found in the middle of the 5 ml gradient. Heat-treated VP2 H93C mutant derived proteins were barely detected in the fractions after treatment at pH 6.2, but however, the strongest signal was seen from fraction 1 after treatment at 56°C and in fraction 3 after pH 5.2 treatment (Figure 3.9B(ii)). In the case of the acid-resistant mutant, VP1 N17D, VP4 S73N, fractions 3 to 5 were observed as positive for expression of the capsid proteins (Figure 3.9B(iii)) after the pH assays, as well as the band upon treatment at pH 5.2. On the other hand, the dot-blot analysis of the acid sensitive mutant VP3 A118V revealed weak signals in all of the fractions analysed (Figure 3.9B(iv)). As for oP1-2A, fractions 2 to 5, fractions 3 and 5, and fractions 3 to 5, including the bands, were revealed as positive after treatment at 56°C, pH 5.2 and pH 6.2 respectively (Figure 3.9B(v)).

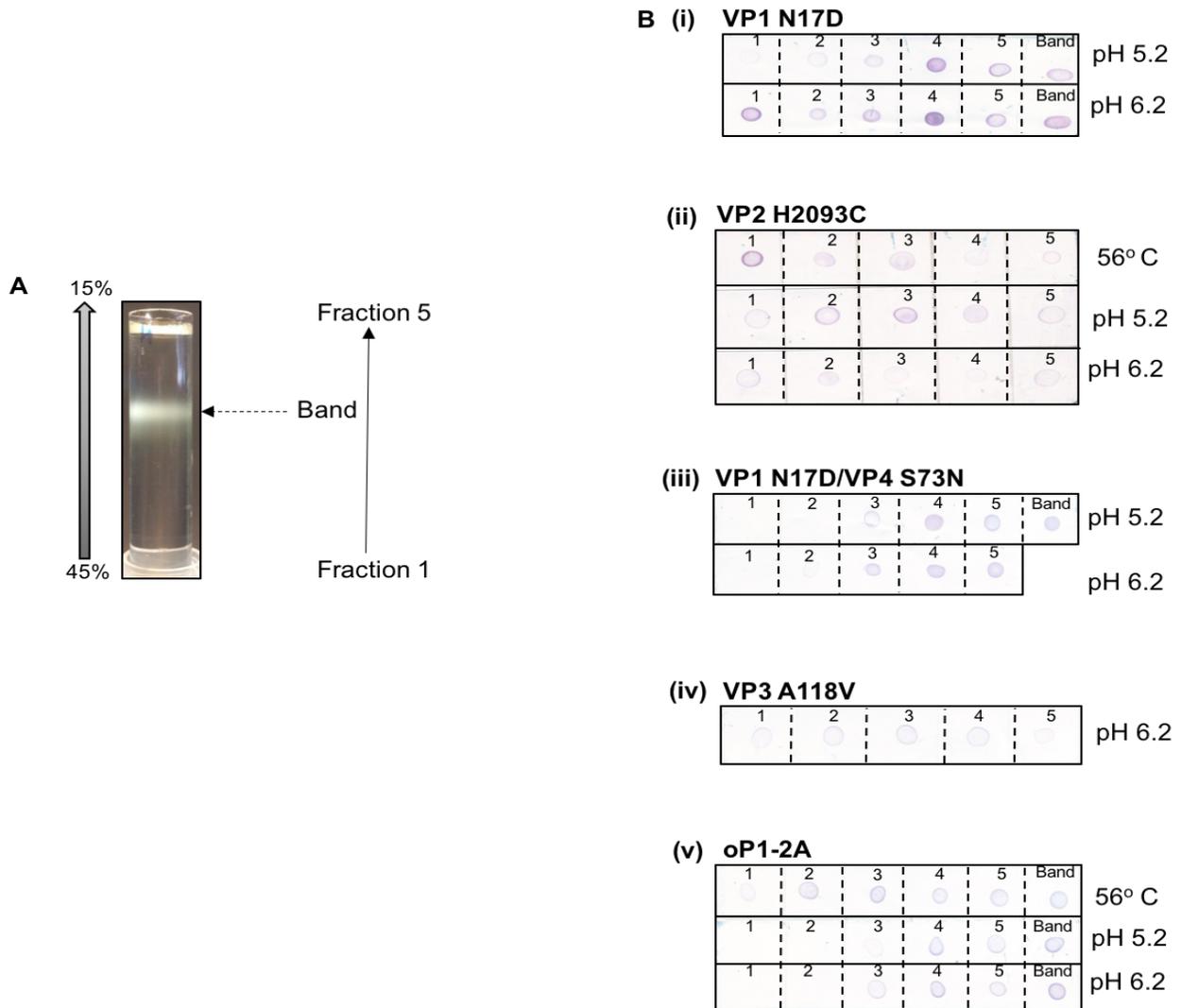


Figure 3.9: Analysis of thermostability and pH stability assays of mutants and wild-type oP1-2A. (A) Linear 15-45% Optiprep™ gradient after repeat sedimentation. (B) Dot-blot analysis of fractions from the gradient and treated with either heat or at different pH, (i) VP1 N17D, (ii) VP2 H93C, (iii) VP1 N17D/VP4 S73N, (iv) VP3 A118V, (v) oP1-2A.

Transmission electron micrographs of the acid resistant mutant VP1 N17D after treatment at pH 6.2 revealed irregularly shaped VLPs (Figure 3.10A, red arrow). Similar results were observed from EM images of VP1 N17D after treatment at pH 5.2, VP1 N17D/VP4 S73N and VP2 H93C treated at pH 5.2 and 6.2 (data not shown). However, Figure 3.10B, representing VP2 H93C, after treatment at 56°C, reveals the presence of many protein aggregates and 1 VLP per field of view (red arrow), for 5 fields of view was recorded.

On the other hand, in the case of the acid sensitive mutant VP3 A118V, no empty capsids were detected by EM analysis (Figure 3.10C). Interestingly, fractions representing oP1-2A (Figures 3.10D and 3.10E) after the pH assays showed the presence of regularly shaped VLPs (red arrows). It was also observed that fewer VLPs were present in the sample of oP1-2A, treated at 56°C (Figure 3.10F), along with other host proteins. The VLP count after treatment of oP1-2A at 56°C, at pH 5.2 and at pH 6.2 resulted in 1 VLP per view, 1 VLP per view and 3 VLPs per view, respectively. The total number of fields of view was kept to 5.

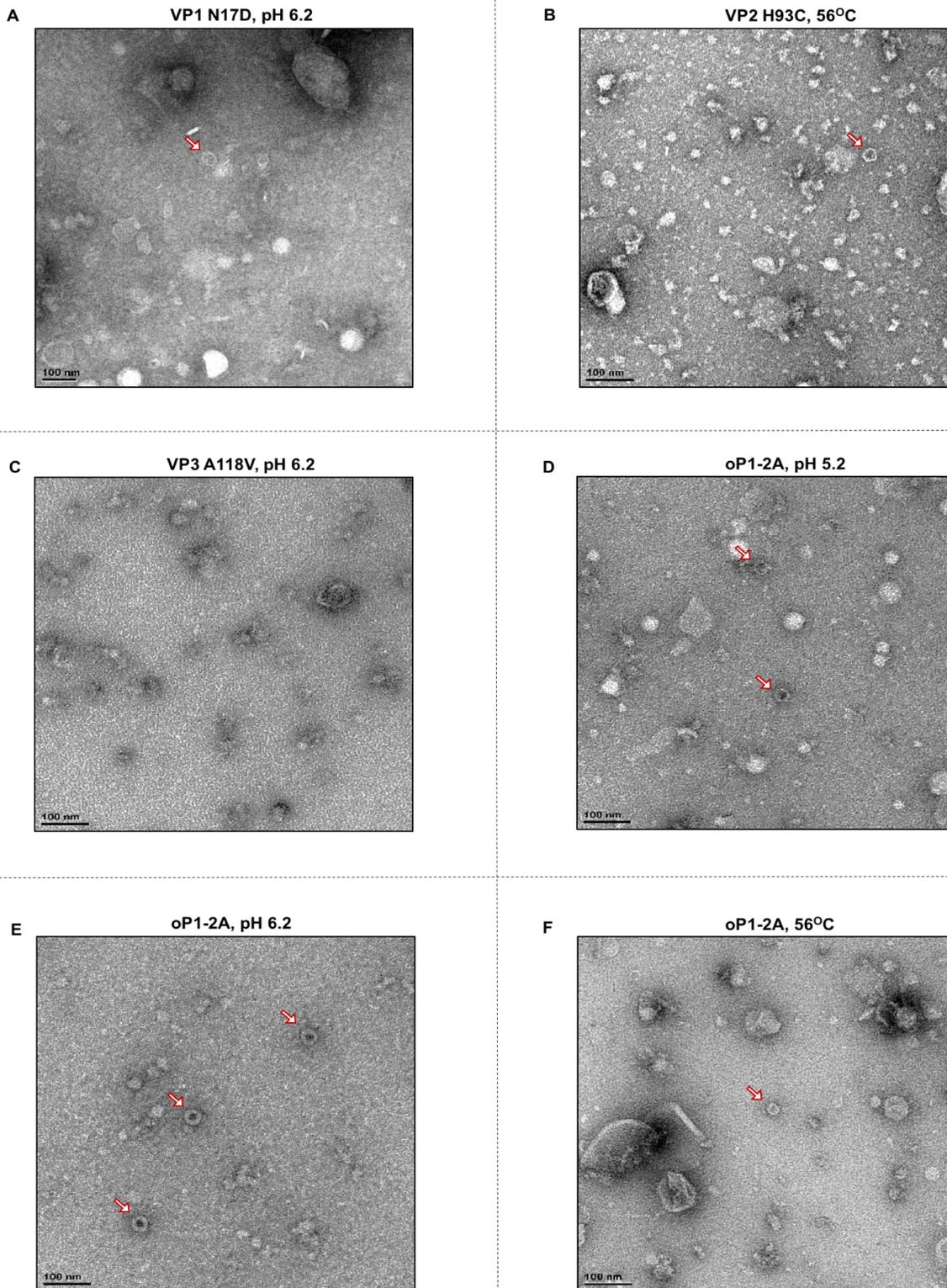


Figure 3.10: EM images of selected purified fractions after heat and pH assays on selected fractions. (A) VP1 N17D, pH 6.2. (B) VP2 H93C, 56°C. (C) VP3 A118V, pH 6.2. (D) oP1-2A, pH 5.2. (E) oP1-2A, pH 6.2. (F) oP1-2A, 56°C. Magnification at 53 000x. Red arrows point out VLPs of ~30 nm.

The acid-treated and heat treated fractions were further analysed on western blots which showed the presence of smears where protein bands were expected to be for the mutants; this could be attributed to degradation of the proteins after treatment at extreme conditions (data not shown). Stability analysis oP1-2A capsid proteins were further confirmed by the detection of the expected bands on a western blot (Figure 3.11). The fraction labelled 'band', corresponding to the opaque band collected after centrifugation, demonstrated the highest amount of all the VPs after treatment at pH 5.2 (Figure 3.11A) and pH 6.2 (Figure 3.11B).

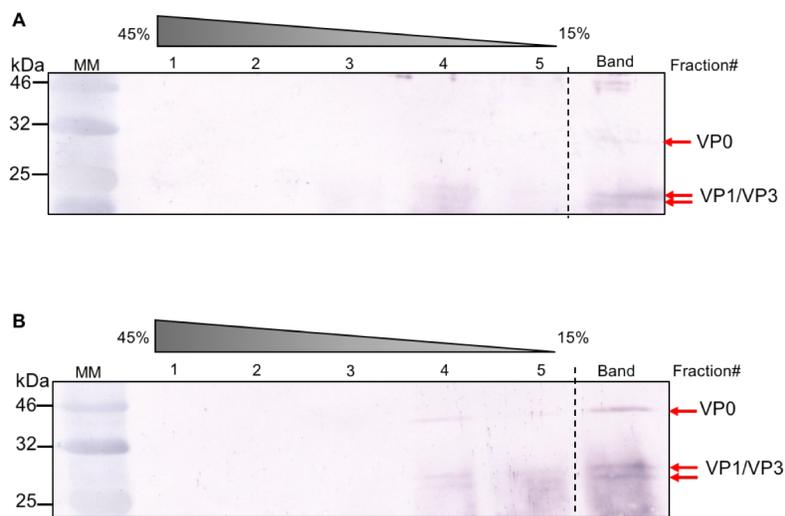


Figure 3.11: Western blot analysis of oP1-2A fractions from pH assays. (A) pH assay was performed at pH 5.2. (B) pH assay was performed at pH 6.2. The capsid proteins are represented as VP0 (~34 kDa), VP1 (~27 kDa) and VP3 (~27 kDa). (MM) Colour Prestained Protein Standard (New England Biolabs).

3.4. DISCUSSION

Understanding the molecular basis underlying the control of capsid stability is relevant for both knowledge of the basic aspects of FMDV and for the design of vaccines based on stable empty capsids (Caridi et al., 2015). In this study, I engineered previously described mutants in order to assess expression in plants: these were two mutants with increased resistance to pH, namely VP1 N17D and VP1 N17D/VP4 S73N (Caridi et al., 2015; Liang et al., 2014), one heat and acid resistant mutant, VP2 H93C (Kotecha et al., 2015; Porta et al., 2013a) and an acid labile mutant VP3 A118V (Caridi et al., 2015; Martin-Acebes et al., 2010). These site-directed mutations directed at the

pentamer interfaces (Figure 3.1) of empty capsids should contribute to the stability and thus to a longer shelf-life of recombinant empty capsid vaccines, as shown by kinetics and the assays in literature. Furthermore, for comparative purposes, the non-mutated oP1-2A was also expressed and tested for thermostability and pH stability under the same conditions as its mutants. Caridi et al. (2015) and Roy and Post (2012) reported that there are functional relationships between distant amino acid residues which have been described for picornavirus uncoating, and thus these mutations were made to analyse whether these replacements could compensate for the heat and acid labile phenotypes. The mutants: VP1 N17D, VP2 H93C, VP1 N17D/VP4 S73N and VP3 A118V and wild-type oP1-2A were expressed in the absence of the FMDV 3C-protease and subjected to extreme pH and temperature conditions.

As postulated in section 1.4.4, upon encapsidation of viral RNA, FMDV VP0 (~34 kDa) is usually cleaved into VP2 (~28 kDa) and VP4 (~6 kDa) (Arnold et al., 2008; Hogle et al., 1985; Jacobson and Baltimore, 1968). According to literature, aberrant cleavage of VP0 in FMDV empty capsids has been demonstrated, thus implying that viral RNA is required for the proper cleavage event to occur, since it is noted that cleavage of VP0 usually should not occur in VLPs. Moreover, the mechanism behind this unusual cleavage of VP0 is not clear and is thought to be linked to an autocatalytic mechanism which could potentially be responsible for the normal process of VP4 cleavage (Curry et al., 1995; Curry et al., 1997; Porta et al., 2013a). This phenomenon was observed on western blots of the time-trials of all the mutants (Figure 3.5), where bands at ~28 kDa, representing VP2 were detected. Furthermore, as cleavage proceeded in the absence of the 3C-protease, the occurrence of other autocatalytic processes should not be disregarded.

Optimum conditions for large-scale infiltration and expression of the mutant derived VPs were determined from analysis of the time-trials and are listed in Table 3.3. To test if these VPs could assemble into VLPs, plants were infiltrated at the desired OD₆₀₀ values and harvested at 5 dpi. Likewise, plants were also infiltrated with oP1-2A and were processed. Purified fractions were further analysed by western blotting and indicated the presence of bands representing only VP2 (~28 kDa) from expression of VP1 N17 (Figure 3.6B) and VP1 N17D/VP4 S73N (Figure 3.6D), with EM analysis showing the presence of numerous protein aggregates (Figures 3.7B and 3.7D). A plausible explanation for the lack of observation of the other VPs (VP1, VP3 and VP4) was the fact

that maturation processes at room temperature and the absence of the 3C-protease, could have led to degradation of the other VPs.

Western blot analyses of the heat and acid resistant mutant VP2 H93C (Figure 3.6C) and oP1-2A (Figure 3.6F) were very similar, as evidenced by bands representing VP0 (~34 kDa), VP1 (~27 kDa) and VP3 (~27 kDa). The substitution of the His residue in the VP2 segment, located at the icosahedral 2-fold axis between adjacent pentamers (Ellard et al., 1999), by a Cys residue, would ultimately help engineer a disulfide bond, which is known to stabilize many extracellular proteins and also some virus capsids (Wikoff et al., 2000). These covalent cross-links are usually more robust than the non-covalent interactions holding protein assemblies together. In this study, however, the conformation of oP1-2A VLPs (Figure 3.7F) were observed to be more uniform than those of VP2 H93C (Figure 3.7C). This could be related to the presence of a relatively high amount of nucleic acids present in the purified fractions (Figure 3.8), supporting the hypothesis that these empty capsids encapsidated heterogeneous plant nucleic acids, sometimes resulting in non-uniform assembly of the mutant VLPs or in the proper assembly of oP1-2A VLPs.

Intriguingly, after thermostability and pH assays were analysed (Figure 3.9), the most stable VLPs were observed for the non-mutated oP1-2A (Figures 3.10D, 3.10E and 3.10F). A close assessment of the VLPs viewed for VP2 H93C was one VLP per field of view for 5 fields of view (Figure 3.10C) after treatment at 56°C compared to 3 VLPs per field of view from a purified oP1-2A fraction. Vaccines composed of empty capsids that can withstand higher temperatures would be very advantageous especially for use in endemic regions where socioeconomic factors impede adequate cold chain distribution. Thus, though the yields of VLPs were low for both the mutant VP2 H93C and oP1-2A, their ability to withstand these non-optimal conditions prove to be beneficial and their expression could possibly be optimised by using a larger number of plants.

Studies pertaining to such acid and heat resistant mutations predominantly implied the testing of mutant forms of FMDV virions whereby Rincón et al. (2014) demonstrated how mutated FMDV virions were shown to be more stable than the parental virion by engineering some mutations, thus ameliorating electrostatic repulsion between pentamers; acid lability is also directly related to thermal instability. Rincón et al. (2014) proposed a model in the form of an energy diagram (Figure 3.12) that acidic-to-neutral mutations will improve electrostatic repulsions. These

mutations are postulated to cause a decrease in the free energy of the 'capsid' state without affecting the free energy of the transition state where unfavorable electrostatic interactions have not yet been formed, resulting in the net effect of an increase in the energy barrier between the two states. Consequently, this gives a reduced rate of irreversible dissociation at a certain temperature. Furthermore, it can be formulated that in the absence of the expression of the 3C-protease and its cleavage properties, oP1-2A could have not only relied on the cleavage of plant proteases as stipulated in Chapter 2 (Veerapen et al., 2017, manuscript submitted for publication), and thus there might have been some random cleavages on the capsid-precursor oP1-2A, conforming to formation of stable covalent bonds between pentamers which are behaving in a similar way as would acid-to-neutral mutants. Taken altogether, the capsid originating from oP1-2A has been kinetically stabilized (Figure 3.12, represented by the greater k_d) (Sivertsson and Itzhaki, 2014), implying that its dissociation into pentamers will be slowed down in an acidic environment or at high temperatures. However, a low amount of VLPs were still observed from EM images (Figures 3.10D, 3.10E and 3.10F) which could be attributed to the low amount of starting biomass used or the presence of other plant proteins in the samples could interfere with the rate of formation of the empty capsids.

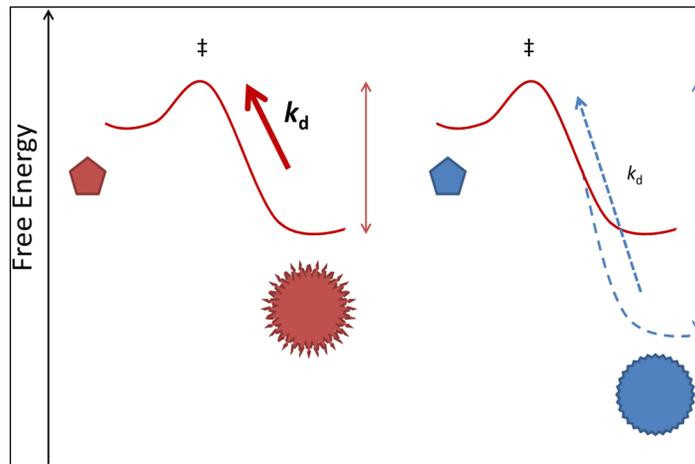


Figure 3.12: Energy diagram illustrating the kinetic stabilization of FMDV by mutation. The parent virus is represented in red and the acidic-to-neutral mutation is in blue. The associated state for irreversible dissociation into pentamers is stabilized by the mutations (the greater k_d), giving a kinetically stabilized capsid. The transition state is represented by (\ddagger). Taken from Sivertsson and Itzhaki (2014).

In conclusion, as it is known that FMDV is acid and heat labile, it was imperative to engineer mutations which would help promote stability and formation of VLPs. However, it was shown in this study that the non-mutated oP1-2A is giving rise to empty capsids that that can tolerate heat and relatively extreme acidic conditions, better than the mutants generated, which further represents a potential candidate vaccine which would promote shelf-life. However, the attempt to acquire more VLPs by stabilizing the bonds at the pentamer interfaces, through these mutations, was not successful. Therefore, it can further be concluded that other external factors, such as the necrosis of leaves, are influencing the expression of the capsid proteins, resulting in low number of VLPs.

CHAPTER 4

Optimisation of P1-2A expression in *N. benthamiana* and preliminary immunogenicity studies of vaccine candidates in mice

4.1. INTRODUCTION

Plant-based and subunit vaccines are considered to be easier to handle than conventional equivalents as their preparation circumvents the high biocontainment issues required for many cell-based vaccines, and especially those made from virulent live viruses (Laere et al., 2016). Moreover, plants as bioreactors are ideal for the manufacture of high-demand vaccines based on the economy of scale offered by agricultural production (Merlin et al., 2014; Rybicki, 2014). In this part of my study, the number of plants used for expression was scaled up to produce sufficient FMDV empty capsids to determine their immunogenicity in mice.

A prophylactic FMDV vaccine in the form of VLPs produced by the expression of the capsid-precursor P1-2A, would be advantageous as literature has shown that the co-expression of P1-2A with 3C-protease could lead to deleterious effects: the protease exhibits “host cell shutoff” phenotype as besides its mode of action on the capsid precursor, it also cleaves host cell proteins (Li et al., 2008; Mignaquí et al., 2013; Porta et al., 2013b). Preliminary testing of such a vaccine in large animals, such as cattle, could prove to be expensive and unwieldy. Molin-Capeti et al. (2013) demonstrated in Brazil how the Balb/C mouse model can be used instead of cattle for quality control of inactivated vaccines. The authors tested the potency of a FMD vaccine by antibody titration against the structural viral proteins in sera obtained from cattle, and compared the results with humoral antibodies produced by Balb/C mice to a FMDV experimental vaccine. Their results showed that even though the mice were vaccinated with a lower volume of the vaccine compared to doses administered to the cattle, an adequate immune response was induced and a similar immune response was obtained at 21 days post vaccination (dpv). Furthermore, Liu et al. (2017) showed how chimaeric FMDV partially- and fully-assembled VLPs provided immunity in guinea-pigs and four out of five animals showed complete protection after challenge, while Jin et al. (2007) demonstrated how FMDV VLPs vaccine elicited high levels of antigen-specific IgG

production, T-cell proliferation, cytotoxic T lymphocyte response and cytokine production in mice. In this study, I evaluated the immunity of a mixed population of P1-2A VLPs and pentamers in female Balb/C mice.

To compensate for the low immunogenicity of non-replicating vaccine antigens, adjuvants are widely co-administered with antigens to enhance the clinical efficacy of the vaccine. Recently, inactivated FMDV vaccines used worldwide have shown an improvement in the level of immunity conferred, and the development of long lasting immune responses, with the use of oil-based adjuvants (Awate et al., 2013; Savelkoul et al., 2015). It is also known that oil-adjuvanted vaccines especially increase humoral immunity (Doel, 1996). Of the many oil adjuvants which are available on the market, a water-in-oil-in-water (W/O/W) type adjuvant, Montanide ISA 206 (Seppic, Paris, France), is used in FMD vaccines. Other oil adjuvants from the same company include the Montanide ISA 50 (water-in-oil [W/O]), ISA 70 (W/O) and ISA 25 (oil-in-water [O/W]), with the main differences between these adjuvants observed in water to oil ratio and in safety, where for instance, Montanides ISA 50 and ISA 70 are better tolerated than the others (Aucouturier et al., 2001).

The objective of this study was to assess the immunogenicity of a FMDV P1-2A vaccine candidate consisting of a mixed population of VLPs and pentamers, in the presence and absence of Montanide ISA 50 V 2 (Seppic, Paris, France) in Balb/C mice.

4.2. MATERIALS AND METHODS

Ultracentrifugal purification using a continuous OptiprepTM gradient resulted in low yields of VLPs from the loss of capsid proteins through each purification step. Therefore, candidate vaccines were prepared by partially purifying P1-2A VLPs. OptiprepTM is an endotoxin-free density medium, which has been shown to be safe for inoculation in animals.

4.2.1. Purification of empty capsids

Empty capsids were purified via two methods for comparison purposes. In both methods, 100 grams of plant biomass was extracted in 2x NTE buffer. Immunogenicity studies were carried out using purified samples from method B.

Method A: Purification of putative VLPs was performed as described in section 2.2.11, using Optiprep™ cushioning and density gradient centrifugation.

Method B: Partial purification of empty capsids consisted of loading clarified crude plant extract onto 30% Optiprep™ cushions and pelleting the VLPs (as described in section 2.2.11). The pellets containing VLPs were resuspended in 7 ml 1x NTE buffer (pH 8.0) and matured as described in section 2.2.11. The matured sample was centrifuged at 30 021 *xg* using a JA-14 rotor (Beckman) at 4°C, for 15 min to remove any insoluble material from the suspension. After centrifugation, the supernatant was collected for analysis and immunogenicity studies.

4.2.2. Protein analysis

SDS-PAGE, western blotting and dot-blots were performed as described in section 2.2.10.1, 2.2.10.2, 2.2.10.3, respectively. Silver staining was performed on 15% SDS-PAGE gels according to the Silver Stain Plus™ (Bio-Rad) protocol as summarized in Table 4.1. All the steps included gentle shaking at room temperature.

The gels were washed in deionized water after the stop step.

Table 4.1: Silver staining steps and reagents

Step	Reagents	Time/min
Fixative	50% V/V methanol 10% V/V acetic acid 10% V/V fixative enhancer concentrate™ 30% deionized distilled water	20
Rinse	400 ml Deionized distilled water	2x 10
Staining and developing	35 ml deionized water 5 ml silver complex solution™ 5 ml reduction moderator solution™ 5 ml image development reagent™ 50 ml development accelerator reagent™	30
Stop	5% acetic acid	15

4.2.4. Transmission Electron Microscopy (TEM)

TEM analysis was performed on selected purified fractions as described in section 2.2.12. Immunogold labelling was also carried out on the partially purified VLPs to further characterize the vaccine preparation. Partially purified fractions were adsorbed onto 200 mesh copper grids for 30 min and washed with water. Grids were probed with rabbit anti-P1-2A (diluted 1:10 in 1% Bovine Serum Albumin [BSA]/ 1x PBS, pH 7.2) for 10 min at room temperature, followed by two washes with 1% BSA/1x PBS, pH 7.2. A 10 min incubation with anti-rabbit IgG (whole molecule)-gold antibody produced in goat (Sigma-Aldrich) (diluted 1:50 in 1% PBS, pH 7.4) was performed which was followed by two washes with water. Grids were negatively stained with 2% uranyl acetate.

4.2.5. Quantification of partially plant-purified P1-2A VLPs and intermediates

E. coli-produced P1-2A quantified via gel densitometry (section 2.2.13) was used as antigen to create a standard curve with which to quantitate the plant-produced P1-2A vaccine antigens.

96-well Maxisorp[®] microtitre plates (Nunc) were coated in triplicate with two-fold doubling dilutions (made in 10 mM Tris, pH 8.5) of the *E. coli*-produced P1-2A standard starting at a concentration of 1 ng/μl and the plant-produced P1-2A vaccine candidates, starting from the undiluted sample, that had to be quantified. Plates were incubated overnight at 4°C, after which the wells were blocked for 1 hour at 37°C in TBS blocking buffer (1x Tris-Buffered Saline [TBS] [0.0083 M Tris, 0.15 M NaCl], 5% Non-Fat Dry Milk [NFDM], pH 7.5) and washed four times with 1x TST (1x TBS with 0.05% Tween-20, pH 7.5). Plates were probed with rabbit anti-P1-2A (1:100 diluted in blocking buffer) for 1 hour at 37°C, followed by four washes with 1x TST, pH 7.5. After washing wells were probed with 1:5000 alkaline-phosphatase conjugated anti-rabbit secondary antibody (Sigma-Aldrich) for 1 hour at 37°C and washed four times in 1x TST, pH 9.0. The substrate, SIGMAFAST[™]p-Nitrophenyl phosphate (pNPP, Sigma-Aldrich) was added to each well and plates incubated in the dark for 30 min after which the absorbance was read at A₄₀₅ using a microplate spectrophotometer PowerWave XS[™] (BioTek[®]). Data were analysed using Microsoft[®] Excel.

4.2.6. Total Soluble Protein (TSP)

TSP of the partially purified plant-produced P1-2A and pEAQ-*HT* samples were determined as described in section 3.2.5. The TSP was performed to ensure the negative control contained equivalent amounts of TSP in comparison to the other P1-2A VLPs vaccine candidates (Table 4.2).

4.2.7. Immunization of mice

Female Balb/C mice obtained from the University of Cape Town (UCT), Research Animal Facility, Specified-Pathogen Free Unit (UCT RAF SPF Unit, Cape Town, South Africa) were kept under Biosafety Level 2 (BSL-2) conditions in the Animal Unit at the Health Science Faculty, UCT. Approval for this study was granted by the Animal Research Ethics Committee at UCT (AEC No. 016-026).

Female mice (8 to 10 weeks old) were immunized with the vaccine candidates prepared as described in section 4.2.1, Method B. *N. benthamiana* leaves infiltrated with pEAQ-*HT* were partially purified using the same method used for the candidate vaccines, and served as a negative control. Vaccination details are summarized in Table 4.2 below.

Table 4.2: Plant-produced vaccine candidates used in the immunogenicity study.

Vaccine	Group number ¹ (n=5)	Adjuvant	Antigen dose (µg)
P1-2A VLPs	1	Montanide ISA 50 V 2 (Seppic, Paris, France)	5
P1-2A VLPs	2	n/a	5
pEAQ- <i>HT</i>	3	n/a	5 µg of TSP

¹n= number of mice per group

The partially purified vaccine antigens were adjusted with NTE buffer, pH 8.0 or Montanide ISA 50 V 2 (Seppic, Paris, France) (1:1 ratio) to contain 5 µg in a total volume of 100 µl per dose.

Mice were subcutaneously injected with 50 µl each into both the right and left flanks. Pre-bleeds of 100 µl were collected on the first day of vaccination (day 0) to account for baseline antibody production and mice were boosted on days 13, 27 and 44 post initial vaccination. The bleeds were collected on days 41 and 58, after which serum was isolated and stored at -20°C.

4.2.8. Detection of anti-P1-2A antibodies in mouse sera

The anti-P1-2A response was determined by indirect ELISA (I-ELISA) as described in section 4.2.5 with some modifications. 96-well Maxisorp[®] microtitre plates (Nunc) were coated with 100 µl of 1 ng/µl *E. coli*-produced P1-2A (section 2.2.13).

To evaluate the anti-P1-2A immune response elicited by each mouse, the prebleed, bleed collected at 41 dpv and final bleed sera were diluted 1:50 in TBS blocking buffer and incubated as described above. After incubation with the mouse sera, plates were washed followed by incubation with 1:10 000 goat anti-mouse IgG alkaline phosphatase conjugate (Sigma-Aldrich), diluted in TBS blocking buffer, followed by detection with pNPP. The plates were left in the dark for 30 minutes and absorbance was read as described in section 4.2.5.

To determine the anti-P1-2A binding titres, mouse sera from each group were pooled into the respective vaccine groups for analysis. The final sera were diluted in TBS blocking buffer in a four-fold series in triplicate ranging from 1:50 dilution to 1:3 276 800. Mouse sera from the group vaccinated with plant-purified pEAQ-*HT* served as a negative control. Positive control wells contained rabbit-raised anti-P1-2A to validate the ELISA. Negative ELISA controls consisted of wells containing antigen, and incubation with secondary antibody only.

The anti-P1-2A binding titres were expressed as a reciprocal of the maximum serum dilution which provided absorbance values that were three times greater than the corresponding pre-bleed serum diluted at 1:50.

4.2.8. Statistical analysis

One-way Analysis of Variance (ANOVA) was used to test the null hypothesis of same means among the three groups of mice (Table 4.2) versus the alternative hypothesis that at least one of the means of the groups differs at a 5% level of significance. The Tukey's HSD test was then used to determine the significance ($p= 0.01$) to identify the difference between the pairs of means of the different groups of mice, using Microsoft[®] Excel.

4.3. RESULTS

4.3.1. Production of P1-2A VLPs production for immunogenicity studies

4.3.1.1. Purification through a continuous gradient

Production of VLPs was scaled up via the use of a higher number of plants (~80 plants) infiltrated with oP1-2A (OD₆₀₀ of 0.5). Approximately 100 grams of biomass was obtained and purified using the method described in section 2.2.11.

Figure 4.1A illustrates a 5 to 20% Optiprep™ gradient onto which a matured oP1-2A sample was loaded and centrifuged (175 000 $\times g$ for 2 h 30, SW 32Ti, 12°C). Essentially, quantification via ELISA requires the detection of the antigen of interest in its non-denatured form. To assess whether the rabbit-raised anti-P1-2A could be used as primary antibody in ELISAs to detect conformationally specific epitopes, a dot-blot was performed using both non-denatured and denatured purified P1-2A capsid proteins (Figure 4.1B). The signal detected for both the plant-produced and *E. coli*-produced oP1-2A was stronger in the non-denatured forms than in the denatured forms, suggesting that the rabbit-raised anti-P1-2A would serve as a good primary antibody.

Western blotting (Figure 4.1C(i)) showed that fractions 4 to 14 were positive for the presence of capsid proteins with the detection of VP0, VP1 and VP3 as indicated with the red arrows. Fractions 9 to 11 showed the most prominent bands representing the VPs. EM analysis of fractions 10 to 14 demonstrated the presence of VLPs of ~30 nm, with a count of 5 VLPs per field of view for five fields of view (data not shown), which were selected for silver staining, which further showed the presence of the capsid proteins, with faint bands representing VP0 at ~34 kDa (Figure 4.1C(ii)).

An I-ELISA using rabbit-raised anti-P1-2A as primary antibody was performed to quantify the fractions containing the putative VLPs. ELISAs performed on selected fractions 10 to 14 showed a range of concentrations from 197 ng/ml to 170 ng/ml, which represents a yield of ~0.0030 μg per gram of fresh leaf material. These yields were extremely low, therefore, plant material infiltrated with oP1-2A was partially purified by pelleting putative VLPs through a 30% Optiprep™ cushion as described in section 4.2.1 (Method B).

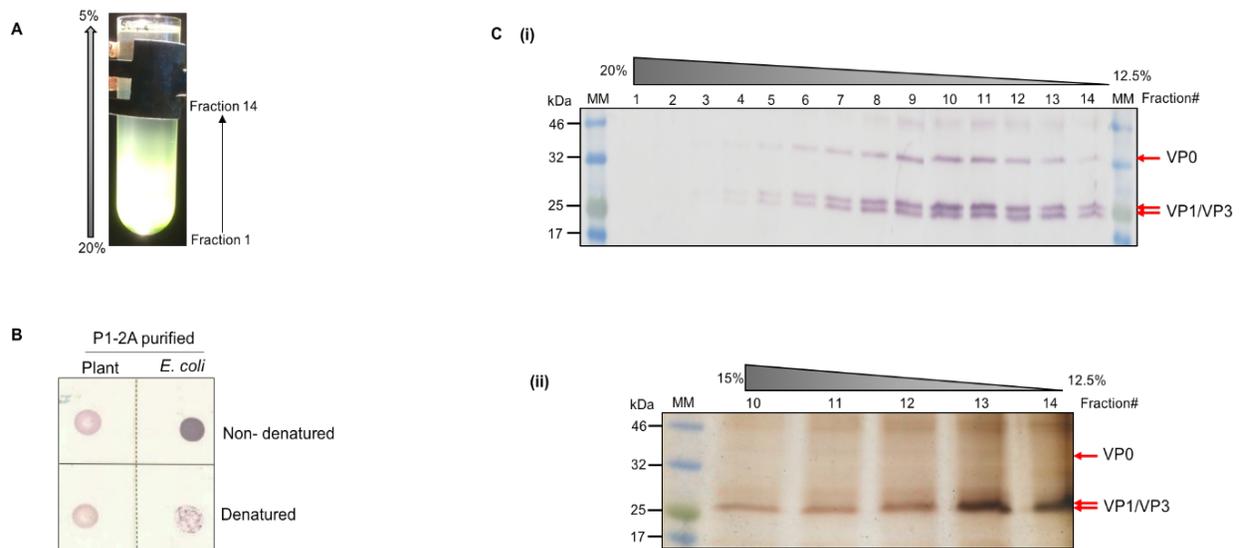


Figure 4.1: Analysis of oP1-2A VLP purification through a continuous gradient. (A) 5 to 20% continuous Optiprep™ gradient, showing the positions of the positive fractions. (B) Dot-blot of non-denatured and denatured plant and *E. coli*-produced oP1-2A detected with rabbit-raised anti-P1-2A. (C) (i) and (ii) Western blot and Silver Stain analyses of purified fractions collected from the continuous gradient respectively. (MM) Colour Prestained Protein Standard (New England Biolabs).

4.3.1.1. Partial purification of putative VLPs

VLPs were pelleted through Optiprep™ cushions, resuspended in 1x NTE buffer, pH 8.0 and resuspended samples pooled (Figure 4.2). Samples were subjected to maturation at room temperature for 30 min and were centrifuged at low speed to further clarify the sample by removing any insoluble material. The pellet and the supernatant collected were both analysed on western blots (Figure 4.2B) and Coomassie-stained gels (data not shown). The VPs of interest VP0 (~34 kDa), VP1 and VP3 (~27 kDa) were present in both the pellet and the supernatant (Figure 4.2B), with a higher concentration of these present in the supernatant as indicated by the intensity of the bands on the western blot.

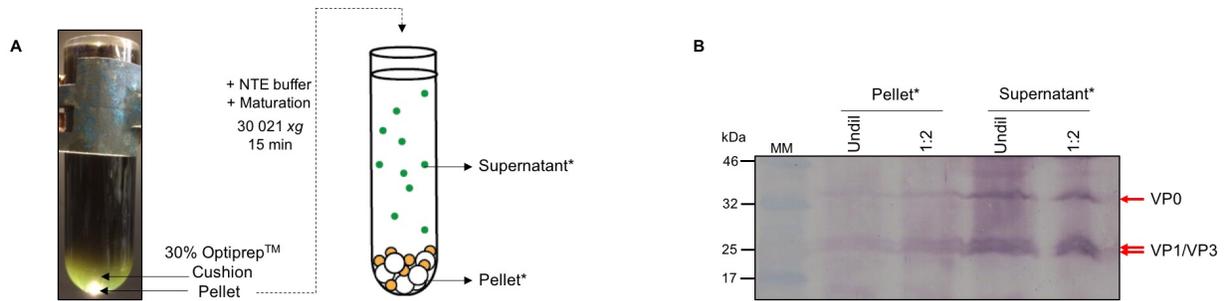


Figure 4.2: Partial purification of oP1-2A. (A) 30% cushion onto which clarified plant extract was loaded and centrifuged at 175 000 xg for 2h 45 at 12°C. Pellets were resuspended in NTE buffer and sample was further clarified. (B) Western blot analysis of pellet and supernatant after centrifugation at 30 021 xg for 15 min at 4°C, VP0 (~34 kDa), VP1 and VP3 (~27 kDa), Undil: undiluted sample. (MM) Colour Prestained Protein Standard (New England Biolabs).

EM analysis (Figure 4.3) illustrated the presence of VLPs measuring ~30 nm (red arrows) in samples diluted 1:50 and at 1:100. Immunogold-labelled (Figure 4.3) grids of the same samples show the presence of gold particles measuring 10 nm (blue arrows), which however were not seen binding to the VLPs, but could be associating with pentamers found in the partially purified samples. The absence of such gold particles was noted from the negative control consisting of an empty grid probed with rabbit-raised P1-2A and with the anti-rabbit IgG (whole molecule) gold antibody produced in goat (Figure 4.3).

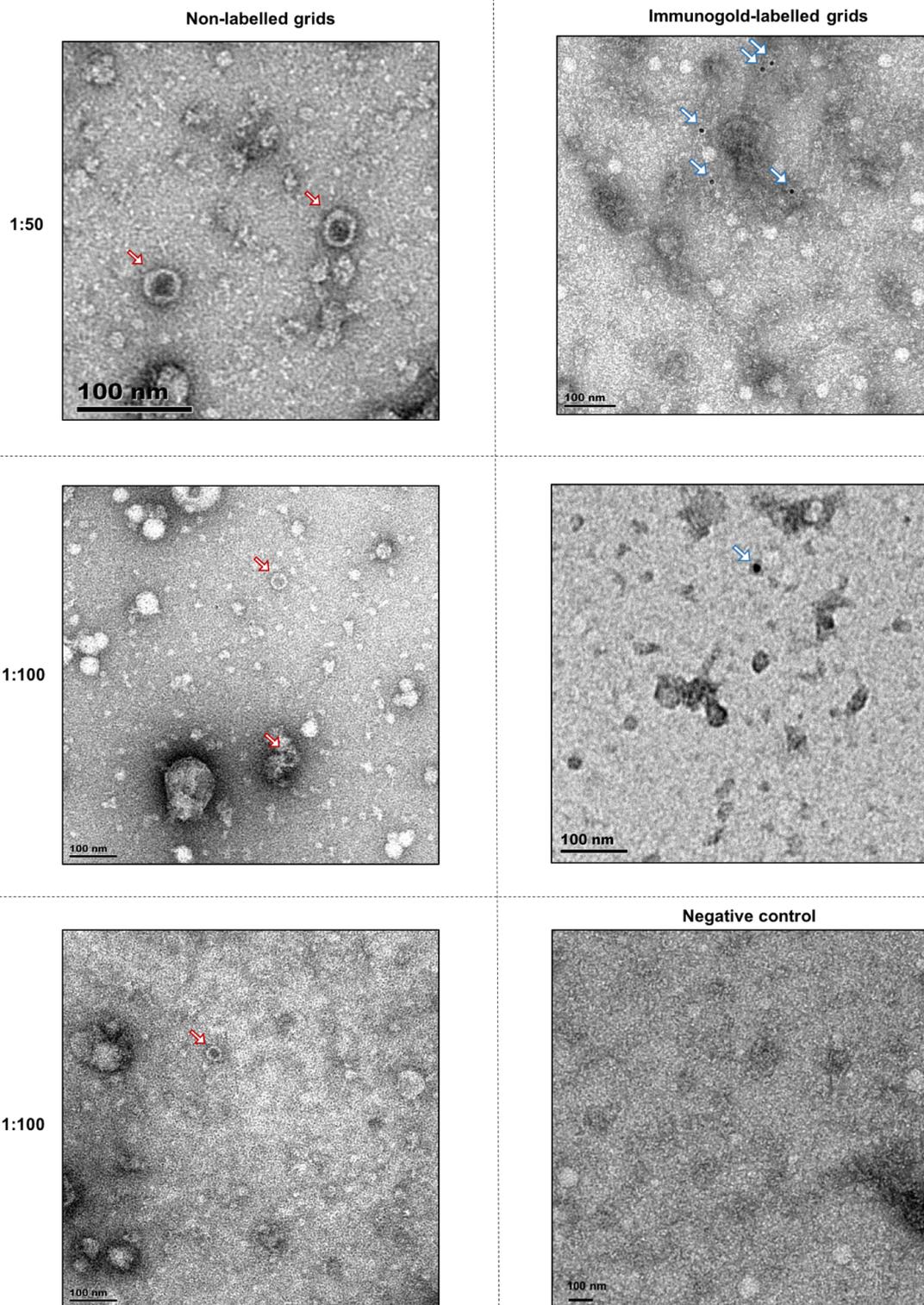


Figure 4.3: TEM analysis at a magnification of 53 000x, VLPs of ~30 nm illustrated by the red arrows (left), 10 nm gold particles (blue arrows) attached to pentamers (right). Negative control consisted of an empty grid probed with anti-P1-2A anti-rabbit and with the anti-rabbit IgG (whole molecule)- gold antibody produced in goat.

4.3.2. TSP quantification of oP1-2A VLPs and pEAQ-HT

The concentration of protein in the partially purified oP1-2A sample was determined to be ~6 mg/ml with the Bio-Rad *DC* assay. Using the same method, it was determined that the negative control, partially purified pEAQ-HT, had a concentration of ~0.3 mg/ml. To more accurately quantitate oP1-2A oP1-2A VLPs, I-ELISAs were carried out, which showed that the concentration of oP1-2A-specific proteins in the sample amounted to 0.104 mg/ml (data not shown), which is lower than the concentration obtained from the Bio-Rad *DC* assay, as expected.

4.3.3. ELISA detection of anti- P1-2A antibodies in mouse sera

To characterize the immunogenicity induced by the P1-2A VLPs, two groups of five mice were initially inoculated with either P1-2A VLPs only, or with P1-2A VLPs formulated with adjuvant. As these VLPs were partially purified, a negative control in the form of partially purified crude extract from plants containing pEAQ-HT was used to inoculate a third group of mice, as summarized in Table 4.2. The animals were bled at 0, 41 and 58 dpv and serum from each mouse was initially analysed by I-ELISA. The error bars indicate the standard deviation which represent the technical repeats of ELISA performed on each mouse serum: each standard deviation shows the standard deviation within the mean of three readings (for the same mouse) from the ELISA plate. The mice vaccinated with P1-2A VLPs (Figure 4.4A) showed a slight increase of anti-P1-2A antibodies at 41 dpv in all mice except for mouse 4, which showed a slight decrease in the response from the prebleed (~0.012) as compared to 41 dpv (~0.006). However, by 58 dpv, the A_{405} for the particular mouse, spiked up to ~0.505. The second group of mice previously inoculated with P1-2A VLPs and adjuvant (Figure 4.4B), showed an increase in A_{405} from 0 dpv to 41 dpv with a steep increase at 58 dpv. The absorbance readings in the final bleed ranged from ~2.155 to ~1.019. The average of the prebleed and 41 dpv readings were ~0.0107 and ~0.0632, respectively. The third group treated with the partially purified pEAQ-HT (Figure 4.4C) demonstrated a decrease from 0 dpv to 41 dpv, except for mouse no. 4 whose A_{405} values showed an increase from ~0.0003 to ~0.0117. At 58 dpv, all mice showed an increase in the A_{405} values with the highest one recorded for mouse no. 5 (~0.319). For mouse no.4, however, a slight decrease from ~0.0117 (41 dpv) to ~0.0107 (58 dpv) was observed. Therefore, considering the mean A_{405} values from the final bleed at 58 dpv,

the group of mice vaccinated with adjuvanted VLPs showed around a four-fold increase in the readings (~ 0.804) compared to the control group (~ 0.194) and those vaccinated with the VLPs, demonstrated around a two-fold increase (~ 0.568).

A ANOVA test was performed on the data collected on the final bleeds, which showed that the means of the A_{405} values of Group 1 (P1-2A VLPs) and Group 2 (P1-2A VLPs and adjuvant) differed from Group 3 ($p < 0.05$). The Tukey HSD test supported the difference of results between Groups 1 and 3 and between 2 and 3 ($p < 0.01$). These results suggested that P1-2A VLPs, and adjuvanted P1-2A VLPs could elicit an effective humoral immune response better than the negative control group.

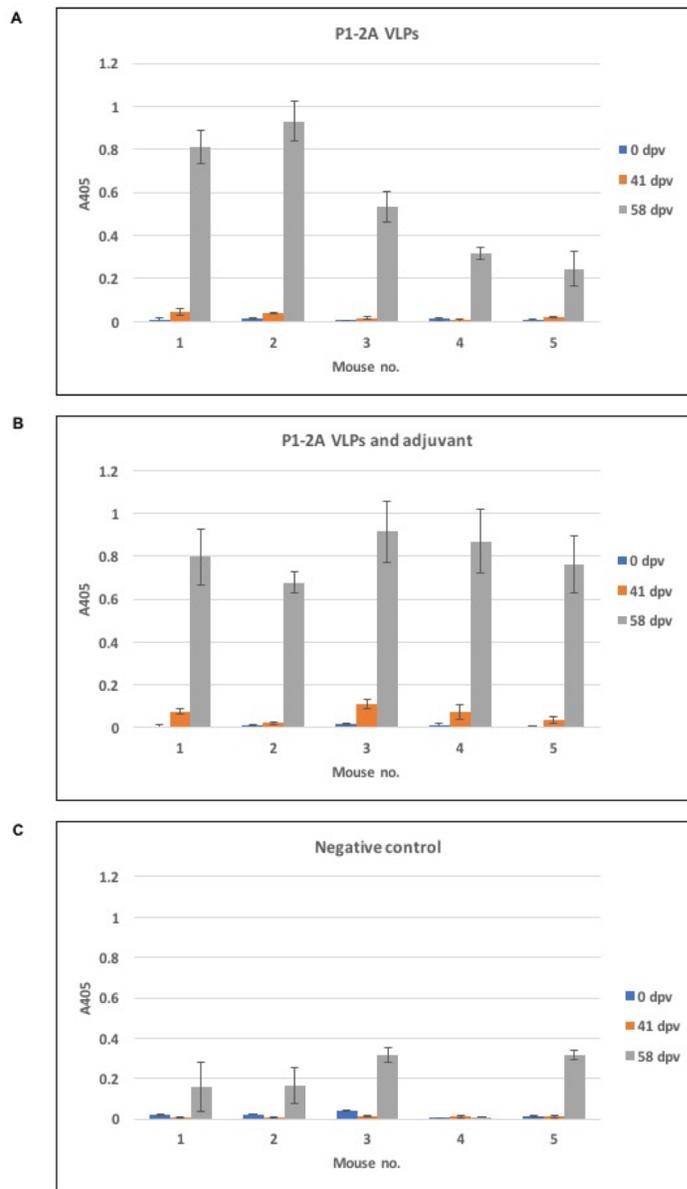


Figure 4.4: Preliminary I-ELISA detection of anti-P1-2A response from mice sera sampled at 0, 41 and 58 dpv. Dilution of antisera at 1:50. (A) Group 1, P1-2A VLPs. (B) Group 2, P1-2A VLPs and adjuvant. (C) Group 3, pEAQ-*HT*. Error bars represent the standard deviation.

4.3.4. Mouse antiserum titration via anti-P1-2A antibody detection

To further corroborate the results obtained from the previous section (section 4.3.3), a four-fold titration was performed on the pooled sera (58 dpv) of the mice (5 mice per group). ELISA plates were coated as described before (section 4.3.3) and anti-P1-2A titres were expressed as the

reciprocal of the maximum dilution containing higher absorbance readings than the pre-bleeds serum at 1:50. The data presented in Figure 4.5 is a representative of three I-ELISA experimental replicates, and each titre was recorded in triplicates. The error bars show the standard deviation calculated from the data collected from all the repeats.

A low antibody response was detected for the negative control (Figure 4.5A, green line) at a titre of 200 at A_{405} of ~ 0.0399 . However, the P1-2A VLPs group (Figure 4.5B, orange line) elicited a higher response with a titre of 3200 at A_{405} of ~ 0.0408 , followed by a good response from P1-2A VLPs with adjuvant group (Figure 4.5C, yellow line), at a value of 51 200 at A_{405} of ~ 0.031 .

To validate the ELISA, the positive control using the anti-P1-2A rabbit sera was also titrated in a similar way, showing a titre of 3200 at a value of ~ 0.108 (Figure 4.5B).

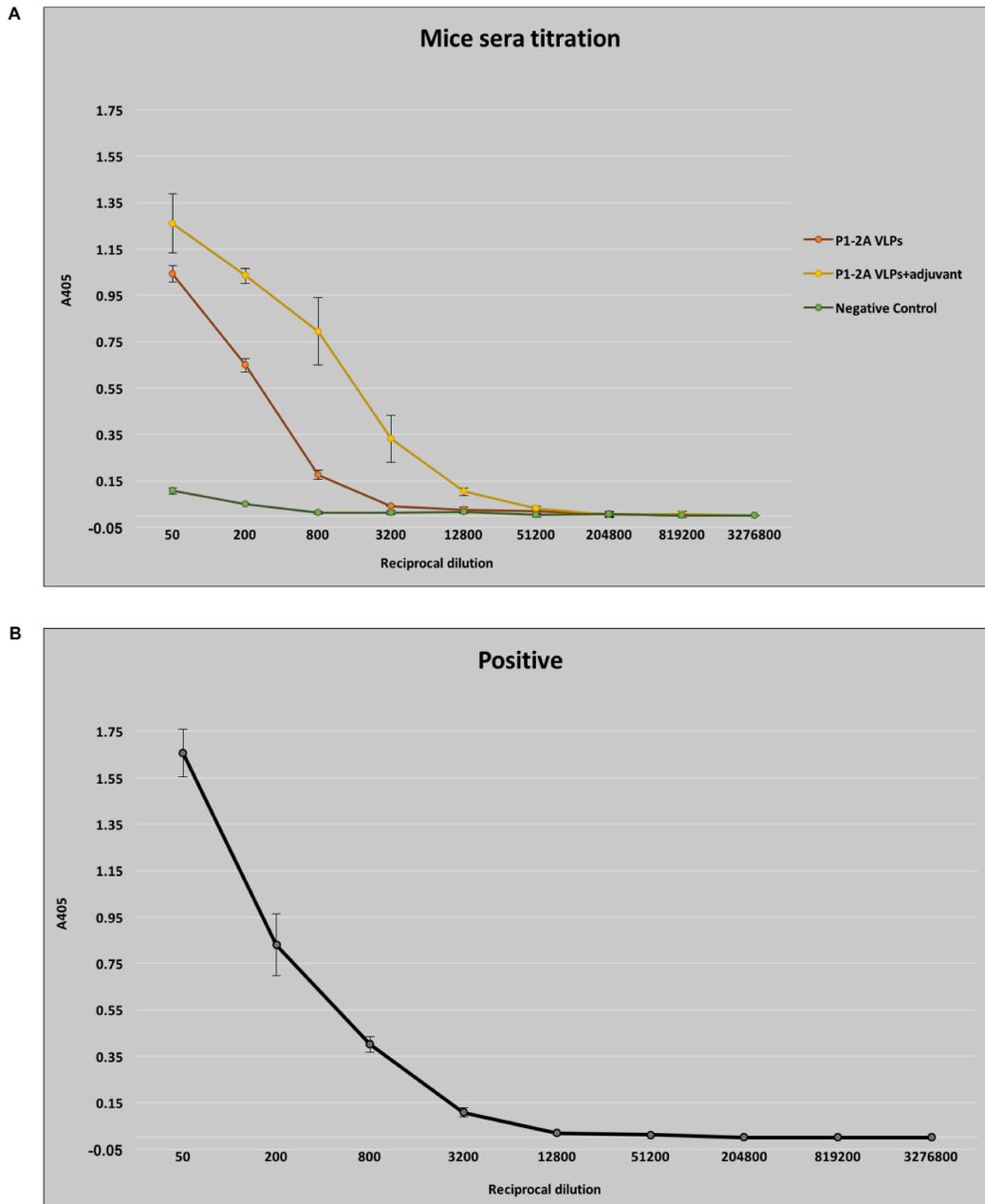


Figure 4.5: Titration of antisera via I-ELISA. (A) Titration of mice sera from experimental groups P1-2A VLPs, P1-2A VLPs with adjuvant and the negative control. (B) Positive control in I-ELISA: anti-P1-2A rabbit sera.

4.3.5. Analysis of humoral immune response via western blotting

The anti-P1-2A response against *E. coli* expressed P1-2A was determined using western blotting. Wells of an SDS-PAGE gel were loaded with equal amount of *E. coli*-produced P1-2A. Mouse sera from their respective groups were pooled and diluted at 1:50 in blocking buffer. The results of

pooled pre-bleed samples from each group were compared to the respective final bleed. Nitrocellulose strips were each incubated individually with the appropriate mouse serum, while a positive control consisted of the use of anti-P1-2A rabbit sera and a negative control consisted of the use of secondary mouse antibody (goat anti-mouse IgG alkaline phosphatase conjugate (Sigma-Aldrich)) only.

Western blot analysis (Figure 4.6) illustrates the detection of the P1-2A capsid precursor at ~83 kDa in the positive control (+) experiment, and the absence of such a band in the negative control (-), as well as in the pre-bleeds of all the groups examined. Similarly, the absence of the ~83 kDa fragment was noted from the negative group. An 83 kDa sized P1-2A band was detected in the two experimental groups inoculated with the P1-2A VLPs or with P1-2A VLPs and adjuvant as indicated by the red arrow. Other bands due to the detection of *E. coli* host proteins were also observed in both the positive control (+) and in the P1-2A VLPs strips.

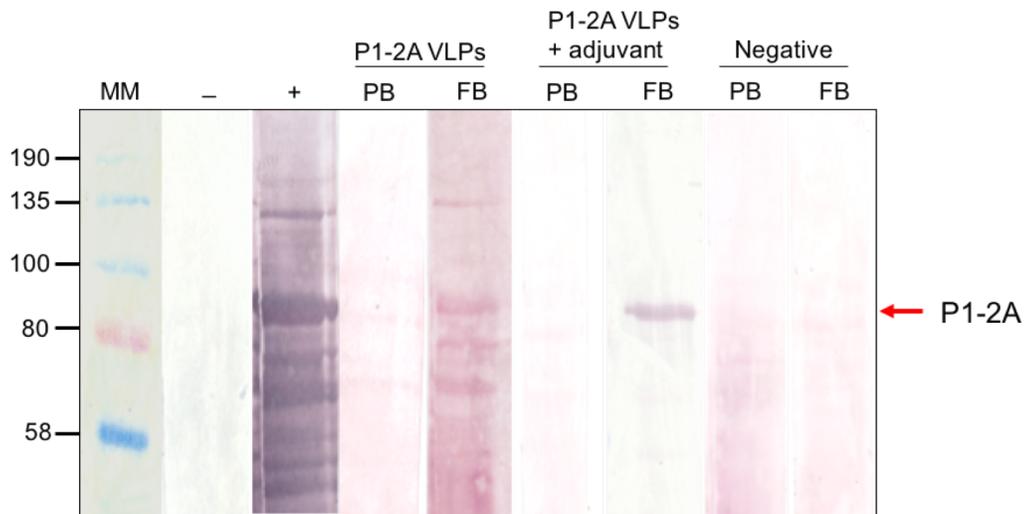


Figure 4.6: Western blot analysis of pooled mice antisera from the three groups: P1-2A VLPs, P1-2A VLPs and adjuvant, and the negative control. (-) antigen detected using the goat anti-mouse IgG alkaline phosphatase conjugate, only. (+) anti-P1-2A rabbit sera. (PB) Pre-bleed. (FB) Final bleed. P1-2A represented at ~83 kDa. (MM) Colour Prestained Protein Standard (New England Biolabs).

4.4. DISCUSSION

As a means of addressing the dire need for prophylactic vaccination to protect animals against FMDV in South Africa, recombinant vaccines in the form of empty capsids are more desirable compared to inactivated vaccines (Bruckner et al., 2002; Jamal and Belsham, 2013).

In this chapter, I demonstrated again (as in Chapter 2) that expression of the capsid proteins from oP1-2A is possible without the co-expression of the FMDV 3C-protease (Figure 4.1C(i)) and (ii)). However, the amount of purified FMDV VLPs produced from ultracentrifugation through a continuous Optiprep™ gradient was low, despite scaling up the amount of biomass processed.

In the past, many studies using plants as an expression platform for the production of FMDV vaccine candidates tested the administration of crude plant extracts in animals as vaccine candidates. Huang et al. (2005) developed a plant-based FMDV vaccine which involved the insertion of an epitope of the VP1 protein into the internal region of the hepatitis B virus core antigen gene (HBcAg). The recombinant construct resulted in the expression of VLPs in transgenic tobacco plants; soluble crude tobacco extract was then used to immunize mice. The vaccinated animals showed antibody responses to both FMDV VP1 and HBcAg and were also protected from viral challenge. In an earlier study conducted by Wigdorovitz et al. (1999b) a recombinant Tobacco mosaic virus bearing the FMDV VP1 was inoculated in *N. benthamiana* leaves, of which the foliar crude extracts were intraperitoneally injected into mice, and showed protection after viral challenge.

Therefore, in order to obtain higher amounts of immunogen in the form of a mixed population of FMDV VLPs and pentamers, partial purification of the vaccine candidates was performed by sedimenting the VLPs using a 30% Optiprep™ cushion, after which the pellets were pooled, resuspended in 1x NTE buffer (pH 8.0) and matured at room temperature. The sample was further clarified by centrifugation (JA-14, 30 021 *xg*, 15 min, 4°C) and the highly soluble FMDV capsid proteins were detected in higher amounts in the supernatant compared to that of the pellet (Figure 4.2B). VLPs measuring ~30 nm were detected in the supernatant (Figure 4.3) while undiluted samples showed a high concentration of pentamers and unassembled capsid proteins

(data not shown) which would still act as viable immunogens upon administration in mice (Liu et al., 2017).

Moreover, the methodology behind the mouse trials focuses on assessing the immunogenicity of the FMDV capsid proteins, primarily because this was a preliminary way of testing whether FMDV capsid proteins produced in the tobacco plants are immunogenic.

The plant-expressed VLPs, when administered both with and without adjuvant, stimulated humoral responses in the mice as determined by I-ELISA. There was a very slight increase in P1-2A-specific antibodies detected 41 dpv but this response was escalated at 58 dpv, after a boost at 44 dpv. An adequate negative control in the form of partially purified plant material agroinfiltrated with *A. tumefaciens* containing the pEAQ-HT vector was administered in another group of mice.

An immune response was observed in mice administered with VLPs, without (Figure 4.4A) and with adjuvant (Figure 4.4B), at 41 dpv. Moreover, the antibody titres escalated at about four-fold in the vaccinated group with the adjuvant (Figure 4.4B) compared to a two-fold increase in the group administered without adjuvant (Figure 4.4A). In this study, I showed that an adjuvant can substantially increase the immune response in the event of a relatively low amount of antigen being available. Considering the number of animals which require FMD vaccination, vaccines formulated with Montanide could provide effective immunization with fewer and potentially lower doses of vaccine. The humoral immune response from mice vaccinated from the adjuvanted vaccine was also shown to be statistically different from the control group ($p < 0.01$).

Furthermore, while the empty pEAQ-HT control vaccinated group initially showed a decrease in apparent immune response from 0 to 41 dpv, there was an apparent slight increase in antibody response at 58 dpv (Figure 4.4C). Such a response was anticipated as the mice have more than likely been pre-exposed to plant proteins and therefore have anti-plant antibodies.

These data and interpretations were visually verified by western blotting (Figure 4.6). A band tentatively representing the uncleaved P1-2A (~83 kDa) protein was detected by mouse serum from animals vaccinated with both the adjuvanted VLPs and VLPs, but was absent in the control group.

In summary, this study illustrates that plant-produced and partially purified FMDV VLPs and intermediates are immunogenic, and represent potential FMD vaccine candidates. I showed that an anti-P1-2A response was elicited in a group of mice vaccinated with the P1-2A VLPs only as well as in a second group administered with adjuvanted VLPs. The immune response from mice administered with an adjuvant formulation showed a better response and a protection to viral challenge would further promote the efficacy of these vaccines.

CHAPTER 5

Conclusions

Foot-and-mouth disease virus (FMDV) is the etiological agent of a highly contagious disease of livestock (Sobrino et al., 2001). It affects mammals in the order Artiodactyla, or cattle, swine, and goats amongst others. Outbreaks of FMD in many parts of the world have had a profound effect on the trade of livestock and its products, leading to significant economic losses. South Africa still has a viral reservoir of the SAT serotypes in the African buffalo population from the Kruger National Park (KNP), although they are asymptomatic. Consequently, protective measures have been taken to monitor for infections and prevent potential outbreaks by vaccination. Animals in the buffer zone next to the KNP are vaccinated yearly. The vaccines available on the market presently consist of inactivated virus, the preparation of which requires high biocontainment facilities for its production. Additionally, the possible presence of live virulent virus in inactivated preparations is also a matter of concern (Grubman and Baxt, 2004; Sobrino et al., 2001).

The main focus of this study was to produce a safe and cost-effective prophylactic FMDV vaccine candidate in the form of virus-like-particles (VLPs), also known as empty capsids. VLPs are multiprotein structures that mimic the organization and conformation of the authentic native virus particle, but lack the viral genome. Thus, such a vaccine would be a potentially safer and cheaper alternative to the currently available inactivated one (Roldao et al., 2010). Previous studies have shown how FMDV VLP production relies on the proteolytic cleavage of the FMDV capsid precursor, P1-2A, by FMDV 3C-protease, to generate the capsid proteins VP0, VP1 and VP3. However, the co-expression of 3C-protease has proved to be deleterious when expressed in recombinant protein production systems (Mignaqui et al., 2013). I therefore aimed at producing and optimising expression of the capsid proteins in the absence of the protease, using *Nicotiana benthamiana* as an expression system. as plants have been shown to be a very suitable platform for generating vaccines.

Firstly, I determined whether codon optimisation of the P1-2A-3C construct expressed in the presence and in the absence of the 3C-protease would result in better expression of the capsid

proteins than its non-codon-optimised (wild-type) oP1-2A. Secondly, I tested the expression of mutated forms of P1-2A, specifically designed to promote acid resistant and/or heat stable VLPs, to try and achieve higher yields. Thirdly, the study culminated in testing the immunogenicity in mice of the candidate VLP vaccine produced from the expression of P1-2A only.

Having previously shown how P1-2A can be expressed in the absence of 3C, I further compared expression of the capsid proteins using a differently codon-optimised *N. benthamiana* P1-2A-3C construct, referred to as mP1-2A-3C in this study. The 'wild-type' construct oP1-2A was also expressed, as well as mP1-2A for comparative purposes. Codon-optimisation was performed on the FMDV type A genes, as proof of concept and as a comparison to a similar study conducted by Mignaquí et al. (2013). I observed that oP1-2A produced a higher VLP count (3 VLPs per field of view) compared to the expression of mP1-2A-3C and mP1-2A (1 VLP per field of view). Therefore, codon-optimisation did not significantly improve expression. It is possible that the recombinant protein was toxic to the plant cells as infiltrated leaves showed necrotic symptoms at 5 dpi. Necrosis which is related to a hypersensitive response (HR) to the viral proteins, generated by the plants (Hao et al., 2006).

I previously determined the optimum conditions for the maximum expression of FMDV capsid proteins' production to occur at 5 days post infiltration (dpi) using an *A. tumefaciens* culture infiltration OD₆₀₀ of 0.5. The establishment of an optimum condition for expression is often derived from fine-tuning the balance between the best dpi, and the best OD while considering the state of the leaves post infiltration. Thus, time-trials at other time intervals (2 and 4 dpi) and at more ODs (0.3, 0.4 and 0.6) would help define other optimum conditions. An alternative to achieving better expression and a higher amount of VLPs, excluding expression of the 3C-protease, would be to co-infiltrate the VPs 0, 1 and 3 in *N. benthamiana* leaves, while varying their ratios. However, the approach should first include the time-trials of the different VPs individually. The use of other plant expression vectors such as pRIC and pTRAc could also be considered. As stipulated by Marsian and Lomonosoff (2016), though viral proteins assemble spontaneously into VLPs, some still require a maturation step. This step was implemented in this study and a maturation time-trial was performed, whereby it was determined a maturation step of 30 to 45 minutes is ideal to generate stable FMDV VLPs.

I also designed and investigated some mutant variants of oP1-2A with specific mutations targeted at the pentamer interface of VLPs or in the N-terminus of VP1. As previously shown by other studies, the acid resistant mutants VP1 N17D (Caridi et al., 2015; Liang et al., 2014) and VP1 N17D/VP4 S73N (Liang et al., 2014), and heat and acid resistant mutant VP2 H93C (Porta et al., 2013a) are known to be more stable when exposed to lower than neutral pH or at a high temperature such as 56°C. I likewise engineered such mutations and performed the pH assays at 5.2 and 6.2 and the heat assay at 56°C. I also compared their expression with that of oP1-2A.

I assessed the stability of VLPs by electron microscopy, and concluded that the number of VLPs per field of view from expression of oP1-2A was marginally higher than its mutants. However, ideally the assays should be extrapolated to a wider range of temperature and pH values. It was also observed that an aberrant cleavage of VP0 into VP2 and VP4 occurred, though VP4 was not visible on the western blot, most probably due to its small size. I further speculated that VLPs produced from expression of oP1-2A and also from its mutants could be encapsidating host nucleic acids. Therefore, in some cases, VP0 is being cleaved into VP4 and VP2, and the process resembles a maturation cleavage that naturally occurs upon encapsidation of viral RNA by the FMDV. To further support this hypothesis, selected purified fractions of the mutants could be run on an agarose gel, to determine whether there are bands representing heterogeneous nucleic acids as shown by a similar study performed by Saunders and Lomonosoff (2015).

My final aim consisted of scaling up the production of VLPs from oP1-2A and testing its immunogenicity in Balb/C mice. A partial purification method was implemented, by loading the clarified plant crude extract onto a 30% Optiprep™ cushion, and the pellets were resuspended in 1x NTE, pH 8.0 buffer. The sample was further clarified by a short centrifugation step, and the supernatant was used for inoculations in mice. This method was preferred compared to the 'complete' purification through a continuous gradient, as extremely low yields obtained were considered inadequate for stimulating a measurable immune response. However, other purification methods such as size exclusion chromatography, which Lin et al. (2015) used successfully to purify Enterovirus 71 VLPs, could be a good alternative to ultracentrifugation. A second method would be purification via precipitation with polyethylene glycol, and anion exchange chromatography could be explored. Nowadays, these substitutes are regarded as

suitable alternatives to the tedious ultracentrifugation methods which also have scalability issues (Koho et al., 2012).

Five micrograms of partially purified P1-2A VLPs were administered to Balb/C mice and a second group of mice received an adjuvanted form of the vaccine. A control group was inoculated with an equal amount of assayed total soluble protein from plant material treated as for the FMDV-containing preparations, previously infiltrated with empty pEAQ-*HT* vector. Sera from the naïve animals were first collected and analysed for the antibody baseline level. The animals were boosted according to the regime described and bled at 41 and 58 dpv. My findings show an induction of an immune response in the two groups of mice inoculated with either the VLPs or the adjuvanted VLPs as from 41 dpv; this increase was also observed at 58 dpv. Besides, while the negative control group did not respond favourably to the inoculations by 41 dpv, they did however show an increase in apparent anti-FMDV antibody titres at 58 dpv. To confirm that this apparent response in the control group was due to the presence of plant proteins in the inoculations, western blots were performed. The antigen used consisted of *E. coli*-expressed P1-2A. The absence of a reactive band representing P1-2A (~83 kDa) from pooled sera of the control-injected mice supported this notion. Furthermore, there was a statistical difference between the groups vaccinated with VLPs, and adjuvanted VLPs compared to the control ($p < 0.01$). Therefore, this showcased the potential of P1-2A VLPs formulated with and without Montanide ISA 50 adjuvant as a candidate prophylactic FMD vaccine.

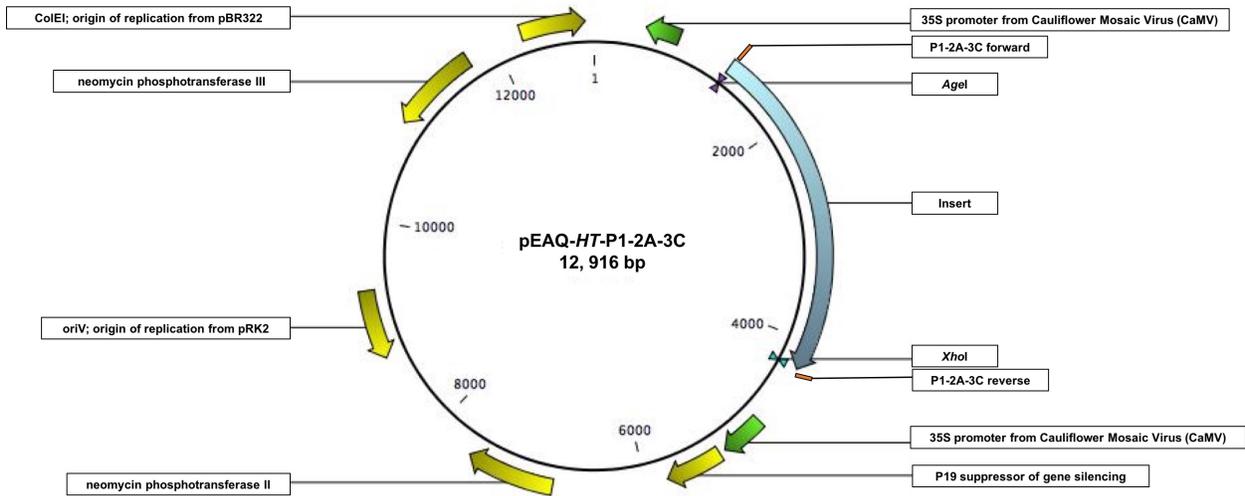
Due to time constraints and lack of facilities, a viral challenge of vaccinated animals was not able to be carried out in order to determine the efficacy of the vaccines. Neutralisation assays would have nonetheless helped to conclude how efficient the candidate vaccines would be. However, due to time, this was not carried out. It would also be more appropriate in the future to repeat the same animal experiments in larger animals, such as cattle which are more prone to FMD (Pega et al., 2013; Pega et al., 2015). I nonetheless demonstrated the possibility of production of VLPs from P1-2A Type A1, which are immunogenic in mice, as a proof of concept. Similar studies should, however, be performed using the P1-2A construct of other serotypes, of particularly the SAT types.

In summary, to my knowledge, this is the first study illustrating a step-by-step investigation of

the expression, production, maturation, stability and immunogenic characteristics of FMDV P1-2A VLPs in *N. benthamiana*. I demonstrated that although codon-optimisation is not the key to increase expression and production of the VLPs, the inclusion of a maturation step at room temperature would improve the stability and shape of the particles. The native P1-2A VLPs compared to its mutants, demonstrated better expression and resistance when subjected to certain extreme conditions. Finally, I showed that these VLPs when formulated with or without the Montanide ISA 50 adjuvant elicited a significant humoral response in mice. These results indicate that FMDV P1-2A VLPs could serve as potential FMDV vaccine candidates, if the production process could be scaled up and yields improved.

APPENDIX I

A



B

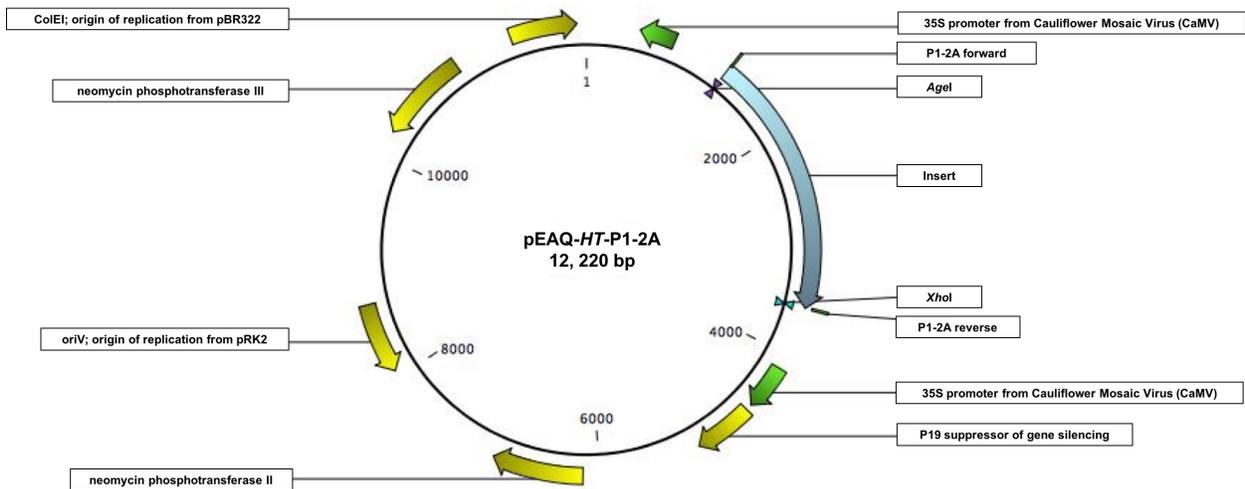


Figure I.1: Recombinant constructs generated from CLC Workbench 6.0 (QIAGEN Bioinformatics) illustrating the features of the pEAQ-HT vector with ColEI: the origin of replication from pBR322, oriV: origin of replication from pRK2, and neomycin phosphotransferase II and III are the kanamycin resistance genes. (A) Recombinant construct pEAQ-HT-P1-2A-3C is a schematic representation of the recombinant pEAQ-HT-oP1-2A-3C and pEAQ-HT-mP1-2A-3C. (B) Recombinant construct pEAQ-HT-P1-2A is a schematic representation of pEAQ-HT-oP1-2A, pEAQ-HT-mP1-2A, pEAQ-HT-VP1 N17D, pEAQ-HT-VP2 H93C, pEAQ-HT-VP1 N17D/VP4 S73N and pEAQ-HT-VP3 A118V.

APPENDIX II

Table II.1: Summary of target genes of FMDV and the respective expression systems used in previous studies for vaccine design. The correlation is established between past data presented by literature and the main aspect of this study.

Miscellaneous expression systems				
Target genes	Expression system	Vaccine delivery system	Animal model	References
P1-3C or P1 only	<i>E. coli</i>	pTP12X3P or p7P12B	Guinea pig	Lewis et al. (1991)
VP0, VP1, VP3	<i>E. coli</i>	SUMO fusion protein	Guinea pig, Swine, Cattle	Guo et al. (2013)
P1-2A, 3C, L-protease	Insect cells	AcMNPV	n/a	Roosien et al. (1990)
P1-2A, 3C	Insect cells	pOPINE and AcMNPV	n/a	Porta et al. (2013b)
P1-2A, 3C	Silkworm	Baculovirus Bm	Cattle	Li et al. (2008)
VP1, VP2, VP3, VP4	Insect cells	Bacmids	Guinea pig	Liu et al. (2017)
P1-2A, 3C	Mammalian cells	pTT5	Mice	Mignaqui et al. (2013)
Plant expression systems (transgenic)				
Target genes	Expression system	Vaccine delivery system	Animal model	References
VP1	<i>Crotalaria juncea</i>	pCAMBIA vector	Guinea pig	Rao et al. (2012)
VP1	<i>Arabidopsis thaliana</i>	pROK1	Mice	Carrillo et al. (1998)
P1-3C	Alfalfa	pROK	Mice	Dus Santos et al. (2005)
VP1	Potatoes	pROK2, pROK3	Mice	Carrillo et al. (2001)
Plant expression systems (transient)				
Target genes	Expression system	Vaccine delivery system	Animal model	References
VP1	<i>Nicotiana benthamiana</i>	<i>Bamboo mosaic virus</i>	Swine	Yang et al. (2007)
VP1	<i>Chenopodium amaranticolor</i> , <i>N. benthamiana</i>	Tobacco necrosis virus A Chinese isolate	Mice	Zhang et al. (2010)
VP1, VP4, 2D, 3D	<i>N. benthamiana</i>	Phytovirus	Mice	Andrianova et al. (2011)
This study				
Target genes	Expression system	Vaccine delivery system	Animal model	References
P1-2A	<i>N. benthamiana</i>	pEAQ-HT	Mice	n/a

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